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Thromboxane receptor stimulation associated with loss of SK_{Ca} activity and reduced EDHF responses in the rat isolated mesenteric artery

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1 The possibility that thromboxane (TXA_2) receptor stimulation causes differential block of the SK_{Ca} and IK_{Ca} channels which underlie EDHF-mediated vascular smooth muscle hyperpolarization and relaxation was investigated in the rat isolated mesenteric artery.

2 Acetylcholine (30 nM–3 μM ACh) or cyclopiazonic acid (10 μM CPA, SERCA inhibitor) were used to stimulate EDHF-evoked smooth muscle hyperpolarization. In each case, this led to maximal hyperpolarization of around 20 mV, which was sensitive to block with 50 nM apamin and abolished by repeated stimulation of mesenteric arteries with the thromboxane mimetic, U46619 (30 nM–0.1 μM), but not the α_1-adrenoceptor agonist phenylephrine (PE).

3 The ability of U46619 to abolish EDHF-evoked smooth muscle hyperpolarization was prevented by prior exposure of mesenteric arteries to the TXA_2 receptor antagonist 1 μM SQ29548.

4 Similar-sized smooth muscle hyperpolarization evoked with the SK_{Ca} activator 100 μM riluzole was also abolished by prior stimulation with U46619, while direct muscle hyperpolarization in response to either levcromakalim (1 μM, K_{ATP} activator) or NS1619 (40 μM, BK_{Ca} activator) was unaffected.

5 During smooth muscle contraction and depolarization to either PE or U46619, ACh evoked concentration-dependent hyperpolarization (to −67 mV) and complete relaxation. These responses were well maintained during repeated stimulation with PE, but with U46619 there was a progressive decline, so that during a third exposure to U46619 maximum hyperpolarization only reached −52 mV and relaxation was reduced by 20%. This relaxation could now be blocked with charybdotoxin alone. The latter responses could be mimicked with 300 μM 1-EBIO (IK_{Ca} activator), an action not modified by exposure to U46619.

6 An early consequence of TXA_2 receptor stimulation is a reduction in the arterial hyperpolarization and relaxation attributed to EDHF. This effect appears to reflect a loss of SK_{Ca} activity.
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Keywords: Acetylcholine; endothelium-derived hyperpolarizing factor; EDHF; endothelium; K-channels; mesenteric artery; U46619; phenylephrine; small conductance calcium-activated potassium channels

Abbreviations: ACh, acetylcholine; BK_{Ca}, large-conductance calcium-activated potassium channels; CPA, cyclopiazonic acid; 1-EBIO, 1-ethyl-2-bezimidazolinone; EDHF, endothelium-derived hyperpolarizing factor; IK_{Ca}, intermediate conductance calcium-activated potassium channels; L-NAME, N^{ω}-nitro-L-arginine methyl ester; NO, nitric oxide; PE, phenylephrine; SERCA, sarcoplasmic endoplasmic reticulum calcium ATPase; SK_{Ca}, small-conductance calcium-activated potassium channels; TXA_2, thromboxane A_2

Introduction

Thromboxane A_2 (TXA_2) is a powerful vasoconstrictor and mitogenic agent, which has both physiological and pathophysiological roles within the cardiovascular system. The ability of TXA_2 potently to stimulate sustained background contraction of vascular smooth muscle is also commonly employed in studies investigating endothelium-dependent dilator pathways (e.g. Fisslthaler *et al*., 1999; McNeish *et al*., 2001). However, stimulation of resistance arteries with high concentrations of the TXA_2 mimetic U46619 is associated with a selective loss of the subsequent relaxation caused by endothelium-derived hyperpolarizing factor (EDHF; Plane & Garland, 1996).

EDHF causes vascular smooth muscle hyperpolarization leading to relaxation, which is independent of the action of both nitric oxide (NO) and prostacylin. The hyperpolarization reflects the action of a factor (EDHF) and/or the spread of current from the endothelium through heterocellular (myoendothelial) gap junctions. Physiologically, EDHF appears to provide an important route to vasodilatation, which assumes increasing importance over NO with decreasing arterial size, predominating in resistance arteries and arterioles and operating *in vivo* (Yamamoto *et al*., 2001; Busse *et al*., 2002; Parkington *et al*., 2002). Therefore, the possibility that an autacoid like TXA_2 could influence the ability of the endothelium locally to modulate the arterial diameter through EDHF is clearly of applied interest.

Whatever the precise factor or structures underlying the EDHF pathway in different arteries, a characteristic is a susceptibility to block with a combination of the potassium channel blockers apamin and charybdotoxin, but not with

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apamin and iberiotoxin (see Dora & Garland, 1998; Busse *et al*., 2002). This reflects a pivotal role for small (SK_{Ca}) and intermediate (IK_{Ca}) conductance, calcium-activated potassium channels, which are restricted to the endothelium (Edwards *et al*., 1998; Doughty *et al*., 1999; Walker *et al*., 2001). Activation of these K channels results in hyperpolarization of the endothelial cells, causing smooth muscle hyperpolarization and relaxation (Busse *et al*., 2002). We have recently reported that the contribution made to the EDHF response by SK_{Ca} and IK_{Ca} activation can vary depending on the extent of smooth muscle depolarization and contraction (Crane *et al*., 2003). While this may reflect a differential localization of these channels within the endothelial cells, it raises the possibility that their respective activities may be modulated independently. The block of EDHF-evoked smooth muscle hyperpolarization and relaxation previously reported with U46619 was both complete and selective, as it did not occur in arteries stimulated with the α_1-adrenergic agonist phenylephrine (PE) (Plane & Garland, 1996). The fact that the smooth muscle cells were still able to contract and relax in this previous study suggested a primary action of U46619 against endothelial cell K channels.

In the present study, we simultaneously measured the changes in smooth muscle membrane potential and tension to investigate the possibility that lower concentrations of U46619 may act differentially against endothelial cell K channels and thus initiate a progressive block of the EDHF pathway. Our data are consistent with an initial and irreversible block by U46619 of SK_{Ca} channels in the endothelium. Some of these results have been presented in preliminary form to the Physiological Society (Crane & Garland, 2003).

Methods

Male Wistar rats (200–250 g) were killed by cervical dislocation and exsanguination (Animals Scientific Procedure Act 1986, U.K.). The mesentery was quickly removed and placed in Krebs buffer at room temperature. A segment (2 mm in length) of a third-order branch of the superior mesenteric artery was carefully cleared of adherent tissue and mounted in Krebs buffer at 37°C in a small vessel myograph (model 400A, Danish Myotechnology, Denmark), at a tension equivalent to that generated at 0.9 times the diameter of the vessel at 100 mmHg (Garland & McPherson, 1992). The functional viability of endothelial cells was then assessed by the ability of 1 μM acetylcholine (ACh) to induce over 95% relaxation following contraction of the arterial segments, with a submaximal concentration of PE (3 μM). The artery was superfused at 3–4 ml min^{-1} with oxygenated Krebs buffer at 37°C. Drugs were then applied under static conditions at 37°C and mixed by continuous gassing with 5% CO_2 in 95% O_2. All experiments were performed in the presence of the NO synthase inhibitor N^{ω}-nitro-L-arginine methyl ester (L-NAME, 100 μM). Individual smooth muscle cells were impaled with sharp glass electrodes (backfilled with 2 M KCl, tip resistances approximately 80 MΩ), to enable measurement of smooth muscle membrane potential and tension simultaneously (Garland & McPherson, 1992).

PE (0.1–1 μM) or U46619 (1 nM–0.1 μM) were applied cumulatively, to evoke smooth muscle depolarization and contraction. In each experiment, the concentration of agonist was titrated to match the extent of depolarization and contraction as closely as possible between each artery and each exposure to either PE and/or U46619 in that artery. This minimized any effect due to the extent of smooth muscle stimulation *per se*. ACh (10 nM–3 μM; cumulatively), or in some cases 300 μM 1-EBIO, was then applied. Following washout for 45–60 min, this cycle was repeated at least twice. In some experiments, a final stimulation with PE was used to assess any cross-depression of ACh responses due to prior exposure to U46619.

U46619 experiments involving K_{Ca} channel inhibitors and the thromboxane receptor antagonist, SQ29548

K_{Ca} channel inhibitors or the TXA_2 antagonist SQ29548 (1 μM) were applied directly to a static bath at least 20 min before and throughout concentration–response determinations to U46619, PE and/or ACh.

Experiments involving cyclopiazonic acid (CPA) and K-channel activator induced smooth muscle hyperpolarization

Smooth muscle hyperpolarization was recorded to the cumulative application of 10 nM–3 μM ACh or single concentrations of CPA (10 μM), NS1619 (40 μM BK_{Ca} channel opener), levcromakalim (1 μM K_{ATP} channel opener), riluzole (100 μM SK_{Ca} channel opener) or 1-EBIO (300 μM IK_{Ca} channel opener). In some experiments, these agents were added after repeated exposure and washout with U46619. In experiments using 50 nM apamin or 100 nM charybdotoxin, toxins were added to the bath at least 20 min before membrane potential changes were recorded.

Solutions and drugs

In all experiments, arterial segments were maintained at 37°C in oxygenated Krebs buffer of the following composition (mM): NaCl 118.0, $NaHCO_3$ 25.0, KCl 3.6, $MgSO_4 \cdot 7H_2O$ 1.2, KH_2PO_4 1.2, glucose 11.0, $CaCl_2$ 2.5. The drugs used were all from Sigma except for charybdotoxin, apamin, iberiotoxin, from Latoxan, 1-ethyl-2-benzimidazolinone (1-EBIO) from Aldrich, and U46619 from Calbiochem. Charybdotoxin was dissolved in 0.9% saline. 1-EBIO (0.1 M), NS1619 (0.1 M), CPA (0.1 M), levcromakalim (0.01 M), and riluzole (0.01 M) were each dissolved in dimethylsulfoxide (DMSO) to make a stock solution for subsequent dilution. Control experiments indicated that DMSO had no direct action in the final concentrations applied. All other stock solutions were dissolved in distilled water.

Analysis of data

Data are given as mean $\pm$ s.e.m. of n replicates, with $n = 1$ for a separate artery, unless stated otherwise. Repeated-measures ANOVA tests are used in cases where protocols were repeated twice. Student's t-tests (paired and unpaired) and ANOVA with Bonferroni multiple comparisons were also used, as indicated. The significance level was $P < 0.05$.

Results

Modulation of EDHF evoked hyperpolarization by U46619 in the mesenteric artery

The outside diameter of the mesenteric arteries ranged between 200 and 300 μm. As previously reported, ACh evoked reproducible, concentration- and endothelium-dependent smooth muscle hyperpolarization (McPherson & Angus, 1991; Garland & McPherson, 1992), increasing the resting potential from -53 ± 1 to -75 ± 2 mV ($n=5$). Over the 3–4 h of an experiment, the magnitude of hyperpolarization decreased slightly with repeated exposure of the tissue to ACh, so that by the third concentration–response determination the maximum increase obtained with 3 μM ACh was reduced by 6 mV, to -69 ± 3 mV, $n=5$, ($P<0.05$).

In marked contrast, if mesenteric arteries were repeatedly stimulated with U46619, followed by washout, and then stimulated with ACh, the normal, robust hyperpolarization was progressively blocked. After three to four separate exposures to U46619 (up to 0.1 μM in each case), the maximum membrane potential attained in the presence of 3 μM ACh was only -55 ± 1 mV. This was not markedly different from the resting potential of -52 ± 1 mV in this series, $n=8$, and represented a marked reduction in hyperpolarization of around 22 mV (from -74 ± 2 mV, $n=13$, Figure 1).

The ability of U46619 to block subsequent endothelium-dependent hyperpolarization to ACh appeared to be receptor mediated, as the smooth muscle hyperpolarization to ACh was not depressed if the TXA$_2$ receptor antagonist SQ29548 (1 μM) was also present during repeated stimulation with 0.1 μM U46619. The concentration of antagonist was sufficient to block completely the contraction to U46619 in these arteries. With 1 μM SQ29548 present, the first exposure to ACh increased the membrane potential to -80 ± 1 mV, $n=6$, while by the third exposure to ACh, the maximum potential attained was -70 ± 4 mV. This potential was not different from time-matched controls which had not been exposed to SQ29548, -69 ± 3 mV.

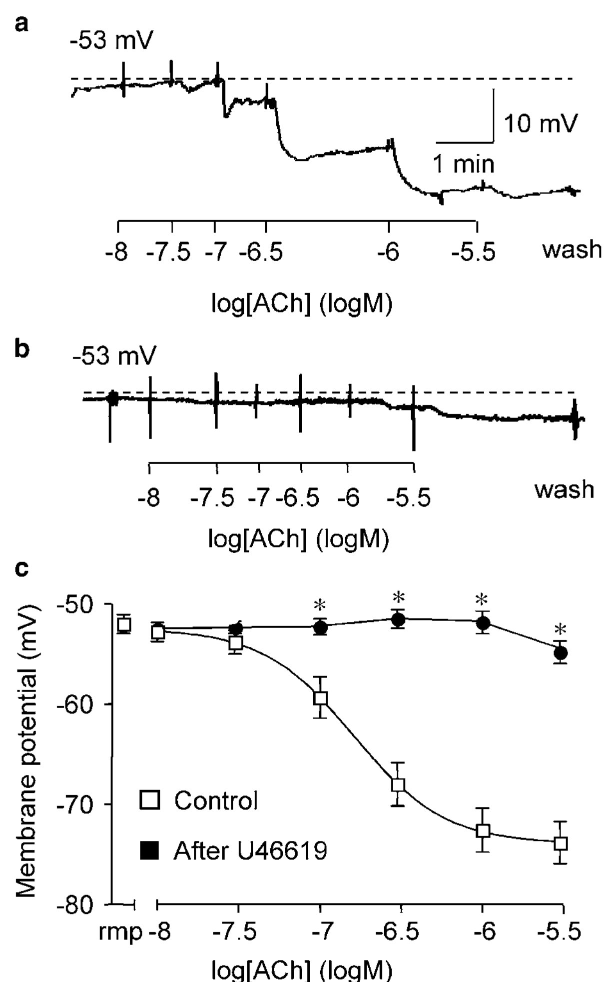

Figure 1 Effect of repeated exposure to U46619 on ACh-evoked hyperpolarization. Representative, original traces show: (a) a typical ACh-evoked smooth muscle hyperpolarization in the mesenteric artery; (b) the depressed response after three repeated exposures and washout of U46619 (30 nM–0.1 μM); (c) ACh cumulative concentration–response curves for smooth muscle hyperpolarization under control conditions, $n=13$, and after 3–4 repeated exposures and washout of U46619, $n=8$, are shown in the right segment (*$P<0.05$, Student's t-test). Resting membrane potential (rmp) for each group is shown in the left segment of the graph.

Influence of U46619 on smooth muscle hyperpolarization evoked with CPA and various K-channel activators

Either CPA, which blocks SERCA, or the putative SK$_{Ca}$ channel activator riluzole, each evoked smooth muscle hyperpolarization with a similar maximum to that in response to ACh. CPA (10 μM) increased the membrane potential from a resting value of -54 ± 2 to -72 ± 3 mV ($n=4$) and 100 μM riluzole from -55 ± 2 mV to -71 ± 3 mV ($n=5$). As with ACh, the extent of hyperpolarization to each agent was reduced markedly after repeated exposure, followed by washout, to U46619, to -56 ± 1 ($n=6$) and -58 ± 2 mV ($n=5$), respectively. In contrast, hyperpolarization either to the K$_{ATP}$ activator, 1 μM levcromakalim ($n=4$) or the BK$_{Ca}$ channel activator 40 μM NS1619 ($n=5$), was unaffected by similar, repeated exposures to U46619. These data are summarized in Figure 2. Consistent with our recent data (Crane *et al.*, 2003), very little smooth muscle hyperpolarization was recorded in control experiments in response to the IK$_{Ca}$ channel activator 300 μM 1-EBIO (5 ± 2 increase to 56 ± 2 mV, $n=6$).

U46619 but not PE depresses smooth muscle hyperpolarization and relaxation to ACh in mesenteric arteries

Ongoing stimulation of mesenteric arteries with U46619 In this series of experiments, the resting membrane potential of the smooth muscle cells was -53 ± 1 mV ($n=5$), which did not vary by more than 1–2 mV throughout each experiment. The application of 30 nM–0.1 μM U46619 stimulated a sustained smooth muscle depolarization (to -42 ± 1 mV) and contraction (to 17 ± 5.0 mN), both of which were reversed with ACh. ACh (10 nM–3 μM) increased the membrane potential beyond normal resting values, to -67 ± 2 mV, and relaxed the artery by 95 ± 1% ($n=5$). Hyperpolarization and relaxation to ACh were blocked in the presence of 50 nM apamin and 100 nM charybdotoxin in

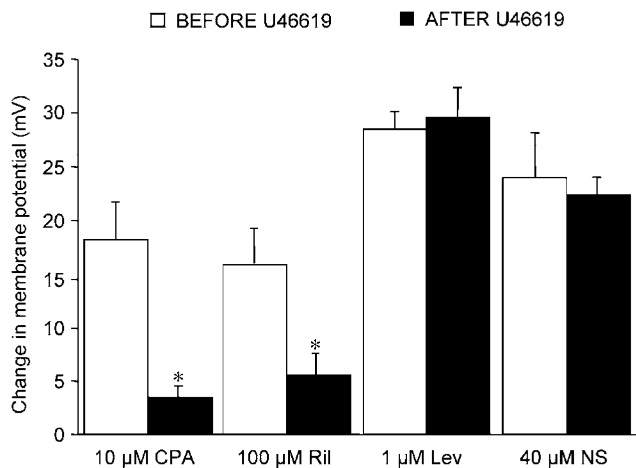

Figure 2 Influence of U46619 exposure on smooth muscle hyperpolarization evoked by CPA and K-channel activators in mesenteric arteries. Smooth muscle hyperpolarization to 10 μM CPA and 100 μM Riluzole (Ril) was significantly reduced after repeated prior exposure to 30 nM–0.1 μM U46619 (*Student's t-test, $P<0.05$). The response to 1 μM Levcromakalim (LEV, $n=4$) and 40 μM NS1619 (NS) was unchanged ($n=5$).

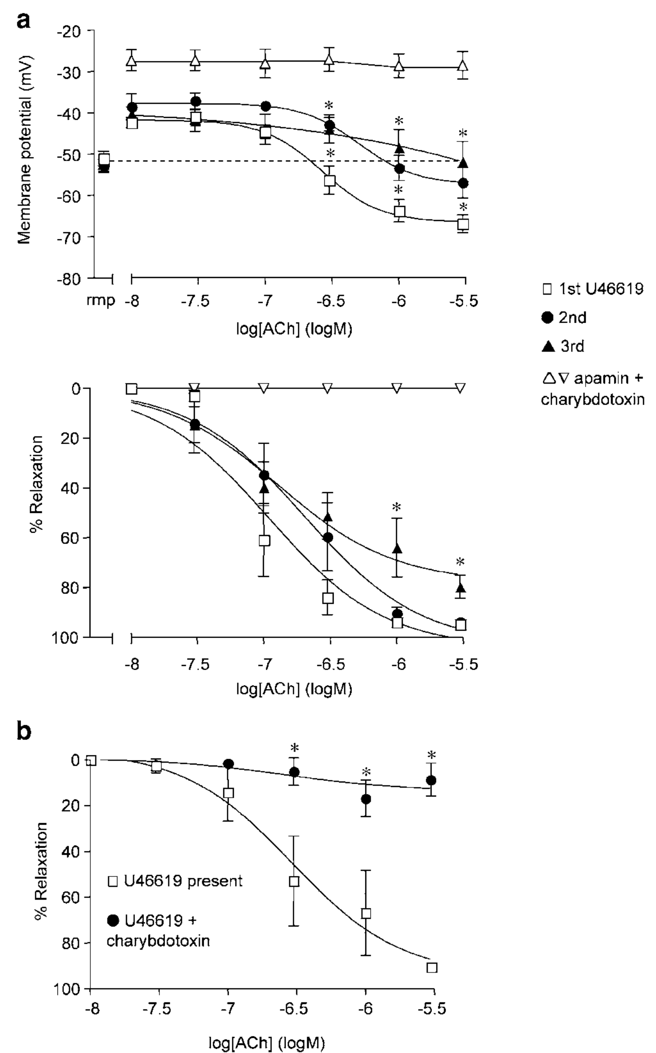

Figure 3 Smooth muscle hyperpolarization and relaxation to ACh during depolarization and contraction to U46619. (a) Simultaneous measurement of changes in smooth muscle membrane potential (upper panel) and relaxation (lower panel) in mesenteric arteries during U46619 (10 nM–0.1 μM) stimulation. Increases in the membrane potential and relaxation with ACh, showed progressive reduction during the second and third stimulation with U46619. By the third exposure, membrane potential did not overshoot the original resting value, *$P<0.05$, ANOVA with Bonferroni multiple comparisons test, $n=5$. Resting membrane potential (rmp) for each group is shown in the left segment of the graph. A small but significant reduction in relaxation was observed during the third stimulation with U46619. The ACh hyperpolarization and relaxation was completely blocked in the presence of 50 nM apamin and 100 nM charybdotoxin combined. (b) Cumulative % relaxation to ACh in U46619 stimulated mesenteric arteries. Membrane potential was not recorded during these experiments. Relaxation to ACh during a forth stimulation with U46619 ($n=6$) was completely blocked with charybdotoxin alone, without a requirement for apamin (*$P<0.05$, $n=5$, Bonferroni multiple comparisons test).

combination ($n=5$, see Figure 3a), but were unaffected in the presence of iberiotoxin (hyperpolarization to -60 ± 4 mV, 97 ± 1% relaxation, $n=5$, data not shown). Following the washout of U46619 and ACh, subsequent exposures to U46619 did not affect the depolarization and contraction of the arteries. However, the hyperpolarization and relaxation in response to cumulative addition of ACh was significantly depressed, so during a third exposure to U46619, ACh only repolarized the smooth muscle membrane potential to -52 ± 5 mV, identical to the original resting potential of -52 ± 2 mV. This effect was associated with a modest but significant decrease in overall relaxation (to 80 ± 5%, $n=5$). In another series of experiments, this persistent relaxation to ACh was effectively abolished in the presence of 100 nM charybdotoxin, during a forth exposure to U46619 (9 ± 7% relaxation, $n=5$), compared to the ACh control (91 ± 2%, $n=6$) (Figure 3b). With higher concentrations of U46619 (3 μM), ACh only stimulated a transient repolarization from –40 mV to circa -49 mV, followed by an immediate depolarization to around -37 mV, changes associated with brief relaxation (4 mN) which rapidly reversed (back to 15–16 mN).

Ongoing stimulation of mesenteric arteries with PE In this series, the resting potential of the smooth muscle cells was -54 ± 1 mV ($n=5$), and again did not change by more than 1–2 mV throughout each experiment. PE (0.6 μM) evoked sustained smooth muscle depolarization (to -43 ± 2 mV) and contraction (to 14 ± 1 mN). ACh (10 nM–3 μM) increased the membrane potential to -72 ± 1 mV and relaxed the artery by 98 ± 1% ($n=5$). Neither the depolarization nor the hyperpolarization to ACh changed significantly overall in the presence of a second or third exposure to PE. Moreover, ACh still caused hyperpolarization to -67 ± 2 mV and relaxed the arteries by 93 ± 1% ($n=5$) during the third exposure to PE, (Figure 4).

Effect of prior exposure to U46619 on the subsequent hyperpolarization and relaxation to ACh during stimulation with PE

PE (1 μM) reduced the smooth muscle resting potential from -54 ± 1 to -40 ± 1 mV, and evoked contraction of 17 ± 1 mN

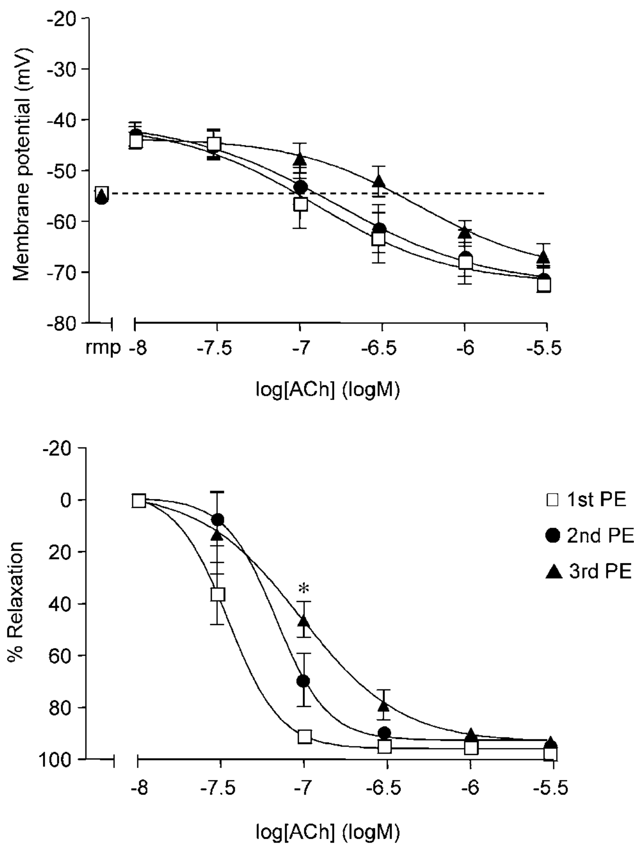

Figure 4 Effect of repeated PE stimulation on hyperpolarization and relaxation to ACh. Simultaneous measurement of changes in smooth muscle membrane potential (upper panel) and relaxation (lower panel) in mesenteric arteries during stimulation with 0.6 μM PE. Mesenteric arteries hyperpolarized and relaxed to ACh to a similar extent during three successive stimulations with PE ($n = 5$). Resting membrane potential (rmp) for each group is shown in the left segment of the graph. Relaxation shifted slightly at intermediate concentrations of ACh during consecutive stimulation with PE, reaching significance with 0.1 μM ACh (*$P<0.05$, Bonferroni multiple comparisons test).

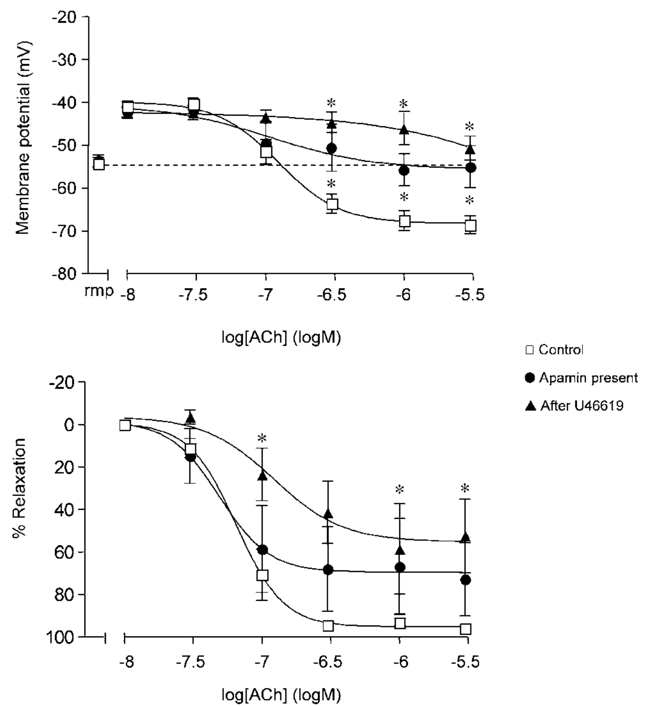

Figure 5 ACh hyperpolarization and relaxation in PE-stimulated mesenteric arteries are depressed by prior U46619 exposures or by apamin. Simultaneous measurement of smooth muscle membrane potential change (upper panel) and relaxation (lower panel) in mesenteric arteries during stimulation with 1 μM PE. ACh hyperpolarization during the first stimulation with PE ($n = 9$) was reduced to a similar extent by the presence of apamin ($n = 4$), as it was in arteries following three previous exposures (and washout) to U46619 ($n = 6$). The loss of membrane potential overshoot was associated with reduced relaxation, which was more extensive in the arteries previously exposed to U46619 (*$P<0.05$, ANOVA with Bonferroni multiple comparisons test). Resting membrane potential (rmp) for each group is shown in the left segment of the graph.

($n = 9$). ACh then hyperpolarized the arteries to -69 ± 2 mV with complete relaxation (96 ± 1%, $n = 9$). Three subsequent exposures, followed by washout, to U46619 (up to 0.1 μM) did not modify the subsequent depolarization to PE, although the contraction was progressively reduced (to 11 ± 3 mN, $n = 6$; $P<0.05$). If these arteries were then stimulated with PE, subsequent responses to ACh were depressed by a similar extent to arteries stimulated with U46619 alone. The membrane potential only repolarized to -51 ± 3 mV, and maximum relaxation at 52 ± 17% ($n = 6$; $P<0.05$) was also reduced. Exposure to 50 nM apamin caused a similar reduction in ACh-evoked hyperpolarization and relaxation to arteries stimulated with U46619. In arteries depolarized and contracted with PE, ACh reversed the membrane potential to -55 ± 5 mV with relaxation of 73 ± 17% ($n = 4$) Figure 5.

Unlike the inhibition of the EDHF response, hyperpolarization to the IK_{Ca} channel opener 1-EBIO (300 μM) was not depressed by prior stimulation with U46619. Relaxation of around 90% was obtained in response to 1-EBIO during each of three exposures to U46619, and then in the presence of a subsequent additional contraction to PE. 1-EBIO, which did not hyperpolarize unstimulated arteries by more than a few mV, evoked repolarization, from -38 ± 2 to -48 ± 2 mV ($n = 4$). During subsequent depolarization in the presence of 1 μM PE, the increase in membrane potential in response to 1-EBIO was additionally associated with slight hyperpolarization. The membrane potential in the presence of PE was -37 ± 5 mV, and increased to -57 ± 1 mV with 1-EBIO ($n = 3$; original resting membrane potential -51 ± 2 mV).

Discussion

The main findings in this study are that the stimulation of TXA_2 receptors in isolated mesenteric resistance arteries results in a progressive loss of smooth muscle hyperpolarization due to EDHF. An early component of this effect appears to reflect the inactivation of the SK_{Ca} channels which are localized on the endothelium. These data are likely to be of physiological relevance in situations where the local concentration of TXA_2 is elevated, for example, during platelet activation, and in the interpretation of experimental studies of EDHF where TXA_2 is used as the background constrictor agonist.

Activation of both SK_{Ca} and IK_{Ca} channels in the arterial endothelium is a key step leading to smooth muscle hyperpolarization and relaxation, independently of both NO

and prostacyclin. This effect is ascribed to EDHF (Bolz *et al*., 1999; Busse *et al*., 2002). In a previous study in rat mesenteric resistance arteries, smooth muscle contraction and depolarization in response to the TXA_2 mimetic U46619 was shown to cause complete loss of EDHF evoked hyperpolarization and relaxation, while relaxation to endothelium-derived NO was not altered. In that study, high (0.5–3 μM) concentrations of U46619 were used (Garland & McPherson, 1992). We now show that a more selective and progressive block can be evoked on repeated stimulation with lower concentrations of U46619.

Our present data show that the first apparent consequence of vascular stimulation with U46619, is a loss of ACh's ability to evoke EDHF-induced hyperpolarization in uncontracted arteries, and of the overshooting hyperpolarization normally obtained during EDHF activation in depolarized and contracted arteries. This indicates an action against SK_{Ca} channels, which underpin this effect in mesenteric resistance arteries (Crane *et al*., 2003). This conclusion is supported by the fact that in the present study apamin alone abolished ACh hyperpolarization in both quiescent arteries and in arteries stimulated with PE (the overshoot of resting membrane potential). The fact that similar hyperpolarization was also stimulated in the mesenteric artery by the SK_{Ca} activator riluzole, and that this hyperpolarization was lost following prior exposure to U46619, is also consistent with an action of the thromboxane–mimetic against SK_{Ca}. Riluzole is an opener of SK_{Ca} channels, although it has other effects, such as inhibition of Na^+ channels and twin-pore domain K channels (Taylor & Meldrum, 1995; Song *et al*., 1997; Duprat *et al*., 2000). The SK_{Ca} channels are restricted to the endothelium, as they are in other arteries (Marchenko & Sage, 1996; Edwards *et al*., 1998; Mistry & Garland, 1998a, b; Burnham *et al*., 2002; Eichler *et al*., 2003).

Whatever the relative cellular locations of SK_{Ca} and IK_{Ca} within the endothelial cells, it is clear that U46619-linked block of SK_{Ca} activity preceded that of the IK_{Ca} channels. The repolarization (and relaxation) remaining after three exposures to U46619 was sensitive to block with charybdotoxin and mimicked by the IK_{Ca} activator, 1-EBIO. The hyperpolarization associated with the action of 1-EBIO $>100\,\mu$M appears to be due to the activation of IK_{Ca} (Walker *et al*., 2001). That block of endothelial cell SK_{Ca} channels, associated with U46619 stimulation, is selective is also supported by unaltered hyperpolarization to the BK_{Ca} channel activator NS1619 and the K_{ATP} channel activator levcromakalim after exposure to U46619. In the mesenteric artery, BK_{Ca} and K_{ATP} channels are restricted to the smooth muscle cells (Mistry & Garland, 1998a; White & Hiley, 2000), whereas the IK_{Ca} channels are on the endothelial not the smooth muscle cells (Walker *et al*., 2001).

How the stimulation of TXA_2 receptors leads to a loss of endothelial K-channel activity, and as a consequence reduced vascular dilatation, is not clear. Neither is it clear if the receptors mediating this effect are on the smooth muscle or the endothelial cells, or both. Obviously, receptors for TXA_2 are present on the smooth muscle to enable contraction (maximum contraction was not significantly changed in endothelium-denuded arteries, data not shown). In another vessel, the porcine coronary artery, the stimulation of dispersed smooth muscle cells with U46619 has been shown to block K channels, but the selectivity of this effect for K channels was not investigated. The channel block required the activation of TXA_2 receptors and involved an alteration in the gating of what were apparently BK_{Ca} channels, but was not reversed by raising intracellular calcium (Scornik & Toro, 1992). In an intact artery, block of BK_{Ca} channels would be expected to increase the overall contraction to constrictor agents (Brayden & Nelson, 1992). As the contraction during repeated exposure to U46619 did not change in our study (range 15–17 mN, data not shown) block of BK_{Ca} channels seems unlikely.

Endothelial cells have been shown to contain TXA_2 receptors, and their stimulation can cause detrimental cellular effects. In human endothelial cells, two isoforms have been reported, α and β, each linked to apoptosis (Gao *et al*., 2000). This effect seems to involve an inhibition of the phosphorylation of Akt kinase, which may involve activation of the protein kinase C isoform PKC-ς (Gao *et al*., 2000; Shizukuda & Buttrick, 2002). The TXA_2 antagonist SQ29548 blocked endothelial cell apoptosis and also reduced the expression of intercellular adhesion molecule-1 (Zou *et al*., 2002). Endothelial cell death may also follow calcium overload, and this effect was used to explain the fact that both U46619 and another TXA_2 agonist, I-BOP, caused time- and concentration-dependent cell death in neuroretinal endothelial cells (Beauchamp *et al*., 2001). The effect appeared to be selective, as associated smooth muscle and astroglial cells, and endothelial cells from nonneuronal tissue, were all unaffected. However, endothelial cell death *per se*, seems an unlikely explanation for our data. The first detrimental action of U46619 was a block of SK_{Ca} and at that time the endothelial cells were still able to cause significant smooth muscle relaxation. Even when U46619 had blocked EDHF-dependent relaxation, NO was able to sustain relaxation (Plane & Garland, 1996).

Other mechanisms to explain the loss of the EDHF response could involve gap junctions or the generation of reactive oxygen species. TXA_2 receptor stimulation has been linked to alterations in the distribution of gap junctions in human endothelial cells, which might impair functional coupling (Ashton *et al*., 1999). This seems an unlikely explanation for the selective loss of SK_{Ca}-evoked hyperpolarization, unless these channels are located on separate endothelial cells to the IK_{Ca} channels, as IK_{Ca}-linked effects persisted. Furthermore, an essential requirement in this case would be that the SK_{Ca} endothelial cells are more susceptible to the effects of U46619, which also seems an unlikely scenario. Finally, TXA_2 receptor stimulation can generate reactive oxygen species, at least under conditions of oxidant stress (Matsuo *et al*., 1996). In retinovascular endothelial cells, U46619 stimulated the formation of hydroperoxides and cell death was prevented by an antioxidant (Beauchamp *et al*., 2001). So reactive oxygen species might possibly underlie the loss of ion channel activity in the endothelium, with more prolonged and sustained periods of exposure progressively affecting other ion channels leading ultimately to endothelial cell death.

Whatever the mechanism, progressive loss of the EDHF pathway during stimulation with U46619 could complicate the interpretation of experimental data obtained when background contraction is evoked by TXA_2 receptor stimulation. In this regard, the use of U46619 is fairly widespread, as it causes stable contraction (Parkington *et al*., 1995; McNeish *et al*., 2001; Bauersachs *et al*., 2002). Interestingly, in the study by McNeish *et al*. (2001), only the IK_{Ca} blocker charybdotoxin was required to abolish EDHF dilatation in the bovine ciliary

vascular bed. The apparent absence of SK_{Ca}-evoked hyperpolarization in these vessels might then reflect their selective loss due to stimulation with U46619. Also, in a recent study with rat mesenteric arteries depolarized and contracted to U46619, ACh and C-type natriuretic peptide each reversed the depolarization without any overshooting hyperpolarization (Chauhan *et al.*, 2003). These data could again reflect SK_{Ca} channel block with U46619. They also suggest that C-type natriuretic peptide may be acting to cause smooth muscle relaxation, at least in part, through the endothelium.

So, in the rat mesenteric artery, SK_{Ca} channels underlie the smooth muscle hyperpolarization evoked with ACh and represent a key component in the EDHF response. Stimulation of TXA_2 receptors, most likely present on the endothelium, is associated with a progressive loss of K-channel activity, starting with SK_{Ca} and ultimately leading to a loss of EDHF-linked changes in smooth muscle membrane potential and tension.

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