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The Activity of Anandamide at Vanilloid VR1 Receptors Requires Facilitated Transport across the Cell Membrane and Is Limited by Intracellular Metabolism*

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The endogenous ligand of CB_1 cannabinoid receptors, anandamide, is also a full agonist at vanilloid VR1 receptors for capsaicin and resiniferatoxin, thereby causing an increase in cytosolic Ca^{2+} concentration in human VR1-overexpressing (hVR1-HEK) cells. Two selective inhibitors of anandamide facilitated transport into cells, VDM11 and VDM13, and two inhibitors of anandamide enzymatic hydrolysis, phenylmethylsulfonyl fluoride and methylarachidonoyl fluorophosphonate, inhibited and enhanced, respectively, the VR1-mediated effect of anandamide, but not of resiniferatoxin or capsaicin. The nitric oxide donor, sodium nitroprusside, known to stimulate anandamide transport, enhanced anandamide effect on the cytosolic Ca^{2+} concentration. Accordingly, hVR1-HEK cells contain an anandamide membrane transporter inhibited by VDM11 and VDM13 and activated by sodium nitroprusside, and an anandamide hydrolase activity sensitive to phenylmethylsulfonyl fluoride and methylarachidonoyl fluorophosphonate, and a fatty acid amide hydrolase transcript. These findings suggest the following. (i) Anandamide activates VR1 receptors by acting at an intracellular site. (ii) Degradation by fatty acid amide hydrolase limits anandamide activity on VR1; and (iii) the anandamide membrane transporter inhibitors can be used to distinguish between CB_1 or VR1 receptor-mediated actions of anandamide. By contrast, the CB_1 receptor antagonist SR141716A inhibited also the VR1-mediated effect of anandamide and capsaicin on cytosolic Ca^{2+} concentration, although at concentrations higher than those required for CB_1 antagonism.

Anandamide (*N*-arachidonoyl ethanolamine, AEA,[1] Ref. 1) was isolated from porcine brain and proposed as an endogenous agonist of cannabinoid CB_1 receptors, which are present in the nervous system as well as in cardiovascular, reproductive, and gastrointestinal tissues (2). Since its discovery, several observations have supported the hypothesis that AEA is an endocannabinoid, *i.e.* a functional activator of CB_1 receptors (see Refs. 3–6 for recent reviews). However, there have been numerous reports showing that AEA is only a partial agonist at these receptors (see Refs. 7, 8 for examples), whereas other observations have suggested that there may be additional molecular targets for this substance (see Refs. 9, 10 for examples, and Ref. 6 for review). Several investigators (11–14) have provided pharmacological and molecular evidence that AEA is a full agonist at the receptor for capsaicin, the substance responsible for the pungent taste of hot chili peppers (15). This receptor is a ligand-gated, nonselective cation channel and was recently cloned and named VR1 (16). It is most abundant in sensory neurons but is also found in select areas of the central nervous system of rats and men (17). AEA, at concentrations similar or higher than those necessary to activate CB_1 cannabinoid receptors, elicits typical VR1-mediated functional responses, such as endothelium-independent vasodilation of small arteries (11), cation inward currents in sensory neurons (12), apoptosis (13), and inhibition of electrically stimulated mouse vas deferens (14). The selective antagonist of vanilloid receptors, capsazepine (18) strongly reduces these effects. Furthermore, AEA activates VR1-mediated cation currents in human embryonic kidney (HEK) 293 cells or *Xenopus* oocytes overexpressing either rat or human VR1 (11, 12), and displaces the high affinity VR1 ligand, [^{3}H]resiniferatoxin, from binding sites in membranes of CHO cells overexpressing rat VR1 (14). These data indicate that, along with its proposed role as an endocannabinoid, AEA might also function as an endovanilloid. However, whether or not AEA behaves as a physiological ligand for VR1 is still being debated (19, 20), and further studies need to be performed to give an answer to this question.

The possible regulation of the activity of AEA at VR1 receptors by biochemical events leading to the physiological inactivation of AEA and by agents that pharmacologically or physiologically modulate these events, could be taken as evidence in favor of a possible role for this compound also as an endovanilloid. Furthermore, the use of antagonists for either CB_1 or

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[1] The abbreviations used are: AEA, arachidonoylethanolamide, anandamide; HEK, human embryonic kidney; CCC, cytosolic Ca^{2+} concentration; PMSF, phenylmethylsulfonyl fluoride; MAFP, methylarachidonoyl fluorophosphonate; SNP, sodium nitroprusside; LOX, lipoxygenase; ETYA, 5,8,11,14-eicosatetraynoic acid; CA, caffeic acid; RP-HPLC, reverse phase-high pressure liquid chromatography; FAAH, fatty acid amide hydrolase; AMT, anandamide membrane transporter; HPETEs, hydroperoxy-eicosatetraenoic acids; VDM11, *N*-(2-methyl-4-hydroxy-phenyl)-arachidonamide; VDM13, *N*-arachidonoyl-5-methoxytryptamine; HETEE, hydroperoxy-eicosatetraenoylethanolamide; RT-PCR, reverse transcriptase-polymerase chain reaction; bp, base pairs.

VR1 receptors, such as SR1417161 (21) and capsazepine (18), respectively, should help in discriminating between those effects of AEA that are mediated by either receptor. Therefore, in the present study we have addressed the question of whether the mechanisms previously shown to cause the inactivation of AEA, *i.e.* uptake by cells facilitated by a selective AEA membrane transporter (AMT), followed by either hydrolysis catalyzed by fatty acid amide hydrolase (FAAH) or oxidation by enzymes of the arachidonate cascade such as lipoxygenases (LOXs) (see Refs. 4, 22, 23 for specific reviews), also modulate the effect of AEA on VR1 receptors. Furthermore, based on previous reports showing that SR141716A, particularly at high concentrations, is not selective for the CB_1 receptor (24, 25), we have investigated the effects of this compound on a typical vanilloid-like, and non-CB_1-mediated, effect of AEA, to assess whether it can be used to discriminate between VR1- and CB_1-mediated AEA actions. Our results suggest that AEA interacts with VR1 at an intracellular site, and, hence, its activity at either VR1 or CB_1 receptors can be regulated via the AMT and is significantly limited by intracellular enzymatic hydrolysis. Moreover, our data show that SR141716A can act as an inhibitor of VR1-mediated signaling, albeit at concentrations higher than those required for CB_1 receptor antagonism and as such should be used with some caution to distinguish between AEA as an endocannabinoid or endovanilloid.

EXPERIMENTAL PROCEDURES

Transfected Cells—Expression of hVR1 cDNA into HEK 293 cells was carried out as described previously (26). Cells were grown as monolayers in minimum essential medium supplemented with nonessential amino acids, 10% fetal calf serum, and 0.2 mM glutamine and maintained under 95:5% O_2/CO_2 at 37 °C.

Compounds—Capsaicin, AEA, and methylarachidonoyl fluorophosphonate (MAFP) were purchased from Cayman Chemicals (Ann Arbor, MI). VDM11 and VDM13 (Fig. 1; Ref. 27) were synthesized from the corresponding amines and arachidonoyl chloride (all from Sigma) in dimethylformamide, in the presence of 1.1 equivalents of triethylamine for 18 h at 4 °C. The reaction was stopped by adding water and by extracting the products with diethyl ether. The compounds were purified by direct phase-high pressure liquid chromatography, and chemical structures were confirmed by means of proton nuclear magnetic resonance and infrared spectroscopy. [^{14}C]AEA (5 mCi/mmol) was synthesized from [^{14}C]ethanolamine and arachidonoyl chloride as described (1). SR141716A was kindly donated by Sanofi Recherche, Montpellier, France. Sodium nitroprusside (SNP), phenylmethylsulfonyl fluoride (PMSF), arachidonic acid, 5,8,11,14-eicosatetraynoic acid (ETYA) and caffeic acid (CA) were purchased from Sigma. Capsazepine and resiniferatoxin were purchased from Alexis Biochemicals. The unselective AMT inhibitor AM404 was purchased from Cayman Chemicals (Ann Arbour, MI). The 5(*S*)-, 11(*S*)-, and 15(*S*)- hydroperoxy derivatives of AEA (Fig. 1) were generated by incubating 40 μM AEA with soybean lipoxygenase-1 (sLOX) or barley lipoxygenase-1 (bLOX), purified as reported (sLOX, Ref. 28; bLOX, Ref. 29). AEA was incubated with lipoxygenase (1 unit per 3 μmol of substrate) in 100 mM sodium borate buffer (pH 9.0 for sLOX and pH 7.0 for bLOX), following the reaction spectrophotometrically at 236 nm. After completion (15 min), the pH was lowered to 4, and the products were purified with SPE columns (Bakerbond 500 mg, J.T. Baker) and then with reverse phase-HPLC as described (30). Reverse phase-HPLC was carried out on a Cosmosil 5C18 AR column (5 μm, 250 × 4.6 mm, Nacalai Tesque, Japan) using tetrahydrofuran/methanol/water/acetic acid (25:40:35:0.1, v/v/v/v) as eluent, at a flow rate of 1 ml/min. 15-hydroperoxy-eicosatetraenoylethanolamide (HETEE) was the major product (~95%) of sLOX, whereas 11- and 5-HEA were the major products (~70% and ~15% respectively) of bLOX. The enantiomeric ratio (*S*/*R*) of each hydroperoxide was found to be 95:5 by chiral separations and CD spectroscopy (30).

Cytosolic Ca^{2+} Concentration (CCC) Assays—The effect of the substances on CCC (27) was determined by using Fluo-3, a selective intracellular fluorescent probe for Ca^{2+}. One day prior to experiments, hVR1-HEK cells were transferred into 6-well dishes coated with poly-L-lysine (Sigma) and grown in the culture medium mentioned above. On the day of the experiment, the cells (50,000–60,000 per well) were loaded for 2 h at 25 °C with 4 μM fluo-3 methylester (Molecular Probes) in Me_2SO containing 0.04% pluoronic. After the loading, hVR1-HEK cells were washed with Tyrode pH = 7.4, trypsinized, resuspended in Tyrode, and transferred to the cuvette of the fluorescence detector (Perkin-Elmer LS50B) under continuous stirring. 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. Capsazepine and SR141716A or EGTA (4 mM), MAFP, PMSF, SNP, ETYA, and CA were added 30 or 10 min, respectively, before AEA or capsaicin or resiniferatoxin, whereas the AMT inhibitors were added 5 min before. Data are expressed as the concentration exerting a half-maximal effect (EC_{50}). The efficacy of the effect was determined by comparing it to the analogous effect observed with 4 μM ionomycin. The inhibitory effects of AMT inhibitors and of antagonists were expressed as IC_{50} calculated by GraphPad software. For the antagonists, K_i values at approximately half-saturating concentrations of agonists were calculated by means of the Cheng-Prusoff equation. K_c values for competitive antagonism were calculated by means of the following equation: $K_c = [ant]/\{(EC_{50}^{+}/EC_{50}^{-}) - 1\}$, where [ant] is the concentration of SR141716A (2.5 μM) and EC_{50}^{+} and EC_{50}^{-} are the EC_{50} values obtained for agonists in the presence and absence of 2.5 μM SR141716A, respectively.

AEA Hydrolase Activity Assays—hVR1-HEK cells were cultured as described above. The effect of VDM11, VDM13, PMSF, and MAFP on the enzymatic hydrolysis of AEA was studied as described previously (31) by using cell membranes incubated with either of the two compounds at different concentrations and [^{14}C]AEA (9 μM) in 50 mM Tris-HCl, pH 9, for 30 min at 37 °C. [^{14}C]Ethanolamine produced from [^{14}C]AEA hydrolysis was measured by scintillation counting of the aqueous phase after extraction of the incubation mixture with 2 volumes of $CHCl_3/CH_3OH$ 2:1 (v/v).

Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) for FAAH—Analysis of the RNA from hVR1-HEK cells for the presence of a FAAH transcript was carried out by means of RT-PCR. Total RNA was extracted from cells (10×10^6/test) as described (32). cDNA synthesis was performed in a 20-μl reaction mixture containing 75 mM KCl, 3 mM $MgCl_2$, 10 mM dithiothreitol, 1 mM dNTPs, 50 mM Tris-HCl pH 8.3, 5 μg of total RNA, 0.125 A_{260} units of hexanucleotide mixture (Roche Molecular Biochemicals) for random priming and 200 units of Superscript II RNase H^- reverse transcriptase (Life Technologies, Inc.). The cDNA reaction mixture was incubated at 25 °C for 10 min and then at 42 °C for 50 min, and the reaction was stopped by heating at 95 °C for 5 min followed by lowering the temperature at 4 °C. RT-PCR amplification was performed by means of a combination of hot-start and touch-down PCR, and using 2 μl of the cDNA and 1.25 units of *Taq* GOLD (PerkinElmer Life Sciences) in 50 μl of its buffer containing 3 mM $MgCl_2$, 250 μM of each dNTPs, and 0.5 μM each of 5′ and 3′ primers. Reactions were performed in a Gene Amp PCR System 9600 thermocycler (PerkinElmer Life Sciences). The amplification profile consisted of an initial denaturation of 10 min at 92 °C and 15 cycles of 30 s at 95 °C, 1 min at 66 °C (annealing) and 1 min at 68 °C (with an annealing temperature stepping down of 1 °C every 3 cycles, from 66 to 61 °C), followed by 25 cycles of 30 s at 95 °C, 1 min at 61 °C, and 1 min at 72 °C. A final extension of 15 min was carried out at 72 °C. The primers used were: FAAH sense primer, 5′-GCCTGGGAAGTGAACAAAGGGACC-3′ and FAAH antisense primer, 5′-CCACTACGCTGTCGCACTCCGCCG-3′. The expected sizes of the amplicon was 202 bp. β2-microglobulin was used as the housekeeping gene (32). PCR primers for FAAH were selected on the basis of the sequence of the *FAAH* human gene (GenBank™/EBI accession number AF098012) by including the intron 497–722. In the presence of contaminant genomic DNA, the expected size of the amplicon would be 425 bp. PCR products (15 μl) were electrophoresed on 2% agarose gel (MS agarose, Roche Molecular Biochemicals) in 1× TAE buffer at 4 V/cm for 4 h. Ethidium bromide (0.1 μg/ml) was included both in the gel and electrophoresis buffer, and PCR products were detected by UV visualization. No PCR product was detected in the absence of cDNA, primers, or Red-hot DNA polymerase. DNA ladder (100-bp molecular ruler, Bio-Rad) was run as a marker.

AEA Transporter Assays—The time- and temperature-dependent uptake of [^{14}C]AEA by intact hVR1-HEK cells was studied as described previously for RBL-2H3 cells (31). The effect of compounds on the uptake of AEA was studied by using 3.6 μM (10,000 cpm) [^{14}C]AEA. Cells were incubated with [^{14}C]AEA for 5 min at 37 °C, with or without varying concentrations of the inhibitors. Residual [^{14}C]AEA in the incubation medium after extraction with $CHCl_3/CH_3OH$ 2:1 (v/v), determined by scintillation counting of the lyophilized organic phase, was used as a measure of the AEA that was taken up by cells. Data are expressed as the concentration exerting 50% inhibition of AEA uptake (IC_{50}).

Receptor Binding Assays—Displacement assays for CB_1 receptors

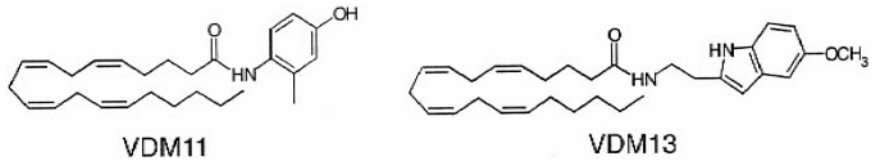

FIG. 1. **Chemical structures of the two new AMT inhibitors, VDM11 and VDM13 that have little or no activity at VR1 receptors (27).**

were carried out by using [^{3}H]SR141716A (0.4 nM, 55 Ci/mmol, Amersham Pharmacia Biotech) as the high affinity ligand and the filtration technique previously described (33) on membrane preparations (0.4 mg/tube) from male CD rat brains (Charles River, Italia) and in the presence of 100 μM PMSF. Specific binding was calculated with 1 μM SR141716A (a gift from Sanofi Recherche, France) and was 84.0%. The K_i value for capsazepine was calculated by applying the Cheng-Prusoff equation to the IC_{50} value (obtained by GraphPad) for the displacement of the bound [^{3}H]SR141716A by increasing concentrations of the test compounds.

RESULTS

Effect of AMT Modulators on AEA Action at VR1 Receptors—In agreement with previous studies (11, 12, 27), we found that AEA, capsaicin, and AM404 are full agonists at human VR1 receptors overexpressed in HEK cells in as much as they elicited a typical VR1 response, *i.e.* the increase of CCC. The EC_{50} for AEA, capsaicin, and AM404 were 0.52 ± 0.12, 0.019 ± 0.009, and 0.035 ± 0.011 μM, respectively. These effects were blocked by EGTA (4 mM, not shown) and by increasing concentrations of the VR1 antagonist capsazepine (see below) and were not observed in nontransfected HEK cells (data not shown).

Two selective and recently developed inhibitors of the AMT, VDM11, and VDM13 (Ref. 27, Fig. 1) strongly inhibited the AEA effect on CCC in hVR1-HEK cells (Fig. 2, Table I). The IC_{50} for VDM11 with a 5-min preincubation was 3.9 ± 1.1 μM, a value similar to that for VDM11 inhibition of the AMT in rat C6 glioma and basophilic leukemia (RBL-2H3) cells (IC_{50} ~10 μM, Ref. 27). VDM11 (4 μM) did not affect the efficacy and potency of capsaicin or resiniferatoxin on CCC (Table I). This compound, at a 10-μM concentration, did inhibit the capsaicin effect if preincubated 5 min prior to the VR1 agonist, but it was inactive when preincubated 30 min before capsaicin (data not shown). It is noteworthy that VDM11 and VDM13 alone (10 μM) (i) exhibited a negligible stimulatory effect on CCC (14.1 ± 5.9 and <5% of the effect of ionomycin, respectively); (ii) had little effect on [^{3}H]SR141716A specific binding from rat brain membranes (11.6 ± 1.8 and 29.1 ± 2.3% displacement, respectively; K_i>20 μM in both cases); and (iii) did not significantly inhibit [^{14}C]AEA hydrolysis by hVR1-HEK cells (<5% and 15.3 ± 4.5% inhibition, respectively) (see also Ref. 27).

Finally, the NO donor SNP enhanced dose-dependently the effect of AEA, but not capsaicin or resiniferatoxin, on CCC (Table I). SNP alone did not produce any significant effect on CCC (<5% of the effect of ionomycin) or [^{14}C]AEA hydrolysis (<5% inhibition).

AEA Uptake by hVR1-HEK Cells—Intact hVR1-HEK cells were shown to uptake [^{14}C]AEA in a time- and temperature-dependent manner (Fig. 3*A*). This process was inhibited dose-dependently by coincubation with VDM11 and AM404 (estimated IC_{50} = 4.0 ± 0.6 and 3.6 ± 0.7 μM, respectively) and VDM13, and was enhanced by SNP (5 mM) (Fig. 3*B* and data not shown).

Effect of FAAH Inhibitors on AEA Action at VR1 Receptors—The general serine protease inhibitor PMSF and the more selective FAAH inhibitor MAFP (34, 35) were used. We found that both compounds, preincubated with cells for 10 min and at concentrations previously found to be fully effective against AEA hydrolysis and FAAH in other cell lines, dose-dependently enhanced the effect of submaximal doses of AEA, but not capsaicin, on VR1-mediated CCC increase (Fig. 2, Table I). Neither PMSF (250 μM) nor MAFP (75 nM) alone exhibited any significant effect on CCC (<5% of ionomycin effect, data not shown).

Effect of FAAH Inhibitors on AEA Hydrolysis by hVR1-HEK Cell Membranes—PMSF and MAFP dose-dependently inhibited the hydrolysis of AEA by hVR1-HEK cell membranes. Basal activity at pH 9 was 27.0 ± 5.8 pmol min^{-1} mg protein^{-1}, which was decreased to 11.1 ± 3.9 and 2.9 ± 0.4 pmol min^{-1} mg protein^{-1} with 100 and 250 μM PMSF, and to 21.1 ± 3.5, 8.8 ± 1.5 and 3.0 ± 0.7 pmol min^{-1} mg protein^{-1}, with 10, 50, and 75 nM MAFP, respectively (n = 3 ± S.E., $p < 0.01$ by analysis of variance with the two highest concentrations of inhibitors).

hVR1-HEK Cells Express a FAAH Transcript—cDNA, obtained by retrotranscription of total RNA from hVR1-HEK cells, was amplified by RT-PCR using probes specific for human FAAH. The amplified fragment was analyzed by agarose gel electrophoresis and showed a single band of the molecular size (202 bp) expected from the human FAAH-encoding cDNA fragment (Fig. 3*C*, *lane 1*). This band could not be attributed to the presence of contaminating DNA, because in this case the expected molecular weight should have been higher (425 bp, Fig. 3*C*, *lane 2*). No band was present in an RNA sample that had not undergone retrotranscription (Fig. 3*C*, *lane 3*).

Effect of Hydroperoxy-eicosatetraenoylethanolamides on VR1 Receptors—5(*S*)-, 11(*S*)-, and 15(*S*)-hydroperoxy-eicosatetraenoylethanolamide were prepared and purified as described in "Experimental Procedures" and were tested on CCC in hVR1-HEK cells. Only the latter compound was found to induce an increase in CCC (Fig. 4), and only at concentrations significantly higher than those necessary to AEA to exert the same effect. We also found that nonselective inhibition of LOX by CA (50 μM) and ETYA (10 μM) did not influence the effect on CCC of either sub- or near-maximal concentrations of AEA, whereas arachidonic acid (10 μM) exerted only a very little effect *per se* (Table I and data not shown).

Effect of SR141716A and Capsazepine on VR1 Receptors—We tested several concentrations of SR141716A (with 30-min preincubation) on the effects of several concentrations of capsaicin, AEA, and AM404 on hVR1-mediated CCC increase in hVR1-HEK cells (Figs. 5 and 6). SR141716A inhibited the effect of the agonists with K_i values of 1.1 ± 0.2, 1.3 ± 0.1, and 1.0 ± 0.1 μM for AEA (0.5 μM), AM404 (32 nM), and capsaicin (20 nM), respectively (means ± S.D., n = 3). SR141716A *per se* also exerted a weak enhancement of CCC but only at high doses (17.9 ± 2.1 and 36.0 ± 3.2% stimulation at 5 and 10 μM, respectively) and in a manner partly sensitive to 5 μM capsazepine (7.1 ± 1.2 and 19.5 ± 1.6% stimulation, respectively, means ± S.D., n = 3, $p < 0.05$ by the t test). The dose-response curve of capsaicin in the presence of the antagonist was shifted to the right to parallel curves (Fig. 5*C*). The dose-response curves for AEA and AM404 were also shifted to the right when they were administered after increasing concentrations of SR141716A (Fig. 5, *A* and *B*), although the maximal effects of these two agonists in the presence of the two highest concentrations of the antagonist did not reach the values observed in its absence. Furthermore, the inhibitory

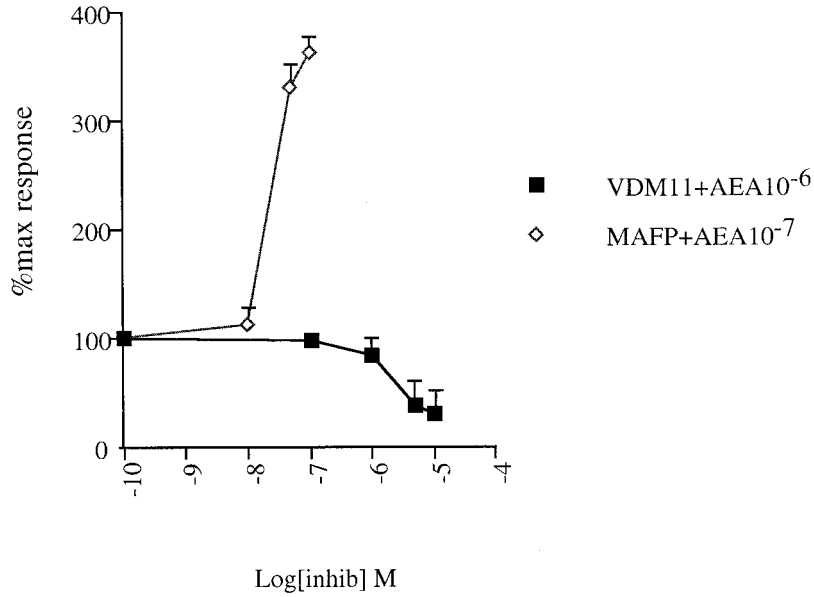

FIG. 2. **Dose-related effect of the AMT inhibitor, VDM11, and the FAAH inhibitor, MAFP, on AEA-induced increase of cytosolic Ca^{2+} concentration in hVR1-HEK cells.** The effects are reported as percent of the effects observed with AEA + vehicle (see Table I). The concentration of AEA used was 0.1 μM with MAFP and 1 μM with VDM11. Data are means ± S.D. of $n = 3$ experiments. VDM11 was added 5 min before AEA; MAFP 10 min before AEA.

effect of SR141716A on capsaicin-induced CCC increase reached a maximum with a 2.5-μM concentration, whereas with AEA and AM404, the effect was maximal at 5 μM (Figs. 5 and 6). With 2.5 μM SR141716A, however, it was possible to calculate K_c values for inhibition of CCC increase induced by the three VR1 agonists, and these values were similar for AEA, AM404, and capsaicin (0.13 ± 0.04, 0.20 ± 0.06, and 0.28 ± 0.03 μM, respectively, means ± S.D., $n = 3$). Capsazepine was ineffective as a CB_1 receptor ligand (K_i>10 μM) in CB_1 receptor binding assays but inhibited AEA effect on CCC with a potency ~30-fold higher than SR141716A (K_i = 35.5 ± 3.2 nM, Fig. 6*C*).

DISCUSSION

We confirmed that AEA acts as a full agonist on VR1 receptors by enhancing CCC in HEK cells transfected with human VR1 receptor cDNA. This effect was due uniquely to activation of VR1, and not CB_1, receptors because it was: 1) observed, at lower doses, also with capsaicin, which does not activate CB_1 receptors (36); 2) completely blocked by the selective VR1 receptor antagonist, capsazepine; and 3) not observed in non-transfected HEK cells. Furthermore, HEK cells do not express CB_1 receptors (see below). One of the aims of this study was to understand whether AEA actions at VR1 receptors are regulated by the mechanisms previously described to lead to AEA metabolism in intact living cells. This issue is not all obvious because, for example, recent studies suggest that the binding site for capsaicin on VR1 receptors is intracellular (37), and not extracellular as for most membrane receptors described to date. Therefore, to activate VR1 receptors, capsaicin must first cross the cell membrane, and it is possible that the transport of AEA into cells via the AMT facilitates rather than terminate AEA action at VR1. Furthermore, a recent report showed that LOX products of arachidonic acid, the hydroperoxyeicosatetraenoic acids (HPETEs), are more potent than AEA as agonists of rat VR1 receptors (38). Therefore, hydrolysis of AEA to arachidonic acid might mediate AEA effect on VR1 receptors. The data presented here demonstrate that AEA-facilitated transport into cells through the AMT is necessary for VR1 activation by this compound; and AEA enzymatic hydrolysis plays an important role in terminating, as opposed to mediating, the vanilloid-like activity of AEA.

We found that two selective and recently developed inhibitors of the AMT, VDM11, and VDM13 (Ref. 27, Fig. 1), strongly inhibit the AEA effect on CCC in hVR1-HEK cells. This is exactly the opposite of what was previously found for the effect of AMT inhibitors on AEA actions that are mediated by CB_1 receptors (39, 40). For example, VDM11 (10 μM) enhances 2-fold the potency of a typical CB_1-mediated effect of AEA (41) that is the mobilization of Ca^{2+} from intracellular stores in N18TG2 cells.[2] Indeed, intact hVR1-HEK cells were shown here to take up AEA via a time- and temperature-dependent mechanism that could be inhibited by VDM11 with exactly the same potency as that observed for the inhibition of AEA-induced increase of CCC. VR1 receptors are readily desensitized following treatment with vanilloid receptor agonists (15). This

TABLE I

Effect of various substances on anandamide (AEA)-, capsaicin (Caps)-, and resiniferatoxin (RTX)-induced increase in cytosolic Ca^{2+} concentration (CCC) in hVR1-HEK cells

The effects are reported as % of the effects observed with agonists + vehicle. In parentheses, the effects of AEA, Caps, RTX, and arachidonic acid are reported as % of maximal possible CCC increase, measured with 4 μM ionomycin. Data are means ± S.E. of $n = 3$ experiments. *, $p < 0.05$; **, $p < 0.01$ *versus* AEA + vehicle, as calculated by the unpaired Students *t* test. SNP, CA, ETYA, MAFP, and PMSF were added 10 min prior to the agonists. VDM11 and VDM13 were added 5 min prior to the agonists.

AEA (1 μM) + vehicle	100 (58.4 ± 5.6)
+ VDM11 (4 μM)	48.8 ± 2.9*
+ VDM13 (4 μM)	59.8 ± 3.7*
+ VDM13 (10 μM)	11.5 ± 0.2*
+ SNP (5 mM)	135.1 ± 11.2*
+ SNP (10 mM)	163.6 ± 11.6*
+ CA (50 μM)	106.7 ± 7.3
+ ETYA (10 μM)	85.4 ± 9.2
AEA (0.1 μM) + vehicle	100 (14.1 ± 8.7)
+ PMSF (100 μM)	257.2 ± 30.6*
+ PMSF (250 μM)	561.5 ± 62.9**
+ MAFP (75 nM)	328.8 ± 25.9**
+ SNP (5 mM)	110.1 ± 4.1*
+ SNP (10 mM)	142.2 ± 10.9*
Caps (0.1 μM) + vehicle	100 (70.0 ± 6.9)
+ VDM11 (4 μM)	97.8 ± 7.8
+ SNP (5 mM)	118.1 ± 10.5
Caps (0.01 μM) + vehicle	100 (21.4 ± 3.1)
+ PMSF (250 μM)	95.2 ± 8.3
+ MAFP (75 nM)	88.3 ± 9.0
RTX (1 μM) + vehicle	100 (71.6 ± 7.7)
+ VDM11 (4 μM)	101.7 ± 9.3
+ SNP (5 mM)	108.4 ± 7
Arachidonic acid (10 μM)	(5.4 ± 5.2)

[2] V. Di Marzo and L. De Petrocellis, unpublished observations.

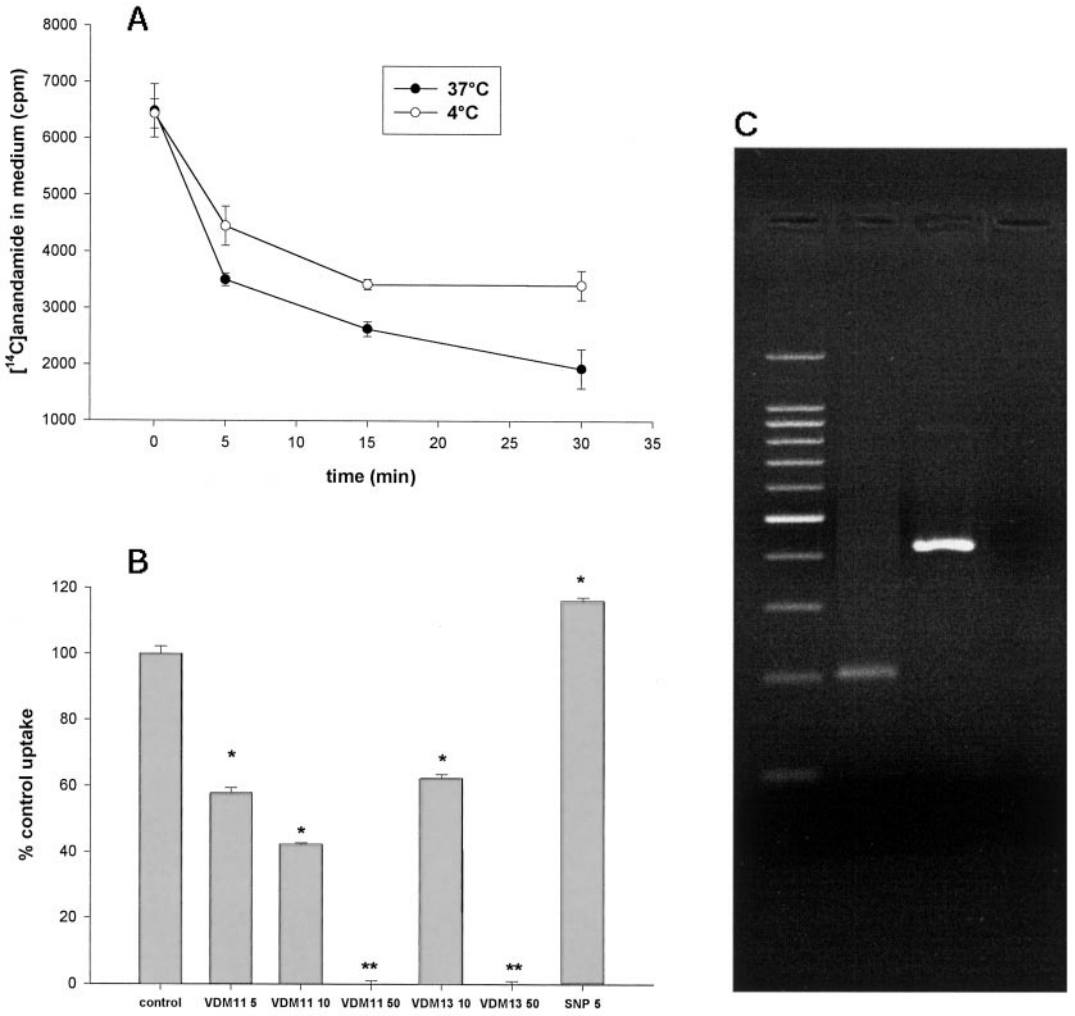

FIG. 3. **Uptake of anandamide and FAAH expression in intact hVR1-HEK cells.** *A,* time-dependent uptake of [^{14}C]anandamide and effect of low temperature. *B,* effect of various substances on the amounts of [^{14}C]anandamide taken up after 5-min incubation with cells. VDM11 5, VDM11 5 μM; VDM11 10, VDM11 10 μM; VDM11 50, VDM11 50 μM; VDM13 10, VDM13 10 μM; VDM13 50, VDM13 50 μM; SNP 5, sodium nitroprusside 5 mM. Data are means ± S.D. of $n = 3$ experiments. *, $p < 0.05$; **, $p < 0.01$ *versus* control, as assessed by analysis of variance. Inhibitors were added 5 min before [^{14}C]anandamide. *C,* agarose gel electrophoresis of RT-PCR transcripts obtained by using cDNA (*lane 2*), DNA (*lane 3*), and RNA (*lane 4*) from hVR1-HEK cells. Amplification was carried out by using oligoprobes for human FAAH. A DNA ladder as bp-molecular ruler is also shown. This figure is representative of three separate experiments.

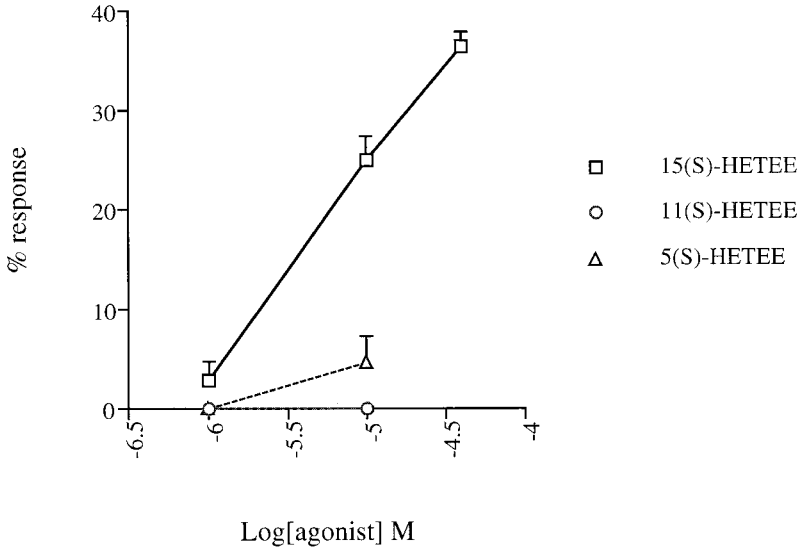

FIG. 4. **Effect of various HETEE on cytosolic Ca^{2+} concentration into hVR1-HEK cells.** The effects are reported as percent of the effects observed with 4 μM ionomycin. Data are means ± S.D. of $n = 3$ experiments.

is the reason why we did not use the previously developed AMT inhibitors, linvanil (40) and AM404 (39), which potently activate *per se* both rat and human VR1 (14, 27, 42–44). With these compounds we might have observed either desensitization or an AMT-independent potentiation of AEA effect on VR1. By contrast, VDM11 and VDM13, whereas being equipotent to the

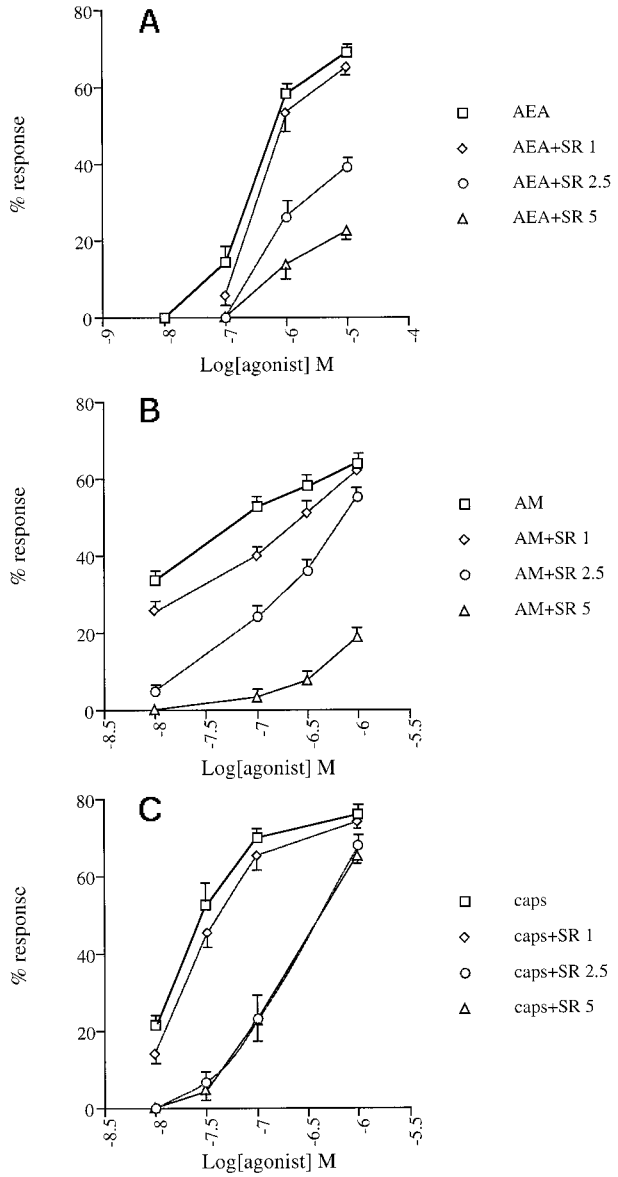

FIG. 5. **Effect of various concentrations of SR141716A on the cytosolic Ca^{2+} concentration in hVR1-HEK cells induced by anandamide (*AEA*, *A*), AM404 (*AM*, *B*), and capsaicin (*caps*, *C*).** The effects are reported as percent of the effects observed with 4 μM ionomycin. SR1, SR141716A 1 μM, SR2.5, SR141716A 2.5 μM, SR5, SR141716A 5 μM. The dose-response curves obtained with 10 μM SR141716A are not shown because they were almost superimposable to those obtained with 5 μM. In all cases, SR141716A was added 30 min before the agonists. Data are means ± S.D. of $n = 3$ experiments.

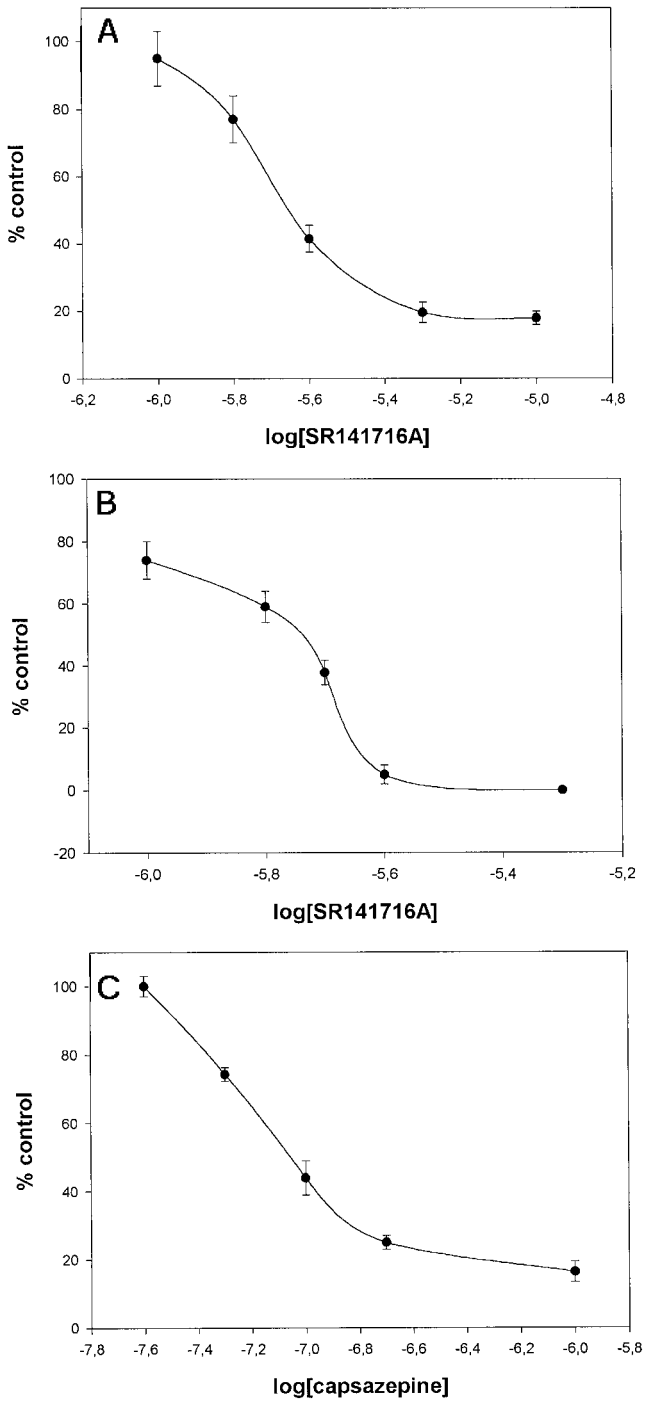

FIG. 6. **Effect of various doses of SR141716A (*A*, *B*) and capsazepine (*C*) on the increase of cytosolic Ca^{2+} concentration in hVR1-HEK cells induced by anandamide (0.5 μM; *A* and *C*) or capsaicin (20 nM; *B*).** The effects are reported as percent of the effects observed with agonist + vehicle (see Table I). Both antagonists were added 30 min before AEA. Data are means ± S.D. of $n = 3$ experiments.

most widely used AMT inhibitor, AM404 (39) in both hVR1-HEK (this study) and other cells (27), are almost inactive *per se* on VR1 receptors (27), thus making it unlikely that their inhibitory action on AEA-induced CCC increase was because of desensitization of these receptors. Furthermore, a half-maximal concentration of VDM11 did not inhibit the effect on CCC produced by either resiniferatoxin or capsaicin, thus arguing against a possible action of this AMT inhibitor through VR1 desensitization or, for example, as a VR1 antagonist. An inhibitory effect on capsaicin-induced CCC increase was observed only with a maximal concentration of VDM11 and with a 5 min pre-incubation. This might be explained with the observation that capsaicin is also recognized by the AMT, although to a smaller extent than AEA (44). In fact, when administered to cells 30 min before capsaicin, VDM11 did not inhibit its effect (27), thus conclusively ruling out desensitization of VR1 receptors, a phenomenon that would last for several hours. Finally, VDM11 and VDM13 are also almost inactive on proteins that might have interfered with the action of AEA on CCC, *i.e.* CB_1 cannabinoid receptors (which, however, are not expressed in the cell model under study) and FAAH. Therefore, to the best of our knowledge, the effect of these two compounds on AEA-induced and VR1-mediated rise in CCC could be due only to interference with the AMT. In further support of the facilitating role of the AMT in the action of AEA on VR1 receptors we found that the NO-donor, SNP, previously shown to activate

the AMT in several cell types (40, 45), significantly enhanced both AEA uptake by hVR1-HEK cells and the stimulatory effect of AEA (but not capsaicin or resiniferatoxin) on VR1-mediated CCC increase in these cells. It is noteworthy that NO donors were previously shown to inhibit a CB_1 receptor-mediated effect of AEA, *i.e.* the inhibition of adenylate cyclase (40).

These data indicate that AEA uptake by cells via the AMT plays a permissive role in the action of exogenous AEA on VR1 receptors, and suggest that AEA site of action on VR1 receptors, like for capsaicin (37), is intracellular. This finding was confirmed by preliminary data obtained in dorsal root ganglia[3] indicating that VDM11 (3 μM) also suppresses another VR1-mediated effect of AEA (1 μM), *i.e.* the release of calcitonin gene-related peptide (46) and has numerous important implications. First, AEA is not stored in and released from preformed vescicles, and the biochemical pathway most likely to generate this compound in stimulated neurons is the phospholipase D-catalyzed hydrolysis of a membrane phospholipid precursor (see Ref. 47 and Ref. 3 for review). Therefore, it is possible that in certain cells AEA synthesized *de novo* acts first as an intracellular endovanilloid and then as an extracellular endocannabinoid, once it is released from the cells, most likely through the AMT itself (22). It is possible that cells have the means of shifting the target (and the subsequent biological effect) of extracellular AEA from CB_1 to VR1 receptors, or of intracellular AEA from VR1 to CB_1 receptors, by regulating the activity of the AMT. This strategy could be also exploited pharmacologically to select the pharmacological action of exogenous AEA or, preferably, of its more stable synthetic analogs such as the CB_1/VR1 hybrid ligand, arvanil (33, 44). This would be important, for example, for the therapeutic treatment of pain, as it has been shown that both CB_1 and VR1 receptor agonists can produce analgesia, although through different mechanisms and under different conditions. On the other hand, AEA was suggested to exert opposite effects on cancer cell apoptosis depending on whether it acts via VR1 or CB_1 receptors (13). Thus, coadministration of AEA and arvanil with either inhibitors or activators of AMT may lead the different effects on pain or cancer cell growth *in vivo*. Finally, the capability of some synthetic VR1 agonists, such as olvanil and arvanil (44), to activate hVR1 more potently than capsaicin (14, 27), may be due in part to their being recognized by the AMT (44, 48) and, hence, penetrating more rapidly into the cell and acting on an intracellular site on VR1. Therefore, one possible strategy for the development of ultrapotent VR1 agonists to be used as therapeutic agents could be the design of compounds that at once activate VR1 and are recognized by the AMT with high efficacy.

The next step in our study was to understand the role of AEA enzymatic hydrolysis in the modulation of AEA activity at VR1 receptors. We studied the effects of two inhibitors of AEA hydrolysis, the general serine protease inhibitor PMSF, and the more selective FAAH inhibitor MAFP (34, 35). We found that both compounds, although being inactive *per se* on CCC, significantly enhanced the activity of submaximal doses of AEA, but not capsaicin, on VR1-mediated CCC increase. At exactly the same concentrations required to enhance AEA activity at VR1, PMSF and MAFP also blocked the hydrolysis of AEA by hVR1-HEK cells, which, as shown here by using the RT-PCR methodology, express a FAAH-like transcript. We cannot rule out the possibility that PMSF enhances the effect of AEA on CCC by directly interacting with VR1 receptors. However, this compound usually behaves as an alkylating agent for Ser and Cys residues, and would be more likely to inactivate VR1 receptors, rather than facilitate their activation by AEA. Therefore, these findings suggest that the effect of PMSF and, particularly, the FAAH-selective MAFP on AEA-induced CCC increase is due to their inhibition of FAAH-catalyzed AEA hydrolysis. Hence, it can be proposed that, as in the case of endocannabinoid AEA (2), also the biological activity of endovanilloid AEA is limited by enzymatic hydrolysis. Furthermore, we could estimate an EC_{50} ~100 nM for AEA effect on VR1-mediated CCC increase in the presence of either 250 μM PMSF or 75 nM MAFP (Table I). This value is not very different from the K_i = 30–50 nM of AEA in CB_1 receptor binding assays carried out in the presence of PMSF (2). Thus, it is possible that AEA potency at VR1 receptors is comparable with that at CB_1 cannabinoid receptors.

Based on the finding that HPETEs behave as full agonists at VR1 receptors (38), it could be possible that also the hydroperoxy derivatives of AEA, the HETEEs, activate these receptors and mediate the vanilloid-like actions of AEA. On the other hand, it is possible that LOX-catalyzed oxidation of AEA, which leads to compounds that are still active on CB_1 receptors (49), is used instead to inactivate AEA as an endovanilloid. Indeed 5(*S*)-, 11(*S*)-, and 15(*S*)-HETEE were found here much less active than AEA on VR1-mediated CCC increase in HVR1-HEK cells. However, it is possible that these compounds could not penetrate the cell membrane and activate the putative intracellular VR1 site for AEA proposed here. In fact, the HETEEs tested here and are not recognized as substrates by the AMT.[4] Nevertheless, because of their lipophilic nature, they might still be capable of diffusing through the cell membrane, which would explain why the 15(*S*)-HETEE did exhibit measurable VR1 activity. Our additional finding that nonselective inhibitors of LOX do not influence the effect on CCC of either sub- or near-maximal concentrations of AEA (whereas arachidonic acid exerted only a very little effect) was not surprising because constitutive LOX activity seems to be low in HEK cells (50, 51). Further studies are needed to fully assess the role of LOXs in AEA activity at VR1 receptors.

A corollary to the findings discussed so far is that truly selective inhibitors of AMT, but not FAAH, can be used to distinguish between the actions of anandamide at VR1 and CB_1 receptors. In principle, also the CB_1 receptor antagonist SR141716A (21) could be employed to discriminate between these two types of AEA pharmacological activity. However, this antagonist, albeit generally selective for CB_1 receptors, acts also on other molecular targets at concentrations higher than 1 μM (24, 25, 52). Furthermore, preliminary experiments revealed that a single high dose of SR141716A can antagonize capsaicin-induced and vanilloid receptor-mediated vasodilation of rat mesenteric arteries (11). Indeed, we found here that SR141716A, at concentrations of >1 μM, inhibits the effects of capsaicin, AEA, and AM404 on hVR1-mediated CCC increase in hVR1-HEK cells. Several lines of evidence suggest that this inhibitory effect of SR141716A is due, at least in part, to an interaction with VR1 but not CB_1 receptors. First, in the cell model used in this study, *i.e.* HEK 293 cells transfected with VR1 receptor cDNA, CB_1 receptors are not expressed, as assessed by RT-PCR,[5] nor is any specific binding for [^{3}H]SR141716A found in nontransfected HEK 293 cells, according to Tao and Abood (53). Second, the effects of AEA, capsaicin, and AM404 on CCC are clearly mediated by VR1 and not CB_1 receptors (see beginning of "Discussion"). Indeed, if AEA were increasing CCC via CB_1 receptors AMT inhibitors should have enhanced, and not inhibited, this effect (see

[3] P. Geppetti and V. Di Marzo, unpublished observations.

[4] M. Maccarrone, unpublished data.

[5] J.B. Davis, unpublished observations.

above). Third, the inhibitory effect of SR141716A on AEA-induced CCC increase was observed at concentrations that were three orders of magnitude higher than those necessary to antagonize CB_1 receptor-mediated effects of AEA (K_c ~0.3 nM, Ref. 33; K_i for human CB_1 = 5.6 nM, Ref. 21; K_d for CB_1 receptors 0.19–1.24 nM, Ref. 2). Finally, SR141716A *per se* exerted a weak agonist effect on CCC in a manner sensitive to the VR1 antagonist capsazepine (shown here to be highly selective for VR1 *versus* CB_1 receptors). This latter observation suggests that SR141716A may act as a partial agonist and, subsequently, competitive antagonist for VR1 receptors. It is unlikely, however, that SR141716A inhibitory action was because of desensitization of VR1 receptors, because the dose-response curves of capsaicin in the presence of the antagonist were shifted to the right to parallel curves. On the other hand, an unusual dose-response curve was observed for the inhibition of capsaicin effect on CCC, with a narrow range of active doses and a ceiling effect at 2.5 μM SR141716A particularly noticeable with concentrations of capsaicin > 30 nM. A similar ceiling effect was also previously observed for another non-CB_1-mediated inhibitory effect of SR141716A (52), and may be due either to the fact that this compound becomes active as a VR1 agonist at ≥5 μM concentrations, or to nonselective effects at high doses. Also with AEA the inhibitory effect of SR141716A was observed in a narrow range of concentrations, and, moreover, could not be overridden by high concentrations of the compound. This may be because of effects of the antagonist also on non-VR1 targets, *i.e.* inhibition of the AMT (39). Although its mechanism of action needs further investigation, the VR1 antagonistic effect of SR141716A, particularly when the antagonist is systemically administered and its local concentrations in tissues cannot be established with accuracy, implies that this compound should be used with some caution when discriminating between CB_1- and VR1-mediated actions of AEA.

In conclusion, the present study supports a role of AEA as an endovanilloid acting upon the VR1 receptor at an intracellular site. Our data also suggest that the activity of AEA at the vanilloid receptor may be controlled by the endocannabinoid pathway components that regulate its internalization and degradation. Hence, targeting of AMT and FAAH may offer indirect means of influencing VR1-mediated sensory signaling and pain. Further studies will be required to determine which of the several pharmacological actions described so far for AEA are exerted through vanilloid receptors; we have shown here that selective AMT inhibitors, but not necessarily the CB_1 receptor antagonist SR141716A, can be used as biochemical tools to distinguish between the vanilloid-like and cannabimimetic actions of AEA.

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The Activity of Anandamide at Vanilloid VR1 Receptors Requires Facilitated Transport across the Cell Membrane and Is Limited by Intracellular Metabolism

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