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Anandamide-induced mobilization of cytosolic Ca^{2+} in endothelial cells

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1 Experiments were designed to determine whether anandamide affects cytosolic Ca^{2+} concentrations in endothelial cells and, if so, whether CB_1 cannabinoid receptors are involved. To this effect, human umbilical vein-derived EA.hy926 endothelial cells were loaded with fura-2 to monitor changes in cytosolic Ca^{2+} using conventional fluorescence spectrometry methods.

2 Anandamide induced an increase in Ca^{2+} in endothelial cells which, in contrast to histamine, developed slowly and was transient. Anandamide caused a concentration-dependent release of Ca^{2+} from intracellular stores without triggering capacitative Ca^{2+} entry, contrary to histamine or the endoplasmic reticulum Ca^{2+}-ATPase inhibitor thapsigargin.

3 Anandamide pretreatment slightly reduced the mobilization of Ca^{2+} from intracellular stores that was evoked by histamine. The mobilization of Ca^{2+} from intracellular stores evoked by anandamide was impaired by 10 mM caffeine.

4 Anandamide and histamine each significantly increased NO synthase activity in EA.hy926 cells, as determined by the enhanced conversion of L-[^{3}H]-arginine to L-[^{3}H]-citruline.

5 The CB_1 cannabinoid receptor antagonist SR141716A (1 μM) only produced a marginal reduction of the mobilization of Ca^{2+} produced by 5 μM anandamide. However, at 5 μM SR141716A elicited the release of Ca^{2+} from intracellular stores. This concentration strongly impaired the mobilization of cytosolic Ca^{2+} evoked by either anandamide, histamine or thapsigargin.

6 Pretreatment of the cells with either 200 μM phenylmethylsulphonyl fluoride (to inhibit the conversion of anandamide into arachidonic acid) or 400 ng ml^{-1} pertussis toxin (to uncouple CB_1 cannabinoid receptors from $G_{i/o}$ proteins) had no significant effect on the mobilization of cytosolic Ca^{2+} evoked by either anandamide, or histamine.

7 Taken together the results demonstrate that anandamide mobilizes Ca^{2+} from a caffeine-sensitive intracellular Ca^{2+} store that functionally overlaps in part with the internal stores mobilized by histamine. However, a classical CB_1 cannabinoid receptor-mediated and pertussis toxin-sensitive mechanism does not mediate this novel effect of anandamide in endothelial cells.

8 The mobilization of cytosolic Ca^{2+} in endothelial cells may account for the endothelium-dependent and NO-mediated vasodilator actions of anandamide. Due to its non-specific inhibition of Ca^{2+} signalling in endothelial cells, SR141716A may not be used to assess the physiological involvement of endogenous cannabinoids to endothelium-dependent control of vascular smooth muscle tone.

Keywords: Cannabinoids; endothelium-derived hyperpolarizing factor; blood vessels; vasodilation; fatty acids

Introduction

Anandamide (arachidonylethanolamide), an endogenous ligand to cannabinoid (CB) receptors, is produced in various organs and found in plasma (see Felder & Glass, 1998; Guiffrida & Piomelli, 1998 and references therein). In addition to its neurobehavioural actions, anandamide induces hypotension *in vivo*. This action is inhibited by the CB_1 receptor antagonist SR141716A (Lake *et al*., 1997; see Felder & Glass for review). Activation of peripheral CB receptors and the generation of anandamide by blood cells may contribute to haemorraghic hypotension (Wagner *et al*., 1997). The synthesis of anandamide has been demonstrated in cultured endothelial cells (Deutsch *et al*., 1994) and in the isolated perfused rat mesentery (Randall *et al*., 1996). CB_1 receptors are expressed in both endothelial and vascular smooth muscle cells (Deutsch *et al*., 1994; Sugiura *et al*., 1998). Hence, it is possible that, under physiological conditions, endogenous cannabinoids, such as anandamide, modulate arterial tone by stimulating endothelial cells to release vasodilator substances or by acting directly on vascular smooth muscle.

In rat cerebral arterioles, anandamide releases vasodilator cyclo-oxygenase products (Ellis *et al*., 1995). In rat juxtamedullary arterioles, the vasodilator response to anandamide is blocked after nitric oxide synthase inhibition (Deutsch *et al*., 1994). However, in bovine coronary arteries the relaxation induced by anandamide results from its conversion into arachidonic acid, which is then converted to vasodilator cyclo-oxygenase and cytochrome P450 metabolites (Pratt *et al*., 1998). In rat hepatic arteries, anandamide causes endothelium-dependent hyperpolarization as well as a direct relaxation of vascular smooth muscle (Zygmunt *et al*., 1997).

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However, in this artery, the relaxation seems independent of hyperpolarization and K^+ channel activation in vascular smooth muscle (Zygmunt *et al*., 1997). In rat mesenteric arteries, anandamide also elicits a hyperpolarization of vascular smooth muscle that is strictly endothelium-dependent (Chataigneau *et al*., 1998). Besides the rat juxtamedullary arterioles study (Deutsch *et al*., 1994), the concentrations of SR141716A required to block the vasodilator actions of anandamide in these *in vitro* models are beyond its selectivity range for CB_1 receptors (Felder *et al*., 1995; Felder & Glass, 1998). Therefore, both the mechanism of the vasodilator action of anandamide and the involvement of CB_1 receptors are still highly controversial.

Since, in certain arteries, anandamide may stimulate the release of either NO (Deutsch *et al*., 1997), of prostaglandins (Ellis *et al*., 1995) or of an endothelium-derived hyperpolarizing factor(s) (Zygmunt *et al*., 1997; Chataigneau *et al*., 1998), it is possible that anandamide mobilizes cytosolic Ca^{2+} in endothelial cells. Indeed, the secretion of these vasodilator mediators by the endothelium is largely dependent on the elevation of cytosolic Ca^{2+} concentration (Adams *et al*., 1989; Graier *et al*., 1994b). Synthetic cannabinoid agonists induce the mobilization of Ca^{2+} in fibroblasts as well as neural cell lines (Felder *et al*., 1995). In the latter, the effect is mediated by CB_1 receptors. Whereas, in fibroblasts this action is not dependent on the expression of CB_1 receptors, since the mobilization of Ca^{2+} could be obtained in cells with no demonstrable specific binding sites (Felder *et al*., 1992). Furthermore, the concentrations of agonists required for this effect were orders of magnitude greater than their potency at CB_1 receptors (Felder *et al*., 1992). In the DDT_1-MF-2 vas deferens-derived smooth muscle cell line, the mobilization of cytosolic Ca^{2+} evoked by cannabinoids is partially blocked by concentrations of SR141716A within its selectivity for CB_1 receptors (Filipeanu *et al*., 1997). Also, cannabinoids evoke capacitative Ca^{2+} entry (Filipeanu *et al*., 1997). In cultured rat striatal astrocytes, the stable anandamide analogue (R)-methanandamide depletes intracellular Ca^{2+} stores through a pertussis toxin-sensitive mechanism (Venance *et al*., 1997). In the present study, experiments were designed to determine whether or not anandamide elicits the mobilization of Ca^{2+} in cultured endothelial cells. Since, such was the case, we also assessed the effects of SR141716A, as a probe for the involvement of CB_1 receptors (Rinaldi-Carmona *et al*., 1995).

Methods

Cell culture

A human umbilical vein-derived endothelial cell line EA.hy926 (a generous gift from Dr Edgell, University of North Carolina, Chapell Hill, NC, U.S.A.) was subcultured in the laboratory in Dulbecco's minimum essential medium (DMEM), containing 10% foetal calf serum and supplemented with 1% HAT (hypoxanthine 5 mM, aminopterin 20 μM, thymidine 0.8 mM).

Ca^{2+} measurements

Determination of changes in intracellular Ca^{2+} levels was performed spectrofluorimetrically using fura-2 as a probe (Graier *et al*., 1995). Cell suspensions were obtained after moderate trypsinization of subconfluent endothelial cells. The cell suspensions were incubated for 30 min at 37°C in DMEM containing 2.5 μmol l^{-1} fura-2/AM to load the fluorescent probe. To study the effects of pertussis toxin, the cells were treated for 3 h with 400 ng ml^{-1} of the toxin before putting them in suspension. After loading with fura-2/AM, the cells were centrifuged, washed and resuspended in 2 ml of Ca^{2+}-free physiological salt solution (composition in mM): NaCl 135, $MgCl_2$ 1, KCl 5, HEPES 10, D-Glucose 10, EGTA 0.1, adjusted to pH 7.4 with NaOH. The cell suspension was transferred to a cuvette, and stirred continuously. To study the effects of the aminohydrolase inhibitor phenylmethylsulphonyl fluoride (PMSF, 200 μM), or the CB_1-receptor antagonist SR141716A, the cell suspension were first treated with either solvent (dimethyl sulphoxide 0.1% v v^{-1}) or the drugs for 4 min before the start of the experiments. All experiments were carried out at room temperature following a 4 min equilibration period. Initial experiments were carried out in physiological salt solution containing $CaCl_2$ 2.5 mM. In a second set of experiments, the cell suspensions were first equilibrated in Ca^{2+}-free physiological salt solution for 4 min. Afterwards, they were challenged (or not) with either anandamide (0.1–10 μM), histamine (100 μM) or thapsigargin (2 μM) to release Ca^{2+} from intracellular stores. Thereafter, $CaCl_2$ 2.5 mM was added to the suspension to record the elevation of cytosolic Ca^{2+} which is caused by Ca^{2+} entry from the extracellular milieu.

Measurement of nitric oxide synthase activity

Activity of endothelial nitric oxide synthase was monitored by measuring the conversion of L-[^{3}H]-arginine into L-[^{3}H]-citrulline as previously described (Graier *et al*., 1996). Briefly, EA.hy926 cells were cultured in 6 well plastic dishes. At confluence, culture medium was removed, the cells were washed twice with phosphate buffer (in mM: NaCl 137, K 2.7, Na_2HPO_4 8, KH_2PO_4 1.5, pH adjusted at 7.4). The experiment was started by the addition of 900 μl HEPES buffer plus 2.5 mM Ca^{2+}, containing 2×10^6 d.p.m. L-[^{3}H]-arginine and 100 μl of either buffer, anandamide (to achieve 5 μM final concentration) or histamine (100 μM final). In parallel series, experiments were repeated in the presence of 300 μM L-N^G-nitro-arginine were performed for each conditions. After 15 min, incubation buffer was discarded, cells were washed three times with chilled Ca^{2+}-free HEPES buffer and were finally lysed with 1 ml 0.01 M HCl. After 1 h at 4°C, the total incorporated radioactivity was measured in a 100 μl aliquot. The remaining 0.9 ml HCl were buffered with 100 μl of 200 mmol l^{-1} sodium acetate buffer (pH = 13.0) containing 10 mM L-citrulline. The amino acids, L-[^{3}H]-arginine and L-[^{3}H]-citrulline were separated using column chromatography as described by Mayer *et al*. (1991).

Data collection and statistical analysis

Due to the uncertainties inherent to measurement of intracellular Ca^{2+} with fluorescent probes (Morgan, 1993; Paltauf-Doburzynska & Graier, 1997; Graier *et al*., 1998), the relative cytosolic concentration of Ca^{2+} is expressed as the ratio of Fura-2 fluorescence that is due to excitation at 340 nm relative to that due to excitation at 380 nm (F_{340}/F_{380}). Activity of endothelial nitric oxide synthase was calculated by the L-N^G-nitro-arginine-sensitive percentage of the conversion of L-[^{3}H]-arginine to L-[^{3}H]-citrulline. Unless otherwise stated, evaluation of the statistical significance between treatments was performed either by a two-tailed Student's *t*-test for unpaired observations or by One-way analysis of variance when more than two treatment groups were assessed. $P < 0.05$

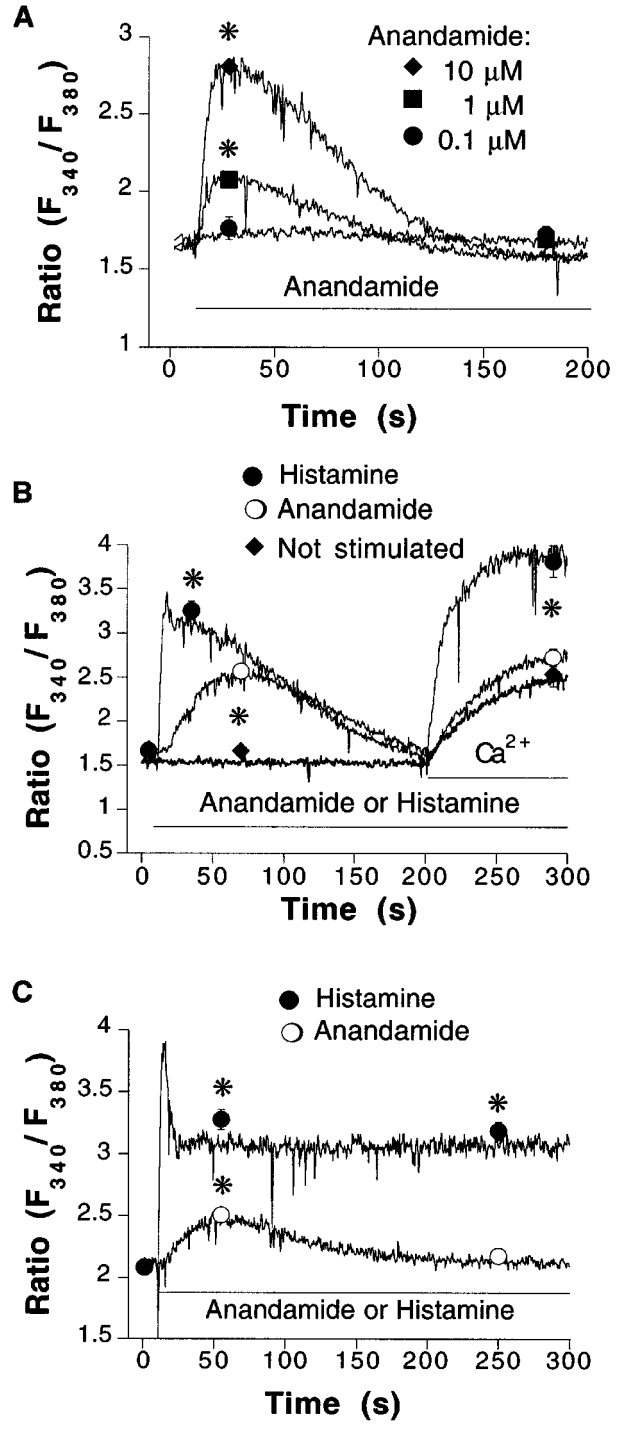

Figure 1 (A) Concentration-dependent mobilization of cytosolic Ca^{2+} induced by anandamide in Ca^{2+}-free physiological salt solution. Representative recordings that were obtained in fura 2-loaded EA.hy926 endothelial cell suspensions challenged with 0.1 μM (circles, $n=5$), 1 μM (squares, $n=6$) and 10 μM (diamonds, $n=7$) anandamide, respectively. 0.1 μM anandamide did not produce a significant release of Ca^{2+} from intracellular stores. (B) Representative recordings from EA.hy926 endothelial cell suspensions that were either not stimulated (bottom trace, diamonds, $n=10$) or stimulated with 100 μM histamine (top trace, squares, $n=5$) or 5 μM anandamide (middle trace, circles, $n=11$) in nominally Ca^{2+}-free physiological. In all panels, horizontal bars indicate application of stimulants or readmission of Ca^{2+}. (C) Representative recordings obtained in cells that were incubated with physiological salt solution containing 2.5 mM Ca^{2+}, followed by a stimulation with either 5 μM anandamide (open circles, $n=4$) or 100 μM histamine (closed circles, $n=4$). Symbols represent mean$\pm$s.e.mean of fluorescence ratio values obtained at indicated times. *$P<0.05$ between changes in cytosolic Ca^{2+} in stimulated *versus* non-stimulated cells. Error bars not visible when the size of the symbol exceeds the spread of s.e.mean.

was considered to represent a statistically significant difference.

Drugs

Medium and supplements used for cell culture were purchased from Gibco BRL (Vienna, Austria), SR141716A (N-piperidino-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-3-pyrazole-carboxamide) was a generous gift from Dr Brelière and Sanofi-Recherche (Montpellier, France), anandamide (Biomol, Plymouth Meeting, PA, U.S.A.), histamine hydrochloride, phenylmethylsulphonyl fluoride (PMSF), pertussis toxin, thapsigargin (Sigma, Vienna, Austria), fura-2/AM (Molecular Probes, Leiden, Netherlands).

Results

In Ca^{2+}-free physiological salt solution, anandamide (0.1–10 μM) evoked concentration-dependent mobilization of

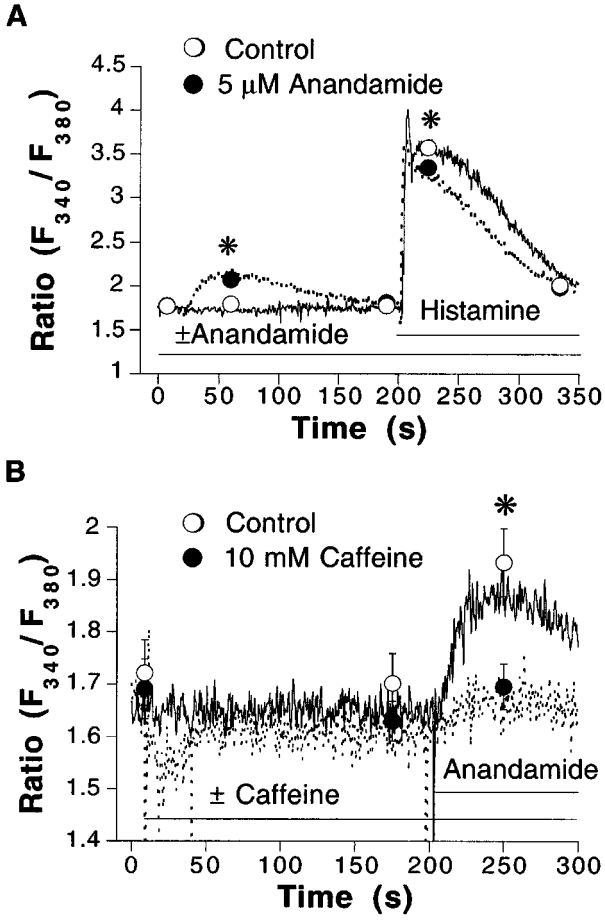

Figure 2 (A) Representative recordings from fura 2-loaded EA.hy926 endothelial cell suspensions that were stimulated with 100 μM histamine in the absence (continuous lines and open circles, $n=4$) and in the presence (dotted lines and closed circles, $n=5$) of 5 μM anandamide, applied as indicated by horizontal bars. Experiments were performed in Ca^{2+}-free physiological salt solution. (B) Recordings obtained in cells that were stimulated with 5 μM anandamide in the absence (continuous lines and open circles, $n=7$) and in the presence (dotted lines and closed circles, $n=7$) of caffeine. Experiments were performed in Ca^{2+}-free physiological salt solution. Symbols represent mean$\pm$s.e.mean of fluorescence ratio values obtained at indicated times. *$P<0.05$ between treatments. Error bars not visible when the size of the symbol exceeds the spread of s.e.mean.

cytosolic Ca^{2+} (Figure 1A). Higher concentrations of anandamide were not tested due to solubility complications, hence, the potency of anandamide (in terms of half-maximally effective concentration) could not be determined. Under similar conditions, histamine elicited an initial short-lived peak of cytosolic Ca^{2+} increase, which was followed by a second slowly developing peak (Figure 1B). Introduction of $CaCl_2$ 2.5 mM into the extracellular milieu evoked an increase in the average cytosolic Ca^{2+} concentration both in the absence and in the presence of endothelial stimulants that is due to Ca^{2+} entry. The influx of Ca^{2+} that was obtained in the absence of stimulant was comparable to that obtained in the presence of anandamide (Figure 1B). Whereas, in the presence of histamine, Ca^{2+} entry was significantly enhanced (Figure 1B). In physiological salt solution already containing $CaCl_2$ 2.5 mM, anandamide induced an elevation of cytosolic Ca^{2+} that still developed slowly and was transient (Figure 1C). By comparison the Ca^{2+} signal elicited by 100 μM histamine comprised a sharp rise, followed by a decrease to a sustained plateau phase (Figure 1C).

As shown in Figure 2A, prior treatment of the cells with anandamide significantly attenuated the mobilization of Ca^{2+} from intracellular stores evoked by histamine. The stimulation of Ca^{2+} release from intracellular stores, without activation of capacitative Ca^{2+} entry is reminiscent of the effects of caffeine in endothelial cells (Graier *et al.*, 1994a). Application of caffeine (10 mM) did not cause any significant increase of average cytosolic Ca^{2+} under the present experimental conditions (Figure 2B). However, the mobilization of cytosolic Ca^{2+} evoked by anandamide was significantly impaired after caffeine (Figure 2B).

The mobilization of Ca^{2+} contributes to the stimulation of endothelium-derived vasodilator mediators such as NO. Therefore, to assess this hypothesis the action of anandamide was determined on EA.hy926 cell NO synthase activity. Anandamide (5 μM) caused a 2 fold increase in L-[^{3}H]-arginine conversion to L-[^{3}H]-citrulline, relative to basal NO synthase activity (Figure 3). In parallel tests, 100 μM histamine provoked a 5 fold enhancement of NO synthase activity (Figure 3).

The mechanism of action of anandamide was further characterized by use of the CB receptor antagonist SR141716A. At 1 μM SR141716A had no significant effect on Ca^{2+} (data not shown, $n=2$). However, the mobilization of cytosolic Ca^{2+} evoked by anandamide was slightly, but significantly, reduced (Figure 4A). Increasing the concentration of SR141716A to 5 μM, further reduced the mobilization of Ca^{2+} by anandamide, and depressed the Ca^{2+} entry obtained after addition of 2.5 mM $CaCl_2$ (Figure 4B). However, at this concentration, SR141716A also elicited the mobilization of Ca^{2+} from intracellular stores (0.56 ± 0.02 Δratio units above basal Ca^{2+} levels, $n=5$). Five μM SR141716A did not significantly affect the Ca^{2+} influx obtained after addition of 2.5 mM $CaCl_2$ (0.87 ± 0.14 ratio units above basal Ca^{2+}levels in the absence, $n=10$, as compared to 0.56 ± 0.16, $n=5$ in the presence of SR141716A). At this concentration, SR141716A significantly depressed the

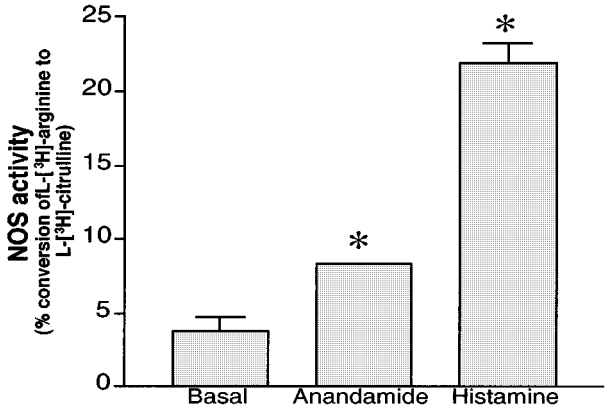

Figure 3 Endothelial NOS activity was determined in confluent EA.hy926 endothelial cells cultured in 6 well plastic dishes. The cells were challenged or not (basal) with 5 μM anandamide or 100 μM histamine, in the absence and in the presence of 300 μM L-N^G-nitro-arginine, as detailed in methods. Activity of endothelial nitric oxide synthase was expressed as the mean $\pm$ s.e.mean of the percentage of the L-N^G-nitro-arginine-sensitive conversion of L-[^{3}H-]-arginine to L-[^{3}H]-citrulline. Error bar for anandamide was too small to be apparent. *$P<0.05$ between control and anandamide- or histamine-treated groups ($n=6$ each).

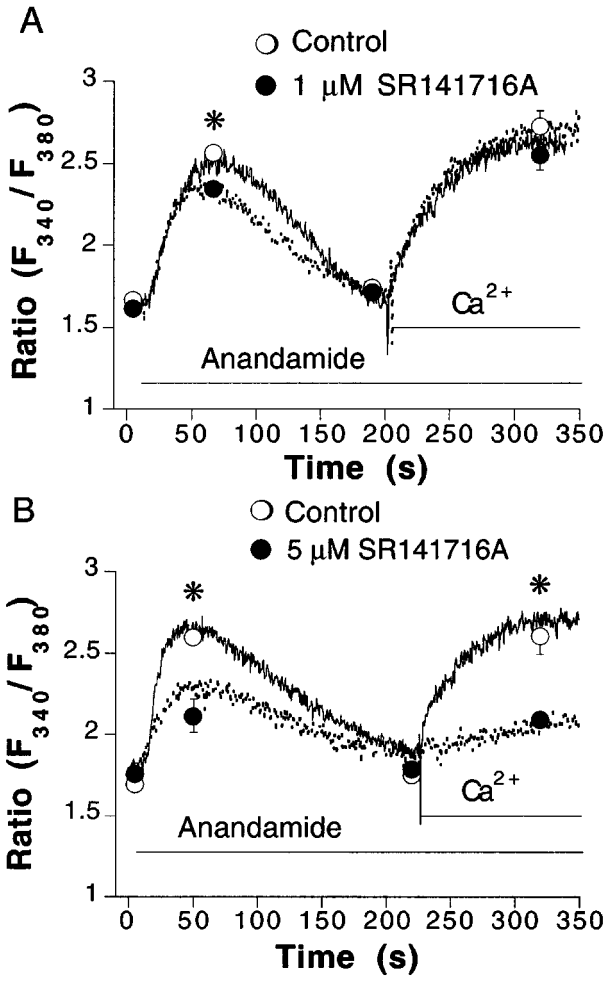

Figure 4 Inhibition by SR141716A of anandamide-induced mobilization of cytosolic Ca^{2+}. Recordings from fura 2-loaded endothelial cell suspensions that were stimulated with anandamide in the absence (continuous lines and open circles) and in the presence (dotted lines and closed circles) of either 1 μM (A) or 5 μM SR141716A (B). The cells were first equilibrated for 5 min with or without SR141716A in Ca^{2+}-free physiological salt solution. Circles represent mean $\pm$ s.e.mean of fluorescence ratio values obtained at indicated times. Error bars not visible when the size of the symbol exceeds the span of the s.e.mean. *$P<0.05$ between control and SR141716A-treated groups ($n=4-6$).

mobilization of Ca^{2+} evoked by histamine from both intra- and extracellular pools (Figure 5A). While SR141716A had no effect on the depletion of Ca^{2+} from intracellular stores that was elicited by thapsigargin, it significantly diminished subsequent influx of Ca^{2+} obtained after addition of 2.5 mM $CaCl_2$ (Figure 5B). Five μM SR141716A by itself also stimulated NO synthase activity, as determined using the L-[^{3}H]-citrulline conversion assay (data not shown).

In addition to inhibition by CB_1 receptor antagonist SR141716A, the actions of anandamide can be affected by amidohydrolase activity and by the uncoupling of the receptors to GTP binding proteins after treatment with pertussis toxin. In the presence of the amidohydrolase inhibitor PMSF (200 μM), the mobilization of cytosolic Ca^{2+} evoked by 5 μM anandamide was not affected (Table 1). Likewise incubation of the cells with pertussis toxin (400 ng ml^{-1} for 3 h) prior to testing, did not significantly change the Ca^{2+} signals induced by anandamide (Table 1). Neither PMSF nor pertussis toxin significantly modified spontaneous Ca^{2+} influx (data not shown).

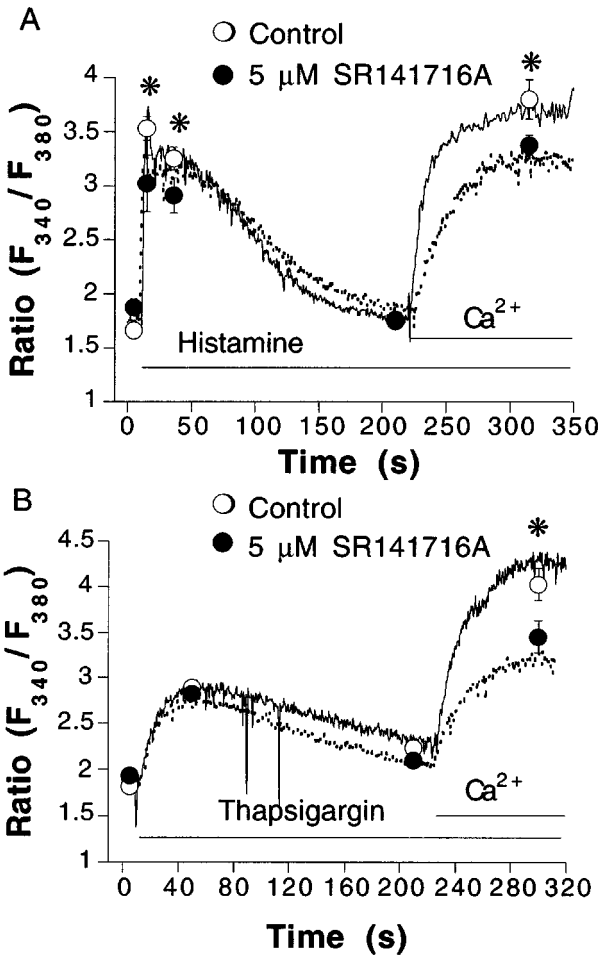

Figure 5 Inhibition by SR141716A of histamine- and thapsigargin-induced mobilization of cytosolic Ca^{2+}. Recordings from fura 2-loaded endothelial cell suspensions that were stimulated with 100 μM histamine (A) or 2 μM thapsigargin (B) in the absence (continuous lines and open circles) and in the presence (dotted lines and closed circles) of 5 μM SR141716A. The cells were first equilibrated for 5 min with or without SR141716A in Ca^{2+}-free physiological salt solution. Circles represent mean$\pm$s.e.mean of fluorescence ratio values obtained at indicated times. Error bars not visible when the size of the symbol exceeds the spread of s.e.mean. *$P<0.05$ between control and SR141716A-treated groups ($n=5-6$).

Table 1 Effects of PMSF and pertussis toxin

Treatment	*Basal Ca^{2+}*	*Ca^{2+} Release*	n
Control to PMSF	1.47$\pm$0.08	0.35$\pm$0.18	4
PMSF	1.48$\pm$0.04	0.34$\pm$0.16	4
Contol to PTX	1.41$\pm$0.06	0.26$\pm$0.09	3
PTX	1.42$\pm$0.07	0.22$\pm$0.07	3

EA.hy926 endothelial cell suspensions were stimulated with 5 μM anadamide in Ca^{2+}-free physiological salt solution. Basal Ca^{2+} levels are expressed in arbitrary ratio units of fluorescence measured at wave lengths 340/384 nm. Ca^{2+} release represents increases in cytosolic Ca^{2+} above basal level, as determined by changes in fluorescence signal (expressed in Δratio units). *n* represents the number of experiments.

Discussion

The present study demonstrates that anandamide mobilizes cytosolic Ca^{2+} from intracellular stores in the human umbilical vein-derived endothelial cells EA.hy926. However, it does not apparently stimulate capacitative Ca^{2+} entry. The mobilization of cytosolic Ca^{2+} evoked by anandamide, which was caffeine-sensitive, somewhat reduced that elicited by histamine. This suggests that functional pools of Ca^{2+} recruited by both agonists may partially overlap. The putative CB_1 receptor antagonist SR141716A inhibited this action. However, SR141716A rather behaved as agonist by stimulating the release of Ca^{2+} from intracellular stores by itself. The actions of anandamide were not affected by either the $G_{i/o}$ protein uncoupler pertussis toxin or the amidohydrolase inhibitor PMSF.

Anandamide mobilized Ca^{2+} from intracellular stores, without stimulating capacitative Ca^{2+} entry. Synthetic cannabinoids mobilize Ca^{2+} from intracellular stores in human fibroblasts and rat mesenteric arteries without activation of Ca^{2+} influx (Felder *et al*., 1992; 1995; White & Hiley, 1998a). Whereas, in DDT_1-MF-2 smooth muscle cells, cannabinoids stimulate capacitative Ca^{2+} entry (Filipeanu *et al*., 1997). The latter mechanism is responsible for the Ca^{2+} influx that is induced following the depletion of intracellular stores (for review see Adams *et al*., 1989; Graier *et al*., 1994b). It accounts for the sustained elevation of cytosolic Ca^{2+} evoked by agonists (such as histamine), or by inhibitors of endoplasmic reticulum Ca^{2+} pumps (e.g. thapsigargin), which deplete intracellular Ca^{2+} stores independently of receptor-dependent transduction mechanisms (Graier *et al*., 1995).

Anandamide slightly, but significantly reduced the mobilization of Ca^{2+} from intracellular stores evoked by histamine. Hence, the functional pools of Ca^{2+} used by anandamide and histamine overlap in part. Intracellular Ca^{2+} stores in endothelial cells can be functionally divided into inositol, 1,4,5-triphosphate (IP_3) and caffeine-sensitive pools (Graier *et al*., 1994a; Sasajima *et al*., 1997). Caffeine blocked the anandamide-induced mobilization of Ca^{2+} from intracellular pools. Caffeine has been shown to deplete certain endothelial Ca^{2+} stores without accompanied elevation of cytosolic Ca^{2+} (Graier *et al*., 1994a). Thus, we suggest that the lack of anandamide to elevate endothelial Ca^{2+} in caffeine-treated cells is due to a 'quiet' depletion of anadamide-sensitive Ca^{2+} stores by caffeine. In rat arterial smooth muscle, caffeine and anandamide also seem to neutralize each other (Zygmunt *et al*., 1997; White & Hiley, 1998a). Taken together with the observations presented herein, we conclude that caffeine and

anandamide compete for the same functional Ca^{2+} pool in these vascular tissues.

The results obtained with the NO synthase assay confirm that anandamide may trigger the release of endothelium-derived vasodilator mediators, since it induces Ca^{2+} signalling. It elicited a 2 fold increase in NO synthase activity, while histamine induced a 5 fold stimulation. These results are commensurate with the ability of the compounds to mobilize Ca^{2+} in EA.hy926 endothelial cells. Deutsch *et al.* (1994) reported that anandamide stimulates the production of NO in perfused renal arteries in the nanomolar range, an effect abolished by selective concentrations of SR141716A. The differences in drug potency may be due to differences in the mechanism of action of anandamide which acts through CB_1 receptors in the renal endothelial cells.

The putative CB receptor antagonist SR141716A evoked the release of Ca^{2+} from intracellular stores by itself. At 1 μM, which is a maximally effective concentration at blocking the actions of agonists at CB_1 receptors (Felder & Glass, 1998; see e.g. Filipeanu *et al.*, 1997). SR141716A marginally inhibited the mobilization of Ca^{2+} elicited by anandamide, without evoking any Ca^{2+} signal by itself. At a higher concentration, SR141716A impaired significantly the mobilization of Ca^{2+} from intracellular stored induced by anandamide and histamine, respectively. Even more intriguing, capacitative Ca^{2+} entry stimulated either by histamine or thapsigargin was impaired by 5 μM SR141716A. By contrast, spontaneous Ca^{2+} influx obtained in the absence of endothelial stimulants was not altered. These findings can not be interpreted on the assumption that SR141716A is acting as a neutral competitive antagonist at CB_1 receptors.

Actually, SR141716A is an inverse agonist at both CB_1 and CB_2 receptors (Bouaboula *et al.*, 1997; MacLennan *et al.*, 1998). This implies that SR141716A induces a conformation of CB receptors that disrupts coupling to certain G proteins; however, it could eventually promote formation of complexes with others (for review on receptor promiscuity see Kenakin, 1996). Hence, the mobilization of Ca^{2+} by SR141716A could possibly reflect agonistic actions at CB-like receptors. Also, SR141716A was able to enhance NO synthase activity like anandamide. In CB receptor-transfected chinese hamster ovarian cells, submicromolar concentrations SR141716A impair cannabinoid-induced inhibition of the accumulation of cyclic AMP stimulated by forskolin (Felder *et al.*, 1995). On the contrary, at micromolar concentrations, SR141716A enhances cyclic AMP elevation (Felder *et al.*, 1995). In this model, CB_1 receptors inhibit adenylate cyclase *via* pertussis toxin-sensitive G proteins, and stimulate the same enzyme through pertussis toxin-resistant G proteins (Felder & Glass, 1998. Accordingly, in the present study pertussis toxin did not influence the mobilization of Ca^{2+} induced by anandamide. Therefore, this finding is consistent with the interpretation that SR141716A preferentially disrupts coupling to pertussis toxin-sensitive transducer mechanisms, and may promote some alternative pathways.

In the context of agonistic properties of SR141716A, the depression of anandamide-induced mobilization of Ca^{2+} could result from the depletion by SR141716A of a common functional pool of Ca^{2+} prior to the addition of anandamide. An agonistic effect of SR141716A can also explain the non-specific actions on the capacitative Ca^{2+} entry. Pilot experiments confirm that anandamide, like SR141716A, attenuates Ca^{2+} entry evoked by histamine (unpublished observations). Indeed, the cytosolic fura-2 signal generated after induction of capacitative Ca^{2+} entry is due in part to the mobilization of Ca^{2+} through the Ca^{2+}-induced Ca^{2+} release mechanism (Mozhayeva, 1996). Hence, depletion of the caffeine-sensitive stores would eliminate this component of the bulk cytosolic Ca^{2+} increase. Alternatively, these compounds may directly or indirectly have non-specific actions on Ca^{2+} channels that mediate the capacitative Ca^{2+} entry. Venance *et al.* (1997) have shown in rat astrocytes that (R)-methanandamide depletes intracellular stores and prevents the subsequent release of Ca^{2+} from intracellular stores that is evoked agonists.

SR141716A depressed Ca^{2+} entry in the presence of anandamide. This effect was unexpected since anandamide alone did not stimulate capacitative Ca^{2+} entry. These results can be explained by assuming anandamide facilitates Ca^{2+} extrusion or inhibits Ca^{2+} influx (e.g. Venance *et al.* (1997)). As an agonist, SR141716A would reinforce Ca^{2+} extrusion or inhibit Ca^{2+} influx. Also, the severe depletion of peripheral anandamide-sensitive stores may induce a steal effect. In such a case, the Ca^{2+} entering the bulk cytoplasm would be shunted in order to first refill the depleted anandamide-sensitive stores. Further investigation are warranted to test these possibilities.

Alternatively to the activation of CB-receptors, anandamide may be acting on some intracellular targets. Thus, the mobilization of Ca^{2+} in human fibroblasts (Felder *et al.*, 1995), the impairment by anandamide of astrocyte junctional coupling (Venance *et al.*, 1997) and the stimulation of mitogen-activated protein kinase in haematopoietic cells (Derocq *et al.*, 1998) occur independently of $CB_{1/2}$ receptors (for review see Hillard & Campbell, 1997). Anandamide may be converted to arachidonic acid, which would in turn be processed into cyclo-oxygenase, lipoxygenase or cytochrome P450 mono-oxygenase metabolites, as shown in bovine coronary endothelial cells (e.g. Pratt *et al.*, 1998; see Felder & Glass, 1998 for review). However, the amidohydrolase inhibitor PMSF (Goparaju *et al.*, 1998) had no effect suggesting that, unless some alternative pathway resistant to PMSF exists in EA.hy926 cells, anandamide is not acting as a precursor. By protecting anandamide from inactivation, PMSF potentiates activation of CB receptors by in numerous tissues. Since, this was not the case in the present study, it is possible that EA.hy926 cells lack amidohydrolase activity, as has been shown in other tissues (Felder *et al.*, 1995).

The proposal that endogenous cannabinoids participate in endothelium-dependent vasodilatation is largely based on the use of SR141716A (see Randall & Kendall, 1998 for review). Inhibition by SR141716A of the mobilization of Ca^{2+} in endothelial cells may account in part for the impairment of endothelium-dependent relaxations (e.g. Randall *et al.*, 1996; White & Hiley, 1997; Randall & Kendall, 1997). In addition, SR141716A may possess other non-specific effects. For example, it inhibits endothelium-independent relaxations evoked by the K_{ATP} channel activator levcromakalim (White & Hiley, 1998b). Taken together, the inverse agonism and its actions not related to CB receptors make SR141716A an inappropriate tool to assess the role of endogenous cannabinoids in the regulation of vascular tone.

At the present stage, it is not clear whether or not receptors are involved in the mobilization of cytosolic Ca^{2+} in endothelial cells, although the involvement of the CB_1 receptor/pertussis toxin-sensitive G protein transduction pathway may be ruled out. It is possible that anandamide acts independently of a receptor to induce mobilization of Ca^{2+} from caffeine-sensitive pools. Nevertheless, the present study raises the intriguing possibility that anandamide may participate in the regulation of the release of Ca^{2+} from caffeine-sensitive pools in cells that synthesize the endogenous cannabinoid upon activation.

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