The endothelial component of cannabinoid-induced relaxation in rabbit mesenteric artery depends on gap junctional communication

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1. We have shown that the endocannabinoid anandamide and its stable analogue methanandamide relax rings of rabbit superior mesenteric artery through endothelium-dependent and -independent mechanisms that are unaffected by blockade of NO synthase and cyclooxygenase.

2. The endothelium-dependent component of the responses was attenuated by the gap junction inhibitor 18α-glycyrrhetinic acid (18α-GA; 50 μM), and a synthetic connexin-mimetic peptide homologous to the extracellular Gap 27 sequence of connexin 43 ([43]Gap 27, SRPTEKTIFII; 300 μM). By contrast, the corresponding connexin 40 peptide ([40]Gap 27, SRPTEKNVFIV) was inactive.

3. The cannabinoid CB_1 receptor antagonist SR141716A (10 μM) also attenuated endothelium-dependent relaxations but this inhibition was not observed with the CB_1 receptor antagonist LY320135 (10 μM). Furthermore, SR141716A mimicked the effects of [43]Gap 27 peptide in blocking Lucifer Yellow dye transfer between coupled COS-7 cells (a monkey fibroblast cell line), whereas LY320135 was without effect, thus suggesting that the action of SR141716A was directly attributable to effects on gap junctions.

4. The endothelium-dependent component of cannabinoid-induced relaxation was also attenuated by AM404 (10 μM), an inhibitor of the high-affinity anandamide transporter, which was without effect on dye transfer.

5. Taken together, the findings suggest that cannabinoids derived from arachidonic acid gain access to the endothelial cytosol via a transporter mechanism and subsequently stimulate relaxation by promoting diffusion of an endothelium-derived hyperpolarizing factor to adjacent smooth muscle cells via gap junctions.

6. Relaxations of endothelium-denuded preparations to anandamide and methanandamide were unaffected by [43]Gap 27 peptide, 18α-GA, SR141716A, AM404 and indomethacin and their genesis remains to be established.

An endothelium-derived hyperpolarizing factor (EDHF) is now widely recognized to mediate endothelium-dependent vascular relaxations that are independent of nitric oxide (NO) and prostanoid synthesis (Mombouli & Vanhoutte, 1997). Although the chemical identity of EDHF remains controversial, there is accumulating evidence that this mediator normally effects relaxation following diffusion from the endothelium to smooth muscle via myoendothelial gap junctions rather than the extracellular space (Chaytor *et al.* 1998; Taylor *et al.* 1998; Dora *et al.* 1999; Hutcheson *et al.* 1999). Anatomically, this hypothesis is supported by the demonstration of myoendothelial gap junction plaques in rabbit conduit arteries (Spagnoli *et al.* 1982), and functional dye transfer experiments confirm direct chemical coupling between endothelium and subjacent smooth muscle (Little *et al.* 1995). Gap junctions are membrane structural proteins which consist of two hemichannels or connexons contributed by apposing cells, with each connexon being formed from six protein subunits or connexins arranged around an aqueous central pore that permits intercellular transfer of electrical current and molecules < 1 kDa in size (Yeager & Nicholson, 1996). Connexin 43 (Cx43) is present in both endothelial and vascular smooth muscle cells, and EDHF-mediated relaxations and hyperpolarizations of rabbit

superior mesenteric artery are attenuated by a short synthetic peptide homologous to a specific region of the second extracellular loop of this connexin ([43]Gap 27, SRPTEKTIFII) (Chaytor *et al.* 1997, 1998; Dora *et al.* 1999; Hutcheson *et al.* 1999). The precise mode of action of [43]Gap 27 peptide remains to be determined but is likely to involve effects on connexon docking (Warner *et al.* 1995; Chaytor *et al.* 1997). EDHF-type responses are also attenuated by the 18α and 18β isoforms of glycyrrhetinic acid, which are lipophilic aglycones that disrupt gap junction plaques (Davidson & Baumgarten, 1988; Taylor *et al.* 1998; Yamamoto *et al.* 1999). Although endothelium-dependent agonists induce endothelial hyperpolarization directly (Mehrke & Daut, 1990; Marchenko & Sage, 1993), at the present time there is little evidence that transmission of a hyperpolarizing electrical current from the endothelium contributes significantly to EDHF-mediated responses, perhaps reflecting the relatively small mass of the endothelium compared to the media (Beny & Pacicca, 1994).

EDHF-mediated responses depend on mobilization of arachidonic acid by a Ca^{2+}-dependent phospholipase A_2 (PLA_2), but the pathways and mechanisms involved distal to this synthetic step remain controversial (Fulton *et al.* 1996; Adeagbo & Henzel, 1998; Hutcheson *et al.* 1999). In the rat mesenteric and coronary vasculature the arachidonic acid derivative *N*-arachidonylethanolamide (anandamide) displays the characteristics of an EDHF in that it stimulates hyperpolarizing calcium-activated potassium (K_{Ca}) channels and evokes NO- and prostanoid-independent relaxations that are attenuated by high extracellular $[K^+]$ (Randall *et al.* 1996, 1997). Relaxations to anandamide are sensitive to the cannabinoid CB_1 receptor antagonist SR141716A, which also attenuates EDHF-type relaxations to muscarinic agonists (Randall *et al.* 1996; White & Hiley, 1997; Randall & Kendall, 1998). Since endothelial cells synthesize anandamide (Deutsch *et al.* 1997), these findings have led to the proposal that this endocannabinoid is an EDHF (Randall & Kendall, 1997). It has become apparent, however, that EDHF- and anandamide-induced relaxations exhibit differential susceptibility to K^+ channel blockers (White & Hiley, 1997; Plane *et al.* 1997; Petersson *et al.* 1997; Zygmunt *et al.* 1997*a*; Randall & Kendall, 1998), and in certain artery types anandamide stimulates endothelium-dependent relaxations that involve NO and/or prostanoids (Ellis *et al.* 1995; Deutsch *et al.* 1997; Bilfinger *et al.* 1998).

In view of the uncertainties regarding anandamide as a putative EDHF, in the present study we have examined the role of CB_1 receptors, the high-affinity anandamide transport mechanism and gap junctional communication in cannabinoid-induced relaxations of the rabbit superior mesenteric artery. As anandamide may be converted into other vasoactive arachidonate derivatives within the endothelial cell (Pratt *et al.* 1998), experiments were also conducted with its stable chiral analogue methanandamide to investigate the possible role of intracellular metabolism (Abadji *et al.* 1994). The effects of two different CB_1 receptor

antagonists (SR141716A and LY320135), an inhibitor of the high-affinity anandamide transporter (AM404), [43]Gap 27 peptide and 18α-glycyrrhetinic acid were evaluated to define the mechanisms contributing to mechanical relaxations. In parallel experiments the ability of these agents to modulate intercellular transfer of Lucifer Yellow dye was investigated in confluent COS-7 cells. This African green monkey kidney fibroblast cell line expresses Cx43 as its only functional gap junction protein (George *et al.* 1998), and Western blot analysis has shown that the only connexin protein expressed in smooth muscle from the rabbit superior mesenteric artery is Cx43 (Chaytor *et al.* 1997). Since mRNA for Cx40 is detectable in the rabbit superior mesenteric artery (Chaytor *et al.* 1997), and in other species Cx40 forms functional channels in vascular cells (Li & Simard, 1999), the effects of a Gap 27 construct possessing homology with Cx40 ([40]Gap 27, SRPTEKNVFIV) were compared to those of [43]Gap 27 peptide.

METHODS

Isolated ring preparations

Male New Zealand White rabbits (2–2·5 kg) were killed by administration of sodium pentobarbitone (120 mg kg^{-1} intravenously). The superior mesenteric artery was removed, stripped of adherent connective and adipose tissue and placed in Holman's buffer (composition, mM: 120 NaCl, 5 KCl, 2·5 $CaCl_2$, 1·3 NaH_2PO_4, 25 $NaHCO_3$, 11 glucose and 10 sucrose). Rings 2–3 mm wide were dissected and suspended in oxygenated (95% O_2–5% CO_2) 3 ml tissue baths at 37 °C and attached using hooks and thread to FT102 force transducers with isometric force being recorded on a MacLab/4e (ADInstruments, Hastings, UK). In experiments investigating the direct smooth muscle effects of anandamide or methanandamide the endothelium was removed by gentle abrasion of the intimal surface with a roughened probe. Each ring was placed under 0·6 g tension and allowed to equilibrate for ~1 h with the baseline being reset at intervals following stress relaxation.

Experimental protocol

Endothelium-intact rings were submaximally contracted by phenylephrine (10 μM) and subsequently allowed to stabilise for 20–30 min before cumulative concentration–response curves to anandamide or methanandamide were constructed in the presence or absence of N^G-nitro-L-arginine methyl ester (L-NAME, 300 μM) and/or indomethacin (10 μM). Following these initial responses and washout of all drugs, a second series of responses was obtained subsequent to incubation with [43]Gap 27 peptide (300 μM) for 20 min. The effects of 18α-glycyrrhetinic acid (18α-GA, 50 μM for 60 min) against anandamide-induced relaxations were similarly determined. Some rings were not challenged with the peptide or 18α-GA in order to obtain time-matched controls. Further experiments were conducted in the presence of the CB_1 receptor antagonists *N*-(piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide hydrochloride (SR141716A) or (6-methoxy-2-(4-methoxyphenyl) benzo[*b*]thien-3-yl)(4-cyanophenyl) methanone (LY320135), or the high-affinity anandamide transporter inhibitor *N*-(4-hydroxyphenyl) arachidonylamide (AM404), each agent being administered at a concentration of 10 μM, 20 min before the response curves were constructed. Responses to cannabinoids were also investigated in endothelium-denuded preparations in the presence or absence of [43]Gap 27 peptide

(300 μM), 18α-GA (50 μM), SR141716A (10 μM), LY320135 (10 μM), AM404 (10 μM) or indomethacin (10 μM). At the end of each experiment involving denuded rings ACh (1 μM) was added to confirm the absence of endothelium.

In a further series of experiments, concentration–relaxation curves were constructed for ACh in endothelium-intact rings submaximally precontracted with phenylephrine (10 μM). Responses to ACh were investigated following preincubation with [43]Gap 27 peptide (300 μM) or [40]Gap 27 peptide (300 μM and 500 μM) for 20 min in the presence of L-NAME (300 μM) and indomethacin (10 μM). Relaxations to ACh were also studied following preincubation for 20 min with SR141716A (10 μM) in the absence or presence of L-NAME (300 μM) and indomethacin (10 μM).

Dye transfer

Lucifer Yellow CH (charge -2, MW 457; 5% w/v in 0·3 M LiCl) was used to evaluate cell–cell coupling in COS-7 cells (ECACC, Wiltshire, UK) following intracytoplasmic injection. Confluent monolayers were formed after 3 days' incubation at 37 °C in 5% CO_2 following seeding at a density of 1×10^6 cells per 60 mm^2 tissue culture dish in complete Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 100 mg l^{-1} penicillin–streptomycin–glutamine and 250 mg l^{-1} amphotericin B (Gibco BRL). The monolayers were washed twice with phosphate-buffered saline (120 mM NaCl; 2·7 mM KCl; 10 mM $Na_2HPO_4.2H_2O$, pH 7·4) and incubated in Leibowitz L-15 medium (supplemented as above) at 37 °C. The cells were then exposed to [43]Gap 27 peptide (300 and 500 μM), [40]Gap 27 peptide (300 μM and 500 μM), anandamide (100 μM), LY320135 (10 μM), SR141716A (10 μM) and AM404 (10 μM) prior to Lucifer Yellow injection. 18α-GA (50 μM) was used to provide a positive control (Munari-Silem *et al.* 1995). In all cases monolayers were exposed to these agents for 60 min at 37 °C.

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 dye injection, the cells were returned to the incubator for 15 min and then fixed in 4% paraformaldehyde. Transfer of Lucifer Yellow was determined by fluorescence microscopy using filter set 05 (395–440 nm/460–470 nm; Zeiss, UK) on an Axiostat microscope (Zeiss, UK). Inhibition of dye transfer was quantified by determining the percentage of injected cells that communicated Lucifer Yellow to < 5, 5–10 and > 10 neighbouring cells (Nadarajah *et al.* 1998).

Materials

Acetylcholine, 18α-glycyrrhetinic acid, indomethacin, L-NAME, phenylephrine and Lucifer Yellow were supplied by Sigma, UK. [43]Gap 27 peptide (SRPTEKTIFII) and [40]Gap 27 peptide (SRPTEKNVFIV) were synthesised by Genosys Biotechnologies (Pampisford, UK). Anandamide was supplied by Tocris, UK and LY320135 from Eli Lilly, UK. We thank Dr A. Makriyannis, School of Pharmacy, University of Connecticut, Connecticut, USA and Dr D. Piomelli, The Neurosciences Institute, San Diego, USA for the gift of AM404. Methanandamide and SR141716A were supplied by Research Biochemicals International, UK. All compounds were dissolved in buffered Holman's solution except AM404, anandamide, methanandamide and SR141716A (all ethanol) and indomethacin (5% bicarbonate solution).

Statistics

Concentration–response curves and Lucifer Yellow dye transfer were analysed using one-way analysis of variance (ANOVA) with the Bonferroni multiple comparisons test as a further method of analysis. Maximal relaxations were compared using Student's paired *t* test. $P < 0.05$ was considered significant. Data are expressed as the mean $\pm$ S.E.M.

RESULTS

Effects of [43]Gap 27 peptide, L-NAME and indomethacin on endothelium-dependent relaxations to anandamide and methanandamide

Phenylephrine (10 μM) induced a constriction of 2·25 $\pm$ 0·14 g in rings of endothelium-intact superior mesenteric artery ($n = 35$; data pooled from all experiments). Anandamide evoked a concentration-dependent relaxation in endothelium-intact rings that was maximal at 10–30 μM, with the maximal response being 41·5 $\pm$ 5·0% of phenylephrine-induced contraction (Figs 1*A* and 2*A*). Preincubation with [43]Gap 27 peptide (300 μM) significantly attenuated this relaxation and caused a rightward shift in the concentration–response curve, such that maximal relaxation was reduced by 47·0 $\pm$ 3·6% and the EC_{50} value increased from 3·3 $\pm$ 1·1 to 9·8 $\pm$ 1·8 μM ($P < 0.05$, $n = 5$; Figs 1*B* and 2*A*). Similarly, methanandamide-induced relaxation of endothelium-intact rings was maximally 55·2 $\pm$ 6·3% of phenylephrine-induced tone at a concentration of 30 μM, and this was reduced by 38·2 $\pm$ 4·8% following preincubation with [43]Gap 27 peptide with a rightward shift in the concentration–response curve such that the EC_{50} value increased from 4·4 $\pm$ 0·8 to 9·7 $\pm$ 1·1 μM ($P < 0.05$, $n = 5$; Fig. 2*C*). Maximal relaxations and EC_{50} values did not differ significantly for the anandamide- and methanandamide-induced responses. Indomethacin (10 μM) had no significant effect on the concentration–response curves to anandamide or methanandamide ($n = 4$; Fig. 2*A* and *C*).

L-NAME (300 μM) had no significant effect on anandamide- or methanandamide-induced relaxations, either alone, or when administered in the presence of [43]Gap 27 peptide (300 μM). In the presence of L-NAME, maximal relaxations were 37·5 $\pm$ 3·6 and 50·5 $\pm$ 5·9% of phenylephrine-induced contraction with EC_{50} values of 3·8 $\pm$ 0·8 and 4·7 $\pm$ 0·9 μM for the two cannabinoids, respectively ($n = 5$; Fig. 2*A* and *C*). When [43]Gap 27 peptide and L-NAME were used in combination the inhibitory effect of the peptide was not enhanced ($n = 5$; Fig. 2*A* and *C*).

Endothelium-independent relaxations to anandamide and methanandamide

Phenylephrine (10 μM) induced a constriction of 2·50 $\pm$ 0·20 g in rings of endothelium-denuded superior mesenteric artery ($n = 18$; data pooled from all experiments). Anandamide and methanandamide also evoked concentration-dependent relaxations in endothelium-denuded rings of superior mesenteric artery, maximal responses being obtained at concentrations of 10–30 μM and representing 22·4 $\pm$ 4·2 and 26·8 $\pm$ 4·7% of phenylephrine-induced contraction, respectively (Fig. 2*B* and *D*). Preincubation with [43]Gap 27 peptide (300 μM) did not significantly affect concentration–response curves to either agent, maximal

relaxations then being $20\cdot3 \pm 4\cdot2$ and $24\cdot8 \pm 3\cdot8\%$, respectively. EC_{50} values were $9\cdot2 \pm 1\cdot2$ and $9\cdot6 \pm 1\cdot1$ μM for control responses and, following preincubation with ^{43}Gap 27 peptide, $9\cdot6 \pm 0\cdot8$ and $10\cdot1 \pm 1\cdot2$ μM for anandamide- or methanandamide-induced relaxations, respectively ($n = 5$; Figs $2B$ and D). No significant inhibitory effect against anandamide- and methanandamide-induced relaxation was found following preincubation with SR141716A (10 μM). In this series of experiments maximal relaxations to the two cannabinoids were $21\cdot6 \pm 4\cdot5$ and $25\cdot3 \pm 4\cdot5\%$ with EC_{50}

values of $10\cdot1 \pm 0\cdot9$ and $10\cdot3 \pm 1\cdot3$ μM, respectively, and these values were not significantly different from each other ($n = 5$; Figs $2B$ and D). Indomethacin (10 μM), LY320135 (10 μM) and AM404 (10 μM) similarly did not affect relaxations to anandamide or methanandamide in endothelium-denuded rings, maximal relaxations being $24\cdot4 \pm 3\cdot8$, $21\cdot8 \pm 5\cdot2$ and $26\cdot2 \pm 5\cdot4\%$ with EC_{50} values of $10\cdot2 \pm 2\cdot6$, $9\cdot8 \pm 1\cdot8$ and $10\cdot1 \pm 2\cdot4$ μM, respectively ($n = 4$ in each case; data not plotted in Fig. 2 for simplicity).
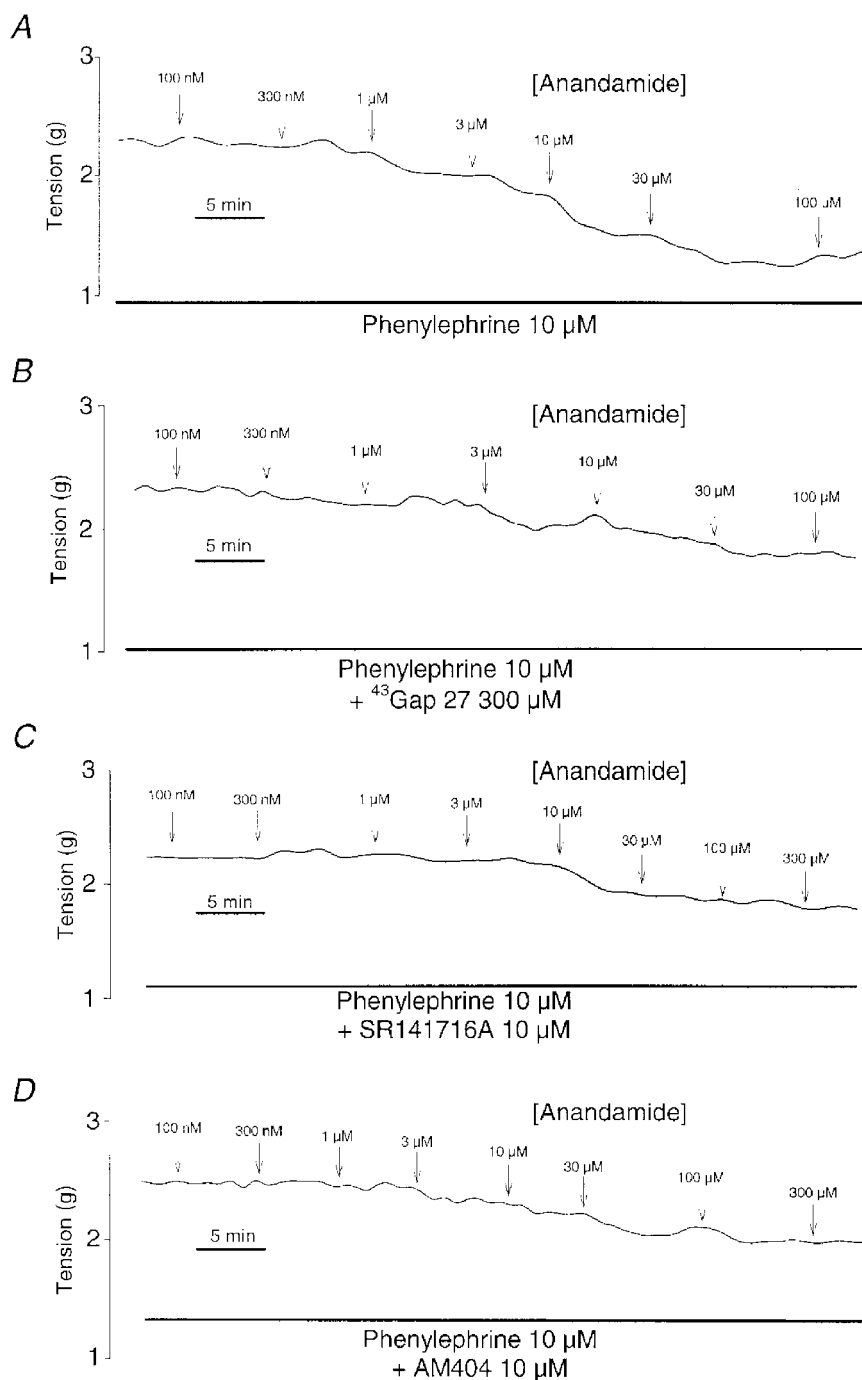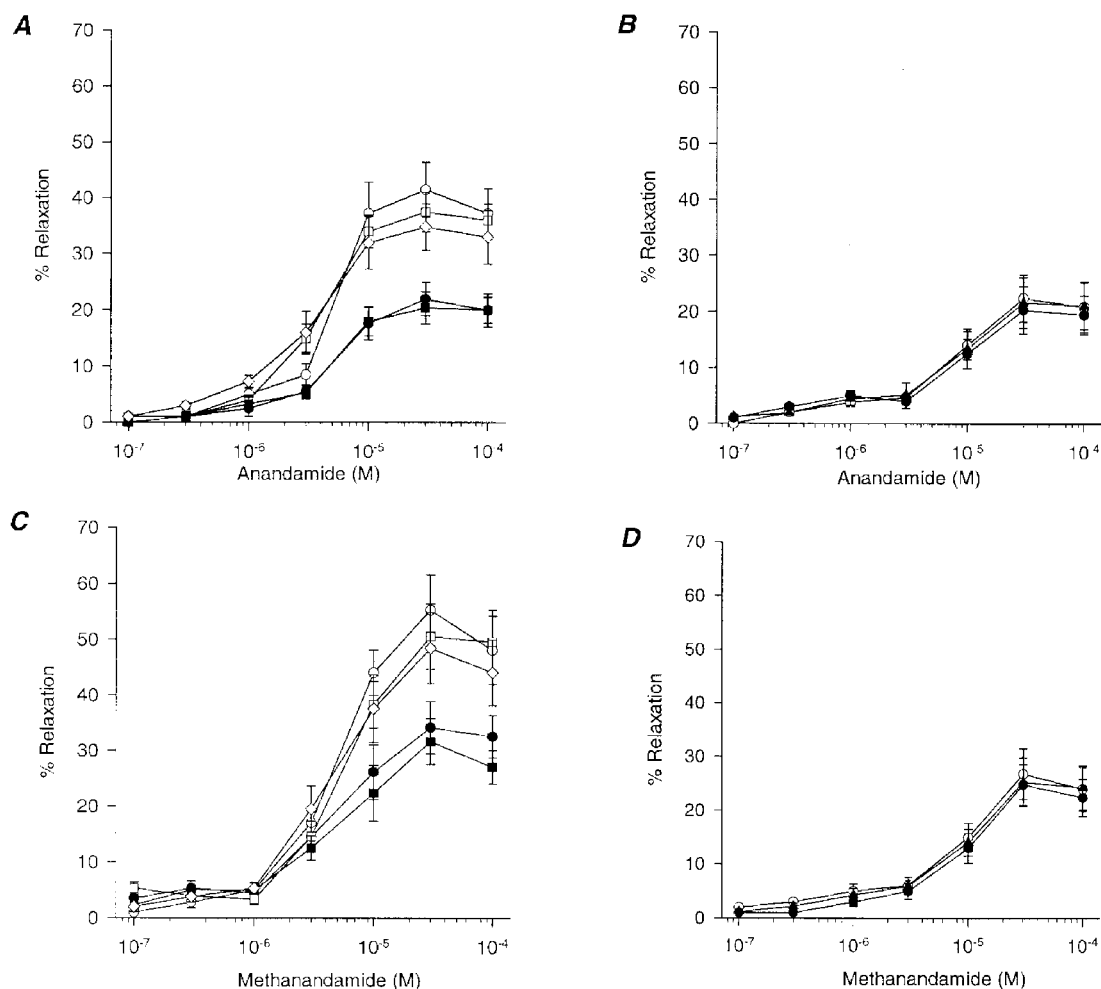

Figure 1. Representative traces showing anandamide-induced relaxation in endothelium-intact rings

Anandamide-induced relaxation in endothelium-intact rings (A) and the effects of preincubation with 300 μM ^{43}Gap 27 peptide (B), 10 μM SR141716A (C) and 10 μM AM404 (D).

Effects of 18α-glycyrrhetinic acid on endothelium-dependent and -independent relaxations to anandamide

The effects of 18α-glycyrrhetinic acid (18α-GA; 50 μM) were also investigated in endothelium-intact and -denuded rings. Preincubation with this gap junction inhibitor significantly attenuated maximal anandamide-induced relaxations by $54 \cdot 3 \pm 4 \cdot 3\%$, with EC_{50} values increasing to $14 \cdot 8 \pm 1 \cdot 2 \ \mu$M ($P < 0 \cdot 05$, $n = 5$; Fig. 3). Anandamide-induced relaxations of endothelium-denuded rings were not significantly affected in magnitude or EC_{50} value following preincubation with 18α-GA (50 μM; $n = 5$; Fig. 3).

Effects of SR141716A, LY320135 and AM404 on endothelium-dependent relaxation to anandamide and methanandamide

The effects of the CB_1 receptor antagonists SR141716A (10 μM) and LY320135 (10 μM) against anandamide- and methanandamide-induced relaxation, were investigated in endothelium-intact rings (Fig. 4). SR141716A caused a rightward shift in the concentration–response curves, such that maximal relaxations were reduced by $52 \cdot 9 \pm 5 \cdot 8$ and $57 \cdot 9 \pm 6 \cdot 2\%$, with the EC_{50} values increasing to $30 \cdot 4 \pm 4 \cdot 1$ and $32 \cdot 0 \pm 2 \cdot 8 \ \mu$M, respectively ($P < 0 \cdot 05$, $n = 4$; Figs 1C, 4A and B). By contrast, LY320135 did not significantly alter

Figure 2. Concentration–response curves showing the effects of ^{43}Gap 27 peptide (300 μM), indomethacin (10 μM) and L-NAME (300 μM) on anandamide- and methanandamide-induced relaxations

A, in endothelium-intact rings ^{43}Gap 27 peptide (300 μM; ●) caused a significant attenuation of responses to anandamide, whereas preincubation with L-NAME (300 μM; $n = 5$; □) or indomethacin (10 μM; $n = 4$; ◇) was without effect when compared to control ($n = 5$; ○). When ^{43}Gap 27 peptide (300 μM) was used in combination with L-NAME (300 μM; ■), responses did not differ significantly from those obtained with the peptide alone ($P < 0 \cdot 05$; $n = 5$). B, in endothelium-denuded rings anandamide-induced relaxation was not attenuated by ^{43}Gap 27 peptide (300 μM; $n = 5$; ●) or SR141716A (10 μM; $n = 5$; ▲) when compared to control (○). C, methanandamide-induced relaxations exhibited similar characteristics to those obtained with anandamide, preincubation with ^{43}Gap 27 peptide (300 μM; $n = 5$; ●) attenuating relaxation in endothelium-intact rings. Relaxations were not affected by L-NAME (300 μM; $n = 5$; □) or indomethacin (10 μM; $n = 4$; ◇). Other symbols as in A. D, in endothelium-denuded rings responses to methanandamide were unaffected by ^{43}Gap 27 peptide (300 μM; $n = 5$; ●) or SR141716A (10 μM; $n = 5$; ▲). ○, control ($n = 5$).

maximal relaxation or EC_{50} values for the anandamide- or methanandamide-induced responses ($n = 4$; Fig. 4C and D).

The cannabinoid transporter inhibitor AM404 caused significant inhibition of both anandamide and methanandamide-induced responses. Maximal relaxations to the two agents were reduced by $42 \cdot 2 \pm 5 \cdot 2$ and $29 \cdot 9 \pm 4 \cdot 1 \%$ and EC_{50} values increased to $34 \cdot 8 \pm 1 \cdot 8$ and $56 \cdot 2 \pm 4 \cdot 1$ μM, respectively ($P < 0 \cdot 05$, $n = 4$; Figs 1D, 4E and F).

Effects of SR141716A on acetylcholine-induced relaxations

Acetylcholine evoked concentration-dependent relaxations that were maximal at 3μM, being then equivalent to $86 \cdot 9 \pm 5 \cdot 4 \%$ of the phenylephrine-induced contraction. Preincubation with SR141716A (10μM) for 20 min caused a significant reduction in maximal relaxation by $48 \cdot 9 \pm 4 \cdot 2 \%$ and a rightward shift in the concentration–response curve with the EC_{50} value increasing from $357 \cdot 4 \pm 28$ to $942 \cdot 0 \pm 56$ nM ($P < 0 \cdot 05$, $n = 4$; Fig. 5). The combination of SR141716A (10μM), L-NAME (300μM) and indomethacin (10μM) caused a further significant decrease in maximal relaxation when compared to the effect of SR141716A alone, with the maximal response being inhibited by $66 \cdot 9 \pm 7 \cdot 2 \%$ ($P < 0 \cdot 05$, $n = 4$; Fig. 5). Although there was a corresponding rightward shift in the concentration–response curve, with the EC_{50} value increasing to 994 ± 62 nM, this did not achieve statistical significance compared to SR141716A alone.

Effects of anandamide, AM404, LY320135, SR141716A, [43]Gap 27 peptide and 18α-GA on Lucifer Yellow dye transfer

Confluent monolayers of COS-7 cells injected with Lucifer Yellow exhibited rapid spread of the dye to neighbouring cells (Fig. 6A and 6A'), with efficient gap junctional coupling permitting $72 \cdot 4 \pm 5 \cdot 7 \%$ of injected cells to transfer the dye to >10 neighbouring cells with $12 \cdot 8 \pm 10 \cdot 3$ and $9 \cdot 0 \pm 2 \cdot 2 \%$ transferring to <5 and 5–10 cells, respectively, in the control situation ($n = 61$ injected cells; Fig. 7). In most instances the dye appeared in 20–30 neighbouring cells within 15 min (Fig. 6A'). Anandamide, the cannabinoid transporter inhibitor AM404 and the CB_1 receptor antagonist LY320135 (100, 10 and 10 μM, respectively) did

not significantly affect the patterns of dye transfer observed in control cells ($n = 21$, 20 and 56 injected cells; Figs 6B–D and 7). By contrast, COS-7 cells incubated with SR141716A (10μM) demonstrated almost complete loss of intercellular communication with dye transfer being restricted to <5 cells in $97 \cdot 9 \pm 1 \cdot 1 \%$ of cases ($n = 103$ injected cells, $P < 0 \cdot 001$; Figs 6E and 7). The known gap junction inhibitor 18α-GA (50μM) caused a significant reduction in cell–cell coupling such that only $38 \cdot 0 \pm 3 \cdot 5 \%$ of cells permitted dye transfer to >10 neighbouring cells with an increase to $53 \cdot 8 \pm 5 \cdot 5 \%$ of cells transferring Lucifer Yellow to <5 cells compared to control ($n = 25$ injected cells, $P < 0 \cdot 005$ and $P < 0 \cdot 005$, respectively; Figs 6I and 7). In a similar fashion, [43]Gap 27 peptide (300 and 500 μM) attenuated dye transfer with $55 \cdot 0 \pm 5 \cdot 0$ and $26 \cdot 8 \pm 3 \cdot 3 \%$ transferring dye to >10 neighbouring cells ($n = 34$ and 17 injected cells, $P < 0 \cdot 05$ and $P < 0 \cdot 01$, respectively; Figs 6H and 7) with a corresponding increase in the number of injections following which dye transferred to <5 cells of $35 \cdot 4 \pm 0 \cdot 4$ and $49 \cdot 7 \pm 2 \cdot 3 \%$, respectively ($P < 0 \cdot 05$ and $P < 0 \cdot 005$, respectively; Figs 6H and 7).

Comparison of the effects of [40]Gap 27 peptide and [43]Gap 27 peptide on endothelium-dependent relaxations to acetylcholine in the presence of L-NAME

In the presence of L-NAME (300μM) and indomethacin (10μM), ACh evoked concentration-dependent relaxations in endothelium-intact rings of superior mesenteric artery that were maximal at 1–3 μM, with the maximal response being $44 \cdot 4 \pm 3 \cdot 6 \%$ of phenylephrine-induced contraction ($n = 5$). Preincubation with [43]Gap 27 peptide (300μM) markedly inhibited this response with maximal relaxations being reduced by $85 \cdot 4 \pm 4 \cdot 2 \%$ and the EC_{50} value increasing to 455 ± 24 nM ($n = 4$; $P < 0 \cdot 05$; Fig. 8A). In marked contrast, preincubation with [40]Gap 27 peptide (300 μM and 500 μM) was completely without effect on the relaxations induced by ACh ($n = 4$; Fig. 8A).

Effects of [40]Gap 27 peptide on Lucifer Yellow dye transfer

In a further set of experiments with COS-7 monolayers, $63 \cdot 7 \pm 10 \cdot 4 \%$ of cells injected with Lucifer Yellow transferred dye to >10 neighbouring cells in the control
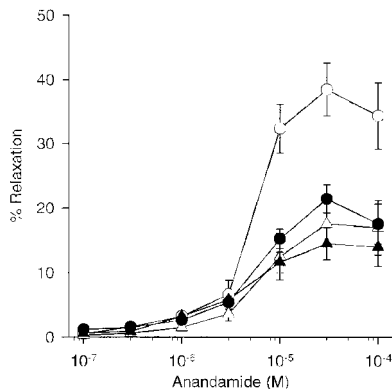
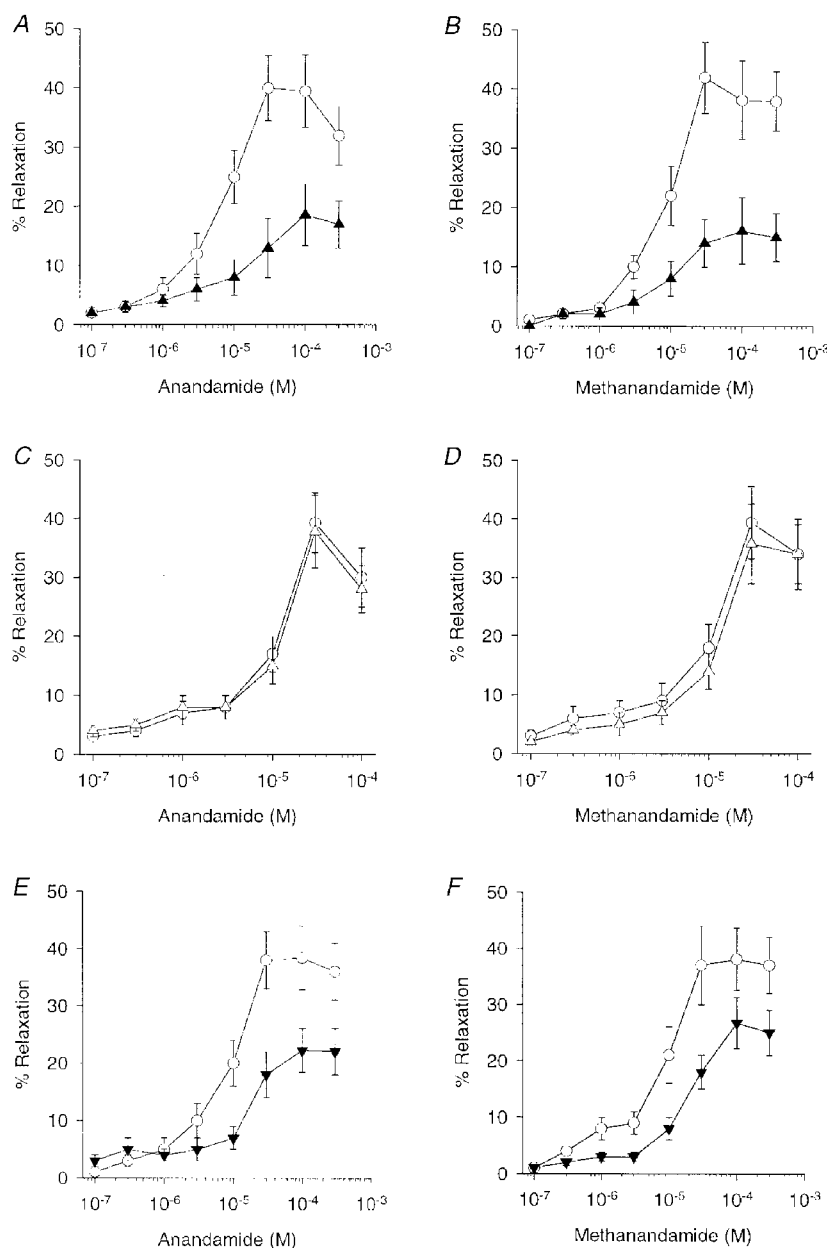**Figure 3. Concentration–response curves showing effects of preincubation with 18α-GA**

18α-GA (50 μM; $n = 5$; $\triangle$) caused attenuation of anandamide-induced relaxation in endothelium-intact rings of superior mesenteric artery when compared to control rings ($n = 5$; $\bigcirc$). In endothelium-denuded preparations 18α-GA ($n = 5$; $\blacktriangle$) did not significantly affect anandamide-evoked responses when compared to denuded controls ($n = 5$; $\bullet$).

situation, with $19\cdot3 \pm 3\cdot5$ and $11\cdot7 \pm 3\cdot5\%$ of cells transferring to <5 and 5–10 neighbours, respectively ($n = 30$ injected cells; Fig. 8B). Preincubation with [40]Gap 27 peptide (300 and 500 μM) did not affect this pattern of dye transfer ($n = 30$ injected cells; Fig. 8B).

DISCUSSION

The major finding of the present study is that direct heterocellular communication, rather than NO or prostanoids, underpins the endothelium-dependent component of the

relaxations evoked by anandamide and methanandamide in the rabbit superior mesenteric artery. Relaxations of endothelium-intact rings in the presence of [43]Gap 27 peptide and 18α-glycyrrhetinic acid, which both interrupt gap junction communication, were thus equivalent in magnitude to those observed in denuded vessels. The study also suggests that the action of cannabinoids can involve facilitated entry into the endothelial cell via a high-affinity transport mechanism, rather than activation of endothelial CB_1 receptors.

Figure 4. Concentration–response curves showing the effects of SR141716A, LY320135 and AM404 on anandamide- and methanandamide-induced relaxations in endothelium-intact rings

A and B, control responses to both agents (○) were significantly attenuated by preincubation with the CB_1 receptor antagonist SR141716A (10 μM; $n = 5$; ▲). C and D, LY320135 (10 μM; $n = 5$; △), also a CB_1 receptor antagonist, did not significantly affect relaxations when compared to control. E and F, preincubation with the transporter inhibitor AM404 (10 μM; $n = 5$; ▼) caused significant attenuation of both anandamide- and methanandamide-induced relaxations.
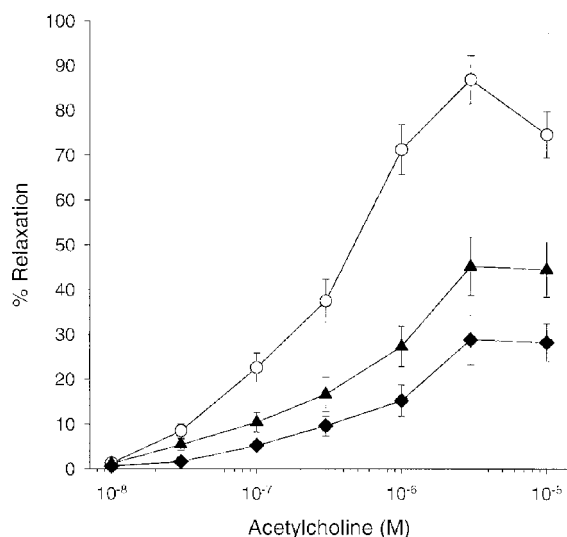
Figure 5. Concentration–response curves to acetylcholine showing the effects of preincubation with SR141716A

SR141716A (10 μM; $n = 4$; ▲) caused attenuation of acetylcholine-induced relaxation when compared to control (○). The combined presence of SR141716A (10 μM), L-NAME (300 μM) and indomethacin (10 μM) caused further inhibition of relaxation ($n = 4$; ◆).

Inhibition of EDHF-type vascular relaxations by SR141716A has previously been attributed to selective blockade of smooth muscle CB_1 receptors (Randall *et al.* 1996; White & Hiley, 1997). An unexpected finding, therefore, was that preincubation with SR141716A at concentrations which inhibited the endothelium-dependent component of responses to anandamide, methanandamide and ACh, markedly attenuated intercellular transfer of Lucifer Yellow in a COS-7 cell system that allows

quantification of intercellular communication via gap junctions constructed from Cx43. The inability of the CB_1 receptor antagonist LY320135 (Felder *et al.* 1998) to attenuate cannabinoid-induced relaxations in either endothelium-intact or -denuded vessels confirms that their action is not dependent on activation of endothelial or smooth muscle receptors in rabbit mesenteric arteries. The findings reveal a previously unsuspected action for SR141716A and question its specificity as a CB_1 receptor
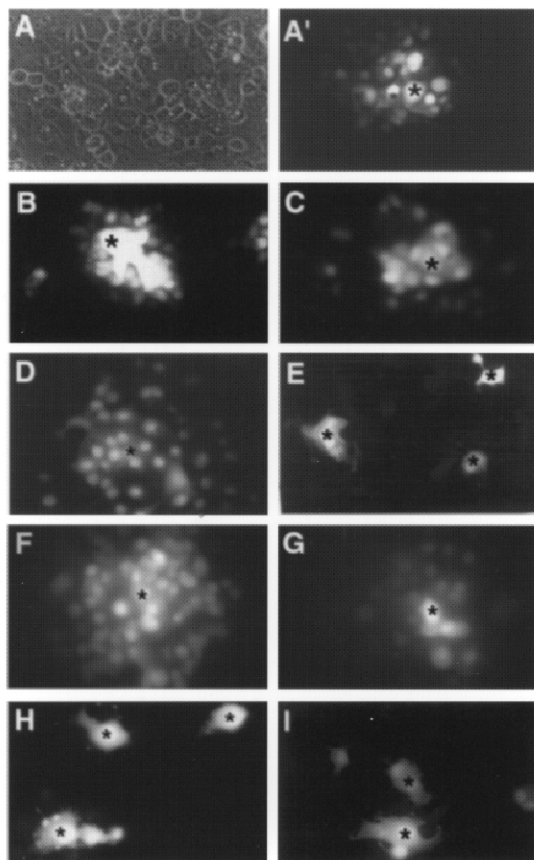

Figure 6. Representative photographs demonstrating the effects of preincubation with anandamide, AM404, LY320135, SR141716A, [40]Gap 27 peptide, [43]Gap 27 peptide and 18α-GA on Lucifer Yellow dye transfer in COS-7 cells

A, typical phase view of COS-7 cells showing confluence. *A′*, Lucifer Yellow dye transfer in the control situation demonstrating efficient gap junctional communication between > 20 cells. Anandamide (100 μM; *B*), the cannabinoid transporter inhibitor AM404 (10 μM; *C*) and the CB_1 receptor antagonist LY320135 (10 μM; *D*) did not affect dye transfer. *E*, preincubation with SR141716A (10 μM) markedly inhibited dye transfer. *F* and *G*, [40]Gap 27 peptide (300 and 500 μM, respectively) did not attenuate Lucifer Yellow dye transfer. *H* and *I*, following preincubation with [43]Gap 27 peptide (500 μM) or 18α-GA (50 μM), dye transfer was markedly reduced. ∗ denotes individual cells injected with Lucifer Yellow. Magnification × 40.

Figure 7. Histogram showing effects on Lucifer Yellow dye transfer in COS-7 cells

Preincubation with SR141716A (10 μM) caused almost complete abolition of dye transfer to more than 5 cells, and 18α-GA (50 μM) and [43]Gap 27 peptide (300 μM and 500 μM, respectively) caused a significant shift in the pattern of dye transfer seen following intra-cytoplasmic injection when compared to control. Anandamide (100 μM), the transporter inhibitor AM404 (10 μM) and the CB_1 receptor antagonist LY321035 (10 μM) did not affect dye transfer.
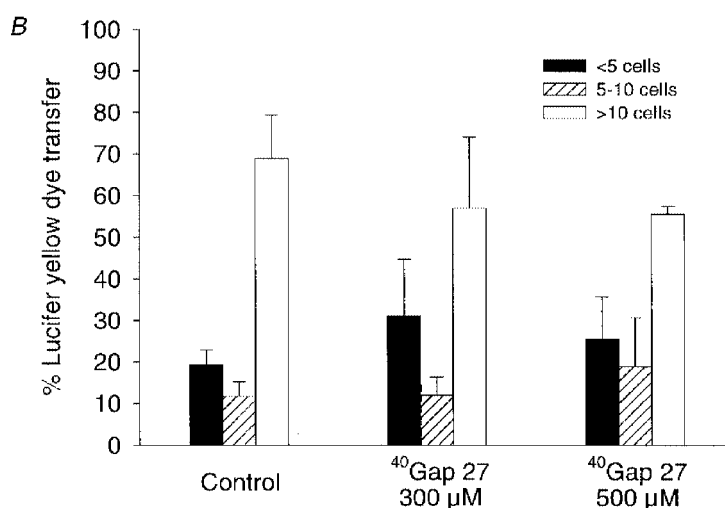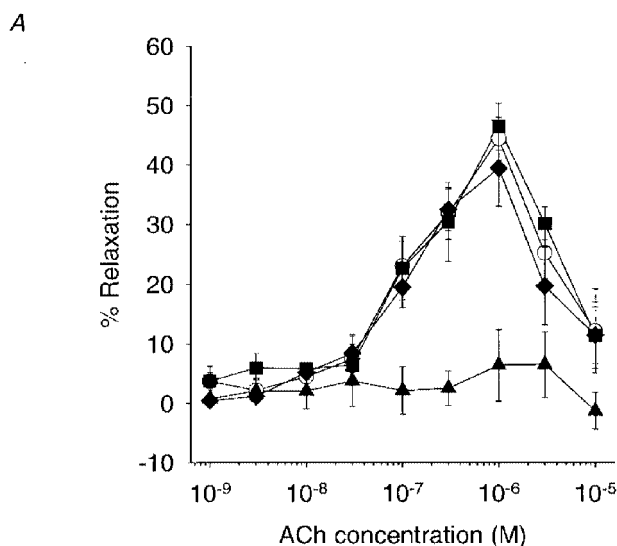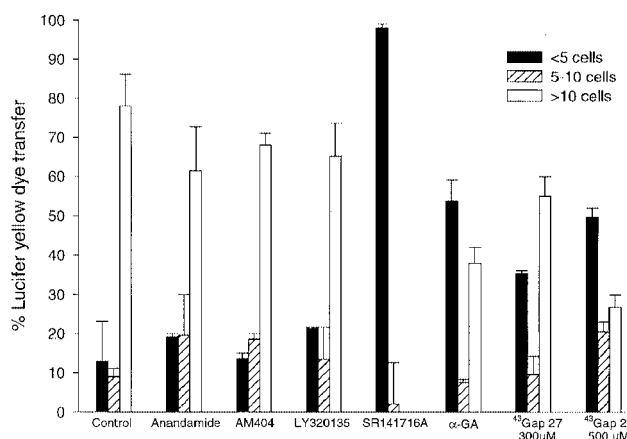
Figure 8. Concentration–response curves and histogram showing the effects of [40]Gap 27 peptide on ACh-induced relaxations in the superior mesenteric artery and Lucifer Yellow dye transfer in COS-7 cells

A, [40]Gap 27 peptide (300 μM; $n = 4$; ■, and 500 μM; $n = 4$; ◆) did not significantly affect relaxation responses evoked by ACh in the presence of L-NAME (300 μM) and indomethacin (10 μM) when compared to control (○; $n = 5$). Preincubation with [43]Gap 27 peptide (300 μM; $n = 4$; ▲) caused marked attenuation of ACh-induced relaxation. *B*, [40]Gap 27 peptide at concentrations of 300 and 500 μM did not significantly alter the pattern of dye transfer following intracytoplasmic injection of Lucifer Yellow.

antagonist in the vascular system. Observations that the antagonism of relaxations to anandamide by SR141716A was non-competitive support this conclusion.

Analogous findings have recently been reported in the perfused rat mesentery in which anandamide induced both endothelium-dependent and -independent relaxations, with only the former component being sensitive to SR141716A (Wagner *et al.* 1999). Furthermore, two potent synthetic CB_1 agonists were devoid of vasodilator activity (Wagner *et al.* 1999). In the literature, however, there are apparently conflicting reports concerning the effects of SR141716A, including: (i) inhibition of EDHF-type responses to acetylcholine and A23187 in the presence of L-NAME and indomethacin (Randall *et al.* 1996), (ii) no effect on anandamide-induced relaxations or EDHF-type responses evoked by acetylcholine and A23187 (Plane *et al.* 1997), (iii) attenuation of anandamide-induced relaxation with no effect on acetylcholine-induced EDHF-type responses (Zygmunt *et al.* 1997*b*), and (iv) no effect on anandamide-induced relaxation but inhibition of the Ca^{2+} ionophore A23187-induced release of radiolabelled arachidonic acid from endothelial cells, which also suggests that SR141716A is not purely a CB_1 receptor antagonist (Pratt *et al.* 1998). Variability in the effects of SR141716A in different vascular beds may be attributable to differences in the contributions of gap junctional communication, populations of CB_1 receptors, or poorly characterized effects on arachidonate metabolism. Indeed, in some vessel types anandamide stimulates NO release through a mechanism that is blocked by SR141716A, consistent with the involvement of endothelial CB_1 receptors (Deutsch *et al.* 1997; Bilfinger *et al.* 1998).

As anandamide does not appear to activate CB_1 receptors in the rabbit superior mesenteric artery, we investigated whether its entry into the endothelial cell was necessary for vasorelaxant activity using a selective inhibitor of the high-affinity anandamide transporter, AM404, that does not activate CB_1 receptors (Beltramo *et al.* 1997). In the guinea-pig, AM404 enhances CB receptor-mediated vascular responses by preventing the uptake and subsequent metabolism of anandamide, thus enhancing its *in vivo* depressor effects (Calignano *et al.* 1997). By contrast, in the present study AM404 selectively abolished endothelium-dependent relaxations to anandamide and methanandamide, suggesting that both are transported into the endothelium before activating more distal mechanisms of relaxation. The observation that AM404 inhibited, rather than enhanced, the endothelium-dependent component of the relaxations induced by the cannabinoids confirms that their action does not involve stimulation of endothelial CB_1 receptors in rabbit mesenteric arteries, given that the COS-7 cell system failed to demonstrate direct inhibition of dye transfer by AM404. By contrast, the direct smooth muscle effects of anandamide and methanandamide did not require facilitated transport, as AM404 did not antagonize relaxation in endothelium-denuded preparations. The mechanisms that underlie this

direct action on smooth muscle remain unknown, but can be distinguished from endothelium-mediated responses by complete resistance to [43]Gap 27 peptide, SR141716A and AM404.

The connexin subtypes that contribute to direct endothelial–smooth muscle communication are presently unknown. Since endothelium expresses Cx37, 40 and 43 proteins, and vascular smooth muscle expresses either Cx43 or Cx40 and 43, a variety of homotypic, heterotypic and heteromeric channels could in theory be formed (Elfgang *et al.* 1995; Carter *et al.* 1996; Chaytor *et al.* 1997; Li & Simard, 1999). In mammals (including rat, human and mouse) the [43]Gap 27 sequence used in the present study is identical to the corresponding Gap 27 sequence in Cx37, so that this peptide cannot distinguish between heterocellular communication via homotypic Cx43/Cx43 channels or heterotypic channels constructed from Cx37/Cx43. However, the lack of effect of [40]Gap 27 peptide against endothelium-dependent relaxations and dye transfer in the present experiments would appear to exclude the participation of Cx40 and is consistent with observations that neither smooth muscle from rabbit mesenteric arteries nor COS-7 cells express Cx40 protein (Chaytor *et al.* 1997; George *et al.* 1998). These findings also suggest that small differences in the Gap 27 peptide sequence leading into the fourth transmembrane domain can markedly affect the connexon selectivity of such peptide constructs.

Differences between freshly isolated arteries and cultured COS-7 cells were nevertheless apparent. Firstly, the attenuation of Lucifer Yellow dye transfer by [43]Gap 27 peptide was concentration dependent but still incomplete at $500~\mu$M, whereas at $300~\mu$M the peptide effectively abolished the EDHF-mediated component of the mechanical relaxations induced by anandamide. Secondly, $10~\mu$M SR141716A was more effective in attenuating dye transfer than $300~\mu$M [43]Gap 27 peptide or $50~\mu$M 18α-glycyrrhetinic acid in COS-7 cells, whereas all three agents effectively abolished EDHF-type relaxations at these concentrations. The explanation for these findings must await a more complete mechanistic understanding of the effects of these agents on gap junction permeability and delineation of the characteristics of the connexon subtypes that couple endothelial and smooth muscle cells. Indeed, in neurones and astrocytes, anandamide and its precursor arachidonic acid have been reported to reduce gap junctional communication through a mechanism that involves altered voltage gating in the case of arachidonic acid (Venance *et al.* 1995; Schmilinsky-Fluri *et al.* 1997; Weingart & Bukauskas, 1998). In the present study, however, dye transfer was unaffected by anandamide in the COS-7 model cell system and anandamide-induced relaxations were not susceptible to inhibition by [43]Gap 27 peptide. These findings indicate that closure of myoendothelial gap junctions is not an important action of anandamide in the arterial wall.

Other endothelial metabolites of arachidonic acid such as cytochrome P450-derived epoxyeicosatrienoic acids (EETs)

also possess the characteristics of EDHFs and may activate large-conductance K_{Ca} channels (Hecker *et al.* 1994; Randall *et al.* 1996; Campbell *et al.* 1996; Mombouli *et al.* 1996; Randall & Kendall, 1998). Indeed, it is possible that the actions of anandamide and EETs are not completely distinct, as anandamide may be converted into EETs following hydrolysis by the fatty acid amide hydrolase (Beltramo *et al.* 1997; Pratt *et al.* 1998). Bovine endothelial cells incubated with radiolabelled anandamide thus demonstrate ∼15 % conversion of anandamide to arachidonic acid, EETs and prostanoids after 30 min (Pratt *et al.* 1998). In the present study, however, maximal relaxations to anandamide, and their potency, were similar to methanandamide, which is not susceptible to breakdown by the fatty acid amide hydrolase (Abadji *et al.* 1994). Furthermore, certain epoxyeicosatrienoic acid regioisomers may stimulate a strictly endothelium-dependent relaxation in rabbit mesenteric arteries that involves synthesis of NO in addition to mechanisms that depend on gap junctional communication, thus exhibiting characteristics that differ from anandamide (Hutcheson *et al.* 1999). The present findings also suggest that there is no significant intracellular metabolism of anandamide to vasoactive prostanoids as both endothelium-dependent and -independent relaxations were unaffected by indomethacin. Taken together, these findings indicate that anandamide hydrolysis is unlikely to contribute to a pool of biologically active EETs or prostanoids that are available for relaxation in rabbit mesenteric arteries.

In conclusion, anandamide stimulates endothelium-dependent relaxations of the rabbit superior mesenteric artery that are exclusively dependent on heterocellular gap junctional communication and likely to involve transport into the endothelial cell rather than activation of CB_1 receptors. Analogous observations were made with the stable analogue methanandamide, thus suggesting that intracellular metabolism is not essential for vasorelaxation. Both cannabinoids also mediate effects on smooth muscle, whose genesis remains to be determined. The role of gap junctional communication in the vascular actions of cannabinoids in other species remains to be evaluated.

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