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SPECIAL REPORT

The endogenous lipid anandamide is a full agonist at the human vanilloid receptor (hVR1)

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The endogenous cannabinoid anandamide was identified as an agonist for the recombinant human VR1 (hVR1) by screening a large array of bioactive substances using a FLIPR-based calcium assay. Further electrophysiological studies showed that anandamide (10 or 100 μM) and capsaicin (1 μM) produced similar inward currents in hVR1 transfected, but not in parental, HEK293 cells. These currents were abolished by capsazepine (1 μM). In the FLIPR anandamide and capsaicin were full agonists at hVR1, with pEC_{50} values of 5.94 ± 0.06 ($n=5$) and 7.13 ± 0.11 ($n=8$) respectively. The response to anandamide was inhibited by capsazepine (pK_B of 7.40 ± 0.02, $n=6$), but not by the cannabinoid receptor antagonists AM630 or AM281. Furthermore, pretreatment with capsaicin desensitized the anandamide-induced calcium response and vice versa. In conclusion, this study has demonstrated for the first time that anandamide acts as a full agonist at the human VR1.
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Abbreviations: AM281, (1-(2,4-dichlorophenyl)-5-(4-iodophenyl)-4-methyl-N-4-morpholinyl-1H-pyrazole-3-carboxamide); AM630, (6-iodo-2-methyl-1-[2-(4-morpholinyl)ethyl]-1H-indol-3-yl](4-methoxyphenyl)methanone);$[Ca^{2+}]_i$ intracellular calcium concentration; CP-55,940, ((−)-cis-3-[2-hydroxy-4-(1,1-dimethylheptyl)phenyl]-*trans*-4-(3-hydroxypropyl)cyclohexanol); DRG, dorsal root ganglion; FI, fluorescence intensity; FLIPR, fluorometric imaging plate reader; hVR1, human VR1; MEM, minimum essential medium; VR1, vanilloid receptor; WIN-55,212-2, ((R)-(+)-[2,3-dihydro-5-methyl-3-(4-morpholinylmethyl)pyrrolo[1,2,3-de]-1,4-benzoxazin-6-yl]-1-naphthalenylmethanone)

Introduction The vanilloid receptor (VR1) has recently been cloned (Caterina *et al*., 1997) and is a ligand-gated ion channel which plays an important role in nociception (Szallasi & Blumberg, 1999). Although the pungent plant extract capsaicin activates VR1 (Caterina *et al*., 1997), the endogenous mammalian ligand remains to be identified (Szallasi & Blumberg, 1999).

We screened a collection of over 1000 bioactive substances for activity at the recombinant VR1, and identified the endogenous cannabinoid anandamide (Devane *et al*., 1992) as an agonist at this receptor. This finding was of particular interest for several reasons. Firstly, the structure of anandamide (Pertwee, 1997) bears a striking similarity to several vanilloids (Szallasi & Blumberg, 1999), most notably olvanil (DiMarzo *et al*., 1998). Secondly, anandamide has vasodilatory effects (Pertwee, 1997), which are not mediated by the cannabinoid receptors (White & Hiley, 1998). Thirdly, olvanil has been shown to inhibit the anandamide transporter (DiMarzo *et al*., 1998; Beltramo & Piomelli, 1999). In addition anandamide has recently been reported to act as a partial agonist at the rat VR1 receptor (Zygmunt *et al*., 1999).

Therefore, in the present study the effects of anandamide, and other cannabinoid ligands (Pertwee, 1997), on HEK293 cells stably expressing the hVR1 have been characterized using electrophysiology and a FLIPR-based calcium influx assay. This study has shown for the first time that anandamide acts as a full agonist at the human VR1.

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Methods *Cloning and expression of VR1 receptors in HEK293 cells* Human VR1 cDNA was identified using the published rat VR1 sequence (GenBank accession AF029310) to search public nucleotide databases. Expressed sequence tag T48002 was identified and its sequence extended by rapid amplification of the cDNA ends using cDNA templates from a number of tissue sources. The full cDNA was amplified from brain cDNA, inserted into the expression vector pcDNA3.1, double strand sequenced, and stably expressed in HEK293 cells. Rat VR1 cDNA was amplified from rat DRG cDNA and similarly expressed in HEK293 cells.

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% O_2/CO_2 at 37°C. Cells were passaged every 3–4 days and the highest passage number used was 20. Dissociated rat neonatal DRG cultures were prepared as described by Skaper *et al*. (1990).

Electrophysiological studies Cells were plated and cultured on glass coverslips at 26,000 cells cm^{-2} and whole-cell voltage-clamp recordings were performed at room temperature (20–24°C), using standard methods. The extracellular solution consisted of (mM): NaCl, 130; KCl, 5; $CaCl_2$, 2; $MgCl_2$, 1; Glucose, 30; HEPES-NaOH, 25, pH 7.3. For anandamide application this solution was supplemented with 0.2% lipid free bovine serum albumin. Patch pipettes of resistance 2–5 MΩ were fabricated on a Sutter Instruments P-87 electrode puller and were filled with the following solution (mM): CsCl, 140; $MgCl_2$, 4; EGTA, 10; HEPES-CsOH, 10, pH 7.3. All recordings were made from single, well isolated, phase bright

cells. Currents were recorded at a holding potential of −70 mV using a Axopatch 200B amplifier. Data acquisition and analysis were performed using the pClamp7 software suite. Drug applications were affected with an automated fast-switching solution exchange device (Warner Instruments SF-77B; time for solution exchange ~30 ms).

Measurements of $[Ca^{2+}]_i$ using the FLIPR hVR1-HEK293 cells were seeded into black walled clear-base 96-well plates (Costar U.K.) at a density of 25,000 cells per well in MEM, supplemented as above, and cultured overnight. The cells were then incubated with MEM containing the cytoplasmic calcium indicator, Fluo-3AM (4 μM; Teflabs, Austin, TX, U.S.A.) at 25°C for 120 min. The cells were washed four times with, and finally cultured in, Tyrode's medium containing 0.2% BSA, before being incubated for 30 min at 25°C with either buffer alone (control) or buffer containing various antagonists. The plates were then placed into a FLIPR (Molecular Devices, U.K.) to monitor cell fluorescence (λ_{EX} = 488 nM, λ_{EM} = 540 nM) (Sullivan *et al*., 1999) before and after the addition of various agonists.

Data analysis Responses were measured as peak fluorescence intensity (FI) minus basal FI, and where appropriate, were expressed as a percentage of a maximum capsaicin-induced response. Data are expressed as mean ± s.e.mean unless otherwise stated. Curve-fitting and parameter estimation were carried out using Graph Pad Prism 3.00 (GraphPad Software Inc., CA, U.S.A.). pK_B values were generated from IC_{50} curves for the antagonist vs a fixed EC_{80} concentration of agonist using the Cheng-Prusoff equation.

Materials All cannabinoids were purchased from Tocris (Bristol, U.K.) and all other ligands were obtained from RBI (Natick, MA, U.S.A.). All cell culture media were obtained from Life Technologies (Paisley, U.K.).

Results Application of either anandamide (10 μM) or capsaicin (1 μM) produced inward currents in HEK293 cells stably transfected with the hVR1 receptor. Neither agent produced responses in parental HEK293 cells. The currents produced by both agents developed slowly with mean time constants of 3.78 ± 0.93 s (1 μM capsaicin, n = 7) and 4.01 ± 0.65 s (10 μM anandamide, n = 9) reading peak amplitudes of 122 ± 27 and 44 ± 13 pA, respectively. The currents produced by either agonist exhibited substantial outward rectification and had similar interpolated reversal potentials (2.0 ± 1.4 mV, anandamide; −1.6 ± 1.2 mV, capsaicin; n = 5). The steady-state currents produced by both anandamide and capsaicin could be completely blocked by subsequent co-application of 1 μM capsazepine (Figure 1A); this antagonism developed with mean time-constants of 941 ± 205 ms (n = 9) and 3406 ± 930 ms (n = 7), respectively (P < 0.05, unpaired Student's t-test). In cultured rat DRG neurons anandamide (10 or 100 μM) produced capsazepine-sensitive inward currents in all capsaicin-responsive cells tested (Figure 1B,C). Even in response to 100 μM anandamide the amplitude of the currents recorded were smaller (~10–50%) than those generated by VR1 activation with a maximum concentration of capsaicin.

In the FLIPR, anandamide (100 pM–10 μM), like capsaicin (100 pM–10 μM), caused a concentration-dependent increase in $[Ca^{2+}]_i$ in hVR1-HEK293 cells (Figure 2), but was without effect in the non-transfected HEK293 cell-line (data not shown). Anandamide displayed a similar efficacy to capsaicin (Figure 2), but was markedly less potent (pEC_{50} values of 5.94 ± 0.06 and 7.13 ± 0.11, respectively, at pH 7.4). Moreover, the anandamide- and capsaicin-induced Ca^{2+} responses had indistinguishable kinetics in the FLIPR, with an initially rapid then slowing onset (peak ~30 s) followed by a gradually declining secondary phase.

The anandamide analogues methanandamide and palmitoylethanolamide also increased $[Ca^{2+}]_i$ in hVR1-HEK293 cells in a concentration-related manner, but were less potent

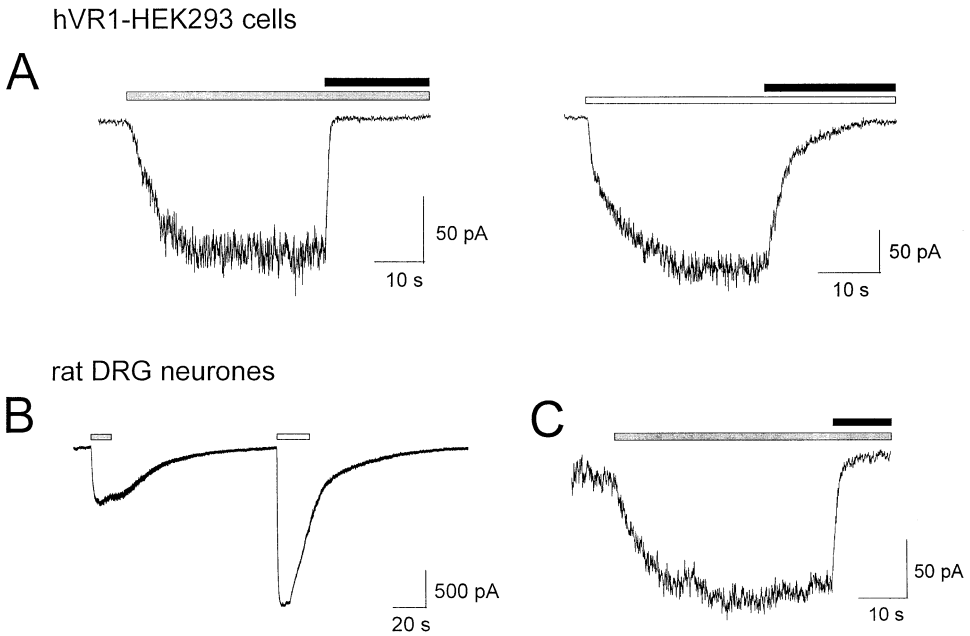

Figure 1 Anandamide-gated currents in hVR1-HEK293 cells and rat DRG neurons are capsazepine sensitive. (A) Application of anandamide (10 μM; grey bar) or capsaicin (1 μM; open bar) led to the appearance of inward currents in cells voltage clamped at −70 mV. These currents were blocked completely by co-application of capsazepine (1 μM; solid bar). Data are representative traces, typical of n = 7–9. (B) Application of anandamide (100 μM; shaded bar) led to the appearance of inward currents in DRG neurons voltage clamped at −70 mV. These cells were also shown to be sensitive to capsaicin (1 μM; open bar). (C) Anandamide-gated currents were blocked by capsazepine (1 μM; solid bar). Data are representative traces, typical of n = 3.

than anandamide, only evoking ~40% responses at 10 μM (Figure 2 and data not shown, $n=3$). Like anandamide both these ligands were without effect in the non-transfected HEK293 cells. The synthetic cannabinoids CP-55,940 (((−)-cis-3-[2-hydroxy-4-(1,1-dimethylheptyl)phenyl]-*trans*-4-(3-hydroxypropyl)cyclohexanol)) and WIN-55,212-2 (((R)-(+)-[2,3-dihydro-5-methyl-3-(4-morpholinylmethyl)pyrrolo[1,2,3-de]-1,4-benzoxazin-6-yl]-1-naphthalenylmethanone)) were without effect in parental or hVR1-expressing HEK293 cells.

Interestingly, lowering the pH of the experimental buffer from 7.4 to 6.4 enhanced the potency of capsaicin (pEC_{50} of 7.13±0.11 at pH 7.4 and 7.86±0.18 at pH 6.4, $n=3$), but had no effect on the potency of anandamide (pEC_{50} of 5.94±0.06 at pH 7.4 and 5.76±0.04 at pH 6.4, $n=3$), or any of the other cannabinoid ligands tested (Figure 2 and data not shown).

The competitive VR1 antagonist (Szallasi & Blumberg, 1999) capsazepine (100 pM–100 μM) inhibited both the anandamide (3 μM)- and capsaicin (100 nM)-induced Ca^{2+} responses, with pK_B values of 7.40±0.02 and 7.31±0.3 respectively ($n=6$). However, the cannabinoid receptor antagonists AM630 ((6-iodo-2-methyl-1-[2-(4-morpholinyl)ethyl]-1H-indol-3-yl](4-methoxyphenyl)methanone)) and AM281 ((1-(2,4-dichlorophenyl)-5-(4-iodophenyl)-4-methyl-N-4-morpholinyl-1H-pyrazole-3-carboxamide)) (100 pM–10 μM) had no effect on either response (data not shown). Furthermore, pretreatment with 1 μM capsaicin for 30 min desensitized the response to 10 μM anandamide (16,271±789 vs 332±26 FI, $n=6$) and 10 μM anandamide desensitized the response to 100 nM capsaicin (15,283±1076 vs 1245±83 FI, $n=6$).

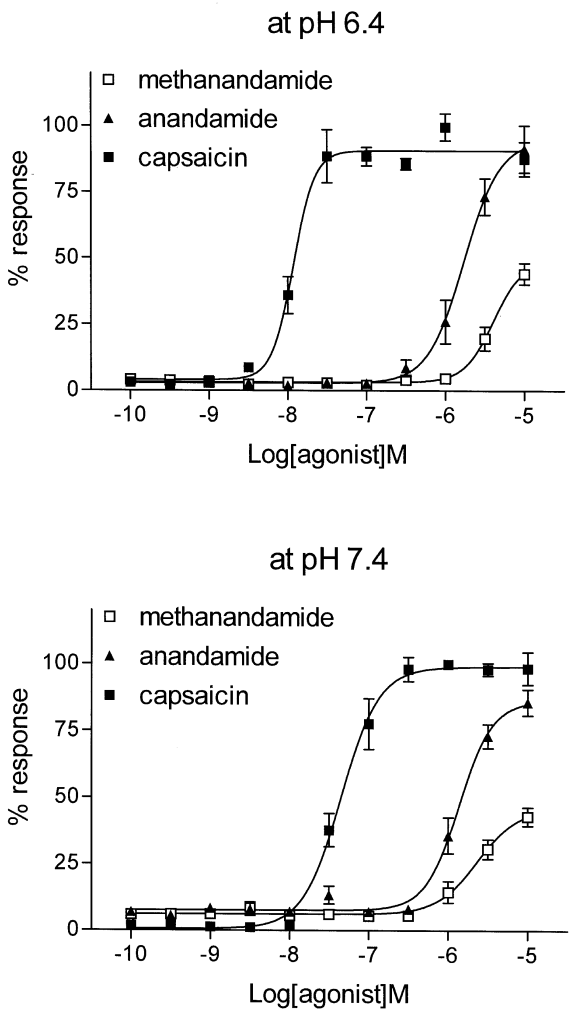

Figure 2 Anandamide-induced Ca^{2+} responses are concentration-dependent $[Ca^{2+}]_i$ was monitored using Fluo-3AM in hVR1-HEK293 cells before and after the addition of capsaicin (100 pM–10 μM), anandamide (100 pM–10 μM) or methanandamide (100 pM–10 μM) at pH 6.4 or 7.4. Responses were measured as peak increase in fluorescence minus basal, expressed relative to the maximum capsaicin response and are given as mean±s.e.mean, where $n=3-8$.

Discussion VR1 is a ligand-gated ion channel which plays an important role in nociceptive signalling (Szallasi & Blumberg, 1999). This receptor is activated by the plant extract capsaicin (Caterina *et al*., 1997), but the identity of the endogenous mammalian ligand remains unclear (Szallasi & Blumberg, 1999). Screening a wide range of bioactive substances revealed that anandamide, an endogenous cannabinoid (Devane *et al*., 1992), acted as an agonist at VR1. Anandamide displays a high structural similarity to the vanilloids, especially olvanil (DiMarzo *et al*., 1998; Beltramo & Piomelli, 1999), and has recently been reported to activate rat VR1 (Zygmunt *et al*., 1999). Therefore, the present study examined the pharmacology of anandamide at the recombinant human VR1 using electrophysiology and a FLIPR-based calcium assay, and has demonstrated for the first time that anandamide acts at human VR1 as a full agonist.

In the present study anandamide activated an inward current in hVR1-expressing, but not in parental, HEK293 cells. This current displayed similar kinetics to the capsaicin-induced current and was inhibited by capsazepine, a VR1 antagonist (Szallasi & Blumberg, 1999). Moreover, the anandamide- and capsaicin-induced currents had similar reversal potentials close to 0 mV, consistent with the gating of a non-selective ion channel and similar to findings for rat VR1 (Caterina *et al*., 1997). Anandamide also produced similar capsazepine-sensitive inward currents in capsaicin-sensitive cultured rat DRG neurons. In both the rat DRG neurones and hVR1-HEK293 cells the peak amplitude of the anandamide-induced current was significantly smaller (<50%) than that of the capsaicin-induced current. Similar results have recently been reported for anandamide at the recombinant rat VR1 (Zygmunt *et al*., 1999). Nevertheless, this apparent partial agonism probably reflects the technical difficulties in applying sufficiently high concentrations of such a lipophilic ligand in electrophysiological studies.

In the FLIPR anandamide and capsaicin both increased $[Ca^{2+}]_i$ in hVR1-expressing, but not non-transfected, HEK293 cells, and these responses displayed virtually identical kinetics. More importantly, the concentration-response relationship for anandamide was defined, and showed that anandamide was a full agonist compared to capsaicin. Capsaicin was more potent than anandamide, with an affinity consistent with that previously reported for rat VR1 (Caterina *et al*., 1997). Indeed, the potency of anandamide at hVR1 was ~20 fold lower than its binding affinity (55 nM) at the cannabinoid receptor (Devane *et al*., 1992), but was more consistent with the affinities (160–540 nM) reported from functional studies (Pertwee, 1997). Moreover, VR1 is structurally related to the transient receptor potential (TRP) channel family (Caterina *et al*., 1997), and certain TRP channels are activated by other lipid messengers with similar potencies (Chyb *et al*., 1999). Interestingly, the potency of capsaicin at hVR1 was enhanced by lowering the pH from 7.4 to 6.4, as reported for the rat VR1 (Caterina *et al*., 1997). In contrast the potency of anandamide was unaffected by pH, suggesting that anandamide and capsaicin may either bind to different

sites on the human VR1 or gate the channel by different mechanisms.

Several lines of evidence demonstrate that the anandamide-induced Ca^{2+} response is mediated by hVR1. Firstly, anandamide has no effect in parental HEK293 cells. Secondly, the anandamide-induced Ca^{2+} response is inhibited by capsazepine, with a pK_B value consistent with the affinity of capsazepine at VR1 (Szallasi & Blumberg, 1999). Thirdly, the cannabinoid receptor antagonists, AM630 and AM281 (Pertwee, 1997) had no effect on the anandamide-induced response. Fourthly, capsaicin causes a homologous desensitization of the anandamide-induced response, and vice versa. Finally, the synthetic cannabinoid agonists, CP-55,950 and WIN-55,212-2 (Pertwee, 1997) were inactive at hVR1. These synthetic cannabinoids also failed to elicit a response in rVR1-HEK293 cells (Zygmunt *et al*., 1999). However, in the present study the putative endogenous peripheral cannabinoid receptor ligand, palmitoylethanolamide (Pertwee, 1997) also activated hVR1, albeit more weakly than anandamide, despite having been reported to be inactive at the rat VR1 (Zygmunt *et al*., 1999). This demonstrates that other endogenous lipids can activate hVR1, suggesting possible roles for many different lipids in nociception. In conclusion, the present study has clearly demonstrated for the first time that the endogenous cannabinoid, anandamide acts as a full agonist at the human VR1.

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