SODIUM CHANNELS IN CARDIAC MYOCYTES

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STATEMENT

The experimental observations and results presented in this thesis were obtained entirely through my own work unless acknowledgement is made.

Most of the contents in this thesis have been published as formal scientific papers or abstracts during the past two years. Some of them have been modified to conform to the rules of university.

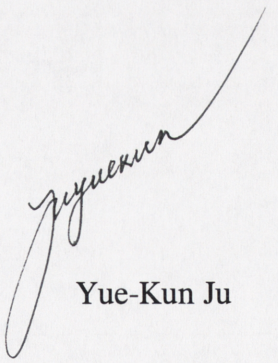

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TABLE CONTENTS

ABSTRACT

It has been known for some time that tetrodotoxin (TTX) reduces the duration of the plateau phase of action potentials in heart muscle (Coraboeuf, Deroubaix & Coulombe, 1979) This implies that there is a TTX-sensitive current, presumably carried by sodium, which contributes to the action potential plateau.

A small, TTX-sensitive persistent current following a much larger, transient current was recorded in single cardiac myocytes isolated from rat ventricular, atrial or toad sinus venosus tissues using the tight seal, whole-cell, voltage clamp technique. These persistent currents were sodium currents because they reversed close to the sodium equilibrium potential and were depressed when choline was substituted for extracellular sodium. The persistent sodium current had characteristics which are significantly different from those of transient sodium currents. It was activated at more negative potentials than the transient sodium current. It was also resistant to inactivation and could be recorded when the transient current had been inactivated with conditioning depolarization. It cannot have been a window current because it could be recorded at positive potentials when the transient current was completely inactivated.

Low concentrations of TTX and class 1 antiarrhythmic drugs (lidocaine & quinidine) blocked the persistent sodium current while having little effect on the transient sodium current. It is suggested that block of the persistent sodium current may be responsible, at least in part, for the antiarrhythmic action of lidocaine and quinidine. The fact that the persistent sodium current is more sensitive to TTX and antiarrhythmic drugs explains well the phenomenon that low concentrations of TTX or local anaesthetics shorten action potential duration.

Single transient and persistent sodium channels elicited by depolarizing voltage pulses were recorded in cell-attached and inside-out patches. A transient burst of channels was followed by late-opening (persistent) channels with low open probability. Conditioning depolarizing pre-pulses that inactivated transient channels and occasional bursting channels had no effect on persistent channels. The average open time of persistent channels increased with depolarization, a characteristic not shared by transient channels. Although conductance of transient and persistent channels was similar, it is suggested that transient and persistent channels have a different structure.

It is believed that there is no voltage-dependent sodium current in single cells isolated from regions of amphibian heart which contain pacemaker cells (Campbell, Rasmusson & Strauss, 1992). However, after calcium current, potassium current and non-selective cation currents activated by hyperpolarization had been blocked, depolarizing voltage steps initiated an inward current which consisted of transient and persistent components in pacemaker cells isolated from toad sinus venosus. Pacemaker cells were identified by their morphology and lack of an inwardly rectifying potassium current. Both components of the inward current were sodium currents. They were abolished by TTX (10 to 100nM), their reversal potential was close to the sodium equilibrium potential and their amplitude and reversal potential were changed as expected for sodium currents when extracellular sodium ions were replaced with choline ions. It was concluded that, in pacemaker cells, TTX-sensitive sodium currents contribute both to diastolic depolarization and to the upstroke of action potentials.

Chapter 1

GENERAL INTRODUCTION

Excitability, autorhythmicity, conductivity, and contractility are four basic physiological properties of cardiac muscle and they are crucial to normal cardiac function (Marshall, 1968).

More than 300 years ago, William Harvey observed that isolated atria beat faster than ventricles (Harvey, 1628). In 1871, Bowditch noted that a frog heart needed a certain period of time after a contraction in order to recover its excitability (Bowditch, 1871). Explanations for these physiological phenomena were not readily available, however, until the 1950's. Studies of the action potential established the important concepts of the ionic hypothesis (Hodgkin & Huxley, 1952a) which were applied to cardiac electrophysiology by Weidmann (Weidmann, 1955). The ionic hypothesis suggested that excitation and conduction of electrical signals in nerve, muscle and other tissues involve the movement of ions, such as Na^+, K^+, Ca^{2+}, and Cl^-, across cell membranes. We now know that the response of membranes to different stimuli, whether electrical, chemical, or mechanical, involves the opening or closing of ion channels. The open channel has the important property of "selective permeability", allowing some restricted class of ions to flow passively down their electrochemical gradients at a very high rate ($>10^6$ ions per second).

Over the past 40 years, ionic channels have been found in the membranes of all living cells. Their known functions include establishing membrane potentials, shaping electrical signals, activating many cell functions by gating the flow of messenger Ca^{2+} ions, controlling cell

volume, and regulating the net flow of ions across epithelial cells of secretory and resorptive tissue (Hille, 1992).

Although Hodgkin & Huxley revolutionized our view of membrane physiology by their fundamental experiments, the ionic basis of cardiac electrical activity at the molecular level in cardiac muscle, was still not well understood however, until three new techniques became available in this decade (Fozzard, January & Makielski, 1985; Kunze & Brown, 1989; Pelzer & Trautwein, 1987).

These three new techniques are: 1) isolation of individual cardiac myocytes: this overcomes the difficulty studying syncytial cardiac muscle tissue with complex geometry (Powell & Twist, 1976; Farmer *et al.*1983); 2) the gigaseal patch clamp technique which allows recording of high resolution currents from single cardiac cells (Sakmann & Neher, 1984; Hamill *et al.*1981); 3) molecular biology techniques which provide ways to reconstitute functioning channels in artificial membranes by using isolated and purified channel proteins (Miller, 1986; 1984), or to express functional cloned channels in various host cells by using cDNA or mRNA sequence techniques (Noda *et al.*1984; Catterall, 1992).

1.1. The properties of sodium currents in cardiac myocytes

1.1.1. Evidence for Na^+ ion involvement in membrane excitability

In 1902, Overton demonstrated that frog muscle became inexcitable when immersed in an isotonic solution containing less than 10% of the normal sodium chloride concentration. Keynes & Lewis (1951) compared the ionic contents of resting and stimulated Sepia axons using radio-isotope techniques. They found that during stimulation there was a net sodium entry of 3.8×10^{-12} mol /cm^2 /impulse and a net potassium loss of 3.6×10^{-12} mol /cm^2 /impulse. Meanwhile, other experiments showed that in squid

axon the action potential was smaller and rose more slowly in solutions containing less than the normal amount of Na^+ (Hodgkin & Katz, 1949). Thus it was demonstrated that the rate of rise and amplitude of action potentials was determined by the concentration of sodium in the external medium (Hodgkin & Katz, 1949).

In 1952, Hodgkin and Huxley first described membrane currents recorded from voltage-clamped squid axons. The effect of sodium ions on the membrane current was investigated by replacing the sodium in the external medium with choline. The results supported the view that depolarization leads to a rapid increase in sodium permeability which allows sodium ions to move into the cell (Hodgkin & Huxley, 1952b).

A few years later, this result was confirmed indirectly in cardiac tissue. Weidmann (1955) investigated the action potentials in single Purkinje fibres and found that there was a S-shaped relationship between the maximal rate of rise of the action potential and the voltage. Because the rate of rise and overshoot of action potential changed with sodium concentration but not potassium concentration, it was suggested that surface membrane underwent an increase in sodium permeability (Weidmann, 1955). The properties of sodium current in cardiac tissues had been assumed similar to those in the squid axon. Thus when Noble (1960) modelled the Purkinje fibre action potential, the mathematical description of the sodium current was very similar to that determined for squid axon, though with significantly reduced inactivation rate constants.

In 1964, Deck, Kern and Trautwein first directly measured membrane current in short Purkinje fibres using the two electrode voltage clamp techniques. Sodium current was found by subtraction of the current recorded in sodium free solution from the current flowing in Tyrode solution. They observed that, in addition to the initial rapidly declining sodium conductance, there was a component of the sodium conductance

which declined slowly (time constant of the order of 100 ms) throughout depolarizations as long as 500 msec. The relatively large sodium current during the plateau explained the striking loss of plateau and the shortening of the Purkinje fibre action potential in choline-Tyrode solution (Deck, Kern & Trautwein, 1964).

Because there was insufficient voltage control within the first 10 ms after a depolarization step in Deck & Trautwein's experiments, Dudel & Rudel (1970) cooled down Purkinje fibre preparations to 4-5 °C in order to slow the activation of the sodium current and separate it from capacitive current. Although the high-quality voltage clamp needed for studies equivalent to those of Hodgkin and Huxley was impossible to achieve in cardiac preparations because of their multicellular nature, they obtained cardiac sodium current recordings and showed that currents decayed with two time constants.

Before the 1980's, voltage clamp data in cardiac tissue were obtained mostly from multicellular Purkinje fibres until the techniques for isolating cardiac myocytes became available. In isolated single rat ventricular myocyte, sodium currents were first recorded by Brown, Lee & Powell (1981a) with a two-suction pipette method. The maximum Na conductance was 25mS cm^{-2} in 145 mM sodium solutions. They also proved that cardiac sodium current is less sensitive to the sodium channel blocker tetrodotoxin (TTX) than other tissues, such as nerve or skeletal muscle in single cells level. (Brown, Lee & Powell, 1981a).

Zilberter and colleagues (1982) were the first to apply patch clamp techniques to measure fast sodium current in isolated cardiac ventricular cells. In 1987, Lark & Giles quantitatively measured the transient inward sodium current in single cells from bullfrog atrium using whole-cell patch clamp techniques. Sodium current was pharmacologically and kinetically isolated from other transmembrane currents. Activation and steady-state

inactivation of sodium current were measured and fitted with single Boltzmann functions with half activation at -33mV and half-inactivation at -71mV (Clark & Giles, 1987).

Although early experiments showed that the slope of the slow diastolic depolarization in the intact pacemaker region of the heart is affected by changes of extracellular sodium concentration (Noma & Irisawa, 1975), amphibian and mammalian myocardium remain excitable in Na-free solution (Irisawa, 1978). The rising rate of action potentials of sino-atrial node cells is very low, and electrophysiological activity seems not affected by TTX (Yamagishi & Sano, 1966; Giles & Shibata, 1985). Therefore it is generally believed that sodium current is not involved in action potentials in heart pacemaker cells (Campbell, Rasmusson & Strauss, 1992; Irisawa, Brown & Giles, 1993). Recently, a fast, TTX-sensitive sodium current was observed in rabbit sino-atrial node cells by Denyer and Brown (1990). They agreed with Kreitner (1975, 1978, 1985), however, that Na channels may be present in pacemaker cells but are inactivated in the normal pacemaking voltage range.

1.1.2. Hodgkin-Huxley model and window current

Hodgkin and Huxley (1952a) found that sodium currents took an appreciable time to reach a maximum, and a change in temperature had a large effect on the rate of rise of sodium conductance but a relatively small effect on its maximum value. They also observed that an "inactivation" process occurs. The inactivated state is different from resting state in that inactivation gradually reduces sodium permeability after it has undergone the initial rise associated with depolarization. At a fixed depolarization, the time course of the sodium current is independent of the magnitude and direction of the current flow through the membrane. These results suggested that depolarization of the membrane is not just simply caused by

electrodiffusion of Na ions through the membrane but involves a change in membrane permeability. Hodgkin and Huxley supposed there are charged particles in the membrane which allow sodium to pass through when they occupy particular sites in the membrane. Movement of these particles is responsible for the changes of sodium conductance after a step depolarization. In classic squid axon experiments, activation follows a sigmoidal time course whilst inactivation process follows an approximately exponential time course.

Because activation and inactivation are two opposing processes, it was suggested that there are two different kinds of particles (gating factors) in the membrane. Hodgkin and Huxley called them m and h. Three m particles control activation and one h particle controls inactivation. The probability that these particles are all in the position which allow sodium current through is m^3h. Here the three power of m is needed to describe a small delay in the rise of Na conductance of the cell membranes after depolarization and its sigmoidal time course. Thus sodium current was described by the simple formulation:

$$I_{Na} = m^3 h g_{Na}(E-E_{Na})$$

where g_{Na} is fraction of maximum conductance; E is membrane potential and E_{Na} is equilibrium potential for sodium. According to Hodgkin and Huxley, the two kinds of particle moved independently. At any given time, m or h are functions of rate constants α_m, $ß_m$ or α_h, $ß_h$. Alternatively, they can be described by voltage dependent time constants τ_m or τ_h where τ_m is the reciprocal of the sum of α_m and $ß_m$, and τ_h is the reciprocal of the sum of α_h and $ß_h$ (Hille, 1992). At the resting membrane potential, m is low and h is high. During depolarization, m rises rapidly and h falls slowly while during repolarization m falls rapidly and h rises slowly. Since h is the probability of an available (closed non-inactivated) Na channel, 1-h represents the probability that the Na channel is inactivated.

According to this formulation, if m^3 or h both approach zero but are not zero over a certain potential range, there will be a steady sodium current. This non-inactivated, time-independent Na current has been called a "window" current (Attwell *et al.*1979). Since m^3 approaches zero at negative potentials and h approaches zero at positive potentials, this window current would occur only over a limited range of potentials and would be zero at very positive and very negative potentials.

In cardiac tissue, this kind of window current had been described by Attwell et al (1979) in cardiac Purkinje fibres. In whole-cell clamp experiments, Kiyosue & Arita (1989) recorded a steady TTX-sensitive current of 12-50pA at -40mV in guinea pig ventricular myocytes. It has been thought that TTX, some local anaesthetics and antiarrhythmic drugs affect action potential duration by blocking this window current (also see next section).

1.1.3. Inactivation of sodium current and persistent sodium current

Inactivation is an important feature of Na current. Inactivation of Na channels accounts for the loss of excitability that occurs if the resting potential of the cell falls by as little as 10 or 15 mV, as occurs when there is an elevated extracellular concentration of K^+ ions, or after prolonged anoxia or metabolic block (Hille, 1992). Once Na channels have been inactivated, the membrane must be repolarized or hyperpolarized, often for many milliseconds, to remove the inactivation.

It has been shown that inactivation can be eliminated by treatment of the cytoplasmic side of the membrane with proteolytic enzymes. After treatment, Na currents activate normally but fail to inactivate, so that during a long depolarizing pulse no current decay can be observed (Armstrong & Bezanilla, 1973; Bezanilla & Armstrong, 1977).

In the Hodgkin-Huxley model, the onset of inactivation is exponential with a single time constant. There is evidence, however, that neither cardiac nor neuronal Na currents behave exactly as described by the classic Hodgkin-Huxley model (Chiu, 1977; Bezanilla & Armstrong, 1977; Dudel & Rudel, 1970). For example, Brown, Lee & Powell (1981b) reported that the kinetics of Na currents in single cardiac ventricular cells are more complex, with the time course of recovery from and development of inactivation exhibiting two exponential components.

Therefore, Na current inactivation has been divided into fast inactivation and slow inactivation, according to two component kinetics (Patlak & Ortiz, 1985; Pelzer & Trautwein, 1987). The fast inactivating sodium current has also been called "transient" sodium current while the slowly inactivating sodium current has been called "persistent" sodium current (Stafstrom, Schwindt & Crill, 1982; Stafstrom *et al.*1985; French & Gage, 1985; Gage, Lamb & Wakefield, 1989).

A slow inactivating persistent sodium current has been observed in many cell types, such as squid axon (Gilly & Armstrong, 1984), mammalian hippocampal neurones (French & Gage, 1985; French *et al.*1990); and mammalian skeletal muscle (Gage, Lamb & Wakefield, 1989). In squid axon, the persistent sodium current activates at a more negative potential than the transient sodium current and therefore has been called a "threshold" sodium current (Gilly & Armstrong, 1984). French (1990) described a voltage-dependent persistent sodium current in mammalian hippocampal neurons. This persistent sodium current was much more resistent to inactivation by depolarization than the transient current and could be recorded at $>50\%$ of its normal amplitude when transient current was completely inactivated.

In cardiac tissue, it has been well established since the study of Coraboeuf and colleagues that TTX reduces the duration of the plateau phase

of action potentials (Coraboeuf, Deroubaix & Coulombe, 1979; Wasserstrom & Salata, 1988). As soon as direct observations of sodium current in voltage-clamped cardiac cells became available, many early studies revealed a slowly inactivating component of sodium current (Reuter, 1968; Dudel & Rudel, 1970; Brown, Lee & Powell, 1981b) and shortening of the action potential duration induced by TTX or some local anesthetics has been attributed in part to a block of such a sodium current by these drugs (Gintant, Datyner & Cohen, 1984; Kiyosue & Arita, 1989).

It has been suggested that a non-inactivating component of sodium current could be a "window" current caused by overlap of the steady-state inactivation and activation curve. Kunze and colleagues (1985) recorded a slowly inactivating sodium current at potentials as positive as -10mV in neonatal rat ventricular myocytes, which implies that the slow current is not a window current (Kunze *et al.*1985). Carmeliet (1987) reported a slowly inactivating sodium current in rabbit cardiac Purkinje fibres. The current-voltage relationship extended over a broad range of potentials, as negative as -85 mV. This result suggests that the slow Na current plays an important role not only in determining the plateau of the cardiac Purkinje action potential but also participates in determining the resting and diastolic potential. The problem that faces investigators who study these slow decaying or steady Na currents is their small size (Fozzard, January & Makielski, 1985) and precise information on the kinetics of such a persistent current in cardiac cells has not been obtained therefore for this reason. Although at the single channel level, some late channel activity in cardiac cells has been described (Patlak & Ortiz, 1985; Kohlhardt, Frobe & Herzig, 1987; Kiyosue & Arita, 1989), it is still not known whether fast inactivation is a multistep process or whether there are two or more populations of sodium channels in cell membranes.

1.2. The properties of sodium channels in cardiac myocytes

1.2.1. Density and distribution of cardiac sodium channels

Patch clamp recording has permitted direct observation of current through single Na channels and calculation of single channel conductance. Cardiac Na channel density is estimated as 2-10 channels/μm^2, compared to 200-500/μm^2 in squid axon (Levinson & Meves, 1975; Strichartz, Rogart & Richie, 1979), 2,000 -10,000/μm^2 in nodes of Ranvier (Rogart, 1981) and 200-300/μm^2 in surface membrane of mammalian skeletal muscle (Richie & Rogart, 1977).

The distribution of Na channels on the surface of cardiac cells is undoubtedly neither uniform nor random. It is generally believed that channels occur in clusters: patches can have so many channels that single events are almost completely obscured or they may have no channels. Such a very high density of channels in occasional patches has not been observed in Purkinje cells, which have no T-tubules. Binding studies (Doyle *et al.*1986) suggest that patches in ventricular cells with a high density of sodium channels may be at the mouth of T-tubules .

1.2.2. Single channel conductance and subconductance states

The single channel conductance (γ) of cardiac Na channels is reported to be between 10 and 20 pS, depending upon the recording conditions (Kunze & Brown, 1989). This represents about $5x10^6$ Na ion sec^{-1} passing through a single channel under normal conditions (Fozzard & Hanck, 1992). There was no obvious difference in single-channel current amplitude between rat heart and rat brain Na channels (Kirsch & Brown, 1989). However, when Patlak measured Na channel conductance in mouse skeletal muscle, γ values were almost twice that for cardiac and neuroblastoma cells. Guo and colleagues (1987) incorporated canine cardiac,

rat brain, and rat skeletal muscle Na channels into planar bilayers, along with batrachotoxin (BTX). Under these conditions, γ values were almost the same for all of the channels.

Most early studies of sodium channels did not report subconductance states (Sigworth & Neher, 1980; Horn, Patlak & Stevens, 1981; Krueger, Worley & French, 1983). In the case of native voltage-dependent Na channels, part of the reason for this is their very fast gating kinetics that make measurement of amplitude very difficult. In some cases (Nagy, Kiss & Hof, 1983; Cachelin *et al.*1983; Kunze *et al.*1985) several sodium channel conductances have been described. However, it is not clear whether they were due to different independent channel populations or subconductance states. For example, Scanley and Fozzard (1987) concluded that the lower conductance channel (about 5 pS) was a separate population of Na channels, which nevertheless showed the same kinetic properties as dominant channel type. Nilius and colleagues (1989) observed a 5 pS channel in guinea pig ventricular myocytes, which they thought was a separate type of channel because it exhibited different kinetics and a lower sensitivity to TTX.

Chinn and Narahashi (1986) were the first to demonstrate a subconductance state in sodium channels of mouse neuroblastoma cells treated with deltamethrin which stabilizes a variety of channel states by reducing the transition rate between them. Since then, evidence for multiple open states has accumulated. Using improved substate resolution techniques, Patlak (1988) found that unmodified Na channels at low temperature had sublevels similar to those of the drug-modified channels. In cardiac cells as many as nine open levels have been reported by using different gating modifiers (Schreibmayer, Tritthart & Schindler, 1989; Schreibmayer & Jeglitsch, 1992; Albitz, Droogmans & Nilius, 1991).

More recently, Benndorf (1993) described multiple conductance levels of native cardiac Na channels at elevated temperatures with a high-bandwidth/low-noise patch clamp. The experiment showed that at 9°C, channel openings were regularly dominated by one open level, above 24°C (filter 10 or 13kHz), however, channel-open levels were heterogeneous with maximum levels of up to 4.5 pA at 50mV in 287 mM sodium, but no regular level patterns were found.

1.2.3. Gating models and modes of gating behaviour

Voltage-sensitive channels have a voltage sensor, a collection of charges or equivalent dipoles that move under the influence of the membrane electric field. The movement of the charges can be measured as a tiny gating current (Armstrong & Bezanilla, 1977). Gating current measurements have led to the development of sequential state models of the Na channel that appear to be superior to the Hodgkin-Huxley model in fitting the experimental data, and they offer predictions about transitions in the actual channel structure (Hille, 1992).

In the Hodgkin-Huxley model, steady-state inactivation is steeply voltage dependent, with a slope corresponding to an equivalent gating valence z_g of 3.5 charges per channel and inactivation is independent of activation. Nevertheless, Armstrong and Bezanilla (1977) found no gating current that they could attribute to inactivation gating. Therefore, they suggested a ball-and-chain model (Armstrong & Bezanilla, 1977; Patlak, 1991) in which the inactivation gate is a ball-and-chain hanging out into the axoplasm. When a channel activates, it provides a cup that the inactivation ball falls into stoppering the channel. In this model, microscopic inactivation has no intrinsic voltage dependence. They also showed that the initial stages of inactivation were briefly delayed and Goldman (1989) proved that the delay in inactivation onset appears to be closely associated

with the time course of activation but not of inactivation itself. The results could be most easily explained if the rate of inactivation depended, at least in part, on the activation state of channel.

It has been demonstrated that the open time of normal Na channels is briefer than expected from the time constant of the macroscopic inactivation (Patlak & Horn, 1982; Aldrich, Corey & Stevens, 1983). An alternative model of inactivation was proposed (Armstrong & Bezanilla, 1977; Aldrich, Corey & Stevens, 1983; Hille, 1992), the so called ABACS model (by Armstrong, Bezanilla, Aldrich, Corey, and Stevens), In this model, microscopic inactivation is comparatively fast and activation is slow in contrast to the classic HH model in which inactivation is slow and follows rapid activation. The ABACS model predicts nearly the same time course for g_{Na} of macroscopic current as HH model except that a much smaller proportion of total number of channels are open at the peak compared to HH model. In single channel recording, more channel openings could be seen with smaller than with large depolarizations. According to the ABACS model, with a smaller depolarized pulse, each open channel inactivates with the same rapid time course, but new channels enter into the open pool slowly and continue to arrive long after the first ones have inactivated. So at more negative potential, the longer time course of g_{Na} reflects slow activation, rather than slow inactivation.

However, slow inactivation has been demonstrated at the single channel level in cardiac tissues (Patlak & Ortiz, 1985; Kohlhardt, Frobe & Herzig, 1987; Nilius, Benndorf & Markwardt, 1986; Nilius, 1987). Unlike fast gating Na channels which show short open times, slow gating Na channels have a longer open time and exhibit reopenings and bursts of openings. Individual later channel openings with short open times have been observed in cardiac tissue as well and were considered to be fast channels reopening from an inactivated state (Kunze *et al.*1985).

Patlak & Ortiz (1986) suggested that bursts of openings are due to the entry of the channel into different kinetic "mode". One mode is characterized by a fast transition of an open state into an absorbing state. This behaviour would account for fast inactivation. The second mode is characterized by the reversible failure of the channel's inactivation gate for prolonged periods, giving so called "bursting" openings. The bursting behaviour may account for slow inactivation of averaged or macroscopic currents.

Bursting openings can be observed in excised patches (Nilius, 1988) and in patches exposed to lysophosphatidylcholine (Burnashev *et al.*1991) or aconitine (Nilius, Benndorf & Markwardt, 1986). Zhou et al (1991) investigated macroscopic and single channel sodium currents in oocytes expressing the μl adult rat skeletal muscle α subunit (SkM1). The results of injection with μl cRNA alone or coinjection with another mRNA showed that the equilibrium among gating modes was influenced by a modulating factor encoded in rat skeletal muscle and brain mRNA. Moorman & Kirsch (1990) also reported fast and slow gating of sodium channels encoded by a single mRNA of rat brain type III Na channels. As patches with purely fast or purely slow gating were not observed, it is unlikely that more than one population of channels was expressed in *Xenopus* oocytes.

1.3. Effects of drugs on sodium channels

1.3.1. Tetrodotoxin (TTX)

TTX is one of the guanidium-containing neurotoxins. It is amazing that more than 4000 years ago, Chinese pharmacopeia had already recommended using tetrodon eggs to arrest convulsive diseases (Wong & Wu, 1936; Kao, 1966) and Captain Cook had an experience of tetrodon poisoning during the second circumnavigational voyage in 1774, which was

described as numbness all over limbs and losts of the sense of feeling (Cook, 1777). Only 30 years ago, however, modern pharmacological experiments revealed that TTX blocks sodium currents selectively. (Narahashi, Moore & Scott, 1964). Since then, TTX has become a magic bullet. Almost all of our knowledge about Na channels has come from studies using TTX as a highly selective tool.

It is believed that TTX, which has a net positive charge, blocks the Na channel by binding to a negatively charged site near its mouth, called site 1 (Narahashi, Anderson & Moore, 1966). The rate of block is linearly proportional to the toxin concentration, and one toxin molecule suffices to block one channel (Hille,1992). The equilibrium dissociation constant (K_d) of TTX binding to Na channels varies in different tissues and species, The K_d for the node of Ranvier is 1.2nM (Hille, 1968), while the K_d for cardiac muscle is 1.5μM (Cribbs *et al.*1990). This has led to a proliferation of terms describing channels by their sensitivities to TTX. Channels are thus described either being as TTX-sensitive or TTX-insensitive (TTX-resistant).

Channels blocked by TTX are believed to continue gating normally since their gating currents are not modified by TTX (Hille, 1992). TTX reduces the frequency of single Na channel openings, thus proving an all or none type block (Quandt, Yeh & Narahashi, 1985).

It has been reported that the TTX block of Na channels in cardiac muscle is voltage-dependent (Vassilev *et al.*1986). Voltage-dependent block by TTX of cardiac Na channels reconstituted along with batrachotoxin (BTX) in lipid bilayers was also reported by Moczydlowski and colleagues (1984). However, Dugas *et al.* (1989) investigated single sodium channels in the presence of germitrine, which prolongs the open state of the Na channel in cultured rat cardiac cells and showed TTX blocking kinetics that were independent of membrane potential between -100 and 0 mV. The membrane electric field does not affect TTX blocking kinetics arguing

against an intramembrane location or voltage dependent alteration of the TTX receptor.

1.3.2. Antiarrhythmic drugs

Drugs affecting cardiac arrhythmias have been conveniently classified according to the pattern of electrophysiological effects. The original classification, introduced by Vaughan-Williams (Vaughan-Williams, 1981; Sheldon, Hill & Duff, 1989), was based upon observations on preparations of canine or bovine cardiac Purkinje fibres (Bigger & Hoffman, 1985). The class I agents are supposed to depress directly the rate of upstroke of action potentials by blockade of fast sodium channels. In terms of their relative ability to slow membrane repolarization, class I drugs are subcategorized into class I_a (prolong repolarization e.g. quinidine), class I_b (shorten repolarization e.g. lidocaine), and class I_c (little effect on repolarization e.g aprindine). Class II includes agents that primarily block ß-adrenergic receptors. Class III agents prolong the period of repolarization by blocking one or more potassium channels (Sheldon, Hill & Duff, 1989). Finally, class IV agents have relatively selective depressant actions on calcium channels.

Drug interaction with the Na channels can be divided into two modes: tonic block, measured as a decrease in sodium current during the first depolarizing pulse which mainly reflects a closed channel block (Snyders, Bennett & Hondeghem, 1992); and phasic or use-dependent block, measured as a progressive reduction in sodium current during repetitive activation. (Chernoff & Strichartz, 1990).

Two major hypotheses, the modulated receptor hypothesis and the guarded receptor hypothesis have been put forth to explain the mechanisms of drugs blocking channels. The modulated receptor hypothesis (Hondeghem & Katzung, 1977; Hille, 1977) proposes a state-dependent drug

affinity for a blocking site. For example, the affinity of the binding site for drug may be higher when the channel is open or inactivated than when in the resting state. The guarded receptor hypothesis (Starmer, Grant & Strauss, 1984) proposes that the affinity is constant but access to the binding site is guarded by the activation and /or inactivation gates, such that the forward binding rate is faster when the channel is open or inactivated than under resting conditions.

Single-channel experiments in cardiac Na channels showed that blocking by antiarrhythmic drug occurs in an all-or-none fashion. All-or-none blocking represents a common principle regardless of the nature and site of action of a particular blocking molecule. Na channels basically fail to conduct when the drug molecule occupies a primary binding site that lies between the gates and the selectivity filter (Hille, 1992). It has been assumed that drug binds more tightly to the inactivated form of the channel than to the resting or activated form. Use-dependent block and the interactions between drug and gating may play a significant role in the antiarrythmic actions.

Bean, Cohen and Tsien (1983) investigated the effect of lidocaine on sodium currents of rabbit Purkinje fibers using a two microelectrode voltage clamp. They found that lidocaine bound very strongly to cardiac sodium channels when the channels were inactivated. Nilius, Benndorf & Markwardt (1987) showed that in single channel recordings lidocaine reduced the averaged current mainly by an increase in the number of null sweeps without changes of the size of the unitary currents. They also found that lidocaine decreased the mean open time of Na channels and the concentration of lidocaine for half maximum block was smaller if it was applied from inside the cell than from outside.

At therapeutic concentrations lidocaine shortens the duration of the action potential while quinidine lengthens it (Colatsky, 1982). This

suggestes that lidocaine may block a TTX-sensitive steady state sodium current while quinidine may have a strong effect on currents responsible for repolarization e.g. a potassium current.

1.4. Structure and function of sodium channels in cardiac muscle

1.4.1. Basic structure and function of the sodium channel

The voltage-sensitive sodium channel is responsible for the increase in sodium conductance resulting from depolarization of cell membranes during the action potential in nerve, skeletal muscle, cardiac muscle, and neuroendocrine cells (Catterall, 1992). Sodium-based action potentials in different excitable tissues are very similar (Hille, 1992). Sodium channels that have been purified from many tissues show extensive homology (Trimmer & Agnew, 1989; Patlak, 1991), which is probably the basis of this functional similarity .

Molecular studies of eel *(Electricus electrophorus)* sodium channels revealed the first primary structure of a voltage-sensitive ion channel (Noda *et al.*1984). Since then, a multigene family has been identified in different tissues and species (Mandel, 1992).

The voltage-sensitive sodium channel exists either as a single protein or as a complex of proteins (Trimmer & Agnew, 1989). Irrespective of the source, all of the channels contain a large α subunit of approximately 260 kDa. The basic primary structure of the alpha subunit in the human heart shows a large polypeptide of 2016 amino acids with calculated molecular weight of 227,159 daltons. It contains four repeated domains (I -IV) of 226 to 288 amino acids (Gellens *et al.*1992). According to the single pore hypothesis (Hille, 1992), each domain is thought to form part of the wall around a central pore. Each domain contains 6 hydrophobic segments (S1-S6) with the potential for formation of transmembrane α helices including a

positively charged amphipathic segment (S4), which is comparable to other sodium channels (Catterall, 1986). Sodium channels in mammalian brain and skeletal muscle, in addition to the alpha subunit, contain one or two smaller beta subunits of 37 and 39 kDa (Catterall, 1986; Barchi, 1988). The primary structures of ß1 subunit have been determined recently (Isom *et al.*1992). Although the function of ß subunits is not clear yet, it may be anticipated that they contribute to the diversity of sodium channel structure and function (Catterall, 1992). It is seems however, that the cardiac sodium channel α-subunit alone can express the pharmacological and kinetic properties of native cardiac sodium channels (Satin *et al.*1992a).

1.4.2. Structures responsible for activation and inactivation

The change in sodium conductance is biphasic, since it is controlled by two processes: activation, which increases the conductance in response to depolarization and inactivation, which subsequently decreases the conductance to the resting level even if depolarization is maintained.

Mutagenesis experiments provided direct evidence about the origin of voltage sensitivity. The S4 segments in sodium channels are thought to be the voltage sensors responsible for voltage-dependence of activation (Kra-Oz *et al.*1992). The S4 segment is one of the putative membrane-spanning segments and it has a striking pattern of positive charged amino acids every third residue (Patlak, 1991). This structure is also typical of all voltage-gated cation channels (Tanabe *et al.*1987; Papazian *et al.*1987). Neutralization of the one to three positively charged amino acid residues in the S4 segment in domain I of sodium channel α-subunit causes a progressive reduction in the steepness of the voltage-dependent activation of sodium channels as expected if these positive charged amino acid residues serve as gating charges (Stuhmer *et al.*1989). In addition, mutation of a hydrophobic residue in the S4 segment in domain II from leucine to

phenylalanine causes a 20 mV shift in the voltage dependence of gating to more positive membrane potentials (Auld *et al.*1990).

The primary structure responsible for inactivation has been identified in the short intracellular loop connecting domains III and IV by application of site specific antibodies in combination with voltage patch clamp techniques. In both whole cell and inside out patches, an antibody directed against this short intracellular segment inhibited sodium channel inactivation (Vassilev, Scheuer & Catterall, 1988; 1989). Mutagenesis experiments have also shown that expression of the sodium channel α-subunit in *Xenopus* oocytes as two pieces corresponding to the first three domains (I-III) and the fourth domain (IV) results in channels that activate normally but have slowed inactivation (Stuhmer *et al.*1989). It is believed that the single phenylalanine in the centre of a three-residue hydrophobic cluster is the critical residue, since its conversion to glutamine is sufficient by itself to nearly completely prevent fast channel inactivation (West *et al.*1992).

1.4.3. Structures responsible for TTX sensitivity

Although the structure and function of sodium channels in different tissues are very similar, the presence of multiple sodium channel isoforms is clearly established (Trimmer & Agnew, 1989; Mandel, 1992). These isoforms of sodium channels can be distinguished by their electrophysiology and pharmacology.

A significant difference between sodium channels in cardiac tissues and other excitable tissues is their sensitivity to TTX. One class of sodium channels, present in brain and adult skeletal muscle, is blocked by application of TTX in the nanomolar range. On the other hand, sodium channels in cardiac muscle and in developing or denervated skeletal muscle, are blocked only by higher TTX concentrations, in the 1-10 micromolar range (Mandel, 1992). Direct binding studies also indicate the presence of

approximately four times more low affinity binding sites than high affinity sites for the TTX analog, saxitoxin (STX) in cardiac tissue (Catterall & Coppersmith, 1981). This raises the possibility that there exist different types of sodium channel even in cardiac tissues themselves.

Three TTX insensitive cDNAs: rSkm2 from rat skeletal muscle (Kallen *et al.*1990), RH1 from rat cardiac tissue (Rogart *et al.*1989) and hH1 from human cardiac tissue (Gellens *et al.*1992) have been identified. The degree of primary structure identity that exists between hH1 and rSkM2 (93.8%) is significantly greater than that between the two rat isoforms rSkM1 (brain) and rSkM2 (59%), while the RH1 appears to be identical to rSkM2 (Rogart *et al.*1989).

The SS2 domain has been postulated to contribute to the binding site for TTX. SS2 is a seven-amino acid segment that forms part of the external loop that connects membrane-spanning segments S5 and S6. Two of the seven amino acids in SS2 of the first NH2-terminal repeat or domain I differ between the TTX-resistant and TTX-sensitive Na channels (Guy & Conti, 1990; Satin *et al.*1992a). In SS2 of TTX-resistant Na channels, asparagine is replaced by arginine and phenylalanine or tyrosine is replaced by cysteine.

Because the asparagine residue that is present in this region in all TTX-sensitive sodium channel isoforms is replaced by arginine in both hH1 and rSkM2, it has been suggested that the charge difference could contribute to the decreased affinity for TTX exhibited by these channels (Gellens *et al.*1992; White *et al.*1991). However, mutation experiments have shown recently that an increase of negative charge by substitution of the positively charged Arg^{377} for a neutral Ans to RH1 Na channel caused a decrease in TTX affinity rather than an increase.

When Cys^{374} in SS2 region was replaced by Tyr, however, it resulted in an 730-fold increase in TTX binding affinity (Satin *et al.*1992a), whereas replacement of phenylalanine (385) of brain sodium channel by

cysteine reduces sensitivity to TTX (Heinemann, Terlau & Imoto, 1992). Therefore it is believed that the cysteine rather than arginine in SS2 of cardiac Na channel is critical to TTX binding. These obsevations also suggest that TTX binding in the Na channel may not be a simple electrostatic attraction between the positively charged toxin guanidinium groups and negatively charged acidic group in Na channel binding site, perhaps involving an ionized hydrogen bond (Satin *et al.*1992a)

1.5. Other ion currents involved in the action potential of cardiac myocytes

1.5.1. Calcium current

Two kinds of voltage-sensitive calcium channels, L-type and T-type, are consistently identified in different cardiac tissues (Nilius *et al.*1985; Mitra & Morad, 1986). Both are thought to play an important role in cardiac pacemaking (Hagiwara, Irisawa & Kameyama, 1988) and in maintaining the plateau of action potentials (Doerr *et al.*1990; Pelzer, Siegried & McDonald, 1992).

In addition, a new third type of Ca channel has been identified in cardiac sarcolemmal membranes, termed the B-type because it may contribute to steady background current at negative membrane potentials (Rosenberg, Hess & Tsien, 1988; Rosenberg & Tsien, 1987). These calcium channel types differ in their sensitivity to depolarization, pharmacology, ionic selectivity, metabolic regulation, and single-channel conductance (Hille, 1992).

L-type Ca channels, the best known class, are presently defined as high-voltage activated (HVA) channels sensitive to dihydropyridine compounds (DHP) such as nifedipine. L-type Ca channels can thus be distinguished pharmacologically from T-type Ca channels which are

generally DHP-resistant. Compared with the transient sodium current, the L-type calcium current is very small and has relatively slow kinetics. In mammalian ventricle, Calcium current is only 1/20 to 1/30 of size of the peak sodium current and its kinetics of activation and inactivation are approximately ten times slower (Giles, 1989). The inactivation of calcium current appears to be more complex than that of sodium current, being not only voltage-dependent but also Ca-dependent. Calcium channel inactivation could result from the local rise of intracellular free Ca^{2+} concentration as Ca^{2+} ions flow into the cell during a depolarizing pulse (Pelzer *et al.*1989). The ion selectivity of L-type Ca channels is extremely high, with $> 10^3$ ratios for divalent over monovalent cations. L-type Ca channels can be blocked by multivalent cations such as La, Cd, Co, Mn and Ni, at concentrations from 10μM to 5 mM (Pelzer *et al.*1989). Block by multivalent cations takes place when blocking ions lodge within the pore, whereas block by organic Ca channel blockers involves a different mechanism, which includes stabilization of the channel in a nonconducting state (Pelzer, Siegried & McDonald, 1992).

T-type Ca channels are also known as low-voltage activated (LVA) Ca channels because they can be opened by small depolarizations from relatively negative holding potential. T-type calcium currents begin to inactivate at -90 mV and inactivation is complete at -40mV; the threshold for activation of this current is near -50mV, and it is fully activated at 0 mV. Due to their negative voltage range of activation and inactivation, and their transient appearance, T-type Ca currents probably contribute very little to the plateau phase of cardiac action potentials. However, it is believed that they may contribute to pacemaker spontaneous depolarization (Irisawa, Brown & Giles, 1993). The sensitivity of T-type Ca channels to block by inorganic multivalent cations seems to be quite variable. Compared with L-type Ca channels, they appear to be less sensitive to block by Cd and more

sensitive to block by Ni, and more or less equally sensitive to Co and Mn (Irisawa, Brown & Giles, 1993; Hille, 1992; Pelzer, Siegried & McDonald, 1992).

1.5.2. Potassium currents

Potassium currents in cardiac cells have long been renowned for their confusing diversity (Trube, 1989; Carmeliet *et al.*1987). To date, at least ten different types of K channels have been described in cardiac tissues, of which four are activated primarily by voltage and the remaining six primarily by agonist action (Carmeliet, 1989). Voltage-dependent K channels can be activated either by depolarization or hyperpolarization (Hille, 1992). Agonist-operated K channels can be activated or inactivated by intracellular ions such as Ca^{2+} (Callewaert, Vereecke & Carmeliet, 1986), Na^{+} (Kameyama *et al.*1984), or intracellular metabolic products, such as ATP (Noma, 1983), arachidonic acid and phosphatidylcholine (Wallert *et al.*1991) or extracellular neurotransmitters, such as acetylcholine (ACh) (Noma & Trautwein, 1978), or somatostatin (Lewis & Clapham, 1989; Sims, Lussier & Kraicer, 1991; White, Schonbrunn & Armstrong, 1991). It is of interest to note that agonists can modify the behaviour of voltage-dependent K channels and that voltage also can affect agonist-operated K channels.

1.5.2.1. Delayed outward current

A delayed outward potassium current, known as *the delayed rectifier*, I_k, contributes to the termination of the action potential plateau. It acts to shorten action potential duration and increase heart rate (Hille, 1992; Giles, 1989).

Noble & Tsien (1969) performed a classic analysis of outward time-dependent currents in sheep Purkinje fibres. They found at least two time-

dependent components of outward current (I_{x1}, I_{x2}), since two exponentials were required to describe the kinetics of current in their multicellular preparations. In contrast, it has been reported from single-cell experiments that there is only one type of delayed rectifying current, and that it can be fitted by standard Hodgkin and Huxley kinetics with two gating particles (Gintant *et al.*1992). I_k activates at around -30 mV and rises slowly to approach a steady state after about 10 sec (Hume *et al.*1986; Simmons, Creazzo & Hartzell, 1986). The differences between single cell and multicellular tissues are thought to arise from K accumulation in the narrow extracellular space of multicellular preparations so that after prolonged membrane depolarization, the electro-chemical gradient for potassium changes (Gintant *et al.*1992). However, it has recently been shown that I_k in single cardiac cells is the sum of two overlapping K currents, I_{ks} (slowly activating) and I_{kr} (rapidly activating) (Balser, Bennett & Roden, 1990; Sanguinetti & Jurkiewicz, 1990; 1991;1992). I_{kr} is strikingly similar to the long-lasting Ca-activated K current described by Baro and Escande (1989) in its voltage-dependence and unique rectification. I_{kr} activates rapidly and is blocked by relatively low concentrations of La^{3+}. I_{ks} is a much larger current that activates very slowly, and is not blocked by low concentrations of La^{3+}.

Although patch clamp techniques have been successfully applied to the study of cardiac inward rectifier K channels (Sakmann & Trube, 1984), measurements of delayed rectifier K channels in cardiac tissue have remained elusive. Shibasaki (1987) examined the properties of a delayed rectifier K channel in the nodal cells of the rabbit heart. The channel was largely K-selective and had a single channel conductance of 11.1 pS with 150 mM external potassium (Shibasaki, 1987). In cell-attached patches, Balser, Bennett & Roden (1990) reported single delayed rectifier channels which had a mean chord conductance of 5.4 pS at 60mV with 4.5 mM

external potassium. However, still unexplained is the extreme rarity of observing these channel in cell-attached patches and the relatively lower density of these channels in membrane patches compared with whole cell recording.

Delayed rectifier K channels in different tissues seem to vary in their pharmacology and gating kinetics. For example, in frog skeletal muscle delayed rectifier K channels require 8 mM TEA for half block while in frog heart I_k channels are hardly affected by 20mM external TEA. The activation kinetics of delayed rectifier K channels in the heart are 1000 times slower than in frog node of Ranvier and this may account for the action potential in heart being almost 1000 times longer than in axons. These phenomenonological differences suggest that delayed rectifier channels could be encoded by a gene family (Paulmichl *et al.*1991).

1.5.2.2. Transient outward current

The upstroke of the action potential of the cardiac tissues is immediately followed by a rapid fall giving what is termed phase 1 of the action potential (Gintant *et al.*1992). Voltage-clamp studies suggest that a transient outward current (I_{to}) is involved in this fall in potential giving this characteristic morphology of the action potential in cardiac tissue. The transient outward current seems to have two components: a voltage-gated potassium current and a calcium-activated potassium (or chloride) current. In many respects, the voltage-gated conductance is similar to the A current (I_A) described in molluscan neurons (Hille, 1992; Gintant *et al.*1992), which can be separated from the total outward current during a voltage step by changing the holding potential, since I_A can be only activated from a hyperpolarized potential (Connor & Stevens, 1971). I_{to} can be blocked by 4-aminopyridine (4-AP) and is less sensitive to block by TEA than I_k (Nakajima, 1966; Thompson, 1977). A calcium-activated component of the

transient outward current has been reported in atrial or ventricular muscle from different species. This component is resistant to 4-AP. However, it is uncertain whether the charge carriers of this component are potassium or chloride ions since it also blocked by anion transport blockers (Zygmunt & Gibbons, 1991).

1.5.2.3. Inward rectifier K current

Inward rectifier K current is of special physiological relevance in heart, as it passes current far more easily in the inward than the outward direction. Absence of I_{ki} at depolarized potentials reduces the amount of inward current necessary to maintain depolarization during the plateau. This rectification can be described as being due to two processes: deactivation and voltage-dependent block of the channel by internal Mg^{2+} (Trube, 1989). Deactivation of I_{ki} channels will cause outward current to decrease to zero for potentials moderately positive to E_K (Kurachi, 1985). The range of potentials where deactivation occurs shifts with $[K^+]_o$, suggesting that the driving force ($V-E_K$) rather than membrane potential alone is the important factor controlling rectification. The single-channel conductance of I_{ki} is proportional to the square root of $[K]_o$. The single channel conductance of inwardly rectifying channels in ventricular cells is about 22 pS and 16 pS at external K^+ concentration of 150 and 40 mM, respectively (Matsuda, 1988). The density of I_{ki} channels is 10-100 time larger in the ventricle than in the S-A or A-V node. The sparsity of I_{ki} channels in nodal tissue may account for their role as dominant pacemakers (Irisawa, Brown & Giles, 1993). Another feature of inward-rectifying K current in cardiac preparations is that the current shows a time-dependent decline upon hyperpolarization. Voltage-clamp experiments have indicated that inactivation could contribute to the time-dependent decay (Payet, Rousseau & Sauve, 1985). Biermans, Vereecke & Carmeliet (1987) demonstrated that

the inactivation during hyperpolarization is largely due to a block of the channel by external Na^+ ions.

1.5.2.4. ATP-sensitive K channels

ATP-sensitive K channels (K_{ATP}) were first described by Noma in 1983 (Noma, 1983; Noma & Shibasaki, 1985). In cyanide-treated mammalian heart cells, he found a specific K channel which was depressed by intracellular adenosine triphosphate (ATP) at concentrations greater than 1 mM. This channel had bigger conductance (65 pS) than inwardly rectifiying K channel (34 pS) recorded in the same patch. The conductance for 5.4, 50 and 100 mM K at the outside of the membrane of the ATP-sensitive channels, was 20, 63, and 80 pS respectively.

The kinetics of the channel were almost voltage-independent. When ATP concentration was increased, the time constants obtained from the open-time histogram decreased and those from the closed-time histogram increased, resulting in a decrease of the open-state probability. The conductance of the channel was not affected by ATP level (Kakei, Noma & Shibasaki, 1985), but the amplitude of the open-channel current was reduced with increasing $[Mg^{2+}]_i$, $[Na^+]_i$ and $[Ca^{2+}]$ in a concentration- and voltage-dependent manner (Horie, Irisawa & Noma, 1987; Findlay, 1987). The effect of ATP on the K channel is most probably mediated by binding of the ATP molecule to its receptor site, the occupation of which induces a modulation of the gating mechanism (Kakei, Noma & Shibasaki, 1985).

The role of ATP-sensitive K channels in cardiac cells may be important in cardiac ischemia and anoxia. A shortening of the cardiac action potential during hypoxia was reported more than three decades ago by Trautwein, Gottstein & Dudel (1954). The direct relationship between anoxia and K_{ATP} has been reported by Benndorf *et al.* (Benndorf, Friedrich & Hirche, 1991; Benndorf *et al.*1992) , who found that 4-35 min anoxia

induced a large time-independent K current, which disappeared within 2-5s following reoxygenation. The conductance, moderate inward rectification, opening/closing kinetics, and glibenclamide sensitivity (Fosset *et al.*1988) further confirmed that anoxia-induced K current is mediated by K_{ATP}. It seems that anoxia leads to a decrease in intracellular ATP concentrations which triggers an activation of ATP-sensitive K channels and an efflux of K^+ through the open channels provoking a shortening of the action potential. It is appear that the drugs which block K_{ATP} are not effective antiarrhythmic agents. Thus even though ATP-sensitive K channels are activated during ischemic condition, they play a small role in the genesis of arrhythmias.

1.5.3. Hyperpolarization-activated cation current

An inward current (I_f) activated by hyperpolarization in cardiac primary pacemaker tissue was first observed independently in two different laboratories (Brown, DiFrancesco & Noble, 1979; Yanagihara & Irisawa, 1980). Like I_{Ki}, I_f is a time-dependent inward cationic current that activates on hyperpolarization rather than on depolarization like most other known cationic currents, and is blocked by low concentrations of caesium (DiFrancesco & Tromba, 1989). Unlike I_{Ki}, I_f channels are almost as permeable to Na^+ as to K^+, not strongly blocked by Ba^+, have a nearly linear instantaneous current-voltage relation with a reversal potential near -20mV which does not shift with changes of $[K]_o$ (Hille, 1992). There is recent evidence that inward Na^+ current through the I_f channel increases on raising the extracellular K^+ concentration. The Na^+ permeability relative to K^+ permeability (P_{Na}/P_K), as measured from the reversal potential, increases and saturates near 5 mM K^+. It is suggested that the I_f channel has multiple, interactive binding sites for cation permeation (Frace, Maruoka & Noma, 1992).

Using two pipettes on the same cell, one for whole-cell recording and the other for recording from cell-attached patches, DiFrancesco found that the single-channel events underlying I_f are only 0.067-0.085pA at potentials between -80 and -102 mV in 70 mM $[k]_o$. Thus their single channel conductance is about 1 pS (DiFrancesco, 1986).

Since I_f has been found only in restricted regions of heart and precisely in those regions where spontaneous activity can normally be recorded (Campbell, Rasmusson & Strauss, 1992) and activation at negative potentials could contribute to the slow pacemaker depolarization, I_f is regarded as a pacemaker current and contributes to the spontaneous diastolic depolarization. However, there is still much controversy concerning the nature of I_f and its role in pacemaking (Irisawa, Brown & Giles, 1993). Major points of dispute are that the kinetics of activation of I_f are too slow and the steady-state activation range of I_f is too negative for this current to play any role in pacemaking (Campbell, Rasmusson & Strauss, 1992). 2mM Cs^+ can block I_f selectively and completely. This concentration Cs^+, however, slows but does not arrest the directly observable spontaneous contractile activity of pacemaking cells (Denyer & Brown, 1990). These observations suggest that I_f is not an essential current required to produce primary pacemaking activity.

I_f is increased by ß-adrenergic agonists and decreased by muscarinic agonists. Acetylcholine, at nanomolar concentrations, inhibits I_f and slows the spontaneous beating rate of the heart, whereas 20 times higher concentrations are required to activate the acetylcholine-dependent potassium current (DiFrancesco, Ducouret & Robinson, 1989). It has been suggested that G proteins directly regulate I_f channels responsible for vagal or sympathetic regulation of the heart rate (Yatani *et al.*1990). It has also been found that I_f can be activated by intracellular cyclic AMP, through a mechanism that is independent of phosporylation, and due to direct

interaction cAMP with the channels at their cytoplasmic side (DiFrancesco & Tortora, 1991).

1.6. The aims of this thesis

In this thesis, a series of studies of transient and persistent currents through sodium channels in isolated cardiac myocytes will be described. First, a persistent sodium current which could contribute to the plateau of action potentials and diastolic depolarization was investigated in mammalian ventricular myocytes and amphibian pacemaker cells. Secondly, the effects of antiarrhythmic drugs on both sodium currents was studied in mammalian ventricular myocytes. Thirdly, the properties of transient and persistent sodium currents were investigated at the single channel level. Finally transient sodium currents involved in action potential activity were investigated in single sinus venosus cells isolated from amphibian heart. These observations challenge some traditional views of cardiac function.

Chapter 2

METHODS & TECHNIQUES

2.1. Isolation of adult cardiac myocytes

2.1.1. The structure of cardiac muscle

The notion that individual cells exist in the adult myocardium has been the subject of protracted historical debate (Dow, Harding & Powell, 1981). The earliest diagram of the catenary structure of myocardium was reported by van Leeuwenhoek in 1695. He believed that the heart tissue is arranged in a catanary fashion and is impossible to separate without breakage. As late as the 1901, it was accepted that no sarcolemma had been proven to exist in the adult heart.

The first electron microscope observation of intercalated discs as two parallel membranous structures was made by Van Breemen in 1953. It is clear from electron microscope studies, with supporting evidence from phase microscopy and histological staining, that the adult myocardium is composed of individual muscle cells, and intercalated discs are the single-cell boundaries (Moore & Ruska, 1957).

The heart's gross structure arises from multiple-layered sheets of muscle laid down in a complexly convoluted manner. Sheets are formed from bundles, and bundles from chains of individual myocytes. Within chains, myocytes are connected by multiple intercellular membrane junctions at their step-shaped transverse ends (intercalated disks) and the chains follow devious, branching paths. On average, each myocyte is connected to at least

two cell-neighbours longitudinally and at least one laterally (Jacobson, 1989).

Three types of intercellular (sarcolemmal) junctions are found at the intercalated discs, where the plasma membranes of adjacent cells come into intimate contact. These are gap junctions, desmosomes and fasciae adherens (Severs *et al.*1985). The gap juctions form low resistance pathways along which the action potential can spread from one cell to the next, and, together with the other type juctions, it ensures mechanical integration of the contractile apparatus of all the cells in a muscle bundle (Severs, 1989).

The presence of junctions whose function is to bond of cells firmly together poses a potential problem for the successful dissociation of functionally intact individual cells. Fortunately, the intercellular core components of the desmosome and fascia adherens are removed by the low calcium/enzyme solutions used in dissociation, so that these junctions simply fall apart. However, the gap junction membranes do not so easily come apart; they are normally torn from the plasma membrane of one cell as it separates from its neighbour (Mazet, Wittenberg & Spray, 1985; Severs *et al.*1985). Inevitably, some cells are irretrievably damaged in the process, but many survive.

Investigation of the properties of the intact functional dissociated myocyte permits novel insight into heart function at the level of its component specialized cells, and bridges the experimental gap between the multicellular models and subcellular fractions obtained from homogenates of whole heart.

2.1.2. History of isolating cardiac myocytes

The history of efforts to isolate cardiac myocytes can be traced back to 1866, when C.J. Eberth used alkaline digestion of myocardial tissue and observed that myocardial fibres could be readily separated along the

sarcolemma and at intercalated disks. Cavanaugh first reported the isolation of single viable cardiac myocytes in 1955 using trypsin digestion of embryonic heart fragments. In the 1960's many reports demonstrated that cardiac myocytes with apparently intact morphology could be isolated from heart of adult mammals. However, it became apparent subsequently that morphological integrity, even at the ultrastructural level, was not a sufficient condition for isolated cardiomyocytes to be biochemically or physiologically normal (Silver & Houser, 1985).

Cells of nearly all early preparations of isolated cardiomyocytes were stable in media with calcium ion concentrations of a few micromolar or in higher concentrations at unphysiologically low temperatures. However, these cells suffered irreversible hypercontraction within seconds after being suspended in media at normal physiological temperature and Ca^{2+} concentration. Such cells are termed "calcium ion intolerant". Another term, "calcium paradox", was applied to describe a phenomenon which appears in the process of isolating myocytes (Farmer *et al.*1983). Reduction of Ca^{2+} concentration to near zero is a requirement for isolation of cells from a variety of tissues, including heart. In this regard, heart tissue is troublesome. When the perfusion for several minutes at near-zero Ca^{2+} concentration is followed by normal Ca^{2+} concentration, massive cellular damage takes place. However, once isolated cells are Ca^{2+} tolerant, they are not subject to calcium paradox damage (Jacobson, 1989).

In the 1970's two ways to isolated Ca^{2+} tolerant adult cardiomyocytes were developed. One was immersion perfusion which is the simultaneous mechanical and enzymatic treatment of small pieces of myocardium; the other was Langendorff perfusion which consists of retrograde perfusion with an enzymatic solution via the aorta of the intact heart, and subsequent mechanical or further enzymatic treatment of pieces of the pretreated heart (Powell & Twist, 1976).

Today, there are numerous procedures for isolating Ca^{2+} tolerant adult cardiomyocytes. Many are modifications of earlier methods. However a few principles of cell isolation are fairly well established, although it is believed that preparing cells is still more art than science.

2.1.3. Langendorff method for isolating ventricular myocytes of adult rats

Excising and cannulating the heart

4 to 6 month-old Wistar rats were given an intraperitoneal injection of 2000 units of heparin, and killed 20-30 min later by cervical dislocation. Blood was discharged from carotid arteries. The rat was placed supine in an ice box , and the chest was opened very quickly. The arch of the aorta was freed and the end of it tied as far as possible from the heart. The heart was quickly excised from surrounding tissues.

The heart was rinsed with Ca^{2+} free Tyrode's solution at 0-4°C bubbled with oxygen for 2-3 min, or until the heart appeared pink in colour. Tyrode's solution contained (mM): NaCl 133, KCl 4.0, NaH_2PO_4 1.2, MgCl 1.2, TES (N-tris [hydroxymethyl] methyl-2-aminoethanesulfonic acid) 10, glucose 11, pH adjusted to 7.4 $\pm$ 0.05 with NaOH (about 6mM) and osmolarity was about 300 mosmol/L measured by a vapour pressure osmometer (Wescor 5100C).

The aorta was cannulated through a cut at the junction connecting the root of the right subclavian artery and ascending aorta, and tied firmly with a cotton thread. The way of cannulating provided a longer segment of aorta and was designed to prevent a deeper cannulation which could interfere with closure of the aortic valve. If the valve is not permitted to close, the entire perfusion pressure would be applied across the wall of the left ventricle

which would cause the collapse of the coronary circulation, thus preventing adequate perfusion and reducing cell yield (Muir, 1967; Farmer *et al.*1983).

I believe that avoiding anoxic injury is a key to obtain Ca^{2+} tolerant myocytes; hence administering heparin, chilling the heart, rapid excising, oxygen bubbling, and correct cannulation etc., became very important "safety factors" in the Langendorff perfusion procedure.

Cleavage of intercellular junctions and disruption of extracellular matrix

The release of myocytes from tissue requires that desmosomal, intermediate, and gap juntions are cleaved and that nonjunctional intercellular connections are eliminated by removal of the extracellular matrix.

Since calcium has a central role in intercellular adhesion, less than 250 μM Ca^{2+} is required if desmosomal and intermediate junctions are to be cleaved (Crevey, Langer & Frank, 1978). After the heart was cannulated, it was perfused with Ca^{2+}- free Tyrode's solution for no longer than 5 min. It has been reported that a long period of Ca^{2+} free perfusion (5-30 min), will damaged the glycocalyx, which correlates with calcium paradox damage (Yates & Dhalla, 1975).

The perfusate was maintained at 37°C, bubbled with oxygen, and was circulated by means of a peristalic pump (Gilson Minipuls 2) at the rate of 9-10ml/min. Normally, after 5 min single-pass perfusion, the perfusate became blood free. The heart was then perfused in a recirculating way with Tyrode's solution containing 25 μM Ca^{2+}, collagenase (80 units /ml, Worthington CLS II), protease (0.1mg/ml, Sigma type XIV) and fetal calf serum (1μl/ml, Cytosystems)

Collagenase and protease were used to disrupt the extracellular matrix. Collagenase is activated by Ca^{2+}. Kono (1969) observed that when

1mM Ca^{2+} was present in crude collagenase during cell isolation, most of the isolated cells were "digested". However, with 100 μM Ca^{2+}, morphologically intact cells were isolated. During enzyme perfusion, the Ca^{2+} concentration was reduced to 25 μM, since it was considered that the Ca^{2+} concentration probably rises to a higher level from endogenous Ca^{2+} release (Jacobson, 1989). Because Ca^{2+} concentration is critical in isolating myocytes, the water which was used to make Tyrode's solution was Ca^{2+} free. The Milli-Q reagent water system (Millipore corporation) is normally satisfactory.

The concentration of enzymes and the time of treatment are another two important factors, as enzyme activity varies from batch to batch. Myocytes could not be isolated if cardiac tissue was underdigested whilst sodium channel density could be reduced if the tissues were overdigested (Howe & Ritchie, 1990). A routine was established whereby small pieces of ventricle were cut consecutively over a period of 3-5 min. The enzyme concentration and perfusion time were based on these empirical data.

Release and harvesting cells and establishing Ca^{2+} tolerance.

After 25 min of enzyme perfusion, the heart became enlarged, pale, and flaccid. The ventricles were then removed piece by piece, normally at 5 min intervals. The ventricular tissue was then chopped into small pieces and a fresh Tyrode solution with 25 μM Ca^{2+} was added. The solution containing the chopped ventricles was collected into a 10 ml centrifuge tube with wide bottom, centrifuged at 1000 rpm for 1 min, and the supernatants were discarded. A fresh 25 μM Ca^{2+} Tyrode's solution was added. The pieces of tissue were gently triturated by using a plastic pipette with a large opening. When the supernatant became cloudy, it was transfered into another clean test tube. This process was repeated three times for each sample.

The cell suspensions were centrifuged, the sedimented myocytes washed with 200 μM Ca^{2+}-Tyrode solution containing 1 mM pyruvic acid, and maintained at room temperature for about 1 hour before being resuspended in 1 mM Ca^{2+}-Tyrode's, since healing of sarcolemmal tears requires the presence of Ca^{2+}. If the Ca^{2+} concentration is too high however, it may promote hypercontraction as Ca^{2+} enters cells before healing is complete. Therefore, Ca^{2+} concentration was increased progressively (25μM - 200μM - 1mM) to give the cells some time to heal any damaged membranes. The myocytes seemed to establish Ca^{2+}-tolerance very well in this way, since the cells still showed normal morphological and electrophysiological properties when they were kept in normal Ca^{2+} Tyrode's solution for up to 24-48 hours at room temperature. Other investigators have used a low Na^{+}-high K^{+} concentration solution, so called KB medium (Isenberg & Klockner, 1982; Bihler, Ho & Sawh, 1984), to establish calcium tolerance. However this method is simpler, reliable and more economics.

2.1.4. Langendorff method for isolating ventricular myocytes of toad.

Toads (*Bufo marinus*) were killed by decapitation and pithed. The heart was removed and perfused through the left subclavian artery with Ca^{2+} free Ringer solution containing (mM), NaCl 110, KCl 2.5, Hepes 10, $CaCl_2$ 2, pH adjusted to 7.2 $\pm$ 0.05 with NaOH, osmolarity to 240 mosmol/L. The perfusion procedure was the same as that outlined above for rat myocytes.

2.1.5. Immersion method for isolating sinus venosus cells from toad.

Isolation of pacemaker cells (e.g. from sino-atrial node of mammals or sinus venosus of amphibia) has proved more difficult, possibly because this region of the heart consists of small cells embedded in a dense, fibrous network of connective tissue (Denyer & Brown, 1990). The procedure which I used was modified from Giles & Shibata (1985).

After the toad was killed, the heart was removed and placed in a dissection dish. The heart was immersed in 2mM-Ca^{2+} Ringer solution and maintained its beating. The heart was fixed with pins under a dissection microscope (Nikon) to expose the sack-like sinus venosus structure. The sinus venosus was carefully dissected free from connected atrial tissue under the microscope. The dissection was judged to be successful when the isolated sinus venosus tissue remained spontaneously beating at approximately the normal heart rate, whilst the remaining heart stopped beating immediately after the sinus venosus tissue was excised. The intact sinus venosus tissue was placed in Ca^{2+} free Ringer solution for 5 min, then placed into 5 ml 25μM Ca^{2+} Ringer solution containing 300 units/ml collagenase (Worthington CSL II) and 40 units/ml elastase (Sigma, Type II_A). The enzyme solution with sinus venosus tissue was maintained at 29°C and gently stirred with a small magnetic bar (1.5 x 8 mm) at a low speed (about 50 r.p.m.) for 60 min. The preparation was washed in 25μM Ca^{2+} Ringer solution, and left at room temperature for 20 min, then triturated gently to dissociate the cells which were allowed to recover for 2 hours in ascending concentrations of Ca^{2+} Ringer solution up to 1mM.

I found with higher enzyme concentrations or longer exposures, cells had normal morphology but no sodium currents could be recorded. This method was therefore modified from that of Giles & Shibata by reducing the duration of enzyme treatment.

2.2. Patch clamp technique

2.2.1. The development of patch clamp technique

For technical reasons, classical electrophysiology of cell membranes was based on the study of the squid axon or giant snail neurons, simply because mammalian cells are much smaller and more difficult to handle (Sakmann & Neher, 1984). The technical challenge of measuring the small current which flows across a single open ion channel led Sakmann & Neher to develop patch clamp techniques in 1976. Using a pipette with a tip diameter of 1 micron and a very sensitive recording system, they found that they could measure the current flow through the single transmembrane channel which was activated by acetylcholine. However, one limitation was the leakiness of the seal between the pipette and the cell surface, which created "noise" that obscured very small currents. The shunt conductance between the pipette interior and the bath was the dominating noise source. In 1980, Neher found by chance that he could produce an electrical seal 100 times better than normal and make the noise level drop to practically zero by using freshly made and fire-polished pipettes and by applying a slight suction. Thereby, a small patch of membrane is sucked into the pipette interior. The electrical resistance across the seal is then in the order of 10-100 gigaohms, and is known as a "gigaseal". The high seal resistance can be exploited to perform high-resolution current measurements and to apply voltage across the membrane. The gigaseal is physically strong enough to allow certain mechanical manipulations to be performed, such as the tearing off patches of membrane from the cell, that remain attached to the recording pipette (Hamill *et al.*1981).

Patch clamp techniques are well suited not only to the study of single-channel currents from a small patch of cell membrane but also to the

study of the electrical activity of small cells. In the past 15 years, different single channel activities and whole cell currents have been described by use of the patch clamp technique. This technique has revolutionized neuroscience and cell biology and Neher and Sakmann won a Noble prize in physiology and medicine for this contribution in 1991.

2.2.2. Four configurations of Patch clamp

Cell-attached configuration

This is the initial configuration of the patch clamp. As soon as the pipette is sealed to the cell membrane and a gigaseal established, one can usually record single channel activity. The cell-attached configuration is thus the easiest to obtain. Current of both voltage and transmitter activated channels can normally be recorded by either varying the membrane voltage, or using pipettes filled with solutions containing the transmitter. The cell-attached configuration has one prominent feature: when seal resistance reaches 50-200 GΩ, the distance between glass and membrane surfaces has been estimated to be no larger than 2-5 Å (Conti & Neher, 1980), so that even small extracellular molecules are unable to diffuse through into a patched membrane area. This unique feature of the cell-attached configuration is well suited to experiments which are designed to test the involvement of second messengers in channel modulation (Franciolini, 1986). However, in this configuration, it is difficult to measure the resting membrane potential in the cell. This problem havs been overcome by using high concentration potassium (140mM) in the external solution so that the the cell membrane potential becomes eventually zero.

Inside-out configuration

As soon as a cell-attached patch is formed, further manipulation is possible because of the high mechanical stability of the glass membrane seal. The so called inside-out configuration is achieved by quickly withdrawing the pipette from the cell, which tears off the patch of membrane, while the glass-membrane seal is maintained. The cytoplasmic (inside) face of the membrane will then be exposed to the bath solution. Once the cytoplasmic side of the membrane is exposed to the bath solution, it can be easily and repeatedly exposed to a variety of solutions.

One problem in my experiments was that myocytes did not easily attach to the glass coverslips which were used for patch clamp experiments. Therefore, it was difficult, sometimes, to tear off membrane from the cell. In order to improve the attachment of cells, ploy-$_L$-lysine hydrobromide (Sigma) was used to coat all glass coverslips before plating myocytes on them.

Whole-cell configuration

Whole-cell configuration is achieved by the rupture of the patch in cell-attached configuration. The whole-cell configuration provides a low resistance pathway between the pipette solution and the cell interior. This feature is used to record macroscopic currents from the entire cell membrane. In cardiac myocytes, I found it was difficult to obtain a whole-cell configuration after tight seal formation, since the membrane of myocytes seemed to reseal easily or to be difficult to break after a tight seal.

Outside-out configuration

The outside out configuration results from pulling off a patch of membrane after the whole-cell configuration has been formed. It yields a patch with its external side in contact with the bath solution. Although the

outside-out configuration is a valuable experimental tool, it appears to be a more delicate and less stable structure. Formation of an outside-out patch may also involve major structural rearrangements of the membrane, since it appears to alter channel kinetics (Sakmann & Neher, 1983).

2.2.3. Pipettes and pipette fabrication

The patch pipettes for the whole-cell configuration recording were made from thin wall borosilicate glass (Modulohm I/S, Micro-haematocrit tubes). The pipettes were pulled in two stages using a vertical microelectrode puller (List-Medical). The temperature for the second stage pull was set and adjusted to obtain a pipette tip opening as large as possible. It is necessary to obtain a low resistance pathway between the pipette solution and myocytes which have relative large sodium currents in order to reduce series resistance. The pipette resistance was typically 1.5-2 MΩ. Polishing of the glass wall at the pipette tip was done with a heated filament under a microscope and observed at x600 magnification. The tip of pipette was brought to within 10-20 μm of heated filament for about 10 seconds.

The patch pipettes for single channel recording were made from thick wall borosilicate glass (Clark electromedical instruments, GC150F-15). The resistance of pipettes was 7-15 MΩ. Before the pipettes were polished, in order to reduce the pipette-bath capacitance, pipette shanks were coated with Sylgard (184 silicone elastomer) to within 50μm from the tip. Care was taken to ensure that the actual tip remained uncoated. Sylgard coating was done under a microscope at x40 magnification, with the pipette mounted in an auto-turning manipulator (made by JCSMR workshop) and cured by bringing the turning pipette close to a heated filament. In this way, an even Sylgard coating could be achieved.

2.2.4. Formation of a tight seal

The pipette was filled with internal solution (depending on the requirements of the experiment) and all air bubbles removed. The pipette was mounted in an electrode holder and connected via a silver chlorided wire to the headstage (CV4 0.1/100, Axon Instruments) of the patch clamp amplifier (Axopatch-1D). Junction potentials were nulled after electrode was lowed into the bath solution. The micro-electrode was moved near the cell using a very stable micromanipulator (JCSMR workshop). For cell-attached and inside-out configurations, gigaseal formation was obtained by pressing the pipette tip gently against the cell membrane, then applying suction. In order to improve the probability of obtaining gigaseal, the solutions in the bath as well as in the pipette were filtered with filter (0.2 μm, Acrodisc). Sometimes, flowing of bath solution could help to form gigaseal. The photomicrogragh on page 46 showns the glass pipette and a myocyte isolated from rat ventricle during tight seal forming.

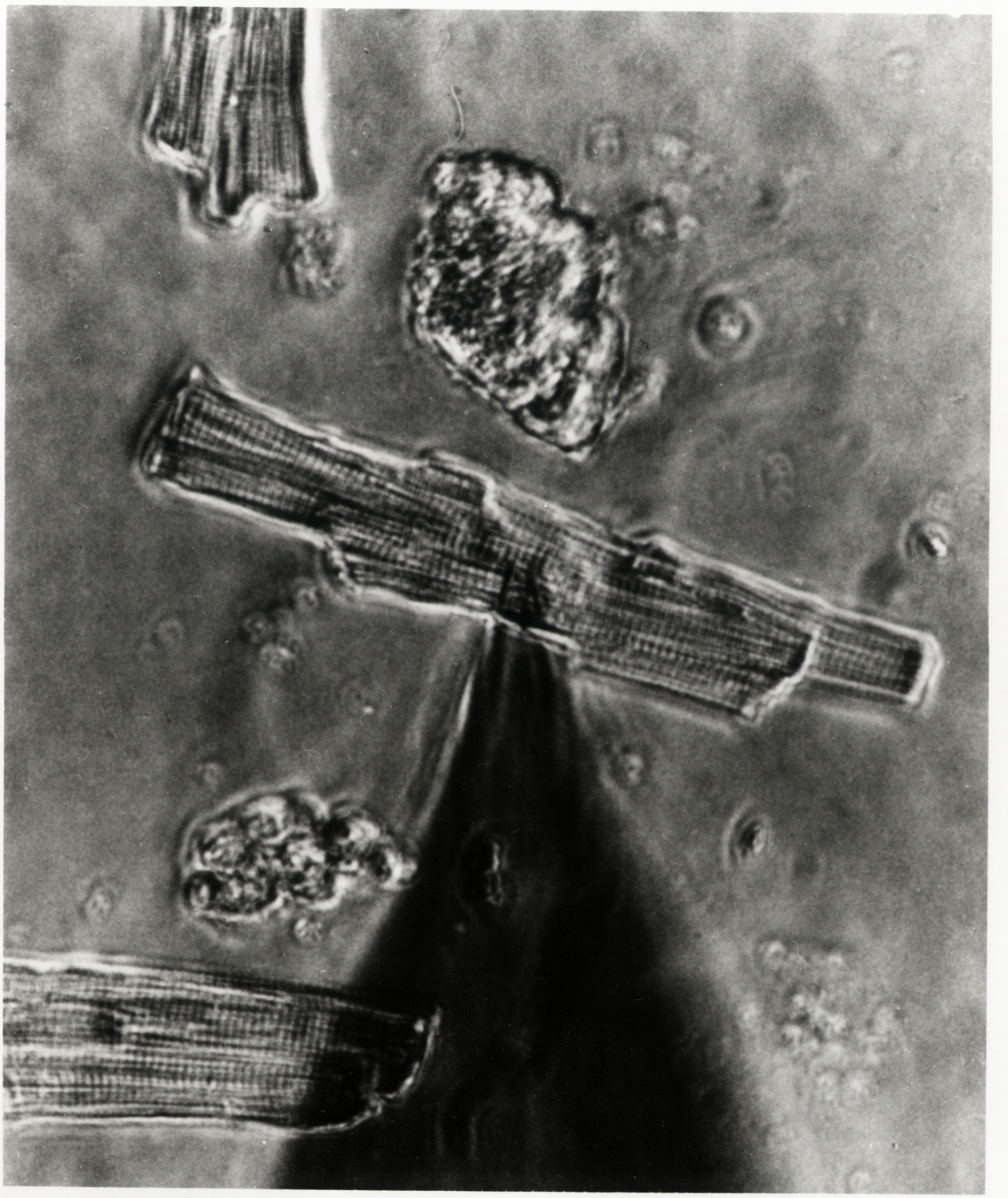
2.2.5. The recording of currents

The currents were recorded with an Axopatch-1D amplifier. The headstage of the amplifier (CV-4 0.1/100) has 50MΩ (ß= 0.1) and 50 GΩ (ß= 100) feedback resistors. A headstage gain (ß) of 100 was chosen for single-channel recording, while a headstage gain of 0.1 was chosen for whole-cell recording. When ß is 0.1, the amplifier can pass up to 200nA current. making it suitable for recording larger sodium currents from cardiac myocytes. Voltage commands were generated by a computer (Osborne 386-SX) which was also used to record currents digitized at 10kHz with a 12-bit A/D converter. Capacitative transients and leakage currents were compensated with the controls on the amplifier. In whole-cell configuration, series resistance compensation was done by careful adjustment of the controls on the amplifier. Currents were normally filtered

at 2 or 5 kHz. Final compensation for residual linear leakage and capacitive current was done digitally at the time of analysis by appropriately scaling and subtracting the current generated by a 20mV hyperpolarizing step immediately preceding the test voltage step.

2.2.6. Data analysis

An IBM-compatible computer (Osborne 386-SX) and a software program "canalyse" written by Colin Mc Culloch were used for data analysis. The recordings of whole-cell current were displayed and the baseline of the current was adjusted with a baseline value subtraction. The selective parts or points of the traces were saved as ASCII data file. In single channels recording, the capacitance was subtracted from original data by using the ensemble trace which was obtained by averaging traces which contained no visible openings. The single channel open time and amplitude were measured using the "channel" program written by M. Smith. In order to generate the amplitude histogram, the data was digitally filtered with a running bin average filter. Gaussian distributions were then fitted using the program "peakfit" (Jandel Scientific).



Chapter 3:

A PERSISTENT SODIUM CURRENT IN CARDIAC MYOCYTES

3.1. Introduction

It has been known for some time that TTX reduces the duration of the plateau phase of action potentials in heart muscle (Attwell *et al.*1979; Coraboeuf, Deroubaix & Coulombe, 1979; Callewaert, Carmeliet & Vereecke, 1984). This implies that there is a TTX-sensitive current, presumably carried by sodium, which contributes to the action potential plateau. Direct observations of sodium currents in voltage-clamped cardiac cells have revealed slow components of inactivation (Reuter, 1968; Dudel & Rudel, 1970; Brown, Lee & Powell, 1981b; Gintant, Datyner & Cohen, 1984).

It has also been suggested that a non-inactivating component of the sodium current could be due to a "window" current caused by overlap of the steady-state inactivation and activation curves (Attwell *et al.*1979) *i.e.* some sodium channels may activate at potentials where inactivation is not complete (see chapter 1). It would be predicted by this hypothesis that a persistent sodium current would not occur at very positive potentials where inactivation is complete.

It has generally been believed that both the rapid and slow phases of the decay of sodium currents can be attributed to the one kind of sodium channel. The slowly decaying current would be caused by some of the channels opening repeatedly or opening after a long latency. If so, it would

be expected that the voltage-dependence of activation, and sensitivity to TTX, of the different components of sodium current should be the same.

More recently, it has been observed that TTX causes a fall in the rate of rise of pacemaker action potentials, as well as causing a fall in heart rate (Bywater *et al.*1989; Edwards, Hirst & Bramich, 1993). This would be consistent with the existence of sodium currents in pacemaker cells which contribute both to action potential upstroke and diastolic depolarization.

A very slowly inactivating, TTX-sensitive sodium current in cardiac myocytes isolated from ventricles is described in this chapter. This current is not a window current since it can be recorded over a wide range of membrane potentials. It is more sensitive to block by TTX, is activated at more negative potentials, and is more resistant to inactivation than the transient current. These results suggest that the transient and persistent sodium currents in these cells may be generated by different kinds of sodium channel. In addition, a persistent sodium current has been recorded in cells isolated from rat atrial myocytes and the pacemaker region of the toad heart, which appears to contribute to pacemaking activity in these cells.

3.2. The persistent current : a slowly inactivating TTX-sensitive current recorded from rat ventricular myocytes

Ventricular myocytes were enzymatically isolated from rat heart using the Langendorff perfusion method (see chapter 2). Myocytes were internally perfused and voltage-clamped using the whole-cell patch clamp technique. The standard pipette solution contained (mM): CsF 50; NaF 70; KEGTA 20; $CaCl_2$ 2; TES 10; ATP 10, pH adjusted to 7.4 $\pm$ 0.05 with KOH. The "control" bath solution contained (mM): NaCl 130; KCl 5.4; $MgCl_2$ 1; $CaCl_2$ 2; $CoCl_2$ 5; CsCl 5; TES 10; NaOH 5; glucose 10, pH adjusted to 7.4 $\pm$ 0.05 with NaOH.

Tetrodotoxin-sensitive currents were usually blocked by using a TTX concentration of 50 μM. As the persistent current is very small and difficult to detect in the presence of other currents, records obtained in the presence of TTX were subtracted from those obtained in the same cell before exposure to TTX. In order to reduce the possibility of time-dependent changes in other currents, TTX was normally washed out and the currents recorded compared with those obtained before exposure to TTX: if any time-dependent changes were detected, the results were not used.

The current recorded from a ventricular myocyte in response to a 140 ms voltage step to -40 mV from a holding potential of -130 mV is illustrated in Fig. 3-1A (control). An early, rapidly decaying (transient) inward current is followed by a much smaller, persistent, inward current which can be seen more clearly at higher current gain (inset). In the presence of TTX (50 μM) the transient current was almost entirely blocked and the persistent inward current was converted to an outward current (upper trace, TTX). By subtracting the trace recorded in the presence of TTX from the control trace, TTX-sensitive currents could be isolated. The trace in Fig. 3-1B was obtained in this way from the currents in Fig. 3-1A. A transient current with a peak amplitude of -53 nA is followed by a persistent inward current that has an amplitude of about -300 pA at the end of the test pulse. The amplitude of the persistent current measured in 13 cells in this way ranged from -107 to -879 pA (mean -427 pA, 1 s.e.m. 58 A). When expressed as a percentage of the amplitude of the transient current, the amplitude of the persistent current was, on average, 0.57% of the amplitude of the transient current (1 s.e.m. 0.05). The effect of TTX on both transient and persistent currents was fully reversible and could be reproduced several times in the same cell.

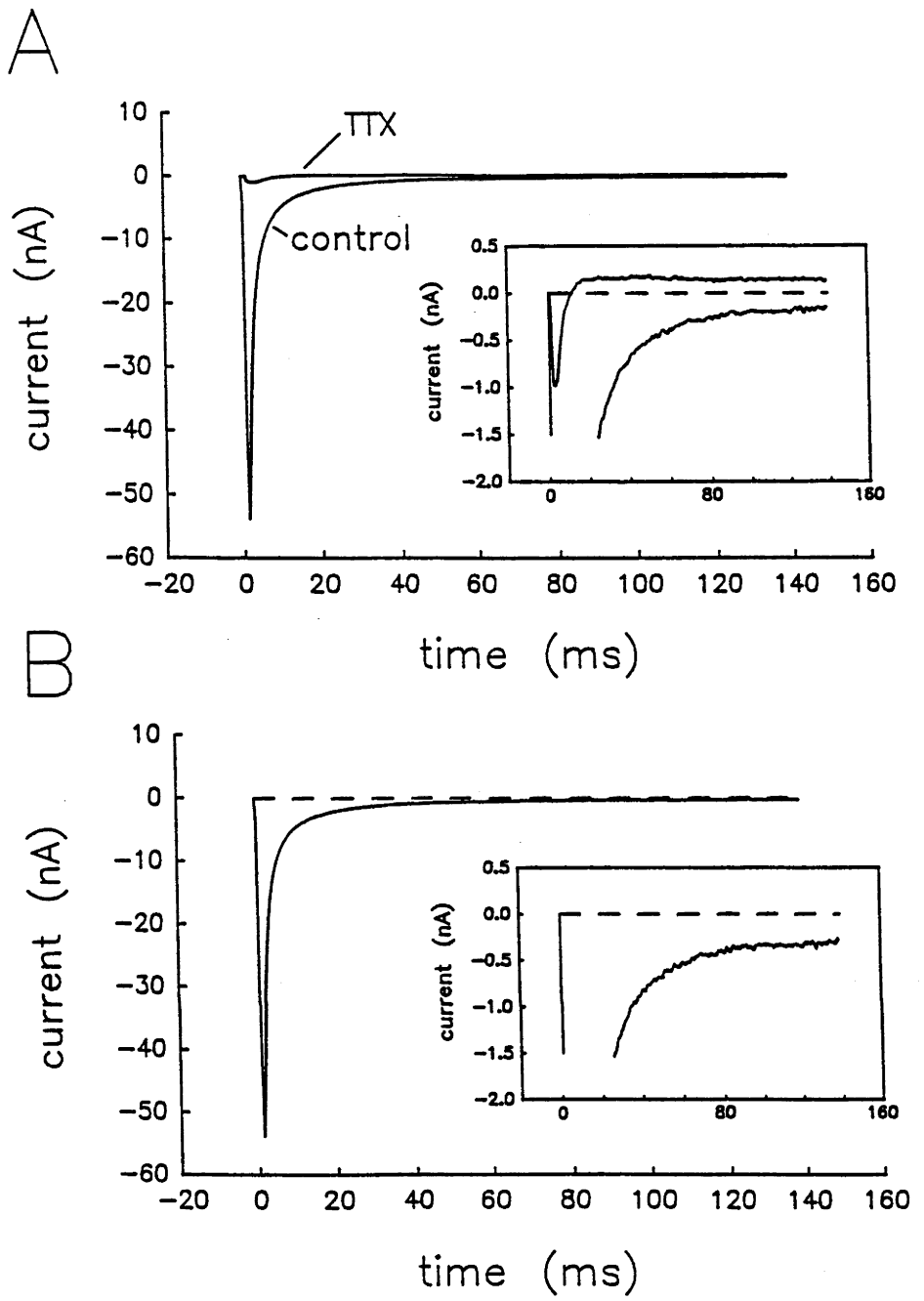


Fig. 3-1. TTX-sensitive currents recorded from the rat ventricular myocyte. **A**: Currents generated by a 150 ms voltage step to -50 mV from a holding potential of -100 mV. The trace showing the prominent transient current was recorded in control solution. The other trace was recorded during exposure of the cell to TTX (50 μM). The same traces are shown inset at higher vertical magnification. **B**: TTX-sensitive current obtained by subtraction of the two traces in A. The inset shows the current at higher vertical magnification.

3.3. The persistent current is a sodium current

Despite an attempt to block all ionic currents except sodium currents, it was still possible that the persistent current could have been due to effects of TTX on channels other than sodium channels. The characteristics of the persistent current were therefore examined further in rat ventricular myocytes. The currents were generated in an isolated myocyte by a voltage step to -40 mV from a prepulse potential of -150 mV (holding potential -100 mV). In the standard extracellular solution, the rapidly decaying transient inward current (peak amplitude -25.3 nA, truncated in Fig. 3-2) was followed by a slowly inactivating persistent current which had an amplitude of about -100 pA after 150 ms. Application of 50 μM TTX blocked the persistent current completely while causing an incomplete block of the transient current (residual peak amplitude -2.8 nA). The effects of TTX were readily reversible, as shown by the trace labelled "wash". That this persistent current blocked by TTX is carried by sodium ions was confirmed by replacing the extracellular sodium ions with choline. This subsititution completely removed both transient and persistent sodium currents (Fig. 3-2).

Further evidence that the persistent current was a voltage-dependent sodium current came from two other kinds of experiment. First, the relationship between the amplitude of the persistent current and membrane potential is shown as Fig. 3-3A. The persistent current was measured at the end of a 150 ms voltage pulse, before (filled circles) and during (open circles) exposure of a myocyte to 50 μM TTX, TTX had no effect on the persistent current at potentials close to +10 mV (Fig. 3-3A). With the solutions used, only sodium ions had an equilibrium potential close to +10 mV (calculated Na equilibrium potential +17 mV).

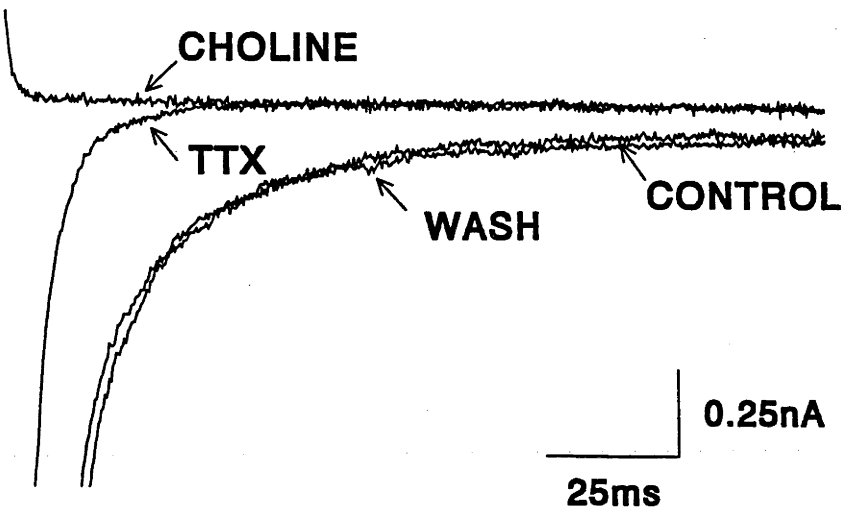


Fig. 3-2. Block of the persistent inward current by TTX (50 μM) and by substitution of choline for sodium in the bath solution. The four current traces were elicited in an isolated myocyte by voltage steps to -40 mV from a pre-pulse potential of -150 mV in the normal bath solution (CONTROL), in bath solution containing 50 μM TTX (TTX), after washing out the TTX with normal bath solution (WASH) and in bath solution containing choline instead of sodium (CHOLINE).

The small discrepancy between the observed null potential (the potential at which the TTX-sensitive current was zero) and the calculated sodium equilibrium potential could have been partly due to the junction potential between the bath and pipette solutions (9 mV, calculated by JPCalc program witten by P.H.Barry) that is initially corrected for but disappears in the whole-cell configuration and partly due to some permeability of the channels to ions other than sodium. No attempt was made to look at this more closely because the result as it stands provides strong evidence that the current is a "sodium" current.

In the second kind of experiment, choline was substituted for extracellular sodium ions and caused changes in the amplitude of both transient and persistent currents. The amplitude of the persistent current measured at the end of a 150 ms test pulse in a cell in control solution is plotted against test potential in Fig. 3-3B (filled circles). Following replacement of the extracellular solution with a solution containing choline instead of sodium (filled triangles), there was a significant change in the current-voltage relationship. The persistent inward current was converted to an outward current at potentials more positive than -60 mV.

Since the persistent inward current was blocked by TTX, had a null potential close to the sodium equilibrium potential and was sensitive to extracellular sodium concentration, it was concluded that the current was a sodium current.

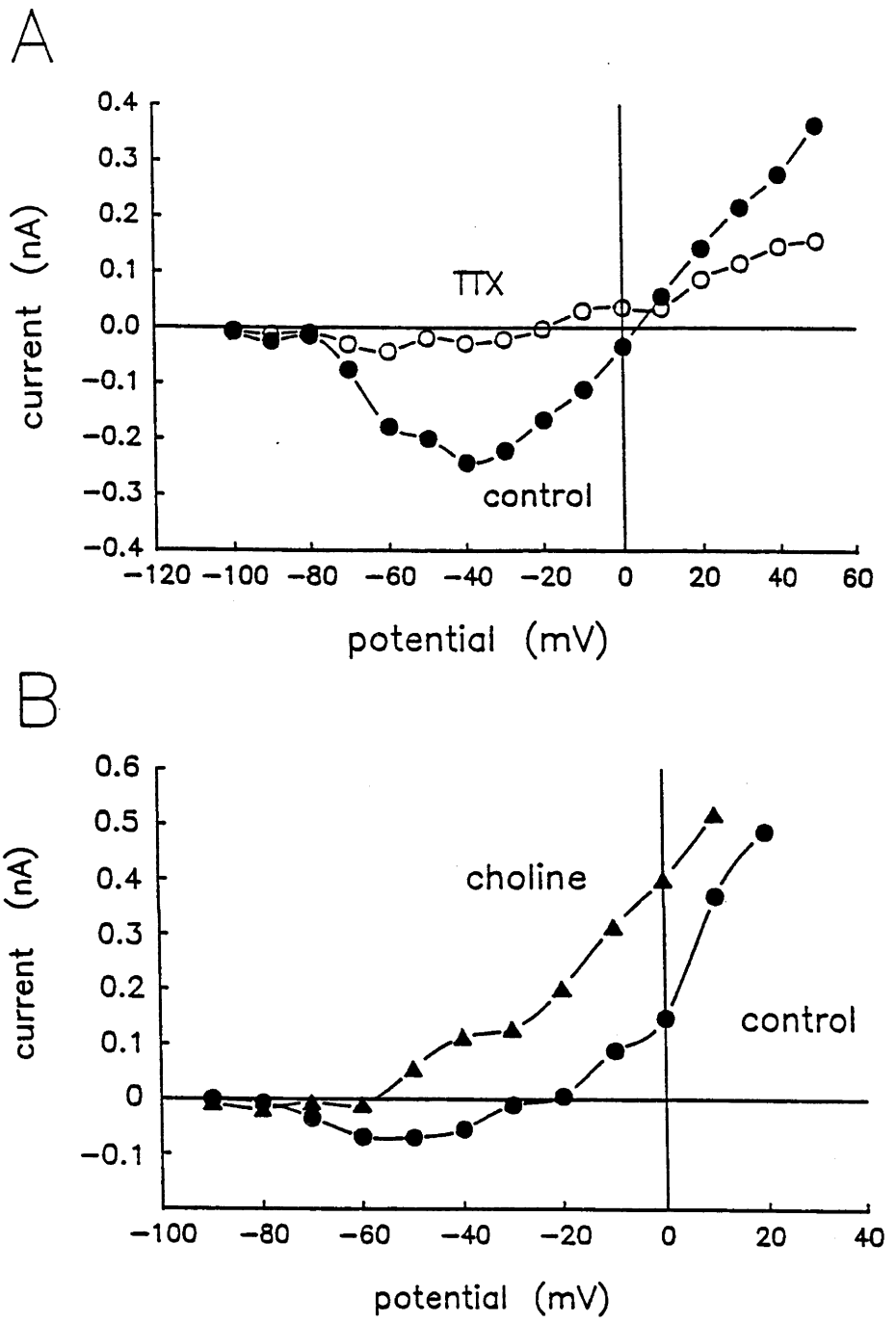


Fig. 3-3. Effects of TTX and choline substitution. Persistent currents were measured at the end of 150 ms test voltage steps (holding potential, -100 mV). The amplitude of the persistent current is plotted against membrane potential. **A**: Currents were recorded in control solution (filled circles), then in a solution containing TTX (50 μM, open circles). **B**: Currents were recorded in control solution (filled circles), then in a solution containing choline chloride in place of sodium chloride (filled triangles).

3.4. The persistent current is not a window current

It has been suggested that overlap of the voltage-dependence of activation and inactivation of sodium channels in cardiac muscle may generate a persistent "window" sodium current (Attwell *et al.*1979). A persistent non-inactivating sodium current would then occur at potentials where some sodium channels are activated but not all are completely inactivated. If the current described here were such a window current, it should not be seen at very positive potentials, since the inactivation of the transient current would then be complete. However, it was possible to record persistent sodium currents generated by voltage steps to very positive potentials, as illustrated in Fig. 3-4. TTX-sensitive currents generated by a voltage step to +40 mV following prepulses to -130 and -50 mV (Fig. 3-4A) are shown in Fig. 3-4B. Note that sodium currents are outward at this potential with the solutions used. There was clearly a persistent sodium current at this positive potential. In addition, although the transient current was almost completely inactivated by the prepulse to -50 mV, the persistent current was essentially unaffected by the prepulse potential. Hence it appears that the persistent sodium current in mammalian ventricular muscle is not a "window" current.

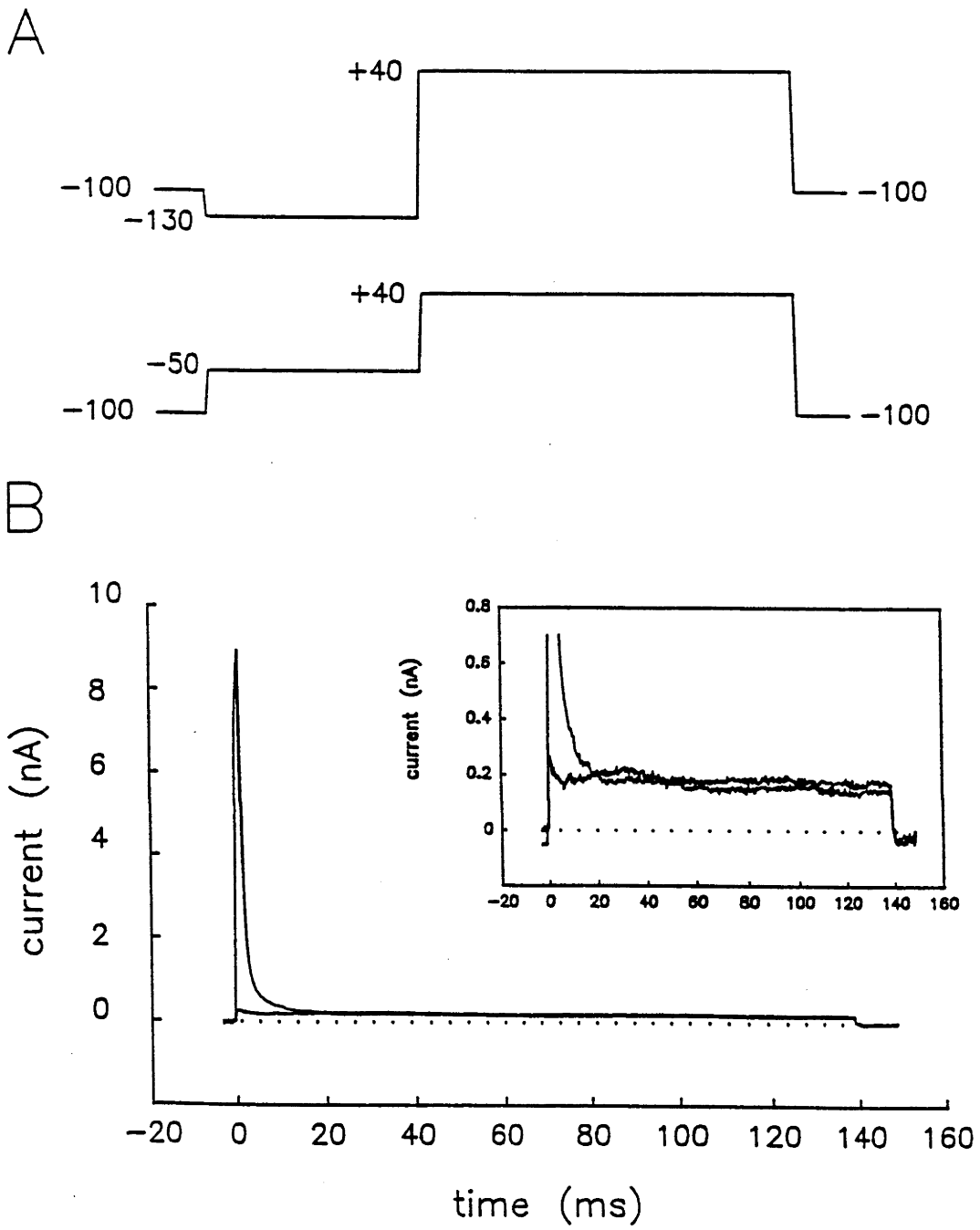


Fig. 3-4. The persistent current recorded at positive potentials. **A**: Illustration of the voltage protocols used to generate the currents shown in (B). **B**: Currents generated by a 140 ms voltage pulse to +40 mV following 80 ms prepulses to -130 mV (large transient current) and -50 mV (small transient current). Currents are shown inset at higher vertical magnification.

3.5. Comparison of the transient and the persistent current

3.5.1. The persistent current activates at more negative potentials

The persistent current appears not to be a window current (Fig. 3-4) and hence may be due to channels different from those which give rise to the transient current. To investigate this possiblity, the amplitude of both transient and persistent sodium currents was measured over a range of test pulse voltages in several myocytes. Typical results obtained in one of these experiments are illustrated in Fig. 3-5.

It can be seen in the current-voltage curves for the transient (Fig. 3-5A) and persistent (Fig. 3-5C) currents that the persistent current was activated at more negative potentials than the transient current. Conductance was calculated from current amplitudes using the equation

$$g = I/(V-E_0)$$

where g is conductance, I is the peak amplitude of the transient current or final amplitude of the persistent current, V is test potential and E_0 the null (zero current) potential. Conductance-voltage curves for the transient and persistent currents are shown in Fig. 3-5, B & D respectively.

There is clearly a significant difference in the voltage-dependence of the two conductance-voltage relationships. The lines through the curves are best fits to the data points (by eye) of the Boltzmann equation

$$g = g_{max}/(1 + \exp((V'-V)/k))$$

where g is conductance, g_{max} the maximum conductance, V' the test potential at which $g = g_{max}/2$, V is test potential and k is a slope factor. V' for the transient current was -34 mV in contrast to a V' of -52 mV for the persistent current.

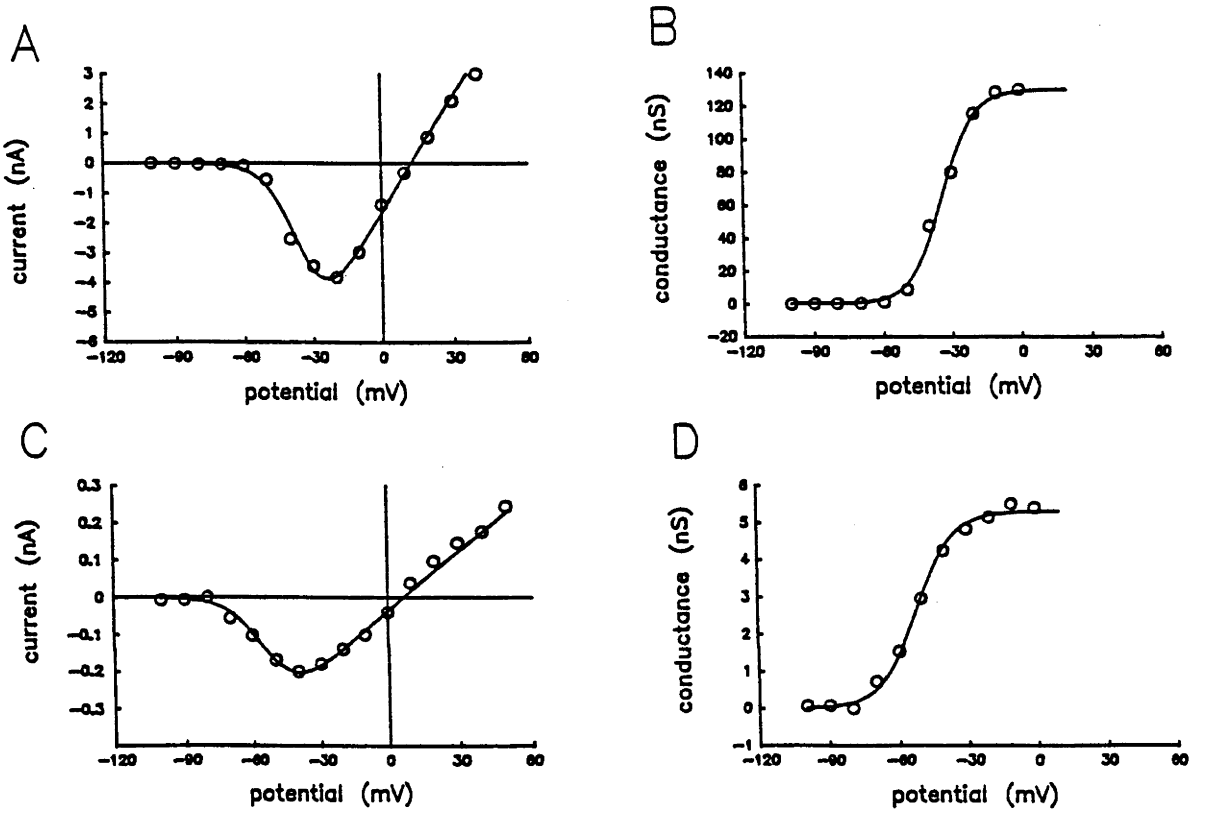


Fig. 3-5. Voltage-dependence of activation of persistent and transient currents. Current-voltage relationships for transient (**A**) and persistent (**C**) TTX-sensitive currents generated by 150 ms voltage pulses from a holding potential of -130 mV. The two sets of currents in **A** and **C** were recorded in the same cell. The amplitude of the transient current was reduced with TTX to reduce series resistance errors. Conductance was calculated from current amplitude as described in the text and plotted against pulse potential for transient (**B**) and persistent (**D**) currents. The solid lines through data points are best fits (by eye) of the Boltzmann equation described in the text. The values for V' and k were -34 and 7 mV in **B**, -52 and 8 mV in **D**.

Normalised conductance-voltage curves obtained from the transient (filled triangles) and persistent (filled circles) currents are shown superimposed in Fig. 3-6 to illustrate the difference in voltage-dependence of the two kinds of conductance.

The current-voltage relationship for the comparatively large transient current (up to 100 nA) obtained in experiments such as these is very susceptible to distortion due to the effects of series resistance (see, for example, Jack, Noble & Tsien, 1975). This problem can be largely circumvented by reducing the size of the currents, either by increasing the steady state inactivation or by pharmacological means. For the data illustrated in Fig. 3-5 and Fig. 3-6, currents were first recorded in control solution then in a solution containing sufficient TTX to reduce the amplitude of the transient current to about 5% of its normal size, then in a solution containing 50 μM TTX. The amplitude of the transient current, shown in Fig. 3-5A, was obtained by subtraction of records obtained with the low concentration of TTX and with 50 μM TTX. The residual errors due to inadequate series resistance compensation in Fig. 3-5 and Fig.3-6 would have been very small and, if corrected entirely, would give a shift to the right of no more than 3 to 4 mV in the conductance-voltage relationship. This would increase the difference in the conductance-voltage relationship for persistent and transient currents.

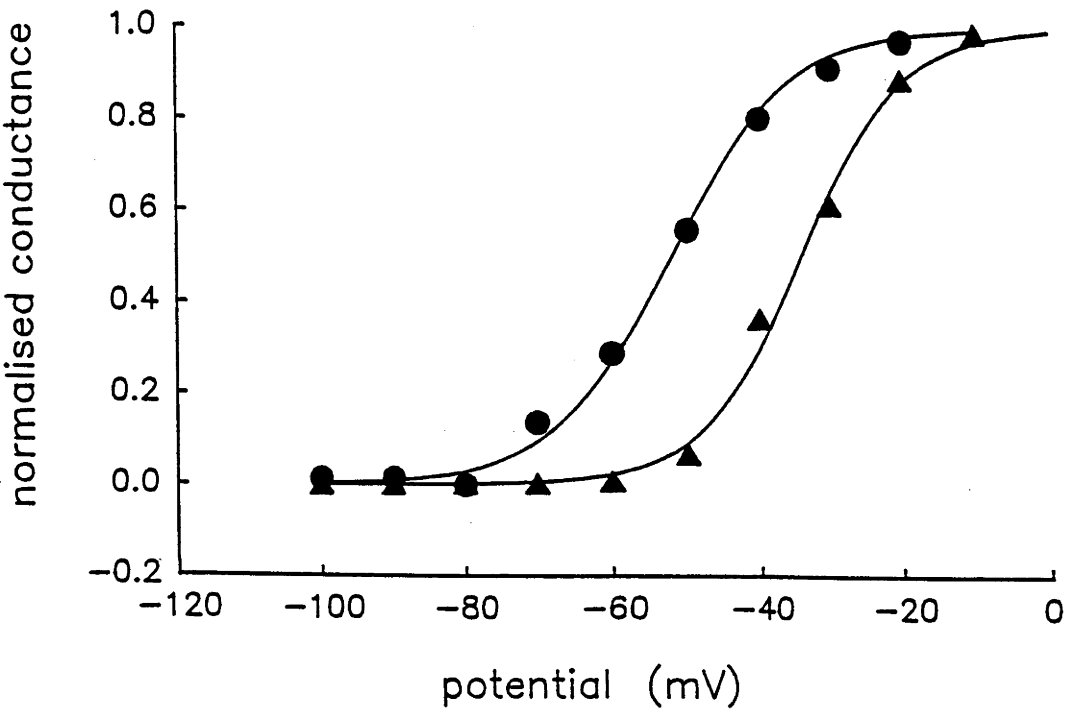


Fig. 3-6. Comparison of normalised conductance-voltage curves for the same transient (filled triangles) and persistent (filled circles) TTX-sensitive currents as in Fig. 3-5.

3.5.2. The persistent current is resistant to inactivation

The fact that the persistent current could be recorded at the end of long depolarizing pulses (Fig. 3-1) indicates that the current is rather resistant to inactivation. The relative resistance to inactivation of the transient and persistent sodium currents was explored in experiments of the kind illustrated in Fig. 3-7.

The voltage protocol used is shown in Fig. 3.7A. An 80 ms prepulse to a conditioning potential of -130 or -50 mV from a holding potential of -100 mV was followed by a test pulse to -40 mV. Currents produced following these prepulses are shown in Fig. 3-7B and at higher current gain in Fig. 3-7C. It can be seen that the prepulse to -50 mV caused almost complete inactivation of the transient current but the persistent current at the end of the test pulse was unaffected. The relative effect of a range of conditioning prepulses on the transient and persistent sodium currents is illustrated in Fig. 3-7D. The persistent current (filled circles) is clearly highly resistant to inactivation by the prepulse at all conditioning potentials tested. It is also clear that the persistent current does not remain a constant fraction of the transient current as the latter becomes progressively more inactivated at increasingly positive conditioning potentials. This makes it unlikely that the persistent current is generated by a small, fixed fraction of the channels that generate the transient current.

Although the graph in Fig. 3-7 shows little voltage-dependent inactivation of the persistent sodium current, some time-dependent inactivation was detected with longer voltage pulses. The slow decay of a persistent sodium current recorded during a 900 ms pulse to -40 mV from a holding potential of -100 mV is shown in Fig. 3-8.

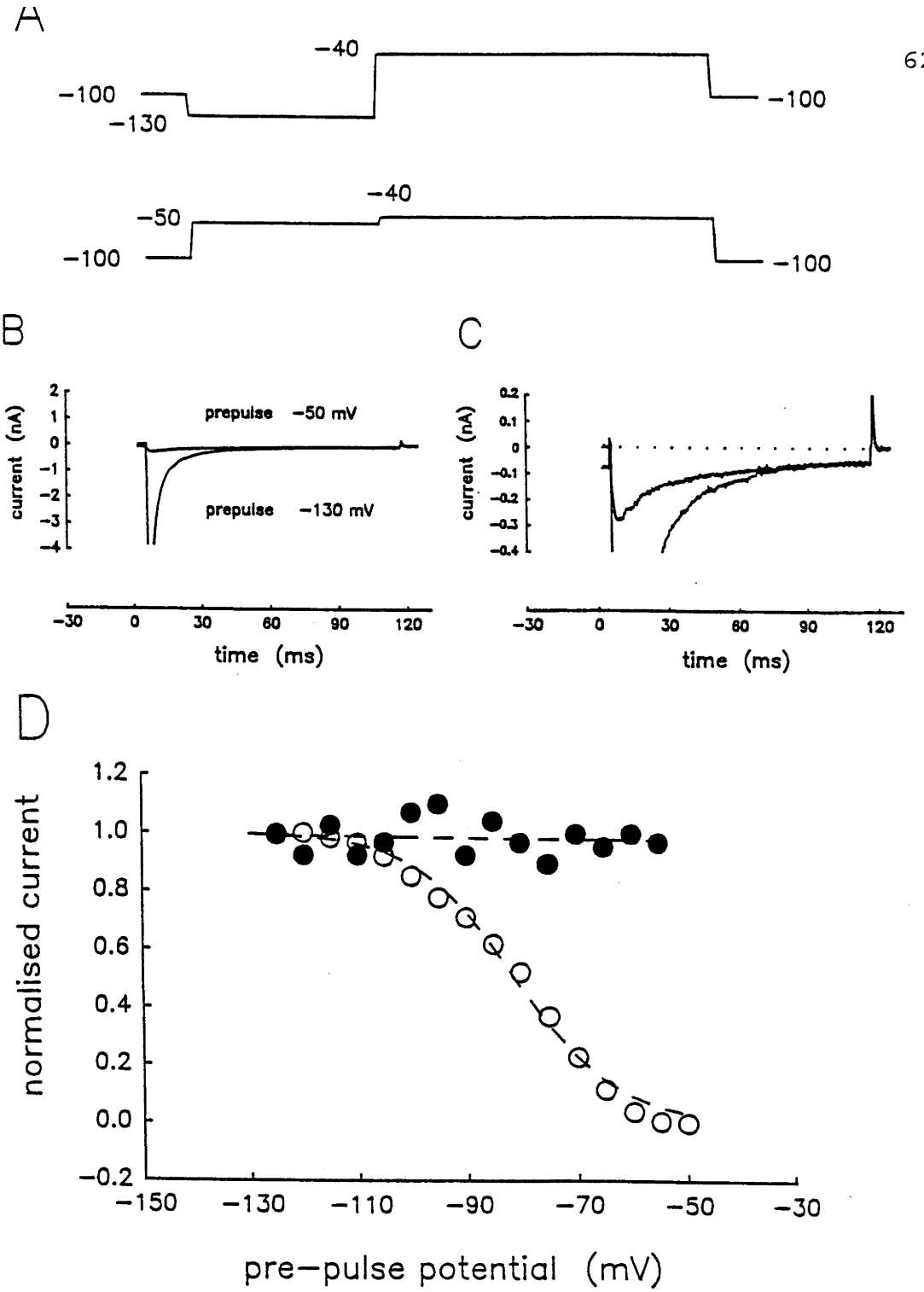


Fig. 3-7. Differential voltage-dependent inactivation of transient and persistent sodium currents. **A**: Illustration of the voltage pulse protocols used to obtain the currents in (B) and (C). **B**: Currents generated by a 120 ms voltage step to -40 mV following 80 ms prepulses to -130 (large transient current) or -50 mV (small transient current). The currents shown were obtained by subtraction of records in the presence and absence of 50 μM TTX. **C**: Traces in B at higher vertical magnification to show the persistent current more clearly. **D**: Normalised amplitude of the persistent (filled circles) and transient (open circles) currents generated by a 120 ms voltage steps to -40 mV following 80 ms pre-pulses to the potentials indicated on the abscissa.

There was a slow decline in the amplitude of the persistent current during the pulse. This can be seen more clearly in the amplified trace from 400 to 900 ms shown in the inset. The decay of this current, at times beyond 400 ms, could be well described by a single exponential with a time constant of about 1 second.

3.5.3. The persistent current is more sensitive to TTX than the transient current

In most experiments in which the persistent current was measured, a high concentration of TTX was used to ensure that the current was completely blocked. However, in seven cells exposed first to a lower concentration of TTX, the persistent current was found to be more sensitive to TTX than the transient current. Currents recorded in one of these cells in control solution and in solutions containing 0.1 μM and 50 μM TTX are shown in Fig. 3-9. The 0.1 μM TTX caused little change in the amplitude of the transient current whereas the persistent current was markedly reduced in amplitude. In the presence of 50 μM TTX, the transient current was almost completely blocked but there was no further reduction in the amplitude of the persistent current. The relative resistance of the transient current to block by 0.1 μM TTX was seen in all seven cells. A similar result also can be see in the current recorded from an atrial cell (Fig. 3-10A). In this experiment, the block of the persistent current reached a maximum with increasing time of exposure to TTX well before the transient, indicating that the persistent current is more sensitive to block by TTX.

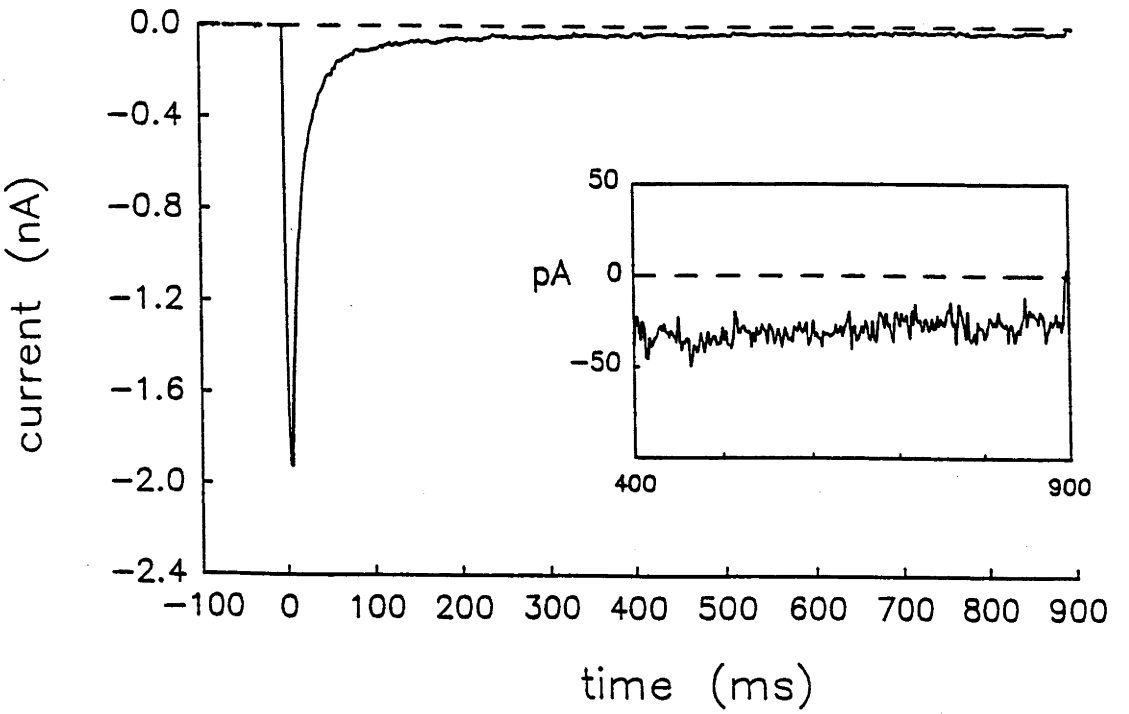


Fig. 3-8. Time-dependent inactivation of the persistent sodium current. The current shown is the TTX-sensitive current generated by a 900 ms voltage step to -40 mV from a holding potential of -100 mV. The transient current in this record has been truncated because of amplifier saturation. Inset is shown the current trace from 400 to 900 ms at higher vertical magnification to illustrate the decay of the persistent current.

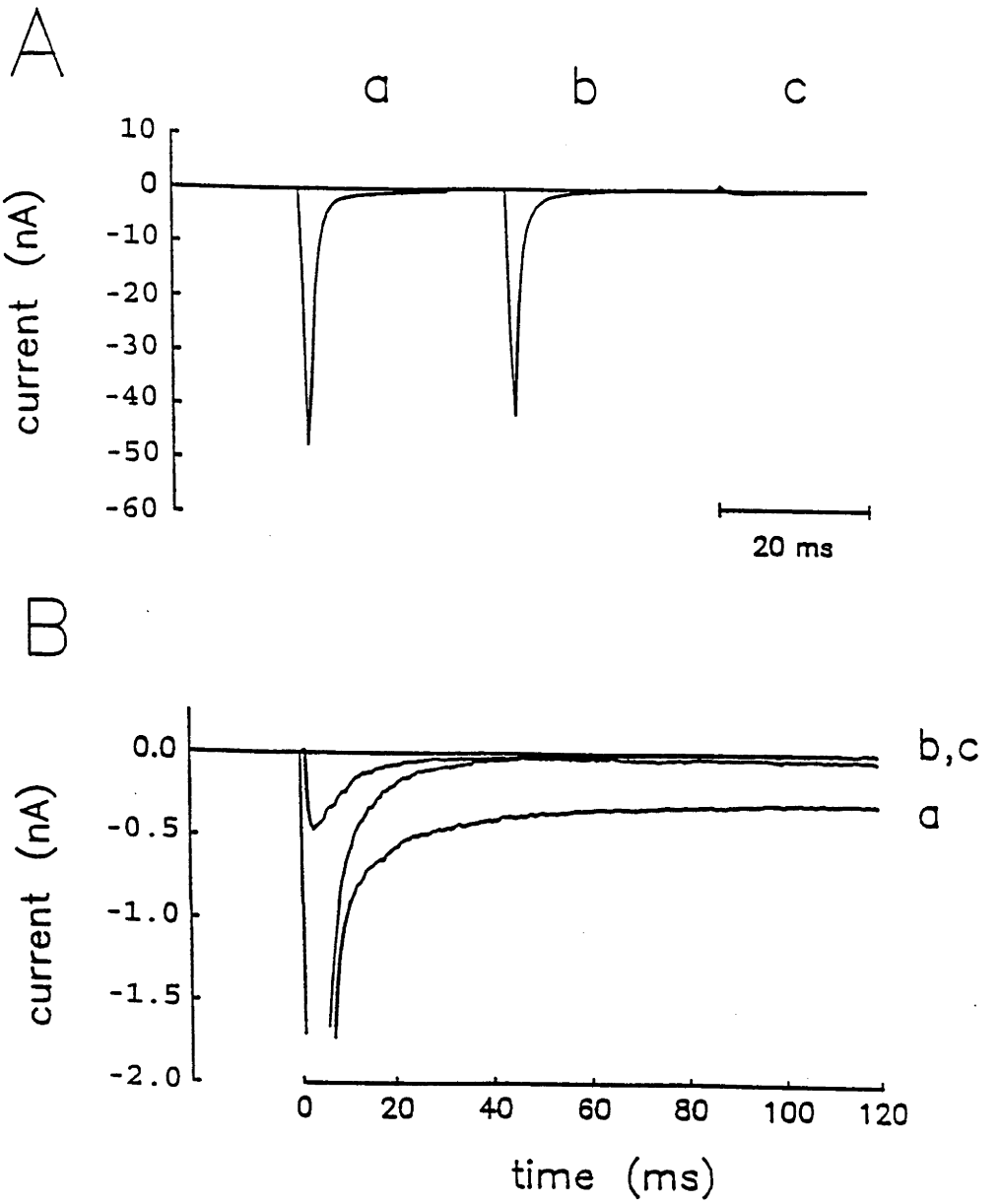


Fig. 3-9. Differential effect of a low concentration of TTX on the transient and persistent inward currents. Currents were recorded in control solution (a), in a solution containing 0.1 μM TTX (b) and in a solution containing 50 μM TTX (c). The currents are shown at low gain in **A** and superimposed at higher gain in **B**.

3.6. Persistent current recorded from rat atrial myocytes

A question arises from these ventricular experiments; does such a small and slow TTX-sensitive current exist in other parts of the heart? Mammalian atrial cells were first chosen to test this possibility. Atrial myocytes were enzymatically isolated from the rat heart using the Langendorff perfusion method and were voltage-clamped using the whole-cell configuration of the patch clamp technique, in the same way as cells from ventricular tissue. The current recorded from an atrial myocyte in response to a 140 ms voltage step to -50 mV from a holding potential of -150 mV is illustrated in Fig. 3-10A (control). After the application of TTX (50 μM), the late outward current gradually increased, which suggested that an inward current was being blocked. Fig. 3-10A (TTX) shows currents recorded after 3, 5, and 8 min TTX application, respectively. The trace in Fig. 3-10B was obtained by subtracting the current recorded in the presence of TTX (after 8 min) from the control current, to reveal a TTX sensitive persistent current. Its amplitude was -240 pA, which was about 0.5% of the amplitude of the transient current recorded in this cell. This result suggested that a persistent current similar to that recorded in ventricular myocytes could also be recorded in atrial myocytes.

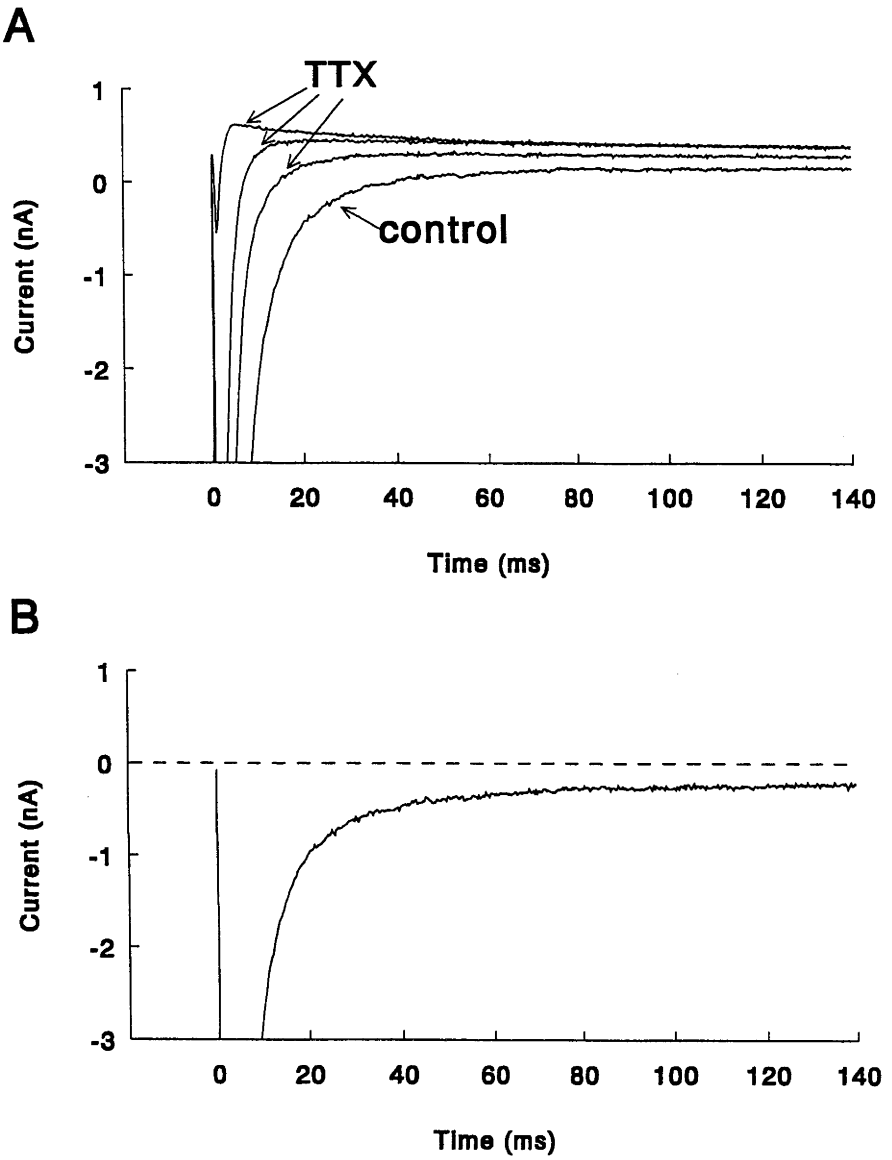


Fig. 3-10. TTX-sensitive currents recorded from a rat atrial myocyte. **A**: The currents generated by 150 ms voltage step to -50 mV from a holding potential of -150 mV is illustrated in control and at 3, 5, 8 min after TTX (50 μM) application, respectively. **B**: The trace was obtained by subtracting the current recorded after 8 min in TTX from the control current.

3.7. Persistent current recorded from toad sinus venosus

The rate of generation of pacemaker action potentials is reduced in intact sinus venosus preparations when TTX is added to the extracellular solution (Bywater *et al.*1989; Edwards, Hirst & Bramich, 1993). This observation suggested that a persistent sodium current may be present in pacemaker cells and contribute to pacemaking activity. This possiblity was investigated by recording currents in cells isolated from the sinus venosus region of the toad heart (see chapter 2). Toads were used for these experiments because the pacemaker region of amphibian heart is more easily isolated than is the pacemaker region of mammalian heart.

The intracellular (patch pipette) solution generally had the following composition (mM): CsF 40; CsCl 60; $MgCl_2$ 2; KEGTA 10; $CaCl_2$ 2; Na_2ATP 5; TES (N-tris(hydoxymethyl) methyl-2-aminoethane-sulphonic acid) 10; pH adjusted to 7.2 with KOH. The solution bathing the cells had the composition (mM): NaCl 100; KCl 2.5; CsCl 5; $CoCl_2$ 5; Hepes (N-2-hydoxyethylpiperazine-N'-2-ethanesulphonic acid) 10; $CaCl_2$ 2, $MgCl_2$ 1, pH adjusted to 7.2 with NaOH. These solutions were designed to block calcium and potassium currents, as well as I_f (see chapter 1). Recordings were made at room temperature (20 to 22°C).

In 6 of 11 pacemaker cells which were identified morphologically and electrophysiologically (see Chapter 6) examined in this way, there was an obvious TTX-sensitive persistent current. In each case, washing the TTX from the bath caused a restoration of the persistent current: it is thus unlikely that the persistent current arose from changes in other currents not related to exposure to TTX. Currents recorded in one of these experiments are shown in Fig. 3-11.

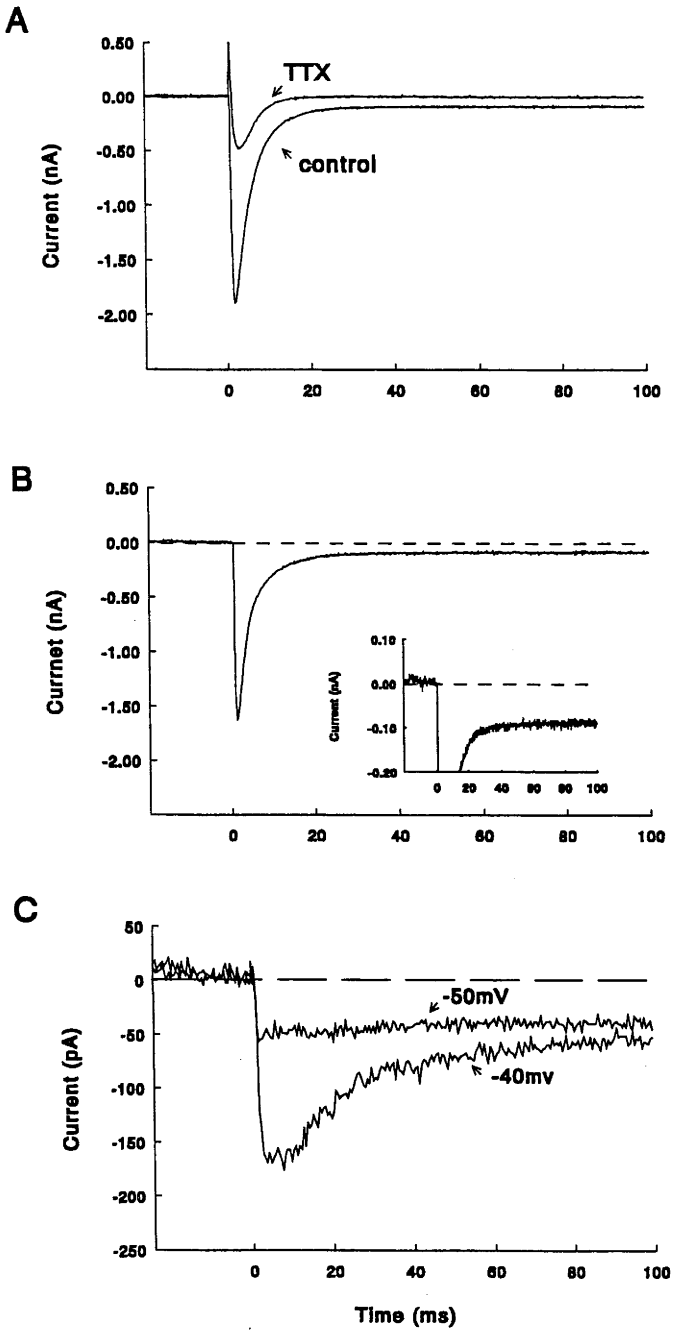


Fig. 3-11. TTX-sensitive persistent sodium current recorded from a toad sinus venosus cell. **A:** currents evoked by a voltage step to +20 mV from a pre-pulse potential of -130 mV. TTX (10^{-8} M) reduced the amplitude of the transient current by about 75% and also blocked a persistent inward current lasting more than 100 ms. **B:** the TTX-sensitive current obtained by subtraction of the current recorded in the presence of TTX from the control current. Inset is the same trace at higher current gain. **C:** TTX sensitive currents, obtained by subtraction, elicited in another cell by voltage steps to -50 and -40 mV from a prepulse potential of -130 mV.

The two traces in Fig. 3-11A show currents evoked by a voltage step from -120 mV to +20 mV, before and after exposure of the cell to TTX (10 nM) for 3 min. It is clear that the TTX depressed both the transient current (also see chapter 6) and a persistent current. Subtraction of the two currents shows the transient and persistent current blocked by TTX (Fig. 3-11B). The persistent current had an amplitude of about -100 pA (insert, Fig. 3-11B), about 4% of the size of the transient current initiated by the same voltage pulse. In the 6 cells in which a persistent current was detected in this way, the average amplitude at +20 mV was -58 $\pm$ 9.3 pA. With small depolarizing steps, e.g. to -50 mV, persistent inward currents could be seen in the absence of a transient current (Fig. 3-11C).

There is some overlap in activation and inactivation of the transient sodium current in sinus venosus cells within a range from -40 mV to 0 mV (see chapter 6, Fig. 6-6). Although a window current should not occur with a voltage step to +20 mV (Fig. 3-11), experiments were done to record the current generated by a step to +60 mV which should have produced rapid and complete inactivation of the transient current in sinus venosus cells. In order to obtain a measurable outward current at this potential, the sodium concentration in the pipette was increased to 90mM and the sodium concentration in the extracellular solution was reduced to 4mM. Examples of currents produced by depolarizing a cell to a potential of +60 mV are shown in Fig. 3-12A. In the upper panel, there are 3 currents, all generated by a voltage step to +60 mV but following prepulses to -130 mV (largest transient current), -40 mV (small transient current) and 0 mV (no transient current). It is clear from the insert in Fig. 3-12 that the conditioning prepulses which inactivated the transient current had little effect on the persistent current. In other words, the transient current shows voltage-dependent inactivation whereas the persistent current does not.

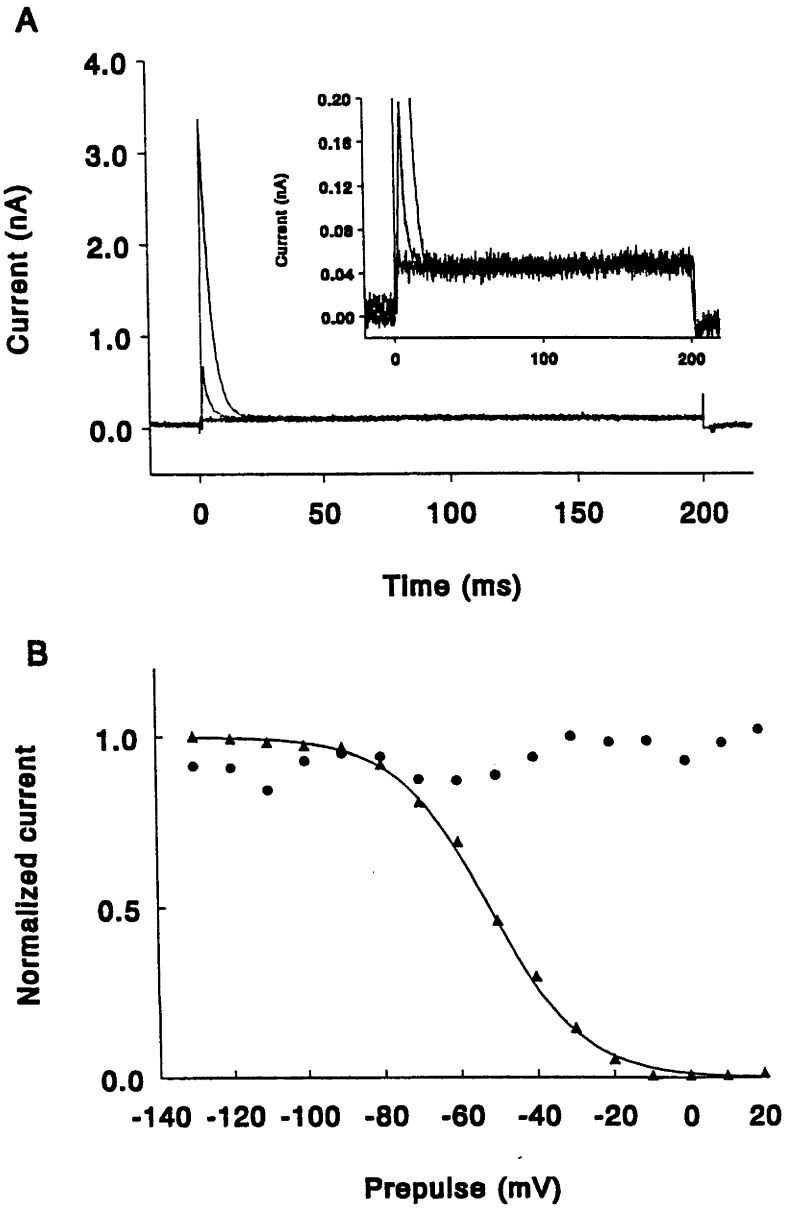


Fig. 3-12. The outward persistent current recorded in sinus venosus cell. **A:** currents were evoked by a voltage step to +60 mV following pre-pulse potentials of -130 mV, -40 mV and 0 mV, first in control solution and then in the presence of $2x10^{-7}$ M TTX. The intracellular and extracellular Na^+ concentrations were 90 and 4 mM, respectively, so that outward currents were generated at these potentials. The currents shown are the TTX-sensitive currents obtained by subtraction of currents recorded before and after exposure to the TTX. As the pre-pulse potential became more positive, the transient current became smaller but the persistent current was unaffected. **B:** Amplitudes (normalised to maximum amplitudes) of TTX-sensitive transient (triangles) and persistent currents (circles, measured 200 ms after the onset of depolarization) evoked by a voltage pulse to +60 mV are plotted against prepulse potential. The transient current shows inactivation whereas the persistent current does not.

This is illustrated more clearly in Fig. 3-12B in which the voltage-dependence of inactivation of the transient and persistent currents recorded in one cell are compared. Similar curves were obtained in 3 other cells in which the effect of a wide range of conditioning prepulses was tested.

3.8. Summary

1. A small, TTX sensitive persistent current following a much larger, transient current can be recorded in single cardiac myocytes isolated from rat ventricular, atrial or toad sinus venosus tissues using the tight seal, whole-cell, voltage clamp technique.

2. The persistent currents were sodium currents because they reversed close to the sodium equilibrium potential and were depressed when choline was substituted for extracellular sodium.

3. The persistent sodium current was activated at more negative potentials than the transient sodium current. It cannot have been a window current because it was recorded at positive potentials when the transient current was completely inactivated.

4. The persistent sodium current was resistant to inactivation and could be recorded when the transient current had been inactivated with conditioning depolarization. Only slight inactivation of the persistent current occurred during depolarizing pulses lasting up to 900 ms.

5. A lower concentration of TTX (0.1 μM) blocked the persistent sodium current while having little effect on the transient sodium current.

Chapter 4

EFFECTS OF ANTIARRHYTHMIC DRUGS ON THE PERSISTENT SODIUM CURRENT

4.1. Introduction

A small, very slowly inactivating, TTX-sensitive sodium current exists in cardiac myocytes was described in Chapter 3. There has been considerable indirect evidence for such a current e.g. the decrease in duration of action potentials in dog Purkinje fibres caused by TTX (Coraboeuf, Deroubaix & Coulombe, 1979). As this current becomes activated close to the resting membrane potential (Chapter 3; Saint, Ju & Gage, 1992), it would contribute to the pacemaker current. Drugs that depress the persistent sodium current could act as antiarrhythmic agents by increasing the interval between action potentials and slowing the heart rate. Indeed, TTX has been shown to have an antiarrhythmic action *in vivo* (Abraham *et al.*1989). But not all of antiarrhythmic drugs action this way, some prolonging the refractory period by blocking potassium currents may contribute to antifibrillatory actions (Woosley, 1991).

Lidocaine and quinidine are widely used in the management of ventricular arrhythmias. Both decrease the maximum rate of rise of ventricular action potentials and have been classified as Class 1 antiarrhythmic agents (Vaughan-Williams, 1981) which share the common property of acting primarily on sodium channels. It is generally considered that these drugs

suppress arrhythmias by blocking the sodium channels that cause rapid depolarization during an action potential and then quickly inactivate. Arrhythmias would be less likely in the presence of such agents because of an increase in threshold or a decrease in conduction velocity of action potentials.

In this chapter, the effects of lidocaine and quinidine on the persistent sodium current are described. Both lidocaine and quinidine block the persistent sodium current at clinically relevant concentrations (Rosen, Hoffman & Wit, 1975; Hoffman, Rosen & Wit, 1975) that have relatively little effect on the transient sodium current.

These observations raise the possibility that the antiarrhythmic effects of these drugs are due, at least in part, to depression of the persistent sodium current.

4.2. Solutions and drugs

For simultanous recording of sodium and potassium currents, the pipette solution contained (mM): KF 120; K-EGTA 10; $MgCl_2$ 1; $CaCl_2$ 2; ATP 10; TES 10, pH adjusted to 7.4 $\pm$ 0.05 with KOH. For recording sodium currents in isolation, the pipette solution contained (mM): CsF 50; NaF 70; K-EGTA 20; $CaCl_2$ 2; TES 10; ATP 10, pH adjusted to 7.4 $\pm$ 0.05 with KOH. Electrodes typically had resistances of 1.5 to 2.0 MΩ when filled with pipette solution. Experiments were performed at room temperature (22 to 25 °C) in a bath solution normally containing (mM): NaCl 130; KCl 5.4; $MgCl_2$ 1; $CaCl_2$ 2; CsCl 5; $CoCl_2$ 5; TES 10; NaOH 5; glucose 10, pH adjusted to 7.4 $\pm$0.05 with NaOH.

Quinidine sulfate dihydrate (Aldrich-chemie D-7924 Steinheim) and lidocaine (Sigma Chemical, St.louis, MO) were dissolved in test solutions.

4.3. The effects of drugs on the action potentials

The effects of tetrodotoxin, lidocaine and quinidine on action potentials in isolated rat ventricular myocytes are shown for comparison in Fig. 4-1.

At the concentrations used, tetrodotoxin and lidocaine had little effect on the amplitude of action potentials but affected their plateau phase. It can be seen in Fig. 4-1 that tetrodotoxin (0.1 μM) and lidocaine (25 μM) shortened the duration of the action potential. Quinidine (10 μM) had a more complicated effect, as shown in Fig. 4-1C: the amplitude of the action potential was slightly reduced and repolarization after the peak was initially more rapid but the plateau phase was prolonged. The opposite effects of lidocaine and quinidine on the duration of the plateau phase of action potentials agree with previous observations (e.g. Colatsky, 1982).

The plateau phase of a cardiac action potential is due to a balance of inward and outward currents at the plateau potential. A drug which disturbs the balance of these currents should affect the plateau. For example, TTX would be expected to affect the plateau because it has been shown at this concentration (0.1 μM) to block a persistent, inward sodium current that would be present at the plateau potential (Chapter 3; Saint, Ju & Gage, 1992). And indeed TTX does shorten the action potential (Fig. 4-1A). The reduced duration of the plateau phase of the action potential caused by lidocaine (Fig. 4-1B) would be explained if lidocaine also blocked the persistent sodium current. This possibility was therefore tested.

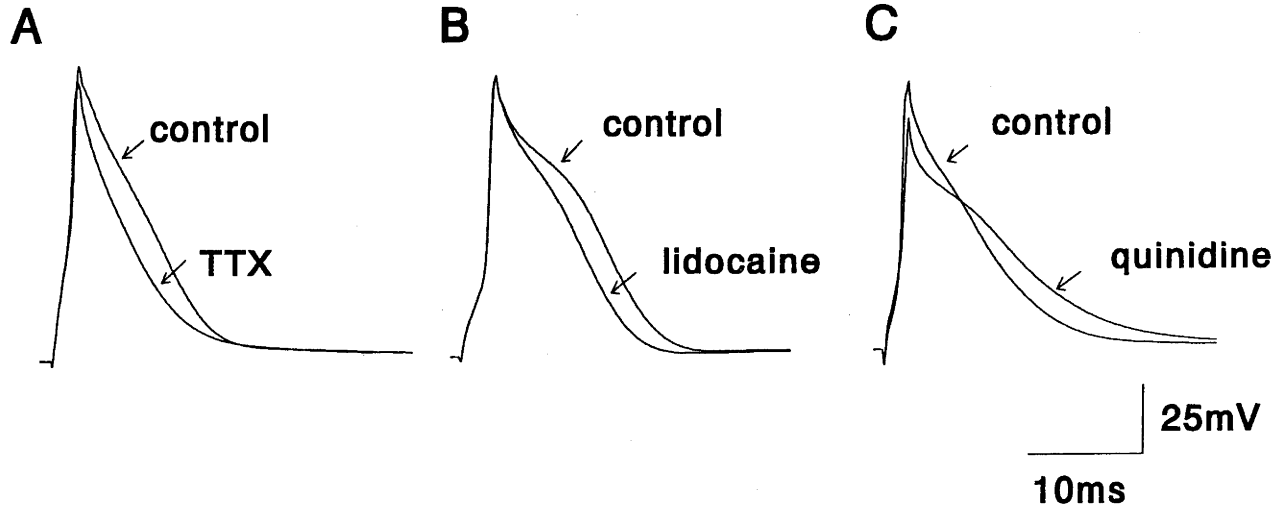


Fig. 4-1. Effects of 0.1 μM TTX (**A**), 25 μM lidocaine (**B**) and 10 μM quinidine (**C**) on action potentials in isolated ventricular myocytes.

4.4. Lidocaine and quinidine block the persistent sodium current

The effect of lidocaine on the persistent sodium current is illustrated in Fig. 4-2A. At a concentration of 200 μM, lidocaine clearly blocked the persistent sodium current while not completely blocking the transient sodium current (the peak amplitude of the transient current was reduced from 30.1 to 2.7 nA). It has been proved in choline substitution experiments (see Chapter 3, Fig. 3-2) that this concentration of TTX (50μM) could completely block the persistent sodium current. Therefore, the degree of block of the persistent sodium current by the lidocaine was checked with 50 μM TTX. Addition of TTX (50 μM) caused no further reduction in the amplitude of the persistent sodium current (Fig. 4-2A) indicating that the lidocaine had completely blocked this current. It can be seen in Fig. 4-2A, however, that the addition of TTX caused a further reduction in the amplitude of the transient sodium current.

Quinidine had a similar effect on the persistent sodium current (Fig. 4-2B). At a concentration of 40 μM, quinidine caused significant depression of the persistent sodium current whereas the transient current was much less affected (the peak amplitude of the transient current was reduced from -68 to -11 nA).

Again, most of the persistent sodium current must have been blocked by the quinidine because there was little further depression of the persistent current when 50 μM TTX was added to the solution (Fig. 4-2B).

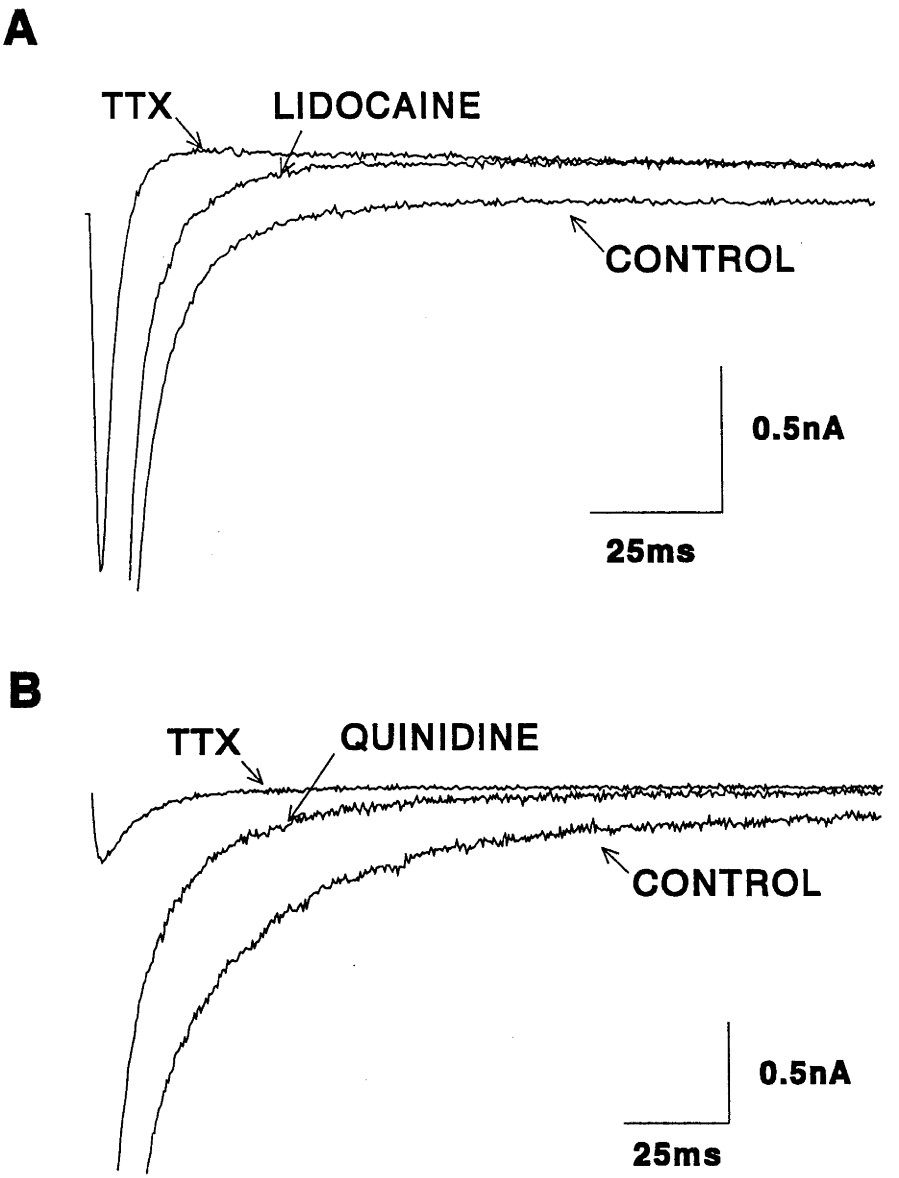


Fig. 4-2. Block of the persistent sodium current by lidocaine and quinidine. All current traces were elicited by voltage steps to -40 mV from a prepulse potential of -150 mV. **A**: the traces shown were recorded in control solution (CONTROL), then in a solution containing 200 μM lidocaine (LIDOCAINE), then in a solution containing 200 μM lidocaine plus 50 μM TTX (TTX). **B**: the traces were recorded in control solution (CONTROL), in a solution containing 40 μM quinidine (QUINIDINE), and in a solution containing 40 μM quinidine plus 50 μM TTX (TTX).

4.5. The persistent current is more sensitive to antiarrhythmic drugs than the transient current.

In experiments such as those illustrated in Fig. 4-2, it appeared that lidocaine and quinidine were blocking the persistent sodium current more effectively than the transient sodium current. The selective effect of these drugs on the persistent sodium current was even clearer when lower concentrations were used. The selective effect of a lower concentration of lidocaine is illustrated in Fig. 4-3.

Currents generated by voltage steps to -50 mV from a pre-potential of -150 mV are shown at both low (A) and high (B) gains so that the transient and persistent currents can be clearly seen. Traces obtained before introduction of the drug are marked C. Lidocaine, at a concentration of 12.5 μM, caused only slight depression of the transient current (from -47.5 to -39 nA) but there was significant depression of the persistent current. With a higher concentration of lidocaine (100 μM), there was much more depression of the transient current which now had a peak amplitude of 16 nA but there was little further depression of the persistent current, indicating that it had been completely blocked by the lower concentration of lidocaine. This was confirmed in several experiments by demonstrating that 50 μM TTX caused no further depression of the persistent sodium current.

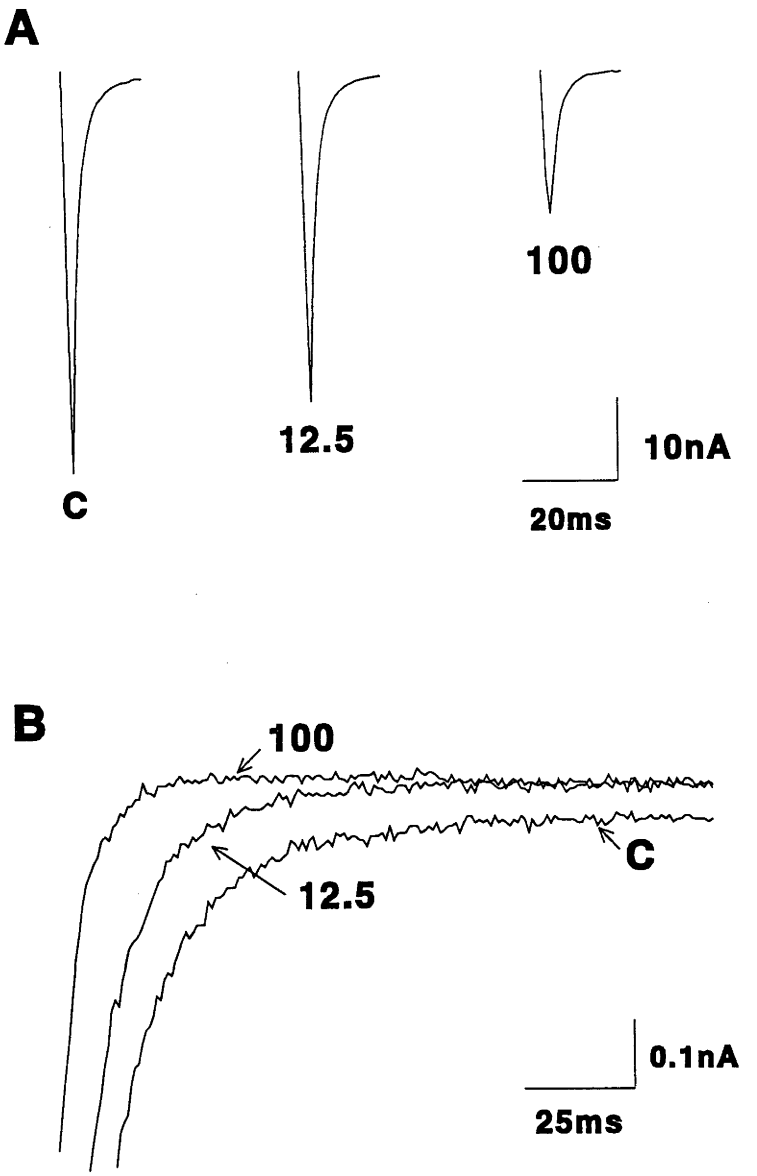


Fig. 4-3. Differential sensitivity of the transient and persistent sodium current to lidocaine. The currents, evoked by voltage steps to -50 mV from a prepulse potential of -150 mV, are shown at low gain in A and at higher gain in B to reveal the persistent current. The records, all from the same cell, were obtained in control solution (labelled C), a solution containing 12.5 μM lidocaine (labelled 12.5) and a solution containing 100 μM lidocaine (labelled 100).

A comparison of the effects of low (12.5 to 25 μM) and high (100 to 200 μM) concentrations of lidocaine on the transient and persistent sodium currents can be seen in Table 1. There was no significant difference ($p > 0.05$, Student's t test) in the depression of the persistent sodium current with low and high concentrations of lidocaine whereas the transient sodium current was significantly smaller in solutions containing the higher concentrations of lidocaine.

Quinidine, at low concentrations, also selectively and reversibly blocked the persistent sodium current, as illustrated in Fig. 4-4. Sodium currents were first recorded in control solution (C). After addition of 5 μM quinidine to the bath, the peak transient current was reduced by only 12% (from -86.5 to -77.5 nA, Fig. 4-4A), but the persistent current was substantially reduced in amplitude (Fig. 4-4B). These effects of quinidine could be reversed by washing the drug out of the bath, as illustrated by the traces marked W in Fig. 4-4.

Effects of 5-10 μM and 40-120 μM quinidine on transient and persistent sodium currents are summarised in Table 2. There was no significant difference ($p > 0.05$, Student's t test) in the amount of persistent sodium current blocked by the low and high concentrations of quinidine. In contrast, the transient sodium current was more effectively blocked by the higher concentrations of quinidine. It is clear from these results that the persistent sodium current is more sensitive to block by quinidine than is the transient sodium current: 5 or 10 μM quinidine caused an essentially complete block of the persistent current but, on average, only a 26% block of the transient sodium current.

Table 1. Depression of transient and persistent sodium currents by lidocaine

cell	lidocaine (μM)	ΔI_p (pA)	I_{tc} (nA)	$\Delta I_p/I_{tc}$ (%)	I_{td} (nA)	$(I_{tc}-I_{td})/I_{tc}$ (%)	V_t (mV)
Low concentration (12.5-25μM)							
1	25	-118	-49.0	0.24	-44.0	10.2	-50
2	25	-149	-44.0	0.34	-38.5	12.5	-40
3	25	-122	-41.5	0.29	-33.5	19.3	-50
4	25	-90	-48.5	0.19	-33.5	30.9	-40
5	12.5	-89	-34.5	0.26	-23.5	31.9	-40
6	12.5	-95	-33.0	0.29	-23.5	28.8	-40
7	12.5	-170	-69.5	0.24	-53.0	23.7	-50
8	12.5	-103	-47.5	0.22	-39.5	16.8	-50
9	25	-103	-35.0	0.29	-30.0	14.3	-40
mean		-115	-44.7	0.26	-35.4	20.9	
1 s.e.mean		9.29	3.73	0.02	3.18	2.74	
High concentration (100-200μM)							
1	200	-115	-28.0	0.41	-13.5	51.8	-40
2	200	-158	-48.5	0.33	-17.0	64.9	-40
3	100	-114	-36.5	0.31	-13.0	64.4	-40
4	200	-264	-78.5	0.34	-4.5	94.3	-50
5	100	-103	-47.5	0.22	-16.5	65.3	-40
6	100	-79	-42.5	0.19	-22.5	47.1	-50
7	100	-103	-45.0	0.23	-28.5	36.7	-50
8	100	-116	-50.0	0.23	-19.5	61.0	-40
9	100	-215	-21.5	1.00	-13.0	39.5	-40
10	200	-142	-30.0	0.47	-2.65	91.2	-40
11	100	-110	-41.5	0.27	21.0	49.4	-40
mean		-138	-42.7	0.36	-15.6	60.5	
1 s.e.mean		16.6	4.54	0.07	2.28	5.66	

The symbols denote: ΔI_p,the change in amplitude of the persistent current (measured at the end of a 150ms test pulse to V_t); I_{tc}, the peak amplitude of the transient current in control solution; I_{td}, the peak amplitude of the transient current in the presence of the drug. The holding potential was -100mV.

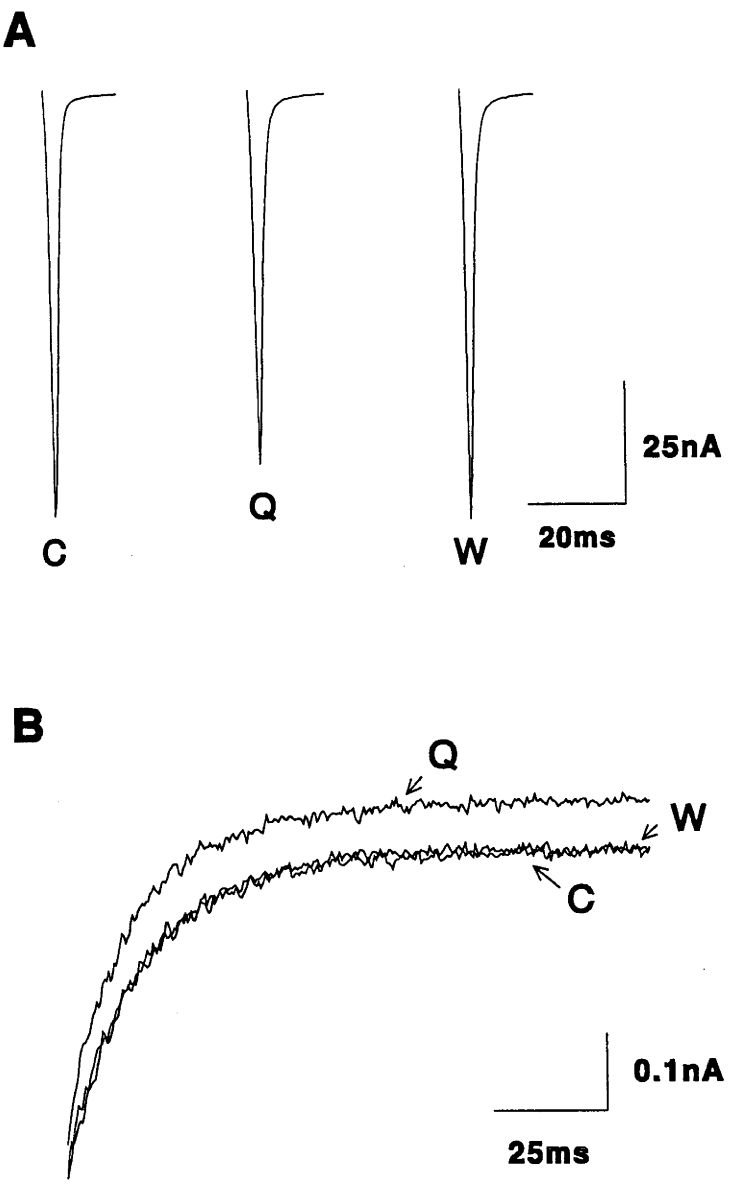


Fig. 4-4. Differential sensitivity of the transient and persistent sodium current to quinidine. The currents, evoked by voltage steps to -50 mV from a prepulse potential of -150 mV, are shown at low gain in **A** and at higher gain in **B** to reveal the persistent current. The records, all from the same cell, were obtained in control solution (labelled C), in a solution containing 5 μM quinidine (labelled Q), and back in control solution after washing out the quinidine (labelled W).

Table 2. Depression of transient and persistent sodium currents by quinidine

cell	quinidine (μM)	ΔI_p (pA)	I_{tc} (nA)	$\Delta I_p/I_{tc}$ (%)	I_{td} (nA)	$(I_{tc}-I_{td})/I_{tc}$ (%)	V_t (mV)
Low concentration (5-10μM)							
1	5	-102	-86.5	0.12	-77.5	10.4	-50
2	10	-86	-32.0	0.27	-26.0	18.8	-60
3	10	-83	-20.0	0.42	-17.0	15.0	-40
4	5	-125	-66.7	0.19	-35.6	46.6	-40
5	5	-161	-76.5	0.21	-61.1	20.1	-40
6	5	-148	-36.5	0.41	-22.8	37.5	-40
7	5	-156	-37.9	0.41	-31.2	17.7	-40
8	5	-155	-55.1	0.28	-30.0	45.6	-40
mean		-127	-51.4	0.29	-37.6	26.5	
1 s.e.mean		11.5	8.32	0.04	7.35	5.11	
High concentration (40-120μM)							
1	40	-95	-31.0	0.31	-10.5	66.1	-40
2	40	-83	-32.5	0.26	-9.00	72.3	-40
3	40	-176	-68.0	0.26	-11.0	83.8	-50
4	80	-122	-66.7	0.18	-5.77	91.3	-40
5	120	-175	-76.5	0.23	-1.50	98.0	-40
6	80	-194	-36.5	0.53	-8.40	77.0	-40
7	80	-150	-41.0	0.37	-4.20	89.8	-40
mean		-142	-50.3	0.30	-7.20	82.6	
1 s.e.mean		16.3	7.29	0.04	1.32	4.30	

The symbols denote: ΔI_p, the change in amplitude of the persistent current (measured at the end of a 150ms test pulse to V_t); I_{tc}, the peak amplitude of the transient current in control solution; I_{td}, the peak amplitude of the transient current in the presence of the drug. The holding potential was -100mV.

4.6. Effect of lidocaine and quinidine on potassium currents.

As both lidocaine and quinidine block the persistent sodium current, why does quinidine not shorten the plateau phase of action potentials? A possible explanation is that quinidine depresses the outward current that balances the inward current and eventually causes repolarization. The effects of tetrodotoxin, lidocaine and quinidine on outward currents were therefore investigated. In these experiments, inward sodium current was blocked by substituting choline for sodium in the extracellular solution. The currents shown in Fig. 4-5 were generated by voltage steps to +40 mV from a holding potential of -140 mV.

They consisted of a transient peak which subsided to a plateau. TTX (50 μM, A: and lidocaine (100 μM, B) caused a slight depression of the outward current which may have been due to block of a small outward sodium current at +40 mV in the extracellular solution containing choline instead of sodium. In contrast, in the presence of quinidine (20 μM, C) the transient outward current was decreased in amplitude and decayed more rapidly. There was also a large decrease in the amplitude of the sustained outward current. Similar effects here have been reported previously (Hiraoka, Sawada & Kawano, 1986; Imaizumi & Giles, 1987; Balser *et al.*1991), and may well explain the prolongation caused by quinidine in the plateau phase of the action potential (Fig. 4-1C).

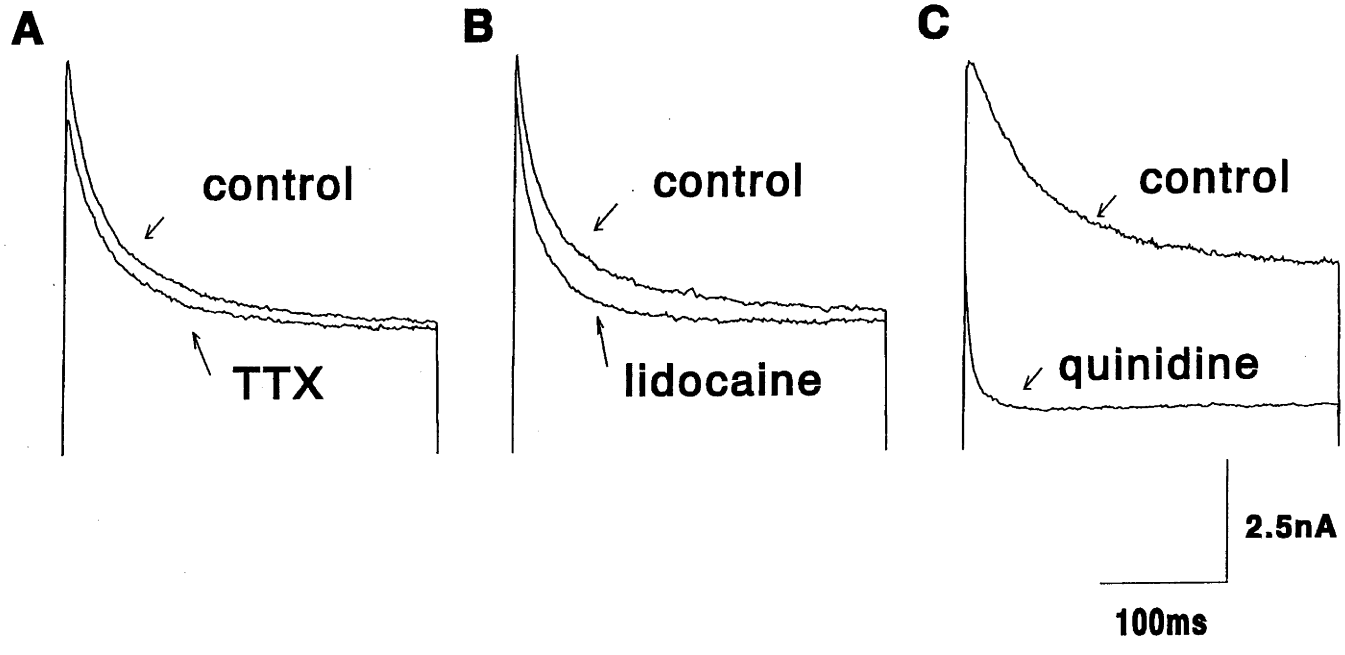


Fig. 4-5. Effects of TTX (50 μM), lidocaine (100 μM) and quinidine (20 μM) on potassium currents. Choline was substituted for extracellular sodium and the currents shown were elicited by a voltage step to +40 mV from a potential of -140 mV. **A** and **B** contain records from the same cell, **C** from another.

4.7. Summary

1. The effects of the Class 1 antiarrhythmic agents lidocaine and quinidine on action potentials, and on sodium currents and potassium currents activated by depolarization, were examined in isolated rat ventricular myocytes using the whole-cell, tight seal recording technique.

2. Tetrodotoxin and lidocaine shortened, whereas quinidine prolonged, the duration of the plateau phase of action potentials.

3. At low concentrations, lidocaine and quinidine blocked a persistent sodium current that was resistant to inactivation but they had only a small effect on the transient sodium current. At higher concentrations, they also blocked the transient sodium current.

4. Quinidine, but not tetrodotoxin or lidocaine, depressed potassium currents activated by depolarization and this could account for the prolongation of the plateau phase caused by quinidine.

5. It is suggested that block of the persistent sodium current may be responsible, at least in part, for the antiarrhythmic action of lidocaine and quinidine.

Chapter 5

SODIUM CHANNELS UNDERLYING THE PERSISTENT CURRENT IN RAT VENTRICULAR MYOCYTES

5.1. Introduction

The observation that tetrodotoxin (TTX) shortens the plateau phase of cardiac ventricular action potentials (Coraboeuf, Deroubaix & Coulombe, 1979) indicates that there is a persistent TTX-sensitive current contributing to the plateau. In chapter 3, such a sodium current has been described. This current was shown to be resistant to inactivation and also to have a voltage dependence for activation different from that of the transient sodium current. This persistent sodium current was also more sensitive to block by TTX (Chapter 3; Saint, Ju & Gage, 1992) and class I antiarrhythmic agents (Chapter 4; Ju, Saint & Gage, 1992) than the transient current.

The different electrophysiological and pharmacological properties of transient and persistent sodium currents raise the possibility that they are generated by different channels. In order to test this hypothesis, experiments were performed in which single-channel currents underlying the two types of macroscopic sodium current could be recorded. The results of these experiments are described in this Chapter.

5.2. Single sodium channel recording

Single ventricular myocytes were prepared by the method described previously (Chapter 2). Cell-attached and inside-out patch clamp techniques (Chapter 2) were used for recording single channel currents. Patch clamp microelectrodes were fabricated from borosilicate glass (Clark Electromedical; GC 150-15) and generally had a resistance of 5 to 10 MΩ when filled with a solution containing (mM): NaCl 130, KCl 5.4, $MgCl_2$ 1,

$CaCl_2$ 2, $CoCl_2$ 5, CsCl 5, TES 10, pH adjusted to 7.4 ± 0.05 with NaOH (usually about 12 mM). To reduce stray capacitance, electrodes were coated with Sylgard (Dow Corning) as close to the pipette tip as possible.

The bath solution for both cell-attached and inside-out configurations normally contained (mM): K-aspartate 140, EGTA 8, $MgCl_2$ 2, Na-ATP 4, CsCl 2, TES 10, pH adjusted to 7.4 ± 0.05 with KOH. In some experiments on inside-out patches, the bath solution was changed to 200 μM Ca^{2+}-Tyrode's solution (133mM NaCl), in order to obtained a sodium equilibrium potential close to 0mM.

Voltage commands were generated by a microcomputer and D/A converter. Currents were recorded with an Axopatch 1D amplifier (Axon Instruments), filtered at 5 kHz (4-pole bessel, -3dB), digitised at 10 kHz with a 12 bit A/D converter and stored on the computer hard disc. All potentials and currents are given with the convention of ground at the extracellular surface of the membrane.

In order to measure the characteristics of transient single-channel currents, only single, non-superimposed openings within 50ms of the onset of a test depolarization were analysed.

5.3. Late sodium channels activated by depolarization

5.3.1. Late channel openings can be recorded with long depolarizing pulses.

In both inside-out or cell-attached patches, when a depolarizing pulse lasting 200ms was preceded by a prepulse to -150 mV for 300ms, early single channel openings could be seen at the beginning of each depolarizing step. This early channel activity decays rapidly to zero within 25 ms. However, late channel openings can be see occasionally after a variable latency as shown in Fig. 5-1A, which shows selected traces from an inside-out patch.

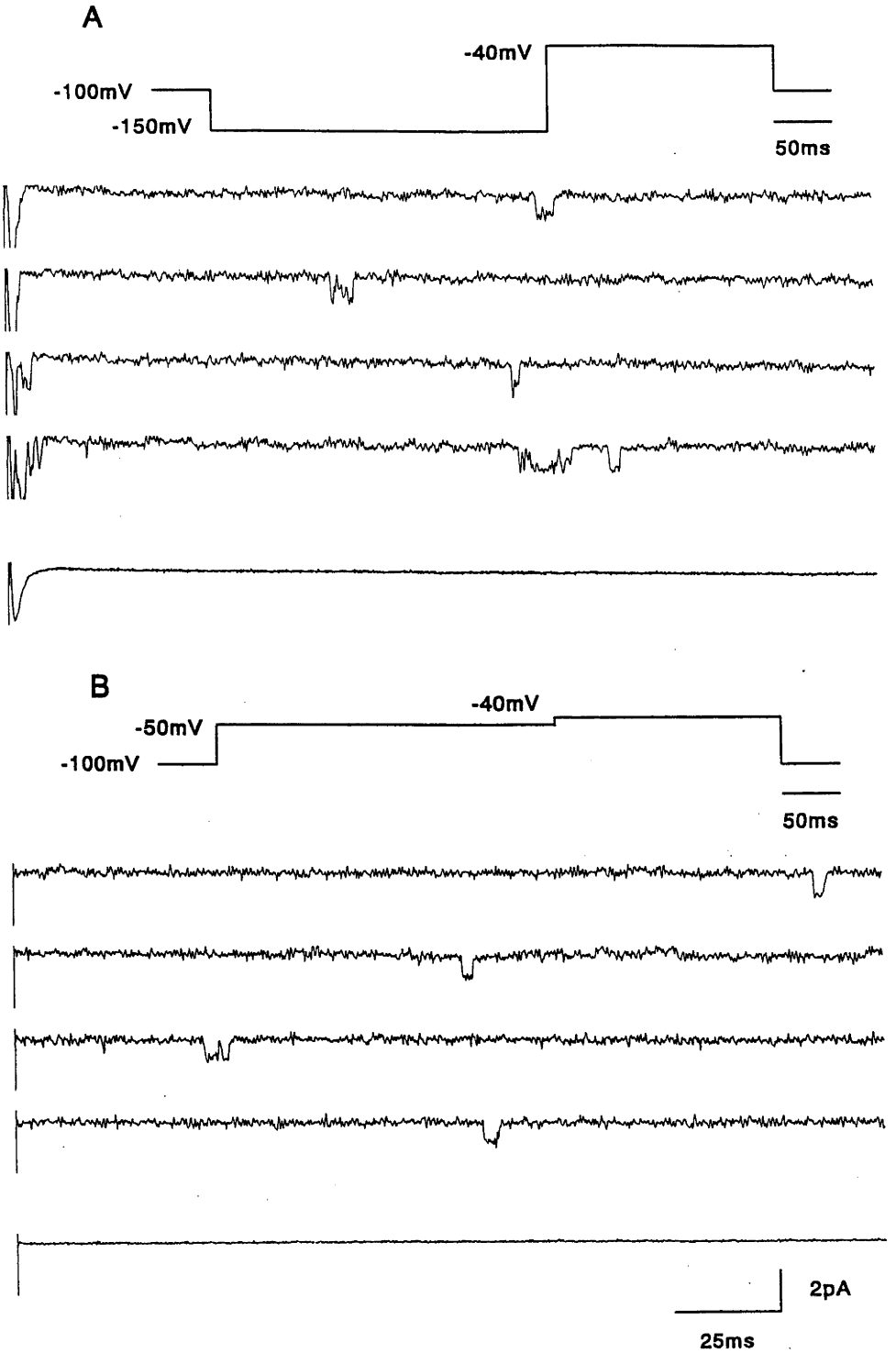


Fig. 5-1. Single channels recorded from an inside out patch. All traces start at the beginning of a 200 ms test pulse to -40mV which was preceded by a hyperpolarising prepulse to -150 mV lasting 300 ms. Capacitive and leakage current have been subtracted. **A**: top panel shows the voltage protocol. Four traces (selected from 300 traces) show early and later channel openings evoked by the test pulse from a prepulse to -150 mV. The bottom trace is an ensemble average of 300 sweeps. **B**: Traces of currents elicited by the same test pulse but from a prepulse potential of -50 mV (as shown in the top panel). The bottom trace is an ensemble average of 300 sweeps. Selected traces show that although the early channel activity (in the first 50 ms) was abolished by this change in the pre-pulse potential, as clearly illustrated by the ensemble average current, the incidence of late opening channels was not affected.

Since these late channel openings appear with a very low frequency, it is very difficult to study and characterise them. It should be noted that in this study "late" channel openings have been defined as those seen later than 50 ms after onset of a depolarizing pulse. The frequencies seen in 19 recordings from 8 cells in which prolonged recordings could be obtained ranged from 0.09 to 1.8 channel openings per second (Table 5-1).

5.3.2. The late channel openings are sodium channels.

Although the solutions used in these experiments were designed to block conduction through other kinds of ion channels, two experiments were designed to confirm that both the early and late channels which were recorded during a depolarization pulse were indeed sodium channels.

In the first experiment, the bath solution was changed to 200 μM Ca^{2+}-Tyrode's solution containing 133mM NaCl, with the pipette solution remaining as before (130mM sodium), to give a sodium equilibrium potential close to 0 mM. With a depolarizing voltage pulse to -70 mV, there was a transient burst of currents within the first 50 ms and then sporadic late channel openings during the remainder of the 200 ms depolarization (Fig. 5-2A). During a depolarization to +60 mV in the same patch (Fig. 5-2B), currents were outwards and of similar amplitude to those in Fig. 5-2A. As the pipette solution contained cobalt and caesium in order to block calcium and potassium channels activated by depolarization, it was concluded that both early and late channel currents were carried by sodium ions. The similarity of the amplitudes of the currents at -70 mV and +60 mV would be expected for sodium channels with an equilibrium potential close to 0 mV.

Table 5-1. Conductance of transient and persistent sodium channels.

cell	Patch type	Frequency persistent (channels/sec)	Conductance persistent (pS) mean	SE (n)	transient (pS) mean	SE (n)	Voltage (mV)
C1	i/o	0.43	6.58	0.41 (13)	6.14	0.20 (47)	-80
C2	i/o	0.49	6.13	0.17 (22)	6.55	0.20 (40)	-80
C3	c/a	1.04	7.14	0.14 (47)	6.17	0.11 (81)	-80
C1	i/o	0.10	6.18	0.09 (3)	6.46	0.10 (130)	-70
C3	c/a	0.91	6.65	0.26 (41)	7.17	0.12 (132)	-70
C4	c/a	0.29	7.31	0.29 (26)	7.13	0.15 (111)	-70
C5	i/o	1.80	7.31	0.10 (243)	7.10	0.13 (160)	-70
C6	i/o	0.29	9.26	0.48 (13)	7.77	0.16 (70)	-70
C1	i/o	0.63	7.94	0.18 (19)	6.81	0.10 (76)	-60
C5	i/o	1.37	7.95	0.10 (246)	7.71	0.07 (298)	-60
C6	i/o	0.09	7.73	0.33 (21)	7.47	0.21 (39)	-60
C7	c/a	0.59	6.87	0.19 (53)	6.70	0.35 (13)	-60
C8	c/a	0.11	10.7	0.70 (20)	9.63	0.12 (133)	-50
C4	c/a	0.32	9.55	0.67 (29)	9.11	0.36 (30)	-50
C5	i/o	0.71	8.37	0.11 (64)	9.40	0.29 (32)	-40
C7	c/a	0.42	8.70	0.21 (38)	8.65	0.27 (18)	-40
C1	i/o	0.17	9.40	0.72 (5)	9.40	0.29 (32)	-40
C8	i/o	0.13	9.70	0.30 (6)	8.13	0.11 (139)	-40
C2	i/o	0.18	9.32	0.68 (8)	8.70	0.14 (12)	-20

The symbols denote: i/o, inside-out patch, c/a cell-attached patch, n, number of channel openings measured.

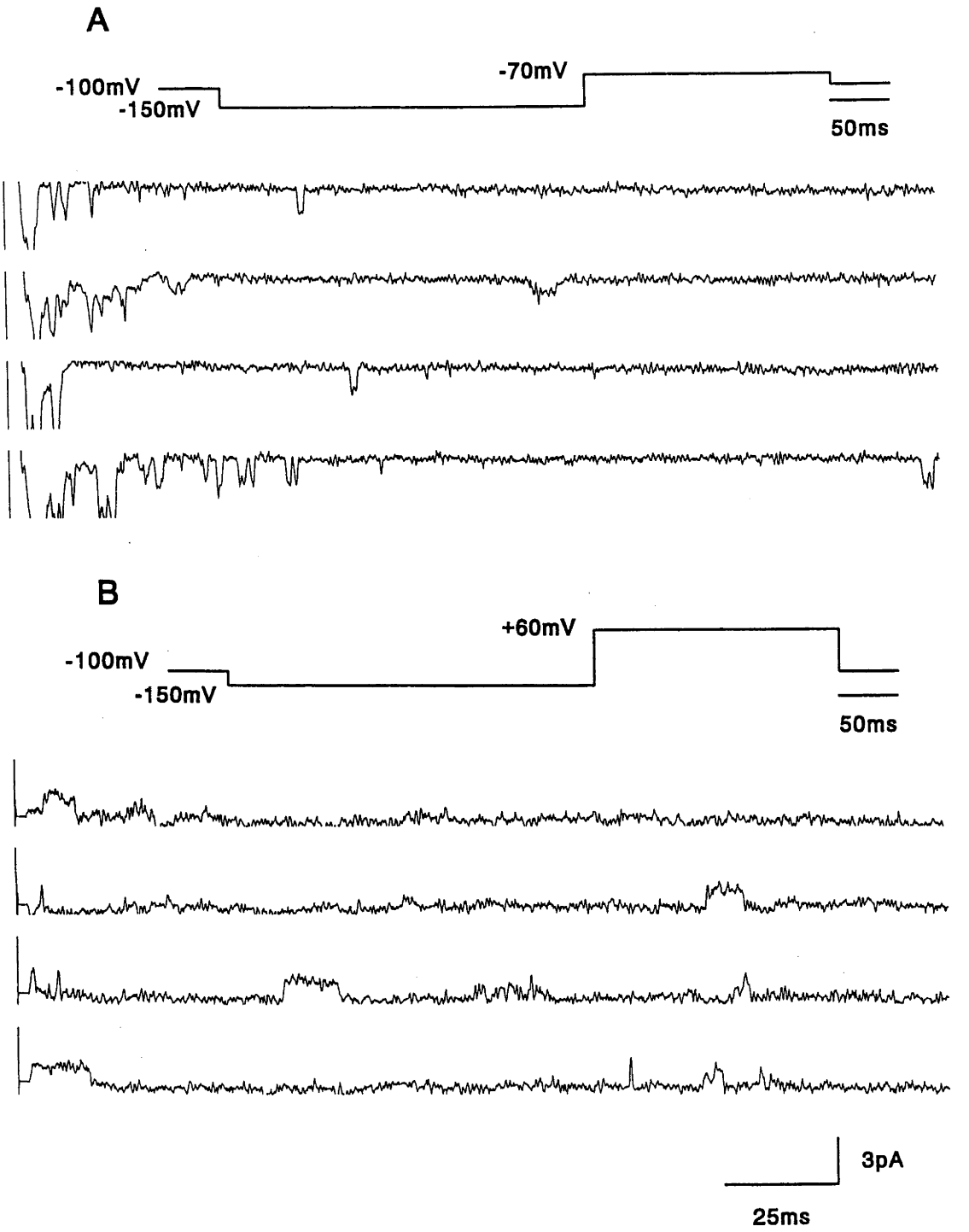


Fig. 5-2. Reversal of channel currents. **A**: The topmost diagram depicts the voltage protocol applied to an isolated inside-out patch. Below are shown selected records, each of 200 ms duration recorded during the depolarizing pulse to -70 mV. **B**: Current records obtained from the same patch during a depolarizing pulse to +60 mV. Both early and late currents are inverted at this potential, which is positive to E_{Na} in the solutions used (see text). The first 5 ms of these traces is blanked due to avoid capacitative transients.

Further evidence that these channels were sodium channels was obtained from experiments with TTX. In these experiments, TTX was injected into the tip of the patch pipette through an internal perfusion system (JCSMR workshop design), after a control recordings of channel activity was obtained.

Fig. 5-3 shows single channel activity (a) before TTX, (b) 2 min after TTX injection into the pipette, and (c) 30min after TTX injection. The currents were recorded in a cell-attached patch during a depolarization pulse to -60mV following a prepulse to -130mV. The ensemble average traces (300 sweeps) are shown at the bottom of each panel. Before injection of TTX, the channel openings can be seen with a high probability at the beginning of each depolarizing step followed by a few later channel openings (31 in 300 sweeps) (Fig. 5-3A). After 2 min of TTX (100 μM) injection, the ensemble trace of 300 sweeps was depressed a little (Fig. 5-3B). The number of late channel openings, was also reduced to 9 in 300 sweeps. 30 min after TTX injection, the ensemble average trace was depressed totally and no late channel openings were seen in 300 sweeps (Fig. 5-3C). Similar results were obtained in another 3 patches. Hence, both early and late channel openings activated by depolarization pulse are sodium channels sensitive to TTX. It also seems that late channel openings are more sensitive to TTX. When a low concentration TTX was in the pipette, few patches showed late channel activity, even though there were early channel activity.

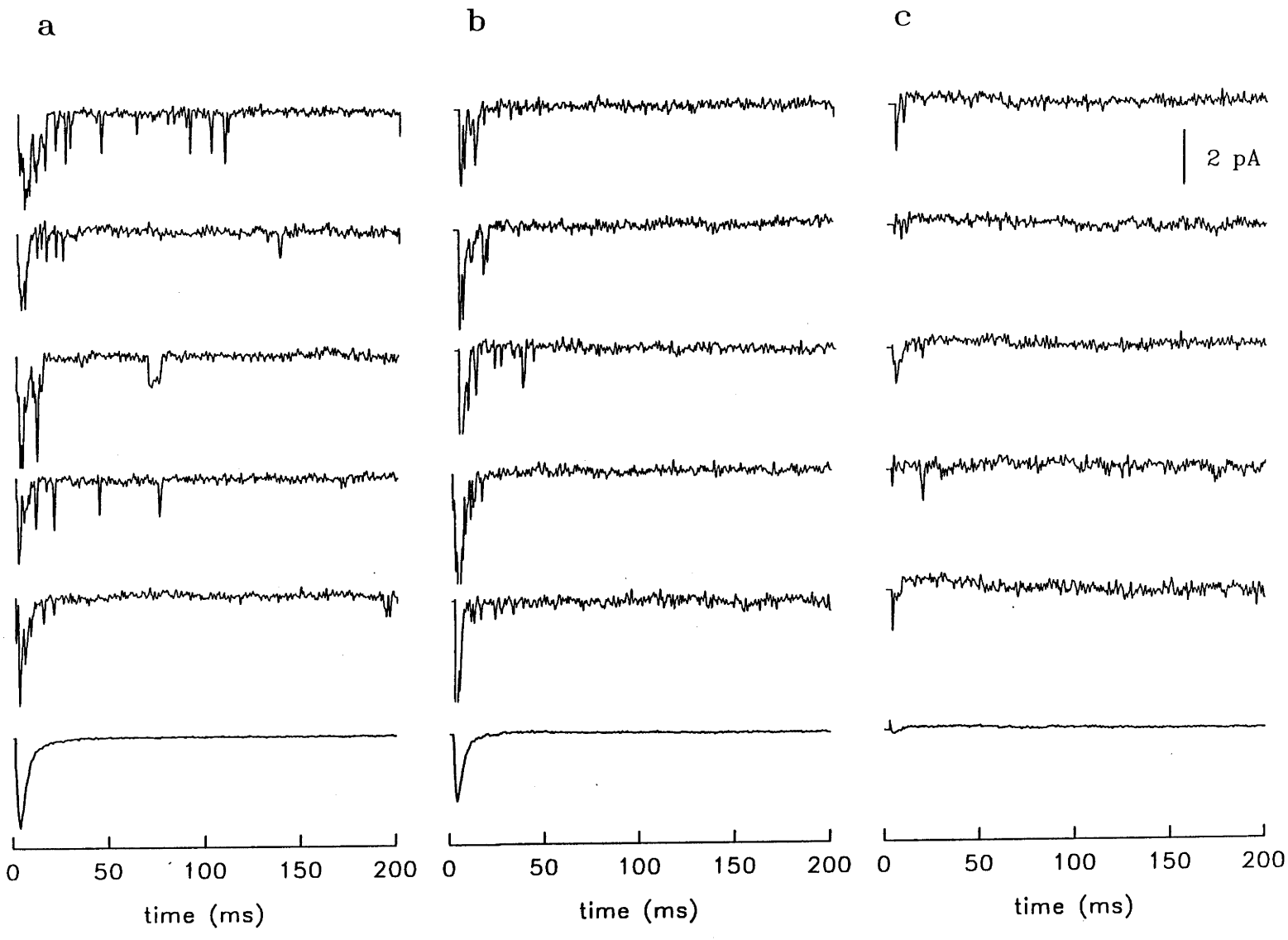


Fig. 5-3. TTX blocks early and late sodium channels The single channel activities recorded during a depolarization pulse to -60mV from prepulse -130mV in a cell-attached patch before (**A**), after 2 min (**B**) and 30min (**C**) of TTX application to the patch pipette.

5.4. Late sodium channel openings are resistant to inactivation.

A characteristic of the persistent sodium current that distinguishes it from the transient sodium current is its resistance to inactivation (Chapter 3; Saint, Ju & Gage, 1992). In order to investigate whether late channel openings are responsible for persistent currents previously recorded in whole-cell, inactivation experiments were carried out in both inside-out and cell-attached patches.

In Fig. 5-1A, when a depolarizing pulse to -40 mV was preceded by a conditioning prepulse to -150mV, sodium channels were activated (Chapter 1). A large ensemble trace was obtained by averaging 300 sweeps in this recording. This average transient current as shown in the lowest trace of Fig. 5-1A was similar in time course to the sodium current recorded in whole-cell configuration (Chapter 3). In addition to the early currents, during these 300 sweeps there were 26 individual channel openings at times later than 50 ms after the onset of the depolarizing pulse. In the same patch, when the depolarizing pulse to -40 mV was preceded by a conditioning prepulse to -50 mV (Fig. 5-1B), the early channel activity within the first 25 ms was no longer seen, as illustrated by the ensemble average current. The late channel activity was largely unaffected by this change in the prepulse: there were 20 single-channel currents after 50 ms in 300 trials with -50 mV prepulse compared with 26 with a -150 mV prepulse. However, because the frequency of late channel openings was very low, it is not possible to see persistent currents in the ensemble current.

It has been shown that the transient sodium channel can change its kinetic behaviour under some circumstances and can undergo occasional long lasting bursts of activity, or "chattering" (Patlak & Ortiz, 1986; Kohlhardt, Frobe & Herzig, 1987; Nilius, 1988). In a large population of channels, this kind of occasional behaviour could give rise to a small macroscopic current such as persistent currents (Chapter 3; Saint, Ju & Gage, 1992). An example of this kind of behaviour can be see in Fig. 5-4A. In this cell-attached patch, a test pulse to -40 mV gave an early burst of channel openings that was occasionally followed by repeated openings of channels chattering. 14 late opening channels were seen in 297 traces not showing chattering channel activity. When the level of the conditioning prepulse was changed from -150 mV (Fig. 5-4A) to -50 mV (Fig. 5-4B), both the transient channels and the chattering channels disappeared but individual late channel openings could still be seen (Fig. 5-4B). Chattering channel activity was seen in another seven inside-out patches and three cell-attached patches. In none of those patches was chattering activity seen when prepulse were changed to -50 mV. As the persistent current is resistant to inactivation (Chapter 3; Saint, Ju & Gage, 1992), it cannot be generated by the chattering channel openings. In contrast, the individual late channel openings are much more resistant to inactivation and they could therefore be responsible for persistent currents recorded in whole cells. I will call the late opening channels "persistent" sodium channels in this chapter, in order to emphasize their resistance to inactivation and distinguish them from the early channel openings which are responsible for the transient sodium current in whole-cell recordings.

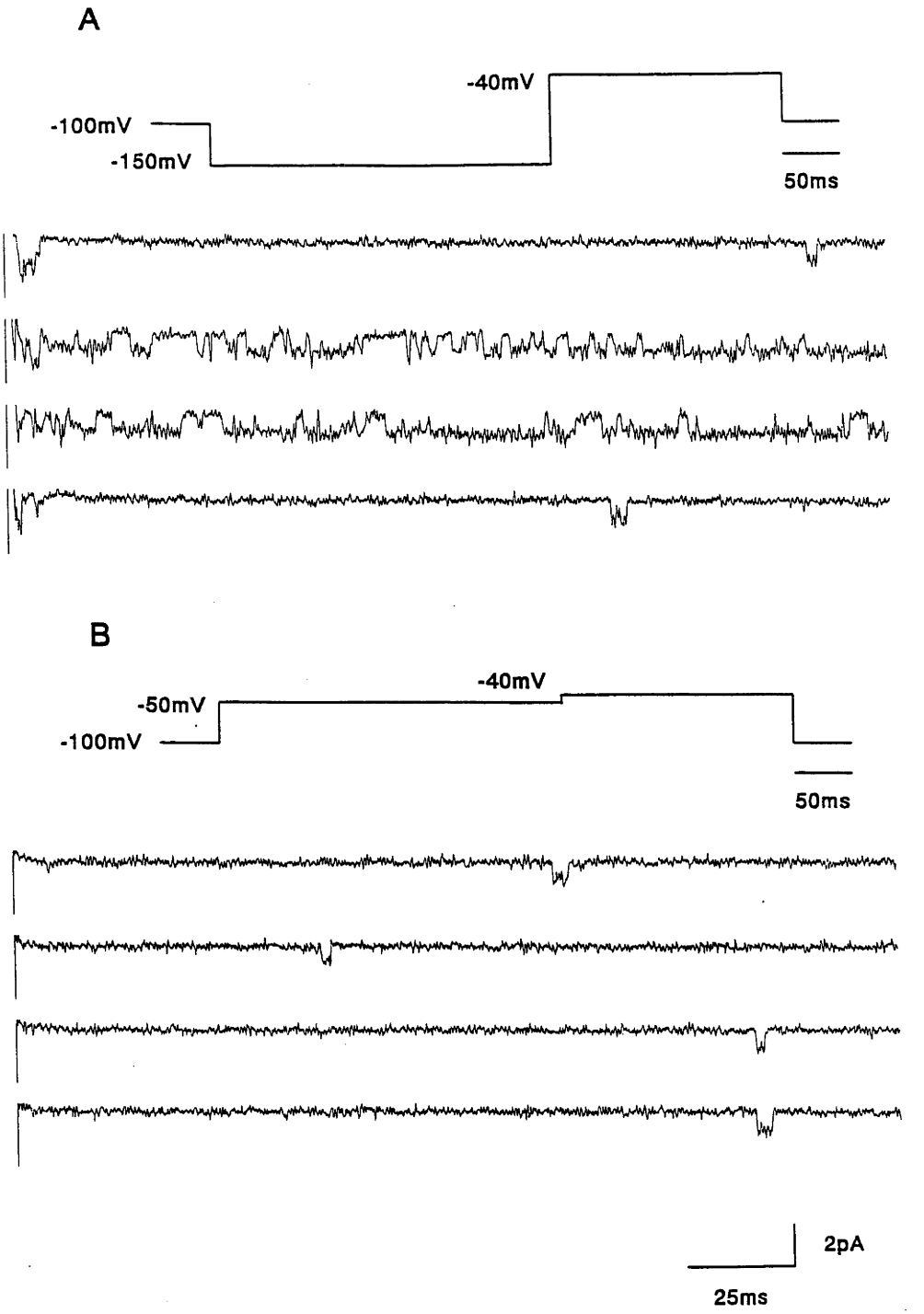


Fig. 5-4. Different inactivation property of late channel openings and "chattering" kinetic behaviour of the transient channels. **A**: Current records obtained from a cell-attached patch during the depolarizing step of the voltage protocol depicted above. The selected traces (2 and 3), as an example, show that the transient channels can occasionally undergo repeated opening and closing (Chattering) for variable lengths of time. Traces 1 and 4 show late channel openings in the same patch along with chattering. **B**: Selected recordes from the same patch with the prepulse to -50 mV. The transient channel activity is inactivated by this prepulse, as is the chattering behaviour. However the number of late channel openings was unchanged (see text).

5.5. Comparison of persistent and transient sodium channels

5.5.1. Persistent sodium channels activate at more negative potentials

Another characteristic of the persistent sodium current that distinguishes it from the transient sodium current is that it can be elicited at more negative potentials (Chapter 3; Saint, Ju & Gage, 1992). This was also seen with single channels, as illustrated in Fig. 5-5. With a depolarizing pulse to -80 mV, there were only two channel openings with a latency greater than 50 ms in 300 records and this can be seen in the forth and fifth trace in Fig. 5-5A. There was also very little transient channel activity at this voltage, as can be seen in from the ensemble average of 300 traces shown below. With a depolarizing pulse to -60 mV (Fig. 5-5b), there were 15 late opening channels in 300 records, but only a little more transient channel activity that is barely detectable in an average of 300 traces (lowest record). With a pulse to -40 mV (Fig. 5-5c), there were 16 late openings in 300 records and a very high probability of transient channel opening so that the average of 300 traces showed an obvious transient current. Hence it appeared that, in this cell-attached patch, the number of late opening channels approached a maximum at about -60 mV whereas the transient channel open probability was still comparatively low at this voltage. The voltage-dependence of activation was not always the same in every patch but persistent channel frequency in any patch always reached a maximum at more negative potentials than transient channels.

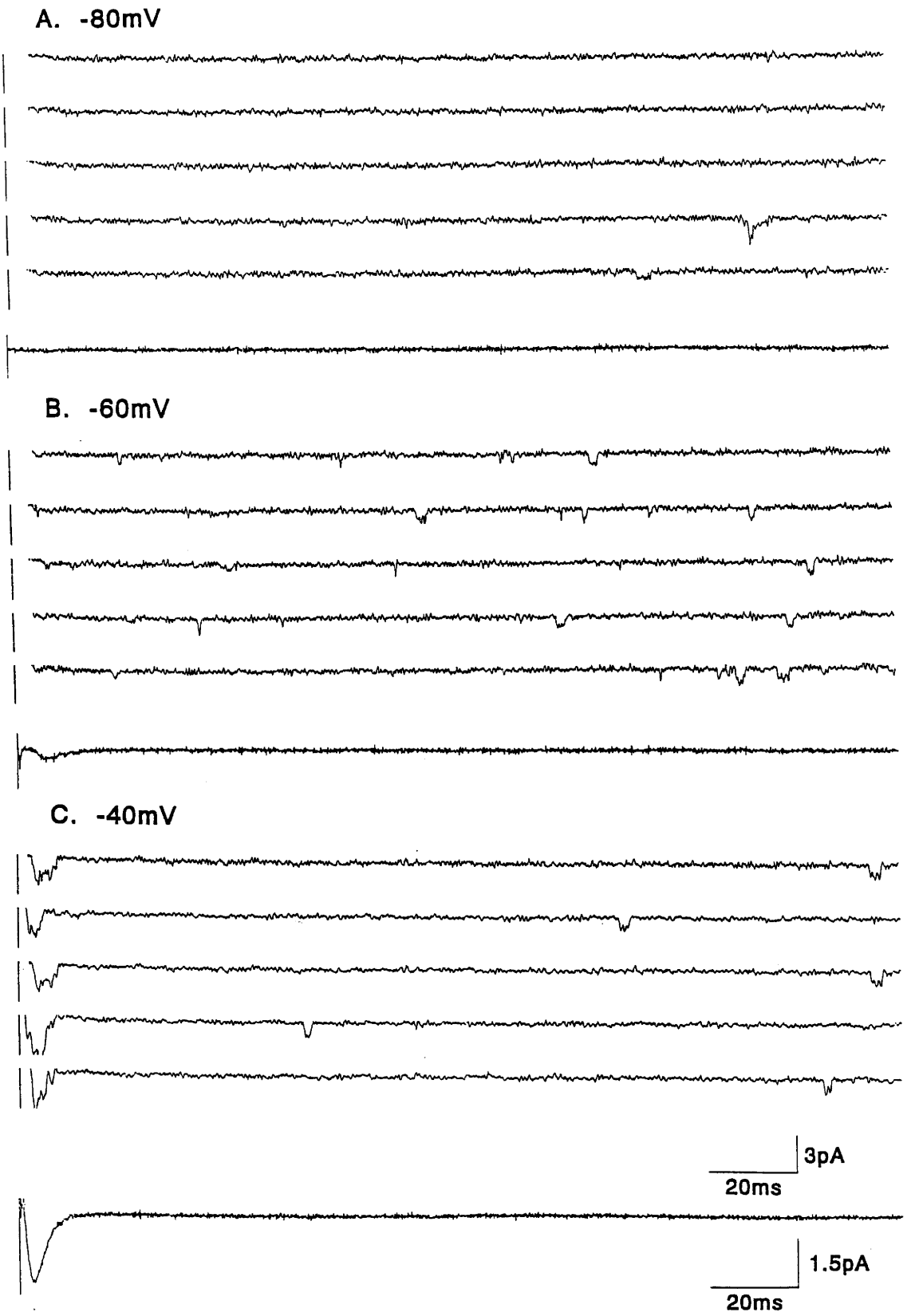


Fig. 5-5. Voltage dependence of activation for the late channels in a cell-attached patch. Selected traces show currents during a 200 ms depolarizing step to -80mV (**A**), -60mV (**B**) and -40 mV (**C**). The ensemble average of 300 sweeps recorded at each voltage is shown at the bottom of each panel. The vertical calibration bar denotes 3pA is that for the single channel records, and 1.5 pA for the ensemble averages.

5.5.2. Open time

The open times of both transient and persistent sodium channels were brief but appeared to be different. The distributions of open times of transient and persistent channels recorded in one patch in response to a voltage step to -60 mV are shown in Fig. 5-6. The average open time (obtained from best fits of a single exponential) was 0.51 ms for the transient channels (Fig. 5-6A) and 1.1 ms for the persistent channels (Fig. 5-6B). Furthermore, the open time of persistent channels, but not of transient channels, appeared to increase with depolarization between -70 mV and -40 mV (Fig. 5-6C). In this patch, there was a significant difference in the open time of the two channels at potentials more positive than -70 mV. Similar results were obtained in another 3 patches.

5.5.3. Conductance and subconductance states

There was no significant difference in the main state conductance of transient and persistent channels as can be seen in Table 5-1 and Fig. 5-6D. The conductance of both kinds of channel appeared to decrease at more negative potentials, a phenomenon which has previously been reported for the transient channel (Kunze *et al.* 1985).

Both transient (Fig. 5-7A) and persistent (Fig. 5-7B) sodium channels showed several conductance levels as illustrated in Fig. 5-7. All-point histograms of transient (Fig. 5-7C) and persistent (Fig. 5-7D) channels recorded at -70 mV in one patch could not be fitted with a single open level represented by a single Gaussian with the same standard deviation (SD) as baseline noise. Reasonable fits (broken lines) were obtained in both cases with Gaussians (solid line) representing 4 open states. The amplitude of the four open states for transient channels were 0.38, 0.67, 1.05 and 1.46 pA, and for persistent channels were 0.34, 0.65, 0.93, and 1.24 pA. The correlation coefficients were 0.999 for both transient and persistent channels.

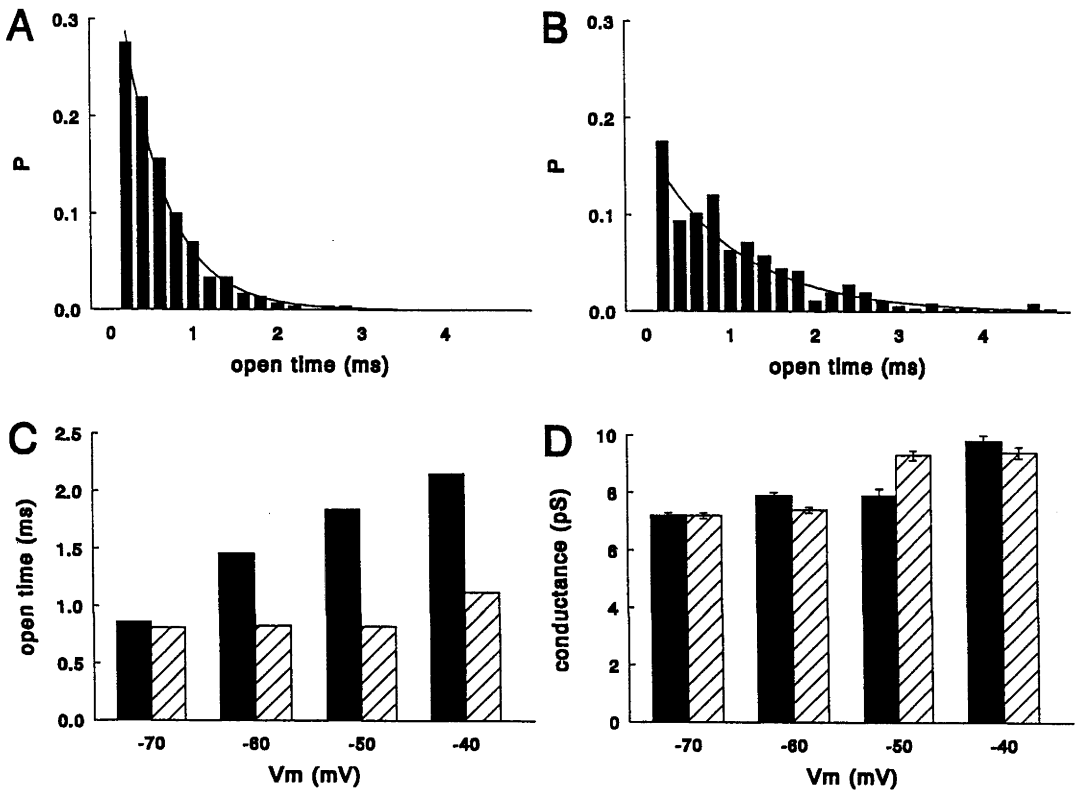


Fig. 5-6. Open time distributions for the transient and the persistent channels. The distributions of open times of transient and persistent channels were recorded in an inside-out patch in response to a voltage step to -60 mV . The lines through the histograms in A and B are the best fits of a single exponentials to the data. The exponential has a time constant of 0.51 ms for the transient channels (**A**) and 1.1 ms for the persistent channels (**B**). **C**: Voltage-dependence of the mean open time of persistent (filled bars), and transient channels (hatched dars). **D**: Voltage-dependence of the mean conductance ($\pm$1SEM) of persistent (filled bars) and transient (hatched bars) channels.

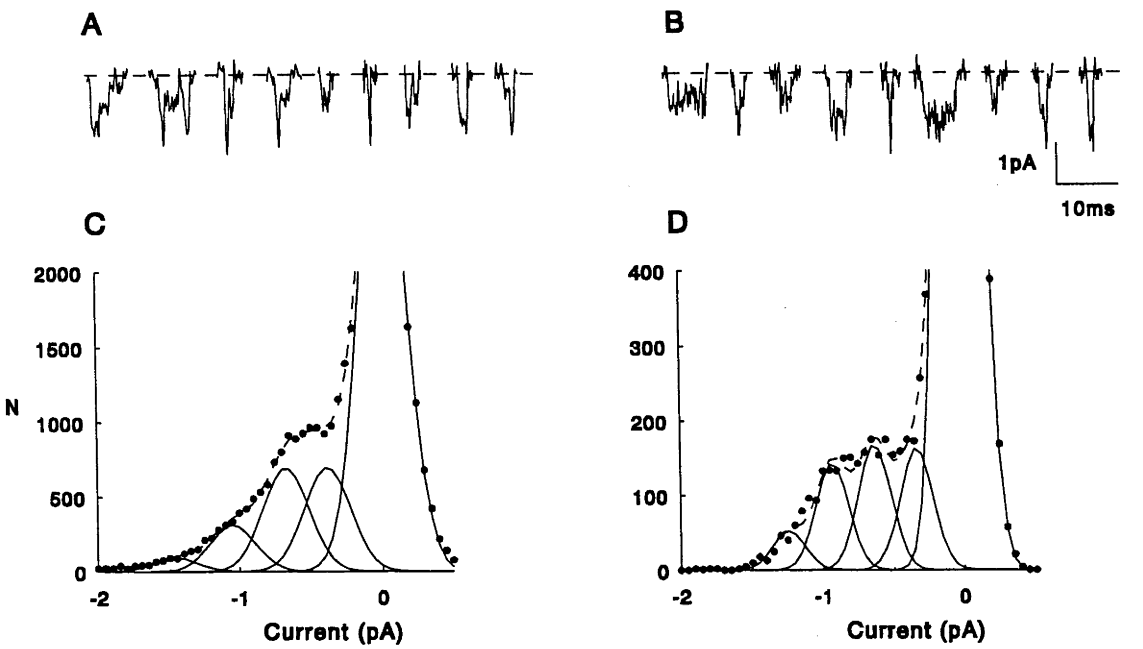


Fig. 5-7. Subconductance states of transient and persistent sodium channels recorded from a inside out patch. Individual transient (**A**) and persistent (**B**) sodium channels selected from the same inside-out patch recorded at -70mV show different subconductance levels. All-point histograms of transient (**C**) and persistent (**D**) sodium channels were fitted with Gaussian distributions using "Peakfit" (Jandel Scientific). In C and D, 5 Gaussians with the same SD as the major (baseline) peak were fitted and their sum is shown by the broken line. The vertical scale has been truncated for clarity.

5.6. Summary

1. Single transient and persistent sodium channels elicited by depolarizing voltage pulses were recorded in cell-attached and inside-out patches. A transient burst of channels was followed by late-opening (persistent) channels with low open probability.

2. TTX blocked both transient and persistent sodium channels when it was applied to the solution in the patch microelectrode.

3. Conditioning depolarizing pre-pulses that inactivated transient channels and occasional bursting channels had no effect on persistent channels.

4. The open probability of persistent channels reached a maximum at more negative potentials than transient channels.

5. The average open time of persistent channels increased with depolarization, a characteristic not shared by transient channels.

6. The conductance of transient and persistent channel was similar: both had several conductance levels and the main-state conductance appeared to increase at more depolarized potentials.

Chapter 6

SODIUM CURRENTS RECORDED FROM SINGLE SINUS VENOSUS CELLS OF THE TOAD

6.1. Introduction

The cells of most regions of the heart generate action potentials that depend on an increase in sodium conductance when depolarized (Fozzard, January & Makielski, 1985; Kunze & Brown, 1989). Thus in ventricular, atrial and purkinje cells, sodium currents become activated at depolarized potentials (Giles, 1989). These currents show a rapid time-dependent inactivation during sustained depolarizations and are only fully reactivated upon returning to membrane potentials more negative than -100 mV (Brown, Lee & Powell, 1981a; Brown, Lee & Powell, 1981b). Characteristically, such sodium channels are blocked by tetrodotoxin, but only when it is applied at high concentrations (Brown, Lee & Powell, 1981a; Kunze & Brown, 1989).

There are conflicting views, however, as to whether voltage-dependent sodium channels are present in cardiac pacemaker cells. As pacemaker cells continue to generate action potentials in the presence of TTX (Yamagishi & Sano, 1966; Kreitner, 1975), an inward TTX-sensitive sodium current is evidently not necessary for the upstroke of the action potential. Indeed, it is believed that there is no voltage-dependent sodium current in single cells isolated from regions of amphibian heart which contain pacemaker cells (Campbell, Rasmusson & Strauss, 1992). In mammalian pacemaker cells, sodium channels were considered to be present but inactivated in the range of membrane potentials normally experienced

during pacemaker activity (Kreitner, 1975). However, more recent studies have indicated that sodium channels are present in the majority of pacemaker cells isolated from mammalian sinoatrial node cells and contribute to the upstroke of the action potential (Denyer & Brown, 1990). In the sinus venosus of the toad, TTX (100 nM) reduced the rate of generation of pacemaker action potentials to about half the control rate. When lower concentrations of TTX were applied, it became apparent that even quite low concentrations of TTX caused bradycardia (Bywater *et al.*1989; Edwards, Hirst & Bramich, 1993). This indicates that sodium channels are present either in pacemaker cells or in the driven cells coupled to the electrical syncytium containing the pacemaker cells.

It has been shown that there is a "persistent" TTX-sensitive sodium current that is activated at negative potentials and probably contributes to diastolic depolarization (Chapter 3; Saint, Ju & Gage, 1992; Ju, Saint & Gage, 1992).

A transient voltage-dependent TTX-sensitive sodium currents in sinus venosus cells of the toad will be described in this Chapter, since a persistent sodium current recorded from sinus venosus cells of the toad under the same conditions has been described in Chapter 3, only for an editing reason. The membrane potentials at which transient and persistent currents are active suggest that they are responsible for the effects of TTX on intact sinus venosus preparations.

6.2. Identification of single isolated pacemaker cells

It has been reported that cells from the central part of the SA node are unaffected by the Na-current blocker TTX, whereas those from the crista terminalis or transitional zone between nodal and atrial cells frequently exhibit TTX-sensitive characteristics. Despite the fact that in intact sinus venosus, recordings were made from cells which had the characteristics of pacemaker cells and it has been shown that TTX influenced heart rate (Edwards, Hirst & Bramich, 1993), it is possible that the real pacemaker cells *in situ* do not contain TTX-sensitive sodium channels: some cells in the pacemaker region that are not the actual pacemaker cells may contain TTX-sensitive sodium channels that can influence heart rate when coupled to the pacemaker cells in a syncytium. Alternatively, the pacemaker cells themselves may indeed contain TTX-sensitive sodium channels. In order to be confident that recordings were made from pacemaker cells, the cells were defined not only morphologically but also electrophysiologically.

Experiments were carried out on cells isolated from preparations of sinus venosus which had been dissected free of adjacent atrial muscle fibres (Chapter 2). Sinus venosus cells then were isolated using the immersion method (Chapter 2). Cells had diameters of 4 to 5 μm and lengths of some 100 to 150 μm. Striations were less obvious in these cells (Fig. 6-1, top) than in ventricular cells prepared from the same animals (Chapter 2) as shown in Fig. 6-1, bottom). Pacemaker cells isolated from other amphibian hearts have been reported to have a size and appearance similar to these cells (Giles & Shibata, 1985).

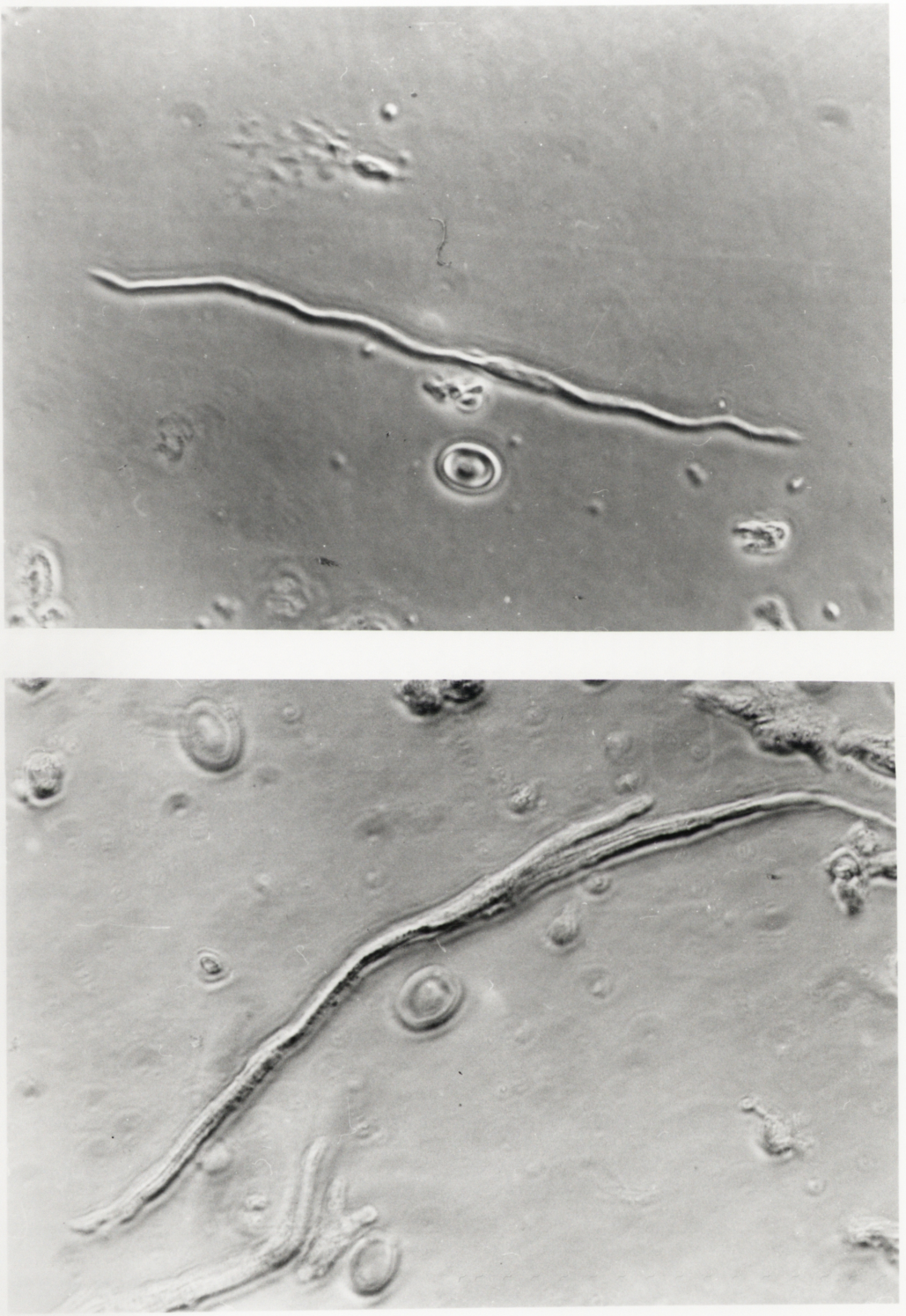


Fig. 6-1. An enzymically isolated single sinus venosus cell from toad (top); and an enzymically isolated single ventricular myocyte from toad (bottom). (Magnification x1760)

When electrophysiological recordings have been made from cardiac myocytes isolated from other amphibian and mammalian hearts, it has been found that cells isolated from either atria or ventricles always have an inwardly rectifying potassium current (Chapter 1) whereas pacemaker cells do not (Noble & Tsien, 1968; Giles & Shibata, 1985; Josephson & Browm, 1986; Irisawa, Brown & Giles, 1993). As a check on the identity of the cells used in this study, some preliminary experiments were carried out with no caesium in solutions and 2.5 mM K^+ in the extracellular solution. The intracellular (patch pipette) solution generally had the following composition (mM): KF 140; $MgCl_2$ 2; KEGTA 10; $CaCl_2$ 2; Na_2ATP 5; TES 10; pH adjusted to 7.2 with KOH. The solution bathing the cells had the composition (mM): NaCl 105; KCl 2.5; $CoCl_2$ 5; Hepes 10; $CaCl_2$ 2, $MgCl_2$ 1, pH adjusted to 7.2 with NaOH. Cells were held at 0 mV and the membrane potential stepped to -30, -60, -90 and -120 mV (Fig. 6-3A). Cells isolated from the sinus venosus displayed no inwardly rectifying current (Fig. 6-2B). No large inward current was seen in any of the 8 pacemaker cells examined in this way. When similar experiments were carried out under identical conditions using ventricular myocytes, prominent inwardly rectifying currents were generated by voltage pulses to negative potentials (Fig. 6-2C).

The voltage-dependence of activation of the inwardly rectifying current in ventricular myocytes was influenced by extracellular K^+ and the rectification was abolished when barium ions were added to the external solution, both characteristics of the inwardly rectifying potassium current (Hume & Giles, 1983; Giles & Shibata, 1985).

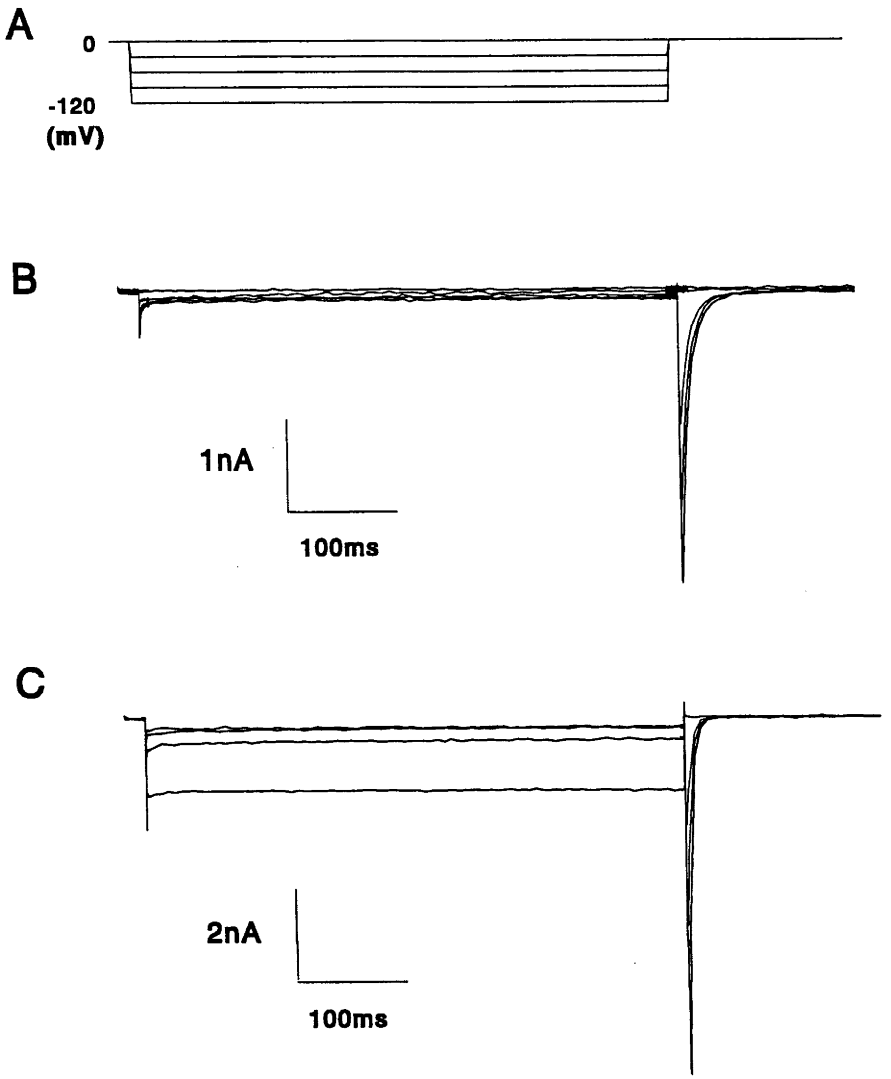


Fig. 6-2. A: the voltage protocol used to demonstrate the inward rectifier. The cells were held at 0 mV and given hyperpolarizing pulses for 500 ms to -30, -60, -90 and -120 mV (A). The currents generated in one cell with this a protocol are shown superimposed. **B**: Currents recorded in a sinus venosus cell in 2.5mM $[K]_o$. For pulses to potentials more negative than -30 mV, a large transient current was evoked when the membrane potential returned to 0 mV at the end of the hyperpolarization. **C**: Inwardly rectifying potassium currents in an isolated toad ventricular myocyte of the toad was evoked during hyperpolarization pulse negative to -90mV. Similar transient currents were recorded when the membrane potential returned to 0 mV at the end of the hyperpolarization.

In Fig. 6-3A, when extracellular K^+ concentration was increased from 2.5mM to 5 mM, the amplitude of the inward rectifier potassium current in ventricular myocyte was increased from 1.5 nA to 3.0 nA (-120 mV, 500 ms). The current was totally blocked when 0.5 mM barium chloride was added in the bath solution. Fig. 6-3B shows a current-voltage relationship (IV curves) for inward rectifier potassium currents in a ventricular cell exposed to 2.5 or 5 mM K^+ and the block by barium. The results demonstrate that the inward currents recorded in ventricular myocytes are indeed inward rectifier potassium currents. A comparison of the IV curves for the inward rectifier currents obtained in a pacemaker cell and ventricular myocytes is shown in Fig. 6-4. Even when the extracellular potassium concentration was changed from 2.5 to 5 mM no inwardly rectifying current could be recorded in sinus venosus cells at any negative potentials (Fig. 6-4A), in contrast to the situation of ventricular cells (Fig. 6-4B).

These observations indicate that cells which were isolated from sinus venosus have the morphological and electrophysiological properties of cells that have been identified as pacemaker cells (Irisawa, Brown & Giles, 1993; Zhou & Lipsius, 1992).

It can be seen in Fig. 6-2B that, in cells which displayed no inwardly rectifying potassium current, there was a prominent, rapid, transient, inward current when the potential was returned to 0 mV from potentials more negative than -30 mV. Ventricular cells, like those dissociated from sinus venosus, also generated transient inward currents under these conditions (Fig. 6-2C). It seemed likely that these transient inward currents were sodium currents that recovered from inactivation during the hyperpolarizing pulse and were then activated on return to 0 mV. The characteristics of the inward current in these cells showing no inward rectification were explored using more conventional pulse protocols.

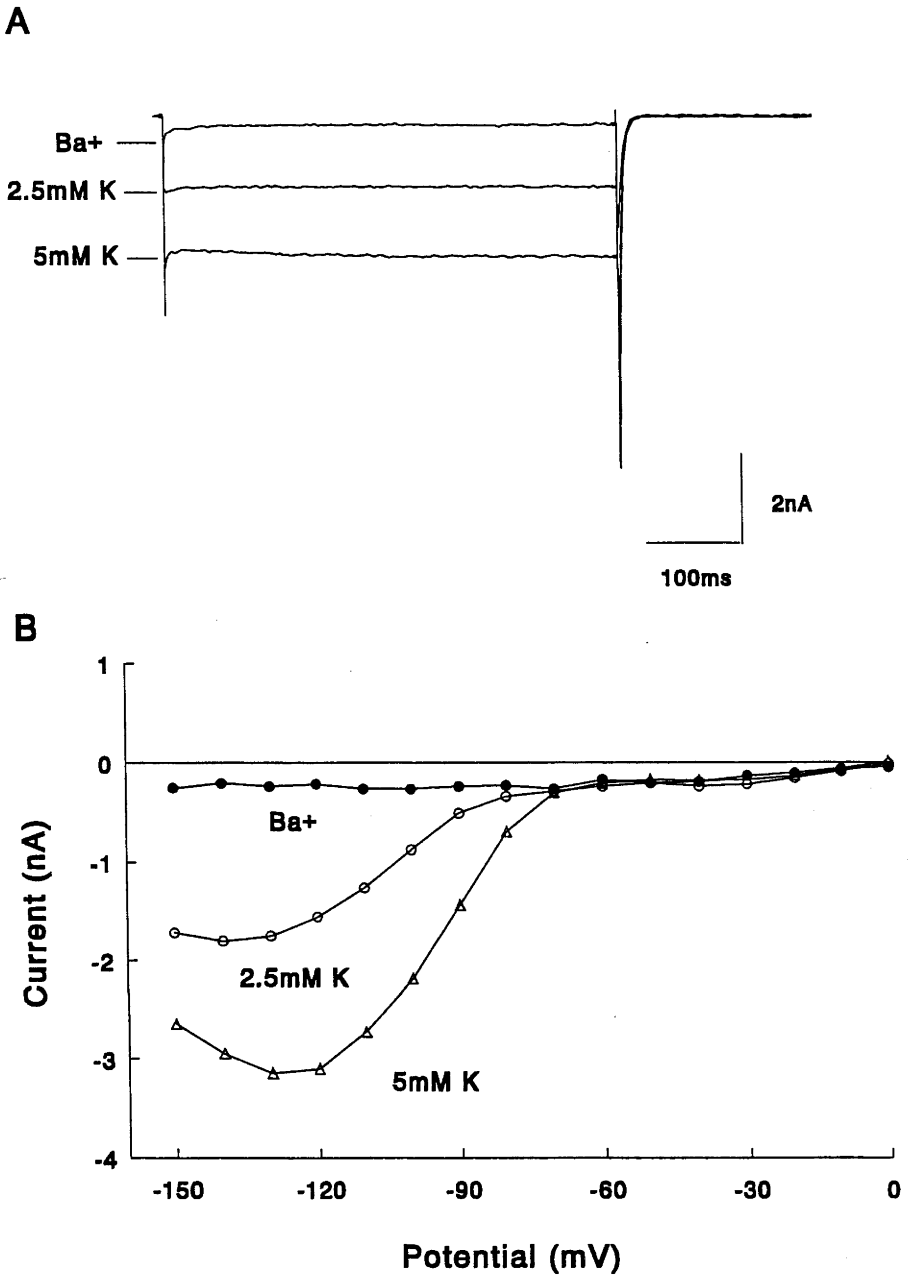


Fig. 6-3. Inward rectifier potassium current recorded in ventricular myocytes isolated from toad. **A**: the currents were elicited by a -120 mV pulse lasting 500ms from a holding potential of 0 mV. The amplitude of the current increased when the extracellular potassium concentration rose from 2.5 mM to 5 mM. The current was blocked by barium chloride (0.5 mM). **B**: Current-voltage relationships for inward rectifier potassium currents measured in 2.5mM K (open circles), 5mM K (open triangles), or 0.5 mM barium chloride (close circles)

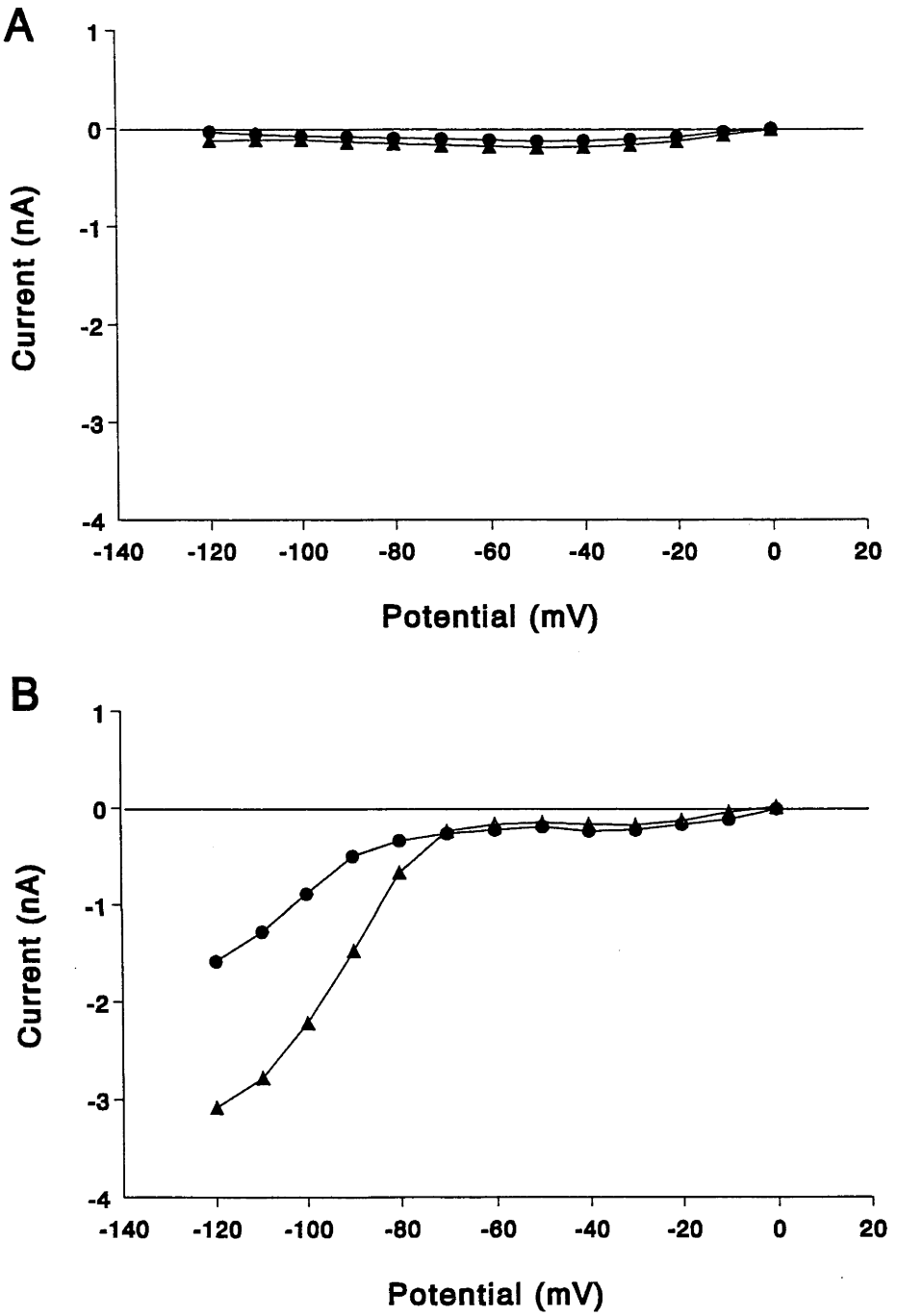


Fig. 6-4. A comparison of currents recorded from a pacemaker cell (**A**) and ventricular cell (**B**). **A**: the current-voltage relationships for inward rectifier potassium currents recorded in sinus venosus cell exposed to 2.5 mM K^+ (circles) or 5 mM K^+ (triangles). **B**: Current-voltage relationships for inward rectifier potassium currents recorded in a ventricular cell exposed to 2.5 mM K^+ (circles) or 5 mM K^+ (triangles).

6.3. The transient inward current is a sodium current

6.3.1. Reversal potential is close to the calculated sodium equilibrium potential

To prevent contamination of the inward currents with potassium currents, all experiments described below were carried out in solutions in which caesium ions were present in both the bathing solution and in the patch pipette (see Chapter 3: section 7). Recordings were made from 23 cells with an $[Na^+]_o$ of 104 mM and $[Na^+]_i$ of 10 mM, and from a further 5 cells with an $[Na^+]_i$ of 70 mM. In 26 of the 28 cells, depolarization to potentials more positive than -40 mV evoked obvious transient inward currents. Currents generated by voltage pulses to potentials between -40 and +60 mV from a holding potential of -130 mV in one cell are shown in Fig. 6-5A. The currents reached a peak within 2 to 3 ms and rapidly inactivated over the following 10 to 20 ms.

The relationship between peak current and membrane potential is shown in Fig. 6-5B. It can be seen that the peak current reached a maximum at a potential of -10 to 0 mV and reversed at +54 mV. Data from 8 pacemaker cells gave an average reversal potential of $+57 \pm 1.2$ mV, close to the calculated sodium equilibrium potential, E_{Na}, of +59 mV. In the 5 experiments carried out with an internal sodium concentration of 70 mM, the average reversal potential for the current was 13.1 ± 3.0 mV, also very close to the calculated E_{Na} of +10 mV. A current-voltage curve for one of these cells can be seen in Fig. 6-6A (open circles).

The inward current in this cell reversed at +10 mV. The shift in the current-voltage curve caused by changing the extracellular $[Na^+]$ from 104 to 54 mM in one of the experiments (choline substituted for Na^+) is illustrated in Fig. 6-6B. Currents were reduced in amplitude and the reversal potential shifted by about the 17 mV expected for a current carried predominantly by sodium ions.

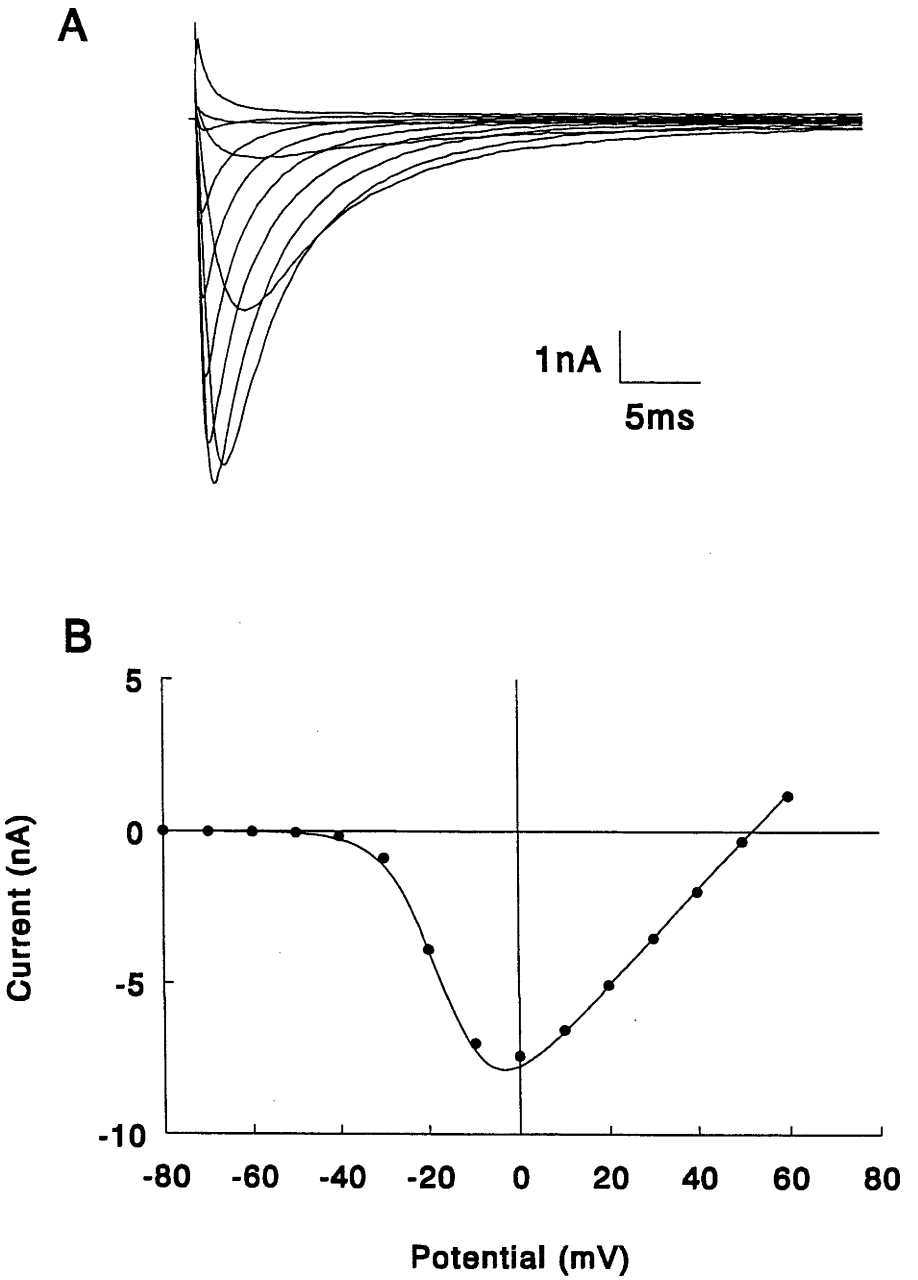


Fig. 6-5. Whole-cell recording of sodium currents from pacemaker cells. **A**: a family of currents evoked by depolarizing pulses to potentials between -40 and +60 mV (in 10 mV steps) from a holding potential of -130 mV. The external solution contained 104 mM sodium and the internal (pipette) solution contained 10 mM sodium. $CoCl_2$ (5 mM) and CsCl (5 mM) were also present in the external solution. **B**: the current-voltage relationship for the currents shown above. Maximum peak current amplitude was -7.4 nA (evoked by a step to 0 mV), and the current reversed at about 54 mV. The line through the data points is $I = G_{max}.(V-E_{Na})/(1+\exp((V'-V)/k))$ where V is clamp potential, E_{Na} = 54 mV, V' = -16 mV and k = 7 mV.

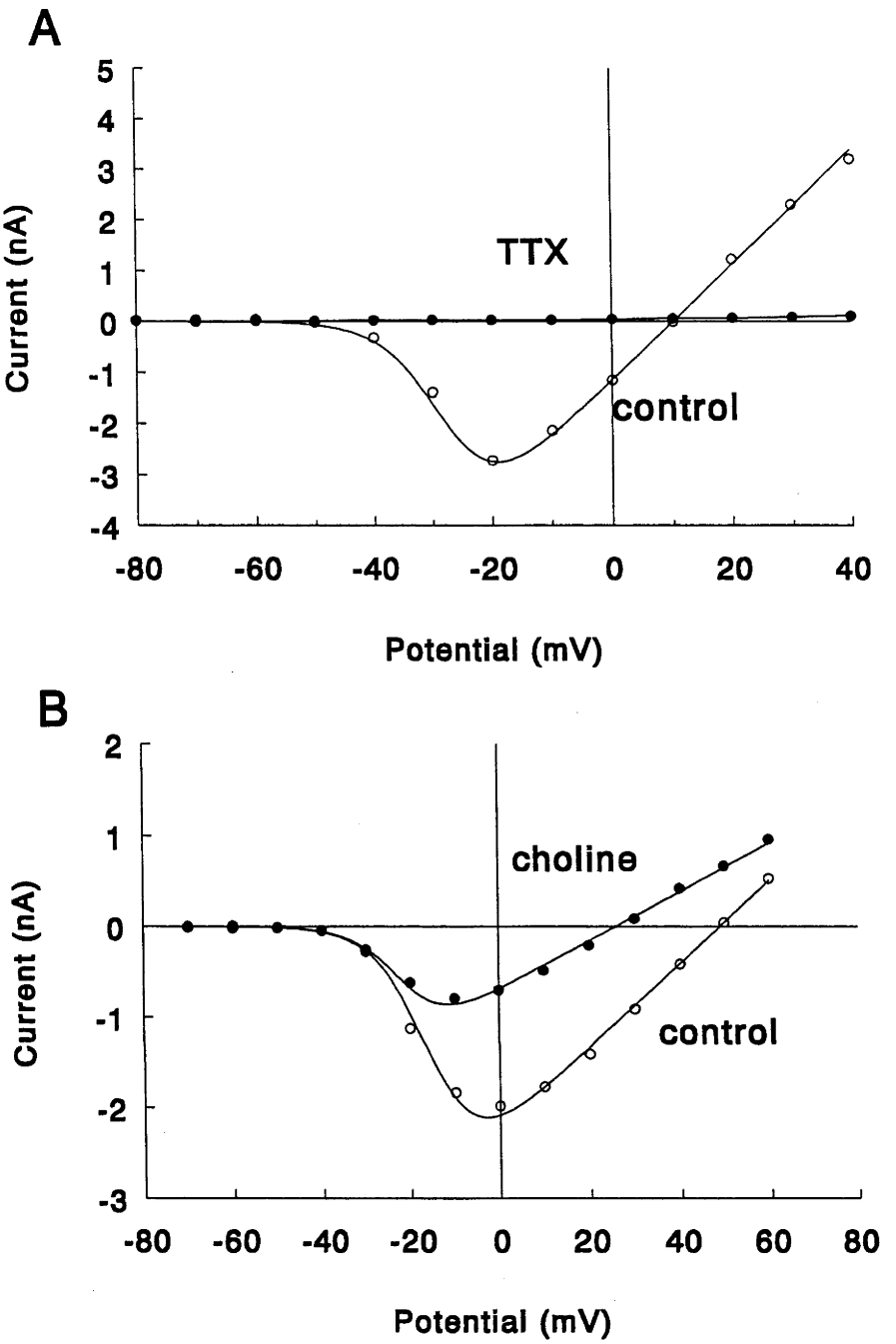


Fig. 6-6. Evidence that the transient current is a sodium current. **A**: current-voltage curves recorded with an internal sodium concentration of 70 mM before (open circles) and after (filled circles) addition of TTX (1 μM) to the extracellular solution. The line through the open circles, fitted as in Fig. 6-5, gave a reversal potential of +10 mV (V' = -27 mV, k = 5 mV). **B**: current-voltage curves recorded with an internal sodium concentration of 10 mM and an extracellular sodium concentration of either 104 mM (open circles) or 54 mM (filled circles, 50 mM choline chloride substituted for 50 mM sodium chloride). The reversal potentials were 48 mV (open circles) and 27 mV (filled circles).

6.3.2. Block of the transient current by TTX

The transient inward current was blocked by TTX (1 μM, filled circles, Fig. 6-7A). At lower concentrations, the Na^+ current was partially blocked, as illustrated in Fig. 6-7A. Exposure of a cell to 10 nM TTX reduced the current by about 50%. Increasing the TTX concentration to 100 nM blocked almost all of the current. The effect was not potential-dependent as can be seen in the current-voltage curves recorded from another cell (Fig. 6-7B) in control solution (open circles), in the presence of 10 nM TTX (filled triangles) and in the presence of 100 nM TTX (inverted triangles). TTX caused no change in the reversal potential or the voltage at which peak current was recorded, a result which also indicates good series resistance compensation in these experiments. Similar observations were made in another 9 cells in addition to the two illustrated in Fig. 6-7.

It is clear that the transient sodium current recorded in these isolated cells was sensitive to the same concentrations of TTX that changed the frequency and rate of rise of action potentials in the intact sinus venosus (Edwards, Hirst & Bramich, 1993).

6.4. Voltage dependence of the transient sodium current

The observations above indicate that the transient inward currents recorded in single sinus venosus cells were TTX-sensitive, voltage-dependent sodium currents. The functional role of this current in pacemaking activity depends, however, on whether the channels responsible can be activated over the range of potentials normally encountered in sinus venosus cells. In normal pacemaker activity, the diastolic potential ranges from about -65 mV to about -50 mV, the threshold potential for action potentials (Bywater *et al.*1989; Bramich, Edwards & Hirst, 1990).

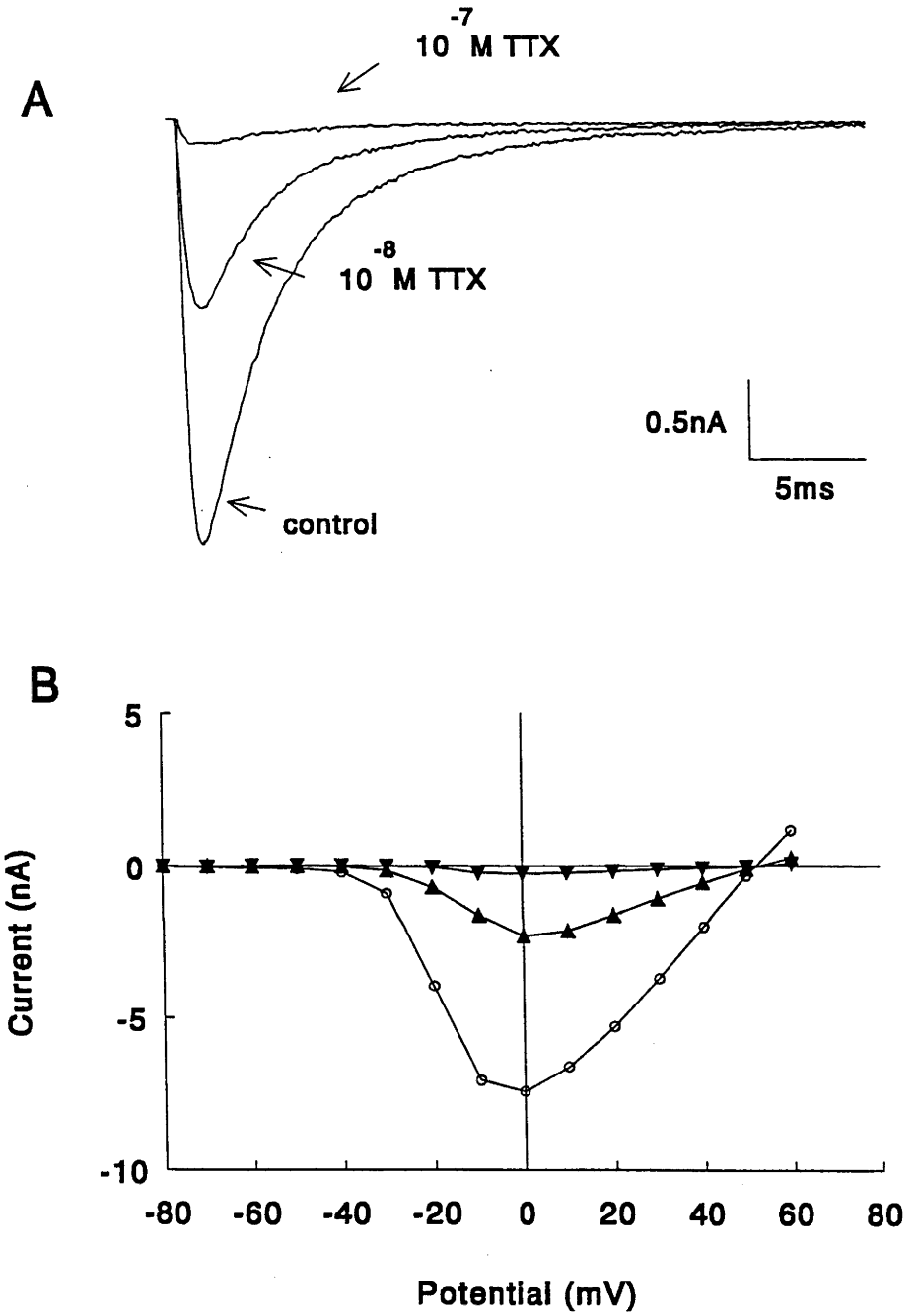


Fig. 6-7. Effect of TTX on the transient sodium current recorded from pacemaker cells. **A**: Currents elicited by a voltage pulse from -130 mV to 0 mV in control solution and after exposure to 10^{-8} M and 10^{-7} M TTX. **B**: Current-voltage curves in control solution (open circles) and in the presence of 10^{-8} M TTX (filled triangles) and 10^{-7} M TTX (filled inverted triangles).

If sodium channels were completely inactivated in this potential range, they could not contribute to the action potential. With this in mind, activation and steady-state inactivation curves for the transient current were determined in 11 cells.

The fraction of channels activated at any potential was measured by stepping the membrane potential from a pre-potential of -130 mV to potentials from -80 to +20 mV. The peak current was converted to conductance and normalised to the maximum conductance obtained at +20 mV. The inactivation curve was obtained from the peak amplitude of the transient current, generated by a voltage pulse to 0 mV following a 100 ms prepulse to potentials from -100 to +20 mV, normalised to the current obtained with the prepulse to -100 mV. Typical activation (triangles) and inactivation (circles) curves obtained in one cell are shown in Fig. 6-8.

The lines through the circles and triangles show the best fits of the Boltzmann equations $I = I_{max}/(1+\exp((V'-V)/k))$ and $I = I_{max}/(1+\exp((V-V')/k))$, respectively, where I is the peak current, I_{max} is the maximum peak current, V is the membrane potential, V' is the potential at which $I = I_{max}/2$ and k is a slope factor. V' values for activation and inactivation in Fig. 6-8 were -13 mV and -42 mV. Average values in 11 cells were -16 $\pm$ 1.9 mV for activation and -43 $\pm$ 2.1 mV for inactivation.

Similar experiments were done on single cells isolated from toad ventricles. These cells, like pacemaker cells, generated inward sodium currents when depolarized and the currents decreased in amplitude when TTX, 10 to 100nM, was added to the extracellular solution. However, the half maximum values for activation and inactivation of the sodium currents in ventricular cells, measured under identical conditions, were obviously more negative than in the pacemaker cells. The mean values of V' for activation and inactivation in 5 ventricular cells were -24 $\pm$ 3.6 mV and -55 $\pm$ 4.1 mV.

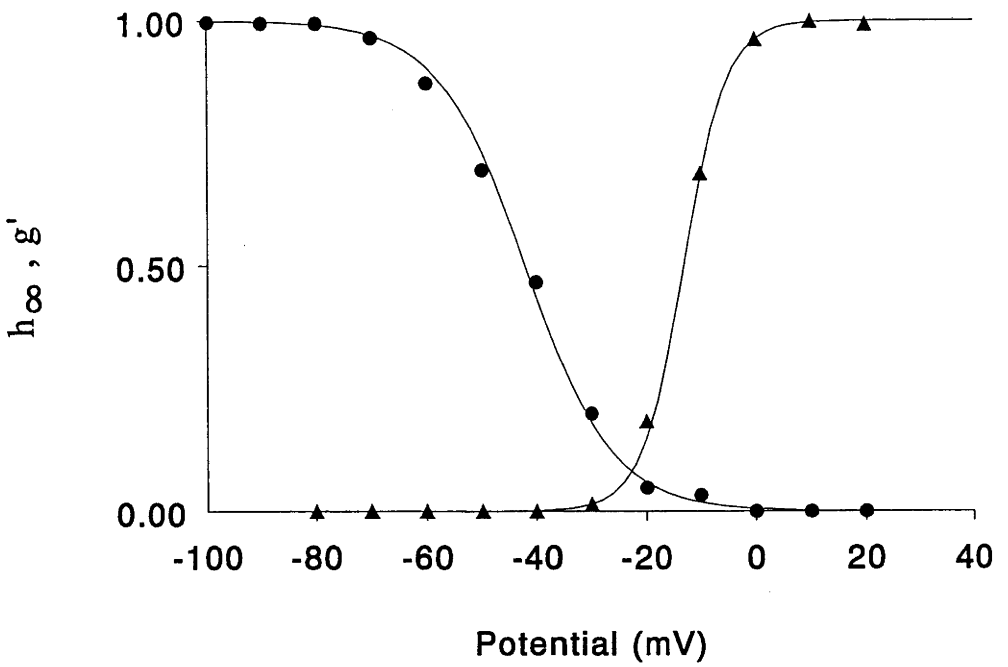


Fig. 6-8. Voltage dependence of activation and inactivation of the transient sodium current in a pacemaker cell. Currents activated by positive voltage pulses from a holding potential of -130 mV were converted to conductance with an E_{Na} = 55 mV, normalised to the maximum conductance and plotted against pulse potential to obtain the activation curve (triangles). The line through the points is the best fit of the Boltzmann equation (see text) with V' = -13 mV and k = 5.5 mV. The inactivation curve (circles) was obtained by plotting the normalised peak amplitude of currents, evoked by a depolarizing step to 0 mV following a 100 ms conditioning pre-pulse to levels from -130 mV to +20 mV, against the pre-pulse potential. Peak current amplitude was normalised by dividing by the maximum peak current. The line through the circles is a best fit of the Boltzmann equation (see text) with E_{Na} = 55 mV, V' = -42 mV and k = 8 mV.

6.5. Persistent sodium currents in sinus venosus cells.

see Chapter 3

6.7. Summary

1. Membrane currents were recorded from single pacemaker cells isolated from sinus venosus of toad using the tight-seal whole-cell voltage clamp technique.

2. Pacemaker cells were identified by their morphology and lack of an inwardly rectifying potassium current.

3. After calcium current, potassium current and non-selective cation currents activated by hyperpolarization had been blocked, depolarizing voltage steps initiated an inward current which consisted of transient and persistent components.

4. Both components of the inward current were sodium currents. They were abolished by tetrodotoxin (10 to 100nM), their reversal potential was close to the sodium equilibrium potential and their amplitude and reversal potential were changed as expected for sodium currents when extracellular sodium ions were replaced with choline ions.

5. The transient current was activated at potentials more positive than -40 mV while the persistent sodium current was seen at more negative potentials.

6. It was concluded that, in pacemaker cells, TTX-sensitive sodium currents contribute both to diastolic depolarization and to the upstroke of action potentials.

Chapter 7

GENERAL DISCUSSION

7.1. The major findings of the thesis

A persistent, TTX sensitive current was recorded from isolated single cardiac myocytes using tight seal whole-cell voltage-clamp techniques (Chapter 3). That the persistent current is carried by Na^+ ions was demonstrated in Chapter 3 (Fig. 3-2, Fig. 3-3). It was also observed, at the single channel level, that a late opening channel could be responsible for the persistent sodium current.

This is first time that persistent sodium currents have been quantitatively analysed in both whole-cell and single channel configurations of isolated cardiac myocytes. The experiments carried out in single myocytes isolated from different species (amphibia and mammals) and from different regions of the heart (ventricle, atrium, and sinus venosus) (see Fig. 3-1, Fig. 3-10, Fig. 3-11) suggest that the persistent sodium current exists extensively in cardiac tissue.

The fact that the persistent sodium current is more sensitive to TTX and antiarrhythmic drugs (Fig. 3-9, Fig. 4-3, Fig. 4-4) explains well the phenomenon that low concentrations of TTX or local anaesthetics shorten action potential duration (Dudel *et al.*1967; Coraboeuf, Deroubaix & Coulombe, 1979; also Chapter 4 Fig. 4-1). The contribution of the persistent current to the action potential has been clearly shown in Fig. 4-1 by using sodium channel blockers (TTX and lidocaine), and will be discussed further in this chapter.

The persistent sodium current has characteristics which are significantly different from that of transient sodium currents. These

characteristics may play an important role in physiological and pathophysiological conditions. A discussion about the possible mechanism of these differences is prompted by the single channel study.

The experiments show that there are sodium currents (both transient and persistent) in pacemaker cells. This observation contravenes a traditional view that pacemaker cells lack voltage-dependent, TTX-sensitive sodium channels (Giles, van Ginneken & Shibata, 1986; Campbell, Rasmusson & Strauss, 1992; Irisawa, Brown & Giles, 1993). A thorough description of transient sodium currents in pacemaker cells (Chapter 6) and the fact that the persistent sodium current could be recorded from cells identified as pacemaker cells both morphologically and electrophysiologically provides strong evidence that sodium current could be involved in pacemaking activity.

7.2. The characteristics of the persistent sodium current and contribution to plateau currents of action potential

The transient Na^+ current, responsible for the upstroke of action potentials, inactivates within a few milliseconds (Colatsky, 1980). Therefore, the shortening of action potential duration by TTX or local anaesthetics has been interpreted as due to block of a sodium window current, persisting during the plateau (Attwell *et al.*1979). Although this hypothesis can explain steady currents over a narrow range near the threshold potential, it is not adequate to explain the relatively large steady currents seen after strong depolarization (Shoukimas & French, 1980) (also see this thesis Chapter 3 Fig. 3-7), whereas persistent sodium currents can be observed at very positive potentials (Fig. 3-4, Fig. 3-12).

Although there have been many descriptions of a slow decay of sodium currents in cardiac muscle, showing more than one decay time constant (Reuter, 1968; Dudel & Rudel, 1970; Brown, Lee & Powell,

1981a; Colatsky, 1980; Ebihara & Johnson, 1980; Zilberter *et al.*1982; Benndorf, Boldt & Nilius, 1985; Fozzard, 1984) there are few reports of components of decay with time constants longer than tens of milliseconds. Isolation of TTX-sensitive currents (Kunze *et al.*1985) has previously revealed a slowly decaying component of sodium current with a time constant of several hundred milliseconds. These authors did not show, however, the voltage-dependency of activation and inactivation of this current. In rabbit Purkinje fibres, an ultra slow Na^+ component which inactivated with time constants varying between a few hundreds of milliseconds to tens of seconds has been described (Carmeliet, 1987). The current-voltage relation of this slow Na^+ current extended over a broad range of potentials, as negative as -85 mV. The voltage-dependence of inactivation, however, was described by a sigmoidal curve with a half maximum potential of -75.6 mV, which implied that the current would inactivate at least partially, at the depolarizing voltage during the plateau period of the action potential . Although it was observed that there were time-independent components, which could be regarded as a window current, a quantitative analysis of this window current was not made because of "secondary effects" (intracellular Na^+ & Ca^+ concentrations changed through the Na^+/Ca^+ and Na^+/K^+ exchange system) occurring during very long voltage clamps on multicellular Purkinje fibres (Carmeliet, 1987).

The experiments described in this thesis show that there is a persistent, TTX-sensitive sodium current in isolated cardiac myocytes, which decays with a time constant of the order of a second (Chapter 3 Fig. 3-8). The properties of the persistent current are incompatible with those expected of a "window" current (Attwell *et al.*1979), since it can be recorded essentially unchanged in magnitude at very positive potentials (Fig. 3-4, Fig. 3-12), well outside the area of possible overlap of the inactivation and activation curves of the transient sodium current. The persistent current is

also resistant to inactivation (Chapter 3, Fig. 3-7 and Fig. 3-12). Since the persistent current activates with membrane depolarization, but dose not inactivate at depolarized membrane potentials, and its time constant of decay is very slow, it could be a component of the plateau current of action potentials. Further evidence shows that drugs which block the persistent currents do shorten the action potential duration (Chapter 4 Fig. 4-1).

7.3. Class I Antiarrhythmic drugs and the persistent sodium current

The experiments showed that the Class I antiarrhythmic drugs, lidocaine and quinidine, block a persistent sodium current in rat ventricular myocytes (Ju, Saint & Gage, 1992; Chapter 4). Lidocaine and quinidine do this at concentrations that are within their therapeutic ranges (Rosen, Hoffman & Wit, 1975; Hoffman, Rosen & Wit, 1975). At the same concentrations, they had much less effect on the transient sodium current. A wide range of concentrations was not tested because accurate determination of smaller depressions of the persistent sodium current would be very difficult. Moreover, my primary interest was in the mechanism of the action of these drugs in the therapeutic range. The results clearly show that the persistent sodium current was much more sensitive than the transient sodium current to both lidocaine and quinidine (Chapter 4 Fig. 4-3, Fig. 4-4).

Although both lidocaine and quinidine blocked the persistent sodium current, they had different effects on the plateau phase of action potentials (Fig. 4-1). This can be attributed to the depression of voltage-activated potassium currents caused by quinidine, an effect that has been well described previously (Hiraoka, Sawada & Kawano, 1986; Imaizumi & Giles, 1987; Balser *et al.*1991).

It would be interesting to know why the persistent sodium current is more sensitive to these drugs than the transient sodium current. One

possible explanation is that the drugs block open channels. If sodium channels were open for a longer time during the persistent sodium current, the probability of block by a "channel blocking" drug would be increased. Alternatively, binding of these drugs to sodium channels may be both time- and potential-dependent, being greater at later times and more depolarized potentials, whether or not channels are open. Indeed, depression of sodium currents by lidocaine in cardiac muscle shows both of these forms of use-dependence (Clarkson *et al.*1988; Makielski, Alpert & Hanck, 1991; Starmer *et al.*1991). As the persistent sodium current is also recorded from pacemaker cells (Fig. 3-11), it would contribute an inward current that would increase in amplitude with progressive depolarization during a pacemaker potential. Block of such a current would tend to slow the rate of depolarization during the pacemaker potential and hence reduce the rate of firing of action potentials. Furthermore, cardiac muscle fibres would be less excitable when the persistent sodium current is blocked. It seems very likely, therefore, that the antiarrhythmic effects of lidocaine and quinidine are due, at least in part, to their effect on the persistent sodium current.

The channels responsible for the persistent current are clearly more susceptible to block by TTX, lidocaine and quinidine than channels responsible for the transient sodium current. Discovery of the reason for this may lead to the introduction of new treatments for arrhythmias.

7.4. TTX-sensitivity and sodium channel diversity

The experiments revealed that the persistent Na^+ current is more sensitive to TTX than transient sodium current recorded from the same cells. It also appears that toad heart muscle is more sensitive to TTX than mammalian heart muscle. Concentrations of TTX greater than 10 μM are normally required to completely block sodium currents in the mammalian heart (Mandel, 1992) whereas sodium currents in both the sinus venosus and

ventricle of the toad were completely blocked by 100 nM TTX. It also seems possible that the persistent sodium current is smaller in mammalian pacemaker cells than in toad sinus venosus. This hypothesis is reinforced by the observations that the rate of generation of pacemaker action potentials is halved by the addition of TTX in the toad, the rate of generation of pacemaker action potentials is only reduced by about 10% when 1 μM TTX is applied in guinea-pig sino-atrial preparations (G.D.S. Hirst and J. Choate, unpublished observations).

Although the presence of multiple sodium channel types has been clearly established (Noda *et al.*1986; Kayano *et al.*1988; Satin *et al.*1992b), it is not clear why the persistent sodium current is more sensitive to TTX than transient sodium current. Again it is not clear why the sodium channels in cane toads are more susceptible to TTX than rat ventricular cells and why amphibians seems to have more persistent sodium current than mammal. However, the finding supports the view that Na channels have tissue or cell-specific and functional-specific expression.

A mixture of TTX sensitivity has been found in other tissue preparations. For example, immature (Kostyuk, Veselovsky & Tsyndrenko, 1981; Fedulova, Kostyuk & Veselovsky, 1991) and adult rat dorsal root ganglion cells (McLean, Bennett & Thomas, 1988; Elliott & Elliott, 1993), and vertebrate neurones (Bossu & Feltz, 1984; Schlichter, Bader & Bernheim, 1991) have different TTX-sensitivity. The localization of different sodium channels to the specific regions in the nervous system supports the possibility that cell-specific regulation of this gene family is at the transcriptional level (Mandel, 1992). The molecular genetics experiments suggest that there are two levels of regulation of sodium channel expression (Mandel *et al.*1988; Mandel, 1992). The first level is a genetic on-off switch, which is flipped on in excitable cells or off in inexcitable cells. In the "off" position, none of the sodium channel genes are expressed.

In the "on" position, a second level of regulation comes into play, controlling which member of the sodium channel gene family should be expressed in a specific excitable cell type. However it is not clear whether TTX-insensitive Na channels in different tissure is always due to expression of the same gene and whether difference in post-translational modifications of α-subunit, or contributions of other subunits, are responsible for the observed differences in channel properties.

In contrast to nerve and skeletal muscle, functional diversity of sodium channels in cardiac muscle is not well documented. More resentely, a cardiac sodium channel α-subunit mutant was found, with Tyr substituted for Cys at position 374. It converts TTX-resistant sodium channel to TTX-sensitive channel (Satin *et al.*1992a). Satin *et al.*(1992b) also reported that Na current obtained from expression of cardiac α-subunits in oocytes shows relative resistance to TTX and has faster kinetics than Na current obtained from expression of the α-subunit of brain or skeletal muscle. TTX-sensitive current obtained by expression in *Xenopus* oocytes of the α-subunits of the rat brain and skeletal muscle Na channels show abnormally slow decay kinetics. It seems likely that a gene level regulation could exist in cardiac Na channels and it is responsible for different drug sensitivity and channels kinetics.

7.5. Single channel events underlying the persistent sodium current

A traditional explanation for slow Na currents is that this current is due to re-opening of some of the channels which produce the transient current (Kunze *et al.*1985), or a single set of Na channels which shifts between two "modes" of behaviour (Patlak & Ortiz, 1986; Duval *et al.*1992; Alzheimer, Schwindt & Crill, 1993). If the persistent currents were due to

repeated openings of channels that contribute to the transient current, it would be expected that the amplitude of the persistent current should be related to the amplitude of the preceding transient current. This was not observed. In contrast, persistent currents can be recorded when the transient current is totally inactivated by changing the prepulse potential (Fig. 3-4, Fig. 3-12).

In addition to late sporadic channel activity, single channel studies did reveal a late chattering channel activity as described by others (Patlak & Ortiz, 1986; Nilius, 1987; Nilius, 1988; Kiyosue & Arita, 1989). It has been suggested that this chattering results from individual channels switching between two modes of gating (fast gating or slow gating). A slow gating behaviour of sodium channels could be responsible for slowly inactiviting sodium currents (Moorman *et al.*1990; Zhou *et al.*1991). It has been suggested that chattering channels are due to activity of the same Na channels that give rise to the transient Na^+ currents at the start of the depolarizing pulse (Patlak & Ortiz, 1986). Moorman *et al.* (1990) suggested that structural mechanisms for fast and slow gating are encoded by a single mRNA. Therefore it is unlikely that more than one population of channels are involved in the different gating behaviour. Experiments described here demonstrated that chattering activity could be inactivated totally by changing the prepulse potential in the same way as early channel activities which are responsible for the transient Na current. Similar results have been reported in frog muscle (Patlak & Ortiz, 1986), and adult rat ventricular myocytes (Burnashev *et al.*1991). Hence it seems unlikely that channel chattering could responsible for persistent sodium current recorded in whole-cell configuration (Fig. 5-4).

An alternative hypothesis uses a second open state, connected directly to the inactivated state, to explain late Na channel activity (Chandler & Meves, 1970; Bezanilla & Armstrong, 1977; Sigworth, 1981; Aldrich,

Corey & Stevens, 1983). However, if late opening channels are due to the normal transient channel occasionally returning from the inactivated state to the open state, one would predict that these late channels would have the same open time as the early channels. Therefore, the late openings of such channels usually would consist of isolated short openings whose mean lifetime should be insensitive to membrane potential (Aldrich, Corey & Stevens, 1983). Patlak & Ortiz (Patlak & Ortiz, 1986) observed a kind of late sporadic channel activity, e.g so called "background" channel in frog sartorius muscle. The background channel had many properties in common with the predictions of the Aldrich et al (1983) model. However, late individual opening sodium channels recorded in my experiments which have characteristics consistent with persistent whole-cell sodium current (Fig. 5-1A) appear to be different from the late opening "background" sodium channels described by Patlak and Ortiz (1986). In contrast to background channels, the frequency of persistent channels did not depend on the presence of transient channels (Fig. 5-1; Fig. 5-4) and a marked contrast in the activation of the two types of channel was observed. In the single channel experiments the open probability reached a maximum at about -60 mV (Fig. 5-5) whereas the transient channels did not reach maximum open probability until more positive potentials (normally about -40 mV). This difference in activation was also seen in the conductance-voltage relationships (g/v curves) for the whole cell currents (Fig. 3-6). However, it should be noted that g/v relationship for persistent current is not a true reflection of the single channel open probability, since the channel open time increases with depolarization (Fig. 5-6). This explains the discrepancy between the voltage dependence of activation at either the single channel or whole cell level. Nevertheless, it is clear that the voltage dependence of activation of the tow channels is different. The late channel openings also appear resistant to inactivation (Fig. 5-1). Kiyosue & Arita (1989) also

observed that hyperpolarization preceding step depolarization was not necessary to induce late sodium channel activities in guinea pig ventricular myocytes.

It seems very likely that the persistent current is generated by channels different from those that are responsible for the transient sodium current, a hypothesis which is strengthened by the demonstration that it is more sensitive to block by TTX and antiarrhythmic drugs than the transient current (Fig. 3-9, Fig. 4-2, Fig. 4-4). In particular, the persistent current had a very different dependence on voltage (Fig. 3-6) and was relatively insensitive to inactivation compared with the transient current (Fig. 3-7, Fig. 3-12). The difference in activation has also been shown in single channel recordings (Fig. 5-5).

The voltage-dependence of the activation of the persistent currents or channels in other tissues is very similar to that reported here for cardiac muscle (Gage, Lamb & Wakefield, 1989; Duval *et al.*1992). It is interesting to note that poneratoxin, a new toxin from an ant venom induced a slow Na current in frog skeletal muscle fibres which starts to activate at -85 mV (40 mV more negative that that of the fast one) and inactivates very slowly (Duval *et al.*1992). Although they believed that the slow Na current was caused by an interconversion between two gating modes of the Na channels, it is hard to explain why the two current components have different kinetics and voltage-dependences.

However, the late opening channels and early opening channels seem to have similar subconductance and conductance states as shown in (Fig. 5-7). Although these results so far do not exclude the possibility that persistent channels represent a different low probability mode of activity of transient channels, a simpler explanation is that transient and persistent channels have a different structure. This hypothesis is consistent with ^{3}H-TTX binding experiments which show that there two distinct binding site in

cardiac tissue, with high and low affinity for TTX (Catterall & Coppersmith, 1981; Renaud *et al.*1983).

There is also evidence for the involvement of more than one mRNA species in controlling the inactivation process, which suggests that a protein, encoded by an mRNA smaller than that for the ß-subunit, may be necessary for the channel inactivation (Krafte *et al.*1988).

7.6. The persistent sodium current could be modulated

The persistent current has characteristics similar in many respects to persistent sodium currents that have been described in squid axon (Gilly & Armstrong, 1984), mammalian hippocampal neurons (French & Gage, 1985; French *et al.*1990) and mammalian skeletal muscle (Gage, Lamb & Wakefield, 1989). In particular, the currents are much smaller than transient sodium currents, are activated close to the resting membrane potential and inactivate very slowly.

In the experiments reported here, the amplitude of persistent currents was measured by subtracting the current recorded in the presence of TTX or antiarrhythmic agents from control records. The measurement thus depends on the assumption that TTX has no secondary effects on other membrane currents. This assumption seems reasonable since a current of the same amplitude was obtained when extracellular Na^+ was replaced by choline (Chapter 3, Fig. 3-2). The amplitude of persistent currents, however, varied from cell to cell, even when calculated as a percentage of transient sodium current (table 4-1, table 4-2). One possibility is that the persistent sodium current may be modulated by some unknow intracellular factors. A preliminary experiment showed that the amplitude of the persistent current increased under anoxic situations. In these experiments Na-cyanide was added to the intracellular solution to depress ATP synthesis. In order to block ATP dependent K^+ current, glibenclamide was present in the

extracellular solution. The ratio of persistent current to transient current under anoxic condition was increased to $0.94 \pm 0.26\%$ (n=4). The results suggest that the amplitude of persistent currents could be modulated by some metabolic products which are increased during anoxic conditions. However, more experiments have to be done before reaching a conclusion.

Recently, more evidence has shown that accumulation of metabolic products during hypoxia or anoxia might influence channel function (Numann, Catterall & Scheuer, 1991; Burnashev *et al.*1991; Wallert *et al.*1991). Ruppersberg *et al.* (1991) proposed a model in which disulfied bridges alter the mobility of the inactivation "ball" (see Chapter 1, section 1.2.3), according to the observation that inactivation of cloned K^+ channels is regulated by the reducing agent. However, in contrast to potassium channels, the possible contribution of cysteine-based disulfide bridges to inactivation of sodium channels is unclear, although there have been reports that oxidizing agents and reducing agents change inactivation of sodium channels (Stampfli, 1974; Strupp *et al.*1992).

It would be interesting to know whether and how metabolic products influence persistent sodium current, since the persistent sodium current may play a role in the generation of cardiac arrhythmias. If the persistent current becomes significant in ischaemic cardiac tissue, it could cause arrhythmias by depolarizing the membrane, thus firing action potentials. If so, the persistent sodium current may prove to be an important target for new antiarrhythmic agents.

7.7. TTX-sensitive sodium currents in pacemaker cells

Single cells, isolated from sinus venosus, failed to display inward rectification (Fig. 6-2. Fig. 6-3, Fig. 6-4), which is regarded as a distinguishing property of pacemaker cells (Irisawa, Brown & Giles, 1993).

Virtually all of these pacemaker cells had a large transient inward TTX-sensitive sodium current when depolarized. Thus, it would appear that pacemaker cells of the sinus venosus contain voltage-dependent sodium channels that influence the frequency of action potentials. At first sight, this observation is surprising since one of the criteria used to identify pacemaker cells has been a lack of TTX-sensitive sodium channels (Irisawa, Brown & Giles, 1993). The basis for this is obscure however. It is well recognised that TTX can slow the heart rate in mammals (see, for example, Abraham *et al.*1989). The continuation of action potentials in the presence of TTX does not indicate that there are no TTX-sensitive sodium channels but merely that other inward currents are sufficient to generate action potentials. When recordings are made from single isolated pacemaker cells in the absence of TTX, sodium currents are readily detected (Denyer & Brown, 1990).

It is possible that failure to record sodium currents in pacemaker cells in some studies may have been due to the isolation procedure. I noted during early experiments in my study that Na channels in toad pacemaker cells were quite labile. If single cells were prepared by prolonged digestion with enzyme, or if a high concentration of enzyme was used, they often lacked sodium currents. It may be that the enzymes depressed the amplitude of sodium currents, as has been reported previously (Howe & Ritchie, 1990).

The sodium currents recorded from pacemaker cells shared many of the characteristics of other TTX-sensitive sodium currents. However, activation and inactivation occurred at more positive potentials than normally found in other cardiac cells (Kunze & Brown, 1989) even compared to the sodium currents recorded from ventricular myocytes from the same animals using identical procedures. The reason for this difference in sinus venosus and ventricular cells is not clear.

It seems very unlikely that isolation procedures selected a population of cells that were not pacemaker cells but influenced electrical activity in pacemaker cells. Rather, I propose that pacemaker cells of the sinus venosus in cane toads generate TTX-sensitive transient and persistent sodium currents. These currents are activated over the range of membrane potentials normally seen in pacemaker cells. Although small, they would have a substantial influence on potential in these cells which have a high input impedance (Campbell, Rasmusson & Strauss, 1992).

7.8. The persistent sodium current could be a pacemaker current

In addition to a transient TTX-sensitive sodium current, about half of the sinus venosus cells examined displayed a clear TTX-sensitive persistent sodium current which has not been reported in pacemaker cells previously. It was mentioned above that the integrity of transient sodium currents was sometimes impaired by the cell isolation procedure. The absence of a persistent sodium current in some preparations may indicate that this current is more labile than the transient sodium current. This may be due to the small amplitude of the persistent current which becomes obvious only when the "difference" current is revealed by subtraction of currents recorded with and without TTX. This current is active in the potential range more negative than the threshold for action potentials and would be expected to contribute to the pacemaker current. It is hardly surprising, therefore, that TTX slows the frequency of action potentials.

The slowing of heart rate caused by TTX can be explained by removal of the persistent current and consequent slowing of the rate of diastolic depolarization. Furthermore, since TTX is as effective as caesium in slowing pacemaking activity in these preparations (Edwards, Hirst & Bramich, 1993), the persistent sodium current must make as significant a contribution to the pacemaking current as the cation channels activated by

hyperpolarization (DiFrancesco, 1985). These experiments give further support to the idea that pacemaking activity results from the coordinated activity of a variety of ion channels (Brown *et al.*1984b).

The current underlying pacemaker activity has been considered as a very complex current system. The evidence so far leads to a general conclusion that no one ionic current system is alone responsible for pacemaking (Irisawa, Brown & Giles, 1993). In addition to large voltage-dependent Ca^+ and K^+ currents and transporter-mediated sarcolemmal currents (I_{Na}/K and I_{Na}/Ca), hyperpolarization-activated cation current (I_f) and inward background current (I_B) have been considered play an important role in the generation of the spontaneous diastolic depolarization.

The cells of the pacemaking region of the heart have a relative positively resting membrane potential. The quiescent sino-atrial (SA) node cell, for example, has a resting potential near -40 mV and the maximum diastolic potential is approximately -50 to -60 mV. In contrast, resting membrane potentials of ventricular cells are around -70 mV to -80 mV (Irisawa, 1989). The low resting potential in pacemaker cells has been attributed to a high ratio of Na permeability to K permeability (P_{Na}/P_K). The absence of inwardly rectifying K channels provides a low P_K (Noma *et al.*1984);(also see Chapter 6 Fig. 6-2, Fig. 6-3) favouring the high P_{Na}/P_K ratio; however, a high value of P_{Na} is still suggested in the pacemaker cells. The theoretical necessity of a background Na (I_B) is acknowledged in nearly all mathematical models of primary pacemaking activity (Bristow & Clark, 1983; Brown *et al.*1984a; Rasmusson *et al.*1990). In these models, a large background leak conductance has usually been assumed (Noble, DiFrancesco & Denyer, 1989) in order to simulate the normal pattern of action potentials. A direct experimental observation of I_B currents has been reported recently from sino-atrial node cells of rabbit heart (Hagiwara *et al.*1992) and from ventricular and atrial cells of the guinea-pig (Kiyosue *et al.*1993)

The persistent sodium current recorded from sinus venosus cells is different from I_B. Firstly, I_B is insensitive to TTX (Hagiwara *et al.*1992), whereas the persistent sodium current is TTX-sensitive (Fig. 3-11a). Secondly, I_B is voltage-independent, its current-voltage relationship is almost linear, whereas the persistent current is activated at depolarizing membrane potentials (Fig3-3; Fig. 3-11c). Thirdly, I_B has poor cation selectivity, The reversal potential of I_B was around -21 mV at physiological concentrations of intracellular K^+ and extracellular Na^+, whereas the reversal potential for persistent current is close to the sodium equilibrium potential. Therefore Na^+ conductance increases in the period of diastolic depolarization could be due to persistent sodium channels in pacemaker cells, especially at more positive potentials close to the I_B current reversal potential.

I_f currents are activitied by hyperpolarization, and have slow kinetics. Furthermore, I_f currents are modulated in opposite ways by ß-adrenergic and cholonergic stimuli that allows a fine rhythm control by the autonomic nervous system. Because of these specific properties, I_f has been considered as a current designed to initiate a slow diastolic depolarization and to control its rate of rise in response to neurotransmitter-induced modulation (DiFrancesco, 1981a; 1981b; 1993). However, on the basis of its negative threshold potential and its slow time course of activation, Yanagihara and Irisawa concluded that I_f plays little role in normal spontaneous pacemaker activity. Nathan (1987) has reported that in cultured SA node cells, the threshold for activation of I_f is approximately -65 mV but that because of its slow time course of activation, it is not measurable during the first 300 ms of a voltage-clamp pulse. At low doses, Cs^+ is a selective blocker of I_f, it causes a substantial slowing of beating rate, indicating a contribution of I_f to the pacing of these cell. However, several independent laboratories have demonstrated that when Cs^+ is applied to SA node cell in concentrations sufficient to completely and selectively block I_f, the rate of spontaneous

pacemaking is slowed (by approximately 10-30%), but pacemaking is not arrested (Denyer & Brown, 1990; Campbell, Rasmusson & Strauss, 1992; Giles, van Ginneken & Shibata, 1986; Noble, 1984). In addition, in frog sinus venosus, Gile and Shibata (1985) failed to record I_f. Nonetheless, sinus venosus cells generate normal spontaneous pacemaker activity under control recording conditions. These observations indicate that I_f is not essential for pacemaking.

As a candidate for pacemaker currents, the persistent sodium current appears superior to I_f in some respects. The persistent current activates more quickly than I_f and it inactivates very slowly, which allows a constant current flow during a depolarization. The amplitude of the persistent current recorded from sinus venosus cells is proportionally bigger than that recorded from ventricular cells. Considering that the input resistance in pacemaker cells is very high (Irisawa, Brown & Giles, 1993), a very small fluctuation in transmembrane current can easily elicit a large deflection of the potential in the pacemaker cells.

Hence it is reasonable to assume that persistent sodium current may play a more important role in diastolic depolarization than both I_B and I_f.

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