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Role of gap junctions in endothelium-derived hyperpolarizing factor responses and mechanisms of K^+-relaxation

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Abstract

We have examined the effects of ouabain (1 mM), the gap junction inhibitors, 18α-glycyrrhetinic acid (100 μM), *N*-(piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1*H*-pyrazole-3-carboxamide hydrochloride (SR141716A; 10 μM) and palmitoleic acid (50 μM), and clotrimazole (10 μM) against endothelium-derived hyperpolarizing factor (EDHF)-mediated and K^+-induced vasorelaxations in the rat mesentery. In the presence of indomethacin (10 μM) and 300-μM N^Gnitro-L-arginine methyl ester (L-NAME), carbachol caused EDHF-mediated relaxations ($R_{max} = 85.3 \pm 4.0\%$). In the presence of ouabain, these responses were substantially reduced ($R_{max} = 11.0 \pm 2.3\%$). 18α-glycyrrhetinic acid, SR141716A, palmitoleic acid and clotrimazole also significantly inhibited these EDHF-mediated responses. K^+ caused vasorelaxation of preparations perfused with K^+-free buffer ($R_{max} = 73.7 \pm 2.4\%$), which were reduced by 10-μM indomethacin ($R_{max} = 56.4 \pm 6.2\%$). K^+ vasorelaxation was essentially abolished by endothelial denudation. Both ouabain and 18α-glycyrrhetinic acid opposed K^+ relaxations, however, neither SR141716A, clotrimazole nor palmitoleic acid had any effect. Direct cell–cell coupling via gap junctions was attenuated by ouabain, clotrimazole and palmitoleic acid. We conclude that: (i) that gap junctional communication plays a major role in EDHF-mediated relaxations, (ii) that K^+-vasorelaxation is endothelium-dependent (thus, K^+ is unlikely to represent an EDHF), and (iii) that the inhibitory actions of ouabain and clotrimazole on gap junctions might contribute towards their effects against EDHF.

Keywords: Endothelium-derived hyperpolarizing factor (EDHF); Ouabain; 18α-Glycyrrhetinic acid; Gap junctions; K^+; Endothelium

1. Introduction

The identity of the endothelium-derived hyperpolarizing factor (EDHF; Feletou and Vanhoutte, 1988; Taylor and Weston, 1988; Garland et al., 1995) is controversial (see Mombouli and Vanhoutte, 1997). There is now evidence that EDHF-type relaxations involve the transfer of a mediator from the endothelium to smooth muscle via myoendothelial gap junctions (Chaytor et al., 1998). EDHF-type responses are thus attenuated by connexin–mimetic peptides (Chaytor et al., 1998; Hutcheson et al., 1999; Dora et al., 1999), and by 18-α and 18-β glycyrrhetinic acids, which inhibit gap junctions (Davidson et al., 1986; Taylor et al., 1998; Yamamoto et al., 1999).

Edwards et al. (1998) have also proposed that EDHF-type relaxations are explained by endothelial K^+ efflux (Gordon and Martin, 1983) through apamin and charybdotoxin-sensitive K^+ channels (Marchenko and Sage, 1996; Doughty et al., 1999), while vasorelaxation to K^+ (Katz and Linder, 1938; Bonaccorsi et al., 1977; Webb and Bohr, 1978) is thought to be due to activation of Na^+/K^+-ATPases and opening of inward rectifier K^+ channels (Chen et al., 1972; Hirst and van Helden, 1982; Edwards and Hirst, 1988; McCarron and Halpern, 1990; Knot et al., 1996). In support of their contention that K^+ may represent an EDHF, Edwards et al. (1998) reported that EDHF release was associated with endothelial K^+ efflux, which was sensitive to apamin and charybdotoxin. In addition, K^+ caused hyperpolarization via an ouabain-sensitive Na^+/K^+-ATPase and Ba^{2+}-sensitive inward rectifier K^+

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channels. However, Quignard et al. (1999) have reported that in guinea-pig carotid and porcine arteries, neither barium, ouabain, nor their combination, inhibited EDHF responses. Similarly, K^+ ions did not mimic EDHF responses in terms of amplitude and time course.

Activation of the Na^+/K^+-ATPase by K^+, leading to relaxation, has been suggested on the basis of the inhibitory effects of ouabain (Henderickx and Casteels, 1974; Webb and Bohr, 1978). In the context of EDHF, Feletou and Vanhoutte (1988) reported that ouabain inhibited endothelium-dependent hyperpolarization (but not relaxation) in canine coronary arteries. However, in rat hepatic arteries, Zygmunt and Hogestatt (1996) found that ouabain did not affect endothelium-dependent relaxations. While Edwards et al. (1998) reported that high concentrations of ouabain plus Ba^{2+} abolished EDHF and K^+-induced hyperpolarization, but only modestly affected vasorelaxation.

In view of the proposal that K^+ might represent an EDHF, the present aim was to compare K^+-induced relaxations and EDHF-mediated responses. Given that myoendothelial gap junctions may play a critical role in EDHF-mediated responses, we have investigated the effects of the gap junction inhibitors, 18-α glycyrrhetinic acid (Davidson et al., 1986; Taylor et al., 1998; Chaytor et al., 1999), palmitoleic acid (Burt et al., 1991; Lavado et al., 1997; Domenighetti et al., 1998) and *N*-(piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1*H*-pyrazole-3-carboxamide hydrochloride (SR141716A) (Chaytor et al., 1999), against EDHF-mediated responses to carbachol and relaxations to K^+. It has previously been shown that the cannabinoid receptor antagonist SR141716A (1 μM) reduces EDHF-type responses (Randall et al., 1996), but this agent potently blocks gap junctional communication (Chaytor et al., 1999). Furthermore, we have also tested whether ouabain or Ba^{2+} affects gap junctional communication by determining their effects on dye transfer through gap junctions. In addition, clotrimazole is a widely used inhibitor of EDHF-mediated responses (McCulloch et al., 1997), and so, we have examined whether this agent may affect gap junctional communication. It has been found by Okazaki et al. (1998), but not by Prior et al. (1998), that removal of the endothelium abolishes responses to K^+, thereby suggesting that K^+ may act via the release of endothelial autacoids. Therefore, we also investigated the effects of endothelial denudation on responses to carbachol and K^+.

2. Methods

2.1. Rat isolated, perfused superior mesenteric arterial bed

Male Wistar rats (250–350 g) were anaesthetized with sodium pentobarbitone (60 mg/kg, i.p.) and killed by exsanguination. Following a mid-line incision, the superior mesenteric artery was cannulated. The arterial vasculature was dissected away from the guts and placed in a jacketed organ bath as previously described (McCulloch et al., 1997) and perfused at 5 ml/min with oxygenated (95% O_2/5% CO_2) Krebs–Henseleit solution (composition, mM; NaCl 118, KCl 4.7, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25, $CaCl_2$ 2, D-glucose 10; 37°C). Indomethacin (10 μM) and N^Gnitro-L-arginine methyl ester (L-NAME; 300 μM) were added to the Krebs–Henseleit buffer in experiments involving carbachol. These inhibitors were included in order to define EDHF as the mediator of NO- and prostanoid-independent relaxations to the endothelium-dependent vasorelaxant, carbachol (McCulloch et al., 1997). In preliminary experiments, it was found that relaxations to K^+ were limited in the buffer containing physiological K^+ concentrations. Accordingly, to optimize the responses to K^+ in most experiments involving K^+-induced relaxations, a K^+-free Krebs–Henseleit solution was prepared by substituting KCl and KH_2PO_4 for equimolar concentrations of NaCl and Na_2HPO_4, respectively. Additional K^+ experiments were carried out in the presence of normal K^+-containing Krebs–Henseleit buffer. Levcromakalim was used as a control endothelium-independent vasorelaxant to examine whether any of the treatments applied exerted non-specific effects against vasorelaxation per se. The perfusion pressure was continuously monitored by means of a pressure transducer coupled to a MacLab 4e recording system (AD instruments, New South Wales, Australia).

2.2. Experimental protocol

Following a 30-min equilibration period, methoxamine (1–2 μM) was added to the buffer to increase perfusion pressure by 100–150 mm Hg. Once a stable tone had been established, the vasorelaxant effects of carbachol (acting via EDHF) or KCl were assessed. Carbachol was added via an injection port in bolus doses of 100 μl or less. KCl was added via the injection port in 1-ml boluses of various doses. Injection of 1 ml of buffer alone did not result in any significant changes in tone (5.30 ± 2.15% relaxation, $n = 3$). The doses of KCl (1–25 μmol) were regarded as stock concentrations of 1–25 mM, and the rapid injection of the boluses of significant volumes in to the perfusion system would have transiently resulted in concentrations in this range (1–25 mM). Accordingly, injection of 1 ml of 1 μmol KCl would have given rise to a concentration of 1 mM through to 1 ml of 25 μmol KCl, giving rise to a concentration of 25 mM. For precision, the data have been presented in terms of the doses applied in moles (not concentrations).

In preparations receiving indomethacin (10 μM), L-NAME (300 μM), flurbiprofen (10 μM), clotrimazole (a cytochrome *P*450 and a widely accepted EDHF inhibitor; 10 μM; McCulloch et al., 1997), barium chloride ($BaCl_2$;

30 μM) or ouabain (1 mM), the agents were added to the buffer to achieve the desired concentration and allowed to equilibrate for 30 min before addition of vasorelaxants. 18α-glycyrrhetinic acid (100 μM), palmitoleic acid (50 μM) and SR141716A (10 μM) were allowed to equilibrate for 1 h. Any loss of established tone following addition of these agents was restored by further addition of methoxamine. In the presence of L-NAME, a lower concentration of methoxamine was required to achieve equivalent tone. In some preparations, the endothelium was removed by perfusion with distilled water for 3 min (Wagner et al., 1999). The endothelium was considered denuded if relaxation to 54.8-nmol carbachol was less than 10%.

2.3. Dye transfer experiments

As previously described (George et al., 1998; Dora et al., 1999; Chaytor et al., 1999), a COS-7 (monkey fibroblast) cell line, which endogenously expresses connexin 43 as its only functional connexin protein (ECACC, Wiltshire, UK), was used to evaluate direct cell–cell coupling following intracytoplasmic injection of Lucifer Yellow CH (charge −2, MW 457 Da; 5% w/v in 0.3 M LiCl). Confluent monolayers of COS-7 cells were formed after a 3-day incubation at 37°C in 5% CO_2, following seeding at a density of 10^6 cells/60 mm^2 culture dish, in complete Dulbecco's Modified Eagles Medium supplemented with 10% fetal bovine serum, 100 mg/l penicillin/streptomycin/glutamine and 250 mg/l amphotericin B (GibcoBRL). The monolayers were washed twice with phosphate buffered saline (120 mM NaCl; 2.7 mM KCl; 10 mM $Na_2HPO_4 \cdot 2H_2O$, pH 7.4) and incubated in Leibowitz L-15 medium (supplemented as above) at 37°C. The cells were subsequently exposed to 0.1 or 1 mM ouabain, 30 μM Ba^{2+}, 1 mM ouabain plus 30-μM Ba^{2+}, 10 μM clotrimazole or 50 μM palmitoleic acid for 60 min at 37°C prior to Lucifer yellow injection. Single cells were selected and Lucifer yellow injected into the cytoplasmic compartment using an Eppendorff micromanipulator 5170 and microinjector 5242 at a pressure of 200 hPa and an injection time of 0.3 s. Following injection, the cultures were returned to the incubator for 15 min before fixation in 4% paraformaldehyde. Transfer of Lucifer yellow was determined by fluorescence microscopy (excitation at 395–440 nm; emission at 460–470 nm) on an Axiostat microscope (Zeiss, UK). Dye transfer was quantified by determining the percentage of injected cells that communicated Lucifer yellow to < 5, 5–10 and > 10 neighboring cells.

2.4. Data and statistical analysis

The dose–response curves were fitted to the logistic equation described by McCulloch et al. (1997). The $-\log ED_{50}$ (pD_2) and maximal relaxation (R_{max}) values obtained were compared by analysis of variance with Bonferroni's post-hoc test. In experiments measuring K^+ responses, Student's *t*-tests were performed at each dose. The response at the maximum dose (25 μmol) was taken as the maximum response.

2.5. Drugs and reagents

Barium chloride was supplied by Aldrich Chemical (Milwaukee, USA) and dissolved in saline. Levcromakalim was obtained from Smithkline Beecham (UK) and dissolved in ethanol. SR141716A was supplied by Sanofi (Montpellier, France) and dissolved in ethanol. All other drugs were supplied by Sigma (UK) and dissolved in saline, except indomethacin, flurbiprofen and clotrimazole (dissolved in ethanol), palmitoleic acid (sonicated into Krebs–Henseleit buffer) and 18α-glycyrrhetinic acid (dissolved in dimethyl sulphoxide; DMSO). The final concentration of DMSO in the perfusion fluid was 0.01% (v/v), and ethanol was 0.025% (v/v), concentrations which do not influence vascular responses. In preliminary experiments, carbachol caused control relaxations ($R_{max} = 80.8 \pm 8.9\%$; $ED_{50} = 0.74 \pm 0.13$ nmol; $n = 3$) which were unaffected by the presence of ethanol ($R_{max} = 87.8 \pm 11.8\%$; $ED_{50} = 0.77 \pm 0.22$ nmol; $n = 3$) or DMSO ($R_{max} = 84.5 \pm 9.0\%$; $ED_{50} = 0.79 \pm 0.02$ nmol; $n = 3$). Similarly, relaxations to K^+ were unaffected (control: $R_{max} = 74.6 \pm 2.3\%$, $n = 3$; in the presence of ethanol, $R_{max} = 78.8 \pm 3.2\%$, $n = 3$, and in the presence of DMSO, $R_{max} = 70.0 \pm 4.4\%$, $n = 3$).

3. Results

3.1. Vasorelaxant responses to carbachol (acting via EDHF)

In the presence of indomethacin (10 μM) and L-NAME (300 μM), carbachol caused dose-dependent relaxations of methoxamine-induced tone (Fig. 1 and Table 1). Addition of the Na^+/K^+-ATPase-inhibitor ouabain (1 mM), in the presence of indomethacin and L-NAME, essentially abolished responses to carbachol (Fig. 1 and Table 1). Ba^{2+} (30 μM) alone produced a modest inhibition of carbachol responses (Fig. 1 and Table 1). In combination, Ba^{2+} and ouabain significantly inhibited relaxation to carbachol (Fig. 1 and Table 1), although, to a lesser extent than ouabain alone.

Addition of clotrimazole (10 μM), an EDHF inhibitor, essentially abolished relaxations to carbachol (Fig. 2 and Table 1). Removal of the endothelium also abolished relaxation to carbachol ($R_{max} = 7.5 \pm 0.6\%$, $P < 0.001$; $n = 5$; Fig. 2).

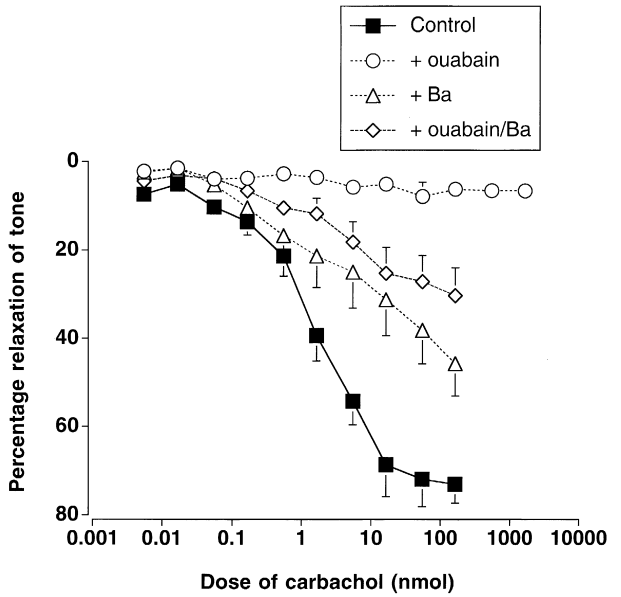

Fig. 1. Vasorelaxation to carbachol in the rat isolated mesenteric arterial bed in the presence of indomethacin (10 μM) and L-NAME (300 μM; control) and either ouabain (1 mM), $BaCl_2$ (30 μM) or the combination of $BaCl_2$ and ouabain. Values are shown as means and vertical lines indicate SEM.

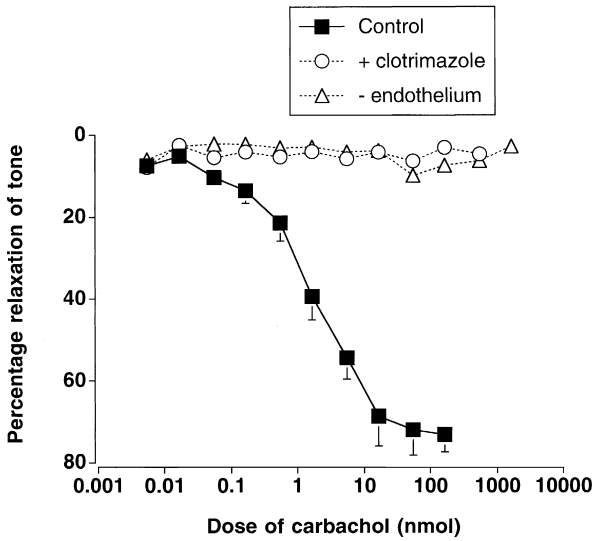

Fig. 2. Vasorelaxation to carbachol in the rat isolated mesenteric arterial bed in the presence of indomethacin (10 μM) and L-NAME (300 μM; control) and clotrimazole (10 μM). Also shown are the effects of endothelial denudation. Values are shown as means and vertical lines indicate SEM.

The gap junction inhibitors, 18α-glycyrrhetinic acid (100 μM), palmitoleic acid (50 μM) and SR141716A (10 μM) all significantly inhibited vasorelaxations to carbachol in the presence of indomethacin and L-NAME (Fig. 3 and Table 1).

Relaxations to control, endothelium-independent vasorelaxants, levcromakalim ($ED_{50} = 2.8 \pm 0.3$ (control) vs. 2.6 ± 0.4 nmol; $R_{max} = 96.8 \pm 2.2\%$ (control) vs. $90.6 \pm 1.4\%$; $n = 5$), or sodium nitroprusside ($ED_{50} = 69.8 \pm 12.6$ vs. 61.4 ± 12.7 pmol; $R_{max} = 88.3 \pm 3.4\%$ vs. $79.5 \pm 3.3\%$; $n = 5$) were unaffected by ouabain. Both 18α-glycyrrhetinic acid ($n = 4$) and palmitoleic acid ($n = 6$) enhanced the ED_{50} values of levcromakalim ($ED_{50} = 0.6 \pm 0.05$ nmol, $P < 0.01$ and 1.1 ± 0.1 nmol, $P < 0.001$) without affecting the maximum response ($R_{max} = 95.2 \pm 1.4\%$ and $89.2 \pm 2.7\%$), whereas SR141716A significantly inhibited levcromakalim responses ($ED_{50} = 8.8 \pm 0.9$ nmol, $P < 0.001$; $R_{max} = 43.9 \pm 1.9\%$, $P < 0.001$; $n = 4$).

3.2. Vasorelaxant responses to K^+

In normal Krebs–Henseleit buffer, potassium chloride (KCl; 1–25 μmol) caused relaxations of methoxamine-induced tone with a maximum relaxation (R_{max}) of $46.6 \pm 8.5\%$ ($n = 4$; Fig. 6) at 25 μmol. In K^+-free Krebs–Henseleit buffer, relaxation was significantly greater ($R_{max} = 71.9 \pm 2.1\%$, $P < 0.001$; $n = 7$; Fig. 4). Addition of indomethacin (10 μM) significantly ($P < 0.05$) inhibited these relaxations ($R_{max} = 56.4 \pm 6.2\%$ at 25 μmol; $n = 6$;

Table 1
The effects of the various inhibitors against the responses to carbachol in the presence of 300 μM L-NAME and 10 μM indomethacin

	Control	1 mM Ouabain	30 μM Barium	1 mM Ouabain +30 μM Barium	10 μM Clotrimazole	100 μM 18-αGA	10 μM SR141716a	50 μM Palmitoleic acid
n	8	6	6	11	5	6	4	6
ED_{50} (nmol)	3.61 ± 1.86	nd	9.3 ± 3.7	5.7 ± 1.7	nd	107 ± 89	nd	14.7 ± 5.2
R_{max} (%)	85.3 ± 4.0	11.0 ± 2.3[a]	48.4 ± 7.6[a]	34.0 ± 6.9[a]	7.9 ± 0.7[a]	44.3 ± 10.4[b]	10.8 ± 1.4[a]	46.7 ± 9.6[a]

The data are given as the ED_{50} and R_{max} values obtained from dose–response curves in Figs. 1–3. The control responses are the relaxations to carbachol in the presence of L-NAME and indomethacin; 18-αGA is 18α-glycyrrhetininc acid.
Where the responses have been almost abolished, the ED_{50} values could not be determined (nd).
[a] Significant ($P < 0.001$) difference between the responses in the presence of the various inhibitors and the control as determined by analysis of variance.
[b] Significant ($P < 0.01$) difference between the responses in the presence of the various inhibitors and the control as determined by analysis of variance.

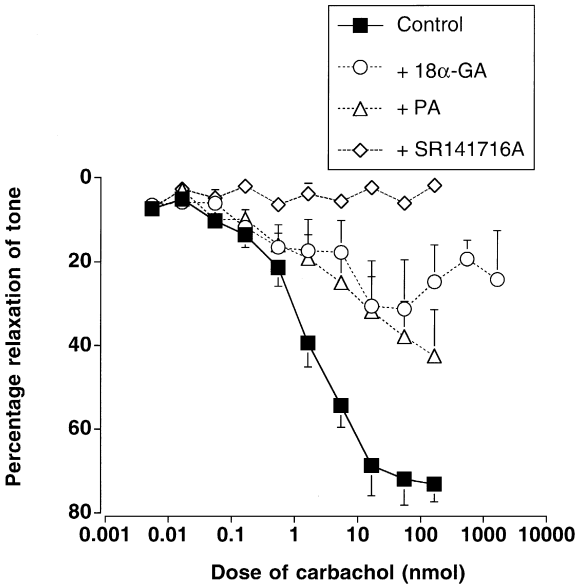

Fig. 3. Vasorelaxation to carbachol in the rat isolated mesenteric arterial bed in the presence of indomethacin (10 μM) and L-NAME (300 μM; control) and either 18α-GA (18α-glycyrrhetinic acid; 100 μM), palmitoleic acid (50 μM) or SR141716A (10 μM). Values are shown as means and vertical lines indicate SEM.

Fig. 4). The combination of indomethacin and L-NAME (300 μM) had no further inhibitory effect on the K^+ relaxation ($R_{max} = 54.6 \pm 3.2\%$, $P < 0.001$; $n = 6$). The cyclooxygenase inhibitor, flurbiprofen (10 μM), also significantly ($P < 0.05$) inhibited K^+-evoked relaxations ($R_{max} = 64.7 \pm 1.5\%$; $n = 5$; Fig. 4). The combination of flurbiprofen and L-NAME attenuated this relaxation ($R_{max} = 47.9 \pm 6.9\%$; $P < 0.001$; $n = 5$). K^+ relaxations in the presence of L-NAME alone were significantly affected ($R_{max} = 63.2 \pm 3.1\%$, $P < 0.05$; $n = 5$; Fig. 4). In the presence of 1 mM ouabain, relaxation to K^+ was essentially abolished (Fig. 5 and Table 2). Ba^{2+} (30 μM) significantly inhibited K^+ responses (Fig. 5 and Table 2), whereas the combination of ouabain and Ba^{2+} further attenuated these responses (Fig. 5 and Table 2). Removal of the endothelium virtually abolished K^+-induced relaxation ($R_{max} = 11.9 \pm 2.6\%$, $P < 0.001$; $n = 6$; Fig. 6). However, the addition of clotrimazole (10 μM) had no effect on relaxation to K^+ (Fig. 6 and Table 2). 18α-glycyrrhetinic acid (100 μM) significantly ($P < 0.01$) inhibited vasorelaxation to K^+ (Fig. 7 and Table 2), while SR141716A (10 μM) and palmitoleic acid (50 μM) had no significant effects on K^+-induced vasorelaxation (Fig. 7 and Table 2).

3.3. Effects of ouabain and Ba^{2+} on intercellular dye transfer

Confluent COS-7 cells injected with Lucifer yellow demonstrated rapid spread of the dye within the monolayer with $73 \pm 5\%$ of injected cells transferring dye to > 10 neighboring cells and $14 \pm 7\%$ and $13 \pm 3\%$ transferring to 5–10 and < 5 cells, respectively, in the control situation ($n = 4$ experiments, 62 injected cells; Fig. 8). As previously reported, in most instances, the dye appeared in 20–30 neighboring cells within 15 min. 30-μM Ba^{2+} did

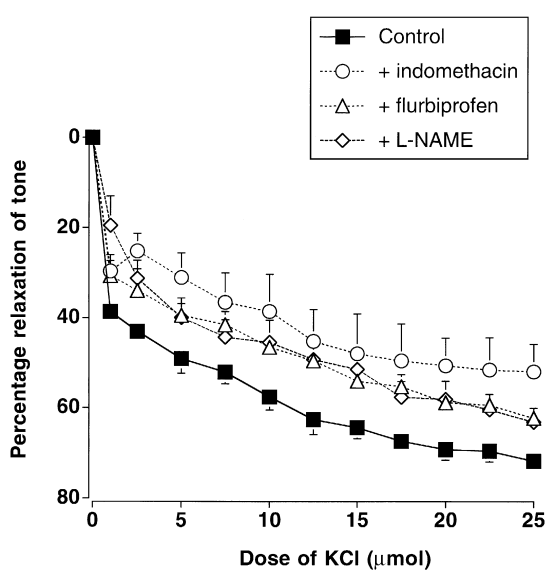

Fig. 4. Vasorelaxation to KCl in the rat isolated mesenteric arterial bed in the presence of either indomethacin (10 μM), flurbiprofen (10 μM) or L-NAME (300 μM). Values are shown as means and vertical lines indicate SEM.

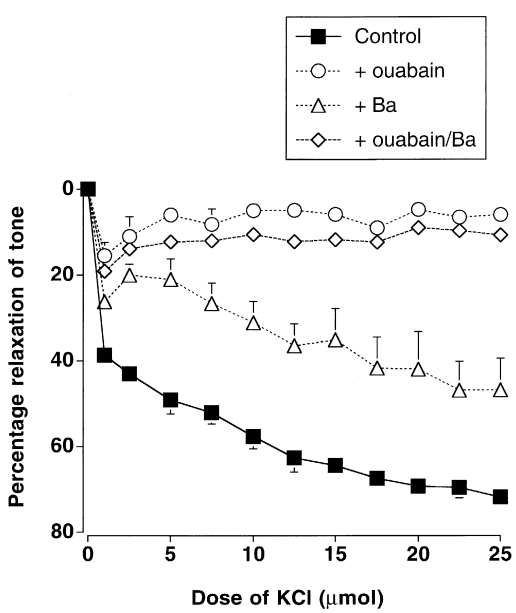

Fig. 5. Vasorelaxation to KCl in the rat isolated mesenteric arterial bed in the presence of either ouabain (1 mM), $BaCl_2$ (30 μM) or the combination of $BaCl_2$ and ouabain. Values are shown as means and vertical lines indicate SEM.

Table 2
The effects of the various inhibitors against the vasorelaxant responses to K^+

	Control	1 mM Ouabain	30 μM Barium	1 mM Ouabain +30 μM barium	10 μM Clotrimazole	100 μM 18-αGA	10 μM SR141716a	50 μM Palmitoleic acid
n	7	6	6	6	5	6	4	6
R_{max} (%)	71.9 ± 2.1	9.7 ± 1.7[a]	46.8 ± 7.8[a]	10.7 ± 2.1[a]	70.5 ± 2.0	48.3 ± 8.0[b]	67.2 ± 5.1	77.8 ± 3.4

The data are given as the R_{max} values (obtained at 25 μmol K^+) from dose–response curves in Figs. 4–7. 18-αGA is 18α-glycyrrhetininc acid.
[a]Significant ($P < 0.001$) difference between the responses in the presence of the various inhibitors and the control as determined by analysis of variance.
[b]Significant ($P < 0.01$) difference between the responses in the presence of the various inhibitors and the control as determined by analysis of variance.

not significantly affect the patterns of dye transfer observed in control cells ($n = 4$ experiments, 98 injected cells; Fig. 8). In the presence of ouabain, there was marked attenuation of intercellular communication with dye transfer being restricted to < 5 cells in 59 ± 19% of cases for 0.1 mM ouabain ($n = 2$ experiments, 82 injected cells, $P < 0.001$) and 69 ± 10% of cases with 1 mM ouabain ($n = 4$ experiments, 143 injected cells; $P < 0.001$). 30-μM Ba^{2+} did not further affect the distribution of Lucifer yellow in the presence of 1 mM ouabain ($n = 4$ experiments, 165 injected cells; Fig. 8).

In the presence of 10 μM clotrimazole, there was substantial attenuation of intercellular communication with dye transfer being restricted to < 5 cells in 100 ± 0% of cases ($n = 4$ experiments, 120 injected cells; $P < 0.001$; Fig. 8). Similarly, 50 μM palmitoleic acid also inhibited intercellular communication with dye transfer being restricted to < 5 cells in 100 ± 0% cases ($n = 4$ experiments, 64 injected cells; $P < 0.001$; Fig. 8).

4. Discussion

In the present study, we have clearly shown that EDHF-mediated responses and vasorelaxation to K^+ show different pharmacological characteristics. The effects of the inhibitors on the EDHF-mediated responses clearly emphasizes the role of gap junctional communication. By contrast, the vasorelaxation to K^+ was found to be endothelium-dependent and partly mediated via prostanoids.

In view of evidence that NO-independent relaxations involve direct heterocellular communication in rabbit arter-

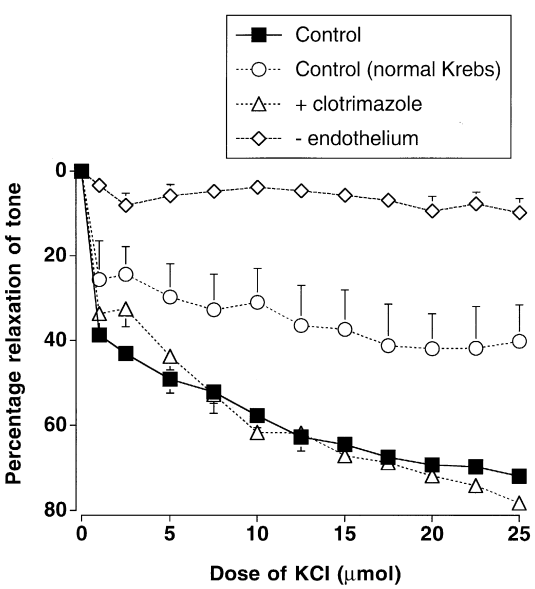

Fig. 6. Vasorelaxation to KCl in the rat isolated mesenteric arterial bed in the presence of clotrimazole (10 μM). Also shown are the effects of endothelial denudation and perfusion with a normal Krebs–Henseleit buffer on vasorelaxation to KCl. Values are shown as mean and vertical lines indicate SEM.

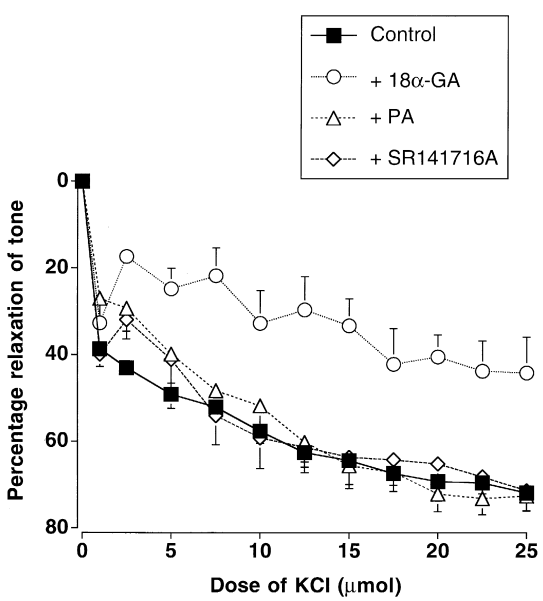

Fig. 7. Vasorelaxation to KCl in the rat isolated mesenteric arterial bed in the presence of either 18α-GA (18α-glycyrrhetinic acid; 100 μM), palmitoleic acid (50 μM) or SR141716A (10 μM). Values are shown as means and vertical lines indicate SEM.

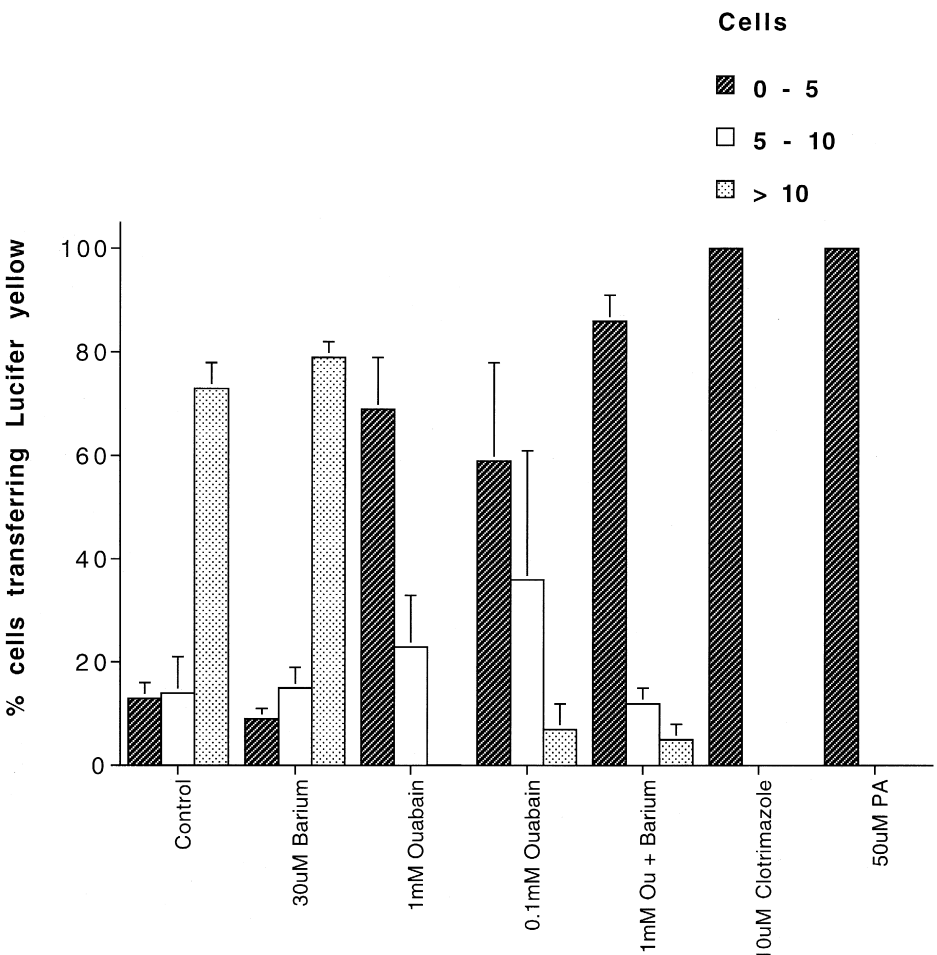

Fig. 8. Effects of either ouabain (0.1 mM; 82 cells injected and 1mM; 143 cells injected), $BaCl_2$ (30μM; 98 cells injected), the combination of $BaCl_2$ and ouabain (165 cells injected), clotrimazole (10 μM; 120 cells injected) or palmitoleic acid (50 μM; 64 cells injected) on the percentage of cells transferring Lucifer Yellow in COS-7 cells. Values are shown as means and vertical lines indicate SEM.

ies, we investigated the effect of the gap junction inhibitor 18α-glycyrrhetinic acid on responses evoked by carbachol in the presence of L-NAME. 18α-glycyrrhetinic acid markedly attenuates EDHF-type relaxations to acetylcholine and cyclopiazonic acid in the rabbit iliac artery (Taylor et al., 1998), and similarly attenuated responses to carbachol in the rat mesenteric bed, where the maximal depressor effect was reduced by ~66%. We also investigated the effects of two additional gap junction inhibitors: SR141716A, which blocks gap junctional communication almost completely at a concentration of 10 μM in fibroblasts (Chaytor et al., 1999), and palmitoleic acid, which we confirmed here attenuates cellular coupling at 50 μM (Domenighetti et al., 1998). In the rat mesenteric bed, 10 μM SR141716A essentially abolished NO- and prostanoid-independent responses to carbachol, whereas 50 μM palmitoleic acid inhibited relaxation to an extent that was approximately equivalent to 100 μM 18α-glycyrrhetinic acid. In addition, in the course of the present study, we also demonstrated that clotrimazole (an acknowledged inhibitor of EDHF responses; McCulloch et al., 1997) is also a potent inhibitor of gap junctional communication. Taken together, these findings add further weight to the proposal that EDHF-type relaxations involve direct endothelial–smooth muscle coupling (Chaytor et al., 1998).

As in the case of carbachol, K^+-evoked relaxations in the mesenteric bed were attenuated by 18α-glycyrrhetinic acid with the response to 25 μmol K^+ being depressed by ~40%. In marked contrast to carbachol, however, SR141716A, palmitoleic acid and clotrimazole were all without effect, leading to the conclusion that gap junctional communication does not contribute to K^+-evoked relaxations. These observations highlight fundamental differences between EDHF- and K^+-mediated mechanisms of vasorelaxation, and also suggest that the action of 18α-glycyrrhetinic acid against K^+ vasorelaxation involves pharmacological effects that are distinct from blockade of gap junctions, such as an ability to inhibit the Na^+/K^+-ATPase (Terasawa et al., 1992).

The experimental protocol employed bolus injections of K^+ into K^+-free buffer, which is likely to involve activation of the Na^+/K^+-ATPase following sudden re-introduc-

tion of K^+ (see Zygmunt et al., 2000). The relaxations to K^+ in normal buffer were less appreciable, but once again pronounced at 1 μmol. The reduced relaxations in normal buffer are certainly consistent with vasorelaxation to K^+ being dependent on activation of the Na^+/K^+-ATPase, as the response was suppressed by raising external K^+. In normal buffer, the Na^+/K^+-ATPase would be expected to be active but not working at full capacity as some of the isoforms show relatively low sensitivity to external K^+ (Blanco and Mercer, 1998). Hence, addition of K^+ would be expected to cause further activation (and relaxation), especially at the lower doses, but in general, responses would be expected to be less than in K^+-free buffer. These data, taken together with the inhibitory effects of ouabain and 18α-glycyrrhetinic acid, appear to implicate a role for the Na^+/K^+-ATPase in relaxations to K^+.

It has recently been proposed that K^+ ions released from the endothelium may function as an EDHF, at least in small mesenteric and hepatic arteries isolated from the rat vasculature (Edwards et al., 1998). The strictly endothelium-dependent nature of the responses to K^+ observed in the present study, however, does not support this hypothesis in the rat isolated perfused mesenteric bed. Furthermore, relaxations, evoked by carbachol or by K^+, were abolished completely by ouabain, which mimicked the effects of endothelial denudation, thus, seemingly excluding a major role for inwardly rectifying K^+ channels. Indeed, it was found that the inhibitory effects of ouabain against carbachol-induced relaxations were reduced when administered in combination with Ba^{2+}, suggesting that Ba^{2+} may, in some way, exert a "protective" effect against the inhibitory actions of ouabain. It has been shown by Condrescu et al. (1997) that Ba^{2+} competitively inhibits Ca^{2+} uptake by Ca^{2+}/Na^+ exchange in transfected Chinese hamster ovary cells. It is possible that Ba^{2+} may prevent the redistribution of Ca^{2+} favoured by ouabain, which is thought to lead to the indirect inhibitory effects against gap junctions (Schirrmacher et al., 1996). This could potentially explain apparent interaction between ouabain and $BaCl_2$. It would also explain why Edwards et al. (1998) obtained partial inhibition, and not abolition, of vasorelaxation with this combination. Given the ability of gap junction inhibitors to attenuate relaxations evoked by carbachol, and observations that ouabain blocks intercellular coupling in non-vascular cell types (Schirrmacher et al., 1996), we investigated the effects of ouabain on dye transfer in a previously described COS-7 cell system that expresses connexin 43 as its only functional connexin protein (Dora et al., 1999; Chaytor et al., 1999). This connexin subtype is found in both vascular smooth muscle and endothelial cells (Chaytor et al., 1997; Yeh et al., 1998; Li and Simard, 1999). Ouabain significantly inhibited the intercellular spread of Lucifer yellow in this model system, suggesting that its inhibitory action against carbachol-evoked relaxations may, at least, in part, be explained by effects on gap junctions. Indeed, at a concentration of 100 μM, ouabain attenuated dye transfer as effectively as previously reported for 50 μM 18α-glycyrrhetinic acid (Chaytor et al., 1999). These findings suggest that ouabain may exert novel pharmacological effects on myoendothelial gap junctional communication whose functional consequences have not previously been recognized. It is possible that similarities in the pharmacological profiles of ouabain and 18α-glycyrrhetinic acid, including the ability to block gap junctions, reflect their common basic steroidal structure. Indeed, 18α-glycyrrhetinic acid has itself been shown to inhibit the Na^+/K^+-ATPase in the canine kidney, albeit with a substantially greater IC_{50} ($\sim$ 70 μM) than ouabain (0.5 μM) (Terasawa et al., 1992). It remains to be determined if the ability of these agents to attenuate gap junctional communication is related to their ability to block the Na^+/K^+-ATPase. It has thus been suggested that the mechanism through which ouabain decreases gap junctional permeability in osteoblasts is secondary to the elevations in intracellular Ca^{2+} that are an expected consequence of Na^+ pump inhibition (Schirrmacher et al., 1996).

The finding that ouabain abolished EDHF-mediated vasorelaxation contrasts with Zygmunt and Hogestatt (1996), who showed that NO/prostanoid-independent relaxations are insensitive to ouabain in rat hepatic arteries. The observation that the combination of ouabain and Ba^{2+} partially inhibited, but did not abolish, EDHF-type responses is consistent with Edwards et al. (1998), who also showed that this combination of agents only partially inhibited EDHF relaxation in the rat mesenteric artery. However, Edwards et al. (1998) also showed that the combination of ouabain and Ba^{2+} abolished EDHF-mediated hyperpolarization. The evidence that hyperpolarization, but not relaxation, may be abolished raises the possibility that another endothelium-dependent mechanism, independent of hyperpolarization, participates in NO- and prostanoid-independent relaxations. Alternatively, the associated hyperpolarization may be an epiphenomenon (see Vanhoutte, 1998). However, Quignard et al. (1999) have recently shown that neither Ba^{2+}, ouabain, nor their combination, exert inhibitory effects on EDHF-mediated hyperpolarization in guinea-pig carotid and porcine coronary arteries, therefore, questioning the proposal of Edwards et al. (1998) in these tissues.

The mechanisms underlying the effects of K^+ ions in the rat mesenteric bed are likely to involve stimulation of the endothelial Na^+/K^+-ATPase and/or activation of inwardly rectifying endothelial K^+ channels (Daut et al., 1988; Laskey et al., 1990). Both actions would be expected to promote membrane hyperpolarization and, thus, increase the electrochemical gradient for Ca^{2+} entry and promote Ca^{2+}-dependent synthesis of endothelial autacoids. In contrast to the partial inhibition seen with Ba^{2+} in the present study, the endothelium-dependent relaxations to K^+ observed by Okazaki et al. (1998) in the rat mesenteric bed were abolished by Ba^{2+}. This could reflect the fact that these workers employed cumulative addition of K^+,

whereas in the present study, bolus administration of K^+ to K^+-free buffer would be expected to activate the endothelial Na^+/K^+-ATPase (Daut et al., 1988). This mechanism should in theory be susceptible to inhibition by 18α-glycyrrhetinic acid, as well as ouabain, as discussed above, thus, explaining the ability of both compounds to attenuate or abolish, respectively, endothelium-dependent relaxations to K^+. The effects of 18α-glycyrrhetinic acid against carbachol-induced relaxations, by contrast, are unlikely to involve a direct action at the level of the endothelial cell, as it has been shown that 18β-GA, an analogue of 18α-glycyrrhetinic acid, which similarly inhibits EDHF-type responses, is completely without effect on the endothelial hyperpolarization induced by acetylcholine (Yamamoto et al., 1999).

The cellular events that lead to EDHF synthesis by the endothelium remain controversial, but in some vessel types, they appear to involve mobilization of arachidonic acid by a Ca^{2+}-dependent phopholipase A_2, which may be further metabolized by the cytochrome *P*450 monooxygenase to products that are involved in vascular relaxation and hyperpolarization in a step that is sensitive to inhibition by clotrimazole (Fulton et al., 1996; McCulloch et al., 1997; Adeagbo and Henzel, 1998; Hutcheson et al., 1999). To date, the precise actions of clotrimazole against EDHF-mediated relaxations have been controversial, with its inhibitory effects being explained by inhibition of cytochrome *P*450 monooxygenase involved in EDHF synthesis (Fulton et al., 1995) or inhibition of potassium channels involved in the EDHF-response (Zygmunt et al., 1996). We have now shown for the first time that clotrimazole acts as a gap junction inhibitor and may inhibit EDHF-mediated responses by interfering with heterocellular communication.

In the present study, clotrimazole abolished EDHF-type relaxations to carbachol but was without effect on the relaxations evoked by K^+, further highlighting major differences in the pathways activated by the two classes of stimuli. The lack of effect of clotrimazole against K^+-induced relaxations would appear to exclude the involvement of the "classical" EDHF pathway that participates in the response to carbachol. Indeed, the cyclooxygenase inhibitors indomethacin and flurbiprofen partially attenuated the responses to K^+ but not carbachol (McCulloch et al., 1997), indicating that endothelium-derived prostanoids, rather than cytochrome *P*450 metabolites, contribute to K^+ relaxations.

In summary, the present investigation has shown that K^+ is unlikely to be an EDHF in the rat isolated mesentery, as its mechanism of action is strictly endothelium-dependent. Experiments with 18α-glycyrrhetinic acid, SR141716A, palmitoleic acid and clotrimazole have identified an important role for direct endothelium–smooth muscle coupling via gap junctions in the relaxations induced by carbachol, but not K^+. Differences in the pathways activated by these stimuli are therefore apparent in that responses to K^+ are insensitive to gap junction inhibitors (except 18α-glycyrrhetinic acid) and in part mediated via endothelial release of prostanoids. The ability of ouabain and clotrimazole to inhibit gap junctional communication may contribute towards their inhibitory effects against EDHF-mediated responses.

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