Pergamon

SCIENCE DIRECT®

Bioorganic & Medicinal Chemistry 11 (2003) 817–825

BIOORGANIC & MEDICINAL CHEMISTRY

N-Morpholino- and *N*-Diethyl-analogues of Palmitoylethanolamide Increase the Sensitivity of Transfected Human Vanilloid Receptors to Activation by Anandamide Without Affecting Fatty Acid Amidohydrolase Activity

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Received 1 October 2002; accepted 5 November 2002

Abstract—The abilities of 19 analogues of palmitoylethanolamide and two analogues of oleoylethanolamide to affect the Ca^{2+} influx into human embryonic kidney cells expressing the human vanilloid receptor (hVR1-HEK293 cells) in response to anandamide (AEA) have been investigated using a FLIPR assay and a bovine serum albumin-containing assay medium. Only palmitoylethanolamide produced any effect in the absence of AEA. The ability of palmitoylethanolamide to potentiate the response to AEA was retained when the *N*-CH_2CH_2OH group was replaced by *N*-CH_2CH_2Cl,whereas replacement with *N*-alkyl substituents [from –H up to $-(CH_2)_{12}CH_3$] resulted either in a reduction or in a complete loss of this activity. The tertiary amide *N*-$(CH_2CH_3)_2$ (**19**) and *N*-morpholino (**20**) analogues of palmitoylethanolamide potentiated the response to 1 μM AEA to a greater degree than the parent compound, whereas the *N*-$(CH_3)_2$ analogue was inactive. **19** and **20** produced leftward shifts in the dose–response curve for AEA activation of Ca^{2+} influx into hVR1-HEK293 cells. EC_{50} values for AEA to produce Ca^{2+} influx into hVR1-HEK293 cells were 1.1, 1.1, 0.54 and 0.36 μM in the presence of 0, 1, 3 and 10 μM **19**, respectively. The corresponding values for **20** were 1.5, 1.3, 0.77 and 0.17 μM, respectively. The compounds did not affect the dose–response curves to capsaicin. The ability of oleoylethanolamide to potentiate AEA is retained by the *N*-CH_2CH_3 and *N*-$CH(CH_3)_2$ analogues (**22** and **23**, respectively). **22** and **23** produced a small (~25%) inhibition of the binding of [^{3}H]-CP55,940 and [^{3}H]-WIN 55,212-2 to CB_1 and CB_2 receptors, respectively, expressed in CHO cells. The compounds inhibited the metabolism of 2 μM [^{3}H]-AEA by rat brain fatty acid amidohydrolase with IC_{50} values of 5.6 and 11 μM, respectively. In contrast, **19** and **20** were without effect on either binding to CB receptors or fatty acid amidohydrolase activity. Minor reductions in the accumulation of 10 μM [^{3}H]-AEA into C6 glioma cells were seen at 10 μM concentrations of **19** and **20**. It is concluded that **19** and **20** selectively enhance AEA effects upon VR1 receptors without potentially confounding effects upon CB receptors or fatty acid amidohydrolase activity.

Introduction

Vanilloid receptors (VR) are ligand-gated ion channels that are located on sensory nerve terminals and play an important role in nociception.[1] VR can be activated not only by heat, protons, vanilloids like capsaicin and resiniferatoxin,[2] but also by the endogenous cannabinoid receptor (CB) agonist anandamide (arachidonoylethanolamide, AEA).[3,4] A number of experiments have indicated that the binding site(s) for capsaicin and anandamide, in contrast to that for protons,[2,5] are located intracellularly.[6–9] In hVR1-HEK cells transfected with VR1 receptors, AEA acts as a full agonist, producing a rapid influx of calcium that is blocked by the VR antagonist capsazepine, and is not seen when untransfected HEK cells are used.[4] AEA is removed from the medium by a process of cellular accumulation followed by hydrolysis by fatty acid amidohydrolase (FAAH) to form arachidonic acid.[10,11] Consistent with the intracellular localization of the AEA activation site on VR,

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PII: S0968-0896(02)00567-9

FAAH inhibitors enhance, and inhibitors of AEA accumulation block, respectively, the Ca^{2+} response to exogenous AEA in hVR1-HEK cells.[8] These cellular processes are shown schematically in Figure 1.

Although it has been established without doubt that AEA activates VR, it is not clear as to whether or not this compound is an endovanilloid, since the potency required for VR activation is usually rather higher than needed for activation of CB receptors.[12] In vivo, however, AEA is synthesised on demand together with other *N*-acyl ethanolamines such as palmitoylethanolamide (**1**), (structure shown in Fig. 2); indeed, in most tissues, the concentrations of these other *N*-acyl ethanolamines are much higher than that of AEA.[13] Addition of these compounds potentiate VR1-mediated effects of AEA both for cells transfected with the human receptor,[14,15] and, in the case of **1**, in perivascular sensory nerves naturally expressing the receptor.[16] For **1** the EC_{50} value for the ability of AEA to produce a Ca^{2+}-influx into hVR1-HEK cells was decreased by a factor of two.[14] The shorter chain homologue lauroylethanolamide was even more dramatic in this respect, reducing the EC_{50} value for AEA more than 8-fold.[15] This property of long chain *N*-acyl ethanolamines may be an important regulator of the actions of AEA upon vanilloid receptors under severe inflammatory conditions, given that their synthesis is increased under such conditions.[17]

Although the abilities of the *N*-acyl ethanolamine homologues of **1** to affect the VR response to AEA in hVR1-HEK cells have been fully investigated,[15] little is known as to whether variations in the ethanolamine side chain of **1** affect its properties in this regard. The only compounds so far evaluated, where the *N*-ethanolamine side chain was replaced by either a primary NH_2,(**2**) *N*-ethylamine (**4**), *N*-butylamine (**6**), *N*-isopropylamine (**8**), *N*-cyclohexylamine (**15**) (structures shown in Fig. 3) or by trifluoromethyl ketone groups, were without obvious effect either per se or upon the Ca^{2+}-response to AEA.[15] In the present study, we have investigated the abilities of 14 additional analogues of **1** and 2 analogues of oleoylethanolamide to potentiate the response to AEA in hVR1-HEK cells. The most interesting compounds have in addition been characterised for their ability to interact with CB receptors, FAAH and the AEA accumulation process.

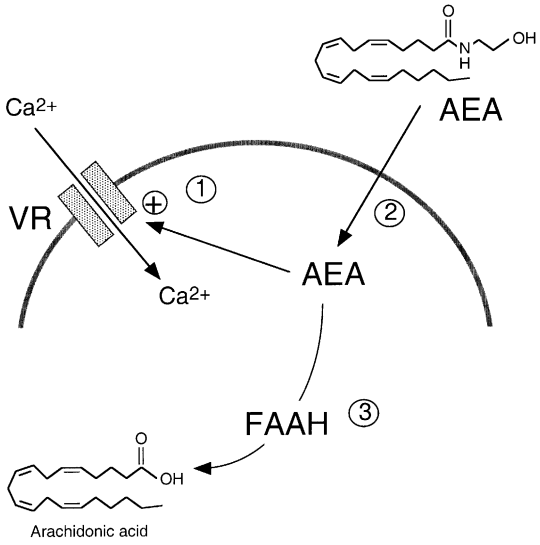

Figure 1. Schematic representation of the action of anandamide at VR1. After intracellular accumulation (2), the compound acts intracellularly upon VR1 (1) to produce an influx of calcium. The intracellular anandamide is metabolised to arachidonic acid by fatty acid amidohydrolase (3). These three sites are thus potential targets whereby novel compounds can potentiate the response of the vanilloid receptor to anandamide.

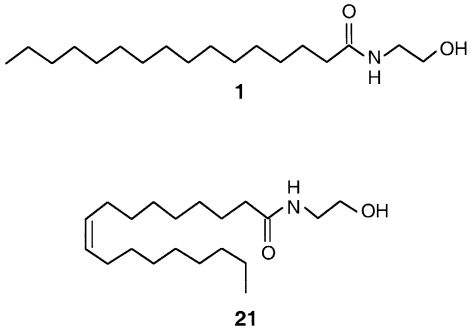

Figure 2. Structures of palmitoylethanolamide (**1**) and oleoylethanolamide (**21**).

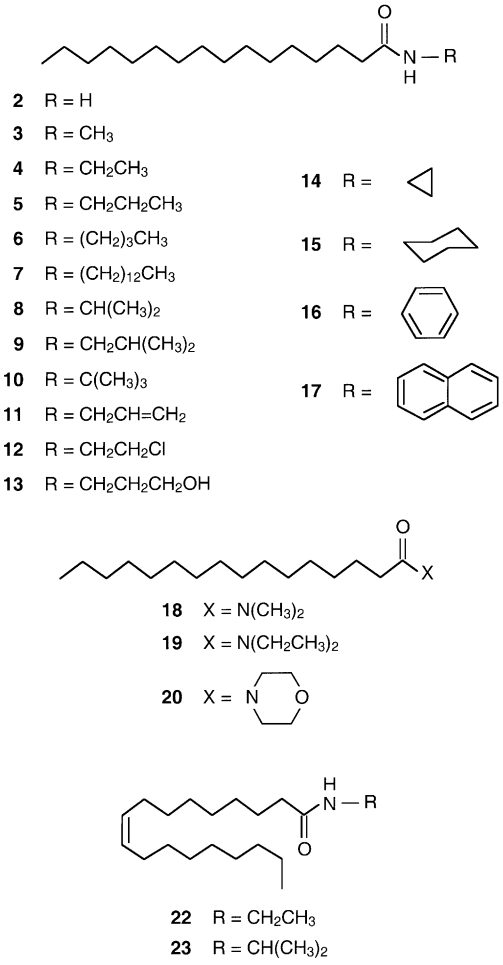

Figure 3. Structures of primary (**2**), secondary (**3**–**17**), tertiary (**18**–**20**) amides deriving from palmitoylethanolamide. Structures of oleoyl-derivatives (**22**,**23**).

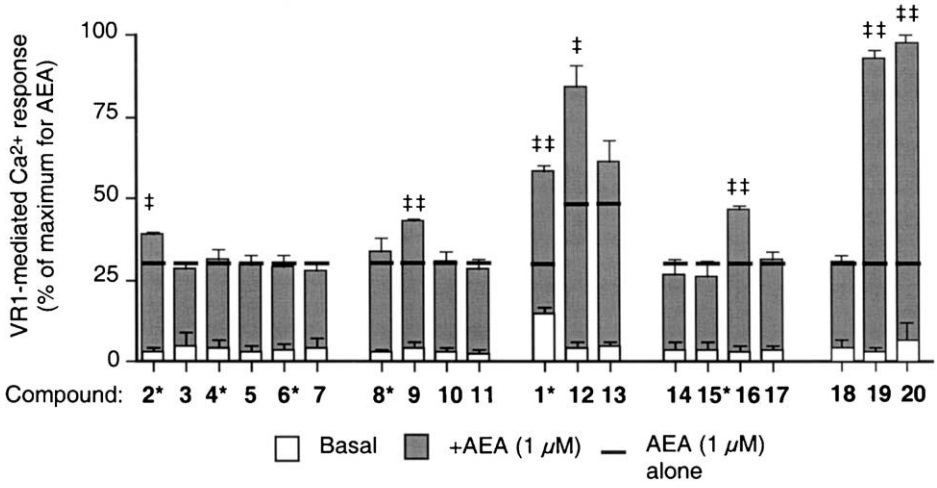

Figure 4. Effects of a series of analogues of **1** upon the Ca^{2+}-influx in hVR1-HEK293 cells in the absence ('basal') and presence of 1 μM AEA. The compounds were used at a concentration of 10 μM. Shown are means ± SE mean, $n = 3$–4. The solid bars indicate the mean response to 1 μM AEA in the absence of the test compounds assayed concomitantly. The means ± SE mean values corresponding to these two solid bars are 30 ± 2.65 and 48 ± 9.6%. Compounds listed with an asterisk indicate data from Smart et al.[15] $^{\ddagger}p < 0.05$, $^{\ddagger\ddagger}p < 0.01$ versus the corresponding response to 1 μM AEA in the absence of the test compound, two-tailed *t*-test.

Results

Effects of amide analogues of palmitoylethanolamide upon AEA-induced activation of VR1 receptors in hVR1-HEK293 cells

A series of primary and secondary amide analogues were tested at a concentration of 10 μM to determine their effects upon Ca^{2+} influx into hVR1-HEK293 cells (Fig. 4). Experiments were conducted to see whether the compounds alone produced a calcium response ('basal') and to determine whether they potentiated the response to a submaximal concentration (1 μM) of AEA. The FLIPR assay methodology required the presence of bovine serum albumin to prevent binding of the AEA to the assay plates. Bovine serum albumin avidly binds AEA and thus affects the observed potency of AEA at VR1.[14] However, the AEA concentration used here (1 μM) is close to its EC_{50} value (~1.3 μM, see below) under the present assay conditions, and is thus on the steepest part of the dose-response curve, and therefore highly sensitive to potentiating effects. The compounds were tested in two separate experimental series. In the first series, AEA gave a response of 30 ± 2.7% of maximal, whereas in the second series, the response was slightly higher (48 ± 10%). This difference simply reflects the fact that the concentration of AEA is on the steepest part of the dose–response curve. In consequence, the appropriate AEA controls are shown for each compound in the figure. The analogues previously published[15] are also shown in the figure for comparative purposes.

Of all the analogues tested, only palmitoylethanolamide (**1**) possessed any large intrinsic activity per se at the concentration of 10 μM. In addition, among the primary and secondary amide analogues, the only compound which completely retained the ability of **1** to potentiate the response to AEA was the compound where the *N*-CH_2CH_2OH group was replaced by *N*-CH_2CH_2Cl (**12**). Replacement with *N*-alkyl substituents [from –H up to –$(CH_2)_{12}CH_3$] and *N*-cyclic substituents (-cyclopropyl, -cyclohexyl, and -naphthyl) resulted either in a reduction or in a complete loss of this activity (Fig. 4).

Three tertiary amide analogues of **1** were tested (Fig. 4). The *N*-$(CH_2CH_3)_2$ and *N*-morpholino analogues (**19** and **20**, respectively) produced a large potentiation of the response to 1 μM AEA, whereas the *N*-$(CH_3)_2$ analogue (**18**) did not. **20** was also tested in the second experimental series (i.e., with the higher response to AEA alone) and gave similar values (5.4 ± 1.3 and 89 ± 3.3% of maximal response for basal and + AEA conditions, respectively).

Two secondary amide analogues of oleoylethanolamide (**21**) were also tested (Fig. 5). Both the *N*-CH_2CH_3 and *N*-$CH(CH_3)_2$ analogues (**22** and **23**, respectively) of **21** potentiated the response to AEA.

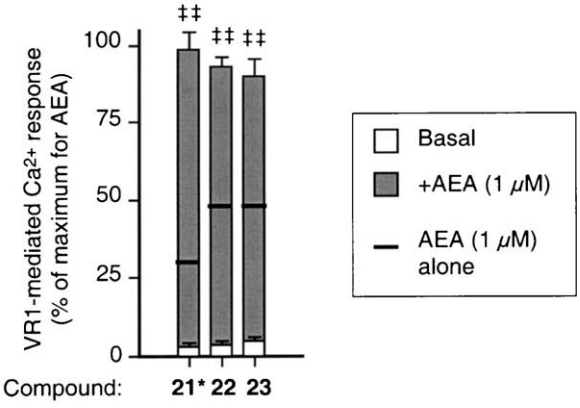

Figure 5. Effects of two secondary amide analogues of **21** upon the Ca^{2+}-influx in hVR1-HEK293 cells in the absence ('basal') and presence of 1 μM AEA. The compounds were used at a concentration of 10 μM. Shown are means ± SE mean, $n = 3$–4. For further details, see legend to Figure 4.

Comparison of the effects of 19 and 20 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 Fig. 6. **19** and **20** produced leftward shifts in the dose-response curve for AEA activation of Ca^{2+} influx into hVR1-HEK293 cells (Fig. 6A and B). EC_{50} values for AEA to produce Ca^{2+} influx into hVR1-HEK293 cells were 1.1, 1.1, 0.54 and 0.36 μM in the presence of 0, 1, 3 and 10 μM **19**, respectively. The corresponding values for **20** were 1.5, 1.3, 0.77 and 0.17 μM, respectively. The compounds did not affect the dose–response curves to capsaicin (Fig. 6C and D).

Interaction of 19, 20, 22 and 23 with CB_1 and CB_2 receptors

The ability of **19**, **20**, **22** and **23** (10 μM) to inhibit the binding of [^{3}H]-CP55,940 to CB_1 receptors and of [^{3}H]-WIN55,212-2 to CB_2 receptors expressed on CHO cells is shown in Table 1. The data are shown as % of specific inhibition of the radioligand. **19** did not affect the binding to the receptors, and **20** produced a small (15.5±0.3%) inhibition of the binding to CB_1 receptors. **22** and **23** produced a modest inhibition (20–28%) of the binding. Thus the analogues behave like their corresponding-ethanolamines with respect to effects at CB receptors.[18]

Interaction of 19, 20, 22 and 23 with FAAH

The ability of the compounds to inhibit the FAAH-catalysed metabolism of [^{3}H]-AEA (2 μM) was investigated using rat brain membranes as enzyme source (Fig. 7A).

Table 1. Effects of **19**, **20**, **22** and **23** upon the binding of [^{3}H]-CP55,940 and [^{3}H]-WIN 55,212-2 to CB_1 and CB_2 receptors, respectively, expressed in CHO cells

Compd (10 μM)	% Inhibition of specific binding	
	Cell line: CHO-CB_1 Ligand: [^{3}H]-CP 55,940	CHO-CB_2 [^{3}H]-WIN 55212,2
19	3.3±0.3	2.7±0.1
20	15.5±0.3	4.2±0.3
22	27.5±0.3	20.1±0.3
23	27.3±0.5	23.1±0.3

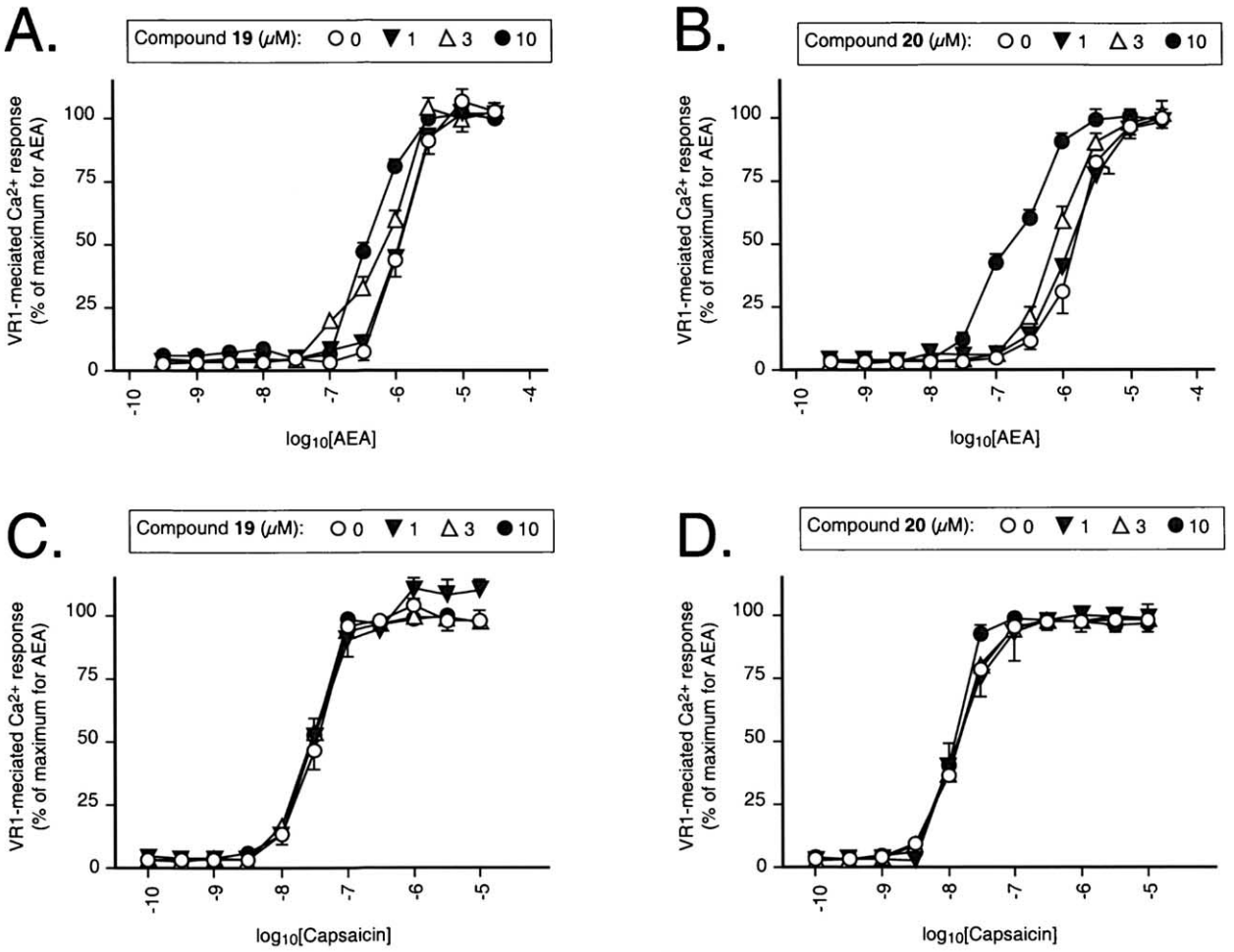

Figure 6. Effects of **19** (**A**,**C**) and **20** (**B**,**D**) upon the Ca^{2+}-influx in hVR1-HEK293 cells produced by (**A**,**B**) AEA and (**C**,**D**) capsaicin. Data are means of four experiments. In (**A**) and (**B**) the SE means are shown (when not enclosed by the symbols) for the sake of clarity only for the curves for 0, 3 and 10 μM compounds; In (**C**) the SE means are shown only for the curves for 0 and 1 μM **19**; and in (**D**) the SE means are shown only for the curves for 1 and 10 μM **20**.

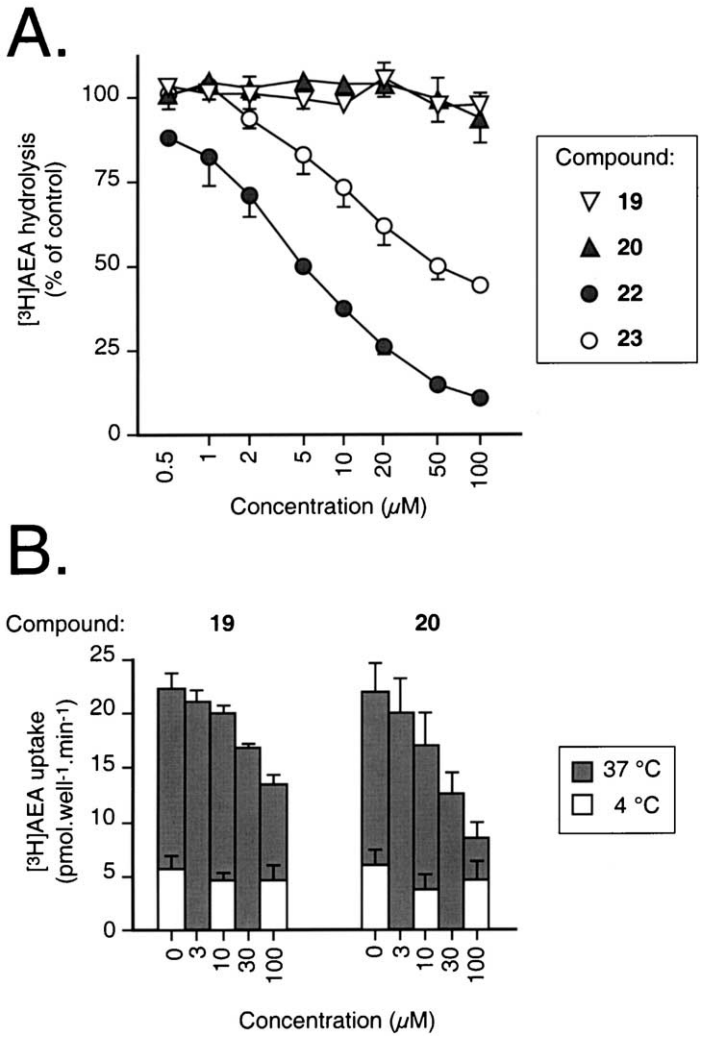

Figure 7. Panel A: Effects of **19**, **20**, **22** and **23** upon the FAAH-catalysed metabolism of 2 μM [^{3}H]-AEA in rat brain homogenates. Panel B: Effects of **19** and **20** upon the uptake of 10 μM [^{3}H]-AEA into C6 glioma cells. Data are means $n = 3$–5, with SE mean values shown when not enclosed by the symbols or columns. In panel (**B**), the effects upon uptake at 4 °C were not tested for 3 and 30 μM concentrations of **19** and **20**.

22 and **23** inhibited [^{3}H]-AEA metabolism with IC_{50} values of 5.6 and 11 μM, respectively. The IC_{50} values refer to the inhibitable fraction of the [^{3}H]-AEA metabolism (100 and 58% of the total for **22** and **23**, respectively). In contrast, **19** and **20** did not inhibit [^{3}H]-AEA metabolism even at a concentration of 100 μM.

Effect of 19 and 20 upon [^{3}H]-AEA accumulation into C6 glioma cells

The effects of **19** and **20** upon the uptake of 10 μM [^{3}H]-AEA into C6 glioma cells are shown in Figure 7B. **19** was a weak inhibitor of uptake at 37 °C, producing 10 ± 8% and 39 ± 7% inhibition at concentrations of 10 and 100 μM, respectively. **20** was a more potent uptake inhibitor (IC_{50} value 49 μM), although at a concentration of 10 μM, the inhibition of uptake was only 23 ± 4%.

Discussion

In the present study, the abilities of a series of palmitoylethanolamide (**1**) and oleoylethanolamide (**21**) analogues to affect the VR-mediated Ca^{2+}-influx in response to AEA has been investigated in hVR1-HEK293 cells. In contrast to the modest partial agonist effect of **1** seen in these cells,[4] none of the compounds produced dramatic VR agonist effects in the absence of AEA. The most interesting finding in this respect is that the *N*-phenyl derivative **16** was without observable efficacy, since the *N*-(3-methoxy-4-hydroxyphenyl) analogue of **1**, palvanil, is as effective and considerably more potent than AEA as a VR agonist in hVR1-HEK293 cells.[19] The lack of efficacy of **21** (and indeed the efficacy per se of **1**) contrasts with the situation for VR in mesenteric arteries.[16] However, this responsiveness is presumably related to a number of factors, not the least being the level of receptor expression. More important for the present study are the reports that **1** not only potentiates AEA responsiveness at VR in transfected cells,[14,15] but also in physiological systems naturally expressing VR.[16]

The structural requirements for the ethyl side chain with respect to the potentiation of the VR response to a submaximal concentration of AEA are rather different to that for an effect per se. Whist the hydroxyl group of

1 can be replaced by a chloride group without loss of efficacy, replacement with a methyl or ethyl group removes the ability to potentiate AEA. Among the analogues of 1, the two compounds showing the most marked potentiation of the response to AEA were 19 and 20. This potentiation was due to a leftward shift in the dose–response curve for AEA. A corresponding leftward shift for the capsaicin dose–response curve was not seen, indicating a selective effect upon the response to AEA. This result is similar to that reported previously for 1[14] and lauroylethanolamide,[15] although the leftward shift for 1 was less marked than for 19 and lauroylethanolamide.

A direct effect on the VR to increase the sensitivity of the receptor to AEA is the simplest explanation of the present data (and supported by previous findings with 1[14]). However, other modes of action should be considered. In theory, compounds can produce a potentiation of Ca^{2+}-influx response to AEA by preventing the breakdown of AEA and thereby increasing its available concentration. FAAH inhibitors potentiate the effects of AEA at VR receptors in some,[8,20] but not all[16] model systems. However, 19 and 20 do not affect FAAH, which would rule out this mode of action, at least for these compounds. Nevertheless, FAAH inhibition can in theory contribute to the potentiating effects of the 21 analogues 22 and 23, since these compounds inhibited FAAH with IC_{50} values of 5.6 and 11 μM, respectively. For comparison, 21 inhibited FAAH (by acting as an alternative substrate[21]) with an IC_{50} value, under the same assay conditions, of 4.7 μM.[18] The corresponding *N*-ethanolamine (1), *N*-CH_2CH_3 (4) and *N*-$CH(CH_3)_2$ (8) compounds in the palmitoyl-series were also FAAH inhibitors with rather similar potencies (IC_{50} values 5.1, 3.6 and 13 μM) (ref 18 and unpublished data).

An alternative explanation would be that the compounds are able to increase the sensitivity of the VR1 to AEA by increasing its rate of cellular accumulation, given that the activation site for AEA is located intracellularly.[8,9] Although no molecular characterization of an anandamide membrane transporter has yet been reported and the detailed mechanism of cellular AEA accumulation is a matter of debate,[22–24] the accumulation process can certainly be modulated either to be less active (by inhibiting FAAH, since removal of AEA intracellularly drives the uptake process[25,26]) or more active (by the addition of NO donors[27,28]). However, this mechanism does not account for the properties of 19 and 20, since they have minor effects on the accumulation of AEA, at least into C6 glioma cells, at the concentrations required to affect the VR response to AEA. In this respect, it is at first sight surprising that 21 produces a potentiation of the VR response to AEA, given the report[22] that the concentration of this compound used here reduces the accumulation of AEA into cerebellar granule cells by 61%. However, the concentration of [^{3}H]AEA used in those experiments was very low (0.2 nM), and at a higher AEA concentration, 10 μM 21 does not affect the accumulation of AEA into C6 cells.[18]

In conclusion, the present study has demonstrated that the Ca^{2+} influx response mediated by VR1 receptors in hVR1-HEK293 cells can be potentiated not only by *N*-acyl ethanolamines, but by the *N*-morpholinyl and *N*-diethyl analogues of 1. These analogues lack potential confounding effects on FAAH and upon CB_1 and CB_2 receptors, and may be useful agents, by enhancing the effects of exogenously added AEA upon VR1 receptors, to explore the physiological properties of this 'endovanilloid'.

Experimental

Compounds

Anandamide [ethanolamine-1-^{3}H] ([^{3}H]-AEA; specific activity 30–60 Ci mmol^{-1}) was obtained from American Radiolabeled Chemicals, Inc. (St. Louis, MO, USA). [^{3}H]-WIN 55,212-2 (specific activity 1.68 TBq/mmol, 45.5 Ci mmol^{-1}) and [^{3}H]-CP55,940 (specific activity 3.737 TBq/mmol, 101 Ci mmol^{-1}) were obtained from New England Nuclear, Boston, MA, USA. The syntheses of the compounds tested, with the exception of the four compounds described below, have either been reported previously[18,29,30] or will be reported elsewhere. Capsaicin, non-radioactive AEA (for the VR1 experiments), HU-210 [(6αR)-*trans*-3-(1,1-dimethylheptyl)-6α,7,10,10α-tetrahydro-1-hydroxy-6,6-dimethyl-6*H*-dibenzo [*b*,*d*]pyran-9-methanol] was purchased from Tocris (Bristol, UK) and non-radioactive WIN 55,212-2 mesylate [(*R*)-(+)-[2,3-dihydro-5-methyl-3-(4-morpholinylmethyl) pyrrolo[1,2,3-*de*]-1,4-benzoxazin-6-yl]-1-naphthalenylmethanone] was purchased from RBI (Sigma-RBI, Boornem, Belgium). Non-radioactive AEA (for the FAAH and AEA accumulation experiments), was purchased from Cayman Chemical Company (Ann Arbor, MI, USA). Fatty acid free bovine serum albumin, polyethyleneimine and phenylmethylsulfonyl fluoride were obtained from the Sigma Chemical Co. (St Louis, MO, USA). Cell culture media were obtained from Gibco/Life Technologies.

Synthesis of *N*,*N*′-diethylhexadecanamide (20, palmitoyl(diethyl)amide), 4-(1-oxohexadecyl) morpholine (21, palmitoylmorpholinamide), *N*-ethyloleamide (22, oleoylethylamide) and *N*-(1-methylethyl)oleamide (23, oleoylisopropylamide)

In all four cases, the same protocol was used. In a two-neck flask, 100 mmol (unless otherwise stated) of diethylamine (for 20), morpholine (for 21), ethylamine (for 22, 5.85 g of a 70% solution in water) or isopropylamine (for 23) were poured into 10 mL of dry methylene chloride. The solutions was cooled in an ice bath and magnetically stirred after which 10 mmol of either palmitoyl chloride (20 and 21) or oleoyl chloride (22 and 23) was added dropwise. The reaction mixture was stirred for 12 h at room temperature and then washed with 5% sodium bicarbonate solution, 1 M HCl and brine. The organic layer was dried over $MgSO_4$ and after filtration and solvent was evaporated under reduced pressure. Yields, chromatographic and spectroscopic data for the compounds are summarised below.

***N*,*N*′-Diethylhexadecanamide 20 (palmitoyl(diethyl)amide).** CA 57303-21-6. Yield: 2.3 g (74%) of a yellowish oil. TLC (chloroform/methanol 6:4 vv^{-1}) R_f = 0.82; spectroscopic data: ^{1}H NMR (*d*-$CDCl_3$) δ (ppm) 0.87 (*t*, *J* = 6 Hz, 3H); 1.09–1.16 (*m*, 6H); 1.22–1.41 (*m*, 26H); 2.27 (*t*, *J* = 7 Hz, 2H); 3.33 (*q*, *J* = 12 Hz, 4H); ^{13}C NMR (*d*-$CDCl_3$) δ (ppm) 13.18; 14.14; 14.49 (CH_3); 22.79; 25.58; 29.64; 29.81; 32.04; 33.17; 40.11; 42.03 (CH_2); 172.11 (C=O); mass spectrometry [M^+·] = 312; IR ν (cm^{-1}): 1646 (C=O).

4-(1-Oxohexadecyl)morpholine 21 (palmitoylmorpholinamide). CA 5299-68-3. Yield: 1.7 g (52%) of a white solid. Mp: 38–39 °C (uncorrected); TLC (chloroform/methanol 6:4 vv^{-1}) R_f = 0.9; spectroscopic data: ^{1}H NMR (*d*-$CDCl_3$) δ (ppm) 0.86 (*t*, *J* = 6 Hz, 3H); 1.22–1.43 (*m*, 26H); 2.29 (*t*, *J* = 7 Hz, 2H); 3.43–3.46 (*m*, 4H); 3.62–3.65 (*m*, 4H); ^{13}C NMR (*d*-$CDCl_3$) δ (ppm) 14.12 (CH_3); 22.71; 25.02; 25.31; 29.39; 29.47; 29.52; 29.55; 29.69; 31.73; 31.96; 33.14; 41.92; 46.13; 66.72; 66.99 (CH_2); 171.89 (C=O); mass spectrometry [$M^{+\bullet}$] = 326; IR ν (cm^{-1}): 1649 (C=O).

***N*-Ethyloleamide 22 (oleoylethylamide).** CA 85075-82-7. Yield: 2.5 g (82%) of a yellowish oil. TLC (ethyl acetate/methanol 8:2 vv^{-1}) R_f = 0.76; spectroscopic data : ^{1}H NMR (*d*-$CDCl_3$) δ (ppm) 0.87 (*t*, *J* = 7 Hz, 3H); 1.12 (*t*, *J* = 7 Hz, 3H); 1.21–1.42 (*m*, 22H); 1.98–2.01 (*m*, 4H); 2.14 (*t*, *J* = 7 Hz, 2H); 3.25–3.28 (*m*, 2H); 5.31–5.35 (*m*, 2H); ^{13}C NMR (*d*-$CDCl_3$) δ (ppm) 14.10; 14.94 (CH_3); 22.71; 25.82; 27.23; 29.31; 29.56; 29.76; 31.89; 34.29; 35.58; 36.87 (CH_2); 129.78; 129.98 (CH); 172.94 (C=O); mass spectrometry [M^+] = 310; IR ν (cm^{-1}): 3288 (NH); 1644 (C=O).

***N*-(1-methylethyl)oleamide 23 (oleoylisopropylamide).** CA 10574-01-3. Yield: 2.42 g (75%) of a yellowish oil. TLC (ethyl acetate/methanol 8:2 vv^{-1}) R_f = 0.82; spectroscopic data: ^{1}H NMR (*d*-$CDCl_3$) δ (ppm) 0.87(*t*, *J* = 7 Hz, 3H) ; 1.12 (*t*, *J* = 3 Hz, 6H); 1.21–1.42 (*m*, 22H); 1.98–2.02 (*m*, 2H); 2.11 (*t*, *J* = 7 Hz, 2H); 3.25–3.28 (*m*, 2H); 4.07–4.09 (*m*, 1H); 5.31–5.34 (*m*, 2H); ^{13}C NMR (*d*-$CDCl_3$) δ (ppm) 14.16; 14.54 (CH_3); 22.71; 22.90; 25.88; 27.23; 29.37; 29.56; 29.82; 31.96; 37.07 (CH_2); 41.21; 129.78; 130.04 (CH); 172.23 (C=O); mass spectrometry [M^+·] = 324; IR ν (cm^{-1}): 3285 (NH); 1641 (C=O).

Cell culture

hVR1-HEK293 cells, grown as monolayers in minimum essential medium (MEM) supplemented with non-essential amino acids, 10% foetal bovine serum, and 0.2 mM L-glutamine, were maintained under 95%/5% O_2/CO_2 at 37 °C. Cells were passaged every 3–4 days and the highest passage number used was 15.

Human CB_1-('CHO-CB_1') and CB_2-('CHO-CB_2') transfected CHO cells (passage range 11–13 and 12–15, respectively), kindly donated by Drs. Nokin and Detheux (Euroscreen, Belgium) were cultured in Ham's F12 medium containing 10% fetal bovine serum, 100 μg/mL streptomycin, 100 U/mL penicillin and 200 μg/mL G418.

Rat C6 glioma cells (passage range 59–60), obtained from the American Type Culture Collection, MD, USA, were grown in 75 cm^2 culturing flasks at 37 °C, 5% CO_2 in air at normal atmospheric pressure. The cells were cultured in Ham's F10 medium, supplemented with 10% foetal bovine serum and 100 units mL^{-1} penicillin + 100 μg mL^{-1} streptomycin.

Measurement of $[Ca^{2+}]_i$ using the FLIPR technology

The method of Smart et al.[4] was used. hVR1-HEK293 cells (density 25 × 10^3 cells well^{-1}) were seeded into black walled clear-base 96-well plates (Costar UK) and cultured overnight in MEM, supplemented as described above. The cells were then incubated with MEM containing the cytoplasmic calcium indicator, Fluo-3AM (4 μM; Teflabs, Austin, TX, USA) at 25 °C for 120 min. After washing the plates four times with Tyrode's medium containing 0.1% bovine serum albumin, they were placed into a FLIPR (Fluorometric Imaging Plate Reader, Molecular Devices, UK). Cell fluorescence (λ_{EX} = 488 nm, λ_{EM} = 540 nm) was monitored before and after the addition of various compounds in the presence or absence of 1 μM AEA. Incubations were routinely run over three min. The calcium responses generally appeared within a few seconds of injection of the anandamide and peaked at about 30 s. Responses were measured as peak fluorescence intensity (FI) minus basal FI, and results are expressed as a percentage of the maximum response obtained with either anandamide or capsaicin, as appropriate. The observed potency of AEA is highly dependent upon the assay conditions, in particular upon the concentration of bovine serum albumin.[14] However, bovine serum albumin is required in the FLIPR assay to prevent binding of the AEA to the plastic wells. Thus, it is important to stress that the screening concentration of AEA was chosen to give a response of about 30–50% max, thus allowing for potentiation to be seen.

Radioligand binding experiments

Membranes (40 μg) from the CB receptor-transfected CHO cells were incubated at 30 °C with 1 nM [^{3}H]-CP55,940 (CHO-CB_1) or 1 nM [^{3}H]-WIN 55,212-2 (CHO-CB_2) for 1 h in 50 mM Tris–HCl with $MgCl_2$ and EDTA (pH 7.4) in the presence of 50 μM phenylmethylsulfonylfluoride. Non-specific binding was determined in parallel samples using either 10 μM HU-210 (CHO-CB_1) or 10 μM WIN 55,212-2 (CHO-CB_2), as appropriate. After incubation, membrane suspensions were rapidly filtered through 0.5% polyethyleneimine-pretreated GF/B glass fibre filters (Whatman), and the radioactivity trapped on the filters was measured by liquid scintillation spectroscopy. Assays were conducted in quadruplicates.

Assay of FAAH activity

The assay of Omeir et al.,[31] adapted to the tritiated substrate[32] was used. Frozen adult rat brains (minus cerebellum) 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 $MgCl_2$. The homogenates were centrifuged twice (36,000 *g*, 20 min, 4 °C). The tissue pellets were resuspended in homogenisation buffer and incubated at 37 °C for 15 min. After further centrifugation (36,000 *g*, 20 min, 4 °C), the membranes were re-suspended in 50 mM Tris–HCl buffer, pH 7.4, containing 1 mM EDTA and 3 mM $MgCl_2$, and stored at −70 °C until used for assay. Upon assay, membranes (~15 μg protein $assay^{-1}$), test compounds or ethanol carrier, [^{3}H]-AEA and assay buffer (10 mM Tris–HCl, 1 mM EDTA, 1% (w/v) fatty acid-free bovine serum albumin, pH 7.6) were incubated for 10 min at 37 °C (final assay volume of 200 μL). Blanks contained homogenization buffer instead of the membranes. The reactions were stopped by placing the tubes in ice followed by addition of chloroform/methanol (1:1 v/v, 400 μL). Samples were thoroughly mixed, the phases were separated by centrifugation and aliquots (200 μL) of the methanol/buffer phase containing the [^{3}H]-ethanolamine product were removed and analyzed for radioactivity by liquid scintillation spectroscopy with quench correction.

[^{3}H]-AEA accumulation into C6 cells

The method of Rakhshan et al.[33] was used. C6 cells were plated on 24-well plates at an initial density of 1.5–2 × 10^5 cells $well^{-1}$ and incubated overnight at 37 °C under an atmosphere of 5% CO_2 in air at normal atmospheric pressure. Cells were then washed once with 0.5 mL warm assay buffer (120 mM NaCl, 4.7 mM KCl, 2.2 mM $CaCl_2$, 10 mM HEPES, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, pH 7.4; gassed with 95% O_2, 5% CO_2) and pre-incubated in 350 μL buffer with the test compounds or ethanol carrier at 37 °C or 4 °C, as appropriate, for 10 min. [^{3}H]-AEA (50 μL, final concentration 10 μM) was added to the wells and the samples were incubated at 37 or 4 °C, as appropriate, for 15 min. The plates were placed on ice and the cells were washed three times with ice-cold buffer containing 1% BSA. After aspiration of the buffer, NaOH (0.2 M, 500 μL $well^{-1}$) was added and the samples were incubated at 75 °C for 15 min. Aliquots (300 μL) of the solubilized cells were transferred to scintillation vials and the tritium content was determined by liquid scintillation spectroscopy with quench correction. Results are expressed as pmol of tritium retained by the cells min^{-1} $well^{-1}$, after subtraction of the binding to the wells, determined concomitantly (12 ± 0.9 and 15 ± 6.6% of the total tritium retained at 37 and 4 °C, respectively).

Determination of pI_{50} values

pI_{50} values [$-\log_{10}$(IC_{50} value)] and hence IC_{50} values were obtained using the built-in equation 'sigmoid dose–response (variable slope)' of the GraphPad Prism computer programme (GraphPad Software Inc., San Diego, CA, USA) as described in detail previously.[18]

Acknowledgements

The authors would like to thank Britt Jacobsson for her expert technical assistance with the FAAH and AEA uptake experiments. This study was supported by grants from the Swedish Research Council (Grant no. 12158), Konung Gustav V's and Drottning Victorias Foundation, the Swedish Asthma and Allergy Association's Research Foundation, the Swedish Psoriasis Association, and the Research Funds of the Medical Odontological Faculty, Umeå University. The chemical work was supported by a grant from the Belgian National Fund for Scientific Research and an FSR grant from the Université catholique de Louvain.

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