Comparison of effects of anandamide at recombinant and endogenous rat vanilloid receptors

J. C. Jerman1*, J. Gray2, S. J. Brough1, L. Ooi2, D. Owen2, J. B. Davis2 and D. Smart2

1Department of Discovery Research and 2Department of Neurology, GlaxoSmithKline, New Frontiers Science Park, Third Avenue, Harlow, Essex CM19 5AW, UK

*Corresponding author. E-mail: jeff_jerman@gsk.com

Background. Anandamide, an endogenous lipid, activates both cannabinoid (CB1) and vanilloid (VR1) receptors, both of which are co-expressed in rat dorsal root ganglion (DRG) cells. Activation of either receptor results in analgesia but the relative contribution of CB1 and VR1 in anandamide-induced analgesia remains controversial. Here we compare the in vitro pharmacology of recombinant and endogenous VR1 receptors using calcium imaging, in clonal and DRG cells, respectively. We also consider the contribution of CB1 and VR1 receptors to anandamide-induced analgesia.

Methods. Using a Flurometric Imaging Plate Reader (FLIPR™), calcium imaging has been used to study the effects of several vanilloid and cannabinoid ligands in rat VR1-transfected HEK293 (rVR1-HEK) cells and in DRG cells. The effect of pre-exposure of several vanilloid and cannabinoids has also been compared in DRG cells.

Results. The VR1 agonists capsaicin, olvanil, (N-(4-hydroxyphenyl-arachinoylamide) (AM404) and anandamide caused a concentration-dependent increase in intracellular calcium concentration ([Ca2+]i), with similar temporal profiles in both rVR1-HEK and DRG cells, and potency (pEC50) values of 8.25 (SEM 0.11), 8.37 (0.04), 6.96 (0.06), 5.85 (0.01) and 7.45 (0.10), 7.55 (0.07), 6.10 (0.13), approximately 5.5, respectively. These responses were inhibited by the VR1 antagonist capsazepine (1 µM). In contrast, application of synthetic cannabinoid antagonists failed to inhibit the anandamide-induced increase in [Ca2+]i. Reapplication of VR1 agonists significantly inhibited a subsequent challenge to either capsaicin or anandamide in either cell type, whilst pre-exposure to cannabinoid agonists were without effect.

Conclusion. Here we provide evidence that the pharmacology of recombinant rVR1 receptors is similar to those endogenously expressed in DRG cells. Moreover, we have shown that VR1, but not CB1, receptors are involved in anandamide-induced responses in dorsal root primary neurones in vitro. Therefore, the analgesic properties of anandamide are likely to be mediated, at least in part, by VR1 activation in DRG cells in vivo.

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The development of novel therapeutics, efficacious in the clinical control of pain but lacking undesirable side-effects, has thus far proved elusive, despite a considerable research effort. The identification of two distinct cannabinoid receptor subtypes,12 termed CB1 and CB2, and cloning of these cannabinoid receptors,3 has facilitated extensive study of their pharmacology and distribution.3 Soon after the cloning of CB1 and CB2 receptors, which are members of the G-protein-coupled receptor superfamily and couple specifically to Gi/o G-proteins, two endogenous lipid
activators, anandamide and 2-arachidonylglycerol (2-AG), were identified in mammalian tissues. Several lines of investigation have led workers to conclude that the typical in vivo actions of cannabinoids—that are analgesia, effects on appetite, mood, hypotension, cognition, spontaneous activity and thermoregulation—are exclusively CB₁ receptor-mediated. However, studies using selective CB₁ receptor antagonists and mice lacking the CB₁ gene provide compelling evidence against CB₁ receptors being solely responsible for the in vivo actions of anandamide. This raises the question of how anandamide exerts non-CB₁ receptor-mediated effects, such as analgesia?

The vanilloid receptor (VR1) is a ligand-gated ion channel expressed on sensory neurones and plays an important role in nociception. Prolonged activation of VR1 results in receptor desensitization and analgesia and it has recently been reported that anandamide is an agonist at VR1 receptors. However, it is difficult, and often misleading, to correlate results derived from in vitro assay systems, using recombinant receptors, with those from isolated tissue preparations, especially when trying to model the situation in vivo.

Therefore, in the present study we have used a fluorometric imaging plate reader (FLIPR™) to measure changes in intracellular calcium concentration ([Ca²⁺]ᵢ) and compared the effects of anandamide at recombinant VR1 receptors with those in physiologically relevant dorsal root ganglion (DRG) primary cell cultures.

Materials and methods

Cloning and expression of rat VR1
Cloning and expression of rat VR1 (rVR1) was performed as described previously. In brief, a rat mammalian expression construct was generated by amplification of cDNA from reverse transcribed adult DRG mRNA, using forward and reverse primers incorporating the restriction sites RVR1F- and RVR1R- and the Xba I and Hin dIII ± sites of pcDNA3.1 (Invitrogen). Adherent human embryonic kidney (HEK293) cells were then transfected with rVR1.pcDNA3.1 using Lipofectamine Plus (Life Technologies) and stable clones generated following application of selection media (genetecin, 400 µg ml⁻¹) and colony cloning.

Cell culture
HEK293 cells stably expressing the recombinant rVR1 receptor, were maintained in culture in minimum essential medium (MEM) supplemented with non-essential amino acids, 10% fetal calf serum and 2 mM L-glutamine. Cells were grown as monolayers under 95:5% air:CO₂ at 37°C and passaged every 3–4 days. The highest passage used was 20. Dissociated rat neonatal DRG cultures were prepared as described by Skaper and co-workers. In brief, DRG cells were prepared from 8-day-old Sprague–Dawley rats and cultured in microtitre plates in DMEM containing N2 supplements, β-NGF (50 ng ml⁻¹), bovine serum albumin (BSA) (0.05%), penicillin (100 units ml⁻¹) and streptomycin (100 units ml⁻¹). Cells were cultured for 3 days and maintained under 95:5% air:CO₂ at 37°C.

Measurement of [Ca²⁺]ᵢ using the FLIPR™
rVR1-HEK cells were seeded into black walled clear-base 96-well plates (Costar, UK) at a density of 25 000 cells per well in MEM, supplemented as above and cultured overnight. Cells were then incubated with the cytoplasmic calcium indicator, Fluo-3 in the acetoxymethyl ester form (4 µM; Teflabs, TX, USA) at 25°C for 2 h. The loaded cells were then washed four times with, and finally incubated in, Tyrode’s medium (NaCl, 145 mM; KCl, 2.5 mM; HEPES, 10 mM; glucose, 10 mM; MgCl₂, 1.2 mM; CaCl₂, 1.5 mM) containing 0.2% BSA, before being incubated for 30 min at 25°C with either buffer alone or buffer containing various antagonists. The plates were then placed into a FLIPR™ (Molecular Devices, UK) to monitor cell fluorescence (λex=488 nm, λem=540 nm) before and after the addition of various vanilloid or cannabinoid agonists.

Dissociated rat neonatal DRG cells were seeded into black walled clear-base 384-well plates (Costar, UK) at a density of 10 000 cells per well and incubated with Tyrode’s buffer containing Fluo-4 at 37°C for 1 h. DRG cells were then washed as described previously before incubation with buffer alone or buffer containing antagonists at 25°C for 30 min. Cells were then placed into the FLIPR™ and fluorescence monitored as described above before and after the addition of various vanilloid or cannabinoid agonists.

Measurement of cyclic AMP production
DRG cells were incubated with cannabinoid agonists (100 nM) for 15 min at 37°C. The cells were then exposed to Forskolin (3 µM) for 20 min and the levels of cAMP determined, using a cAMP enzyme immunoassay kit system (Amersham, cat. no. RPN 225).

Data analysis
Functional responses using FLIPR were measured as peak fluorescence intensity minus basal fluorescence and, where appropriate, expressed as a percentage of a maximum capsaicin-induced response on the same plate. Iterative curve-fitting and parameter estimations were carried out using a four parameter logistic model and Microsoft Excel. Antagonist potency values (IC₅₀) were converted...
to apparent pKB values (functional antagonist affinity equivalent to pKi) using a modified Cheng–Prusoff correction.16

\[
\text{Apparent pKB} = -\log \frac{[IC_{50}]}{[agonist]} + \frac{[EC_{50}]}{[agonist]}
\]

Data are expressed as mean (SEM) unless otherwise stated. Statistical tests for significance were conducted using two-way analysis of variance.

Materials
Anandamide (arachidonoyl-ethanolamide), AM404 (N-(4-hydroxyphenyl-arachinoylilamide) and all other cannabinoids (AM630, AM251, AM281, HU210, WIN55, ZIZ-Z, CP55, G40) were obtained from Tocris (Bristol, UK). Capsaicin, capsazepine, olvanil (N-vanillyl-cis-9-octadecenoamide) and all bulk reagents were obtained from Sigma-Aldridge Ltd (Poole, Dorset, UK). All cell culture media were obtained from Life Technologies (Paisley, UK).

Results
Capsaicin, olvanil, anandamide and AM404 increased [Ca\textsuperscript{2+}]\textsubscript{i} when applied to rVR1-HEK and to rat DRG cells (Fig. 1). The temporal profiles of responses to capsaicin and olvanil in both cell types were comparable and were typified by an initially rapid then slowing onset (peak ~30 s) followed by a slowly declining phase (Fig. 1). The temporal profile of responses to anandamide and AM404 were also similar in both cell types although responses in DRG cells were more sustained than that in the recombinant system. Responses to all agonists were concentration dependent, with a rank order of potency of capsaicin = olvanil > AM404 > anadamide in both cell types, although potencies determined in rVR1-HEK cells tended to be higher (~0.8 log units) than those in DRG cells (Fig. 2 and Table 1). In the rVR1-HEK cells all agents were full agonists compared with capsaicin whereas AM404 was a partial agonist, compared with capsaicin, when tested in rat DRG cells (Fig. 2). Similarly, anandamide was less efficacious in DRG cells compared with rVR1-HEK cells, although the absolute efficacy of anandamide in DRG cells is difficult to determine as concentration–response curves were bell shaped. The absence of a defined saturable response for anandamide in DRG cells, complicates determination of a meaningful potency value (Table 1).

The competitive VR1 antagonist capsazepine and the poreblocker ruthenium red inhibited capsaicin-induced responses in a concentration-dependent manner with pKB values of 7.21 (0.06) and 8.54 (0.08) in rVR1-HEK cells and 6.42 (0.02) and 8.60 (0.06) in rat DRG cells, respectively. In rVR1-HEK cells the antagonist affinity of capsazepine was not significantly different when inhibiting capsaicin-, olvanil-, AM404- or anandamide-induced responses with pKB values of 7.21 (0.06), 7.35 (0.06), 7.18 (0.03) and 7.40 (0.12), respectively.

Incubation with capsazepine (10 μM) or ruthenium red (1 μM) significantly inhibited (P<0.01) subsequent responses to capsaicin (300 nM) and anandamide (10 μM) in rat DRG cells (Table 2). In contrast to this, pre-incubation with the cannabinoid antagonists AM630, AM251, AM281,17 or cannabinoid agonists HU210, WIN 55,212-2 or CP 55,94031 (10 μM) did not significantly alter the magnitude of subsequent capsaicin- or anandamide-induced responses (Table 2).

Application of the synthetic cannabinoid agonists HU210, WIN 55,212-2 or CP 55,940 (1 μM) failed to induce an increase in [Ca\textsuperscript{2+}]\textsubscript{i} in either cell type (data not shown), whereas pre-application of either capsaicin (1 μM)
or anandamide (10 µM) significantly reduced (P<0.01) the magnitude of subsequent capsaicin (300 nM) and anandamide (10 µM) responses in DRG cells (Table 2).

In DRG cells, application of the cannabinoid agonist HU210 or CP 55,940 (1 µM) did not significantly (P<0.01) inhibit forskolin-induced cAMP production, with cAMP levels of 359 (103), 373 (101) and 376 (43) fmol, respectively (n=3).

**Discussion**

VR1 receptors have been identified as a receptor for the endogenous cannabinoid anandamide in studies using both recombinant receptors and isolated tissue preparations. In the present study, using a common methodology we have compared the in vitro pharmacology of recombinant rat VR1 receptors with that of endogenous VR1 in primary DRG cells. We have also considered the relative contribution of CB1 and VR1 receptors to anandamide-induced responses.

In the present study capsaicin, olvanil, anandamide and AM404 induced a concentration-dependent increase in [Ca2+]i, and the kinetics of responses in rVR1-HEK cells were virtually identical compared with those in rat DRG cells (Fig. 1). Similarly, agonist rank order of potency was also maintained between the two cell types and was consistent with that of previous reports. The observation that agonist potencies were higher in rVR1-HEK cells (~0.8 log units) and the fact that anandamide and AM404 were less efficacious in DRG cells compared with rVR1-HEK cells, is readily explained by accepted receptor theory and suggests the presence of receptor reserve in the latter. However, the relatively low efficacy of anandamide in the DRG cells, and the fact that concentration–response curves to anandamide were bell-shaped in these cells, complicates determination of maximal efficacy. Moreover, in the present study it is impossible to determine if anandamide is a partial agonist in the DRG cells. However, it is likely that the bell-shaped concentration–response curves to anandamide reflect the relative insolubility of this compound at higher concentrations. In the present study responses to 30 µM anandamide, which represents the highest achievable concentration without encountering solubility problems, were approximately 60% relative to that of capsaicin (1 µM). The data are in agreement with data from electrophysiological studies using VR1 expressing HEK293 cells and *Xenopus* oocytes. The physiological significance of the relative efficacy of anandamide in this study, and the relevance to

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**Table 1** Agonist potencies (pEC50) or per cent stimulation (30 µM, relative to capsaicin) of increases in [Ca2+]i, in rVR1 expressing HEK293 cells and in dissociated rat neonatal DRG cultures. Data are mean (SEM) from four to eight independent experiments

<table>
<thead>
<tr>
<th></th>
<th>rVR1-HEK293</th>
<th>Rat DRG</th>
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<tbody>
<tr>
<td>Capsaicin</td>
<td>8.25 (0.11)</td>
<td>7.45 (0.10)</td>
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<tr>
<td>Anandamide</td>
<td>5.85 (0.01)</td>
<td>63%</td>
</tr>
<tr>
<td>AM404</td>
<td>6.96 (0.06)</td>
<td>6.10 (0.13)</td>
</tr>
<tr>
<td>Olvanil</td>
<td>8.37 (0.04)</td>
<td>7.55 (0.07)</td>
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**Table 2** Effects of 30 min pre-incubation with vanilloid and cannabinoid ligands on subsequent capsaicin- or anandamide-induced [Ca2+]i responses in DRG cells. Data are mean (SEM); n=4; *P<0.01 vs control

<table>
<thead>
<tr>
<th></th>
<th>vs Capsaicin (300 nM)</th>
<th>vs Anandamide (10 µM)</th>
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<tbody>
<tr>
<td>Control</td>
<td>100 (3)</td>
<td>100 (5)</td>
</tr>
<tr>
<td>Capsazepine (10 µM)</td>
<td>3 (5)*</td>
<td>9 (1)*</td>
</tr>
<tr>
<td>Ruthenium red (1 µM)</td>
<td>28 (3)*</td>
<td>38 (8)*</td>
</tr>
<tr>
<td>Capsaicin (1 µM)</td>
<td>12 (2)*</td>
<td>23 (2)*</td>
</tr>
<tr>
<td>Anandamide (10 µM)</td>
<td>24 (4)*</td>
<td>16 (4)*</td>
</tr>
<tr>
<td>AM630 (1 µM)</td>
<td>102 (10)</td>
<td>120 (9)</td>
</tr>
<tr>
<td>AM251 (1 µM)</td>
<td>99 (5)</td>
<td>102 (19)</td>
</tr>
<tr>
<td>AM281 (1 µM)</td>
<td>115 (5)</td>
<td>100 (12)</td>
</tr>
<tr>
<td>WIN 55,212-2 (1 µM)</td>
<td>108 (8)</td>
<td>107 (10)</td>
</tr>
<tr>
<td>CP 55,940 (1 µM)</td>
<td>105 (7)</td>
<td>101 (11)</td>
</tr>
<tr>
<td>HU210 (1 µM)</td>
<td>91 (11)</td>
<td>116 (16)</td>
</tr>
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the efficacy in man, has yet to be determined. It is noteworthy that the apparent affinity of capsaizpine, but not ruthenium red, was lower in the DRG preparation than that in rVR1-HEK293 cells. The reason(s) for this remain unclear, but may reflect secondary modulation of the binding sites associated with the differing cellular environment.

Taken collectively the data are consistent with VR1-mediation of responses seen in both recombinant and DRG cells. However, several aspects of the present study provide evidence against the involvement of cannabinoid receptors in responses in the DRG cells. No inhibition of forskolin-induced cAMP production, nor any increase in [Ca\(^{2+}\)], was seen following the application of potent CB\(_1\) receptor agonists,\(^3\) despite immunocytochemical evidence of CB\(_1\) expression in DRG neurones.\(^20\) Furthermore, neither the cannabinoid antagonists AM630, AM251 and AM281,\(^17\) nor the CB\(_1\) receptor agonists HU210, WIN 55,212-2 and CP 55,429\(^13\) had any effect on either capsaicin- or anandamide-induced responses in DRG cells. In addition, the potency of anandamide in the present study (>10 \(\mu\)M) is considerably lower than the binding affinity (55 nM) reported for anandamide at CB\(_1\) receptors.\(^4\) In contrast, pre-application of known VR1 antagonists such as capsaizpine\(^9\) and ruthenium red\(^21\) significantly inhibited a subsequent response to capsaicin and anandamide.

In the present study we have provided evidence that vanilloid pharmacology is similar in recombinant and physiologically relevant systems in vitro. We have also provided evidence that anandamide induces response in DRG cells that are VR1, but not CB\(_1\) receptor-mediated. It is reasonable, therefore, to conclude that anandamide may interact with vanilloid receptors in vivo leading to receptor desensitization and analgesia, as proposed previously,\(^22\) but this remains controversial.\(^24\) Overcoming the technical difficulties in handling poorly soluble lipid compounds such as anandamide, will contribute greatly to the resolution of this controversy.

Nevertheless, it is clear that regarding the cannabinoid and vanilloid receptor systems as two completely separate entities, at least in their respective contribution to analgesia, is unwise. It could be argued that these two receptors share a common pharmacophore and that this is purely coincidental. On the other hand, though purely speculative, it is also tempting to consider that these receptors represent different classes of the same receptor system, somewhat analogous to the ionotropic and metabotropic classes of glutamate receptors.

Evidence that a physiological link exists between VR1 and CB\(_1\) receptors is lacking in our data. In the present study, selective CB\(_1\) agonists and antagonists had no feedback effects on subsequent VR1 activity. However, considering the possibility that the cannabinoid and the vanilloid systems are interlinked in the same physiological responses and share common pharmacophores, affords the possibility of development of novel analgesics with high therapeutic potential. A non-pungent VR1 receptor agonist that is also a CB\(_1\) receptor agonist for example, may be efficacious in the treatment of pain.

Alternative approaches are also being considered.\(^25\) For example, modulation of anandamide biosynthesis and inactivation, such as inhibition of anandamide hydrolysis, may also provide useful therapeutics.\(^25\) However, as is the case with all cannabinoid targeted drug therapies the contribution of the vanilloid system must not be overlooked.

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