



Inhibition of the production of endothelium-derived hyperpolarizing factor by cannabinoid receptor agonists

*¹I. Fleming, ¹B. Schermer, ¹R. Popp & ¹R. Busse

¹Institut für Kardiovaskuläre Physiologie, Klinikum der J.W. Goethe-Universität, Theodor-Stern-Kai 7, D-60590 Frankfurt am Main, Germany

1 The endogenous cannabinoid, anandamide, has been reported to induce an 'endothelium-derived hyperpolarizing factor (EDHF)-like' relaxation *in vitro*. We therefore investigated the effects of cannabinoid CB1 receptor agonists; HU 210, Δ^9 -tetrahydrocannabinol (Δ^9 -THC) and anandamide, and a CB1 antagonist/inverse agonist, SR 141716A, on nitric oxide (NO) and EDHF-mediated relaxation in precontracted rings of porcine coronary, rabbit carotid and mesenteric arteries.

2 In rings of mesenteric artery HU 210 and Δ^9 -THC induced endothelium- and cyclo-oxygenase-independent relaxations which were sensitive to SR 141716A. Anandamide (0.03–30 μ M) induced a slowly developing, endothelium-independent relaxation which was abolished by diclofenac and was therefore mediated by cyclo-oxygenase product(s). None of the CB1 agonists tested affected the tone of precontracted rings of rabbit carotid or porcine coronary artery.

3 In endothelium-intact segments, HU 210, Δ^9 -THC and anandamide did not affect NO-mediated responses but under conditions of continuous NO synthase/cyclo-oxygenase blockade, significantly inhibited acetylcholine and bradykinin-induced relaxations which are attributed to the production of EDHF. The effects of HU 210 and Δ^9 -THC were not observed when experiments were performed in the presence of SR 141716A suggesting the involvement of the CB1 receptor.

4 In a patch clamp bioassay of EDHF production, HU 210 decreased the EDHF-mediated hyperpolarization of detector smooth muscle cells when applied to the donor segment but was without effect on the membrane potential of detector cells. The inhibition of EDHF production was unrelated to alterations in Ca^{2+} -signalling or cytochrome P450 activity.

5 These results suggest that the activation of endothelial CB1 receptors appears to be negatively coupled to the production of EDHF.

Keywords: Endothelium-derived hyperpolarizing factor (EDHF); nitric oxide (NO); cannabinoid CB1 receptor; electrophysiology

Abbreviations: ACh, acetylcholine; CB1, cannabinoid CB1 receptor; CYP, cytochrome P450; Δ^9 -THC, Δ^9 -tetrahydrocannabinol; EDHF, endothelium-derived hyperpolarizing factor; HUVEC, human umbilical vein endothelial cells; L-NA, N^G-nitro-L-arginine; NO, nitric oxide

Introduction

Δ^9 -Tetrahydrocannabinol (Δ^9 -THC) has long been known to exert distinct effects on the cardiovascular system inducing a maintained decrease in blood pressure (Graham & Li, 1973; Estrada *et al.*, 1987). Δ^9 -THC- and the endogenously generated cannabinoid, anandamide, induce hypotension and bradycardia which are generally attributed to a centrally mediated reduction in sympathetic tone (Berdyshev *et al.*, 1996; Varga *et al.*, 1995). However, in anaesthetized rats the cannabinoid CB1 receptor agonist, HU 210, induces a long-lasting hypotension and bradycardia which cannot be attributed to sympathetic withdrawal (Vidrio *et al.*, 1996). Indeed, cannabinoid receptors have recently been reported in the peripheral organs (Galiegue *et al.*, 1995; Munro *et al.*, 1993) as well as in endothelial cells (Deutsch *et al.*, 1997). Thus, the activation of the peripheral CB1 receptor on endothelial cells may well influence vascular tone and the generation of endothelium-derived autacoids. While some of the effects of anandamide may be attributed to its metabolism to arachidonic acid and subsequently to cyclo-oxygenase or cytochrome P450 (CYP) products *via* a receptor-independent mechanism (Ellis *et al.*, 1995; Pratt *et al.*, 1998), others are clearly mediated by CB1 receptor activation (Hunter & Burstein, 1997). The recent discovery of cannabinoid

receptors and some of their endogenous ligands has in turn focused attention on the possible signalling pathways that may be activated by these seven transmembrane, G protein-coupled receptors. Evidence for at least three, apparently different pathways has been proposed and depending on the cell type under investigation, cannabinoids are able to stimulate eicosanoid synthesis to inhibit the activation of adenylyl cyclase and to affect Ca^{2+} signalling (Pertwee, 1997). CB1 receptors are also involved in G protein receptor cross talk and anandamide is, for example, able to inhibit the activation of 5-HT₃ receptors in rat nodose ganglion neurones (Fan, 1995).

Endothelial cells generate and release anandamide (Deutsch *et al.*, 1997) as well as a second endogenous cannabimimetic molecule, 2-arachidonoylglycerol (Sugiura *et al.*, 1998), and as such may also be involved in the local regulation of vascular responsiveness. Indeed, just such a role for anandamide was recently proposed when it was suggested that a CB1 agonist produced by endothelial cells could account for the, as yet still elusive, endothelium-derived hyperpolarizing factor (EDHF) (Randall *et al.*, 1996). The aim of the present study was to determine whether a crosstalk occurs between CB1 receptor activation or inactivation and endothelial autacoid production. To this end we studied the effects of CB1 receptor agonists (HU 210, Δ^9 -THC and anandamide) and the selective antagonist/inverse agonist SR 141716A (Bouaboula *et al.*,

* Author for correspondence; E-mail: Fleming@em.uni-frankfurt.de

1997) on the vascular tone of three different arteries all of which synthesize both nitric oxide (NO) and an EDHF which is characterized as a CYP-dependent metabolite of arachidonic acid (Bauersachs *et al.*, 1994; Popp *et al.*, 1996; 1998).

Methods

Materials

Bradykinin was from Bachem Biochemica GmbH (Heidelberg, Germany), N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid (HEPES) and N^G-nitro-L-arginine (L-NA) from Serva (Heidelberg, Germany). Diclofenac (Voltaren injection solution) was from Ciba-Geigy, (Wehr, Germany), M-199 medium from GIBCO (Berlin, Germany), U46619 (9,11-dideoxy-11 α ,9 α -epoxymethano-prostaglandin F_{2 α}) was provided by Upjohn (Ann Arbor, MI, U.S.A.) and SR 141716A was provided by Sanofi Research (Montpellier, France). Rp-cyclic AMPS was from Alexis Biochemicals (Grünberg, Germany), 3-(1,1-dimethylheptyl)-(-)-11-hydroxy- Δ^8 -tetrahydrocannabinol (HU 210) was purchased from Tokris Cookson (Langford, England). Arachidoylethanolamide (anandamide), Δ^9 -tetrahydrocannabinol (Δ^9 -THC), acetylcholine (ACh), isobutylmethyl-xanthine (IBMX), sodium nitroprusside and all other chemicals were purchased from Sigma (Deisenhofen, Germany).

Vessel preparation

Porcine coronary arteries: Porcine hearts were obtained from a local slaughterhouse, placed immediately into ice-cold Krebs-Henseleit solution and transported to the laboratory. The coronary arteries were dissected, cleaned of adventitial adipose and connective tissue and cut into rings (4 mm) for organ bath studies. Coronary segments (40 mm length) were used for the bioassay of EDHF.

Rabbit carotid and mesenteric arteries: New Zealand White rabbits of either sex (1.5–2.5 kg body weight) were anaesthetized with pentobarbitone sodium (90 mg kg⁻¹ i.v.) and exsanguinated by cutting through both the abdominal aorta and vena cava. The carotid arteries and the main mesenteric artery were dissected, cleaned of adventitial adipose and connective tissue, and cut into rings (4 mm). In a number of experiments the endothelium was removed mechanically using blunt forceps.

Vascular reactivity studies Porcine coronary artery, rabbit carotid or mesenteric artery rings were mounted between force transducers (Scaime, France) and a rigid support for measurement of isometric force and incubated in organ baths containing warmed (37°C), oxygenated (95% O₂, 5% CO₂) Krebs-Henseleit solution (pH 7.4) of the following composition (mM): NaCl, 119; NaHCO₃, 25.0; KCl, 4.7; CaCl₂, 1.6; KH₂PO₄, 1.2; MgSO₄, 1.2; glucose 12.

Porcine coronary arteries Passive tension was adjusted over a 60 min equilibration period to 5 g; thereafter the segments were exposed to a Krebs-Henseleit solution rich in KCl (80 mM) until stable contractions were obtained. After washing, arterial rings were exposed to U46619 (0.1–0.3 μ M) to achieve a stable contraction which was approximately 80% of the maximal KCl-induced contraction. Thereafter, the integrity of the endothelium was assessed by the application of bradykinin (1 μ M) and vessels exhibiting less than 90% relaxation were discarded.

Rabbit carotid and mesenteric arteries Passive tension was adjusted over a 60 min equilibration period (2 g for carotid arteries and 2.5 g for mesenteric arteries), thereafter the segments were exposed twice to K⁺-rich Krebs solution. After washing and a further equilibration period of 40–60 min the arterial rings were contracted to 80% of the maximal KCl response using phenylephrine (0.1–1 μ M). The integrity of the endothelium was assessed using ACh (1 μ M) and vessels exhibiting less than 90% relaxation were discarded. As some of the relaxant responses observed were transient, the concentration-response curves presented represent the peak response observed to cumulative applications of the agonists.

Cell culture Human umbilical vein endothelial cells were seeded on fibronectin-coated culture dishes containing medium M-199 and 20% heat-inactivated foetal calf serum (Greiner) supplemented with penicillin (50 u ml⁻¹), streptomycin (50 μ g ml⁻¹) and L-glutamine (1 mM). CYP activity, cyclic AMP levels and [Ca²⁺]_i were estimated in confluent cells.

Rat aortic smooth muscle cells were isolated and cultured as described (Popp *et al.*, 1996). Experiments were performed using confluent smooth muscle cells grown on glass coverslips between passages 6 and 16.

Electrophysiological measurements The membrane potential of cultured rat aortic smooth muscle cells was recorded using the slow whole-cell configuration of the patch-clamp technique. The patch pipettes used had an input resistance of 8–10 M Ω when filled with standard KCl solution (mM: KCl, 140; MgCl₂, 1; CaCl₂, 1.3; HEPES, 10; D-glucose, 5; pH 7.4). G Ω seals (approximately 10 G Ω) were established by gentle suction. The slow whole-cell configuration was obtained using nystatin (100 μ g ml⁻¹) in the pipette. This nystatin concentration provides a low resistance access to the cytosol to measure intracellular potentials under current clamp conditions. The electrical contact with the cytosol was established within 1–2 min of seal formation, during this time the input resistance decreased to approximately 1 G Ω . The cell membrane potential, measured in the current clamp mode, was recorded continuously and only detector smooth muscle cells which had a stable resting membrane potential for more than 2 min and exhibited no further change in the input resistance were used.

Detection of EDHF release The release of EDHF was detected by recording changes in membrane potential of cultured rat aortic smooth muscle cells exposed to the effluve of a perfused porcine coronary artery. The transit time between the coronary artery and the detector smooth muscle cells was approximately 2 s. Briefly, a segment of porcine coronary artery was cannulated at both ends, mounted in an organ chamber and perfused intraluminally (1 ml min⁻¹) with HEPES-modified Tyrode's solution of the following composition (in mM): NaCl, 132; KCl, 4; CaCl₂, 1; MgCl₂, 0.5; HEPES, 9.5 and glucose, 5 (pH 7.4, 37°C) containing the NO synthase inhibitor L-NA (100 μ M) and diclofenac (10 μ M). The perfusate from a bradykinin-stimulated, L-NA/diclofenac-treated porcine coronary artery was directed over cultured rat aortic smooth muscle cells, the membrane potential of which was recorded using the slow whole-cell configuration of the patch clamp technique as described above. The resting membrane potential as determined in the whole cell configuration was approximately -48.2 mV ($n=30$) in cultured rat aortic smooth muscle cells. Continuous superfusion with HEPES-Tyrode (1 ml min⁻¹) did not alter the resting

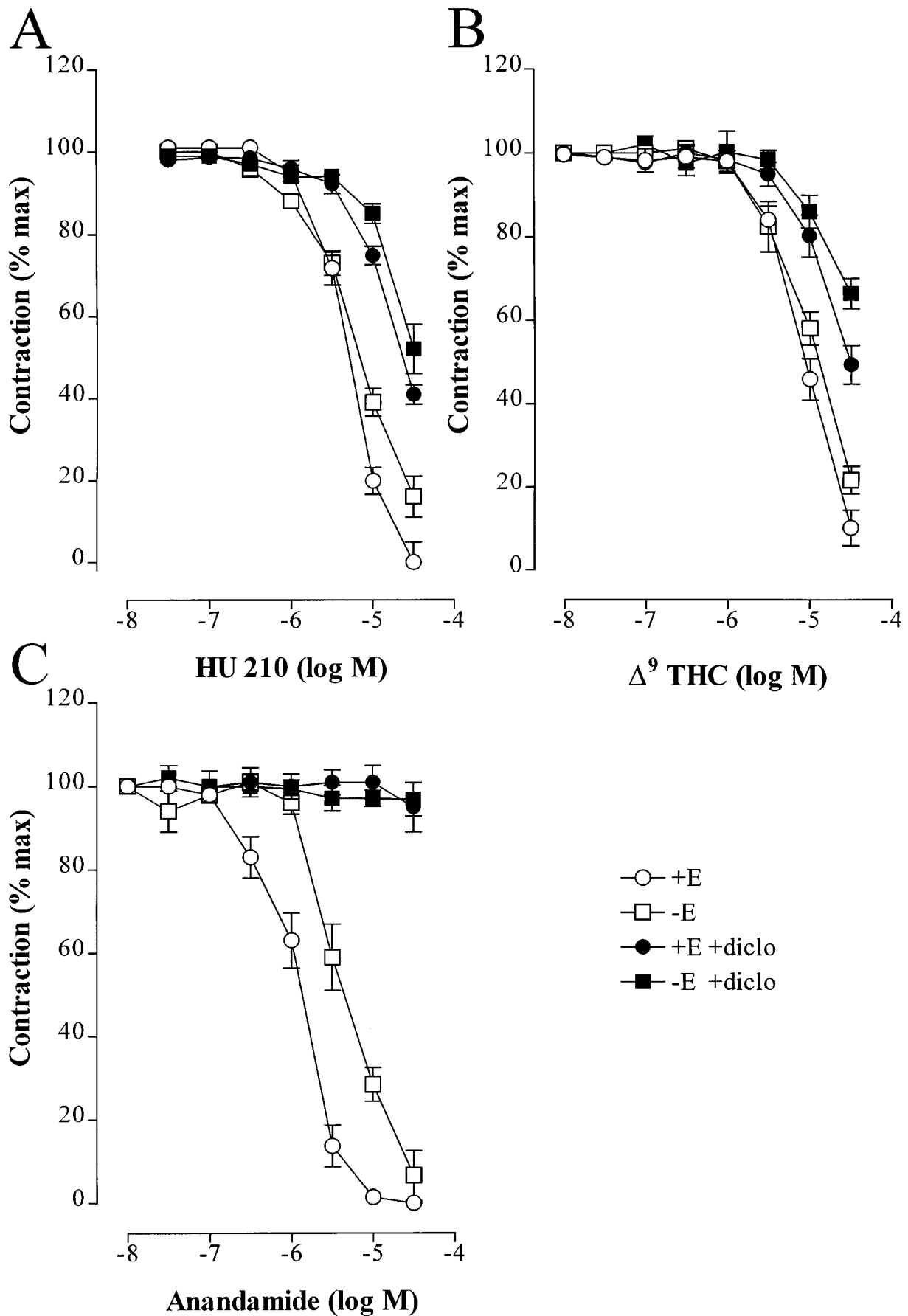


Figure 1 Effect of CB1 agonists on the tone of phenylephrine-precontracted mesenteric artery rings. Concentration-response curves showing the relaxant effect of (A) HU 210, (B) Δ^9 -THC and (C) anandamide on rings of rabbit mesenteric artery precontracted to 80% of the maximal KCl-induced response with phenylephrine. Experiments were performed using endothelium-intact (+E) and -denuded (-E) arteries in the absence and presence of diclofenac (diclo, 10 μ M). Results are presented as the mean \pm s.e. mean of 12 separate experiments.

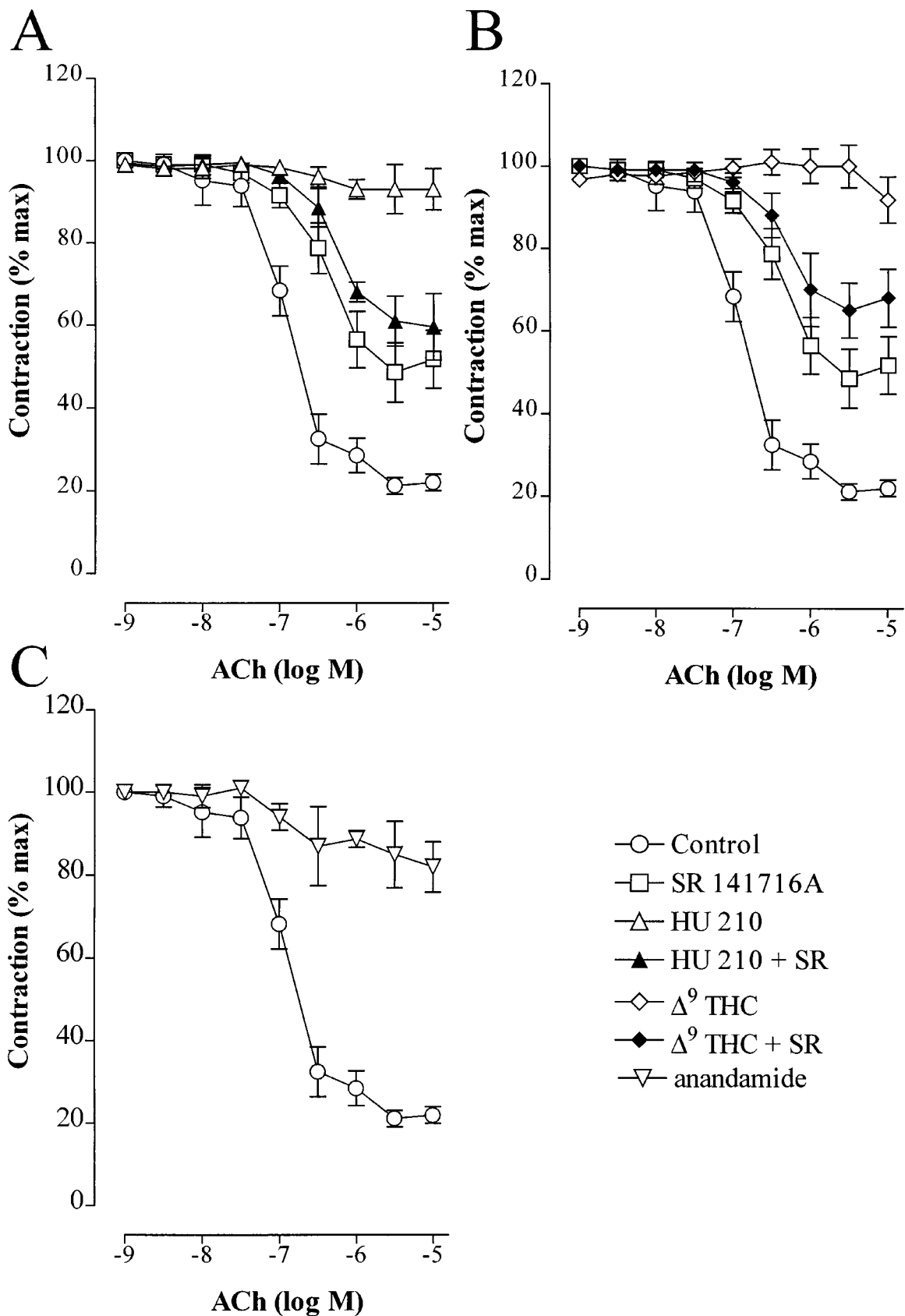


Figure 2 Effect of CB1 agonists on the EDHF-mediated relaxation of the rabbit mesenteric artery. Concentration-response curves to acetylcholine (ACh) were obtained using phenylephrine precontracted mesenteric artery rings in the presence of either solvent (0.06% ethanol), SR 141716A (30 μ M), HU 210 (30 μ M; A), Δ^9 -tetrahydrocannabinol (Δ^9 -THC, 30 μ M; B), anandamide (30 μ M; C) or combinations of CB1 agonist and SR 141716A. All experiments were performed in the continuous presence of N^G -nitro-L-arginine (100 μ M) and diclofenac (10 μ M) and the results are presented as the mean \pm s.e. mean of nine separate experiments.

membrane potential. We have previously reported that freshly isolated porcine coronary smooth muscle cells and cultured rat aortic smooth muscle cells respond in a similar manner to the luminal incubate from endothelium-intact porcine coronary arteries (Popp *et al.*, 1998).

Cell culture Human umbilical vein endothelial cells (HUVEC), isolated from umbilical cords as described previously (Hopkins & Gorman, 1981) were seeded on coverslips in culture dishes containing medium M-199 and 20% heat-inactivated foetal calf serum supplemented with penicillin (50 u ml⁻¹), streptomycin (50 µg ml⁻¹), L-glutamine (1 mM), glutathione (5 mg ml⁻¹) and L(+)-ascorbic acid (5 mg ml⁻¹) (GIBCO) and cultured until confluent.

Measurement of [Ca²⁺]_i For the measurement of [Ca²⁺]_i, HUVEC were loaded with the fluorescent Ca²⁺-sensitive dye fura 2 by incubation with 3 µM fura 2-AM and 0.025% (w/v) Pluronic F-127 at 37°C for 60 min. Thereafter the coverslips were washed in HEPES-Tyrode solution and [Ca²⁺]_i was determined fluorometrically in thermostatically controlled cuvette as described previously (Fleming *et al.*, 1996).

Measurement of cytochrome P450 (CYP) activity CYP-dependent metabolic activity was assayed as the dealkylation of 7-ethoxyresorufin (Yatomi *et al.*, 1992) in confluent HUVEC in which P450 activity had been induced by incubation with β-naphthoflavone (3 µM, 48 h). Briefly, cells were washed twice with HEPES-Tyrode and thereafter incubated with 7-ethoxyresorufin (3.4 µM) for 10 min. The supernatant was then removed and the resorufin formed was measured at an excitation of 522 nm and emission at 586 nm using a fluorescence spectrophotometer (Deltascan, Photon-Technology, Wedel, Germany). Cell-mediated conversion of ethoxyresorufin to resorufin was linear over at least 30 min and was abolished by the P450-inhibitor clotrimazole (3 µM).

Determination of cyclic AMP concentration In order to assess the effects of CB1 agonists on the isoprenaline-induced increase in HUVEC cyclic AMP, cells were pretreated with the phosphodiesterase inhibitor IBMX (0.1 mM) in the absence and presence of either HU 210 (30 µM) or Δ⁹-THC (50 µM). Cells were then incubated with isoprenaline (0.3 µM, 37°C), and after 5 min the incubation was stopped by the aspiration of the buffer and addition of 600 µl of ice cold 6% (v/v) trichloroacetic acid. Samples were left on ice for 30 min, cells and supernatants were then harvested by scraping and the precipitates centrifuged at 10,000 × g (15 min, 4°C). There-

after, the supernatants were extracted four times with 5 volumes of water-saturated diethyl ether and the concentration of cyclic AMP was determined by a specific radioimmunoassay. Cyclic AMP content is expressed as pmol per mg protein.

Statistical analysis Unless otherwise indicated data are expressed as mean ± s.e.mean. Statistical evaluation was performed using Student's *t*-test for unpaired data, one-way analysis of variance (ANOVA) followed by a Bonferroni *t*-test, or ANOVA for repeated measures where appropriate. Values of *P* < 0.05 were considered statistically significant.

Results

Effects of HU 210, Δ⁹-THC and anandamide on vascular tone

In phenylephrine-precontracted rabbit mesenteric artery rings, the selective non-hydrolysable CB1 agonist, HU 210, induced an endothelium-independent decrease in mesenteric artery tone which was slightly, but not significantly, attenuated by diclofenac (Figure 1A) and was abolished by the CB1 antagonist SR 141716A (data not shown). Δ⁹-THC also induced an endothelium-independent relaxation which was mostly insensitive to diclofenac (Figure 1B). The endocannabinoid, anandamide, elicited a slowly developing relaxation, with a threshold of 0.1 µM (Figure 1C). Although the removal of the endothelium induced a distinct rightward shift in the concentration-response curve to anandamide (EC₅₀ -6.0 ± 0.05 and -5.39 ± 0.02 M, in endothelium-intact and endothelium denuded rings respectively, *P* < 0.05), the maximal relaxation (R_{max}) of vessels precontracted to 80% of the maximal KCl-induced contraction was comparable in both the presence and absence of the endothelium. Inclusion of diclofenac (10 µM) in the organ bath abolished the anandamide-induced relaxation of both endothelium-intact and -denuded mesenteric artery rings (Figure 1C).

Neither HU 210, Δ⁹-THC nor anandamide relaxed rabbit carotid or porcine coronary artery rings (data not shown).

Effect of SR 141716A, HU 210, Δ⁹-THC and anandamide on EDHF-mediated relaxations

In endothelium-intact phenylephrine-contracted rabbit mesenteric artery rings under conditions of combined NO synthase/cyclo-oxygenase blockade (L-NA 100 µM; diclofenac 10 µM), ACh induced a concentration-dependent relaxation which was

Table 1 Effect of CB1 agonists and a CB1 antagonist on the ACh-induced, EDHF-mediated relaxation of the rabbit mesenteric and carotid arteries

	Mesenteric artery		Carotid artery	
	EC ₅₀ (M)	R _{max} (%)	EC ₅₀ (M)	R _{max} (%)
Control	-7.10 ± 0.25	78.00 ± 2.00	-7.18 ± 0.40	78.02 ± 4.06
SR 141716A	-6.41 ± 0.10	48.21 ± 6.20***	-6.49 ± 0.28*	65.23 ± 7.63
HU 210	-	3.30 ± 5.03***, †	-6.59 ± 0.25	28.00 ± 8.02***, †
HU 210 + SR 141716A	-6.31 ± 0.12	40.4 ± 7.6***	-6.53 ± 0.20	64.04 ± 8.25
Δ ⁹ -THC	-	8.2 ± 5.0***, †	-	4.44 ± 2.50***, †, ††
Δ ⁹ -THC + SR 141716A	-6.47 ± 0.31	33.8 ± 7.03***	-6.66 ± 0.33	45.25 ± 2.54
Anandamide	-	18.6 ± 6.33***	-6.58 ± 0.36	40.00 ± 8.31*

The results are presented as the mean ± s.e.mean of nine (mesenteric artery) and ten (carotid artery) experiments. *Indicates a significant difference from control (**P* < 0.05, ***P* < 0.01, ****P* < 0.001) and † a significant difference between experiments performed in the absence and presence of SR 141716A (†*P* < 0.05, †††*P* < 0.001).

attributed to the synthesis and release of EDHF (Figure 2). The CB1 antagonist, SR 141716A (30 μM) did not alter the basal tension of mesenteric arteries or significantly affect tone

in precontracted arteries. However, SR 141716 (30 μM) attenuated the ACh-induced EDHF-mediated relaxation (Figure 2, Table 1).

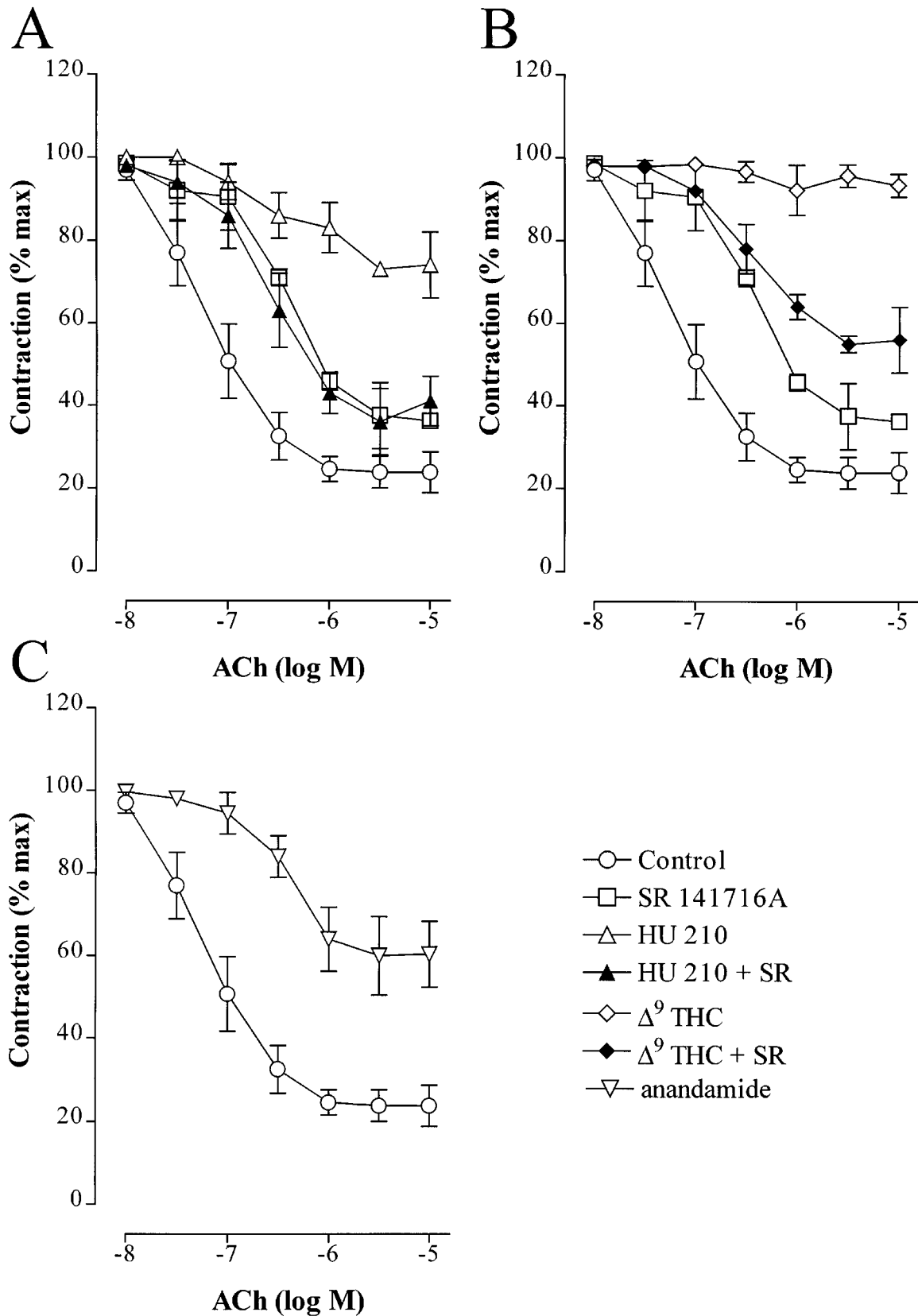


Figure 3 Effect of CB1 agonists on the EDHF-mediated relaxation of the rabbit carotid artery. Concentration-response curves to acetylcholine (ACh) were obtained using phenylephrine precontracted carotid artery rings in the presence of either solvent (0.06% ethanol), SR 141716A (30 μM), HU 210 (30 μM ; A), Δ^9 -tetrahydrocannabinol (Δ^9 -THC, 30 μM ; B), anandamide (30 μM ; C) or combinations of CB1 agonist and SR 141716A. All experiments were performed in the continuous presence of N^G -nitro-L-arginine (100 μM) and diclofenac (10 μM) and the results are presented as the mean \pm s.e.mean of ten separate experiments.

The ACh-induced, EDHF-mediated relaxations of mesenteric arteries was concentration-dependently inhibited by HU 210 (1–30 μM) and was abolished at the highest concentration studied. This effect was not observed in experiments performed in the presence of SR 141716A (Figure 2A, Table 1). The sensitivity of arterial rings to an exogenous NO donor (sodium nitroprusside, 1 μM) was unaffected by HU 210 (R_{max} was $103 \pm 6\%$ and $98 \pm 10\%$ in vessels with solvent and HU 210 respectively, $n=9$). Similarly, Δ^9 -THC (30 μM ; Figure 2B) also resulted in the SR 141716A-sensitive inhibition of EDHF-mediated relaxation, while the anandamide-induced inhibition (30 μM ; Figure 2C) was not sensitive to SR 141716A (not shown).

In the rabbit carotid artery, which did not relax in response to either HU 210, Δ^9 -THC or anandamide, all of these agents inhibited EDHF-mediated relaxation to a similar extent as in the rabbit mesenteric artery (Figure 3, Table 1). Similar results were obtained using the porcine coronary artery.

To determine whether the effect of the CB1 agonists on EDHF-mediated relaxations was specific, the NO-mediated relaxation to ACh was determined in the absence and presence of HU 210 (30 μM , Figure 4). The CB1 agonist failed to affect the ACh-induced, NO-mediated relaxation of vessels precontracted to 80% of the maximal KCl-induced contraction, demonstrating that these compounds selectively affect EDHF production. The subsequent addition of L-NA completely abolished ACh-induced relaxation (not shown). Although the second concentration response curve to ACh was shifted slightly to the right with respect to the first (Figure 4), this was the case in vessels treated with either solvent or HU 210. Similar results were obtained using the rabbit carotid artery (data not shown).

Effect of HU 210 on the production and activity of EDHF

A patch clamp bioassay for EDHF production was used to assess the effects of the CB1 agonists on the production and actions of EDHF. In this model the perfusate from a bradykinin-stimulated, L-NA/diclofenac-treated porcine coronary artery is directed over cultured rat aortic smooth muscle

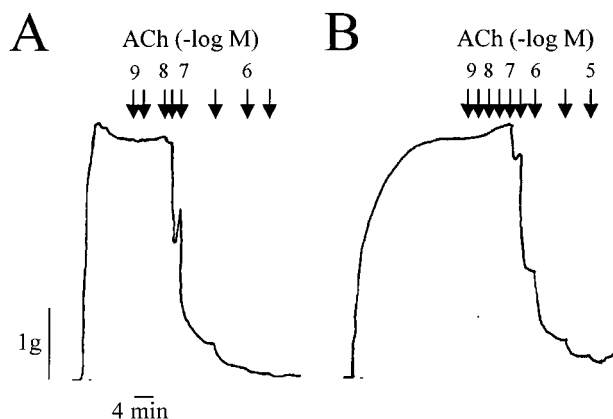


Figure 4 Effect of HU 210 on the acetylcholine-induced, NO-mediated relaxation of precontracted rabbit mesenteric arteries. Concentration-response curves to acetylcholine (1 nM to 3 μM) were obtained in rings of rabbit mesenteric artery precontracted to 80% of the maximal KCl-induced tone with phenylephrine. Experiments were performed in (A) the absence (0.06% ethanol) and (B) the presence of HU 210 (30 μM). Diclofenac (10 μM) was present throughout. The tracings shown are representative of results obtained in four separate experiments.

cells, the membrane potential of which is continuously monitored using the patch clamp technique (Popp *et al.*, 1996; Bauersachs *et al.*, 1996).

The effects of 'authentic EDHF', anandamide and HU 210 on smooth muscle membrane potential were determined. The EDHF present in the perfusate of bradykinin-stimulated arteries induced an immediate biphasic change in smooth muscle membrane potential which consisted of an initial hyperpolarization of -11.4 ± 1.0 mV, followed by a depolarization of 4.7 ± 1.0 mV, as previously described (Popp *et al.*, 1996). Anandamide used at a concentration (30 μM) which induced complete relaxation of rabbit mesenteric artery rings induced a small and slowly developing smooth muscle cell hyperpolarization which reached a maximum of -3.04 ± 0.5 mV approximately 3 min after its application (Figure 5A). Neither the resting membrane potential nor the magnitude of the EDHF-induced hyperpolarization were markedly altered when detector cells were pre-treated with HU 210 (30 μM) or the CYP inhibitor miconazole (3 μM , Figure 5B). However, incubation of donor segments with either HU 210 or miconazole attenuated the production of EDHF, as demonstrated by a decrease in the hyperpolarization of detector smooth muscle cells induced by the perfusate from HU 210 or miconazole-treated, bradykinin-stimulated porcine coronary artery segments (Figure 5B).

Effects of CB1 agonists on endothelial $[Ca^{2+}]_i$, CYP activity and cyclic AMP levels

Since HU 210 decreased the synthesis and/or release of EDHF we determined its effect on endothelial $[Ca^{2+}]_i$ and CYP activity. These responses were assessed using human umbilical vein endothelial cells which produce an EDHF which we have previously characterized as a CYP-dependent metabolite of arachidonic acid (Popp *et al.*, 1996). In fura 2-loaded human umbilical vein endothelial cells, bradykinin (10 nM) induced a biphasic increase in $[Ca^{2+}]_i$ as previously described (Fleming *et al.*, 1995). Neither basal $[Ca^{2+}]_i$ nor the bradykinin-induced Ca^{2+} -response were altered by HU 210 (50 μM , Figure 6A) irrespective of whether experiments were performed in the absence or presence of L-NA and diclofenac (data not shown).

As the production of EDHF by all of the arteries tested as well as that produced by cultured endothelial cells stimulated with bradykinin (Bauersachs *et al.*, 1994; 1996; Popp *et al.*, 1996), could be attenuated by inhibitors of CYP we determined whether the CB1 agonists affected P450 activity in cultured human umbilical vein endothelial cells. CYP activity, assayed as the dealkylation of 7-ethoxyresorufin, was slightly attenuated by anandamide and Δ^9 -THC. HU 210 (30 μM) did not affect ethoxyresorufin dealkylase activity in intact endothelial cells, whereas miconazole inhibited the formation of resorufin by approximately 70% (Figure 6B). None of the CB1 agonists tested inhibited CYP activity in purified endothelial cell microsome preparations (data not shown).

As CB1 receptors are negatively coupled to adenylyl cyclase, we determined the effect of the CB1 agonists on the isoprenaline-induced increase in endothelial cyclic AMP. In confluent human umbilical vein endothelial cells HU 210 markedly reduced cyclic AMP levels under basal conditions and following stimulation with isoprenaline. Δ^9 -THC induced a similar, though less pronounced effect on cyclic AMP levels (Figure 6C).

In order to determine whether an increase in cellular cyclic AMP is a prerequisite for the production of EDHF we performed experiments using a specific protein kinase A inhibitor. Rp-cyclic AMPs (25 μM) was without effect on the

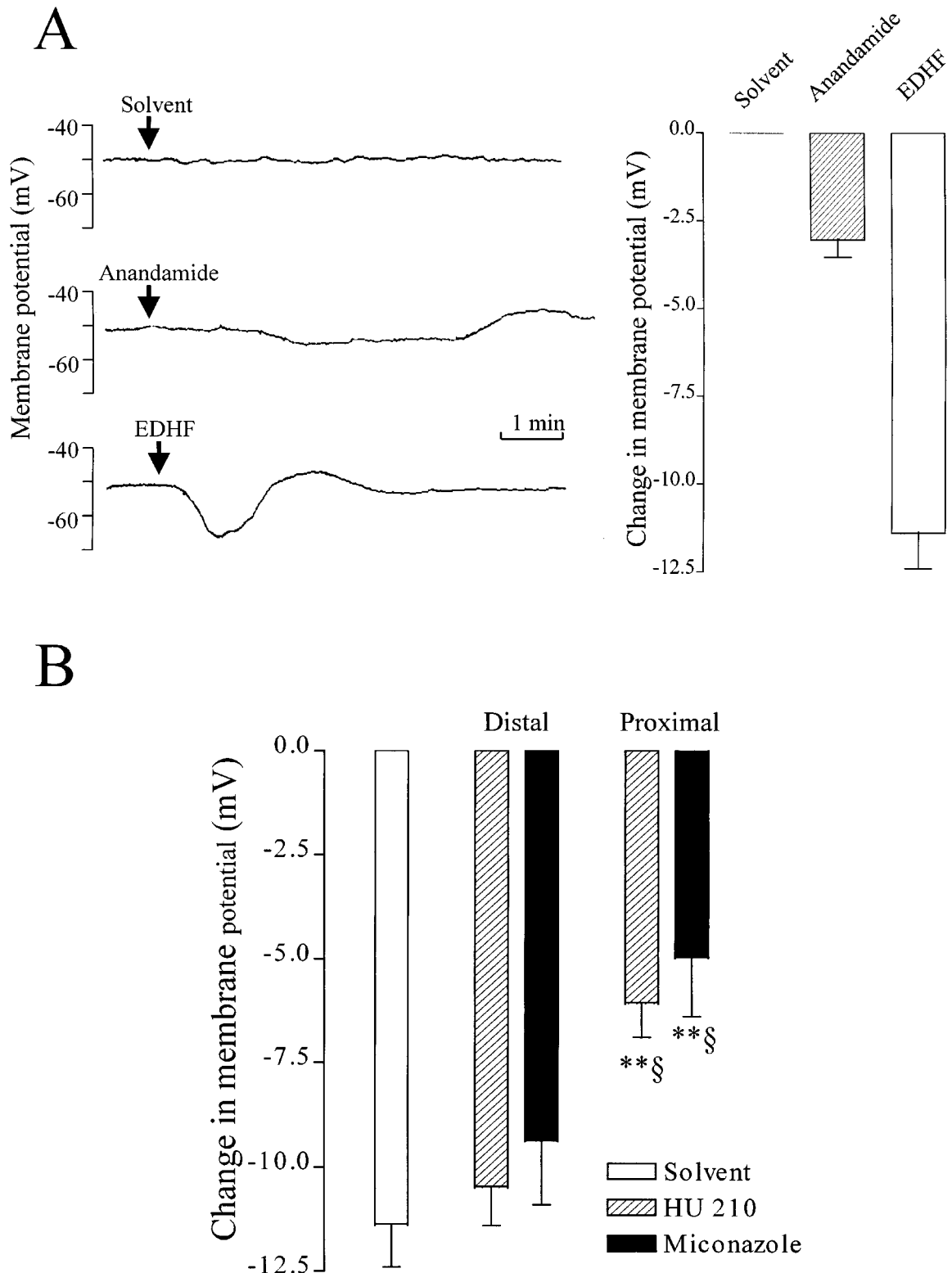


Figure 5 Effect of anandamide and HU 210 on smooth muscle membrane potential and the generation of EDHF from porcine coronary arteries. (A) Comparison of the effects on anandamide and 'authentic EDHF' on the membrane potential of cultured rat aortic smooth muscle cells. Original tracings (left panel) and statistical summary (right panel) illustrating the hyperpolarization of rat aortic smooth muscle cells induced by either the direct application of solvent (0.06% ethanol), anandamide (30 μ M) or the luminal perfusate removed from bradykinin-stimulated porcine coronary artery segments in the continuous presence of N^G-nitro-L-arginine (100 μ M) and diclofenac (10 μ M). Results are expressed as the mean \pm s.e.mean of four separate experiments. (B) Effect of HU 210 and miconazole on the generation of EDHF by bradykinin-stimulated porcine coronary arteries. The membrane potential of cultured vascular smooth muscle cells superfused with the effluate from bradykinin-stimulated porcine coronary arteries was monitored (open columns). Experiments were performed in the absence (0.06% ethanol) and presence of HU 210 (30 μ M) and miconazole (3 μ M) added to the perfusate either proximal or distal to the donor segment (incubation time 15 min). Results are presented as the mean \pm s.e.mean of six separate experiments, ** P < 0.01 vs control and § P < 0.05 vs response when the substance was applied distal to the donor artery.

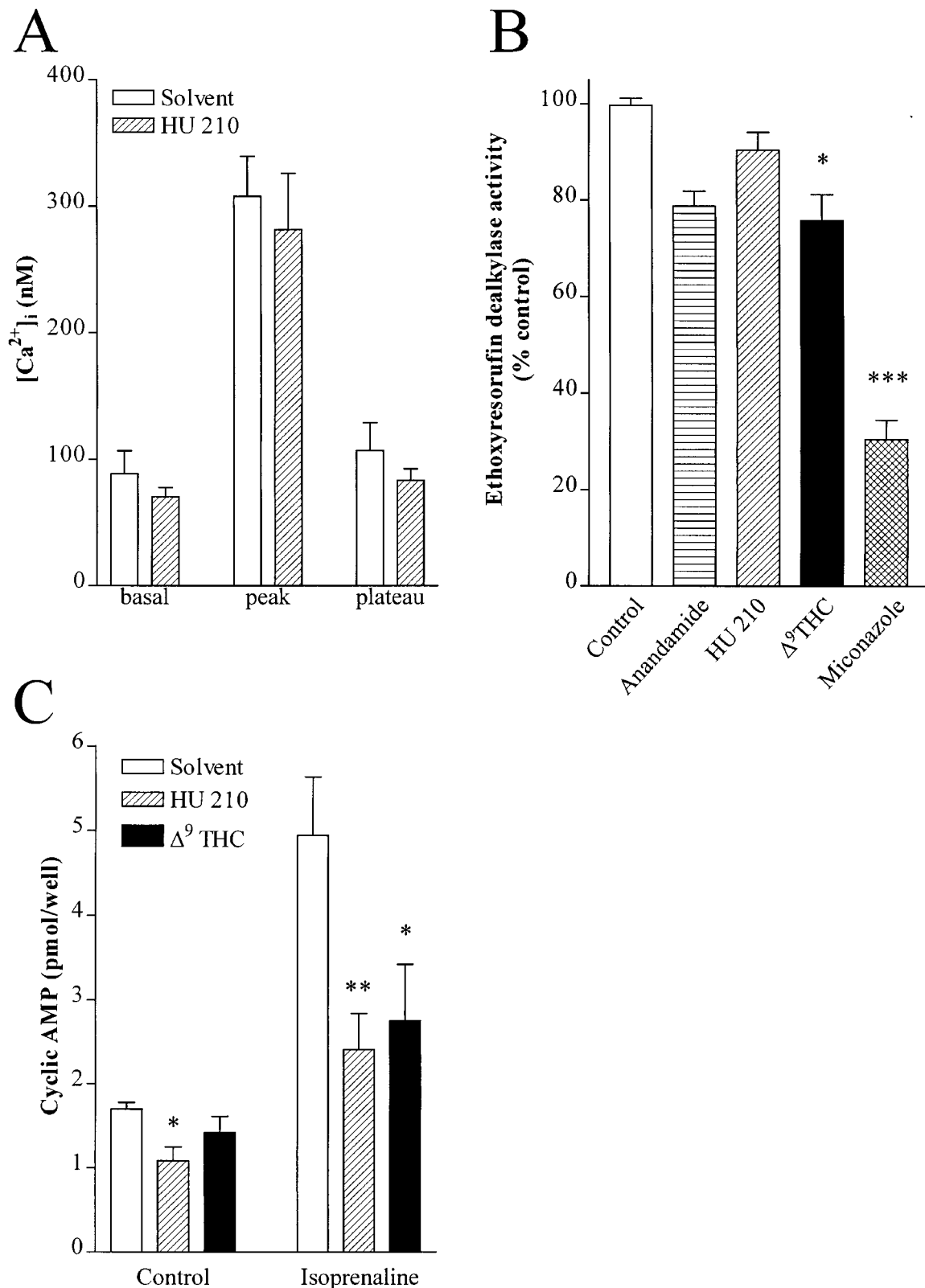


Figure 6 Effect on CB1 agonists on endothelial $[Ca^{2+}]_i$, P450 activity and cyclic AMP levels. (A) Basal $[Ca^{2+}]_i$ and the bradykinin (10 nM)-induced Ca^{2+} response in fura 2-labelled human endothelial cells. Experiments were performed in the absence (0.06% ethanol) and presence of HU 210 (30 μ M). Results are presented as the mean \pm s.e. mean of eight separate experiments. (B) Ethoxyresorufin dealkylase activity was assessed in endothelial cells pre-incubated with either solvent, anandamide (30 μ M), HU 210 (30 μ M), Δ^9 -THC (30 μ M) or miconazole (3 μ M). Results are presented as the mean \pm s.e. mean of five separate experiments. (C) Intracellular cyclic AMP levels in endothelial cells under basal and isoprenaline (0.3 μ M)-stimulated conditions. Experiments were performed in the absence and presence of HU 210 (30 μ M) or Δ^9 THC (30 μ M). Results are presented as the mean \pm s.e. mean of six separate experiments * P <0.05, ** P <0.01, *** P <0.001 vs the appropriate solvent.

EDHF-mediated relaxation of the phenylephrine-precontracted rabbit mesenteric artery (data not shown).

Discussion

The results of the present study demonstrate that in rabbit mesenteric and carotid arteries as well as porcine coronary arteries, the CB1 agonists HU 210, Δ^9 -THC and anandamide inhibit the production of EDHF without affecting the synthesis of NO. The inhibitory effect of these agonists on EDHF production was not related to changes in endothelial $[Ca^{2+}]_i$ or CYP activity and could be reversed by the inclusion of the CB1 receptor antagonist, SR 141716A. Thus, the activation of endothelial CB1 receptors appears to be negatively coupled to the production of EDHF.

Anandamide has been reported to dilate rat mesenteric and coronary vascular beds *via* an EDHF-like (i.e. NO/PGI₂-independent) mechanism (Randall *et al.*, 1996; Randall & Kendall, 1997). The observation that the selective CB1 antagonist SR 141716A inhibited EDHF-mediated relaxations in these preparations led to the suggestion that EDHF may be an endocannabinoid. This hypothesis is however a matter of debate, as other investigators have since reported that anandamide-induced relaxations are not sensitive to SR 141617A and appear not to be mediated by the CB1 receptor or Ca²⁺-dependent K⁺ channel activation (Plane *et al.*, 1997; Chataigneau *et al.*, 1998). Although anandamide does hyperpolarize vascular smooth muscle cells, the response obtained in the present study bore characteristics markedly different to that induced by EDHF, being delayed in onset and exhibiting a different kinetic. Anandamide also relaxed rabbit mesenteric arteries but as this effect was sensitive to diclofenac the reduction in vascular tone was more likely to be mediated by a cyclo-oxygenase product. Indeed, the cyclo-oxygenase-dependent metabolism of anandamide underlies its vasodilator action in cerebral arterioles (Facci *et al.*, 1995) and bovine coronary arteries (Pratt *et al.*, 1998; Ellis *et al.*, 1995).

While the discussion relating to the role played by anandamide in EDHF-mediated relaxations seems to be concluded, endocannabinoids do appear to play a significant role in the regulation of vascular tone in certain vascular beds. In the rat kidney, which has previously been reported to express both anandamide amidase and the CB1 receptor (Deutsch & Chin, 1993; Shire *et al.*, 1995), anandamide vasodilates juxtamedullary afferent arterioles by a process involving the generation of endothelium-derived NO (Deutsch *et al.*, 1997). This anandamide-induced production of NO inhibited the release of norepinephrine from sympathetic nerve endings in the renal artery (Deutsch *et al.*, 1997) demonstrating that anandamide possesses both vasorelaxant and neuromodulatory properties. Similarly, anandamide is also reported to stimulate the production of NO from the vascular endothelium of the rat portal plexus (Prevot *et al.*, 1998). The effects of CB1 agonists appear to vary with the vascular bed under investigation since none of the CB1 agonists used affected tone in rabbit carotid or porcine coronary arteries. Only rings from the rabbit mesenteric artery relaxed in response to CB1 agonists. HU 210 and Δ^9 -THC relaxed both endothelium-intact and denuded mesenteric arterial rings to a similar extent, thus excluding a role for endothelium-derived NO. The inclusion of diclofenac shifted the concentration-response curve of both agonists to the right suggesting that both cyclo-oxygenase-dependent and -independent processes were involved in mediating the response observed. However, the fact that the relaxation induced by HU 210 and Δ^9 -THC was

resistant to L-NA and diclofenac does not suggest that these agents represent an EDHF-like substance as neither compound hyperpolarized vascular smooth muscle cells.

The data in Figures 2 and 3 clearly show that SR 141716A alleviated (between 70 and 100%) the inhibition of EDHF-mediated responses by HU 210 and Δ^9 -THC suggesting that this effect may be mediated by the CB1 receptor. However, since high concentrations of the inverse agonist (i.e., a compound which exhibits biological activity by blocking the signal transduction mediated by constitutively activated receptors) were required to observe any effect, we cannot rule out the possibility that the effects observed may be indirect. Indeed, high concentrations of SR 141716A have recently been reported to block the agonist-induced release of arachidonic acid (Pratt *et al.*, 1998) and to hyperpolarize guinea-pig carotid arteries and thus attenuate the hyperpolarization observed in response to ACh (Chataigneau *et al.*, 1998).

The patch clamp bioassay, in which HU 210 attenuated EDHF-mediated hyperpolarizations only when applied to the donor artery, provided the most convincing evidence that CB1 agonists interfere with the generation of EDHF by endothelial cells rather than its effect on smooth muscle membrane potential. Exactly how the CB1 agonists interfere with the production of EDHF is at the moment unclear but cannabinoids are able to intercalate into cell membranes and have been reported to alter the properties of cellular proteins including G protein-coupled receptors. Such effects are however unlikely to account for the results observed in the present study as neither anandamide nor HU 210 markedly altered basal $[Ca^{2+}]_i$ or the bradykinin-induced Ca²⁺-response in cultured human umbilical vein endothelial cells, which express mRNA for the CB1 receptor (data not shown), and neither Δ^9 -THC nor HU 210 affected ACh or bradykinin-induced, NO-mediated relaxations.

Given that the exact chemical identity of EDHF remains to be elucidated, it is difficult to investigate the signal transduction cascade which results in the activation of the 'EDHF synthase'. Current opinion is that the synthesis of EDHF is secondary to the activation of phospholipase A₂ (PLA₂), and the liberation of arachidonic acid (Quilley *et al.*, 1997). Indeed, the synthesis of EDHF is thought to be Ca²⁺-dependent by virtue of the reported Ca²⁺-dependency of PLA₂. Thereafter, the signalling pathway is obscure and although EDHF may be generated from arachidonic acid by CYP enzymes in some vascular beds this is almost certainly not the case in others (Mombouli & Vanhoutte, 1997; Quilley *et al.*, 1997). Since the EDHF produced in all of the arteries under investigation has been pharmacologically characterized as a CYP-dependent metabolite of arachidonic acid, it seemed plausible to suggest that the CB1 agonists attenuated EDHF production by decreasing the activity of the 'EDHF synthase'. However, although cannabidiol has been reported to inactivate several CYP enzymes (Bornheim *et al.*, 1993), and anandamide and Δ^9 -THC did attenuate ethoxyresorufin dealkylase activity in intact endothelial cells, HU 210 was without effect. Thus CYP inhibition seems unlikely to be the major mechanism by which the CB1 agonists attenuate the production of EDHF. It is nonetheless impossible to rule out the possibility that HU 210, Δ^9 -THC and anandamide affect the activity of CYP enzymes which do not metabolise 7-ethoxyresorufin.

The binding of anandamide to cannabinoid receptors leads to the inhibition of adenylyl cyclase and anandamide has been shown to inhibit forskolin-stimulated cyclic AMP accumulation in a number of cell systems expressing the CB1 receptor (Felder *et al.*, 1993; Vogel *et al.*, 1993; Childers *et al.*, 1994). One previous study showed that isoprenaline induced an

endothelium-dependent relaxation in rat aortic rings which appeared to be mediated by the release of a CYP-dependent metabolite of arachidonic acid (Satake *et al.*, 1997). Although HU 210 and Δ^9 -THC attenuated both basal cyclic AMP levels as well as the isoprenaline-stimulated increase in cyclic AMP in cultured endothelial cells, a selective inhibitor of protein kinase A did not affect EDHF-mediated dilation.

Novel effects of CB1 receptor activation are continuously being described, for example it was recently reported that coupling exists between CB1 receptors and receptor tyrosine kinase-activated transduction pathways (Bouaboula *et al.*, 1997). Since SR 141716A has been shown to interfere with the activation of mitogen-activated protein (MAP) kinases following receptor tyrosine kinase activation by insulin and IGF1 (Bouaboula *et al.*, 1997), it is feasible that CB1 agonists affect signalling pathways which may be involved in the generation of EDHF. Although it is tempting to suggest that CB1 agonists interfere with the signal transduction pathway initiated by ACh and bradykinin, such a general effect cannot account for the selective inhibition of EDHF production. The only way that signal crosstalk could influence the production of EDHF selectively is if the pathways for EDHF production are regulated independently of that resulting in an increase in NO production. Such an idea would contradict the general concept that EDHF synthesis is activated simply in response to an increase in $[Ca^{2+}]_i$.

Assuming that an increase in $[Ca^{2+}]_i$ is the essential signal to initiate the generation of EDHF, one might speculate that the CB1 agonists interfere with EDHF-mediated responses by uncoupling cell-cell communication. The possibility that

EDHF is a substance which is capable of passing through gap junctions is not necessarily at odds with the observations made using bioassay models, as it is conceivable that this factor may spill over into the extracellular fluid. Anandamide has been reported to inhibit gap junction conductance and dye permeability in astrocytes in a pertussis toxin-sensitive manner (Venance *et al.*, 1995; Schmilinsky-Fluri *et al.*, 1997). Moreover, cell-cell communication in connexin 43-expressing cells is rapidly and transiently disturbed by G protein-coupled receptors (Postma *et al.*, 1998) via a process involving the activation of p60^{src}. Other indications that CB1 agonists interfere with gap junction conductance can be indirectly inferred from observations that MAP kinases are involved in the regulation of connexin 43-based cell-cell communication (Kanemitsu & Lau, 1993) and that anandamide (Wartmann *et al.*, 1995) and HU 210 (authors unpublished observations) can activate MAP kinase in cells expressing the CB1 receptor. Given that peptides homologous to extracellular loop motifs of connexin 43 attenuate EDHF-mediated relaxations (Chaytor *et al.*, 1997), an inhibitory effect of CB1 agonists on myo-endothelial and/or inter-endothelial gap junctions is an attractive hypothesis, currently under investigation, which may explain the abolition of EDHF-mediated relaxations by HU 210 and Δ^9 -THC.

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