

'Entourage' effects of *N*-acyl ethanolamines at human vanilloid receptors. Comparison of effects upon anandamide-induced vanilloid receptor activation and upon anandamide metabolism

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1 The abilities of a series of saturated *N*-acyl ethanolamines and related compounds to affect the ability of anandamide (AEA) to produce a Ca^{2+} influx into human embryonic kidney cells expressing the human vanilloid receptor (hVR1-HEK293 cells) has been investigated.

2 The C3:0, C4:0, C6:0 and C10:0 ethanolamides neither affected basal Ca^{2+} -influx, nor the influx in response to a submaximal concentration of AEA (1 μM). In contrast, the C12:0, C17:0, C18:0 ethanolamides and the monounsaturated compound oleoylethanolamide (C18:1) greatly potentiated the response to AEA. Palmitoylethanolamide (C16:0) produced both a response *per se* and an augmentation of the response to AEA.

3 Lauroylethanolamide (C12:0) produced a leftward shift in the dose-response curve for AEA. EC_{50} values for AEA to produce Ca^{2+} influx into hVR1-HEK293 cells were 1.8, 1.5, 1.1 and 0.22 μM in the presence of 0, 1, 3 and 10 μM lauroylethanolamide, respectively. Lauroylethanolamide did not affect the dose-response curves to capsaicin.

4 Palmitoylethylamide was synthesized and found to be a mixed-type inhibitor ($K_{i(\text{slope})}$ 4.1 μM , $K_{i(\text{intercept})}$ 66 μM) of [^3H]-AEA metabolism by rat brain membranes.

5 The -amide, -ethylamide, -isopropylamide, -butylamide, -cyclohexamide and -trifluoromethyl ketone analogues of palmitoylethanolamide had little or no effect on the Ca^{2+} influx response to 1 μM AEA.

6 There was no obvious relation between the abilities of the compounds to enhance the Ca^{2+} influx response to 1 μM AEA into hVR1-HEK293 cells and to prevent the hydrolysis of AEA by rat brain membranes.

7 It is concluded that although palmitoylethanolamide has entourage-like effects at VR1 receptors expressed on hVR1-HEK293 cells, other *N*-acyl ethanolamines have even more dramatic potentiating effects. It is possible that they may play an important role under conditions where their synthesis is increased, such as in severe inflammation.

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Abbreviations: AEA, anandamide; FAAH, fatty acid amidohydrolase; MEM, minimal essential medium; OEA, oleoylethanolamide; PA-Et, palmitoylethylamide; PA-NH₂, palmitamide; PBA, palmitoylbutylamide; PCA, palmitoylcyclohexamide; PEA, palmitoylethanolamide; PIA, palmitoylisopropylamide; PTMK, palmitoyl trifluoromethyl ketone; VR, vanilloid receptors

Introduction

It is now well established that the *N*-acyl ethanolamine anandamide (arachidonoyl ethanolamide C20:4, AEA) not only acts as an endogenous ligand at cannabinoid receptors (Devane *et al.*, 1992), but is also capable of activating vanilloid receptors (VR) (Zygmunt *et al.*, 1999; Smart *et al.*, 2000). In contrast to the activation produced by hydrogen ions (Caterina *et al.*, 1997; McLatchie & Bevan, 2001), the VR agonist capsaicin is believed to activate VR1 receptors intracellularly (Jung *et al.*, 1999; Welch *et al.*, 2000), and a similar mechanism may exist for AEA, since blockade of

AEA facilitated transport into hVR1-HEK cells transfected with VR1 receptors inhibits AEA-induced influx of calcium into the cell (De Petrocellis *et al.*, 2001a). Intracellular AEA is metabolized to arachidonic acid by the enzyme fatty acid amide hydrolase (FAAH) (Deutsch & Chin, 1993), and inhibition of the enzyme potentiates the effects of AEA upon VR1 receptors (De Petrocellis *et al.*, 2001a; Ross *et al.*, 2001).

Whilst it is clear that AEA activates VR1 receptors, it is not as yet proven beyond doubt that AEA acts as an 'endovanilloid' in the same way as it acts as an endocannabinoid (see Di Marzo *et al.*, 2001). The main obstacle to the acceptance of AEA as an endovanilloid has been its relatively low potency (Zygmunt *et al.*, 1999; Smart *et al.*, 2000), even

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after FAAH inhibition (Ross *et al.*, 2001). However, there is evidence that the potency of AEA towards VR1 receptors can be regulated (Di Marzo *et al.*, 2001). Thus, the naturally-occurring *N*-acyl ethanolamine palmitoylethanolamide (PEA, C16:0) potentiates the effects of AEA and resiniferatoxin at VR1 receptors (De Petrocellis *et al.*, 2001b). It is not known, however, whether these effects of PEA are shared by other *N*-acyl ethanolamines. In consequence, in the present study, the effects of a series of PEA analogues and homologues have been investigated with respect to their ability to potentiate the actions of AEA at VR1 receptors.

Methods

Compounds

Anandamide [ethanolamine-1-³H] ([³H]-AEA; specific activity 60 Ci mmol⁻¹) was obtained from American Radiolabeled Chemicals, Inc. (St. Louis, MO, U.S.A.). The syntheses of propanoylethanolamide (C3:0), butanoylethanolamide (C4:0), hexanoylethanolamide (C6:0), octanoylethanolamide (C8:0), decanoylethanolamide (C10:0), lauroylethanolamide (C12:0), myristoylethanolamide (C14:0), palmitoylethanolamide (C16:0), palmitamide (C16:0), palmitoylbutylamide (C16:0), palmitoylisopropylamide (C16:0), palmitoylcyclohexamide (C16:0) and oleoylethanolamide (C18:1) have been described earlier (Lambert *et al.*, 1999; 2001; Jonsson *et al.*, 2001). Capsaicin and non-radioactive AEA (for the VR1 experiments) was purchased from Tocris (Bristol, U.K.). Heptadecanoylethanolamide (C17:0), stearoylethanolamide (C18:0), palmitoyl trifluoromethyl ketone and non-radioactive AEA (for the FAAH experiments) were purchased from Cayman Chemical Company (Ann Arbor, MI, U.S.A.). Fatty acid free bovine serum albumin was obtained from the Sigma Chemical Co. (St Louis, MO, U.S.A.). Cell culture media were obtained from Gibco/Life Technologies.

Synthesis of palmitoylethylamide (PA-Et)

In a two-neck flask, 4.17 g of ethylamine (92.6 mmoles) were poured into 20 ml of dry methylene chloride. The solution was cooled in an ice bath and magnetically stirred. Palmitoyl chloride (2.74 g, 10 mmoles) was added dropwise. The reaction mixture was stirred for 12 h at room temperature and then washed with 5% sodium bicarbonate solution, HCl 1 M and brine. The organic layer was dried (MgSO₄) and the solvent evaporated under reduced pressure to give 2.34 g (83%) of a white solid.

m.p.: 62–63°C (uncorrected); TLC (chloroform : methanol 6 : 4 v v⁻¹) R_f = 0.86; spectroscopic data: ¹H NMR (*d*-CDCl₃) δ (p.p.m.) 0.86 (*t*, *J* = 7 Hz, 3 H); 1.14 (*t*, *J* = 7 Hz, 3 H); 1.16–1.24 (*m*, 26 H); 2.1 (*t*, *J* = 7 Hz, 2 H); 3.2–3.33 (*m*, 2 H); 5.4 (NH); ¹³C NMR (*d*-CDCl₃) δ (p.p.m.) 14.1; 14.95 (CH₃); 22.7; 25.8; 29.26; 29.37; 29.5; 29.63; 29.64; 29.65; 29.66; 29.68; 29.69; 29.8; 31.94; 34.3; 36.94 (CH₂); 172.97 (C=O); mass spectrometry [M⁺] = 283; IR ν (cm⁻¹): 3298 (NH); 1633 (C=O).

Cell culture

hVR1-HEK293 cells were grown as monolayers in minimum essential medium (MEM) supplemented with non-essential

amino acids, 10% foetal calf serum, and 0.2 mM L-glutamine, and maintained under 95%/5% O₂/CO₂ at 37°C. Cells were passaged every 3–4 days and the highest passage number used was 27.

Measurement of [Ca²⁺]_i using the FLIPR technology

The method of Smart *et al.* (2000) was used. hVR1-HEK293 cells were seeded (density 25 × 10³ cells well⁻¹) into black walled clear-base 96-well plates (Costar, U.K.) and cultured overnight in MEM, supplemented as described above. The cells were then incubated at 25°C for 120 min with MEM containing the cytoplasmic calcium indicator, Fluo-3AM (4 μM; Teflabs, Austin, TX, U.S.A.). After washing the plates four times with Tyrode's medium containing 0.1% bovine serum albumin, they were placed into a FLIPR (Molecular Devices, U.K.) to monitor cell fluorescence (λ_{EX} = 488 nm, λ_{EM} = 540 nm) before and after the addition of various compounds in the presence or absence of 1 μM AEA. Incubations were routinely run over 3 min, and the responses generally appeared within a few seconds of injection of the anandamide, peaking at about 30 s. Responses were measured as peak fluorescence intensity (FI) minus basal FI, and are expressed as a percentage of the response obtained with either anandamide or capsaicin, as appropriate.

Assay of FAAH activity

The assay used was that of Omeir *et al.* (1995) adapted to the tritiated substrate (Fowler *et al.*, 1997). Briefly, frozen brains (minus cerebellum) from adult rats that had been stored at –70°C were thawed and homogenized at 4°C in 20 mM HEPES buffer, pH 7.0, with 1 mM MgCl₂. The homogenates were centrifuged twice at 36,000 × *g* for 20 min at 4°C. Tissue pellets were resuspended in homogenization buffer and incubated at 37°C for 15 min, after which they were centrifuged at 36,000 × *g* for 20 min at 4°C. Membranes were re-suspended in 50 mM Tris-HCl buffer, pH 7.4, containing 1 mM EDTA and 3 mM MgCl₂, and stored at –70°C until used for assay. Upon assay, membranes [12.5 μg protein assay⁻¹], palmitoylethylamide (PA-Et) or ethanol carrier, [³H]-AEA and assay buffer (10 mM Tris-HCl, 1 mM EDTA, 1% (w v⁻¹) fatty acid-free bovine serum albumin, pH 7.6) (final assay volume of 200 μl) were incubated for 10 min at 37°C. Reactions were stopped by placing the tubes in ice and thereafter adding 400 μl of chloroform : methanol (1 : 1 v v⁻¹). Phases were separated by centrifugation in a bench centrifuge after the samples had been thoroughly mixed. Aliquots (200 μl) of the methanol/buffer phase containing the [³H]-ethanolamine product were removed and analysed for radioactivity by liquid scintillation spectroscopy with quench correction. Blanks contained distilled water instead of the membranes.

Calculation of EC₅₀, K_{i(slope)} and K_{i(intercept)} values

EC₅₀ values were calculated from the mean values at each AEA or capsaicin concentration using the built-in equation 'sigmoid dose-response (variable slope)' of the GraphPad Prism computer programme (GraphPad Software Inc., San Diego, CA, U.S.A.). Top and bottom values were set to 100

and 0, respectively. Apparent K_M and V_{max} values for inhibition of [3H]-AEA metabolism by PA-Et were calculated from the mean data using the Direct Linear Plot analysis (Eisenthal & Cornish-Bowden, 1974) and the Enzyme Kinetics v 1.4 software package (Trinity Software, Campton, NH, U.S.A.). These values were used in secondary replots of [PA-Et] vs $K_{Mapp}/V_{max\ app}$ and $1/V_{max\ app}$ to determine $K_{i(slope)}$ and $K_{i(intercept)}$ values, respectively.

Results

Effect of N-acylethanolamines upon AEA-induced activation of VR1 receptors in hVR1-HEK293 cells

A series of *N*-acylethanolamines ranging from 3 carbons (propanoylethanolamide) up to 18 carbons (stearoylethanolamide [C18:0], oleoylethanolamide (OEA, [C18:1]) were investigated (Figure 1). The compounds were tested at a concentration of 10 μM for effects *per se*, and for their ability to affect the Ca^{2+} -influx response produced by a submaximal concentration of AEA (1 μM). Two series of experiments were undertaken, with AEA, PEA and OEA being tested in both experiments. Very similar values were seen for these compounds in both sets of experiments. For simplicity, the second of these values are shown in the figures.

With the exception of PEA, none of the *N*-acyl ethanolamines produced a Ca^{2+} -response *per se* in the hVR1-HEK293 cells (Figure 1a). The C3:0, C4:0, C6:0 and C10:0 compounds were also without effect upon the response to AEA. In contrast, the response to AEA was greatly potentiated by the C12:0, C17:0, C18:0 and C18:1 compounds, so that in the case of the C12:0 compound (lauroylethanolamine), the response to 1 μM AEA became maximal (Figure 1a). In Figure 1b, the ability of the compounds to inhibit the FAAH-catalyzed metabolism of AEA is shown. There was no obvious relation between the abilities of the compounds to inhibit AEA metabolism and to potentiate the VR1-mediated Ca^{2+} influx in the hVR1-HEK293 cells. Thus, for example, the C18:0 and C18:1 compounds potentiate the Ca^{2+} influx response to AEA to about the same extent, but have greatly differing abilities to inhibit [3H]-AEA metabolism. Conversely, the C12:0 and C14:0 compounds produce rather similar inhibitions of [3H]-AEA metabolism, but differ greatly in their ability to potentiate the Ca^{2+} influx response to AEA (Figure 1a, b).

Comparison of the effects of lauroylethanolamide (C12:0) upon the Ca^{2+} influx response to AEA and capsaicin in hVR1-HEK293 cells

Dose-response curves for the stimulation of Ca^{2+} influx in hVR1-HEK293 cells by AEA and capsaicin are shown in Figure 2. Lauroylethanolamide produced a leftward shift in the AEA dose-response curve, so that the potency of AEA was increased approximately 10 fold at the highest lauroylethanolamide concentration tested (10 μM) (Figure 2a). Thus, the EC_{50} values for AEA calculated from the curves were 1.8, 1.5, 1.1 and 0.22 μM in the presence of 0, 1, 3 and 10 μM lauroylethanolamide, respectively. In contrast to the effects on AEA, lauroylethanolamide did not affect the dose-response curves to capsaicin, with EC_{50} values in

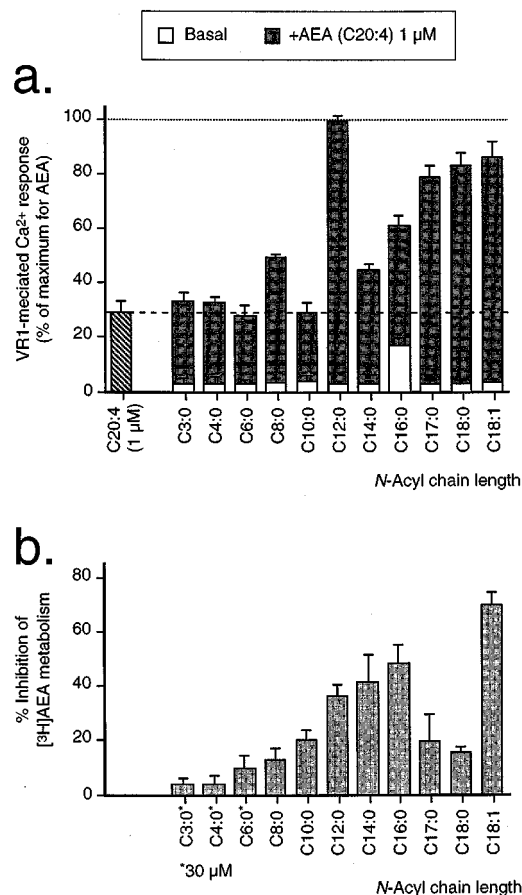


Figure 1 (a) Effects of a series of *N*-acyl ethanolamines upon the Ca^{2+} -influx in hVR1-HEK293 cells in the absence and presence of 1 μM AEA. The compounds were used at a concentration of 10 μM . Shown are means $\pm$ s.e.mean of three experiments. The 'C' numbers given in the abscissa indicate the acyl chain length and degree of saturation, respectively. Thus, for example, C20:4 indicates a 20 carbon length side chain with four double bonds, i.e. the arachidonoyl group in AEA. (b) Inhibition of the metabolism of 2 μM [3H]-AEA by rat brain membranes by the *N*-acyl ethanolamines at concentrations of 10 μM (unless otherwise shown). The data are means $\pm$ s.e.mean and are taken from the raw data of Figure 3 of Jonsson *et al.* (2001).

the range of 10–13 nM being found for the four curves (Figure 2b).

Effects of PEA analogues upon FAAH activity and the Ca^{2+} influx response to AEA and capsaicin in hVR1-HEK293 cells

Six analogues to PEA where the ethanolamine group was replaced by -amide (PA-NH₂), -ethylamide (PA-Et), -isopropylamide (PIA), -butylamide (PBA), -cyclohexamide (PCA) and -trifluoromethyl ketone (PTMK) groups were investigated. Of these, no data is available on the ability of PA-Et to interact with FAAH, so the effects of this compound upon [3H]-AEA by rat brain membranes was determined. PA-Et was found to be a mixed-type inhibitor, with a $K_{i(slope)}$ value of 4.1 μM and a $K_{i(intercept)}$ value of 66 μM (Figure 3).

In Figure 4, the effects of the six PEA analogues upon basal and AEA-induced Ca^{2+} influx in hVR1-HEK293 cells

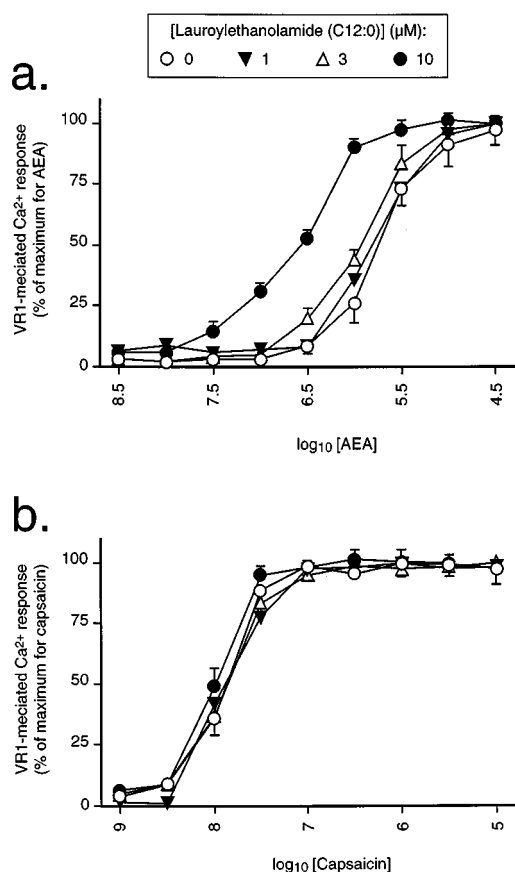


Figure 2 Effects of lauroylethanolamide (C12:0) upon the the Ca^{2+} -influx in hVR1-HEK293 cells produced by (a) AEA and (b) capsaicin. Data are means of three experiments. In (a) the s.e.means are shown (when not enclosed by the symbols) for the sake of clarity only for the curves for 0, 3 and 10 μM lauroylethanolamide. In (b) the s.e.means are shown only for the curves for 0 and 10 μM lauroylethanolamide.

are shown together with data on the abilities of the compounds to affect $[\text{H}]\text{-AEA}$ metabolism. The experiments were conducted concomitantly with the experiments shown in Figure 1, so the data for AEA is the same in both figures. None of the compounds produced marked effects either *per se*, or upon the Ca^{2+} influx response to 1 μM AEA (Figure 4a), despite a large variation in the abilities of the compounds to prevent $[\text{H}]\text{-AEA}$ metabolism (Figure 4b).

Discussion

In the short time since the publication by Zygmunt *et al.* (1999) that AEA was able to activate VR1 receptors, a number of studies, not the least in this journal, have demonstrated the involvement of these sensory nerve receptors in the pharmacological effects of exogenously applied AEA in nociceptive (Gauldie *et al.*, 2001; Tognetto *et al.*, 2001) and pulmonary (Craib *et al.*, 2001; Smith & McQueen, 2001) function as a consequence of its effects on sensory neurotransmission (Zygmunt *et al.*, 1999; Ralevic *et al.*, 2001; Ross *et al.*, 2001; Mang *et al.*, 2001). However, it is still a matter of debate as to whether AEA acts as an endogenous vanilloid (see Di Marzo *et al.*, 2001).

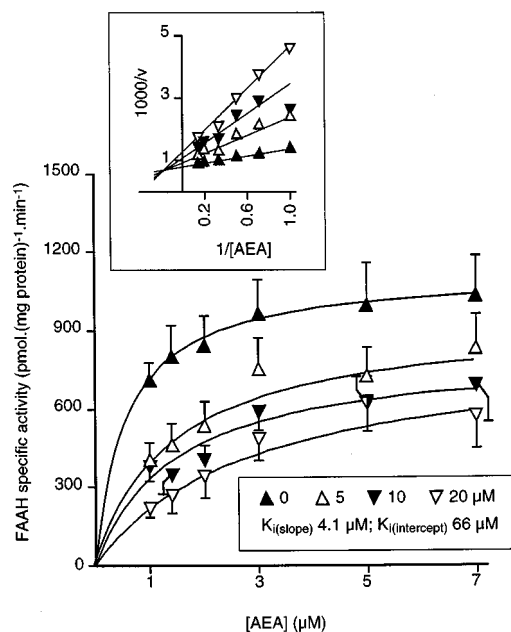


Figure 3 Mode of inhibition of $[\text{H}]\text{-AEA}$ metabolism in rat brain membranes by palmitoylethylamide (PA-Et). Means $\pm$ s.e. mean of three experiments are shown in the main plot. A secondary Lineweaver-Burk replot of the mean data is shown in the insert to illustrate the mixed-type nature of the inhibition. The $K_{i(\text{slope})}$ and $K_{i(\text{intercept})}$ values were calculated from the mean data as described in Methods.

In a recent study, it was reported that the *N*-acylethanolamine palmitoylethanolamide (C16:0) was able to increase the VR1-mediated Ca^{2+} influx in response to AEA in hVR1-HEK293 cells (De Petrocellis *et al.*, 2001b). These authors found that PEA produced a 2 fold decrease in the EC_{50} value for AEA and was also able to potentiate the responses to the VR1 stimulators capsaicin and resiniferatoxin, although the stimulation of the response to resiniferatoxin was more marked than for capsaicin. At a concentration of 1 μM AEA (the concentration used in the present study), PEA was without effect upon the response to AEA, whereas a potentiation was seen at AEA concentrations < 250 nM (De Petrocellis *et al.*, 2001b). The apparent potency of AEA is highly dependent upon the assay conditions, in particular upon the concentration of bovine serum albumin (De Petrocellis *et al.*, 2001b) (which is required in the FLIPR assay to prevent binding of the AEA to the plastic wells). However, the AEA concentration used here (1 μM) relative to its EC_{50} value (1.8 μM) corresponds to a concentration of ~ 240 nM under the conditions used by De Petrocellis *et al.* (2001b) relative to their EC_{50} value of 0.44 μM . In addition to its effects on AEA-induced Ca^{2+} -influx, the authors found that PEA produced a 5 fold decrease in the K_i for the inhibition of $[\text{H}]\text{-resiniferatoxin}$ binding by AEA (De Petrocellis *et al.*, 2001b). The authors suggested that this action of PEA could be described as an 'entourage' effect, similar to that seen for the ability of the 2-arachidonoyl glycerol homologues 2-palmitoylglycerol and 2-linoleoyl-glycerol to potentiate the cannabinoid receptor-mediated effects of 2-arachidonoyl glycerol without themselves having affinities for cannabinoid receptors (Ben-Shabat *et al.*, 1998).

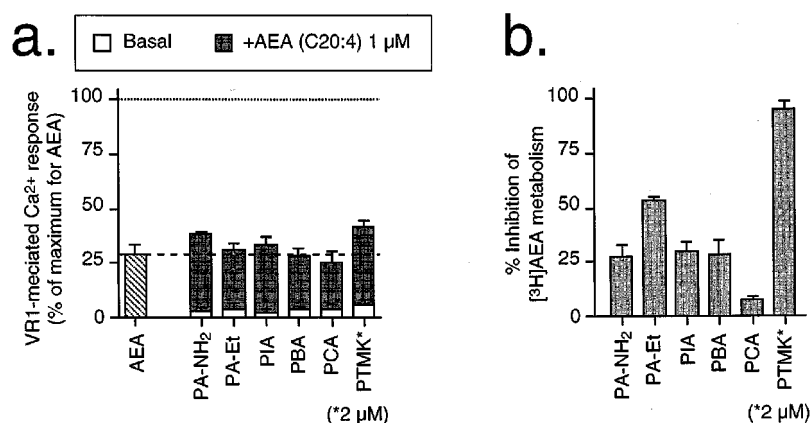


Figure 4 (a) Effects of a series of palmitoylethanolamide analogues upon the Ca^{2+} -influx in hVR1-HEK293 cells in the absence and presence of 1 μM AEA. Shown are means $\pm$ s.e. mean of three experiments. The concentrations of the palmitoylethanolamide analogues were in all cases 10 μM , except for PTMK where a concentration of 2 μM was used. (b) Inhibition of the metabolism of 2 μM [^3H]-AEA by rat brain membranes by the palmitoylethanolamide analogues at concentrations of 10 μM (unless otherwise shown). The data are means $\pm$ s.e. mean and are taken from the raw data of Figure 4 of Jonsson *et al.* (2001), with the exception of PA-Et and PA-NH₂, which are from unpublished data.

In the present study, we have investigated a series of palmitoylethanolamide homologues and analogues in order to investigate whether such an entourage effect is unique for PEA, or whether other *N*-acylethanolamines produce even more dramatic effects. In addition, in view of the finding that PEA acts as a partial agonist at VR1-receptors overexpressed in hVR1-HEK293 cells (Smart *et al.*, 2000), effects of the compounds *per se* upon VR1-receptors were investigated. PEA was found to produce effects both *per se* and upon the response to AEA, essentially in agreement with the study of De Petrocellis *et al.* (2001b). These effects are lost when the -ethanolamine group was replaced by -ethylamine (PA-Et), -isopropylamine (PIA), -butylamine (PBA), -cyclohexamine (PCA) and -trifluoromethyl ketone (PTMK) groups, suggesting a degree of specificity in the entourage actions of PEA, although it should be pointed out as a caveat that the modest inhibitory effects of PIA and PCA on the transport of AEA, at least in rodent cell lines (Jonsson *et al.*, 2001), may mask small augmentations of the response to AEA, given that in hVR1-HEK cells AEA uptake inhibitors block the Ca^{2+} -response to this compound (De Petrocellis *et al.*, 2001a). The finding that palmitamide (PA-NH₂) has at best modest effects in this assay is important. This compound is a good substrate of FAAH (Boger *et al.*, 2000), and its low activity would suggest that the effects of PEA are not a result of its metabolism to the free fatty acid.

PEA, however, was not the best entourage compound at the assay concentration of AEA used, since lauroylethanolamide (C12:0), heptadecanoylethanolamide (C17:0), stearoylethanolamide (C18:0) and oleoylethanolamide (C18:1) produced much more robust potentiations of the effect of AEA without producing Ca^{2+} -influxes in the cells in its absence. The C12:0 compound potentiated the effects of AEA but not of capsaicin, suggesting that in some way it increases the affinity of the VR1 receptor for the endogenous compound but not the exogenous compound. At first sight, this differs from the observations with PEA (De Petrocellis *et al.*, 2001b), and might suggest that the C12:0 and C16:0 compounds may exert their effects in different ways. However, the effect of PEA upon the

response to capsaicin reported by De Petrocellis *et al.* (2001b) was rather modest.

It has long been known that *N*-acyl ethanolamines are substrates for amidohydrolase activities that are presumed to be identical to fatty acid amide hydrolase (FAAH, Epps *et al.*, 1982; Schmid *et al.*, 1985; Deutsch & Chin, 1993), and that they will thus compete with AEA for this enzyme (Jonsson *et al.*, 2001). In theory, such a potentiation of the response to AEA, but not capsaicin, could be a result of competition for FAAH, so that more AEA is available for interaction with the VR1 receptor. Certainly, the FAAH inhibitors phenylsulphonyl methyl fluoride and methyl arachidonoyl fluorophosphonate potentiate the Ca^{2+} -response to a low concentration (100 nM) of AEA but not capsaicin (10 nM) in hVR1-HEK cells (De Petrocellis *et al.*, 2001a). However, in that study, the FAAH inhibitors were preincubated with the cells for 10 min prior to addition of the agonists, whereas in the present study, they were added concomitantly. Given the very short incubation times used in the FLIPR methodology (the peak response to AEA occurs at about 30 s after administration, Smart *et al.*, 2000), together with the finding by De Petrocellis *et al.* (2001b), that PEA at the concentrations used did not affect [^{14}C]-AEA hydrolysis by hVR1-HEK293 cell membranes, it is unlikely that FAAH inhibition is involved in the actions of the NAEs. Consistent with this conclusion, the present data show no relationship between the ability of the compounds tested to inhibit [^3H]-AEA metabolism and their ability to potentiate the VR1-mediated actions of AEA. The best illustrations of this are PTMK, stearoylethanolamide and OEA. PTMK produces an almost complete inhibition of FAAH but does not affect the VR1 response to AEA; Stearoylethanolamide and OEA produce similar robust augmentations of the VR1 response to AEA despite large differences in their abilities to affect AEA metabolism. Of course, it should be mentioned as a caveat that the FAAH data was obtained using rat brain membranes, whereas the HEK cells are of human origin. However, rat and human FAAH have very similar sequences (82% amino acid identity and only one divergent amino acid in the amidase signature sequence) and are able to hydrolyze

a series of *N*-acyl amides with rather similar relative specificities (Cravatt *et al.*, 1996; Giang & Cravatt, 1997). Thus, in agreement with the conclusion of De Petrocellis *et al.* (2001b), FAAH inhibition does not account for the potentiation of AEA effects upon Ca^{2+} -influx in hVR1-HEK293 cells produced by the *N*-acyl ethanolamines.

In conclusion, the present study has confirmed that PEA has entourage-like effects at VR1 receptors expressed on hVR1-HEK293 cells and demonstrated that other *N*-acyl ethanolamines have even more dramatic potentiating effects. It is of course hazardous to extrapolate data obtained from transfected cells to the situation *in vivo*. However, one important property of *N*-acyl ethanolamines in the body is that their synthesis is dramatically increased under certain conditions of cellular stress, ischaemia and inflammation (see e.g. Epps *et al.*, 1979; Kondo *et al.*, 1998; Berdyshev *et al.*, 2000; Hansen *et al.*, 2001). Thus, for example, 9 h following cadmium chloride-induced inflammation in the rat testis, the

levels of the C16:0 and C18:0 *N*-acyl ethanolamines were increased 39 and 21 fold (Kondo *et al.*, 1998). Although there is no apparent increased sensitivity of peripheral nociceptors to exogenously applied AEA in arthritic rat knee joints (Gauldie *et al.*, 2001), it is nevertheless tempting to speculate that *N*-acyl ethanolamines may be an important regulator of the actions of AEA upon vanilloid receptors under severe inflammatory conditions.

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