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K^+-induced hyperpolarization in rat mesenteric artery: identification, localization and role of Na^+/K^+-ATPases

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1 Mechanisms underlying K^+-induced hyperpolarizations in the presence and absence of phenylephrine were investigated in endothelium-denuded rat mesenteric arteries (for all mean values, $n=4$).

2 Myocyte resting membrane potential (m.p.) was -58.8 ± 0.8 mV. Application of 5 mM KCl produced similar hyperpolarizations in the absence (17.6 ± 0.7 mV) or presence (15.8 ± 1.0 mV) of 500 nM ouabain. In the presence of ouabain +30 μM barium, hyperpolarization to 5 mM KCl was essentially abolished.

3 In the presence of 10 μM phenylephrine (m.p. -33.7 ± 3 mV), repolarization to 5 mM KCl did not occur in the presence or absence of 4-aminopyridine but was restored (-26.9 ± 1.8 mV) on addition of iberiotoxin (100 nM). Under these conditions the K+-induced repolarization was insensitive to barium (30 μM) but abolished by 500 nM ouabain alone.

4 In the presence of phenylephrine + iberiotoxin the hyperpolarization to 5 mM K^+ was inhibited in the additional presence of 300 nM levcromakalim, an action which was reversed by 10 μM glibenclamide.

5 RT–PCR, Western blotting and immunohistochemical techniques collectively showed the presence of α_1-, α_2- and α_3-subunits of Na^+/K^+-ATPase in the myocytes.

6 In K^+-free solution, re-introduction of K^+ (to 4.6 mM) hyperpolarized myocytes by 20.9 ± 0.5 mV, an effect unchanged by 500 nM ouabain but abolished by 500 μM ouabain.

7 We conclude that under basal conditions, Na^+/K^+-ATPases containing α_2- and/or α_3-subunits are partially responsible for the observed K^+-induced effects. The opening of myocyte K^+ channels (by levcromakalim or phenylephrine) creates a 'K^+ cloud' around the cells which fully activates Na^+/K^+-ATPase and thereby abolishes further responses to $[K^+]_o$ elevation.

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Abbreviations: EDHF, endothelium-derived hyperpolarizing factor

Introduction

Small increases in extracellular potassium concentration ($[K]_o$) can generate a vasodilation which results in an increase in local blood flow of physiological significance. Thus, the loss of K^+ from contracting skeletal muscle cells is believed to increase blood flow to active muscle bundles (Skinner & Powell, 1967; Juel *et al*., 2000). Similarly, the efflux of K^+ from neurones in active regions of the CNS produces a local vasodilatation which helps to ensure an increased supply of oxygen and nutrients to 'busy' regions (Kuschinsky & Wahl, 1978). More recently, Edwards *et al*. (1998) proposed that the activation of vascular endothelial K^+ channels and the resulting outflow of K^+ could hyperpolarize and relax the underlying vascular smooth muscle in some rat arteries. Therefore in these vascular beds, K^+ acts as the endothelium-derived hyperpolarizing factor (EDHF), the identity of which has been the subject of much speculation (see reviews by Taylor & Weston, 1988; Garland *et al*., 1995; Edwards & Weston, 1998; Félétou & Vanhoutte, 2000).

The direct smooth muscle vasodilator action of K^+ in rat mesenteric arteries and the proposed identity of EDHF in this vessel as ionised K^+ have both been questioned following myograph studies in phenylephrine-contracted rat vessels (Andersson *et al*., 2000; Doughty *et al*., 2000; Lacy *et al*., 2000). Subsequently, however, detailed microelectrode experiments have shown that the opening of smooth muscle K^+ channels by phenylephrine produces a 'K^+ cloud' around the muscle and that this phenomenon prevents the vasodilator action of exogenously-applied K^+ (Richards *et al*., 2001). This leaves open the possibility that under physiological conditions of moderate to low vascular tone, endothelium-derived K^+ can indeed function as a local intravascular vasodilator (Richards *et al*., 2001).

A key component in K^+-induced vasodilation is the smooth muscle Na^+/K^+-ATPase (McCarron & Halpern, 1990; Prior *et al*., 1998), a family of proteins consisting of ion-transporting α- and modulatory β- and γ-subunits. The different isoforms of the Na^+/K^+-ATPase display different affinities for $[K^+]_o$. The α_1-containing isoforms (which in the rat are relatively insensitive to ouabain) may be considered as

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ubiquitous, 'housekeeping' forms which are fully activated by physiological concentrations of $[K^+]_o$ (~5.9 mM). In contrast, the α_2-and α_3-containing isoforms are further activated by small increases in $[K^+]_o$ and may act as 'reserves' (Blanco & Mercer, 1998; Crambert *et al*., 2000).

The purpose of this study was to confirm the involvement of Na^+/K^+-ATPase in K^+-induced hyperpolarization of the rat mesenteric artery smooth muscle and to identify the α-subunit isoforms present in this preparation. A preliminary account of some of these observations has been presented (Burnham *et al*., 2000).

Methods

Tissue dissection

Male Sprague–Dawley rats were killed by stunning and cervical dislocation. Whole mesenteric beds were placed in ice-cold Krebs solution (mM) NaCl 118, KCl 3.4, $CaCl_2$ 2.5, KH_2PO_4 1.2, $MgSO_4$ 1.2, $NaHCO_3$, 25, glucose 11, gassed with 5% CO_2 in O_2, and arteries were dissected free of surrounding tissue. Endothelium-intact vessels were used to provide samples for RT–PCR and Western blots.

Microelectrode studies

Small mesenteric arteries were pinned to the Sylgard base of a heated bath (10 ml) and superfused with Krebs solution containing 10 μM indomethacin and 300 μM N^G-nitro-L-arginine (10 ml min^{-1}, 37°C). Smooth muscle cells were impaled from the adventitial side using microelectrodes filled with 3 M KCl (tip resistance 50–80 MΩ). Recordings were made using a conventional high-impedance amplifier (Intra 767; WPI Instruments) and microelectrode recordings were digitized and analysed using a MacLab system (AD Instruments). Endothelial cells were removed by perfusing vessels with distilled water, and the lack of a functional endothelium was confirmed by an absence of response to acetylcholine. Levcromakalim, added directly to the bath to produce a transient, calculated concentration of 10 μM, was used to indicate the ability of the smooth muscle cells to hyperpolarize following endothelium removal and drug treatments. Phenylephrine (10 μM), 4-aminopyridine (5 mM), iberiotoxin (100 nM), levcromakalim (300 nM) and ouabain (500 nM or 500 μM) were added to the Krebs superfusing the bath. KCl (16.6 μl of 3 M stock) was added as a bolus directly into the 10 ml bath, close to the Krebs inflow, to produce (assuming rapid mixing) a calculated transient 5 mM elevation in extracellular K^+ concentration. In some experiments, tissues were incubated in K^+-free Krebs solution to inhibit all isoforms of Na^+/K^+-ATPase and the hyperpolarization induced by re-introduction of K^+ (ie. superfusion with normal Krebs solution) was monitored in the absence or presence of ouabain. For these experiments, KCl and KH_2PO_4 were replaced by appropriate, equimolar concentrations of NaCl or NaH_2PO_4, respectively, in the Krebs solution. Endothelium-denuded segments of artery were incubated for at least 30 min in K^+-free Krebs solution before the start of the experiment and, where appropriate, were exposed to ouabain for at least 10 min before the re-introduction of K^+.

Gene-specific RT–PCR

Total RNA was isolated from rat mesenteric arteries and brain using QIAGEN RNEasy Mini kits according to the manufacturer's instructions. Following DNAse treatment, cDNA synthesis was performed using Superscript II reverse transcriptase. RT–PCR primers, designed to amplify bases 3252-3410 of rat α_1 (NCBI accession number NM_012504), 4797-5040 of rat α_2 (NM_012505) and 3324-3529 of rat α_3 (NM_012506), comprised: α_1-forward: 5′GAAGCTCATCATCAGGCGACG3′, α_1-reverse: 5′CCAGGGTAGAGTTCCGAGCTC3′, α_2-forward: 5′GGGCCTGACTAATTTGAGATCACTG3′, α_2-reverse: 5′GTCTCACAGAAGGTCACCAGTAAGG3′, α_3-forward: 5′CCACACCTCGGTTACCTCTCAC3′, α_3-reverse: 5′CAGATTTAGAACCGGAGATGGC3′.

Hot-start RT–PCR was performed for 35 cycles with an annealing temperature of 60°C and 1.5 mM $MgCl_2$. RT–PCR products were visualized using 1.5% agarose-ethidium bromide gels. To confirm identity, PCR products were cloned using a TA cloning kit (Invitrogen) according to the manufacturer's instructions and sequenced using a Big Dye Terminator Kit (PE Applied Biosystems). Reaction products for sequencing were submitted to the central sequencing facility in the School of Biological Sciences, University of Manchester for analysis.

Western blotting

Rat kidney, heart, brain and mesenteric arteries were homogenized on ice in homogenization buffer (20 mM Tris pH 7.5, 0.25 M sucrose, 5 mM EDTA, 10 mM EGTA, Sigma protease inhibitor cocktail Sigma P2714) using ground-glass homogenizers. Homogenates were cleared by centrifugation for 10 s at $12{,}000 \times g$. Mesenteric artery samples were prepared as particulate fractions by centrifuging post-nuclear supernatants at $100{,}000 \times g$ for 30 min at 4°C before resuspending pellets in homogenization buffer. Protein concentrations were determined by the method of Bradford (1976) using Bio-Rad reagent. Western blot samples were prepared in 5 fold concentrated Laemmli sample buffer (Laemmli, 1970) and heated to 100°C for 5 min.

Sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS–PAGE), on 6% (w v^{-1}) acrylamide separating gels, and electrophoretic transfer to polyvinylidene fluoride membranes were performed as previously described (Laemmli, 1970; Towbin *et al*., 1979). Membranes were blocked for 1 h at room temperature in 50 mg ml^{-1} non-fat dried milk in Tween-Tris buffered saline (1 μl ml^{-1} Tween-20, 20 mM Tris pH 8.0 (pH 6.8 for α_2), 150 mM NaCl). Blots were probed with monoclonal antibodies M8P1A3 (anti-α_1, dilution 1 : 1000), McB2 (anti-α_2, dilution 1 : 250) and XVIF9-G10 (anti-α_3, dilution 1 : 4000) for 1 h at room temperature, except α_2 which was incubated overnight at 4°C. Detection was achieved using horseradish peroxidase-conjugated secondary antibodies and ECL reagents.

Immunofluorescence histochemistry

Arteries were immersed in periodate-lysine-paraformaldehyde fixative (McClean & Nakane, 1974) for 20 min and cryoprotected overnight in 0.3 g ml^{-1} sucrose in phosphate-buffered saline (PBS). After embedding in OCT compound,

4 μm cryostat sections were collected on silanated microscope slides. Sections were treated with 1 mg ml^{-1} SDS in PBS for 30 min, washed in PBS and blocked for 1 h with blocking buffer (50 μl ml^{-1} normal goat serum, 10 mg ml^{-1} bovine serum albumin in PBS). Primary antibodies (dilutions: anti-α_1, 1 : 50, anti-α_2, 1 : 5 and anti-α_3, 1 : 50; anti-von Willebrand's factor and anti-muscle actin, each 1 : 100) were applied for 1 h and secondary antibodies conjugated to Texas Red (α_1) or Cy3 (α_2 and α_3) were applied for 30 min together with 5 μg ml^{-1} 4,6-diamidino-2-phenylindole (DAPI) as a blue-fluorescent nuclear stain. Sections were viewed with a Zeiss epifluorescence Axioplan 2 microscope. Image capture and pseudocolouring were performed using Zeiss KS300 software. Identical microscope, camera and software settings were used when imaging labelled sections and negative controls.

Materials

For the microelectrode studies the following substances were used: synthetic iberiotoxin (Latoxan, France) and levcromakalim (SmithKline Beecham). All other reagents were supplied by Sigma U.K.

RNeasy Mini Kits (Qiagen), Superscript II RNase H^- reverse transcriptase (Life Technologies), DNase (Life Technologies, amplification grade) Ex-Taq polymerase (TaKaRa Biomedicals) and custom oligonucleotides (Sigma-Genosys) were used in cDNA production and PCR. Original TA cloning kit (Invitrogen) and Big Dye Terminator sequencing kit (Perkin Elmer) were used for PCR product cloning and sequencing. Primary antibodies anti-α_1 and anti-α_3 were obtained from Affinity Bioreagents. Anti-α_2 was kindly provided by K.J. Sweadner. Anti-von Willebrand's factor was from Novocastra and anti-muscle actin (mouse monoclonal; clone HHF35) was purchased from Dako). Secondary antibody conjugates were purchased from Jackson Immunoresearch (Stratech Scientific Ltd, U.K). OCT® compound was obtained from R.A. Lamb Ltd (East Sussex, U.K.). All other reagents were supplied by Sigma U.K.

Data analysis

All values are given as mean$\pm$s.e.mean; n indicates the number of arteries from which membrane potential recordings were made. Student's t-test (paired observations) was used to assess the probability that differences between mean values had arisen by chance. $P<0.05$ was considered to be significant.

Results

Microelectrode studies

The basal resting membrane potential of smooth muscle cells in endothelium-denuded vessels was -58.8 ± 0.8 mV ($n=4$). A transient increase in extracellular K^+ by 5 mM produced a smooth muscle cell hyperpolarization of 19.4 ± 1.4 mV ($n=4$). The hyperpolarization to 5 mM KCl in four arteries was similar in the absence (17.6 ± 0.7 mV) or presence of 500 nM ouabain (15.8 ± 1.0 mV) (Figure 1). However, in the additional presence of 30 μM barium (which itself depolarized the membrane by 3.4 ± 0.6 mV) the hyperpolarization to 5 mM KCl was almost abolished (1.1 ± 0.9 mV, $n=4$) (Figure 1).

In the presence of 10 μM phenylephrine, the smooth muscle cell membrane depolarised to -33.7 ± 2.8 mV ($n=4$) and a subsequent transient increase in extracellular K^+ by 5 mM was without effect (see Figure 2a). In the continued presence of phenylephrine, the inclusion of 100 nM iberiotoxin in the bathing solution produced a further depolarization (5.6 ± 1.1 mV, $n=4$). This was, however, larger than the small, but significant, depolarization produced by iberiotoxin in the absence of phenylephrine (1.6 ± 0.4 mV, $n=8$). In the presence of phenylephrine and iberiotoxin, a transient increase in extracellular K^+ by 5 mM partially repolarized the muscle cells (by 26.9 ± 1.8 mV, $n=4$). Subsequent exposure to 500 nM ouabain produced a further small depolarization of smooth muscle cell membranes (3.9 ± 1.7 mV, $n=4$) and abolished the response to a transient increase (5 mM) in extracellular K^+ (see Figure 2b), whereas 10 μM levcromakalim produced a hyperpolarization of 39.1 ± 0.4 mV ($n=4$). The extent to which ouabain depolarized the smooth muscle in the presence of phenylephrine and iberiotoxin was dependent on the membrane potential prior to its addition. Thus, in the presence of phenylephrine, the response to ouabain was small in artery segments in which iberiotoxin had produced a marked depolarization (see Figure 2a). However, in artery segments depolarized by

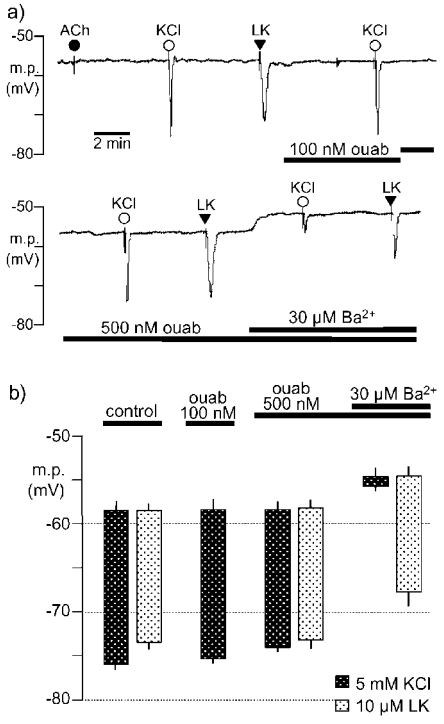

Figure 1 Effects of ouabain and barium on hyperpolarizations induced by 5 mM KCl and levcromakalim in rat endothelium-denuded mesenteric arteries. (a) Typical trace showing the initial smooth muscle hyperpolarization induced by transient application of 5 mM KCl and 10 μM levcromakalim (LK) in the absence (control) and presence of ouabain (ouab) and barium (Ba^{2+}) as indicated by the horizontal bars. In all experiments, removal of the endothelium was confirmed by a lack of response to 10 μM acetylcholine (ACh). (b) Graphical representation of data from four separate experiments of the type shown in (a). Each column represents the mean membrane potential (m.p.) before (+s.e.mean) and after (−s.e.mean) addition of 5 mM KCl or 10 μM levcromakalim, in the absence (control) and sequential presence of ouabain (ouab) and barium (Ba^{2+}).

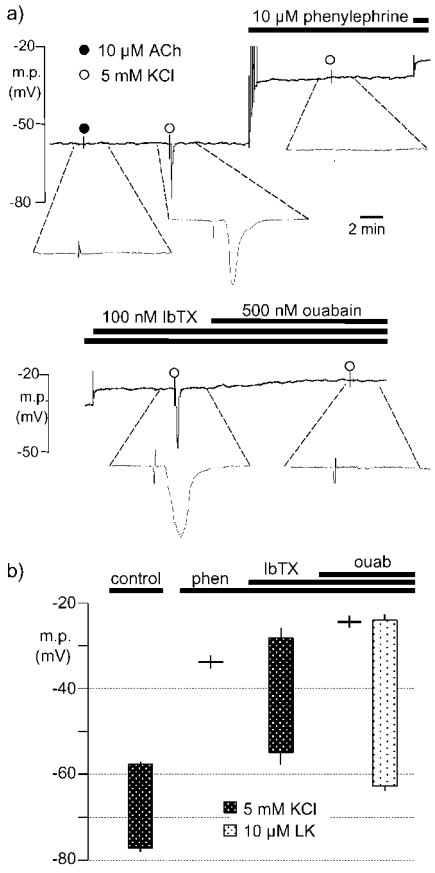

Figure 2 Effect of phenylephrine, iberiotoxin and ouabain on hyperpolarizations induced by 5 mM K^+ in rat endothelium-denuded mesenteric arteries. (a) Typical, single trace showing changes in smooth muscle membrane potential (m.p.) in response to transient application of 5 mM KCl (open circle) in the absence and presence of phenylephrine, iberiotoxin (IbTX) and ouabain as indicated by the horizontal bars. For clarity, the trace has been divided with some overlap such that the depolarization on addition of iberiotoxin is duplicated. In addition, 1 min portions of the trace have been expanded to allow the responses to be distinguished from the injection artefacts. (b) Graphical representation of data from four separate experiments of the type shown in (a). Each column represents the membrane potential before (+s.e.mean) and after (−s.e.mean) addition of 5 mM K^+, in the absence (control) and sequential presence of 10 μM phenylephrine (phen), 100 nM IbTX and 500 nM ouabain (ouab).

10 μM phenylephrine (which depolarized the membrane from -58.5 ± 0.5 to -30.5 ± 1.8 mV, $n=4$) but in the absence of iberiotoxin, the depolarizing effect of 500 nM ouabain was more marked (8.2 ± 0.9 mV, $n=4$).

In contrast to ouabain, 30 μM barium was without effect on the membrane potential in the presence of 10 μM phenylephrine +100 nM iberiotoxin and did not inhibit the subsequent hyperpolarization to 5 mM KCl (Figure 3). However, under these conditions, a low concentration of levcromakalim (which itself repolarized the smooth muscle by 8.4 ± 0.4 mV; $n=4$) markedly inhibited the hyperpolarization to 5 mM KCl (Figure 4). Depolarization and elevation of intracellular calcium potentially lead to the opening of both BK_{Ca} and delayed rectifier K^+ channels (K_V). Since, in the presence of phenylephrine, the hyperpolarization to K^+ could be restored by iberiotoxin (an inhibitor or BK_{Ca}), the effect of 4-aminopyridine (an inhibitor of K_V) was also determined. In the presence of 10 μM phenylephrine (m.p. -29.1 ± 0.4 mV, $n=4$), application of 5 mM 4-aminopyridine produced a further depolarization of 4.0 ± 0.7 mV, but 5 mM KCl had no effect in either the absence or presence of 4-aminopyridine. In the additional presence of 100 nM iberiotoxin the hyperpolarizing effect of KCl (30.3 ± 1.7 mV, $n=4$) was restored.

Re-introduction of K^+ following a period of at least 30 min in K^+-free Krebs hyperpolarized the smooth muscle (Figure 5). Essentially, neither the resting membrane potential nor this hyperpolarization was modified in segments of artery which had been subjected to 20 min incubation with 500 nM ouabain. This contrasts with the ability of such a concentration of ouabain to abolish the hyperpolarization induced by a 5 mM transient elevation of extracellular K^+ as shown in Figures 1 and 2 (although in the absence of phenylephrine there was a requirement for the additional presence of 30 μM barium to inhibit the inwardly-rectifying K^+ channel; Figure 1). However, the higher concentration of ouabain (500 μM) used in these K^+ re-introduction experiments depolarized the smooth muscle to -56.8 ± 0.4 mV and almost abolished the effect of K^+ re-introduction (Figure 5). Neither 500 nM ouabain nor 500 μM ouabain modified the membrane potential to which 10 μM levcromakalim hyperpolarized the smooth muscle (Figure 5).

Analysis of α-subunit expression

The expression of Na^+/K^+-ATPase α subunit isoforms in rat mesenteric arteries was examined by RT–PCR and Western blotting. RT–PCR, using primer pairs designed for the detection of each isoform, was performed in rat mesenteric artery cDNA preparations obtained from three rats. Messenger RNA for Na^+/K^+-ATPase α_1-, α_2- and α_3-subunits (159, 244 and 206 base-pair products, respectively) was detected in all three mesenteric artery samples (Figure 6a). Rat brain cDNA, which contains all three isoforms, was included as a positive control and detection of GAPDH housekeeping gene in each mesenteric artery sample verified cDNA integrity (not shown). The identity of each product was confirmed by cloning and sequencing. No products were amplified from template which was prepared without reverse transcription, thus excluding contamination by genomic DNA.

Samples of rat mesenteric artery were prepared for Western blotting, together with control samples from rat kidney (which predominantly expresses the α_1 isoform) skeletal muscle (which predominantly expresses the α_2 isoform) and brain (which expresses all three isoforms) (see Blanco & Mercer, 1998). The monoclonal, isoform-specific antibodies detected proteins of approximately the size expected for Na^+/K^+-ATPase α subunits (97.5 kDa) in the appropriate control tissues (Figure 5b), supporting the well-documented specificity of these antibodies towards rat α-subunit isoforms (Malik *et al.*, 1993; Arystarkhova & Sweadner, 1996). In rat mesenteric artery samples, the α_1, α_2 and α_3 isoforms were detected using 5, 60 and 25 μg protein loadings, respectively (Figure 6b).

Immunofluorescence labelling

Cryostat sections of rat mesenteric artery were labelled using the monoclonal antibodies specific to the α-subunit isoforms. Visualization of primary antibody binding was achieved using

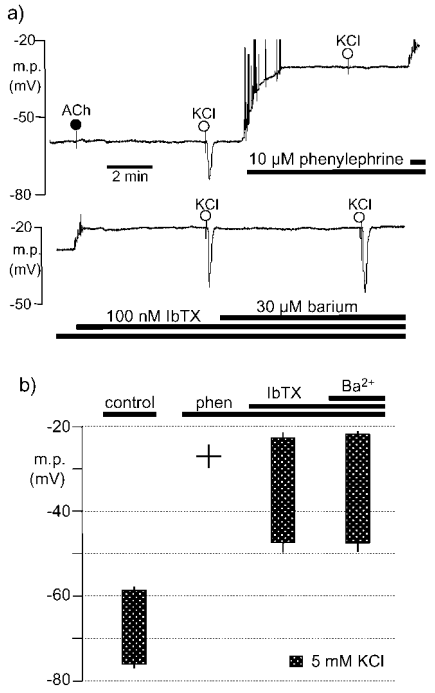

Figure 3 Lack of inhibitory effect of barium on hyperpolarizations induced by 5 mM K^+, in the presence of phenylephrine and iberiotoxin, in rat endothelium-denuded mesenteric arteries. (a) Typical single trace showing the smooth muscle hyperpolarization induced by transient application of 5 mM KCl in the absence (control) and presence of phenylephrine, iberiotoxin and barium as indicated by the horizontal bars. For clarity, the trace has been divided with some overlap such that the depolarization on addition of iberiotoxin is duplicated. In all experiments, removal of the endothelium was confirmed by a lack of response to 10 μM acetylcholine (ACh). (b) Graphical representation of data from four separate experiments of the type shown in (a). Each column represents the membrane potential (m.p.) before (+s.e.mean) and after (−s.e.mean) addition of 5 mM KCl, in the absence (control) and sequential presence of 10 μM phenylephrine (phen), 100 nM iberiotoxin (IbTX) and 30 μM barium (Ba^{2+}).

secondary antibodies conjugated to Texas Red or Cy3 (pseudo-coloured red, Figure 7); nuclei labelled with DAPI appear blue (Figure 7). The intrinsic autofluorescence of the internal elastic lamina was visible through the green filter-set, and was included in images to indicate the smooth muscle-endothelium boundary. This green autofluorescence did not interfere with the red signal from the secondary antibodies. Examination of multiple mesenteric artery sections from three rats indicated that the α_1, α_2, and α_3 isoforms were all expressed in the smooth muscle. Endothelial labelling for the α_1 and α_3 isoforms was also apparent (Figure 7).

Discussion

The role of the Na^+/K^+-ATPase in K^+-induced hyperpolarizations of rat mesenteric artery has been investigated together with the expression of Na^+/K^+-ATPase α_1-, α_2- and α_3-subunits. The Na^+/K^+-ATPase α_4-subunit was not investigated in this study, as it is believed to be restricted to the testis and epididymis (Shamraj & Lingrel, 1994; Underhill *et al*., 1999).

In agreement with previous work (Richards *et al*., 2001), the present study showed that pre-stimulating endothelium-denuded rat mesenteric arteries with 10 μM phenylephrine abolished the smooth muscle hyperpolarization in response to transient 5 mM increases in $[K^+]_o$. However, this observation was extended by the demonstration that K^+-induced hyperpolarizations were restored by iberiotoxin alone and that 4-aminopyridine was totally without effect. Thus, the restoration of the hyperpolarization to K^+ by the combination of iberiotoxin and 4-aminopyridine, which was previously reported (Richards *et al*., 2001), is due solely to the effect of iberiotoxin. This suggests that the formation of a 'K^+ cloud' by K^+ efflux *via* the large-conductance calcium-sensitive K^+ channel (BK_{Ca}) plays a major role in preventing the hyperpolarizing effects of transient increases in $[K^+]_o$ in phenylephrine-stimulated preparations.

Although we have not directly measured an increase in the $[K^+]_o$ between the smooth muscle cells, such an increase is consistent with changes in the hyperpolarizing effect of levcromakalim. Thus, 10 μM levcromakalim hyperpolarized to -73.3 ± 0.8 mV ($n=4$) in the presence of 10 μM phenylephrine but to -82.1 ± 0.7 mV ($n=12$) in its absence. At this concentration of 10 μM we assume that levcromakalim hyperpolarizes to the K^+ equilibrium potential (E_K), and thus it appears that phenylephrine modifies E_K. If it assumed that this concentration (10 μM) of levcromakalim hyperpolarizes to E_K, phenylephrine (10 μM) must shift E_K by approximately 10 mV in a depolarizing direction. Using the Nernst equation (see Hamilton *et al*., 1986) such a shift in E_K could be explained by an increase in extracellular K^+ of approximately 5 mM.

The depolarization induced on exposure to iberiotoxin in the presence of phenylephrine suggests that some BK_{Ca} channels are contributing to the net membrane potential. The fact that the membrane remains very depolarized under these conditions indicates that the dominant conductance is likely to be due to open Cl^- channels (Large & Wang, 1996) rather than to BK_{Ca}.

An interesting observation from the present study was that when 10 μM phenylephrine was present, the membrane potential in the additional presence of 500 nM ouabain (21.9 ± 1.2 mV, $n=4$) was not significantly different from that in the presence of 100 nM iberiotoxin (-20.3 ± 2.9 mV, $n=8$), of iberiotoxin + 500 nM ouabain (-23.5 ± 1.2 mV, $n=4$) or of iberiotoxin + 4-aminopyridine (-22.1 ± 1.9 mV, $n=8$). Thus, despite the fact that ouabain, 4-aminopyridine and iberiotoxin each produce depolarization by different mechanisms, the steady-state membrane potential is approximately −22 mV. The calculated Cl^- equilibrium potential (E_{Cl}) in smooth muscle lies in the range −25 to −20 mV (Aickin & Brading, 1983; Large & Wang, 1996). It thus appears that the dominant membrane conductance in the presence of phenylephrine is due to the presence of open chloride channels. Almost certainly, the proximity of the membrane potential to E_{Cl} contributes to the lack of depolarization to K^+ observed. Under conditions in which hyperpolarization to K^+ does not occur (which we assume is due to saturation of Na^+/K^+-ATPase), it would be expected that elevation of $[K^+]_o$ would have a depolarizing effect due to the change in E_K. Such an effect was never observed, presumably partially because of the dominance of the Cl^- conductance, but perhaps also due to the opening of additional BK_{Ca} channels in response to the depolarization.

The inability of K^+ to induce hyperpolarization in the presence of phenylephrine could be explained by a reduction

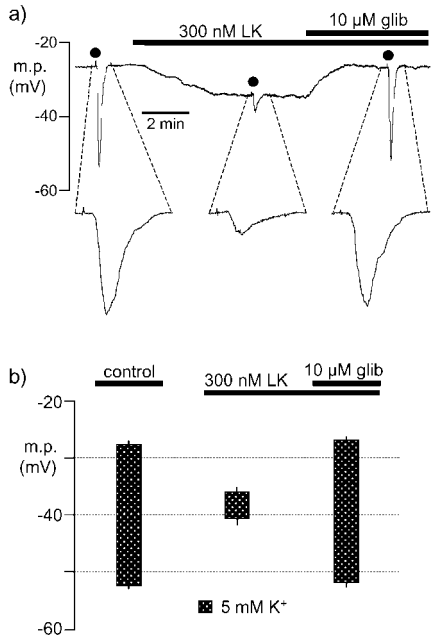

Figure 4 Inhibitory effect of levcromakalim on hyperpolarizations induced by 5 mM K^+, in the presence of phenylephrine and iberiotoxin, in rat endothelium-denuded mesenteric arteries. (a) Typical trace showing the smooth muscle hyperpolarization induced by transient application of 5 mM KCl in the presence of phenylephrine (10 μM) and iberiotoxin (100 nM). The response was inhibited by exposure to 300 nM levcromakalim (LK) and restored by 10 μM glibenclamide (glib) in the continued presence of levcromakalim. In all experiments, removal of the endothelium was confirmed by a lack of response to 10 μM acetylcholine (ACh). (b) Graphical representation of data from four separate experiments of the type shown in (a). Each column represents the membrane potential (m.p.) in the presence of phenylephrine and iberiotoxin before (+s.e.mean) and after (−s.e.mean) addition of 5 mM KCl, in the absence (control) and sequential presence of 300 nM levcromakalim (LK) and 10 μM glibenclamide (glib).

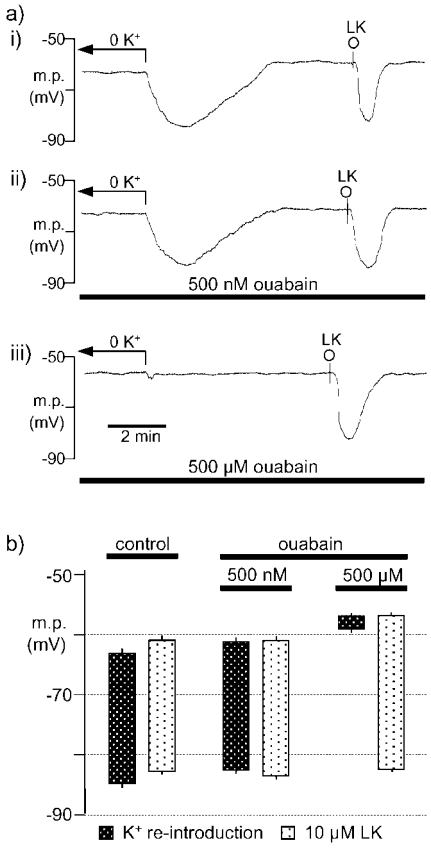

Figure 5 Effect of ouabain on hyperpolarization induced by K^+-reintroduction (to 4.6 mM) following a period of at least 30 min in K^+-free Krebs (0 K^+). (a) Typical traces showing the hyperpolarization in the absence (i) or in the presence of 500 nM (ii) or 500 μM (iii) ouabain. Levcromakalim (10 μM; LK) was added as a positive control. (b) Graphical representation of data from four separate experiments of the type shown in (a). The columns represent either the membrane potential (m.p.) in K^+-free Krebs and the peak hyperpolarization following the switch to normal Krebs or that in normal Krebs before (+s.e.mean) and after (−s.e.mean) addition of 10 μM levcromakalim, in the absence (control) and presence of ouabain.

in membrane resistance due to the presence of open chloride channels such that, despite the current carried by Na^+/K^+-ATPase, there was no voltage change (since V = IR : Ohm's law). However, previous studies were able to exclude a reduction in smooth muscle input resistance as the primary cause underlying the loss of K^+-induced hyperpolarization (Richards *et al*., 2001). In addition, ouabain (500 nM) produced a further depolarization in the presence of phenylephrine, suggesting that the Na^+/K^+-ATPase is indeed contributing to the membrane potential. Collectively, the data favour the interpretation that the ouabain-sensitive, K^+-responsive isoforms of Na^+/K^+-ATPase are maximally activated in the presence of phenylephrine due to the presence of a 'K^+ cloud' in the interstitial spaces.

The present study confirms that in non-depolarized preparations, a combination of 30 μM barium and 500 nM ouabain was required to inhibit K^+-induced hyperpolarization of rat hepatic arteries, thus implicating both an inward-rectifier K^+ channel and Na^+/K^+-ATPase in the response (Edwards *et al*., 1999). However, in the presence of phenylephrine, when K^+-induced hyperpolarizations were restored by iberiotoxin, 500 nM ouabain alone completely inhibited the hyperpolarizing effects of transient increases in $[K^+]_o$. Since phenylephrine produced a large depolarization, the inward-rectifier K^+ channel (which is inhibited by 30 μM barium) should not conduct outward K^+ current. Indeed the present data showed a complete lack of effect of barium in the presence of the spasmogen, confirming unexplained earlier observations (Dora & Garland, 2001). Thus, whereas hyperpolarization due to activation of the Na^+/K^+ ATPase by elevations in $[K^+]_o$ can occur in both resting and phenylephrine-stimulated preparations (provided that the proposed formation of a K^+ cloud is prevented), inwardly-rectifying potassium channels only contribute to hyperpolarization in non-stimulated vessels. The activation of Na^+/K^+-ATPase by the K^+ cloud is further supported by the intrinsic depolarizing effect of a low concentration of ouabain in the presence but not in the absence of phenylephrine.

To test further the 'K^+ cloud' hypothesis, vessels pretreated with phenylephrine in the presence of iberiotoxin were exposed to levcromakalim, an opener of ATP-sensitive K^+ channels (K_{ATP}). Under these conditions, a K^+ cloud was presumably generated by K^+ efflux through K_{ATP} and the hyperpolarizing effects of added K^+ were virtually abolished.

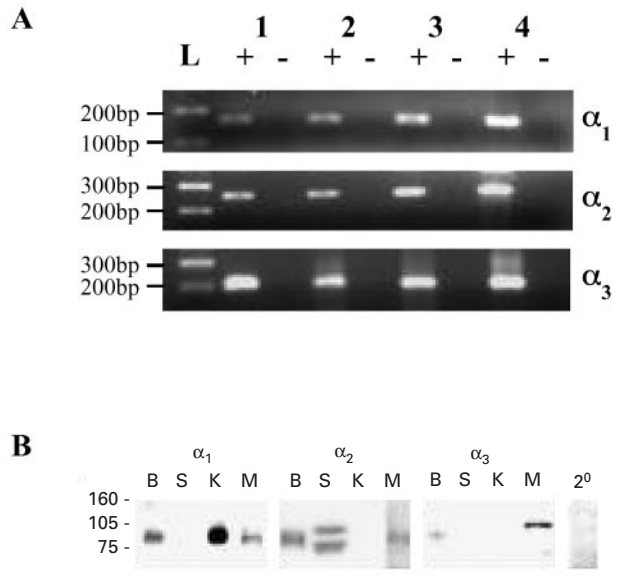

Figure 6 Detection of Na^+/K^+ ATPase α-subunits by RT–PCR and Western blot analysis. (a) RT–PCR products of the expected sizes were detected in rat mesenteric artery (lanes 1–3) and rat brain (positive control, lane 4) cDNA samples prepared with (+), but not without (−), reverse transcription. For size comparison, a 100 base-pair DNA ladder is indicated (L). (b) Western blot analysis of rat mesenteric artery samples (M), together with control samples from rat kidney (K; which predominantly expresses the α_1 isoform), skeletal muscle (S; which predominantly expresses the α_2 isoform) and brain (B; which expresses all three isoforms). The lack of reactivity of secondary antibody alone with the mesenteric artery (2^o) is evident. Molecular weight markers are indicated (kDa).

In the additional presence of the K_{ATP} inhibitor glibenclamide the hyperpolarizing effect of K^+ elevation was restored.

Although this observation is consistent with the 'cloud' hypothesis, it is possible (as discussed earlier) that the opening of K_{ATP} reduced membrane resistance sufficiently to diminish the hyperpolarizing effects of the current generated by Na^+/K^+-ATPase. However, a low concentration of levcromakalim was chosen to minimise any decrease in membrane resistance and earlier experiments (Richards *et al*., 2001) provided no evidence that a decrease in membrane resistance could account for the loss of K^+-induced hyperpolarization in the presence of phenylephrine. Collectively, therefore, the results favour the view that the loss of K^+-induced hyperpolarization in the presence of phenylephrine alone or following exposure to phenylephrine + iberiotoxin + levcromakalim are mainly the result of K^+ cloud formation rather than of a lowering of membrane resistance.

Rat Na^+/K^+ ATPase α-subunits show differing sensitivities to inhibition by ouabain, such that the ouabain IC_{50} is approximately 50 μM for the α_1-subunit, whereas for the α_2 or α_3 isoforms the IC_{50} values are in the nanomolar range (O'Brien *et al*., 1994). In addition, the different Na^+/K^+-ATPase α subunits show a varying degree of activation in response to changes in extracellular K^+, a phenomenon which may be modulated by the β subunit (for references see Hasler *et al*., 1998). Studies of the kinetic characteristics of rat Na^+/K^+-ATPase isozymes in insect Sf-9 cells suggest that whereas α_1-containing isoforms are maximally activated at physiological $[K^+]_o$, the α_2-and α_3-containing isoforms can increase their activity in response to small elevations in K^+ concentration (Blanco & Mercer, 1998). The functional significance of this observation has been verified in guinea-pig mesenteric resistance artery smooth muscle cells in which an increase in $[K^+]_o$ from 5 to 10 mM increases Na^+/K^+-ATPase currents (Nakamura *et al*., 1999).

In the present study, 500 nM ouabain had little effect on the resting membrane potential in the absence of phenylephrine but produced depolarization in the presence of this agonist. This suggests that the isoform of Na^+/K^+-ATPase which is stimulated by elevation of extracellular K^+ (either by exogenous KCl or by K^+ which effluxes from smooth muscle BK_{Ca} channels: see Richards *et al*., 2001) is not active under the basal conditions but is active in the presence of phenylephrine. In contrast, the hyperpolarization produced by reintroduction of potassium ions after incubation in potassium-free solution was ouabain-resistant and this electrical change therefore appears to result predominantly from re-activation of the α_1 isoform. A high concentration of ouabain (500 μM), which would inhibit the α_1-containing isoform of Na^+/K^+-ATPase (Blanco & Mercer, 1998), did produce smooth muscle depolarization, indicating that this isoform is active under basal conditions. We have previously shown in the rat hepatic artery that 1 mM ouabain has very little effect on the hyperpolarizing action of K^+ on de-endothelialised vessels in the absence of barium (Edwards *et al*., 1998). Barium alone (30 μM) also only had a small inhibitory effect on the response to K^+, although together barium + ouabain abolished the hyperpolarization to K^+ (Edwards *et al*., 1998). This suggests that depolarizing effects of high concentrations of ouabain are due to selective inhibition of Na^+/K^+-ATPase, with little, if any, effect which is attributable to inhibition of inwardly-rectifying K^+ channels (which may also contribute to the resting membrane potential; Nelson & Quayle, 1995). Thus, the hyperpolarizing Na^+/K^+-ATPase currents which have been recorded from vascular smooth muscle cells 'at rest' (Quinn *et al*., 2000) are almost certainly due to the activity of an α_1-isoform, whereas (as previously proposed, Edwards *et al*., 1999) α_2- and/or α_3-isoforms are likely to be responsible for the K^+-induced hyperpolarization.

Our molecular studies indicate that rat mesenteric arteries do indeed express mRNA transcripts for Na^+/K^+-ATPase isoforms responsive to raised K^+ (α_2 and α_3) as well as the 'housekeeping' isoform (α_1). Furthermore, the α_1, α_3, and to a lesser extent α_2, proteins were detected in this tissue and localized to the myocytes. Previous studies of cultured rat mesenteric artery myocytes detected the α_1 and α_3 Na^+/K^+-ATPase isoforms by Western blot analysis but, possibly due to cell culturing, failed to detect the α_2 isoform (Juhaszova & Blaustein, 1997a). However, expression of α_2 and/or α_3 isoforms on the myocytes suggests that the machinery is present for the generation of smooth muscle hyperpolarizations in response to increases in extracellular K^+. The work of Juhaszova & Blaustein (1997a,b) showed α_1 subunits had a homogeneous distribution across the smooth muscle cell surface, whereas the α_3 protein seemed to correspond to the underlying sarcoplasmic reticulum (SR), in close association with the Na^+/Ca^{2+} exchanger. The same workers observed a similar pattern in neurones. They suggested that the α_3-containing isozymes may act in concert with the Na^+/Ca^{2+} exchanger to control intracellular Na^+ and Ca^{2+} concentrations in the vicinity of the SR, thereby controlling Ca^{2+}

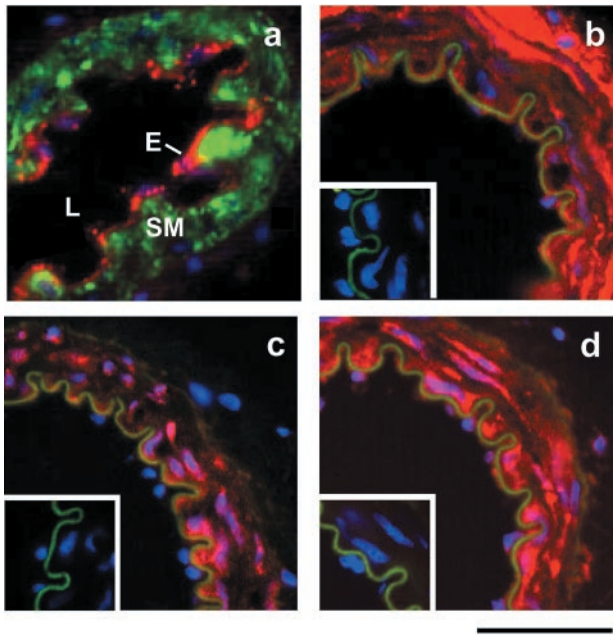

Figure 7 Localization of Na^+/K^+ ATPase α-subunits by immunofluorescence labelling. The positions of the lumen (L), endothelium (E) and smooth muscle (SM) are as indicated in (a) in which sections were dual-labelled with anti-von Willebrand's factor (red) and anti-muscle actin (green). (b) α_1-, (c) α_2- and (d) α_3- labelling was present in the smooth muscle cell layers of rat mesenteric arteries. Negative controls (no primary antibody) are shown as insets. In all sections, DAPI-stained nuclei appear blue and in (b), (c) and (d) anti-α-subunit immunoreactivity appears red and the autofluorescence of the internal elastic lamina is green. Scale bar indicates 50 μm.

mobilisation, and cellular excitability. In addition Blanco & Mercer (1998) have suggested that α subunits that are less sensitive to K^+ (i.e. α_2 and α_3) might be activated to restore ionic gradients after nerve impulses. The results of the present study suggest that the α_2 and α_3 isoforms may also have an important role in tempering contractile responses following agonist-induced depolarization of vascular smooth muscle.

In the presence of phenylephrine, but in the absence of iberiotoxin, K^+ induced smooth muscle hyperpolarization occurs in intact but not in endothelium-denuded arteries (Richards *et al*., 2001; present study). This suggests that under these conditions, endothelial cells are still able to respond to elevation of K^+ and to influence the smooth muscle membrane potential. We previously proposed that the phenylephrine-induced K^+ cloud does not fully envelop the endothelial cells, thus allowing some response to elevation of extracellular K^+ (Richards *et al*., 2001). To explore an alternative possibility, namely that Na^+/K^+-ATPase subunits with different K^+ sensitivities are present on endothelial and smooth muscle cells, the distribution of the subunits was assessed using an immunohistochemical approach. Surprisingly, the α2 isoform was predominantly found in the smooth muscle and there was no essential difference between these cells with respect to the expression of the isoform (α_3) which has the lowest affinity for K^+ (Blanco & Mercer, 1998).

In a variety of tissues, Na^+/K^+-ATPase activity is enhanced by α_1-adrenoceptor activation (Viko *et al*., 1997; Holtback & Eklof, 1999; Mallick *et al*., 2000), possibly by dephosphorylation of this enzyme (Mallick *et al*., 2000). Since the effects of phenylephrine in arteries are restricted to the myocytes (Dora *et al*., 1997), it is possible that the degree of activation of the ouabain-sensitive isoforms of Na^+/K^+-ATPase in the myocytes prior to elevation of $[K^+]_o$ may have been greater than that of the same isoforms in endothelial cells. This would explain the lower sensitivity of the smooth muscle Na^+/K^+-ATPase to the hyperpolarizing effect of raising extracellular K^+ in comparison to the endothelial cells (in which phenylephrine had not stimulated Na^+/K^+-ATPase).

Na^+/K^+-ATPase expression and activity is regulated by a variety of moieties including the cytoskeleton, endogenous inhibitors, hormones and protein kinases (for review see Therien & Blostein, 2000) and different subunit isoforms may be heterogeneously regulated. In addition to the multiple α-subunit isoforms there are also at least three isoforms of β-subunit which also show tissue-specific expression. Little is known about the endogenous dimerization of specific subunit isoforms but the opportunity for functional diversity of Na^+/K^+-ATPases seems to be considerable.

Conclusion

The results of the present study show that rat mesenteric artery myocytes possess α_1-, α_2- and α_3-subunits isoforms of Na^+/K^+-ATPase. Furthermore, those comprising α2- or α3-subunits, which are sensitive to low concentrations of ouabain, are responsible for the hyperpolarization induced by small increases in $[K^+]_o$ above the basal physiological level. We conclude that in the presence of phenylephrine, the elevation of intracellular Ca^{2+} and depolarization of the smooth muscle stimulates K^+ efflux, predominantly through BK_{Ca}. Under these conditions the accumulation of an extracellular K^+ 'cloud' could occur and prevent any further activation of α_2- and/or α_3-type Na^+/K^+-ATPases in response to elevation of extracellular K^+.

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