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SPECIAL REPORT

Suppression of K^+-induced hyperpolarization by phenylephrine in rat mesenteric artery: relevance to studies of endothelium-derived hyperpolarizing factor

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In intact mesenteric arteries, increasing $[K^+]_o$ by 5 mM hyperpolarized both endothelial and smooth muscle cells. Subsequent exposure to 10 μM phenylephrine depolarized both cell types which were then repolarized by a 5 mM increase in $[K^+]_o$. In endothelium-denuded vessels, increasing $[K^+]_o$ by 5 mM hyperpolarized the smooth muscle but K^+ had no effect after depolarization by 10 μM phenylephrine. On subsequent exposure to iberiotoxin plus 4-aminopyridine, the repolarizing action of 5 mM K^+ was restored. In endothelium-intact vessels exposed to phenylephrine, pretreatment with a gap junction inhibitor (gap 27) reduced K^+-mediated smooth muscle repolarization without affecting the endothelial cell response. It is concluded that phenylephrine-induced efflux of K^+ *via* smooth muscle K^+ channels produces a local increase in $[K^+]_o$ which impairs repolarization to added K^+. Thus, studies involving vessels precontracted with agonists which increase $[K^+]_o$ maximize the role of gap junctions and minimize any contribution to the EDHF pathway from endothelium-derived K^+.
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Abbreviations: EDHF, endothelium-derived hyperpolarizing factor; $[K^+]_o$, extracellular K^+ concentration

Introduction When stimulated by acetylcholine, substance P or bradykinin, the vascular endothelium releases an endothelium-derived hyperpolarizing factor (EDHF) which is distinct from both nitric oxide and prostacyclin (Edwards & Weston, 1998). In rat hepatic and mesenteric arteries, Edwards *et al.* (1998) showed that the initial phase in the EDHF pathway was endothelial cell hyperpolarization following ligand-induced activation of charybdotoxin- and apamin-sensitive K^+ channels on the endothelial cells. These workers proposed that EDHF was in fact the K^+ which effluxed through these channels. The resulting local elevation in K^+ concentration ($[K^+]_o$) in the myoendothelial space activated smooth muscle Na^+/K^+ ATPases and inwardly-rectifying K^+ channels, thus producing the observed smooth muscle hyperpolarization (Edwards *et al.*, 1998).

Lacy *et al.* (2000) have questioned the role of K^+ as a smooth muscle hyperpolarizing factor in rat mesenteric arteries. Using endothelium-intact vessels pre-contracted with phenylephrine 10 μM, these workers only obtained relaxations to elevated K^+ in 30–40% of vessels studied. Furthermore, endothelium removal abolished the relaxant responses of phenylephrine-precontracted vessels to increases in $[K^+]_o$.

To clarify these findings, the effects of raising $[K^+]_o$ on endothelial and smooth muscle membrane potential have now been examined in rat mesenteric small arteries in the presence and absence of phenylephrine.

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Methods Male Sprague-Dawley rats (150–200 g) were killed by stunning and cervical dislocation. Small mesenteric arteries were dissected, pinned to the base of a heated bath (10 ml) and superfused (10 ml min^{-1}), at 37°C, with Krebs solution containing 10 μM indomethacin and 300 μM N^G-nitro-L-arginine and gassed with 95% O_2/5% CO_2. Cells were impaled from the adventitial (smooth muscle) or intimal (endothelial cell) sides (Edwards *et al.*, 1998). Transmembrane potential was recorded using glass capillary electrodes (tip resistance 50–80 MΩ) filled with 3 M KCl. In some experiments, endothelium was removed by perfusing the vessel with distilled water, and the lack of a functional endothelium was confirmed in each preparation by the absence of a response to acetylcholine. Levcromakalim (10 μM) was employed to assess the ability of smooth muscle cells to hyperpolarize following endothelium removal and drug treatments. Smooth muscle membrane input resistance in de-endothelialized vessles, was derived from the mean membrane potential changes generated by 1 nA current pulses, each of duration 1 s and delivered at 10 s intervals *via* the recording electrode. Prior to insertion into the cell, microelectrode tip resistance and capacitance were conventionally negated using the microelectrode preamplifier (Intra 767; WPI Instruments). To investigate the effects of gap 27, vessels were preincubated with the peptide at 37°C for 60 min after which impalements were made during the washout of the inhibitor. Drugs (including KCl dissolved in Krebs) were added as bolus injections (10–20 μl) directly into the 10 ml bath in quantities calculated to give (transiently) the final concentrations indicated. Microelectrode recordings

were digitized and analysed using a MacLab system (AD Instruments).

Solutions and drugs Krebs solution comprised (mM): Na^+ 143, K^+ 4.6, Ca^{2+} 2.5, Mg^{2+} 1.2, Cl^- 126.4, $H_2PO_4^-$ 1.2, SO_4^{2-} 1.2, HCO_3^- 25, glucose 11.1. The following substances were used: acetylcholine (Sigma), gap 27 (Chaytor *et al*., 1998; amino-acid sequence SRPTEKTIFII, purity >99%, Severn Biotech), phenylephrine (Sigma), levcromakalim (SmithKline Beecham), synthetic iberiotoxin (Latoxan, France) and 4-aminopyridine (Sigma).

Statistics Data are shown as mean±s.e.mean; *n* indicates the number of cells in which membrane potential recordings were made. Student's *t*-test (paired or unpaired as appropriate) was used to assess the probability that differences between the mean values had arisen by chance. $P<0.05$ was considered to be significant.

Results *Effects of K^+ on smooth muscle cells* The basal resting membrane potential of smooth muscle cells in endothelium-intact mesenteric arteries was -59.7 ± 1.0 mV ($n=4$). On exposure to 5 mM K^+, a hyperpolarization of 12.9 ± 0.6 mV ($n=4$) was recorded (Figure 1b). Subsequent removal of the endothelium produced a slight depolarization (to -57.9 ± 0.8 mV, $n=4$) and 5 mM K^+ again hyper-polarized the smooth muscle by 14.2 ± 0.5 mV ($n=4$; Figure 1b).

In endothelium-intact preparations, 10 μM phenylephrine produced a marked smooth muscle cell depolarization (to -34.2 ± 1.0 mV; $n=4$) and fast, spike-like potentials were often observed (Figure 1c). Under these conditions, 5 mM K^+ increased the membrane potential by 23.0 ± 0.8 mV ($n=4$). However, following endothelium removal in these vessel segments, 5 mM K^+ was without effect (Figure 1c).

In all these experiments, removal of the endothelium abolished acetylcholine-induced hyperpolarizations of the smooth muscle cells but had no significant effect on membrane responses to 10 μM levcromakalim (Figure 1b,c).

Effects of K^+ on endothelial cells The basal resting membrane potential of endothelial cells was -57.2 ± 0.3 mV ($n=4$) and on exposure to 5 mM K^+, a hyperpolarization of 15.4 ± 0.7 mV ($n=4$) developed (Figure 1a). Subsequent application of 10 μM phenylephrine depolarized the endothelial cells by 22.8 ± 1.7 mV ($n=4$; $P<0.05$), an action often accompanied by fast spike-like depolarizations. In the presence of phenylephrine, 5 mM K^+ was still able to increase endothelial cell membrane potential (by 31.1 ± 1.2 mV, $n=4$; Figure 1a). In all of the above conditions, 10 μM acetylcholine produced marked hyperpolarization of endothelial cells (Figure 1a).

Restoration of K^+-induced membrane potential changes by iberiotoxin+4-aminopyridine Experiments were undertaken to clarify the finding that the hyperpolarizing action of 5 mM K^+ was lost in the presence of 10 μM phenylephrine (Figure 1b–d). In these experiments, K^+-induced hyperpolarizations (18.1 ± 0.9 mV, $n=4$) were first generated in endothelium-denuded vessels under basal conditions (input resistance 22.5 ± 1.1 MΩ, $n=4$). Phenylephrine (10 μM) was then applied and in the presence of the ensuing depolarization (25.7 ± 1.8 mV, $n=4$; input resistance 14.8 ± 0.8 MΩ, $n=4$),

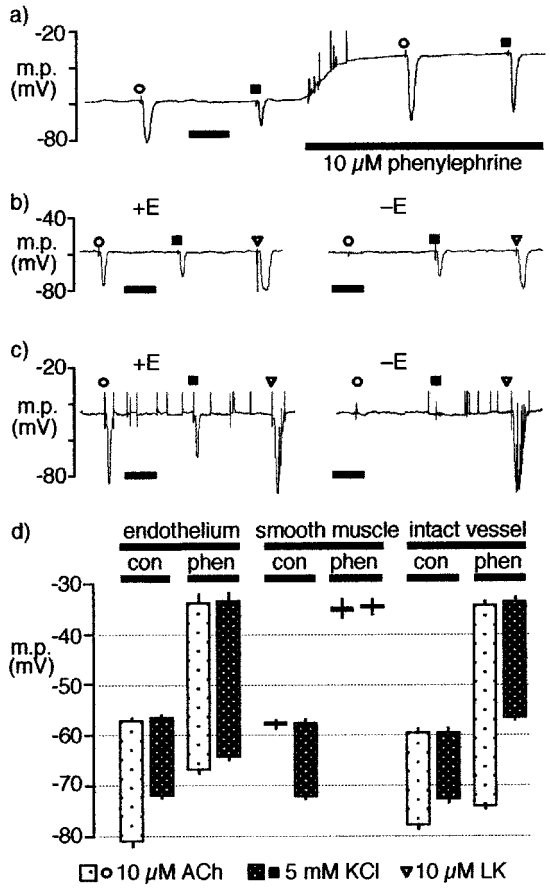

Figure 1 Effect of phenylephrine (phen) on hyperpolarizations induced by acetylcholine (ACh), KCl or levcromakalim (LK) in rat mesenteric arteries. (a) endothelial responses, (b) and (c) smooth muscle responses in the presence (+E) or absence (−E) of the endothelium. Horizontal time markers represent 2 min. (d) Graphical representation of data from four separate experiments of the types shown in (a)–(c). Each column represents the membrane potential (m.p.), + or −s.e.mean, before and after exposure to acetylcholine, KCl or levcromakalim and in the absence (con) and presence of 10 μM phenylephrine. Note that in (a) and (c) the amplitude of some spike-like deflections has been reduced for clarity.

exposure to 5 mM K^+ was (as previously observed, see Figure 1c,d) completely without effect (Figure 2). Exposure to a mixture of 100 nM iberiotoxin plus 5 mM 4-aminopyridine produced a further membrane depolarization of 9.8 ± 0.3 mV ($n=4$). In the four vessels examined, a small, mean increase in input resistance (to 15.9 ± 1.1 MΩ, $n=4$) ensued but this did not achieve statistical significance. Subsequent increases in $[K^+]_o$ by 5 mM in the presence of iberiotoxin plus 4-AP increased smooth muscle membrane potential by 20.2 ± 2.9 mV (Figure 2, $n=4$), a change similar to that seen prior to addition of phenylephrine ($P>0.05$).

Effects of gap 27 Incubation of endothelium-intact vessels for 60–90 min with 500 μM gap 27 had no effect on the resting membrane potential of endothelial cells (-57.6 ± 0.33 mV, $n=4$) or on the magnitude of their hyperpolarizations to 5 mM KCl (15.9 ± 0.7 mV, $n=4$) or 10 μM acetylcholine (21.4 ± 1.4 mV, $n=4$). However, endothelial cells became less depolarized in the presence of phenylephrine (to -38.9 ± 0.5 mV, $n=4$) than in controls (to

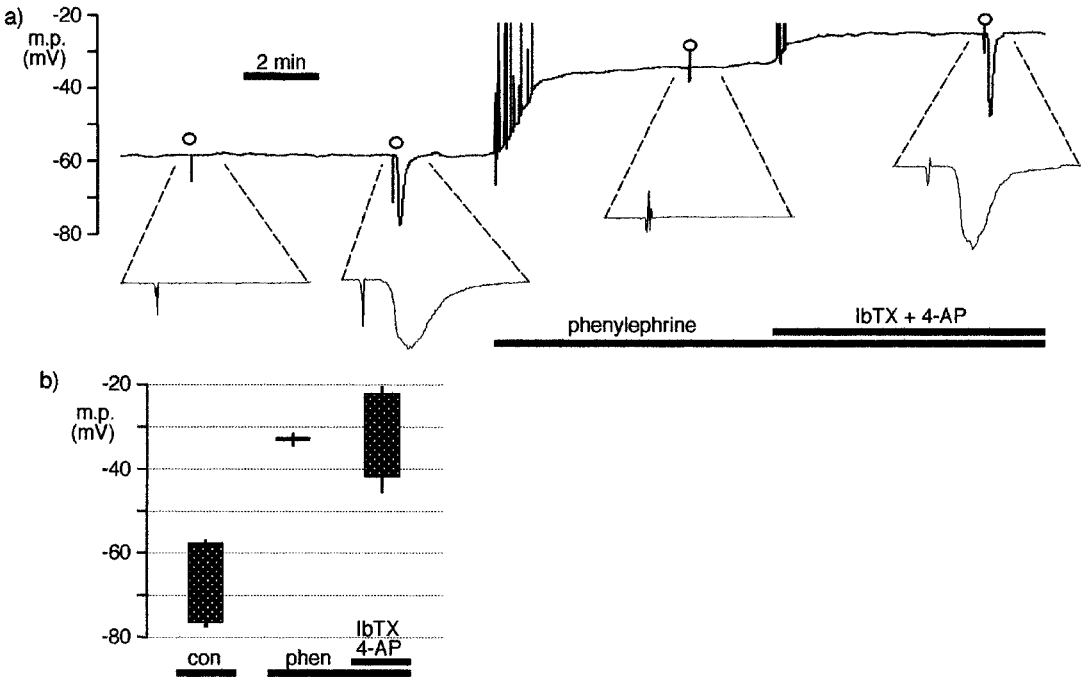

Figure 2 Restoration of KCl-induced hyperpolarization in the presence of 10 μM phenylephrine by 100 nM iberiotoxin (IbTX) + 5 mM 4-aminopyridine (4-AP) in an endothelium-denuded vessel. One min segments of trace have been expanded to allow responses and injection artifacts to be distinguished. Some spike-like deflections have been truncated for clarity. (b) Graphical representation of data from four separate experiments of the types shown in (a) in which each column shows the membrane potential (m.p.), + or − s.e.mean, before and after exposure to 5 mM KCl under control conditions (con) or in the presence of 10 μM phenylephrine (phen) with or without IbTX and 4-AP.

-33.6 ± 1.5 mV, $n=4$; $P<0.05$). In addition, the spike-like depolarizations which especially characterized the initial depolarizing phase of the action of phenylephrine were of reduced amplitude.

In endothelium-intact vessels in the presence of 1 μM phenylephrine, gap 27 had no effect on the membrane potential of smooth muscle cells (without gap 27, -48.7 ± 1.2 mV, $n=4$; with gap 27, -48.5 ± 0.5 mV, $n=4$). However, even in the presence of this lower concentration of phenylephrine, hyperpolarizations to 5 mM K^+ and to 10 μM acetylcholine were reduced (K^+ control, -19.4 ± 0.8 mV, $n=5$; gap 27, -11.5 ± 0.8 mV, $n=4$; $P<0.05$), (acetylcholine control, 27.1 ± 0.7 mV, $n=5$; gap 27, -14.6 ± 0.6 mV, $n=4$: $P<0.05$).

Discussion In rat hepatic and mesenteric arteries, Edwards *et al*. (1998) suggested that EDHF was K^+ liberated from the vascular endothelium. This view was recently challenged (Andersson *et al*., 2000; Doughty *et al*., 2000; 2001; Lacy *et al*., 2000). Although none of these workers measured membrane potential changes, their myograph studies in precontracted vessels did not support the view (Edwards *et al*., 1998) that EDHF was endothelium-derived K^+.

Does an increase in $[K^+]_o$ hyperpolarize rat mesenteric artery smooth muscle? In the absence of phenylephrine, raising the Krebs solution $[K^+]$ by 5 mM always hyperpolarized the membrane of both the endothelial and smooth muscle cells. On endothelium removal, 5 mM K^+ still hyperpolarized the smooth muscle cells so that in this vessel, the hyperpolarizing action of K^+ does occur in the absence of an endothelium.

These direct membrane potential measurements clearly show, contrary to the conclusions of Doughty *et al*. (2001), that the hyperpolarizing action of raising $[K^+]_o$ is not exclusively exerted on endothelial cells. This action of K^+ is presumably caused by the stimulation of Na^+/K^+ ATPases and/or inwardly-rectifying K^+ channels, both of which are present on both the smooth muscle and the endothelial cells of this vessel. Thus, as proposed by Edwards *et al*. (1998), it is theoretically possible for K^+ ions liberated from the vascular endothelium to hyperpolarize the surrounding vascular smooth muscle cells and to contribute to the EDHF response.

Is endothelial cell membrane potential dominated by a Cl^- conductance? In their myograph experiments, Doughty *et al*. (2001) found that most rat mesenteric arteries were unresponsive to elevation of extracellular K^+, but that relaxation to K^+ was always observed in the presence of the non-selective (see Kato *et al*., 1999) ion channel modulator, NPPB (5-nitro-2-(3-phenylpropylamino)-benzoic acid).

In the present study, the mean resting membrane potential of endothelial cells was approximately -60 mV, similar to that previously recorded in the rat mesenteric artery (-53 mV, $n=60$; White & Hiley, 2000). Furthermore, in de-endothelialized vessels, K^+-induced smooth muscle hyperpolarizations were observed in the presence of phenylephrine and the K^+ channel inhibitors when the mean membrane potential was very depolarized (-22.2 ± 1.7 mV, $n=4$). Thus direct measurements do not support the conjecture (Doughty *et al*., 2001) that endothelial cell membrane potential is

dominated by a chloride conductance and that K^+-induced hyperpolarization only occurs when the membrane potential is close to E_K.

Is the hyperpolarizing action of K^+ dependent on resting membrane potential? Effects of 4-aminopyridine and iberiotoxin In contrast to its ability directly to hyperpolarize smooth muscle cells in non-stimulated mesenteric arteries, K^+-induced increases in smooth muscle membrane potential were reduced in the presence of 1 μM phenylephrine (Edwards *et al*., 1999; present study) and eliminated when the phenylephrine concentration was increased to 10 μM (present study). However, despite further depolarization of the myocytes, K^+-induced changes were restored by blocking delayed rectifier (K_V) and large conductance, Ca^{2+}-sensitive K^+ (BK_{Ca}) channels using 4-aminopyridine and iberiotoxin.

To determine whether the loss of K^+-induced membrane potential changes in the presence of phenylephrine and their return after exposure to iberiotoxin + 4-aminopyridine simply reflected the associated changes in membrane resistance, measurements of smooth muscle input resistance were performed. Phenylephrine (10 μM) reduced input resistance, presumably reflecting the agonist-induced opening of chloride, calcium and/or non-specific cation channels as well as calcium-sensitive and delayed rectifier K^+ channels. Such a decrease could itself reduce the magnitude of any pump-induced hyperpolarization following elevation of extracellular K^+ and be responsible for the observed loss of K^+-induced hyperpolarization under these conditions. However, iberiotoxin + 4-aminopyridine restored the hyperpolarization to K^+ in the presence of phenylephrine with no significant increase in input resistance.

Measurement of input resistance reflects not only transmembrane resistance but also that of lower-resistance pathways between the muscle cells. However, a decrease in resistance was detectable on exposure to phenylephrine and thus the failure to see a significant increase after K^+ channel blockade probably reflects the relatively small contribution to total membrane conductance made by calcium-sensitive and delayed rectifier K^+ channels in the presence of phenylephrine. Collectively, therefore, the most obvious explanation for these findings is that the smooth muscle depolarization and increase in intracellular Ca^{2+} produced by the adrenoceptor agonist activates K_V and BK_{Ca} as part of a general 'excitation brake' mechanism. Rather than merely reducing membrane resistance, the functionally-important consequence of this K^+ channel opening, which increases K^+ efflux (see, for example Bolton & Clapp, 1984), is to generate a local increase in $[K^+]_o$. This 'K^+ cloud' around the muscle cells fully activates Na^+/K^+ ATPases (see Edwards *et al*., 1998) and prevents any hyperpolarizing actions of added K^+.

Role of gap junctions Gap junctions have been implicated in the EDHF pathway in precontracted vessels following the use of peptides such as gap 27 (Chaytor *et al*., 1998). Indeed, Edwards *et al*. (1999) showed that the smooth muscle hyperpolarizing action of raised $[K^+]_o$ in rat intact mesenteric artery exposed to phenylephrine was markedly inhibited by pretreatment with gap 27.

In the present study, the lack of any inhibitory effect of gap 27 on smooth muscle hyperpolarization to K^+ under basal conditions suggests that such membrane potential changes result, even in endothelium-intact vessels, from a direct action of this ion on the muscle cells. However, in the presence of a spasmogen which itself raises $[K_o]$ around the muscle cells, the hyperpolarizing effects of added K^+ on endothelial cells assume greater importance since the smooth muscle hyperpolarization becomes sensitive to gap 27. Presumably the K^+- (and acetylcholine-) induced endothelial cell electrical changes are transmitted to the muscle *via* myoendothelial gap junctions which thus assume a greater role when the vessel is in a markedly contracted state. These findings explain why K^+-induced relaxations in de-endothelialized vessels cannot be detected in myograph experiments (Lacy *et al*., 2000; Doughty *et al*., 2001).

Conclusions Collectively, the data from the present study together with the results of others, indicates that both 'K^+ coupling' and gap junctional communication play a role in the rat mesenteric artery with the former the more important the lower the degree of on-going vasoconstriction.

It is only possible to speculate about which of these two parallel mechanisms is functionally the more important *in vivo*. However, Buus *et al*. (1994) have shown that spasmogens are more potent in pressurized vessels than in conventional myographs. If this is associated with a reduced K^+ cloud around the muscle cells, it should favour K^+ coupling between endothelium and muscle. Furthermore, Bang *et al*. (1998) reported that infusions of iberiotoxin had no effect on blood pressure in otherwise untreated, conscious rats. Such a finding suggests that the general level of vascular smooth muscle depolarization and intracellular Ca^{2+} concentration *in vivo* is insufficient to activate BK_{Ca} and, by extrapolation, unlikely to be associated with an inhibitory 'K^+ cloud' around the vascular smooth muscle. Thus, the possibility of K^+ coupling is again favoured although gap junctions could become very important in vasospastic conditions.

The findings of the present study clarify many of the current controversies in the literature concerning the EDHF pathway. In particular, they strongly suggest that studies of EDHF which rely on the measurement of relaxations in precontracted vessels minimize or even totally prevent the detection of the direct effects of endothelium-derived K^+ ions on the vascular smooth muscle cells. The axiomatic importance of measuring membrane potential changes when investigating the features of a hyperpolarizing phenomenon is clearly indicated.

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