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Anandamide initiates Ca^{2+} signaling *via* CB_2 receptor linked to phospholipase C in calf pulmonary endothelial cells

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1 The endocannabinoid anandamide has been reported to affect neuronal cells, immune cells and smooth muscle cells *via* either CB1 or CB2 receptors. In endothelial cells, the receptors involved in activating signal transduction are still unclear, despite the fact that anandamide is produced in this cell type.

2 The present study was designed to explore in detail the effect of this endocannabinoid on Ca^{2+} signaling in single cells of a calf pulmonary endothelial cell line.

3 Anandamide initiated a transient Ca^{2+} elevation that was prevented by the CB2 receptor antagonist SR144528, but not by the CB1 antagonist SR141716A. These data were confirmed by molecular identification of the bovine CB2 receptor in these endothelial cells by partial sequencing.

4 The phospholipase C inhibitor 1-[6-[[(17β)-3-methoxyestra-1,3,5(10)-trien-17-yl]amino]hexyl]-1*H*-pyrrole-2,5dione and the inositol 1,4,5-trisphosphate receptor antagonist 2-aminoethoxydiphenylborate prevented Ca^{2+} signaling in response to anandamide.

5 Using an improved cameleon probe targeted to the endoplasmic reticulum (ER), fura-2 and ratiometric-pericam, which is targeted to the mitochondria, anandamide was found to induce Ca^{2+} depletion of the ER accompanied by the activation of capacitative Ca^{2+} entry (CCE) and a transient elevation of mitochondrial Ca^{2+}.

6 These data demonstrate that anandamide stimulates the endothelial cells used in this study *via* CB2 receptor-mediated activation of phospholipase C, formation of inositol 1,4,5-trisphosphate, Ca^{2+} release from the ER and subsequent activation of CCE. Moreover, the cytosolic Ca^{2+} elevation was accompanied by a transient Ca^{2+} increase in the mitochondria. Thus, in addition to its actions on smooth muscle cells, anandamide also acts as a powerful stimulus for endothelial cells.
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Keywords: Cameleon; CB2 receptor; Ca^{2+} mobilization; endoplasmic reticulum Ca^{2+}; mitochondrial Ca^{2+}; phospholipase C; ratiometric-pericam-mt: SR141716A; SR144528

Abbreviations: $[Ca^{2+}]_{cyto}$, cytosolic Ca^{2+} concentration; $[Ca^{2+}]_{er}$, endoplasmic reticulum lumenal Ca^{2+} concentration; $[Ca^{2+}]_{mito}$, mitochondrial Ca^{2+} concentration; ER, endoplasmic reticulum; 2-APB, 2-aminoethoxydiphenylborate; U73122, (1-[6-[[(17β)-3-methoxyestra-1,3,5(10)-trien-17-yl]amino]hexyl]-1*H*-pyrrole-2,5dione

Introduction

Arachidonylethanolamide (anandamide) was first isolated from porcine brain in 1992 and represents a putative endogenous ligand of the so-called cannabinoid receptors (Devane *et al*., 1992). This receptor family was pharmacologically characterized with Δ^9-tetrahydrocannabinol and its derivates, which led to the differentiation of two receptor isoforms, CB1 and CB2. The isoforms were finally confirmed by the cloning and sequencing of the receptors in humans and mice (Matsuda *et al*., 1990; Munro *et al*., 1993). These two cannabinoid receptors display a diverse tissue expression pattern. Notably, the CB1 receptor has been found to be mainly distributed in the nervous system and has been implicated in the decrease in blood pressure that follows hemorrhagic and endotoxic shock. In this respect, macrophages and platelets are thought to generate two different types of endocannabinoids, 2-arachidonyl glycerol and anandamide, which may contribute to septic shock-associated hypotension, possibly *via* the CB1 receptor (Wagner *et al*., 1997; Varga *et al*., 1998). CB1 receptors are mainly found in the central nervous system, where they are proposed to be involved in neurobehavioral actions (Di Marzo, 1998), whereas CB2 receptors are primarily expressed in the immune system.

Remarkably, endothelial cells have been identified as a source of anandamide (Deutsch *et al*., 1997; Sugiura *et al*., 1998), which is released from the A2 position of *N*-arachidonoyl-phosphatidylethanolamide by phospholipase D upon elevation of intracellular-free Ca^{2+} or condensation of phospholipase A2-released arachidonic acid with ethanolamide (Schmid, 2000). This endothelial anandamide has been suggested to act as an endothelium-derived hyperpolarizing factor in resistance vessels (Randall *et al*., 1996; Randall & Kendall, 1997; White & Hiley, 1997; Harris *et al*., 2000; 2002). It has been shown to initiate relaxation in guinea-pig basilar arteries (Zygmunt *et al*., 1997) and bovine coronary artery

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(Pratt *et al*., 1998), but in certain conduit blood vessels, such as rat carotid arteries (Holland *et al*., 1999), no relaxation in response to anandamide was observed. Various mechanisms for the action of anandamide in promoting smooth muscle relaxation have been described, including CB1 receptor-independent hyperpolarization (Plane *et al*., 1997), inhibition of smooth muscle Ca^{2+} mobilization (Zygmunt *et al*., 1997; Chantaigneau *et al*., 1998) and direct attenuation of vascular smooth muscle Ca^{2+} channels *via* G-protein-coupled CB1 receptors (Gebremedhin *et al*., 1999). Anandamide has also been found to initiate blood vessel dilatation *via* the activation of endothelial cells (Deutsch *et al*., 1997; Chaytor *et al*., 1999). In human umbilical vein endothelial cells, anandamide and the CB1 receptor agonist HU210 have been found to upregulate iNOS (MacCarrone *et al*., 2000). In addition, in the human umbilical vein endothelial cell line EA.hy926, anandamide initiated the elevation of the intracellular Ca^{2+} concentration ($[Ca^{2+}]_{cyto}$), which leads to the formation of nitric oxide, but the mechanisms by which anandamide initiates endothelial $[Ca^{2+}]_{sub}$ signaling are still unknown (Mombouli *et al*., 1999) Nevertheless, although anandamide is obviously also able to activate vascular endothelial cells, the actual CB receptor isoform(s) in this type of cell has not been molecularly characterized and differentiation is entirely based on the use of CB1 or CB2 agonists/antagonists that are chemically very distinct from anandamide (Howlett *et al*., 2002).

Thus, the present study was designed to explore in detail the effect of anandamide on a calf pulmonary endothelial cell line (Dedkova & Blatter, 2002). The identity of the CB receptor in these cells was characterized using CB1 and CB2 receptor-selective compounds and by molecular sequencing. In addition, the mechanism of anandamide-initiated Ca^{2+} signaling was elucidated. Finally, in view of the recent findings on the particular importance of the endoplasmic reticulum (ER; Graier *et al*., 1998; Frieden *et al*., 2002; Malli *et al*., 2003) and the mitochondria (Sedova & Blatter, 2000; Pozzan *et al*., 2000a; Malli *et al*., 2003) to many cell functions and phenomena (Pozzan & Rizzato, 2000b), the effect of this endocannabinoid on the ER and mitochondrial Ca^{2+} ($[Ca^{2+}]_{mito}$) concentrations was tested by transient expression of the organelle-targeted Ca^{2+} sensor protein vYC4-ER in the ER (Miyawaki *et al*., 1997; Miyakawa *et al*., 1999; Frieden *et al*., 2002; Malli *et al*., 2003) and ratiometric-pericam-mt in the mitochondria (Nagai *et al*., 2001; Thyagarajan *et al*., 2002).

Methods

Chemicals, supplies and materials

Fura-2/AM was obtained from Molecular Probes Europe (Leiden, Netherlands). [^{3}H]anandamide was obtained from APB (Vienna, Austria). Anandamide (arachidonylethanolamide), *R*-(+)-methandamide, 1-[6-[[(17β)-3-methoxyestra-1,3,5(10)-trien-17-yl]amino]hexyl]-1*H*-pyrrole-2,5dione (U73122), 2-aminoethoxydiphenylborate (2-APB) and HU210 ((6a*R*)-*trans*-3-(1,1-dimethylheptyl)-6a,7,10,10a-tetrahydro-1hydroxy-6,6-dimethyl-6H-dibenzo[*b*,*d*]pyran-9-methanol) were purchased from Tocris Cookson Ltd (Northpoint, Avonmouth, Bristol, U.K.). SR144528 and SR141716 were kind gifts from Sanofi-Recherche (Montpellier, France). Bradykinin, arachidonic acid and adenosine 5′-triphosphate were obtained from Sigma Chemicals (St Louis, MO, U.S.A.). The RNeasy Mini kit and the EndoFree Plasmid Maxi kit were purchased from Qiagen (VWR, Vienna, Austria). RQ1 RNase-free Dnase I and TransFast™ were obtained from Promega (Mannheim, Germany). RNAguard and random hexamer primers were from APB. First-strand buffer, Moloney murine leukemia virus reverse transcriptase, cell culture media and media substitutes were obtained from Life Technologies Inc. (Gibco, Invitrogen, Vienna, Austria). Fetal calf serum was obtained from PAA (Linz, Austria). DyNAzyme II DNA polymerase was obtained from Finnzymes Oy (Vienna, Austria). Primers were synthesized at MWG Biotech (Ebersberg, Germany). Restriction enzymes and T4 DNA ligase were purchased from New England BioLabs (Frankfurt, Germany) or Promega. The expression vectors pcDNA 3 and pBudCE4.1 were obtained from Invitrogen (Vienna, Austria). The dNTPs and all other chemicals were obtained from Roth (Karlsruhe, Germany).

Cell culture

Calf pulmonary artery endothelial (CPAE) cells from the American Type Culture Collection (ATCC CCL-209; Manassas, VA, U.S.A.) of passage 54 and higher were grown on glass coverslips in Dulbecco's minimum essential medium containing 20% fetal calf serum and 1% nonessential amino acids (catalog number 1140–050, Gibco, Invitrogen, Vienna, Austria).

Plasmids and transfection

In this work, an improved version of the original ER-targeted cameleon probe YC4-ER (Miyawaki *et al*., 1997; Miyakawa *et al*., 1999) for Ca^{2+} measurement by fluorescence resonance energy transfer (FRET) was used. We exchanged the original yellow fluorescent protein (YFP) in the YC4-ER construct for the more pH and bleaching-resistant mutant venus (Nagai *et al*., 2002). Using PCR, an *Sac*I restriction site and the KDEL ER remaining/re-entry sequence followed by an *Eco*RI restriction site were added to the original venus at the 5′ and 3′ ends, respectively. With the respective restriction enzymes, this sequence was introduced into the YC4-ER construct in place of YFP and cloned into the expression vector pBudCE4.1. This improved Ca^{2+} sensing protein (vYC4-ER) was nicely targeted to the ER and offered increased brightness and enhanced FRET dynamics. The ratiometric-pericam-mt in pcDNA 3 was a gift from Dr Atsushi Miyawaki (Riken, Japan; Nagai *et al*., 2001). Following the guidelines for using this reagent, endothelial cells of approximately 80% confluency were transiently transfected with 1.5–3 μg of purified plasmid DNA using TransFast™.

Ca^{2+} measurements

Between 24 and 36 h after transfection, cells were used for experiments. Prior to the experiment, the cells were washed twice with storage buffer (SB; in mM: 2 $CaCl_2$, 135 NaCl, 1 $MgCl_2$, 5 KCl, 10 HEPES, 2.6 $NaHCO_3$, 0.44 KH_2PO_4 and 10 D-glucose (pH adjusted to 7.4)). All the experiments were performed at room temperature (20–25°C).

Cytosolic Ca^{2+} ($[Ca^{2+}]_{cyto}$) concentration $[Ca^{2+}]_{cyto}$ was monitored in single endothelial cells using the fluorescent ratiometric Ca^{2+} indicator fura-2 as described previously (Graier *et al*., 1998; Paltauf-Doburzynska *et al*., 1998; 2000). Briefly, cells were loaded for 45 min at room temperature in the dark in SB containing 3 μM fura-2/AM, washed twice and equilibrated for a further 30 min in SB. The coverslip was mounted in an experimental chamber and perfused ($\sim$1.5 ml min^{-1}) with HEPES-buffered solution (HBS) containing (in mM): 145 NaCl, 5 KCl, 1 $MgCl_2$ and 10 HEPES acid (pH adjusted at 7.4) with either 2 mM $CaCl_2$ or 10 μM EGTA.

ER lumenal Ca^{2+} ($[Ca^{2+}]_{er}$) concentration For measuring $[Ca^{2+}]_{er}$, the Ca^{2+}-dependent FRET in the improved venus-modified cameleon construct vYC4-ER was monitored as described previously (Frieden *et al*., 2002; Malli *et al*., 2003). The coverslip was washed twice, transferred to the experimental chamber and perfused with HBS.

Mitochondrial Ca^{2+} concentration ($[Ca^{2+}]_{mito}$) To monitor $[Ca^{2+}]_{mito}$, ratiometric-pericam-mt (Nagai *et al*., 2001) was used as described previously (Thyagarajan *et al*., 2002).

Data acquisition and analysis Experiments were performed using a deconvolution microscope as recently described (Graier *et al*., 1998; Paltauf-Doburzynska *et al*., 1998; 2000). Briefly, the microscope consisted of a Nikon inverted microscope (Eclipse 300TE, Nikon, Vienna, Italy) equipped with CFI Plan Fluor $\times$40 oil-immersion objective (NA 1.3, Nikon, Vienna, Austria), an epifluorescence system (150 W XBO; Optiquip, Highland Mills, NY, U.S.A.), a computer-controlled z-stage (Ludl Electronic Products, Hawthrone, NY, U.S.A.) and a liquid-cooled CCD camera (−30°C; Quantix KAF 1400G2, Roper Scientific, Acton, MA, U.S.A.). Excitation wavelengths were selected using a computer-controlled filter wheel (Ludl Electronic Products). All devices were controlled either by Metafluor 4.0 (Visitron Systems, Puchheim, Germany) for Ca^{2+} measurements or ImagePro 3.0 (Media Cybernetics, Silver Spring, MA, U.S.A.) for deconvolution imaging.

To measure $[Ca^{2+}]_{cyto}$, $[Ca^{2+}]_{er}$ or $[Ca^{2+}]_{mito}$, cells were illuminated alternately at 340 and 380 nm (340HT15 and 380HT15; Omega Optical, Brattleboro, VT, U.S.A.), 440 nm (440AF21; Omega Optical) or 433 and 485 nm (433DF15 and 485DF15; Omega Optical) for fura-2, vYC4-ER and ratriometric-pericam-mt, respectively. Emission was monitored at 510 nm (510WB40; Omega Optical) for fura-2, 480 and 535 nm for vYC4-ER (480AF30 and 535AF26; Omega Optical) and at 535 nm for ratiometric-pericam-mt (535AF26; Omega Optical).

RT–PCR

Total RNA from mouse and bovine spleen, human acute monocytic leukemia cells (THP-1), the human B lymphoblastoid cell line (DAUDI; Galiegue *et al*., 1995) and CPAE was isolated using the RNeasy Mini Kit according to the manufacturer's protocol. Samples (3 μg) were treated with 1 U of RQ1 RNase-free Dnase I for 15 min at 37°C and used as a template for the first-strand cDNA synthesis. Reverse transcription was performed for 1 h at 37°C in a reaction mixture containing 0.5 mM dNTPs, 15 U RNAguard, 3.3 μM random hexameric primers, 10 mM dithiothreitol and 200 U Moloney murine leukemia virus-reverse transcriptase in the first-strand buffer. For the amplification of the CB2 receptor, 2.5 μl cDNA was used as a template in a 50 μl reaction mixture containing 0.2 mM dNTPs, 10 μM specific primers, 1 $\times$ PCR buffer and 1 U of DyNAzyme II DNA polymerase in appropriate buffer. Following an initial denaturation step at 94°C for 10 min, PCR was carried out for 40 cycles with denaturation for 30 s at 94°C, annealing for 30 s at 57°C and extension for 1 min at 72°C followed by a final extension of 10 min at 72°C. If necessary, 1–2.5 μl of the PCR products were taken for reamplification under the same conditions. The oligonucleotide primers based on the human or bovine CB2 sequence were forward primer, 5′-GGCAGCGTGACTATGACCTT-3′; and reverse primer, 5′-GTCTTGGCCAACCTCACATC-3′ or forward primer, 5′-GCGCGTAGGTGTAGATGATG-3′; and reverse primer, 5′-GCAGCGTGACTATGACCTTC-3′, respectively. The primers for the amplification of the CB1 receptor based on the human sequence were forward primer, 5′-GCATCATCATCCACACGTCT-3′; and reverse primer, 5′-CTGTGAACACTGGCTGCATT-3′. The same primers were used for automated sequencing of the PCR products obtained.

Anandamide uptake

For testing the uptake of anandamide in CPAE cells, the cells were grown in six-well plates until confluence. Prior to the experiments, the culture medium was removed and the cells were equilibrated for 30 min in HBS at 37°C. The experiment was started by the addition of 1, 3 or 10 μM anandamide, including [^{3}H]-anandamide (10^6 d.p.m. per well) for 10 min. Alternatively, a time course experiment was performed, with 1 μM anandamide/[^{3}H]-anandamide being added for 1, 3, 5, 7, 10, 20 and 30 min. At the end of the experiment, the incubation medium was aspirated, the cells were washed three times with ice-cold HBS and 0.5 ml 1 N NaOH was added. After 15 min, the supernatant was neutralized with 0.5 ml 1 N HCl and transferred to a 10 ml vial, where 6 ml scintillation cocktail was added. Radioactivity was measured for 10 min in a β-counter.

Statistics

If not otherwise indicated, data represent the means $\pm$ s.e.m. Analysis of variance was performed and statistical significance was evaluated using Scheffe's *post hoc* F-test. The level of significance was defined as $P<0.05$. Curve fitting was performed using GraphPad Prism 3.0 for Macintosh.

Results

In the presence of 2 mM extracellular Ca^{2+}, 10 μM anandamide evoked a rapid and transient elevation in the $[Ca^{2+}]_{cyto}$, but this was not followed by a significant or long-lasting Ca^{2+} plateau (Figure 1a). The EC_{50} for initiation of endothelial Ca^{2+} signaling by anandamide was 8.4 (7.3–9.7) μM (Figure 1b) Under the same conditions, 0.1 μM bradykinin induced a similar initial elevation in cytosolic Ca^{2+}, but the following plateau phase was more pronounced compared with that found in cells stimulated with anandamide (Figure 1a). Thus, although the initial elevation in $[Ca^{2+}]_{cyto}$ induced by

anandamide was similar to that produced by bradykinin, the subsequent Ca^{2+} plateau was considerably lower in anandamide-stimulated cells.

Anandamide initiated endothelial Ca^{2+} signaling with a slightly higher potency than its analog methanandamide, which elevated endothelial $[Ca^{2+}]_{cyto}$ with an EC_{50} of 15.6 (14.0–17.4) μM (Figure 1b). In order to test whether the effect of anandamide was specific to the arachidonic acid ethanol amines, anandamide and methanandamide, the effect of arachidonic acid on endothelial $[Ca^{2+}]_{cyto}$ was tested. As shown in Figure 1c, 10 μM arachidonic acid failed to elevate $[Ca^{2+}]_{cyto}$. Moreover, prior exposure to 10 μM arachidonic acid had no effect on the increase in Ca^{2+} signaling elicited by anandamide.

The effect of anandamide on endothelial Ca^{2+} signaling was further explored to determine the receptor involved. For this purpose, the inhibitory effects of the antagonists for the CB1 and the CB2 receptors, SR141716 (Rinaldi-Carmona *et al*., 1994) and SR144528 (Rinaldi-Carmona *et al*., 1998), on the anandamide-initiated Ca^{2+} signaling was studied. The CB1 antagonist SR141716 (1 μM), failed to alter the cytosolic Ca^{2+} response to 10 μM anandamide (Figure 2a). Moreover, the EC_{50} for the initiation of endothelial Ca^{2+} signaling by anandamide in the presence of 1 μM SR141716 was 7.3 (4.6–11.7) μM, which was not different from that of anandamide alone (Figure 2b).

In contrast, the CB2 receptor antagonist SR144528 diminished the effect of 10 μM anandamide on endothelial $[Ca^{2+}]_{cyto}$ by about 90% (Figure 2a and c; Table 1). A full concentration–response curve for anandamide in the presence of SR144528 could not be obtained due to the high concentration of ethanol in the solution with anandamide concentrations >100 μM (Table 1).

In order to confirm the above data indicating that anandamide initiates endothelial Ca^{2+} signaling *via* the CB2 receptor in CPAE cells, the expression of CB2 was verified using RT–PCR. Based on the published sequences for human and mouse CB2 receptors, a set of primers was designed to amplify CB2 receptor in human THP-1 cells (Figure 3a, lane 2), the human B lymphoblastoid cell line (DAUDI; Figure 3a, lane 3), mouse spleen (Figure 3a, lane 4) and bovine spleen (reamplified with an additional 40 cycles; Figure 3a, lane 5). Using this set of primers, we could not identify CB2 receptor in the CPAE cells, even after reamplification (Figure 3a, lane 6). To ensure that the primers used were homologous with the bovine sequence, the bovine RT–PCR construct was sequenced (Figure 4). The amplified region spanned 407 base pairs (bp) between position 457–863 of the human sequence (gi 407806) and position 446–852 of the mouse sequence (gi 791081). Blast analysis of the sequencing result of 367 bp (position 477–843 in the human sequence) revealed that the

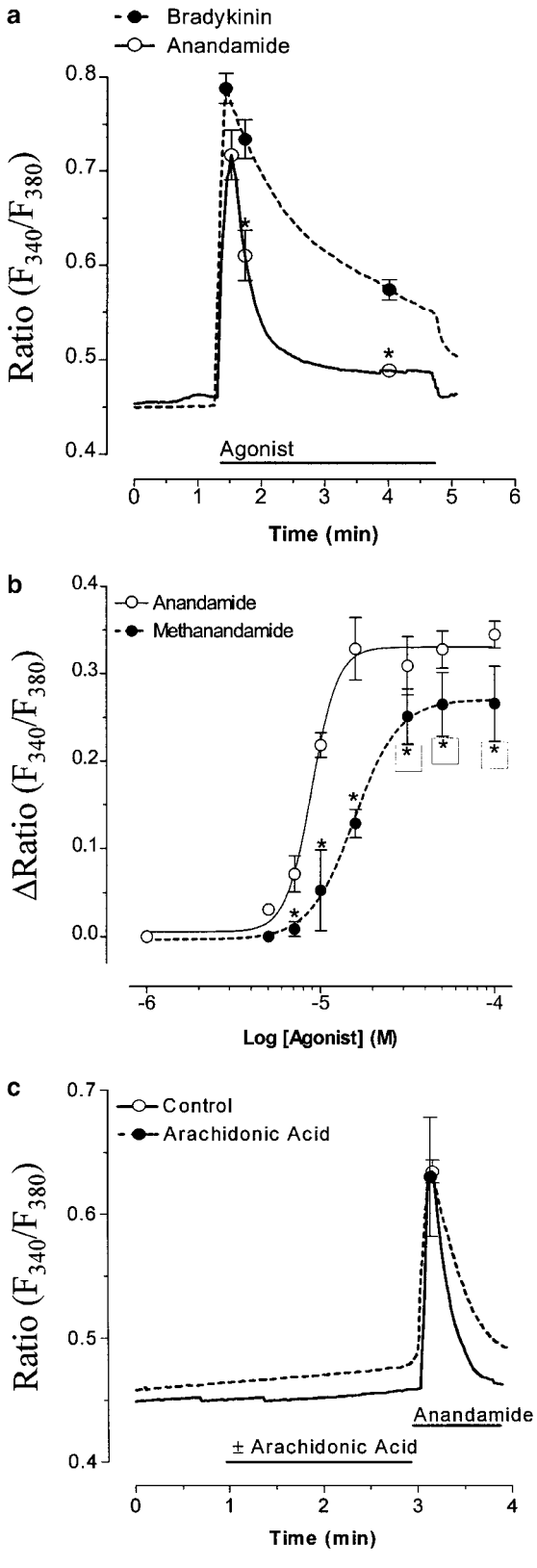

Figure 1 Anandamide evoked increases in $[Ca^{2+}]_{cyto}$ in a calf pulmonary endothelial cell line. Panel a: Comparison of the effect of anandamide on $[Ca^{2+}]_{cyto}$ in CPAE cells with that of bradykinin. As indicated, cells were stimulated with 10 μM anandamide (continuous lines and open circles, $n=23$) or 0.1 μM bradykinin (dotted lines and closed circles, $n=25$) in the presence of 2 mM extracellular Ca^{2+}. $^*P<0.001$ *vs* the effect of anandamide. Panel b: Concentration–response curves for anandamide (continuous curve and open circles each point, $n=10$) and its analogue methandamide (dotted curve and closed circles; $n=8$) in a physiological salt solution containing 2 mM $CaCl_2$. Points represent the peak $[Ca^{2+}]_{cyto}$ response at each concentration. $^*P<0.001$ *vs* effect of anandamide at the same concentration. Panel c: Effect of 10 μM anandamide in cells prestimulated with 10 μM arachidonic acid (dotted lines and closed circles, $n=26$) or solvent (0.1% ethanol, continuous lines and open circles, $n=18$) as indicated by the bars.

bovine CB2 was 86% homologous with human CB2 and 83% homologous with mouse CB2. Thus, a new pair of primers was designed based on the bovine sequence correlating with position 458–757 of the human sequence and position 447–746 of the mouse sequence. This new set of primers revealed an RT–PCR product of the correct size in bovine spleen and CPAE cells, which was confirmed to be a part of the bovine CB2 receptor by sequencing. In contrast, no mRNA for the CB1 receptor could be found. These results, and our findings that there was no uptake of anandamide by CPAE cells (data not shown), support the data from our experiments indicating that anandamide initiates Ca^{2+} signaling in CPAE cells *via* the CB2 receptor.

Since the change in the Ca^{2+} signal in response to either anandamide or bradykinin is substantial (Figure 1a), the mechanisms involved in anandamide-triggered Ca^{2+} elevation were further investigated. In order to identify the source of the Ca^{2+} involved in the cytosolic Ca^{2+} elevation, a classical Ca^{2+} re-entry protocol was performed, in which cells were stimulated in the absence of extracellular Ca^{2+} and following the readdition of extracellular Ca^{2+}. As shown in Figure 5, anandamide (10 μM) initiated a transient Ca^{2+} elevation in the absence of extracellular Ca^{2+} that mirrored the one evoked by 0.1 μM bradykinin under the same conditions. Moreover, the initial cytosolic Ca^{2+} elevation in response to the readdition of 2 mM extracellular Ca^{2+} was virtually identical in cells previously stimulated with 10 μM anandamide or 0.1 μM bradykinin (Figure 5). However, after anandamide stimulation, recovery upon the addition of extracellular Ca^{2+} was faster than after bradykinin stimulation (Figure 5).

In order to investigate whether or not anandamide elevated $[Ca^{2+}]_{cyto}$ *via* the activation of phospholipase C, the effect of the phospholipase C inhibitor U73122 was assessed. Preincubation of CPAE cells with 2 μM U73122 for 3 min in Ca^{2+}-containing HBS abolished the effect of 10 μM anandamide on endothelial Ca^{2+} (Figure 6a). The effect of bradykinin was also prevented by U73122 (Figure 6b). In line with these findings, indicating that anandamide and bradykinin both initiate endothelial Ca^{2+} signaling *via* the activation of phospholipase C, 2-APB (50 μM), an inhibitor of the inositol-1,4,5 trisphosphate receptor, prevented endothelial Ca^{2+} elevation in response to both agonists (Figure 7).

These data indicate that the anandamide-induced Ca^{2+} elevation is due to the activation of phospholipase C and, consequently, to the formation of inositol-1,4,5 trisphosphate, which triggers intracellular Ca^{2+} release from the ER. To confirm this suggestion, CPAE cells were transiently transfected with vYC4-ER, an improved cameleon construct targeting the ER. A drop in the Ca^{2+} content of the ER measured by vYC4-ER (Figure 8a) indicated that anandamide (20 μM) initiated a strong depletion of the ER. While the actual decrease in ER Ca^{2+} content upon stimulation with 20 μM anandamide did not differ from that achieved with 0.1 μM

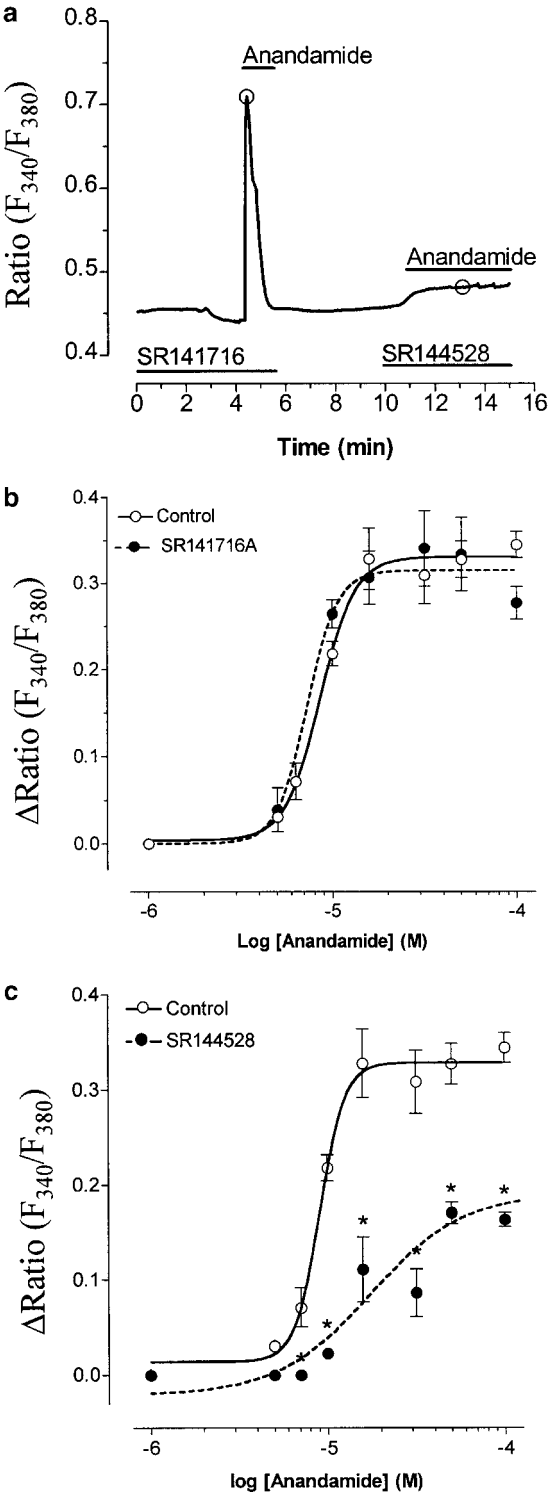

Figure 2 CB2 receptor antagonist SR144528 diminished the effect of anandamide on endothelial $[Ca^{2+}]_{cyto}$, but the CB1 receptor antagonist SR141716A did not. Panel a: Concentration–response curves of anandamide in the absence (continuous curve and open circles, $n=10$) and the presence of 1 μM SR141716 (CB1 antagonist; dotted curve and closed circles, $n=7$). Panel b: CPAE cells were stimulated with 10 μM anandamide in the presence of 1 μM SR141716A as indicated. After washout, the same cells were activated again with 10 μM anandamide in the presence of 1 μM SR144528 ($n=7$). Panel c: Concentration–response curves of anandamide in the absence (continuous curve and open circles, $n=10$) and presence of 1 μM SR144528 (dotted curve and closed circles, $n=7$). Owing to the potential effects of the solvent at higher anandamide concentrations, a maximal concentration of 100 μM anandamide was used. Points represent the peak response at the given anandamide concentration expressed as the change (Δ) in the fluorescence ratio (F_{340}/F_{380}). $^*P<0.001$ *vs* effect of anandamide alone.

Table 1 Comparison of the EC_{50} values of anandamide in the presence of the CB1 and CB2 receptor antagonists SR141716A and SR144528 and with methanandamide

	EC_{50} (μM) (95% CI)	*10 (μM)*	*15 (μM)*	*30 (μM)*	*50 (μM)*	*100 (μM)*
Anandamide						
Control	8.4 (7.3–9.7)	0.22±0.01	0.33±0.03	0.31±0.03	0.32±0.02	0.34±0.01
+SR141716A (1 μM)	7.3 (4.6–11.7)	0.26±0.02*	0.31±0.03	0.34±0.04	0.33±0.04	0.27±0.02*
+SR144528 (1 μM)	ND	0.02±0.004*	0.10±0.03*	0.08±0.02*	0.17±0.01*	0.16±0.007*
Methanandamide	15.6 (14–17.4)	0.05±0.04*	0.13±0.01*	0.25±0.03	0.26±0.04	0.26±0.04

EC_{50} values represent the mean (95% confidential interval, CI). Other values represent the means±s.e.m. of the maximal peak Ca^{2+} elevation (Δ_{max} ratio (F_{340}/F_{380}) in response to the agonist concentration indicated. *$P<0.05$ *vs* anandamide; ND, not determined.

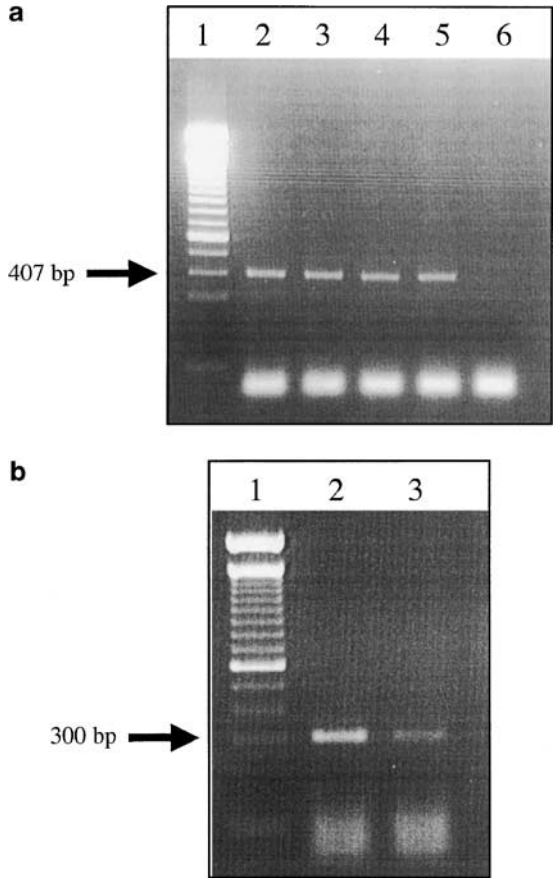

Figure 3 RT–PCR analysis of CB2 receptor expression. Panel a: mRNA expression of CB2 was determined by RT–PCR (407 bp) of mRNA from human monocytic leukemia cells (THP-1, lane 2), the human B lymphoblastoid cell line (DAUDI, lane 3), mouse spleen (lane 4), bovine spleen (reamplified with an additional 40 cycles; lane 5) and CPAE cells (reamplified with an additional 40 cycles; lane 6) as described in Methods. Lane 1 shows the 100-bp standard. Panel b: Based on the sequence analysis of the PCR product in bovine spleen, a new set of primers was designed for mRNA expression analysis of CB2 receptor in CPAE cells by RT-PCR (300 bp). Lane 1 shows the 100 bp standard, lane 2 is bovine spleen and lane 3 represents CPAE cells. PCR products were separated on 2% TAE–agarose.

bradykinin (Figure 8a), the kinetics of ER depletion and ER refilling differed for both agonists. Maximal ER depletion in response to bradykinin was significantly faster than in response to anandamide (Figure 8a; Table 2). Likewise, ER refilling in the presence of extracellular Ca^{2+} after agonist washout was faster after anandamide than after bradykinin stimulation ($n=5$ each, $P<0.05$; Figure 8a; Table 2).

As for most other inositol 1,4,5-trisphosphate-generating agonists, the cytosolic Ca^{2+} elevation in response to 20 μM anandamide was associated with a mitochondrial Ca^{2+} transient (Figure 8a and c). Notably, while neither the kinetics of the onset of the initial Ca^{2+} transient nor its decline towards basal level differed from the kinetics of the response to bradykinin, the elevation in $[Ca^{2+}]_{mito}$ with anandamide was more pronounced than with bradykinin (Figure 8c).

Discussion

While neuronal cells, immune cells and smooth muscle cells have been clearly found to be targets for anandamide, the reports on endothelial cells are controversial. Apart from the principal findings that endothelial cells are activated by anandamide, the receptors involved and the underlying signal transduction are still unclear. In the present work, we have identified the bovine CB2 receptor in the CPAE cell line by partial molecular sequencing, but have not identified the CB1 receptor. In addition to these findings, we have performed a functional analysis that points to CB2 receptors initiating signal transduction *via* phospholipase C and inositol 1,4,5-trisphosphate (IP_3) in response to anandamide. The IP_3 further initiates intracellular Ca^{2+} release from the ER accompanied by the activation of capacitative Ca^{2+} entry (CCE) and a transient elevation of mitochondrial Ca^{2+}. Thus, in addition to its actions on other vascular cells, anandamide acts as a powerful stimulus for endothelial cells that is distinct from, for example, bradykinin.

In agreement with our previous work on suspended cells from the human umbilical vein endothelial cell line EA.hy926 (Mombouli *et al*., 1999), anandamide also initiated Ca^{2+} signaling in single CPAE cells that was comparable, at least in terms of the initial Ca^{2+} elevation, to the effect of bradykinin. Remarkably, the effect of anandamide was not mimicked by arachidonic acid, which has been found to activate Ca^{2+}-permeable plasma membrane ion channels in other types of endothelial cells (Fiorio Pla & Munaron, 2001). Furthermore, preincubation with arachidonic acid failed to affect anandamide-induced Ca^{2+} signaling, while methanandamide mimicked the effect of anandamide on endothelial Ca^{2+}. Thus,

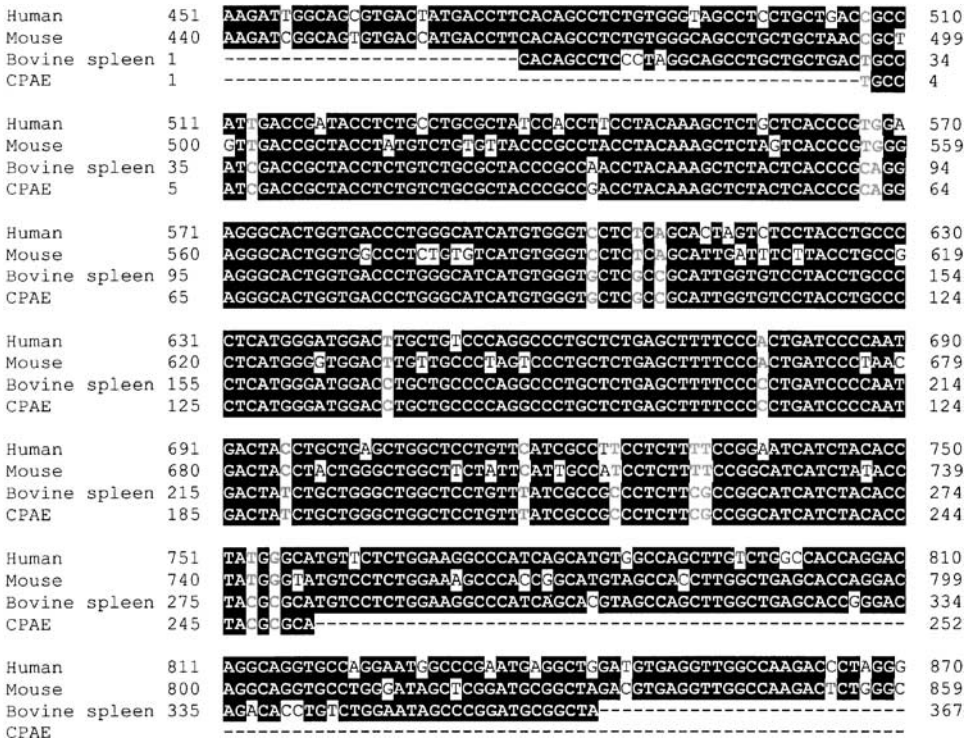

Figure 4 Sequence alignment of parts of the human, mouse, bovine spleen and CPAE CB2 receptors. The Genbank accession numbers are gi 407806 for human CB2 and gi 791081 for mouse CB2; the bovine sequence has not yet been published. Bases in the bovine sequence that are identical to either or both of the human and murine sequences are indicated in bold. Bases highlighted in gray indicate regions that are conserved in the human and mouse sequences, but differ from the bovine sequence.

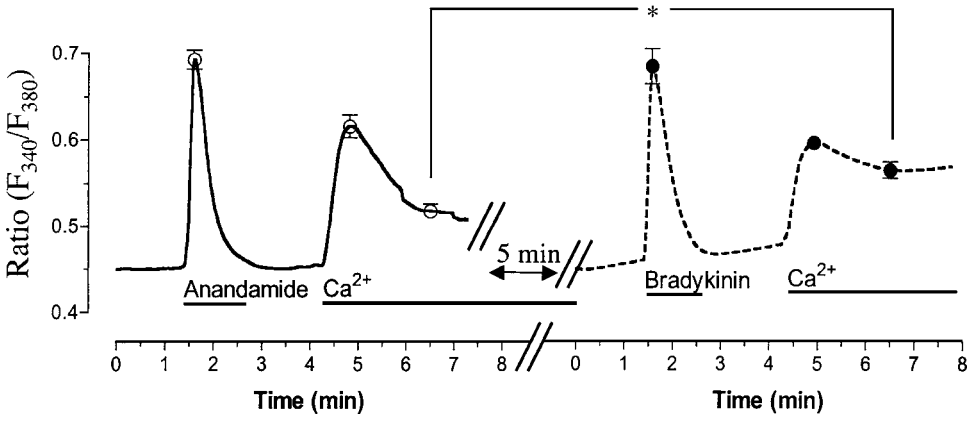

Figure 5 Measurements of $[Ca^{2+}]_{cyto}$ during anandamide- and bradykinin-initiated intracellular Ca^{2+} release and CCE. Representative recordings of the effect of 10 μM anandamide (continuous lines and open circles, $n=8$) on intracellular Ca^{2+} release in the absence of extracellular Ca^{2+} and following the addition of 2 mM extracellular Ca^{2+} at the times indicated. After approximately 10 min in a Ca^{2+}-containing solution, the same experiment was repeated with 0.1 μM bradykinin (dotted lines and closed circles, $n=8$). Except for the faster recovery following the addition of Ca^{2+} in anandamide-stimulated cells compared with that found in cells stimulated with bradykinin ($^*P<0.01$), no differences were found. Results were identical when the order of testing the autacoids was reversed.

our data suggest that the effect of anandamide on endothelial cells differs from that of arachidonic acid and might be due to its cannabinoid 3D-structure rather than to any similarities with arachidonic acid.

Notably, methanandamide, which has been found to be more selective for the CB1 receptor and less selective for the CB2 receptor (Goutopoulos *et al*., 2001) than anandamide, was approximately half as potent at initiating Ca^{2+} signaling in CPAE cells. In line with these findings, the CB1 receptor antagonist SR141716A (Rinaldi-Carmona *et al*., 1994) failed to affect anandamide-initiated Ca^{2+} signaling in endothelial cells. Moreover, in this study the CB2 receptor antagonist SR144528 (Rinaldi-Carmona *et al*., 1998) prevented anandamide-initiated Ca^{2+} signaling, thus pointing to an involvement of the CB2 receptor in the stimulation of CPAE cells by endocannabinoids. In contrast to the data reported here, in human umbilical vein endothelial cells anandamide has been characterized using the same antagonists and found to initiate

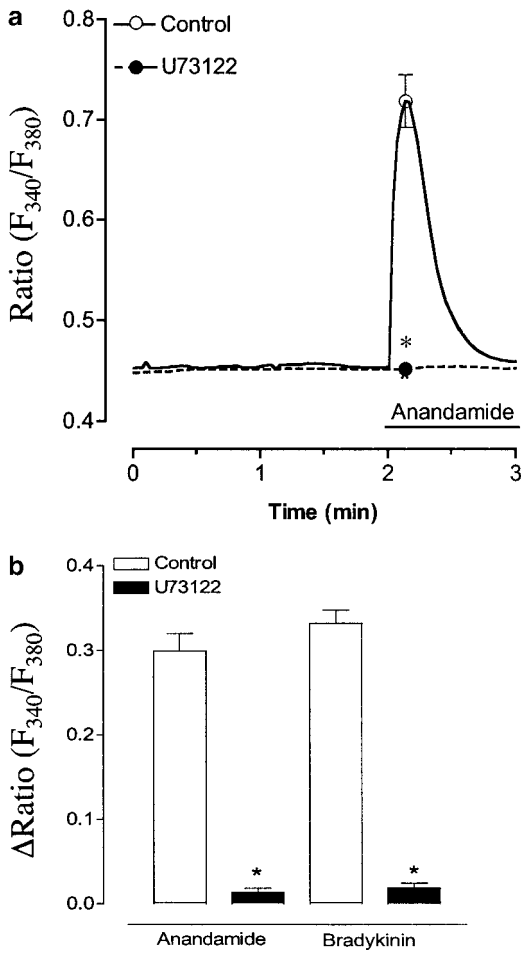

Figure 6 Effect of anandamide on endothelial $[Ca^{2+}]_{cyto}$ was prevented by U73122, an inhibitor of phospholipase C. Panel a: Single endothelial cells were stimulated with 10 μM anandamide in the absence (continuous lines and open circles, $n=32$) and presence of 2 μM U73122 (dotted lines and closed circles, $n=29$). Points represent the means$\pm$s.e.m. $^*P<0.0001$ *vs* in the absence of U73122. Panel b: Comparison of the effects of 10 μM anandamide and 0.1 μM bradykinin on endothelial $[Ca^{2+}]_{cyto}$ in the absence (white columns; anadamide $n=33$, bradykinin $n=41$) and presence of 2 μM U73122 (black columns; anadamide $n=29$, bradykinin $n=31$). $^*P<0.0001$ *vs* in the absence of U73122.

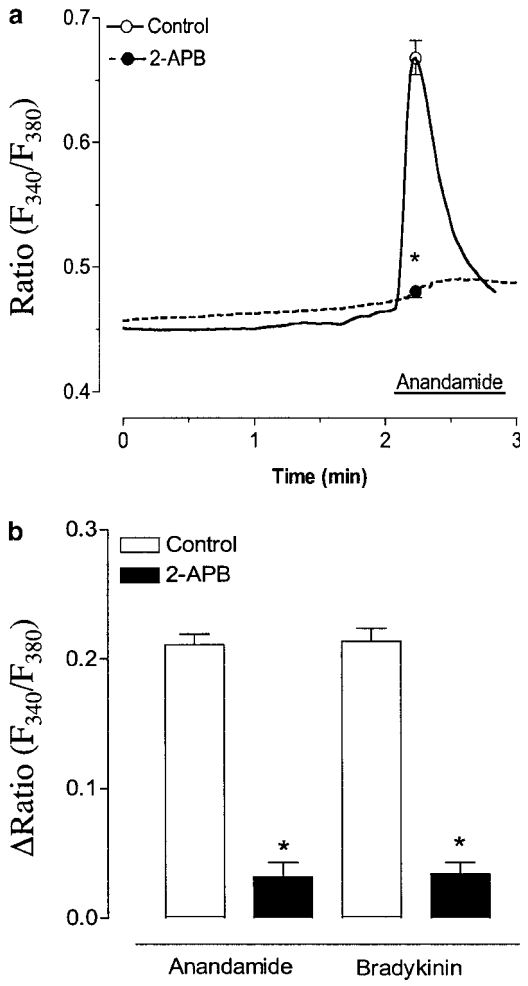

Figure 7 The inhibitor of inositol 1,4,5-trisphosphate receptors 2-APB prevented the anandamide-induced elevation in $[Ca^{2+}]_{cyto}$ in CPAE cells. Panel a: Single endothelial cells were stimulated with 10 μM anandamide in the absence (continuous lines and open circles, $n=24$) and presence of 50 μM 2-APB (dotted lines and closed circles, $n=29$). $^*P<0.0001$ *vs* in the absence of 2-APB. Panel b: Comparison of cytosolic Ca^{2+} response to 10 μM anandamide and 0.1 μM bradykinin stimulation in the absence (white columns; anadamide $n=40$, bradykinin $n=36$) and presence of 2 μM U73122 (black columns; anadamide $n=29$, bradykinin $n=34$). $^*P<0.0001$ *vs* in the absence of 2-APB.

the upregulation of iNOS *via* the CB1 receptor (Fimiani *et al*., 1999; Maccarrone *et al*., 2000). In addition, the expression of the CB1 receptor in human umbilical vein endothelial cells has been shown using RT–PCR (Sugiura *et al*., 1998). In rabbit aortic rings, anandamide has been found to initiate endothelium-dependent relaxation that is sensitive to SR141716A, thus pointing to an involvement of CB1 receptors in endothelial cells. On the other hand, in rat mesenteric arteries, anandamide has been found to initiate endothelial-dependent relaxation *via* an SR141716A-sensitive receptor that is distinct from CB1 (Wagner *et al*., 1999). This conclusion is supported by reports of SR141716A-sensitive anandamide- and Ca^{2+}-induced relaxation in CB1 receptor knockout mice (Jarai *et al*., 1999; Bukoski *et al*., 2002). Overall, these results emphasize the existence of a non-CB1, but SR141716A-sensitive cannabinoid receptor on endothelial cells.

Although the involvement of this type of nonclassical CB receptor seems unlikely in view of our findings that the effects of anandamide were prevented by the CB2 antagonist SR144528, but not the CB1 receptor antagonist SR141716A, the involvement of the CB2 receptor in CPAE cells was confirmed by molecular identification. Since, in contrast to the CB1 receptor, the CB2 receptor is not highly conserved across species (Shire *et al*., 1999) and the sequence of bovine CB2 is unknown, primers homologous to the human and mouse sequences for CB2 were used to amplify the bovine CB2 receptor from bovine spleen, as this tissue is supposed to have a high expression level of this receptor. The product was sequenced and a new pair of primers was designed based on the bovine CB2 receptor found in the CPAE cells. A further sequence analysis revealed identical mRNA for this region of the CB2 in bovine spleen and CPAE cells that was 86, 83 and 82% homologous to published sequences of the CB2 receptor in human, mouse or both, respectively. Thus, these data complete our pharmacological characterization of the cannabinoid receptor involved in the CPAE cells and

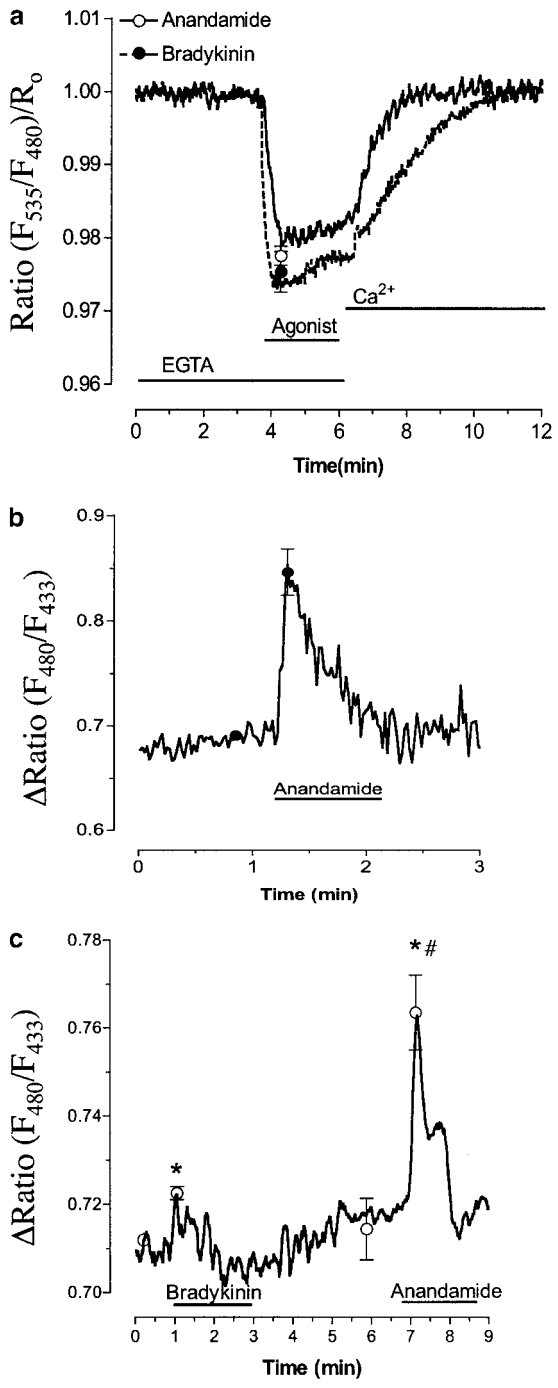

Figure 8 Anandamide initiated Ca^{2+} depletion of the ER and evoked a transient mitochondrial Ca^{2+} signal in CPAE cells. Endothelial cells were transiently transfected with vYC4-ER (panel a) or ratiometric-pericam-mt (panel b). Panel a: As indicated by the bars, cells were stimulated with 20 μM anandamide or 0.1 μM bradykinin and the effect on the ER Ca^{2+} content ($n=5$; panel a) was monitored. Panel b: Transiently transfected endothelial cells were stimulated with 20 μM anandamide and the mitochondrial Ca^{2+} concentration ($n=8$) was followed as described in Methods. Panel c: Comparison of the effect of maximally effective concentrations of bradykinin (0.1 μM) and anandamide (20 μM) on $[Ca^{2+}]_{mito}$. As indicated by the bars, cells were stimulated with bradykinin and anandamide in the presence of extracellular Ca^{2+} ($n=8$). $^*P<0.01$ *vs* basal and $^{\#}P<0.01$ *vs* the effect of bradykinin.

demonstrate the expression of CB2 receptors on this type of endothelial cell.

Remarkably, in many tissues the stimulation of cannabinoid receptors has been shown to produce a lowering of cAMP *via* the activation of $G_{i/o}$ protein (for a review, see Howlett *et al*., 2002). However, in neurons anandamide has been found to initiate Ca^{2+} signaling *via* the CB1 receptor, which activates the inositol 1,4,5-trisphosphate pathway and subsequent ER depletion (Venance *et al*., 1997; Mukhopadhyay *et al*., 2002). Similar results have been obtained in human umbilical vein endothelial cells, where the Ca^{2+} elevation was associated with the stimulation of eNOS (Fimiani *et al*., 1999; Mombouli *et al*., 1999). In contrast, the present study provides the first evidence to our knowledge for anandamide-initiated Ca^{2+} signaling *via* the CB2 receptor in endothelial cells. In CPAE cells, anandamide was as potent as bradykinin at initiating intracellular Ca^{2+} release from the ER. This was shown by a similar elevation in $[Ca^{2+}]_{cyto}$ in response to either anandamide or bradykinin in the absence of extracellular Ca^{2+}. The intracellular Ca^{2+} release in response to bradykinin is known to be mediated by phospholipase C-mediated activation of inositol 1,4,5-trisphosphate (Graier *et al*., 1991). In our experiments, the phospholipase C inhibitor U73122 (Aschner *et al*., 1997) was found to prevent anandamide-induced Ca^{2+} signaling with the same potency as bradykinin. Furthermore, the inositol 1,4,5-trisphosphate inhibitor 2-APB (Koganezawa & Shimada, 2001) prevented anandamide- and bradykinin-induced Ca^{2+} signaling in the presence and absence (data not shown) of extracellular Ca^{2+}. Although 2-APB has proven effects on plasma membrane ion channels and CCE (Putney, 2001), these actions cannot account for the inhibition of the intracellular Ca^{2+} release in response to anandamide. Overall, these data strongly support the hypothesis that anandamide initiates intracellular Ca^{2+} release *via* the generation of inositol 1,4,5-trisphosphate due to the activation of phospholipase C.

In line with our findings described above, anandamide triggers a fast depletion of the ER. This event was monitored in cells that were transiently transfected with an improved ER-targeted cameleon probe (vYC4-ER), that is more pH and bleaching resistant, and offers a larger dynamic range in the FRET signal than YFP. Anandamide evoked Ca^{2+} ER depletion that was similar to the effect of bradykinin. However, the kinetics of ER depletion were approximately two times faster in response to bradykinin than to anandamide, while ER refilling after agonist washout was approximately 2.5 times faster in cells stimulated with anandamide than with bradykinin (Table 2). The enhanced capacity of the ER to become refilled after/during anandamide stimulation might result in the discontinuation of CCE and could be the reason for the very transient nature of the anandamide-triggered cytosolic Ca^{2+} signal. This hypothesis is further supported by our findings using the classical Ca^{2+} re-entry protocol shown in Figure 5 that there was no difference between the initial Ca^{2+} entry in anandamide- and bradykinin-stimulated cells. Using this protocol, the ER is depleted in the absence of extracellular Ca^{2+} and Ca^{2+} entry is monitored by the addition of extracellular Ca^{2+} after washout of the agonist (Figure 5). These data emphasize that, compared with bradykinin, anandamide-induced depletion of the ER and triggered CCE, but allowed faster ER refilling that in turn terminated CCE. Very similar findings have been obtained in porcine coronary artery cells, where substance P initiated a

Table 2 Comparative analysis of the kinetics of ER Ca^{2+} depletion and refilling in response to anandamide and bradykinin

ER Ca^{2+} kinetics (s)	*ER Ca^{2+} depletion*	*ER Ca^{2+} refilling*	*τ of ER Ca^{2+} refilling (s)*
Anandamide	48.2 ± 8.2 s*	101.4 ± 6.6 s*	36 ± 9.6 s*
Bradykinin	29.4 ± 1.6 s	241.6 ± 10.4 s	101.0 ± 18.4 s

ER Ca^{2+} depletion, the time until the ER was maximally depleted after the addition of the agonist; ER Ca^{2+} refilling, the time needed for ER refilling after agonist washout in Ca^{2+}-containing buffer; τ of ER Ca^{2+} refilling, the time until the ER was 50% refilled after agonist washout in Ca^{2+}-containing buffer. Each time represents the means $\pm$ s.e.m. *$P<0.05$ vs bradykinin.

transient Ca^{2+} elevation, whereas bradykinin achieved a long-lasting Ca^{2+} plateau (Frieden *et al*., 1999). In the latter study, the two agonists activated two different types of Ca^{2+}-activated Ca^{2+} channels, indicating that there may be diverse downstream signaling pathways and/or different spatial Ca^{2+} signaling in response to substance P and bradykinin in these cells.

In line with this hypothesis, further differences between the actions of anandamide and bradykinin were obtained when their effects on the mitochondrial Ca^{2+} signal were studied. Notably, despite the transient nature of the effect of anandamide on $[Ca^{2+}]_{cyto}$, a strong mitochondrial Ca^{2+} signal in response to this substance was observed in CPAE cells. These findings are in agreement with the data from other endothelial cells (Sedova & Blatter, 2000) showing that the mitochondrial Ca^{2+} concentration transiently elevates due to Ca^{2+} release from the ER Ca^{2+} pool (Szabadkai *et al*., 2003). In contrast, the effect of bradykinin on $[Ca^{2+}]_{mito}$ was significantly weaker than that of anandamide (Figure 8c). While this finding is surprising considering the more pronounced cytosolic Ca^{2+} elevation and ER Ca^{2+} depletion obtained in response to bradykinin compared with anandamide, these findings might help one to explain the observed differences in the kinetics of CCE and ER Ca^{2+} depletion/refilling in response to these autacoids. Since elevation of mitochondrial Ca^{2+} has been reported to facilitate fast ER Ca^{2+} refilling (Szabadkai *et al*., 2003), one can speculate that the large elevation in $[Ca^{2+}]_{mito}$ caused by anandamide contributes to the faster Ca^{2+} refilling of the ER and, in turn, to the more transient CCE activation compared with braydkinin found in this study. Nevertheless, the mechanisms involved in the contribution of mitochondria to the Ca^{2+} signaling response to anandamide and bradykinin in CPAE cells remain unresolved and should be explored in detail in future.

In conclusion, our data demonstrate that the endocannabinoid anandamide initiates Ca^{2+} signaling in CPAE cells *via* the activation of the CB2 receptor. This receptor was identified by molecular sequencing and found to be 86 and 83% homologous to the corresponding regions of the human and mouse CB2 receptor sequences. Although the extent of the initial Ca^{2+} elevation induced by anandamide, and its initiation by inositol 1,4,5-trisphosphate and the consequences on ER Ca^{2+} content and mitochondrial Ca^{2+} concentration are similar to those produced by bradykinin, considerable differences in the kinetics of the cytosolic Ca^{2+} elevation and ER depletion responses exist. Whether or not these differences are the functional result of different stimulation mechanisms by these agonists is unknown and needs to be further explored in more detail.

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