Palmitoylethanolamide enhances anandamide stimulation of human vanilloid VR1 receptors

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Received 11 July 2001; revised 14 September 2001; accepted 16 September 2001

First published online 26 September 2001

Edited by Judit Ovádi

Abstract In human embryonic kidney cells over-expressing the human vanilloid receptor type 1 (VR1), palmitoylethanolamide (PEA, 0.5–10 μM) enhanced the effect of arachidonoylethanolamide (AEA, 50 nM) on the VR1-mediated increase of the intracellular Ca^{2+} concentration. PEA (5 μM) decreased the AEA half-maximal concentration for this effect from 0.44 to 0.22 μM. The PEA effect was not due to inhibition of AEA hydrolysis or adhesion to non-specific sites, since bovine serum albumin (0.01–0.25%) potently inhibited AEA activity, and PEA also enhanced the effect of low concentrations of the VR1 agonists resiniferatoxin and capsaicin. PEA (5 μM) enhanced the affinity of AEA for VR1 receptors as assessed in specific binding assays. These data suggest that PEA might be an endogenous enhancer of VR1-mediated AEA actions. © 2001 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: Endocannabinoid; Vanilloid; Receptor; Cannabinoid; Capsaicin; Calcium

1. Introduction

Anandamide (arachidonoylethanolamide, AEA) was the first endogenous substance to be proposed as a cannabinoid receptor ligand in 1992 [1]. Yet, AEA belongs to a family of lipids known since the late 1950s, the *N*-acylethanolamines (reviewed in [2]), which also includes the anti-inflammatory compound palmitoylethanolamide (PEA). Recently there has been renewed interest in the properties of PEA ([3], and [4] for review), and it was proposed that this lipid could act as an agonist for the cannabinoid receptor type 2 (CB_2) [5]. However, PEA exhibits very little, if any, affinity for the cloned CB_1 and CB_2 cannabinoid receptors from rat, mouse or man [6–8]. The hypothesis that this compound owes its properties to CB_2-like cannabinoid receptors, proposed by some authors to explain why PEA analgesic effects were antagonized by a CB_2 receptor blocker [9,10], has not found any molecular support to date. Other authors have suggested that PEA may act as an 'entourage' compound, i.e. by inhibiting the inactivation of endogenous cannabinoids such as AEA, thereby increasing their levels [4]. However, although PEA is hydrolyzed by the enzyme mostly responsible for AEA degradation, the fatty acid amide hydrolase (FAAH, see [11] for a recent review), this compound is not a very efficacious inhibitor of AEA hydrolysis [12]. A recent study showed that, rather than directly inhibiting AEA hydrolysis by substrate competition, PEA affects the degradation of AEA by down-regulating the expression of FAAH [13]. PEA is co-synthesized with AEA in most of the cells analyzed so far in amounts 5–10-fold higher [4,14], and could thus play a role as an 'entourage' substance for AEA when the two substances are endogenously produced.

A possibility that could explain some of the anti-inflammatory and analgesic properties of PEA is that this compound interacts with and immediately desensitizes a particular type of nociceptor expressed in most peripheral C-fibers, the type 1 vanilloid receptors (VR1), which are activated by protons, heat and plant toxins such as capsaicin and resiniferatoxin (RTX) [15]. Although leading to painful responses and local vasodilation, activation of these receptors is immediately followed by desensitization, so that they become refractory to subsequent stimulation by nociceptive stimuli. Therefore, some VR1 ligands have been used paradoxically to induce analgesia and inhibit inflammation [16]. AEA was recently found to fully activate, and subsequently desensitize, both rat and human VR1, although at concentrations 5–20-fold higher than those necessary for capsaicin to exert the same action, or for AEA to activate CB_1 receptors [17,18]. However, several regulatory events are being found that render AEA equipotent at VR1 and CB_1 receptors [19–21] and, hence, a potential 'endovanilloid' [22]. Here we investigated the possibility that PEA, previously shown to exert per se only a negligible effect on VR1 even at high concentrations [18,23], enhances the potency of AEA at this receptor, thus possibly behaving as an endogenous modulator of endovanilloid activity.

2. Materials and methods

2.1. Materials and cells

AEA and PEA were synthesized from arachidonic acid or palmitic

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Abbreviations: AEA, arachidonoylethanolamide; PEA, palmitoylethanolamide; HEK, human embryonic kidney; hVR1, human vanilloid receptor type 1; CB_1, cannabinoid receptor type 1; CB_2, cannabinoid receptor type 2; FAAH, fatty acid amide hydrolase; BSA, bovine serum albumin; RTX, resiniferatoxin; $[Ca^{2+}]_i$, intracellular calcium concentration

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PII: S0014-5793(01)02934-9

acid and ethanolamine, as described in [1], whereas RTX and capsaicin were purchased from Calbiochem. Fatty acid-free bovine serum albumin (BSA) was from Sigma. Over-expression of human VR1 cDNA into human embryonic kidney (HEK) 293 cells was carried out as described previously [24]. Cells were grown as monolayers in minimum essential medium supplemented with non-essential amino acids, 10% fetal calf serum and 0.2 mM glutamine, and maintained under 95% O_2/5% CO_2 at 37°C.

2.2. Intracellular Ca^{2+} assay

The effect of the substances on intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) was determined using Fluo-3, a selective intracellular fluorescent probe for Ca^{2+} [18,23]. One day prior to experiments cells were transferred into six-well dishes coated with poly-L-lysine (Sigma) and grown in the culture medium mentioned above. On the day of the experiment the cells (50–60 000 per well) were loaded for 2 h at 25°C with 4 μM Fluo-3 methyl ester (Molecular Probes) in dimethyl sulfoxide containing 0.04% Pluronic. After the loading, 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 ($\lambda_{ex} = 488$ nm, $\lambda_{em} = 540$ nm) before and after the addition of the test compounds at various concentrations. Capsazepine (10 μM) was added 30 min before AEA. PEA was added 5 min before AEA, RTX or capsaicin. Either a single concentration of PEA before varying concentrations of the VR1 ligands, or varying concentrations of PEA before a sub-threshold concentration of the ligands, was tested. 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.

2.3. VR1 receptor binding assays

The affinity of AEA for human VR1 receptors was assessed by means of displacement assays carried out with membranes (50 μg/tube) from HEK-hVR1 cells, prepared as described previously [25] but in the absence of FAAH inhibitors, and the high affinity VR1 ligand [^{3}H]RTX (48 Ci/mmol, NEN-Dupont), using the incubation conditions described previously [25]. Under these conditions the K_d and B_{max} for [^{3}H]RTX were 0.5 nM and 1.39 pmol/mg protein. The K_i for the displacement of 1 nM [^{3}H]RTX by increasing concentrations of AEA, in the presence or absence of PEA 5 μM, was calculated from the IC_{50} values (obtained by GraphPad Software) using the Cheng–Prusoff equation. Specific binding was calculated with 1 μM RTX (Alexis Biochemicals) and was 78.1 ± 3.7%.

2.4. AEA hydrolase activity assays

HEK-hVR1 cells were cultured as described above. The effect of PEA (5 μM) on the enzymatic hydrolysis of AEA was studied as described previously [20] by using cell membranes incubated with either of the two compounds 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).

3. Results and discussion

In agreement with previous studies [18,20,21,23], AEA induced a dose-dependent ($EC_{50} = 0.44 \pm 0.11$ μM) increase in $[Ca^{2+}]_i$ in HEK-hVR1 cells, which was blocked by capsazepine and was absent in wild-type HEK cells (not shown). PEA (0.5–10 μM), incubated with cells 5 min prior to AEA (50 nM), dose-dependently (estimated $EC_{50} \sim 0.7$ μM) enhanced the effect of the latter compound from 9.2 ± 4.4 to 24.6 ± 4.5% of the effect of 4 μM ionomycin (Fig. 1A). PEA alone also induced a response, which was, however, negligible compared to that observed with similar doses of AEA alone (up to 13.3 ± 3.2% of the effect of ionomycin with 10 μM PEA, mean ± S.E.M., $n = 10$), and had returned to baseline when AEA was added to the cells after PEA pretreatment (not shown). When increasing concentrations of AEA were tested

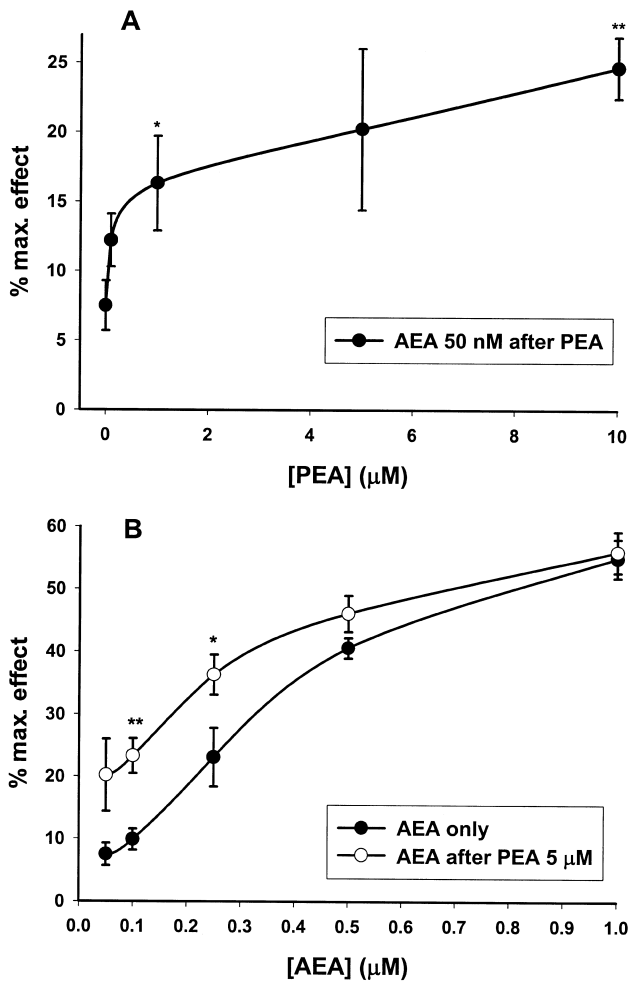

Fig. 1. Effect of PEA on the VR1-mediated stimulation of $[Ca^{2+}]_i$ by AEA in HEK-hVR1 cells. A: Effect of increasing concentrations of PEA on the effect of 50 nM anandamide. B: Effect of 5 μM PEA on the effect of varying concentrations of AEA. PEA was pre-incubated with cells 5 min prior to AEA treatment. The effect was measured as the % of the effect on $[Ca^{2+}]_i$ of 4 μM ionomycin. Data are means ± S.D. of three separate determinations. *$P < 0.05$; **$P < 0.01$, unpaired Student's *t*-test.

after PEA (5 μM), the half-maximal concentration of the former compound for the stimulation of $[Ca^{2+}]_i$ was decreased from 0.44 to 0.22 μM (Fig. 1B). The effect of PEA was more marked, and statistically significant, only with concentrations of AEA < 0.25 μM, i.e. at concentrations at least 20-fold lower than the concentration of PEA used, which reflects to some extent the molar ratio at which PEA and AEA are found in most tissues.

We next wanted to determine if the effect of PEA was due to inhibition of AEA enzymatic hydrolysis. In fact, it has been previously shown that degradation, very probably by FAAH, significantly minimizes AEA activity at VR1 [20,25], and that HEK-hVR1 cells do express FAAH and can hydrolyze AEA [20]. However, no effect was found here of PEA on the hydrolysis of [^{14}C]AEA by HEK-hVR1 cell membranes (not shown). Nevertheless, we decided to investigate if the enhancement of AEA-induced activation of hVR1 could also be observed with other VR1 ligands, i.e. RTX and capsaicin. These compounds, while being several-fold more potent than AEA on VR1, are not substrates for FAAH and their effect

on $[Ca^{2+}]_i$ is insensitive to inhibitors of AEA hydrolysis [20,25]. Indeed, we found that, when using concentrations of RTX <4 pM, and of capsaicin <2 nM, PEA (5 μM) also significantly enhanced the effect of these two potent VR1 agonists (Table 1). Interestingly, the effect was more marked with RTX than with capsaicin. It is noteworthy that for many years it was believed that distinct binding sites existed for these two compounds [16], but recent data have ruled out this possibility [26]. However, it is likely that RTX and capsaicin interact with different amino acid residues within the ligand binding site of VR1. Although these data rule out the participation of FAAH in PEA-induced enhancement of VR1 ligand activity, we cannot exclude that other 'entourage' compounds [27] that are better substrates for the enzyme, such as oleoylethanolamide or linoleoylethanolamide, or oleamide, also potentiate AEA activity at vanilloid receptors. Preliminary studies carried out in our laboratories seem to indicate that this might be the case.

We also wanted to ascertain that PEA was not enhancing AEA action by merely preventing this compound from binding to the cuvette used for the fluorimetric analysis. Therefore, we assayed the effect on AEA-induced increase of $[Ca^{2+}]_i$ of BSA, which is normally used to avoid lipophilic substances from sticking to plastic and glass ware. We were surprised to find that BSA did not enhance but instead potently inhibited the AEA effect on hVR1 (Fig. 2). This finding can be explained by suggesting that BSA prevents the uptake of AEA by HEK-hVR1 cells, thus interfering with the carrier-mediated internalization of this compound, which is necessary to observe activation of an intracellular hVR1 site by AEA [20]. Indeed, previous studies have shown that BSA is necessary to observe AEA release from cells because otherwise this compound is immediately taken up from the incubation medium [28]. These data rule out the possibility that PEA enhancement of AEA action on VR1 is due to inhibition of binding to non-specific sites, and explains why a potency 5–10-fold lower than that reported by us ([19,20,23] and this study) has been observed for AEA on VR1 by other authors [18,25,29] who all introduced BSA in their VR1 assay protocols.

Table 1
Effect of PEA on the VR1-mediated stimulation of $[Ca^{2+}]_i$ by low doses of RTX and capsaicin in HEK-hVR1 cells

Substance	% effect of ionomycin (4 μM)
RTX 1 pM	9.8 ± 2.3
PEA 0.01 μM+RTX 1 pM	10.9 ± 4.1
PEA 0.05 μM+RTX 1 pM	14.8 ± 2.1*
PEA 0.1 μM+RTX 1 pM	15.2 ± 3.9*
PEA 1 μM+RTX 1 pM	23.0 ± 3.5**
PEA 5 μM+ RTX 1 pM	23.5 ± 4.9**
PEA 10 μM+RTX 1 pM	26.5 ± 5.1**
RTX 2 pM	16.1 ± 3.0
PEA 5 μM+RTX 2 pM	34.0 ± 7.2**
RTX 4 pM	33.9 ± 3.1
PEA 5 μM+ RTX 4 pM	44.9 ± 4.1*
Capsaicin 1 nM	7.6 ± 1.2
PEA 1 μM+capsaicin 1 nM	14.1 ± 1.2*
Capsaicin 4 nM	11.9 ± 3.1
PEA 1 μM+capsaicin 4 nM	23.1 ± 3.2**

PEA was preincubated with cells 5 min prior to RTX and capsaicin treatment. The effect was measured as the % of the effect on $[Ca^{2+}]_i$ of 4 μM ionomycin. For capsaicin, only the doses of PEA and capsaicin that yielded maximal enhancement are shown. Data are means ± S.D. of three separate determinations. *$P<0.05$; **$P<0.01$, unpaired Student's t-test.

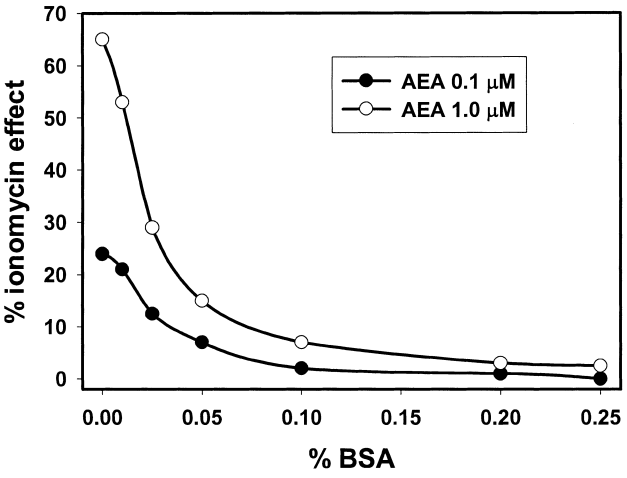

Fig. 2. Effect of BSA on the VR1-mediated stimulation of $[Ca^{2+}]_i$ by AEA in HEK-hVR1 cells. The effect of increasing concentrations of BSA was assessed on two concentrations of AEA. BSA was preincubated with cells 5 min prior to AEA treatment. Data are means of three separate determinations. S.D. bars are not shown for the sake of clarity.

Finally, we investigated if the PEA effect was due to enhancement of AEA binding to VR1. We studied the effect of PEA (5 μM) on AEA displacement of [^{3}H]RTX from membranes from HEK-hVR1 cells and found a decrease of the K_i of AEA from 18.8 to 3.9 μM (Fig. 3). In this case, the dose–response curve of AEA was shifted leftwards, suggesting that PEA might act as an allosteric factor for AEA binding to hVR1, and that this effect may be responsible, at least in part, for PEA-induced enhancement of the AEA effect on $[Ca^{2+}]_i$. We were not surprised to observe a high K_i value for the displacement of [^{3}H]RTX by AEA, since Ross et al. [25] have reported an even higher value when using membranes from CHO cells over-expressing the rat VR1 in the absence of AEA hydrolysis inhibitors.

In conclusion, we have shown here that PEA can significantly enhance the VR1-mediated action of AEA on $[Ca^{2+}]_i$. This effect was not due to inhibition of AEA hydrolysis or of AEA binding to non-specific sites during the experiments, but more likely to stimulation of AEA interaction with a RTX

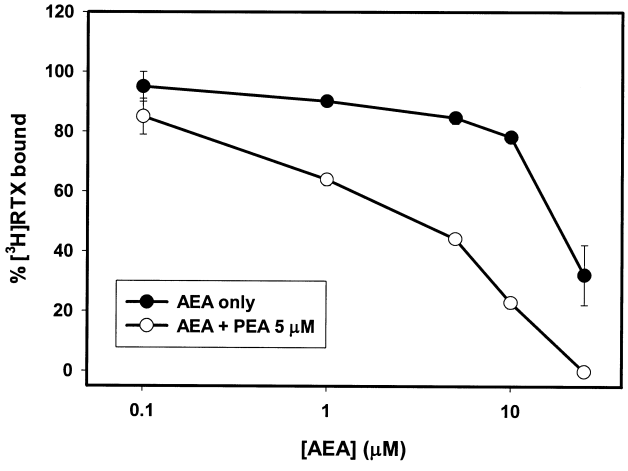

Fig. 3. Effect of PEA on the displacement by AEA of [^{3}H]RTX from HEK-hVR1 cell membranes. Data are means ± S.D. of three separate determinations.

binding site within hVR1. Nevertheless, the effect reported here can still be described as an 'entourage' [4,27] action inasmuch as it was observed at molar ratios of AEA and PEA (from 1:10 to 1:50) similar to those found in tissues and cells (usually from 1:5 to 1:20). Likewise, 2-palmitoylglycerol was shown to enhance the effects on cannabinoid receptors of the other endocannabinoid 2-arachidonoylglycerol without inhibiting its inactivation by cells [30]. It is possible that, by interacting directly with VR1, PEA also enhances other known biological effects of VR1 ligands. Indeed, we have found (D. Melck and V. Di Marzo, unpublished results) that PEA significantly potentiates the antiproliferative action of capsaicin and RTX on human breast cancer cells, an effect that was previously reported to be counteracted by the VR1 blockers capsazepine and ruthenium red [31].

Acknowledgements: The authors are grateful to Ines Brandi, Aniello Schiano Moriello, Alessia Ligresti and Tiziana Bisogno for their valuable assistance. Supported in part by MURST (Grant 3933 to V.D.M.).

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