Properties of smooth muscle hyperpolarization and relaxation to $K^+$ in the rat isolated mesenteric artery

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Dora, Kim A., and Christopher J. Garland. Properties of smooth muscle hyperpolarization and relaxation to $K^+$ in the rat isolated mesenteric artery. Am J Physiol Heart Circ Physiol 280: H2424–H2429, 2001.—Smooth muscle membrane potential and tension in rat isolated small mesenteric arteries (inner diameter 100–200 μm) were measured simultaneously to investigate whether the intensity of smooth muscle stimulation and the endothelium influence responses to exogenous $K^+$. Variable smooth muscle depolarization and contraction were stimulated by titration with 0.1–10 mM phenylephrine. Raising external $K_1^+$, contraction were stimulated by titration with 0.1–10 mM phenylephrine. Raising external $K^+$ to 10.8 mM evoked contraction to 13.8 mM still hyperpolarized and relaxed the smooth muscle. Relaxation to endothelium-derived hyperpolarizing factor, released by ACh, was not altered by the level of stimulation. In endothelium-denuded arteries, the concentration-relaxation curve to $K^+$ was shifted to the right but was not depressed. In denuded arteries, relaxation to $K^+$ was unaffected by the extent of prior stimulation and was blocked with 0.1 mM ouabain but not with 30 μM Ba$^{2+}$. The ability of $K^+$ to stimulate simultaneous hyperpolarization and relaxation in the mesenteric artery is consistent with a role as an endothelium-derived hyperpolarizing factor activating inwardly rectifying $K^-$ channels on the endothelium and Na$^+$-$K^-$-ATPase on the smooth muscle cells.

endothelium-derived hyperpolarizing factor; membrane potential; acetylcholine; vascular smooth muscle

THE IDENTITY OF THE HYPERPOLARIZING factor that mediates vascular smooth muscle relaxation independently of nitric oxide and prostacyclin remains controversial. One recent suggestion is that endothelium-derived hyperpolarizing factor (EDHF) may simply be $K^+$ released from endothelial cells during stimulation with ACh (8). This suggestion was based on the similarity between smooth muscle hyperpolarization and relaxation evoked with $K^+$ or ACh (to release EDHF) in the rat hepatic and mesenteric arteries and the pharmacological profile of these responses. In addition, direct recording from endothelial cells showed that the hyperpolarization of these cells to ACh was abolished in the presence of apamin and charybdotoxin, but not apamin and iberiotoxin. This combination of $K^+$ channel blockers provides a characteristic block of the EDHF pathway (4, 19, 23). This suggests that agents such as ACh, which release EDHF, do so as a consequence of their ability to raise endothelial cell Ca$^{2+}$ levels and thereby activate small- and intermediate-conductance Ca$^{2+}$-activated $K^+$ ($K_{Ca}$) channels, allowing $K^+$ to efflux and act as EDHF. In this way, Edwards et al. (8) suggested that $K^+$ could act universally as an EDHF, with the caveat that other pathways could also contribute to a greater or lesser extent in different vessels. This point was illustrated in the mesenteric artery, which, unlike the hepatic artery, did not appear to rely entirely on $K^+$ as an EDHF. Therefore, a significant component of the EDHF-evoked relaxation in this vessel was due to an additional pathway. It was suggested that this may reflect the spread of hyperpolarization through myoendothelial gap junctions, a theory that was subsequently supported by experimentation (6, 9).

However, recent measurements of smooth muscle relaxation have questioned the role of $K^+$ as an EDHF in the rat small mesenteric artery (14). These workers were able to obtain only modest $K^+$-evoked relaxation in 30–40% of the vessels they studied. Furthermore, the relaxation was very transient compared with ACh-evoked relaxation and was abolished by removal of the endothelium. Clearly, these data question the role of $K^+$ in the EDHF response of the mesenteric artery. We now provide the first simultaneous membrane potential and tension data showing robust hyperpolarization and relaxation to $K^+$. Our data indicate that a failure by others to record consistent relaxation to $K^+$ in the mesenteric artery can be explained simply by the intensity of stimulation against which $K^+$ is applied. Our observations are consistent with the EDHF pathway in the mesenteric artery being explained in part by $K^+$ acting directly as an EDHF and in part by the myoendothelial spread of endothelial cell hyperpolarization, reflecting $K^+$ activation of inwardly rectifying $K^+$ ($K_{IR}$) channels and an efflux of this ion through these channels and $K_{Ca}$ channels.

METHODS

Male Wistar rats (200–250 g) were killed by cervical dislocation and exsanguination following procedures required...
under Schedule 1 of the Animals Scientific Procedure Act 1986 (United Kingdom) and monitored by the Home Office. A segment (2 mm long) of a third-order branch of the superior mesenteric artery was mounted in a Mulvany-Halpern myograph (model 400A, J. P. Trading) at a tension equivalent to that generated at 0.9 times the diameter of the vessel at 100 mmHg. Simultaneous recording of smooth muscle membrane potential and tension was achieved with glass electrodes (filled with 2 M KCl, tip resistances ~100 MΩ) (10). Endothelial cell viability was assessed as the ability to induce >95% relaxation with 1 μM ACh in arterial segments pre-constricted with a submaximal concentration of phenylephrine and in the presence of the nitric oxide synthase inhibitor Nω-nitro-arginine methyl ester (L-NAME, 100 μM) and indomethacin (2.8 μM). Endothelial cells were removed where applicable by gentle rubbing with a human hair. Subsequently, phenylephrine was titrated to induce variable levels of depolarization and contraction against which repolarization and relaxation could be assessed to single concentrations of exogenous K⁺ to 10–15 mM final bath concentration.

**Solutions and drugs.** Tissues were maintained at 37°C in oxygenated Krebs buffer composed of (in mM) 118.0 NaCl, 25.0 NaHCO₃, 3.6 KCl, 1.2 MgSO₄·7H₂O, 1.2 KH₂PO₄, 11.0 glucose, and 2.5 CaCl₂, which was continuously aerated with 95% O₂-5% CO₂. All drugs used were purchased from Sigma (Poole, UK).

**Analysis of data.** Relaxation and repolarization are expressed as percent decreases in the respective levels of tone and depolarization. Values are means ± SE of n experiments.

**RESULTS**

In the absence of phenylephrine, smooth muscle cells in the mesenteric artery had a resting membrane potential of −51.4 ± 1.5 mV (n = 12). The application of increasing concentrations of phenylephrine stimulated smooth muscle depolarization associated with rhythmic spike discharges followed by contraction. Raising the bath concentration of K⁺ to 10.8 mM reversed the depolarization and contraction, an effect that was sustained for ≥2 min (Fig. 1A). However, the ability of K⁺ to evoke repolarization and relaxation was reduced if the smooth muscle cells were further depolarized and contracted by phenylephrine (Fig. 1B). There was a strong correlation between the hyperpolarization and relaxation to 10.8 mM K⁺ (Fig. 1C), with both responses being markedly depressed with increasing levels of depolarization and contraction (Fig. 1, D and E). The influence of the extent of stimulation on the responses to K⁺ varied with the steady-state concentration of this ion. Increasing the extent of stimulation depressed the relaxation evoked by 7.8 mM K⁺ in a way similar to 10.8 mM K⁺ (Fig. 2A). However, neither the relaxation with 13.8 mM K⁺ (Fig. 2C) nor the hyperpolarization (118.2 ± 22.2% reversal of depolarization, n = 5) was reduced in the same tissue under the same conditions. Unlike the re-
responses to $K^+$, the relaxation to different concentrations of ACh was not depressed by the intensity of stimulation (Fig. 2, D–F).

In the absence of a functional endothelium, the concentration-response curve for relaxation to $K^+$ was shifted to the right, but the maximal relaxation was unaffected (Fig. 3A). However, the dependence of $K^+$-evoked relaxation on the intensity of stimulation was now absent (Fig. 3, B–D). In intact arteries, 30 µM Ba$^{2+}$ (n = 6) or 0.1 mM ouabain (n = 7) shifted the relaxation curve to $K^+$ to the right. Although the maximal relaxation was reduced slightly, $K^+$ was still able to evoke ~80% relaxation (Fig. 4, A and B). However, in endothelium-denuded arteries, the relaxation to $K^+$ was abolished in the presence of 0.1 mM ouabain but was unaffected by 30 µM Ba$^{2+}$ (Fig. 4C).

![Fig. 2. Influence of phenylephrine-induced contraction on smooth muscle relaxation to $K^+$ and to endothelium-derived hyperpolarizing factor (EDHF) released by ACh. Relaxation to 7.8 mM K$^+$ (A) and 10.8 mM K$^+$ (B) was reduced as the level of stimulation increased, whereas relaxation to 13.8 mM K$^+$ (C) was unaffected by these changes. In contrast, concentrations of ACh evoking submaximal [30 nM (D) and 100 nM (E)] and maximal relaxations [300 nM (F)] were not modified by increasing the level of stimulation. Values are means ± SE of 3–9 responses and were analyzed nonparametrically with the Kruskal-Wallis test. *Statistically significant differences between values for percent relaxation, $P < 0.05$.](http://ajpheart.physiology.org/)

![Fig. 3. A: effect of endothelial cell (EC) removal on the K$^+$ concentration-response curve [+EC (n = 10), and –EC (n = 14)]. Values are means ± SE and were analyzed nonparametrically with the Mann-Whitney test. *Statistically significant differences after removal of the endothelium for each concentration of K$^+$, $P < 0.05$. B–D: relationship between tension and the ability of K$^+$ [10.8 mM (B), 13.8 mM (C), and 16.8 mM (D)] to evoke relaxation in endothelium-denuded arteries. Various levels of tension (expressed as change from basal) were stimulated by phenylephrine (0.1–3 µM) but did not depress the ability of K$^+$ to evoke relaxation. Values are means ± SE. Values were not statistically different by Kruskal-Wallis test.](http://ajpheart.physiology.org/)
DISCUSSION

This study provides the first recordings of correlated smooth muscle hyperpolarization and relaxation in response to increases in extracellular K⁺. Most importantly, the results show that the ability of K⁺ to evoke these smooth muscle responses depends critically on the extent of prior contraction and the concentration of K⁺ applied in vessels with an intact endothelium. Furthermore, the mechanisms underlying relaxation are influenced by the endothelium.

The fact that K⁺ could stimulate smooth muscle hyperpolarization and relaxation in the mesenteric artery was a key piece of evidence in support of the suggestion that this ion acts as an EDHF (8). However, the membrane potential measurements in the original study were made at or close to the resting membrane potential, while the relaxation measurements required prior contraction. The demonstration that hyperpolarization and relaxation to K⁺ occur simultaneously is therefore very important. It extends the previous work, showing that both responses can be stimulated under exactly the same conditions, and as with the equivalent responses to EDHF (released by ACh) (22), they are closely correlated and sustained.

These data also unequivocally demonstrate that the degree of stimulation with phenylephrine influences the ability of extracellular K⁺ to evoke smooth muscle hyperpolarization and relaxation, providing the endothelium is intact. Hyperpolarization and relaxation to K⁺ were evoked every time this ion was applied, but only if a submaximal concentration of phenylephrine was used for stimulation. This contrasts dramatically with recent work in the same artery, where only modest relaxation (maximum ~35% with 10 mM K⁺) could be obtained in 30–40% of the arteries studied (14). This discrepancy simply relates to the degree of stimulation, inasmuch as in the latter study, 10 μM phenylephrine or 10 μM norepinephrine was used as the stimulant. The traces show that this resulted in contraction well in excess of 10 mM, a level where we show that 10.8 mM K⁺ cannot evoke hyperpolarization or relaxation. Why these workers failed to record K⁺-induced relaxation in endothelium-denuded arteries is not clear. It may be explained by endothelium removal abolishing an already very small response, or, alternatively, it may reflect the prevailing extracellular concentration of K⁺ at the surface of the smooth muscle cells, which will increase during contraction. During contraction with phenylephrine, an efflux of K⁺ occurs through large-conductance Ca²⁺-activated K⁺ channels in the smooth muscle cells, which is sufficient to suppress the level of contraction by 75% (5). With higher levels of stimulation, the concentration of K⁺ at the surface of smooth muscle cells could increase to the extent that Na⁺-K⁺-ATPase activity was maximal before the addition of any exogenous K⁺. This would then reduce the ability of added K⁺ to evoke hyperpolarization and relaxation. This effect would occur in endothelium-intact and -denuded preparations but would be most apparent, in terms of a loss of relaxation, in the latter, which lack a significant route to relaxation through KIR channels in the endothelial cells.

Removal of the endothelium significantly shifted the relaxation curve to K⁺ to the right (Fig. 3). We previously showed in endothelium-intact mesenteric arteries that relaxation to K⁺ is shifted to the right by a combination of Ba²⁺ to block KIR channels and ouabain to block Na⁺-K⁺-ATPase (8). We now show that removal of the endothelium removes sensitivity to Ba²⁺ and enables ouabain to completely block relaxation (Fig. 4C). This suggests, first, that Ba²⁺ is acting on the endothelium and, second, that the endothelium appears to influence the action of ouabain. We recently demonstrated that KIR2.1 is expressed predominantly on the endothelium in mesenteric arteries, which is consistent with these functional data (11). Transcript of KIR2.1 has recently been reported in mesenteric smooth muscle cells, but no evidence for functional...
expression was presented (2). It is interesting to note that, with Ba\(^{2+}\) present, the relaxation curve to K\(^{+}\) in intact arteries is very similar to that in denuded arteries (cf. Figs. 3A and 4A). Ouabain, by itself, also shifted the relaxation curve for K\(^{+}\) to the right in intact arteries. However, the fact that in combination these agents do not further depress this relaxation (8) does indicate that K\(^{+}\) is acting through an additional mechanism. Inasmuch as ouabain totally blocked relaxation in denuded arteries, any additional mechanism is unlikely to reflect the release of dilator agents from perivascular nerves, which would be present in intact and denuded preparations.

The involvement of K\(_{IR}\) will confer voltage sensitivity on the relaxation responses to K\(^{+}\), inasmuch as the activity of this channel is a function of voltage and extracellular K\(^{+}\) concentration (17). The ability of 13.8 mM K\(^{+}\) to evoke hyperpolarization and relaxation at a time when 10.8 mM K\(^{+}\) was ineffective is consistent with these properties. It also presumably explains why the presence of ouabain does not block relaxation when the endothelium is functional. K\(^{+}\) activating endothelial cell K\(_{IR}\) and evoking hyperpolarization to spread passively and relax the smooth muscle before the direct smooth muscle depolarizing action of K\(^{+}\) predominates. However, it does not explain why relaxation persists in the additional presence of Ba\(^{2+}\) (8). When Ba\(^{2+}\) and ouabain are present, the action of K\(^{+}\) on the endothelial and smooth muscle cells should be prevented. It is possible that smooth muscle depolarization coupled with the addition of K\(^{+}\) is able to overcome the block with Ba\(^{2+}\). Block of K\(_{IR}\) does decrease with depolarization, but the half-block constant at \(-40\) mV is \(-8\) \(\mu\)M (2), so unless exogenous K\(^{+}\) adds dramatically to the effect of depolarization, this possibility seems unlikely. Alternatively, K\(^{+}\) may facilitate endothelial cell hyperpolarization by modulating another type of K\(^{+}\) channel on the endothelium, to evoke spreading hyperpolarization and then smooth muscle relaxation. For example, extracellular K\(^{+}\) is required for outward current flow through some forms of K\(_{V}\) channels (18). This suggestion remains to be tested, but it is interesting to note that charybdotoxin blocks K\(_{V}\) channels (12).

Depolarization with phenylephrine would be predicted to increase the amplitude of the current attributed to Na\(^{-}\)-K\(^{+}\)-ATPase, inasmuch as membrane potential moves further from the reversal potential (16). The \(\alpha_2\)- and \(\alpha_3\)-isoforms of Na\(^{-}\)-K\(^{+}\)-ATPase are present in mesenteric artery smooth muscle cells, which have apparent affinities (\(K_{0.5}\)) for activation with K\(^{+}\) ranging from 3.6 to 6.2 mM (\(\alpha_2\beta_1, \alpha_2\beta_2, \alpha_3\beta_1, \alpha_3\beta_2\)) (1, 3). In endothelium-denuded arteries, these characteristics will clearly allow for enzyme activation by raised K\(^{+}\), an explanation supported by the sensitivity of the relaxation to ouabain and the lack of inhibition with increasing levels of stimulation with phenylephrine.

The fact that K\(^{+}\) can evoke correlated smooth muscle hyperpolarization and relaxation is very important, as it extends the available evidence that it can act as an EDHF in the mesenteric artery. The fact that these responses can be blocked depending on the extent of smooth muscle depolarization evoked with phenylephrine appears to apply only with lower effective concentrations of K\(^{+}\) (7.8 and 10.8 mM). So, despite the prevailing level of smooth muscle stimulation, if the concentration of K\(^{+}\) is sufficient, it should be able to mimic the hyperpolarization and relaxation to ACh. Discrepancies between relaxation to K\(^{+}\) and ACh, in terms of magnitude and reproducibility, do not negate a role for K\(^{+}\) as an EDHF. The finding that relaxation to ACh was not influenced by the extent of prior stimulation could be explained by K\(^{+}\) accumulating between the endothelial and smooth muscle cells in concentrations where relaxation is also uninfluenced by the extent of stimulation (\(-13\) mM). In addition, ACh probably evokes smooth muscle relaxation by more than one route. Endothelial cell hyperpolarization, which follows the activation of muscarinic receptors with ACh, could passively hyperpolarize and relax the adjacent smooth muscle cells, through the gap junctions that exist between these cells in the mesenteric artery (6, 20). An additional consideration arising from the present study is that K\(^{+}\) will amplify this effect through the activation of K\(_{IR}\) channels on the endothelium. However, it is important to note that this may not be the scheme in all arteries. In coronary and cerebral arteries, the available evidence indicates that a K\(_{IR}\) channel linked to relaxation is present on the smooth muscle cells (9).

It is not known whether K\(^{+}\) can act as an EDHF in vivo. However, the evidence available from in vitro experiments with pressurized arteries is consistent with such a role. First, the inhibitory action of K\(^{+}\) is not solely restricted to the mesenteric vasculature. Raising external K\(^{+}\) from 6 to 16 mM evoked a pronounced endothelium-independent hyperpolarization (12–14 mV from potentials around \(-45\) mV) and vasodilation in cerebral and coronary arteries (13). Second, increasing extracellular K\(^{+}\) concentration evoked an endothelium-independent vasodilation in the isolated, perfused mesenteric vascular bed. As with individual mesenteric arteries, this effect was blocked in the presence of a combination of Ba\(^{2+}\) and ouabain. This combination also significantly depressed the endothelium-dependent vasodilation to EDHF (released by ACh in the presence of inhibitors for nitric oxide synthase and cyclooxygenase), again consistent with a role for K\(^{+}\) (15). In the latter study, it is perhaps surprising that K\(^{+}\)-evoked dilation could be evoked, as the concentrations of methoxamine used to stimulate the mesenteric bed were relatively high (\(>4\) \(\mu\)M, giving perfusion pressures of \(\sim100\) mmHg). The fact that K\(^{+}\) did evoke dilation presumably reflects the fact that the pressure at the level of the small mesenteric resistance arteries would be much less. In addition, agonist stimulation would not evoke as much smooth muscle depolarization or tension in pressurized arteries as in isometrically mounted arteries. For example, in isolated mesenteric arteries, even with pressures as high as 120 mmHg, the smooth muscle resting potential was close...
to −50 mV and wall tension decreased as the diameter of the artery decreased (21, 24).

However, as with isometrically mounted arteries, the extent of agonist stimulation does appear to be a relevant consideration when dilator responses are recorded in pressurized arteries. In isolated mesenteric arteries pressurized (no flow) to 80 mmHg and stimulated with phenylephrine, dilation to 10.58 mM K⁺ was recorded in only 30% of the vessels studied (7). In contrast to previous studies with the same preparation (24), these vessels failed to develop myogenic tone. Despite this finding, presumably the wall tension would still be relatively low, so that the extent of phenylephrine-evoked depolarization would explain the inability of K⁺ to evoke reproducible smooth muscle relaxation. In contrast, ACh consistently and fully reversed the phenylephrine-evoked constriction. However, for the reasons given above, this observation alone does not argue against K⁺ acting as an EDHF in this preparation. It simply indicates that, under the experimental conditions employed, the primary mechanism for relaxation was the spread of hyperpolarization from the endothelium. The fact that relaxation to ACh was depressed with gap junction inhibitors supports this view (7). It would clearly be interesting to show if relaxation to K⁺ was more pronounced and reproducible under similar experimental conditions, if the pressure was reduced below 80 mmHg.

In summary, these data clearly show that it is possible to stimulate reproducible and direct smooth muscle hyperpolarization and relaxation to K⁺ in the absence of a functional endothelium, consistent with a possible role for K⁺ as an EDHF in the mesenteric artery. Furthermore, they stress the importance of the experimental conditions to reveal these responses with ≤11 mM extracellular K⁺ and the importance of the endothelium in modulating the responses.

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REFERENCES