

Research Article

Dual effect of cannabinoid CB₁ receptor stimulation on a vanilloid VR1 receptor-mediated response

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Abstract. Cannabinoid CB₁ receptors and vanilloid VR1 receptors are co-localized to some extent in sensory neurons of the spinal cord and dorsal root ganglia. In this study, we over-expressed both receptor types in human embryonic kidney (HEK)-293 cells and investigated the effect of the CB₁ agonist HU-210 on the VR1-mediated increase in intracellular Ca²⁺ ([Ca²⁺]_i), a well-known response of the prototypical VR1 agonist capsaicin. After a 5-min pre-treatment, HU-210 (0.1 μM) significantly enhanced the effect of several concentrations of capsaicin on [Ca²⁺]_i in HEK-293 cells over-expressing both rat CB₁ and human VR1 (CB₁-VR1-HEK cells), but not in cells over-expressing only human VR1 (VR1-HEK cells). This effect was blocked by the CB₁ receptor antagonist SR141716A (0.5 μM), and by phosphoinositide-3-kinase

and phospholipase C inhibitors. The endogenous agonist of CB₁ and VR1 receptors, anandamide, was more efficacious in inducing a VR1-mediated stimulation of [Ca²⁺]_i in CB₁-VR1-HEK cells than in VR1-HEK cells, and part of its effect on the former cells was blocked by SR141716A (0.5 μM). Pre-treatment of CB₁-VR1-HEK cells with forskolin, an adenylate cyclase activator, enhanced the capsaicin effect on [Ca²⁺]_i. HU-210, which in the same cells inhibits forskolin-induced enhancement of cAMP levels, blocked the stimulatory effect of forskolin on capsaicin. Our data suggest that in cells co-expressing both CB₁ and VR1 receptors, pre-treatment with CB₁ agonists inhibits or stimulates VR1 gating by capsaicin depending on whether or not cAMP-mediated signalling has been concomitantly activated.

Key words. Anandamide; capsaicin; cannabinoid; vanilloid; receptor; signalling; pain.

Recent evidence points to the existence of functional relationships between the brain G protein-coupled receptor for the psychoactive principle of marijuana, Δ⁹-tetrahydrocannabinol (THC), i.e. the cannabinoid CB₁ receptor [1, 2], and the membrane cation channel gated by heat, protons and the pungent hot chilli pepper ingredient, capsaicin, i.e. the vanilloid VR1 receptor [3]. The two receptors are co-localized in many, although not all, small-diameter, non-myelinated sensory C fibres, both at the level

of the spinal cord, and in dorsal root ganglia (DRGs) as well as, apparently, in the peripheral terminals of C fibres [4]. In these neurons, CB₁ and VR1 receptors play opposite roles in the control of nociception. VR1 appears to be partly responsible for the transmission of pain during thermal and inflammatory hyperalgesia [5, 6], whereas CB₁ receptors were suggested to counteract hyperalgesia, at least in part by inhibiting VR1-mediated nociception [7, 8]. However, VR1 activation by potent synthetic agonists is immediately followed by desensitization, thereby leading to powerful analgesic effects in vivo [3]. There is now evidence for the co-existence of CB₁ and VR1 re-

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ceptors also in brain nuclei and areas involved in the control of motor (substantia nigra, striatum, cerebellum), cognitive and mnemonic (hippocampus, cortex), emotional (amygdala) and nociceptive (periaqueductal grey) functions [9, 10].

Several connections have also been identified between the endogenous ligands of CB₁ and VR1 receptors in the brain. Anandamide (N-arachidonoyl-ethanolamine, AEA), the first endogenous cannabinoid receptor ligand discovered [11], acts as a full agonist at VR1 receptors [12, 13; for a review see ref. 14] at concentrations that are normally higher than those required to activate CB₁, but that can be significantly decreased under certain conditions [15, 16; for a review see ref. 17]. Furthermore, some long-chain homologues of capsaicin, and synthetic VR1 agonists, can indirectly activate CB₁ receptors either by retarding the cellular uptake and inactivation of endogenous AEA [via inhibition of the AEA membrane transporter (AMT)] [18, 19], or by triggering AEA formation [20].

Stimulation of CB₁ receptors on sensory neurons with CB₁-selective agonists can induce inhibition of VR1-mediated thermal hyperalgesia [7, 8, 21]. However, agents capable of activating both CB₁ and VR1 receptors, such as the AEA/capsaicin structural 'hybrid' arvanil [22] and its analogues [23], are more potent analgesics [23, 24], and produce a much stronger response in the mouse vas deferens assay [25] than 'pure' CB₁ and VR1 agonists. Therefore, functional cross-talk between CB₁ and VR1 receptors, localized in the same or neighbouring neurons, might explain the different impact that CB₁ receptor stimulation has so far been found to have on VR1-mediated signalling.

Here we investigated the effect of CB₁ receptor stimulation on the VR1-induced increase in intracellular calcium concentration ($[Ca^{2+}]_i$) by using human embryonic kidney (HEK)-293 cells stably transfected with cDNAs encoding the CB₁ and VR1 receptors, and therefore co-expressing both receptor types. We report that, depending on whether or not the cAMP cascade is activated, CB₁ receptor stimulation may either inhibit or enhance inhibit VR1-mediated biological responses.

Materials and methods

Drugs

HU-210 and SR141716A were kind gifts from Prof. R. Mechoulam, Hebrew University of Jerusalem and from Sanofi Recherche, respectively. 3-Isobutyl-1-methylxanthine (IBMX) and forskolin were purchased from Sigma (Deisenhofen, Germany) and WIN 55,212-2 was purchased from Tocris (Cologne, Germany). These compounds were prepared as 10 mM stock solutions in 100% dimethylsulfoxide (DMSO; in the case of HU-210,

SR141716A, WIN 55,212-2 and forskolin) or in phosphate-buffered saline (PBS) (136.8 mM NaCl, 2.7 mM KCl, 1.5 mM KH₂PO₄, 10.2 mM Na₂HPO₄, pH 7.4; in the case of IBMX). Ionomycin was purchased from Sigma. AEA was synthesized as previously described [19]. The phosphatidylinositol-3-kinase (PI-3-K) inhibitors, wortmannin and LY294002 were purchased from Alexis Biochemicals (Lausen, Switzerland). The phosphatidylinositol-selective phospholipase C (PI-PLC) inhibitors ET-18 and U73122, and the phosphatidylcholine-selective PLC (PC-PLC) inhibitor D609 were obtained from Biomol Research Laboratories (Plymouth Meeting, Pa, USA).

Construction of the pZeoSV-CB₁ plasmid

The pcDNA3 plasmid containing the N-terminal haemagglutinin (HA)-tagged cDNA of rat CB₁ (pcDNA3-CB₁) was a kind gift from Dr. K. Mackie. pcDNA3-CB₁ was linearized with *Xho*I and overhangs were blunted with Klenow. CB₁ was released with *Acc*65I and subcloned into pZeoSV containing resistance against zeocin (Invitrogen, Karlsruhe, Germany) linearized with *Acc*65I and *Pvu*II to obtain pZeoSV-CB₁. The plasmid was checked by sequencing. For transfection into HEK-293 cells, pZeoSV-CB₁ was linearized with *Not*I [all molecular biology methods were performed as described in ref. 26].

Cell culture and transfection

HEK-293 cells stably expressing human VR1 (hVR1) were obtained from J. Davis (GlaxoSmithKline, Harlow, UK). Cells were maintained in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal calf serum, 2 mM glutamine and, to prevent bacterial and fungal contamination, 1% antibiotic-antimycotic (penicillin/streptomycin/amphotericin; Gibco BRL, Karlsruhe, Germany), at 37°C in a humidified 5% CO₂ incubator. Cell lines were generated by transfection of linearized pZeoSV-CB₁ into HEK-293 cells already stably expressing hVR1 by electroporation as described elsewhere [26]. Stable transfectants were selected in medium containing zeocin (0.6 mg/ml) for CB₁ selection and geneticin (G418, 2 mg/ml) for VR1 selection. Zeocin is an antibiotic that causes cell death by cleaving DNA, and resistance to it is conferred by the *Sh ble* gene product, which binds the antibiotic and prevents its action. Geneticin is instead an antibiotic that interferes with 80S ribosomes, thus blocking protein synthesis, and resistance to it is conferred by the *Tn5* or *Tn601* aminoglycoside phosphotransferase. Colonies of about 500 cells were picked (about 2 weeks after transfection) and allowed to expand, then tested for expression of CB₁ mRNA and protein by Northern and Western blot, respectively. CB₁-VR1-HEK clones containing high levels of CB₁ mRNA and protein were tested for functional receptor properties by measurement of a forskolin-stimulated decrease in cAMP [27]. CB₁-VR1-

HEK cells were maintained under selection by adding antibiotics to culture medium every third passage. No difference in the levels of hVR1 mRNA transcripts, assessed by reverse transcriptase-polymerase chain reaction, between cells expressing only hVR1 and cells expressing both hVR1 and rat CB₁ was observed (data not shown).

Northern blot analysis

Standard Northern blotting protocols were used [27]. Briefly, total RNA (20 µg) was loaded onto formaldehyde-containing 1% agarose gels, blotted onto nylon membranes (Hybond NX; Amersham, Freiburg, Germany), and immobilized by UV cross-linking (UV Stratalinker 2400; Stratagen, Amsterdam, The Netherlands). Blots were pre-hybridized in rapid-hyb buffer (Amersham) and hybridized in the same solution containing [³²P]dCTP-labelled probe at 70°C according to the manufacturer's instructions. Probe labelling of the full-length cDNA of CB₁ was carried out with a random primer DNA labelling system (Gibco BRL). Blots were exposed at -80°C for 1–2 days to Kodak Biomax films with intensifying screens.

Western blot

For detection of the CB₁ receptor protein we used the Western immunoblotting technique, by exploiting the tag with the short HA epitope (corresponding to an internal 9-amino-acid sequence of the influenza HA) attached to the N terminus of the CB₁ receptor (see Construction of the pZeoSV-CB₁ plasmid), and hence using an anti-HA monoclonal antibody. Transfected HEK-293 cells were solubilized in a glass homogenizer with 20 mM Tris-HCl, pH 7.4, containing protease inhibitors (Complete Mini tablets; Roche, Basel, Switzerland). The lysate was centrifuged for 5 min at 1000 g, and the supernatant collected and assayed for protein content (Bio-Rad, Munich, Germany). Loading buffer (Roti-load 1; Roth, Karlsruhe, Germany) was added to protein samples which were denatured for 5 min at 95°C, centrifuged and loaded (20 µg/lane) on a 7.5% polyacrylamide gel. After electrophoresis, proteins were transferred overnight at 4°C onto a cellulose nitrate membrane (Schleicher & Schüll, Dassel, Germany) with transfer buffer (48 mM Tris, 390 mM glycine, 0.1% SDS, 20% methanol), using a Bio-Rad Blot apparatus. The membrane was blocked for 1 h with blocking buffer (10% non-fat milk powder, 20 mM Tris-HCl, 140 mM NaCl, 0.1% Tween-20, pH 7.6). To detect the HA-CB₁ fusion protein, blots were incubated with an anti-HA monoclonal antibody (Santa Cruz, Heidelberg, Germany), diluted 1:200 in blocking buffer overnight at 4°C. After incubation with anti-mouse IgG-horseradish peroxidase as secondary antibody (Dako, Glostrup, Denmark) diluted 1:2000 in blocking buffer, chemiluminescence was performed using the Lumi GLO reagent (Cell Signaling, Frankfurt, Germany)

according to the manufacturer's instructions, and the blots were exposed to Biomax films for 1–10 min. Pre-adsorption of the anti-HA antibody with the corresponding immunizing peptide (Santa Cruz) was carried out to test the specificity of the antibody, and produced no band on the gel.

cAMP accumulation assay

The cAMP assay was performed as described elsewhere [27] with slight modifications. One day before the experiment, CB₁-VR1-HEK cells were plated into 48-well plates in 500 µl of complete DMEM at a density of 4 × 10⁵ cells/ml. On the next day, cells were washed twice with DMEM to remove serum, and incubated for 1 h. Reaction was initiated by adding stimulation buffer containing 20 mM HEPES, 0.1 mg/ml BSA, 5 µM forskolin, 0.5 mM IBMX and the CB₁ agonists WIN 55,212-2 and HU-210. Forskolin, WIN 55,212-2 and HU-210 were dissolved in DMSO. DMSO alone served as a vehicle control and had no effect on cAMP accumulation (data not shown). Reactions were terminated 10 min later by aspiration of the medium and the addition of 500 µl ice-cold 6% trichloroacetic acid followed by incubation overnight at 4°C. To remove the trichloroacetic acid, the extracts were treated twice with 3 ml diethylether, dried overnight in a lyophilizator and reconstituted in DMEM. Intracellular cAMP levels were measured with a competitive protein-binding assay (non-acetylated procedure; Perkin Elmer, Boston, MA, USA). Data obtained in the cAMP accumulation assay were expressed as the percentage of forskolin-stimulated cAMP accumulation. Samples were measured in triplicate and data are given with the standard error of the mean (SE).

[Ca²⁺]_i assays

The effect of test substances on [Ca²⁺]_i in CB₁-VR1-HEK and VR1-HEK cells was determined using Fluo-3 methylester (Molecular Probes, Leiden, The Netherlands), a selective intracellular fluorescent probe for Ca²⁺. Cells were prepared and loaded as described previously [19]. Experiments were carried out by measuring cell fluorescence at 25°C (λ_{EX} = 488 nm, λ_{EM} = 540 nm) before and after the addition of the test compounds at various concentrations. HU-210 (100 nM) or forskolin (5 µM) were added, alone or together, 5 min before capsaicin. SR141716A (0.5 µM) was also added 5 min before HU-210 or AEA. The PI-3-K and PLC inhibitors were added 5 min before HU-210. The efficacy of the effect of each treatment was determined by normalizing it to the analogous effect observed with 4 µM ionomycin in each single experiment. A typical experiment consisted in suspending in a quartz cuvette the cells pre-loaded with Fluo-3, followed by measuring cell fluorescence for 5 min while the response became stable. This was followed by addition of capsaicin or AEA (the 'stimulant'). In the case of

pre-treatments, HU-210 or forskolin or SR141716A or the phospholipase and/or kinase inhibitors, or their combinations, were added 5 min prior to incubation with the stimulant. During this pre-treatment, fluorescence was measured so that the effect, if any, of the pre-treatment on basal $[Ca^{2+}]_i$ could be observed. After the addition of the stimulant, fluorescence was measured for 10–20 min, after which ionomycin (4 μ M) was always added to calculate the maximal inducible $[Ca^{2+}]_i$ in those conditions. The effect of the stimulant was then normalized to the effect of ionomycin, which in turn depends almost uniquely on the amount of viable cells present in each incubation. Data for the compounds tested at varying concentrations were expressed as the concentration exerting a half-maximal effect (EC_{50}), calculated using GraphPad software.

Results

CB₁-VR1-HEK cells express functional CB₁ receptors

Northern blots of CB₁-VR1-HEK clones produced single discrete bands of the same, expected size (1.6 kb), while no band was observed using RNA of cells transfected only with hVR1 (fig. 1A). To test whether receptor mRNA is effectively translated into receptor protein, Western blot analysis was carried out and showed a band of the expected size of 80 kDa for the HA-CB₁ fusion protein (fig. 1B), which was not observed by blocking of the antigen recognition site of the antibody with the immunizing peptide (data not shown). Two clones of CB₁-VR1-HEK cells (no. 10 and no. 15) expressing high levels of mRNA and protein were tested for functional receptor properties. Both clones exhibited functional coupling of CB₁ receptors to G_i proteins, as demonstrated by the inhibition of forskolin-stimulated intracellular cAMP accumulation by HU-210 and WIN 55,212-2 (fig. 1C, D),

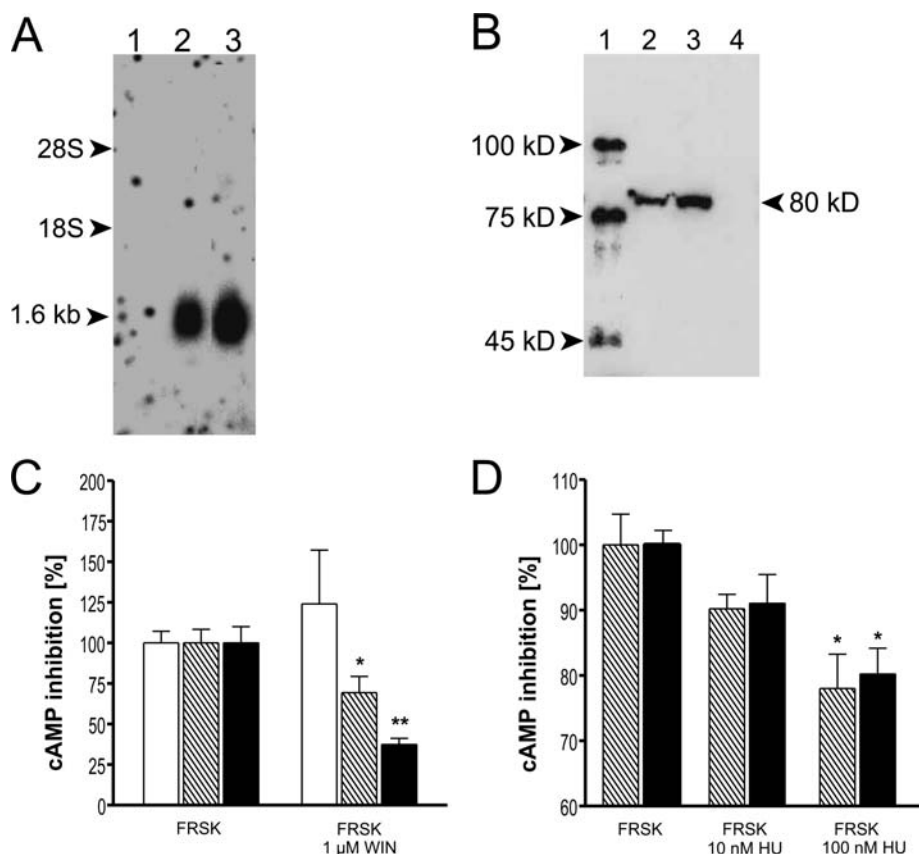


Figure 1. Analysis of CB₁ expression in CB₁-VR1 double-transfected HEK-293 cells. (A) Northern blot showing CB₁ mRNA in the two different clones of CB₁-VR1-HEK cells (lane 2, clone no. 10; lane 3, clone no. 15); VR1-HEK cells served as a negative control (lane 1). (B) Western blot showing CB₁ protein in the same CB₁-VR1-HEK clones (lane 2, clone no. 10; lane 3, clone no. 15); VR1-HEK cells served as negative control (lane 4); HA-tagged protein as molecular-weight standard (lane 1). (C) Effect of WIN 55,212-2 (WIN) on forskolin-induced cAMP accumulation in clone no. 10 (striped bars) and clone no. 15 (black bars) of CB₁-VR1-HEK cells, and in cells expressing only VR1 (white bars). (D) HU-210 (HU)-induced inhibition of cAMP accumulation in clone no. 10 (striped bars) and clone no. 15 (black bars) of CB₁-VR1-HEK cells. Data are expressed as percentages of the effect of forskolin (FRSK) and are means \pm SE of $n = 3$ experiments. * $p < 0.05$ vs FRSK only clone no. 10; ** $p < 0.01$ vs FRSK only clone no. 15, calculated by ANOVA followed by the Bonferroni test.

whereas VR1-HEK cells did not show any response upon stimulation with WIN 55,212-2 (fig. 1C). The two CB₁-VR1-HEK clones were subsequently used for the experiments carried out in this study.

Effect of HU-210 on capsaicin response in CB₁-VR1-HEK cells

The effect of capsaicin, the prototypical VR1 agonist, on [Ca²⁺]_i in CB₁-VR1 HEK cells (clone no. 10) is shown in figure 2A. The compound enhanced [Ca²⁺]_i in a dose-dependent manner, with an EC₅₀ = 35.0 ± 4.0 nM (mean ± SE, n = 3) that was indistinguishable from that observed in HEK cells over-expressing only VR1 (EC₅₀ = 32.1 ± 5.0 nM, n = 3). The CB₁ receptor agonist

HU-210, at a concentration (100 nM) previously shown to be fully effective on CB₁ receptors [1], and shown here to inhibit forskolin-induced cAMP formation in the same cells (fig. 1C), significantly enhanced the effect on [Ca²⁺]_i of 10–50 nM capsaicin (fig. 2A), without having any effect per se on basal [Ca²⁺]_i (data not shown). The EC₅₀ for the effect of capsaicin was lowered from 35.0 ± 4.0 to 17.0 ± 2.1 nM, n = 6, p < 0.05 by ANOVA). This effect was antagonized by the CB₁ receptor antagonist, SR141716A (fig. 2B), at a dose (0.5 μM) selective for CB₁ receptors and devoid per se of any effect on [Ca²⁺]_i (data not shown). The effect of HU-210 was not observed in VR1-HEK cells (data not shown). The HU-210 effect was also observed in a second clone (clone no. 15) of CB₁-VR1 HEK cells, which again responded to capsaicin to the same extent as VR1-HEK cells (EC₅₀ = 27.7 ± 4.3 nM, n = 3). In these cells, the CB₁ agonist decreased the EC₅₀ for the effect of capsaicin to 14.5 ± 1.5 nM, (n = 3, p < 0.05 by ANOVA). Interestingly, simultaneous treatment of CB₁-VR1-HEK cells (clone no. 10) with HU-210 and capsaicin did not lead to a potentiation of the effect on [Ca²⁺]_i of the latter compound (data not shown).

Effect of various inhibitors on HU-210 potentiation of the capsaicin response

The two selective inhibitors of PI-3-K, wortmannin (10 μM) and LY294002 (20 μM), the two selective inhibitors of PI-PLC, ET-18 (20 μM) and U73122 (10 μM), and the selective PC-PLC inhibitor D609 (20 μM) strongly attenuated the effect of capsaicin (20 nM) on [Ca²⁺]_i in CB₁-VR1-HEK cells (clone no. 10) (fig. 3), while exhibiting no effect per se on basal [Ca²⁺]_i (not shown). Moreover, when cells were pre-incubated with HU-210 (100 nM), and the inhibitors were tested at concentrations (1–10 μM) that were inactive per se on the response induced by capsaicin alone, a complete blockade of HU-210 potentiation of the capsaicin effect was observed (fig. 3).

Effect of AEA on [Ca²⁺]_i in CB₁-VR1-HEK and VR1-HEK cells

We compared the effect of the endogenous agonist of CB₁ and VR1 receptors, AEA, on [Ca²⁺]_i in CB₁-VR1-HEK and VR1-HEK cells. Unlike capsaicin, AEA was significantly more efficacious in CB₁-VR1 HEK cells (clone no. 10) than in VR1-HEK cells at the two highest concentrations tested (fig. 4). Importantly, after pre-treatment of CB₁-VR1-HEK cells with a concentration of SR141716A (0.5 μM) selective for CB₁ versus VR1 receptors [15], the effect of AEA became identical to that observed in VR1-HEK cells (fig. 4). AEA was also more potent and efficacious in clone no. 15 of CB₁-VR1-HEK cells than in VR1-HEK cells (data not shown).

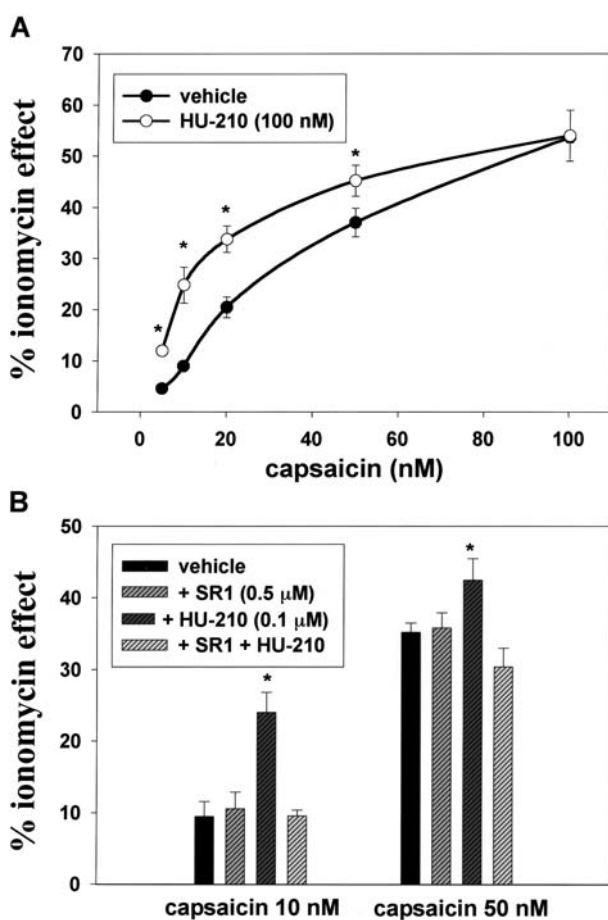


Figure 2. HU-210 enhances the VR1-mediated capsaicin effect on [Ca²⁺]_i in CB₁-VR1-HEK cells via a CB₁ receptor-mediated mechanism. (A) Dose-response for the VR1-mediated effect of capsaicin on [Ca²⁺]_i with (○) or without (●) pre-treatment of cells (clone no. 10) with HU-210 (100 nM). (B) Reversal of HU-210-induced potentiation of the VR1-mediated capsaicin effect on [Ca²⁺]_i by the CB₁ antagonist SR141716A (SR1, 0.5 μM). Data are expressed as percent of the effect of ionomycin (4 μM) and are means ± SE of at least n = 3 independent experiments carried out in duplicate. *p < 0.05 by ANOVA followed by the Bonferroni test. Neither HU-210 nor SR141716A per se caused any significant change in basal intracellular calcium (data not shown).

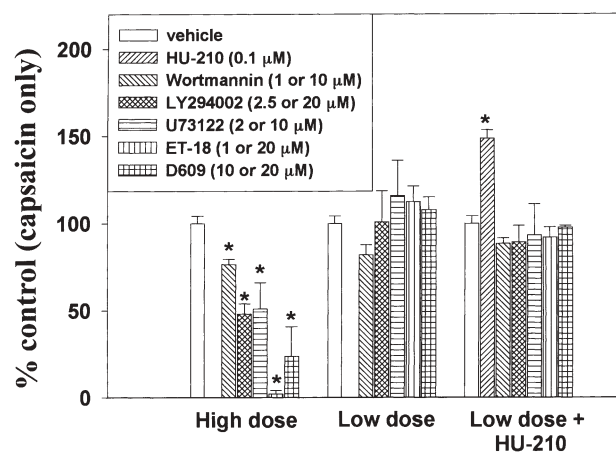


Figure 3. Effect of various inhibitors of PI-3-K (wortmannin, LY294002), PI-PLC (U73122, ET-18) and PC-PLC (D609) on capsaicin action on $[Ca^{2+}]_i$ in CB_1 -VR1-HEK cells. Clone no. 10 of CB_1 -VR1-HEK cells was used in these experiments. The dose of capsaicin used was 20 nM and led to a stimulation of $[Ca^{2+}]_i$ of $22.9 \pm 1.1\%$ (mean \pm SE, $n = 12$) of the effect of ionomycin (4 μ M). The effects of the inhibitors, which were given to cells 5 min before capsaicin, are expressed as a percent of the effect of capsaicin alone and are means \pm SE of at least $n = 3$ independent experiments carried out in duplicate. The high dose of the inhibitors was tested only on capsaicin alone and was 10 μ M for wortmannin and U73122, and 20 μ M for LY294002, ET-18 and D609. The low dose of the inhibitors was tested both on capsaicin alone and on capsaicin + HU-210 (100 nM), and was 1 μ M for wortmannin and ET-18, 2 μ M for U73122, 2.5 μ M for LY294002 and 10 μ M for D609. The effect of HU-210 (100 nM, 5 min pre-treatment) on capsaicin is also shown as a percent of the effect of capsaicin alone. * $p < 0.05$ vs. vehicle (i.e. capsaicin only), calculated by ANOVA followed by the Bonferroni test, and using the raw data (i.e. expressed as percent of the ionomycin effect and not as percent of capsaicin alone). None of the inhibitors per se caused any significant change in basal $[Ca^{2+}]_i$.

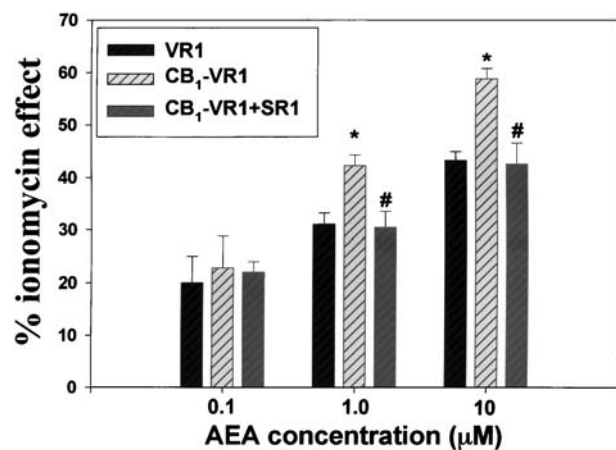


Figure 4. Dose-dependent effect of anandamide (AEA) on $[Ca^{2+}]_i$ in CB_1 -VR1-HEK and VR1-HEK cells. The effect on $[Ca^{2+}]_i$ was expressed as a percent of the effect of ionomycin (4 μ M) and, in CB_1 -VR1-HEK cells (clone no. 10), measured after 5 min pre-treatment with SR141716A (SR1, 0.5 μ M). Data are means \pm SE of at least $n = 3$ independent experiments carried out in duplicate. * $p < 0.05$ vs VR1-HEK cells; # $p < 0.05$ vs CB_1 -VR1-HEK cells without SR1, as calculated by ANOVA followed by the Bonferroni test.

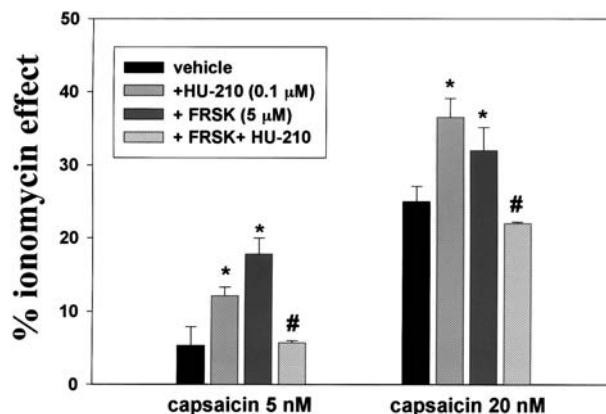


Figure 5. HU-210 inhibits the effect of capsaicin on $[Ca^{2+}]_i$ in CB_1 -VR1-HEK cells pre-treated with forskolin. Cells (clone no. 10) were treated with vehicle, forskolin (FRSK, 5 μ M), HU-210 (100 nM) or with both HU-210 and FRSK 5 min prior to stimulation with capsaicin (5 or 20 nM). FRSK or HU-210+FRSK caused no significant change in basal $[Ca^{2+}]_i$. Data are expressed as percent of the effect of ionomycin (4 μ M) and are means \pm SE of at least $n = 3$ independent experiments carried out in duplicate. * $p < 0.05$ vs control; # $p < 0.05$ vs FRSK, calculated by ANOVA followed by the Bonferroni test.

Effect of HU-210 on forskolin-induced potentiation of the capsaicin response in CB_1 -VR1-HEK cells

In agreement with a previous study carried out with VR1-HEK cells [16], we found that 5 min pre-treatment with forskolin, at a dose (5 μ M) inactive per se on basal $[Ca^{2+}]_i$, led to a significantly enhanced effect of capsaicin on $[Ca^{2+}]_i$ in CB_1 -VR1-HEK cells (clone no. 10) (fig. 5). When cells were pre-treated with both forskolin and HU-210 (100 nM), however, the overall response on $[Ca^{2+}]_i$ was not significantly different from that observed with capsaicin alone (fig. 5).

Discussion

The results reported here indicate that, at least in our in vitro model, stimulation of cannabinoid CB_1 receptors exerts a dual regulatory effect on VR1-induced biological responses, and that the final outcome of this effect depends on the state of activation of cAMP-mediated signalling. We found that a 5-min pre-treatment with the CB_1 agonist, HU-210, of HEK-293 cells co-expressing functionally active CB_1 and VR1 receptors (CB_1 -VR1-HEK cells) significantly enhances the capsaicin-induced, and VR1-mediated, increase in $[Ca^{2+}]_i$. When using a 100 nM concentration of HU-210, the EC_{50} for the capsaicin effect in these cells was decreased twofold. This effect was counteracted by the CB_1 -selective antagonist, SR141716A, and was not observed in HEK-293 cells expressing only VR1 receptors (VR1-HEK cells), thus conclusively demonstrating the involvement of CB_1 receptors

in HU-210 action. Interestingly, simultaneous treatment of CB₁-VR1-HEK cells with HU-210 and capsaicin did not lead to a similar potentiation of the effect on [Ca²⁺]_i by the latter compound. The time dependency of the effect suggests that (i) CB₁-coupled intracellular signalling events, rather than a direct interaction between the two receptors, might be necessary to observe the enhancement of VR1-induced biological effects and (ii) endogenous substances, like AEA or N-arachidonoyl-dopamine [28], which are capable of activating both receptor types, might produce different overall biological effects depending on which of the two receptors they activate first.

To investigate the first of the above possibilities, we carried out some pilot experiments. We started from the recent findings that VR1 activity can be enhanced by protein phosphorylation catalysed by protein kinase C (PKC) [16, 29, 30], and inhibited by phosphatidylinositol-bis phosphate (PIP₂) [31], and that CB₁ receptors are coupled to activation of PLC (possibly via the βγ subunits of G_{i/o} proteins [32, 33]) and stimulation of PI-3-K [34, 35]. Therefore, we tested the effects of PLC and PI-3-K inhibitors on the enhancement by HU-210 of the capsaicin effect on [Ca²⁺]_i. We found that, per se, two PI-3-K inhibitors and two PI-PLC inhibitors significantly reduced the effect of capsaicin on [Ca²⁺]_i at the concentrations previously reported to inhibit PI-3-K and PLC,

respectively. Furthermore, these four compounds, at concentrations per se inactive on the capsaicin-induced response, abolished the potentiation of the capsaicin effect caused by pre-treatment with HU-210. PI-3-K is not only responsible for the formation of PIP₂, but it also catalyses its phosphorylation to phosphatidylinositol-tris-phosphate, whereas PI-PLC catalyses PIP₂ hydrolysis. Therefore, on the basis of these experiments, one can hypothesize that, when over-expressed in HEK-293 cells, VR1 is under the negative influence of PIP₂ [31], whose concentration and turnover are in turn controlled by tonic PI-PLC and PI-3-K activity, respectively. When these two enzymes are inhibited, PIP₂ remains associated with VR1 and the effect of capsaicin on [Ca²⁺]_i is therefore reduced. Conversely, further stimulation of PI-PLC [32] and PI-3-K [34] by CB₁ receptors leads to an enhanced turnover of PIP₂, with subsequent release of VR1 from the tonic inhibitory action exerted by this lipid (fig. 6). We also found that the PC-PLC inhibitor, D609, inhibited the potentiation of the capsaicin effect caused by pre-treatment with HU-210. Thus, CB₁ stimulation may also lead to the activation of PC-PLC [33]. This enzyme, together with PI-PLC, causes the release of diacylglycerols and the subsequent activation of PKC, which can then sensitise VR1 to capsaicin [16, 29, 30].

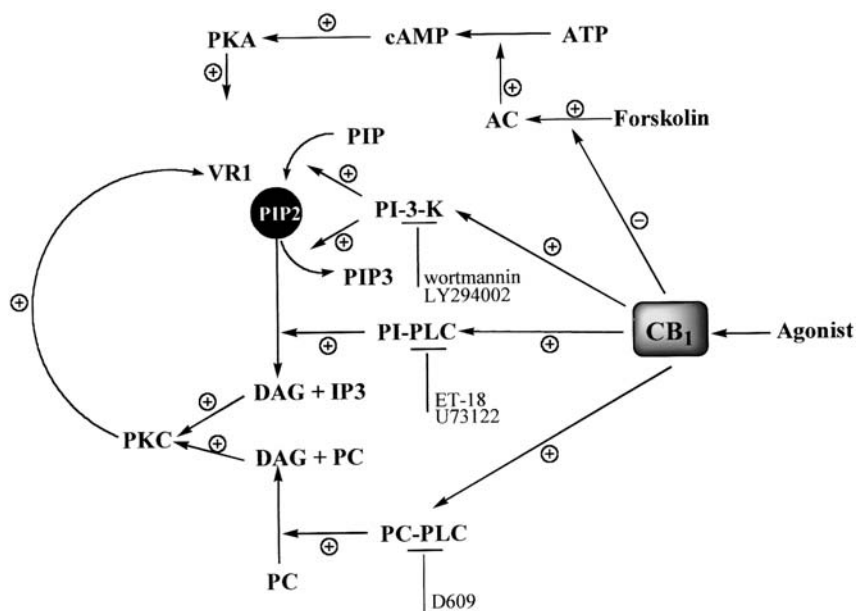


Figure 6. Schematic representation of the possible intracellular pathways underlying the CB₁ receptor-mediated control of VR1 activity. VR1 is tonically inhibited by PIP₂, which in turn can be produced by the action of PI-3-K on phosphatidylinositol-mono-phosphate (PIP), and transformed by the same enzyme into phosphatidylinositol-tris-phosphate (PIP₃), or into diacylglycerols (DAG) and inositol-tris-phosphate (IP₃) by PI-PLC. Thus, inhibitors of PI-3-K and PI-PLC (whose action is indicated by blunt arrows) stabilize the VR1-PIP₂ complex, leading to the inhibition of VR1 sensitivity to capsaicin, or to the inhibition of CB₁-mediated activation of the two enzymes. The potentiation of capsaicin activity by HU-210 observed in this study may involve this signalling pathway. Tonic, or CB₁-induced, stimulation of VR1 by PC-PLC, and subsequent stimulation of DAG release and PKC activity, might also explain why a selective PC-PLC inhibitor (D609), as well as PI-PLC inhibitors, attenuate both basal and HU-210-enhanced activity of capsaicin at VR1. Finally, stimulation of adenylylate cyclase (AC) and protein kinase A (PKA) by forskolin, or during e.g. inflammation, might lead to sensitization (or inhibition of desensitization) of VR1. In this case, activation of CB₁ receptors by agonists, by leading to inhibition of AC, would lead to VR1 inhibition.

A second set of experiments was carried out with exogenous AEA, an endogenous mediator capable of activating both CB₁ and VR1 receptors. Indeed, since the binding sites of CB₁ and VR1 for AEA are extra- and intracellular, respectively [1, 15, 36], and AEA can be rapidly transported into HEK cells [15], treatment of CB₁-VR1-HEK cells with this lipid is likely to produce the sequential stimulation of CB₁ and VR1 receptors, which we found here to be necessary for the enhancement of VR1 activity. Indeed, we observed that AEA was significantly more efficacious on [Ca²⁺]_i in CB₁-VR1-HEK cells than in VR1-HEK cells, and that its effect in the former cells was reduced by the CB₁ antagonist SR141716A to an extent indistinguishable from that observed in VR1-HEK cells. These findings might open the possibility that extracellular AEA exerts a more efficacious action on VR1 in those cells that naturally co-express this receptor together with CB₁ receptors, such as some DRG neurons in culture [4]. Indeed, in sensory neurons, either a strong excitatory effect, or a weaker excitatory effect that is enhanced by CB₁ antagonists have been observed on VR1-mediated cation currents or neuropeptide release [37–39]. In other cells and tissues also, AEA was found to exhibit varying potency at VR1 receptors. In general, one can hypothesize that when a strong VR1-mediated effect is observed, as in the case of mesenteric sensory neurons [12], some DRG preparations [38] and hippocampal slices [40], CB₁ and VR1 receptors are co-expressed in the majority of the cells. Conversely, when both an inhibitory, CB₁-mediated effect (observed at low AEA doses) and an excitatory, VR1-mediated action (observed at high AEA doses and strengthened by CB₁ receptor antagonists) are seen [37, 41, 42], the two receptor types might be co-expressed only in a minority of neurons. Finally, when substances that selectively activate CB₁ receptors, such as HU-210, inhibit the biological effects of substances that selectively activate VR1 receptors, such as capsaicin [21], this might be due to the lack of coupling of CB₁ receptors to those intracellular signalling pathways that facilitate the gating of VR1 (i.e. PI-PLC or PI-3-K; see above), or to their inhibition of signalling events that instead lead to sensitization of VR1 activity [i.e. protein kinase A (PKA); see below].

Recent studies have in fact shown that the sensitivity of VR1 receptors to ligands can be enhanced by substances that stimulate adenylyl cyclase and subsequently activate the cAMP-dependent protein kinase (PKA), thus leading to VR1 phosphorylation [16, 43, 44]. Since CB₁ receptors are coupled to inhibition of adenylyl cyclase via the α subunits of G_{i/o} proteins [1], we reasoned that, in CB₁-VR1-HEK cells, where we found here that stimulation with two distinct CB₁ receptor agonists inhibits the forskolin-induced formation of cAMP, HU-210 would inhibit, rather than enhance the previously reported enhancement of capsaicin VR1-mediated effect on [Ca²⁺]_i

by forskolin. In fact, we found that, in agreement with previous studies carried out with VR1-HEK cells [16], a 5-min pre-treatment with forskolin enhanced the effect of capsaicin on [Ca²⁺]_i also in CB₁-VR1-HEK cells, and that, when incubated together with forskolin, HU-210 totally abolished this enhancement of capsaicin activity. This finding might provide an explanation for the previously reported inhibition of capsaicin-induced thermal and inflammatory hyperalgesia by prior CB₁ receptor stimulation [7, 8, 21]. It is in fact possible that during inflammation, cAMP levels are enhanced, PKA is activated, and VR1 phosphorylated and up-regulated, and that CB₁ receptor agonists inhibit the effects of capsaicin (or of inflammatory stimuli that indirectly gate the VR1 receptors) by inhibiting adenylyl cyclase. By contrast, in other experimental systems, such as the electrically stimulated mouse vas deferens [25], VR1 is possibly not over-activated by the cAMP-signalling cascade, and thus substances that stimulate both CB₁ and VR1 receptors can exert a very strong effect on VR1.

In conclusion, we have demonstrated for the first time that when cannabinoid CB₁ receptors and vanilloid VR1 receptors are co-expressed in the same cells, pre-treatment of cells with CB₁ receptor agonists leads to inhibition of VR1 activity or to its enhanced stimulation depending on whether or not the cAMP-signalling pathway is concomitantly activated. Sequential CB₁-VR1 stimulation occurs *in vitro* when cells are treated first with HU-210 and then with capsaicin, and might occur *in vivo* with extracellular AEA or other endogenous mediators, such as N-arachidonoyl-dopamine, that are capable of activating both receptor types [28]. These findings provide an explanation for the often discrepant effects of AEA on sensory neurons, and strengthen the hypothesis that CB₁ and VR1 receptors can be regarded as interacting metabotropic and ionotropic receptors for this endogenous compound and some of its congeners [45].

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