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# Characterisation using FLIPR of human vanilloid VR1 receptor pharmacology

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**Abstract**

A full pharmacological characterisation of the recently cloned human vanilloid VR1 receptor was undertaken. In whole-cell patch clamp studies, capsaicin (10 μM) elicited a slowly activating/deactivating inward current in human embryonic kidney (HEK293) cells stably expressing human vanilloid VR1 receptor, which exhibited pronounced outward rectification (reversal potential $-2.1 \pm 0.2$ mV) and was abolished by capsazepine (10 μM). In FLIPR™-based $Ca^{2+}$ imaging studies the rank order of potency was resiniferatoxin > olvanil > capsaicin > anandamide, and all were full agonists. Isovelleral and scutigeral were inactive (1 nM–30 μM). The potencies of capsaicin, olvanil and resiniferatoxin, but not anandamide, were enhanced 2- to 7-fold at pH 6.4. Capsazepine, isovelleral and ruthenium red inhibited the capsaicin (100 nM)-induced $Ca^{2+}$ response ($pK_B = 6.58 \pm 0.02$, $5.33 \pm 0.03$ and $7.64 \pm 0.03$, respectively). In conclusion, the recombinant human vanilloid VR1 receptor stably expressed in HEK293 cells acted as a ligand-gated, $Ca^{2+}$-permeable channel with similar agonist and antagonist pharmacology to rat vanilloid VR1 receptor, although there were some subtle differences.


*Keywords:* Vanilloid; Capsaicin; $Ca^{2+}$; Ligand-gated ion channel; FLIPR; Nociception

## 1. Introduction

Capsaicin, the pungent chemical present in hot chilli peppers (Szallasi and Blumberg, 1999), activates a ligand gated, $Ca^{2+}$-permeable, non-selective ion channel present in nociceptors (Bevan and Szolcsanyi, 1990), resulting in a sensation of burning pain (Simone et al., 1987; Park et al., 1995). This channel, termed the vanilloid receptor (Szallasi and Blumberg, 1999), has been proposed as a potential target for the treatment of pain (Dray, 1995).

The recent cloning (Caterina et al., 1997) and pharmacological characterisation (Tominaga et al., 1998; Jerman et al., 2000) of the rat vanilloid VR1 receptor has been a major advance in this field. Furthermore, the recent disruption of the mouse vanilloid VR1 receptor gene has provided further evidence for the utility of this target in the treatment of pain (Davis et al., 2000; Caterina et al., 2000).

More recently the human homologue of vanilloid VR1 receptor has been cloned and expressed (Hayes et al., 2000). Human vanilloid VR1 receptor has 839 amino acids, with six putative transmembrane domains, and displays 92% homology to rat vanilloid VR1 receptor (Hayes et al., 2000). Like rat vanilloid VR1 receptor, human vanilloid VR1 receptor is also related to the transient receptor potential channel family (Hayes et al., 2000; Caterina et al., 1997). Furthermore, the distribution of human vanilloid VR1 receptor, namely high expression levels in dorsal root ganglion neurons, particularly the small to medium diameter cells, and relatively low levels in a variety of brain regions (Hayes et al., 2000) is consistent with that reported for rat vanilloid VR1 receptor (Caterina et al., 1997). In addition, when expressed in *Xenopus* oocytes or human embryonic kidney (HEK293) cells human vanilloid VR1 receptor acts as a non-selective cation channel, with similar electrophysiological properties to both the native vanilloid-gated ion channel and rat

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vanilloid VR1 receptor (Hayes et al., 2000; Bevan and Yeats, 1991). Indeed, human vanilloid VR1 receptor was not only activated by capsaicin, but also by the same endogenous agonists as rat vanilloid VR1 receptor, e.g. anandamide, noxious heat and low pH (Hayes et al., 2000; Smart and Jerman, 2000).

Although the endogenous vanilloid receptors in human and rat dorsal root ganglion neurons display similar pharmacology (Baumann et al., 1996; Szallasi and Blumberg, 1999), only a limited number of studies have been conducted using human dorsal root ganglion neurons (Szallasi and Blumberg, 1999). Furthermore, differences in the pharmacology of recombinant rat vanilloid VR1 receptor compared to that of the endogenous vanilloid receptor in rat dorsal root ganglion neurons have been reported (Caterina et al., 1997; Jerman et al., 2000). Therefore, given the limited extent of the pharmacological characterisation of human vanilloid VR1 receptor to date (Hayes et al., 2000; Smart et al., 2000) we have undertaken a detailed characterisation of the pharmacology of human vanilloid VR1 receptor stably expressed in HEK293 cells using both vanilloid and the non-vanilloid activators of the capsaicin receptor expressing nociceptors, including capsaicin, resiniferatoxin, olvanil, phorbol 12,13-didecanote-20-homovanillate (PPAHV), scutigeral, isovelleral and the endocannibinoid anandamide. This has confirmed and expanded this pharmacology, and identified subtle differences in the pharmacology of human vanilloid VR1 receptor compared to rat vanilloid VR1 receptor. Furthermore, these data demonstrate that vanilloid VR1 receptor can account for most of the putative vanilloid receptor subtypes previously identified pharmacologically in native tissues (Baumann et al., 1996; Szallasi and Blumberg, 1999).

## 2. Methods

### 2.1. Cloning and expression of human vanilloid VR1 receptor receptors in HEK293 cells

The cloning of human vanilloid VR1 receptor was conducted as described previously (Hayes et al., 2000). Briefly, human vanilloid VR1 receptor cDNA was identified using the published rat vanilloid VR1 receptor 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.

### 2.2. Cell culture

Human vanilloid VR1 receptor-HEK293 cells were routinely grown as monolayers in minimum essential medium 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 28.

### 2.3. Whole-cell patch clamp electrophysiology

All recordings were performed at room temperature (20–24°C) using an Axopatch 200B amplifier controlled via the pClamp7 software suite (Axon Instruments). Standard whole-cell patch-clamp methods were employed, as described previously (Gunthorpe et al., 2000). Cells were plated onto glass coverslips coated with poly-D-lysine at a density of $\sim$26,000 cells $cm^{-2}$ and used after 16–48 h. Cells were visualised with standard phase-contrast optics and recordings were made from well isolated single phase bright cells. The extracellular solution consisted of (mM) NaCl, 130; KCl, 5; $CaCl_2$, 2; $MgCl_2$, 1; Glucose, 30; HEPES–NaOH, 25, pH 7.3. Patch pipettes (resistance 2–5 M$\Omega$) 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. Capsaicin and capsazepine were prepared as 10-mM stocks in dimethylsulphoxide prior to dilution in extracellular solution and drug applications were carried out using an automated fast-switching solution exchange system (Warner Instruments SF-77B; time for solution exchange $\sim$30 ms).

### 2.4. Measurement of intracellular $Ca^{2+}$ concentrations using the FLIPR™

Intracellular $Ca^{2+}$ concentrations was monitored using FLIPR™ (Molecular Devices, UK) as described previously (Smart et al., 2000). Briefly, human vanilloid VR1 receptor-HEK293 cells, seeded into 96-well plates (25,000 cells/well), were incubated with culture medium containing the cytoplasmic $Ca^{2+}$ indicator, Fluo-3 (4 $\mu$M; Teflabs, Austin, TX) at 25°C for 120 min. The cells were then washed four times with Tyrode's medium, before being incubated for 30 min at 25°C with either Tyrodes alone (control) or Tyrodes containing various antagonists or signal transduction modifying agents. In some studies $Ca^{2+}$ was omitted from the buffer. The plates were then placed into a FLIPR™ to monitor cell fluorescence ($\lambda_{ex}$ = 488 nm, $\lambda_{EM}$ = 540 nm) (Jerman et al., 2000) before and after the addition of various agonists.

### 2.5. Data analysis

Responses were measured as peak minus basal fluorescence intensity, and where appropriate were expressed as a percentage of a maximum capsaicin-induced response. Data are expressed as mean $\pm$ S.E.M. unless otherwise stated.

Curve-fitting and parameter estimation were carried out using Graph Pad Prism 3.00 (GraphPad Software, California, USA). Statistical comparisons were made where appropriate using Student's *t*-test.

### 2.6. Materials

PPAHV, resiniferatoxin, anandamide, scutigeral and isovelleral were obtained from Alexis Biochemicals (Nottingham, UK). Olvanil was purchased from Tocris (Bristol, UK) and all other ligands were obtained from RBI (Natick, MA, USA). All signal transduction modifying agents were purchased from Calbiochem (Nottingham, UK). All cell culture media were obtained from Life Technologies (Paisley, UK).

## 3. Results

Application of capsaicin (1 μM) to human vanilloid VR1 receptor-HEK293 cells voltage clamped at −70 mV led to the appearance of inward currents which exhibited little, or no, macroscopic desensitisation (mean current = 417 ± 24 pA, $n = 19$; Fig. 1). In contrast, there was no response to the application of 10 μM capsaicin to parental HEK293 cells ($n = 7$, Fig. 1). Current–voltage relationships established for capsaicin-gated currents in human vanilloid VR1 receptor-HEK293 cells exhibited substantial outward rectification and a reversal potential close to 0 mV (−2.1 ± 0.2 mV, $n = 5$, Fig. 1); furthermore, these

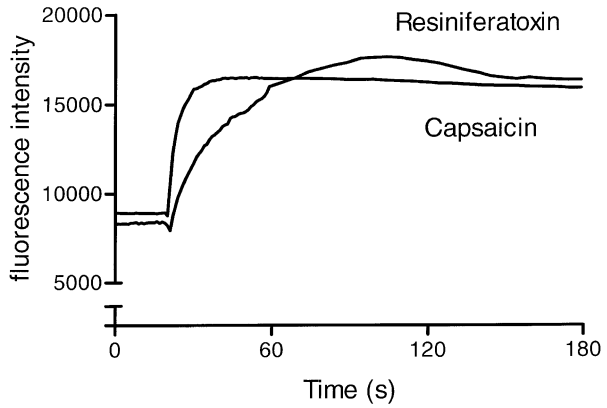


Fig. 2. Resiniferatoxin- and capsaicin-induced $Ca^{2+}$ responses have different kinetics in human vanilloid VR1 receptor-HEK293 cells. Intracellular $Ca^{2+}$ concentration (as fluorescent intensity units) was monitored using Fluo-3 in HEK293 cells stably expressing human vanilloid VR1 receptor before and after the addition of resiniferatoxin (30 nM) or capsaicin (100 nM). Data shown are representative traces, typical of at least $n = 60$.

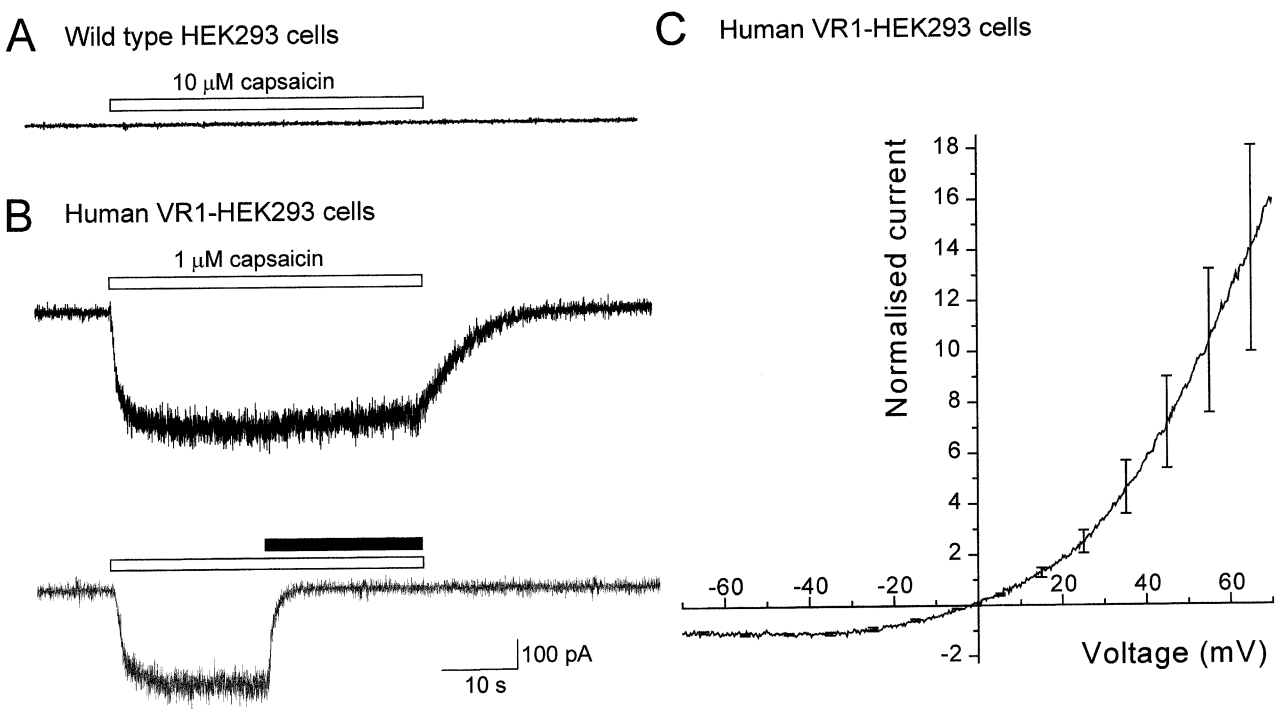


Fig. 1. Capsaicin-gated currents in human vanilloid VR1 receptor-HEK293 cells. Whole-cell patch clamp electrophysiology was used to investigate the properties of capsaicin-gated currents in parental HEK293 cells and human vanilloid VR1 receptor-HEK293 cells. (A) Capsaicin (10 μM) was without effect when applied to wild-type HEK293 cells voltage clamped at −70 mV ($n = 7$). (B) In contrast, under the same conditions, application of 1 μM capsaicin to human vanilloid VR1 receptor-HEK293 cells led to the appearance of slowly activating and deactivating inward currents in all cells tested ($n = 19$). These capsaicin-gated currents were completely inhibited by subsequent co-application of the vanilloid VR1 receptor antagonist capsazepine (10 μM, $n = 6$). (C) The current–voltage relationship for capsaicin-gated currents in human vanilloid VR1 receptor-HEK293 cells was studied using a voltage ramp protocol (−70 to +70 mV at 0.14 mV $ms^{-1}$) applied during the plateau phase of the response. Current responses at each holding potential were normalised to the current recorded at −70 mV. The resulting current–voltage relationship obtained shows that capsaicin-gated human vanilloid VR1 receptor currents exhibit pronounced outward rectification and a reversal potential of −2.1 ± 0.2 mV ($n = 5$).

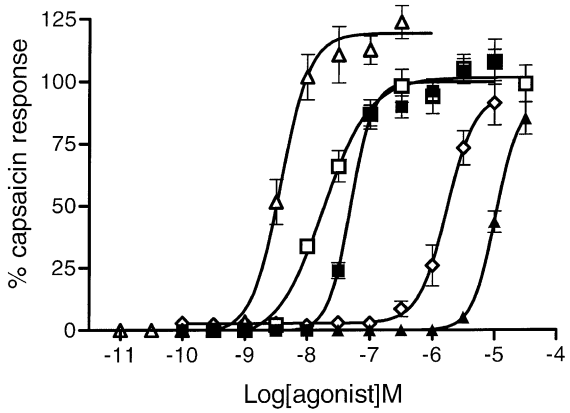


Fig. 3. The agonist-induced $Ca^{2+}$ responses are concentration-dependent. Intracellular $Ca^{2+}$ concentration was monitored using Fluo-3 in human vanilloid VR1 receptor-HEK293 cells before and after the addition of resiniferatoxin (△; 10 pM–0.3 μM), capsaicin (■; 100 pM–10 μM), olvanil (□; 100 pM–30 μM), anandamide (◇; 100 pM–10 μM) or PPAHV (▲; 100 pM–30 μM). Responses were measured as peak increase in fluorescence minus basal, expressed relative to the maximum capsaicin response and are given as mean ± S.E.M., where $n = 5–8$.

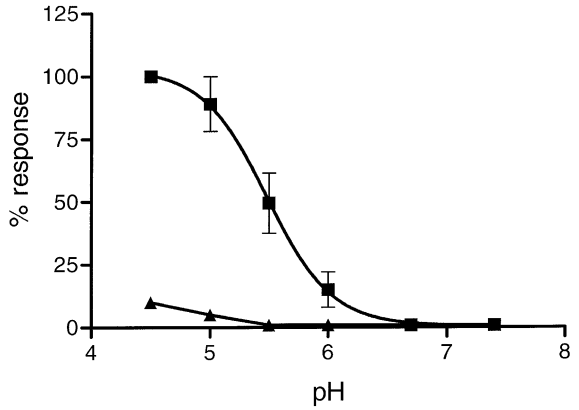


Fig. 4. Protons induce a human vanilloid VR1 receptor-mediated $Ca^{2+}$ response. Intracellular $Ca^{2+}$ concentration was measured (as fluorescent intensity units) using Fluo-3 in human vanilloid VR1 receptor-HEK293 cells before and after the addition of HCl (1.5–4.2 mM). In some studies, the cells were preincubated with capsazepine (▲; 1 μM) for 30 min. Data are mean ± S.E.M., where $n = 5$.

currents were completely blocked by subsequent co-application of the vanilloid VR1 receptor antagonist capsazepine (10 μM, $n = 6$, Fig. 1).

Capsaicin (100 nM) caused an increase in the intracellular $Ca^{2+}$ concentration in human vanilloid VR1 receptor-HEK293 cells, which was typified by an initially rapid then slowing onset (peak ~ 30 s), followed by a gradually declining secondary phase (Fig. 2). PPAHV, olvanil and anandamide also evoked $Ca^{2+}$ responses with similar kinetics (data not shown). However, the resiniferatoxin (30 nM)-induced $Ca^{2+}$ response had different kinetics, with a more gradual onset (peak ~ 90 s) followed by a similar slowly declining secondary phase (Fig. 2).

All agonist-induced responses were concentration-dependent (Fig. 3) and were full agonists compared to capsaicin, although the resiniferatoxin-induced response obtained a greater maximum than the other compounds (Fig. 3). The rank order of potency obtained (resiniferatoxin > olvanil > capsaicin > anandamide > PPAHV) was consistent with the established pharmacology of vanilloid VR1 receptor (Table 1). However, scutigeral and isovelleral (30 μM) displayed no agonist activity at human vanilloid VR1 receptor (Table 1). Interestingly, all the ligands except olvanil displayed positive cooperativity, with Hill coefficients of ~ 2 (Table 1).

Lowering the pH to 6.4 enhanced (2- to 7-fold) the potencies of all the vanilloid ligands, but not that of anandamide (Table 1). However, the Hill coefficients were unchanged (Table 1). The potency of carbachol at the endogenous muscarinic $M_3$ receptor expressed in HEK293 cells was unaffected at pH 6.4 (Table 1). Lowering the pH further directly increased intracellular $Ca^{2+}$ concentrations in human vanilloid VR1 receptor-HEK293 cells in a pH-dependent manner, with an $EC_{50}$ of 5.5. Capsazepine or ruthenium red (1 μM) abolished the pH-induced response in the human vanilloid VR1 receptor transfected cells (Fig. 4 and data not shown).

Table 1
Acidification enhances the potency of vanilloid, but not cannabinoid, vanilloid VR1 receptor agonists

| | At pH 7.4 | | At pH 6.4 | |
|---|---|---|---|---|
| | $pEC_{50}$ | slope | $pEC_{50}$ | slope |
| Capsaicin | 7.29 ± 0.04 | 2.50 ± 0.44 | 7.98 ± 0.04[a] | 2.41 ± 0.39 |
| Olvanil | 7.73 ± 0.06 | 1.19 ± 0.18 | 8.24 ± 0.08[a] | 1.24 ± 0.13 |
| Resiniferatoxin | 8.43 ± 0.07 | 1.86 ± 0.31 | 8.63 ± 0.04[a] | 1.94 ± 0.26 |
| PPAHV | 4.97 ± 0.06 | 2.36 ± 0.26 | 5.72 ± 0.10[a] | 2.17 ± 0.18 |
| Anandamide | 5.60 ± 0.10 | 1.91 ± 0.09 | 5.74 ± 0.05 | 2.07 ± 0.23 |
| Carbachol | 5.31 ± 0.03 | 1.04 ± 0.22 | 5.35 ± 0.03 | 1.10 ± 0.23 |
| Scutigeral | Inactive | | Inactive | |
| Isovelleral | Inactive | | Inactive | |

Data are mean ± S.E.M., $n = 5–8$.
[a] Denotes $P < 0.05$ increased compared to at pH 7.4.

Table 2
Vanilloid VR1 receptor agonists do not display cooperativity

| Agonist | % Response |
|---|---|
| Resiniferatoxin (1 μM) | 123.5 ± 4.8 |
| Capsaicin (10 μM) | 100.0 ± 0.0 |
| Resiniferatoxin (10 nM) | 106.0 ± 9.5 |
| PPAHV (10 μM) | 35.6 ± 4.6 |
| Capsaicin (100 nM) | 84.0 ± 6.1 |
| Olvanil (100 nM) | 91.8 ± 4.8 |
| Resiniferatoxin (10 nM) + PPAHV (10 μM) | 126.3 ± 5.7 |
| Resiniferatoxin (10 nM) + Capsaicin (100 nM) | 121.0 ± 5.1 |
| Resiniferatoxin (10 nM) + olvanil (100 nM) | 124.6 ± 6.4 |
| Capsaicin (100 nM) + PPAHV (10 μM) | 102.7 ± 5.8 |
| Capsaicin (100 nM) + olvanil (100 nM) | 103.6 ± 9.1 |
| PPAHV (10 μM) + olvanil (100 nM) | 98.6 ± 8.3 |

Data are expressed relative to the maximum capsaicin (10 μM) response and are given as mean ± S.E.M., where $n = 5$.

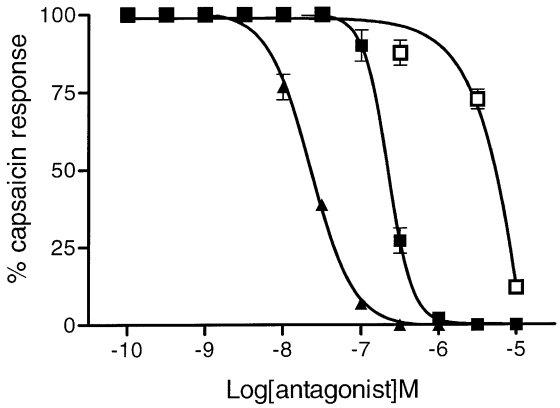


Fig. 5. Vanilloid VR1 receptor antagonists inhibit the capsaicin-induced $Ca^{2+}$ response. In the upper panel, Fluo-3 loaded human vanilloid VR1 receptor-HEK293 cells were preincubated with buffer (control), capsazepine (■; 0.1 nM–10 μM), isovelleral (□; 1 nM–10 μM) or ruthenium red (▲; 0.1 nM–10 μM) for 30 min at 25°C, and then the intracellular $Ca^{2+}$ concentration was monitored before and after the addition of capsaicin (100 nM). Responses were measured as peak increase in fluorescence minus basal, expressed relative to the control capsaicin response and are given as mean ± S.E.M., where $n = 6$.

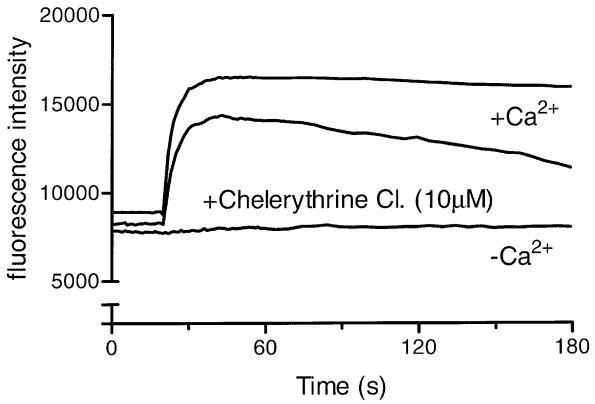


Fig. 6. The role of extracellular $Ca^{2+}$ and protein kinase C in the human vanilloid VR1 receptor-mediated $Ca^{2+}$ response. Intracellular $Ca^{2+}$ concentration was monitored (as fluorescent intensity units) using Fluo-3 in human vanilloid VR1 receptor-HEK293 cells, incubated in $Ca^{2+}$-containing or $Ca^{2+}$-free buffer, in the presence or absence of chelerythrine chloride (10 μM), before and after a capsaicin (1 μM) challenge at 20 s. Data are representative traces, typical of at least $n = 5$.

The potential for cooperativity between agonists was also examined by measuring the responses to sub-maximal concentrations of resiniferatoxin (10 nM), PPAHV (10 μM), capsaicin (100 nM) and olvanil (100 nM), alone or in combination. All four agonists on their own produced sub-maximal $Ca^{2+}$ responses and in combination were simply additive (Table 2).

Capsazepine (0.1 μM–10 μM), isovelleral (300 nM–10 μM) and ruthenium red (10 nM–10 μM) inhibited the capsaicin (100 nM)-induced $Ca^{2+}$ response in human vanilloid VR1 receptor-HEK293 cells in a concentration-dependent manner (Fig. 5). Capsazepine, isovelleral and ruthenium red also antagonised the resiniferatoxin (30 nM)-induced response (Table 3). None of these antagonists displayed any agonist-like activity (Table 1 and data not shown). The rank order of affinities obtained was ruthenium red > capsazepine > isovelleral (Table 3).

Consistent with human vanilloid VR1 receptor being a $Ca^{2+}$-permeable ligand-gated ion channel, removal of extracellular $Ca^{2+}$ abolished the capsaicin (100 nM)-induced response in human vanilloid VR1 receptor-HEK293 cells (Fig. 6). Pretreatment with tetrodotoxin (3 μM), nimodipine (10 μM), ω-GVIA conotoxin (1 μM), thapsigargin (1 μM), {1-[6-((17β-3-methoxyestra-1,3,5(10)-trien-17-yl)amino)hexyl]-1*H*-pyrrole-2,5-dione} (U73122; 3 μM) or {*N*-[2-((*p*-bromocinnamyl)amino)ethyl]-5-isoquinolinesulfonamide,2HCl} (H-89; 3 μM) had no effect on the capsaicin (100 nM)-induced response. However, the protein kinase C inhibitor, chelerythrine chloride (10 μM) partially (~20%) inhibited the peak response and appeared to increase the rate of decline of the secondary phase (Fig. 6).

Table 3
Antagonist affinities at recombinant human vanilloid VR1 receptor expressed in HEK cells

| | $pK_B$ values | | |
|---|---|---|---|
| | Capsazepine | Isovelleral | Ruthenium red |
| Vs. capsaicin | 6.58 ± 0.02 | 5.33 ± 0.03 | 7.64 ± 0.03 |
| Vs. resiniferatoxin | 6.50 ± 0.05 | 5.35 ± 0.02 | 8.11 ± 0.03 |

Data are mean ± S.E.M., $n = 5$.

## 4. Discussion

Vanilloid receptors are important potential targets for the treatment of pain (Dray, 1995). The cloning (Caterina et al., 1997) and characterisation (Tominaga et al., 1998; Jerman et al., 2000) of rat vanilloid VR1 receptor significantly advanced this field, and more recently a further breakthrough has occurred with the cloning of the human orthologue (Hayes et al., 2000). However, only a limited pharmacological characterisation of human vanilloid VR1 receptor has been conducted to date (Hayes et al., 2000; Smart et al., 2000). The present study has confirmed and expanded this pharmacology, utilising all the major classes of vanilloid and non-vanilloid ligands, and identified subtle differences in the pharmacology of human vanilloid VR1 receptor compared to rat vanilloid VR1 receptor. Furthermore, these data demonstrate that human vanilloid VR1 receptor can account for most of the putative vanilloid receptor subtypes proposed previously on pharmacological grounds in native tissue studies (Baumann et al., 1996; Szallasi and Blumberg, 1999), e.g. the R and C receptor subtypes (Szallasi, 1994).

Capsaicin-activated inward currents in human vanilloid VR1 receptor expressing, but not in parental, HEK293

cells, consistent with our previous studies (Hayes et al., 2000). Furthermore, this current was blocked by the vanilloid VR1 receptor antagonist, capsazepine (Bevan et al., 1992), and had a reversal potential similar to that reported for capsaicin-induced currents in human dorsal root ganglion neurons (Baumann et al., 1996) and rat vanilloid VR1 receptor expressing HEK293 cells (Caterina et al., 1997; Gunthorpe et al., 2000).

In the present study, capsaicin increased intracellular $Ca^{2+}$ concentrations in human vanilloid VR1 receptor expressing, but not in parental, HEK293 cells, and the kinetics of this response were consistent with those previously reported for the endogenous vanilloid receptor (Szallasi and Blumberg, 1999) and rat vanilloid VR1 receptor (Jerman et al., 2000). PPAHV, olvanil and anandamide also evoked similar responses. However, the resiniferatoxin-induced $Ca^{2+}$ response displayed different kinetics, with a markedly slower onset. Furthermore, the magnitude of the maximum $Ca^{2+}$ response induced by resiniferatoxin was $\sim$20% greater than that induced by capsaicin. Similar findings have been reported from native tissues (Petersen et al., 1996) and rat vanilloid VR1 receptor (Jerman et al., 2000). Interestingly, isovelleral and scutigeral, which have been reported to be vanilloid receptor agonists in native tissues (Szallasi et al., 1996, 1999a), had no agonist effect at any concentration (100 pM–30 $\mu$M) tested. Isovelleral has also been shown to be devoid of agonist activity at rat vanilloid VR1 receptor (Jerman et al., 2000).

All the vanilloid-induced $Ca^{2+}$ responses were concentration-dependent, yielding affinities and a rank order of potency generally in keeping with the established pharmacology of the endogenous receptor (Baumann et al., 1996; Szallasi and Blumberg, 1999), although the $EC_{50}$ for capsaicin was higher than that previously reported for human vanilloid VR1 receptor expressed in *Xenopus* oocytes (Hayes et al., 2000), possibly reflecting relative expression levels and/or other methodological differences. As previously reported for the recombinant rat vanilloid VR1 receptor (Caterina et al., 1997; Jerman et al., 2000), resiniferatoxin was only $\sim$20-fold more potent than capsaicin, contrary to the several thousand-fold higher affinity resiniferatoxin displayed in binding studies in native tissues (Szallasi, 1994; Szallasi and Blumberg, 1996). Different vanilloid receptor subtypes for resiniferatoxin and capsaicin have been proposed (Szallasi, 1994), and there is some evidence which suggests that the resiniferatoxin-binding site is relatively unimportant in the activation of the cation channel (Szallasi and Blumberg, 1999). However, a recent study has identified both the resiniferatoxin and capsaicin binding sites on the recombinant rat vanilloid VR1 receptor (Szallasi et al., 1999b). Another important feature of the vanilloid-induced responses in the present study was that all the agonists tested, except olvanil, displayed Hill coefficients of $\sim$2, indicative of positive cooperativity. Similar Hill coefficients have been obtained for both recombinant (Caterina et al., 1997; Jerman et al., 2000) and endogenous (Szallasi et al., 1993; Oh et al., 1996) rat vanilloid VR1 receptor.

It has been proposed that protons associated with low extracellular pH are the endogenous activators of the vanilloid receptor (for review, see Kress and Zeilhofer, 1999), although others have suggested that they merely play a modulatory role (Szallasi and Blumberg, 1999). In the present study, the potency of capsaicin was enhanced $\sim$3-fold by lowering the pH to 6.4. The effect of pH was specific to the vanilloid receptor as the muscarinic receptor-mediated $Ca^{2+}$ response in the same cells was unaffected. The responses to olvanil, resiniferatoxin and PPAHV were also enhanced at pH 6.4, whereas those induced by anandamide were not, indicating that the modulatory effects of pH are agonist-dependent. Moreover, further acidification directly activated the cation channel and so increased intracellular $Ca^{2+}$ concentrations in the human vanilloid VR1 receptor-HEK293 cells in the absence of capsaicin, in a pH-dependent manner, with an $EC_{50}$ of 5.5. This response was capsazepine- and ruthenium red-sensitive, confirming it was mediated by the activation of human vanilloid VR1 receptor. Similar findings have been reported for both the recombinant rat vanilloid VR1 receptor (Tominaga et al., 1998; Jerman et al., 2000) and the endogenous vanilloid receptor (Petersen and LaMotte, 1993; Baumann et al., 1996).

As acidic conditions potentiated the responses to some VR1 agonists, and others have suggested that different ligands may bind to different sites on the vanilloid receptor (Szallasi and Blumberg, 1999), the possibility of cooperativity between different agonists was tested. The responses to sub-maximal concentrations of resiniferatoxin, capsaicin, olvanil and PPAHV were simply additive, and the maximum response to a given agonist was unaltered in the presence of another agonist, demonstrating that the ligands tested were not cooperative. Similar findings have been reported for PPAHV and resiniferatoxin in dorsal root ganglion neurons (Szallasi et al., 1996), and all four ligands in rat vanilloid VR1 receptor-HEK293 cells (Jerman et al., 2000).

In the present study, capsazepine and ruthenium red inhibited the capsaicin- and resiniferatoxin-induced $Ca^{2+}$ responses in a concentration-dependent manner, with affinities similar to that reported for the endogenous vanilloid receptor (Bevan et al., 1992; Acs et al., 1997; Wardle et al., 1997). Others have previously reported that capsazepine inhibited the capsaicin-induced response in human vanilloid VR1 receptor expressing cells, but did not examine the concentration-dependency of this effect (Hayes et al., 2000). Isovelleral also acted as an antagonist at human vanilloid VR1 receptor, inhibiting capsaicin- and resiniferatoxin-induced $Ca^{2+}$ responses at micromolar concentrations. This suggests that the capsaicin-like effects of isovelleral in rat sensory neurones (Szallasi et al., 1996) may not be mediated by vanilloid VR1 receptor and is consistent with our previous findings that isovelleral is as a

competitive antagonist at rat vanilloid VR1 receptor (Jerman et al., 2000).

Although in general human vanilloid VR1 receptor displayed similar pharmacology to that previously reported for rat vanilloid VR1 receptor (Jerman et al., 2000), there were some, relatively subtle, differences. Firstly, PPAHV displayed a ~10-fold lower potency at human vanilloid VR1 receptor (11 μM) compared to rat vanilloid VR1 receptor (1 μM) (Jerman et al., 2000). Secondly, olvanil was more potent than capsaicin at human vanilloid VR1 receptor, yet at rat vanilloid VR1 receptor this rank order of potency was reversed (Jerman et al., 2000). Thirdly, in human vanilloid VR1 receptor-HEK293 cells moderately acidic conditions enhanced the potency of all the vanilloid ligands, whereas in rat vanilloid VR1 receptor-HEK293 cells only the potencies of capsaicin and olvanil were enhanced under the same conditions (Jerman et al., 2000). Finally, although the rank order of affinities for the three vanilloid VR1 receptor antagonists was identical for human and rat vanilloid VR1 receptor, the actual affinities were ~3-fold lower at human vanilloid VR1 receptor. Taken collectively, these data suggests that, although human vanilloid VR1 receptor has 92% homology to rat vanilloid VR1 receptor (Hayes et al., 2000), the few differences in amino acid sequence there are do have functional consequences.

Both the recombinant human vanilloid VR1 receptor (Hayes et al., 2000) and the endogenous human vanilloid receptor (Baumann et al., 1996) have been reported to act as non-selective cation channels with substantial $Ca^{2+}$ permeability. Consistent with this, the removal of extracellular $Ca^{2+}$ abolished the capsaicin-induced $Ca^{2+}$ response in human vanilloid VR1 receptor-HEK293 cells, whilst thapsigargin and U73122 were without effect, indicating that activation of vanilloid VR1 receptor promoted $Ca^{2+}$ influx, but did not mobilise $Ca^{2+}$ from intracellular stores (Berridge, 1993). Furthermore, it has been suggested that tetrodatoxin-sensitive sodium channels and/or voltage-sensitive $Ca^{2+}$ channels are involved in the effects of capsaicin in sensory neurones (Maggi et al., 1988). However, neither tetrodatoxin, nimodipine nor ω-GVIA conotoxin had any effect on the capsaicin-induced response in the present study nor on capsaicin-induced neuropeptide release in native tissue studies (Szolcsanyi et al., 1998). Taken collectively these data confirm human vanilloid VR1 receptor acts as a $Ca^{2+}$-permeable ligand-gated ion channel. Inhibition of protein kinase C with chelerythrine chloride inhibited the capsaicin-induced $Ca^{2+}$ response in human vanilloid VR1 receptor-HEK293 cells, whilst inhibition of protein kinase A with H-89 was without effect. This was somewhat surprising as both protein kinase C and protein kinase A modulate the capsaicin receptor-mediated response in sensory neurons (Sluka et al., 1997; Lopshire and Nicol, 1998; Cesare et al., 1999), and human vanilloid VR1 receptor has both putative protein kinase A and protein kinase C phosphorylation sites (Hayes et al., 2000). However, modulation of protein kinase A also failed to effect rat vanilloid VR1 receptor activity in recombinant systems (Lee et al., 2000; Jerman et al., 2000), suggesting the actions of protein kinase A in sensory neurones may be indirect or that there may be differences in tonic protein kinase A activity in dorsal root ganglion neurons compared to HEK293 cells.

In conclusion, the present study has confirmed and extended the pharmacological characterisation of human vanilloid VR1 receptor, identifying for the first time agonist-dependent aspects of the human vanilloid VR1 receptor-mediated response, and identified subtle differences in the pharmacology of human vanilloid VR1 receptor compared to rat vanilloid VR1 receptor. Furthermore, these data suggest the pharmacology of human vanilloid VR1 receptor can account for most, but not all, of the putative vanilloid receptor subtypes identified pharmacologically in native tissues.

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