Depolarization evoked by acetylcholine in mesenteric arteries of hypertensive rats attenuates endothelium-dependent hyperpolarizing factor

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Objective During blockade of endothelium-dependent hyperpolarizing factor (EDHF), acetylcholine evoked larger and faster depolarization in mesenteric arteries of spontaneously hypertensive rats (SHR) than normotensive Wistar–Kyoto (WKY) rats. We studied the mechanism underlying this response and its role in the attenuation of EDHF.

Methods Electrophysiology, computational modelling and myography were used to study changes in membrane potential and effects on contractility.

Results The large acetylcholine-evoked depolarization in SHR was accompanied by contraction, but this was not seen in WKY rats. The depolarization depended on release of intracellular Ca^{2+} but was unaffected by nonselective cation channel inhibitors, gadolinium, lanthanum or amiloride. The depolarization was significantly reduced by the Ca^{2+} -dependent Cl^- channel inhibitors, niflumic acid or flufenamic acid, or alterations in Cl^- gradients using bumetanide ($\text{Na}/\text{K}/\text{Cl}$ transporter inhibitor) or external Cl^- replacement with isethionate. These drugs altered the time course of EDHF-evoked hyperpolarizations in SHR, making them indistinguishable from those in WKY rats. EDHF-induced relaxation was less sensitive to acetylcholine in SHR than in WKY rats, but this difference was eliminated following artery pretreatment with bumetanide.

Introduction

Vascular endothelial cells release several relaxing factors such as nitric oxide, prostacyclin and endothelium-dependent hyperpolarizing factor (EDHF) [1–3]. Although the nature of EDHF may be heterogeneous depending on the animal species and vascular bed [4], there is a general consensus that endothelial hyperpolarization, resulting from the opening of small-conductance and intermediate-conductance Ca^{2+} -dependent K^+ channels (SK_{Ca} and IK_{Ca}), is an essential prerequisite for EDHF-mediated hyperpolarization of smooth muscle in many vascular beds [4]. Since EDHF appears to be a dominant vasodilator in resistance arteries [5], any change in EDHF could alter the vascular tone.

In superior mesenteric arteries of spontaneously hypertensive rats (SHR), EDHF-mediated hyperpolarization is

Computational modelling in which the SHR fast depolarizing response was selectively modulated mimicked physiologically acquired results obtained in SHR and WKY rats during Cl^- -channel blockade.

Conclusions Acetylcholine evokes a fast depolarization in SHR but not in WKY rats, mediated by the opening of Ca^{2+} -dependent Cl^- channels. The depolarization is responsible for a constriction that reduces EDHF-mediated relaxation. Data suggest that Ca^{2+} -dependent Cl^- channels may provide a novel therapeutic target for improvement of endothelial dysfunction during hypertension. *J Hypertens* 25:345–359 © 2007 Lippincott Williams & Wilkins.

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decreased compared with that of normotensive Wistar–Kyoto (WKY) rats [6]. Impairment of EDHF-mediated responses during hypertension has also been reported in other vascular beds; however, the underlying mechanism is unknown [7]. Our studies in mesenteric resistance arteries of SHR have recently demonstrated that, after blockade of EDHF with K_{Ca} channel inhibitors, acetylcholine evoked a membrane depolarization that was effectively absent in WKY rat arteries [8]. Since the overall hyperpolarization induced by acetylcholine was significantly reduced in SHR [8], we hypothesized that the acetylcholine-evoked depolarization may counteract acetylcholine-induced, EDHF-mediated hyperpolarization and relaxation in this vascular bed. In the present study we have tested this possibility directly and investigated the mechanism underlying the acetylcholine-evoked depolarization in mesenteric arteries from SHR and WKY rats and its interaction with EDHF-mediated hyperpolarization using electrophysiological and computational modelling techniques.

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Methods

Blood pressure was measured in both WKY rats and SHR using the noninvasive tail-cuff method on animals acclimatized for 1 week to the apparatus.

Preparation of arteries

All experiments were approved by the Australian National University Animal Experimentation Ethics Committee under guidelines of the National Health and Medical Research Council of Australia. Twelve-week-old male SHR and age-matched normotensive male WKY rats were used. Rats were anaesthetized with ether and killed by decapitation. A 10 mm segment of a primary branch of the mesenteric arteries was removed, cleaned of fat and pinned to the base of a recording chamber. In some preparations, the endothelium was selectively disrupted in one-half of the segment length by gently rubbing the intimal surface with a human hair, as previously described [8]. Disruption of the endothelium was confirmed by the absence of acetylcholine-induced hyperpolarization. Tissues were superfused (3 ml/min) with Krebs' solution (NaCl 120 mmol/l, KCl 5 mmol/l, NaHCO₃ 25 mmol/l, NaH₂PO₄ 1 mmol/l, CaCl₂ 2.5 mmol/l, MgCl₂ 2 mmol/l, glucose 11 mmol/l) at 34–35°C, and gassed with 95% O₂:5% CO₂. In low-chloride experiments, the NaCl was replaced with an equimolar quantity of sodium isethionate or sodium methyl sulphate. *N*^w-nitro-L-arginine methyl ester (L-NAME 100 µmol/l) and indomethacin (10 µmol/l) were present in all experiments to inhibit nitric oxide and prostanoid production, respectively. Each preparation was taken from a different animal.

Electrophysiological experiments

After at least 40 min of equilibration, membrane potentials of vascular smooth muscle cells were recorded with conventional microelectrodes (tip resistance, 120–240 MΩ [9]) containing 0.2% propidium iodide in 0.5 mol/l KCl for identification of impaled cells. Acetylcholine (1 mol/l; 500 ms, 500 nA) was ionophoresed from a micropipette (tip diameter ~1 µm) onto the adventitial surface of the arteries, and the change in membrane potential was recorded [8]. A retaining current (200 nA) was used to prevent continuous release of acetylcholine from the pipette [8]. Ionophoretic application permitted some delineation of the kinetic properties of the different conductances activated by acetylcholine. In contrast, the slow steady changes in membrane potential evoked by bath application of acetylcholine mask the characteristics of the individual components. The peak concentration of acetylcholine at the tissue surface produced by the current ejection conditions used here (500 ms, 500 nA) was calculated to be between 4 µmol/l and 170 nmol/l with the ionophoretic electrode at a distance of 20–50 µm from the adventitial surface (see equation A13 in [10]).

For the depolarization study, apamin (0.5 µmol/l SK_{Ca} inhibitor) and charybdotoxin (60 nmol/l, large-

conductance and intermediate-conductance K_{Ca} inhibitor) were superfused for at least 15 min before the application of acetylcholine. In preliminary experiments, apamin and the selective IK_{Ca} inhibitor, TRAM-34 (100 nmol/l, 1 µmol/l) were used. The combination of these drugs, however, often produced an incomplete block of the hyperpolarization and a variably sized depolarization. We therefore chose to use the combination of apamin plus charybdotoxin as this produced a rapid and complete abolition of the hyperpolarization in both SHR and WKY rats, with only a small 2–3 mV change in membrane potential (see Results section and [8]).

Both 1-(6-((17β-3-methoxyestra-1,3,5(10)-trien-17-yl)amino)hexyl)-1*H*-pyrrole-2,5-dione (U-73122) and cyclopiazonic acid (CPA) were superfused for 30–40 min, as control experiments showed that superfusion of U-73122 (10 µmol/l) or CPA (10 µmol/l) for 30 min abolished the acetylcholine-induced hyperpolarization. The other drugs were superfused for 10–25 min. In low-chloride experiments, records of membrane potential were corrected for the change in liquid junctional potential of the electrode by direct measurement of the effects of these solutions before cell impalements. In some experiments, vessels were preconstricted with phenylephrine (1 µmol/l), and simultaneous changes in membrane potential and changes in vessel diameter [9] were measured to evaluate the functional role of the acetylcholine-evoked depolarization.

The peak response (mV, µm) and the time from acetylcholine application to the peak amplitude of the response [time to peak (TTP), s] were measured for the acetylcholine-induced responses. Acetylcholine was delivered ionophoretically, and therefore diffusion delays caused dispersion in the times of arrival of acetylcholine at any cell as well as influencing the peak concentration reached at each cell [10]. The decays of the membrane potential transients were therefore complicated functions of diffusion rates, both within the extracellular domain in the syncytium and outside the tissue, and of the rate of hydrolysis of acetylcholine. Furthermore, the preparation was not electrically isopotential and so a range of electrotonic conduction delays influenced the membrane potential response. The duration of the membrane potential transient was therefore not included as a measured or simulated characteristic in this study.

Measurement of relaxation

EDHF-mediated relaxation was measured in arteries from WKY rats and SHR under isometric conditions using a Mulvany–Halpern-style myograph. One-millimetre-long segments of vessel were mounted between two 40 µm wires and equilibrated for 30 min prior to stretching to an equivalent tension of 80 mmHg. After a further 30 min equilibration period, preparations were constricted with three sequential concentrations of phenylephrine (1, 2 and

10 $\mu\text{mol/l}$) and then subsequently with phenylephrine (1–2 $\mu\text{mol/l}$) to approximately 80% of maximal constriction. After the constriction reached steady state, the contribution of EDHF to the vasodilation induced by acetylcholine was assessed by adding acetylcholine cumulatively (3 nmol/l–0.1 $\mu\text{mol/l}$) in the presence of L-NAME (100 $\mu\text{mol/l}$) and indomethacin (10 $\mu\text{mol/l}$), to inhibit nitric oxide and prostaglandins, respectively. After wash-out of phenylephrine and acetylcholine, preparations were incubated in bumetanide (10 $\mu\text{mol/l}$), L-NAME and indomethacin for a further 30 min before repeating the dose–response curve to acetylcholine. The extent of relaxation was expressed as the percentage reversal of the constriction evoked by phenylephrine.

Modelling of electrical responses

A segment of arterial smooth muscle including endothelium was approximated as an electrically isopotential compartment and its electrical behaviour was modelled as a single equivalent cell. Acetylcholine-evoked hyperpolarization was considered as a two-component potassium conductance, the characteristics of which were based on results obtained in the present study. The acetylcholine-evoked depolarization, revealed after inhibition of the hyperpolarization with apamin and charybdotoxin, was modelled as two inward conductances with characteristics defined by the results in WKY rats and SHR. The equivalent resting membrane conductance was represented as an ohmic leak conductance (g_{Rest}) with an equilibrium potential equal to the resting membrane potential (RMP) (E_{Rest}) of -47 mV (average of control values, $n = 59$). The absolute value of g_{Rest} (50 nS) was chosen to present an input resistance for the preparation of 20 M Ω [11]. The membrane capacitance (C_m) was set to 5 nF, yielding a membrane time constant of 100 ms [11]. In some simulations, the RMP was set to -56 mV [average of membrane potential values in niflumic acid, flufenamic acid and 5-nitro-2-(3-phenyl-propylamino) benzoic acid (NPPB), $n = 24$] and the input resistance of the preparation was concurrently increased by 50% to mimic the blocking of Cl^- conductances in the membrane [12].

The rapid component of the acetylcholine-induced depolarization was modelled as a transient increase in membrane conductance (g_{Fast}) having a reversal potential of -18 mV (E_{Fast} ; Table 1). The time course of the conductance was defined as the difference between two exponential functions, raised to a power, N , as shown

in the following equation:

$$g = M(e^{-t/A} - e^{-t/B})^N \text{ for } t > 0 \quad (1)$$

For the rapid component, g_{Fast} , M scaled the peak conductance to 18.3 nS, and values for A , B and N are presented in Table 1. These and other model parameter values were established by a simplex least-squares optimization to fit peak polarization/TTP point pairs for 11 experimental situations. The more slowly developing component of the acetylcholine-induced depolarization was modelled as an additional slower increase in membrane conductance, g_{Slow} . Equation (1) was again used to generate this modulation, and values for A , B and N together with peak conductance and reversal potential are presented in Table 1.

The acetylcholine-induced hyperpolarization was most easily modelled as a composite potassium conductance having fast and slow components sharing an equilibrium potential of -90 mV (E_K). Equation (1) was used to generate each component of the hyperpolarization with parameter values as shown in Table 1. The equation describing the membrane potential (E_m) of the equivalent cell is

$$dE_m/dt = \sum I/C_m \quad (2)$$

where $\sum I$ is the sum of the currents resulting from the imposed conductance transients and the resting current.

The degree of attenuation of g_{Fast} at -47 mV to simulate the effects of isethionate, methyl sulphate or bumetanide in SHR and that at -56 mV to simulate the effects of niflumic acid or flufenamic acid in SHR were also varied in the optimization. The best fitting reduction factor values were 0.49 for the cases at -47 mV and 0.22 for those at -56 mV . Peak polarizations and the corresponding TTP values generated by the optimized model at the appropriate RMPs are shown for comparison with the averaged physiological data. Paired t -tests showed that amplitudes ($P > 0.99$) and TTP values ($P > 0.75$) were not significantly different from those measured from the physiologically acquired data for the 11 sets of conditions considered.

Simulations and optimizations were carried out using Matlab 7.1.0.246 software (The Mathworks, Inc., Natick, Massachusetts, USA).

Drugs and solutions

The following drugs were used: acetylcholine hydrochloride, atropine sulphate, amiloride hydrochloride, apamin, bumetanide, charybdotoxin, CPA, chelerythrine chloride, flufenamic acid, gadolinium chloride, indomethacin, lanthanum chloride, myristoylated protein kinase C peptide, L-NAME, niflumic acid, NPPB, phenylephrine hydrochloride, U-73122 (Sigma Chemical Co., St Louis, Missouri, USA), isethionic acid, sodium salt

Table 1 Model parameters

Conductance	Equilibrium potential (mV)	Peak conductance (nS)	A (s)	B (s)	N
g_{Fast}	−18	18.3	3.5	3.4	2.4
g_{Slow}	−45	58.6	11.3	11.2	2.9
g_{KF}	−90	21.6	4.3	4.2	4.5
g_{KS}	−90	65.1	9.7	9.6	3.0

(Acros Organics, Geel, Belgium), and sodium methyl sulphate (Aldrich Chemical, Milwaukee, Wisconsin, USA). Ca^{2+} -free Krebs' solution was made by omitting CaCl_2 . Stock solutions of bumetanide, chelerythrine chloride, indomethacin, niflumic acid and U-73122 were dissolved in dimethyl sulphoxide; flufenamic acid and NPPB were dissolved in ethanol ($\times 10000$ stock). All other drugs were made up as $\times 1000$ stock solutions in distilled water and diluted directly into Krebs solution. Control experiments showed no significant effects of dimethyl sulphoxide (0.01%) or ethanol (0.01%) on acetylcholine-induced electrical responses. Solutions of light-sensitive drugs were protected from light through the use of long wavelength ($> 610\text{ nm}$) light. Concentrations of drugs known to affect specific ion channel activity were chosen according to data provided in reviews and primary articles [13–18].

Statistical analysis

Data were expressed as the mean $\pm$ SEM; n refers to the number of animals tested. Statistical significance was determined by paired or unpaired Student's t -test. Linear regression analysis was used to test the correlation between the membrane potential and the amplitude of the depolarization, and two-way analysis of variance was used to test significance among dose–response curves to acetylcholine. A value of P less than 0.05 was considered statistically significant.

Results

Systolic blood pressure was higher in SHR compared with WKY rats (187 ± 6 vs 130 ± 5 mmHg, respectively; $P < 0.01$; $n = 8-9$). The RMP of smooth muscle cells in the presence of indomethacin ($10\text{ }\mu\text{mol/l}$) and L-NAME ($100\text{ }\mu\text{mol/l}$) was similar for mesenteric arteries from SHR and WKY rats, although a small difference could be identified when data were pooled from a large group of impalements (SHR, $-49 \pm 0.4\text{ mV}$, $n = 41$; WKY, $-51 \pm 0.9\text{ mV}$, $n = 14$; $P < 0.05$).

In both SHR and WKY rats, acetylcholine evoked a hyperpolarization in the presence of indomethacin ($10\text{ }\mu\text{mol/l}$) and L-NAME ($100\text{ }\mu\text{mol/l}$). Preincubation in apamin and charybdotoxin abolished the hyperpolarization and revealed a depolarization that was substantially larger in SHR than in WKY rats, confirming our previous results (Fig. 1a and [8]).

Since iontophoresis at a point source produces responses whose time course is principally determined by the opening and closing of ion channels rather than by diffusion [10], we could observe that the time course of the depolarization varied between arteries taken from SHR and WKY rats. In SHR, the depolarization had a rapidly rising phase and a slower falling phase (Fig. 1a, SHR), while in WKY rats the depolarization had a slower and symmetrical time course (Fig. 1a, WKY rats). These

Fig. 1

Acetylcholine (ACh)-evoked depolarization in mesenteric arteries of spontaneously hypertensive rats (SHR). (a) ACh-evoked depolarization was larger in amplitude and faster in time course in SHR than in Wistar–Kyoto (WKY) rats. (b) Relationship between the resting membrane potential and amplitude of the ACh-evoked depolarization in SHR. Estimated reversal potential was -18 mV . Data are responses in control solution only ($n = 59$). (c) Role of the endothelium in ACh-evoked depolarization. In the endothelium-intact half of the artery (unshaded), ACh produced both hyperpolarization and depolarization (upper panel), the latter revealed in the presence of apamin ($0.5\text{ }\mu\text{mol/l}$) and charybdotoxin (60 nmol/l). In the endothelium-disrupted half of the artery (shaded), neither hyperpolarization nor depolarization was observed. All recordings were from the same preparation. Indomethacin ($10\text{ }\mu\text{mol/l}$) and N^G -nitro-L-arginine methyl ester ($100\text{ }\mu\text{mol/l}$) were present throughout. Arrows, application of ACh. EC, endothelial cells; Em, membrane potential.

data suggested the existence of a larger, fast and a smaller, slow component to the depolarization in SHR but only a small, slow component in WKY rat arteries. Since the depolarization in WKY rats was often less than 1 mV ($1.2 \pm 0.4\text{ mV}$, $n = 6$), it was only possible to investigate the depolarization further in SHR.

Acetylcholine-evoked depolarization in SHR

The acetylcholine-evoked depolarization was studied in the presence of apamin plus charybdotoxin to abolish the hyperpolarization (RMP -47 ± 0.4 mV; $n = 59$). The amplitude and TTP of the depolarization were 7.9 ± 0.2 mV and 3.1 ± 0.1 s, respectively ($n = 59$). There was a significant negative correlation between the RMP and the amplitude of the acetylcholine-evoked depolarization, and the estimated reversal potential for the acetylcholine-evoked depolarization was -18 mV (Fig. 1b). While these experiments do not provide a reliable value for the reversal potential of the current, they suggest that the conductance increase will not be dramatically changed by drugs that produce a moderate depolarization.

The essential role of the endothelium in the acetylcholine-evoked depolarization in SHR is demonstrated in Fig. 1c. Acetylcholine produced both hyperpolarization and depolarization in the endothelium-intact half of the artery (Fig. 1c, upper panel). In contrast, neither hyperpolarization nor depolarization was observed in the endothelium-disrupted half of the artery (Fig. 1c, lower panel).

Role of the IP_3 pathway and calcium in the acetylcholine-evoked depolarization in SHR

The muscarinic acetylcholine receptor antagonist atropine ($1 \mu\text{mol/l}$) abolished acetylcholine-evoked depolarization without altering the RMP ($n = 3$, Fig. 2a). The phospholipase C inhibitor U-73122 ($10 \mu\text{mol/l}$) abolished acetylcholine-induced hyperpolarization (Fig. 2b, left and middle panels) and acetylcholine-evoked depolarization when incubated in the presence of apamin and charybdotoxin (Fig. 2b, right panel) (RMP, -45 ± 0.7 mV in U-73122; $n = 3$).

The acetylcholine-evoked depolarization was not reduced by the protein kinase C inhibitor chelerythrine chloride ($5 \mu\text{mol/l}$) [19]. In contrast, the amplitude increased (6.2 ± 0.7 vs 12.1 ± 1.5 mV for control and chelerythrine chloride, respectively; $n = 3$, $P < 0.05$; Fig. 2c) and the membrane hyperpolarized (-46 ± 0.7 vs -57 ± 2.2 mV for control and chelerythrine chloride, respectively; $n = 3$, $P < 0.05$). Another protein kinase C inhibitor, myristoylated protein kinase C peptide ($10 \mu\text{mol/l}$) [19], also failed to inhibit the acetylcholine-evoked depolarization (amplitude, 8.4 ± 1.8 vs 10.4 ± 0.8 mV for control and drug, respectively; $n = 3$, $P > 0.05$; RMP, -47 ± 0.9 vs -54 ± 3.2 mV for control and drug, respectively; $n = 3$, $P < 0.05$).

Depletion of the intracellular Ca^{2+} stores by inhibiting the Ca^{2+} -ATPase pump of the endoplasmic reticulum with CPA ($10 \mu\text{mol/l}$) [20] markedly reduced the acetylcholine-evoked depolarization (amplitude, 8.4 ± 1.0 vs 2.2 ± 0.9 mV for control and CPA, respectively; $n = 3$,

Fig. 2

Dependency of the acetylcholine-evoked depolarization in mesenteric arteries of spontaneously hypertensive rats (SHR) on the IP_3 pathway and Ca^{2+} . (a) Acetylcholine did not evoke depolarization after treatment with atropine. (b) The phospholipase C inhibitor U-73122 abolished both the acetylcholine-evoked hyperpolarization (middle panel) and depolarization (right panel). The acetylcholine-evoked depolarization was not reduced by the protein kinase C inhibitor chelerythrine chloride (c), while it was abolished by the Ca^{2+} -ATPase inhibitor, cyclopiazonic acid (CPA) (d) or Ca^{2+} -free solution (e, right panel). Ca^{2+} -free solution significantly reduced the acetylcholine-evoked hyperpolarization (e, middle panel). Recordings in each pair were obtained from the same cell (a, c) or the same preparation (b, d, e). Apamin ($0.5 \mu\text{mol/l}$) and charybdotoxin (60 nmol/l) were present as indicated. Indomethacin ($10 \mu\text{mol/l}$) and N^G -nitro-L-arginine methyl ester ($100 \mu\text{mol/l}$) present throughout. Arrows, application of acetylcholine.

$P < 0.01$; Fig. 2d). This was accompanied by significant membrane depolarization (-48 ± 1.2 vs -41 ± 1.7 mV for control and CPA, respectively; $n = 3$, $P < 0.01$; Fig. 2d). Acetylcholine-evoked depolarization was also

dramatically reduced in Ca^{2+} -free solution (amplitude, $1.8 \pm 0.5 \text{ mV}$; $n=3$; Fig. 2e, right panel) in which the RMP was significantly depolarized to $-35 \pm 0.7 \text{ mV}$ ($n=3$). Ca^{2+} -free solution reduced, but did not abolish, acetylcholine-induced hyperpolarization (Fig. 2e, middle panel).

Role of chloride and nonselective cation channels in the acetylcholine-evoked depolarization in SHR

The preceding experiments suggested that the depolarization was Ca^{2+} dependent. Since the reversal potential was considerably more positive of the RMP, the underlying conductance was likely to be an inward current. Closure of an outward current (e.g. a K^+ conductance) would be expected to produce a voltage change with a reversal potential more negative of the RMP. It should also be noted that Ca^{2+} -dependent K^+ channels were already blocked by apamin and charybdotoxin in these experiments.

Candidates for the Ca^{2+} -dependent inward current were Ca^{2+} -dependent Cl^- channels and nonselective cation channels. The Ca^{2+} -dependent Cl^- channel inhibitors, niflumic acid ($50 \mu\text{mol/l}$) and flufenamic acid ($100 \mu\text{mol/l}$) [13], reduced the amplitude by approximately 40% and increased the TTP of the depolarization (Fig. 3a,b and Table 2), suggesting a selective effect on the fast, rather than the slow, component of the depolarization observed in SHR. These drugs produced a significant hyperpolarization of the cell membrane (Fig. 3a,b and Table 2). In contrast, the volume-regulated Cl^- channel inhibitor NPPB had no effect on the acetylcholine-evoked depolarization at either 1 or $100 \mu\text{mol/l}$ [14,16], although the latter concentration resulted in significant hyperpolarization of the membrane (Fig. 3c and Table 2).

Replacement of Cl^- in the superfusion solution with an impermeant anion isethionate (120 mmol/l) substantially reduced the amplitude of the fast component of the acetylcholine-evoked depolarization (Fig. 3d and Table 2). In contrast to niflumic acid and flufenamic acid, isethionate had no effect on the TTP but the RMP was slightly but significantly depolarized (Table 2). Inhibition of the $\text{Na}^+/\text{K}^+/\text{2Cl}^-$ cotransporter with bumetanide ($10 \mu\text{mol/l}$) [15] also produced a significant reduction in the amplitude of the acetylcholine-evoked depolarization, without altering the RMP or TTP (Fig. 3e and Table 2).

The nonselective cation channel inhibitors, gadolinium ($50 \mu\text{mol/l}$), lanthanum ($50 \mu\text{mol/l}$) and amiloride ($100 \mu\text{mol/l}$) [14,18], the latter of which is also an antagonist of mechanosensitive Na^+ channels expressed in vascular smooth muscle [21], were without effect on the acetylcholine-evoked depolarization or on membrane potential (Fig. 3f,g and Table 2).

Fig. 3

Effects of ion channel inhibitors on acetylcholine-evoked depolarization in mesenteric arteries of spontaneous hypertensive rats. Acetylcholine-evoked depolarization was diminished by Ca^{2+} -dependent Cl^- channel inhibitors niflumic acid (a) and flufenamic acid (b), but not by the nonspecific Cl^- channel inhibitor, 5-nitro-2-(3-phenyl-propylamino) benzoic acid (c). Replacement of external Cl^- ions with an impermeant anion, isethionate, or treatment with the $\text{Na}^+/\text{K}^+/\text{2Cl}^-$ cotransporter inhibitor, bumetanide, reduced the depolarization (d, e). Nonselective cation channel inhibitors, gadolinium or lanthanum, were without effect (f, g). Recordings in each pair were obtained from the same cell (a, b, d, f, g) or the same preparation (c, e). Indomethacin ($10 \mu\text{mol/l}$), N^G -nitro-L-arginine methyl ester ($100 \mu\text{mol/l}$), apamin ($0.5 \mu\text{mol/l}$) and charybdotoxin (60 nmol/l) present throughout. Arrows, application of acetylcholine.

Modelling of depolarizing responses in SHR and WKY rats

The data presented in the previous sections suggested that the acetylcholine-evoked depolarization in SHR might consist of a fast and a slow component, while in

Table 2 Effects of drugs on acetylcholine-induced depolarization in the mesenteric artery of spontaneously hypertensive rats

Drug	Acetylcholine-induced depolarization			<i>n</i>
	Amplitude (mV)	Time to peak (s)	Membrane potential (mV)	
Control	8.8 ± 0.8	2.3 ± 0.4	-45 ± 0.6	3
Niflumic acid 50 µmol/l	5.5 ± 0.7*	5.8 ± 0.2 [†]	-53 ± 1.7 [†]	3
Control	7.3 ± 0.7	2.8 ± 0.1	-50 ± 1.3	4
Flufenamic acid 100 µmol/l	4.1 ± 0.6*	4.3 ± 1.6	-57 ± 2.5 [†]	4
Control	8.1 ± 1.5	3.4 ± 0.6	-46 ± 1.7	3
NPPB 1 µmol/l	7.1 ± 1.7	3.9 ± 0.6	-46 ± 1.5	3
Control	6.2 ± 1.0	3.8 ± 0.6	-46 ± 1.5	3
NPPB 100 µmol/l	5.9 ± 0.9	3.7 ± 0.7	-57 ± 2.1*	3
Control	6.9 ± 0.4	3.5 ± 0.2	-47 ± 1.8	3
Isethionate 120 mmol/l	3.1 ± 0.5*	2.9 ± 0.4	-44 ± 1.9 [†]	3
Control	8.9 ± 0.3	3.0 ± 0.2	-45 ± 0.9	3
Gadolinium 50 µmol/l	9.4 ± 1.4	3.5 ± 0.3	-44 ± 0.7	3
Control	10.6 ± 0.7	3.3 ± 0.2	-48 ± 2.8	3
Lanthanum 50 µmol/l	10.3 ± 0.9	3.7 ± 0.2	-47 ± 3.3	3
Control	6.7 ± 1.3	3.8 ± 0.7	-47 ± 0.7	3
Amiloride 100 µmol/l	6.6 ± 1.2	3.8 ± 0.4	-46 ± 1.2	3
Control	8.1 ± 1.1	2.9 ± 0.2	-47 ± 2.4	4
Bumetanide 10 µmol/l	5.1 ± 1.0*	2.9 ± 0.5	-47 ± 2.5	4

Values presented as the mean ± SEM. NPPB, 5-nitro-2-(3-phenyl-propylamino) benzoic acid. **P* < 0.01 vs control; [†]*P* < 0.05 vs control.

WKY rats only a slow depolarization was present. A reversal potential of -18 mV was used for the fast component, as described above, and a similar plot of RMP versus amplitude produced an estimate of -45 mV for the slow component. The membrane potential responses due to the two model conductance transients (Fig. 4a) at RMPs of -47 and -56 mV can be seen individually in Fig. 4b and combined in Fig. 4c. These levels of RMP were chosen to correspond to averaged physiologically acquired data as described in the Methods. The slow component alone at -47 mV (Fig. 4b, left panel) resembled the response recorded in WKY rats in control (Fig. 1a, right panel; Table 3). The combined responses at the two different membrane potentials mimic those seen in SHR in control and chelerythrine chloride (Fig. 2c and Table 3, SHR + PKC inhibitors).

Attenuation of the fast component by 51% at -47 mV produced the response in Fig. 4d (left panel), resembling the response in bumetanide and isethionate (Fig. 3d,e and Tables 2 and 3), while attenuation of the fast component by 78%, accompanied by hyperpolarization (RMP -56 mV) and an increase in input resistance due to closure of Cl⁻ channels, produced the response shown in Fig. 4d (right panel). The amplitude and time course of this composite response resembled that seen in SHR after incubation in niflumic acid or flufenamic acid (Fig. 3a,b and Tables 2 and 3).

Interaction between the acetylcholine-induced depolarization and hyperpolarization in SHR and WKY rats

In mesenteric arteries of SHR, the TTP of the acetylcholine-induced hyperpolarization was significantly longer than that in WKY rat arteries (8.7 ± 0.4 vs 5.8 ± 0.9 s for SHR and WKY rats, respectively; *n* = 5–6, *P* < 0.05; Fig. 5a vs Fig. 5b). Inspection of the time

course of the hyperpolarization in WKY rats revealed an initial rapid component with a slower component superimposed on the repolarizing phase (Fig. 5b). Control SHR hyperpolarizations appeared to comprise an attenuated fast component and an intact slow component (Fig. 5a). Our hypothesis was that the absence of the fast hyperpolarizing component in SHR was due to the presence of the fast depolarization described in the previous sections. Incubation of SHR arteries in niflumic acid (50 µmol/l) or flufenamic acid (100 µmol/l), to reduce the fast depolarization, reinstated the fast component of the acetylcholine-induced hyperpolarization, leading to a significant reduction of the TTP without effect on the amplitude, although the RMP became hyperpolarized (Fig. 5a,c and Table 4). In contrast, in WKY rat arteries, niflumic acid (50 µmol/l) did not alter the TTP of the acetylcholine-induced hyperpolarization (Fig. 5b,d; *n* = 6, *P* > 0.05), although the amplitude was reduced (Fig. 5b,d; *n* = 6, *P* < 0.01) and the RMP was hyperpolarized (Table 3; *n* = 6, *P* < 0.01).

Substitution of Cl⁻ with isethionate (120 mmol/l) or methyl sulphate (120 mmol/l), or inhibition of the Na⁺/K⁺/2Cl⁻ cotransporter with bumetanide (10 µmol/l) – procedures that also reduced the amplitude of the fast depolarization – produced similar reduction in the TTP of the hyperpolarization in SHR arteries (Fig. 6). Under these conditions, however, the amplitude of the acetylcholine-induced hyperpolarization was increased and the RMP was not hyperpolarized (Fig. 6 and Table 4; RMP, -49 ± 0.9 vs -48 ± 1.1 mV for control and sodium methyl sulphate, respectively; *n* = 5, *P* > 0.05). In contrast, neither NPPB (100 µmol/l), gadolinium (50 µmol/l) or lanthanum (50 µmol/l) altered the TTP of the acetylcholine-induced hyperpolarization in SHR, although the amplitude was significantly reduced and the RMP hyperpolarized in NPPB (Table 4).

Fig. 4

Computational modelling of depolarization. (a) Electrical circuit used to simulate acetylcholine-induced depolarizations in the rat mesenteric artery. The passive properties of the membrane were modelled as the combination of the membrane conductance (g_{Rest} , 50 nS) at the resting membrane potential (RMP) (E_{Rest} -47 mV) in parallel with the membrane capacitance (C_m , 5 nF), giving a membrane time constant of 100 ms. The rapid component of the acetylcholine-induced depolarization was modelled as a transient increase in membrane conductance (g_{Fast}) with a peak value of 18.3 nS and an equilibrium potential of -18 mV (E_{Fast}). The time course of the modulation was defined by equation (1) (see Methods). The slow component was modelled as a more slowly developing transient increase in membrane conductance (g_{Slow}) with a peak value of 58.6 nS and an equilibrium potential of -45 mV (E_{Slow}). The time course of the modulation was again defined by equation (1). (b) Voltage changes due to the individual fast and slow conductances at RMPs of -47 and -56 mV. Since the latter case occurred when Cl^- channels in the membrane had been blocked, the input resistance of the preparation ($1/g_{Rest}$) was concurrently increased by 50%. (c) Combinatory effect of both fast and slow conductances at -47 and -56 mV. (d) Combinatory effect of the two conductances under conditions in which the fast component was attenuated to 49% of control level at -47 mV to simulate spontaneously hypertensive rats (SHR) arteries in isethionate, bumetanide, and to 22% of control level at -56 mV to simulate SHR arteries in niflumic acid or flufenamic acid. Note that the peak potential of the combined responses is similar to that of the fast component except in (d) at -56 mV, where the response is dominated by the slow component.

Table 3 Physiologically acquired data and model predictions

Condition	Physiological data			Model data		
	Amplitude	Time to peak	Resting membrane potential	Amplitude	Time to peak	Resting membrane potential
Depolarization						
WKY rats	1.2 ± 0.4 (6)	11.2 ± 0.6 (6)	-49 ± 1.0 (6)	2.2	11.3	-49
SHR	7.9 ± 0.2 (59)	3.1 ± 0.1 (59)	-47 ± 0.4 (59)	6.9	3.1	-47
SHR + PKC inhibitors	11.2 ± 0.8 (6)	3.2 ± 0.2 (6)	-55 ± 1.8 (6)	12.5	3.3	-55
SHR + bumetanide	5.1 ± 1.0 (4)	2.9 ± 0.5 (4)	-47 ± 2.5 (4)	4.0	3.2	-47
SHR + NFA	5.5 ± 0.7 (3)	5.8 ± 0.2 (3)	-53 ± 1.7 (3)	5.3	5.8	-53
Hyperpolarization						
WKY rats	-16.1 ± 0.8 (6)	5.8 ± 0.9 (6)	-50 ± 1.2 (6)	-16.1	5.0	-50
WKY rats + NFA	-10.6 ± 1.2 (6)	5.9 ± 1.3 (6)	-60 ± 1.4 (6)	-11.5	4.1	-60
SHR	-13.7 ± 0.4 (39)	8.7 ± 0.3 (39)	-49 ± 0.4 (39)	-14.0	8.7	-49
SHR + NPPB	-6.8 ± 1.7 (4)	8.5 ± 1.2 (4)	-59 ± 2.4 (4)	-7.6	8.4	-59
SHR + bumetanide	-16.6 ± 1.0 (8)	6.9 ± 0.4 (8)	-50 ± 0.8 (8)	-14.2	6.7	-50
SHR + NFA	-13.2 ± 0.6 (5)	3.6 ± 0.2 (5)	-56 ± 1.3 (5)	-12.6	5.0	-56

Paired *t*-test comparisons of model data with physiologically acquired data show no significant differences. NPPB, 5-nitro-2-(3-phenyl-propylamino) benzoic acid; PKC, protein Kinase C; SHR, spontaneously hypertensive rats; WKY, Wistar-Kyoto. *P* > 0.99 for amplitude; *P* > 0.75 for time to peak.

Fig. 5

Effects of niflumic acid on acetylcholine-induced, endothelium-dependent hyperpolarizing factor-mediated hyperpolarization in mesenteric arteries of spontaneously hypertensive rats (SHR) and Wistar-Kyoto (WKY) rats. Niflumic acid (50 μmol/l) reduced the time to peak (TTP) of the acetylcholine-induced hyperpolarization in SHR (a, c) but not in WKY rats (b, d). In contrast, the amplitude of the hyperpolarization was reduced in WKY rats but not in SHR. Group data represent the mean ± SEM; $n = 6$. * $P < 0.01$ compared with control. Indomethacin (10 μmol/l) and *N*^ω-nitro-L-arginine methyl ester (100 μmol/l) present throughout. Arrows, application of acetylcholine.

Modelling of hyperpolarizing responses and interaction between depolarization and hyperpolarization in SHR and WKY rats

Figure 7a shows the circuit diagram describing the fast and slow membrane conductances, modelled as potassium conductances, underlying the hyperpolarization evoked by acetylcholine in addition to the two depolarizing currents described previously. The behaviour of the fast and slow components of the hyperpolarization (g_{KF} and g_{KS} , respectively) can be seen individually in Fig. 7b at -47 and -56 mV. In order to

mimic the control hyperpolarizing response in SHR, the two hyperpolarizing currents were combined with the two depolarizing currents at -47 mV (Fig. 7c, left panel). This produced a potential of 15.5 mV with a TTP of 8.8 s and a time course very similar to that seen in SHR in control solution (e.g. Fig. 6). When all four currents were combined at -56 mV (Fig. 7c, right panel) there was little change in the TTP (8.5 s), although the amplitude was reduced to 10 mV. This resembled the effect of NPPB in which the cell membrane was hyperpolarized, the amplitude of the hyperpolarization was reduced but the TTP was unaltered (Tables 3 and 4).

To mimic the hyperpolarization in control solution in WKY rats, the two hyperpolarizing currents were combined at -47 mV with the slow depolarization only (Fig. 7d, left panel). In contrast to the combination of all four currents, the TTP (5.3 s) was substantially reduced and the amplitude (18 mV) increased, resembling the hyperpolarization in WKY rats (Fig. 5b, control and washout). When these three currents were combined at -56 mV (Fig. 7d, right panel), the TTP (4.4 s) and amplitude (14 mV) were both reduced. Interestingly, if the currents were combined at -60 mV, corresponding to the response of WKY rats with niflumic acid (Fig. 5b, middle panel), the amplitude (11.5 mV) was further reduced, corresponding to the physiologically acquired data (Table 3).

To mimic the hyperpolarization in SHR in isethionate, methyl sulphate or bumetanide (Fig. 6a–c), the two hyperpolarizing currents and the slow depolarizing current were combined at -47 mV with the fast depolarizing component reduced by 51% (Fig. 7e, left panel). The resulting response showed a reduction in the TTP (6.9 s) and an increase in amplitude (16.1 mV) compared with SHR in control solution. When these conductance modulations were combined at -44 mV, as occurred in isethionate (Table 4), the amplitude of the response was further enhanced (18.3 mV).

Combination of the two hyperpolarizing currents and the slow depolarizing current at -56 mV, with the fast depolarizing component reduced by 78% (Fig. 7e, right panel) to mimic responses in SHR after incubation in niflumic acid or flufenamic acid (Table 4), produced a response with reduced TTP (5 s) corresponding with the physiological data (Table 3).

Functional consequences of the acetylcholine-evoked depolarization

Acetylcholine evoked membrane depolarization in SHR arteries even under conditions of significant depolarization with 1 μmol/l phenylephrine (amplitude of depolarization, 4.7 ± 0.4 mV; $n = 3$). Under these conditions, the RMP or resting diameter of the mesenteric arteries did not differ between SHR and WKY rats (RMP, -33 ± 0.7

Table 4 Effects of drugs on acetylcholine-induced hyperpolarization in the mesenteric artery of spontaneously hypertensive rats

Drug	Acetylcholine-induced hyperpolarization			n
	Amplitude (mV)	Time to peak (s)	Membrane potential (mV)	
Control	13.6 ± 0.7	8.7 ± 0.4	-47 ± 0.9	5
Niflumic acid 50 µmol/l	13.2 ± 0.6	3.6 ± 0.2*	-56 ± 1.3 [†]	5
Washout	13.1 ± 0.8	8.5 ± 0.6	-49 ± 1.2	5
Control	12.2 ± 0.8	7.8 ± 0.7	-47 ± 0.2	5
Flufenamic acid 100 µmol/l	10.2 ± 1.4	4.4 ± 0.1*	-53 ± 1.4 [†]	5
Washout	13.1 ± 0.9	8.0 ± 0.9	-50 ± 1.8	5
Control	13.3 ± 0.8	8.1 ± 1.2	-47 ± 1.3	4
NPPB 100 µmol/l	5.8 ± 1.7 [†]	8.5 ± 1.2	-59 ± 2.4*	4
Washout	10.7 ± 1.6	7.9 ± 1.3	-54 ± 1.2 [†]	4
Control	13.8 ± 1.2	9.2 ± 0.6	-47 ± 0.9	6
Isethionate 120 mmol/l	18.4 ± 1.4*	5.4 ± 0.4*	-44 ± 0.5	6
Washout	13.9 ± 0.8	8.4 ± 0.5	-47 ± 0.8	6
Control	13.7 ± 1.2	10.5 ± 1.0	-50 ± 1.8	4
Gadolinium 50 µmol/l	10.9 ± 1.9	11.3 ± 1.3	-51 ± 2.1	4
Washout	13.2 ± 0.6	11.5 ± 1.1	-50 ± 1.4	4
Control	14.0 ± 0.7	9.3 ± 0.8	-52 ± 1.9	4
Lanthanum 50 µmol/l	10.5 ± 2.3	10.9 ± 1.7	-54 ± 2.7	4
Washout	12.6 ± 1.2	10.9 ± 1.0	-50 ± 0.6	4
Control	13.8 ± 0.7	7.7 ± 0.2	-50 ± 0.8	8
Bumetanide 10 µmol/l	16.6 ± 1.0*	6.9 ± 0.4 [†]	-50 ± 0.8	8

Values presented as the mean ± SEM. NPPB, 5-nitro-2-(3-phenyl-propylamino) benzoic acid. * $P < 0.01$ vs control; [†] $P < 0.05$ vs control.

vs -34 ± 2.1 mV for SHR and WKY rats, respectively; $n = 3$, $P > 0.05$; resting diameter, 273 ± 10 vs 267 ± 14 µm for SHR and WKY rats, respectively; $n = 3$, $P > 0.05$). Moreover, the acetylcholine-evoked depolarization in SHR was accompanied by vasoconstriction (amplitude, 16.2 ± 1.4 µm; $n = 3$). In contrast, acetylcholine failed to evoke depolarization or vasoconstriction in WKY rats under the same depolarized conditions ($n = 3$).

Functional consequences of the interaction between depolarization and hyperpolarization

Bumetanide (10 µmol/l) was used to test the effect of a reduction in the depolarization on acetylcholine-evoked vasodilation. Unfortunately these experiments were not possible in flufenamic acid or niflumic acid due to the substantial loss of phenylephrine-induced tone produced by these drugs.

Relaxation was significantly less sensitive to the cumulative application of increasing concentrations of acetylcholine in SHR than in WKY rats (Fig. 8, $P < 0.05$, two-way analysis of variance). Responses in SHR were also often transient at submaximal concentrations of acetylcholine compared with the persistent responses found in WKY rats (Fig. 8a, peak responses vs Fig. 8b, persistent or plateau responses). Preincubation in bumetanide (10 µmol/l) reduced the subsequent contraction to phenylephrine to $80 \pm 7.3\%$ (for WKY rats) and $74 \pm 5.2\%$ (for SHR) of that in the absence of bumetanide, but these contractions were not significantly different ($P > 0.05$). Treatment with bumetanide significantly increased the sensitivity of both the peak and plateau relaxation to acetylcholine in SHR ($P < 0.05$), but had no effect in WKY rats ($P > 0.05$, two-way analysis of variance).

Discussion

The present study has shown that acetylcholine can activate different ionic currents in mesenteric arteries from normotensive and hypertensive rats. In vessels from normotensive WKY rats, application of acetylcholine evoked two outward membrane currents of differing time course, as well as a slow inward current. An additional fast inward current, which could be differentially blocked with drugs affecting Ca^{2+} -dependent Cl^- channels or Cl^- movement across the cell membrane, was activated in arteries from hypertensive SHR rats. Computational modelling of these conductances provided a good description of the experimental data and confirmed that the faster inward currents were unique to the SHR. Differences in the time course of EDHF-mediated hyperpolarization between SHR and WKY rats were eliminated by drugs affecting Ca^{2+} -dependent Cl^- channels or Cl^- movement across the cell membrane. These effects could be predicted by our computational model under conditions in which the fast depolarization was substantially reduced. Importantly, when the membrane potential was depolarized to mimic physiological conditions, acetylcholine evoked a depolarization only in SHR and this was accompanied by constriction. Furthermore, EDHF-mediated relaxation, which is reduced in SHR compared with WKY rats, was significantly improved by the inhibitor of $\text{Na}^+/\text{K}^+/\text{2Cl}^-$ cotransport, bumetanide, which the present study shows can attenuate the depolarization.

Abolition of the acetylcholine-evoked depolarization in SHR with atropine demonstrated the involvement of muscarinic, acetylcholine receptors, while inhibition of the response in preparations treated with U-73122 or CPA or incubation in Ca^{2+} -free solution provided evidence of

Fig. 6

Effect on acetylcholine-induced, endothelium-dependent hyperpolarizing factor-mediated hyperpolarization in spontaneously hypertensive rats of lowering external chloride ions or inhibiting the $\text{Na}^+/\text{K}^+/\text{2Cl}^-$ cotransporter with bumetanide. Representative traces (a, b, c) and group data (d, e, f) of the effect of replacing external NaCl with sodium isethionate (a, d) or sodium methyl sulphate (b, e) or treatment with bumetanide (c, f). All treatments significantly augmented the amplitude and diminished the time to peak (TTP) of the acetylcholine-induced hyperpolarization. Recordings in each drug were obtained from the same cell. Indomethacin (10 μ mol/l) and N^G -nitro-L-arginine methyl ester (100 μ mol/l) present throughout. Data are mean $\pm$ SEM; $n = 6$ (d), $n = 5$ (e), $n = 8$ (f). * $P < 0.01$, † $P < 0.05$ compared with control. Arrows, application of acetylcholine.

Ca^{2+} dependency and a role for the IP_3 pathway. In contrast, neither of the protein kinase C inhibitors, chelerythrine chloride or myristoylated protein kinase C peptide, affected the acetylcholine-evoked depolarization, suggesting that this arm of the pathway was not involved. In Ca^{2+} -free solution and CPA, the RMP was depolarized by 12 and 7 mV, respectively. A similar depolarizing influence of Ca^{2+} -free solution [16,22,23] and CPA [24] on vascular smooth muscle cells has been reported, perhaps due to opening of nonselective cation channels activated by intracellular Ca^{2+} depletion [24].

Fig. 7

Computational modelling of hyperpolarization and depolarization. (a) Electrical circuit used to simulate acetylcholine-induced hyperpolarizations as well as depolarizations in the rat mesenteric artery. A rapidly acting (g_{KF} , peak value 21.6 nS) and a slower (g_{KS} , peak value 65.1 nS) potassium conductance pathway have been added to the circuit previously described (Fig. 4a). They share an equilibrium potential of -90 mV (E_K). For some simulations E_{Rest} was changed to -56 mV and the input resistance of the preparation ($1/g_{Rest}$) was concurrently increased by 50% as before. (b) Voltage changes due to the individual fast and slow hyperpolarizing conductances (g_{KF} and g_{KS}) at resting membrane potentials of -47 and -56 mV. For these calculations, the depolarizing conductances (g_{Fast} and g_{Slow}) were set to zero. (c) Combined effect of all four fast and slow, depolarizing and hyperpolarizing conductance transients at -47 mV to simulate spontaneously hypertensive rats (SHR) and -56 mV to simulate SHR arteries in 5-nitro-2-(3-phenyl-propylamino) benzoic acid. (d) Combined effect of both hyperpolarizing conductances and the slow depolarizing conductance (g_{Slow}) at -47 mV to simulate Wistar-Kyoto rats and at -56 mV. During these calculations, the value of g_{Fast} was set to zero. (e) Combined effects of g_{KF} and g_{KS} and the slow depolarizing conductances, under conditions in which the fast depolarizing conductance (g_{Fast}) was attenuated to 49% of control level at -47 mV to simulate SHR arteries in isethionate, methyl sulphate, bumetanide, and to 22% of control level at -56 mV to simulate SHR arteries in niflumic acid or flufenamic acid. Dotted lines represent 15 mV.

Considering the reversal potential of the fast depolarization was more positive than the RMP, the effects of CPA and Ca^{2+} -free solution might arise from the membrane depolarization itself. If this were the case, however, the

Fig. 8

Effect of bumetanide on acetylcholine (ACh)-evoked relaxation in spontaneously hypertensive rats (SHR) and Wistar-Kyoto (WKY) rats. Cumulative dose-response curves to ACh of 1 mm-long segments of mesenteric artery from SHR or WKY rats, mounted in a wire myograph. Peak and plateau (persistent) responses are shown since submaximal responses to ACh in SHR, but not in WKY rats, were often transient.

estimated amplitude of the acetylcholine-evoked depolarization in preparations depolarized to a similar degree would be between 5 and 6 mV, some threefold larger than the experimentally obtained values. Indeed, when the membrane potential was depolarized to -30 mV by phenylephrine, acetylcholine still evoked a 5 mV depolarization.

The time course of the depolarization in SHR arteries suggested the involvement of a dominant fast inward current with a slower component on the falling phase. In contrast, the depolarization in WKY rats consisted only of a small, slow inward current. Inhibition of Ca^{2+} -dependent Cl^{-} channels with niflumic or flufenamic acid, the $\text{Na}^{+}/\text{K}^{+}/2\text{Cl}^{-}$ cotransporter with bumetanide, or external Cl^{-} replacement with isethionate significantly reduced the fast component in SHR. Taken together with the Ca^{2+} dependency of the response and a reversal potential of -18 mV, which agrees closely with that reported for Cl^{-} ions [25,26], the data support the conclusion that the fast component of the depolarization in SHR is mediated, at least in part, by a Ca^{2+} -dependent Cl^{-} conductance. While the inhibitory effect of bumetanide is likely to result from a decrease in Cl^{-} conductance since inhibition of the $\text{Na}^{+}/\text{K}^{+}/2\text{Cl}^{-}$ cotransporter with bumetanide reduces intracellular Cl^{-} concentration [15], the replacement of external Cl^{-} might be expected to produce an initial potentiation of the depolarization followed by a reduction as the intracellular Cl^{-} concentration becomes reduced. In contrast, we only recorded a time-dependent decrease in the depolarization. Similar results have been obtained in the anococcygeus muscle, where it was suggested that anions such as isethionate might block Cl^{-} channels rather than simply altering Cl^{-} gradients [27]. Alternatively, the effect of Cl^{-} replacement on Cl^{-} fluxes may be obscured in a multilayered syncytial preparation, such as the mesenteric artery, in which rapid changes in Cl^{-} concentration are unlikely to occur simultaneously in all cells.

While the acetylcholine-evoked depolarization was demonstrated to be initiated in the endothelium, the question arises as to the site of the ion channels, functional Ca^{2+} -dependent Cl^{-} channels having been reported in both vascular smooth muscle cells [13] and endothelial cells [14,28]. Unfortunately, since myoendothelial gap junctions exist and are functional in rat

Fig. 8 (Continued)

After the initial dose-response curve in control solution, arteries were incubated in bumetanide (BT; $10 \mu\text{mol/l}$) and then exposed to a second concentration-response curve to ACh. Note that arteries from WKY rats were more sensitive to ACh than those from SHR ($P < 0.05$, two-way analysis of variance) and that bumetanide significantly increased the sensitivity to ACh in SHR but not in WKY rat segments ($P < 0.05$, two-way analysis of variance). Indomethacin ($10 \mu\text{mol/l}$) and N^{ω} -nitro-L-arginine methyl ester ($100 \mu\text{mol/l}$) were present throughout. $n = 4$ rats for SHR and WKY rats. PE, phenylephrine.

mesenteric resistance arteries [29,30], the acetylcholine-evoked depolarization observed here in smooth muscle cells could result from the spread of electrical signals from endothelial cells or from the movement of Ca^{2+} through these channels. Alternatively, diffusible factors other than cyclooxygenase metabolites might evoke the depolarization through the opening of smooth muscle channels. At the present time this issue cannot be resolved by recording from endothelial cells in intact arteries since no selective inhibitors of myoendothelial gap junctions are currently available to prevent current from spreading back into the endothelium from the smooth muscle. A systematic patch clamp analysis of the inward currents present in isolated endothelial cells from normotensive and hypertensive rats may therefore be required to shed further light on this issue.

Since the fast depolarization was not completely abolished by any of the treatments that targeted Cl^- channels or Cl^- fluxes, we considered that there may be additional components. Given that closure of an outward current was not likely, since Ca^{2+} -dependent K^+ currents were already inhibited in these experiments, potential candidates were the nonselective cation channels of the transient receptor potential family. Although there are few selective blockers of known molecular subtypes within this growing superfamily [18], we tested lanthanum, gadolinium and amiloride, which inhibit some members of the transient receptor potential family as well as mechanosensitive Na^+ channels expressed in vascular smooth muscle [21]. None of these drugs, however, reduced the fast depolarization or altered the time course of the hyperpolarization. While gadolinium-resistant and lanthanum-resistant cation channels may be involved in the residual response, it should be noted that the equilibrium potential for these channels whose predominant permeant ions are Na^+ and Ca^{2+} is considerably more positive [18] than that estimated in the present study.

An alternative explanation for the incomplete block of the depolarization in SHR by Cl^- channel antagonists was provided by our computational modelling based on experimentally observed values for the fast and slow depolarizations in SHR and WKY rats, respectively. The simulations with this model showed that a similar incomplete reduction in amplitude and an increase in the TTP of the fast depolarizing component could be reproduced after the fast component had been reduced by 78%, provided that the membrane was simultaneously hyperpolarized, as was observed in niflumic acid or flufenamic acid. The substantial residual response could be explained by augmentation of the slow depolarizing component at hyperpolarized potentials. Results obtained with isethionate and bumetanide, which did not hyperpolarize the membrane, could be mimicked with a 51% inhibition of the fast component. An incomplete block of the depolarization would be expected for

drugs such as these that simply reduce, rather than abolish, Cl^- gradients.

Further investigation of the mechanism underlying the slow component of the depolarization was beyond the scope of the present study since our experimental and modelling data suggested that this component was common to both SHR and WKY rats, and hence was unlikely to contribute to the impairment in vasodilation observed here in SHR. It is interesting, however, that a similar reversal potential to that estimated here for WKY rat arteries has been described for the cation current, I_h , in neurons of the rat brain [31] and for the Ca^{2+} -activated cation conductance in myenteric neurons showing after hyperpolarization [32]. This conductance is reported to be carried by members of the hyperpolarization-activated nucleotide-gated (HCN) family, of which HCN2 and HCN4 are expressed in these myenteric neurons [33]. Although the slow conductance in the mesenteric arteries of WKY rats can be activated by acetylcholine in the absence of hyperpolarization, HCN2 channels have been reported to be expressed in arteriolar endothelial cells [33] and may be appropriate targets for future functional studies conducted in normotensive arteries.

We have previously speculated that the overall hyperpolarization in mesenteric arteries of SHR was significantly less than that in WKY rats due to antagonism by the acetylcholine-evoked depolarization, and that this would contribute to the impairment of vasodilation in hypertensive arteries [8]. If this hypothesis were true, then drugs that selectively reduce the acetylcholine-evoked depolarization in SHR should augment the acetylcholine-induced hyperpolarization and decrease the TTP. In the present study, we tested this hypothesis directly and found that augmentation of the acetylcholine-induced hyperpolarization occurred in SHR but not in WKY rats following reduction of external Cl^- ions or treatment with bumetanide. In contrast, the hyperpolarization was not significantly increased in the presence of the Ca^{2+} -dependent Cl^- channel inhibitors, niflumic acid or flufenamic acid, although the TTP was significantly reduced and the membrane potential was hyperpolarized. Our computational modelling has confirmed that reduction in the fast depolarization could modify the form of the hyperpolarization to produce these effects in SHR if the membrane potential was also altered. Whether the two outward conductances that comprise the hyperpolarization represent those of IK_{Ca} and SK_{Ca} channels is beyond the scope of the present study. Taken together, the experimental and modelling data strongly support the hypothesis that the acetylcholine-induced hyperpolarization is reduced by the fast but not the slow depolarization and that these changes are only seen in the SHR. Furthermore, drugs that interfere with chloride handling reduced these effects

and, more importantly, the impaired acetylcholine-mediated relaxation recorded in SHR was improved.

In the present study we used isolated segments of the primary branches of mesenteric arteries that were stretched and pinned flat. These preparations had the advantage that we were able to make stable impalements of smooth muscle cells and continuously monitor membrane potential changes in the same cell in spite of the mechanical activity that accompanied both hyperpolarizations and depolarizations. Caution should be exercised, however, when extrapolating our results to in-vivo conditions since the arterial segments were not intraluminally perfused and the stimulus employed here was a brief pulse of acetylcholine, which may have no counterpart *in vivo*. Nevertheless, the results cannot simply be attributable to the experimental conditions since the membrane potentials of our vessels were not substantially different from those reported for pressurized rat mesenteric arteries [34]. Furthermore, when arteries were further depolarized by phenylephrine, acetylcholine evoked depolarization and constriction in SHR but not WKY rat vessels.

In conclusion, acetylcholine evokes two inward currents in mesenteric arteries of hypertensive rats, but only one of these is present in arteries from normotensive rats. The current unique to the SHR depends predominantly on the opening of Ca^{2+} -dependent Cl^- channels and acts to reduce EDHF-mediated hyperpolarization and relaxation of the smooth muscle of mesenteric arteries. Interestingly, an increase in Cl^- concentration in smooth muscle cells of deoxycorticosterone acetate salt hypertensive rats has been reported [15]. While such an increase may also occur in mesenteric arteries of SHR, it is unlikely that this would produce the alteration in temporal characteristics we observed for the current present in SHR. Alternatively, there may be upregulation in the expression of a novel molecular subtype of Ca^{2+} -dependent Cl^- channel in the SHR. Putative candidates for the Ca^{2+} -dependent Cl^- channels are members of the CLCA gene family, of which there are currently six subtypes cloned from mice and four from human tissues. Recent reviews [28,35–37], however, have cast doubts on the role of these molecules as the molecular equivalents of the physiological Ca^{2+} -dependent Cl^- channels due to their involvement in other cellular functions, such as cell adhesion and oncology, and the inability of the cloned subtypes to produce channels with biophysical properties that mimic those of the channels recorded from smooth muscle preparations. The recent identification of a conserved hydrolase domain has also led to the suggestion that this family of proteins are actually membrane-anchored or secreted metal-dependent hydrolases [38]. An alternative molecular counterpart for the Ca^{2+} -dependent Cl^- channels has been suggested to be the recently cloned bestrophin family, which is associated with

chloride transport in the retinal pigment epithelium and is responsible for a form of visual macular dystrophy [28,35,39]. Apart from involvement in retinal degeneration, however, the physiological function of these proteins has not yet been identified and expressed bestrophin channels, like the CLCA proteins, do not exhibit the voltage sensitivity of the native channels [28]. The absence of a clear molecular identity for the physiological Ca^{2+} -dependent Cl^- channels therefore prevents further investigation at this time.

Endothelial dysfunction in hypertension has been reported in both animals and humans (see [7]). In animal models of experimental hypertension, several studies suggest that impairment of EDHF may account, at least in part, for the reduced endothelial function [6,7]. Interestingly, endothelial dysfunction observed in the mesenteric artery of the SHR was improved by treatment with bumetanide. Our present findings therefore suggest that an increased activity of Cl^- channels may be responsible for endothelial dysfunction and impairment of EDHF in hypertension.

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