THE HEPATIC ACTION OF α-ADRENERGIC AGONISTS

by

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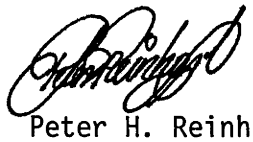
Statement

The design and interpretation of all experiments reported in this thesis was carried out by myself, except for those relating to gluconeogenesis which were performed largely by Dr W.M. Taylor. The execution of many experiments was expedited by the help of Dr W.M. Taylor and Mrs E. van de Pol.



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List of Publications

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7. Taylor, W.M., Reinhart, P.H. and Bygrave, F.L.

   On the role of calcium in the mechanism of action of α-adrenergic agonists in rat liver.

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8. Taylor, W.M., Reinhart, P.H. and Bygrave, F.L.

Stimulation by α-adrenergic agonists of calcium fluxes, mitochondrial oxidation and gluconeogenesis in perfused rat liver.

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9. Reinhart, P.H., Taylor, W.M. and Bygrave, F.L.

The effect of A23187 on calcium fluxes and α-adrenergic agonist action in perfused rat liver.

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Abstracts Presented

1. Reinhart, P.H., Taylor, W.M. and Bygrave, F.L.

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   Early metabolic responses to α-adrenergic agonists may involve calmodulin.

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3. Reinhart, P.H., Taylor, W.M. and Bygrave, F.L.

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4. Bygrave, F.L., Reinhart, P.H. and Taylor, W.M.

   Control by Glucagon and -adrenergic agonists of organellar calcium fluxes, mitochondrial respiration and glycogenolysis in situ in perfused rat liver.

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5. Reinhart, P.H., Taylor, W.M. and Bygrave, F.L.

   The role of calcium in the hepatic action of α-adrenergic agonists.

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6. Reinhart, P.H., van de Pol, E., Taylor, W.M. and Bygrave, F.L.

   An assessment of the in situ calcium content in rat liver mitochondria.

   Proc. Austr. Biochem. Soc. (1983) 15, 513.

7. Bygrave, F.L., Epping, R.J., Reinhart, P.H., Taylor, W.M., and E. Van de Pol

   Calcium fluxes in perfused rat liver in situ induced by the action of α-adrenergic agonists.

   Fourth Int. Symp. on Calcium Binding Proteins in Health and Disease, Trieste, Italy, May 16-19, 1983, p32.

Abstract

The mechanism of α-adrenergic agonist action has been examined in perfused rat liver. Aspects studied included 1) α-agonist-receptor interactions, 2) the redistribution of cellular calcium which follows hormone-receptor binding and 3) the role this redistribution plays in mediating numerous responses to α-agonists. Most of these studies involved the development of new techniques that revealed novel aspects of α-agonist action.

Firstly, the implementation of a sensitive Ca^{2+}-electrode technique, allowed the accurate determination of α-agonist-induced Ca^{2+}-fluxes under physiologically relevant conditions. Furthermore a small, intracellular hormone-sensitive pool of calcium was characterized. The ability to specifically deplete this pool, revealed the obligatory role of α-agonist-induced calcium mobilization in mediating many hormone responses.

Secondly, the development of a rapid single-step fractionation procedure, using Percoll-density gradient centrifugation, allowed an examination of the location of the hormone-sensitive intracellular calcium pool. The results indicate that two pools may exist, one located within mitochondria and the other associated with plasma membrane and/or endoplasmic reticular membranes. This approach also revealed that a significant redistribution of mitochondrial calcium may occur during the isolation of the organelle. Mitochondria isolated under conditions of minimal calcium redistribution, hence possibly reflecting the in vivo state, were found to contain only 2 nmol.calcium.mg $protein^{-1}$.

Thirdly, the α-agonist-induced movement of Ca^{2+} across the plasma membrane of cells was examined by the simultaneous measurement of ^{45}Ca-uptake rates, and net Ca^{2+}-movements using the Ca^{2+}-electrode. It was

found that as well as mobilizing intracellular calcium, α-agonists also stimulate the rate of Ca^{2+} entry into cells, leading to an enhanced rate of Ca^{2+}-cycling across the plasma membrane of cells. This Ca^{2+}-cycle appears to play an important role in the maintenance of sustained responses to α-agonists.

Finally, a technique for examining α-adrenergic agonist-receptor interactions in intact perfused liver was developed. Liver was shown to rapidly accumulate circulating adrenaline, probably by receptor-mediated internalization. This response is not dependent on the redistribution of cellular calcium. Only a small proportion of α-adrenergic receptors appear to be coupled to a signal-transducing mechanism, and the effect of pharmacological agents which can interfere with binding to these sites, can be determined using an in situ competitive binding assay.

Outline of Presentation

The body of this thesis has been organized into two main sections. The first of these entitled 'Introductory Review' represents an overview of the current state of knowledge on the action of α-adrenergic agonists in rat liver. Hence established information, major recent advances in the field, as well as some of the findings made during this study are juxtaposed, allowing the reader to assess how the results of this study mesh into the wider framework of research into α-agonists. It is hoped that an unbiased view has been presented, especially in cases where data can be interpreted in more than one way.

The second part of this thesis consists of the experimental data and interpretations, obtained during this study. Since these results have for the most part been accepted in refereed journals, they have been presented in the form of research papers. It is hoped that the relationship between these papers will become obvious from the introductory review, and from the discussions in each of the papers.

The final few pages are devoted to a brief description of the general directions into which α-adrenergic agonist research appears to be moving.

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1. Introduction

A. Historical Perspective

The beginning of adrenaline research was marked by the discovery in 1895 that an extract from the suprarenal gland can induce vasopressor effects (Oliver & Schafer, 1895). Following the preparation of synthetic adrenaline in 1904 (Stoltz, 1904), progress in the field was rapid with the mechanism of hormone action explained in terms of "receptive substances" (Langley, 1905; Dale, 1906). Adrenergic receptors were later identified in a variety of tissues and species, and the diverse actions of this hormone explained in terms of tissue-specific receptor properties (Clark, 1937). This view was expanded by Ahlquist (1948) who showed that adrenergic receptors could be separated into two groups, α and β, on the basis of differences in the order of potencies of numerous sympathetic amines. The terminology of α- and β-receptors forms the basis for current adrenergic receptor classifications.

One of the first effects of adrenaline to be studied in detail was the stimulation of glycogenolysis in liver (McChesney et al., 1949; Sutherland, 1950). These studies contributed to the discovery of cAMP, and indicated that the glycogenolytic effect of adrenaline in this tissue is mediated by a β-adrenergic receptor-mediated increase in the concentration of cAMP (Rall et al., 1957; Rall & Sutherland, 1958; Makman & Sutherland, 1964; Sutherland et al., 1965; Robison et al., 1967). Subsequent studies, however, indicated that in rat liver, adrenaline-induced effects differ from those observed for classical β-adrenergic agonists (Hynie et al., 1961; Ellis et al., 1967; Arnold & McAuliff, 1968; Exton et al., 1971). Later, Sherline et al., (1972) presented the first evidence that hepatic glycogenolysis could be stimulated by a cAMP-independent α-adrenergic mechanism. Since then,

work with either perfused rat liver or hepatocytes has substantiated the role of α-adrenergic mechanisms in adrenaline-mediated glycogenolysis or gluconeogenesis (Tolbert et al., 1973; Kneer et al., 1974; Tolbert & Fain, 1974; Exton & Harper, 1975; Hutson et al., 1976; Cherrington et al., 1976). These initial observations generated much interest, and induced numerous workers to study the mechanism of α-adrenergic agonist action.

The remainder of this review sets out to describe current views about α-adrenergic agonist action in liver. Topics considered include α-agonist-receptor interactions, how such interactions are thought to induce a redistribution of cellular calcium, and how this in turn may mediate many of the physiological responses of these agonists. To complement and extend the above, aspects of hepatic calcium homeostasis, and current second messenger proposals are also briefly treated. The review is focussed primarily at a discussion of rat liver, and other tissues will be mentioned only to reinforce information available for this organ.

2. Adrenergic Receptors

A. Classification of hepatic adrenergic receptors

Following Ahlquist's proposal that catecholamine-induced responses can be grouped into α- and β-classes (Ahlquist, 1948), numerous investigations revealed that the type of response appears to be species-dependent (see Schmelck & Hanoune, 1980; Exton, 1981). Hence whereas catecholamine-induced responses in rabbit and dog liver can be correlated with a β-receptor-induced activation of adenyl cyclase (Newton & Hornbrook, 1972; Lacombe et al., 1976; Strauss et al., 1979; Kawai & Arinze, 1983), responses in guinea pig and rat liver are

indicative of a role for α-adrenergic mechanisms (Hornbrook, 1970; Haylett & Jenkinson, 1972; Sherline et al., 1972; Jenkinson, 1973; Tolbert et al., 1973; Hutson et al., 1976; Kuo et al., 1977; Proost et al., 1978). Furthermore, in rabbit, human and rat liver, both α- and β-receptor types appear to be present (Lacombe et al., 1976; Guellaen et al., 1978; Minneman et al., 1979; Pecker et al., 1979; Rizza et al., 1980; Malbon, 1981; Rufo et al., 1981; Kawai & Arinze, 1983).

The chemical synthesis of a large number of β-adrenergic agonists and antagonists has allowed the subclassification of β-receptors into β_1 and β_2 (Langer, 1974; Berthelson & Pettinger, 1977). In particular the use of [^{3}H]-dihydroalprenolol, a β-receptor antagonist shown to label both β_1- and β_2-receptors with high affinity (Hancock et al., 1979; Lefkowitz & Hoffman, 1980), β_1-selective antagonists such as atenolol and practolol, together with β_2-selective antagonists such as IPS 339 and butoxamine (Lefkowitz & Hoffman, 1980; Minneman et al., 1979, 1980), has allowed the classification of rat, human and rabbit liver β-receptors as belonging to the β_2-subclass (Lefkowitz, 1975; Lacombe et al., 1976; Munnich et al., 1981; Kawai & Arinze, 1983). In rat liver these receptors number between 60 and 75 fmol.mg of plasma membrane protein^{-1}, which represents less than 5% of the total adrenergic receptor population (Guellaen et al., 1978; Munich et al., 1981; Kawai & Arinze, 1983). The physiological significance of these receptors has not been clearly established, since the administration of adrenaline to either the perfused rat liver or hepatocytes results in only small and transient increases in either the activity of adenyl cyclase or the concentration of cAMP (Bitensky et al., 1970; Exton et al., 1971; Leray et al., 1973; Assimacopoulos-Jeannet et al., 1977). Thus, while hormone responses in rat liver may be predominantly of the α-type, (see Hems & Whitton, 1980), under certain conditions β-receptors may also play a

role (Pointer et al., 1976; Birnbaum & Fain, 1977; Kemp & Clark, 1978). Furthermore, the finding that in murine S49 lymphoma cells, β-receptor-mediated Mg^{2+} transport occurs independent of changes in the concentration of cAMP (Erdos et al., 1981), indicates that even some hepatic responses to β-adrenergic agonists may not be mediated by cAMP.

B. α-adrenergic receptor subtypes

While responses to adrenergic agonists have for a long time been separated into α- and β-, only recently has it been possible to demonstrate α-adrenergic receptors in hepatic plasma membrane fragments (Guellaen et al., 1978; Clarke et al., 1978). This was brought about by the availability of [^{3}H]-dihydroergocryptine, a potent competitive α-adrenergic antagonist, labelled to a high specific radioactivity (Williams & Lefkowitz, 1976).

Analogous to the subclassification of β-receptors into β_1- and β_2-, α-receptors have also been separated into α_1- and α_2- on the basis of both agonist (adrenaline > phenylephrine > clonidine) and antagonist (prazosin > yohimbine) potency series (Doxey et al., 1977; Langer, 1977; Starke et al., 1977). Such a pharmacological classification scheme has recently been substantiated by a characterization of physical properties of receptors. Hence both the purification of adrenergic receptors (Guellaen et al., 1979; Graham et al., 1982; Kunos et al., 1983) and structural analyses using specific affinity labels, monoclonal antibodies, auto-antibodies and radiation inactivation, have confirmed the distinct nature of α_1- and α_2-receptors (see Venter & Fraser, 1983). The number of α-adrenergic receptors estimated from studies using [^{3}H]-dihydroergocryptine is thought to be between 850 and 1400 fmol.mg of membrane protein^{-1}

(Guellaen et al., 1978; El Refai et al., 1979; Hoffman et al., 1980a), with approx. 80% of these receptors comprising the α_1-type (Hoffman et al., 1980c). However, using [^{3}H]-adrenaline, El-Refai et al., (1979) found that binding of this agent was saturable at about 120 fmol. mg of membrane protein^{-1}, and that this subclass of α-receptors (i.e. α_1-) appears to mediate physiological responses to adrenergic agonists. This discrepancy may, in part, be explained by proposing the existence of distinct receptor-subtype states (El-Refai et al., 1979; Hoffman et al., 1980a, b; Geynet et al., 1981).

Hence α_2-receptors have been further subclassified into high and low affinity states, α_{2H}-and α_{2L}-respectively (Hoffman et al., 1980a, b), while α_1-receptors have been thought to exist in precursor form, or as coupled receptors (El-Refai et al., 1979; Geynet et al., 1981). Low concentrations of [^{3}H]-adrenaline (10 nM) apparently bind predominantly to α_{2H}-receptors, while higher concentrations of the hormone (40 to 100 nM) appear to also bind to coupled α_1-receptors (Hoffman et al., 1980a, b; Geynet et al., 1981). In contrast, [^{3}H]-dihydroergocryptine appears to bind to the entire population of α-adrenergic receptors (Hoffman et al., 1980a).

It should be stressed that this complex model was generated from in vitro experiments using membrane fragments enriched in plasma membranes, incubated under non-physiological conditions. As will be discussed below, tissue-related factors may considerably alter current concepts of α-adrenergic agonist-receptor interactions.

C. Hormone-receptor interactions in intact cells

A series of experiments performed using adrenaline covalently linked to a soluble copolypeptide, showed that adrenaline-receptor

binding at the plasma membrane may be the primary event in α-adrenergic receptor-mediated glycogenolysis (Dehaye et al., 1980). Hence, internalization or metabolism of the hormone does not appear to play a significant role in generating a "second messenger", thereby giving rise to the suggestion that the α-receptor-linked transducing signal is generated in or near the plasma membrane (Dehaye et al., 1980).

Intact cells were further used in an attempt to relate hormone responses to data from binding studies carried out with purified plasma membrane fragments. Responses examined include the adrenaline-induced activation of phosphorylase a (El-Refai et al., 1979); Aggerbeck et al., 1980; Hoffman et al., 1980a), glucose output and respiration (Reinhart et al., 1982a), phosphatidylinositol turnover (Tolbert et al., 1980; Prpic et al., 1982) and alterations in Ca^{2+} redistributions (Reinhart et al., 1982b). All of these effects are more sensitive to inhibition by prazosin than by yohimbine, indicating the operation of an α_1-adrenergic mechanism.

However, cautious interpretation of such comparisons is required, since the experimental conditions for studies on intact cells are very different to those employed in binding assays. For example observations on the activation of phosphorylase performed by adding 100nM adrenaline for 60s to hepatocytes treated for 20min with α-adrenergic antagonists, may not be comparable to binding assays performed by a 30min treatment of membrane fragments with either 10nM (Hoffman et al., 1980a) or 40nM (El-Refai et al., 1979) adrenaline in hypotonic Tris buffer. Furthermore the possibility exists that isolated plasma membrane-enriched fractions contain only a sub-population of the total plasma membrane receptor pool, or that functional alterations of adrenergic receptors occur during membrane isolation.

Recently, a more direct comparison of hormone-receptor interactions with physiological responses was carried out in intact perfused liver (Reinhart et al., 1983e). It was shown that adrenaline-induced responses on glucose output and mitochondrial respiration (Reinhart et al., 1982b) were associated with the rapid and continuous uptake of [^{3}H]-adrenaline. Both hormone uptake and physiological responses could be inhibited by prazosin or yohimbine, indicating that hormone uptake follows binding to α-receptors, which are coupled to an undefined transducing mechanism. The presence of a large number of receptors not 'coupled' to the transducing signal was inferred from data showing that the complete inhibition of physiological responses could be correlated with only a partial inhibition of prazosin-sensitive [^{3}H]-adrenaline uptake. Hence most hepatic responses to adrenaline appear to be mediated by a relatively small population of 'coupled' α_1-receptors.

D. Alterations in the receptor subtype number or coupling mechanism

Evidence is accumulating that either the total number of adrenergic receptors, or the coupling mechanism linking receptor binding and hormone responses may change due to a number of physiological or pathological conditions.

Firstly, the relative importance of α- and β-receptors in mediating the regulation of glucose output, glycolysis and pyruvate kinase activity has been shown to change during development (Bitensky et al., 1970; Blair et al., 1979a,b; Morgan et al., 1983a,b). Blair et al., 1979a,b) presented data indicating that in young rats (100 to 150 g) both α- and β-receptor systems contribute to the regulation of glycogen metabolism, while maturation of the rat ($\simeq$ 300 g) is accompanied by a loss of functional β-receptors. These workers also proposed, that

in the adult rat, α-adrenergic receptors can induce both an elevation of the cAMP concentration, and a cAMP-independent stimulation of glucose output (Blair et al., 1979a). Morgan et al., (1983a) confirmed and extended these observations by showing that while either α_1- or α_2-adrenergic agonists can stimulate glucose output in young (80-110 g) rats, only α_1-adrenergic stimulation may be important physiologically.

Secondly, sex-related differences in adrenergic receptor subtypes have also been reported (Bitensky et al., 1970; Studer & Borle, 1982, 1983). Adrenergic agonist-induced responses in hepatocytes prepared from either young male or female rats were found to be similar (Studer & Borle, 1982). Hence in female rats adrenaline may utilize both - and β-adrenergic mechanisms to stimulate glycogenolysis.

Thirdly, the number of β-adrenergic receptors is increased following adrenalectomy (Wolfe et al., 1976; Guellaen et al., 1978; Goodhardt et al., 1982), while the total number of α-adrenergic receptors is not altered (Guellaen et al., 1978; Chan et al., 1979). Thus, the administration of adrenaline to adrenalectomized rats results in a greater stimulation of cAMP accumulation and an increase in the cAMP-dependent conversion of phosphorylase b to phosphorylase a. Furthermore α-adrenergic responses are inhibited by this treatment (Chan et al., 1979), an effect attributed to an impairment in the coupling of α_1-receptors to their effector system (Goodhardt et al., 1982), rather than a decrease in receptor number. A similar shift of α-adrenergic to β-adrenergic responses has also been observed in primary cultures of rat hepatocytes (Okajima & Ui, 1982).

Hypothyroidism and cholestatis have also been shown to increase the hepatic β-adrenergic response (Malbon et al., 1978; Preiksaitis & Kunos, 1979) by increasing the number of β-adrenergic receptors (Malbon, 1980, Aggerbeck et al., 1983). In addition these treatments decrease the

number of α_1-receptors, as indicated by binding studies utilizing [^{3}H]-prazosin (Preiksaitis et al., 1982; Aggerbeck et al., 1983).

Finally, an effect of the nutritional state of the rat on adrenergic receptors, has recently been documented (El-Refai & Chan, 1982). Plasma membranes isolated from 24h-fasted rats were shown to contain 25% fewer α_1-binding sites and 50% fewer α_2-binding sites, although the total number of [^{3}H]-dihydroergocryptine binding sites was not altered.

Hence the recent development of specific adrenergic agonists and antagonists has revealed much about the complexities associated with the binding of adrenaline to adrenergic receptors. Current trends indicate that the receptor classification scheme, α_1, α_2, β_1, β_2, may need to be extended to differentiate between receptors coupled to effector systems and 'uncoupled' receptors. Therefore binding data needs to be correlated with a hormone response elicited under identical conditions either in situ (Reinhart et al., 1983e) or in vitro. In the past, lack of knowledge about the α-receptor coupling system has curtailed in vitro correlations between binding and effects. However, the demonstration of an α_2-receptor-mediated inhibition of adenyl cyclase activity (Jard et al., 1981), and an α_1-receptor-mediated stimulation of phosphatidylinositol metabolism (Harrington & Eichenberg, 1983) in plasma membrane fragments, should provide a powerful approach to differentiate between coupled and uncoupled receptors. Information gained from such studies will be invaluable in the elucidation of the mechanism whereby hormone receptor binding is translated into physiological responses.

E. Hepatic nerves and α-adrenergic receptors

The contribution of hepatic nerves in mediating α-adrenergic responses has received little attention in the past, although it is known that electrical stimulation of these nerves can elevate the rate of glucose and lactate release, as well as altering the activity of numerous hepatic enzymes in a range of species (see Himms-Hagen, 1967; Harris, 1974; Shimazu & Amakawa, 1975; Sawchenko and Friedman, 1979; Shimazu, 1979; Lautt, 1980; Hartmann et al., 1982). The majority of hepatic nerves enter the liver in association with the blood vessels and the bile duct (Sutherland, 1965; Lautt, 1980) innervating blood vessels, parenchymal cells, Kupfer cells, endothelial lining cells, and fat storing cells (Mikhail & Salek, 1961; Ito & Shibasaki, 1968; Skaaring & Bierring, 1976; Forssman & Ito, 1977; Lautt, 1980; Moghimzadeh et al., 1983). Considerable controversy still surrounds the identification of nerve types within the liver, due in part to pronounced species-dependent variations in nerve type and innervation density (Ungvarih & Donath, 1969; Forssman & Ito, 1977; Moghimzadeh et al., 1983). In man, rhesus monkey, guinea pig and cat, adrenergic nerves are thought to extensively innervate parenchymal cells, while in the rat and mouse innervation densities may be much lower (Yamada, 1965; Forssman & Ito, 1977; Nobin et al., 1978; Jarhult et al., 1980; Moghimzade et al., 1983). However, the proposal by Forssman & Ito (1977), that an inverse relationship may exist between the density of parenchymal cell innervation, and gap junction-mediated electrotonic coupling, raises the possibility that responses induced by adrenergic nerve activation may be relatively independent of innervation density. More recent findings by Seydoux et al., (1979) and Hartmann et al., (1982), working with perfused mouse and rat liver, respectively, are consistent with such a proposal.

Seydoux et al., (1979) showed that the electrical stimulation of perivascular nerve bundles rapidly increased the rate of glucose output by the liver, and that this stimulated rate was inhibited by α-adrenergic but not β-adrenergic antagonists. These workers concluded that liver glycogenolysis may be regulated by norepinephrine released as a result of increased nervous activity in the vicinity of parenchymal cells, rather than by circulating catecholamines. Hartmann et al., (1982) using a more physiological perfusion medium with respect to the oxygen, glucose and lactate concentration, extended these observations by showing that the α-adrenergic action of nerve stimulation was not due to haemodynamic effects or due to overflow of neurotransmitters from the vasculature, but rather due to the direct innervation of parenchymal cells. It was further shown that the enhanced rate of glycogenolysis induced by nerve stimulation could be correlated with an increase in glycogen phosphorylase activity, and was dependent on the extracellular calcium concentration. Hence these workers proposed a model whereby the signal generated due to the release of α-adrenergic neurotransmitter, at the few hepatocytes innervated, is propagated via electrotonic coupling through gap junctions.

Much further work is needed to characterize this class of α-adrenergic receptors, and the possibility that the transducing mechanism for these receptors is different from that for circulating catecholamines, cannot be ruled out.

3. Regulation of Cellular Calcium

A. Calcium Compartmentation

In order to evaluate the role of calcium ions in the hepatic action of α-adrenergic agonists, a brief overview of current ideas about the

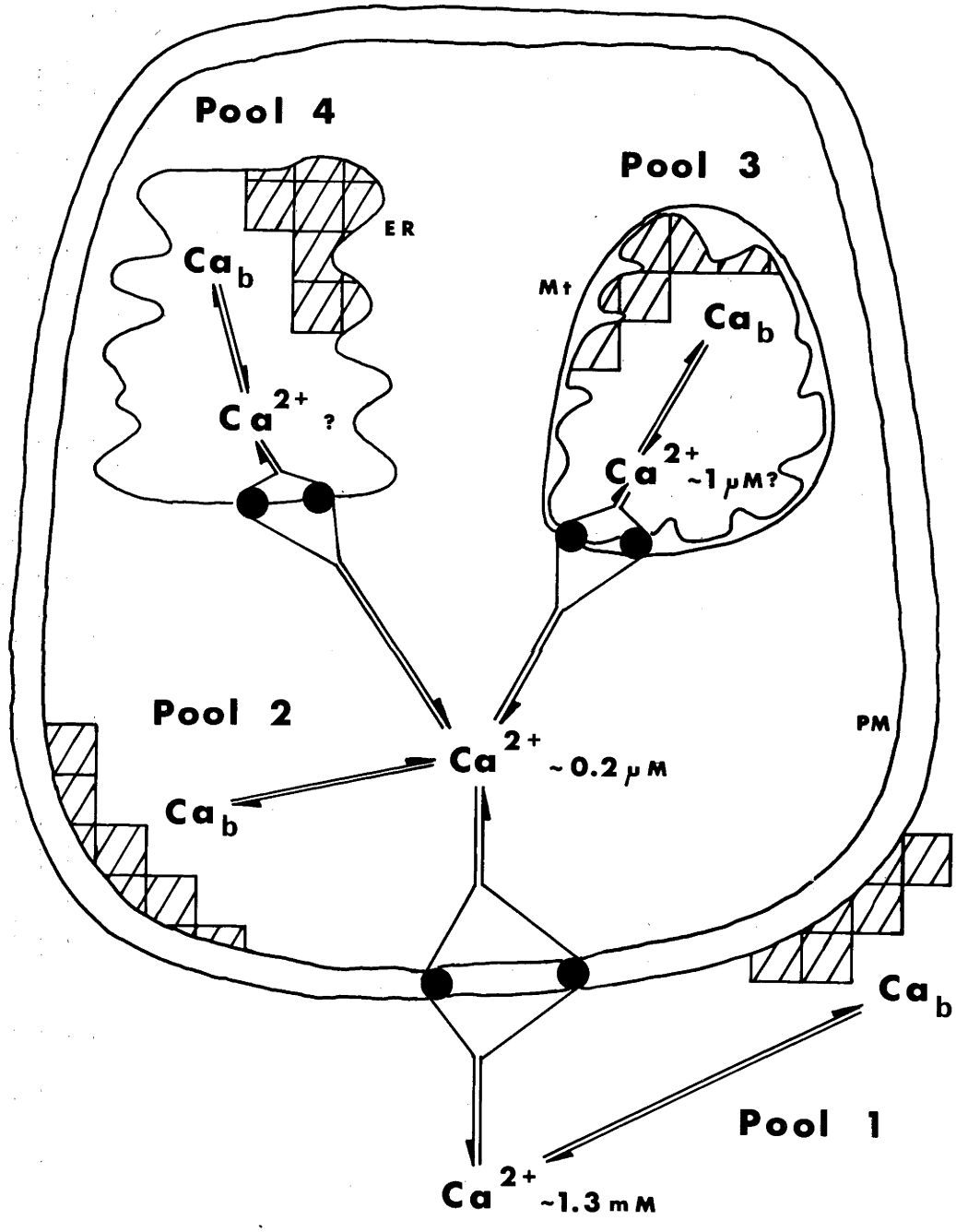
regulation of cellular calcium is warranted. Aspects of this topic have been reviewed recently (Denton & McCormack, 1980; Borle, 1981; Williamson et al., 1981; Barritt, 1982; Akerman & Nicholls, 1983).

The total calcium content of liver (approx. 2 μmol.g wet wt^{-1}; Borle, 1981), is located in discrete cellular compartments (Claret-Berthon et al., 1977; Barritt et al., 1981). The simplest model of cellular calcium regulation, consistent with currently available data, considers the activity of Ca^{2+} transporters located in three membranes; the plasma membrane, the mitochondrial inner membrane and the endoplasmic reticulum (see Fig. 1). These activities lead to the compartmentation of calcium into four pools located in the extracellular space, the cytoplasm, the mitochondrial matrix and endoplasmic reticulum lumen. Within each of these compartments calcium can exist in two forms, either bound to membranes, macromolecules or other ions (Dawson & Hauser, 1970; Williams, 1974), or as the free ion, Ca^{2+}. Although it is Ca^{2+} which plays an important role in the activation of many enzymes, only a small proportion of the total calcium content of any intracellular compartment may be in this form (Borle, 1981; Coll et al., 1982).

The largest pool of cellular calcium, constituting approx. 800 nmol.g $liver^{-1}$, is located on the outside of the plasma membrane (Van Rossum, 1970; Claret-Berton et al., 1977) bound to the coat of proteins and mucopolysaccharides called the glycocalyx (see Barritt, 1982). In intact liver, a portion of this bound pool of Ca^{2+} is in equilibrium with free Ca^{2+} (approx. 1 mM), giving liver cells access to a relatively large extracellular Ca^{2+} pool (Pool 1 in Fig. 1). The total size of the intracellular Ca^{2+} pools is more difficult to determine since these measurements rely on the fractionation of the tissue or cells, and this may give rise to the artificial redistribution of Ca^{2+} (Reinhart et al., 1983b). Previously, mitochondria were thought to contain the largest

Fig. 1 *Schematic representation of calcium compartmentation in hepatocytes*

The simplest model consistent with current experimental evidence considers 4 pools of calcium and 3 Ca^{2+}-translocation cycles. Each pool consists of varying proportions of free (Ca^{2+}) and bound (Ca_B) calcium. Bound pools are represented graphically by cross-hatching. Shown are: pool 1 : the extracellular space and binding sites on the exterior face of the plasma membrane (PM), pool 2: the cytoplasm and the interior face of the plasma membrane, pool 3: mitochondria (Mt), pool 4: the endoplasmic reticulum (ER). Ca^{2+}-translocation cycles may consist of distinct Ca^{2+} uptake and Ca^{2+} efflux activities. Further details are discussed in the main body of this review.


Pool 4
Pool 3
ER
Mt
Ca_b
Ca_b
Ca^{2+} ?
Ca^{2+} ~1 μM?
Pool 2
PM
Ca^{2+} ~0.2 μM
Ca_b
Ca_b
Pool 1
Ca^{2+} ~1.3 mM

cellular pool of calcium (Claret-Berthon et al., 1977; Borle, 1981). However, a recent re-examination of the total calcium content in mitochondria (Reinhart et al., 1983b), revealed that this pool of calcium may be much smaller than previous estimates of 650 nmol.g wet wt^{-1} (Claret-Berthon et al., 1977). By using a rapid, single-step isolation procedure (Reinhart et al., 1982b) to prepare mitochondria under a variety of conditions, it was shown that large movements of Ca^{2+} can occur during isolation of the organelle, and that inhibitors of mitochondrial Ca^{2+} fluxes, such as Ruthenium Red and nupercaine, are only partially effective in preventing these movements. Only by adjusting the Ca^{2+} concentration of all isolation media close to the mitochondrial 'set point', were these Ca^{2+} movements minimized. Mitochondria isolated under these conditions contain only approx. 2 nmol.mg protein, indicating that the total mitochondrial Ca^{2+} pool may be approx. 120 nmol.g wet wt^{-1} (Reinhart et al., 1983b).

The properties of other intracellular pools of calcium have not been clearly defined. This is partly due to the limitations of current fractionation approaches, since intact endoplasmic reticulum and plasma membrane cannot be isolated, and partly due to the limitation of kinetic approaches to adequately differentiate between intracellular Ca^{2+} pools. Most information about these pools has been obtained from membrane fractions enriched in marker enzymes for the endoplasmic reticulum or the plasma membrane. However, such fractions are often heavily contaminated with membranes from a variety of organelles, and the proportion of vesicles in these fractions is often not determined. Furthermore, lack of knowledge about the intactness or orientation of vesicles, or about the redistribution of Ca^{2+} during isolation, limits the usefulness of this approach. An alternative approach, using intact hepatocytes, was

recently adopted by Joseph et al., (1983). These workers used FCCP to deplete the mitochondrial Ca^{2+} pool and A23187 to deplete the total hepatic pool of Ca^{2+}. The difference between the two measurements was taken to represent Ca^{2+} in the endoplasmic reticulum. This approach, however, may give misleading results since FCCP has been shown to also release Ca^{2+} from the microsomal pool (Bygrave, 1978a), and the actions of A23187 in liver are complex, since some bound pools of Ca^{2+} may not be mobilized by this ionophore (Reinhart et al., 1983a). Hence the total pool size of calcium in the endoplasmic reticulum remains an open question. The application, to liver, of techniques such as electron probe analysis in conjunction with cryo-ultramicrotomy (Shuman et al., 1976; Somlyo et al., 1978, 1979) allowing the quantitation of the composition of cellular organelles in situ, may represent a useful approach to quantitate hepatic Ca^{2+} pools.

B. The role of Ca^{2+}-translocation cycles in the regulation of intracellular Ca^{2+} compartments

Considerable interest has recently been focussed on the relative contribution of Ca^{2+}-translocation systems located in the plasma membrane, the mitochondrial membrane, and the endoplasmic reticulum, to the regulation on intracellular Ca^{2+} (Becker et al., 1980; Denton & McCormack, 1980; Joseph et al., 1983; Nicholls & Akerman, 1983; Reinhart et al., 1983b,c,d). Due to the presence of numerous Ca^{2+}-sensitive enzymes in the cytoplasmic compartment, considerable emphasis has been placed on defining the regulation of Ca^{2+} in this compartment (see Denton & McCormack, 1980; Borle, 1981; Williamson et al., 1981; Nicholls & Akerman, 1982). A model implicating mitochondria in this regulation was first proposed by Drahota et al., (1965), and has since been extended by

numerous workers (Claret-Berthon et al., 1977; Bygrave, 1978b; Nicholls, 1978; Borle, 1981; Coll et al., 1982; Joseph et al., 1983). Consistent with such a model, were the findings that mitochondria contain Ca^{2+} transporters for both Ca^{2+} uptake and Ca^{2+} efflux, resulting in the generation of a Ca^{2+}-'translocation cycle' across the inner mitochondrial membrane (see Bygrave, 1978b; Nicholls & Akerman, 1982) and that this cycle resulted in the buffering of the extramitochondrial Ca^{2+} concentration at values close to those thought to exist in the cytoplasm (Nicholls, 1978; Becker et al., 1980; Joseph et al., 1983).

However, recent data indicate that the ability of mitochondria to buffer cytosolic Ca^{2+} is dependent on the total mitochondrial calcium content (Joseph et al., 1983). Mitochondria containing less than 10 nmol calcium.mg protein $^{-1}$ buffer the extramitochondrial Ca^{2+} concentration poorly, and even between 12 and 20 nmol calcium.mg protein^{-1}, less than complete buffering is observed (Joseph et al., 1983). This incomplete buffering is believed to be due to an increase in the rate of mitochondrial Ca^{2+} efflux, in parallel with increased total calcium content (Joseph et al., 1983; Akerman & Nicholls, 1983). The finding that the proportion of free mitochondrial Ca^{2+} increases linearly as a function of the total calcium content in vitro (Coll et al., 1982), is consistent with such a proposal. Half-maximal rates of efflux were observed at a matrix free Ca^{2+} concentration of 9.7 μ M (equivalent to 13.8 nmol. total calcium.mg protein^{-1}). Hence to effectively buffer the cytoplasmic Ca^{2+} concentration, mitochondria in vivo need to contain sufficient total calcium to maintain the rate of the Ca^{2+} efflux pathway constant for a range of total calcium contents. The recent finding, that mitochondria isolated under conditions of minimal Ca^{2+} redistribution and hence approximating the in vivo state, contain only 2 nmol calcium.mg protein^{-1} (Reinhart et al., 1983b), is not consistent with the view that

mitochondria play a predominant role in regulating cytosolic Ca^{2+}. However, it should be stressed that *in vivo* the relationship between the rate of Ca^{2+} efflux and the total calcium content may be significantly different to *in vitro* observations. Hence endogenous Ca^{2+}-complexing ligands, such as phosphate, may effectively buffer the matrix Ca^{2+} concentration, thereby making the rate of Ca^{2+} efflux less dependent on the total calcium content (Akerman & Nicholls, 1983). The role of mitochondria, even containing only 2 nmol calcium.mg protein^{-1}, in regulating the cytoplasmic Ca^{2+} concentration, remains to be established.

Denton & McCormack (1980) have described an alternative role for the mitochondrial Ca^{2+}-translocation cycle. These authors propose that this cycle may regulate the matrix free Ca^{2+} concentration, and hence the activity of numerous Ca^{2+}-sensitive, matrix-located enzymes (Denton *et al.*, 1980; McCormack & Denton, 1980, 1981; Hansford & Castro, 1981, 1982). Although the Ca^{2+} sensitivity of such enzymes has been documented for both the isolated enzymes, and for intact, uncoupled mitochondria (Denton *et al.*, 1972, 1975; McCormack & Denton, 1980; Denton *et al.*, 1980) the question whether or not such regulation is operative *in vivo* is still a controversial issue (Coll *et al.*, 1982; Hansford, 1982; Joseph *et al.*, 1983; Akerman & Nicholls, 1983; Reinhart *et al.*, 1983b). However, recent evidence showing that the ratio of mitochondrial total to free Ca^{2+} is constant at approx. 7×10^4 (hence more than 99.9% of mitochondrial Ca^{2+} is in the bound form), and that mitochondria *in vivo* may contain only approx. 2 nmol.mg protein, indicates that the free matrix Ca concentration may approximate 1.4 μM. The presence of endogenous Ca^{2+}-complexing ligands could reduce this value even further, indicating that the mitochondrial matrix Ca^{2+} concentration *in vivo* may be in a range consistent with the regulation of matrix enzymes.

A further finding of potential significance to intracellular Ca^{2+} homeostasis is that a population of vesicles of non-mitochondrial origin can buffer the ambient Ca^{2+} concentration at a value lower than the 'set point' attained by isolated mitochondria (Becker et al., 1980). This indicates that under the experimental conditions employed, a non-mitochondrial Ca^{2+}-translocation cycle is the primary determinant of the final free Ca^{2+} concentration, raising the possibility that in vivo such a cycle may regulate the cytoplasmic Ca^{2+} concentration. Unfortunately neither the intracellular location of this cycle nor the orientation of these vesicles has, as yet, been defined. However, the finding that digitonin-treated intact hepatocytes buffer the ambient Ca^{2+} concentration at a constant value (approx. 0.1 μ M) in either the presence or absence of Ruthenium Red (Coll et al., 1982) indicates that the final cytoplasmic 'set point' achieved even in the absence of a functional plasma membrane Ca^{2+}-translocation cycle, is independent of the mitochondrial Ca^{2+} uptake system.

Post-mitochondrial microsomal fractions have been shown to consist of a mixture of endoplasmic reticular membranes and plasma membranes (Reinhart & Bygrave, 1981). Hence to correlate Ca^{2+} transport in this fraction with the endoplasmic reticulum only (Joseph et al., 1983) may be misleading since rapid Ca^{2+} movements across the plasma membrane have been demonstrated (see Barritt et al., 1981; Reinhart et al., 1982b; Taylor et al., 1983b).

Clearly much further work is required to examine the regulation of cellular Ca^{2+}, particularly in terms of non-mitochondrial Ca^{2+}-translocation cycles, using intact tissue, or membrane fractions sufficiently enriched in vesicles from a single membrane type to allow the interpretation of Ca^{2+} transport data in terms of a single distinct Ca^{2+}-translocation cycle (see Fig. 1). It should be stressed that much

of this information on cellular Ca^{2+} homeostasis is still incomplete, highlighting the difficulty of interpreting the effects of α-agonists on Ca^{2+} fluxes, against a background of only partial knowledge about basal Ca^{2+} metabolism.

4. α-adrenergic agonist-induced redistributions of cellular Ca^{2+}

A. Initial evidence implicating calcium

There is now much evidence indicating that in rat liver α-adrenergic agonists can stimulate the rate of glycogenolysis or gluconeogenesis without increases in the cAMP concentration, or activation of cAMP-dependent protein kinase (Sherline et al., 1972; Exton & Harper, 1975; Cherrington et al., 1976; Assimacopoulos-Jeannet et al., 1977; Birnbaum & Fain, 1977; Van der Werve et al., 1977). These findings have generated much interest about the mechanism of α-adrenergic action. A role for Ca^{2+} in such a scheme was initially inferred from three pieces of data. Firstly, it was shown that the activity of the glycogenolytic enzyme phosphorylase kinase, can be regulated by micromolar concentrations of Ca^{2+}, as well as by cAMP (Shimazu & Amakawa, 1975; Khoo & Steinberg, 1975; Van der Werve et al., 1977; Sakai et al., 1979). This finding provided an important link between the cytoplasmic Ca^{2+} concentration, and the rate of glycogenolysis. Secondly, the glycogenolytic effect of α-adrenergic agonists was severely impaired by the prolonged incubation of hepatocytes in media where the extracellular free Ca^{2+} concentration was reduced to micromolar levels (De Wulf & Keppens, 1976; Assimacopoulos-Jeannet et al., 1977; Whitton et al., 1977). Thirdly, in a Ca^{2+}-containing medium the ionophore A23187 had been shown to mimic some adrenaline-induced responses (Selinger et al.,

1974; De Wulf & Keppens, 1976; Assimacopoulos-Jeannet et al., 1977 Keppens et al., 1977).

These findings led to the formulation of the hypothesis that the glycogenolytic effects of α-adrenergic agonists in rat liver, are mediated by an increase in the cytoplasmic Ca^{2+} concentration (De Wulf & Keppens, 1976; Assimacopoulos-Jeannet et al., 1977). Much recent work has been aimed at evaluating this postulate by defining α-adrenergic agonist-induced Ca^{2+} redistributions, particularly in relation to glycogenolysis (see Exton, 1981; Williamson et al., 1981; Bygrave et al., 1983; Taylor et al., 1983a).

B. ^{45}Ca measurements

Initial attempts to examine α-agonist-induced Ca^{2+} redistributions were carried out by following the movement of ^{45}Ca added to the extracellular medium (Assimacopoulos-Jeannet et al., 1977; Foden & Randle, 1978). These experiments indicated that α-agonists can stimulate the rate of Ca^{2+} uptake into hepatocytes. However, further studies, using hepatocytes prelabelled with ^{45}Ca, showed that the rate of ^{45}Ca efflux is also stimulated by adrenaline (Blackmore et al., 1978; Chen et al., 1978; Babcock et al., 1979; Poggioli et al., 1980; Kimura et al., 1982). Hence the interpretation of ^{45}Ca data in terms of net Ca^{2+} movements is difficult due to the simultaneous movement of Ca^{2+} into and out of cells (Barritt et al., 1981; Reinhart et al., 1983d). Furthermore, the possibility that experiments were performed under non-steady-state conditions with respect to net Ca^{2+} movements (Borle, 1975), or that ^{45}Ca movements were only a reflection of altered pool sizes, could not be ruled out.

To date a number of distinct experimental approaches have revealed useful information about α-adrenergic agonist-induced Ca^{2+}-redistributions. Firstly, a detailed kinetic analysis of exchangeable Ca^{2+} pools in hepatocytes was performed by Barritt et al., (1981). It was shown that under steady-state conditions the administration of adrenaline for 15 mins, stimulated both the rate of inflow exchange, and the loss of ^{45}Ca from hepatocytes. The ^{45}Ca exchange curves generated under basal and hormone-challenged conditions were analyzed by fitting them to the simplest exponential equation to accurately describe the data. This allowed the definition of three kinetic compartments (as opposed to physiological compartments, Fig. 1) of exchangeable calcium, which were postulated to describe extracellular calcium, cytoplasmic calcium, and calcium sequestered by intracellular organelles such as mitochondria and the endoplasmic reticulum. The effects of adrenaline on ^{45}Ca exchange curves were interpreted as indicating a decrease in the size of the mitochondrial plus endoplasmic reticulum pool, and an increase in the cytoplasmic exchangeable calcium pool, mediated by both the inflow of extracellular Ca^{2+} and the release of intracellular Ca^{2+}. While such a kinetic analysis represents an important approach to define hormone-induced calcium redistributions using intact cells or tissues, it does have the limitations that only cells in steady-state can be analyzed. Hence transient events that occur in the interval between the control state and the new hormone-induced steady-state after hormone-administration (i.e. some 15 min in Barritt et al., 1981) cannot be examined. This is an important point since most adrenaline-induced effects are maximally stimulated by 1 to 2 min after administration, and many effects are transient, occurring only during the first 3 min (Reinhart et al., 1982b, 1983c,d).

C. Net Ca^{2+} Movements

An alternative approach used to define adrenaline-induced Ca^{2+} redistributions was the determining of net movements of Ca^{2+} across the plasma membrane of liver cells or tissue. Using either atomic absorption spectroscopy (Blackmore et al., 1978; 1979) or Ca^{2+}-sensitive electrodes (Chen et al., 1978; Althaus-Salzmann et al., 1980; Sies et al., 1981) to continuously measure Ca^{2+} movements, it was shown that adrenaline induced a net loss of Ca^{2+} from hepatocytes, apparently ruling out a contribution of extracellular Ca^{2+} in the mediation of adrenaline-induced responses. However, a major problem in measuring net Ca^{2+} fluxes is that small and transient net Ca^{2+} movements are difficult to detect. Previous workers have overcome this problem by lowering the extracellular Ca^{2+} concentration to between 5 and 50 μM (Blackmore et al., 1978; 1979; Althaus-Salzmann et al., 1980; Sies et al., 1981). However, such non-physiological conditions have been shown to significantly alter adrenaline-induced Ca^{2+} fluxes (Barritt et al., 1981) and physiological responses (Reinhart et al., 1983c).

The determination of adrenaline-induced net Ca^{2+} movements in intact liver, perfused with media containing 1.3mM added Ca^{2+}, was made possible by the development of a sensitive Ca^{2+} electrode technique (Reinhart et al., 1982b). A resolution of $\pm$1 μM Ca^{2+}, against a background of 1.3 mM Ca^{2+}, was achieved by coupling the electrode output to a microprocessor-ionanalyzer linked in turn to a computing integrator through a bucking voltage device similar to that described by Madeira (1975). Using this approach it was shown that α-adrenergic agonists rapidly stimulate the transient efflux of Ca^{2+} from the liver. The onset was rapid occurring at approx. 4-7 s after phenylephrine administration; maximal rates of efflux were observed at 35 to 45s of treatment,

thereafter declining to be no longer detectable after 2 to 3 min. The total amount of Ca^{2+} lost (approx. 120 nmol Ca^{2+}.g liver $^{-1}$, Table 1) constitutes between 5 and 10% of total cellular calcium (see Section 3A). Within seconds of terminating infusion of the α-agonist, Ca^{2+} was rapidly accumulated, the total amount of Ca^{2+} entering the liver being similar to the amount initially lost. The re-administration of phenylephrine under these conditions resulted in another cycle of Ca^{2+} efflux, and was again followed by Ca^{2+} uptake. This process can be repeated more than 20 times without any decrease in the rate of Ca^{2+} efflux or the stimulation of glycogenolysis. Hence even at 1.3 mM Ca^{2+}, adrenaline stimulates the net efflux of Ca^{2+} rather than uptake.

When these experiments were repeated in the presence of only approx. 6 μM Ca^{2+} in the perfusate, a number of important points emerged (Reinhart et al., 1982b). Firstly, neither the rate nor the amount of Ca^{2+} efflux is diminished by reducing the perfusate Ca^{2+} concentration. However, after removal of the α-agonist no reuptake of Ca^{2+} can be detected. Further administrations of the hormone under these conditions result in much reduced rates of Ca^{2+} efflux until by the fifth 'pulse' of phenylephrine very little Ca^{2+} efflux can be detected. Hence the repeated administration of α-adrenergic agonists to liver perfused with media containing only approx. 6 μM Ca^{2+}, together with sensitive Ca^{2+} electrode measurements, represents a powerful approach to selectively deplete the hormone-sensitive pool of intracellular Ca^{2+}. By quantitating the total amount of Ca efflux the size of the hormone-sensitive pool of intracellular Ca^{2+} can be assessed (Table 1). Furthermore an assessment of the role played by this pool in mediating adrenaline-induced responses can be made (see Section 5A).

D. Characterization of the intracellular pool of Ca^{2+}.

Although many properties of the hormone-sensitive pool of Ca^{2+} have been determined (see Table 1), considerable controversy still exists with respect to the intracellular location of this pool. In large part this appears to be related to the limitations of currently available experimental approaches. Some of these approaches are detailed below:

(i) - fractionation studies - ^{45}Ca contents

These experiments have involved the preincubation of cells or tissue with ^{45}Ca in either the presence or absence of α-adrenergic agonists, followed by cell fractionation and the determination of the ^{45}Ca content in a mitochondrial and/or microsomal pellet (Foden & Randle, 1978; Althaus-Salzmann et al., 1980; Poggioli et al., 1980; Barritt et al., 1981; Berthon et al., 1981; Kimura et al., 1982). The results of these studies are consistent only in that all workers report an effect of α-adrenergic agonists on the mitochondrial ^{45}Ca content. Foden & Randle (1978) found that incubating hepatocytes in media containing 1.2 mM Ca^{2+}, phenylephrine and ^{45}Ca for either 25 or 45 min, increased the mitochondrial ^{45}Ca content, a finding confirmed by Althaus-Salzmann et al., (1980) using liver perfused with media containing only 10 μM Ca^{2+} and examining the ^{45}Ca content 1 and 10 min after hormone administration.

In contrast to these reports, Poggioli et al., (1980) and Berthon et al., (1981) using hepatocytes suspended in 1.8 mM Ca^{2+}, found that α-agonists induce a transient increase in the mitochondrial ^{45}Ca content which is maximal at 15 to 30 s after hormone administration, and returns towards basal levels within 1 to 8 min (Poggioli et al., 1980; Berthon et al., 1981).

Barritt et al., (1981) and Kimura et al., (1982) working at 0.1 mM and 1 mM Ca^{2+} respectively, found that at 3, 5 or 30 min after hormone administration the mitochondrial ^{45}Ca content had significantly decreased.

These data have resulted in a range of interpretations with the plasma membrane (Althaus-Salzmann et al., 1980; Kimura et al., 1982), the endoplasmic reticulum (Poggioli et al., 1980; Barritt et al., 1981; Berthon et al., 1981) and mitochondria (Barritt et al., 1981; Kimura et al., 1982) each having been implicated as containing part of the hormone-sensitive pool of calcium. It appears that variations in the experimental regime employed by various workers can significantly alter the results obtained, thus creating difficulties in interpreting mitochondrial ^{45}Ca content data.

(ii) - fractionation studies - total calcium contents

An alternative approach to identifying the location of the hormone-sensitive pool of intracellular Ca^{2+} is to use atomic absorption spectroscopy to measure the total calcium content in liver fractions isolated before or after α-adrenergic agonist challenge (Babcock et al., 1979; Blackmore et al., 1979; Murphy et al., 1980; Taylor et al., 1980; Reinhart et al., 1982b). Although again, the experimental conditions used by different workers differ significantly, all of the studies are in agreement that at least a portion of the hormone-sensitive pool is mitochondrial in origin. However, a problem with any fractionation study is that significant redistributions of Ca^{2+} may take place during homogenization and fractionation of the tissue (Reinhart et al., 1983b). Such redistributions are only partially inhibited by the presence of mitochondrial Ca^{2+} flux inhibitors and by low temperatures. In an effort to minimize such redistributions a novel fractionation procedure was developed (Reinhart et al., 1982b). This procedure allows

the complete fractionation of perfused liver in a single 30s centrifugation step, by utilizing Percoll as an iso-osmotic density-gradient medium. The ability to rapidly fractionate tissue, together with information about isolation conditions minimizing the redistribution of mitochondrial Ca^{2+} (Reinhart et al., 1983b), allowed an examination of α-adrenergic agonist-sensitive pools of Ca^{2+} (Reinhart et al., 1982b). Whereas an earlier conventional fractionation study indicated that phenylephrine decreases the total calcium content of all fractions examined (Blackmore et al., 1979), the rapid fractionation study indicated that two distinct pools of intracellular Ca^{2+} were being mobilized (Reinhart et al., 1982c). Approximately 50% of the total amount of Ca^{2+} mobilized by α-agonists was correlated with a decrease of the mitochondrial calcium content, while the other 50% was derived from a fraction enriched in both plasma membrane and endoplasmic reticulum vesicles. Significant decreases in the calcium content of these fractions were observed after 25s of hormone treatment, with near-maximal effects being evident between 45 and 60s of treatment, consistent with the time course of Ca^{2+} efflux detected by Ca^{2+} electrode measurements in the perfused liver (Reinhart et al., 1982c). Although it is virtually impossible to totally rule out Ca^{2+} redistributions during any fractionation study, the above data are consistent with two distinct pools of intracellular calcium, one of which appears to be mitochondrial in origin.

(iii) - intact cell studies

A number of workers have used alternative approaches, not involving tissue fractionation, to define the hormone-sensitive pool of Ca^{2+} (Chen et al., 1978; Babcock et al., 1979; Barritt et al., 1981; Blackmore et al., 1982; Kimura et al., 1982; Reinhart et al., 1982b; Whiting & Barritt, 1982).

Babcock et al., (1979) used the fluorescent probe chlortetracycline as an indicator of mitochondrial membrane-bound Ca^{2+} (Luthra & Olsen, 1978). α-adrenergic agonists were found to induce a decrease in chlortetracycline fluorescence within 0.5 to 1.5 min of hormone administration. While these results are consistent with a mitochondrial location of the hormone-sensitive pool of Ca^{2+}, the possibility that in intact cells chlortetracycline fluorescence is in part non-mitochondrial in origin, cannot be ruled out.

A number of workers have used inhibitors of mitochondrial function to show that these agents can inhibit the effects of α-adrenergic agonists (Chen et al., 1978; Barritt et al., 1981; Blackmore et al., 1982; Reinhart et al., 1982a). Compounds used include the uncoupling agents DNP and FCCP, mitochondrial respiratory chain inhibitors such as amobarbital, antimycin A and rotenone, the mitochondrial ATP synthetase inhibitor oligomycin, and the ionophores A23187 and valinomycin. Although the use of ionophores or uncouplers is of little value in identifying the location of the hormone-sensitive pool of Ca^{2+} due to the non-specificity of their action, the use of respiratory chain inhibitors at concentrations similar to those required to inhibit uncoupler-stimulated respiration (Reinhart et al., 1982a), is again consistent with a role for mitochondria in α-agonist-induced responses.

Finally, some workers (Althaus-Salzmann et al., 1980; Barritt et al., 1981; Kimura et al., 1982) have attempted to identify the

TABLE 1

α-adrenergic agonist-sensitive pool of intracellular calcium

Total pool size	:	≃ 160 nmol.g liver^{-1}
Amount mobilized by a single administration of α-agonist	:	≃ 120 nmol.g liver^{-1}
Source	:	a) mitochondria ≃ 70 nmol.g liver^{-1}
	:	b) PM/ER (?) ≃ 50 nmol.g liver^{-1}
State	:	probably bound calcium
Temporal Details of mobilization	:	a) onset 4-7s[a] (≃ 1s?)[b]
	:	b) maximal rate 45s
	:	c) complete 180s
Maximal rate of efflux	:	120 nmol.min^{-1}.g liver^{-1}
Repleted by	:	Extracellular calcium
Depleted by	:	a) Repeated α-adrenergic administrations in media containing only μM Ca^{2+} concentrations 90-95%
		b) EGTA + A23187 80-90%
		c) Antimycin A 40-50%

[a] Measured by Ca^{2+}-electrode in perfused liver.

[b] Measured by spectrofluorometry using Quin-2 loaded hepatocytes.

hormone-sensitive pool of Ca^{2+} by correlating the kinetic properties of this pool with properties of one of the kinetically defined cellular Ca^{2+} pools described by Claret-Berthon et al., (1977). Althaus-Salzmann et al., (1980) interpreted their data as being consistent with the mobilization of extracellular plasma membrane-bound Ca^{2+}. However, since these workers perfused livers for 50 min, with medium containing less that 10 μM Ca^{2+}, yet containing 2 mM EGTA (resulting in a calculated free Ca^{2+} concentration of 1.9×10^{-11}M) it is likely that both intracellular and extracellular pools of Ca^{2+} were depleted. Kimura et al., (1982) have examined the amount of ^{45}Ca effluxed by the administration of α-adrenergic agonists to perfused livers equilibrated with ^{45}Ca. By varying the Ca^{2+} wash out time prior to α-agonist treatment these workers concluded that the hormone-sensitive pool of Ca^{2+} consists of mitochondrial as well as plasma membrane-bound Ca^{2+}. Similarly, Barritt et al., (1981), by comparing rate constants for $^{45}Ca^{2+}$ outflow in isolated mitochondria with those for hormone-challenged perfused liver, found that α-adrenergic agonists can induce the mobilization of mitochondrial Ca^{2+}.

Hence, while much of the supporting evidence is indirect, at least part of the hormone-sensitive pool of Ca^{2+} appears to be mitochondrial in origin. While evidence for a second intracellular pool is increasing, this has as yet not been clearly defined.

E. Role of both intracellular and extracellular calcium pools - plasma membrane Ca^{2+}-cycling.

While much interest has been focussed on defining the intracellular pools of hormone-sensitive Ca^{2+}, the role of extracellular Ca^{2+} has received little attention. This is due, in part, to observations that

the brief removal of extracellular Ca^{2+} does not inhibit the effect of α-adrenergic agonists on activating phosphorylase or stimulating the rate of Ca^{2+} release, glucose output or mitochondrial respiration (Blackmore et al., 1979; Blackmore et al., 1982; Reinhart et al., 1982b, 1983c). Hence, it has been suggested that the mediation of α-adrenergic-responses does not require extracellular Ca^{2+} (Blackmore et al., 1982).

In view of earlier reports showing that α-agonists may increase the rate of ^{45}Ca uptake into cells (Assimacopoulos-Jeannet et al., 1977; Foden & Randle, 1978) even under steady-state conditions (Barritt et al., 1981), the role of extracellular Ca^{2+} has recently been re-examined. By using a combination of ^{45}Ca and Ca^{2+}-electrode techniques it was shown that Ca^{2+} movements across the plasma membrane are regulated by distinct Ca^{2+} uptake and Ca^{2+} efflux activities (Reinhart et al., 1983d). These activities lead to the generation of a Ca^{2+}-translocation cycle, net Ca^{2+} movements being regulated by the ratio of Ca^{2+} uptake to Ca^{2+} efflux.

α-adrenergic agonists were shown to alter the activities of both the rate of Ca^{2+} uptake and efflux in a time-dependent manner. The initial effect, occurring within 7 s of hormone administration is an increase in the rate of Ca^{2+} efflux from liver, presumably in response to the mobilization of intracellular Ca^{2+} within 1 to 2s (Charest et al., 1983). While this movement of Ca^{2+} out of the cell is still occurring, the rate of Ca^{2+} entry into the cell increases, leading to an increased rate of Ca^{2+}-cycling across the plasma membrane. Within 3 to 4 min, net Ca^{2+} efflux has ceased while the rate of Ca^{2+}-cycling has reached a new steady-state, significantly higher than the basal rate of cycling. This steady-state is maintained for the duration of α-agonist administration. Both the basal and hormone-stimulated rates of Ca^{2+}-cycling were shown to be dependent on the extracellular Ca^{2+} concentration. After removal of

the α-adrenergic agonist a large transient net uptake of Ca^{2+} into cells is observed, while the rate of cycling is again transiently stimulated. Within 5 to 7 min of hormone removal net Ca^{2+} movements have ceased, while the rate of Ca^{2+}-cycling has returned towards basal levels.

Hence extracellular Ca^{2+} appears to fulfil two important roles in the α-adrenergic agonist-induced redistribution of cellular Ca^{2+}. Firstly, it allows the maintenance of a significantly higher rate of plasma membrane Ca^{2+}-cycling, and secondly, extracellular Ca^{2+} repletes the hormone-sensitive pool of Ca^{2+} after removal of the α-agonist. The possible significance of plasma membrane Ca^{2+}-cycling in mediating α-adrenergic agonist-induced responses is discussed in Section 5B.

F. The cytoplasmic free Ca^{2+} concentration.

The underlying feature of all models of α-adrenergic agonist-induced Ca^{2+} redistributions in liver is that the cytoplasmic free Ca^{2+} concentration increases during α-agonist administration (see Exton, 1981; Williamson et al., 1981; Bygrave et al., 1983; Taylor et al., 1983a). However, due to the lack of suitable techniques, this has been difficult to determine experimentally. A kinetic analysis of adrenaline-induced Ca^{2+} redistributions revealed that the size of a small intracellular pool was increased (Barritt et al., 1981). However, this pool could not be unequivocally identified as representing cytoplasmic free Ca^{2+}. An alternative approach was adopted by Murphy et al., (1980). These workers developed a null-point titration technique whereby hepatocytes were incubated in Ca^{2+}-free medium supplemented with digitonin to permeabilize the plasma membrane. The final free Ca^{2+} concentration attained was assumed to represent the cytoplasmic Ca^{2+} concentration. This technique is subject to a number of potential

problems. These include the possibilities that the low extracellular Ca^{2+} concentration may have reduced the size of the intracellular hormone-sensitive pool of Ca^{2+}, that the contributing role of extracellular Ca^{2+} has not been considered, that the dilution of the cytoplasm with the extracellular medium may have altered the kinetics of Ca^{2+}-transporting enzymes, and that the presence of digitonin precludes the assessment of the plasma membrane Ca^{2+}-translocation cycle in the maintenance of the cytoplasmic Ca^{2+} concentration. Hence the final free Ca^{2+} concentration established, will be a reflection of the set-point attained by intracellular Ca^{2+}-translocation cycles only. Nevertheless these workers showed that the cytoplasmic free Ca^{2+} concentration does appear to transiently increase from 0.15 μM to 0.45 μM due to α-agonist treatment (Murphy et al., 1980).

Recently, the development of a new fluorescent Ca^{2+} indicator, Quin-2, with high selectivity for Ca^{2+} over Mg^{2+} or H^{+} (Tsien, 1980) has allowed the development of a fluorescent technique for cytoplasmic Ca^{2+} determinations (Tsien, 1981; Lew et al., 1982; Tsien et al., 1982a,b; Pozzan et al., 1982; Hesketh et al., 1983). The technique involves loading cells or tissues with the acetoxymethyl tetraester (Quin-2 A/M) of Quin-2. Acetylesterases within the cell regenerate the indicator, which responds to Ca^{2+} binding with a 5 to 6-fold enhancement of fluorescence (Tsien, 1980; Tsien et al., 1982b). The dissociation constant of 114 nM, indicates that maximum fluorescence changes will occur at Ca^{2+} concentrations close to those thought to exist in the cytoplasm.

This technique has recently been applied to liver by Charest et al., (1983), to show that within 6 to 10s of administration, α-adrenergic agonists can maximally stimulate Quin-2 fluorescence. After correcting for hormone-induced fluorescence changes in the absence

of Quin-2, these workers concluded that the cytoplasmic Ca^{2+} concentration increases from 0.2 to 0.6 μM in the presence of α-adrenergic agonists. While these results are in close agreement with the null-point titration data (Murphy et al., 1980), the possibilities that Quin-2 can accumulate in Ca^{2+} compartments other than the cytoplasm, or that Quin-2 itself may perturb intracellular Ca^{2+} homeostasis, cannot be ruled out.

G. Current model of α-agonist-induced Ca^{2+} redistributions.

The data generated to date give rise to a model of hormone-induced Ca^{2+} redistributions which can be described in three distinct phases (see Fig. 2).

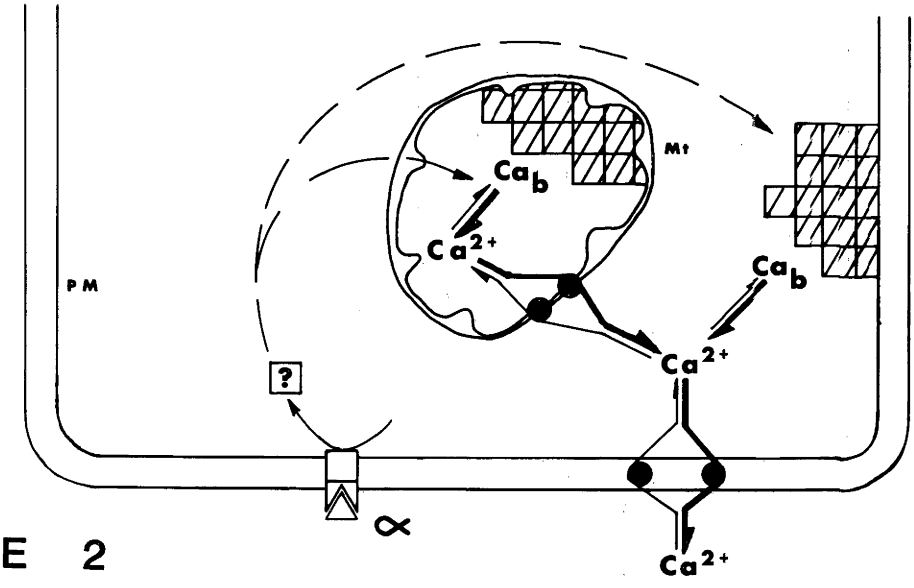
- Phase 1

The 'first' phase is characterized by the mobilization of intracellular Ca^{2+}, possibly from two distinct pools. A large proportion of the Ca^{2+} mobilized appears to represent bound Ca^{2+} as judged by the amount of the ion mobilized (see Table 1), and differences between A23187-induced and α-agonist-induced rates of Ca^{2+} efflux (Reinhart et al., 1983a). This mobilization may increase the free Ca^{2+} concentration in the cytoplasm (Murphy et al., 1980; Charest et al., 1983) and within other intracellular Ca^{2+} compartments. Possibly as a result of such an elevation of the cytoplasmic Ca^{2+} concentration, the rate of net Ca^{2+} efflux from the cell is stimulated (Reinhart et al., 1982b). As the pool of hormone-sensitive Ca^{2+} is being depleted, net Ca^{2+} efflux diminishes. During this period, the rate of Ca^{2+}-cycling at the plasma membrane increases significantly. Within 3 to 5 min of hormone-administration Ca^{2+}-cycling has reached a new steady-state, while net Ca^{2+} fluxes are no longer detectable (Reinhart et al., 1983b).

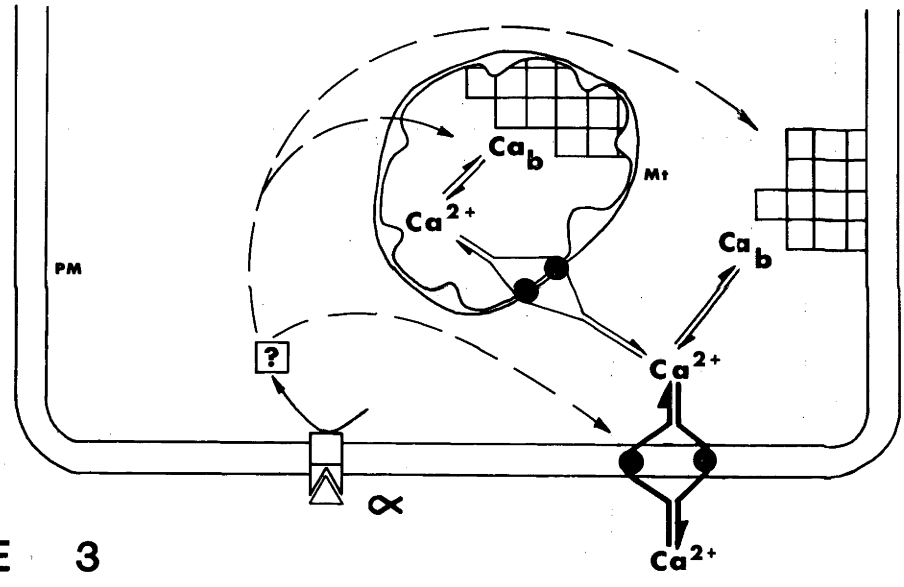
Fig. 2 Three phases of calcium redistribution induced by α-adrenergic agonists

Phase 1 is characterized by the rapid mobilization of intracellular, presumably bound calcium (Ca_B) by one or more undefined 'second messengers'. Part of this calcium appears to be derived from the mitochondria, while a second site of mobilization has not been clearly characterized, but may include a plasma membrane (PM) or endoplasmic reticular pool (for the sake of simplicity only the former is shown in the Fig.). This mobilization may elevate the cytoplasmic Ca^{2+} concentration, stimulate the rate of net Ca^{2+} efflux from the cell, and lead to the depletion of the hormone-sensitive pool of calcium. Phase 2 occurs within 2 to 3 min of hormone administration, by which time cells have returned to a steady-state with respect to net Ca^{2+} movements. During this phase, Ca^{2+}-cycling across the plasma membrane, and possibly across other membranes, appears to be elevated. This cycling may allow the maintenance of an elevated cytoplasmic Ca^{2+} concentration. Phase 3 is initiated by the removal of α-adrenergic agonists from the extracellular space. The discontinuation of 'second messenger' generation results in the net uptake of Ca^{2+} by the cell, leading to the repletion of the hormone-sensitive pool of calcium.

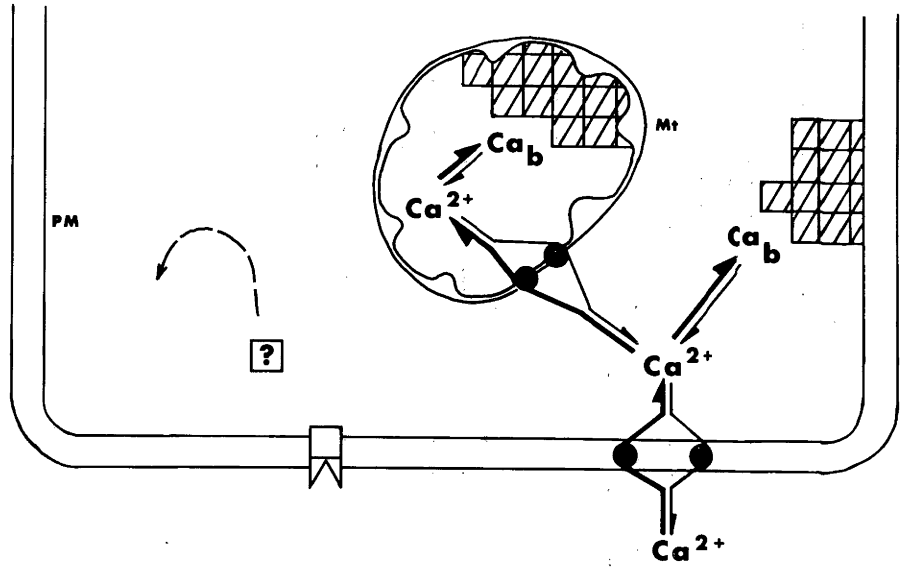
PHASE 1



PHASE 2



PHASE 3



- Phase 2

Ca^{2+} movements during this 'phase' are dependent on the extracellular Ca^{2+} concentration. At concentrations above 100 μM the continued presence of the hormone results in an elevated rate of Ca^{2+}-cycling across the plasma membrane, which may contribute to the maintenance of the elevated cytoplasmic Ca^{2+} concentration. This new steady-state appears to be sustained for the duration of hormone administration. During this 'phase' the hormone-sensitive pool of Ca^{2+} remains depleted.

- Phase 3

The sequence of Ca^{2+} movements during the third 'phase' are triggered by the termination of α-adrenergic agonist administration. The constraints keeping the hormone-sensitive pool of Ca^{2+} in the depleted state are removed, and the pool refills (Reinhart et al., 1982b). The net movement of Ca^{2+} into the cell is transiently stimulated, yet the cytoplasmic Ca^{2+} concentration appears to be returning towards basal values. The rate at which the hormone-sensitive pool of Ca^{2+} is being repleted is dependent on the extracellular Ca^{2+} concentration. Hence even at 100 μM extracellular Ca^{2+}, the hormone-sensitive pool can be repleted if sufficient time (7 to 10 min) is allowed (P.H. Reinhart, unpublished observations). At the end of this phase the cell is again in a basal state with respect to Ca^{2+} homeostasis.

5. Role of Ca^{2+} redistributions in mediating α-adrenergic agonist-induced responses.

A. Ca^{2+}-dependent effects.

Although much effort has been expended on defining α-agonist-induced Ca^{2+} redistributions, the role played by such redistributions in

mediating hormone responses has only recently been examined in detail (Reinhart et al., 1983d; Taylor et al., 1983b). Initial studies examining the Ca^{2+} dependence of responses have either used A23187 to show this ionophore can mimic adrenaline-induced responses (Selinger et al., 1974; Assimacopoulos-Jeannet et al., 1977; Keppens et al., 1977; Chen et al., 1978; Foden & Randle, 1978; Babcock et al., 1979; Whiting & Barritt, 1982) or used high concentrations of EGTA to deplete cells of endogenous Ca^{2+} (De Wulf & Keppens, 1976; Whitton et al., 1977; Assimacopoulos-Jeannet et al., 1977; Garrison et al., 1979; Althaus-Salzmann et al., 1980). However, both of these approaches have a number of shortcomings in that the action of A23187 at the cellular level is very complex (Reinhart et al., 1983a), and Ca^{2+}-sensitive enzymes may respond to this ion only within a tightly-defined range (Van der Werve et al., 1977; Chrisman et al., 1982; Reinhart et al., 1983a). In the majority of studies using A23187, no attempt was made to examine the rate or extent of Ca^{2+} redistributions. Furthermore, the use of high concentrations of EGTA may induce non-specific damage to cells by depleting total intracellular Ca^{2+}, rather than just the hormone-sensitive pool. A more sophisticated approach was made possible by the development of techniques to inhibit the rate of plasma membrane Ca^{2+}-cycling, and to allow the relatively specific depletion of the small intracellular hormone-sensitive pool of Ca^{2+} (Reinhart et al., 1982b, 1983d). The Ca^{2+}-dependence of a number of adrenergic responses including the rate of glucose output, lactate, pyruvate, β-hydroxybutyrate and acetoacetate appearance, alterations in the mitochondrial and cytoplasmic redox ratios, the rate of mitochondrial respiration, plasma membrane K^+ fluxes, [^{3}H]-adrenaline uptake, and gluconeogenesis from a range of substrates, was examined (Reinhart et al., 1982b, 1983c,d,e; Taylor et al., 1983b). It was found that all

responses, except the rate of [^{3}H]-adrenaline uptake, were obligatorily dependent on the redistribution of the hormone-sensitive pool of Ca^{2+}. Hence the depletion of this intracellular pool, representing only 5 to 10% of the total cellular Ca^{2+}, was sufficient to almost totally inhibit α-agonist-induced responses. A further feature revealed by these studies is that phenylephrine-induced responses can be separated into 'transient' and 'sustained' responses.

B. "Transient" and "sustained" responses.

Transient α-adrenergic agonist-induced responses are characterized by a rapid onset of the response (≃ 10s) rising to maximal levels within 30 to 60 s of hormone treatment. This is followed by a decay of the response towards basal values within the next 3 to 5 min, even though the hormone is continuously being administered (Reinhart et al., 1983c). Responses classified as transient include changes in both net Ca^{2+} and K^+ movements (Blackmore et al., 1979; Althaus-Salzmann et al., 1980; Reinhart et al., 1982b, 1983c) and alterations in the cytoplasmic and mitochondrial redox ratios (Scholz & Schwabe, 1980; Sugano et al., 1980; Reinhart et al., 1983c; Taylor et al., 1983b).

Sustained responses also appear to be rapid in onset, however, these responses remain near-maximally stimulated for the duration of hormone administration. Examples of such responses include the adrenaline-stimulated rate of glucose output (Reinhart et al., 1980; Kimura et al., 1982; Reinhart et al., 1982b, 1983c), mitochondrial respiration (Jacob & Diem, 1975; Sugano et al., 1978; Scholz & Schwabe, 1980; Reinhart et al., 1980, 1982a) glycolysis, gluconeogenesis and ketogenesis (Reinhart et al., 1983c; Taylor et al., 1983b) and the rate

of ^{45}Ca exchange across cellular membranes (Barritt et al., 1981; Reinhart et al., 1983d).

These two response types are further characterized by differences in their dependence on cellular Ca^{2+} redistributions. Transient responses appear to be independent of extracellular Ca^{2+}, while being obligatorily dependent on the mobilization of intracellular Ca^{2+} (Reinhart et al., 1983c). Sustained responses, on the other hand, are dependent on both extracellular and intracellular Ca^{2+}. In the absence of extracellular Ca^{2+}, adrenaline is still able to maximally stimulate sustained responses, but rather than remaining stimulated the responses return towards basal values within 3 to 5 min of hormone administration. Hence it appears as though the mobilization of the intracellular hormone-sensitive pool of Ca^{2+} is limited to mediating adrenaline-induced responses only during the first 3 to 5 min of hormone treatment, extracellular Ca^{2+} being required to sustain these responses. Consistent with such a scheme, is the finding that re-elevating the extracellular Ca^{2+} concentration to 1.3 mM, after the decay of sustained responses, results in the rapid re-activation of these responses (Reinhart et al., 1983c). Thus a close relationship may exist between extracellular Ca^{2+}, plasma membrane Ca^{2+}-cycling, and the maintenance of sustained responses. If an increased cytoplasmic free Ca^{2+} concentration does indeed mediate some prolonged effects, then it is tempting to speculate that the plasma membrane Ca^{2+}-translocation cycle can regulate the cytoplasmic Ca^{2+} concentration under these conditions.

C. Intracellular sites of calcium action.

Although many adrenaline-induced responses are mediated by the redistribution of cellular Ca^{2+}, in most cases the mechanism or site of

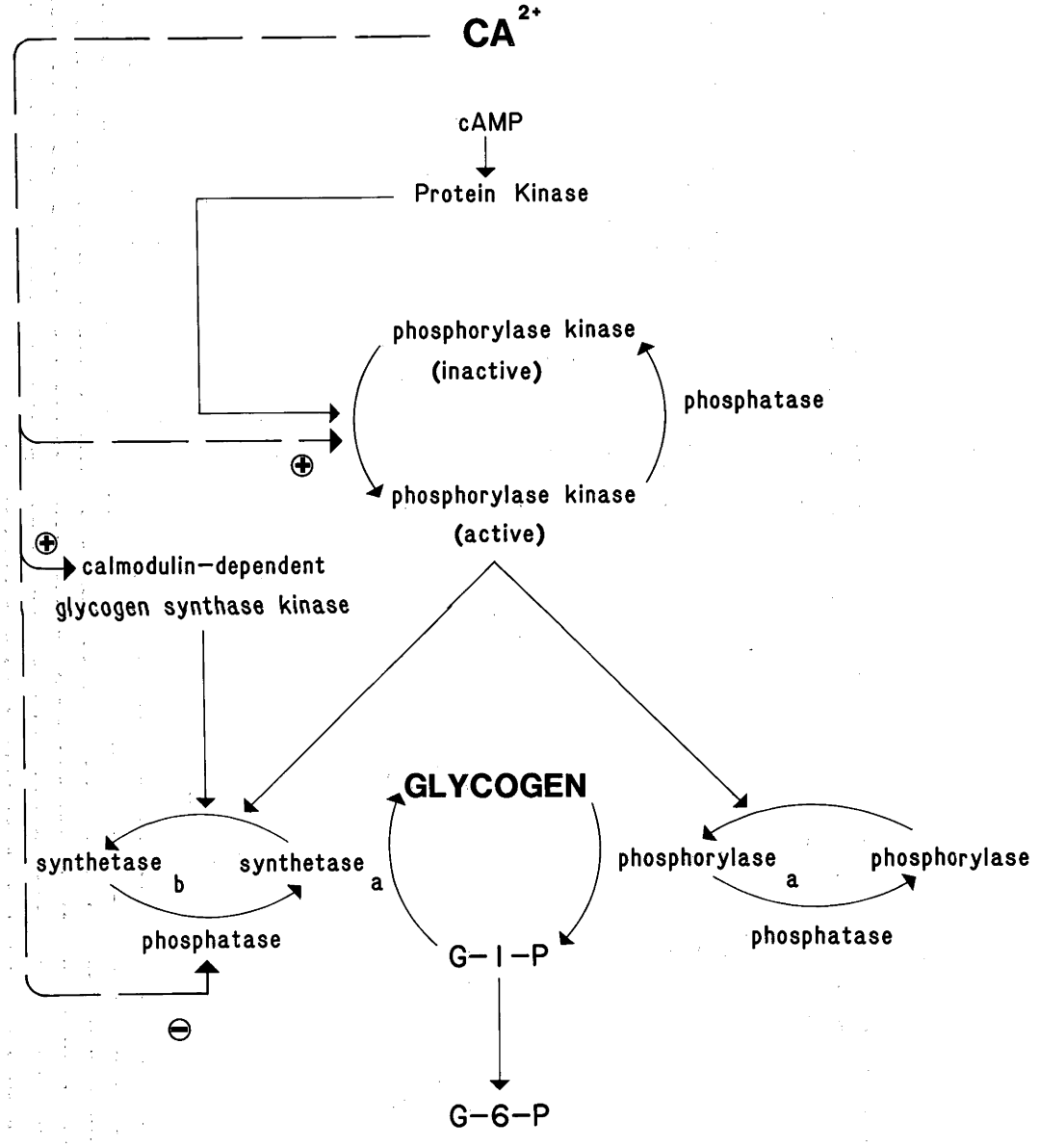
Ca^{2+} action is not known. The hepatic response to α-adrenergic agonists examined in most detail is the glycogenolytic effect, and hence the Ca^{2+} dependence of this response may serve as a useful model for examining other Ca^{2+}-dependent responses.

A simplified outline of the regulation of glycogen metabolism is shown in Fig. 3. From this scheme it can be seen that both Ca^{2+} ions and cAMP-dependent protein kinase are thought to stimulate the rate of glycogenolysis by interacting with phosphorylase kinase, in such a way so as to convert the inactive form into an active form (see Huijing, 1975; Hers, 1976; Stalmans, 1976; Krebs & Beavo, 1979; Hems & Whitton, 1980). Whereas cAMP-dependent protein kinase increases the catalytic activity of this enzyme by phosphorylating both the α-and β-subunits (Cohen, 1973; Hayakawa et al., 1973), the Ca^{2+}-sensitivity of this enzyme activity (Khoo & Steinberg, 1975; Shimazu & Amakawa, 1975; Assimacopoulos-Jeannet et al., 1977; Keppens et al., 1977; Van de Werve et al., 1977) has only recently been interpreted in terms of the calcium-binding protein calmodulin (Schenolikar et al., 1979; Cohen, 1980a; Picton et al., 1980; Walsh et al., 1980; Chrisman et al., 1982).

Calmodulin, a small heat- and acid-stable protein, possesses 4 divalent cation binding sites for Ca^{2+} (see Wang & Waisman, 1979; Brewer, 1980; Cheung, 1980; Klee et al., 1980; Marx, 1980; Means & Dedman, 1980). Binding of Ca^{2+} to any one of these sites results in a conformational change which appears to be required for calmodulin to regulate numerous enzyme systems (Wolff & Brostrom, 1979; Klee et al., 1980; Means & Dedman, 1980). Phosphorylase kinase isolated from skeletal muscle (Cohen et al., 1978; Shenolikar et al., 1979) has been shown to consist of a tetramer of four subunits $(\alpha\beta\gamma\delta)_4$, where the δ-subunit is identical to calmodulin. More recently Chrisman et al., (1982) have confirmed the presence of calmodulin in rat liver phosphorylase kinase, and shown that

Fig. 3 The role of Ca^{2+} in regulating glycogen metabolism.

This simplified representation of the major regulatory enzymes of glycogen metabolism highlights the role of Ca^{2+} in stimulating or inhibiting enzyme activities (broken lines). Ca^{2+} stimulates the rate of glycogenolysis by promoting the conversion of phosphorylase kinase from the inactive to the active form. This in turn leads to the activation of phosphorylase, and possibly the inactivation of glycogen synthetase. Other potential sites for Ca^{2+} regulation include the stimulation of a calmodulin-dependent glycogen synthase kinase and the inhibition of glycogen synthetase phosphatase.


CA 2+
cAMP
Protein Kinase
phosphorylase kinase
(inactive)
phosphatase
phosphorylase kinase
(active)
calmodulin–dependent
glycogen synthase kinase
GLYCOGEN
synthetase b
synthetase a
phosphatase
phosphorylase a
phosphorylase b
phosphatase
G–1–P
G–6–P

the liver enzyme may be activated by lower concentrations of Ca^{2+} than the muscle form, yet be inhibited by higher than optimal concentrations of Ca^{2+} (5×10^{-6}M). However, one point which requires further attention (see Taylor et al., 1983a) is the determination of phosphorylase kinase activities under conditions more closely resembling in vivo conditions. The interaction of Ca^{2+} with the 4 cation-binding sites on calmodulin has been shown to proceed by an ordered unique binding sequence where each calmodulin-Ca^{2+} complex exhibits a specific conformation (Klee, 1977; Walsh et al., 1979). Other cations such as K^+, Mg^{2+} and Mn^{2+} have been shown to decrease the affinity of Ca^{2+} for specific calmodulin binding sites (Wolff et al., 1977; Haiech et al., 1981) or alter the affinity of the calmodulin-Ca^{2+} complex for enzymes (Brostrom & Wolff, 1976). Hence the Ca^{2+}-induced activation of phosphorylase kinase may be altered by the presence of physiological concentrations of other ions such as K^+ and Mg^{2+}. Nevertheless since liver phosphorylase kinase can be activated by Ca^{2+} concentrations (Khoo & Steinberg, 1975; Van de Werve et al., 1977; Sakai et al., 1979; Chrisman et al., 1982) within the range of 0.1 to 0.4 μM thought to exist in the cytoplasm (Murphy et al., 1980; Charest et al., 1983), then the mechanism whereby adrenaline stimulates the rate of glycogenolysis appears to be established.

For some tissues it has been proposed that the active form of phosphorylase kinase may also phosphorylate, and thereby inactivate, glycogen synthase (Roach et al., 1978; Embi et al., 1979; Soderling et al., 1979a; Walsh et al., 1979). The findings that the activity of liver glycogen synthase is sensitive to Ca^{2+} (Strickland et al., 1980), and the liver phosphorylase kinase can alter the activity of muscle glycogen synthase (Soderling et al., 1979b), indicates that a similar mechanism may operate in liver tissue. However, Ca^{2+} has also been shown

to inhibit glycogen synthase activity by activating an alternative glycogen synthase kinase, again involving calmodulin (Payne & Soderling, 1980), and a Ca^{2+}-dependent inhibition of glycogen synthase phosphatase has also been reported (Van de Werve, 1981).

Hence the regulation of glycogen metabolism by Ca^{2+} appears to be quite complex, possibly involving the concerted action of a number of Ca^{2+}-calmodulin dependent kinases and phosphatases (Cohen, 1982; Stewart et al., 1982 Ingebritsen & Cohen, 1983).

While much has been learned about the relationship between adrenaline-induced Ca^{2+}-redistributions and the activation of glycogenolysis, the mechanistic basis for numerous other Ca^{2+}-dependent reactions stimulated by this hormone, has not been established. One possibility receiving much current attention is that the Ca^{2+}-sensitive phosphorylation and dephosphorylation of enzymes is not restricted simply to carbohydrate metabolism, but forms a general mechanism whereby intracellular events are regulated by extracellular stimuli (Greengard, 1978; Krebs & Beavo, 1979; Cohen, 1980b; Garrison et al., 1980; Cohen, 1982). A number of workers have now shown that α-adrenergic agonists alter the phosphorylation state of between 7 and 11 cytoplasmic proteins (Avruch et al., 1978; Garrison & Borland, 1978; Garrison et al., 1979; Van den Berg et al., 1980; Garrison & Wagner, 1982; Vargas et al., 1982).

Three of these proteins have been identified as phosphorylase, glycogen synthase and pyruvate kinase (Garrison & Wagner, 1982). Although it may be expected that adrenaline can alter the phosphorylation state of membrane-associated proteins, as well as cytosolic proteins, a recent investigation did not detect any such alterations (Vargas et al., 1982). However, much further work is required in this area since many studies examined only a single time of incubation with the hormone, even though many adrenaline-induced effects are transient (Reinhart et al.,

1983c). Furthermore in studies where whole cells are treated with [^{32}P]Pi (Garrison & Borland, 1978; Garrison et al., 1979; Garrison & Wagner, 1982; Vargas et al., 1982) great care must be taken to avoid the artefactual phosphorylation and dephosphorylation of proteins during fractionation of the cells or tissue.

To date a number of calcium-sensitive protein kinases and phosphatases have been identified. These include posphorylase kinase (Chrisman et al., 1982), Ca^{2+}-calmodulin-dependent glycogen synthase kinase, (Payne & Soderling, 1980), protein kinase C (Takai et al., 1979), myosin light chain kinase in both muscle and non-muscle cells (Scholey et al., 1980) and protein phosphatase-2B (Ingebritsen & Cohen, 1983). However, the relative contribution of these enzymes in mediating the hepatic effects of adrenaline is not known. Calmodulin appears to play a central role as the Ca^{2+}-sensitive receptor protein in many of these effects. One notable exception appears to be protein kinase C, where data showing that calmodulin-inhibitors such as trifluoperazine, chlorpromazine and fluphenazine can inhibit protein kinase C activity (Mori et al., 1980; Schatzman et al., 1981), has been interpreted as representing an interaction with phospholipids, rather than calmodulin. Other secondary effects of calmodulin inhibitors such as trifluoperazine, chlorpromazine and penfluoridol (Gietzen et al., 1980) have also been noted in intact cell or tissue studies (Blackmore et al., 1981; Joseph et al., 1981; Reinhart et al., 1980, 1983c). Hence the effect of these agents in inhibiting α-adrenergic agonist-induced respiration and glycogenolysis (Reinhart et al., 1980) may be related to interference with hormone-receptor binding (Blackmore et al., 1981; Reinhart et al., 1981), rather than to an involvement of calmodulin.

An important role for calmodulin, as a mediator for many effects induced by the redistribution of cellular Ca^{2+}, appears to be

established. However, a number of responses may not require this Ca^{2+}-binding protein, raising the possibility that other as yet undefined Ca^{2+}-binding proteins may be involved, or that Ca^{2+} itself interacts with regulatory sites on enzymes.

6. Second messenger proposals for α-adrenergic agonists

A. Criteria

There is now good evidence that the binding of adrenaline to α-adrenergic receptors on the plasma membrane of cells, mediates the numerous intracellular effects of this hormone (Dehaye et al., 1980). Such a scheme necessitates the existence of some information transfer mechanism between the plasma membrane and target enzymes analogous to the role played by cAMP in β-adrenergic systems. Criteria which must be met by any proposed 'second messenger' for α-adrenergic agonists include:

1) the messenger must be generated in all cell types containing functional α_1-adrenergic receptors,
2) it must be generated within 1 to 3 s of hormone-receptor binding,
3) a mechanism for the rapid removal of the messenger must exist,
4) messenger generation must be independent of hormone-induced Ca^{2+} redistributions,
5) agonist concentrations giving rise to physiological responses should generate the messenger, and
6) all α_1-agonist-induced responses must be obligatorily dependent on the generation of this messenger.

While considerable effort is currently being expended on the identification of such a messenger for α-adrenergic agonists, to date no proposals have satisfied all of the above criteria. Candidates which

have so far been considered include Na^+ ions, redox ratio changes, natural Ca^{2+} ionophores, cyclic GMP, and metabolites associated with inositol phospholipid turnover such as diacylglycerol, phosphatidate or arachidonic acid metabolites (Pointer et al., 1976; Goldberg & Haddox, 1977; Jones & Michell, 1978; Barritt, 1981; Exton, 1981; Williamson et al., 1981; Taylor, et al., 1983a). Of these, inositol phospholipid turnover is by far the strongest candidate (see Schultz et al., 1977; Hughes et al., 1980; Mellian et al., 1981; Whiting & Barritt, 1982; Taylor et al., 1983a for discussions tending to rule out some of the other proposals).

B. Phosphatidylinositol turnover

Inositol phospholipid turnover, until recently referred to as phosphatidylinositol (PI) breakdown or resynthesis (Jones & Michell, 1978; Michell, 1979; Barritt, 1981; Berridge, 1981; Takai et al., 1981; Exton, 1982; Prpic et al., 1982) has been found to accompany not only α_1-adrenergic agonist action in a range of tissues, but also the action of numerous other agonists thought to induce effects in a Ca^{2+}-dependent, cAMP-independent manner (see Michell & Kirk, 1981; Downes & Michell, 1982). This led to the postulate that PI turnover was in some way generating a messenger giving rise to the redistribution of cellular Ca^{2+} (Michell, 1979; Barritt, 1981; Berridge, 1981; Michell & Kirk, 1981). While this hypothesis appears to fulfill the first criteria, workers found it more difficult to demonstrate that some of the other criteria could be met. Hence the Ca^{2+}-independence of α-adrenergic agonist-induced PI breakdown (criterion 4) has not yet been firmly established (see Billah & Michell, 1979; Tolbert et al., 1980; Cockroft et al., 1981; Prpic et al., 1982; Moore et al., 1983; Creba et al., 1983). Evidence

for the Ca^{2+}-independence of this event is usually of three sorts. Firstly the inability of A23187 to induce PI turnover has been interpreted as indicating that an increase in the cytoplasmic Ca^{2+} concentration does not stimulate PI turnover (Billah & Michell, 1979; Tolbert et al., 1980). However, there are numerous problems associated with such an approach. Firstly, the actions of A23187 in intact cells are very complex, many effects are transient, and particularly at longer times of treatment, Ca^{2+}-independent (Reinhart et al., 1983a). Furthermore some Ca^{2+}-dependent enzymes may respond to Ca^{2+} only within a narrow concentration range (Chrisman et al., 1981; Reinhart et al., 1983a), which A23187 may not be able to mimic. Since studies utilizing A23187 have usually involved prolonged incubation times (Billah & Michell, 1979; Tolbert et al., 1980) and very high ionophore concentrations (Tolbert et al., 1980;Creba et al., 1983), while making no attempt to actually measure Ca^{2+}-movements induced by this agent, then interpretations of this work must be treated with caution.

A second approach is to examine α-adrenergic agonist-induced PI breakdown in cells depleted of Ca^{2+} by incubating them with varying concentrations of EGTA (Billah & Michell, 1979; Tolbert et al., 1980; Prpic et al., 1982). Again this approach has not yielded unequivocal data since this treatment inhibited PI hydrolysis by between 50 and 100% (Billah & Michell, 1979; Tolbert et al., 1980; Prpic et al., 1982), leading to interpretations that PI breakdown is either Ca^{2+}-independent (Billah & Michell, 1979; Tolbert et al., 1980; Creba et al., 1983) or Ca^{2+}-dependent (Prpic et al., 1982). Since EGTA depletes total cell Ca^{2+}, rather than just the hormone-sensitive pool of intracellular Ca^{2+}, and no attempt was made to quantitate the degree of Ca^{2+} depletion in these studies, then again the role of Ca^{2+} in PI breakdown cannot be assessed by this approach. A possible alternative approach might be to

examine inositol phospholipid breakdown in tissue depleted of only the small intracellular pool of hormone-sensitive Ca^{2+} (Reinhart et al., 1982b).

A third approach to examine the Ca^{2+}-dependence of PI breakdown has recently been adopted by Wallace et al., (1982) and Harrington & Eichenberg (1983). These workers used plasma membrane-enriched fractions isolated from rat liver to demonstrate that α-adrenergic agonists can stimulate PI breakdown in vitro, even in the presence of excess EGTA. Even though very high concentrations of α-agonists (50 μM) and prolonged incubation times (30 min) were used in these experiments, this approach has generated the most convincing evidence for the Ca^{2+}-independence of phosphatidylinositol breakdown.

However, to date it has not been possible to show that PI breakdown (or resynthesis) can occur within the time constraints of criterion 2. Hence while α-adrenergic agonist-induced increases in the cytoplasmic Ca^{2+} concentration, the rate of glucose output and mitochondrial respiration, and glycogen phosphorylase activities could be detected within seconds of α-agonist administration (Blackmore et al., 1982; Reinhart et al., 1982b; Charest et al., 1983) it is difficult to measure an elevated rate of PI turnover within 5 min (Billah & Michell, 1979; Tolbert et al., 1980; Prpic et al., 1982). This observation represents a major stumbling block for the acceptance of the theory that PI hydrolysis is linked to the 'second messenger' generation.

C. *Polyphosphoinositide breakdown*

Recent evidence indicates that the preoccupation of examining only phosphatidylinositol turnover may have been misplaced, and that the initial event following hormone-receptor binding is the breakdown of

inositol phospholipids other than phosphatidylinositol (Kirk et al., 1981a; Michell et al., 1981; Kirk, 1982; Berridge, 1983; Creba et al., 1983). Three types of inositol phospholipids have been identified in mammalian tissues, of which the most predominant form is phosphatidylinositol (PI), while phosphatidylinositol 4-phosphate (PI 4P), and phosphatidylinositol 4,5-biphosphate ($PI(4,5)P_2$) together constitute between 2 and 10% of the total inositol phospholipids (Brockerhoff & Ballou, 1961, 1962; Michell, 1975; Hokin-Neaverson, 1977; Agranoff, 1978; Wells & Eisenberg, 1978; Downes & Michell, 1982; Berridge, 1983; Creba et al., 1983). Agents previously shown to stimulate PI breakdown such as α-adrenergic agonists, vasopressin, and angiotensin in hepatocytes and 5-hydroxytryptamine in insect salivary glands, were found also to induce the breakdown of PI 4P and $PI(4,5)P_2$, although the time course of breakdown differed between inositol phospholipids (Kirk et al., 1981a; Downes & Michell, 1982; Kirk, 1982; Berridge et al., 1983; (Creba et al., 1983). Similar observations have been made in a range of other tissues including iris muscle (Abdel-Latif et al., 1977), parotid gland (Weiss et al., 1982) and platelets (Billah & Lapetina, 1982). Detailed examinations of the time course of inositol phospholipid breakdown, by measuring the decrease of $[^3H]$-PI in lipids extracted from hepatocytes labelled to equilibrium with $[^3H]$-inositol (Kirk et al., 1981b; Kirk, 1982) or in the $[^{32}P]$ radioactivity of $[^{32}P]$-PI 4P and $[^{32}P]$-$PI(4,5)P_2$ extracted from hepatocytes allowed to equilibrate with $[^{32}P]P_i$ Creba et al., 1983), indicated that PI 4P and $PI(4,5)P_2$ breakdown was much faster than PI breakdown, occurring within seconds of hormone administration.

This approach has been extended by a kinetic analysis of the rates at which inositol phosphates, partial products of inositol phospholipid

hydrolysis, are formed when salivary gland cells are stimulated (Berridge et al., 1983; Berridge, 1983).

It was found that within the earliest timepoint examined (5s), levels of inositol (1,4)-bisphosphate and inositol (1,4,5)-triphosphate, possible products of PI 4P and PI(4,5)p_2 hydrolysis respectively, were already 4 to 5 times higher than control levels. However, unlike the findings made in liver that both PI 4P and Pi(4,5)P_2 contents rapidly decreased (Kirk et al., 1981a; Kirk, 1982; Creba et al., 1983), in salivary gland only the PI(4,5)P_2 concentration was shown to decrease rapidly, leading to the conclusion that PI(4,5)P_2 breakdown to form a diacylglycerol and inositol (1,4,5)-triphosphate, forms the initial response to hormonal stimulation. The rapid accumulation of inositol (1,4)-bisphosphate was assumed to be derived from inositol (1,4,5) triphosphate (Berridge, 1983).

Hence these studies have revealed that polyphosphoinositide breakdown may be able to satisfy criteria 1 to 4 (p 50) although evidence for the Ca^{2+}-independence of the event is again based on either EGTA-induced Ca^{2+}-depletion or A23187 experiments discussed above (Creba et al., 1983) yielding conflicting data (Rhodes et al., 1983). To date lack of suitable experimental approaches have prevented the verification of criteria 5 and 6.

Nevertheless polyphosphoinositide breakdown products remain the strongest candidates for the elusive second messenger not only for α-adrenergic agonists, but for a range of agonists thought to act independently of cAMP. Both, inositol (4,5)-biphosphate and inositol (1,4,5)-triphosphate have been implicated as second messengers whose function is the mobilization of intracellular Ca^{2+} pools (Berridge, 1983), although as yet there is no experimental evidence for such a scheme.

An alternative proposal (Takai et al., 1979; Kishimoto et al., 1980; Nishizuka & Takai, 1981; Takai et al., 1981) is that the diacylglycerol generated by inositol phospholipid hydrolysis may activate a Ca^{2+} and phospholipid-sensitive protein kinase (protein kinase C, see Section 5C), which may be part of an activation cascade, analogous to cAMP-dependent protein kinase in β-adrenergic agonist action. This is an attractive hypothesis, however, the assumption that in vivo the Ca^{2+} requirement of the kinase is met by membrane-bound Ca^{2+} needs careful appraisal, especially in light of the observations of Garrison et al., (1979) that the phosphorylation of a number of cytoplasmic proteins by Ca^{2+}-mobilizing hormones is inhibited by the pre-incubation of hepatocytes in Ca^{2+}-free media. Also, as the properties of protein kinase C have been determined using partially-purified enzyme preparations, the question whether or not the small quantities of diacylglycerol generated due to polyphosphoinositide breakdown are sufficient to activate C-kinase in vivo, remains to be established.

Hence current evidence favours polyphosphoinositide breakdown products such as inositol (1,4,5)P_3, inositol (4,5)P_2 or diacylglycerol as the most likely 'second messenger' candidates. However, two points which need emphasizing are firstly, that the proposed mechanisms need not be mutually exclusive, i.e. there may be more than one 'second messenger' for α-adrenergic agonists. This possibility is enhanced by the finding that the redistribution of cellular Ca^{2+} is initiated at two sites with distinct temporal aspects (Reinhart et al., 1983c,d). Secondly, the validity of the inositol phospholipid 'second messenger' theory relies on the demonstration that α-adrenergic agonist-induced responses are obligatorily dependent on this event. Until such evidence is obtained the possibility that inositol phospholipid cycling represents an event parallel to, rather than in series with, the 'second messenger'

generation, cannot be ruled out. A combination of lively interest in this area, and the recent development of suitable experimental approaches, should reveal much information over the next few years.

7. The current model of α-adrenergic agonist action in rat liver

From the above it should be obvious that many aspects of α-adrenergic agonist action in rat liver remain to be established. Hence any model of α-agonist action needs to be viewed as a working model only, which will need to be modified as new experimental data comes to hand. Such a 'working model' is depicted in Fig. 4.

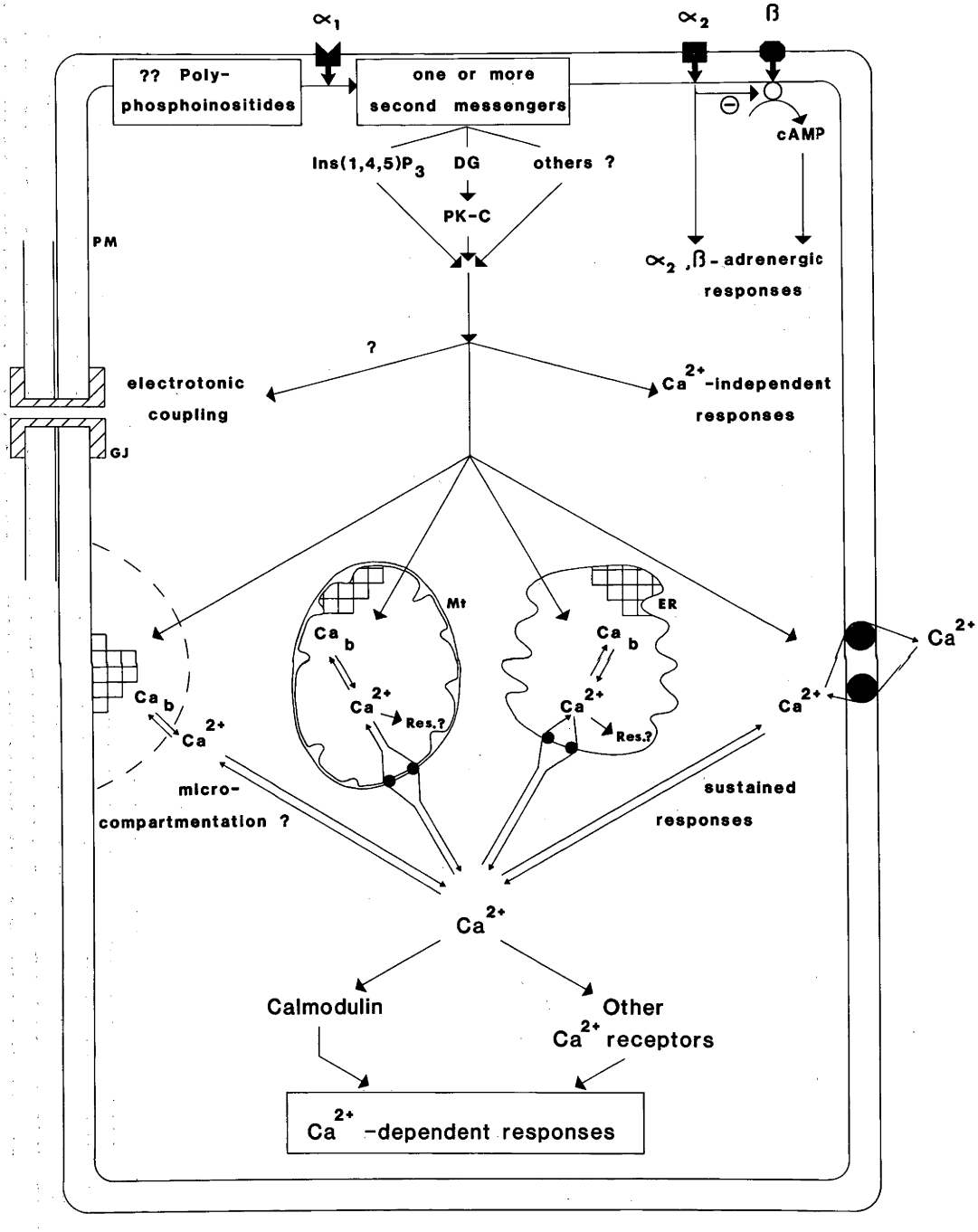
Of the two types of α-receptors (α_1- and α-$_2$) most physiological responses appear to be mediated by a sub-population of coupled α_1-adrenergic receptors (Section 2C). Such binding is believed to induce the generation of one or more 'second messengers', as well as leading to the internalization and metabolism of the hormone. Current candidates fulfilling at least some of the criteria required of 'second messengers' (Section 6A) are diacylglycerol, and breakdown products of polyphosphoinositides such as inositol (4,5)P_2 and inositol (1,4,5)P_3, (however also see Rhodes et al., 1983).

There appear to be at least two intracellular targets for the 'second messenger(s)'. Firstly, a bound pool of intracellular Ca^{2+} is mobilized. At least a portion of this pool appears to represent bound Ca^{2+} in the mitochondrial matrix (Section 4D). This mobilization leads to an increase in the concentration of cytoplasmic Ca^{2+} (Section 4F) and the net extrusion of Ca^{2+} from the cell, possibly by a plasma membrane-located Ca^{2+}-sensitive Ca^{2+} pump. Whether or not this mobilization increases the Ca^{2+} concentration in other cellular compartments such as the mitochondrial matrix and endoplasmic reticulum lumen, has not been clearly defined. Virtually all of the α-adrenergic agonist-mediated

Fig. 4 A current model for the action of adrenaline in rat liver.

Most adrenaline-mediated effects in mature, healthy rats appear to be mediated by coupled α_1-receptors. Hormone-receptor binding may lead to the generation of one or more as yet undefined 'second messengers'. Current evidence indicates that products of polyphosphoinosite hydrolysis such as Inositol (1,4,5)P_3 and diacylglycerol (DG), or protein kinase C (PK-C) may be part of the second messenger system. Under some conditions signal transduction may be by electrotonic coupling through gap junctions.

A major response to this messenger(s) appears to involve the redistribution of both intracellular and extracellular calcium, resulting in an elevation of the cytoplasmic Ca^{2+} concentration, and the stimulation of Ca^{2+}-dependent responses. The alteration of the bound/free calcium ratio (Ca_B/Ca^{2+}) within organelles such as mitochondria (Mt) or the endoplasmic reticulum (ER) may induce responses (Res.?) within these organelles. While all transient responses require only the mobilization of intracellular calcium, sustained responses are dependent on the extracellular Ca^{2+} concentration, and the Ca^{2+}-translocation cycle across the plasma membrane of cells.


α1
α2
β
?? Poly-
phosphoinositides
one or more
second messengers
cAMP
Ins(1,4,5)P3
DG
others ?
PK-C
PM
α2 ,β-adrenergic
responses
?
electrotonic
coupling
Ca2+-independent
responses
GJ
Mt
ER
Ca b
Ca2+
Res.?
micro-
compartmentation ?
sustained
responses
Ca2+
Calmodulin
Other
Ca2+ receptors
Ca2+ -dependent responses

effects examined to date appear to be obligatorily dependent on the mobilization of intracellular Ca^{2+} (Section 5A). Within 3 to 4 min of hormone treatment the intracellular pool of hormone-sensitive Ca^{2+} has become virtually depleted.

The second target for α-agonist induced 'second messenger(s)' appears to be a plasma membrane-located Ca^{2+} gate or channel. Even while net Ca^{2+} is still being extruded, an increase in the Ca^{2+} permeability of the plasma membrane leads to an enhanced rate of Ca^{2+}-cycling (Section 4E). This Ca^{2+}-cycle appears to maintain the elevated cytoplasmic Ca^{2+} concentration, indicating that sustained responses to α-adrenergic agonists are dependent predominantly on extracellular Ca^{2+}.

After removal of α-adrenergic agonists from the extracellular space, a large net movement of Ca^{2+} into the cell occurs, refilling the previously depleted intracellular pool of Ca^{2+}, while the cytoplasmic Ca^{2+} concentration, the rate of plasma membrane Ca^{2+}-cycling and all sustained hormone responses, return towards basal values.

While many aspects of this 'working model' still require experimental verification, it is hoped that over the next few years significant advances will be made in the identification of α-adrenergic agonist-induced 'second messenger(s)' and the way in which they can induce the redistribution of cellular Ca^{2+}. A long-term goal is the examination of the mechanism(s) whereby the redistribution of cellular Ca^{2+} can induce many α-adrenergic agonist-induced responses.

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SECTION B

Publications

Section B.1

Trifluoperazine, an inhibitor of calmodulin action, antagonises phenylephrine-induced metabolic responses and mitochondrial calcium fluxes in liver.

(Reinhart et al., 1980)

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TRIFLUOPERAZINE, AN INHIBITOR OF CALMODULIN ACTION, ANTAGONISES PHENYLEPHRINE-INDUCED METABOLIC RESPONSES AND MITOCHONDRIAL CALCIUM FLUXES IN LIVER

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1. Introduction

The stimulation of glycogenolysis and gluconeogenesis by α-adrenergic agonists in liver may be associated with a transient increase in the concentration of cytoplasmic Ca^{2+} (see [1]). Some uncertainty appears to exist as to the source of this Ca^{2+} (cf. [2–4] and [5,6]), although the ability of mitochondria of participate in the sequestration of this pulsed Ca^{2+} now seems established [7].

In view of the apparent involvement of calmodulin in numerous Ca^{2+}-dependent metabolic events [8–10], it seemed important to consider whether α-adrenergic agonist-induced changes in liver metabolism involve this protein. This study, using the perfused rat liver system, provides evidence that trifluoperazine, an agent used to assess the action of calmodulin [8,9,11], antagonises the α-adrenergic agonist-induced stimulation of glucose output, oxygen consumption and mitochondrial Ca^{2+} fluxes.

2. Methods

Livers from female Wistar strain albino rats (~200 g body wt) were perfused with Krebs-Henseleit bicarbonate medium equilibrated with 95% O_2–5% CO_2 essentially as in [12]. Minor modifications were the use of a non-recirculating medium at 32°C [13] containing 1.65 mM $CaCl_2$. The flow rate was maintained at 15 ml/100 g body wt. The effluent medium was monitored for oxygen content using a Rank oxygen electrode, and the flow rate through the liver determined by measuring the volume of the effluent collected in a fixed time. Glucose released by the liver was estimated using the glucose oxidase method (Sigma assay kit 510-A). 'Heavy' mitochondria [14] were isolated from separate lobes at 0–4°C. Each lobe was completely homogenised within 10 s of its removal from the intact organ. Measurement of mitochondrial Ca^{2+} influx, Ca^{2+} retention and the protonmotive force was as in [7,15,16]. Mitochondrial protein was determined as in [17]. All reagents were of analytical reagent grade. Hormones were purchased from the Sigma Chemical Co., St Louis, MO. Trifluoperazine was a generous gift of Smith, Kline and French.

3. Results

Fig.1. shows the effect of phenylephrine on glucose output and oxygen consumption by the livers of fed rats, pre-perfused for 10–15 min with or without trifluoperazine (3.5×10^{-6} M). Data in fig.1a indicate that the output of glucose from liver is enhanced immediately following the administration of phenylephrine (2×10^{-6} M). Such output is maximal by ~2 min and continues for the duration of the experiment. The data also show that pre-perfusion of the liver for 10 min with trifluoperazine reduces this response to phenylephrine by ~80%.

Oxygen consumption by the liver increases immediately following infusion of phenylephrine to reach maximal values by ~2 min and continues for at least 7 min (fig.1b). This response is diminished in magnitude by ~50% in the presence of trifluoperazine.

By contrast, glucagon administration induced slower responses in respect of both glucose output and oxygen consumption and these were unaffected

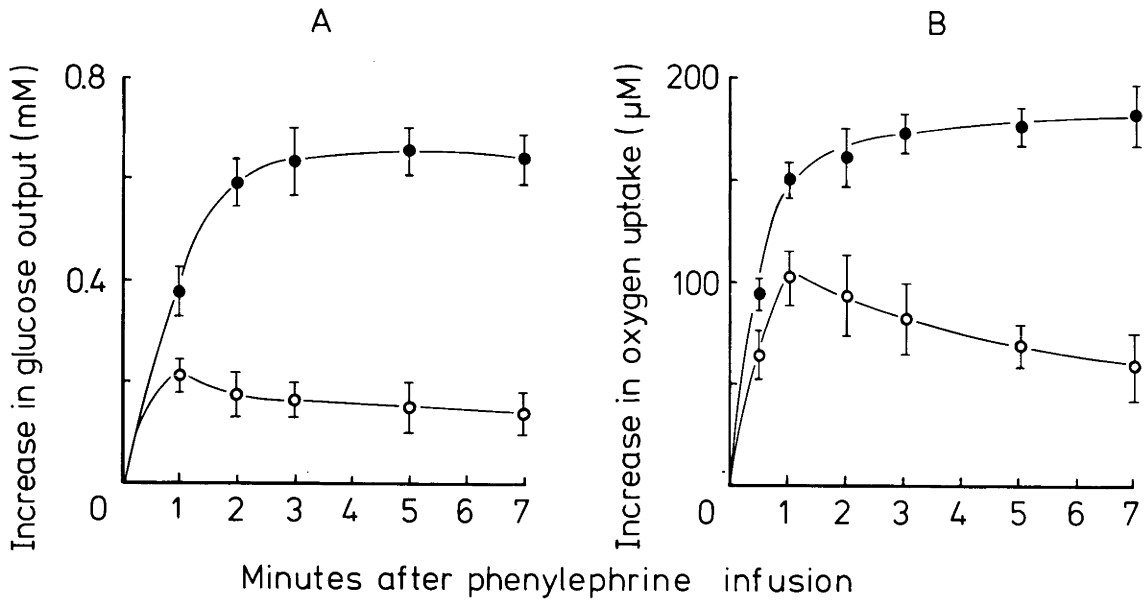

Fig.1. Effect of trifluoperazine on phenylephrine-induced glucose release and oxygen output in perfused rat liver. Livers of fed rats were perfused initially for 5 min with oxygenated Krebs-Henseleit buffer as in section 2. Trifluoperazine (3.5×10^{-6} M) was then infused where indicated and 15 min later, phenylephrine (2×10^{-6} M) was infused for a further 7 min. Glucose release (A) was estimated by collecting 600 μl samples of the effluent medium, removing any contaminating erythrocytes by centrifugation, and assaying portions (50–200 μl) of the supernatant. The amount of glucose release prior to phenylephrine infusion was 0.4–0.8 m Oxygen consumption (B) was estimated from the difference between influent and effluent oxygen concentrations. The amount of oxygen consumed prior to infusion of phenylephrine was 300–500 μM. Results shown are the mean of 5 separate expt. (○) Trifluoperazine infused; (●) trifluoperazine absent.

by pre-perfusion with the calmodulin inhibitor (not shown).

Fig.2a shows the mean of 5 individual expt. in which Ca^{2+} influx was determined in mitochondria isolated from the lobes of livers perfused with or without phenylephrine. The initial rate of Ca^{2+} transport is stimulated ~80% over that of the control (cf. [7]). Fig.2b shows that perfusion of the liver with trifluoperazine itself does not alter the control rate of Ca^{2+} influx but completely prevents the stimulation induced by the hormone. Similar effects of the α-agonist and trifluoperazine were observed on mitochondrial Ca^{2+} influx when the initial [Ca^{2+}] was reduced to 5 μM. Perfusion with glucagon did not alter initial rates of Ca^{2+} influx, and pretreatment of livers with trifluoperazine was without effect (not shown).

The time for which mitochondria were able to retain a fixed amount of Ca^{2+} [15] was also increased following pre-perfusion for 7 min with 2×10^{-6} M phenylephrine (3.5 ± 0.3 vs 6.9 ± 0.9 min, $n = 4$). Trifluoperazine 3.5×10^{-6} M) exerted effects similar to those described above for Ca^{2+} influx. The calmodulin inhibitor had no effect on Ca^{2+} retention alone but inhibited the effect of phenylephrine by $39 \pm 14\%$ ($n = 4$).

In some experiments the components of the protonmotive force, the mitochondrial transmembrane pH gradient and membrane potential, were also dete mined since these parameters are indicators of both the functional integrity of the organelle and under some conditions, of the capacity of the mitochondri to sequester and retain Ca^{2+} [7,18]. The transmembrane pH gradient and membrane potential of mitochondria prepared from livers perfused for 7 min wi phenylephrine (2×10^{-6} M) (see fig.2), is increased b $27 \pm 6\%$ and $3 \pm 0.5\%$ ($n = 3$), respectively, when cor pared to their appropriate controls (control transmer brane pH gradient, 75.3 ± 1.7 mV ($n = 3$); membran potential, 139.6 ± 2.8 mV ($n = 3$).

By contrast, pre-perfusion of livers with glucagon or trifluoperazine alone had no effect on the mitochondrial transmembrane pH gradient or the membrane potential in subsequently prepared mitochon-

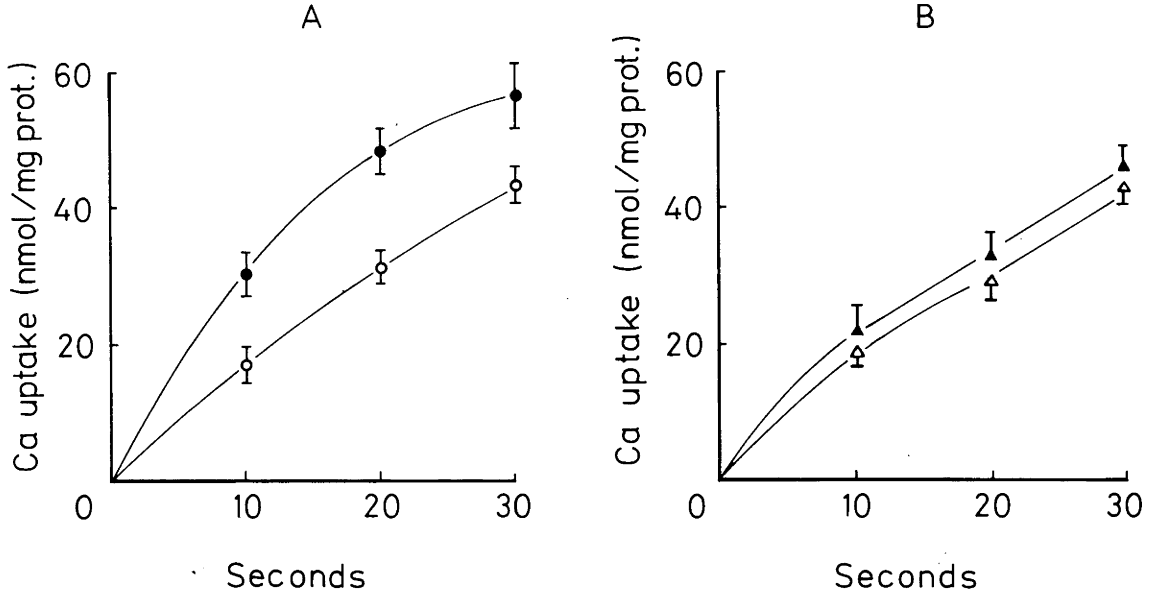

Fig.2. Effect of trifluoperazine on phenylephrine-induced stimulation of the initial rate of mitochondrial Ca^{2+} influx. Livers of fed rats were perfused as indicated in fig.1 and section 2. Prior to infusing phenylephrine, the median lobe was removed, homogenised, and the mitochondria isolated. The liver was allowed to recover for 3 min, and then phenylephrine was infused into the remaining lobes for 7 min. At this time the papilliform and left main lobes were removed, homogenised and the mitochondria isolated. The initial rate of mitochondrial Ca^{2+} transport was then measured [7] using 50 μM $CaCl_2$ containing 0.3 μCi $^{45}Ca^{2+}$. In (A) trifluoperazine was absent. In (B) trifluoperazine (3.3×10^{-6} M) was infused for 15 min prior to the infusion of phenylephrine. Results shown are the mean of 5 separate expt. (○,△) refer to mitochondria isolated from median lobes. (●,▲) mitochondria isolated from papilliform and left main lobes.

dria. Pre-perfusion of livers with trifluoperazine prior to administration of phenylephrine, however, reduced the stimulatory effect of the α-agonist on the transmembrane pH gradient by 52 ± 8% (n = 3).

4. Discussion

Phenothiazine antipsychotic agents such as trifluoperazine have been used in a number of studies to assess the potential involvement of calmodulin in intracellular Ca^{2+}-dependent reactions [8,11,19,20]. Experiments in this report establish that pre-perfusion of rat liver with trifluoperazine, antagonises the stimulation by phenylephrine of glucose output and oxygen consumption. The studies in [21] indicate that the bulk of this oxygen consumption is attributable to mitochondria, and other workers have observed that several mitochondrial energy-linked reactions are sensitive to prior hormone treatment of the intact tissue [22–25]. Here we show that trifluoperazine also antagonises the stimulation of the protonmotive force induced by phenylephrine. The data also confirm our findings that α-adrenergic agonists stimulate mitochondrial Ca^{2+} influx [7], an effect we show here to be antagonised by the calmodulin inhibitor.

On the other hand, trifluoperazine failed to antagonise glucagon-induced metabolic changes in the perfused liver. This, as well as its inability to alter the protonmotive force in the subsequently isolated mitochondria, provides support for a specific action of the inhibitor. The data also support the view that α-adrenergic agonists act through a mechanism different from that of glucagon [26,27].

Finally, if one assumes that trifluoperazine specifically inhibits the action of calmodulin, then some of the physiological responses of α-adrenergic agonists must involve calmodulin. Since one of these early responses is a redistribution of cytoplasmic Ca^{2+} [1–7,26,27], it is of some interest that trifluoperazine also antagonises phenylephrine-induced stimulation of mitochondrial Ca^{2+} transport. Thus calmodulin appears to be involved in the mechanism of action

of the α-adrenergic agonists, and may be a regulator of mitochondrial Ca^{2+} fluxes in liver. The interrelationships of these calmodulin-mediated events is currently under further investigation.

Acknowledgements

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Section B.2

The action of anti-psychotic agents in inhibiting α -adrenergic agonist-induced hepatic metabolic responses.

(Reinhart et al., 1981)

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THE ACTION OF ANTI-PSYCHOTIC AGENTS IN INHIBITING α-ADRENERGIC AGONIST INDUCED HEPATIC METABOLIC RESPONSES

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SUMMARY

Phenylephrine-induced increases in hepatic glucose output, oxygen uptake and mitochondrial adenine nucleotide content, were half-maximally inhibited when approx. 10^{-6}M trifluoperazine (TFP) was preperfused into rat liver. Because the calmodulin inhibitors TFP and the butyrophenone penfluoridol also inhibited specific epinephrine binding to isolated hepatic plasma membranes, the potential involvement of calmodulin in intracellular α-adrenergic responses is still an open question.

INTRODUCTION

Antipsychotic agents such as the phenothiazines trifluoperazine, fluphenazine, and chlorpromazine have been shown to selectively bind to calmodulin in a Ca^{2+}- dependent fashion (1), and hence inhibit the Ca^{2+}-calmodulin-mediated stimulation of cyclic nucleotide phosphodiesterase (2), phosphorylase b kinase (3), rabbit liver cAMP-independent glycogen synthase kinase(4) and protein phosphorylation in insuloma cytosol (5). Previously we have shown that trifluoperazine (TFP) also antagonises a number of physiological responses to the α-adrenergic agonist phenylephrine (6). More recently we have presented data indicating that, closely linked with the initial events in

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the mechanism of action of α-adrenergic agonists, is an increase in oxygen consumption by the liver, and an elevation in the concentration of mitochondrial adenine nucleotides (7). The effects of TFP on initial events associated with α-adrenergic agonist action are the subject of the present study.

MATERIAL AND METHODS

Livers from female Wistar strain albino rats, weighing between 200 to 300 g were perfused with Krebs-Henseleit bicarbonate medium equilibrated with 95% O_2- 5% CO_2 as described in (8), with minor modifications as detailed in (6). Glucose released by the liver was determined by the glucose oxidase method (Sigma assay kit 510-A). Oxygen uptake by the perfused liver was monitored using a Rank oxygen electrode. "Heavy" mitochondria (9) were isolated from separate liver lobes as described previously (10). Mitochondrial adenine nucleotide concentrations were determined essentially by the method of Sutton and Pollak (11), using an LKB 1250 Luminometer.

Plasma membranes used for receptor-binding studies were isolated from the livers of female Wistar rats by the method of Song et al., (12), as modified (13). Binding assays were carried out as described elsewhere (13), with minor modifications. Briefly, plasma membranes, at a final protein concentration of 2mg/ml, were incubated in a medium consisting of 50 mM-Tris buffer (pH 7.5), 0.8 mM-ascorbate, 3 mM-catechol, 5 mM-$MgCl_2$, 10 μM-propanolol, and 40 μM [^{3}H]-epinephrine in a final volume of 300 μl. Incubations were carried out for 30 min at 25°C. It has previously been established that under these assay conditions the degradation of [^{3}H]-epinephrine is negligible (13). At 30 min the reaction was stopped by filtering through Gelman GF/AE glass fibre filters, and rapidly washed with 10 ml of incubation buffer at 4°C. Filters were dried, and radioactivity counted in a Beckman LS-330 liquid scintillation counter to within 1% error. Specific binding was defined as the difference between binding observed in the presence or absence of 10 μM-phenoxybenzamine, an α-antagonist. Competitive binding assays were carried out by preincubating the membrane fraction with TFP for 4 min prior to the addition of [^{3}H]-epinephrine. Non-specific ligand displacement by TFP was corrected for in assays in which both TFP and phenoxybenzamine were present together. Further experiments detailing the validity of these binding assays have been described (13). Protein assays were carried out by a modification (14) of the method of Lowry et al., (15). (±)- [7-^{3}H]-epinephrine-(+) bitartrate (13-15 Ci/mmol) was obtained from New England Nuclear, and was diluted with 50 mM-Tris buffer (pH 7.5) plus 0.8 mM-ascorbate, just prior to each experiment. Trifluoperazine and penfluoridol were a generous gift from Smith Kline and French and Janssen Pharmaceutica respectively. Phenoxybenzamine and propanolol were kindly supplied by Dr. J. H. Exton.

RESULTS AND DISCUSSION

Figs. (1a) and (1b) show that TFP over a range of concentrations inhibits the phenylephrine-induced stimulation of 0_2 uptake, and glucose output when administered 10 min prior to phenylephrine infusion to perfused rat liver. For both hormone responses, inhibition is significant at 3.3×10^{-7} M TFP, half-maximal at $\simeq 10^{-6}$ M TFP, and near-maximal at $\simeq 10^{-5}$ M TFP. These concentrations of TFP are similar to those previously used to examine calmodulin-sensitive reactions (4).

Data in Fig. 2 indicate that the phenylephrine-induced stimulation of the mitochondrial adenine nucleotide pool (7), is also inhibited by the preperfusion of 10^{-5} M TFP. Hence both of the α-adrenergic agonist-induced responses previously shown to be closely linked with the initial events of these hormones are inhibited by TFP, indicating that this phenothiazine is acting at some early step in the action of α-agonists. Hence we examined the effect of TFP on α-agonist receptor binding.

Results from competitive binding assays (Fig. 3), indicate that, TFP inhibits the specific receptor-binding of [^{3}H]-epinephrine. The inhibitory concentrations are similar to those used to inhibit the phenylephrine-induced stimulation of oxygen uptake, glucose output, and mitochondrial adenine nucleotide content (see above).

The butyrophenone penfluoridol was recently shown to interact with calmodulin and to inhibit the calmodulin stimulated Ca^{2+}-ATPase activity of red blood cells (16). It was of additional interest therefore to find that penfluoridol also inhibits the specific receptor-binding of [^{3}H]-epinephrine

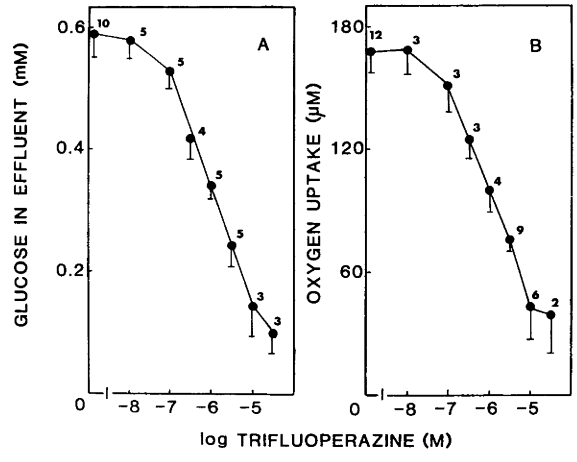

Fig. 1. Effect of trifluoperazine on phenylephrine-stimulated increase in glucose output and oxygen consumption by perfused rat liver.

Livers of fed rats were perfused initially for 5 min with oxygenated Krebs-Henseleit medium at 32°C in a non-recirculating mode. Some livers were then infused with TFP, at the concentrations indicated, using a Braun Perfusor Spritze. 15 min later phenylephrine (2×10^{-6} M final concentration) was infused for a further 3 min. We have previously shown (6) that at 3 min after phenylephrine infusion, the rates of both glucose output (Fig. a) and oxygen uptake (Fig. b) are maximally stimulated. Glucose output and oxygen uptake were assayed as previously described (6). Basal glucose output was 0.21 mM $\pm$ 0.14 (n=7), and basal O_2 uptake was 365 µM $\pm$ 62.1 (n=12). Results shown are the means $\pm$ SEM for the number of separate experiments shown alongside each point.

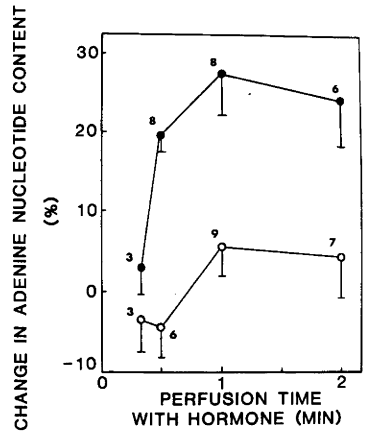

Fig. 2. Effect of trifluoperazine on phenylephrine-induced stimulation of mitochondrial adenine nucleotide content.

Perfusion details were as described in the legend to Fig. 1. with the modifications shown below. Prior to infusing with phenylephrine, the median lobe was ligated, removed, homogenized, and mitochondria isolated as described in (9). The liver was allowed to recover for 3 min, and then phenylephrine (2×10^{-6} M final concentration) was infused into the remaining lobes for the times indicated. Mitochondria were then prepared from the left main lobe and the papilliform lobes, and the adenine nucleotide content assayed as described in Methods and Materials. The basal adenine nucleotide content was 12.61 $\pm$ 0.25 nmol per mg protein (n=25). Assays were carried out in duplicate and the results shown are the means $\pm$ SEM for the number of separate experiments shown alongside each point.

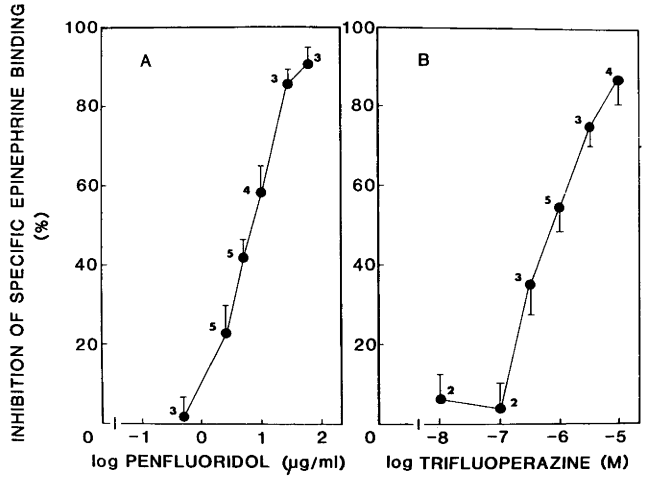

Fig. 3. The inhibition by trifluoperazine and penfluoridol of specific [^{3}H]-epinephrine receptor binding.

Rat liver plasma membranes were isolated and assayed for [^{3}H]-epinephrine receptor binding as described in Methods and Materials. Specific binding (52.61 $\pm$ 6.5 nmol per mg protein; n=15) is defined as that portion of [^{3}H]-epinephrine removed by 10 µM phenoxybenzamine. Non-specific displacement of [^{3}H]-epinephrine by TFP or penfluoridol was corrected for in binding assays containing both TFP or penfluoridol and phenoxybenzamine (10 µM). Assays were carried out in triplicate for the number of separate experiments shown in parentheses. The concentrations of TFP (Fig. a), and penfluoridol (Fig. b) were varied as indicated. Assays were carried out in triplicate for the number of separate experiments shown alongside each point.

(Fig. 3). The concentrations giving 50% inhibition of binding were $\simeq 10^{-6}$ μM TFP and 10 μg/ml penfluoridol, respectively.

CONCLUDING COMMENTS

The data presented here show that derivatives of both phenothiazines and butyrophenones that are structurally quite dissimilar, inhibit specific receptor-binding of [^{3}H]-epinephrine to isolated hepatic plasma membranes. The concentrations of TFP inhibiting this binding were similar to those required to inhibit early phenylephrine-induced metabolic responses in perfused livers.

Several inferences may be drawn from these observations. We have previously concluded, that, assuming TFP to specifically inhibit the action of calmodulin, some of the physiological responses of α-adrenergic agonists must involve calmodulin (6). The present data showing inhibition of specific receptor-binding of [^{3}H]-epinephrine by TFP raises the question whether such inhibition is attributable to interaction with calmodulin or simply, involves some non-specific effect of the phenothiazine. Such effects are known to include the electrical stabilisation and protection of membranes, membrane expansion and the displacement of membrane-bound Ca^{++}(17). Indeed Blackmore et al., (18) who also have recently demonstrated the inhibitory effect of TFP on binding of α-agonists to the α-adrenergic receptor, have argued that such an interaction of TFP with the receptor renders invalid the use of phenothiazines in the exploration of the role of calmodulin in α-adrenergic cellular responses.

On the other hand it is of some interest to note that

such structurally diverse antipsychotic agents as TFP and penfluoridol which are known to inhibit calmodulin-mediated reactions (3-5), also inhibit specific receptor-binding of [^{3}H]-epinephrine. Hence the possibility, that calmodulin and Ca^{2+} themselves may be closely associated with the active site of the α-adrenergic receptor cannot be ruled out. Furthermore the involvement of calmodulin in intracellular responses to α-adrenergic agonists is still an open question.

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Section B.3

Hormonal control of calcium fluxes in rat liver.

(Bygrave et al., 1982)

HORMONAL CONTROL OF CALCIUM FLUXES IN RAT LIVER

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AUSTRALIA

1. Introduction

The physiological responses of a number of hormones in a range of tissues are closely associated with the redistribution of intracellular Ca^{2+}. This redistribution appears to involve changes in the rate of Ca^{2+} transport in subcellular organelles known to contain appreciable "pools" of Ca^{2+} (Claret-Berthon et al., 1977) and may result in an alteration in either the cytoplasmic or the intraorganellar Ca^{2+} concentration. As most mammalian cell types contain many Ca^{2+}-dependent reactions (Carafoli and Crompton, 1978), the hormonal regulation of Ca^{2+} transport activity in subcellular organelles may have an important role in mediating the responses to the hormones. Following a brief account of current information about mitochondrial and microsomal Ca^{2+} transport activities, this review proceeds to consider our understanding of how adrenergic agonists and glucagon may regulate these activities and examines the possible role this regulation may play in mediating cellular hormone responses. The discussion is purposely confined to a consideration of liver tissue only.

Both mitochondria and microsomes have been shown to actively sequester Ca^{2+} in vitro, and when incubated together are able to maintain the ambient Ca^{2+} concentration close to 0.2 μM (Becker et al., 1980). As this concentration of Ca^{2+} was also maintained by suspensions of digitonin-treated hepatocytes (Becker et al., 1980: Murphy et al., 1980), this value may approximate the actual cytoplasmic free Ca^{2+} concentration in situ, and strongly implicates mitochondria and the endoplasmic reticulum in the maintenance of cytoplasmic Ca^{2+}.

One feature that most readily distinguishes mitochondrial from microsomal Ca^{2+} transport activities is the difference in sensitivity to ruthenium red; the former activity is almost totally abolished by low concentrations of the compound (K_i = 30 pmoles/mg protein) while the

latter is unaffected by concentrations several orders of magnitude greater. This fact has proved to be most useful in the experimental analysis of mitochondrial (i.e., ruthenium red-sensitive) and nonmitochondrial (i.e., ruthenium red-insensitive) Ca^{2+} transport in liver. In digitonin-treated hepatocytes for example, greater than 90% of the Ca^{2+} uptake activity is ruthenium red-sensitive (Babcock et al., 1979; Murphy et al., 1980) as is the case in a rat liver homogenate (Ash and Bygrave, 1977). This supports the idea that mitochondria play a major role in Ca^{2+} homeostasis. Further support derives from the observation that the initial rates of Ca^{2+} transport are significantly greater in mitochondria than microsomes.

The driving force for electrophoretic Ca^{2+} uptake by mitochondria occurs in response to the membrane potential negative inside, generated either by the hydrolysis of ATP or by electron transport activity (Nicholls and Crompton, 1980). In contrast, microsomal Ca^{2+} transport is coupled to the hydrolysis of MgATP only (Moore et al., 1975; Bygrave, 1978a).

Several features, on the other hand, appear to be common to both transport systems. These include the existence of specific carriers in the respective membranes, the cyclic nature of Ca^{2+} transport, the high affinity for free Ca^{2+}, and stimulation by permeant weak acids (Bygrave, 1978b; Carafoli and Crompton, 1978). Of special interest in the present context is the observation that both transport systems respond to physiological stimuli such as tissue development and changes in the circulating hormone concentration (Bygrave, 1978b; Reinhart and Bygrave, 1981).

2. Action of α-Adrenergic Agonists and Glucagon on Mitochondrial Ca^{2+} Transport

A number of laboratories have demonstrated that stable changes in Ca^{2+} transport occur in mitochondria following their isolation from liver administered *in situ* with glucagon or α-adrenergic agonists. Besides adding strength to a role of mitochondrial Ca^{2+} in the α-agonist and glucagon-induced responses, this phenomenon also provides a useful means of examining details of the mechanism involved in Ca^{2+} mobilization and in the action of the hormones.

Early experiments involved the intraperitoneal or intravenous injection of the hormone into an intact animal, followed by isolation of the liver mitochondria and assay of Ca^{2+} transport. More recent studies have adopted the perfused liver system or hepatocytes. Experiments with mitochondria isolated from liver 30 to 60 min after the intraperitoneal injection of glucagon (Hughes and Barritt, 1978; Prpic *et al.*, 1978) and those with mitochondria isolated from liver 7 to 10 min after perfusion with hormone (Taylor *et al.*, 1980a) have yielded similar information with respect to stable changes in Ca^{2+} transport. The most pronounced changes so far detected are an enhanced ability of the mitochondria to retain high concentrations of Ca^{2+} and an increase in the initial rate of Ca^{2+} influx, although these latter effects are not as reproducible as the former. This hormone-sensitive Ca^{2+} transport appears to be enriched in mitochondria sedimented at relatively low centrifugation forces (Prpic *et al.*, 1978).

Other stable changes seen, that are of potential relevance to the mechanism of mitochondrial Ca^{2+} fluxes, include an increase in the transmembrane pH gradient, an increase in the adenine nucleotide concentration (Taylor *et al.*, 1980a; Halestrap, 1978), and a reduced ability for oxaloacetate-induced NADPH oxidation (Prpic and Bygrave,

1980). These changes are seen also in mitochondria isolated from liver perfused with α-agonists for 7 to 10 min and suggest that the actions of the two hormones on several energy-linked mitochondrial reactions including Ca^{2+} fluxes, have many features in common.

Because the longer-term effects of the two hormones may not directly bear on the early action of the hormones, it has been important to assess the earliest times at which such effects are detectable. Recently we have investigated the action of cAMP and phenylephrine at very early times after their administration to the perfused liver (i.e., in the time span of 10 to 20 sec). These short-term perfusion studies, in contrast to the relatively longer-term ones discussed above, reveal important differences in the early action of the two hormones on energy-linked reactions in liver mitochondria (Taylor et al., 1981). Phenylephrine is much more rapid in its action than glucagon or cAMP. By 30 sec after phenylephrine administration, levels of mitochondrial adenine nucleotides are elevated approximately 35%, while cAMP has little effect on this parameter even after 4 min. Whereas Ca^{2+} retention is increased by 50% 20 sec after administration of phenylephrine, no significant change is seen until 120 sec following perfusion with cAMP. Initial rates of Ca^{2+} transport are stimulated at about 120 sec after the administration of either hormone.

While these studies were in progress, Yamazaki et al., (1980) reported a rapid action of glucagon on hepatic mitochondrial metabolism. Within 1 min of the intravenous injection of glucagon into anesthetized rats, Ca^{2+} retention by isolated mitochondria was increased to near-maximal extents, an effect shown to be independent of the Ca^{2+} content of the mitochondria. The first-order rate constant for Ca^{2+} influx was also increased significantly.

Because of the strong possibility that changes in mitochondrial Ca^{2+} fluxes are closely associated with the cellular metabolic network (see below), it is of additional interest that a number of other rapid changes in mitochondrial energy-linked reactions occur following incubation of hepatocytes with glucagon or α-agonists. These include a stimulation of uncoupler-dependent ATPase (Titheradge et al., 1979), increases in ADP-stimulated respiration and succinate dehydrogenase activity, as well as a shift to the reduced in the oxidation-reduction state (Siess and Wieland, 1980). The effects of the α-agonists were more rapid than those of glucagon (Titheradge et al., 1979).

Thus, while the longer-term actions of the two hormones on mitochondrial energy-linked reactions, including Ca^{2+} fluxes, resemble each other in many respects, their early actions differ, particularly with regard to the rapidity with which they induce their responses.

3. Action of Hormones on Microsomal Ca^{2+} Transport

The experimental approach used to study the action of hormones on microsomal Ca^{2+} transport in liver, like that described in the previous section, has essentially involved administration of the hormone to the intact animal or perfused liver followed by isolation of the microsomal fraction and the measurement of Ca^{2+} transport. Isolated liver cells have been equally useful in this study because of the relative ease of preparing functionally intact microsomes from these cells following their incubation with the hormone. In contrast to studies examining mitochondrial energy-lined reactions, the majority of studies with microsomes have involved administration of either glucagon or cAMP and its analogs (see Reinhart and Bygrave, 1981).

The principal action of glucagon administration on the intact tissue or isolated cells is stimulation of the initial rate of Ca^{2+}

transport as measured in vitro. The degree of stimulation is greatest with the "heavy" populations of microsomes (Reinhart and Bygrave, 1981) and is dependent on the concentration of glucagon administered and the time for which the tissue or cells are incubated with the hormone. The earliest effects are seen 5 to 10 min after hormone administration to hepatocytes and can be prevented by coadministration of insulin (Taylor et al., 1980b; Andia-Waltenbaugh et al., 1980). Elevation of intracellular cAMP by inhibitors of phosphodiesterase also leads to a stimulation of Ca^{2+} transport (Taylor et al., 1980b; Reinhart and Bygrave, 1981). When the microsomal membrane is incubated in vitro for short periods with small amounts of the supernatant fraction in the presence of ATP and cAMP, the initial rate of Ca^{2+} transport is stimulated approximately 40%. Exclusion of cAMP alone from the medium prevents any such stimulation (Reinhart and Bygrave, 1981). The evidence would suggest that the mechanism of stimulation of the initial rate of Ca^{2+} transport by glucagon involves a cAMP-dependent protein kinase that in turn phosphorylates a component on the microsomal membrane on or near the Ca^{2+} transport protein as appears to take place in sarcoplasmic reticulum (Tada et al., 1979).

4. Action of Hormones on Plasma Membrane Ca^{2+} Transport

Considerable experimental difficulties are associated with the study of Ca^{2+} fluxes across the plasma membrane (Racker, 1980). These partly relate to the perturbation of such fluxes by intracellular organelles. Thus, little definitive information is available regarding the action of hormones on plasma membrane Ca^{2+} transport. However, in a recent kinetic analysis of the effects of epinephrine on Ca^{2+} distribution in hepatocytes, Barritt et al., (1981) concluded that a major effect of the hormone is to increase the rate constant for influx

of Ca^{2+} to a small intracellular compartment of exchangeable Ca^{2+}, a finding at variance with reports showing a rapid efflux of Ca^{2+} from perfused liver (Althaus-Saltzmann et al., 1980; Blackmore et al., 1979).

Reports that calmodulin activates plasma membrane Ca^{2+} pumps in some tissues (e.g. Lynch and Cheung, 1979; Pershadsingh et al., 1980) raise the question whether this protein might be involved also in liver plasma membrane Ca^{2+} transport.

5. Physiological Role of Hormone-Induced Changes in Intracellular Ca^{2+} Fluxes

Unlike glucagon, the α-adrenergic agonists are thought not to give rise to an increase in cAMP concentration. Instead the action of these hormones is considered to be mediated by a rise in intracellular Ca^{2+} (see Blackmore et al., 1979), which in turn may stimulate the conversion of phosphorylase a and hence increase glycogenolysis. Independent studies from several laboratories have suggested that mitochondria are the source of this Ca^{2+} (Babcock et al., 1979; Blackmore et al., 1979; Taylor et al., 1980a) although this view has been questioned (Poggioli et al., 1980; Althaus-Saltzmann et al., 1980). The recent kinetic analysis of Barritt et al., (1981) suggests all three membranes (mitochondrial, microsomal, and plasma membrane) are involved in the epinephrine-induced Ca^{2+} redistribution in liver.

A role for microsomal Ca^{2+} fluxes in the regulation of cytoplasmic Ca^{2+} is suggested from considering (1) the ability of the organelle to maintain a low steady-state concentration of Ca^{2+} (Becker et al., 1980), (2) the lowering of the Ca^{2+} content in the microsomal fraction following glucagon or phenylephrine treatment (Blackmore et al., 1979), and (3) the close correlation between glucagon-stimulated microsomal Ca^{2+} flux activity and glycogenolysis during liver development (Reinhart and

Bygrave, 1981). These data point strongly to a role of microsomal Ca^{2+} fluxes in the regulation of glycogen metabolism in liver.

Rapid cycling of Ca^{2+} across the inner mitochondrial membrane (Nicholls and Crompton, 1980) provides a means of controlling not only cytoplasmic Ca^{2+} but also intramitochondrial Ca^{2+} (Denton and McCormack, 1980). In this way changes in mitochondrial Ca^{2+} fluxes are suggested to control the activities of several Ca^{2+}-sensitive matrix dehydrogenases (Denton and McCormack, 1980). For example, Hems et al., (1978) consider that vasopressin may bring about an increase in pyruvate dehydrogenase through an increase in intramitochondrial Ca^{2+} (see also Sugden et al., 1980).

The possible involvement of Ca^{2+}-regulated events in the control of hydrogen transfer between mitochondria and cytoplasmic compartments during the stimulation of gluconeogenesis by α-agonists and glucagon has been emphasized recently (Kneer et al., 1979). Moreover, changes in Ca^{2+} fluxes may play a role in the hormal control of gluconeogenesis through increases in the rate of mitochondrial ATP formation. The increases in mitochondrial Ca^{2+} fluxes (Yamazaki et al., 1980; Taylor et al., 1981), respiratory chain activity (Yamazaki et al., 1980), and mitochondrial ATP content (Taylor et al., 1981) brought about by the action of α-agonists all appear closely related events. Of additional interest are reports that shifts of the ATPase-inhibiting peptide to and from the ATPase (Tuena de Gomez-Puyou et al., 1980; Carafoli et al., 1980) may in turn regulate the rate at which mitochondria take up Ca^{2+} .

Some important questions that remain to be elucidated relate to the nature of any purported secondary messenger to the α-receptor and its interaction with any of the intracellular organelle membranes. Also, although the action of glucagon appears to involve the activation of

cAMP-dependent protein kinase, the link between this activation and the observed alteration to cellular Ca^{2+} fluxes is not known.

One possible common focal point for both classes of hormones is their ability to phosphorylate the same 12 cytoplasmic proteins (Garrison, 1978). Presumably these must be capable of transferring information to the target organelles. Thus, there may exist a cAMP-independent protein kinase linked in some way to the generation of an α-agonist second messanger. It is of some interest that recently a cAMP-independent protein kinase linked to the turnover of phosphatidylinositol has been described (Kishimoto et al., 1980). As the turnover of this phospholipid in tissues has been associated with α_1-receptors (Fain and Garcia-Sainz, 1980), the possibility is raised that this protein kinase is associated with such a second messenger and hence the physiological action in response to this hormone.

The role of Ca^{2+} redistribution in the molecular events linking the action of α-agonists and glucagon in liver tissue with their physiological responses poses an important challenge for the future.

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Section B.4

Studies on α-adrenergic agonist-induced respiration and glycogenolysis in perfused rat liver.

(Reinhart et al., 1982a)

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Studies on α-Adrenergic-induced Respiration and Glycogenolysis in Perfused Rat Liver*

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Phenylephrine (1.5×10^{-6} M) administered to perfused livers from fed rats gave rise to a rapid, parallel increase in oxygen uptake and glucose output. The time of onset for oxygen uptake was 9.9 ± 0.4 s following phenylephrine administration, and immediately preceded glucose output which occurred at 11.6 ± 0.5 s. Near-maximal effects were observed 50 s following α-agonist treatment. Both responses appear to be mediated by α_1-adrenergic receptors.

The mitochondrial respiratory chain blockers antimycin A and rotenone, inhibited the α-agonist-induced oxygen uptake and glycogenolytic responses at inhibitor concentrations similar to those required to block uncoupler-stimulated respiration in the intact perfused liver. Oligomycin and carboxyatractyloside also inhibited the phenylephrine-induced respiratory response.

Vasopressin (1 milliunit/ml), and angiotensin II (6×10^{-9} M) had effects similar to phenylephrine in the perfused liver which also were prevented by the prior administration of antimycin A and rotenone.

In contrast, glucagon-induced (10^{-8} M) glycogenolysis proceeded in the absence of large changes in respiration, was slower in onset (26.1 ± 4.2 s following hormone administration), and was not inhibited by mitochondrial respiratory blockers.

These data indicate that glycogenolysis induced by α-adrenergic agonists, vasopressin, and angiotensin II is associated with a large increase in mitochondrial respiration, that may play a role in a general, as yet undefined mechanism whereby these agents stimulate glycogenolysis in rat liver.

Considerable evidence now suggests that most, if not all, of the physiological effects of glucagon, including glycogenolysis in rat liver, are mediated through elevations in the concentration of cAMP (reviewed in Refs. 1 and 2). In contrast, the glycogenolytic effects of epinephrine, vasopressin, and angiotensin II, appear to be mediated not through modulation of the cAMP concentration but rather through the direct activation of phosphorylase kinase activity, by an increase in the concentration of cytosolic free Ca^{2+} (3–8). No clear consensus has yet been reached as to the source of this hormone-sensitive pool of Ca^{2+}, but evidence indicates that intracellular organelles, such as mitochondria and the endoplasmic reticulum may be involved (9–13). This view is supported by findings that both mitochondria and the endoplasmic reticulum appear to play a role in the regulation of free cytosolic Ca^{2+} concentrations (14–17), and that both α-adrenergic agonists and glucagon, induce numerous stable functional alterations in these organelles isolated following hormonal challenge to either the intact animal (18–23), perfused liver (11, 13, 24), or isolated liver cells (25). Some of these stable changes include the rate of ADP-stimulated respiration, the total adenine nucleotide content, the initial rate of Ca^{2+} uptake, the time of Ca^{2+} retention, the total Ca content, the uncoupler-stimulated ATPase, and energy-linked transhydrogenase activity (11, 13, 19, 26, 27). However, the full significance of these stable changes, and their relation to the metabolic effects of these hormones, remains uncertain, partly due to the difficulty of relating results obtained with subcellular fractions, to the intact cell or organ.

Previously, we (28, 29) and others (30–32), have reported that the glycogenolytic action of the α-adrenergic agonist, phenylephrine, is associated with an increase in oxygen uptake by the perfused rat liver. This hormone response is of particular interest since it appears to occur independently of changes in the concentration of cAMP, and can be measured directly in the intact perfused organ.

In the present study we show that the phenylephrine-mediated increase in oxygen uptake is a very rapid response, and precedes glucose release by the liver. Our data indicate that oxygen uptake induced by the agonist is largely due to enhanced mitochondrial respiration, and that, inhibition of this respiratory response results in the virtual abolition of the agonist-induced glycogenolytic response. Similar results were obtained with vasopressin and angiotensin II. It is proposed that mitochondria represent a functional component in a general, as yet undefined mechanism, whereby cAMP-independent hormones such as α-adrenergic agonists, vasopressin, and angiotensin II, activate glycogenolysis in rat liver.

EXPERIMENTAL PROCEDURES

Animals and Perfusions—Female Wistar strain albino rats, weighing between 200 and 250 g, and having free access to food, were used for all experiments. Rats were anaesthetized with sodium pentobarbitone (50 mg/kg), and the livers perfused with Krebs-Henseleit bicarbonate medium (33) equilibrated with 95% O_2–5% CO_2 and containing 1.30 mM added Ca^{2+}, essentially as described (13). A nonrecirculating mode of perfusion was used at a flow rate of 3.5 ml/g liver (30) and all animals were routinely perfused for 15 min prior to phenylephrine treatment. Perfusions were usually carried out between 0800 and 1200 h to minimize diurnal fluctuations in the amount of basal glucose output.

Oxygen Uptake Determinations—The oxygen consumption by the liver was calculated from the difference between influent and effluent oxygen concentrations measured with a Clark type oxygen electrode, modified for a flow-through mode of operation. The dead volume of the electrode was kept at less than 400 μl, and the electrode was calibrated prior to every experiment. The linearity of the response was established by the colorimetric oxygen assay of Hamlin and

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Lambert (34) using the photo-reduced form of methylene blue as the phenothiazine dye oxidized by oxygen.

After each experiment, the lag time from the point of hormone infusion to the oxygen electrode was determined using sodium dithionite. Lag times varied as a function of the flow rate and liver weight, but were usually from 6 to 9 s. Within any one experiment lag times could be reproducibly measured to within 0.1-s error. The electrode response was routinely examined and was always at least 40 times faster than the most rapid hormone-induced response.

Glucose Output Determinations—Glucose determinations were carried out on effluent samples using the glucose oxidase, peroxidase method. Briefly, effluent samples (≃500 μl) were collected at 1-s intervals and centrifuged immediately in an Eppendorf microfuge for 2 min to remove any erythrocytes. 200-μl samples were assayed for glucose content. Deproteinization was found to be unnecessary due to the low protein content of the samples. Lag times of glucose release were determined after every experiment by infusing ^{3}H-inulin at the site of hormone infusion. These times varied as a function of the flow rate and liver weight and were usually from 11 to 14 s. Within any one experiment the lag time could be reproducibly measured to within 0.5-s error. The reproducibility of the assay was such that changes of 5 to 10 μM could be detected.

Inhibitors and Uncouplers—Antimycin A, oligomycin, and atractyloside were solubilized in ethanol. Rotenone was solubilized in a mixture of ethanol acetone (19:1, v/v). The final concentration of ethanol in the perfusate never exceeded 0.05%.

Care was taken to ensure that neither glucose release nor oxygen uptake induced by hormones was affected by the ethanol solvents used, even though previous workers have used higher concentrations of these solvents with hepatocytes and synaptosomes (35, 36). All agents were infused into the inflow line near to the liver, at flow rates of less than 1% of the total perfusate flow.

Chemicals—Phenylephrine, glucagon, [8-arginine] vasopressin and [5-valine] angiotensin II, carboxyatractyloside, and the glucose assay kit (510-A) were obtained from Sigma. Dinitrophenol was obtained from E. Merck A. G., Darmstadt. Prazosin and yohimbine were a gift from Dr. N. H. Hunt, John Curtin School of Medical Research. Other chemicals used were of analytical grade.

Expression of Data—All experiments were performed at least three times, and data are expressed as means ± S.E. for the number of experiments described.

RESULTS

Time Course of Phenylephrine- or Glucagon-induced Increases in the Rate of Glucose Output and Oxygen Uptake—A number of previous reports have indicated that the administration of phenylephrine to perfused livers from fed animals, results in a stimulation of glucose release (11, 28, 30, 31, 37) and oxygen uptake (28–31) 3 to 5 min following infusion of the α-adrenergic agonists. However, a recent report (32) indicates that the onset of these metabolic responses may occur more rapidly. In initial experiments this point was examined in detail.

Increases in oxygen consumption and glucose output during the first 60 s of phenylephrine administration to the perfused rat liver are shown in Fig. 1. The lag times, from the point of infusion to either the oxygen electrode, or to the point at which effluent was collected for glucose determinations, were calculated separately for each experiment, and all data shown have been corrected for these lag times (see "Experimental Procedures"). The results shown indicate a rapid, parallel increase occurs for both glucose output and oxygen uptake by phenylephrine-treated livers. The corrected time of onset for phenylephrine-stimulated oxygen uptake is 9.9 ± 0.4 s (n = 11) following agonist infusion, while the time of onset for glucose output is 11.6 ± 0.5 s (n = 9). While these values are similar to those reported by Scholz and Schwabe (32), the present data indicate that both collectively and in individual experiments the oxygen response always precedes glucose output. It is important to point out that because extracellular measurements are being made of intracellular events, the observed differences in times of onset of glucose output and oxygen uptake may only approximate the actual intracellular difference. Of special interest is the finding that the rate of both the oxygen and glucose responses are already maximal from the time of onset. Both responses were almost fully stimulated after 50 s of phenylephrine infusion. This time sequence of phenylephrine-induced glucose release is more rapid than has been previously reported, possibly because other workers have not corrected for lag times inherent in the perfusion apparatus, and apparently have not sampled the effluent until 30 or 60 s following agonist administration (11, 28, 30, 31, 37).

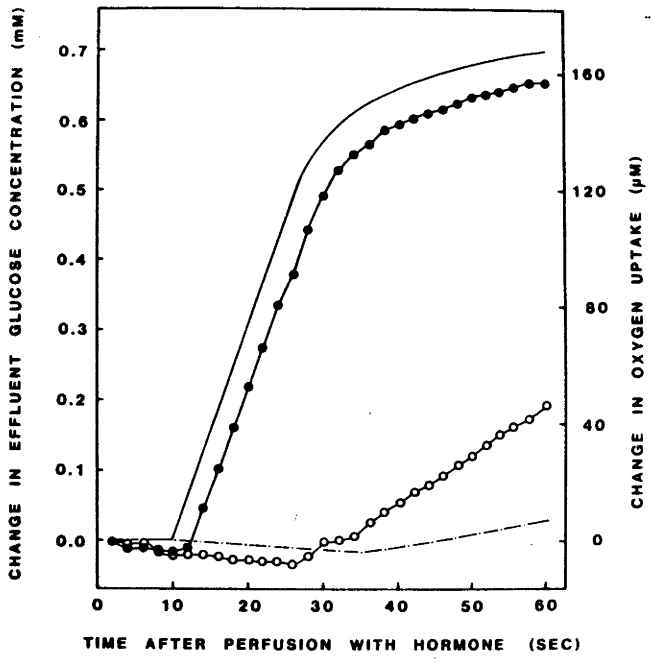

FIG. 1. **Time course of phenylephrine-or glucagon-induced increases in the rate of glucose output and oxygen uptake.** Livers of fed rats were perfused initially for 15 min with Krebs-Henseleit buffer equilibrated with carbogen (95% O_2, 5% CO_2) as described under "Experimental Procedures." Some livers were then infused with either phenylephrine (1.5×10^{-6} M) or glucagon (10^{-8} M). Oxygen uptake (*solid line*) was estimated from the difference between influent and effluent oxygen concentrations. Effluent samples were collected at 1-s intervals, contaminating erythrocytes removed by centrifugation, and samples of the supernatant assayed for glucose. Lag times for both glucose and oxygen measurements were determined separately for each experiment. For simplicity, data points on the graph are shown at 2-s intervals. Shown in Fig. 1 are the corrected response times (*i.e.* lag times subtracted). Basal glucose release at 15 min of perfusion was 0.31 mM ± 0.08 (n = 9), and basal oxygen uptake was 394 μM ± 42 (n = 11). Glucose output data shown are the means of 9 experiments for phenylephrine (●——●) and 6 experiments for glucagon (○——○). Error bars have been omitted for clarity. However, the S.E. was never greater than 0.04 mM glucose or 4 μM oxygen. The oxygen traces shown are from one typical experiment of 11 performed for phenylephrine (*solid line*) and 6 performed for glucagon (*dashed line*).

In contrast to these rapid phenylephrine-mediated effects, the infusion of glucagon results in a much slower stimulation of glycogenolysis, and has little effect on oxygen uptake by the perfused liver. The onset of glucose release occurs at 26.1 ± 4.2 s (n = 4), and 120 ± 7.1 s (n = 4) are required to achieve half-maximal stimulation. However, the response is of greater magnitude and more prolonged than the biphasic phenylephrine-induced glucose output (data not shown).

Effect of α_1- and α_2-Adrenergic Antagonists on Phenylephrine-stimulated Respiration and Glucose Output—α-Adrenergic receptors have been grouped into two categories (α_1 and α_2) based on the order of potency of a number of agonists and antagonists (38). This pharmacological classification appears to be of functional significance with activation of α_1-

receptors having been proposed to be linked to changes in cytosolic Ca^{2+} concentrations, phosphatidylonositol turnover, and the activation of phosphorylase (38-41), whilst stimulation of α_2-receptors in some cases has been linked with inhibition of adenyl cyclase (40, 42). Prazosin has been shown to be a specific antagonist for α_1-receptors, while yohimbine is a more potent inhibitor of α_2-effects (38).

Data shown in Fig. 2 indicate that prazosin is approximately 2 orders of magnitude more potent than yohimbine in inhibiting both phenylephrine-stimulated oxygen uptake and glucose output. Prazosin half-maximally inhibits phenylephrine-induced glucose release or oxygen uptake at 1.3×10^{-8} M and 1.8×10^{-8} M, respectively, while half-maximal effects of yohimbine on either glucose release or oxygen uptake are observed at 1.1×10^{-6} M and 1.8×10^{-6} M, respectively. Hence both of the agonist-induced responses appear to be largely mediated through the activation of α_1-adrenergic receptors.

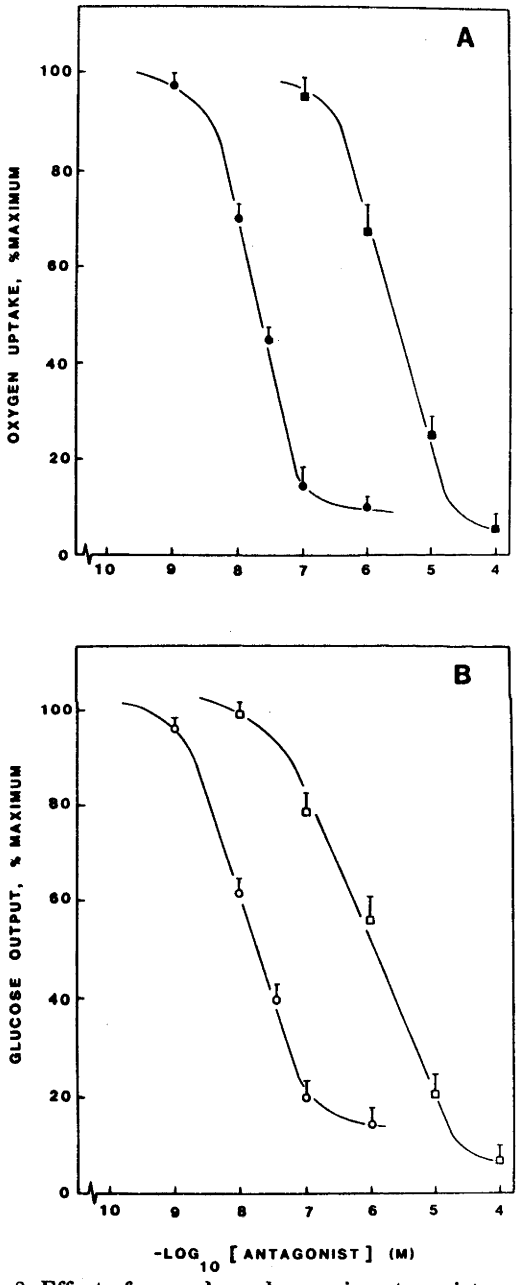

FIG. 2. **Effect of α_1- and α_2-adrenergic antagonists on phenylephrine induced stimulation of glucose release (*A*) and oxygen uptake (*B*).** Livers of fed rats were perfused as described in the legend to Fig. 1. Three min prior to phenylephrine (1.5 μM) infusion, either prazosin (●, ○) or yohimbine (■, □) was infused into the livers, at the concentrations indicated. The maximum oxygen uptake response induced by phenylephrine in the absence of α-adrenergic blockers was 162 μM ± 6.1 (n = 12) and the maximum amount of glucose released was 0.61 mM ± .10 (n = 8). Basal glucose output and oxygen uptake were as described in the legend to Fig. 1. Data shown are means ± S.E. for between 4 and 8 separate experiments.

The Effect of Mitochondrial Effectors on the Phenylephrine-induced Increase in Oxygen Uptake and Glucose Output—Data in Fig. 3 show that the mitochondrial respiratory chain blockers antimycin A at 2.5 μM and rotenone at 7.5 μM inhibit the α-adrenergic agonist-induced increase in oxygen uptake. Preliminary experiments had indicated that 3 min of preperfusion with the inhibitors was the shortest time at which reproducible results could be obtained, hence this preinfusion period was routinely used for all experiments. The ethanol solvent used to solubilize either antimycin A or rotenone did not significantly affect the maximal extent of the phenylephrine-induced oxygen uptake (162 ± 6.1 μM, n = 12, *versus* 176 ± 9.3 μM (n = 6) for 3-min solvent pretreated livers). Furthermore, amo-barbital, a water-soluble respiratory blocker, also inhibited phenylephrine-induced respiration by approximately the same extent as either antimycin A or rotenone (Table I).

The specificity of these mitochondrial respiratory inhibitors was examined using the respiratory chain uncoupler 2, 4-dinitrophenol. DNP[1]-stimulated oxygen uptake was maximal by 120 to 140 s following infusion, the extent of stimulation being strictly concentration-dependent in the range of 10 to 80 μM DNP. Consequently, for all further experiments a concentration of DNP (24 μM) was used which resulted in a similar increases in oxygen uptake to those induced by maximal doses (1.5 μM) of phenylephrine.

Since DNP presumably stimulates only mitochondrial respiration, we compared the concentration dependence of antimycin A or rotenone on either phenylephrine- or DNP-stimulated respiration. The data in Fig. 4 show that the stimulation of oxygen uptake induced by either agent is inhibited to the same degree by any given concentration of antimycin A or rotenine (r = 0.98 and 0.90, respectively). This highly significant correlation constitutes good evidence that phenylephrine is acting at the same locus as DNP, presumably the mitochondria. This was confirmed by experiments using either oligomycin or carboxyatractyloside. These agents, known indirectly to block respiration in coupled mitochondria, inhibit the phenylephrine-induced respiratory response in a similar manner to antimycin A or rotenone (Table I).

Since the phenylephrine-mediated oxygen response appears to be mediated by the same receptor system (Fig. 2), is as least as rapid in onset, and follows the same temporal progression as the α-agonist-stimulated glucose output (Fig. 1), it was important to assess whether or not this response is functionally related to the glycogenolytic response.

Previous workers (3) have shown that glycogen breakdown in fed rats accounts for essentially all of the glucose and lactate output. In these experiments we have used glucose output as an indicator of glycogenolysis even though data obtained in our laboratory[2] and by others (31, 37), have shown that phenylephrine also stimulates the production of lactate and the rate of respiration. Thus, the measurement of glucose release will tend to underestimate the total extent of glycogenolysis. In our hands, agonist-induced glucose release represents greater than 70% of glucose + ½ lactate produced. Glucose data alone are presented since the ethanol solvent used to solubilize some of the respiratory inhibitors altered

[1] The abbreviation used is: DNP, 2,4-dinitrophenol.

[2] P. H. Reinhart, W. M. Taylor, and F. L. Bygrave, unpublished observations.

the basal concentration of lactate and pyruvate release by the liver, but not the amount of glucose released.

Data in Fig. 4 further reveal that either of these agents inhibits most of the agonist-induced glucose output, at concentrations similar to those previously used (Fig. 3) to inhibit oxygen uptake. Inhibition by antimycin A was apparent at approximately 0.3 μM, half-maximal at approximately 0.75 μM, and maximal at 2.5 μM (Fig. 4*A*). The correlation coefficient for the inhibition of respiration and glucose release was 0.92.

Fig. 4*B* shows that qualitatively similar results were obtained for rotenone, although this agent was less potent, on a molar basis, than antimycin A. Inhibition was apparent at 0.5 μM, half-maximal at approx 2.5 μM, and maximal at 7.5 μM.

For each concentration of either antimycin A or rotenone used, the appropriate control experiments were carried out to determine the effects of the inhibitors alone on either oxygen uptake or glucose output. At the short pre-infusion times used, these effects were minimal even at the highest respiratory inhibitor concentrations (data not shown) and were corrected for in each experiment. Neither antimycin A nor rotenone ever totally inhibited the α-adrenergic agonist-induced glycogenolytic response even at inhibitor concentrations significantly higher than those shown in Fig. 4 (data not shown).

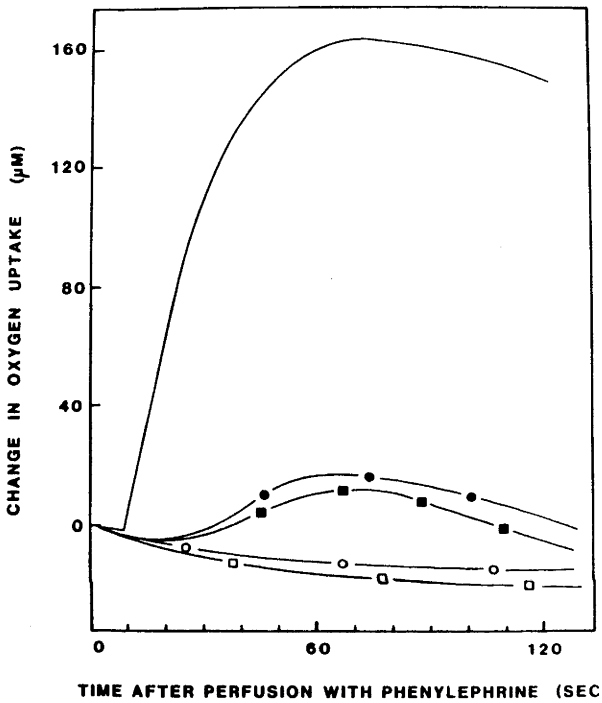

FIG. 3. **Effect of antimycin A or rotenone on phenylephrine-stimulated increase in oxygen uptake.** Livers of fed rats were perfused as described in the legend to Fig. 1. At 15 min of perfusion either antimycin A (2.5 μM final concentration), or rotenone (7.5 μM final concentration) was infused for 3 min, at which time phenylephrine (1.5 μM) was infused into livers where indicated. The infusion of the ethanol solvent used to solubilize either antimycin A or rotenone had no significant effect on the phenylephrine-induced oxygen response. Data shown are continuous traces from a typical experiment of 9 experiments for phenylephrine alone (*solid line*), 6 experiments for antimycin A plus phenylephrine (●—●), 11 experiments for rotenone plus phenylephrine (■—■), 5 experiments for antimycin A (○—○) or rotenone (□—□) alone.

Specificity of Hormone-stimulated Increase in Oxygen Uptake—Previous work has shown that vasopressin and angiotensin II stimulate hepatic glycogenolysis apparently in a cAMP-independent manner (4, 43). Data in Fig. 5, *B* and *D* show that vasopressin and angiotensin II also induce a rapid stimulation of oxygen uptake by the liver. The time of onset, the rate, and the maximal extent of oxygen uptake by these hormones is similar to that induced by phenylephrine (Fig. 1), epinephrine, or norepinephrine (data not shown). In contrast, neither glucagon (Fig. 5*F*) nor dibutyryl cAMP or the β-adrenergic agonist isoproterenol (data not shown) significantly stimulated respiration.

The respiratory response induced by vasopressin and angiotensin II was also largely abolished by concentrations of antimycin A or rotenone that abolished phenylephrine-induced respiration. Consequently, it was important to examine the effects of these respiratory blockers on glucose release induced by these hormones. Data in Fig. 5, *A* and *C* show that both antimycin A and rotenone inhibit the glycogenolytic response to either vasopressin or angiotensin II. As has been previously observed for phenylephrine (Fig. 4), this inhibition is not total but reaches approximately 70 to 80%. Separate control experiments were carried out to establish that the ethanol solvent (0.05% v/v) affected neither hormone-induced glucose release nor oxygen uptake. A further point of interest is that the maximal extent of glucose release induced by phenylephrine, epinephrine, vasopressin, and angiotensin II is very similar (0.6 to 0.8 mM), as is the rapidity of onset of the response (data not shown).

Because glucagon-induced glycogenolysis proceeds in the absence of any large, rapid increase in respiration, at least in the perfused liver, it was of interest to examine whether or not mitochondrial respiratory inhibitors prevent glucagon-induced glycogenolysis. Data in Fig. 5*E* indicate that the maximal amount of glucose output induced by 10^{-8} M glucagon (0.85 ± 0.06 mM, $n = 6$) is slightly larger than that induced by phenylephrine (Fig. 1). This glucose release is only moderately decreased (0.81 ± 0.04 mM, $n = 4$) by 3 min of preperfusion with the ethanol solvent used to solubilize the respiratory inhibitors.

Since glucagon-induced glucose release is only near-maximal following 10 min of hormone administration (data not shown), careful measurements of the amount of glucose released by the respiratory inhibitors themselves had to be

TABLE I

Effect of mitochondrial respiratory inhibitors on either phenylephrine-induced or dinitrophenol-induced oxygen uptake

Experimental details are as described in the legend to Fig. 4. Carboxyatractyloside was infused for 10 min prior to phenylephrine or DNP treatment, while the preperfusion time for oligomycin was 5 min.

	Maximal stimulation	% Inhibition of maximal stimulation				
		Amo-barbital	Rotenone	Antimycin A	Oligomycin	Carboxyatractyloside
	(μM)	*0.6 mM*	*7.5 μM*	*2.5 μM*	*10 μM*	*30 μM*
Phenylephrine (1.5 μM)	162 ± 6.1 ($n = 12$)	84 ± 6.7 ($n = 3$)	88 ± 3.2 ($n = 5$)	92 ± 4.5 ($n = 6$)	85 ± 7.1 ($n = 3$)	79 ± 6.4 ($n = 5$)
Dinitrophenol (24 μM)	189 ± 13 ($n = 6$)	90 ± 5.3 ($n = 3$)	92 ± 3.1 ($n = 4$)	95 ± 4.5 ($n = 3$)	9.8 ± 6.5 ($n = 3$)	10.8 ± 7.2 ($n = 6$)

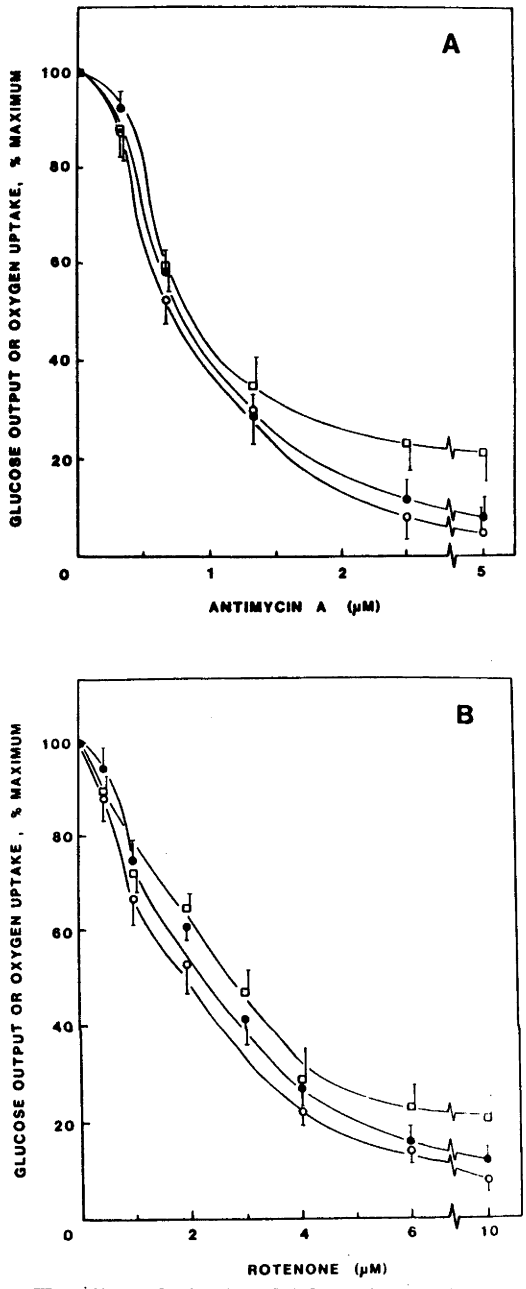

FIG. 4. **The effect of mitochondrial respiratory inhibitors on phenylephrine-induced glycogenolysis and oxygen uptake or on DNP-stimulated respiration.** Perfusion details are as outlined in the legend to Fig. 1. Following a 15-min preperfusion antimycin A (4*A*) or rotenone (4*B*) at the concentrations shown were infused for 3 min. At this time phenylephrine (2 μM) was infused, and changes in the rate of oxygen uptake and amount of glucose release assayed. In a separate set of experiments, DNP (24 μM) was infused and changes in the rate of oxygen uptake, assayed. Maximal changes occur at between 60 and 120 s of agonist treatment, hence results shown are 90-s values. Separate control experiments were carried out to establish that the ethanol solvent used diminished neither the phenylephrine-induced stimulation of glucose output nor respiration. Results shown have been corrected for basal effects of either inhibitor. Basal glucose output and oxygen uptake were as described in the legend to Fig. 1. Changes in glucose output (□—□) and oxygen uptake (●—●, phenylephrine; ○—○, DNP) are the means ± S.E. for between 3 and 8 separate experiments.

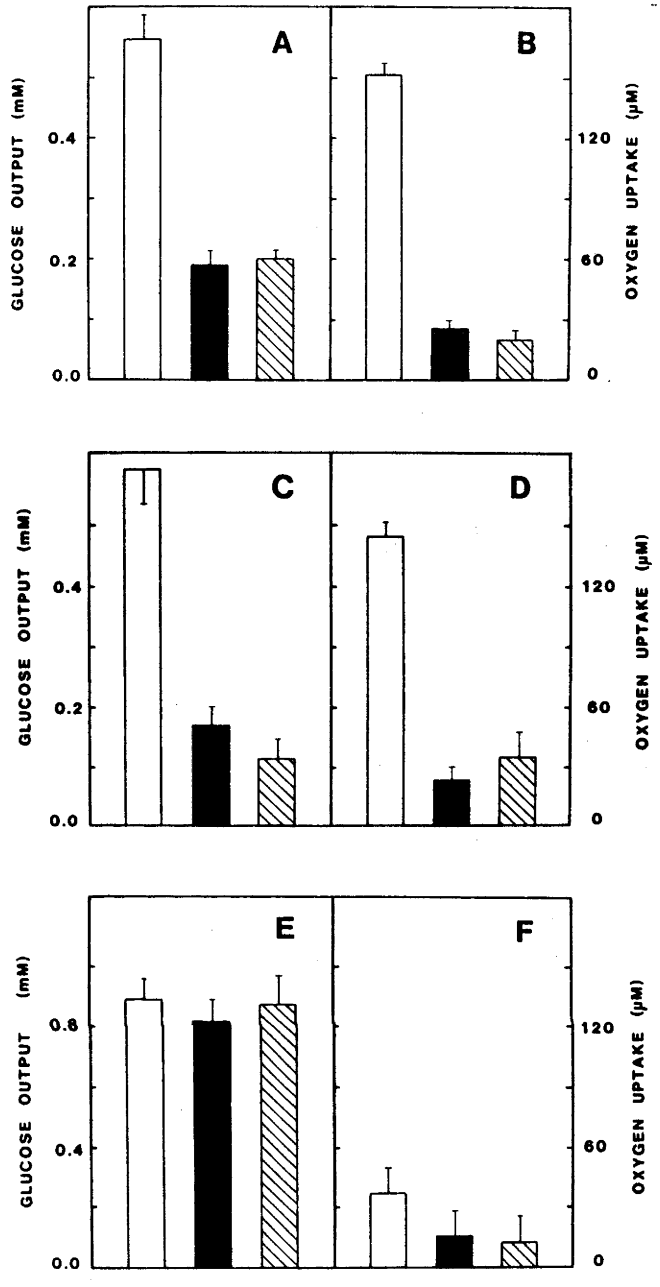

FIG. 5. **Effect of mitochondrial respiratory inhibitors on glucose release and oxygen uptake induced by vasopressin, angiotensin II, and glucagon.** Perfusions and glucose assays were carried out as described in the legend to Fig. 1. At 15 min of perfusion some livers were infused for 3 min with rotenone (7.5 μM, *solid bars*), antimycin A (2.5 μM, *cross-hatched bars*), or the ethanol solvent used to solubilize the respiratory inhibitors (*open bars*). Then either vasopressin (1 munit/ml), angiotensin II (6×10^{-9} M), or glucagon (10^{-8} M) was infused. All data shown for vasopressin (*A* and *B*) and angiotensin II (*C* and *D*) were obtained after 2 min of hormone treatment, while glucagon data (*E* and *F*) shown is for 10 min of hormone treatment. For all hormones, corresponding control experiments were carried out to determine basal effects of the inhibitors, and the data was corrected accordingly. Results shown are the means ± S.E. for between 3 and 5 separate experiments. Note change of scale on *E*. Basal glucose output and oxygen uptake were as described in the legend to Fig. 1.

made in order to allow a valid determination of the effects of these inhibitors on glucagon-stimulated glucose release. Data in Fig. 5*E* show that neither rotenone nor antimycin A, at doses sufficient maximally to inhibit phenylephrine-stimulated glucose output, has any significant effect on glucagon-mediated glucose output. Antimycin A itself gives rise to a 0.17 ± 0.04 nM (n = 3) increase in glucose release, while glucagon in the presence of antimycin A (2.5 μM) induces a

further 0.80 ± 0.3 mM (n = 6) increase in effluent glucose concentration. Similarly, rotenone (7.5 μM) gives rise to a 0.27 ± 05 mM (n = 4) increase in glucose release at 10 min of treatment while glucagon further increases this output by 0.84 ± 0.06 nM (n = 4). Hence glucose release induced by glucagon is not inhibited by the respiratory inhibitors.

DISCUSSION

The experiments reported in this study provide evidence that a) the major portion of the phenylephrine-induced increase in oxygen uptake by the perfused liver is attributable to mitochondria, b) there exists a tight association between this oxygen uptake response and agonist-induced glucose output, c) that the responses involve the α_1- class of α-adrenergic receptors, and d) that vasopressin and angiotensin II induce similar rapid responses to those induced by α-adrenergic agonists, which differ from those induced by either glucagon, or dibutyryl cAMP.

Although previous reports have indicated that epinephrine (31, 32), norepinephrine (30), and phenylephrine (28, 32), all stimulate the rate of oxygen uptake by the perfused rat liver, neither the intracellular location of this response, nor its relation to the glycogenolytic response of these hormones has been elucidated. We have obtained evidence for the underlying involvement of mitochondria in the phenylephrine-induced oxygen uptake response by first studying the influence, in the perfused liver, of inhibitors known to block the electron transport chain in isolated mitochondria. Thus antimycin A and rotenone, at concentrations similar to those used with isolated mitochondria (44, 45), virtually abolished the phenylephrine-induced increase in oxygen uptake (Figs. 3 and 4). The possibility that these respiratory inhibitors were acting in a nonspecific manner was lessened by employing the shortest preperfusion time consistent with reproducible results (3 min).

Previous studies have indicated that the short exposure of intact hepatocytes (35), synaptosomes (46), and Ehrlich ascites tumour cells (47), to these inhibitors has no significant effect on the mitochondrial membrane potential. Similarly, the administration of amytal, dinitrophenol, or carboxyatractyloside to the perfused liver from fed rats increases the mitochondrial ATP/ADP ratio and only moderately decreases the cytosolic ATP/ADP ratio (48), indicating that in the absence of mitochondrial respiratory chain activity, anaerobic glycolysis is essentially able to maintain the cytosolic free energy charge at levels similar to those maintained by oxidative phosphorylation.

Furthermore, the conclusion that mitochondria are the principal site of action of these inhibitors was substantiated by the finding that the concentrations of either antimycin A or rotenone required to inhibit phenylephrine-stimulated respiration were similar to those required to inhibit uncoupler-stimulated respiration.

A third piece of evidence implicating mitochondrial respiration is that agents such as carboxyatractyloside and oligomycin, which inhibit respiration in tightly coupled mitochondria (44, 45), also largely prevented the α-adrenergic agonist-mediated respiratory response (Table I). The inhibition of phenylephrine-induced respiration by these compounds clearly distinguishes the phenylephrine response from that of the uncoupler DNP, which was unaffected by these agents (Table I). This suggests that the phenylephrine-induced respiratory response does not reflect an alteration in the coupling between respiration and oxidative phosphorylation.

A second major point revealed in this study is that the phenylephrine-induced mitochondrial oxygen uptake is intimately associated with the glycogenolytic response to this agent. This conclusion is made in light of a) the similar α_1-receptor specificity for either oxygen uptake or glucose output, as revealed by the greater inhibition of the responses by prazosin than by yohimbine, b) the very similar times of onset and parallel rates of response, and c) their similar sensitivities to antimycin A and rotenone.

Data further substantiating this view was obtained using vasopressin and angiotensin II, hormones postulated to stimulate hepatic glycogenolysis in a cAMP-independent manner (4, 43). Both of these hormones induce responses similar to those of phenylephrine in that they give rise to a rapid increase in oxygen uptake by the perfused liver, paralleled by glucose output, and that each of these responses is inhibited by mitochondrial respiratory inhibitors (Fig. 5). In contrast, glucagon (Fig. 5), dibutyryl cAMP, or isoproterenol (data not shown) stimulate glucose output in the absence of large changes in respiration, and this effect is not inhibited by mitochondrial respiratory chain inhibitors. Hence, there appear to be common aspects of the mechanisms whereby α-adrenergic agonists, vasopressin, and angiotensin II stimulate hepatic glycogenolysis, an observation in agreement with data presented by Blackmore *et al.* (11) showing that each of these hormones also stimulate Ca^{2+} output by the perfused liver. The finding that glucagon-induced glycogenolysis is not inhibited by respiratory chain inhibitors supports the notion that these inhibitors have no direct effect on the glycogenolytic process itself.

A point of note is that a small portion of both the glucose output response and the respiratory response induced by phenylephrine, vasopressin, and angiotensin II, is insensitive to even the highest concentrations of antimycin A or rotenone used. Experiments with phenylephrine in the presence of 20 μM propanolol (a β-adrenergic antagonist) indicated that this residual activity was not due to a weak β-component of phenylephrine action (data not shown). Whether this residual oxygen uptake response represents mitochondrial or nonmitochondrial respiration is not known.

The observation that in the perfused liver glucagon stimulates glycogenolysis in the absence of large changes in respiration is difficult to integrate with previous data showing that mitochondria isolated following glucagon challenge to either the intact animal (13, 23) or hepatocytes (25), exhibit stimulated rates of state 3 respiration. This respiratory response is not observed with mitochondria isolated from phenylephrine-treated hepatocytes (25), and only a small and variable effect is observed with mitochondria isolated from phenylephrine-challenged perfused liver (13). Efforts to observe a glucagon-induced respiratory response dependent on the presence of glutathione (see Ref. 25) have proved unsuccessful in the intact perfused liver.[2] These apparently contradictory observations highlight the difficulties of relating results obtained with isolated organelles to the intact tissue.

A final point arising from our data is the rapidity of glucose release induced by phenylephrine, vasopressin, and angiotensin II. Since the onset of oxygen uptake and glucose output occurs by 10 to 12 s following hormone administration, and immediately exhibits maximally-stimulated rates, this provides a critical temporal framework against which the glycogenolytic action of α-adrenergic agonists, vasopressin, and angiotensin II must now be considered. For instance, if, as has been suggested (11, 13, 37), a redistribution of intracellular Ca^{+} is an important messenger in the glycogenolytic action of these hormones in the perfused liver, then this would have to occur within 10 s of the hormone interacting with the receptor on the cell surface. Similarly phosphatidylinositol turnover, which has been suggested to play a role in the generation of a second messenger for hormones such as the α-adrenergic

agonists, vasopressin and angiotensin II, (40, 41, 49–52) must be accommodated within this same temporal framework.

The mechanism by which α-adrenergic agonists induce rapid mitochondrial oxygen uptake, and the relationship of this response to a redistribution of intracellular Ca^{2+}, phosphatidylinositol metabolism, and glycogenolysis are the subject of further investigation.

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Section B.5

A procedure for the rapid preparation of mitochondria from rat liver.

(Reinhart et al., 1982b)

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A procedure for the rapid preparation of mitochondria from rat liver

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A technique for the rapid preparation of mitochondria from rat liver is described. Tissue fractionation is performed by a single centrifugation step with a discontinuous Percoll density gradient. Total preparation times of 5–6 min are achieved by using this method. The mitochondrial fraction obtained is relatively free of contaminating organelles, as judged by marker-enzyme activity determinations. Mitochondria isolated by Percoll-density-gradient centrifugation differ from mitochondria obtained by differential centrifugation [Taylor, Prpić, Exton & Bygrave (1980) *Biochem. J.* **188**, 443–450] in that the former exhibit a higher acceptor control ratio and a higher calcium content. Values obtained for the protonmotive force are not significantly different between the two preparations. The technique described may be widely applicable for studies requiring the rapid preparation of functionally intact and relatively uncontaminated mitochondria.

A number of differential centrifugation procedures have been developed for the preparation of rat liver mitochondria (Schneider & Hogeboom, 1950; Appelmans *et al.*, 1955; Johnson & Lardy, 1967; Schnaitman & Greenawalt, 1968; Harada & Sato, 1974; Bustamante *et al.*, 1977). Although these allow for the isolation of coupled mitochondria in high yields, the final fractions are usually contaminated to various degrees with other organelles, and require preparation times of between 1 and 5 h (Baudhuin & Beaufay, 1963; Harada & Sato, 1974).

Because these long preparation times may allow a significant redistribution of metabolites or ions between organelles, a number of techniques for the rapid fractionation of cells have been developed. These involve the disruption of cells by shear forces created by passing isolated liver cells through a small-diameter needle under high pressure (Tischler *et al.*, 1977), or by using digitonin (Zuurendonk & Tager, 1974; Booth & Clark, 1979), or by a combination of both procedures (Scott *et al.*, 1980; Murphy *et al.*, 1980). The subsequent fractionation is achieved by centrifuging through silicone oil at 12000 *g*. These new techniques, however, are not applicable to the fractionation of intact tissue, and as presently employed result in a crude 'heavy particulate' fraction, rather than a purified mitochondrial fraction (Murphy *et al.*, 1980; Scott *et al.*, 1980).

In the present paper we describe a rapid simple method for the isolation of relatively uncontaminated intact mitochondria from perfused rat liver by the use of a discontinuous density gradient of iso-osmotic Percoll. Mitochondrial integrity and purity were examined by determining the protonmotive force, the acceptor control ratio and the distribution of marker enzymes.

Experimental

Liver perfusion and homogenization

Livers of male Wistar-strain albino rats weighing 200–250 g were perfused with Krebs & Henseleit (1932) bicarbonate medium equilibrated with O_2/CO_2 (19:1) and containing 1.30 mM added $CaCl_2$, essentially as described by Reinhart *et al.* (1982). After a 15 min pre-perfusion period, the median lobe was rapidly excised, placed into a chilled glass/Teflon tissue disintegrator (size C; A. H. Thomas Co., Philadelphia, PA, U.S.A.) containing 10 ml of ice-cold homogenization medium {210 mM-mannitol, 60 mM-sucrose, 10 mM-KCl, 10 mM-sodium succinate, 1 mM-ADP, 0.25 mM-dithiothreitol and 0.1 mM-EGTA in 10 mM-Hepes [4-(2-hydroxyethyl)-1-piperazine-ethane sulphonic acid]/KOH buffer, pH 7.4} and homogenized by using two rapid strokes of a pestle driven at 900 rev./min. Succinate and ADP were present to maintain the protonmotive force during isolation of the mitochondria. In addition ADP has been shown to lower the rate of Ca^{2+} efflux in isolated mitochondria (Harris, 1979). For experiments in which the mitochondrial calcium content was determined, EGTA was replaced with 0.5 μM-

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Ruthenium Red and 1 mM-Nupercaine, to minimize mitochondrial Ca^{2+} uptake (Moore, 1971) and efflux (Dawson & Fulton, 1980) respectively during cell fractionation. The time taken to complete the tissue disruption was 4–6 s.

Isolation procedure

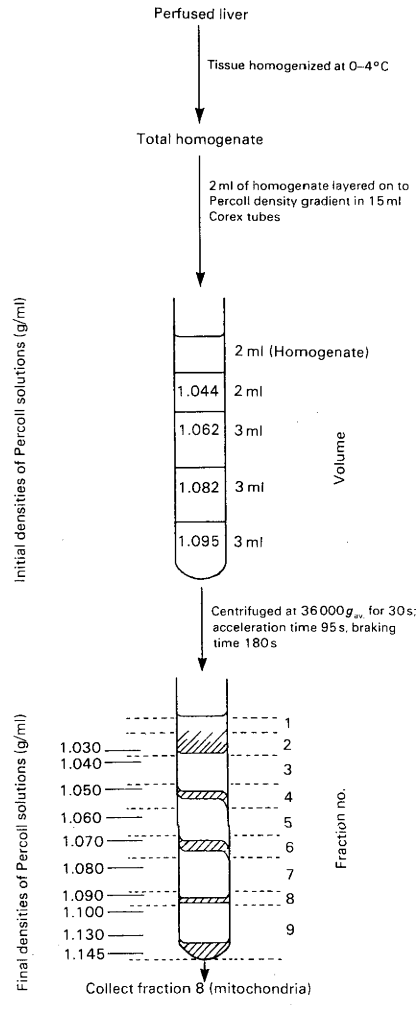
Commercially obtained Percoll was as a routine passed through a Chelex-100 column to remove contaminating cations. Step gradients, prepared in 15 ml Corex tubes, corresponded to approx. 52%, 42%, 31% and 19% (v/v) Percoll in medium containing 210 mM-mannitol, 60 mM-sucrose, 10 mM-KCl, 10 mM-sodium succinate, 1 mM-ADP and 0.25 mM-dithiothreitol in 10 mM-Hepes/KOH buffer, pH 7.4, to yield the densities shown in Scheme 1. Gradients were prepared on ice and used within 15 min of preparation. Density-marker beads were used to calibrate the gradients. The density values supplied for Percoll mixed with 0.25 M-sucrose were corrected for the slightly higher osmolarities of the solutions used in these experiments.

Portions of the liver homogenates (2 ml containing 127 ± 16 mg of protein; $n = 9$) were rapidly layered on to the gradients and immediately centrifuged at 20000 rev./min (36900 $g_{av.}$) for 30 s in a Sorvall RC-5B refrigerated centrifuge fitted with an SS-34 rotor. Preliminary experiments had established that this procedure is suitable for collecting mitochondria at the 42%/52% interface. Gradients were fractionated into nine fractions as shown in Scheme 1, and the distribution of organelles was analysed, with cytochrome *c* oxidase, glucose 6-phosphatase, 5′-nucleotidase, acid phosphatase and catalase as marker enzymes for mitochondria, endoplasmic reticulum, plasma membrane, lysozomes and peroxisomes respectively. For experiments in which only mitochondria were required, the gradient was aspirated to the 42%/52% interface, and the mitochondrial fraction was removed by automatic pipette and, without addition of medium, resuspended to a final protein concentration of 22.8 ± 3.1 mg/ml ($n = 12$). All operations were performed at 0–4°C.

Mitochondria prepared by differential centrifugation (Taylor *et al.*, 1980) were isolated in homogenization medium, except for experiments involving the assay of total calcium, in which case EGTA was replaced with 1 mM-Nupercaine and 0.5 μM-Ruthenium Red.

Analytical procedures

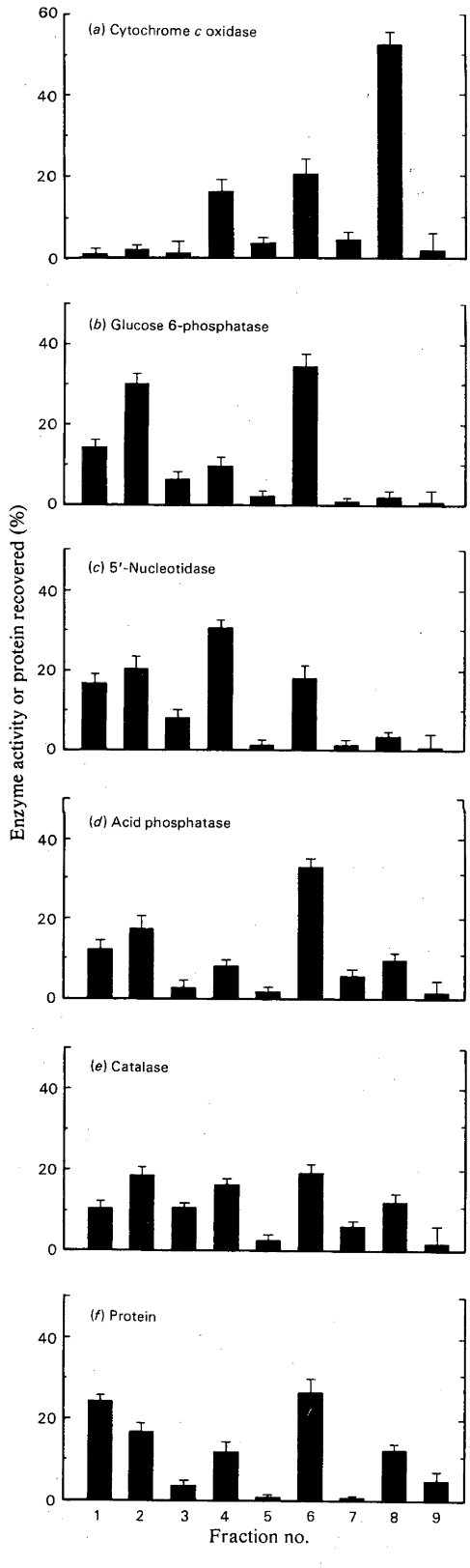
Respiration in the absence or in the presence of ADP was measured polarographically by using a Clark-type oxygen electrode (Rank Brothers, Bottisham, Cambs., U.K.), in a medium containing 100 mM-sucrose, 50 mM-KCl, 10 mM-KH_2PO_4, 2 mM-$MgSO_4$, 1 mM-EDTA and 10 mM-sodium succinate in 15 mM-Tris/HCl buffer pH 7.4. The temperature was 25°C. The components of the protonmotive force were determined by the ion-distribution technique of Nicholls (1974) as described by Taylor *et al.* (1980).



Scheme 1. *Flow diagram for the rapid preparation of mitochondria from perfused rat liver*

For full details see the Experimental section. As Percoll spontaneously forms a gradient when centrifuged at high speed, the final densities differ slightly from the starting densities.

Calcium concentrations were determined by atomic-absorption spectroscopy with a nitrous oxide/acetylene flame. Medical-grade gases only were used for these determinations. Glucose 6-phosphatase and 5′-nucleotidase activities were assayed as described by Bygrave & Tranter (1978), and cytochrome oxidase was assayed as described



by Bygrave *et al.* (1978). Acid phosphatase activity was measured by the method of Baudhuin (1974), after solubilization of approx. 100 μg of protein with 100 μl of 2% (w/v) Triton X-100 for 2 min. Inorganic phosphate was assayed by a modification (Dulley, 1975) of the procedure described by Baginski *et al.* (1967), after centrifuging the acid-treated samples at 12000 ***g*** for 2 min to remove the Percoll. Catalase activity was assayed by measuring the decrease in absorbance at 240 nm as described by Aebi (1974). Protein was assayed by the method of Lowry *et al.* (1951), with bovine serum albumin (fraction 5; Sigma) as the standard. Results are expressed as the means $\pm$ S.E.M. for the numbers of independent experiments shown in parentheses.

Materials

Percoll and the density-marker beads were obtained from Pharmacia Fine Chemicals, Uppsala, Sweden. Ruthenium Red was from Sigma Chemical Co., St. Louis, MO, U.S.A. Chelex-100 was from Bio-Rad Laboratories, Richmond, CA, U.S.A. Corex centrifuge tubes were supplied by Du Pont Instruments, Newtown, CN, U.S.A. Nupercaine (dibucaine hydrochloride) was obtained from Astra Chemicals, Sydney, N.S.W., Australia.

Results and discussion

Scheme 1 is a flow diagram for the rapid preparation of mitochondria isolated from perfused liver. Purification is achieved by density-gradient centrifugation through iso-osmotic Percoll. This isolation procedure has several advantages. First, the preparation is rapid and involves a single centrifugation step. Fractionation is begun within 20 s of

Fig. 1. *Distribution of marker enzymes and protein in fractions obtained from Percoll density gradients*

Portions of liver homogenate (2 ml) were fractionated and marker-enzyme activities were assayed as described in the Experimental section. Results are expressed as percentages of the total activity recovered. Enzyme activities in the liver homogenate were 82.5 ± 9.6 ($n = 5$) nmol/min per mg of protein for cytochrome *c* oxidase, 34.6 ± 4.5 ($n = 5$) nmol of P_i/min per mg of protein for glucose 6-phosphatase, 41.6 ± 5.6 ($n = 4$) nmol of P_i/min per mg of protein for 5'-nucleotidase, 77.3 ± 9.4 ($n = 5$) nmol of P_i/min per mg of protein for acid phosphatase and 1.61 ± 0.32 ($n = 4$) $\Delta A_{240}^{1\,cm}$/min per mg of protein for catalase. The recovery of marker enzyme activities, or of protein, in the nine fractions was at least 90%. Results are presented as means $\pm$ S.E.M. for between four and eight independent experiments.

Table 1. *Comparison of properties of different mitochondrial preparations*

Mitochondria were prepared either by Percoll-density-gradient centrifugation (Scheme 1) or by differential centrifugation (Taylor *et al.*, 1980). Respiration in the presence (State 3) or in the absence (State 4) of added ADP was measured polarographically. The acceptor control ratio is defined as the State 3/State 4 (Chance & Williams, 1956) respiration ratio. Determination of total calcium content and the components of the protonmotive force were performed as described in the Experimental section.

	Mitochondria prepared by	
	Density-gradient (Percoll) centrifugation	Differential centrifugation
State 3 respiration (ng-atoms of O/min per mg of protein)	108.11 ± 3.49 ($n = 6$)	86.56 ± 0.82 ($n = 15$)
State 4 respiration (ng-atoms of O/min per mg of protein)	17.64 ± 0.55 ($n = 6$)	20.56 ± 0.82 ($n = 15$)
Acceptor control ratio	6.15 ± 0.34 ($n = 6$)	4.20 ± 0.16 ($n = 15$)
ΔpH (mV)	76.7 ± 2.4 ($n = 4$)	76.2 ± 2.1 ($n = 6$)
ΔE (mV)	125.5 ± 2.0 ($n = 4$)	133.5 ± 3.3 ($n = 6$)
Protonmotive force (mV)	200.2 ± 4.1 ($n = 4$)	209.7 ± 4.1 ($n = 6$)
Calcium content (nmol/mg of protein)	6.75 ± 0.33 ($n = 6$)	4.95 ± 0.48 ($n = 5$)

excising the liver lobe and is complete within 6 min. Secondly, a mitochondrial fraction only minimally contaminated with other organelles is obtained (see below). Thirdly, mitochondria are not subjected to high hydrostatic pressures or hyperosmotic conditions, as may occur in sucrose-density-gradient centrifugation (Wattiaux *et al.*, 1971).

As shown in Scheme 1 and Fig. 1, the bulk of the mitochondria are recovered at a density of 1.085–1.095 g/ml (fraction 8). This density range is similar to those quoted for fat-cell mitochondria (Belsham *et al.*, 1980) and liver mitochondria (Jenkins *et al.*, 1979; Blume, 1979). The density steps were chosen in order to obtain a relatively pure mitochondrial fraction in high yield (30 mg of mitochondrial protein/g wet wt. of liver). This compares favourably with mitochondrial yields obtained by using differential centrifugation (Bustamante *et al.*, 1977). However, lower yields were obtained when, in an attempt to decrease contamination, the density of the 1.082 g/ml step was increased to 1.087 g/ml (results not shown).

Fig. 1 shows that fraction 8 is relatively free of contaminating endoplasmic reticulum or plasma membranes, as evidenced by the low recovery of glucose 6-phosphatase or 5′-nucleotidase activity. The distributions of acid phosphatase and catalase are far more heterogeneous for the density range 1.040–1.108 g/ml, and hence lysosomes and peroxisomes are a more significant contaminant of fraction 8. This extent of contamination by organelles of non-mitochondrial origin is comparable with, and in some cases less than, that which occurs in conventional mitochondrial preparations (see, e.g., Baudhuin & Beaufay, 1963; Harada & Sato, 1974; Gellerfors & Nelson, 1979; F. L. Bygrave & T. P. Heaney, unpublished work).

The data in Table 1 show that rapidly prepared mitochondria have a significantly higher acceptor control ratio than do mitochondria prepared by differential centrifugation. This was due largely to a higher rate of respiration in the presence of added ADP by rapidly prepared mitochondria, and is indicative of a higher state of coupling between oxidation and phosphorylation. The components of the protonmotive force (ΔE and ΔpH) were measured in the absence of any added substrate, and were not significantly different ($P < 0.1$) between the two preparations of mitochondria.

The calcium content of rapidly prepared mitochondria is significantly higher than that measured in mitochondria isolated by conventional means, emphasizing the possibility that the redistribution of ions, including Ca^{2+}, may occur during the isolation of mitochondria by conventional methods (even in the presence of 0.5 μM-Ruthenium Red and 1 mM-Nupercaine). Hence the rapid isolation procedure described should be useful for studying relatively rapid changes in the concentrations of mitochondrial metabolites and ions.

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Section B.6

Calcium fluxes induced by the action of α-adrenergic agonists in perfused rat liver.

(Reinhart et al., 1982c)

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Calcium ion fluxes induced by the action of α-adrenergic agonists in perfused rat liver

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Phenylephrine (2.0 μM) induces an α_1-receptor-mediated net efflux of Ca^{2+} from livers of fed rats perfused with medium containing physiological concentrations (1.3 mM) of Ca^{2+}. The onset of efflux (7.1 ± 0.5 s; $n = 16$) immediately precedes a stimulation of mitochondrial respiration and glycogenolysis. Maximal rates of efflux are observed between 35 s and 45 s after α-agonist administration; thereafter the rate decreases, to be no longer detectable after 3 min. Within seconds of terminating phenylephrine infusion, a net transient uptake of Ca^{2+} by the liver is observed. Similar effects were observed with vasopressin (1 m-unit/ml) and angiotensin (6 nM). Reducing the perfusate $[Ca^{2+}]$ from 1.3 mM to 10 μM had little effect on α-agonist-induced Ca^{2+} efflux, but abolished the subsequent Ca^{2+} re-uptake, and hence led to a net loss of 80–120 nmol of Ca^{2+}/g of liver from the tissue. The administration at 5 min intervals of short pulses (90 s) of phenylephrine under these conditions resulted in diminishing amounts of Ca^{2+} efflux being detected, and these could be correlated with decreased rates of α-agonist-induced mitochondrial respiration and glucose output. An examination of the Ca^{2+} pool mobilized by α-adrenergic agonists revealed that a loss of Ca^{2+} from mitochondria and from a fraction enriched in microsomes accounts for all the Ca^{2+} efflux detected. It is proposed that the α-adrenergic agonists, vasopressin and angiotensin mobilize Ca^{2+} from the same readily depleted intracellular pool consisting predominantly of mitochondria and the endoplasmic reticulum, and that the hormone-induced enhanced rate of mitochondrial respiration and glycogenolysis is directly dependent on this mobilization.

The stimulation of hepatic glycogenolysis by α-adrenergic agonists, vasopressin and angiotensin appears to be mediated by a cyclic AMP-independent mechanism, possibly involving the direct activation of phosphorylase *b* kinase through an elevation of the cytosolic free Ca^{2+} concentration (Sherline *et al.*, 1972; Tolbert *et al.*, 1973; Hutson *et al.*, 1976; Cherrington *et al.*, 1976; Birnbaum & Fain, 1977; Chan & Exton, 1977; Van de Werve *et al.*, 1977; Blair *et al.*, 1979). The pool of Ca^{2+} mobilized during this activation, however, remains a controversial issue. Some investigators have proposed that α-adrenergic agonists can induce the inflow of Ca^{2+} from the external medium into the cell (Assimacopoulos-Jeannet *et al.*, 1977; Foden & Randle, 1978; Poggioli *et al.*, 1980; Barritt *et al.*, 1981*a*), whereas others have suggested that the pool is derived from stores located at, or close to, the plasma membrane (Althaus-Salzmann *et al.*, 1980). Also, a number of reports indicate that a major portion of the Ca^{2+} may be released from mitochondria or other intracellular organelles (Chen *et al.*, 1978; Blackmore *et al.*, 1979; Babcock *et al.*, 1979; Murphy *et al.*, 1980; Barritt *et al.*, 1981*a*,*b*; Berthon *et al.*, 1981).

Several features of the experimental conditions employed in the different studies cited may account for the seemingly conflicting conclusions. First, recent work has established that the effects of α-adrenergic agonists on hepatic metabolism are very rapid, and in many instances transient (Scholz & Schwabe, 1980; Reinhart *et al.*, 1981, 1982*a*). Hence studies involving, for example, the pre-incubation of isolated liver cells with adrenaline for up to 60 min (Foden & Randle, 1978) may give misleading information.

A second point is that isolated cells or perfused livers have been exposed in most studies to low Ca^{2+} concentrations (10–50 μM) in order to facilitate detection of Ca^{2+} flux exchanges. In those studies,

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Ca^{2+} release from hepatocytes or perfused livers has been measured by using atomic-absorption spectroscopy, chlortetracycline fluorescence or Ca^{2+}-sensitive electrodes (Chen *et al.*, 1978; Blackmore *et al.*, 1979*a*; Babcock *et al.*, 1979; Althaus-Salzmann *et al.*, 1980). Since 10μM-Ca^{2+} represents only about 1% of unbound extracellular Ca^{2+}, the prolonged use of such media considerably depletes the Ca^{2+} content of the whole liver, or of subsequently prepared liver fractions (Blackmore *et al.*, 1979*a*,*b*), as well as altering the cellular response to α-adrenergic agonists (Chan & Exton, 1977; Van de Werve *et al.*, 1977; Blair *et al.*, 1979; Assimacoupoulos-Jeannet *et al.*, 1977). A third point is that studies attempting to correlate changes in cellular Ca^{2+} fluxes with the Ca^{2+} content of subsequently prepared liver organelles have utilized extended fractionation procedures that allow a possible Ca^{2+} redistribution between organelles during their isolation (Blackmore *et al.*, 1979*a*,*b*; Babcock *et al.*, 1979; Althaus-Salzmann *et al.*, 1980; Murphy *et al.*, 1980; Poggioli *et al.*, 1980; Barritt *et al.*, 1981*a*,*b*; Berthon *et al.*, 1981).

In an attempt to overcome these problems, we have used a Ca^{2+}-specific electrode to continuously measure rapid cellular Ca^{2+} flux changes in response to α-adrenergic agonists and other hormones, in livers perfused with media containing 1.3mM-added Ca^{2+}. In addition, we have correlated the extent and direction of cellular Ca^{2+} fluxes with the Ca^{2+} content of liver organelles prepared by a recently-developed rapid fractionation procedure (Reinhart *et al.*, 1982*b*). The rapid hormone-induced changes in liver O_2 consumption and glucose output were also determined, and correlated with changes in Ca^{2+} flux and Ca^{2+} content.

We conclude that the earliest measurable effect of α-adrenergic agonists on perfused liver is to stimulate the rate of Ca^{2+} efflux from cells. The source of this mobilized Ca^{2+} appears to be intracellular organelles, predominantly mitochondria and the endoplasmic reticulum. Both α-adrenergic agonist-induced glycogenolysis and mitochondrial respiration (Reinhart *et al.*, 1982*a*) are dependent on the mobilization of Ca^{2+} from this pool.

Experimental

Animals and perfusions

Male Wistar-strain albino rats, weighing between 200 and 250g and having free access to food, were used for all experiments. Rats were anaesthetized with sodium pentobarbitone (50mg/kg body wt.), and the livers were perfused with Krebs–Henseleit bicarbonate medium (Krebs & Henseleit, 1932) equilibrated with O_2/CO_2 (19:1) and containing either 1.30mM-added $CaCl_2$ or no added $CaCl_2$ (Reinhart *et al.*, 1982*a*). To reduce the time for which livers were exposed to a low Ca^{2+} concentration, $CaCl_2$ was administered by infusion syringe, the $CaCl_2$ infusion being terminated only 5min before a low-Ca^{2+} medium was required. At 3–4min after the termination of Ca^{2+} infusion, the perfusate Ca^{2+} concentration had decreased from 1300μM to approx. 10μM, as determined both by atomic-absorption spectroscopy and Ca^{2+}-electrode measurements. All experiments were carried out between 08:00h and 12:00h to minimize diurnal fluctuations in the amount of basal glucose output.

Perfusate Ca^{2+} and O_2 determinations

The perfusate Ca^{2+} concentration was continuously monitored with a Radiometer F2112 Ca^{2+}-selective electrode, coupled to a Radiometer GK 2401 C combination electrode via an agarose/KCl salt bridge. Signal amplification was achieved by connecting both electrodes to an Orion model 901 microprocessor ionanalyzer, set to the grounded-solution mode. The output of the ionanalyzer was modified by Orion Research, Sydney, Australia, to yield a 2mV output signal per mV displayed. Data was recorded by coupling the microprocessor ionanalyzer to a Spectra-Physics SP4100 computing integrator through a bucking-voltage device similar to that described by Madeira (1975). Shielded cables were used for all connections. The computing integrator was programmed to display both changes in perfusate Ca^{2+} concentration (peak height) and the total amounts of Ca^{2+} taken up or released by the liver (integration mode).

The Ca^{2+}-sensitive electrode was placed in a small flow-through reaction vessel mounted above a magnetic stirring unit, as close as practicable to the liver. Both the Ca^{2+} electrode, and the reference combination electrode vessels were kept at 20°C in a Faraday cage. For each experiment the Ca^{2+} electrode response was calibrated during the initial 15min of perfusion, by infusing known amounts of $CaCl_2$, at a constant flow-rate, between the liver and the electrode. In this way changes in mV output could be related to increases or decreases in the total Ca^{2+} concentration. Electrode membranes were discarded if the voltage change deviated by more than 15% from theoretical (Nernstian) values.

Preliminary experiments indicated that under some conditions the Ca^{2+}-sensitive electrode responds to changes in the concentration of perfusate O_2. This was traced to the existence of earth loops, and could be overcome by electrically isolating the perfusion circuit from any earth leaks. The only earth connection is from the solution in which the reference combination electrode is submerged, to the microprocessor ionanalyzer. Thereafter, altering the O_2 concentration in the perfusion medium between 50μM and 800μM did not alter the Ca^{2+} electrode signal.

A second initial observation was that the basal Ca^{2+} electrode potential is dependent on the flow rate of medium through the liver. As the vasoconstrictive action of some of the hormones and agonists used in the present study tend to transiently reduce the flow rate through the liver (P. H. Reinhart, W. M. Taylor & F. L. Bygrave, unpublished work), the perfusion circuit was changed from constant pressure to constant flow-rate.

For some experiments the performance of the Ca^{2+} electrode was independently checked by using atomic-absorption spectroscopy (see Fig. 6). Perfusate samples were extracted with 2 M-$HClO_4$ and analysed in 0.1% (w/v) KCl using an N_2O/acetylene flame (medical-grade gases).

The O_2 consumption by the liver was calculated from the difference between influent and effluent O_2 concentrations, measured with a Clark-type oxygen electrode modified for a flow-through mode of operation as described previously (Reinhart *et al.*, 1982*a*). The electrode was calibrated before every experiment, and after each experiment the lag time, from the point of hormone infusion to either the Ca^{2+} or oxygen electrode, was determined by using 10mM-$CaCl_2$ and 50mM-$Na_2S_2O_4$ respectively. Lag times varied as a function of flow rate and liver weight, but were usually from 5 to 8s for Ca^{2+}, and 8 to 12s for O_2. Within any one experiment, lag times could be reproducibly measured to within 0.1s. All rates of O_2 and Ca^{2+} concentration changes are expressed as μM. To express data as μmol/g wet wt. of liver per min, simply multiply by the constant 0.0035 [since the flow rate for all perfusions was constant at 3.5ml/g of liver per min, μM = μmol/liver wt. (g) per 1000/3.5 liver wt. (g)/(min)].

Glucose-output determinations

Effluent perfusate was assayed for glucose by using the glucose oxidase/peroxidase method as previously described (Reinhart *et al.*, 1982*a*).

Liver fractionation and Ca^{2+} content assay

In some experiments the liver was fractionated by a rapid procedure, recently developed in this laboratory (Reinhart *et al.*, 1982*b*). Briefly, this involved excising the median lobe and homogenizing in a medium consisting of 210mM-mannitol, 60mM-sucrose, 10mM-KCl, 10mM-sodium succinate, 1mM-ADP, 0.25mM-dithiothreitol, 2μM-EGTA, 5μM-Ruthenium Red, 1mM-nupercaine and 10mM-Hepes/KOH (pH7.4). A portion of the resulting homogenate (2ml) was layered on to a discontinuous density gradient of iso-osmotic Percoll, and centrifuged for 30s at 39600 $g_{av.}$ in a Sorvall RC-5B refrigerated centrifuge with the SS 34 rotor. In this way nine liver fractions were obtained within a total preparation time of 6min. In the present study only five of the nine fractions (fractions 1, 2, 4, 6 and 8) were further assayed, since more than 90% of the total protein and Ca^{2+} could be recovered in these five fractions (see Fig. 1 in Reinhart *et al.*, 1982*b*). The recovery of marker enzyme activities for mitochondria, endoplasmic reticulum, plasma membrane, peroxisomes and lysosomes in the nine fractions has previously been described (Reinhart *et al.*, 1982*a*). Ruthenium Red and nupercaine had no significant effects on the distribution of marker enzyme activities (results not shown). Immediately after isolation, fractions 1, 2, 4, 6 and 8 were extracted with ice-cold 2M-$HClO_4$ for 30min. Portions of the supernatant were analysed for Ca^{2+} using the atomic-absorption spectroscopy procedure described above.

Chemicals and materials

Phenylephrine, glucagon, [Arg^8]vasopressin, [Val^5]-angiotensin, Ruthenium Red and the glucose assay kit (510-A) were obtained from Sigma. Percoll and density-marker beads were from Pharmacia Fine Chemicals AG, Uppsala, Sweden. Nupercaine (cinchocaine hydrochloride) was supplied by Astra Chemicals, Sydney, N.S.W., Australia, Ca^{2+}-electrode membranes (F2002) and filling solution S 43316 were obtained from Radiometer, Copenhagen, Denmark. Prazosin and yohimbine were gifts from Dr. N. H. Hunt, John Curtin School of Medical Research, Australian National University. Other chemicals used were of analytical grade.

Expression of data

All experiments were performed at least three times, and data are expressed as means ± S.E.M. for the number of independent experiments described.

Results

Effect of α-adrenergic agonists and other glycogenolytic hormones on liver Ca^{2+} fluxes

As the experimental approaches, previously used to examine the effects of α-agonists on liver Ca^{2+} fluxes, have serious shortcomings, we have investigated these flux changes in more detail. In the present study using a Ca^{2+}-sensitive electrode system, as described in the Experimental section, we show that phenylephrine induces a rapid and transient net efflux of Ca^{2+} from livers of fed rats perfused with medium containing physiological concentrations (1.3mM) of Ca^{2+}. Data in Fig. 1 indicate the time of onset of phenylephrine-induced Ca^{2+} efflux is very rapid, occurring at just 7.1 ± 0.5s ($n = 16$) after α-agonist administration. Ca^{2+} efflux occurs just before detection of phenylephrine-induced increases in O_2 consumption and glucose output, which, in this series of experiments, was

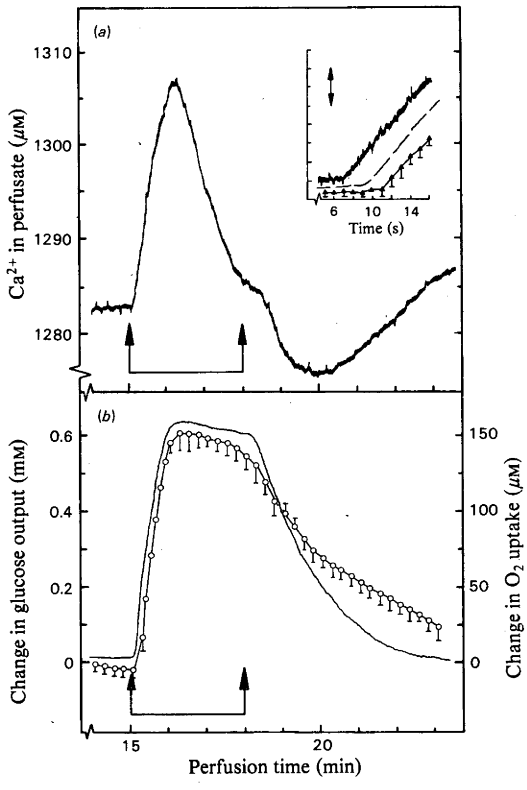

Fig. 1. *Effect of phenylephrine on effluent Ca^{2+} concentration (a), O_2 uptake and glucose release (b)*
Livers of fed rats were perfused initially for 15 min with Krebs–Henseleit buffer equilibrated with Carbogen (O_2/CO_2, 19:1) and containing 1.3 mM added $CaCl_2$ as described in the Experimental section. Livers were then infused with phenylephrine (2.0 μM, final concentration) for 3 min. (*a*) The effluent Ca^{2+} concentration was measured using a Ca^{2+}-selective electrode coupled to a microprocessor/ionalyzer and a computing integrator as described in the text. (*b*) O_2 uptake (continuous trace) was estimated from the difference between influent and effluent O_2 concentrations. Glucose (○) was assayed at 1 s intervals after removing contaminating erythrocytes by centrifugation. The inset in (*a*) shows the initial corrected time course (i.e. lag times subtracted) of phenylephrine-induced Ca^{2+} efflux (continuous trace), O_2 uptake (broken trace) and glucose output (▲). The interval shown represents 4 μM-Ca^{2+}, 20 μM-O_2 or 0.1 mM-glucose. Ca^{2+} and O_2 traces shown are from one typical experiment of 16 performed. Glucose output data are means ± S.E.M. for nine independent experiments.

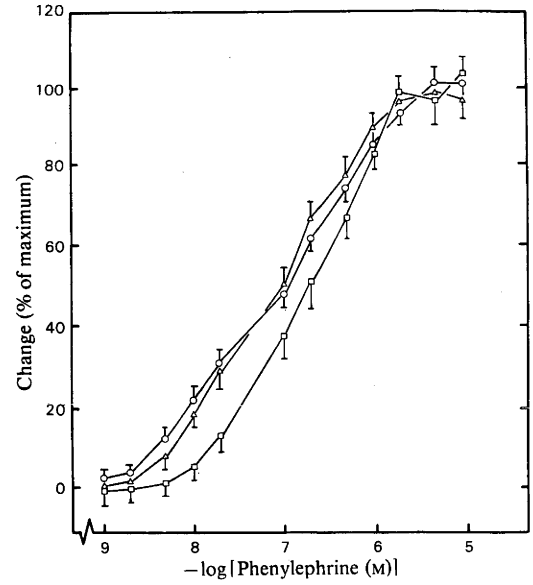

Fig. 2. *Dose–response data for the effect of phenylephrine on Ca^{2+} efflux, O_2 uptake and glucose output*
Livers of fed rats were perfused as described in the legend to Fig. 1. At 15 min of perfusion, phenylephrine, at the concentrations indicated, was infused and the maximal changes in Ca^{2+} efflux (○), O_2 uptake (△) and glucose output (□) were determined as described in the legend to Fig. 1. Maximal changes (100%) for Ca^{2+} efflux, O_2 uptake and glucose output were 21.5 ± 0.4 μM ($n = 8$), 164 ± 4.1 μM ($n = 12$), and 0.615 ± 0.04 mM ($n = 7$) respectively. Data are means ± S.E.M. for between four and 12 independent experiments.

shown to occur at 9.6 ± 0.6 s ($n = 16$) and 11.5 ± 0.4 s ($n = 12$) respectively, after α-agonist infusion (Fig. 1; Reinhart *et al.*, 1982*a*). No detectable influx of Ca^{2+} was seen before the Ca^{2+} efflux. The rate of Ca^{2+} efflux increased, to reach a maximum of 20–25 μM at 35–45 s of phenylephrine treatment, thereafter declining until being no longer detectable after 2–3 min. In contrast, phenylephrine-induced increases in O_2 consumption and glucose output were slower in reaching maximal values, requiring 50–60 s after α-agonist infusion, and were maintained near these rates for at least 3 min. Within 10–15 s of terminating the infusion of phenylephrine, a compensatory net uptake of Ca^{2+} by the liver is observed. The rate of this Ca^{2+} influx was slower but more prolonged (up to 5 min) than the preceding efflux rate and hence the total net amounts of Ca^{2+} involved in the efflux and influx phases (80–120 nmol/g wet wt. of liver) were approximately similar. Phenylephrine-stimulated rates of respiration and glucose output both declined toward basal values during this period of Ca^{2+} influx.

Fig. 2 shows the dose–response curves for the effect of phenylephrine on Ca^{2+} efflux, glucose output and O_2 uptake. The Ca^{2+} efflux and O_2 uptake dose–response curves are very similar,

showing significant stimulation at 8 nM-phenylephrine and half-maximal and maximal responses at 0.1 and 2 μM-phenylephrine respectively. Glucose output is slightly less sensitive to phenylephrine, with significant stimulation observed at 20 nM-phenylephrine and half-maximal and maximal responses evident at agonist concentrations of 0.2 and 1 μM respectively.

The effect of a range of hormones, agonists or analogues on the maximal rate of net Ca^{2+} efflux is shown in Fig. 3. Vasopressin (3 m-units/ml) and angiotensin (10 nM), hormones thought to stimulate hepatic glycogenolysis in a cyclic AMP-independent manner (Hems & Whitton, 1973; Kirk & Hems, 1974; Keppens & De Wulf, 1976; Keppens *et al.*, 1977; Fain, 1978), and adrenaline (0.2 μM) all induce a rapid efflux of Ca^{2+} similar to that described for phenylephrine. For each of these agents the Ca^{2+} efflux response was transient, maximal effects of 19–26 μM being observed at between 35 and 50 s of treatment, and basal Ca^{2+} concentrations being re-established by 3 min (results not shown). In contrast, the administration of glucagon or of cyclic AMP analogous at concentrations producing maximal effects on glycogenolysis (Reinhart *et al.*, 1982*a*) had comparatively small effects only.

The effect of α_1- and α_2-adrenergic binding inhibitors on phenylephrine-stimulated Ca^{2+} efflux

Livers cells have been shown to contain both α_1- and α_2-adrenergic receptors (Hoffman *et al.*, 1980), and the activation of either receptor class may lead to metabolic responses (Wikberg, 1979; Hoffman *et al.*, 1980; Fain & Garcia-Sainz, 1980; Tolbert *et al.*, 1980; Jard *et al.*, 1981). Stimulation of both glycogenolysis, and of mitochondrial respiration by phenylephrine, adrenaline or noradrenaline has previously been shown (Reinhart *et al.*, 1982*a*) to be mediated via the α_1-receptor system in studies detailing the relative sensitivity of these responses to specific α_1- or α_2-receptor antagonists. Phenylephrine-induced Ca^{2+} efflux is also apparently an α_1-receptor-mediated event, since the addition of the specific α_1-antagonist prazosin at either 20 nM or 0.2 μM almost completely abolished the response (Fig. 4). In contrast, the administration of 0.2 μM-yohimbine (an α_2-binding antagonist) had essentially no effect on either phenylephrine-induced (Fig. 4) or adrenaline-induced (results not shown) Ca^{2+} efflux.

The effect of perfusate Ca^{2+} concentration on α-adrenergic agonist or hormone-stimulated Ca^{2+} efflux, respiration and glucose output

The effects of phenylephrine (2 μM) on Ca^{2+} efflux, O_2 consumption and glucose output in rat livers perfused with either 1.3 mM- or approx. 10 μM-Ca^{2+}

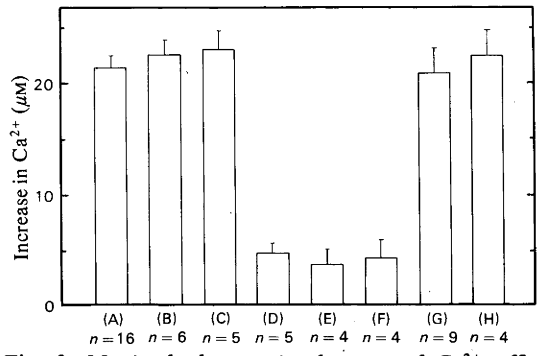

Fig. 3. *Maximal changes in the rate of Ca^{2+} efflux induced by hormones or agonists from livers perfused with Krebs–Henseleit medium containing 1.3 mM added $CaCl_2$*

Livers of fed rats were perfused as described in the legend to Fig. 1. At 15 min of perfusion hormones or agonists were infused at these final concentrations: phenylephrine (A), 2 μM; adrenaline (B), 0.2 μM; noradrenaline (C), 0.2 μM; glucagon (D), 10 nM; dibutyryl cyclic AMP (E), 0.1 mM; 8-bromo-cyclic AMP (F), 0.1 mM; vasopressin (G), 3 munits/ml; angiotensin (H), 10 nM. Maximal rates of Ca^{2+} efflux were determined as described in the legend to Fig. 1. Data are means ± S.E.M. for the numbers of independent experiments indicated.

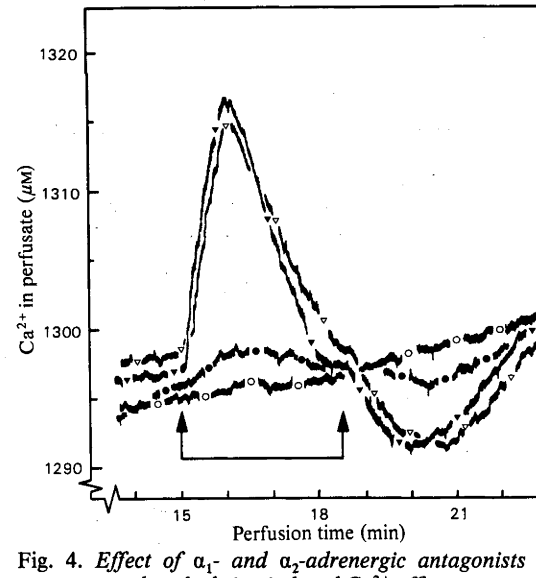

Fig. 4. *Effect of α_1- and α_2-adrenergic antagonists on phenylephrine-induced Ca^{2+} efflux*

Perfusion details are as outlined in the legend to Fig. 1. At 15 min of perfusion prazosin at 20 nM (●) or 0.2 μM (○) final concentration, or yohimbine at 20 nM (▼) or 0.2 μM (▽) were infused for 5 min before phenylephrine (2.0 μM) administration. Changes in the rates of Ca^{2+} efflux were determined as described in the legend to Fig. 1. Data for each agent are typical experiments for between four and six independent experiments performed.

are shown in Figs. 5(*a*) and 5(*b*) and Figs. 5(*c*) and 5(*d*) respectively. At each concentration the initial response to the first pulse of phenylephrine administration is very similar, with times of onset and maximal responses of Ca^{2+} efflux and enhanced O_2 consumption and glucose output being essentially as detailed in Fig. 1. Differences in Ca^{2+} efflux became evident, however, approx. 1 min after α-agonist administration. In perfusions performed with media containing approx. 10 μM-Ca^{2+}, the efflux of the ion was more prolonged and did not return to basal levels until 4–5 min after phenylephrine was first administered. In addition, no compensatory uptake of Ca^{2+} after efflux was observed in this medium (Fig. 5*c*). The absence of any observed Ca^{2+} uptake is not due to the possibility that the concentration of Ca^{2+} in the perfusate is below the detection limit of the Ca^{2+} electrode, since, at the experimental conditions employed, the relationship between pCa and mV remains linear up to approx. 0.1 μM (results not shown).

Further marked differences in response to phenylephrine were also observed when up to five consecutive short infusions of the agonist were performed at the two Ca^{2+} concentrations described. Whereas the stimulation of Ca^{2+} efflux, O_2 consumption and glucose output was essentially the same as described in Fig. 1 during each of five successive administrations of phenylephrine to livers perfused with 1.3 mM-Ca^{2+}, in livers perfused with 10 μM-Ca^{2+} the responses were all increasingly reduced with successive phenylephrine infusions. Under these latter conditions increases in the rate of Ca^{2+} efflux, O_2 consumption and glucose output were all inhibited to approximately the same degree, being virtually abolished after the third phenylephrine pulse. This inhibition could be overcome by exposing the liver to 1.3 mM-Ca^{2+} for 90 s before another pulse of phenylephrine infusion (Figs. 5*c* and 5*d*).

The effect of successive short infusions of different hormones or agonists on Ca^{2+} efflux in livers perfused with approx. 10 μM-Ca^{2+} is shown in Fig. 6. Clearly, prior infusion of phenylephrine diminishes not only the responses to the later re-administration of the agonist (Fig. 5) but also to vasopressin or angiotensin as well. Similarly the responses to phenylephrine are diminished after prior vasopressin (Fig. 6) or angiotensin (results not shown) infusion.

In contrast, the glycogenolytic action of glucagon was still near-maximal after prior infusion of phenylephrine and vasopressin. Furthermore the responses to phenylephrine, vasopressin or angiotensin were not altered appreciably by prior infusion of glucagon. [Values for glucose output were partially masked by the prolonged glycogenolytic effect of glucagon (Reinhart *et al.*, 1982*a*) in these latter experiments; results not shown.]

The effect of phenylephrine on the Ca^{2+} content of liver fractions

The data in Figs. 1–6 indicate that phenylephrine induces a rapid and transient release of Ca^{2+} from a cellular pool in the perfused liver. We attempted to locate the intracellular pool(s) by using a rapid liver fractionation technique (Reinhart *et al.*, 1982*b*). During these studies perfusions were carried out in media containing 10 μM-Ca^{2+}, since under these conditions the administration of phenylephrine results in a net loss of Ca^{2+} from cells, as efflux is not followed by uptake (Fig. 5). 'Low-Ca^{2+}' perfusions also have the effect of decreasing the total basal Ca^{2+} content of all subcellular fractions (Blackmore *et al.*, 1979*a*). However, it is not known whether this difference represents a depletion of Ca^{2+} in 10 μM-Ca^{2+}, or a sequestration of Ca^{2+} in 1.3 mM-Ca^{2+}-containing medium.

Fig. 7 shows the effect of phenylephrine on the Ca^{2+} content of five fractions isolated from liver treated for between 10 and 180 s with the α-agonist. The largest loss of Ca^{2+} induced by phenylephrine occurs in fraction 8, which we have previously shown to consist of relatively uncontaminated mitochondria (Reinhart *et al.*, 1982*b*). Significant

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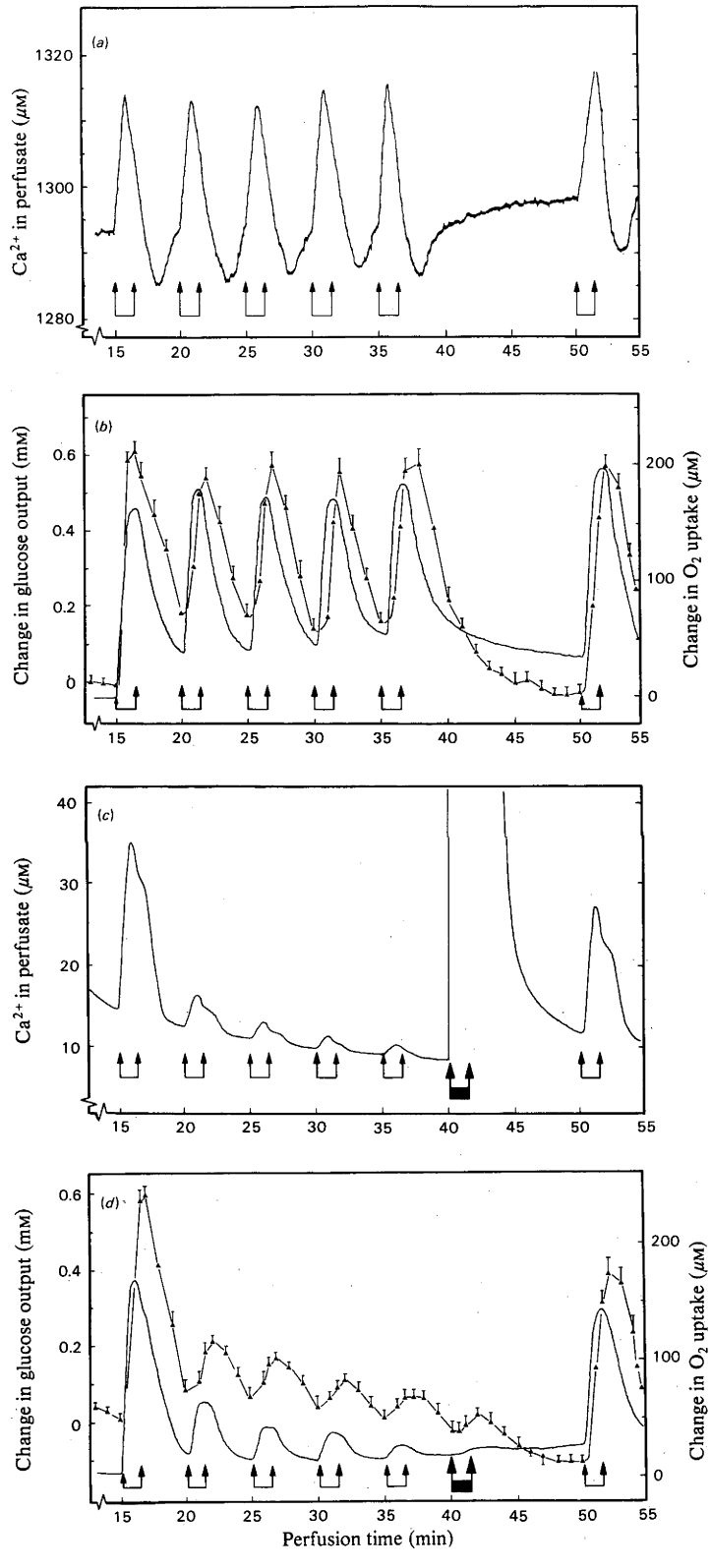
Fig. 5. *A comparison of the effects of short repeated pulses of phenylephrine on Ca^{2+} efflux, O_2 uptake and glucose output measured in 'high-Ca^{2+}' and 'low-Ca^{2+}' perfusion medium*

For 'high-Ca^{2+}' experiments (*a* and *b*) livers were perfused with media containing 1.3 mM added Ca^{2+} for 15 min, at which time phenylephrine (2.0 μM) was infused for 90 s; 3.5 min later another 90 s phenylephrine pulse was administered and this process was repeated three more times. At 50 min of perfusion, a sixth 90 s pulse of phenylephrine was administered. For 'low-Ca^{2+}' experiments (*c* and *d*), the $CaCl_2$ in the Krebs–Henseleit buffer was replaced with NaCl. During the first 10 min of perfusion 1.3 mM-$CaCl_2$ (final concentration) was administered by infusion syringe. At 10 min of perfusion time $CaCl_2$ infusion was terminated and at 15 min perfusion time, 90 s pulses of phenylephrine (2.0 μM) were administered every 5 min. At 40 min of perfusion, a 90 s pulse of $CaCl_2$ (1.3 mM final concentration) was administered (arrows) and 5 min later a final pulse of phenylephrine (2.0 μM). (*b*) and (*d*) show changes in O_2 uptake (continuous trace lines) and glucose output (▲). Ca^{2+} and O_2 traces are from representative experiments of between three and five performed independently. Glucose data are expressed as means ± S.E.M. for three to six independent experiments.


(a)
1320
1300
1280
Ca^{2+} in perfusate (μM)
15 20 25 30 35 40 45 50 55
(b)
0.6
0.4
0.2
0
Change in glucose output (mM)
200
100
0
Change in O_2 uptake (μM)
15 20 25 30 35 40 45 50 55
(c)
40
30
20
10
Ca^{2+} in perfusate (μM)
15 20 25 30 35 40 45 50 55
(d)
0.6
0.4
0.2
0
Change in glucose output (mM)
200
100
0
Change in O_2 uptake (μM)
15 20 25 30 35 40 45 50 55
Perfusion time (min)

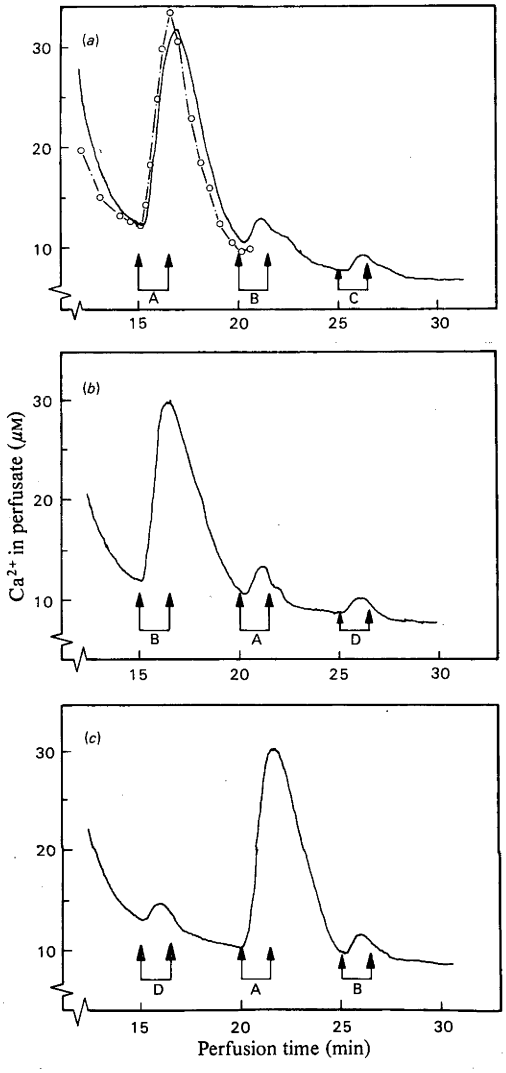


Fig. 6. *Effects of successive short infusions of different agonists or hormones on Ca^{2+} efflux*

Livers of fed rats were perfused with Krebs–Henseleit medium in which the $CaCl_2$ had been replaced with NaCl. During the first 10 min of perfusion, $CaCl_2$ (1.3 mM final concentration) was administered by perfusion syringe. At 15, 20 and 25 min of perfusion, 90 s pulses of different hormones or agonists (A, phenylephrine; B, vasopressin; C, angiotensin; D, glucagon) were administered at the concentrations described in the legend to Fig. 3. Ca^{2+} efflux was measured as described in the legend to Fig. 1 and the results shown are representative experiments for between three and five performed independently. Alternatively atomic-absorption spectroscopy was used to assay Ca^{2+} in the perfusate (O) as described in the Experimental section. Data are means from nine independent experiments. For the sake of clarity, standard errors of the mean have been omitted; however, $\pm 2.4\,\mu$M was never exceeded.

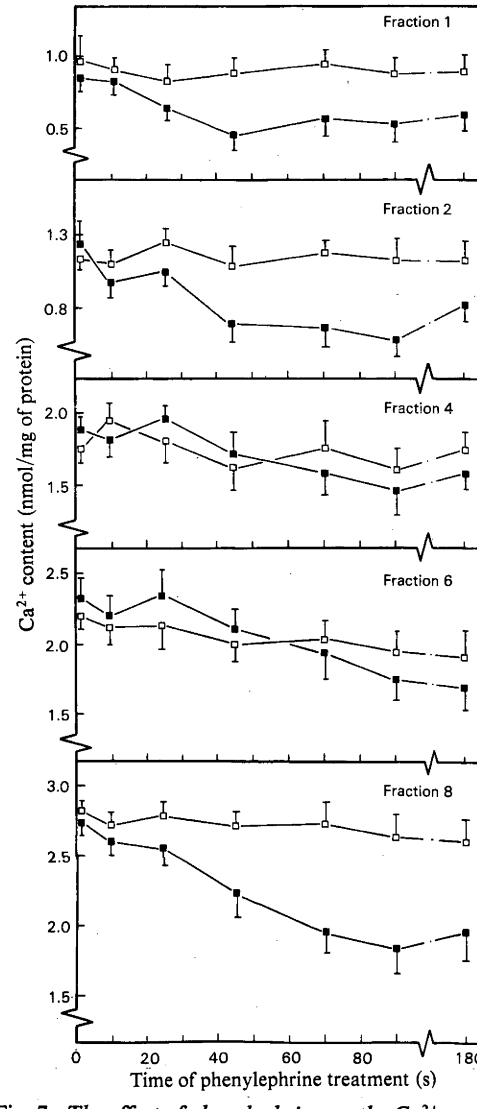


Fig. 7. *The effect of phenylephrine on the Ca^{2+} content of liver fractions*

Livers of fed rats were perfused as described in the legend to Fig. 6, with the infusion of $CaCl_2$ (1.3 mM final concentration) being terminated at 10 min of perfusion time. At 15 min of perfusion phenylephrine (2.0 μM; ■) or Krebs–Henseleit medium (□) was infused for the times indicated. At these times the median and left main lobes were excised and rapidly fractionated as described in the Experimental section. The Ca^{2+} content of the fractions shown was determined by atomic-absorption spectroscopy. Data are means ± S.E.M. for between three and nine independent experiments for each time point and condition.

decreases in the Ca^{2+} content of this fraction can be measured after 25 s of phenylephrine treatment with maximal effects observed after between 45 and 60 s

of treatment. The maximal loss of Ca^{2+} from this pool is 0.82 ± 0.2 ($n = 6$) nmol/mg of protein, which corresponds to approx. 50 nmol/g of liver or approx. 50% of the Ca^{2+} detected by using the Ca^{2+} electrode. From Fig. 7 it is also evident that a small decrease in Ca^{2+} occurs in fractions 1 and 2 over a similar time course. The maximal amount of Ca^{2+} lost from these glucose 6-phosphatase-enriched fractions is 0.4 nmol/mg, but as the protein yield is higher, the total amount of Ca^{2+} lost from these two fractions is similar to that lost from fraction 8 (approx. 40 nmol/g of liver). No significant phenylephrine-induced decreases in Ca^{2+} content were observed in any of the other fractions (results for fractions 3, 5, 7 and 9 not shown), even though fraction 6 contains significant amounts of both cytochrome *c* oxidase and glucose 6-phosphatase activities. Presumably fraction 6 contains a hormone-insensitive subpopulation of these organelles (Prpić *et al.*, 1978; Taylor *et al.*, 1980; Reinhart & Bygrave, 1981) or non-intact organelles. Qualitatively similar data were obtained when perfusions were carried out in media containing 1.3 mM added $CaCl_2$, although quantitatively the changes were smaller and the experimental error was larger (Ca^{2+} content in fraction 8: control, 6.81 ± 0.53 mmol/mg of protein, $n = 3$; 3 min phenylephrine-treated, 6.07 ± 0.44 nmol/mg of protein, $n = 3$).

Discussion

The present study demonstrates that α-adrenergic agonists, vasopressin and angiotensin all induce a transient net efflux of Ca^{2+} within seconds after their administration to rat liver perfused with media containing physiological concentrations of Ca^{2+}. The onset of Ca^{2+} efflux immediately precedes the onset of hormone-induced increases in both respiration and glycogenolysis, a finding consistent with our previous report (Reinhart *et al.*, 1982*a*), showing that these hormones stimulate mitochondrial respiration just before glycogenolysis. Hence the mobilization of a cellular Ca^{2+} pool represents one of the earliest and most transient effects of α-adrenergic agonists on rat liver.

Previous attempts to determine the effects of α-agonists on cellular Ca^{2+} fluxes have yielded conflicting data and interpretations (Foden & Randle, 1978; Blackmore *et al.*, 1979*a*,*b*; Babcock *et al.*, 1979; Poggioli *et al.*, 1980; Althaus-Salzmann *et al.*, 1980; Chen *et al.*, 1978; Murphy *et al.*, 1980; Barritt *et al.*, 1981*a*,*b*; Berthon *et al.*, 1981). Hence a critical examination of some of the experimental details employed in previous studies is now warranted. Investigators using $^{45}CaCl_2$ to estimate net Ca^{2+} flux changes have suggested that noradrenaline or phenylephrine may stimulate cellular Ca^{2+} uptake (Assimacopoulos-Jeannet *et al.*, 1977; Foden & Randle, 1978), Ca^{2+} efflux (Barritt *et al.*, 1981*a*) or Ca^{2+} uptake followed by efflux (Poggioli *et al.*, 1980). These discrepancies may in part be due to unknown changes in pool sizes and specific radioactivities of $^{45}Ca^{2+}$, as well as the fact that in some studies $^{45}Ca^{2+}$ flux changes were not determined during the first 60 s of α-agonist administration, the time when we observe maximal changes in Ca^{2+} fluxes (Fig. 1). Furthermore, a rigorous analysis of the effects of adrenaline on $^{45}Ca^{2+}$ exchange curves in hepatocytes revealed both long-term (steady-state) and short-term (transient) effects of the hormone (Barritt *et al.*, 1981*a*), emphasizing the importance of correlating the temporal progression of Ca^{2+} flux changes with Ca^{2+}-regulated metabolic pathways such as glycogenolysis. Both the extent and the time-dependence of these responses were markedly altered by decreasing the extracellular Ca^{2+} concentration from 1.3 mM to 0.1 mM (Barritt *et al.*, 1981*a*), further highlighting the importance of the experimental conditions employed.

Previous studies using atomic-absorption spectroscopy (Blackmore *et al.*, 1979*b*), chlortetracycline fluorescence (Babcock *et al.*, 1979) or ion-sensitive electrodes (Althaus-Salzmann *et al.*, 1980; Chen *et al.*, 1978) to follow α-adrenergic agonist-induced Ca^{2+} flux changes have resorted to using media containing only very low concentrations of Ca^{2+} (10–50 μM). Flux changes observed under these conditions may not accurately reflect changes at more physiological Ca^{2+} concentrations. A dependence of α-adrenergic agonist-induced Ca^{2+} fluxes on the extracellular Ca^{2+} concentration was evident in the present study, in which we found that reducing the perfusate calcium concentration from 1.3 mM to 10 μM for only 1–2 min distinctly alters not only the Ca^{2+} efflux response, but also the respiratory and glycogenolytic responses, particularly at times of α-agonist administration longer than 60 s (see Fig. 5). The finding that the initial responses (<60 s) to α-agonists are not significantly altered by reducing the perfusate Ca^{2+} concentration indicates that extracellular Ca^{2+} appears not to play a role in the primary hormone signal. This conclusion was confirmed by experiments in which the extracellular Ca^{2+} concentration was further reduced from 10 μM to less than 0.1 μM by infusing 0.2 mM-EGTA for 3 min before phenylephrine treatment (results not shown). In these experiments the extent of Ca^{2+} efflux and glycogenolysis was only slightly less than that observed in 1.3 mM-Ca^{2+}-containing medium, a finding consistent with results presented by Blackmore *et al.* (1978), who showed that phenylephrine activates phosphorylase *a* and induces a decrease in the total Ca^{2+} content of hepatocytes incubated in the presence of stoichiometric concentrations of EGTA.

However, extracellular Ca^{2+} does appear to play

an important role in the longer term (>60s) effects of α-agonists, since both the respiratory and the glycogenolytic responses are more transient in 10 μM-Ca^{2+}-containing medium, and no re-influx of Ca^{2+} is observed under these conditions (Fig. 5). This last finding is of particular interest, since this hormone-induced irreversible loss of the Ca^{2+} allows the depletion of the α-agonist-sensitive pool of Ca^{2+} by three short consecutive pulses of hormone.

Assuming that the short-term perfusion of livers with media containing 10 μM-Ca^{2+} does not in itself decrease the size of the hormone-sensitive pool of Ca^{2+}, then by integrating the Ca^{2+} efflux peaks, we estimate that the total α-adrenergic agonist-mobilized pool contains approx. 140nmol of Ca^{2+}/g wet wt. of liver, a value that may be compared with 160–180 nmol/g wet wt. for the mitochondrial pool of exchangeable Ca^{2+} (Claret-Berthon *et al.*, 1977; Barritt, 1981). Our calculated pool size may be a slight overestimate, since we cannot rule out the possibility that a very slow Ca^{2+} uptake by cells is taking place between consecutive α-agonist pulses. This hormone-sensitive Ca^{2+} pool is apparently in rapid equilibrium with extracellular Ca^{2+}, since the re-administration of Ca^{2+} for 90s to livers whose hormone-sensitive Ca^{2+}-pool had been depleted by three consecutive phenylephrine pulses re-establishes the α-agonist-induced responses.

In contrast, consecutive phenylephrine pulses did not decrease the Ca^{2+} efflux response in livers perfused with 1.3 mM-Ca^{2+}, even when, in one series of experiments, 24 consecutive phenylephrine pulses were administered (results not shown). Hence it appears as though α-agonists rapidly mobilize an intracellular pool of Ca^{2+} that is expelled from the cell. Although the mechanism by which this Ca^{2+} is expelled is unclear, a recently characterized Ca^{2+}-dependent ATPase present in the plasma membrane, whose activity is stimulated by Ca^{2+} in the range 1–100nM (Lotersztajn *et al.*, 1981), may play a role. Presumably it is during the expulsion of Ca^{2+} from the cell that the cytosolic Ca^{2+} concentration is transiently increased, hence allowing the activation of phosphorylase. Subsequent to this mobilization, the hormone-sensitive pool is repleted by extracellular Ca^{2+}. The cytoplasmic Ca^{2+} concentration does not appear to be elevated during this period of Ca^{2+} uptake, since glucose release is decreasing rather than increasing.

The pools of Ca^{2+} sensitive to α-agonists, vasopressin and angiotensin are apparently identical, or at least in rapid equilibrium with each other, since depletion of these Ca^{2+} pools by multiple pulses of any one of these agents abolished the efflux of Ca^{2+} in responses to the subsequent addition of any other of these hormones. This depletion of the hormone-sensitive Ca^{2+} pool is unlikely to result from Ca^{2+}-dependent changes in hormone receptor binding, since all three different classes of hormone are equally affected, and we have found that adrenaline receptor binding in liver plasma-membrane preparations is unaffected by as much as 1 mM-EGTA in a medium containing no added Ca^{2+} (results not shown). Hence all these classes of hormones may have similar Ca^{2+}-dependent post-receptor mechanisms for stimulating hepatic glycogenolysis, and mitochondrial respiration.

Previous reports have suggested that glucagon and cyclic AMP analogues enhance liver glycogenolysis by a mechanism different to that of the agents described above. Our data reinforce this view, as we have consistently found that glucagon or 8-bromo-cyclic AMP elicits only a very small increase in Ca^{2+} efflux. In addition, under low-Ca^{2+} conditions, the prior stimulation of glycogenolysis by glucagon or 8-bromo-cyclic AMP does not deplete the Ca^{2+} pool(s) affected by α-agonists, vasopressin or angiotensin, as judged by their unimpaired ability to stimulate Ca^{2+} efflux. Likewise prior depletion of this Ca^{2+} pool by repeated administrations of α-adrenergic agonists or vasopressin does not appreciably diminish the glycogenolytic effect of subsequently administered glucagon or 8-bromo-cyclic AMP. Hence α-adrenergic agonists, and more importantly adrenaline, the physiologically relevant hormone, appear to stimulate liver glycogenolysis by a cyclic AMP-independent mechanism. This conclusion appears to be valid even after repeated pulses of hormone in medium containing 10 μM total Ca^{2+}, since under these conditions α-agonist-induced glycogenolysis was greatly diminished, although glucagon-induced glycogenolysis was unimpaired.

In only one previous study (Blackmore *et al.*, 1979*b*) has any attempt been made to examine the effect of α-agonists on the Ca^{2+} content of all subcellular fractions recovered, and to correlate these fractions with organellar marker-enzyme activities. Unfortunately the results of that study were not clear, since phenylephrine induced a loss of Ca^{2+} from all fractions examined. This may have been due to a redistribution of Ca^{2+} during the isolation of the fractions, as extended fractionation times were employed and the isolation medium was devoid of Ca^{2+} flux inhibitors such as Ruthenium Red and nupercaine, although containing very high (2mM) concentrations of EGTA for a perfusion medium containing only 50 μM total Ca^{2+}.

In the present study we have further utilized the observation (Fig. 4) that the administration of phenylephrine to livers perfused with 10 μM-Ca^{2+}-containing medium results in the irreversible depletion of the hormone-sensitive Ca^{2+} pool to show that this pool is located in mitochondria and to a lesser extent in the endoplasmic reticulum. Since the maximal loss of Ca^{2+} from the mitochondrial

fraction amounts to approx. 60nmol/g wet wt. of liver, and the Ca^{2+} lost from the endoplasmic-reticulum-enriched fraction is about 40nmol/g wet wt. of liver, together these pools approximately correlate with the amount of Ca^{2+} efflux detected using the Ca^{2+}-sensitive electrode (80–120nmol/g wet wt. of liver) after a single pulse of phenylephrine. The time course of Ca^{2+} decrease in these fractions also correlates well with the Ca^{2+} efflux detected using the Ca^{2+} electrode. After 25s of α-agonist treatment we can detect significant decreases in mitochondrial Ca^{2+} content and maximal effects are observed at between 60 and 90s of treatment. The slightly longer times required until Ca^{2+} efflux can no longer be detected using the Ca^{2+} electrode is probably the result of signal spreading due to the finite electrode-response time (particularly at low Ca^{2+} concentrations) and due to flow characteristics of the perfusion tubing. Hence we predict that the actual Ca^{2+} fluxes at the cellular membranes are even more rapid and transient than recorded. After this work was completed, Blackmore *et al.* (1982) confirmed the rapidity of α-adrenergic agonist-induced Ca^{2+} efflux by showing that the total $^{45}Ca^{2+}$ content of hepatocytes decreases after only 10s of agonist treatment.

In conclusion, we suggest that α-adrenergic agonists, vasopressin and angiotensin all rapidly mobilize a small intracellular pool of Ca^{2+}, consisting predominantly of mitochondria and endoplasmic reticulum. Most of this mobilized Ca^{2+} is extruded from the cell during the first minute of agonist administration, to be subsequently reaccumulated. Hence, although extracellular Ca^{2+} appears not to play a role in the initial response of α-agonists, it does play a role in replenishing the depleted pool. This leads us to postulate that within seconds of α_1-adrenergic receptors becoming occupied, some as yet undefined mechanism ensures the transfer of information from the plasma membrane to mitochondria and the endoplasmic reticulum, resulting sequentially in Ca^{2+} release from these organelles, efflux of the ion across the plasma membrane, an increase in respiration and a stimulation of glycogenolysis. The mechanism of α_1-adrenergic-activated Ca^{2+} release and the relationship of this response to mitochondrial respiration requires further investigation.

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Section B.7

On the role of calcium in the mechanism of action of α-adrenergic agonists in rat liver.

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ON THE ROLE OF CALCIUM IN THE MECHANISM OF ACTION OF α-ADRENERGIC AGONISTS IN RAT LIVER

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1. INTRODUCTION

A number of hormones including the α-adrenergic agonists, vasopressin and angiotensin II appear to exert their physiological effects in liver by mechanism(s) independent of cyclic 3′,5′ AMP (for reviews see Exton 1979, 1980, 1981; Williamson *et al.*, 1981). An elevation of the cytoplasmic calcium ion (Ca^{2+}) concentration seems to be an integral part of the action of these hormones (Exton, 1980). The precise nature of this Ca^{2+} involvement is not fully understood but evidence for its important role in the action of α-adrenergic agonists will be detailed. In addition, we shall outline the use which has been made of a variety of drugs in the investigation of these Ca^{2+}-dependent mechanisms. Aspects of the topic have been the subject of recent reviews (Exton 1980; Williamson *et al.*, 1981).

2. α-ADRENERGIC AGONISTS, ANTAGONISTS AND RECEPTORS

Catecholamine receptors were originally divided into two classes, α and β (Ahlquist, 1948; see Wikberg, 1979) and more recently subdivided into α_1, α_2, β_1, and β_2 receptors on the basis of pharmacological potency series and radioligand binding studies. The activation of β-receptors is now fairly well understood in terms of the cyclic AMP phosphorylation cascade mechanism (Robison *et al.*, 1971; Cohen, 1979) while as yet no such unifying theory is available for α-receptors.

The study of α-receptor binding and function has relied on a group of compounds called α-adrenergic agonists, which consist of the naturally-occurring catecholamines epinephrine and norepinephrine, as well as pharmacological agents like phenylephrine, norephenylephrine, methoxamine, naphazoline, oxymetazoline, clonidine and xylazine (Fig. 1).

Receptors for α-agonists reside on the outer surface of the plasma membrane. The evidence for this stems from observations that isolated plasma membranes bind labeled catecholamines with a finite number of high-affinity sites (see below); that epinephrine, covalently fixed to a large molecular weight polypeptide, can produce all of the α-adrenergic responses of the free hormone when administered to liver tissue (Dehaye *et al.*, 1980), that isolated mitochondria and microsomes from rat liver do not possess receptors for α-agonists (El-Refai *et al.*, 1979), and that α-adrenergic receptors have recently been solubilized from rat liver plasma membranes (Guellaen *et al.*, 1979). Receptors of the α_1-class are characterized by having relatively higher affinities for phenylephrine and norphenylephrine than for clonidine, xylazine and tramazoline, while the order of affinities is reversed for α_2-adrenergic receptors. Further evidence for a distinction between α_1- and α_2-receptors comes from studies using a range of antagonists including dihydroergocryptine, phenoxybenzamine, phentolamine, prazosin and yohimbine (Fig. 2). Of these,

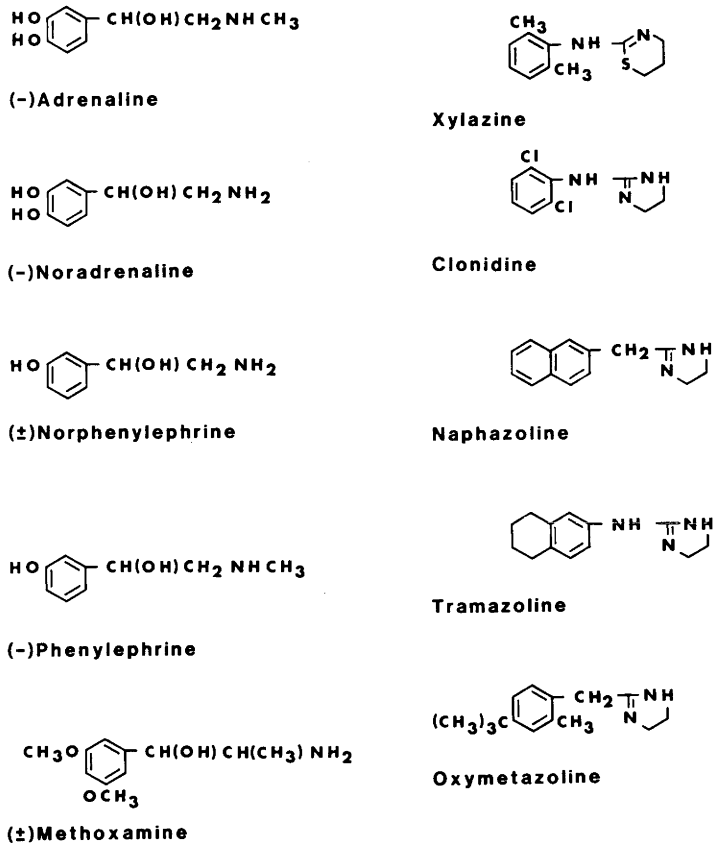


FIG. 1. Chemical structure of the α-adrenergic agonists referred to in the text.

the latter two are proving to be especially useful due to their high affinity for α_1 and α_2-receptors, respectively (Doxey *et al.*, 1977).

Alpha-receptors in rat liver plasma membranes have been characterized further by studying the "receptor-binding" of various radioactive ligands including agonists like [^{3}H]-epinephrine and [^{3}H]-norepinephrine or antagonists such as [^{3}H]-dihydroergocryptine, [^{3}H]-prazosin and [^{3}H]-yohimbine (El Refai *et al.*, 1979; El Refai and Exton, 1980; Hoffman *et al.*, 1979, 1980, 1981). There is some concern, however, over the choice of radio-ligand used to identify the binding sites and whether such binding reflects an interaction with the physiologic receptor. For example, although the radioligand [^{3}H]-dihydroergocryptine may bind to the entire α-receptor population in rat liver plasma membrane preparations (see Hoffman *et al.*, 1980), a considerable proportion of these binding sites appear to have little physiological relevance. Furthermore it is now known that low concentrations (i.e. approx 10 nM) of the endogenous ligand [^{3}H]-epinephrine, bind predominantly to α_2-receptors, whereas higher concentrations apparently bind to both α_1- and α_2-receptors (Hoffman *et al.*, 1980). Consequently early studies which employed low concentrations of [^{3}H]-epinephrine to study α_1-receptor binding, may have given erroneous results (see Hoffman *et al.*, 1980). The most precise method for quantifying the α-receptors would appear to result from using either high concentrations of [^{3}H]-epinephrine in the presence of yohimbine to mask any α_2-sites, or [^{3}H]-prazosin, the specific α_1-antagonist.

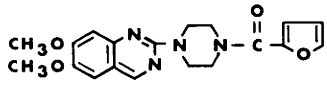
From studies involving competitive binding and the use of the selective radiolabeled antagonists [^{3}H]-prazosin and [^{3}H]-yohimbine (Hoffman *et al.*, 1981), it has been concluded that of the total population of α-receptor sites, which according to Hoffman *et al.* (1981) number about 550 fmol/mg of protein, approx 80% are of the α_1-type and approx 20% are of the α_2-type.

Prazosin and yohimbine now have been widely used to show that it is the α_1-type of receptor that mediates the rapid physiological responses of α-adrenergic agonists in rat liver. Such effects include increased rates of glycogenolysis (Hoffman *et al.*, 1980; Reinhart *et al.*, 1982a), gluconeogenesis (Kneer *et al.*, 1979), respiration (Reinhart *et al.*, 1982a), Ca^{2+} mobilization (Reinhart *et al.*, 1982c) and phosphatidylinositol turnover (Fain and Garcia-Sainz, 1980). The physiological role of α_2-receptors in liver remains unclear although there is evidence that epinephrine acts through these receptors to inhibit adenylate cyclase activity (Jard *et al.*, 1981).

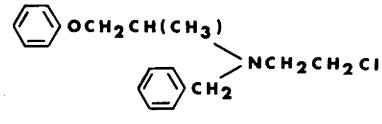
As initimated in the Introduction, α_1-receptor occupancy leads to a rapid redistribution of cell Ca^{2+} which in turn results in the various physiological responses. The evidence for this important role of Ca^{2+} in liver tissue is examined below.

3. EVIDENCE FOR A ROLE OF Ca^{2+} IN HEPATIC α-AGONIST ACTION

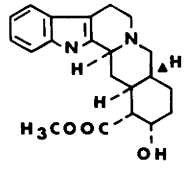
A role for Ca^{2+} in the glycogenolytic and gluconeogenic actions of the catecholamines in rat liver was initially inferred from experiments where the responses obtained in the presence or absence of added medium-Ca^{2+} were compared. Stimulation of gluconeogenesis by norepinephrine using a range of substrates was greatly diminished when hepatocytes or perfused livers were maintained for 30 min in medium containing no added Ca^{2+}. Normal gluconeogenic responses were re-established following Ca^{2+} re-addition (Kneer *et al.*, 1979; Sugano *et al.*, 1980). Similarly the stimulation of glycogenolysis by α-adrenergic agonists was partially inhibited in hepatocytes or livers pretreated



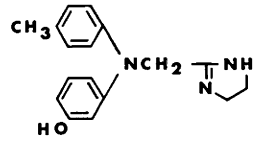
Prazosin



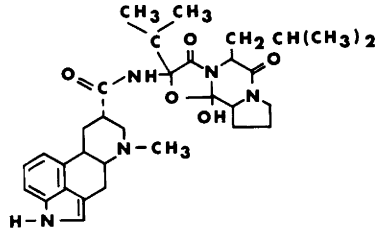
Phenoxybenzamine



Yohimbine



Phentolamine



Dihydroergocryptine

FIG. 2. Chemical structure of the α-adrenergic antagonists referred to in the text.

for varying periods of time in buffers containing low concentrations of Ca^{2+} (Garrison and Borland, 1979; Assimacopoulos-Jeannet *et al.*, 1977; Keppens *et al.*, 1977; Hue *et al.*, 1978; Van de Werve *et al.*, 1979; Althaus-Salzmann *et al.*, 1980). The divalent cation chelator EGTA [Ethylene *bis* (oxyethylene nitrolo-tetra-acetic)] was employed to chelate Ca^{2+} and so maintain very low external Ca^{2+} concentrations in a number of these studies (Kneer *et al.*, 1979; Assimacopoulos *et al.*, 1977; Keppens *et al.*, 1977; Hue *et al.*, 1978; Althaus-Salzmann *et al.*, 1980). The most severe inhibition of α-adrenergic responses was observed when EGTA concentrations up to 2.5 mM were employed or when lower concentrations of EGTA were used for prolonged periods. In those circumstances intracellular stores of Ca^{2+} may have been considerably depleted as well. Thus while the use of EGTA confirmed the requirement for Ca^{2+}, this agent alone, could not effectively discriminate between extracellular, plasma membrane-bound or intracellular pools of Ca^{2+}.

The divalent cation ionophore A23187 has also been employed to define the possible involvement of Ca^{2+} in α-adrenergic responses. When a relatively high concentration of Ca^{2+} was present in the external medium, A23187 mimicked the action of α-agonists by increasing phosphorylase a activity and stimulating glycogenolysis (Assimacopoulos-Jeannet *et al.*, 1977; Keppens *et al.*, 1977; Friedmann *et al.*, 1979). This effect was correlated with an uptake of Ca^{2+} by the perfused liver (Friedmann *et al.*, 1979). However, when low concentrations of Ca^{2+} or A23187 were employed, an efflux of Ca^{2+} was observed, presumably because the Ca^{2+} gradient across the mitochondrial membrane is dissipated and the outwardly-directed plasma membrane Ca^{2+} pump activated prior to breakdown of the plasma membrane Ca^{2+} gradient (Babcock *et al.*, 1976). In hepatocytes, low concentrations of the ionophore deplete intracellular Ca^{2+}, again mimicking the response of the isolated cells to the α-adrenergic agonists, and such pretreatment with A23187 reduced the subsequent Ca^{2+} efflux and stimulation of gluconeogenesis observed after catecholamine treatment (Chen *et al.*, 1978).

It has been reported that A23187, by depleting mitochondrial Ca^{2+} and Mg^{2+}, gives rise to secondary effects including phosphate depletion and a lowered ATP/ADP ratio Pfeiffer *et al.*, 1976). Such indirect effects may be involved where gluconeogenesis is inhibited by the ionophore (Friedmann *et al.*, 1979).

Calcium channel blockers such as verapamil (Kolhardt *et al.*, 1972) and diltiazem (Saikawa *et al.*, 1977) also inhibit the glycolytic and glycogenolytic responses to α-agonists (Kimura *et al.*, 1981). However, since these agents inhibit α-agonist receptor binding under some conditions (Blackmore *et al.*, 1979a), the effect of these agents on α-agonist action may be due to inhibition of α-receptor binding rather than Ca^{2+} channel blockade.

4. MEASUREMENT OF CELLULAR Ca^{2+} FLUXES

Initial attempts to measure α-agonist-induced changes in Ca^{2+} fluxes were performed using $^{45}Ca^{2+}$ and gave seemingly conflicting results. Some investigators proposed that α-agonists stimulated the rate of Ca^{2+} uptake by liver cells (Foden and Randle, 1978; Assimacopoulos-Jeannet *et al.*, 1977) while other workers measured either an increase in the rate of Ca^{2+} efflux (Chen *et al.*, 1978; Blackmore *et al.*, 1979b; Babcock *et al.*, 1979; Barritt *et al.*, 1981; Berthon *et al.*, 1981) or observed no effect on net cellular Ca^{2+} fluxes (Murphy *et al.*, 1980). It now appears that many of these discrepancies are due to problems associated with the use of $^{45}Ca^{2+}$ in these studies. The unequivocal interpretation of such data is made difficult due to the potential problems of non-equilibrium conditions, and α-agonist-induced alterations in the specific activity of $^{45}Ca^{2+}$.

A comprehensive kinetic analysis of the effect of α-agonists on $^{45}Ca^{2+}$ fluxes under steady-state conditions (Barritt *et al.*, 1981) has revealed that epinephrine stimulates both the long-term Ca^{2+}–Ca^{2+} cycling across the plasma membranes of hepatocytes and also transiently increases the loss of $^{45}Ca^{2+}$ from prelabeled hepatocytes. Hence α-agonist changes in hepatocyte Ca^{2+} content will depend on the net difference between Ca^{2+}

uptake and efflux pathways. It should be stressed that such differences may be quite transient.

Numerous workers have attempted to determine the effects of α-agonists on the net Ca^{2+} fluxes across the plasma membrane of hepatocytes (Chen *et al.*, 1978; Babcock *et al.*, 1979; Blackmore *et al.*, 1982) or perfused liver (Blackmore *et al.*, 1979b; Berthon *et al.*, 1981; Reinhart *et al.*, 1982c) by using either atomic absorption spectroscopy, Ca^{2+}-sensitive electrodes or Ca^{2+}-indicator dyes such as murexide and Arsenazo III. In most cases α-agonists have been shown to stimulate the net efflux of Ca^{2+} from liver, indicating that a cellular pool of the ion is being mobilized. However, a major shortcoming of these studies is that the very low (10–50 μM) extracellular Ca^{2+} concentrations used, in order to measure these Ca^{2+} flux changes, may have modified the α-agonist-induced effects. For example the kinetic analysis of $^{45}Ca^{2+}$ flux changes shows that the epinephrine-induced Ca^{2+} inflow exchange, and the transient $^{45}Ca^{2+}$ loss, are both significantly altered by reducing the extracellular Ca^{2+} concentration from 1.3 mM to 100 μM (Barritt *et al.*, 1981). Hence to bear a closer relation to the situation *in vivo*, Ca^{2+} flux-changes should be determined in the presence of physiological Ca^{2+} concentrations.

Recently a sensitive Ca^{2+}-electrode technique has been employed to measure the effect of α-agonists on net Ca^{2+} fluxes in the presence of 1.3 mM Ca^{2+} (Reinhart *et al.*, 1982c). The results of this study revealed that within seconds of administering phenylepharine to perfused liver, a transient stimulation in the rate of net Ca^{2+} efflux could be measured, resulting in the loss of approx 120 nmoles Ca^{2+}/g liver. After removal of the α-agonist, a transient stimulation of net Ca^{2+} *influx* was observed (see Fig. 3). When the extracellular Ca^{2+} concentration was reduced to 6 μM, phenylephrine induced a transient efflux of Ca^{2+} similar to that observed in 1.3 mM Ca^{2+}-containing medium but after α-agonist removal no compensatory re-uptake could be observed. Hence these studies confirm that just prior to the physiological responses, α-agonists mobilize a cellular pool of Ca^{2+}, which is rapidly expelled from the cell. Subsequent re-accumulation of Ca^{2+} apparently only occurs when the concentration of extracellular Ca^{2+} is greater than 100 μM. Presumably it is during the period of Ca^{2+} mobilization that the cytoplasmic Ca^{2+} concentration is transiently elevated. This view is supported by observations made by Murphy *et al.* (1980) who used a null-point titration technique to show that in isolated hepatocytes, phenylephrine transiently increased the cytoplasmic free Ca^{2+} concentration from 0.15 μM to 0.45 μM.

5. POOLS OF Ca^{2+} MOBILIZED BY α-AGONISTS

The pool(s) of Ca^{2+} mobilized during α-agonist stimulation remains a controversial issue, with an extracellular plasma membrane-bound pool (Althaus-Salzmann *et al.*, 1980; Kimura *et al.*, 1982) mitochondria (Blackmore *et al.*, 1979b; Babcock *et al.*, 1979; Chen *et al.*, 1978; Barritt, 1981; Murphy, *et al.*, 1980; Reinhart *et al.*, 1982c) and the endoplasmic reticulum (Berthon *et al.*, 1981) all having been implicated. The evidence implicating a plasma membrane-bound pool rests on the finding that high concentration of EGTA (2 mM) severely inhibited the phenylephrine-induced Ca^{2+} fluxes in hepatocytes suspended in a medium containing only 10 μM Ca^{2+} (Althaus-Salzmann *et al.*, 1980). However, as this EGTA treatment was carried out for a period of 30 min, it is possible that intracellular Ca^{2+} pool(s) may have been depleted by this procedure. The treatment of livers with 2 mM EGTA for 3 min, a time sufficient to reduce the extracellular free Ca^{2+} concentration to less than 10^{-9} M and displace all of the extracellular EGTA-accessible pool of Ca^{2+}, inhibits the phenylephrine-induced pulse of Ca^{2+} efflux, respiration and glucose output by only 15–20% (Reinhart *et al.*, 1983). This indicates that the α-agonist-sensitive Ca^{2+} pool is intracellular, a finding supported by a number of other workers. For instance, Babcock *et al.* (1979) used chlorotetracycline fluorescence as an indicator of Ca^{2+} associated with mitochondria, to show that norepinephrine decreases the fluorescence signal in hepatocytes, indicating a loss of mitochondrial Ca^{2+}. This finding was supported in studies where the total Ca^{2+} content of subsequently isolated

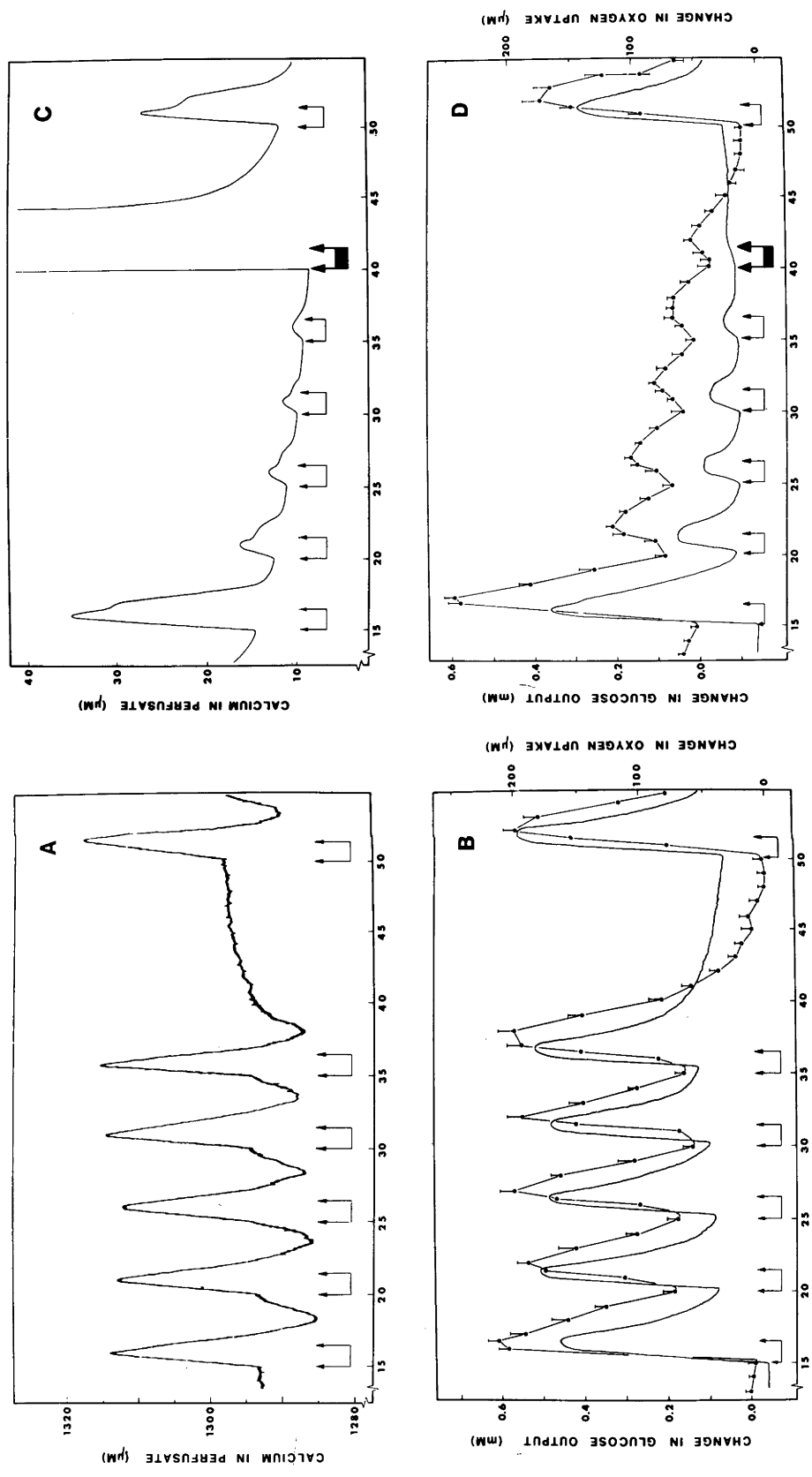


FIG. 3. Effect of the external calcium concentration on the responses to repeated administration of phenylephrine to perfused rat liver. Graphs A and B show the experiment carried out in 1.3 mM Ca^{2+}; graphs C and D show the experiment carried out in 6.8 μM Ca^{2+}. Faint arrows show the time of phenylephrine administration; bold arrows show the time of 1.3 mM Ca^{2+} administration. In graphs B and D, the continuous trace shows the respiratory response. For further details see Reinhart *et al.* (1982c). Reproduced with permission from The Biochemical Society.

mitochondria was determined (Blackmore *et al.*, 1979b; Taylor *et al.*, 1980). Similar data were obtained by Murphy *et al.* (1980) using a combination of digitonin and disruptive shear forces to rapidly fractionate hepatocytes to obtain a heavy particulate fraction enriched in mitochondria. The total Ca^{2+} content in this fraction was decreased after 2.5 min of α-agonist treatment. Recently, in an attempt to minimize the possible redistribution of Ca^{2+} during tissue disruption, a rapid fractionation technique using Percoll-density gradient centrifugation was applied to the perfused liver (Reinhart *et al.*, 1982b) and the Ca^{2+} content of all fractions in the liver correlated with marker enzyme activities (Reinhart *et al.*, 1982c). During fractionation, Ruthenium Red and the local anesthetic nupercaine (Dawson *et al.*, 1979; Dawson and Fulton, 1980) were used to block both mitochondrial Ca^{2+} uptake and efflux, respectively. It was found that by 20–40 sec following phenylephrine administration, the Ca^{2+} content in two cell fractions was significantly decreased with maximal effects being observed after 90 sec of α-agonist administration. The largest loss of Ca^{2+} occurred in a fraction consisting predominantly of mitochondria, while a significant loss of the ion also occurred in a fraction containing both endoplasmic reticulum and plasma membrane fragments. The total amount of Ca^{2+} lost from these fractions closely correlated with the total amount of Ca^{2+} efflux measured using the Ca^{2+} electrode (Reinhart *et al.*, 1982c).

Although the studies described so far indicate an involvement of Ca^{2+} in α-agonist action and some of the long-term Ca^{2+}-depletion and EGTA experiments suggest that Ca^{2+} is essential for these hormonal responses (Whitton *et al.*, 1977; Althaus-Salzmann *et al.*, 1980) these treatments may cause more fundamental alterations to hepatic metabolism which in turn could influence the responses to α-agonists. Stronger support for an obligatory role of Ca^{2+} comes from studies in which the α-agonist-sensitive pool of Ca^{2+} was depleted in relatively short-term experiments. This was achieved by perfusing rat livers with medium containing 6 μM Ca^{2+} for less than 5 min prior to repeatedly administering 90 sec pulses of phenylephrine (Fig. 3). Under these conditions α-agonists induce a net efflux of Ca^{2+} without re-accumulation of the ion and the agonist-sensitive Ca^{2+} pool is rapidly depleted. Concomitantly, α-agonist stimulation of glycogenolysis, gluconeogenesis and respiration is inhibited by up to 90% (Reinhart *et al.*, 1982a; Taylor *et al.*, 1983). A single 90 sec administration of Ca^{2+} (1.3 mM) essentially restores to normal the glycogenolytic, glucogenogenic, respiratory and Ca^{2+} efflux responses to phenylephrine. These experiments suggest that mobilization of the intracellular pool of Ca^{2+} is essential for the expression of these responses, and that extracellular Ca^{2+} plays an important role in maintaining the 'hormonally' sensitive Ca^{2+} pool.

The complete description of α-agonist-induced Ca^{2+} changes will necessarily require information regarding both net Ca^{2+} flux changes and alterations in the rate of Ca^{2+}-cycling, across all cellular Ca^{2+}-transporting membranes such as the plasma membrane, the inner mitochondrial membrane and the endoplasmic reticulum. The 'free' and 'total' Ca^{2+} concentrations in the various Ca^{2+} pools will also need to be established. An initial attempt to establish the 'total' and 'free' Ca^{2+} concentrations in mitochondria following α-agonist treatment of hepatocytes has been made (Coll *et al.*, 1982). However, these workers used Ruthenium Red-treated and uncoupled mitochondria *in vitro*, and therefore their findings may not mirror the situation *in vivo*.

6. METABOLIC CONSEQUENCE OF ALTERED CELLULAR Ca^{2+} DISTRIBUTION

In the case of α-adrenergic activation of glycogenolysis, the Ca^{2+}-sensitivity of phosphorylase b kinase provides the link between an elevated cytoplasmic Ca^{2+} concentration and the physiological response. For a recent review see Exton (1980). The activation constant $K_{(0.5)}$ for Ca^{2+} of liver phosphorylase kinase is approx 0.3 μM (Khoo and Steinberg, 1975; Van de Werve *et al.*, 1977; Sakai *et al.*, 1979) and lies within the range of cytoplasmic free Ca^{2+} concentrations recently estimated in rat hepatocytes (Murphy *et al.*, 1980). A general note of caution should be added here concerning the determination

of the activation constant $K_{(0.5)}$ for Ca^{2+} of various enzymes. To date such determinations have not been performed in media containing an ionic composition approximating that of the intracellular milieu. The studies of Haiech *et al.* (1981) clearly demonstrate that the $K_{(0.5)}$ for Ca^{2+} binding to calmodulin is increased as the Mg^{2+} and K^+ ion concentrations are elevated toward their respective intracellular concentrations. Consequently, the activation constant $K_{(0.5)}$ for Ca^{2+} of enzymes like phosphorylase kinase which involve interaction with Ca^{2+} and calmodulin need to be re-examined under the appropriate ionic conditions.

Mammalian phosphorylase kinase catalyses the Ca^{2+}-dependent phosphorylation and activation of phosphorylase b to phosphorylase a, thereby increasing the activity of the rate limiting enzyme in the glycogenolytic process (Brostrom *et al.*, 1971). Increased phosphorylase kinase activity may also be involved in the phosphorylation and inhibition of hepatic glycogen synthase by α-agonists (Strickland *et al.*, 1980) although increases in the activity of other Ca^{2+}-sensitive glycogen synthase kinases may also be involved (Srivastava *et al.*, 1979). Detailed studies concerned with the tertiary and quaternary structure of phosphorylase kinase from skeletal muscle have shown the enzyme to consist of a tetramer of four sub-units $(\alpha, \beta\ \gamma\ \delta)_4$ where the δ sub-unit is identical to the Ca^{2+}-binding protein calmodulin (Cohen *et al.*, 1978; Cohen, 1980).

Phenothiazine antipsychotic agents such as chlorpromazine, trifluoperazine and fluphenazine which bind to calmodulin and prevent the formation of the activating conformation in the presence of Ca^{2+} (Levin and Weiss, 1979; Klevit *et al.*, 1980) and thereby prevent phosphorylase kinase activation (Walsh *et al.*, 1980), have recently been used to assess the role of Ca^{2+} and calmodulin in the action of α-adrenergic agonists. These agents were shown to inhibit α-adrenergic action in the perfused liver including the stimulation of glycogenolysis and respiration (Blackmore *et al.*, 1981; Reinhart *et al.*, 1981). Conclusions drawn from these studies, however, should be treated with caution since phenothiazine derivatives stabilize membranes and decrease ion flux (Landry *et al.*, 1981) and may therefore alter metabolic responses as a result of nonspecific alterations of membrane function. In addition, these antipsychotic agents inhibit α-agonist-receptor binding (Blackmore *et al.*, 1981; Reinhart *et al.*, 1981). These binding studies are also inconclusive, however, since under the conditions employed, α_2-receptor binding was most likely measured and the effect on the more relevant α_1-receptor binding (see section on α-adrenergic receptors) has yet to be examined under the appropriate conditions.

The possible site(s) at which altered cellular Ca^{2+} concentrations may act to mediate the α-adrenergic activation of gluconeogenesis have not been clearly defined. It has been suggested that α-adrenergic agonists may stimulate gluconeogenesis by phosphorylation, and consequent inhibition, of pyruvate kinase (Garrison, 1978; Garrison *et al.*, 1979) perhaps as a result of an increase in the activity of a cytoplasmic Ca^{2+}-dependent protein kinase (Garrison *et al.*, 1980). An activation of phosphoenolpyruvate carboxykinase, indirectly mediated by Ca^{2+}, and involving the release of mitochondrial Fe^{2+}, has also been proposed (Merryfield and Lardy, 1982). Alternatively, it has been argued that a decrease in intramitochondrial Ca^{2+} may allow the activation of pyruvate carboxylase (Williamson *et al.*, 1981).

Recent studies with α-agonists in hepatocytes indicate that a Ca^{2+}-dependent exchange of reducing equivalents between cytoplasmic and mitochondrial compartments may be involved in the stimulation of gluconeogenesis from reduced substrates (Kneer *et al.*, 1979; Yip and Lardy, 1981). In those studies α-agonists induced an oxidation of the cytoplasmic NADH/NAD and it was suggested that Ca^{2+} may stimulate the glycerophosphate/dihydroxyacetone phosphate redox shuttle. These authors demonstrated that rat liver mitochondrial glycerophosphate dehydrogenase was activated by Ca^{2+} *in vitro* with an activation constant $K_{0.5}$ for Ca^{2+} of approx 0.1 μM (Wernette *et al.*, 1981).

However, in the perfused liver, Ca^{2+}-dependent activation of gluconeogenesis by α-agonists can be observed with a range of substrates without concomitant oxidation of the cytoplasmic NADH/NAD (Taylor *et al.*, 1983). Relatively oxidized substrates such as fructose and dihydroxyacetone which enter the gluconeogenic pathway at the level of the

triose phosphates were among the substrates which exhibited enhanced conversion to glucose in the presence of α-agonists in these studies.

Richards and Uyeda (1982) have described a Ca^{2+}-dependent inhibition of fructose 6-P, 2-kinase in hepatocytes following adrenaline administration. The product of this reaction, fructose 2,6-*bis*phosphate, itself a potent activator of phosphofructokinase, was also present at much lower concentrations following adrenaline treatment. Thus, stimulation of gluconeogenesis by α-agonists may also involve inhibition of phosphofructokinase and a decreased rate of cycling of fructose phosphates.

The precise role of Ca^{2+} in the stimulation of respiration by catecholamines in perfused liver is still unknown. Inhibitors of oxidative phosphorylation including rotenone, antimycin A, oligomycin and carboxyatractyloside, as well as the uncoupler 2,4-dinitrophenol have been used to identify the mitochondrial respiratory chain as the site of α-adrenergic-enhanced respiration (Reinhart *et al.*, 1982a).

The finding that the intramitochondrial enzymes pyruvate dehydrogenase (Randle *et al.*, 1974), iso-citrate dehydrogenase and α-ketoglutarate dehydrogenase (McCormack and Denton, 1979) could be activated by Ca^{2+} in cardiac muscle has led to the proposal that an increase in the intramitochondrial free Ca^{2+} concentration could stimulate mitochondrial oxidative metabolism (Denton and McCormack, 1980). Present data concerning absolute levels and changes in intramitochondrial free Ca^{2+} concentrations, however, are insufficient to rigorously test this proposal. Williamson *et al.* (1981), in a recent review, suggest that such a proposed regulatory mechanism is likely to be limited since the activation constant $K_{0.5}$ for Ca^{2+} of the intramitochondrial dehydrogenases ranges from 0.1 to 1.0 μM whilst the intramitochondrial free Ca^{2+} concentration is likely to be from 5 to 10 μM (Coll *et al.*, 1982). It should be emphasized, however, that the quoted concentrations for intramitochondrial free Ca^{2+} calculated by these authors were determined using mitochondria *in vitro* and may therefore not accurately reflect the situation *in vivo*. Nevertheless, it has been argued that control of the mitochondrial electron transport rate may reside at the level of dehydrogenase activity and NADH supply (Williamson *et al.*, 1981) since an increased reduction of pyridine nucleotides has been observed following catecholamine treatment (Sugano *et al.*, 1980). In this regard it is interesting to note that recent experiments in our laboratory show that α-agonist-induced increases in respiration in perfused livers from 48 hr-fasted rats are accompanied by elevated mitochondrial NADH/NAD and enhanced rates of ketogenesis (Taylor *et al.*, 1983).

A number of other α-agonist-induced changes have also been linked to altered Ca^{2+} fluxes. Hue *et al.* (1981) suggested that α-agonist-induced increases in glycolysis may result from an activation of phosphofructokinase following Ca^{2+}-dependent activation of glycogenolysis. These authors provided evidence that an increased glycogenolytic flux led to elevated fructose 6-phosphate levels and in turn to increased concentrations of fructose 2,6-*bis*phosphate, an activator of phosphofructokinase.

Changes in K^+ fluxes have also been reported following α-agonist treatment (Althaus-Salzmann *et al.*, 1980; Jakob *et al.*, 1980). The possible interrelationship between altered fluxes of this ion and corresponding changes in Ca^{2+} fluxes and physiological responses have received relatively little attention, although some evidence suggests that such altered K^+ ion fluxes may be dependent on Ca^{2+} flux changes and Ca^{2+} flux effects on the plasma membrane Na^+, K^+-ATPase (Burgess *et al.*, 1981; Capiod *et al.*, 1982).

Carbamoyl-phosphate synthetase and citrulline synthesis, mitochondrial components of the urea cycle, are inhibited by Ca^{2+} *in vitro* (Garrison and Borland, 1979; Meijer *et al.*, 1980; Titherage and Haynes, 1980). Coll *et al.*, (1982) have presented some evidence for an α-agonist-induced decrease in intramitochondrial free Ca^{2+} concentration and it has been argued that such a decrease in the mitochondrial free Ca^{2+} concentration may therefore account for the stimulation of the urea cycle by α-agonists (Williamson *et al.*, 1981).

Inhibition of acetyl CoA carboxylase by α-agonists has also been described (Hems,

1977) and this may result from enhanced phosphorylation catalyzed by a Ca^{2+}-dependent protein kinase (Williamson *et al.*, 1981).

Other enzymes shown to be sensitive to changes in Ca^{2+} concentration include phospholipase C and CDP diacylglycerolinositol transferase, enzymes linked to the turnover of phosphatidylinositol (see below; Irvine and Dawson, 1980). However, no direct evidence is availabe concerning their possible participation in the action of α-agonists.

7. CURRENT VIEWS ON THE NATURE OF THE α-AGONIST-INDUCED 'SIGNAL'

The present concept of α-agonist action (see Fig. 4) envisages that interaction between α-agonists and the α-receptor leads to a generation of a signal at the plasma membrane which is transmitted to intracellular organelles and results in a transient release of Ca^{2+}. The Ca^{2+} concentration in the cytoplasm is presumably increased during this phase of release (Murphy *et al.*, 1980; Barritt *et al.*, 1981) and at least some of the Ca^{2+} is expelled from the cell resulting in a net loss of the ion (Blackmore *et al.*, 1982; Reinhart *et al.*, 1982c). The mobilization of this hormone-sensitive pool of intracellular Ca^{2+} apparently forms an obligatory step in the mechanism whereby α-agonists stimulate glucose output, respiration (Reinhart *et al.*, 1982a), lactate and pyruvate formation, ketogenesis, citrate cycle activity, and changes in both the cytoplasmic and mitochondrial redox ratio (Reinhart *et al.*, 1983).

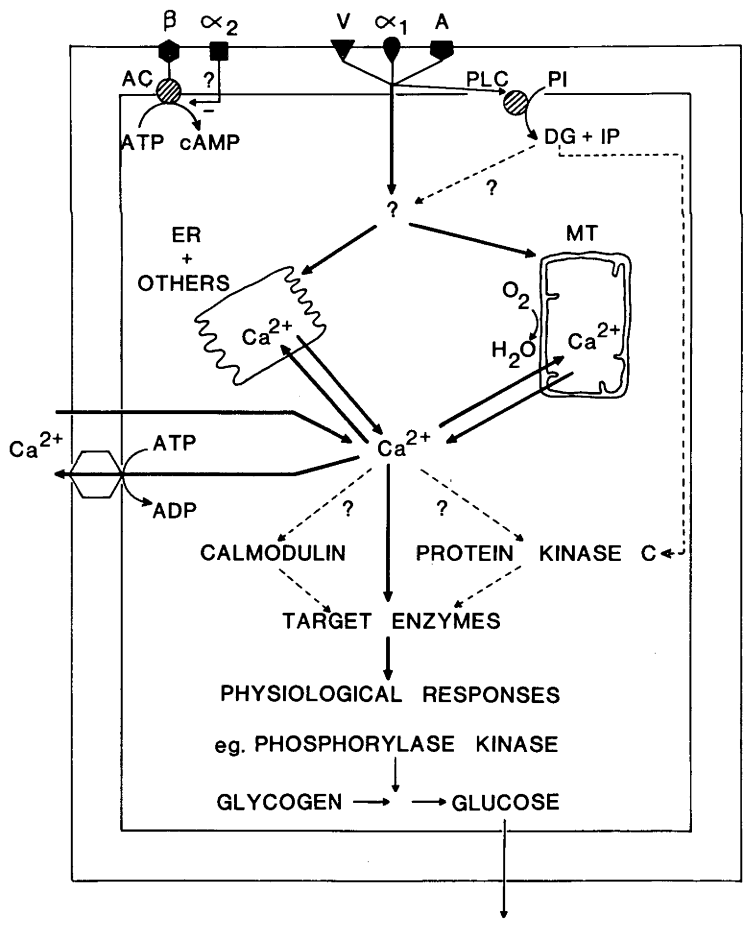


FIG. 4. Possible mechanisms of action of α-adrenergic agonists in rat liver. Abbreviations are: AC, adenyl cyclase; PLC, phospholipase C; PI, phosphatidylinositol; DG, diacylglycerol; IP, inositol phosphate; ER, endoplasmic reticulum; MT, mitochondria.

Currently, considerable effort is being directed towards the identification of the signal responsible for Ca^{2+} mobilization, and a number of metabolites or metabolic changes have been considered. They include Na^+ ions, redox ratio changes, natural Ca^{2+} ionophores, protein kinase C activation and products of phosphatidylinositol turnover such as diacylglycerol phosphatidate or arachidonic acid metabolites.

Although Na^+ has recently been shown to induce Ca^{2+} efflux from isolated rat liver mitochondria (Haworth *et al.*, 1980; Heffron and Harris, 1981; Nedergaard and Cannon, 1980) there is little evidence indicating α-agonist-induced mobilization of Ca^{2+} occurs through this mechanism. For instance the alteration of cytoplasmic Na^+ concentrations by either the Na^+ ionophore monensin, or an inhibitor of the Na^+, K^+-ATPase, digitoxin, did not affect phosphorylase kinase activity and thus did not mimic the action of α-agonists (Hughes *et al.*, 1980).

An alternative proposal suggests that mitochondrial Ca^{2+} release may be initiated by the oxidation of intramitochondrial NADPH. Isolated mitochondria release Ca^{2+} following NADPH oxidation induced by oxaloacetate, acetoacetate or hydroperoxides (Lehninger *et al.*, 1978; Prpic and Bygrave, 1980; Lötscher *et al.*, 1979, 1980). Similarly in studies with perfused livers from phenobarbitol-treated rats, the oxidation of cytoplasmic NADPH by cytochrome P-450 during the metabolism of drugs like aminopyrine, ethylmorphine, hexobarbital and t-butyl hydroperoxide also leads to Ca^{2+} mobilization and release (Sies *et al.*, 1981). However, the physiological relevance of this proposal has been questioned since the treatments used may damage the inner mitochondrial membrane prior to Ca^{2+} release (Nicholls and Brand, 1980; Beatrice *et al.*, 1980; Wolkowicz and McMillin-Wood, 1980; Bardsley and Brand, 1982). In addition, effects of α-agonists on NADPH oxidation have not been reported, while NADH/NAD^+ ratios in perfused liver (Sugano *et al.*, 1980) or isolated hepatocytes (Balaban and Blum, 1982) are increased rather than decrease after hormone treatment.

It has also been proposed that a Ca^{2+} ionophore, generated in response to α-agonists, may account for the mobilization of intracellular Ca^{2+}. This notion has received some support from studies using the ionophores A23187 and ionomycin in which these agents mimic some of the actions of α-agonists (Assimacopoulos-Jeannet *et al.*, 1977; Blackmore *et al.*, 1978; Chen *et al.*, 1978; Whiting and Barritt, 1982). However, if a natural ionophore is to rapidly mobilize intracellular Ca^{2+} by equilibration of intracellular Ca^{2+} concentration gradients, then sufficiently large gradients must be present in the relevant cellular compartments. It has recently been estimated that the Ca^{2+} concentration on the matrix side of the inner mitochondrial membrane may be only 2–3 fold greater than on the cytoplasmic side (Denton *et al.*, 1980; McCormack and Denton, 1980; Hansford and Castro, 1981). On this point, a recent comparison of the effects of A23187 and α-agonists carried out in our laboratory (Reinhart *et al.*, 1983), shows that α-agonist-induced mobilization and efflux in perfused livers is considerably more rapid than the corresponding changes induced by a range of A21387 concentrations. This contrasts with a recent report that A23187 mobilizes $^{45}Ca^{2+}$ as rapidly as α-agonists in isolated hepatocytes (Whiting and Barritt, 1982).

Considerable attention has recently been focussed on the possibility that a metabolite associated with phosphatidylinositol (PI) degradation may be part of the α-signal. Phosphatidylinositol, a quantitatively minor membrane phospholipid, is known to be rapidly hydrolysed (and resynthesised) as a result of α-agonist binding to α-receptors (Jones and Michell, 1978; Billah and Michell, 1979; Tolbert *et al.*, 1980). Hydrolysis of PI occurs by a reaction analogous to that catalyzed by phospholipase C and initially yields diacylglycerol and inositol phosphate. In liver, PI contains the highest arachidonate content of the different phospholipids, and consequently it has been proposed that the product(s) of arachidonic acid metabolism may induce the mobilization of Ca^{2+} from intracellular sources (Barritt, 1981).

Recent experimental evidence indicates that although arachidonic acid can induce a release of Ca^{2+} from hepatocytes, the concentrations required are very high and inhibitors of the cyclooxygenase and lipoxygenase pathways of arachidonic acid metabolism

did not block the effect of adrenaline on Ca^{2+} mobilization (Whiting and Barritt, 1982). The possibility that other products of PI hydrolysis are involved in the generation of some 'signal' by a mechanism other than ionophoresis, has not been excluded. For example, Takai *et al.* (1981) consider that PI turnover may be linked to the activation of the recently identified Ca^{2+}-sensitive protein kinase (C-kinase) isolated from a diverse set of tissues including liver (Takai *et al.*, 1979; Takai *et al.*, 1981). C-kinase requires Ca^{2+}, phospholipid (particularly phosphatidyl serine) and unsaturated diacylglycerol such as diolein to exhibit full catalytic activity *in vitro* (Kishimoto *et al.*, 1980). Since the effect of diolein is to increase the apparent affinity of the enzyme for phospholipid as well as for Ca^{2+}, it has been argued that PI hydrolysis may generate diacylglycerol necessary for activation of C-kinase, which in turn may phosphorylate various regulatory proteins and enzymes. It has not yet been established that diacylglycerol is an activator of C-kinase *in vivo* or if an increase in the concentration of Ca^{2+}, such as occurs after α-adrenergic stimulation (Murphy *et al.*, 1980; Barritt *et al.*, 1981) may activate the enzyme. This latter possibility is supported by a recent report that C-kinase can be activated by Ca^{2+} in the absence of any added phospholipids *in vitro* (Zabrenetzky *et al.*, 1981). An implication of this finding is that C-kinase-induced protein phosphorylation may be a response to α-adrenergic agonist-induced Ca^{2+} mobilization rather than the stimulus giving rise to this mobilization. Indeed, Garrison *et al.*, (1979) have demonstrated that the phosphorylation of a number of cytoplasmic proteins by the Ca^{2+}-mobilizing hormones vasopressin and angiotension II is inhibited by the pre-incubation of hepatocytes in Ca^{2+}-free medium. A number of antipsychotic drugs such as trifluoperazine, chlorpromazine, fluphenazine, dibucaine and haloperidol which have previously been shown to antagonise the calmodulin-induced stimulation of enzymes (Levin and Weiss, 1976; Gietzen *et al.*, 1980; Roufagalis, 1981), also appear to inhibit C-kinase activity (Mori, *et al.*, 1980; Schatzmann *et al.*, 1981). Hence, some of the effects of these drugs may relate to perturbations of Ca^{2+} metabolism through both calmodulin- and C-kinase-mediated reactions. More recently, melittin, a primary component of bee venom, has been shown to inhibit C-kinase from a range of tissues (Katoh *et al.*, 1982). The use of such inhibitors may help to clarify the possible role of C-kinase in α-agonist-induced responses.

If PI hydrolysis plays a role in the generation of the α-adrenergic signal, whatever the exact mechanism may be, then it is essential to demonstrate that (1) PI breakdown does not occur as a consequence of Ca^{2+} mobilization, (2) PI breakdown must be at least as rapid as the earliest detectable hormone response, and (3) all hormone responses must be obligatorily dependent on PI hydrolysis.

In liver tissue, evidence that PI hydrolysis can occur independently of Ca^{2+} mobilization has been based on two types of experiments. In the first, isolated hepatocytes were depleted of Ca^{2+} by prolonged treatment with EGTA until vasopressin administration no longer resulted in the activation of glycogen phosphorylase (Tolbert *et al.*, 1980). Under these conditions vasopressin-induced PI turnover, although diminished was still evident. However, α-adrenergic agonist-induced phosphorylase activation could not be inhibited even in the Ca^{2+}-free incubation medium and hence the Ca^{2+}-independence of α-agonist-induced PI turnover could not be established. In other experiments the ionophore A23187 was found to have only small variable effects on PI turnover in hepatocytes (Billah and Michell, 1979; Tolbert *et al.*, 1980). However, interpretation of these results is made difficult since phosphorylase activation was assayed 1 min after agonist treatment whilst PI turnover was not determined until 15–60 min later. Furthermore, in all cases, the resynthesis of PI rather than the initial hydrolysis was measured, and no attempt was made to ensure that under the particular experimental conditions employed A23187 did in fact elevate the cytosolic calcium concentration. Thus the Ca^{2+} independence of PI hydrolysis in liver remains to be firmly established. Recent experiments indicate that α-agonist-induced increases in Ca^{2+} mobilization and release in hepatocytes or perfused liver occur within 5–7 secs of agonist administration (Reinhart *et al.*, 1982c). Although there are reports that PI hydrolysis occurs within 10 secs in other cell types including red blood cells and leukocytes, as yet such rapid responses have not been

demonstrated in hepatocytes. Recently, Michell *et al.* (1982) presented evidence that the initial effect of Ca^{2+}-mobilizing hormones may be to stimulate the breakdown of phosphatidylinositol-4,5 *bis*phosphate rather than PI. They suggest that PI breakdown may be involved in the resynthesis of phosphatidylinositol-4,5 bisphosphate. Finally, to date there are no reports of any α-agonist-induced response being obligatorily dependent on either PI or phosphatidylinositol-4,5 bisphosphate hydrolysis.

Thus although there is much evidence indicating that PI turnover is associated with Ca^{2+} mobilizing hormones, a 'second messenger' function for this phenomenon remains to be demonstrated.

8. CONCLUDING COMMENTS

A role for Ca^{2+} in the mechanism of action of α_1-adrenergic agonists and catecholamines in rat liver now seems firmly established. When responses to these agents are mediated via α_1-receptors, the mechanism involves the mobilization of intracellular pools of Ca^{2+}. One of these is mitochondrial in origin, while the other(s) may be identified with the endoplasmic reticulum and/or plasma membrane.

The chemical nature of the α_1-agonist-induced signal linking the α_1-receptor with the intracellular Ca^{2+} pools has not been defined although the transduction of this signal must occur extremely rapidly.

An important consequence of the mobilization of intracellular Ca^{2+} is the apparent elevation of the cytoplasmic Ca^{2+} concentration. This leads to the activation of the Ca^{2+}-sensitive enzyme phosphorylase b kinase and thereby, stimulation of the glycogenolytic cascade.

Similar changes in Ca^{2+} fluxes are also involved in the stimulation by α_1-adrenergic agonists of gluconeogenesis, respiration, ureogenesis and glycolysis. However, the precise site(s) of Ca^{2+} involvement in these metabolic responses remain largely unresolved.

It is hoped that future studies will define these sites and elucidate the effects of α-agonists on changes in the free Ca^{2+} concentration in different cell compartments.

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Section B.8

Stimulation by α-adrenergic agonists of calcium fluxes, mitochondrial oxidation and gluconeogenesis in perfused rat liver.

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Stimulation by α-adrenergic agonists of Ca^{2+} fluxes, mitochondrial oxidation and gluconeogenesis in perfused rat liver

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Glucose output from perfused livers of 48h-starved rats was stimulated by phenylephrine (2μM) when lactate, pyruvate, alanine, glycerol, sorbitol, dihydroxyacetone or fructose were used as gluconeogenic precursors. Phenylephrine-induced increases in glucose output were immediately preceded by a transient efflux of Ca^{2+} and a sustained increase in oxygen uptake. Phenylephrine decreased the perfusate [lactate]/[pyruvate] ratio when sorbitol or glycerol was present, but increased the ratio when alanine, dihydroxyacetone or fructose was present. Phenylephrine induced a rapid increase in the perfusate [β-hydroxybutyrate]/[acetoacetate] ratio and increased total ketone-body output by 40–50% with all substrates. The oxidation of [1-^{14}C]octanoate or 2-oxo[1-^{14}C]glutarate to $^{14}CO_2$ was increased by up to 200% by phenylephrine. All responses to phenylephrine infusion were diminished after depletion of the hepatic α-agonist-sensitive pool of Ca^{2+} and returned toward maximal responses after Ca^{2+} re-addition. Phenylephrine-induced increases in glucose output from lactate, sorbitol and glycerol were inhibited by the transaminase inhibitor amino-oxyacetate by 95%, 75% and 66% respectively. Data presented suggest that the mobilization of an intracellular pool of Ca^{2+} is involved in the activation of gluconeogenesis by α-adrenergic agonists in perfused rat liver. α-Adrenergic activation of gluconeogenesis is apparently accompanied by increases in fatty acid oxidation and tricarboxylic acid-cycle flux. An enhanced transfer of reducing equivalents from the cytoplasmic to the mitochondrial compartment may also be involved in the stimulation of glucose output from the relatively reduced substrates glycerol and sorbitol and may arise principally from an increased flux through the malate–aspartate shuttle.

Ca^{2+} ions appear to be involved in the mechanism of action of α-adrenergic agonists in liver in both the fed and starved states (for reviews see: Exton, 1981; Williamson *et al.*, 1981; Taylor *et al.*, 1983). Although it is generally agreed that α-adrenergic agonists enhance glycogenolysis through a Ca^{2+}-dependent activation of phosphorylase *b* kinase, such clearly defined regulatory site(s) have not been elucidated for the stimulation of the gluconeogenic pathway by these agonists. Proposed sites of action of α-adrenergic agonists include pyruvate carboxylase (Garrison & Borland, 1979; Williamson *et al.*, 1981), phosphoenolpyruvate carboxykinase (Merryfield & Lardy, 1982) and pyruvate kinase (Garrison *et al.*, 1979, 1981). It has also been suggested that these agonists may stimulate gluconeogenesis from reduced substrates such as glycerol and sorbitol in hepatocytes, by promoting the exchange of reducing equivalents from the cytoplasmic to the mitochondrial compartment (Kneer *et al.*, 1979; Yip & Lardy, 1981). A Ca^{2+}-dependent activation of the mitochondrial glycerol phosphate dehydrogenase was purported to play a major role in this exchange mechanism (Yip & Lardy, 1981). However, α-adrenergic agonists have also been reported to stimulate gluconeogenesis by a mechanism apparently independent of Ca^{2+} (Kneer *et al.*, 1979).

Previously we described a method for depleting the α-adrenergic-sensitive Ca^{2+} pool in the perfused rat liver (Reinhart *et al.*, 1982*a*). In the present study this technique is employed to examine further the role of Ca^{2+} in the stimulation of gluconeogenesis by α-adrenergic agonists in the perfused liver of starved

rats. This study provides insights into the general role of Ca^{2+} and examines the possible role of mitochondrial respiration and transfer of reducing equivalents across the mitochondrial inner membrane.

Experimental

Animals and perfusions

Wistar-strain albino rats (200–280g body wt.) were starved for 48h before use. Rats were anaesthetized with sodium pentobarbitone (50mg/kg), and the livers perfused with Krebs–Henseleit (1932) bicarbonate medium equilibrated wtih O_2/CO_2 (19:1) and modified to contain 1.3mM added $CaCl_2$ unless otherwise indicated. All other perfusion details were as described previously (Reinhart *et al.*, 1982*a*).

Perfusate Ca^{2+} and oxygen determinations

Perfusate Ca^{2+} and oxygen concentrations were continuously monitored wtih a Radiometer F2112 Ca^{2+}-selective electrode and Clark-type oxygen elctrode, respectively, as detailed by Reinhart *et al.* (1982*a*). Lag times for Ca^{2+} and oxygen responses were determined as described previously (Reinhart *et al.*, 1982*b*).

Other analytical procedures

Effluent perfusate was assayed for glucose by the glucose oxidase/peroxidase method, and lag times for glucose output response were measured with [^{3}H]inulin, both as described by Reinhart *et al.* (1982*b*). In some experiments, larger volumes of perfusate (7ml) were collected in graduated centrifuge tubes, centrifuged to remove contaminating erythrocytes, and the supernatants (6ml) mixed with 0.2ml of 2M-$HClO_4$ containing 5mM-Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid]. Samples were left on ice for 10min and then neutralized with 0.2ml of 2M-KOH, and the resulting $KClO_4$ was removed by centrifugation at 2000*g* for 5min. Portions were taken for assay of pyruvate, lactate, acetoacetate and β-hydroxybutyrate, essentially as described by Bergmeyer (1974). Where relatively large volumes of perfusate were required for these multiple assays, the values obtained necessarily represented the average attained over the time of sampling (7ml corresponding to 10–15s sampling time), and corrections for response lag times were not made.

Production of $^{14}CO_2$ from labelled substrates was estimated from perfusate samples (1ml) taken by syringe from either the inflow or outflow cannulae close to the liver. Perfusate samples were injected into sealed vials containing 2ml of 100mM-sodium phosphate/citric acid buffer (pH3.0), and fitted with centre wells containing filter paper impregnated with 0.1ml of Hyamine hydroxide. After 15min, the filter papers were transferred to scintillation vials containing 10ml of scintillant {0.6% (w/v) butyl-PBD [5-(biphenyl-4-yl)-2-(4-t-butylphenyl)-1-oxa-3,4-diazole] in 2-methoxyethanol/toluene (2:3, v/v)} and their radioactivity was determined in a Beckman LS 300 scintillation counter. Samples of perfusion medium taken before passage through the liver were used for background correction and specific-radioactivity determination.

Chemicals and materials

Phenylephrine, noradrenaline hydrochloride, adrenaline bitartrate, [Arg^8]vasopressin, amino-oxyacetate and the glucose assay kit (510-A) were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A. Ca^{2+}-electrode membranes (F2002) and filling solution (S43316) were obtained from Radiometer, Copenhagen, Denmark. Lactate dehydrogenase and β-hydroxybutyrate dehydrogenase were from Boehringer, Mannheim, West Germany. Hyamine hydroxide, [1-^{14}C]octanoate and 2-oxo[1-^{14}C]-glutarate were supplied by New England Nuclear, Boston, MA, U.S.A. All other chemicals were of analytical grade.

Results

Although the glycogen contents in livers of starved rats are reported to be low (Exton & Park, 1967; Hems & Whitton, 1973) and therefore unlikely to contribute significantly to glucose output by the liver, it was important to establish this point at the outset of the present experiments. Use was made of the finding that the transaminase inhibitor amino-oxyacetate, which blocks the malate–aspartate shuttle (Borst, 1963), inhibits gluconeogenesis from lactate (Arinze *et al.*, 1973; Rognstad, 1980).

Data in Fig. 1 show that the presence of 0.2mM-amino-oxyacetate essentially abolished the increase in glucose output induced by lactate infusion in livers of 48h-starved rats. In addition, glucose output in the absence of added lactate was very low (cf. that observed in the perfused liver of fed rats; Reinhart *et al.*, 1982*a*,*b*), and was also decreased by approx. 40% by amino-oxyacetate. Moreover, phenylephrine-induced increases in glucose output in either the presence or the absence of lactate were largely prevented by the transaminase inhibitor. The maximal rate of glucose output induced by phenylephrine in the absence of lactate was only 5–10% of that observed in the fed rat (see Reinhart *et al.*, 1982*b*), and substantial increases in glucose output were only induced by phenylephrine in the presence of lactate. In other control experiments amino-oxyacetate was shown to have little effect on basal oxygen consumption by the liver and to inhibit only slightly phenylephrine-induced in-

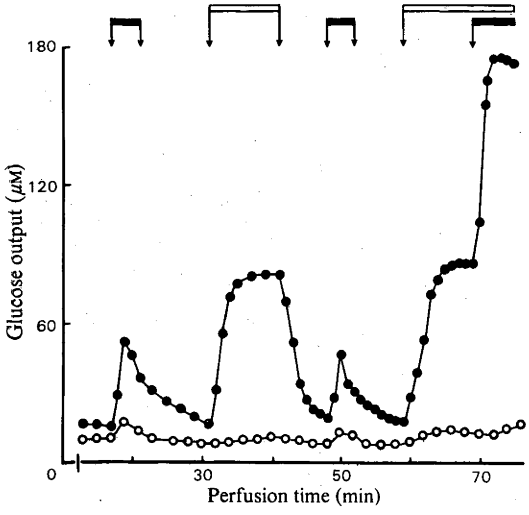

Fig. 1. *Effects of lactate, amino-oxyacetate and phenylephrine on glucose output from perfused liver*
Livers of 48h-starved rats were perfused with Krebs–Henseleit buffer as described in the Experimental section in either the presence (○) or the absence (●) of 0.2mM-amino-oxyacetate. Phenylephrine (▬) and L(+)-lactate (▭) were infused for the times indicated to give final concentrations of 2μM and 2.5mM respectively. Effluent samples were collected, contaminating erythrocytes removed by centrifugation, and samples of supernatant assayed for glucose. Data shown are from a typical experiment of three experiments performed in the presence of amino-oxyacetate and four experiments in its absence.

creases in oxygen uptake and Ca^{2+} efflux ($138 \pm 21\mu M$ versus $106 \pm 18\mu M$, and $20.6 \pm 2.1\mu M$ versus $19.8 \pm 2.0\mu M$ respectively; ±S.E.M., $n = 3$). Finally, we showed that glycogenolysis is apparently insensitive to amino-oxyacetate, since phenylephrine-induced glucose output from perfused livers of fed rats was only slightly inhibited by the transaminase inhibitor ($628 \pm 54\mu M$ versus $562 \pm 41\mu M$, $n = 4$).

We would thus conclude that the substrate- and hormone-induced increase in glucose output observed in Fig. 1 and in the data reported below for the livers of 48h-starved rats reflects that arising from gluconeogenesis.

Effect of phenylephrine on Ca^{2+} efflux, oxygen uptake and glucose output

Livers perfused with 2.5mM-lactate were treated with a maximally effective concentration of phenylephrine (2μM) and showed rapid changes in Ca^{2+} efflux, oxygen uptake and glucose output, as detailed in Fig. 2. Phenylephrine-induced increases in Ca^{2+} efflux, oxygen uptake and glucose output were

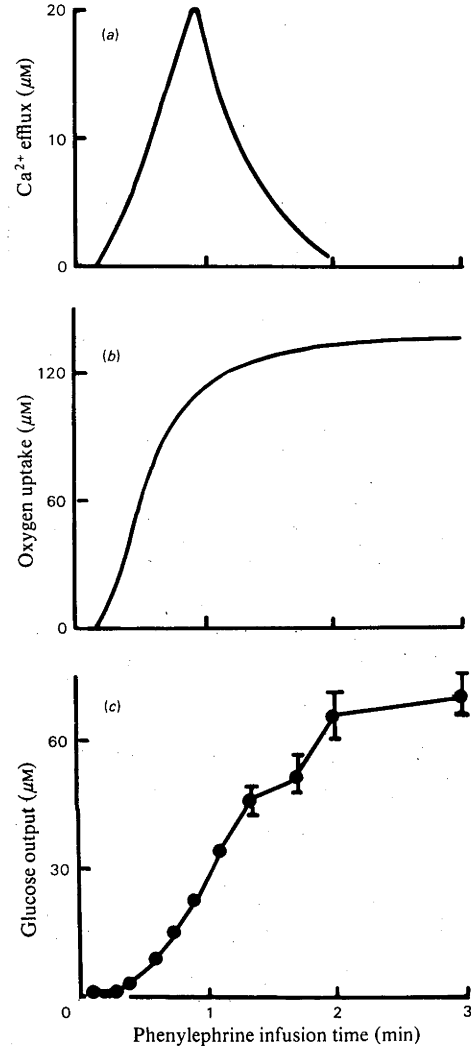

Fig. 2. *Time course for the effect of phenylephrine on Ca^{2+} efflux, oxygen uptake and glucose output*
Livers of 48h-starved rats were perfused as described in Fig. 1 in the presence of 2.5mM-lactate for 15min, and then phenylephrine (2μM final concn.) was infused. Perfusate Ca^{2+} (*a*), oxygen uptake (*b*) and glucose output (*c*) were monitored as described in the Experimental section. Increases in these during the initial 3min of phenylephrine infusion are shown. Lag times for Ca^{2+}, oxygen and glucose responses were determined separately for each experiment and data shown were corrected for these. Just before phenylephrine infusion, basal oxygen uptake was $382 \pm 38\mu M$ ($n = 8$), basal glucose output was $84 \pm 9\mu M$ ($n = 5$) and perfusate Ca^{2+} was 1.3mM. Data shown for Ca^{2+} efflux and oxygen uptake are continuous traces from one of six experiments that gave similar results. Variation between experiments was small, with S.E.M. values less than 10% of mean. Glucose-output data are the means ± S.E.M. for five separate experiments.

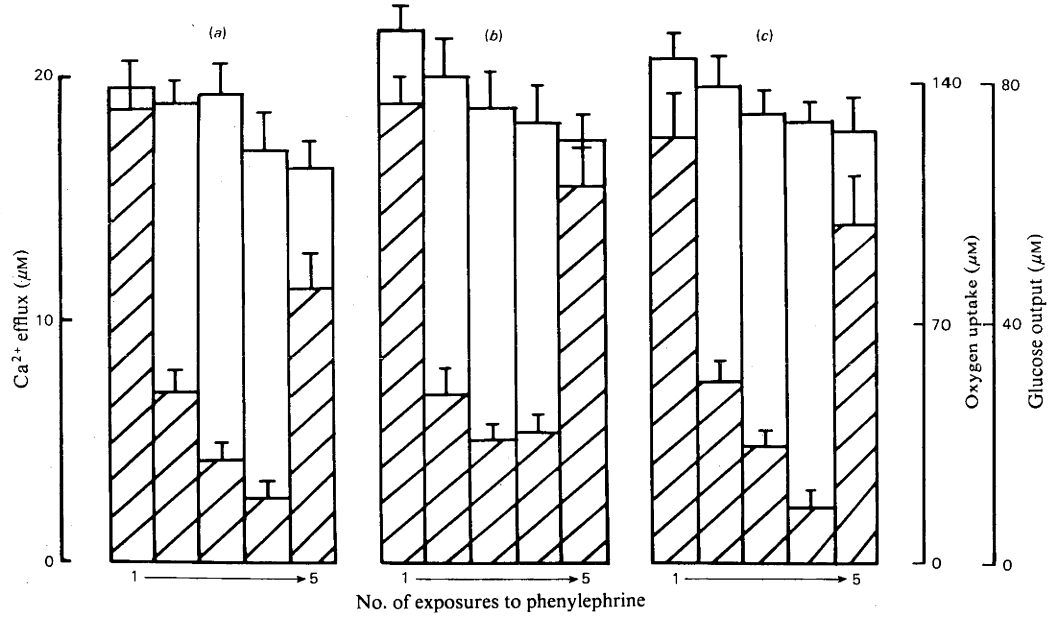

Fig. 3. *Dependence on Ca^{2+} of phenylephrine-induced increases in Ca^{2+} efflux, oxygen uptake and glucose output*
Livers were initially perfused for 15 min as described in Fig. 2. Phenylephrine (2 μM) was then infused in five pulses each of 4 min duration and at 4 min intervals except the last, which was given 8 min after the fourth pulse. In some experiments (hatched columns) the 1.3 mM-$CaCl_2$ present in the perfusion medium (administered by infusion syringe) was removed 4 min before the commencement of the phenylephrine infusions. In those experiments, 1.3 mM-$CaCl_2$ was re-administered for 2 min, 6 min before the fifth phenylephrine infusion. All other experiments were performed with 1.3 mM-$CaCl_2$ present throughout the entire perfusion (open columns). Perfusate Ca^{2+} (*a*), oxygen uptake (*b*) and glucose output (*c*) were determined as described in the Experimental section and the maximal increase induced by each phenylephrine infusion is shown. Basal values for oxygen uptake and glucose output were 345 ± 54 μM and 87 ± 11 μM respectively at a perfusate Ca^{2+} concentration of 1.3 mM, and 341 ± 50 μM and 84 ± 12 μM respectively in experiments where the $CaCl_2$ infusion was terminated and perfusate Ca^{2+} decreased to approx. 6 μM just before infusion of phenylephrine. These basal values remained essentially unchanged throughout the experimental period. Data shown are the means ± S.E.M. for five separate experiments.

evident after 9.5 ± 0.8, 10.5 ± 1.1 and approx. 20 s respectively. The increase in Ca^{2+} efflux was transient, with a maximal increase of 21.7 ± 2.3 μM being attained after 75 s of phenylephrine infusion. Ca^{2+} efflux declined thereafter and reached basal values again by 2.5 min (Fig. 2). In contrast with the transient effect on Ca^{2+} efflux, phenylephrine-induced increases in oxygen uptake and glucose output were maintained near maximal values until the α-agonist infusion was discontinued (results not shown). Near-maximal increases in oxygen uptake and glucose output were achieved after 1.5 and 2 min of phenylephrine infusion respectively (Fig. 2). The responses in terms of Ca^{2+} efflux and oxygen consumption to phenylephrine seen here are virtually identical with those seen with perfused livers from fed rats (Reinhart *et al.*, 1982*a*,*b*).

The next set of experiments involved utilizing conditions that lead to the depletion of the α-adrenergic-agonist-sensitive pool of Ca^{2+} in the perfused liver of the fed rat and were designed to test the hypothesis that intracellular Ca^{2+} is essential for α-adrenergic-agonist-induced increases in gluconeogenesis. The procedure involves perfusing the liver without added Ca^{2+} in the medium and to infuse phenylephrine by repeated pulses (see Fig. 5 of Reinhart *et al.*, 1982*a*). As shown in that paper, four such pulses, each of 90 s duration and given at 5 min intervals, were sufficient essentially to deplete the liver of the α-adrenergic-sensitive intracellular Ca^{2+} pool.

Data in Fig. 3(*a*) show that when the livers of 48 h-starved rats, perfused with buffer containing no added Ca^{2+}, were given four repeated pulses of phenylephrine, the maximum rate of Ca^{2+} efflux was decreased from 20 μM to approx. 2 μM, reflecting a loss of phenylephrine-sensitive intracellular Ca^{2+}. The response to the fifth pulse, given after a 2 min re-infusion of 1.3 mM-$CaCl_2$ to the liver, was approx. 70% of that which followed the first pulse. A parallel diminution in oxygen uptake and gluconeogenic responses was observed after the loss of intra-

Table 1. *Dependence on Ca^{2+} of phenylephrine-induced increases in glucose output*

Livers were initially perfused for 15 min as described in Fig. 2, except that the gluconeogenic substrates (each at 2.5 mM final concn.) were present as shown. Phenylephrine was administered for 4 min, and after a further 4 min the 1.3 mM-$CaCl_2$ in the perfusion medium (administered by infusion syringe) was removed. The liver was then infused with five successive pulses of phenylephrine (2 μM) each of 4 min duration as described in Fig. 3. A 2 min pulse of 1.3 mM-$CaCl_2$ was administered 6 min before the final phenylephrine infusion. Samples of perfusate were taken for glucose assay, and maximal increases in glucose output induced by phenylephrine were determined before $CaCl_2$ removal (+1.3 mM-Ca^{2+}) during the fourth infusion of the α-agonist in buffer containing no added Ca^{2+} (Ca^{2+}-depleted) and during the last infusion of phenylephrine, 4 min after a 2 min re-administration of 1.3 mM-Ca^{2+} (Ca^{2+} re-added). For each substrate the rate of basal glucose output was determined after the initial 15 min perfusion and was essentially unchanged throughout the period of perfusion. Data shown are means ± S.E.M. for at least four separate experiments.

	Glucose output (μM)			
		Phenylephrine-induced increase in output		
Substrate	Basal output	+1.3 mM-Ca^{2+}	Ca^{2+}-depleted	+1.3 mM-Ca^{2+} re-added
Alanine	33 ± 5	68 ± 4.8	10 ± 2.7	57 ± 6
Pyruvate	41 ± 6	33 ± 5.7	15 ± 3.0	28 ± 4
Lactate	83 ± 7	93 ± 7.7	12 ± 2.3	71 ± 9
Glycerol	71 ± 6	81 ± 7.4	22 ± 6.8	53 ± 8
Dihydroxyacetone	152 ± 24	67 ± 9.0	23 ± 8.0	59 ± 11
Sorbitol	78 ± 11	114 ± 15	13 ± 3.9	92 ± 13
Fructose	177 ± 34	57 ± 11	17 ± 5.0	38 ± 8
None	14 ± 4	16 ± 2.1	3 ± 0.8	7 ± 2

cellular Ca^{2+} (Figs. 3*b* and 3*c*); these responses also were largely restored after a short re-infusion of 1.3 mM-$CaCl_2$. Fig. 3 also shows that in control experiments, where 1.3 mM-$CaCl_2$ was continuously present, responses to repeated pulses of phenylephrine were only slightly diminished.

Stimulation of glucose output by phenylephrine was also observed with a range of gluconeogenic substrates, as shown in Table 1. Increases in glucose output ranged from 28% and 31% with fructose and dihydroxyacetone as substrate to 160% and 180% with sorbitol and alanine as substrate. Basal glucose output was substantially higher with fructose or dihydroxyacetone than with the other substrates, and consequently the absolute increases in glucose output induced by phenylephrine with fructose or dihydroxyacetone approached those observed with the other substrates. With all substrates tested, increases in glucose output induced by phenylephrine were substantially decreased when livers were perfused under conditions which deplete the α-agonist-sensitive Ca^{2+} pool (Table 1). In these Ca^{2+}-depletion experiments, increases in glucose output in response to phenylephrine approached maximal values again when 1.3 mM-$CaCl_2$ was re-infused for 2 min before phenylephrine treatment. Data similar to those shown for phenylephrine in Table 1 were also obtained with 50 nM-adrenaline, 50 nM-noradrenaline and 2.5 munits of vasopressin/ml (results not shown). It should be noted that, although glucose output was still enhanced by adrenaline or noradrenaline at higher concentrations of hormone, oxygen consumption was inhibited at catecholamine concentrations greater than 0.5 μM.

Thus, and by analogy with information gained earlier from experiments with fed rats (Reinhart *et al.*, 1982*a*), the present experiments provide strong evidence that intracellular Ca^{2+} is required for phenylephrine-induced stimulation of gluconeogenesis.

Effect of phenylephrine on perfusate [lactate]/[pyruvate] and [β-hydroxybutyrate]/[acetoacetate] ratios

Since it has been suggested that α-adrenergic agonists may increase gluconeogenesis from relatively reduced substrates by enhancing the transfer of reducing equivalents from the cytoplasmic to the mitochondrial compartment (Kneer *et al.*, 1979; Yip & Lardy, 1981), the effects of phenylephrine on perfusate [lactate]/[pyruvate] and [β-hydroxybutyrate]/[acetoacetate] ratios, indicators of the cytoplasmic and mitochondrial [NADH]/[NAD^+] ratios respectively, were examined with a range of gluconeogenic substrates.

Changes in [lactate]/[pyruvate] ratios were evident 30–40 s after phenylephrine infusion, to near-maximal values by 90 s (Fig. 4). The [lactate]/[pyruvate] ratio was decreased by phenylephrine when the relatively reduced substrates glycerol or sorbitol were employed and the initial ratios were relatively high (Fig. 4). With all other substrates tested, phenylephrine induced an increase in the

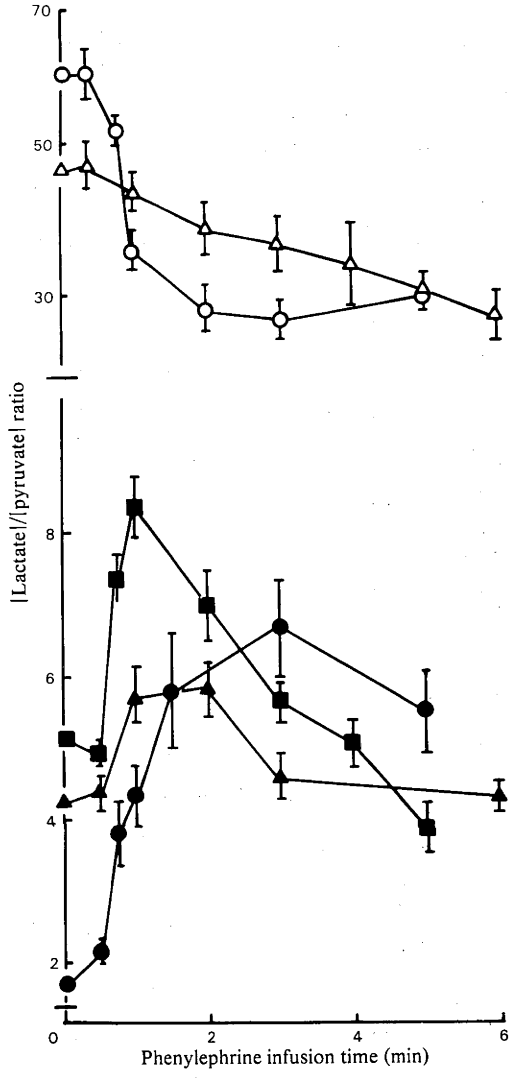

Fig. 4. *Effect of phenylephrine on the [lactate]/[pyruvate] ratio*

Livers were initially perfused for 15 min as described in Fig. 2, except that lactate was replaced by the gluconeogenic substrates (○) glycerol, (△) sorbitol, (■) dihydroxyacetone, (▲) fructose or (●) alanine, each at a final concentration of 2.5 mM. Phenylephrine (2 μM) was then infused for 6 min. Perfusate lactate and pyruvate were assayed as described in the Experimental section. Data shown are the means ± S.E.M. for five separate experiments.

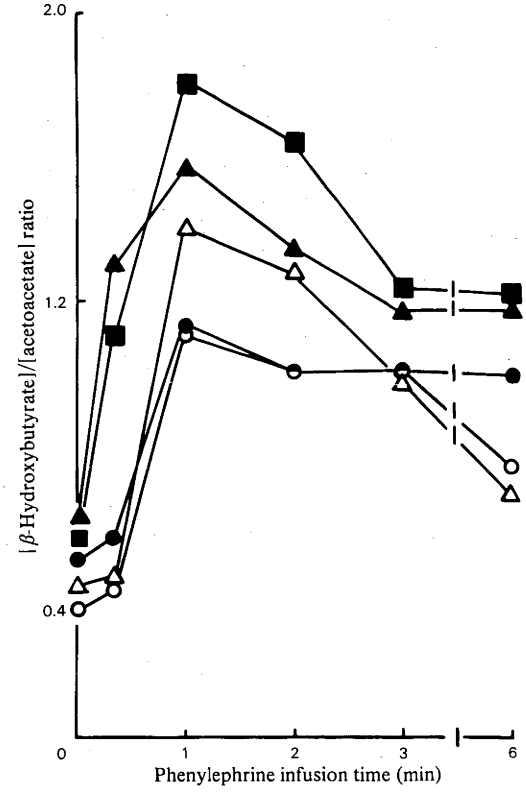

Fig. 5. *Effect of phenylephrine on the [β-hydroxybutyrate]/[acetoacetate] ratios*

Samples of perfusate obtained as in Fig. 4 were assayed for β-hydroxybutyrate and acetoacetate as described in the Experimental section. Substrates were (○) glycerol, (△) sorbitol, (■) dihydroxyacetone, (▲) fructose and (●) alanine, each at a final concentration of 2.5 mM. Data shown are the means of five separate experiments. For simplicity, S.E.M. values are not shown; however, they never exceeded 0.2 and were generally less than 0.1.

[lactate]/[pyruvate] ratio. Where fructose or dihydroxyacetone was employed, the increase in the [lactate]/[pyruvate] ratio was transient and had returned close to initial values by 6 min of phenylephrine administration.

[β-Hydroxybutyrate]/[acetoacetate] ratios were increased by phenylephrine irrespective of the substrate employed (Fig. 5). The ratio increased within 20–30 s of phenylephrine infusion, was near maximal by 90 s and decreased slightly thereafter, but was still significantly above initial values at the end of the phenylephrine infusion (6 min). Effects similar to those described for phenylephrine in Figs. 4 and 5 were also obtained after perfusion with adrenaline, noradrenaline or vasopressin (results not shown). However, effects of phenylephrine on ([lactate]/[pyruvate] and [β-hydroxybutyrate]/[acetoacetate] ratios were not observed after livers were perfused under conditions that deplete the α-agonist-sensitive Ca^{2+} pool as described by Reinhart *et al.* (1982*a*) and used in Fig. 3 and Table 1 (results not shown).

Effect of agents that alter intracellular redox ratios on glucose output

The transaminase inhibitor, amino-oxyacetate, blocks the mitochondrial malate–aspartate shuttle and thereby diminishes the transfer of reducing equivalents between the cytoplasmic and mitochondrial compartments. Data presented in Fig. 6 and Table 2 indicate that amino-oxyacetate (0.2 mM) inhibits basal glucose output from glycerol and sorbitol by 34% and 55% respectively. Moreover, phenylephrine-induced increases in glucose output from glycerol or sorbitol were inhibited by this agent by 66% and 75% respectively. Amino-oxyacetate, however, had little effect on either basal or phenylephrine-induced glucose output from fructose or dihydroxyacetone.

Phenazine methosulphate, an agent that rapidly re-oxidizes reduced nicotinamide nucleotides, and dinitrophenol, an uncoupler of oxidative phosphorylation, both increased glucose output from glycerol and sorbitol. With glycerol as substrate, phenazine methosulphate (30 μM) and dinitrophenol (25 μM) increased glucose output from 73 ± 8 to 116 ± 13 and 134 ± 18 μM ($n = 4$) respectively. With sorbitol, however, dinitrophenol was much less effective, and variable responses were obtained (average increase of 15%), whereas phenazine methosulphate was still an effective activator (112 ± 10 versus 198 ± 16 μM, $n = 4$). Phenazine methosulphate and dinitrophenol both inhibited glucose output from fructose or dihydroxyacetone and increased total pyruvate and lactate outputs (results not shown).

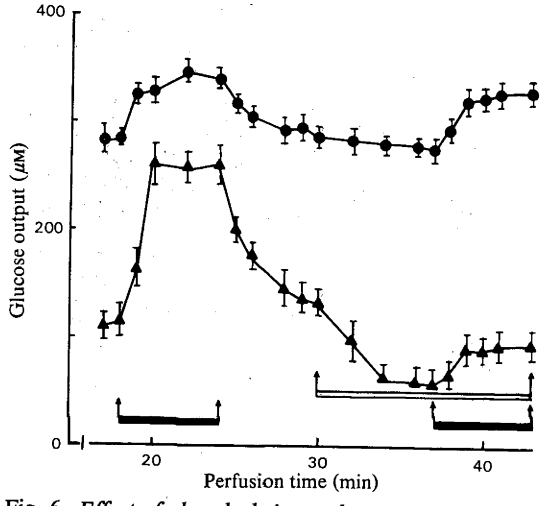

Fig. 6. *Effect of phenylephrine and amino-oxyacetate on glucose output*

Livers were initially perfused for 15 min as described in Fig. 2, except that 2.5 mM-fructose (●) or 2.5 mM-sorbitol (▲) was used as gluconeogenic substrate. Phenylephrine (2 μM, ▬) or amino-oxyacetate (0.2 mM, ▭) was then administered as indicated and perfusate samples were taken for glucose determination. Data shown are the means ± S.E.M. for four separate experiments.

Effect of phenylephrine on ketogenesis and oxidation of [1-^{14}C]octanoate and 2-oxo[1-^{14}C]glutarate

The rate of oxidation of tracer amounts of [1-^{14}C]octanoate to $^{14}CO_2$ has been used as a measure of tricarboxylic acid-cycle flux in the perfused rat liver (Soboll *et al.*, 1981). In the present study, tricarboxylic acid-cycle flux was also monitored by following $^{14}CO_2$ generation from 2-oxo-[1-^{14}C]glutarate and gave results similar to those obtained with [1-^{14}C]octanoate. Relatively low rates of $^{14}CO_2$ production were observed in the absence of gluconeogenic substrates when rates of ketone-body output were relatively high (see Table 3). For example, rates of octanoate oxidation were increased from a basal value of 0.38 ± 0.09 ng-atom/min ($n = 3$) to 0.80 ± 0.11 or 1.5 ± 0.20 ng-atom/min ($n = 4$) on addition of sorbitol or fructose respectively. Rates of ketogenesis were correspondingly decreased on addition of sorbitol or fructose (Table 3).

Table 2. *Effect of phenylephrine and amino-oxyacetate on glucose output*

Livers were perfused as described in Fig. 6 with the gluconeogenic substrates shown, each at a final concentration of 2.5 mM. Perfusate samples were taken just before and during infusion of phenylephrine or amino-oxyacetate (final concns. 2 μM and 0.2 mM respectively). Maximal changes in glucose output induced by these agents were determined as described in the Experimental section. Data shown are means ± S.E.M. for four separate experiments.

Substrate	Glucose output (μM)			
	Additions ... None	Phenylephrine	Amino-oxyacetate	Amino-oxyacetate + phenylephrine
Sorbitol	107 ± 10	254 ± 16*	59 ± 10*	97 ± 11
Glycerol	70 ± 9	146 ± 19*	46 ± 7*	71 ± 13
Fructose	281 ± 11	338 ± 14*	273 ± 9	324 ± 13
Dihydroxyacetone	196 ± 14	251 ± 18*	185 ± 16	253 ± 20

* $P < 0.01$ compared with no additions.

The effect of phenylephrine on [1-^{14}C]octanoate oxidation is shown in Fig. 7(*a*). A rapid increase in octanoate oxidation was observed within 10–15 s of phenylephrine infusion when either fructose or sorbitol was present. Maximal increases were observed between 30 and 60 s of α-agonist infusion, and rates of oxidation declined thereafter. Some variability in this latter portion of the phenylephrine response was observed. In some experiments rates of oxidation were maintained at approx. 70% of the maximal value, whereas in others the rate fell to approx. 10–20% above the basal value at the completion of the phenylephrine infusion. Essentially similar data were obtained when [1-^{14}C]-octanoate was replaced with 2-oxo[1-^{14}C]glutarate (Fig. 7*b*). Effects of phenylephrine on oxidation of [1-^{14}C]octanoate or 2-oxo[1-^{14}C]glutarate were inhibited by over 80% after livers were perfused under conditions which deplete the α-agonist-sensitive Ca^{2+} pool (results not shown).

Ketone-body output was measured during gluconeogenesis from a range of substrates shown in Table 3. Phenylephrine induced a rapid increase in ketogenesis, with maximal increases of 40–50% being observed within 2 min of α-agonist infusion. In

Table 3. *Effect of phenylephrine on total ketone-body output*

Perfusate samples were taken immediately before and 2 min after infusion of phenylephrine (2 μM) as described in Fig. 4. Ketone bodies were determined as described in the Experimental section. Data shown are the means ± S.E.M. for five separate experiments.

Substrate	Additions	Total ketone-body output (μM) None	Phenylephrine
None		103 ± 5.8	111 ± 6.1
Alanine		29 ± 1.4	48 ± 6.4*
Glycerol		38 ± 1.0	55 ± 1.4*
Dihydroxyacetone		39 ± 4.3	55 ± 3.8*
Fructose		34 ± 2.5	51 ± 2.2*
Sorbitol		36 ± 1.4	51 ± 1.5*

* $P < 0.01$ compared with respective control.

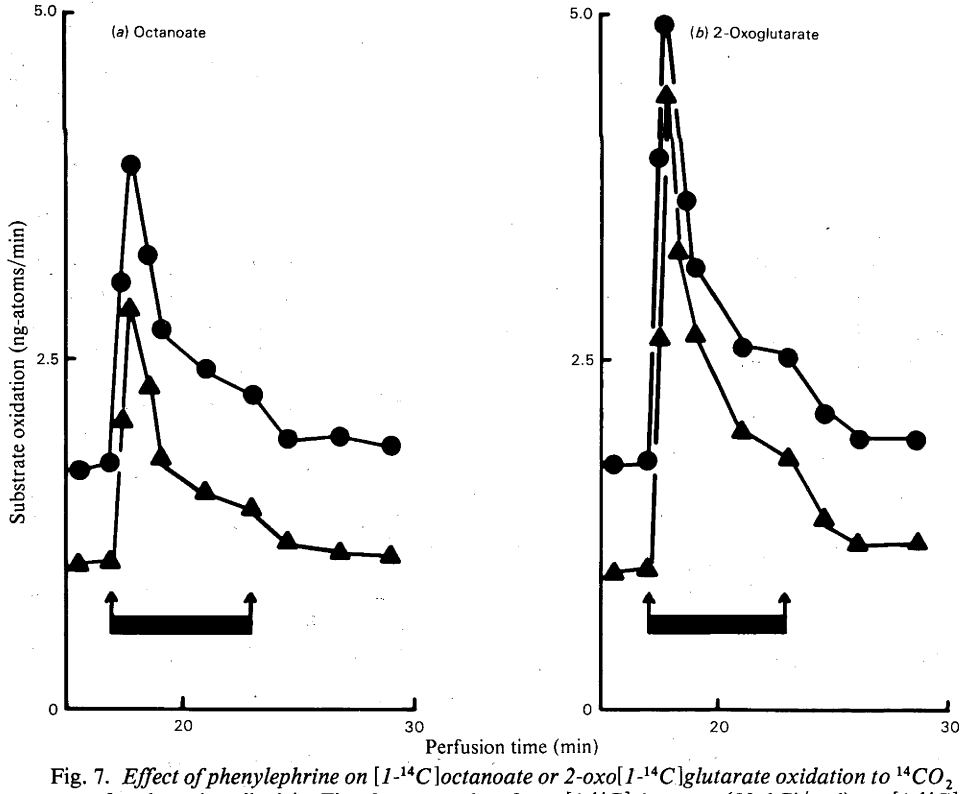

Fig. 7. *Effect of phenylephrine on [1-^{14}C]octanoate or 2-oxo[1-^{14}C]glutarate oxidation to $^{14}CO_2$*

Livers were perfused as described in Fig. 6, except that 2-oxo[1-^{14}C]glutarate (53.6 Ci/mol) or [1-^{14}C]octanoate (25.1 Ci/mol) was continuously infused to give final concentrations of 0.20 and 0.55 μM respectively. The substrates fructose (●) or sorbitol (▲) were used at final concentrations of 2.5 mM and phenylephrine (▬) was infused at a final concentration of 2 μM as indicated. Samples of perfusate were assayed for $^{14}CO_2$ as described in the Experimental section. Data shown are from one experiment of four, each of which gave similar results.

the absence of gluconeogenic substrates, ketone-body output was elevated and phenylephrine-induced increases were not significant under those conditions. Phenylephrine did not affect ketone-body output after livers had been perfused under conditions that deplete the α-agonist-sensitive Ca^{2+} pool (results not shown).

Discussion

Among the major points revealed in this study of α-agonist-induced gluconeogenesis in the perfused liver of starved rats are (*a*) the obligatory role of Ca^{2+} in the gluconeogenic response, (*b*) the variety of metabolic events seemingly associated with the gluconeogenic response and which are also Ca^{2+}-dependent, and (*c*) the rapidity with which the responses are seen. Of particular interest in this regard was the observation that ketogenesis and tricarboxylate-cycle activity are each increased by α-agonists concomitant with enhanced rates of gluconeogenesis.

An obligatory role for Ca^{2+} in α-agonist-induced gluconeogenesis could be deduced from experiments in which the α-agonist-sensitive intracellular pool of Ca^{2+} was manipulated by the administration of phenylephrine through repeated pulses to the liver perfused with bicarbonate buffer containing no added Ca^{2+} (Fig. 3, Table 1). In this way it could be shown that the smaller the α-agonist-induced Ca^{2+} efflux (and presumably the greater the depletion of the α-agonist-sensitive Ca^{2+} pool), the smaller the gluconeogenic response. It was noteworthy that the Ca^{2+}-dependence of the α-agonist-induced increase in glucose output was observed with all gluconeogenic substrates tested (Table 1 and Fig. 3).

An additional feature of our experimental approach (Reinhart *et al.*, 1982*a*), hitherto apparently not undertaken in studies on gluconeogenesis, was the ability to measure rapid and continuous changes in Ca^{2+} fluxes across the plasma membrane and in whole-tissue respiration; such respiration is attributable in large part to mitochondria (Reinhart *et al.*, 1982*b*). In this way we were able to show that such changes precede phenylephrine-induced increases in glucose output. Indeed the time-course and magnitude of Ca^{2+} efflux induced by phenylephrine in these perfusions with livers from starved rats were virtually identical with the phenylephrine-induced Ca^{2+} efflux previously shown to precede glycogenolysis in perfused livers of fed rats (Reinhart *et al.*, 1982*a*). The important conclusion can thus be drawn that such changes in Ca^{2+} efflux are independent of the nutritional status of the experimental animal. The findings also suggest that altered Ca^{2+} fluxes may be linked to the initial events in the mechanism of action of α-adrenergic agonists in rat liver.

The important role of Ca^{2+} in the responses to α-adrenergic agonists in rat liver was further highlighted by the findings that the changes induced by phenylephrine in the redox state of the cytoplasm (Fig. 4) and mitochondria (Fig. 5), as well as in ketogenesis (Table 3) and the oxidation of [1-^{14}C]octanoate or 2-oxo[1-^{14}C]glutarate (Fig. 7), were all considerably diminished after depletion of the α-agonist-sensitive Ca^{2+} pool. As with gluconeogenesis (Fig. 3, Table 1), re-infusion of livers with 1.3 mM-$CaCl_2$ restored all of these responses.

A requirement for Ca^{2+} in the stimulation of gluconeogenesis by α-adrenergic agonists has been reported previously and a number of possible sites for Ca^{2+} action have been suggested. Tolbert & Fain (1974) reported a Ca^{2+}-dependent catecholamine-induced increase in glucose output from a range of gluconeogenic substrates with rat hepatocytes. However, in those studies, basal rates of gluconeogenesis were markedly decreased by the omission of Ca^{2+}. This is in contrast with the present study and earlier studies with perfused liver (Friedmann & Rasmussen, 1970; Sugano *et al.*, 1980) or hepatocytes (Kneer *et al.*, 1979), where Ca^{2+} omission did not cause appreciable changes in the basal rates of gluconeogenesis. More recently, Garrison *et al.* (1979, 1981) have suggested that a cytoplasmic Ca^{2+}-dependent protein kinase may catalyse the phosphorylation and subsequent inhibition of pyruvate kinase in response to catecholamines. In addition, an activation of phosphoenolpyruvate carboxykinase, indirectly mediated by Ca^{2+}, and involving the release of mitochondrial Fe^{2+}, has been proposed by Merryfield & Lardy (1982). It has also been argued that Ca^{2+} may regulate pyruvate carboxylase activity (Williamson *et al.*, 1981).

A Ca^{2+}-sensitive exchange of reducing equivalents between cytoplasmic and mitochondrial compartments involving an increased mitochondrial glycerol phosphate dehydrogenase activity has been proposed to account for the Ca^{2+}-dependent increase in gluconeogenesis from reduced substrates such as glycerol and sorbitol in hepatocytes prepared from 24 h-starved rats (Kneer *et al.*, 1979; Yip & Lardy, 1981). Concentrations of amino-oxyacetate that block the malate–aspartate shuttle reportedly had no effect on noradrenaline-induced glucose output from these relatively reduced substrates (Kneer *et al.*, 1979).

Some of the findings reported by Kneer *et al.* (1979) and Yip & Lardy (1981) differ substantially from the data obtained with perfused livers as documented in the present study. The different results obtained may in part reflect differences between the hepatocytes and perfused liver preparations used, as well as differences in the time of α-agonist treatment and the techniques employed to deplete the relevant Ca^{2+} pool(s). In this regard, it is

noteworthy that hepatocyte preparations have previously been reported to become depleted of malate and aspartate, and consequently exhibit low malate–aspartate-shuttle activities (Cederbaum *et al.*, 1977). In addition, in relatively long-term experiments such as those of Kneer *et al.* (1979), inhibition of reducing-equivalent transfer by amino-oxyacetate may be by-passed as glycerol phosphate concentrations rise and flux through the mitochondrial glycerophosphate dehydrogenase increases (Williamson *et al.*, 1971).

In our hands amino-oxyacetate significantly decreased basal glucose output from sorbitol and glycerol and effectively blocked the phenylephrine-induced increases in glucose output observed with these reduced substrates. Data in the present study then indicate that α-adrenergic agonists may increase gluconeogenesis from glycerol and sorbitol by promoting the removal of cytoplasmic reducing equivalents by the operation of the malate–aspartate shuttle, although some involvement of the glycerol phosphate–dihydroxyacetone phosphate shuttle cannot be ruled out. The mechanism whereby α-agonists may stimulate the malate–aspartate shuttle is unknown, but may be related either to changes in total amounts of shuttle intermediates or to a change in the energization of the mitochondrial inner membrane, which may in turn affect the electrogenic exchange of aspartate for glutamate.

Other data presented are consistent with the suggestion that glucose output from glycerol and sorbitol may be enhanced by an increase in the rate of oxidation of cytoplasmic nicotinamide nucleotides. The electron acceptor phenazine methosulphate, and to a lesser extent dinitrophenol, an uncoupler of oxidative phosphorylation, enhanced glucose output from both of these reduced substrates. In addition, phenylephrine-induced increases in glucose output with glycerol or sorbitol were accompanied by a decrease in the [lactate]/[pyruvate] ratio and an increase in the [β-hydroxybutyrate]/[acetoacetate] ratio.

This study also shows an α-agonist-induced elevation of the [β-hydroxybutyrate]/[acetoacetate] ratio (the mitochondiral redox ratio) with every substrate tested. Sugano *et al.* (1980) reported that noradrenaline increased the total cellular reducing equivalents in perfused liver of starved rats with either pyruvate of lactate as substrate, and presented some evidence for a mitochondrial site of reducing-equivalent generation. In studies with hepatocytes where lactate was the substrate, Siess *et al.* (1978) demonstrated an increase in the mitochondrial redox ratio and a lowered mitochondrial 2-oxoglutarate content in response to catecholamines. Our studies of the conversion of [1-^{14}C]-octanoate or 2-oxo[1-^{14}C]glutarate into $^{14}CO_2$ suggest that α-adrenergic agonists rapidly stimulate tricarboxylic acid-cycle flux. We also observed a 40–50% increase in total ketone-body output. Sugden *et al.* (1980) have reported the stimulation of conversion of [1-^{14}C]oleate into $^{14}CO_2$ in rat hepatocytes after administration of adrenaline or vasopressin. Data from the present study and that of Sugden *et al.* (1980) suggest that α-agonists may increase the mitochondrial redox ratio mainly as a result of an increased rate of β-oxidation of fatty acids and an increased flux through the tricarboxylic acid cycle. A significant contribution of cytoplasmic reducing-equivalent transfer to the mitochondrial compartment may only occur when the initial cytoplasmic [NADH]/[NAD$^+$] ratio is relatively high, for example when glycerol or sorbitol is employed.

Although Ca^{2+} has been reported to stimulate both isocitrate dehydrogenase and 2-oxoglutarate dehydrogenase (McCormack & Denton, 1981), and a Ca^{2+}-dependent inhibition of acetyl-CoA carboxylase has been observed in hepatocytes treated with α-adrenergic agonists (Ly & Kim, 1981), it is not known if these effects are involved in the stimulation of tricarboxylic acid-cycle flux and ketogenesis described in the present study.

We also report the Ca^{2+}-dependent activation by α-adrenergic agonists of glucose output from fructose and dihydroxyacetone, substrates which enter the gluconeogenic pathway at the triose phosphate level. Since these changes were observed in the absence of significant changes in glycolysis (lactate + pyruvate output unchanged), α-adrenergic agonists may stimulate the gluconeogenic pathway at other site(s) located between the triose phosphates and glucose. A Ca^{2+}-dependent inhibition of fructose 6-phosphate 2-kinase, and lowered concentration of the activator of phosphofructokinase, fructose 2,6-bisphosphate, have been described in hepatocytes after treatment with adrenaline (Richards & Uyeda, 1982). Thus α-agonists may also inhibit phosphofructokinase and decrease the rate of cycling of fructose phosphates. However, such a proposed action may be limited in the starved state, as phosphofructokinase activity and the cycling of fructose phosphate are already extremely low in starved-rat liver (Kummel, 1982; Van Schaftingen *et al.*, 1980). Consequently, a future study should examine the possibility that α-adrenergic agonists may stimulate fructose 1,6-bisphosphatase or glucose 6-phosphatase activities.

We are grateful to Mrs. J. Lindley for assistance in some of these experiments. This work was supported by a grant to F. L. B. from the National Health and Medical Research Council of Australia.

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Section B.9

The effect of A23187 on calcium fluxes and α-adrenergic agonist action in perfused rat liver.

(Reinhart et al., 1983a)

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The effect of ionophore A23187 on calcium ion fluxes and α-adrenergic-agonist action in perfused rat liver

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The effect of ionophore A23187 on cellular Ca^{2+} fluxes, glycogenolysis and respiration was examined in perfused liver. At low extracellular Ca^{2+} concentrations ($<4\mu M$), A23187 induced the mobilization of intracellular Ca^{2+} and stimulated the rate of glycogenolysis and respiration. As the extracellular Ca^{2+} concentration was elevated, biphasic cellular Ca^{2+} fluxes were observed, with Ca^{2+} uptake preceding Ca^{2+} efflux. Under these conditions, both the glycogenolytic response and the respiratory response also became biphasic, allowing the differentiation between the effects of extracellular and intracellular Ca^{2+}. Under all conditions examined the rate of Ca^{2+} efflux induced by A23187 was much slower than the rate of phenylephrine-induced Ca^{2+} efflux, although the net amounts of Ca^{2+} effluxed were similar for both agents. The effect of A23187 on phenylephrine-induced Ca^{2+} fluxes, glycogenolysis and respiration is dependent on the extracellular Ca^{2+} concentration. At concentrations of less than $50\mu M$-Ca^{2+}, A23187 only partially inhibited α-agonist action, whereas at 1.3 mM-Ca^{2+} almost total inhibition was observed. The action of A23187 at the cellular level is complex, dependent on the experimental conditions used, and shows both differences from and similarities to the hepatic action of α-adrenergic agonists.

The study of Ca^{2+} fluxes across membranes was greatly facilitated by the finding that the carboxylic antibiotic A23187 is a bivalent cation ionophore with a high association constant for Ca^{2+}, catalysing the electroneutral exchange of Ca^{2+} for two protons through the formation of a charge-neutral lipid-soluble complex (Reed & Lardy, 1972; Deber & Pfeiffer, 1976). Although initially used to examine Ca^{2+} fluxes in isolated mitochondria (Reed & Lardy, 1972; Wong *et al.*, 1973; Schuster & Olson, 1974; Sordahl, 1974), A23187 is now extensively employed in more complex experimental systems involving the use of whole cells (Kleineke & Stratman, 1974; Chen *et al.*, 1978; Akerman & Nicholls, 1981; Peaucellier *et al.*, 1982) or tissues (Friedmann *et al.*, 1979; Hoye *et al.*, 1979; Bihler *et al.*, 1980; Sugden, 1980; Hasper *et al.*, 1982).

Although the ionophore has been used extensively in studies with perfused liver (Friedmann *et al.*, 1979), or with hepatocytes (Blackmore *et al.*, 1978; Foden & Randle, 1978; Chen *et al.*, 1978), its effect on the redistribution of cellular Ca^{2+} has not been clearly resolved, with some investigators reporting Ca^{2+} efflux (Kleineke & Stratman, 1974; Chen *et al.*, 1978), whereas others report Ca^{2+} uptake (Friedmann *et al.*, 1979) or biphasic responses with efflux following uptake (Burgess *et al.*, 1979) or uptake following efflux (Blackmore *et al.*, 1978).

The interpretation of results from experiments of this type, however, is often difficult, since, although the basis for the action of A23187 is usually ascribed to the dissipation of the Ca^{2+} gradient across the plasma membrane and hence an elevation in the concentration of cytosolic Ca^{2+}, this has rarely been verified by direct measurement.

Variability may be due also to the particular experimental conditions used by different groups, such as the pretreatment of livers or cells, the Ca^{2+} content in incubation media, the ionophore concentration, the length of time for which cells are exposed to the ionophore and the technique used to assay Ca^{2+} fluxes.

Recently we developed a technique (Reinhart *et al.*, 1982*a*) that allows the continuous and quantitative measurement of Ca^{2+} fluxes in rat liver perfused with Ca^{2+} concentrations ranging from only several μM to 1.3 mM. In the present study we have used this technique to examine the effect of A23187 on the redistribution of Ca^{2+} in the perfused rat liver under a variety of experimental conditions. Additional information about the effects of this

agent on cell metabolism was obtained by monitoring the rate of glycogenolysis and respiration, each of which, in liver, appears to be sensitive to the redistribution of intracellular Ca^{2+} (Khoo & Steinberg, 1975; Shimazu & Amakawa, 1975; Assimacopoulos-Jeannet *et al.*, 1977; Sakai *et al.*, 1979). Furthermore, in light of suggestions that A23187 alters Ca^{2+} fluxes in a manner similar to flux changes induced by α-adrenergic agonists (Whiting & Barritt, 1982), we have compared the ability of these two agents to induce changes in Ca^{2+} fluxes and metabolism in the perfused rat liver.

Experimental

Animals and perfusions

Male Wistar-strain albino rats weighing between 230 and 270g and having free acess to food were used for all experiments. Rats were anaesthetized with sodium pentobarbitone (50mg/kg body wt.), and the livers were perfused with Krebs–Henseleit (1932) bicarbonate medium equilibrated with O_2/CO_2 (19:1) as described previously (Reinhart *et al.*, 1982*b*). Livers were perfused at a flow rate of 3.5ml/min per g wet wt. of liver, at 35°C. All livers were preperfused for at least 10min with medium containing 1.3mM added $CaCl_2$. At 10min, the free Ca^{2+} concentration in the perfusate was altered either by decreasing the concentration of added $CaCl_2$, or by adding low concentrations of EGTA to the perfusate, and 5min later A23187 was administered by infusion syringe at a flow rate of 0.05% of the total flow, at the final concentrations indicated. The ionophore was solubilized in either ethanol or dimethyl sulphoxide. Preliminary experiments had shown that at concentrations above 0.1% dimethyl sulphoxide significantly alters both mitochondrial respiration and the basal rate of glycogenolysis, and ethanol stimulates the rate of respiration (P. H. Reinhart, W. M. Taylor & F. L. Bygrave, unpublished work). Hence with ethanol as solvent corrections were made for minor ethanol-induced respiratory changes. When dimethyl sulphoxide was used, this agent was infused at less than 0.002% (v/v), at which concentration effects on respiration and glycogenolysis were insignificant. All experiments were performed between 08:00h and 12:00h to minimize diurnal fluctuations of basal metabolism.

Perfusate Ca^{2+} determinations

For most experiments the perfusate Ca^{2+} concentration was measured continuously with a Radiometer F2112 Ca^{2+}-selective electrode as described previously (Reinhart *et al.*, 1982*a*). For each experiment the electrode was calibrated by infusing known amounts of $CaCl_2$, at a constant flow rate, between the liver and the electrode. Neither oxygen- nor glucose-concentration changes in the perfusate interacted with the Ca^{2+}-electrode response (results not shown).

The ionophore induced a slight decrease in the Ca^{2+}-electrode baseline signal (results not shown), and all data shown have been corrected for this response. The electrode response to standard $CaCl_2$ solutions was not altered by the presence of A23187 (results not shown). Electrode membranes were discarded if voltage changes deviated more than 15% from theoretical (Nernstian) values. Other control experiments are detailed by Reinhart *et al.* (1982*a*). As the logarithm of the electrode response becomes non-linear at Ca^{2+} concentrations below 1μM, atomic-absorption spectroscopy was used to determine total Ca^{2+} concentration changes in experiments where EGTA was used to lower the Ca^{2+} concentration below 1μM. For this procedure, samples of perfusate (4ml) were assayed in 0.1% KCl with a N_2O/acetylene flame (medical grade gases).

Calculations of free Ca^{2+} concentration

This was done with an algorithm of the program (Comics) developed by Perrin & Sayce (1967). The logarithms of the total formation constants (Sillen & Martell, 1971) for the following complexes were considered: (pH = 7.4): $PO_4^{3-} + H^+$ (11.8), $EGTA^{4-} + Ca^{2+}$ (11.0), $CO_3^{2-} + H^+$ (10.25), $EGTA^{4-} + H^+$ (9.54), $HEGTA^{3-} + H^+$ (8.93), $HPO_4^{2-} + H^+$ (7.15), $HEGTA^{3-} + Ca^{2+}$ (5.3), $EGTA^{4-} + Mg^{2+}$ (5.2), $HEGTA^{3-} + Mg^{2+}$ (3.4), $HCO_3^- + Mg^{2+}$ (3.4), $CO_3^{2-} + Ca^{2+}$ (3.2), $HPO_4^{2-} + Ca^{2+}$ (2.77), $H_2EGTA^{2-} + H^+$ (2.73), $HPO_4^{2-} + Mg^{2+}$ (2.5), $H_2PO_4^- + Ca^{2+}$ (1.5).

Perfusate glucose and oxygen determinations

Glucose release by the liver was determined by the glucose oxidase/peroxidase method as previously described (Reinhart *et al.*, 1982*b*) and perfusion-circuit lag-times were corrected for also as described in that paper.

Chemicals and materials

A23187 was obtained from Calbiochem-Behring Corp., La Jolla, CA, U.S.A. Phenylephrine and the glucose assay kit (510-A) were obtained from Sigma. Ca^{2+}-electrode membranes (F2002) and filling solution S43316 were obtained from Radiometer, Copenhagen, Denmark. Dimethyl sulphoxide was obtained from Ajax Chemicals, Sydney, N.S.W., Australia. Other chemicals used were of analytical grade.

Expression of data

Data are expressed as means ± S.E.M. for the numbers of independent experiments described in the legends to the Figures.

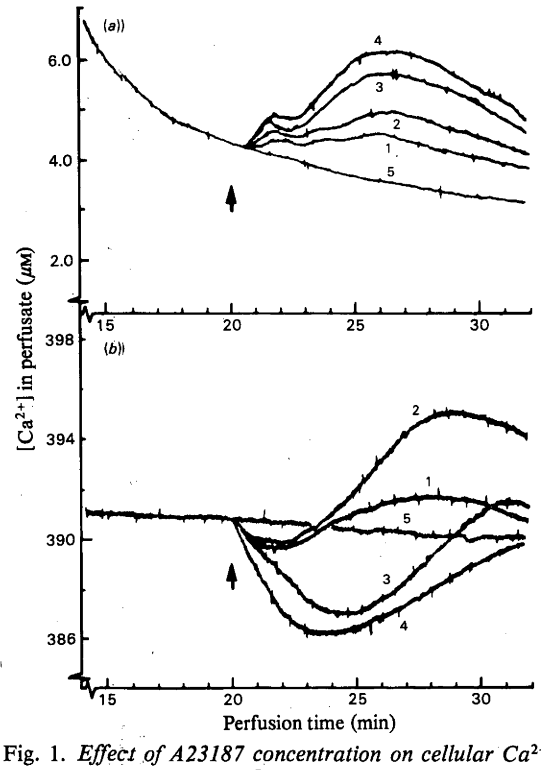

Fig. 1. *Effect of A23187 concentration on cellular Ca^{2+} fluxes*

Livers of fed rats were perfused with medium containing 1.3mM-Ca^{2+} for 12min as described in the Experimental section. Changes in the perfusate Ca^{2+} concentration were measured continuously by using a Ca^{2+} electrode modified for a flow-through mode of operation. At 15min the perfusate Ca^{2+} concentration was decreased to either 4.0μM (*a*) or 390μM (*b*). At 8min thereafter (arrowed), A23187 was infused at final concentrations of 0.25μM (1), 0.5μM (2), 1.0μM (3) or 2.0μM (4). Trace 5 is a recording in which no infusions were performed. Traces shown are typical of those obtained for between 5 and 11 independent experiments performed for each concentration of the ionophore.

Results

Effect of A23187 on cellular Ca^{2+} fluxes, glycogenolysis and respiration

Previous studies with either isolated organelles (Reed & Lardy, 1972) or intact cells (Babcock *et al.*, 1976; Blackmore *et al.*, 1978; Akerman & Nicholls, 1981) have indicated that a number of responses mediated by A23187 show differential sensitivities to either the concentration of the ionophore or the ambient Ca^{2+} concentration. Hence in the present investigation of the action of A23187 in perfused liver we have systematically altered both of these variables.

The data in Fig. 1 show the effects of a range of A23187 concentrations on the net movement of cellular Ca^{2+}. The effects of the ionophore were examined at two different extracellular Ca^{2+} concentrations, firstly to separate predominantly intracellular effects of A23187 from effects owing to the inflow of extracellular Ca^{2+}, and secondly to examine whether or not the A23187 dose–response curve is sensitive to the extracellular Ca^{2+} concentration. With approx. 4.0μM free Ca^{2+} (4.4μM total Ca^{2+}) (Fig. 1*a*) in the perfusate, A23187 induces a net efflux of Ca^{2+} at all ionophore concentrations examined (0.25–2.0μM). A dose of 2μM-A23187 induces a loss of 92 ± 9.7 ($n = 5$)nmol of Ca^{2+}/g of liver, at a maximal rate of 21 ± 4.2 ($n = 5$)nmol of Ca^{2+}/min per g of liver (Fig. 1*a*). The onset of Ca^{2+} efflux is rapid, occurring at 24 ± 3.9s ($n = 4$) after administration, with maximal rates of efflux occurring between 5 and 7min of treatment. The rate of Ca^{2+} efflux remains above control values for at least 10min. At ionophore concentrations below 2μM the rate of Ca^{2+} efflux decreases; however, the efflux response appears to become more prolonged. Hence, although the exact duration of Ca^{2+} efflux is difficult to determine, owing to the low rates of Ca^{2+} efflux involved, the total net loss of Ca^{2+} may be similar for all ionophore concentrations examined. Similar data were obtained when the Ca^{2+} concentrations were increased to 50μM (results not shown).

When the perfusate free Ca^{2+} concentration is increased to 390μM ($\simeq$400μM total Ca^{2+}) (Fig. 1*b*), A23187 induces a different pattern of Ca^{2+}-flux changes. Low concentrations of the ionophore (<0.5μM) induce a slow transient influx of Ca^{2+} lasting between 3 and 4min, and involving the movement of approx. 15nmol of Ca^{2+}/g of liver. This is followed by a more prolonged phase of Ca^{2+} efflux from the liver, similar in magnitude to that observed with higher concentrations of the ionophore at 3.2μM-Ca^{2+} (Fig. 1*a*). When the ionophore concentration is increased to 1μM the initial phase of Ca^{2+} uptake becomes larger and more prolonged, about 40nmol of Ca^{2+}/g of liver entering the cell during the first 5–6min of ionophore administration. This is still followed by a transient efflux of Ca^{2+}; however, the magnitude of this efflux response decreases as the ionophore concentration is increased. Hence at 390μM-Ca^{2+}, a 4-fold change in the concentration of the ionophore is sufficient to reverse what is essentially a Ca^{2+}-efflux response to a Ca^{2+}-uptake response. Results similar to those obtained at 390μM-Ca^{2+} were also observed when the total Ca^{2+} concentration was increased to 1.3mM (results not shown).

A23187-induced changes in hepatic respiration and glycogenolysis were also examined and found to be dependent on both ionophore concentration and

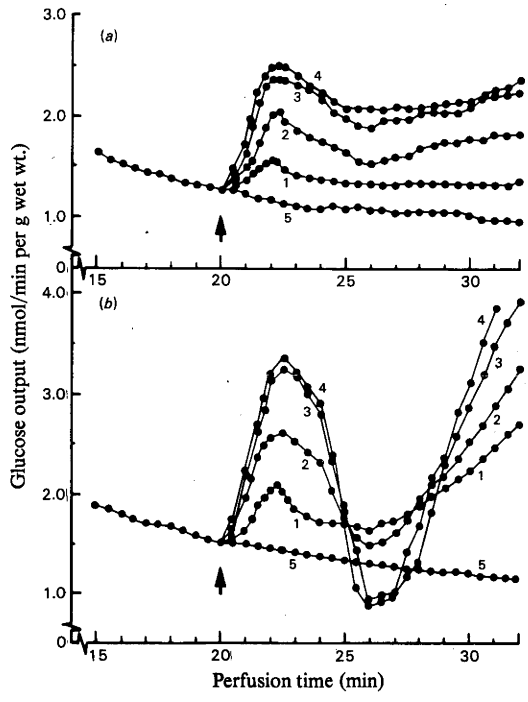

Fig. 2. *Effect of A23187 concentration on the rate of glucose output*

Perfusion details were as described in the legend to Fig. 1. Glucose output was assayed by using glucose oxidase and peroxidase as described in the Experimental section. The extracellular free Ca^{2+} concentration was either 4.0 μM (*a*) or 390 μM (*b*). Trace numbers are as in the legend to Fig. 1. The arrows indicate the point at which A23187 was infused. Results shown are means for between 5 and 11 independent experiments performed for each concentration of the ionophore; S.E.M. values are omitted for the sake of clarity.

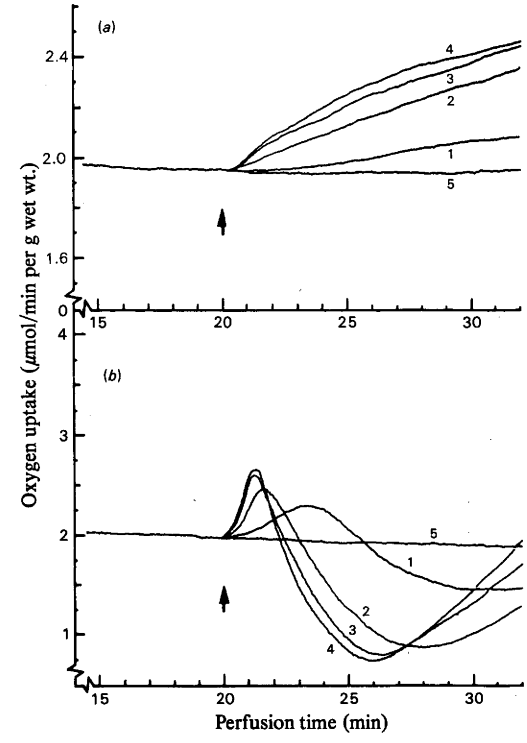

Fig. 3. *Effect of A23187 concentration on the rate of oxygen uptake*

Perfusion details were as described in the legend to Fig. 1. Oxygen uptake was assayed continuously with an oxygen electrode modified for a flow-through mode of operation as described in the Experimental section. The extracellular free Ca^{2+} concentration was either 4.0 μM (*a*) or 390 μM (*b*). Trace numbers are as in the legend to Fig. 1. The arrows indicate the point at which A23187 was infused. All traces shown have been corrected for the small stimulation of oxygen uptake produced by the ethanol solvent used to solubilize the ionophore. Traces shown are typical of those obtained for between 5 and 11 independent experiments performed for each ionophore concentration.

Ca^{2+} concentration. The data in Fig. 2(*a*) show that when the perfusate Ca^{2+} concentration is 4.0 μM all concentrations of A23187 examined significantly stimulate the rate of glucose output, maximal effects being observed at an ionophore concentration of 2 μM. Maximal rates of glucose efflux are observed 2–3 min after administration, thereafter declining to a constant rate which is maintained for at least 10 min. At a perfusate Ca^{2+} concentration of 390 μM, small changes in the ionophore concentration determine both the qualitative and quantitative metabolic responses (Fig. 2*b*). At 0.5 μM-A23187 the stimulation of glycogenolysis is very similar to that observed with maximally effective concentrations of A23187 at 4.0 μM-Ca^{2+}. As the ionophore concentration is increased to the maximally effective dose of 2 μM, the initial glycogenolytic stimulation increases in magnitude and also becomes more transient.

The data in Fig. 3(*a*) show that oxygen uptake by the liver is only slightly stimulated by A23187 at a perfusate Ca^{2+} concentration of 4.0 μM. At a perfusate Ca^{2+} concentration of 390 μM (Fig. 3*b*) the respiratory responses were larger, more rapid and biphasic, with the concentration of A23187 again determining both the direction and the magnitude of the response. As the ionophore concentration was increased, the stimulatory response became more transient, and the subsequent inhibitory response more prolonged and larger. At the near-maximal concentration of 2 μM-A23187, respiration was

inhibited by more than 50% after 5–6 min of ionophore administration.

In all of the experiments described above, it was found that concentrations of A23187 greater than 2 μM did not induce further changes in any of the parameters studied (results not shown).

Comparison of the ability of A23187 and of phenylephrine to induce Ca^{2+}-flux changes, respiration and glycogenolysis in perfused rat liver

Previously we have shown that α-adrenergic agonists rapidly induce large changes in Ca^{2+} fluxes and metabolism in perfused rat liver (Reinhart *et al.*, 1982*a*,*b*). In light of this, and of reports suggesting that α-adrenergic agonists mobilize intracellular Ca^{2+} pools via an undefined natural Ca^{2+} ionophore (Selinger *et al.*, 1974; Dehaye *et al.*, 1980; Blackmore *et al.*, 1982), we have investigated in more detail the relative abilities of A23187 and of the α-adrenergic agonist, phenylephrine, to induce changes in Ca^{2+} fluxes and metabolism. Since maximal effects of A23187 were observed at 2 μM in the previous experiments, this ionophore concentration was used in all experiments described below. Ca^{2+} concentrations of 50, 100 and 400 μM were used, since (*a*) the responses to A23187 were similar in media containing 50 μM- or 4 μM-Ca^{2+} (cf. Figs. 1*a* and 4*a*) and (*b*) the responses to A23187 were near-maximal in media containing 400 μM-Ca^{2+} (Fig. 1*b*).

Data in Fig. 4(*a*) show that when the extracellular Ca^{2+} concentration is 50 μM, pretreatment with A23187 only slightly inhibits the Ca^{2+} efflux induced by phenylephrine. A point of note is that at this concentration of Ca^{2+} variable results were obtained, in terms of both the net amount of Ca^{2+} mobilized by A23187 and the extent to which the presence of the ionophore inhibited phenylephrine-induced Ca^{2+} efflux.

Increasing the extracellular Ca^{2+} concentration further to 100 μM (Fig. 4*b*) significantly alters the pattern of A23187-induced Ca^{2+} fluxes from one of Ca^{2+} efflux to one of biphasic Ca^{2+} fluxes involving the transient uptake and subsequent efflux of Ca^{2+} by the liver. The amount of Ca^{2+} taken up is small (20 nmol/g of liver), and is followed by a more prolonged phase of Ca^{2+} efflux. At this Ca^{2+} concentration, a subsequent administration of phenylephrine induces a rate and amount of Ca^{2+} efflux clearly less than that observed in the absence of A23187.

As the extracellular Ca^{2+} concentration is elevated to 400 μM (Fig. 4*c*) or 1.3 mM (results not shown), the pattern of ionophore-induced Ca^{2+} fluxes changes from one of biphasic responses to one of predominantly Ca^{2+} uptake, with little (400 μM) or no (1.3 mM-Ca^{2+}) Ca^{2+} efflux. Similarly the rate and amount of efflux induced by phenylephrine decreases as the extracellular Ca^{2+} concentration increases.

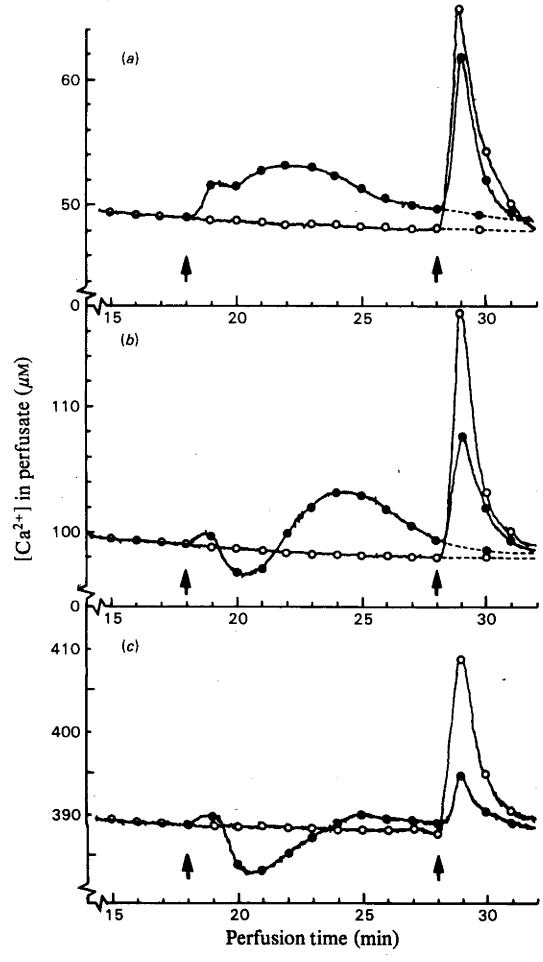

Fig. 4. *Effect of A23187 on phenylephrine-induced cellular Ca^{2+} fluxes at various extracellular Ca^{2+} concentrations*

Livers of fed rats were perfused with medium containing 1.3 mM-Ca^{2+} for 10 min, and perfusate Ca^{2+} was measured continuously, as described in the Experimental section. At 10 min the perfusate Ca^{2+} concentration was decreased to 50 μM (*a*), 100 μM (*b*) or 400 μM (*c*). At 18 min some livers were infused with A23187 (final concn. 2 μM) (●), while others served as controls (○). Then 10 min later phenylephrine (final concn. 2 μM) was infused into some animals, while others served as controls (dashed traces). Traces shown are typical of those obtained for between four and seven independent experiments performed for each condition.

Increases in glycogenolysis and respiration induced by phenylephrine were also inhibited by prior treatment with A23187. The inhibition of phenyl-

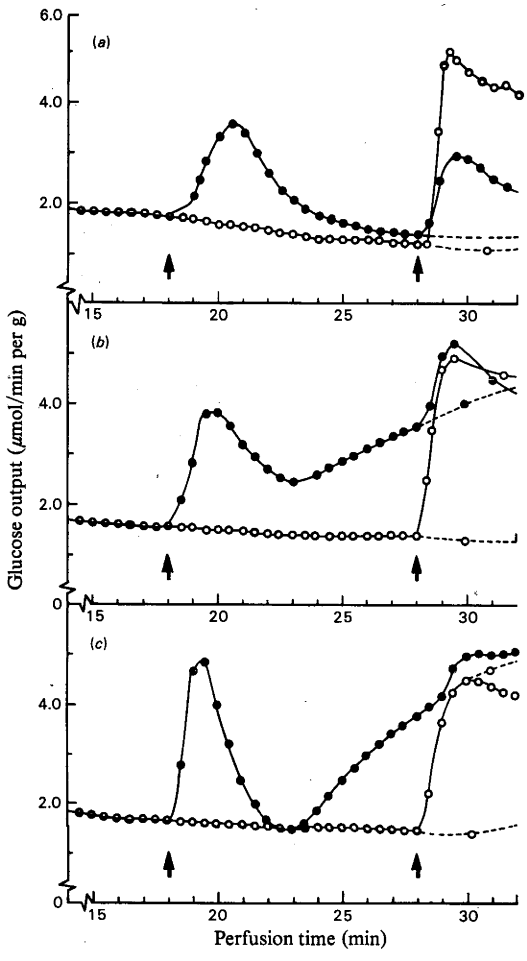

Fig. 5. *Effect of extracellular Ca^{2+} on A23187-induced rates of glucose output*

Perfusion details were as described in the legend to Fig. 4. The perfusate Ca^{2+} concentration was 50 μM (*a*), 100 μM (*b*) or 400 μM (*c*). At 18 min some livers were infused with A23187 (final concn. 2 μM) (●), while others served as controls (○). Then 10 min later phenylephrine (final concn. 2 μM) was infused into some animals, while others served as controls (dashed traces). The data shown represent means for between four and seven independent experiments performed for each condition.

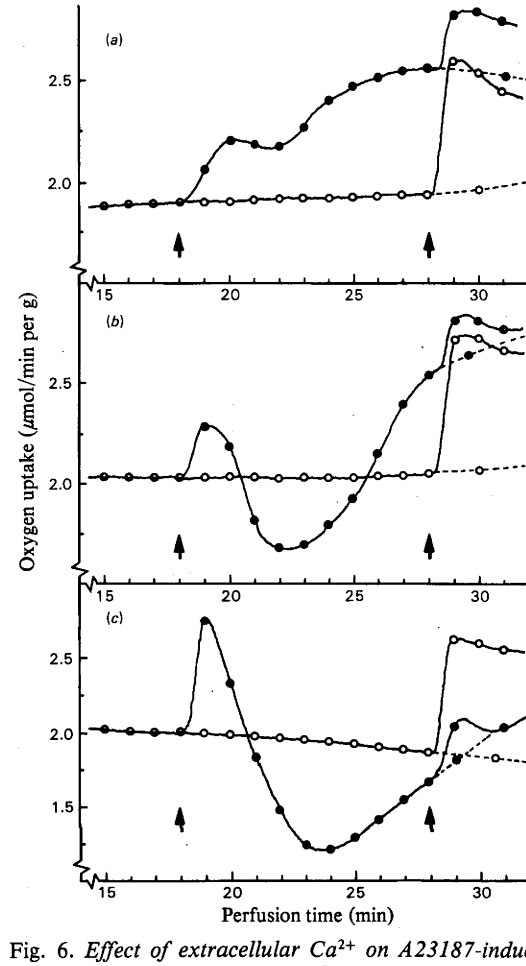

Fig. 6. *Effect of extracellular Ca^{2+} on A23187-induced oxygen uptake*

Perfusion details were as described in the legend to Fig. 4. Oxygen uptake by livers was measured with an oxygen electrode modified for a flow-through mode of operation and described in the Experimental section. The perfusate free Ca^{2+} concentration was 50 μM (*a*), 100 μM (*b*) or 400 μM (*c*). At 18 min some livers were infused with A23187 (final concn. 2 μM) (●), while others served as controls (○). Then 10 min later phenylephrine (final concn. 2 μM) was infused into some animals, while others served as controls (dashed traces). Traces shown are typical of those obtained for between four and seven independent experiments performed for each condition.

ephrine-induced responses was more severe at higher Ca^{2+} concentrations (Figs. 5 and 6).

Discussion

A major point revealed by this study is that the action of A23187 at the cellular level is both complex and dependent on the particular experimental conditions used. Hence to facilitate the assessment as to whether or not the use of A23187 is justified for a particular experimental regime requires an analysis of how this ionophore alters the cellular Ca^{2+} compartmentation. Such an analysis should at least involve the measurement of both the net cellular Ca^{2+} fluxes and the activity of Ca^{2+}-sensitive

enzymes and pathways in the compartment of interest.

In the present study we have separated those actions of A23187 attributable to the equilibration of Ca^{2+} gradients across intracellular compartments from those attributable to an influx of intracellular Ca^{2+} into the cell. This was achieved by altering the magnitude of the Ca^{2+} gradient across the plasma membrane of liver cells and concomitantly measuring net Ca^{2+} fluxes, glycogenolysis and respiration.

When the extracellular concentration of Ca^{2+} was decreased to approach that thought to exist in the cytoplasm (Murphy *et al.*, 1980), A23187 effectively acted to equilibrate Ca^{2+} gradients across the membranes of intracellular organelles, and, through the action of the plasma-membrane Ca^{2+}-efflux mechanism, induced a net efflux of Ca^{2+} from the cell. At least part of this Ca^{2+} may have originated from the mitochondrial matrix, since (*a*) a significant portion of the total intracellular Ca^{2+} is associated with mitochondria, (*b*) previous work has shown that A23187 induces Ca^{2+} efflux from isolated mitochondria (Reed & Lardy, 1972; Whiting & Barritt, 1982), and (*c*) in hepatocytes the ionophore decreases chlortetracycline fluorescence (Babcock *et al.*, 1979), thought to reflect a decrease in mitochondrial membrane-bound Ca^{2+} (Chandler & Williams, 1978).

During the ionophore-induced Ca^{2+} mobilization the cytoplasmic Ca^{2+} concentration appears to be transiently elevated, as reflected by a stimulation in the rate of glycogenolysis. This finding contrasts with previous reports, indicating that in the absence of extracellular Ca^{2+} A23187 has only a small effect on the activity of phosphorylase *a*, or on the rate of glycogenolysis (Assimacopoulos-Jeanett *et al.*, 1977; Friedmann *et al.*, 1979). This may have been due in part to the depletion of Ca^{2+} from intracellular stores in these experiments, since cells were exposed to Ca^{2+}-free media for extended periods of time. In the present study such a depletion was minimized by using low concentrations of EGTA (25 μM) and decreasing the duration for which livers were exposed to such low-Ca^{2+}-containing media. In contrast with the rapid and transient effects of the ionophore on Ca^{2+} fluxes and the rate of glycogenolysis, the effect on stimulating oxygen uptake was slow and prolonged (Fig. 3*a*). Since the large stimulation of respiration in isolated mitochondria (Reed & Lardy, 1972) has been related to an increase in the rate of Ca^{2+} cycling across the mitochondrial inner membrane (Reed & Lardy, 1972; Heaton & Nicholls, 1976), the tentative conclusion can be drawn that such cycling is not occurring here to any significant extent. An important corollary to this is that increases in the concentration of cytoplasmic Ca^{2+} sufficient to stimulate glycogenolysis and the plasma-membrane Ca^{2+} efflux mechanism are not sufficient to increase significantly the rate of the mitochondrial Ca^{2+} uniporter.

Even in the presence of the significant gradient of Ca^{2+} across the plasma membrane at 50 μM perfusate Ca^{2+}, A23187 is still able to induce the biphasic efflux of Ca^{2+} from the liver associated with a stimulation in the rate of glycogenolysis similar to that observed at 4 μM-Ca^{2+}. Although it is difficult to interpret these findings with any precision, it appears as though the plasma-membrane Ca^{2+}-efflux system has sufficient reserve capacity to counteract the ionophore-induced inflow of Ca^{2+}. Consistent with such a Ca^{2+} cycle is the significantly higher rate of ionophore-stimulated respiration at 50 μM perfusate Ca^{2+} than at 4 μM. Such complications may be partly responsible for some of the divergent results previously obtained with A23187 in liver, since either sub-maximal doses of the ionophore or small changes in the gradient of Ca^{2+} across the plasma membrane may significantly alter the effect of this agent in liver. In hepatocytes such variations may be magnified by the possibility that the prolonged perfusion of livers with Ca^{2+}-free media during the isolation of the cells may induce significant alterations in the distribution of Ca^{2+} within the cells.

At higher extracellular [Ca^{2+}], A23187 induces Ca^{2+} uptake by the liver. Under these conditions both the rate of respiration and that of glucose output are transiently elevated above those rates observed in the absence of a Ca^{2+} gradient across the plasma membrane. At longer times the rates of both responses are significantly inhibited, raising the possibility that some Ca^{2+}-sensitive enzymes are stimulated only within a tightly defined range of Ca^{2+} concentrations, and increasing the Ca^{2+} concentration above some optimum is inhibitory. Alternatively, in these conditions, A23187 may have toxic effects through decreasing ATP in the liver (Friedmann *et al.*, 1979).

A second major point revealed by the present study is the usefulness of ionophores in the study of α-adrenergic action; similarities as well as differences in the way these two agents induce a redistribution of cellular Ca^{2+} were shown to exist. The ionophore-induced rate of Ca^{2+} efflux, and to a lesser extent the amount of Ca^{2+} effluxed, are very much less than that achieved either by α-adrenergic agonists or by other Ca^{2+}-mobilizing hormones (Reinhart *et al.*, 1982*a*). This is consistent with other reports indicating that *in vivo* the gradient of Ca^{2+} across the inner mitochondrial membrane may be as low as 2–20-fold (Denton *et al.*, 1980; Coll *et al.*, 1982; McCormack *et al.*, 1982). Hence even the total equilibration of such a gradient would result in only a relatively low rate and amount of Ca^{2+} efflux from the cell. The result of the short-term Ca^{2+}-depletion experiments reported in the present paper

indicate that only a portion of the hormone-sensitive Ca^{2+} pool is susceptible to mobilization by A23187 at low Ca^{2+} concentrations. Thus only under conditions where the ionophore has induced a large change in Ca^{2+} fluxes across the hepatic plasma membrane are the effects of α-agonists largely inhibited.

In summary, then, it is clear from this work that considerable caution needs to be exercised when studies are to be undertaken with the ionophore A23187, especially when complex systems such as perfused liver are under examination.

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Section B.10

An assessment of the calcium content of rat liver mitochondria *in vivo*.

(Reinhart et al., 1983b)

AN ASSESSMENT OF THE CALCIUM CONTENT OF RAT LIVER MITOCHONDRIA IN VIVO

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Short Title: Calcium content of rat liver mitochondria

Proofs to: Dr. F.L. Bygrave

Synopsis

The effect of systematically altering the isolation conditions on the total calcium content of mitochondria isolated from perfused rat liver was examined. We showed that under most isolation conditions, significant redistributions of mitochondrial calcium occurred resulting in up to 5-fold changes of the total calcium content. Mitochondrial Ca^{2+} flux inhibitors such as Ruthenium Red and nupercaine were only partially effective in inhibiting such redistributions. We present evidence indicating that the total calcium content of rat liver mitochondria *in situ* may approximate 2nmol.mg protein^{-1}.

Introduction

Most studies on Ca^{2+} homeostasis in hepatic tissue have suggested that mitochondria play a predominant role in the regulation of the cytoplasmic free Ca^{2+} concentration (Claret-Berthon et al., 1977; Bygrave, 1978; Nicholls, 1978; Baker et al., 1980; Nicholls & Crompton, 1980; Borle, 1981; Coll et al., 1982; Joseph et al., 1983). This view is supported by the finding that isolated mitochondria can buffer the extramitochondrial Ca^{2+} concentration at values close to those thought to exist in the cytoplasm (Nicholls, 1978; Becker et al., 1980; Joseph et al., 1983), due to the establishment of a dynamic equilibrium between separate Ca^{2+} influx and Ca^{2+} efflux pathways (Bygrave, 1978; Carafoli & Crompton, 1978; Nicholls & Crompton, 1980; Akermann & Nicholls, 1983).

An alternative role for the mitochondrial Ca^{2+}-translocation cycle has been suggested by Denton & McCormack (1980). These authors propose that this cycle may be a means by which the matrix free Ca^{2+} concentration and hence the activity of numerous Ca^{2+}-stimulated matrix enzymes can be regulated (Denton et al., 1980; McCormack & Denton, 1980). In order to more closely define the role of this translocation cycle, detailed information about the total and free mitochondrial Ca^{2+} concentrations in vivo is required, and an attempt to obtain this data forms the basis of the present study. The ability to precisely manipulate the external Ca^{2+} concentration in the perfused liver system (Reinhart et al., 1982a), to accurately determine the free Ca^{2+} concentration in Krebs-Ringer bicarbonate media (Reinhart et al., 1983a) and to rapidly isolate mitochondria from an intact liver by Percoll density-gradient-centrifugation (Reinhart et al., 1982b), has provided a means for closely monitoring Ca^{2+} movements during the isolation of mitochondria. Our results indicate that the value for the mitochondrial calcium content is dependent on the concentration of the circulating

external Ca^{2+}, and on the presence or absence of inhibitors of mitochondrial Ca^{2+}-cycling. Based on these findings we present an argument that the concentration of total calcium in rat liver mitochondria *in situ* is of the order of 2 nmol.mg of mitochondrial protein^{-1}. Some implications of this conclusion are discussed in terms of the matrix free Ca^{2+} concentration and the role of this organelle in the regulation of Ca^{2+} homeostasis.

Experimental procedures

Animals and perfusions - Male Wistar strain albino rats, weighing between 200 and 250 g, and having free access to food, were used for all experiments. Rats were anaesthetized with sodium pentobarbitone (50 mg/kg body weight), and the livers perfused with Krebs-Henseleit (1932) bicarbonate medium equilibrated with O_2/CO_2 (19:1) in the absence or presence of added $CaCl_2$ (Reinhart *et al.*, 1982*a*). A non-recirculating mode of perfusion was used at a flow rate of 3.5 ml.min^{-1}.g liver^{-1} (Reinhart *et al.*, 1982*c*). In experiments where Ca^{2+}-free Krebs-Henseleit medium was used, $CaCl_2$ (1.3mM) was administered by infusion syringe during the first 10 min of perfusion. At various times after 10 min the removal or readministration of $CaCl_2$ into the perfusion circuit allows the rapid manipulation of the extracellular Ca^{2+} concentration (see the Results Section). All perfusions were carried out between 0800 and 1200 h.

Liver fractionation - The liver was fractionated by a rapid Percoll density-gradient-centrifugation procedure recently developed in this laboratory (Reinhart *et al.*, 1982*b*). The median lobe was excised and homogenised in a medium consisting of 210 mM-mannitol, 60 mM-sucrose, 10 mM-KCl, 10 mM-sodium succinate, 0.25 mM-dithiothreitol, 1 mM-KH_2PO_4,

2 mM-$MgSO_4$, 10 μM-EGTA and 10 mM-Hepes/KOH (pH 7.4). Where indicated in the legend to the Figs., 1.6 μM-Ruthenium Red and 0.4 mM-nupercaine (cinchocaine hydrochloride) were also present in the homogenising medium. A portion (2ml) of the resulting homogenate was layered on to a discontinuous density-gradient of iso-osmotic Percoll and centrifuged for 30 s at 39,600 g_{av} in a Sorvall RC-5B refrigerated centrifuge with the SS 34 rotor. In this way a fraction enriched in mitochondria (Reinhart et al., 1982b) could be obtained within a total preparation time of 6 min.

Assay of total calcium a) Mitochondria - immediately after isolation, the mitochondrial fraction was extracted with ice-cold 2 M-perchloric acid/5 mM-Hepes and centrifuged for 6 min in an Eppendorf microfuge to sediment insoluble material. Portions of the supernatant were analysed for calcium in 0.1% (w.v) KCl using a N_2O/acetylene flame (medical grade gases) in a Varian Atomic Absorption Spectrophotometer. All solutions were prepared using deionised distilled H_2O (Milli-Q reagent-grade Water system; resistance > 15 Megohms.cm^{-1}). Calcium concentrations were determined by internal standard curve calibration, since Percoll was found to decrease the absorbance response due to calcium (P.H. Reinhart, unpublished observations).

b) Perfusate calcium was measured by collecting samples from the outflow side of the liver. These were then centrifuged in an Eppendorf microfuge for 2 min, and portions of the supernatant assayed for calcium as described above.

Free calcium concentration calculations - Free Ca^{2+} concentrations were calculated using an algorithm of the program (Comics) developed by Perrin & Sayce (1967). The logarithm of the total formation constants (Sillen & Martell, 1971) for the following complexes were considered

(pH = 7.4) : $PO_4^{3-} + H^+$ (11.8), $EGTA^{4-} + Ca^{2+}$ (11.0), $CO_3^{2-} + H^+$ (10.25), $EGTA^{4-} + H^+$ (9.54), $HEGTA^{3-} + H^+$ (8.93), $HPO_4^{2-} + H^+$ (7.15), $HEGTA^{3-} + Ca^{2+}$ (5.3), $EGTA^{4-} + Mg^{2+}$ (5.2), $HEGTA^{3-} + Mg^{2+}$ (3.4), $HCO_3^- + Mg^{2-}$ (3.4), $CO_3^{2-} + Ca^{2+}$ (3.2), $HPO_4^{2-} + Ca^{2+}$ (2.77), $H_2EGTA^{2-} + H^+$ (2.73), $HPO_4^{2-} + Mg^{2+}$ (2.5). $H_2PO_4^- + Ca^{2+}$ (1.5).

Chemicals and materials - Percoll was obtained from Pharmacia Fine Chemicals, AG, Uppsala, Sweden. Ruthenium Red was from The Sigma Chemical Company, St. Louis, Mo., and nupercaine from Astra Chemicals, Sydney, N.S.W., Australia. All other chemicals used were of analytical reagent grade.

Results

The effect of systematically altering the isolation conditions on the total calcium content of liver mitochondria is shown in Fig. 1. The mitochondrial calcium content varies from 2 to 10 nmol.mg of protein^{-1} depending on the Ca^{2+} content of both the liver perfusion medium and the Percoll density gradient solutions, and on the presence of inhibitors of mitochondrial Ca^{2+} uptake or efflux.

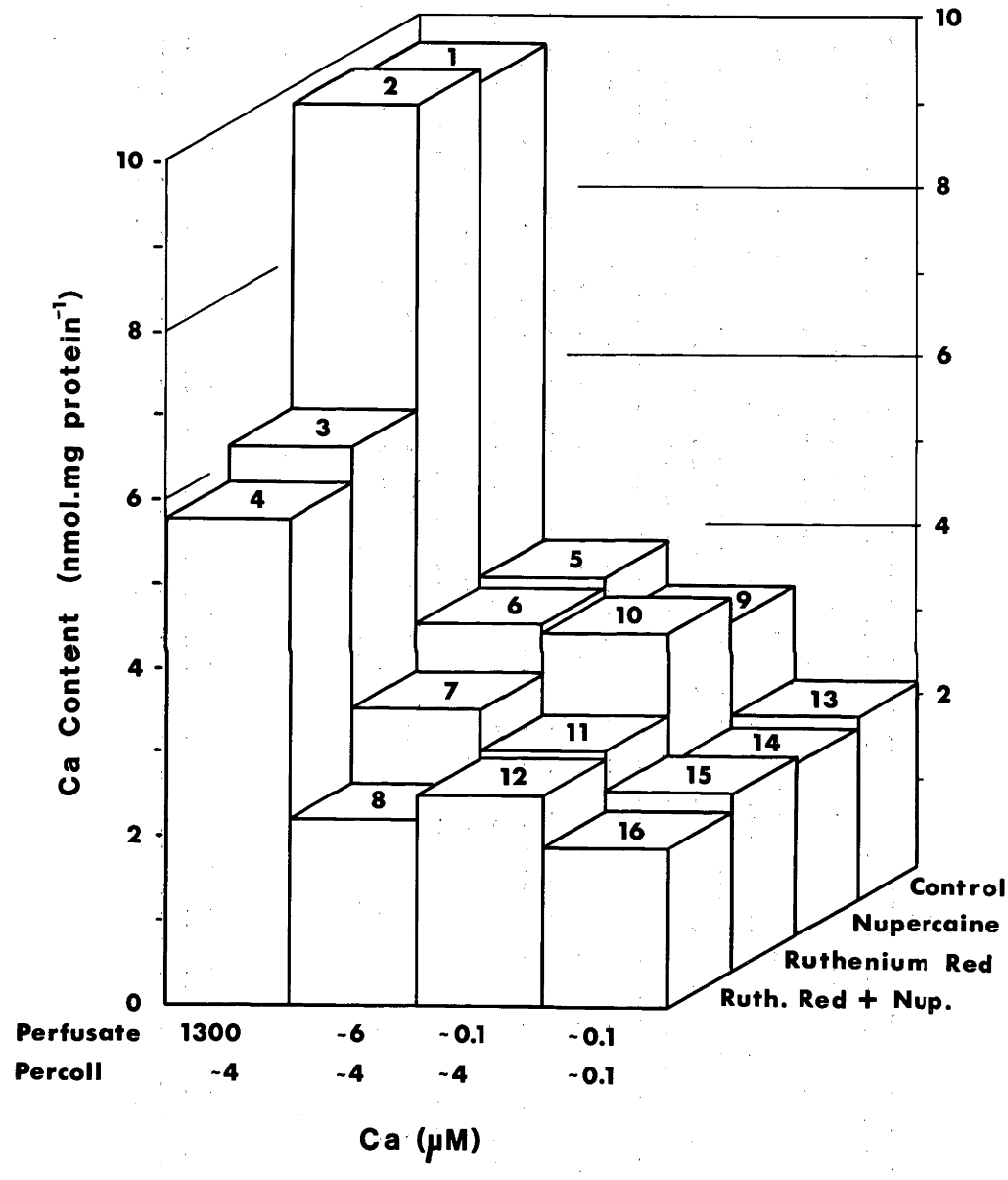
The highest values for the calcium content (approaching 10 nmol.mg of protein^{-1}) were observed when the perfusate contained 1.3 mM Ca^{2+}, and mitochondrial Ca^{2+} flux inhibitors were absent (column 1). The addition of 0.4 mM nupercaine, an inhibitor of mitochondrial Ca^{2+} efflux (Dawson et al., 1979; Dawson & Fulton, 1980) had essentially no effect (col. 2), while the presence of either 1.6 μM Ruthenium Red (col. 3) or Ruthenium Red plus nupercaine (col. 4) resulted in a 40% decrease of mitochondrial calcium content.

An even greater effect on the calcium content was observed when the perfusate Ca^{2+} concentration was reduced from 1.3 mM to either approx. 6 μM (col. 5-8) or approx. 0.1 μM (col. 9-12) prior to homogenisation of

Figure 1. The effect of different isolation conditions on the mitochondrial total calcium content.

Livers of fed rats were perfused as described in Experimental. At 10 min of perfusion the perfusate free Ca^{2+} concentration was adjusted to the values shown in the Fig., and 5 min later the median lobe was excised and mitochondria prepared by Percoll density-gradient centrifugation as described in Experimental. The free Ca^{2+} concentration of the Percoll solutions was adjusted as shown in the Fig. and in some experiments nupercaine (0.4mM) or Ruthenium Red (1.6 μM) was present as indicated. For all conditions, free Ca^{2+} concentrations were determined by computer analysis.

Total calcium contents were determined by Atomic Absorption spectroscopy, as described in Experimental. The data shown represent the means of results from between 3 and 5 independent experiments performed for each condition. For the sake of clarity S.E.M.'s have been omitted, however, 0.75 for columns 1-4, 0.53 for columns 5-8, 0.28 for columns 9-12, and 0.14 for columns 13-16 was never exceeded.


Ca Content (nmol.mg protein^{-1})
0
2
4
6
8
10
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
Control
Nupercaine
Ruthenium Red
Ruth. Red + Nup.
Perfusate 1300 ~6 ~0.1 ~0.1
Percoll ~4 ~4 ~4 ~0.1
Ca (μM)

the tissue. Under these conditions mitochondrial calcium contents of between 3 and 4 nmol.mg of protein^{-1} were recorded, while the inclusion of Ruthenium Red decreased the content further to between 2 and 3 nmol.mg of protein^{-1} (col. 7).

The reduction of the free Ca^{2+} concentration to 0.1 μM in all the density gradient solutions, as well as in the perfusate (col. 13) resulted in the recovery of 2 nmol.calcium mg of protein^{-1}. Under these conditions neither Ruthenium Red nor nupercaine had any significant effect on the mitochondrial calcium content (col. 14-16).

These data suggest that large scale Ca^{2+} redistributions may occur during the isolation of mitochondria, even when using a very rapid isolation procedure. Hence further experiments were designed to examine these redistributions in more detail, with the aim of establishing conditions of minimal Ca^{2+} movements during the isolation procedure.

Firstly, the effect of reducing the perfusate Ca^{2+} concentration from 1.3mM to approx. 0.1 μM on the calcium content of subsequently isolated mitochondria was examined in more detail. Data in Fig. 2 show that as the perfusate Ca^{2+} concentration is decreasing from 1.3mM to 0.1 μM the mitochondrial calcium content also decreases rapidly, basal values of between 2 and 2.5 nmol.mg of protein^{-1} being reached within 3 to 5 min after withdrawing $CaCl_2$ from the perfusion medium. Half-maximal effects on either the calcium content or the perfusate Ca^{2+} concentration are observed by approx. 30 s after $CaCl_2$ removal. A close correlation between the perfusate Ca^{2+} concentration and the mitochondrial calcium content was observed (correlation coefficient = 0.9668). The lowest mitochondrial calcium content recorded (2.1 nmol.mg of protein), was not decreased further by either increasing the time of pre-perfusion at 0.1 μM Ca^{2+} or by increasing the EGTA concentration to 100 μM (data not shown).

Figure 2. Effect of the perfusate Ca^{2+} concentration on the mitochondrial total calcium content.

Livers of fed rats were perfused with media containing 1.3 mM Ca^{2+} for 10 min as described in Experimental. At 10 min the infusion of $CaCl_2$ was terminated and 25 μM EGTA administered. At specified times thereafter, as indicated in the Fig, the median lobe was excised and mitochondria prepared as described for column 13 in Fig. 1. The mitochondrial total calcium content (●) and the perfusate Ca^{2+} concentration (O) was determined as described in Experimental. The data shown represent the results of individual experiments (●) or the means of 3 independent experiments performed (O).

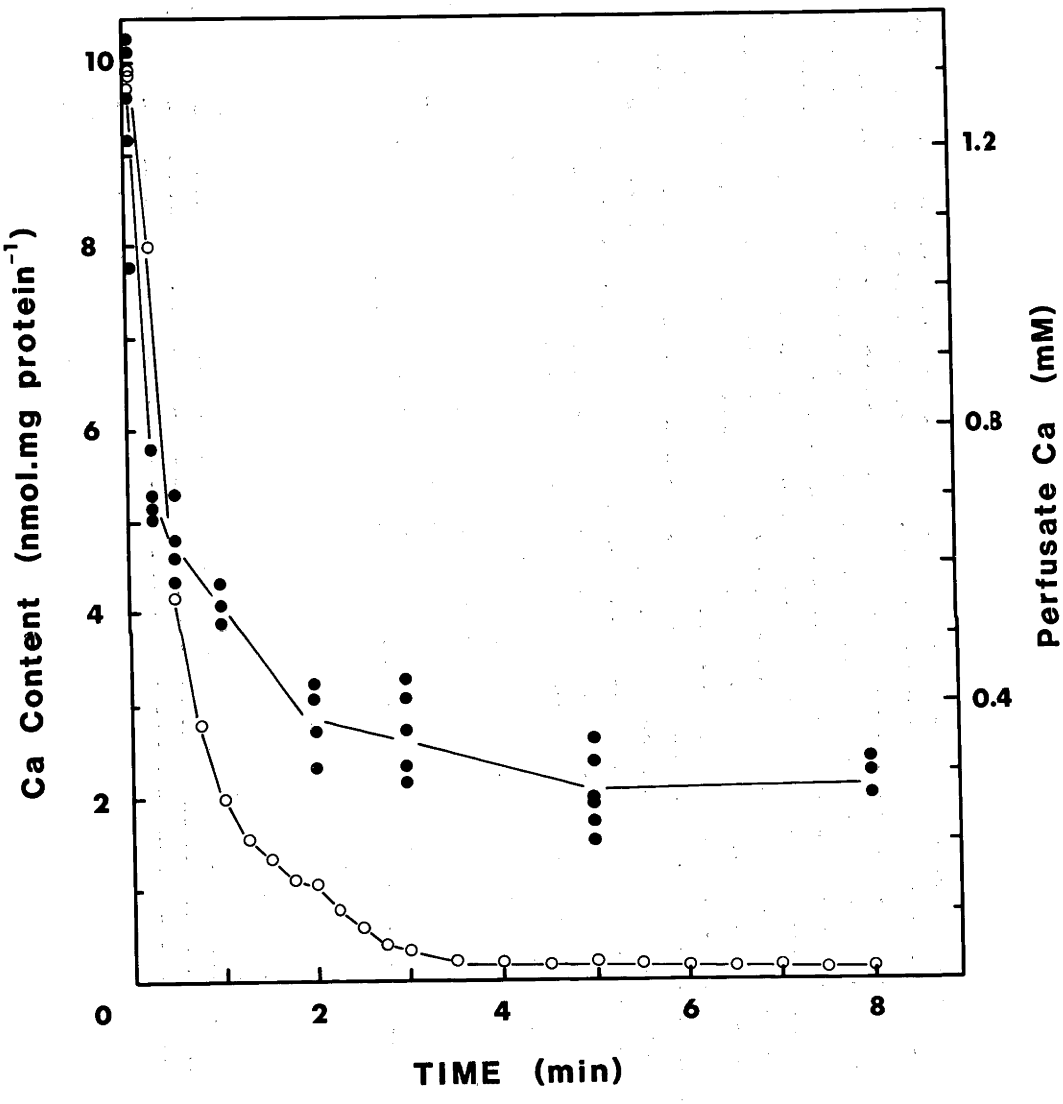

Ca Content (nmol.mg protein^{-1})
Perfusate Ca (mM)
TIME (min)
0
2
4
6
8
10
0.4
0.8
1.2

TABLE 1

Effect of EGTA on the mitochondrial calcium content in vitro

Livers of fed rats were perfused with media containing 1.3 mM Ca^{2+} for 15 min, at which time mitochondria were prepared as described for column 1 in Fig. 1. Half of the mitochondrial fraction (approx. 20 mg of protein) was resuspended in 2 ml of isolation medium and the other half resuspended in 2 ml of isolation medium containing 25 μM EGTA. Fractions were incubated at 37°C for the times indicated, the mitochondria pelleted by centrifugation, and a portion of the resulting supernatant (0.4 ml) assayed for the calcium content. The data shown represent the mean ± S.E.M. for 3 independent experiments.

Incubation time (min)	Mitochondrial calcium content (nmol.mg of protein $^{-1}$)	
	Control	+ EGTA
0	9.85 ± 0.75	
1	9.69 ± 0.07	9.04 ± 0.09
2	9.58 ± 0.09	8.68 ± 0.11
5	9.02 ± 0.06	7.85 ± 0.13
8	8.99 ± 0.10	7.85 ± 0.12

Secondly, the effect of low extramitochondrial Ca^{2+} concentrations on the mitochondrial calcium content was further examined by incubating isolated mitochondria with the same concentration of EGTA as that used during the isolation procedure (approx. 30 μM). The data in Table 1 show that this concentration of EGTA induces only a very slow rate of Ca^{2+} efflux corresponding to 1.1 nmol.mg of protein^{-1} during 8 min of incubation.

Discussion

The results of this study indicate that significant Ca^{2+} movements may occur during the isolation of mitochondria, and furthermore they allow a realistic estimation of the *in vivo* calcium content to be made. Previous estimates of the total calcium content in isolated mitochondria have yielded values ranging between 5 and 25 nmol.mg of protein^{-1} (Carafoli & Lehninger, 1971; Reed & Lardy, 1972; Claret-Berthon *et al.*, 1977; Borle & Studer, 1978; Hughes & Barritt, 1978; Yamazaki *et al.*, 1980; Murphy *et al.*, 1980; Taylor *et al.*, 1980; Reinhart *et al.*, 1982*b*; Coll *et al.*, 1992; Joseph *et al.*, 1983). The reasons for these large variations, however, have not been investigated.

Those studies which have assessed the movement of Ca^{2+} during mitochondrial isolation, have used either Ca^{2+} - loaded mitochondria, or examined only the ability of isolated mitochondria to maintain a given Ca^{2+} load (Claret-Berthon, 1977; Berthon *et al.*, 1981) and hence have not determined the mitochondrial calcium content *in vivo*. Even so, earlier work does suggest that Ca^{2+} can be accumulated by mitochondria during homogenisation and fractionation (Slater & Cleland, 1953; Cohn *et al.*, 1967; Chen *et al.*, 1974; Hughes & Barritt, 1978). Although the inclusion of EGTA or La^{3+} in homogenisation media decreases the mitochondrial

calcium content (Chen et al., 1974; Hughes & Barritt, 1978), the possibility of Ca^{2+} movements from the interstitial space or the outside of the plasma membrane into mitochondria, or Ca^{2+} movements during the isolation period, cannot be ruled out by such an approach. Our data are consistent with and extend reports of Ca^{2+} movements during isolation, by showing that Ca^{2+} movements leading to 5-fold changes in the mitochondrial calcium content may occur during preparation, and that mitochondrial Ca^{2+} flux inhibitors are only partially effective in minimising these fluxes.

In attempting to prepare mitochondria with minimally disturbed total calcium contents, data obtained in this study provide circumstantial evidence indicating that under most isolation conditions mitochondria are accumulating rather than releasing Ca^{2+}.

Firstly, Ruthenium Red was found to lower the mitochondrial calcium content when the concentration of free Ca^{2+} in either the perfusate or the density gradient solutions is above 0.1 μM, with maximal effects observed at 1.3 mM Ca^{2+} (Fig. 1). Nupercaine, however, is without effect indicating that while significant net Ca^{2+} uptake is taking place under these conditions, Ca^{2+} efflux is negligible. The minimal net loss of calcium is consistent with the relatively low Vmax (4.7 nmol.min^{-1}.mg of protein^{-1}) of this activity (Coll et al., 1982) coupled to the known effects of P_i and low temperatures in depressing Ca^{2+} efflux rates (Zoccarato & Nicholls, 1981). In contrast, the maximal activity of the Ca^{2+} uniporter is over 150 times greater, being limited only by the respiratory chain activity (Vinogradov & Scarpa, 1973), and is known to be operative even at sub-zero temperatures (Chance et al., 1979).

Secondly, the rapidity with which a reduction of the extracellular Ca^{2+} concentration results in a lowered mitochondrial calcium content (half-maximal effects are at 30 s, Fig. 2) makes it unlikely that

measured calcium content values of less than 10nmol.mg of protein^{-1} represent a loss of Ca^{2+}. The rate of such a loss would be approx, 15 nmol.mg. min^{-1} which is three times greater than the measured Vmax for mitochondrial Ca^{2+} efflux (Coll et al., 1982).

Thirdly, the direct addition of 30 μM EGTA to mitochondria, resulting in an extramitochondrial free Ca^{2+} concentration well below the set point, induced only a small decrease in the total calcium content (Table 1) confirming that in the presence of P_i the rate of mitochondrial Ca^{2+} efflux is very low (Zoccarato & Nicholls, 1981). Hence it appears that mitochondrial Ca^{2+} efflux is minimal under the isolation conditions examined, while significant rates of Ca^{2+} uptake occur under all conditions except where the Ca^{2+} content of all solutions used to isolate the organelle is close to the mitochondrial set point.

Mitochondria isolated under these conditions contain approx. 2 nmol calcium .mg of protein^{-1} . Such a low endogenous calcium content is consistent with data obtained for smooth muscle using electron probe analysis in conjunction with cryo-ultramicrotomy as an alternative technique for quantitating the composition of cellular organelles in situ (Shuman et al., 1976; Somlyo et al., 1978, 1979).

A mitochondrial calcium content of only 2 nmol.mg of protein^{-1} has important ramifications as to the role mitochondria play in cellular Ca^{2+} homeostasis, since it has been shown that the ability of isolated mitochondria to buffer the ambient Ca^{2+} concentration is dependent on the total calcium content of the organelle (Joseph et al., 1983). These workers showed that by experimentally altering the total mitochondrial calcium content between 1.5 and 20.5 nmol.mg of protein^{-1} , the Ca^{2+} set point could be increased by more than three-fold. Such a change has been attributed to an increase in the matrix free Ca^{2+} concentration, in

parallel with an increase in the total calcium content (Coll et al., 1982). Hence a necessary condition for mitochondria to effectively buffer the cytoplasmic Ca^{2+} concentration is that the rate of mitochondrial Ca^{2+} efflux is constant (Nicholls & Akerman, 1982; Akerman & Nicholls, 1983). Under the conditions employed by Joseph et al., (1983) this does not occur until mitochondria contain greater than 10 nmol calcium.mg of protein^{-1}. Hence mitochondria containing only 2 nmol calcium.mg of protein^{-1} may be expected to play little role in the regulation of cytoplasmic Ca^{2+} .

However, while such a conclusion may be valid for specific conditions in vitro, the presence of P_i or other Ca^{2+}-complexing ligands in vivo may significantly alter the relationship between the total calcium content, and the Ca^{2+} setpoint. Hence the formation of matrix Ca^{2+} complexes in vivo may result in a constant rate of Ca^{2+} efflux, by buffering the matrix free Ca^{2+} concentration. Indeed the addition of P_i to isolated mitochondria has recently been shown to decrease the rate of Ca^{2+} efflux, presumably by Ca^{2+}-P_i complex formation (Zoccarato & Nicholls, 1981). The regulation of the cytoplasmic Ca^{2+} concentration by mitochondria containing even low concentrations of total Ca^{2+} thus remains an open question until more it known about Ca^{2+}-complexing ligands in vivo.

A further consequence of both a low mitochondrial calcium content and the formation of matrix Ca^{2+} complexes, is that the matrix free Ca^{2+} concentration in vivo may be much lower than the previously estimated value of 16 μM (Joseph et al., 1983). The application of a null-point titration technique to isolated mitochondria in vitro has yielded a Ca^{2+} activity coefficient of 7.10^{-4} (Coll et al., 1982) indicating that even in the absence of any added permeant anions, more than 99.9% of the total mitochondrial calcium is bound to endogenous ligands. Thus mitochondria

containing 2nmol calcium.mg of protein^{-1} may have a matrix free Ca^{2+} concentration of 1.4 μM *in vitro* and possibly even lower free Ca^{2+} concentrating *in vivo*. Consequently, the finding that a number of matrix located enzymes are regulated by micromolar Ca^{2+} concentrations (McCormack & Denton, 1980; Denton *et al.*, 1980; Hansford, 1981; Hansford & Castro, 1981) may be of physiological significance.

We conclude that both the mitochondrial total and free Ca^{2+} concentrations are lower than previously thought and that non-mitochondrial Ca^{2+}-translocation cycles may play a significant role in the maintenance of the cytoplasmic Ca^{2+} concentration, while the mitochondrial Ca^{2+}-translocation cycle may regulate the matrix free Ca^{2+} concentration. However, under some conditions, such as during α-adrenergic agonist administration (Reinhart *et al.*, 1982*a*: 1983*b*), the release of bound mitochondrial calcium may contribute to the elevation of the cytoplasmic Ca^{2+} concentration indicating that the function of the mitochondrial Ca^{2+}-translocation cycle may vary, depending on the ratio of free/bound calcium on either side of the inner mitochondrial membrane. Finally these studies highlight the need to examine mitochondrial properties under conditions relating more closely to the *in vivo* state.

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Section B.11

The contribution of both extracellular and intracellular calcium to the action of α-adrenergic agonists in perfused rat liver.

(Reinhart et al., 1983c)

THE CONTRIBUTION OF BOTH EXTRACELLULAR AND INTRACELLULAR CALCIUM TO THE ACTION OF α-ADRENERGIC AGONISTS IN PERFUSED RAT LIVER

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Short Title: Calcium in the hepatic action of α-adrenergic agonists

Synopsis

The role of both intracellular and extracellular Ca^{2+} pools in the expression of α-adrenergic agonist-mediated responses was examined in perfused rat liver. Responses studied included glycogenolysis, respiration, lactate and pyruvate formation, ketone body production, changes in the cytoplasmic and mitochondrial redox ratio and cellular K^{+} fluxes. Each of these was shown to be dependent on the mobilization of intracellular Ca^{2+} and can be grouped into one of two response types. Transient responses (ion fluxes and the redox ratios) are obligatorily dependent on the mobilization of intracellular Ca^{2+} and occur irrespective of the extracellular Ca^{2+} concentration. Sustained responses on the other hand initially require intracellular Ca^{2+} and, subsequently, extracellular Ca^{2+}.

The data indicate that α-adrenergic agonists mobilize extracellular Ca^{2+} as well as intracellular Ca^{2+} and that both pools are required for the full expression of hormone-induced responses in rat liver.

Introduction

A redistribution of cellular Ca^{2+} is involved in the action of α-adrenergic agonists in liver (reviewed in Exton, 1981; Williamson et al., 1981; Taylor et al., 1983b). The earliest detectable movement of Ca^{2+} induced by α-adrenergic agonists is the mobilization of an intracellular pool of the ion (Blackmore et al., 1982; Reinhart et al., 1982b). Although some responses to α-adrenergic agonists are dependent on the mobilization of intracellular Ca^{2+} stores (Reinhart et al., 1982b) there is also some evidence for an additional role of extracellular Ca^{2+} in α-adrenergic effects. Firstly, extracellular Ca^{2+} is in rapid equilibrium with the intracellular hormone-sensitive pool of Ca^{2+}; for example after cessation of hormone administration to the perfused rat liver, extracellular Ca^{2+} rapidly repletes the intracellular hormone-sensitive pool (Reinhart et al., 1982b). Secondly, some of the prolonged α-agonist-induced responses become more transient in the absence of extracellular Ca^{2+} and thirdly, a kinetic analysis of $^{45}Ca^{2+}$ fluxes in isolated hepatocytes has indicated that α-adrenergic agonists stimulate the flux of Ca^{2+} from the extracellular to the cytoplasmic compartment (Barritt et al., 1981).

In the present study we set out to examine the relative contribution of both intracellular and extracellular Ca^{2+} pools in the expression of a number of α-adrenergic agonist-induced responses in the perfused rat liver. We conclude that both extracellular and intracellular Ca^{2+} pools are important in the full expression of the α-agonist-induced effects, and that the regulation of Ca^{2+} fluxes across the plasma membrane constitutes an important event in the action of these agonists.

Experimental

Animals and perfusions - Male Wistar-strain albino rats weighing between 230 and 270 g and having free access to food, were used for all experiments. Rats were anaesthetized with sodium pentobarbitone (50 mg/kg body wt.), and the livers perfused with Krebs-Henseleit bicarbonate medium (Krebs & Henseleit, 1932) equilibrated with O_2/CO_2 (19:1) as described previously (Reinhart et al., 1982a). All livers were preperfused for 15 min with medium containing 1.3 mM added $CaCl_2$ at 35°C and at a flow-rate of 3.5 ml/min/g wet liver. At 15 min the free Ca^{2+} concentration in the perfusate was altered either by decreasing the concentration of added $CaCl_2$ or by adding low concentrations of EGTA to the perfusate. All experiments were performed between 08:00h and 12:00h to minimise diurnal fluctuations of basal metabolism.

Perfusate calcium determinations - For some experiments the perfusate Ca^{2+} concentration was continuously monitored exactly as described previously (Reinhart et al., 1982b). A Radiometer F2112 Ca^{2+}-selective electrode was placed into a small flow-through chamber close to the liver, and coupled to a Radiometer GK 2401 C combination electrode via an agarose/KCl salt bridge. Signal amplification was achieved by connecting both electrodes to an Orion model 901 microprocessor ion-analyzer set to the grounded-solution mode. Output was channeled through a bucking-voltage device (Madeira, 1975) to a Spectra-Physics SP4100 computing integrator which was programmed (Autolab BASIC) to display changes in the perfusate Ca^{2+} concentration (peak height) and the total amounts of Ca^{2+} taken up or extruded by the liver (integration mode). Further details are described in Reinhart et al., (1982b, 1983a). As the logarithm of the electrode response becomes non-linear at Ca^{2+} concentrations below 10^{-6}M, atomic absorption spectroscopy was used to determine total Ca^{2+} concentration changes in experiments where EGTA was

used to lower the Ca^{2+} concentration below 10^{-6}M. For this procedure samples of perfusate (4 ml) were assayed in 0.1% KCl (5 ml total volume) using a NO_2/Acetylene flame (medical grade gases). Standard $CaCl_2$ solutions were prepared in 80% (v/v) Krebs-Henseleit medium plus 0.1% KCl.

Free calcium concentration calculations - Free Ca^{2+} concentrations were calculated using an algorithm of the program (Comics) developed by Perrin & Sayce (1967). The logarithm of the total formation constants (Sillen & Martell, 1971) for the following complexes were considered pH = 7.4: $PO_4^{3-} + H^+$ (11.8). $EGTA^{4-} + Ca^{2+}$ (11.0), $CO_3^{2-} + H^+$ (10.25), $EGTA^{4-} + H^+$ (9.54), $HEGTA^{3-} + H^+$ (8.93), $HPO_4^{2-} + H^+$ (7.15), $HEGTA^{3-} + Ca^{2+}$ (5.3), $EGTA^{4-} + Mg^{2+}$ (5.2), $HEGTA^{3-} + Mg^{2+}$ (3.4), $HCO_3^- + Mg^{2+}$ (3.4), $CO_3^{2-} + Ca^{2+}$ (3.2), $HPO_4^{2-} + Ca^{2+}$ (2.77), $H_2EGTA^{2-} + H^+$ (2.73), $HPO_4^{2-} + Mg^{2+}$ (2.5), $H_2PO_4^- + Ca^{2+}$ (1.5).

Perfusate glucose and oxygen determinations - Glucose release by the liver was determined by the glucose oxidase/peroxidase method as previously described (Reinhart et al., 1982b). Perfusion circuit lag-times, from the point of hormone infusion to the sampling point, were routinely determined using [^{3}H]-inulin. All data shown have been corrected for this lag-time. The oxygen consumption by the liver was calculated from the difference between influent and effluent oxygen concentrations measured with a Clark-type oxygen electrode modified for a flow-through mode of operation as described previously (Reinhart et al., 1982a). The linearity of the electrode response was established using the colorimetric oxygen assay of Hamlin & Lambert (1971). Perfusion circuit lag-times were determined using sodium dithionite, and data shown were corrected for this lag-time.

Other analytical procedures - For some experiments 1.5 ml perfusate samples were centrifuged to remove contaminating red blood cells and the supernatants mixed with 0.2 ml of 2 M perchloric acid containing 5 mM Hepes. After standing on ice for 10 min, samples were neutralized with 0.2 ml KOH and the precipitate removed by centrifugation. Portions of the supernatant were taken to assay pyruvate, lactate, (Gutman & Wahlefeld, 1974), β-hydroxybutyrate and acetoacetate (Williamson & Mellanby, 1974).

Results

Previously we have shown that responses to α-adrenergic agonists, such as a stimulation in the rates of glycogenolysis, respiration (Reinhart et al., 1982b) and gluconeogenesis (Taylor et al., 1983a), are obligatorily dependent on the mobilization of an intracellular pool of Ca^{2+}. To assess the extent to which such a mobilization forms part of a more general mechanism whereby α-adrenergic agonists induce intracellular events, we have examined whether or not a number of other α-agonist-induced metabolic changes are similarly dependent initially on intracellular Ca^{2+}. In these experiments we have employed a technique whereby the ability to rapidly alter both the perfusate Ca^{2+} concentration and the duration of α-adrenergic agonist administration, allows the manipulation of a small intracellular hormone-sensitive pool of Ca^{2+} (Reinhart et al., 1982b). Experiments were carried out therefore under conditions either facilitating the depletion of the hormone-sensitive Ca^{2+} pool (4 μM perfusate Ca^{2+}) or under conditions allowing the continued repletion of the pool (1.3 mM perfusate Ca^{2+}; Reinhart et al., 1982b).

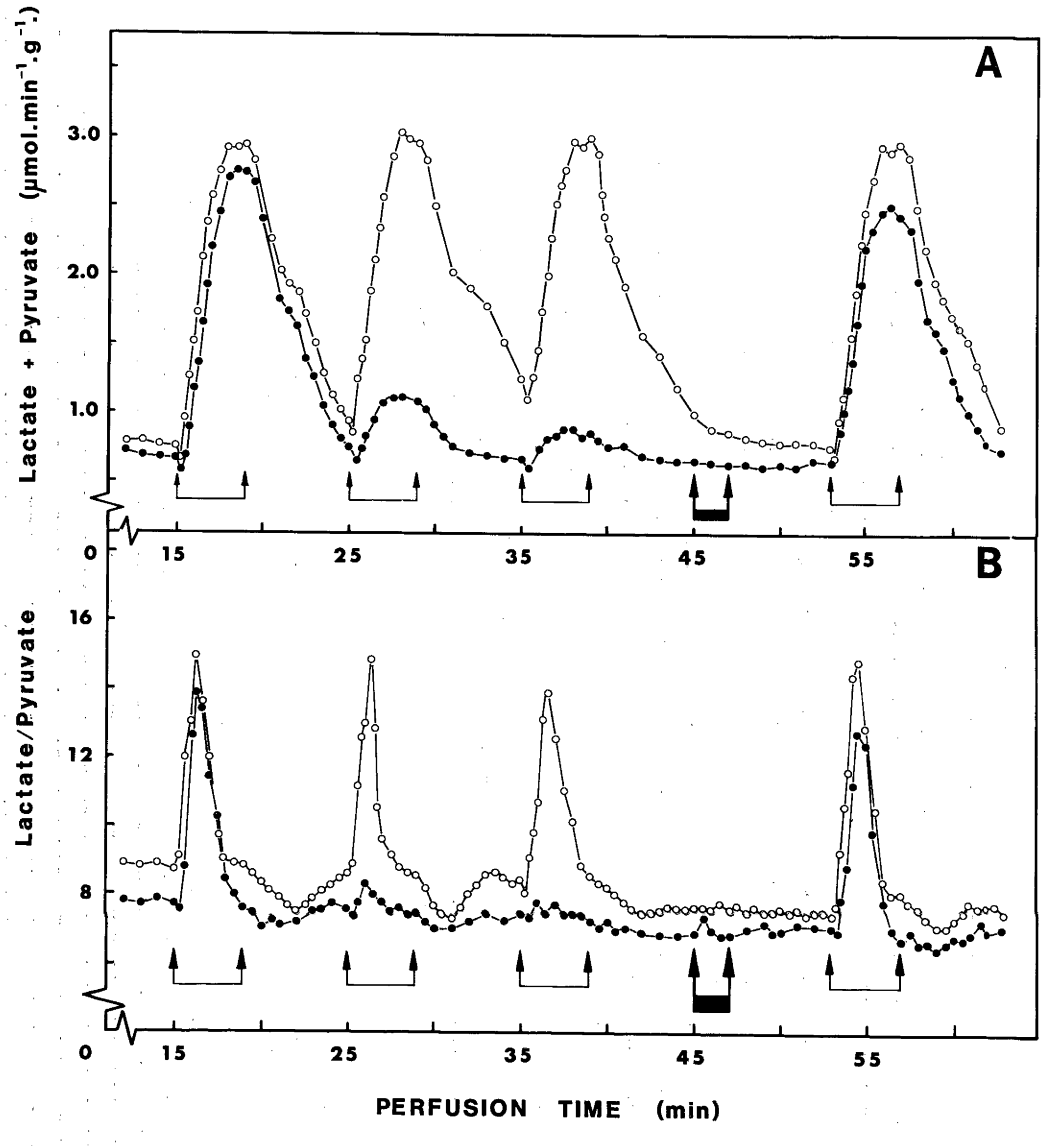
Lactate plus pyruvate formation and the cytoplasmic redox ratio.

Consistent with previous observations (Althaus-Saltzman et al., 1980; Scholz & Schwabe, 1980) the data in Fig. 1A indicate that phenylephrine rapidly stimulates the rate of lactate plus pyruvate formation. By 5 to 6 min after the termination of hormone infusion, the rates of output return to basal values. The present data in addition show that at an extracellular Ca^{2+} concentration of 1.3 mM, repeated pulses of phenylephrine administration induce repeated stimulations (by almost 3-fold) of lactate plus pyruvate output. When the experiment is carried out at an extracellular Ca^{2+} concentration of 4 μM, only the first of the 3 pulses of phenylephrine induces significant production of lactate plus pyruvate. A single 2 min administration of 1.3 mM $CaCl_2$ is sufficient to almost totally restore the response.

The data in Fig. 1B show that as well as stimulating total lactate plus pyruvate output, phenylephrine also raises by almost 2-fold the lactate/pyruvate ratio, thought to reflect the cytosolic redox ratio (Williamson et al., 1967). However, it is noteworthy that in contrast to the sustained effect of the α-agonist on lactate and pyruvate output (Fig. 1), respiration and glucose output (Reinhart et al., 1982b), the effect on the cytosolic redox ratio is transient. Maximum changes are observed between 45 and 60 s of α-agonist infusion; thereafter the lactate/pyruvate ratio returns to basal values even while phenylephrine is still being infused. Subsequent brief administrations of phenylephrine result in qualitatively similar responses when the perfusate Ca^{2+} concentration is 1.3 mM. When the perfusate Ca^{2+} concentration is reduced to 4 μM, only the first of the 3 pulses of phenylephrine induces a significant increase in the lactate/pyruvate ratio. Again a single 2 min administration of 1.3 mM $CaCl_2$ is sufficient to restore the response.

Figure 1. *The effect of brief repeated infusions of phenylephrine on liver lactate plus pyruvate formation and on the cytoplasmic redox ratio.*

Livers of fed rats were perfused as described in Experimental. The perfusion medium contained 1.3 mM Ca^{2+} for the first 10 min. At 10 min the perfusate Ca^{2+} concentration was reduced to 4 μM for some livers (●) while for others it remained at 1.3 mM (○). At 15, 25, and 35 min, livers were infused with phenylephrine (2 μM) for 4 min (faint arrows). At 45 min, livers perfused with medium containing 4 μM Ca^{2+} were infused with 1.3 mM $CaCl_2$ for 2 min (bold arrows). At 53 min livers were again infused with phenylephrine for 4 min. Perfusate lactate and pyruvate, were determined as described in Experimental. Results shown are the means from between 3 to 6 independent experiments. For the sake of clarity S.E.M.'s have been omitted.


A
B
Lactate + Pyruvate (µmol.min⁻¹.g⁻¹.)
Lactate/Pyruvate
3.0
2.0
1.0
0
16
12
8
0
15
25
35
45
55
PERFUSION TIME (min)

β-hydroxybutyrate plus acetoacetate formation and the mitochondrial redox ratio.

Having observed that changes in the cytoplasmic redox ratio induced by repeated administration of α-agonists, are dependent on extracellular Ca^{2+}, we carried out a similar set of experiments examining the mitochondrial redox ratio. Measurements of β-hydroxybutyrate and acetoacetate are considered to provide such an assessment (Williamson et al., 1967).

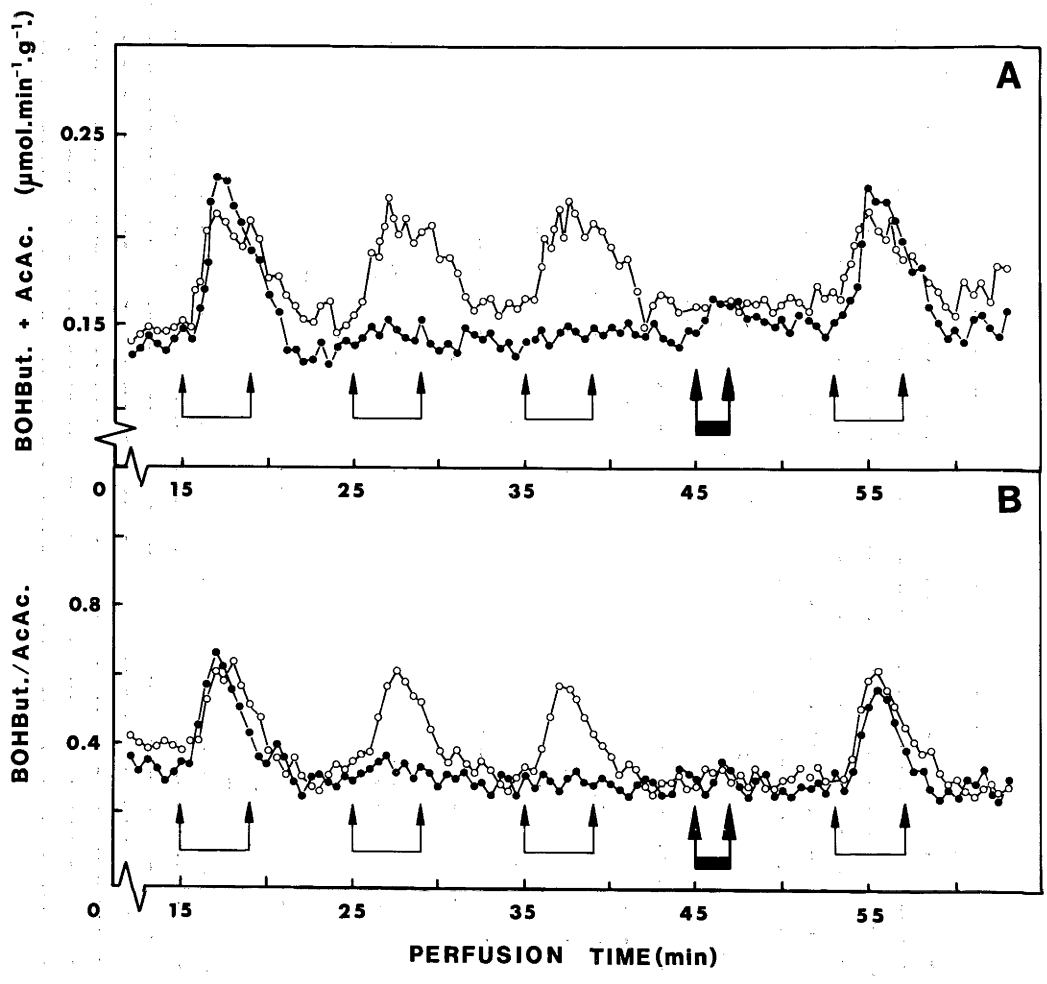
The data in Fig. 2A show the effect of repeated administration of phenylephrine on ketone body formation. With 1.3 mM Ca^{2+} in the perfusion medium, the concentration of these metabolites almost doubled following each pulse of phenylephrine. With 4 μM Ca^{2+} in the perfusion medium, however, only the first of the 3 pulses of hormone induces any significant formation of these metabolites. As shown in Fig. 1A, a short exposure to 1.3 mM Ca^{2+} fully restores the phenylephrine-induced response.

Fig. 2B shows that the ratio β-hydroxybutyrate/acetoacetate doubles following each pulse of phenylephrine in the presence of 1.3 mM Ca^{2+}; like the lactate/pyruvate ratio (Fig. 1B) these also are transient, and are decreased to be approx. 40% above basal values by 4 min of phenylephrine administration. If the duration of phenylephrine treatment is increased to 5 min or longer the redox ratios return to basal values (data not shown). These data are qualitatively similar to the effects of α-adrenergic agonists on mitochondrial redox ratios previously observed in livers of fasted rats (Taylor et al., 1983a). At 4 μM perfusate Ca^{2+}, only the first pulse of hormone induces a response and this is restored following a short exposure to 1.3 mM Ca^{2+}.

The data in Figs. 1 and 2 thus provide evidence that the mobilization of intracellular Ca^{2+} forms part of a general mechanism

Figure 2. The effect of brief repeated infusions of phenylephrine on the formation of β-hydroxybutyrate and acetoacetate and the mitochondrial redox ratio in perfused rat liver.

The experiments were carried out exactly as described for Fig. 1 except that perfusate β-hydroxybutyrate and acetoacetate were determined as described in Experimental. The perfusate Ca^{2+} concentration was either 1.3 mM (O) or 4 μM (●). Faint and bold arrows show the infusion of phenylephrine (2 μM) or Ca^{2+} (1.3 mM), respectively. Results shown are the means of between 3 to 6 independent experiments. For the sake of clarity S.E.M.'s have been omitted.


A
B
BOHBut. + AcAc. (µmol.min⁻¹.g⁻¹.)
BOHBut./AcAc.
0.25
0.15
0
0.8
0.4
0
15
25
35
45
55
PERFUSION TIME(min)

whereby α-adrenergic agonists induce hepatic responses, and that some of these responses are transient whereas others are prolonged.

K^+ fluxes.

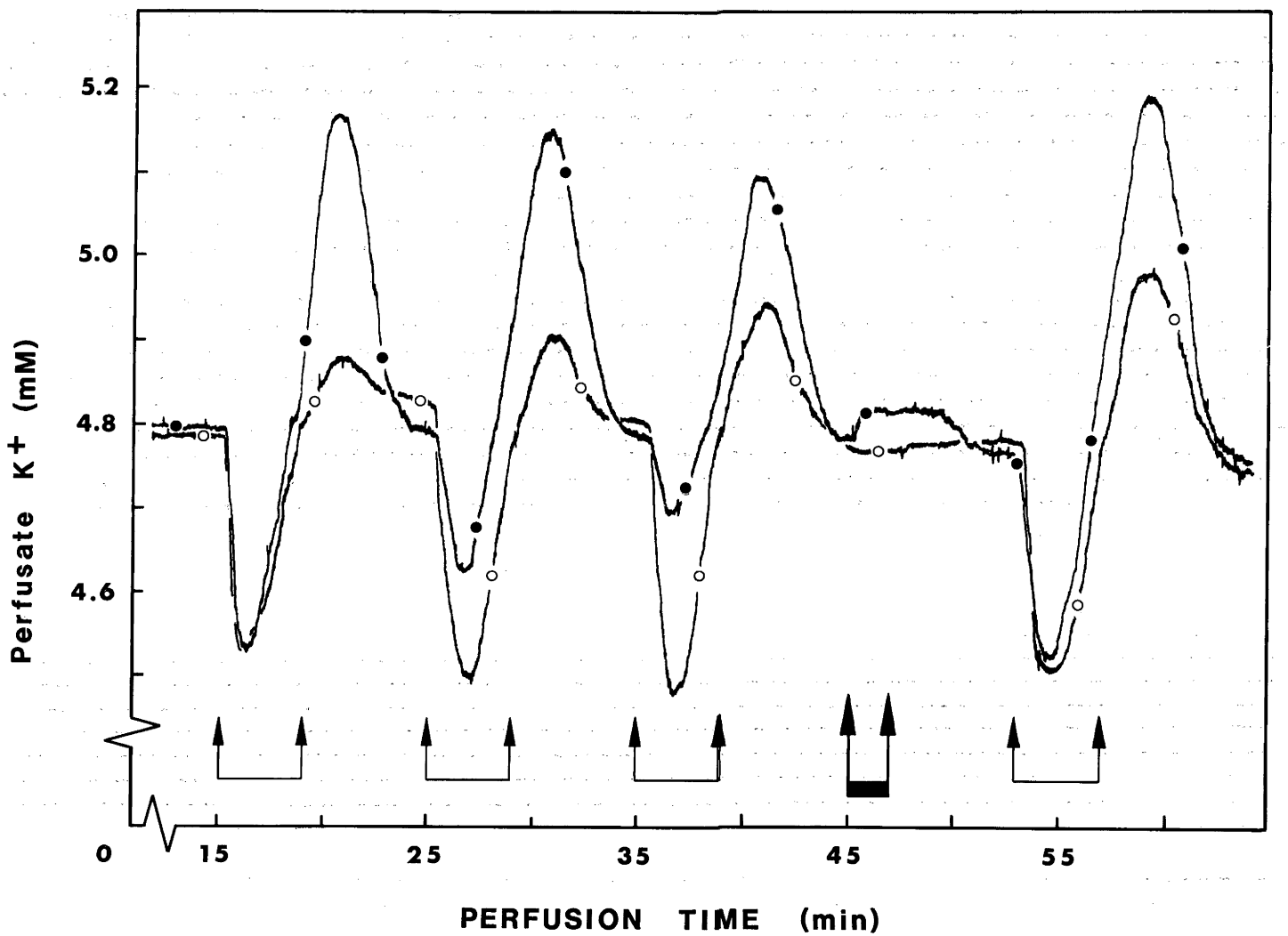
Evidence exists that administration of phenylephrine induces rapid changes in K^+ fluxes in rat liver (Jacob & Diem, 1975; Burgess et al., 1979; Blackmore et al., 1979; Althaus-Saltzman et al., 1980; Burgess et al., 1981; Capiod et al., 1982). Consistent with these reports the data in Fig. 3 show that at 4 μM Ca^{2+}, phenylephrine induces significant K^+ flux changes consisting of 2 components; K^+ influx followed by a more prolonged phase of K^+ efflux from the cell. Repeated administrations of phenylephrine under these conditions result in the inhibition of predominantly the K^+ influx phase, with little effect on the secondary K^+ uptake phase. A single 2 min administration of 1.3 mM $CaCl_2$ fully restores the magnitude of the initial K^+ influx phase. With 1.3 mM Ca^{2+} in the perfusate a different pattern of K^+ fluxes can be seen. Although the initial K^+ influx phase is very similar, the magnitude of K^+ efflux is relatively small during the first phenylephrine administration and increases during each successive phenylephrine treatment. The significance of these differences in K^+ fluxes was not further examined in this series of experiments.

Ca^{2+} fluxes, glycogenolysis and respiration.

Further experiments were designed to define the role of extracellular Ca^{2+} in the action of α-agonists. The data in Fig. 4 show how the magnitude of the Ca^{2+} gradient across the plasma membrane influences the expression of phenylephrine-induced Ca^{2+} fluxes, glucose output and respiration. When the Ca^{2+} gradient is high (i.e. 1.3 mM Ca^{2+} is perfused through the liver), phenylephrine stimulates respiration (4B) and glucose output (4C) for the duration of α-agonist infusion. The effect on Ca^{2+} fluxes (4A) however, occurs in two phases; firstly there

Figure 3. *The effect of brief repeated infusions of phenylephrine of liver K^+ fluxes.*

The experiments were carried out exactly as described for Fig. 1 except that the K^+ concentration in the effluent medium was continuously monitored with a K^+ electrode as described in Experimental. The perfusate Ca^{2+} concentration was either 1.3 mM (O) or 4 μM (●). Faint and bold arrows show the infusion of phenylephrine (2 μM) or Ca^{2+} (1.3 mM), respectively. Results shown are representative recordings from between 3 to 5 independent experiments.


5.2
5.0
4.8
4.6
Perfusate K+ (mM)
0
15
25
35
45
55
PERFUSION TIME (min)

is a transient efflux of Ca^{2+}, and, only after removal of the agonist, a transient reuptake of Ca^{2+}. When the Ca^{2+} gradient is largely removed by reducing the perfusate Ca^{2+} concentration and adding a slight excess of EGTA (30 μM), a number of different effects are observed. Firstly, EGTA mobilizes a pool of Ca^{2+} consisting of between 50 and 60 nmol . g $liver^{-1}$ within three min of its administration. Presumably this pool predominantly represents Ca^{2+} bound to the outside of the plasma membrane (Claret-Berthon et al., 1977). However, neither the loss of this pool nor the low extracellular Ca^{2+} concentration dimishes the rate or amount of Ca^{2+} mobilized by phenylephrine, indicating that EGTA-accessible plasma membrane-bound Ca^{2+} does not form part of the cellular hormone-sensitive pool of Ca^{2+}. Secondly, no compensatory reuptake of Ca^{2+} is observed after removal of the α-agonist. However, the major metabolic difference relates to the maintenance of the sustained glycogenolytic and respiratory responses. In the absence of extracellular Ca^{2+} these responses become transient, and while the maximal extent of stimulation is very similar to that observed at 1.3 mM Ca^{2+}, the responses gradually return to basal values within 5 to 6 min.

The data in Fig. 5 further show the relationship between extracellular Ca^{2+} and phenylephrine on the sustained effects of the agonist. The transient glucose output (A) or oxygen uptake (B) responses observed in perfusions with 4 μM Ca^{2+} can be converted to prolonged responses, by simply returning the perfusate Ca^{2+} concentration to 1.3 mM while the α-adrenergic agonist is still being administered. The extent of restoration is dependent on the extracellular Ca^{2+} concentration. At 1.3 mM Ca^{2+} almost total restoration of the response is observed, while at 400 μM Ca^{2+} partial restoration is observed (Fig. 5) with little restoration apparent at 100 μM Ca^{2+} or less (data not shown). Both the time of onset, and the time taken to reach maximal rates of respiration

Figure 4. *The effect of the extracellular Ca^{2+} concentration on phenylephrine-stimulated Ca^{2+} fluxes, glucose output and respiration in perfused rat liver.*

Livers of fed rats were perfused as described in Experimental. For all livers the perfusion medium contained 1.3 mM Ca^{2+} for the first 12 min. At 12 min the perfusate Ca^{2+} concentration was either reduced to 4 μM (●) and at 15 min reduced further to less than 10^{-7}M by the continuous infusion of a small excess (30 μM) of EGTA, or maintained at 1.3 mM (○). At 19 min all livers were infused with phenylephrine (2 μM) for a total of 8 min. The perfusate Ca^{2+} concentration (A) was determined either by atomic absorption spectroscopy (●) or Ca^{2+}-selectrode (○). Changes in perfusate glucose and oxygen concentration were determined as described in Experimental. The continuous recordings (A;○ and C; ○,●) are from single recordings representative of between 3 and 8 independent experiments. All other data are expressed as means, the S.E.M.'s having been omitted for the sake of clarity.

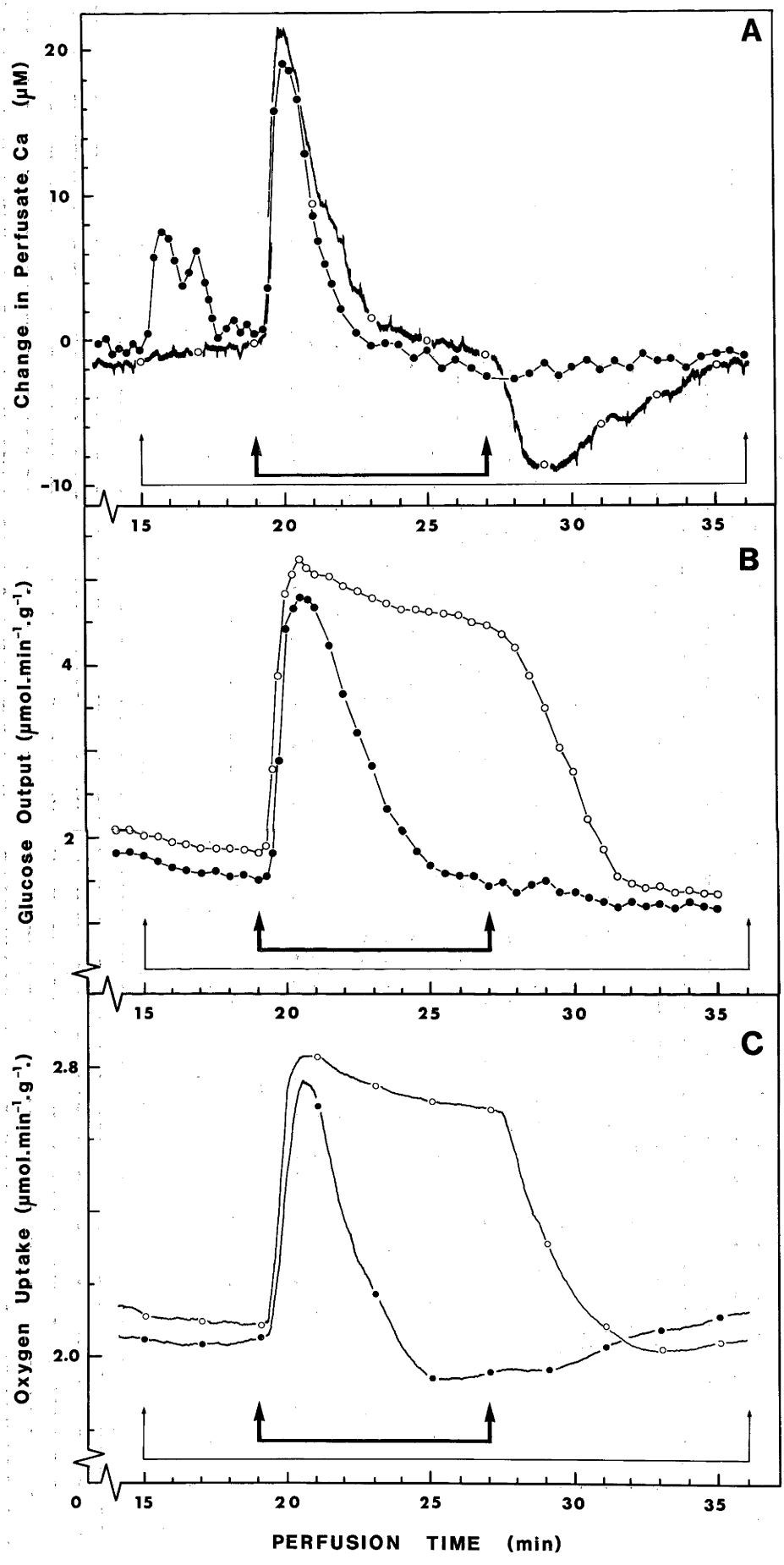
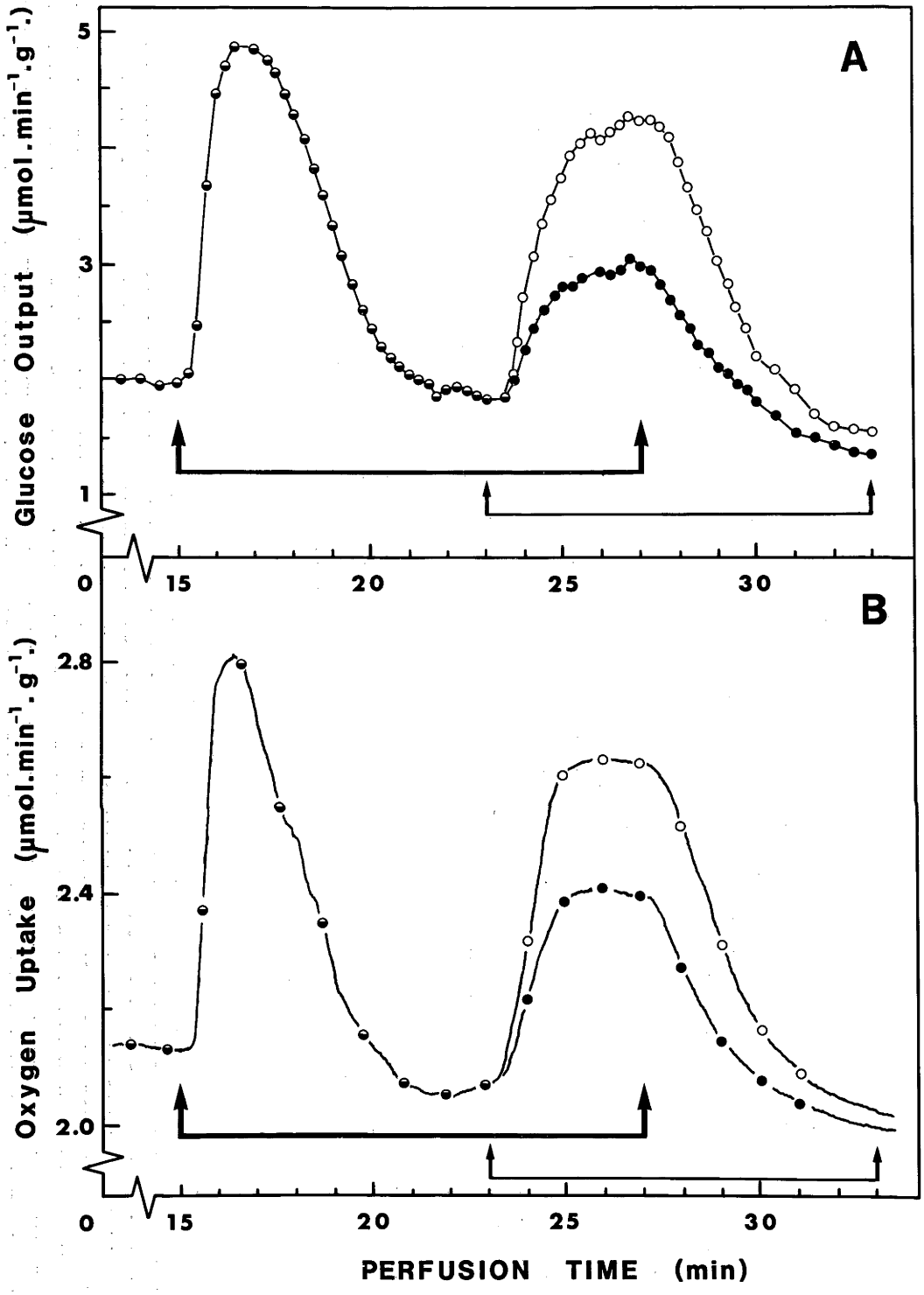

A
Change in Perfusate Ca (μM)
20
10
0
-10
15
20
25
30
35
B
Glucose Output (μmol.min⁻¹.g⁻¹.)
4
2
C
Oxygen Uptake (μmol.min⁻¹.g⁻¹.)
2.8
2.0
0
PERFUSION TIME (min)

Figure 5. *The effect of Ca^{2+} infusions on glucose output and respiration in phenylephrine-challenged perfused rat liver.*

Livers of fed rats were perfused as described in Experimental. At 10 min the perfusate Ca^{2+} concentration was reduced to less than 0.1 μM. At 15 min all livers were infused with phenylephrine (2 μM). At 23 min the extracellular Ca^{2+} concentration was elevated to either 400 μM (●) or 1.3 mM(○), and 4 min later the infusion of phenylephrine terminated. The data in A are means from between 3 and 5 independent experiments, and the data in B representative recordings from between 3 and 5 independent experiments.


A
B
Glucose Output (µmol.min⁻¹.g⁻¹.)
Oxygen Uptake (µmol.min⁻¹.g⁻¹.)
5
3
1
2.8
2.4
2.0
0
15
20
25
30
PERFUSION TIME (min)

or glucose output, is longer for the Ca^{2+}-induced restoration, than for phenylephrine-induced rates, consistent with the interpretation that initial α-adrenergic induced responses are due to the mobilization of intracellular Ca^{2+}. Hence this experimental approach allows the separation of phenylephrine-induced responses due to intracellular Ca^{2+}, from those dependent on extracellular Ca^{2+}. Further aspects of this are discussed in the accompanying paper (Reinhart et al., 1983b).

Discussion

The data presented in this communication have revealed new insights about the redistribution of Ca^{2+} induced by α-adrenergic agonists and the role these play in the expression of α-agonist-induced effects.

Firstly, by using a previously described technique to gently deplete the intracellular hormone-sensitive pool of Ca^{2+} we have shown that the mobilization of this pool is obligatory for the expression of α-agonist-induced responses such as cellular Ca^{2+} efflux, mitochondrial respiration, glycogenolysis (Reinhart et al., 1982a,b), cytoplasmic and mitochondrial redox ratio changes, glycolysis, ketogenesis, K^+ uptake (this paper) and gluconeogenesis (Taylor et al., 1983a). The mechanism whereby a redistribution of Ca^{2+} alters the rate of these responses has not been fully elucidated but an elevation in the concentration of cytoplasmic Ca^{2+} (Barritt et al., 1981; Murphy et al., 1980) probably plays a role.

Secondly, the hepatic responses to α-adrenergic agonists appear to be either "transient" or "sustained", depending on the temporal progression of the response during the first 3 to 5 min of α-agonist administration in medium containing mM Ca^{2+} concentrations. The cytoplasmic and mitochondrial redox ratios, and Ca^{2+} and K^+ flux changes appear to be transient. Changes in the rates of total metabolite release

on the other hand appear to be sustained. The mechanistic basis for this has not yet been established, but because of the Ca^{2+}-dependence, it is tempting to speculate that transient responses are triggered by the initial change in cytoplasmic Ca^{2+} concentration, while sustained responses may require a continually elevated cytoplasmic Ca^{2+} concentration.

The third major observation made in this study is that although virtually all hepatic responses to α-agonists are dependent on the redistribution of intracellular Ca^{2+}, this alone is not sufficient for the full expression of α-agonist-induced effects. The continued expression of sustained effects appears to be dependent on an extracellular pool of Ca^{2+}. Hence the redistribution of Ca^{2+} induced by α-adrenergic agonists appears to follow a strict temporal sequence wherein predominantly intracellular Ca^{2+} is mobilized during the first 1 to 2 min of α-agonist action, and extracellular Ca^{2+} is utilized at longer times. Such a scheme is consistent with a steady-state kinetic analysis of α-agonist-induced ^{45}Ca flux changes (Barritt et al., 1981), and furthermore indicates that any long-term responses i.e. responses activated after 3-4 min of α-agonist administration, should be entirely dependent on extracellular Ca^{2+}.

A further point highlighted by these studies is the important role played by Ca^{2+}-transporting enzymes located in the plasma membrane of liver cells. Although these enzymes have not been clearly defined, both the Ca^{2+} uptake and the Ca^{2+} efflux components are regulated by α-adrenergic agonists, and this may have important consequences in maintaining both the total cellular Ca^{2+} content, and the free Ca^{2+} concentration in one or more intracellular compartments. The Ca^{2+} efflux mechanism appears to be operating well below maximal rates as judged by the reserve capacity for Ca^{2+} efflux observed after hormonal stimulation

(Reinhart et al., 1982b), consistent with observations made for the red blood cell Ca^{2+} pump (Lew & Ferreira, 1978; Schazman & Burgin, 1978).

The regulation of Ca^{2+} uptake by liver is complex, since apparently this activity plays a role both during the administration and after the removal of α-adrenergic agonists (Reinhart et al., 1983b; accompanying paper). Fine regulation is essential since markedly elevated cytoplasmic Ca^{2+} concentrations may induce cell damage (Reinhart et al., 1983a).

We thus propose that an obligatory part of the mechanism whereby α-adrenergic agonists stimulate both transient and sustained responses in liver, is the redistribution of cellular Ca^{2+}. This redistribution appears to progress in two stages. Firstly, a bound pool of intracellular Ca^{2+} (Reinhart et al., 1983a) is mobilized and at least partially extruded from the cell, and secondly, the Ca^{2+} uptake/efflux mechanism at the plasma membrane is altered is such a way as to allow the utilization of extracellular Ca^{2+} for the maintenance of some hormone-induced redistribution of intracellular Ca^{2+} (which may include an elevation in the concentration of cytoplasmic Ca^{2+}; Barritt et al., 1981; Murphy et al., 1980). Thus the signal(s) generated in response to α-adrenergic agonist/receptor binding appears to mobilize both intracellular and extracellular Ca^{2+}.

Acknowledgement

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Section B.12

The action of α-adrenergic agonists on plasma membrane calcium fluxes in perfused rat liver.

(Reinhart et al., 1983d)

THE ACTION OF α-ADRENERGIC AGONISTS ON PLASMA MEMBRANE CALCIUM FLUXES IN PERFUSED RAT LIVER

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Proofs to: Dr F.L. Bygrave

Short Title: α-adrenergic agonists and liver Ca^{2+} fluxes

Synopsis

The effect of α-adrenergic agonists on Ca^{2+} fluxes was examined in the perfused rat liver using a combination of Ca^{2+} electrode and $^{45}Ca^{2+}$ uptake techniques. We showed that net Ca^{2+} fluxes can be described by the activities of separate Ca^{2+} uptake and Ca^{2+} efflux components, and that α-adrenergic agonists modulate the activity of both components in a time-dependent manner. Under resting conditions Ca^{2+} uptake and efflux activities are balanced resulting in Ca^{2+}-cycling across the plasma membrane. α-adrenergic agonists, vasopressin and angiotensin, but not glucagon, stimulate the rate of both Ca^{2+} efflux and Ca^{2+} uptake. During the first 2 to 3 min of α-agonist administration the effect on the efflux component is the greater, the net effect being efflux of Ca^{2+} from the cell. After 3 to 4 min of phenylephrine treatment net Ca^{2+} movements are essentially complete, however, the rate of Ca^{2+}-cycling is significantly increased. After removal of the α-agonist a large stimulation in the rate of Ca^{2+} uptake leads to the net accumulation of Ca^{2+} by the cell. The potential role of these Ca^{2+} flux changes in the expression of α-adrenergic agonist-mediated effects is discussed.

Introduction

A redistribution of cellular Ca^{2+} plays a vital role in the mechanism whereby α-adrenergic agonists induce many hepatic responses (reviewed in Exton, 1981; Williamson et al., 1981; Taylor et al., 1983a). Aspects of this redistribution which so far have been described are the mobilization of two intracellular pools of Ca^{2+} (Reinhart et al., 1982a), a rise in the cytoplasmic free Ca^{2+} concentration (Barritt et al., 1981; Murphy et al., 1980) and a transient stimulation in the rate of net Ca^{2+} efflux from the cell even at an extracellular Ca^{2+} concentration of 1.3 mM (Blackmore et al., 1982; Reinhart et al., 1982a; Siess et al., 1983).

A further feature, revealed in the previous paper (Reinhart et al., 1983b), is that both intracellular and extracellular Ca^{2+} are necessary for the full expression of α-adrenergic agonist-induced responses in perfused rat liver. This feature carries with it the implication that Ca^{2+} movements across the plasma membrane are modulated by α-adrenergic agonists. Consequently we considered it important to attempt to define these movements and the nature of the control imposed by these agonists. To achieve this we have used a technique that allows the simultaneous determination of both net Ca^{2+} movements, and of the Ca^{2+} uptake rate in intact perfused rat liver.

We report here that the movement of Ca^{2+} across the plasma membrane is regulated by the activity of a Ca^{2+}-translocation cycle that consists of Ca^{2+} efflux and Ca^{2+} influx components. α-adrenergic agonists modulate the activity of both components, the net flow of Ca^{2+} being determined by the time course and magnitude of stimulation of each. The possibility is discussed that this cycle contributes, among other things, to the regulation of the cytoplasmic free Ca^{2+} concentration.

Experimental

Animals and perfusions - Male Wistar-strain albino rats weighing between 230 and 270g and either having free access to food, or being starved for 48 h (Taylor et al., 1983b) were used (see Legend to Figs). Rats were anaesthetized with sodium pentobarbitone (50 mg/kg body wt.), and the livers perfused with Krebs-Henseleit bicarbonate medium (Krebs & Henseleit, 1932) equilibrated with O_2/CO_2 (19:1) using a non-recirculating mode as described previously (Reinhart et al., 1982b).

Perfusate calcium concentration determination - This was continuously monitored using a Radiometer F2112 Ca^{2+}-selective electrode placed in a flow-through chamber. Signal amplification was achieved using an Orion model 901 microprocessor ionanalyzer coupled to a Spectra-Physics SP4100 computing integrator. Further details are described in Reinhart et al., (1982a, 1983a). Free Ca^{2+} concentrations were calculated from total concentrations using an algorithm of the program Comics (Perrin & Sayce, 1967) as described previously (Reinhart et al., 1983a).

$^{45}Ca^{2+}$ uptake assay - A trace amount of $^{45}CaCl_2$ (approx. 3×10^7 d.p.m./ml) was infused by means of a glass/glass infusion syringe (EVA superglass μ- micromatic) mounted on a Braun Perfusor IV, into either the inflow cannula or the outflow cannula of the perfused liver system. Plastic or metal/glass syringes resulted in uneven infusion rates. Samples of perfusate (ca. 1 ml) were collected every 5 s between 10 s and 4.0 min after the infusion of $^{45}CaCl_2$. For each time point, two 200 μl samples were added to scintillation vials containing 10 ml of scintillant (6 g of Butyl PBD in 400 ml of 2-methoxyethanol and 600 ml of toluene), and the radioactivity determined to within 0.1% error in a Beckman LS-330 liquid scintillation counter. All counts obtained were corrected for the background radio-activity in the vials plus scintillant. For the

purposes of calculating the rate of $^{45}Ca^{2+}$ removal by the liver, the average of radioactivities for samples collected between 3.5 and 4 min. only was calculated. This short sampling period (30s) was chosen as representing a compromise between the error due to single point determinations on the one hand, and error due to changes in the specific activity of intracellular $^{45}Ca^{2+}$ during prolonged sampling periods, on the other. The difference between the radioactivity in the perfusate following infusion into the inflow and outflow cannulae respectively, was taken to represent the $^{45}Ca^{2+}$ removed by the liver. During the course of these experiments the extent of net Ca^{2+} movement was continuously monitored with the Ca^{2+}-selectrode.

Materials

$^{45}CaCl_2$ was obtained from The Radiochemical Centre Amersham, Bucks., U.K. Ca^{2+}-electrode membranes (F2002) and filling solution S43316 were obtained from Radiometer, Copenhagen, Denmark. Phenylephrine, glucagon, [8-Arginine] vasopressin and [5-valine] angiotensin II were obtained from Sigma Chemical Co., St. Louis, MO. U.S.A. Other chemicals used were of analytical grade.

Expression of Data

All experiments were performed at least three times and data are expressed as means $\pm$ S.E.M. for the number of independent experiments described.

Results

Basal $^{45}Ca^{2+}$ removal

An examination of Ca^{2+} movements in the perfused rat liver by the simultaneous analysis of $^{45}Ca^{2+}$ uptake rates and Ca^{2+}-selective electrode

recordings is shown in Fig. 1. Data in Fig. 1A show that a significant proportion of the $^{45}Ca^{2+}$ infused into the inflow perfusion medium is removed by the liver, a relative plateau of radioactivity usually being observed within 3 to 4 min of infusing the $^{45}Ca^{2+}$. The rate of $^{45}Ca^{2+}$ uptake corresponds to 47 $\pm$ 9 nmol Ca^{2+} min^{-1} .g $liver^{-1}$(n $\pm$ 5) and is represented by Δb in the Fig.

Fig. 1B shows the Ca^{2+}-selectrode recording for the same experiment. It can be seen that during $^{45}Ca^{2+}$ administration the perfusate total Ca^{2+} concentration remains constant indicating that the $^{45}Ca^{2+}$ removal observed in Fig. 1A represents the exchange of $^{45}Ca^{2+}$ for $^{40}Ca^{2+}$.

Because liver contains large extracellular as well as intracellular pools of Ca^{2+} (Claret-Berthon et al., 1977), it was important to differentiate between that proportion of $^{45}Ca^{2+}$ exchanging with extracellular Ca^{2+} from that exchanging with intracellular Ca^{2+}. This was carried out by a procedure similar to that described by Dubinsky & Cockrell (1975) using glycogen-depleted livers (Taylor et al., 1983b) treated with digitonin and antimycin A (Fig. 1C). Under these conditions the equilibration of extracellular $^{45}Ca^{2+}$ with intracellular Ca^{2+} is complete within 2 to 3 min of $^{45}Ca^{2+}$ administration (Dubinsky & Cockrell, 1975). Fig. 1C shows that livers treated in this way remove $^{45}Ca^{2+}$ at a rate of 15 $\pm$ 4 nmol min^{-1} .g $liver^{-1}$ (n = 3). This low rate of $^{45}Ca^{2+}$ removal presumably reflects $^{45}Ca^{2+}$ exchange for extracellular $^{40}Ca^{2+}$ and thus when subtracted from rates of $^{45}Ca^{2+}$ removal measured under basal conditions (Fig. 1A) yields a value of 32 nmol Ca^{2+} min^{-1} .g $liver^{-1}$. This rate, representing $^{45}Ca^{2+}$ exchange with intracellular $^{40}Ca^{2+}$, is a measure of basal Ca^{2+}-cycling since there is no net movement of Ca^{2+} during this time (Fig. 1B).

Figure 1. *Removal of $^{45}Ca^{2+}$ by perfused rat liver in absence of net Ca^{2+} movements.*

Livers of fed (A,B) or starved (C) rats were perfused for 10 min in media containing 1.3 mM Ca^{2+} as described in Experimental. At 10 min the Ca^{2+} concentration was reduced to 600 μM Ca^{2+}, and 5 min later a trace amount of $^{45}CaCl_2$ was infused into the outflow cannula for 4 min. The radioactivity in the perfusate was determined by assaying 200 μl samples, in duplicate, every 5 s. Within 4 min of terminating the $^{45}CaCl_2$ infusion, perfusate radioactivity returns to basal levels (not shown). At 30 min of perfusion trace amounts of $^{45}CaCl_2$ were infused into the inflow cannula. In (B) net changes in the perfusate Ca^{2+} concentration were determined using a Ca^{2+}-specific electrode as described in Experimental. In (C) livers were treated with digitonin (15 μg/ml) and Antimycin A (5 μM) at 24 min and $^{45}Ca^{2+}$ infused as described for A. Results shown are typical experiments chosen from either 4 (A,B) or 3(C) independent experiments performed.

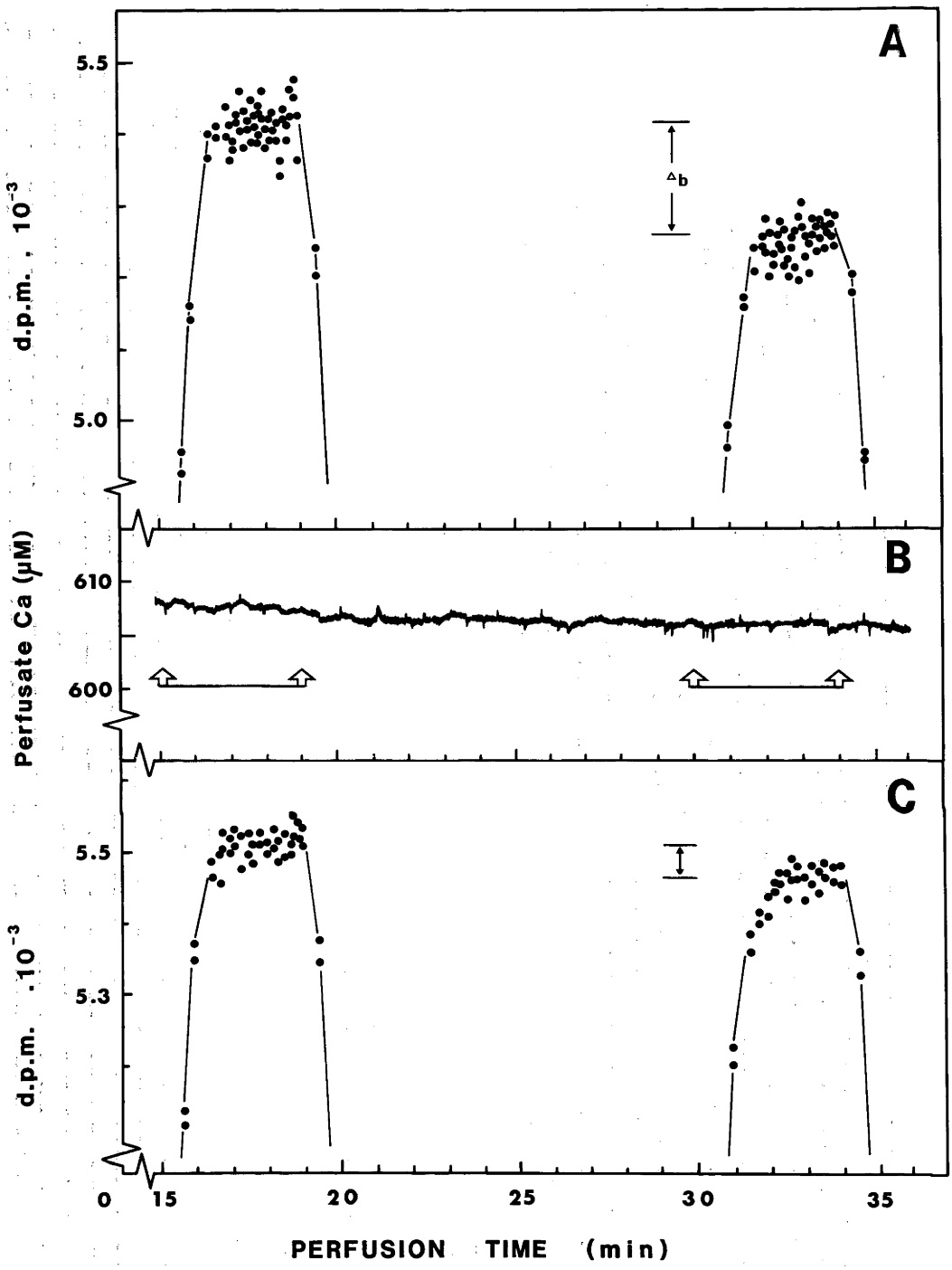
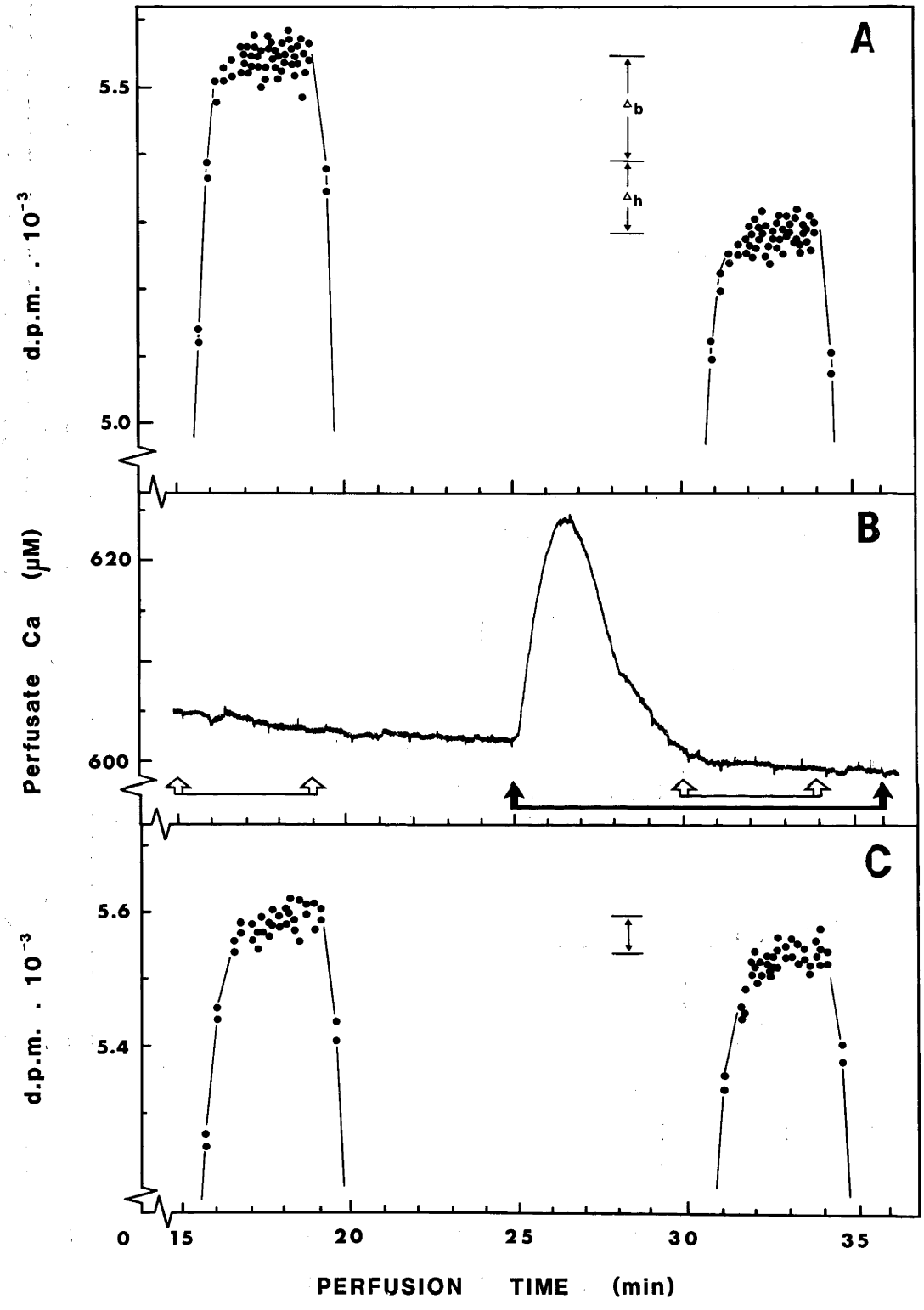

A
B
C
5.5
5.0
610
600
5.5
5.3
d.p.m. , 10^{-3}
Perfusate Ca (μM)
d.p.m. $.10^{-3}$
Δ_b
0
15
20
25
30
35
PERFUSION TIME (min)

Figure 2. Phenylephrine-induced removal of $^{45}Ca^{2+}$ by perfused rat liver.

Livers of either fed (A,B) or starved (C) rats were perfused as described in the legend to Fig. 1. Livers received a continuous infusion of phenylephrine (2 μM) at 25 min of perfusion. All other additions are as described in the legend to Fig. 1.


A
B
C
5.5
5.0
620
600
5.6
5.4
d.p.m. . 10⁻³
Perfusate Ca (μM)
Δb
Δh
0
15
20
25
30
35
PERFUSION TIME (min)

Effect of hormones on $^{45}Ca^{2+}$ removal by the liver.

The effect of the α-adrenergic agonist phenylephrine on liver Ca^{2+} fluxes is shown in Fig. 2A,B,C. As reported previously (Reinhart et al., 1982 a), phenylephrine induces the net efflux of approx. 120 nmol Ca^{2+} .g $liver^{-1}$ within 3 to 4 min of agonist administration. However, by 5 min after agonist administration, the net movement of Ca^{2+} is complete (Fig. 2B). The rate of $^{45}Ca^{2+}$ removal by liver following its infusion at this time has increased significantly above basal values (referred to as Δh in Fig. 2A). An analysis of the rate of $^{45}Ca^{2+}$ exchanging with extracellular $^{40}Ca^{2+}$ (Fig. 2C), carried out as described for Fig. 1C, revealed that this was only slightly increased by phenylephrine administration from 15 ± 4 to 18 ± 5 nmol. min^{-1} .g $liver^{-1}$. Hence by correcting for this exchange with extracellular Ca^{2+} it can be calculated that phenylephrine administration induces an elevation in the rate of $^{45}Ca^{2+}$ uptake from 32 to 61 nmol.min^{-1} .g $liver^{-1}$. Since this increase occurs in the absence of net Ca^{2+} fluxes (Fig. 2A) then this exchange again represents the cycling of extracellular for intracellular Ca^{2+}.

The effect of several other hormones on the rate of $^{45}Ca^{2+}$ removal by the perfused rat liver was also examined. Fig. 3 shows that vasopressin (3 mU/ml) and angiotensin II (10^{-8}M) stimulate the rate of $^{45}Ca^{2+}$ uptake to an extent similar to that induced by phenylephrine. By contrast, glucagon has only a very small effect on $^{45}Ca^{2+}$ uptake, consistent with the minimal effect of this agent on stimulating the rate of net Ca^{2+} efflux from liver (Reinhart et al., 1982a).

Role of extracellular Ca^{2+} in $^{45}Ca^{2+}$ removal.

Fig. 4 shows that both basal (Δb) and hormone-stimulated (Δh) rates of $^{45}Ca^{2+}$ removal respond to extracellular Ca^{2+} over a similar concentration range. $^{45}Ca^{2+}$ removal is not significant below 50 μM Ca^{2+}, is half-maximal at approx 250 μM Ca^{2+} and is near-maximal at 600 μM

Figure 3. Ability of various hormones to induce removal of $^{45}Ca^{2+}$ by perfused rat liver.

Livers of fed rats were perfused and removal of $^{45}Ca^{2+}$ measured as described in the legend to Fig. 2. For each rat, $^{45}Ca^{2+}$ uptake rates were determined by analyzing the average of samples taken every 5 s between 3.5 and 4 min of $^{45}CaCl_2$ administration. Concentrations of hormones used were 2 x 10^{-6}M for phenylephrine (column A), 10^{-8}M for glucagon (column B), 3 mU/ml for Vasopressin (column C) and 10^{-8}M for Angiotensin II (column D). Between 3 and 5 independent experiments were performed for each agent shown.

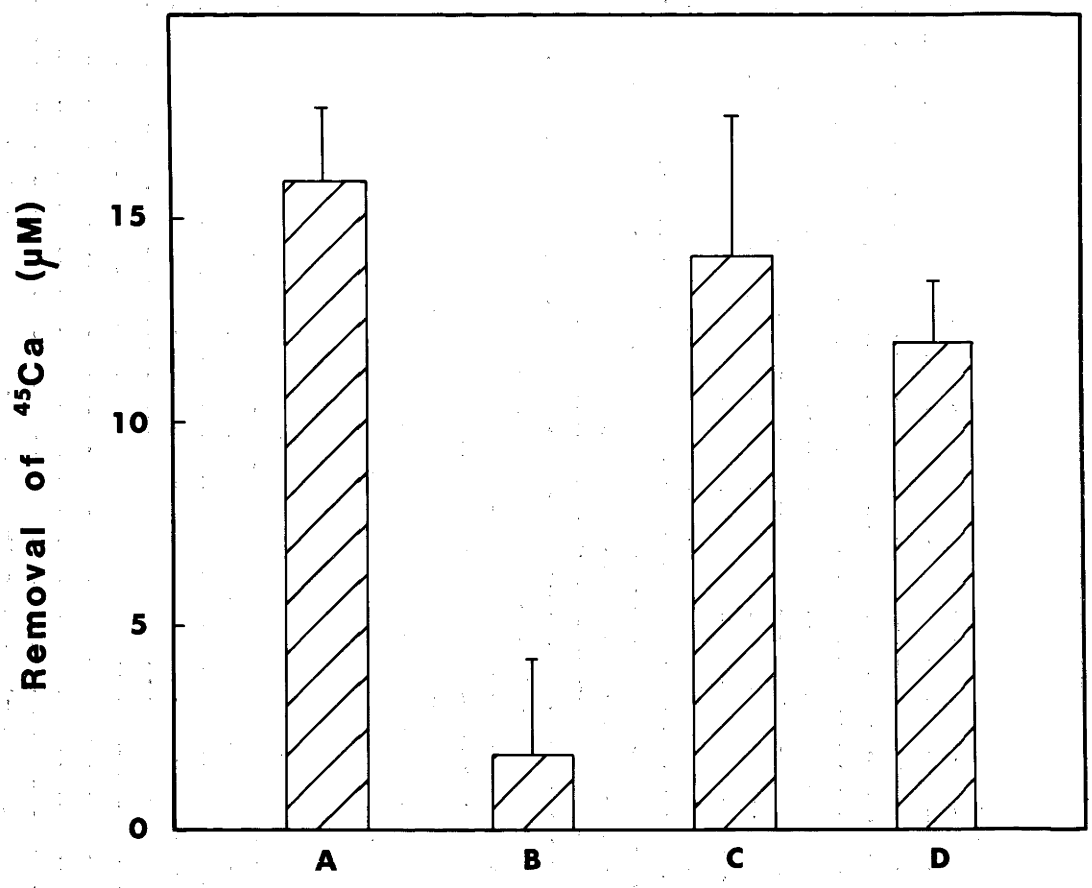
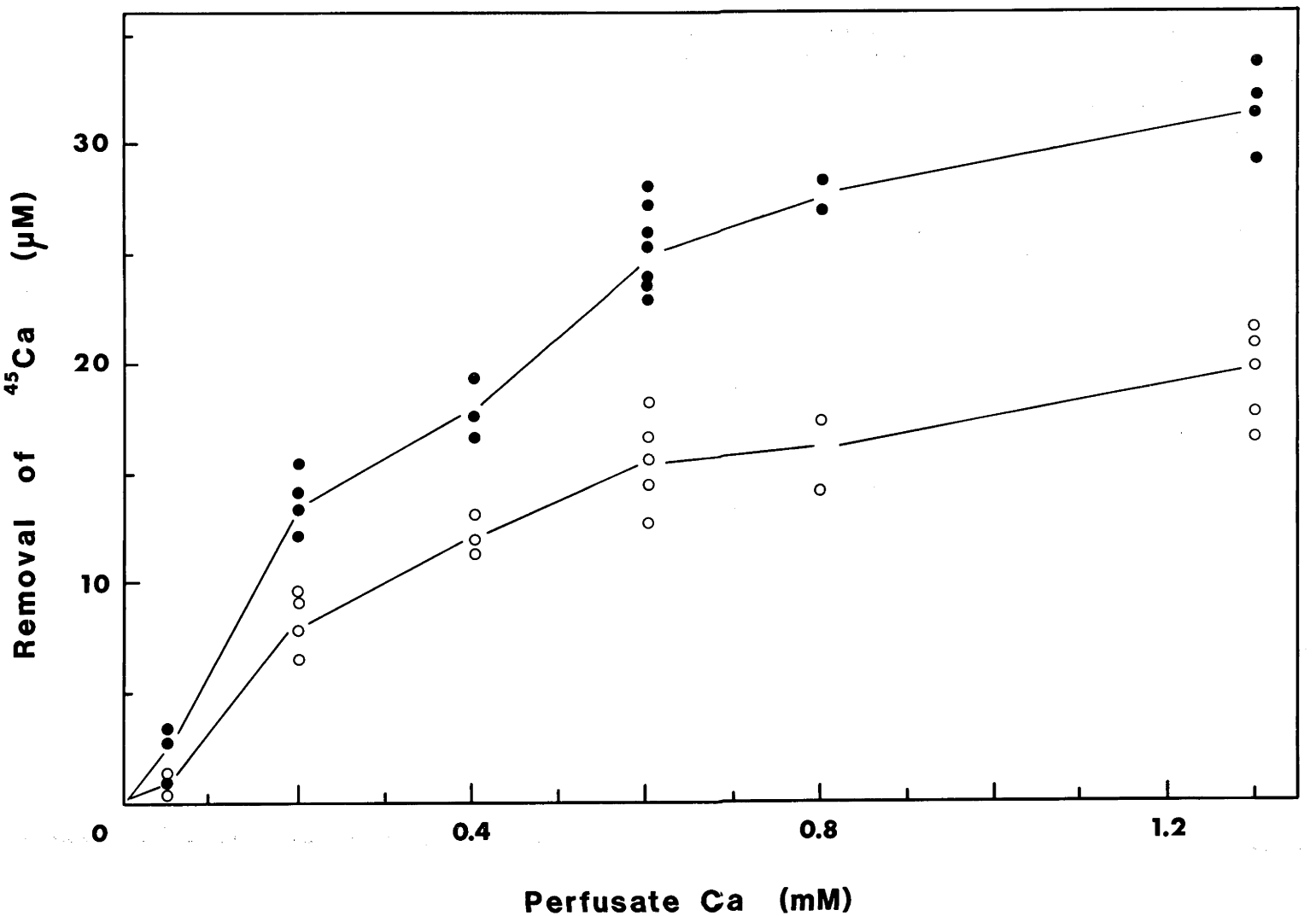

Removal of ^{45}Ca (μM)
15
10
5
0
A
B
C
D

Figure 4. The effect of extracellular Ca^{2+} concentration on the rate of $^{45}Ca^{2+}$ removal by perfused rat liver.

Livers of fed rats were perfused as described in Experimental. At 10 min of perfusion the Ca^{2+} concentration of the perfusate was adjusted to the values indicated. Rates of $^{45}Ca^{2+}$ removal were measured as described in the legend to Fig. 2. Each data point shown is the result of one independent experiment for either phenylephrine-treated (●) or control (○) livers.


Removal of ^{45}Ca (μM)
30
20
10
0
0.4
0.8
1.2
Perfusate Ca (mM)

Ca^{2+} . At all concentrations of Ca^{2+} above 50 μM the ratio of phenylephrine-induced to basal $^{45}Ca^{2+}$ removal is similar.

Time course of phenylephrine-stimulated $^{45}Ca^{2+}$ removal.

The time course of phenylephrine-stimulated $^{45}Ca^{2+}$ removal is depicted in Fig. 5. Between 30 s and 60 s following α-agonist administration (i.e. the earliest measurements possible) the rate of $^{45}Ca^{2+}$ removal into livers is significantly stimulated. Half-maximal effects are observed at between 60 s and 90 s and near-maximal effects at 2.5 min. The effect on $^{45}Ca^{2+}$ removal is sustained for the duration of phenylephrine administration in contrast to the numerous transient responses elicited by the agonist (Reinhart et al., 1983b). Following withdrawal of the α-agonist, a further stimulation in the rate of $^{45}Ca^{2+}$ removal is observed, coinciding with a net accumulation of Ca^{2+} by the liver (Reinhart et al., 1982a). Maximal effects are observed by approx. 90 s after hormone removal and thereafter the rate of Ca^{2+} removal slowly declines to reach basal values after 5 to 10 min.

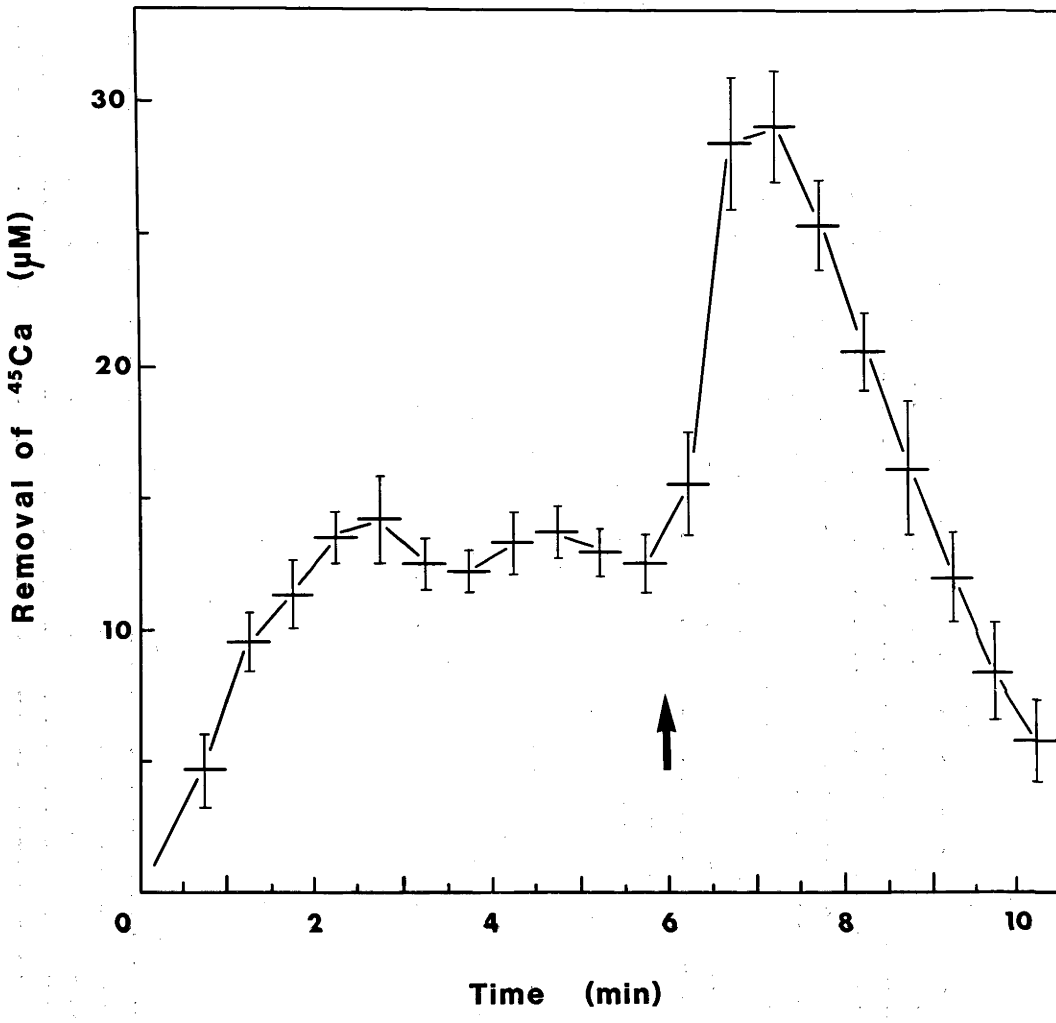
It is important to recall that during the first 3 to 4 min after either the addition of phenylephrine or its removal, there is significant net movement of Ca^{2+} out of or into the liver (Reinhart et al., 1982a; Fig. 2). Under these circumstances therefore, liver Ca^{2+} fluxes are not in a steady-state, i.e. the rate of Ca^{2+} uptake is not equal to the rate of Ca^{2+} efflux.

Discussion

The data presented have revealed the effects of α-adrenergic agonists on the complex interplay between rates of $^{45}Ca^{2+}$ removal and rates of net Ca^{2+} movements in perfused rat liver. $^{45}Ca^{2+}$ removal was defined in terms of rates, requirement for extracellular Ca^{2+}, hormone

Figure 5. *Temporal progression of the onset and decay of phenylephrine-stimulated rates of $^{45}Ca^{2+}$ removal by perfused rat liver.*

Livers of fed rats were perfused as described in the legend to Fig. 1, with the exception that for each time-point the infusion of $^{45}CaCl_2$ through the liver was initiated 3.5 min prior to the administration of phenylephrine. Hence for each timepoint, the rates of $^{45}Ca^{2+}$ uptake between 3.5 and 4 min of $^{45}CaCl_2$ infusion are determined, as described in the legend to Fig. 3. Phenylephrine was removed at 6 min (arrow). The data shown represent the mean $\pm$ S.E.M. for between 3 and 6 independent experiments performed for each time point.


Removal of ^{45}Ca (μM)
30
20
10
0
2
4
6
8
10
Time (min)

specificity, and time-dependent changes following either the administration or withdrawal of the hormone.

One potential complication with the experimental approach used in this paper is the possibility that $^{45}Ca^{2+}$ removal by the liver represents $^{45}Ca^{2+}$ exchange with extracellular $^{40}Ca^{2+}$, rather than with intracellular $^{40}Ca^{2+}$. This problem can be minimized, however, since previous kinetic analyses of cellular Ca^{2+} fluxes have indicated that $^{45}Ca^{2+}$ exchange with extracellular Ca^{2+} is approx. one order of magnitude faster than with intracellular Ca^{2+} (see Borle, 1975, 1981). Hence within 2 to 3 min of $^{45}Ca^{2+}$ infusion, the rapidly-exchangeable extracellular Ca^{2+} pool in cell suspensions (Borle, 1975; Seversen et al., 1976; Foden & Randle, 1978; Martin et al., 1975), perfused rat liver (Claret-Berthon et al., 1977), or isolated liver plasma membrane fragments (Shlatz & Marinetti, 1972) is almost totally equilibrated with $^{45}Ca^{2+}$. The direct examination of this exchange under our experimental conditions (Figs. 1,2) is consistent with these reports, indicating that within 3 to 4 min after $^{45}Ca^{2+}$ addition more than 70% of $^{45}Ca^{2+}$ removal represents $^{45}Ca^{2+}$ uptake by the liver.

Thus after correcting for exchange with extracellular $^{45}Ca^{2+}$ (Fig. 1C), the analysis of measurements of both $^{45}Ca^{2+}$ uptake rates and of the perfusate Ca^{2+} concentration as adopted here, represents a powerful procedure whereby (1) net Ca^{2+} movements may be interpreted in terms of changes in the rate of Ca^{2+} efflux or Ca influx^{2+} components, and (2) transient changes in the rates of each of these components can be examined, something not possible in kinetic studies which must be performed under steady-state conditions (Borle, 1975).

An analysis of $^{45}Ca^{2+}$ uptake rates under conditions where there are no net Ca^{2+} movements (Figs. 1,2) has revealed that Ca^{2+} is continually recycling across the hepatic plasma membrane, indicating that the net

movement of Ca^{2+} is the resultant of Ca^{2+} efflux and Ca^{2+} uptake activities. This rate of cycling, corresponding to approximately 190 fmoles/cm^2/s (assuming 2.8^2.mg wet wt $liver^{-1}$; Claret & Mazet, 1972) is very similar to the basal flux of $^{45}Ca^{2+}$ uptake previously observed in a kinetic analysis of liver Ca^{2+} fluxes (Claret-Berthon et al., 1977), and in a range of other tissues (reviewed in Borle, 1981).

A second point is that α-adrenergic agonists were found to have complex effects on this Ca^{2+}-translocation cycle since the activities of both Ca^{2+} influx and Ca^{2+} efflux components are modulated. We reported previously (Reinhart et al., 1982a) that one of the earliest responses to α-adrenergic agonists is an increase in the rate of net Ca^{2+} efflux from the perfused rat liver occuring within 5 s of agonist administration. However, the rate of $^{45}Ca^{2+}$ influx is also stimulated by the agonist within 30 s of its administration. This clearly indicates that not only are both components of the Ca^{2+}-translocation cycle activated, but also that the efflux component is stimulated to a far greater extent. This finding may be relevant for the interpretation of data obtained by Assimacopoulos-Jeannet et al., (1977) and Foden & Randle (1978) who showed that α-adrenergic agonists stimulate $^{45}Ca^{2+}$ uptake by rat liver cells. By examining only one component of the translocation cycle, these data have been misinterpreted as being indicative of net Ca^{2+} fluxes.

A third point relevant to the action of α-adrenergic agonists is that after the termination of net Ca^{2+} efflux within 3 to 4 min of α-agonist administration, the rate of Ca^{2+}-cycling across the plasma membrane has almost doubled. This observation is consistent with the data of Foden & Randle (1978) showing that phenylephrine stimulates $^{45}Ca^{2+}$ uptake in hepatocytes treated with the agonist for longer than 5 min, and with a kinetic analysis of $^{45}Ca^{2+}$ fluxes showing that 15 min after α-agonist administration the rate of both $^{45}Ca^{2+}$ influx and $^{45}Ca^{2+}$

efflux exchange has increased (Barritt et al., 1981). The finding that (1) the rate of both hormone-induced-and basal-cycling is dependent on the extracellular Ca^{2+} concentration, and (2) that maximal rates of cycling are not observed until 2 to 3 min after α-agonist administration, allows us to suggest that this cycling activity is responsible for the maintenance of the sustained responses described in the preceding publication (Reinhart et al., 1983b). These sustained responses were shown also to be dependent on the extracellular Ca^{2+} concentration and to exert their effect 3 to 4 min after phenylephrine treatment. Thus if intracellular Ca^{2+} pools are capable of only transiently elevating the cytoplasmic Ca^{2+} concentration, then it is possible that the Ca^{2+}-translocation cycle at the plasma membrane may act to maintain this elevated level of Ca^{2+}. The findings that the addition of extracellular Ca^{2+} to livers perfused with media containing low Ca^{2+} concentrations (less than 1 μM) restores the expression of sustained phenylephrine-induced responses (Reinhart et al., 1983a) and that phenylephrine induces a transient elevation of the cytoplasmic Ca^{2+} concentration in hepatocytes incubated in low Ca^{2+} media (Murphy et al., 1980), are consistent with such a proposal. Whether the extracellular Ca^{2+} pool that is mobilized represents bulk phase Ca^{2+}, or extracellular bound Ca^{2+} remains to be established.

The analysis of the temporal progression of α-adrenergic agonist-induced Ca^{2+} fluxes (Fig. 5) has revealed that a further pattern of Ca^{2+} flux changes is observed after removal of the α-agonist from the perfusion medium. Within seconds of withdrawing the agonist there occurs a rapid increase in the rate of both $^{45}Ca^{2+}$ uptake and net Ca^{2+} influx into the liver. Since the rate of $^{45}Ca^{2+}$ uptake is greater than the measured rate of net Ca^{2+} influx, it appears that an inhibition of the rate of Ca^{2+} efflux does not contribute to the net inflow of Ca^{2+}, but

rather that this is due to the opening of a Ca^{2+} channel or gate. Furthermore, since the sustained effects of phenylephrine on glycogenolysis and respiration are returning towards basal levels during this time, then it appears that the consequence of this Ca^{2+} inflow is not to elevate the cytoplasmic Ca^{2+} concentration, but rather to replete the intracellular hormone-sensitive Ca^{2+} pools (Reinhart et al., 1982a). As this pool is being repleted the rates of both net Ca^{2+} inflow, and of Ca^{2+}-cycling are returning towards basal levels. Thus there appears to be some close communication between the hormone-sensitive pool(s) of intracellular Ca^{2+}, and the rates of plasma membrane Ca^{2+} fluxes, both during and after stimulation with α-adrenergic agonists. The hormone specificity of these effects is similar to that found previously for net Ca^{2+} efflux (Reinhart et al., 1982a) and is such as to suggest that these Ca^{2+} movements are part of a more general mechanism whereby hormone receptor interactions are translated into cellular responses.

Hence we propose that both basal and hormone-stimulated cellular Ca^{2+} movements involve a Ca^{2+}-translocation cycle across the hepatic plasma membrane. Short term (< 2 min) effects of α-agonists are dependent mainly on the mobilization of a small pool of intracellular Ca^{2+} (Reinhart et al., 1982a, 1983 a,b,) which is, at least in part, extruded from the cell. Sustained effects appear to be dependent on the activity of Ca^{2+}-cycling across the plasma membrane and hence the extracellular Ca^{2+} concentration. Under these conditions this cycle may act to keep the cytoplasmic Ca^{2+} concentration elevated (Barritt et al., 1981; Murphy et al., 1980). By analogy with numerous cell types (reviewed in Borle, 1981; Barritt, 1982) such a cycle may consist of an outwardly directed Ca^{2+} ATPase Ca^{2+} pump (Lotersztajn et al., 1981), and one or more types of Ca^{2+} uptake channels. Attempts to identify these components in molecular terms, and to relate Ca^{2+} flux alteration to

changes in the cytoplasmic free Ca^{2+} concentration are currently in progress.

Acknowledgements

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Section B.13

Defining the calcium fluxes in perfused rat liver induced by the action of α-adrenergic agonists.

(Bygrave et al., 1983)

DEFINING THE CALCIUM FLUXES IN PERFUSED RAT LIVER INDUCED BY THE ACTION OF α-ADRENERGIC AGONISTS

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Introduction

Calcium ions are not distributed homogeneously throughout the eukaryotic cell but rather exist in a number of cell compartments at different concentrations. The largest concentration gradient of calcium ions (Ca^{2+}) appears to exist across the plasma membrane, where the external concentration is greater than 1 mM and that within the cytoplasm is probably less than 1 μM (1). The magnitude of the Ca^{2+} gradient across organellar membranes is not known with any certainty largely because precise values for the Ca^{2+} concentration in these organelles is generally unavailable. However, it does seem that significant "pools" of Ca^{2+} are located within mitochondria and the endoplasmic reticulum (1,2). Carriers, specifically involved in transmembrane Ca^{2+} fluxes, are known to be present in several of these organellar membranes as well as in the plasma membrane (reviewed in 1,2).

Evidence has been accumulating that the intracellular free Ca^{2+} concentration in the cytoplasm and perhaps in other intracellular compartments plays an important role in controlling a range of physiological events (see eg. 3). In this regard the redistribution of intracellular Ca^{2+} is seen to be an integral component of the mechanism whereby cell metabolism may be regulated by Ca^{2+} (4). The logical question that then follows and is examined in the present paper is, what are the factors that control the free Ca^{2+} concentration in the various cell compartments.

In liver, carriers for Ca^{2+} exist in the mitochondrial, endoplasmic reticular and plasma membranes (1,2). Only mitochondrial Ca^{2+} fluxes have been studied in any detail (for reviews see 1,4,5). Studies on the Ca^{2+} transporters located in the endoplasmic reticulum (6,7) and in the plasma membrane (8,9) were initiated more recently. A major problem in such experiments, is that isolated plasma or endoplasmic reticular

membranes are often grossly contaminated with other organellar membranes also supposedly capable of transporting Ca^{2+}. Hence it has been difficult to unequivocally assign properties to non-mitochondrial Ca^{2+} carriers in a specific membrane.

Information about each of these transport systems, potentially relevant to their having a role in regulating Ca^{2+} fluxes would need to include: their apparent affinity for free Ca^{2+}, how rapidly they transport Ca^{2+}, the amount of Ca^{2+} they retain and whether physiological substances influence Ca^{2+} transport (1,2). These features, however, may still bear only little relation to the events that occur in situ.

Cellular Calcium Fluxes in the Perfused Rat Liver System

In order to overcome many of the limitations inherent in examining Ca^{2+} transport in vitro (see above), we have begun to study Ca^{2+} fluxes in the intact organ. In particular we have studied glycogenolysis in the perfused rat liver induced by the action of α-adrenergic agonists. The experimental details and conceptual background to this work are reported elsewhere (3,10-12). Among the features that make this a useful system for examining the role of Ca^{2+} fluxes in the regulation of cell metabolism are: (a) the existence of a readily measurable physiological response apparently sensitive to changes in the free Ca^{2+} concentration, (b) provision for appropriate stimuli (ie. hormones) to initiate and terminate the physiological response, (c) direct physiological relevance since the tissue is intact, and (d) the ability to allocate a specific temporal sequence to events pertaining to a physiological response.

The following summarises the sequence of Ca^{2+} movements that ensue when α-adrenergic agonists are admitted to a perfused rat liver:

1.	Ca^{2+} loss from intracellular pools:	One of these is mitochondrial and accounts for ca. 60% of the total. The other(s) is endoplasmic reticulum and plasma membrane and accounts for the remaining 40%.
2.	Net Ca^{2+} efflux from the cell:	This occurs by 7 sec. It is apparently a response to increases in the cytoplasmic Ca^{2+} concentration.
3.	Increase in Ca^{2+}-cycling across the plasma membrane in the absence of net Ca^{2+} fluxes:	This occurs soon after hormone administration.
4.	Net Ca^{2+} influx into the cell of the Ca^{2+} previously extruded:	This occurs almost immediately after the hormone is removed. The intracellular calcium pools and transmembrane Ca^{2+} fluxes are restored to the pre-induced states.

Experimental Evidence Relating to this Scheme

a) Evidence of net Ca^{2+} efflux consequent to administration of α-adrenergic agonists: Fig. 1A shows an experiment in which changes in the Ca^{2+} concentration in the perfusate was continuously monitored

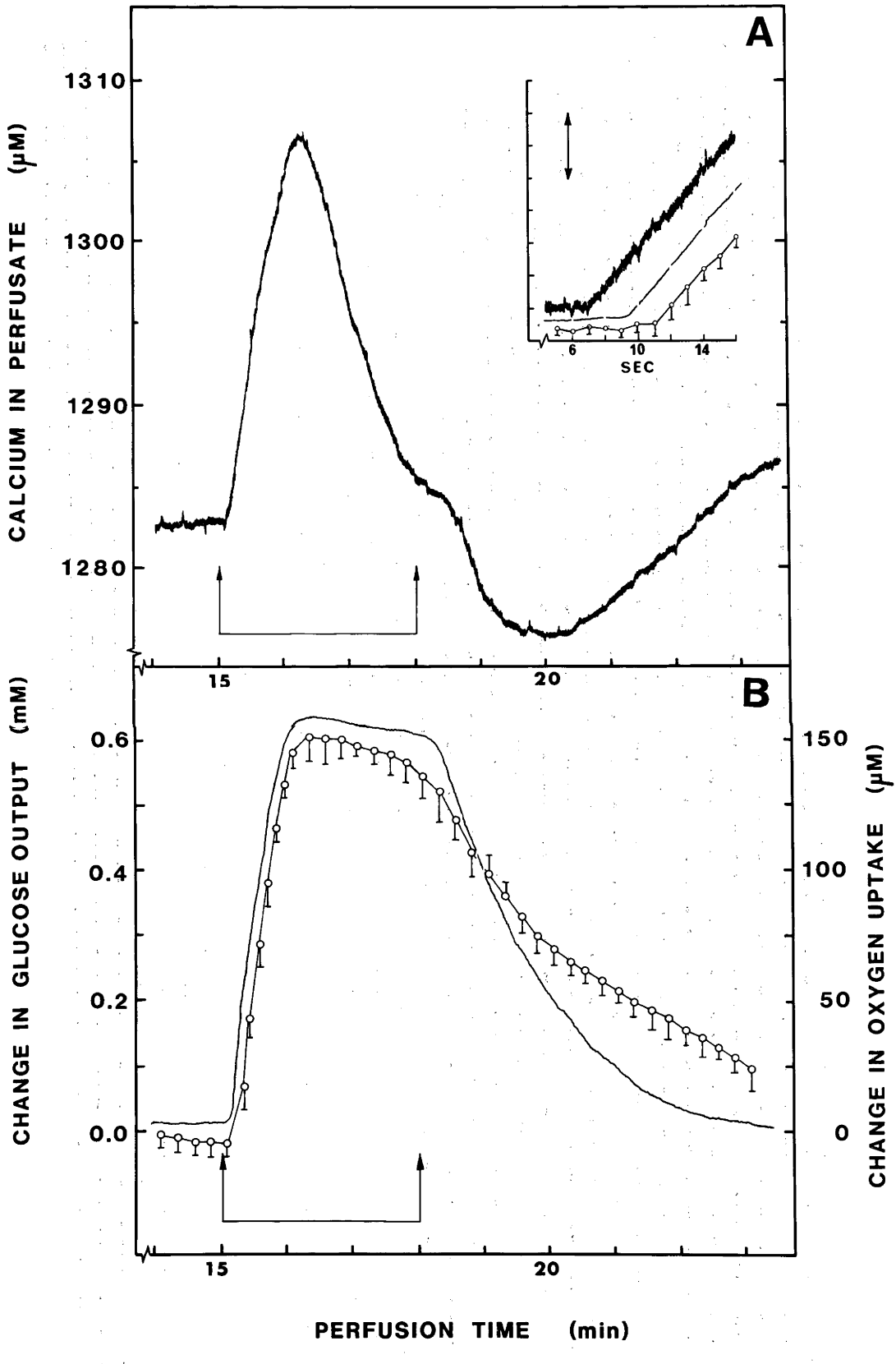
following administration of 2 μM phenylephrine to the perfused liver. These measurements were made possible by the development of an apparatus in which a Ca^{2+}-sensitive electrode was placed in the effluent medium and connected to a microprocessor ionanalyser. This in turn was coupled to a computing integrator programmed to display both changes in perfusate Ca^{2+} (peak height) and the total amount of Ca^{2+} taken up or released by the liver (integration mode). The system was able to detect changes of less than 1 μM Ca^{2+} in a medium containing physiological concentrations of Ca^{2+}, viz. 1.3 mM.

It is seen that the agonist induces a rapid and transient net efflux of Ca^{2+} from the liver. The time of onset occurs by about 7 sec and efflux reaches a maximum of 20-25 μM at about 40 sec. It thereafter declines to be no longer detectable after 2-3 min. Fig. 1A also shows, as will be mentioned later, that re-entry of the ion does not occur until the agonist is removed. This pattern of events is seen also with vasopressin, angiotensin II, adrenaline, and noradrenaline administration, but not with glucagon or analogues of cyclic AMP (12).

b) Intracellular source of moblized Ca^{2+} : In these experiments we used a newly-developed fractionation technique that permits subcellular organelles to be isolated from the intact liver in less than 6 minutes (11). Using a perfusion medium containing only low concentrations of Ca^{2+} (12), livers were infused for 90 sec with phenylephrine, immediately subfractionated and the fractions assayed for calcium content. The data (Table 1) were compared with those obtained from livers not exposed to the agonist. It is seen that substantial amounts of calcium are lost from the mitochondria and from a fraction enriched in endoplasmic reticulum and plasma membranes. The amount of calcium lost from the

Figure 1: *Ca^{2+} efflux, oxygen uptake and glucose output induced by phenylephrine administration to perfused rat liver.*

For details see ref. 12. The continuous line in Fig. 1B represents oxygen uptake. The perfusate Ca^{2+} concentration was 1.3mM.


A
1310
1300
1290
1280
CALCIUM IN PERFUSATE (μM)
6
10
14
SEC
15
20
B
0.6
0.4
0.2
0.0
CHANGE IN GLUCOSE OUTPUT (mM)
150
100
50
0
CHANGE IN OXYGEN UPTAKE (μM)
15
20
PERFUSION TIME (min)

combined pools correlates well with that amount effluxed in Fig. 1A, viz. approx. 80-120 nmol/g liver (12).

TABLE 1

LOSS OF CALCIUM FROM INTRACELLULAR POOLS FOLLOWING 90 SECOND PHENYLEPHRINE ADMINISTRATION TO PERFUSED RAT LIVER

(For experimental details see 12)*

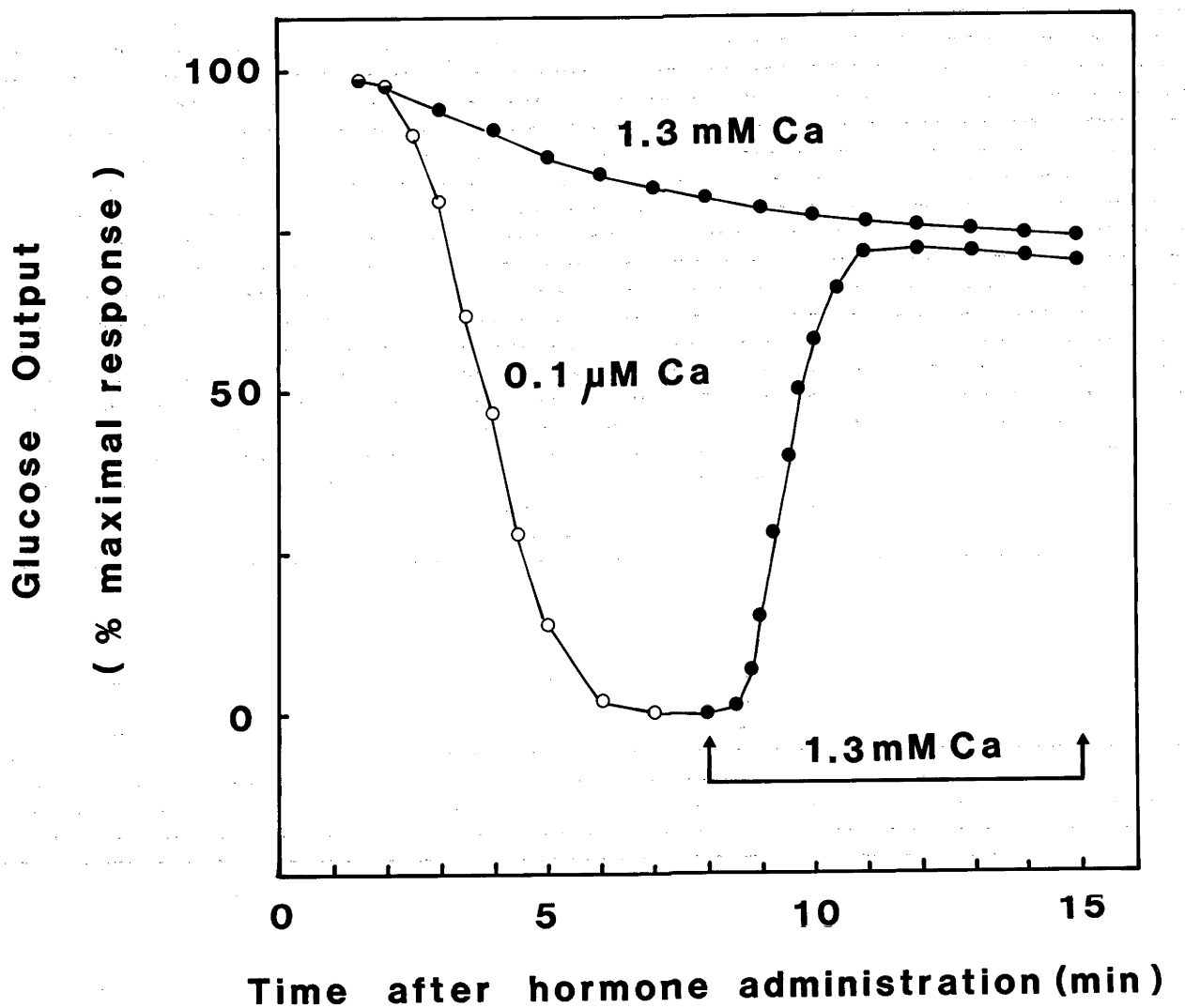
Subcellular fraction	Calcium content (nmol/mg protein)	
	Control	Hormone-treated
Mitochondria	2.8 ± 0.3	1.9 ± 0.2
Endoplasmic reticulum plus Plasma membrane	1.1 ± 0.2	0.6 ± 0.1

c) Correlation of Ca^{2+} efflux with glycogenolysis: Fig. 1B shows that soon after the commencement of Ca^{2+} efflux from the liver, there occurs a rapid increase in glucose output (glycogenolysis) and associated oxygen uptake which has been shown to be mitochondrial in origin (10). However, whereas the increased rate of Ca^{2+} efflux is transient, glucose output and oxygen uptake remain stimulated as long as the agonist is present. It is not until the agonist is removed that these responses begin to return to preinduced values.

* NB: 'n' in the data of Tables 1-3 was always 5 or greater.

Figure 2: Requirement of extracellular Ca^{2+} for long-term physiological responses.

Phenylephrine was added to rat livers perfused with 1.3 mM Ca^{2+} (closed circles) or 0.1 μM Ca^{2+} (open circles). At the arrow 1.3mM Ca^{2+} was infused.


Glucose Output
(% maximal response)
100
50
0
1.3 mM Ca
0.1 μM Ca
1.3mM Ca
0
5
10
15
Time after hormone administration (min)

d) A role for extracellular Ca^{2+}: Elsewhere (12) we showed that when repeated pulses of the hormone are administered to livers in the presence of 1.3 mM perfusate Ca^{2+}, the responses shown in Fig. 1 are repeated following each single pulse. With only 10 μM perfusate Ca^{2+}, however, normal Ca^{2+} efflux and metabolic responses are induced by only the first pulse of hormone. This suggests a role for extracellular Ca^{2+} in the prolonged physiological responses to α-adrenergic agonists in perfused liver. This is shown also by the data in Fig. 2. Livers were perfused with media containing 1.3mM or 0.1 μM Ca^{2+} and glucose output measured after phenlyephrine was administered. With 0.1 μM extracellular Ca^{2+}, glucose output became progressively less responsive to hormone stimulation. However, infusion at 8 min of 1.3mM Ca^{2+} led to an immediate stimulation of glycogenolysis.

e) Evidence for Ca^{2+}-cycling across the plasma membrane: High specific activity $^{45}Ca^{2+}$ was infused through the liver in the presence of 600 μM Ca^{2+} and the effluent measured for radioactivity. When compared with the control (i.e. $^{45}Ca^{2+}$ infusion past the liver), it is seen that $^{45}Ca^{2+}$ is indeed being removed by the liver (Δb in Fig. 3) at an estimated rate of 40 nmol/min/g of liver. In these conditions net flux of Ca^{2+} was not detected by Ca^{2+} selectrode measurements (data not shown). Because significant amounts of $^{45}Ca^{2+}$ are being removed by the liver unaccounted for by binding, and without net movement of Ca^{2+}, it seems that Ca^{2+}-cycling must be taking place. Further studies have revealed that the process requires energy, is dependent on the external Ca^{2+} concentration and is stimulated by α-adrenergic agonists, vasopressin and angiotensin II but not by glucagon (13).

f) Ca^{2+} transport by a plasma membrane-rich fraction from rat liver: That the plasma membrane from rat liver does in fact contain a Ca^{2+}-

sensitive ATPase and transporter capable of responding to changes in the cytoplasmic free Ca^{2+} concentration is shown in Table 2. This fraction, enriched approx. 20-fold in 5'-nucleotidase and relatively free of endoplasmic reticulum and Golgi membranes (14), responds to changes in free Ca^{2+} particularly near the apparent physiological concentration of cytoplasmic Ca^{2+} (approx. 1 μM free Ca^{2+}).

TABLE 2

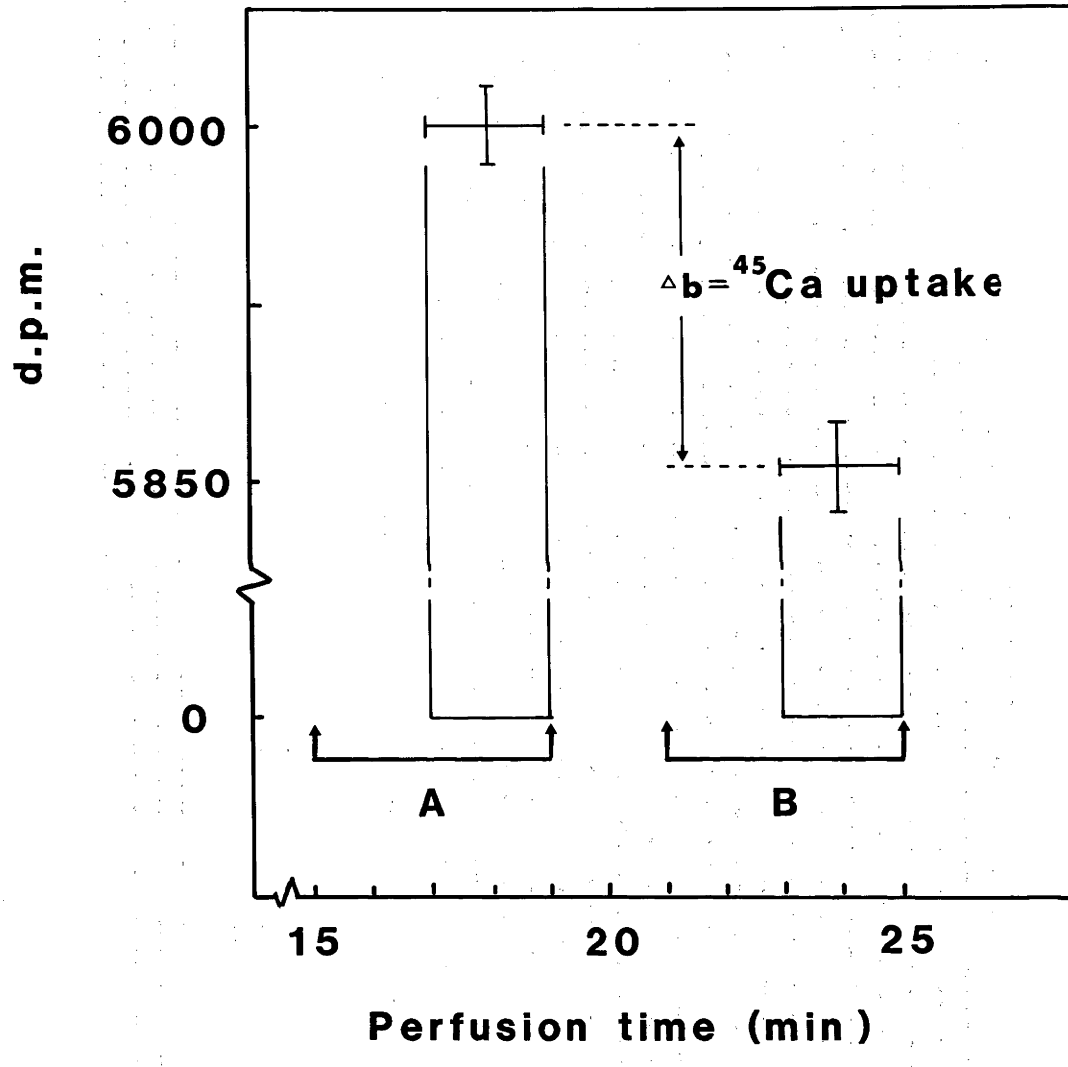
CALCIUM TRANSPORT AND CALCIUM ATPASE IN A PLASMA MEMBRANE-ENRICHED FRACTION FROM RAT LIVER

Free Ca^{2+} concentration (μM)	Ca^{2+} transport (nmol/min/mg protein)	Ca^{2+}-ATPase (nmol/10min/mg protein)
0.01	0.05 $\pm$ 0.05	0.31 $\pm$ 0.01
0.1	1.2 $\pm$ 0.5	0.34 $\pm$ 0.01
1.0	4.0 $\pm$ 1.4	0.39 $\pm$ 0.02
10.0	16.4 $\pm$ 4.5	0.65 $\pm$ 0.01

g) Calcium content of rat liver mitochondria: The data in Table 1 revealed that in our experiments the liver mitochondrial calcium content was considerably less than the generally assumed value of 10-20 nmol/mg of mitochondrial protein (see e.g. 15). Because this value will determine in part the matrix free Ca^{2+} concentration and in turn the gradient of free Ca^{2+} across the inner mitochondrial membrane, we have examined this point in some detail. Livers were perfused with 1.3mM or 0.1 μM Ca^{2+} and subfractionated using the rapid fractionation technique (11). Table 3 shows that when precautions are taken to minimize artefactual entry of Ca^{2+} into the cell consequent to disruption of the

Figure 3: *$^{45}Ca^{2+}$ uptake by perfused rat liver in the absence of net Ca^{2+} fluxes.*

During the period 'A' $^{45}Ca^{2+}$ was infused beyond the liver in media containing 600 μM Ca^{2+} while during the period 'B' it was infused through the liver.


6000
5850
0
d.p.m.
△b=45Ca uptake
A
B
15
20
25
Perfusion time (min)

cell membrane, the content is in fact only approx. 2 nmol/mg of protein. Higher values for the mitochondrial calcium content are seen only when the perfusate Ca^{2+} concentration also is high. Indeed as we show elsewhere (16) a good correlation exists between the Ca^{2+} concentration in the circulating perfusion medium and that in the subsequently isolated mitochondria.

TABLE 3

CALCIUM CONTENT OF MITOCHONDRIA ISOLATED FROM LIVER PERFUSED WITH DIFFERENT CONCENTRATIONS OF EXTRACELLULAR CALCIUM

The concentration of Ca^{2+} in the perfusate was varied as indicated and mitchondria isolated using the rapid tissue fractionation technique (11).

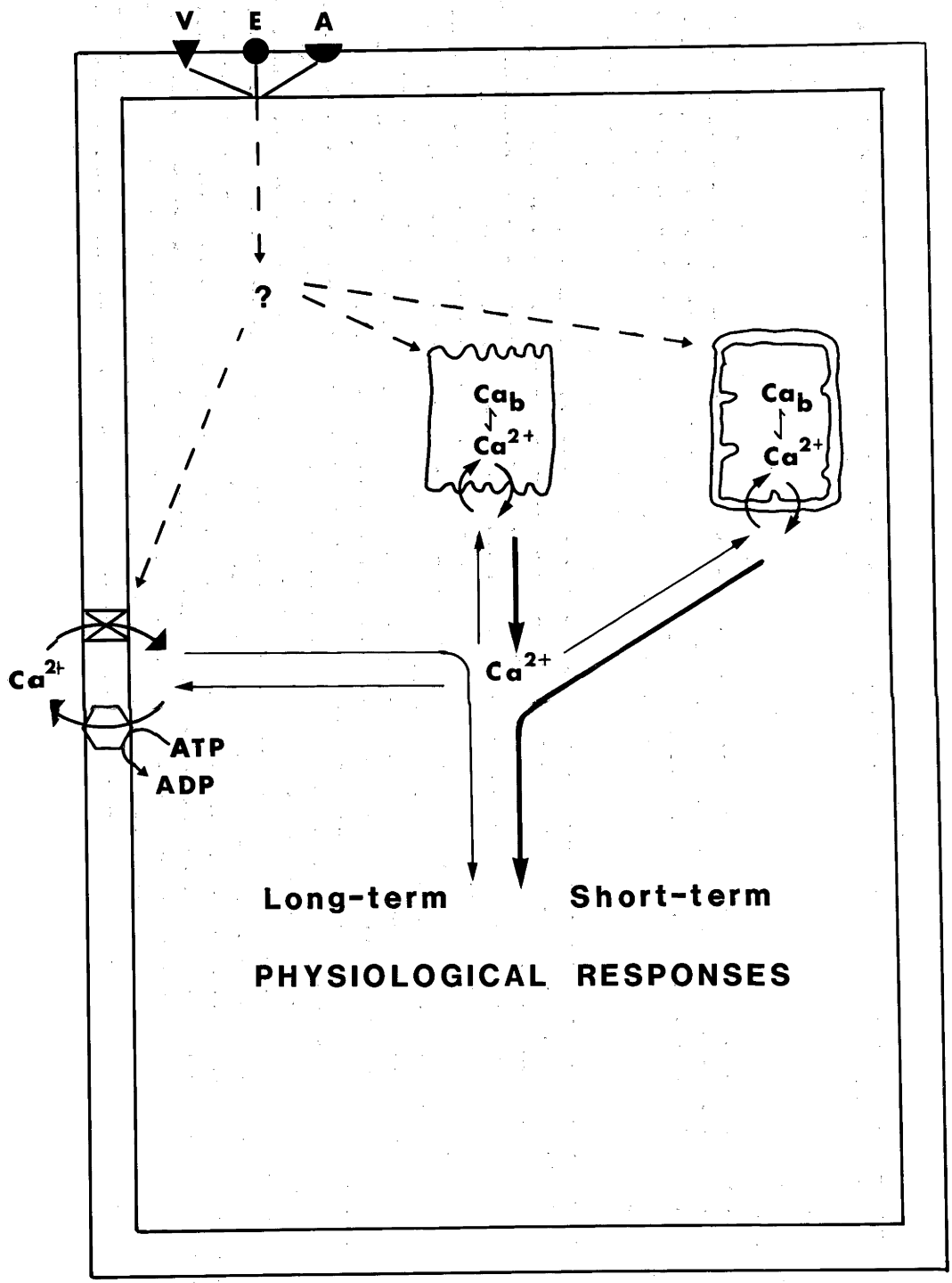
Perfusate Ca^{2+} concentration (μM)	Calcium content (nmol/mg of mitochondrial protein)
1300	9.01 $\pm$ 0.75
6	3.56 $\pm$ 0.53
0.1	2.14 $\pm$ 0.14*

* In these experiments approximately 20 μM EGTA was included in the perfusate and isolation media.

Concluding Comments

The development of a sensitive technique to examine quite small changes in Ca^{2+} fluxes under physiological conditions has allowed us to begin to define in quite precise terms the inter-relation between Ca^{2+} flux changes and the physiological responses induced by α-adrenergic agonists in rat liver (Fig. 4). These hormones, as well as vasopressin and angiotensin II, each trigger a sequence of events that include,

Figure 4: Model summarising Ca^{2+} flux changes in perfused rat liver induced by the action of α-adrenergic agonists (E), vasopressin (V) and angiotensin II (A).


V
E
A
?
Ca_b
Ca^{2+}
Ca_b
Ca^{2+}
Ca^{2+}
Ca^{2+}
ATP
ADP
Long-term
Short-term
PHYSIOLOGICAL RESPONSES

mobilization of intracellular Ca^{2+}, net Ca^{2+} efflux from the cell, cycling of the ion across the plasma membrane, and, immediately upon removal of the hormone, re-entry of the Ca^{2+} into the cell to replete the internal stores.

The revelation, in this work, of a co-ordinated interplay of intracellular and extracellular Ca^{2+} in these physiological responses highlights the specific involvement of plasma membrane Ca^{2+} fluxes. The role of such fluxes in the regulation of liver cell Ca^{2+} has been given only scant attention in the past. Our data show that rapid, energy-dependent cycling of the ion occurs across the plasma membrane and that this is enhanced by α-adrenergic agonists. These factors suggest the concerted action of a hormone-sensitive Ca^{2+} 'gate' and the Ca^{2+}-ATPase/transport system which our data clearly show to be present.

Calcium fluxes across the inner mitochondrial membrane also must be involved in the regulation of both cytoplasmic and the matrix free Ca^{2+} concentration since a large proportion of the mitochondrial calcium is mobilized soon after hormone administration to the perfused liver (Table 1). The finding that the mitochondrial calcium content is probably lower than previously thought, indicates that the Ca^{2+} gradient across this membrane may be quite small. It has been suggested, largely on the basis of the sensitivity of matrix enzymes to Ca^{2+}, that the matrix free Ca^{2+} concentration is of the order of 1 μM (17,18), a point currently under consideration in this laboratory.

The molecular nature of the proposed Ca^{2+} gate and of the initial signal apparently transmitted to the intracellular Ca^{2+} pools remain to be elucidated.

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Section B.14

Binding and Uptake of [^{3}H]-adrenaline by perfused rat liver.

(Reinhart et al., 1983e)

BINDING AND UPTAKE OF [^{3}H]-ADRENALINE BY PERFUSED RAT LIVER

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Short Title: Adrenaline Uptake by Rat Liver

Synopsis

The binding and uptake of [^{3}H]-adrenaline by the intact perfused rat liver was investigated. We showed that the administration of [^{3}H]-adrenaline to liver resulted in the rapid uptake of the radioligand, and that such uptake was independent of any Ca^{2+}-redistributions induced by the hormone. At low adrenaline concentrations (50 nM) uptake was largely inhibited by prazosin, while at higher hormone concentrations a significant proportion of total [^{3}H]-adrenaline uptake could not be inhibited by this antagonist. [^{3}H]-adrenaline uptake could be directly correlated with adrenaline-induced responses such as an increased rate of respiration and glycogenolysis. The partial inhibition (approx. 25%) of [^{3}H]-adrenaline uptake by antagonists was sufficient for the total inhibition of hormone-induced responses. The effect of various pharmacological agents on [^{3}H]-adrenaline uptake was investigated, and the contribution of tissue-related factors on α-adrenergic agonist-antagonist interactions in vivo, is discussed.

Introduction

Liver plasma membrane preparations have been shown to contain at least two types of α-adrenergic receptors, classified as α_1 and α_2 on the basis of pharmacological potency series (Wikberg, 1979; Wood et al., 1979; El-Refai & Exton, 1980; Hoffman et al., 1981; Graham et al., 1982). Most α-adrenergic agonist-induced responses appear to be mediated through binding of the agonist to α_1-receptors (El-Refai & Exton, 1979; Garcia-Sainz et al., 1980; Hoffman et al., 1980; Reinhart et al., 1982a,b; Aggerbeck et al., 1980), while binding to α_2-receptors has been implicated with an inhibition of adenyl cyclase activity (Jard et al., 1981; Limbird, 1983). Furthermore, in vitro competition binding assays have revealed that rat liver contains a high proportion of α_1-receptors, that a single subtype may exist in more than one form (Hoffman et al., 1980; Geynet et al., 1981), and that binding data obtained for α-antagonists such as [^{3}H]-dihydroergocryptine differ from data obtained for α-agonists like [^{3}H]-adrenaline (El-Refai & Exton, 1979; Hoffman et al., 1980; Geynet et al., 1981).

However, the full understanding of agonist-receptor interactions in vivo requires knowledge about drug-related factors such as the efficacy and affinity of agonists and antagonists, as well as tissue-related factors such as the coupled-receptor number, and the rate of agonist or antagonist removal from the receptor compartment (Kenakin, 1983). In liver there has been much interest in examining drug-related factors, using isolated fragments enriched in plasma membranes (see Schmelck & Hanoune, 1980; Hanoune et al., 1981; Exton, 1982), despite the fact that virtually nothing is known about tissue-related factors. Hence we have attempted to extend the information obtained from in vitro binding

studies by examining the [^{3}H]-adrenaline-receptor interaction in the intact perfused liver.

Our studies show that [^{3}H]-adrenaline is rapidly taken up by the liver, and that this uptake can be inhibited by α-adrenergic antagonists such as prazosin or yohimbine, agents previously used to distinguish between binding to α_1- and to α_2-adrenergic receptors, respectively (Doxey et al., 1979; Hoffman et al., 1979).

Inhibition of [^{3}H]-adrenaline uptake by α-adrenergic antagonists is paralleled by inhibition of hormone-induced responses. The dynamic nature of α-adrenergic agonist-receptor interactions, and possible applications of in situ receptor-binding studies are discussed.

Methods

Animals and perfusions - Male Wistar strain albino rats weighing between 230 and 270 g and having free access to food, were used for all experiments. Rats were anaesthetized with sodium pentobarbitone (50mg.kg body wt.$^{-1}$), and the livers perfused with Krebs-Henseleit bicarbonate medium (Krebs & Henseleit, 1932) equilibrated with O_2/CO_2 (19:1) as described previously (Reinhart et al., 1982a). Briefly, livers were perfused at a flow-rate of 3.5 ml.min^{-1}.g wet liver^{-1} and at 34°C. All livers were preperfused for 15 min with medium containing 1.3 mM added $CaCl_2$. In some experiments the perfusate $CaCl_2$ concentration was reduced to approx. 6 μM at 15 min (Reinhart et al., 1982b). All experiments were performed between 08:00 h and 12:00 h to minimise diurnal fluctuations of basal metabolism.

[^{3}H]-adrenaline uptake assay - [^{3}H]-adrenaline (approx. 2 x 10^6 cpm.ml^{-1}) was prepared just prior to each experiment in degassed Krebs-

Henseleit buffer containing 250 μM catechol. The hormone was infused into either the inflow cannula or the outflow cannula using a foil-covered glass/glass infusion syringe (EVA superglass μ-micromatic) mounted on a Braun Perfusor IV infusion pump. Perfusate samples were collected at the times indicated in the legend to Figs. and the radioactivity determined by adding 200 μl perfusate samples to scintillation vials containing 10 ml of scintillant (6 g butyl PBD in 400 ml of 2-methoxyethanol and 600 ml of toluene) and counting to within 0.1% error in a Beckman LS-330 liquid scintillation counter. All counts obtained were corrected for background radioactivity.

For all experiments, catechol (0.6 mM) was also infused 3 min prior to [^{3}H]-adrenaline administration, since this was found to significantly decrease the non-specific uptake of [^{3}H]-adrenaline by the liver (data not shown).

The difference between the radioactivity in the perfusate following infusion into the inflow and outflow cannulae respectively, was taken to represent the [^{3}H]-adrenaline taken up by the liver.

Perfusate glucose and oxygen determinations - Glucose release by the liver was determined by the glucose oxidase/peroxidase method as previously described (Reinhart et al., 1982b). Perfusion circuit lag-times, from the point of hormone or ionophore infusion to the sampling point, were routinely determined using [^{3}H]-inulin. All data shown have been corrected for this lag-time. The oxygen consumption by the liver was calculated from the difference between influent and effluent oxygen concentrations measured with a Clark-type oxygen electrode modified for a flow-through mode of operation as described previously (Reinhart et al., 1982a). The linearity of the electrode response was established using

the colorimetric oxygen assay of Hamlin & Lambert (1971). Perfusion circuit lag times were determined using sodium dithionite, and data shown were corrected for this lag time.

Materials

DL-[7-^{3}H(N)]-adrenaline hydrochloride was obtained from New England Nuclear, prazosin from Pfizer Pty. Ltd., Brooklyn, N.Y., yohimbine and antimycin A from the Sigma Chem. Co., St. Louis, MO, U.S.A. Trifluoperazine and R24571 were gifts from Smith, Kline & French and Janssen Pharmaceutica, respectively. Verapamil was obtained from Knoll-AG, Munich, West Germany. All other chemicals used were of analytical grade.

Expression of Data

All experiments were performed at least three times, and the data expressed as means ±S.E.M. for the number of experiments described.

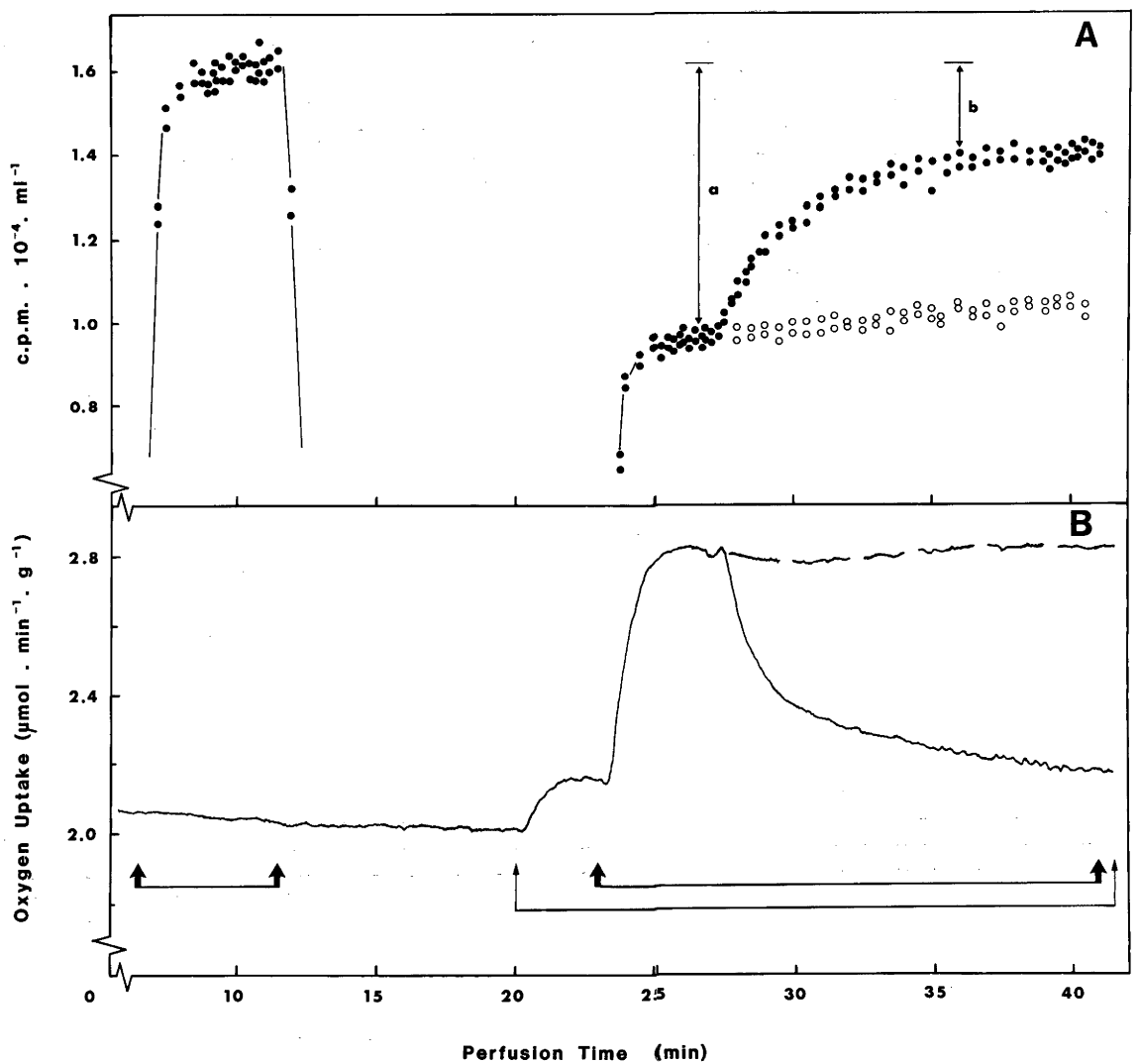
Results

Time course of [^{3}H]-adrenaline uptake by perfused rat liver

The data in Fig. 1 show changes in the perfusate radioactivity (A) and oxygen concentration (B) due to the administration of 60 nM [^{3}H]-adrenaline to the intact perfused liver. The specific activity of [^{3}H]-adrenaline was determined by infusing [^{3}H]-adrenaline into the outflow cannula (A) between 6.5 and 11.5 min of perfusion. The subsequent infusion, at 23 min, of [^{3}H]-adrenaline through the liver results in a significant reduction of the perfusate radioactivity, indicative of a continuous uptake of the α-agonist by the tissue (A). From the initial plateau of radioactivity observed at between 2 and 4 min of [^{3}H]-

Figure 1: *[^{3}H]-adrenaline uptake by perfused rat liver.*

Livers of fed rats were perfused as described in Experimental. Between 6.5 and 11.5 min of perfusion [^{3}H]-adrenaline was infused into the outflow cannula and the specific radioactivity of the perfusate determined. At 20 min of perfusion catechol (fine arrows; 0.6 mM final conc.) was infused through the liver and 3 min later [^{3}H]-adrenaline (6×10^{-8} M final conc.) was administered (bold arrows). The rate of [^{3}H]-adrenaline uptake was usually calculated by determining the radioactivity of 12 samples collected between 3 and 4 min after adrenaline administration (see a). In some experiments prazosin (2×10^{-6} M final conc.) was administered at 27 min (●), while other animals served as controls (○). The proportion of [^{3}H]-adrenaline uptake inhibited by prazosin is calculated by determining the radioactivity of 12 samples collected between 9 and 10 min after prazosin administration (see △ b). Perfusate radioactivities (A) and oxygen concentrations (B) were determined as described in Experimental. The data shown are from a typical experiment of 5 independent experiments performed for prazosin and 3 performed as controls.


A
B
a
b
c.p.m. . 10^{-4}. ml^{-1}
1.6
1.4
1.2
1.0
0.8
Oxygen Uptake (µmol . min^{-1}. g^{-1})
2.8
2.4
2.0
0
10
15
20
25
30
35
40
Perfusion Time (min)

adrenaline administration the rate of uptake can be estimated at 82.1 ± 2.5 pmoles.min^{-1}.g liver^{-1} (n=11). This rate remains virtually constant for at least 15 min. The onset of [^{3}H]-adrenaline uptake is rapid, since maximal rates are observed at the earliest timepoint measurable (approx. 2 min). The addition of prazosin (2 x 10^{-6}M) at 27 min rapidly increases the perfusate radioactivity, indicating a reduction in the rate of α-adrenergic agonist uptake by the liver. Half-maximal effects are observed after approx. 3 min and maximal effects of 65 to 70% inhibition occur between 8 and 10 min following prazosin infusion.

The rate of oxygen uptake by the liver during the same experiment is shown in Fig. 1B. While the infusion of catechol induces a small stimulation in the rate of oxygen uptake, the previously reported stimulation of respiration by adrenaline (Scholz & Schwabe, 1980; Reinhart et al., 1982a) is also observed in the present study.

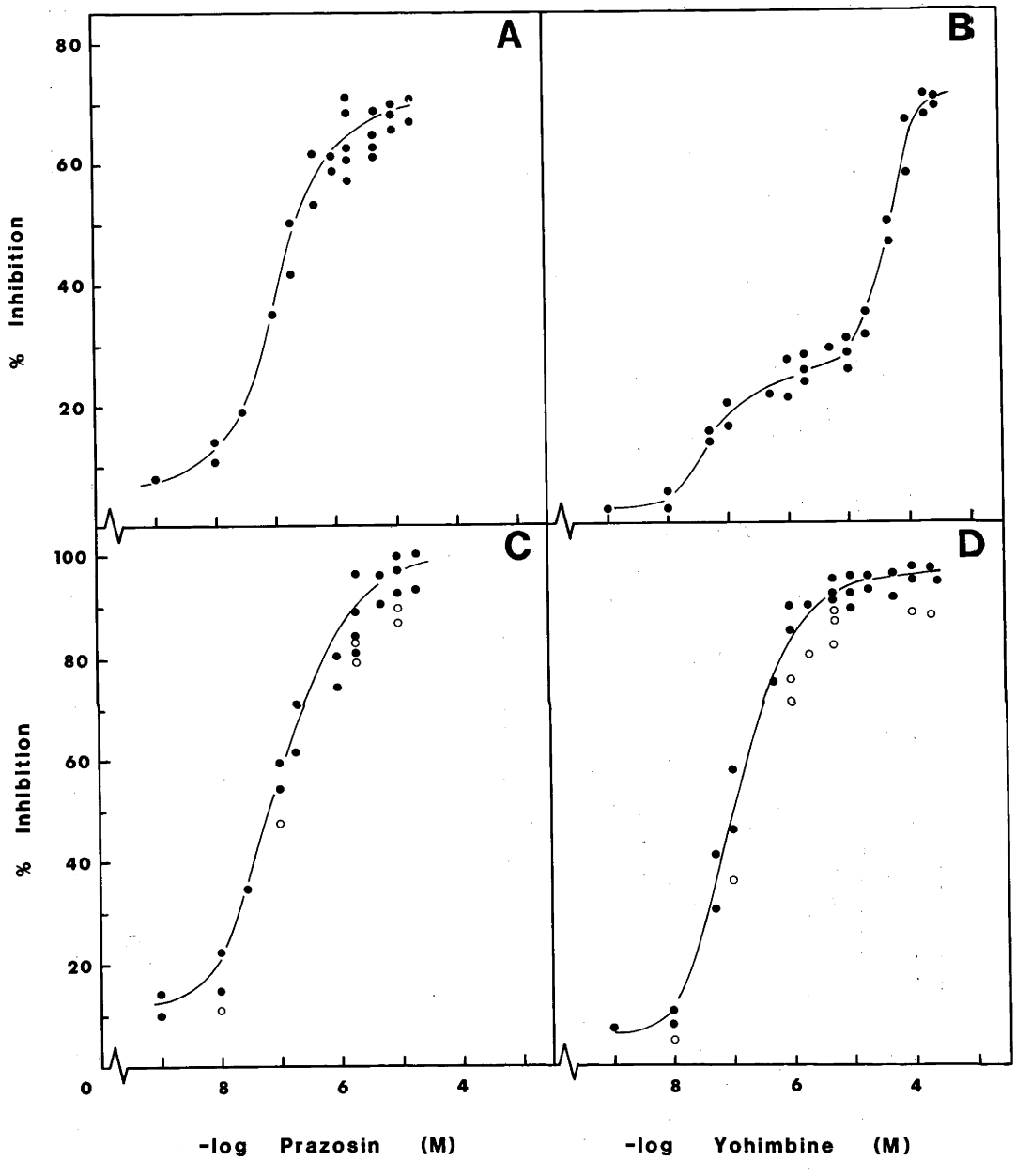
The addition of prazosin, however, rapidly and completely inhibits the adrenaline-induced respiratory response, with a time-course very similar to that observed on the effect on [^{3}H]-adrenaline uptake. The adrenaline-stimulated rate of glucose output was similarly inhibited by prazosin (data not shown).

Effect of α-adrenergic antagonists on [^{3}H]-adrenaline uptake

The data in Fig. 2 show the effect of changing the concentration of either prazosin or yohimbine on the rate of [^{3}H]-adrenaline uptake (A,B), oxygen uptake and glucose output (C,D). Prazosin inhibition of [^{3}H]-adrenaline uptake is significant at 10^{-9} M, half-maximal at 8 x 10^{-8} M and maximal at 2 x 10^{-6} M (A). Prazosin concentrations as high as 10^{-5} M do not inhibit more than 70% of the [^{3}H]-adrenaline uptake. Prazosin also inhibits adrenaline-induced responses such as the stimulation of

Figure 2: The effect of α-adrenergic antagonists on [^{3}H]-adrenaline uptake, glucose output and oxygen uptake.

Livers of fed rats were perfused as described in Experimental. The specific activity of [^{3}H]-adrenaline in the pefusate was determined as described in the legend to Fig. 1. At 20 min of perfusion catechol (0.6 mM) was infused, and at 23 min [^{3}H]-adrenaline (60 nM) was administered. 4 min later either prazosin or yohimbine was infused at the concentrations shown. The rate of [^{3}H]-adrenaline uptake (A,B) was determined by calculating the average radioactivity in samples collected both between 3 and 4 min of [^{3}H]-adrenaline administration, and between 9 and 10 min of antagonist administration (see Fig. 1). The rate of adrenaline-stimulated oxygen uptake (C,D:●) and glucose output (C,D:○) was determined as described in Experimental. Each data point shown was obtained from a separate experiment.


A
B
C
D
% Inhibition
% Inhibition
80
60
40
20
100
80
60
40
20
0
8
6
4
8
6
4
-log Prazosin (M)
-log Yohimbine (M)

respiration and of glycogenolysis by up to 90% over the same concentration range (C).

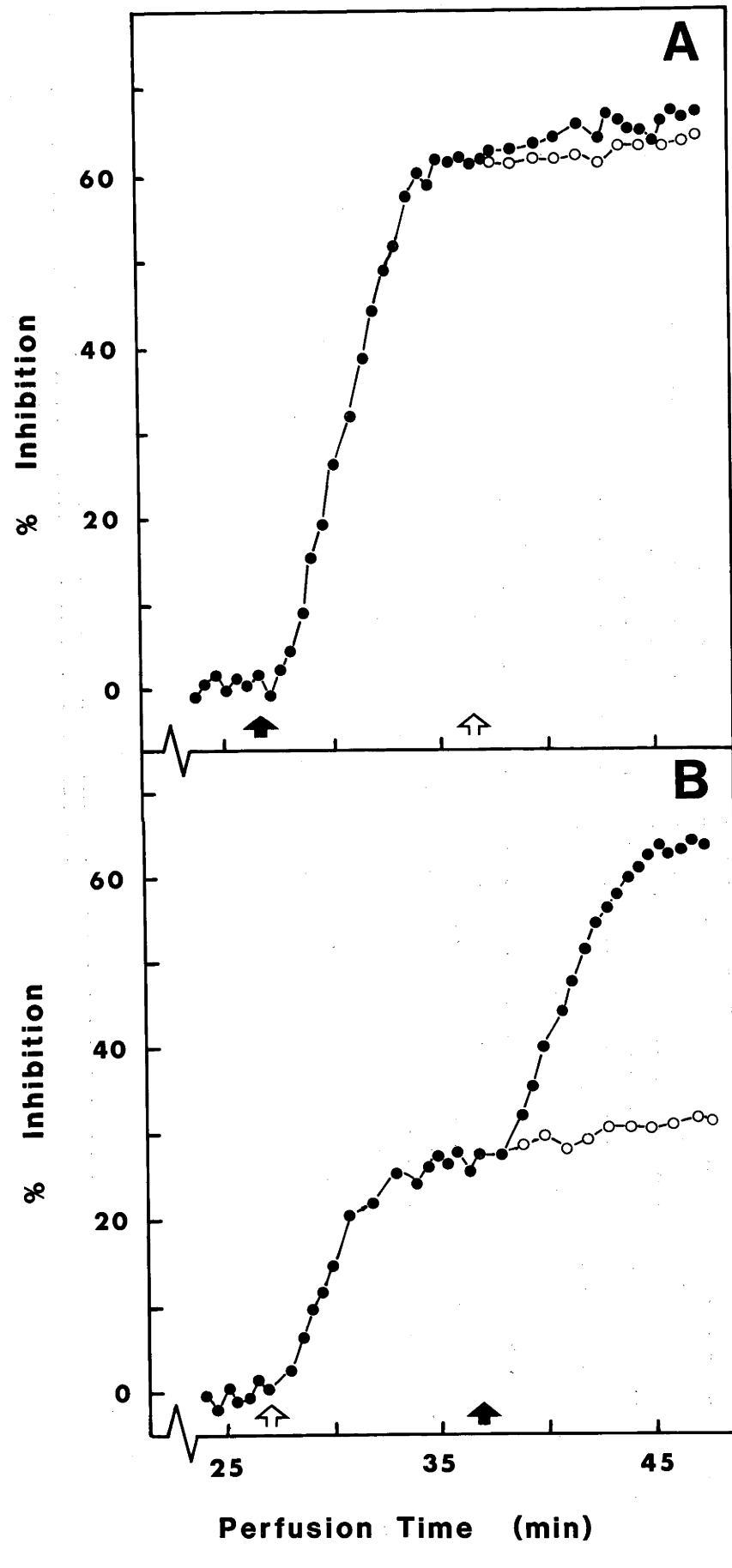
The effects of yohimbine are more complex since [^{3}H]-adrenaline uptake is inhibited in a biphasic manner (B). A relative plateau of approx. 25% inhibition is observed at between 10^{-7} M and 10^{-5} M yohimbine. At higher concentrations of the α-antagonist the rate of [^{3}H]-adrenaline uptake is further inhibited until the maximal inhibition of 70% is observed at approx. 10^{-4} M yohimbine. Maximal inhibitory effects of yohimbine on α-adrenergic agonist-induced respiration and glucose output (D) are observed at concentrations of the antagonist which inhibit only approx. 25% of [^{3}H]-adrenaline uptake. Hence inhibition of α-adrenergic agonist-induced rates of glucose output and respiration in the intact organ are induced by similar concentrations of prazosin and yohimbine.

To determine whether or not yohimbine and prazosin are acting at separate receptor sites, we investigated the inhibition of [^{3}H]-adrenaline uptake by the sequential addition of these antagonists.

The data in Fig. 3A show that the administration of yohimbine after 10 min of prazosin treatment results in virtually no further inhibition of [^{3}H]-adrenaline uptake than observed in the presence of prazosin alone. Similarly the addition of prazosin after yohimbine (Fig. 3B) does not increase the inhibition of [^{3}H]-adrenaline uptake above 70%. In addition the time course of yohimbine-induced [^{3}H]-adrenaline uptake is very similar to that for prazosin where maximal effects were observed after 8 to 10 min.

Figure 3: Additivity of prazosin- and yohimbine-mediated inhibition of [^{3}H]-adrenaline uptake.

Livers of fed rats were perfused as described in the legend to Fig. 2. After [^{3}H]-adrenaline administration either prazosin (closed arrow) or yohimbine (open arrow) was infused at 2×10^{-6} M. In addition, some livers were then treated with either yohimbine (open arrow) or prazosin (closed arrow), while others served as controls (O). [^{3}H]-adrenaline uptake was measured as described in Experimental. Each data point shown represent means for either 3 (A) or 4 (B) independent experiments. S.E.M.'s have been omitted for the sake of clarity but $\pm$7.6% was never exceeded.


A
B
% Inhibition
% Inhibition
0
20
40
60
0
20
40
60
25
35
45
Perfusion Time (min)

Effect of adrenaline concentration in [^{3}H]-adrenaline uptake

The rate of [^{3}H]-adrenaline uptake is directly proportional to the perfusate [^{3}H]-adrenaline concentration over the range 4 to 90 nM (Fig. 4). However, even at concentrations up to 1 μM no saturation of the [^{3}H]-adrenaline uptake rate is observed (data not shown). Higher concentrations of adrenaline were not used since deleterious effects of the agonist were evident. However, the proportion of [^{3}H]-adrenaline uptake which is inhibited by prazosin decreases from 80% at 10 nM to less than 50% at 80 nM, a concentration which induces near-maximal metabolic effects. Thus at high hormone concentrations, a large proportion of [^{3}H]-adrenaline uptake appears to occur independently of α-adrenergic receptor interactions.

Lack of a role for Ca^{2+} in [^{3}H]-adrenaline uptake

Further experiments were designed to examine the Ca^{2+} dependence of [^{3}H]-adrenaline uptake, since previous work has indicated that all other α-adrenergic agonist-induced responses are obligatorily dependent on the redistribution of cellular Ca^{2+} (Reinhart et al., 1982b, 1983). The data in Fig. 5 show that the repeated administration of [^{3}H]-adrenaline, to liver perfused with media containing only μM Ca^{2+} concentrations, does not decrease the rate of [^{3}H]-adrenaline uptake. We have previously shown that this experimental regime depletes the hormone-sensitive pool of cellular Ca^{2+}, and hence the expression of all Ca^{2+}-dependent responses to α-agonists (Reinhart et al., 1982b, 1983). Thus it appears that neither the interaction between adrenaline and α-receptors, nor the internalization of the agonist, is dependent on a redistribution of cellular Ca^{2+}.

Figure 4: Effect of the perfusate adrenaline concentration on the rate of [^{3}H]-adrenaline uptake.

Livers of fed rats were perfused as described in the legend to Fig. 1, except that the adrenaline concentration was varied as shown in the Fig. The rate of [^{3}H]-adrenaline uptake (●) was measured between 3 and 4 min after adrenaline administration, as shown in Fig. 1. The percentage of this rate inhibited by the infusion of 2×10^{-6} M prazosin (0) was calculated as described in Fig. 1. Data points shown are the means of 12 samples collected between 3 and 4 min after [^{3}H]-adrenaline administration (0), or the means of between 3 and 6 independent experiments performed in the presence of prazosin (0).

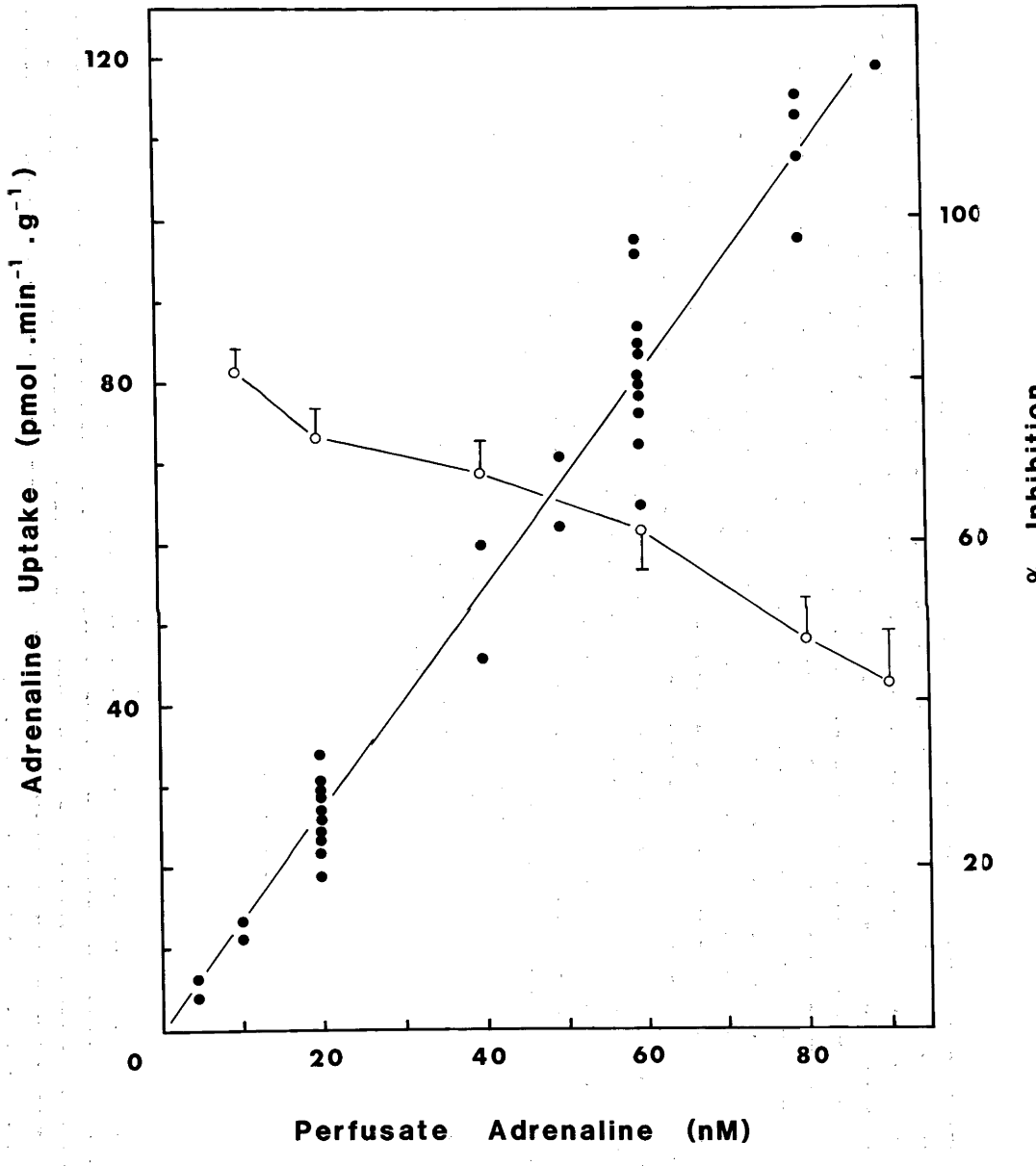

120
80
40
0
20
40
60
80
100
60
20
Adrenaline Uptake (pmol .min^{-1} .g^{-1})
% Inhibition
Perfusate Adrenaline (nM)

Figure 5: *The effect of multiple hormone administrations on the rate of [^{3}H]-adrenaline uptake.*

Livers of fed rats were perfused with media containing 1.3mM added Ca^{2+} as described in Experimental. At 14 min of perfusion the perfusate Ca^{2+} concentration was reduced to approx 6 μM (as determined by atomic absorption spectroscopy). At 10 min and 64 min of perfusion [^{3}H]-adrenaline was administered into the outflow cannula for 4 min to allow determination of the specific activity of perfusate [^{3}H]-adrenaline. At 19,28,37,46 and 55 min of perfusion [^{3}H]-adrenaline was infused through the liver for 4 min and the perfusate radioactivity determined as described in Experimental. Data shown are from a typical experiment chosen from 3 independent experiments.

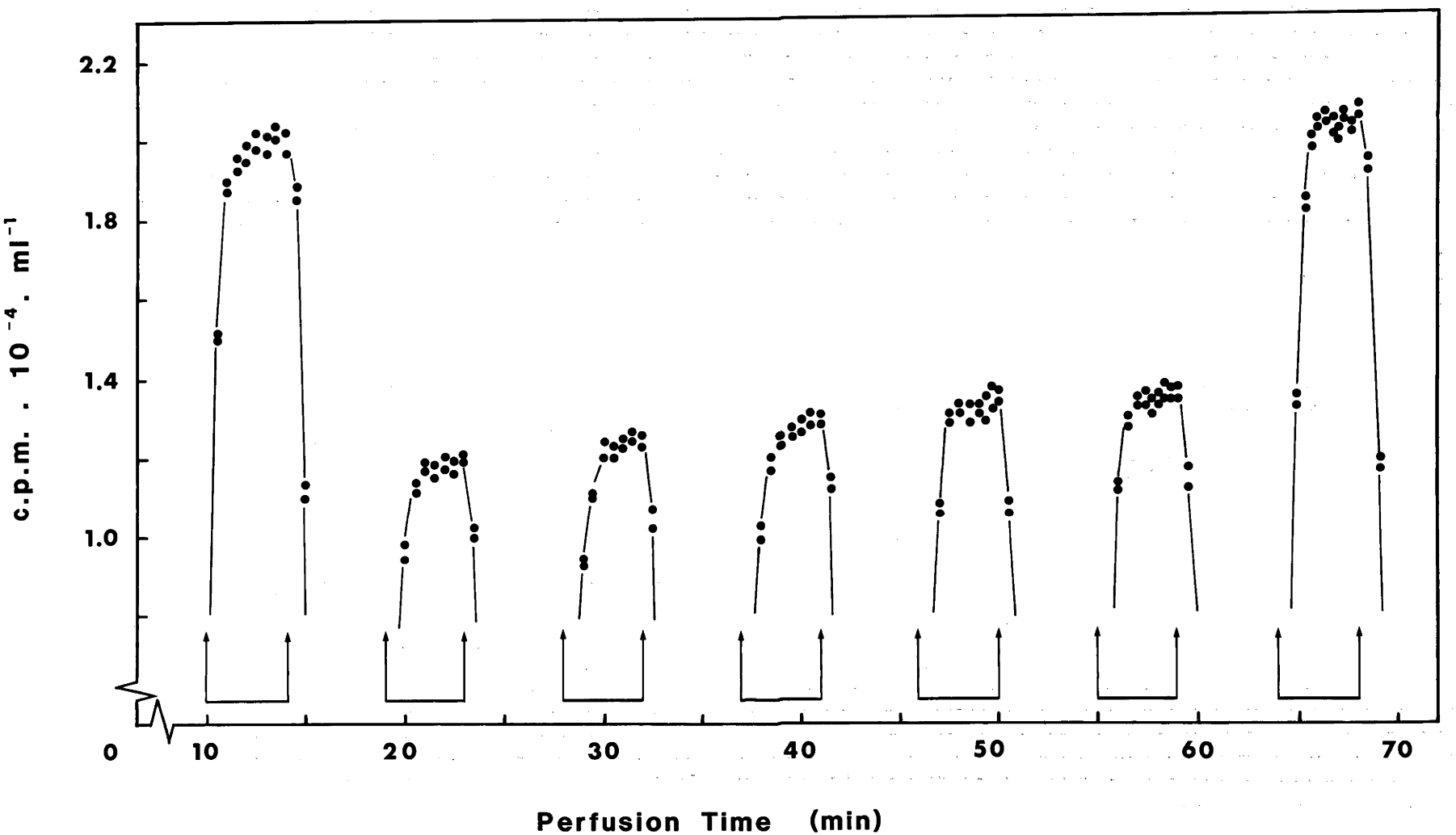

2.2
1.8
1.4
1.0
0
10
20
30
40
50
60
70
c.p.m. . 10^{-4} . ml^{-1}
Perfusion Time (min)

Table 1

The effect of pharmacological agents on the rate of [^{3}H]-adrenaline uptake.

Livers of fed rats were perfused as described in the legend to Fig. 1. At 23 min of perfusion [^{3}H]-adrenaline was administered, and 4 min later the agents shown in the Table were infused for 10 min. Rates of [^{3}H]-adrenaline uptake were determined for 10 min. At this time yohimbine (2 x 10^{-6}M) was administered for 10 min. Rates of [^{3}H]-adrenaline uptake were determined between 9 and 10 min of infusing either the pharmacological agent, or between 9 and 10 min of infusing the agent plus yohimbine. Control experiments indicated that yohimbine alone inhibited 26.7 ± 1.3 (n=8)% of [^{3}H]-adrenaline uptake. Hence by subtracting the inhibition due to the agent from the inhibition due to the agent plus yohimbine an assessment of the additivity of these two agents was made (Δ). The number of independent experiments performed for each condition is shown in the Table.

Agent Infused	Final conc (M)	Inhibition of [^{3}H]-adrenaline uptake due to:		Δ	%Inhibition of yohimbine sites.
		Agent	Agent + yohimbine		
Dinitrophenol	$3x10^{-5}$	1.2 ± 2.2(n=3)	29.3 ± 3.2(n=3)	28.1	≃ 0
Insulin	$1.6x10^{-8}$	4.5 ± 1.5(n=3)	31.8 ± 4.0(n=3)	27.3	≃ 0
R24571	10^{-6}	38.0 ± 2.7(n=4)	70.2 ± 4.2(n=4)	32.2	≃ 0
Antimycin A	$5x10^{-6}$	43.6 ± 1.8(n=3)	71.5 ± 3.1(n=3)	27.9	≃ 0
Trifluoperazine	10^{-5}	49.1 ± 2.9(n=4)	56.0 ± 3.3(n=4)	6.9	≃ 75
Verapamil	10^{-4}	74.1 ± 2.9(n=3)	81.4 ± 4.3(n=3)	7.3	≃ 73

Effect of pharmacological agents on [^{3}H]-adrenaline uptake.

Previous studies have indicated that numerous pharmacological agents, not usually regarded as α-adrenergic antagonists, can inhibit [^{3}H]-adrenaline receptor binding *in vitro* (Blackmore *et al.*, 1978, 1981; Reinhart *et al.*, 1981; Huerta-Bahena *et al.*, 1983). Hence further experiments were designed to examine the effects of a number of pharmacological agents on both the rate of [^{3}H]-adrenaline uptake, and on hormone-induced metabolic effects. The data in Table 1 show that with the exception of dinitrophenol and insulin, all other agents tested significantly inhibit the rate of [^{3}H]-adrenaline uptake. However, only trifluoperazine and verapamil appear to inhibit at coupled receptor sites, as judged by the ability to compete with yohimbine in blocking the rate of [^{3}H]-adrenaline uptake. Hence the inhibitory effect of these two agents on adrenaline-induced respiration does appear to be related to their α-adrenergic antagonist properties, while the inhibitory effect of antimycin A on adrenaline-induced respiration cannot be interpreted in this way. Neither insulin nor R24571, a potent inhibitor of calmodulin-mediated effects (Van Belle, 1981), appear to inhibit [^{3}H]-adrenaline binding to coupled α-adrenergic receptors.

Discussion

The data presented reveal new information about α-adrenergic agonist-antagonist interacions *in situ*. The most striking difference between data obtained from previous *in vitro* binding assays, and the administration of [^{3}H]-adrenaline to the intact perfused liver, is the rapid, large and prolonged uptake of the radioligand *in situ*. The lack of any observable recycling of radiolabel indicates that [^{3}H]-adrenaline and/or the labelled portion of metabolites formed, are not released over

the 10 to 20 min duration of the experiment. Furthermore the uptake of [^{3}H]-adrenaline appears to progress by at least 2 distinct routes. The first of these occurs through the binding of adrenaline to α-adrenergic receptors, as judged by the effectiveness of prazosin in inhibiting uptake, and may involve the internalization of the agonist-receptor complex (see Goldstein et al., 1979; Schlesinger, 1980; Brown & Orci, 1983; Lienhard, 1983). This route appears to predominate at low adrenaline concentrations, while uptake of adrenaline not inhibited by prazosin and hence possibly independent of receptor binding, predominates at agonist concentrations greater than 60 nM.

A second major observation made in this study is that the inhibition of receptor-mediated adrenaline uptake can be directly correlated with the almost complete inhibition of adrenaline-induced responses, such as increased rates of glycogenolysis and respiration. While previous studies with either hepatocytes (Aggerbeck et al., 1980; Blackmore et al., 1978, 1981) or the perfused liver (Reinhart et al., 1981a, 1982b) have shown that numerous pharmacological agents can inhibit hormone-induced responses, these effects were correlated only with binding data obtained using isolated membrane fragments enriched in plasma membranes, and therefore may not be relevant to conditions in situ (Taylor et al., 1983).

A further feature revealed by these in vivo studies is that the binding and internalization of [^{3}H]-adrenaline occurs independently of Ca^{2+} redistributions. The depletion of the hormone-sensitive Ca^{2+} pool had no effect on the rate of [^{3}H]-adrenaline uptake whereas all other α-adrenergic agonist-induced responses are inhibited by this treatment (Reinhart et al., 1982b, 1983). Hence the interaction of adrenaline with α-adrenergic receptors may give rise to both the uptake and metabolism

of the α-agonist (McKillop & Powis, 1976), as well as to the generation of some messenger that gives rise to the redistribution of cellular Ca^{2+}. The finding that adrenaline linked to sepharose can still induce physiological responses (Dehaye et al., 1980) makes it unlikely that [^{3}H]-adrenaline uptake per se plays a role in the generation of some intracellular messenger.

The observed inhibitory effects of prazosin and yohimbine on the rate of [^{3}H]-adrenaline uptake and hormone-induced responses, indicates that tissue-related factors play a significant role in the interactions between these antagonists, adrenaline and α-receptors. Binding assays carried out in vitro have shown that hepatic α-receptors are predominantly of the α_1 subtype and that prazosin is more potent than yohimbine in preventing hormone-induced responses in isolated hepatocytes (Hoffman et al., 1979; Aggerbeck et al., 1980; El-Refai & Exton, 1980; Hoffman et al., 1980).

In the intact tissue a different pattern of responses is observed since relatively high concentrations of prazosin are required to inhibit either [^{3}H]-adrenaline uptake or hormone-induced responses, and the effects of yohimbine are complex, occurring at two concentration ranges of the antagonist. These results may in part be due to the rapid uptake and metabolism of prazosin by the intact liver (Rubin & Reid, 1983) resulting in a decreased concentration of this antagonist at the α-receptor site. Since this effect appears to be less apparent in hepatocytes (Aggerbeck et al., 1980; Hoffman et al., 1980) non-parenchymal cells may then contribute to the uptake of prazosin. In the intact liver yohimbine maximally inhibits adrenaline-induced responses at concentrations inhibiting only a portion of the total prazosin-sensitive rate of [^{3}H]-adrenaline uptake. If prazosin-sensitive hormone uptake

involves the binding of adrenaline to α-adrenergic receptors then the data indicate that only a proportion of the receptor pool bound by adrenaline is coupled to the intracellular signal-transducing mechanism. Such a view is consistent with previous proposals that α-receptor subtypes may exist in more than one form (El-Refai & Exton, 1979, 1980; Hoffman et al., 1980a,b; Geynet et al., 1981) but our data do not allow us to differentiate between binding to high and low affinity forms of α_2-receptors (Hoffman et al., 1980a,b) or precursor forms of α_1-adrenergic binding sites (Geynet et al., 1981). Nevertheless, these data highlight the importance of examining tissue-specific effects when considering α-adrenergic agonist-antagonist interactions in vivo, particularly in liver, the organ involved in the uptake and metabolism of most pharmacological agents. Thus our study has revealed that many pharmacological agents can inhibit the uptake of [^{3}H]-adrenaline, and hence presumably adrenaline-receptor interactions. However, it is important to differentiate between inhibition at coupled receptors, and inhibition at receptors not involved in mediating physiological responses. Many of the agents examined in this study appear to interact at these latter sites, while trifluoperazine and verapamil inhibit hormone-uptake at both coupled and presumably uncoupled receptors. The development of pharmacological agents with a high affinity for coupled α-adrenergic receptors should reveal much information about receptor binding in vivo.

We conclude that the interaction of adrenaline with hepatic α-adrenergic receptors is far more dynamic than indicated by in vitro receptor binding assays, and that as assessment of hormone-receptor interactions in situ, as described in this work, may represent a powerful approach to understanding the action of many drugs used in the treatment

of hypertension and cardic failure (Rubin & Reid, 1983), or in examining the effects of fasting, thyroidectomy and cholestasis on adrenaline-induced responses (El-Refai & Chan, 1982; Preiksaitis et al., 1982; Aggerbeck et al., 1983).

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SECTION C

Future Directions

Work on the hepatic action of α-adrenergic agonists appears to be moving in a number of discrete directions, although general trends common to all may also be identified.

One direction receiving increasing attention is the characterization of hormone-receptor interactions. The implementation of improved methods for the isolation of α-adrenergic receptors currently being developed, should yield much information about this receptor at the molecular level. Studies examining functional aspects of hormone-receptor binding may make use of purified receptors inserted into liposomes to examine such interactions under tightly defined conditions in vitro, possibly leading to the extension of the current α_1, α_2 classification system. Alternatively, the development of highly purified plasma membrane vesicles, of defined orientation may allow the correlation of hormone-receptor interactions with responses such as inositol phospholipid cycling, or alterations in the rate of Ca^{2+}-transport across this membrane. The study of hormone-binding in intact cells or tissues, although complex, has been shown to be feasible, and future studies should provide much information of relevance in vivo. The availability of more specific α-agonists and antagonists would have a significant impact on the field.

A second direction relates to the elucidation of the proposed 'second messenger(s)' for α-adrenergic agonists. Unfortunately only a single event, polyphosphoinositide breakdown, meets even some of the criteria which can be set for such a messenger, while considerable controversy still surrounds the ability to meet other criteria such as the Ca^{2+}-independence of this phenomenon. Nevertheless the identification and preparation of polyphosphoinositide breakdown-products, currently in progress, should reveal whether or not inositol metabolism is related to 'second messenger(s)' generation, or simply a

parallel effect of undefined function. While there is little evidence to support other 'second messenger(s)' proposals, alternative transduction mechanisms which may receive increasing attention are gap junction-mediated electrotonic coupling, and the microtrabecular lattice incorporating microtubules and microfilaments.

The third obvious direction relates to the action of α-adrenergic agonists on inducing a redistribution of cellular calcium. One of the major findings presented in this thesis was the demonstration that virtually all α-agonist-mediated hepatic effects examined are obligatorily dependent on Ca^{2+} redistributions occuring within seconds of hormone-receptor binding. Hence, one initial effect of the 'second messenger(s)' is to mobilize intracellular, probably bound, calcium. The further characterization of hormone-induced calcium redistributions will allow not only the examination of Ca^{2+}-dependent responses in the relevant compartments, but also lead to an assay system for the 'second messenger(s)', analogous to protein kinase for cAMP. Currently, the study of such redistributions is hampered by only a partial understanding of basal cellular calcium homeostasis, and advances in this area are likely to occur with the development of new techniques, or the successful implementation of those presently available such as electron probe analyses of tissue sections, Ca^{2+}-indicators such as Quin-2 and chlortetracycline which may be specific for distinct calcium compartments, and microelectrode techniques.

Increasing attention is presently being focussed on integrating information for individual Ca^{2+}-transporters in terms of Ca^{2+}-translocation cycles across membranes under conditions approaching those found in vivo.

A fourth direction relates to studies focussed at identifying and rationalizing the Ca^{2+}-sensitivity of numerous cellular reactions. It

appears likely that calmodulin will be found to play a major role in such reactions, and the isolation and characterization of 'calmodulin-binding proteins' analogous to phosphorylase kinase, appears imminent. Similarly other, as yet undefined, Ca^{2+}-binding proteins may be important.

Increasing emphasis is now also being placed on extrapolating experimental observations to the in vivo state. While in the past many workers have utilized isolated hepatocytes incubated in non-physiological media, it is hoped that the present study has emphasized the merit of examining the intact tissue, perfused with media containing ion concentrations similar to those of blood plasma. Future studies are likely to make increasing use of such experimental approaches, a point further highlighted by recent interest in non-invasive techniques to measure metabolic events. It appears likely that approaches such as topical magnetic resonance (TMR) utilizing magnetic fields, surface fluorometric techniques using fluors such as Quin-2, and electric field measurements utilizing either microelectrodes or charged tracer molecules which are partitioned by electric fields, will receive increasing attention in α-adrenergic agonist research.

Clearly our understanding of α-adrenergic mechanisms is still in its infancy, nevertheless current progress is such that adolescence is within sight.

APPENDIX 1

Free Ca^{2+} determinations

The following listing shows the stability constants (total formation constants) used for the generation of free Ca^{2+} concentrations in various media, as well as the implementation of BUFFA used on the UNIVAC 1100/82 computer (see Reinhart et al., 1983a).

EXPERIMENTALLY DETERMINED STABILITY CONSTANTS ($-LOG_{10}K$)

FOR METAL ION COMPLEXES

	$EDTA^{4-}$	$EGTA^{4-}$	ATP^{4-}	ADP^{4-}	$OXAL^{2-}$	$*PO_4^{3-}$	NTA^{3-}	CO_3^{2-}
H^+	9.91	9.54	6.52	3.73	3.81	11.80	9.73	10.25
$2H^+$	5.98	8.93	3.87	-	-	7.15	2.50	-
$3H^+$	2.86	2.73	-	-	-	2.10	-	-
Ca^{2+}	10.11	11.00	3.94	2.80	3.00	-	6.53	3.2
$H^+ + Ca^{2+}$	3.51	5.30	2.13	-	-	2.77	-	-
Mg^{2+}	8.73	5.20	4.28	3.30	2.39	-	5.46	3.4
$H^+ + Mg^{2+}$	2.28	3.40	2.29	-	-	2.5	-	-
Co^{2+}	16.31	12.50	4.55	4.12				
$H^+ + Co^{2+}$	9.15	7.99	2.24	-				
Mn^{2+}	14.04	12.28	4.63	4.06				
$H^+ + Mn^{2+}$	6.90	7.02	2.30	-				
Ni^{2+}	18.62	13.55	4.90	4.42				
$H^+ + Ni^{2+}$	11.56	9.19	2.59	2.22				
Sr^{2+}	8.29	8.10	3.45	2.43				
$H^+ + Sr^{2+}$	-	4.37	2.00	-				

* values are highly variable

```
 00010 REM  THIS PROGRAM IS MODIFIED TO BE RUN ON A UNIVAC 1100
SYSTEM.
 00020 REM  THE PROGRAM IS STORED IN THE FILE "PHANTOM" .
 00030 REM  THE ELEMENT NAME OF THE PROGRAM IS "COMICS" .
 00040 REM  TO RUN THE PROGRAM ENTER FOLLOWING COMMANDS :
 00050 REM         @ASG,AXZ PHANTOM.
 00060 REM         @BASIC PHANTOM.
 00070 REM          OLD:COMICS
 00080 REM          ALT * PRINTOUT
 00090 REM          R     (ENTER REQUIRED VALUES)
 00100 REM          SYM PRINTOUT,1,PR1
 00110 REM  THIS WILL GIVE BOTH THE TERMINAL DISPLAY AS WELL AS
HARD-COPY.
 00120 REM  IF HARD-COPY IS NOT REQIRED DELETE "ALT" AND "SYM"
INSTRUCTIONS.
 00130 REM  IF MORE THAN ONE HARD-COPY RUN IS MADE, MUST USE NEW
"ALT"-IDENT.
 00140 PRINT "PROGRAM TO CALCULATE FREE METAL ION CONCENTRATIONS"
 00150 PRINT "IN SOLUTIONS CONTAINING UP TO 3 METALS & 5 LIGANDS"
 00160 PRINT "USING STABILITY CONSTANTS FROM SILLEN & MARTELL,
1964,1971."
 00170 PRINT
 00180 PRINT "MODIFIED BY RON EPPING AND PETER H. REINHART, JULY
1982."
 00190 PRINT
 00200 PRINT
 00210 DIM M$(3),L$(5),N(30,9),B(30),S(30),F(9),T(9),X(9),C(3)
 00220 PRINT "NUMBER OF METALS IN REACTION"
 00230 PRINT "MIXTURE  (MAX = 3)              = ";
 00240 INPUT M
 00250 IF M <= 0 THEN 1410
 00260 FOR I = 1 TO M
 00270 PRINT "  METAL  ",I," = ";
 00280 INPUT M$(I)
 00290 NEXT I
 00300 PRINT
 00310 PRINT "NUMBER OF COMPLEXING AGENTS IN "
 00320 PRINT "REACTION MIXTURE  (MAX = 5)     = ";
 00330 INPUT L
 00340 IF L <= 0 THEN 400
 00350 FOR I = 1 TO L
 00360 PRINT " LIGAND ", I , " = ";
 00370 INPUT L$(I)
 00380 NEXT I
 00390 PRINT
 00400 PRINT "NUMBER OF METAL COMPLEXES AND "
 00410 PRINT "PROTONATED LIGAND SPECIES TO BE"
 00420 PRINT "CONSIDERED  (MAX = 30)          = ";
 00430 INPUT P
 00440 IF P <= 0 THEN 1410
 00450 PRINT
 00460 PRINT
 00470 PRINT "INSERT STOICHIOMETRY OF EACH SPECIES"
 00480 PRINT
 00490 FOR I = 1 TO P
 00500 PRINT TAB(2), "SPECIES", I
```

```
00510 PRINT
00520 PRINT TAB(3), "H", TAB(15), "= ";
00530 INPUT N(I,1)
00540 FOR J = 1 TO M
00550 PRINT TAB(3), M$(J), TAB(15), "= ";
00560 INPUT N(I,(J + 1))
00570 NEXT J
00580 IF L <= 0 THEN 640
00590 K = M + 1
00600 FOR J = 1 TO L
00610 PRINT TAB(3), L$(J), TAB(15), "= ";
00620 INPUT N(I,(J + K))
00630 NEXT J
00640 PRINT TAB(3), "LOG CONSTANT = ";
00650 INPUT B(I)
00660 B(I) = EXP (B(I) * 2.303)
00670 PRINT
00680 PRINT
00690 NEXT I
00700 PRINT
00710 PRINT "PH OF REACTION SOLUTION = ";
00720 INPUT H
00730 PRINT
00740 PRINT
00750 F(1) = EXP ( - H * 2.303)
00760 K = 1 + M
00770 IF L <= 0 THEN 830
00780 FOR I = 1 TO L
00790 PRINT "CONCENTRATION OF ", L$(I) , " = ";
00800 INPUT T(I + K)
00810 NEXT I
00820 PRINT
00830 PRINT "LOWEST CONCENTRATION OF ",M$(1)," = ";
00840 INPUT C(1)
00850 PRINT "HIGHEST CONCENTRATION OF ",M$(1)," = ";
00860 INPUT C(2)
00870 PRINT
00880 PRINT "INCREMENT REQUIRED = ";
00890 INPUT C(3)
00900 FOR J = 2 TO M
00910 PRINT
00920 PRINT "CONCENTRATION OF ",M$(J)," = ";
00930 INPUT T(J + 1)
00940 NEXT J
00950 FOR I = 1 TO 10
00960 PRINT
00970 NEXT I
00980 PRINT "INSERT CONDITIONS",
00990 INPUT Z$
01000 PRINT
01010 PRINT
01020 PRINT TAB(3), "METAL", TAB(13), "TOTAL CONCN", TAB(21), "FREE
CONCN"
01025 PRINT
01030 K = 1 + M + L
01040 T(2) = C(1)
```

```
01050 FOR J = 2 TO K
01060 F(J) = T(J)
01070 NEXT J
01080 Z = 0
01090 FOR I = 1 TO P
01100 S(I) = B(I)
01110 FOR J = 1 TO K
01120 S(I) = S(I) * F(J)^N(I,J)
01130 NEXT J
01140 NEXT I
01150 FOR J = 2 TO K
01160 X(J) = F(J)
01170 FOR I = 1 TO P
01180 X(J) = X(J) + N(I,J) * S(I)
01190 NEXT I
01200 R = SQR (X(J) / T(J))
01210 IF ( ABS (R - 1)) > 1.0E - 04 THEN 1230
01220 Z = Z + 1
01230 F(J) = F(J) / R
01240 NEXT J
01250 IF Z >= (K - 1) THEN 1270
01260 GOTO 1080
01270 FOR J = 1 TO M
01280 PRINT TAB(3), M$(J), TAB(10), T(J + 1), TAB(18), F(J + 1)
01290 NEXT J
01300 C(1) = C(1) + C(3)
01310 IF C(1) <= C(2) THEN 1030
01320 FOR I = 1 TO 5
01330 PRINT
01340 NEXT I
01350 PRINT  "NEW CONCENTRATIONS REQUIRED (Y/N) ?";
01360 INPUT A$
01370 FOR I = 1 TO 5
01380 PRINT
01390 NEXT I
01400 IF A$ = "Y" THEN 710
01410 PRINT "END OF CALCULATIONS"
01420 CLOSE 1
01430 END
```