Characterization using FLIPR of rat vanilloid receptor (rVR1) pharmacology

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1   The vanilloid receptor (VR1) is a ligand-gated ion channel, which plays an important role in nociceptive processing. Therefore, a pharmacological characterization of the recently cloned rat VR1 (rVR1) was undertaken.

2   HEK293 cells stable expressing rVR1 (rVR1-HEK293) were loaded with Fluo-3AM and then incubated at 25°C for 30 min with or without various antagonists or signal transduction modifying agents. Then intracellular calcium concentrations ($[Ca^{2+}]_i$) were monitored using FLIPR, before and after the addition of various agonists.

3   The rank order of potency of agonists (resiniferatoxin (RTX) > capsaicin > olvanil > PPAHV) was as expected, and all were full agonists. The potencies of capsaicin and olvanil, but not RTX or PPAHV, were enhanced at pH 6.4 (pEC_{50} values of 7.47 ± 0.06, 7.16 ± 0.06, 8.19 ± 0.06 and 6.02 ± 0.03 respectively at pH 7.4 vs 7.71 ± 0.05, 7.58 ± 0.14, 8.10 ± 0.05 and 6.04 ± 0.08 at pH 6.4).

4   Capsazepine, isovelleral and ruthenium red all inhibited the capsaicin (100 nM)-induced Ca^{2+} response in rVR1-HEK293 cells, with pK_B values of 7.52 ± 0.08, 6.92 ± 0.11 and 8.09 ± 0.12 respectively ($n = 6$ each). The response to RTX and olvanil were also inhibited by these compounds. None displayed any agonist-like activity.

5   The removal of extracellular Ca^{2+} abolished, whilst inhibition of protein kinase C with chelerythrine chloride (10 μM) partially (~20%) inhibited, the capsaicin (10 μM)-induced Ca^{2+} response. However, tetrodotoxin (3 μM), nimodipine (10 μM), ω-GVIA conotoxin (1 μM), thapsigargin (1 μM), U73122 (3 μM) or H-89 (3 μM) had no effect on the capsaicin (100 nM)-induced response.

6   In conclusion, the recombinant rVR1 stably expressed in HEK293 cells acts as a ligand-gated Ca^{2+} channel with the appropriate agonist and antagonist pharmacology, and therefore is a suitable model for studying the effects of drugs at this receptor.
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Abbreviations: ASIC, acid-sensitive ion channel; $[Ca^{2+}]_i$ intracellular calcium concentration; DRG, dorsal root ganglion; FI, fluorescence intensity; FLIPR, fluorometric imaging plate reader; MEM, minimum essential medium; PKA, protein kinase A; PKC, protein kinase C; RTX, resiniferatoxin; rVR1, rat VR1; TTX, tetrodotoxin; VR1, vanilloid receptor

Introduction

Improving the treatment of acute, and more especially chronic, pain is a major challenge for the pharmaceutical industry. Most current treatments either block prostaglandin production or activate opioid receptors, but both of these mechanisms are associated with significant side effects (Kress & Zeilhofer, 1999). A more promising alternative approach would be to block the activity of nociceptor-specific receptors. The term nociceptor is used to describe the afferent C- and A-δ fibres, which are activated by a wide range of noxious chemical, mechanical and thermal stimuli and are responsible for the first order transmission of nociceptive signalling from the periphery to the central nervous system (Dray, 1995; Furst, 1999).

One characteristic of nociceptors is that they are activated by capsaicin, a pungent chemical present in hot peppers (Bevan & Szolcsanyi, 1990; Szallasi & Blumberg, 1999). Moreover, this activation is specific to the nociceptors, long-lasting and, in mammals, results in a sensation of burning pain (Simone et al., 1987; Park et al.,

1995). As capsaicin is not endogenous to mammals it presumably mimics the actions of an endogenous ligand or another physiological stimulus (Dray, 1995; Tominaga et al., 1998). Studies using ^{3}H-labelled resiniferatoxin (RTX), another plant-derived noxious vanilloid, and the competitive antagonist capsazepine have demonstrated that nociceptors express a specific capsaicin-binding site, referred to as the vanilloid receptor (Bevan et al., 1992; Rinder et al., 1996; Szallasi & Blumberg, 1999). This receptor has subsequently been further characterized using a range of vanilloid ligands including olvanil, PPAHV, isovelleral and ruthenium red (Jung et al., 1998; Szallasi & Blumberg, 1999). The vanilloid receptor acts as a ligand-gated ion channel, allowing Na^{2+} and Ca^{2+} influx into the sensory neurones (Bevan & Szolcsanyi, 1990).

More recently a major breakthrough in the study of vanilloid receptor pharmacology occurred when the rat vanilloid receptor (rVR1) was cloned from the dorsal root ganglion (DRG) (Caterina et al., 1997). rVR1 consists of 838 amino acids, with six putative transmembrane domains, and is most closely related to the transient receptor potential channel family (Caterina et al., 1997; Tominaga et al., 1998).

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The molecular mass (95 kDa) of rVR1, and its distribution are consistent with that of the native vanilloid receptor (Caterina *et al.*, 1997; Tominaga *et al.*, 1998). Furthermore, when expressed in *Xenopus* oocytes (Caterina *et al.*, 1997) or HEK293 cells (Tominaga *et al.*, 1998) rVR1 acts as a non-selective cation channel, with similar electrophysiological properties to the native vanilloid-gated ion channel. Indeed, rVR1 was not only activated by capsaicin, but also by noxious heat and low pH (Caterina *et al.*, 1997; Tominaga *et al.*, 1998). Furthermore, the capsaicin-induced response was enhanced at pH 6.3 (Caterina 1997; Tominaga *et al.*, 1998). Taken collectively these data confirm the important integratory role vVR1 plays for multiple pain-producing stimuli (Tominaga *et al.*, 1998; Kress & Zeilhofer, 1999). However, there were some discrepancies between the pharmacology of rVR1 and the native receptor, most notably the low relative potency of RTX compared to capsaicin at rVR1 (Szallasi & Blumberg 1999). Moreover, as both studies of rVR1 only used a very limited range of vanilloid ligands (Caterina *et al.*, 1997; Tominaga *et al.*, 1998), they did not address the issue of potential receptor subtypes raised by the various different agonist-dependent responses reported in native tissues (Kress & Zeilhof, 1999; Szallasi & Blumberg 1999).

Therefore, the present study was undertaken to address these issues. rVR1 was cloned, stably expressed in HEK293 cells, and a detailed pharmacological characterization conducted using the Ca^{2+}-sensitive dye, Fluo-3AM in a fluorometric imaging plate reader (FLIPR). The pharmacology of the recombinant rVR1 was confirmed and extended, with some agonist-dependent aspects of the rVR1-mediated response being identified for the first time in a homogenous system. This suggests that the pharmacology of rVR1 can account for some, but not all, of the putative vanilloid receptor subtypes identified pharmacologically in native tissues.

Methods

Cloning and expression of rVR1 receptors in HEK293 cells

A rat VR1 mammalian expression construct was prepared by amplifying cDNA from reverse transcribed adult rat DRG mRNA, using forward and reverse primers incorporating the restriction sites shown, RVR1F-*Hind*III (5′-cataagcttgccgc-catggaacaacgggctagcttagactcagagg) and RVR1R-*Xba*I (5′-cattctagaccattatttctcccctgggaccatgg). Reaction products were cloned into pBSSKII⁺ (Stratagene), confirmed by sequencing and then subcloned into the *Hind*III-*Xba*I sites of pcDNA3.1 (Invitrogen). HEK 293 cells were transfected with rVR1.pcDNA3.1 using Lipofectamine Plus (Life Technologies), according to the manufacturer's instructions, followed by selection in 400 μg ml^{-1} geneticin (Life Technologies) and colony cloning. Stable clones were chosen for further analysis on the basis of rVR1 mRNA expression levels and functional expression of rVR1.

Cell culture

rVR1-HEK293 cells were routinely 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.

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

rVR1-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, Texas, U.S.A.) at 25°C for 120 min. The cells were washed four times with, and finally resuspended in, Tyrode's medium, before being incubated for 30 min at 25°C with either buffer alone (control) or buffer containing various antagonists or signal transduction modifying agents. In some studies calcium was omitted from the buffer. The plates were then placed into a FLIPR (Molecular Devices, U.K.) to monitor cell fluorescence $\lambda_{ex} = 488$ nM, $\lambda_{EM} = 540$ 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., California, U.S.A.). Statistical comparisons were made where appropriate using Student's *t*-test.

Materials

PPAHV, RTX and isovelleral were obtained from Alexis Biochemicals (Nottingham U.K.). Olvanil was purchased from Tocris (Bristol, U.K.) and all other ligands were obtained from RBI (Natick, MA, U.S.A.). All signal transduction modifying agents were purchased from Calbiochem (Nottingham, U.K.). All cell culture media were obtained from Life Technologies (Paisley, U.K.).

Results

Capsaicin caused an increase in $[Ca^{2+}]_i$ in rVR1-HEK293 cells, which was typified by an initially rapid then slowing onset (peak ~30 s), followed by a gradually declining secondary phase (Figure 1). PPAHV and olvanil also increased $[Ca^{2+}]_i$ with similar kinetics (data not shown). However, the RTX-induced Ca^{2+} response had different kinetics, with a more gradual onset (peak ~90 s) followed by a similar slowly declining secondary phase (Figure 1).

All agonist-induced responses were concentration-dependent (Figure 2), and were full agonists compared to capsaicin, although the RTX-induced response obtained a greater maximum than the rest (Figure 2). Nevertheless, the rank order of potency obtained (RTX > capsaicin > olvanil > P-PAHV) was consistent with the established pharmacology of the VR1 (Table 1). Interestingly, capsaicin and RTX had a Hill coefficient of ~2 (Table 1).

Lowering the pH to 6.4 enhanced (2–3 fold) the potencies of capsaicin and olvanil, but not RTX or PPAHV (Table 1), yet had no effect on the Hill coefficients (Table 1). The potency of carbachol at the endogenous muscarinic M_3 receptor was unaffected at pH 6.4 (data not shown). Lowering the pH further (5.5) directly increased $[Ca^{2+}]_i$ in both rVR1 transfected and wild type HEK293 cells, although the pH-induced Ca^{2+} response was prolonged in the transfected cells (Figure 3). Preincubation of the cells at pH 6.7 for 30 min

abolished the response to pH 5.5 in the wild type HEK cells but had no effect on the response in the VR1 expressing cells (Figure 3). Using these conditions the pH-induced activation of VR1 was concentration-dependent, with an EC_{50} of 5.49 (Figure 3). Capsazepine (100 nM) had no effect on the pH-induced Ca^{2+} response in the non-transfected HEK293 cells, but abolished the response in the rVR1 transfected cells (data not shown).
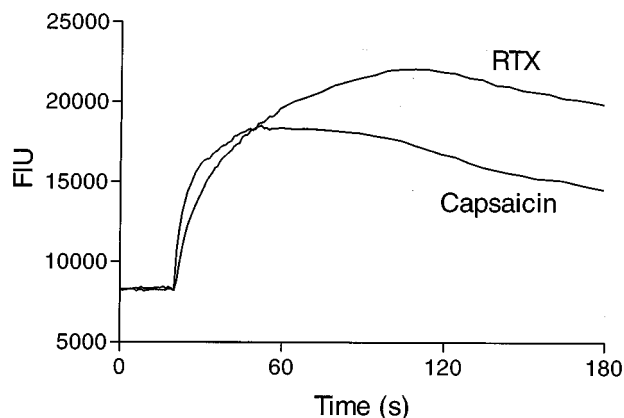

Figure 1 RTX- and capsaicin-induced Ca^{2+} responses have different kinetics in rVR1-HEK293 cells. $[Ca^{2+}]_i$ (as fluorescent intensity units) was monitored using Fluo-3AM in HEK293 cells stably expressing rVR1 before and after the addition of RTX (100 nM) or capsaicin (100 nM). Data shown are representative traces, typical of at least $n = 100$.
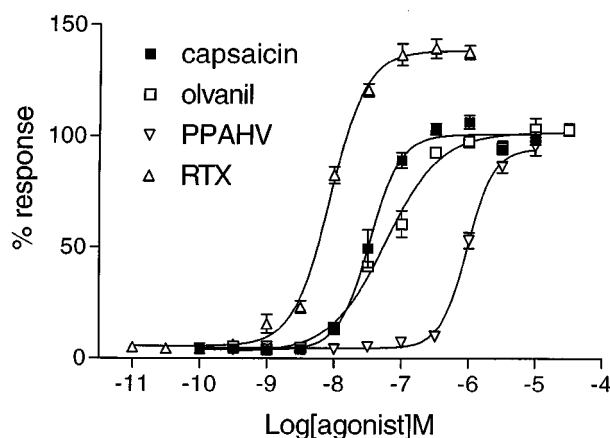

Figure 2 The agonist-induced Ca^{2+} responses are concentration-dependent. $[Ca^{2+}]$ was monitored using Fluo-3AM in rVR1-HEK293 cells before and after the addition of RTX (10 pM–1 μM), capsaicin (100 pM–10 μM), olvanil (300 pM–30 μM), or PPAHV (100 pM–10 μM). Responses were measured as peak increase in fluorescence minus basal, expressed relative to the maximum capsaicin response and are given as mean±s.e.mean, where $n = 6–8$.

The potential for cooperativity between agonists was also examined, by measuring the responses to sub-maximal concentrations of RTX (30 nM), PPAHV (1 μM), capsaicin (100 nM) and olvanil (300 nM), alone or in combination. All four agonists on their own produced sub-maximal Ca^{2+} responses, and in combination were simply additive (Figure 4).

Capsazepine (0.1 nM – 10 μM), isovelleral (1 nM – 10 μM) and ruthenium red (0.1 nM – 10 μM) inhibited the capsaicin (100 nM)-induced Ca^{2+} responses in rVR1-HEK293 cells in a concentration-dependent manner (Figure 5). Capsazepine, isovelleral and ruthenium red also antagonized the RTX (30 nM)– and olvanil (300 nM)-induced responses (Table 2). 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 2). Isovelleral caused concentration-dependent, parallel rightward shifts of the capsaicin concentration-response curve (Figure 5), indicating it was acting as a competitive antagonist. Capsazepine (0.1 μM) also caused a parallel, rightward shift of the capsaicin concentration-response curve (Figure 5).

Consistent with rVR1 being a ligand-gated ion channel, removal of extracellular calcium abolished the capsaicin (100 nM)-induced response in rVR1-HEK293 cells (Figure 6). Pretreatment with tetrodotoxin (3 μM), nimodipine (10 μM), ω-GVIA conotoxin (1 μM), thapsigargin (1 μM), U73122 (3 μM) or H-89 (3 μM) had no effect on the capsaicin (100 nM)-induced response. However, the PKC inhibitor, chelerythrine chloride (10 μM) partially (~20%) inhibited the peak response and increased the rate of decline of the secondary phase (Figure 6).

Discussion

Vanilloid receptors are important potential targets for the treatment of pain (Dray, 1995; Kress & Zeilhofer, 1999). The recent cloning of VR1 from rat DRG neurones (Caterina et al., 1997) was a major breakthrough, but only a limited pharmacological characterization of this receptor was undertaken (Caterina et al., 1997; Tominaga et al., 1998). The present study has confirmed and expanded this pharmacology, identifying for the first time in a homogenous recombinant system, apparent agonist-dependent aspects of the rVR1-mediated response. Furthermore, these data indicate that the pharmacology of rVR1 can account for some, but not all, of the putative vanilloid receptor subtypes previously identified pharmacologically in native tissues (Szallasi & Blumberg, 1999).

In the present study capsaicin increased $[Ca^{2+}]_i$ in rVR1 expressing HEK293 cells, and this response was typified by an initial rapid onset which peaked at ~30 s, followed by a gradual decline towards basal values. Such kinetics are consistent with those previously reported for the endogenous vanilloid receptor (Kress & Zeilhofer, 1999; Szallasi &

Table 1 Acidification enhances the potency of some VR1 agonists

	At pH 7.4		At pH 6.4	
	pEC_{50}	Slope	pEC_{50}	Slope
Capsaicin	7.47±0.06	1.93±0.29	7.71±0.05*	2.11±0.22
Olvanil	7.16±0.06	1.16±0.15	7.58±0.14*	1.24±0.19
RTX	8.19±0.06	1.71±0.34	8.10±0.05	1.92±0.18
PPAHV	6.02±0.03	2.21±0.19	6.04±0.08	2.04±0.15
Isovelleral	Inactive		Inactive	

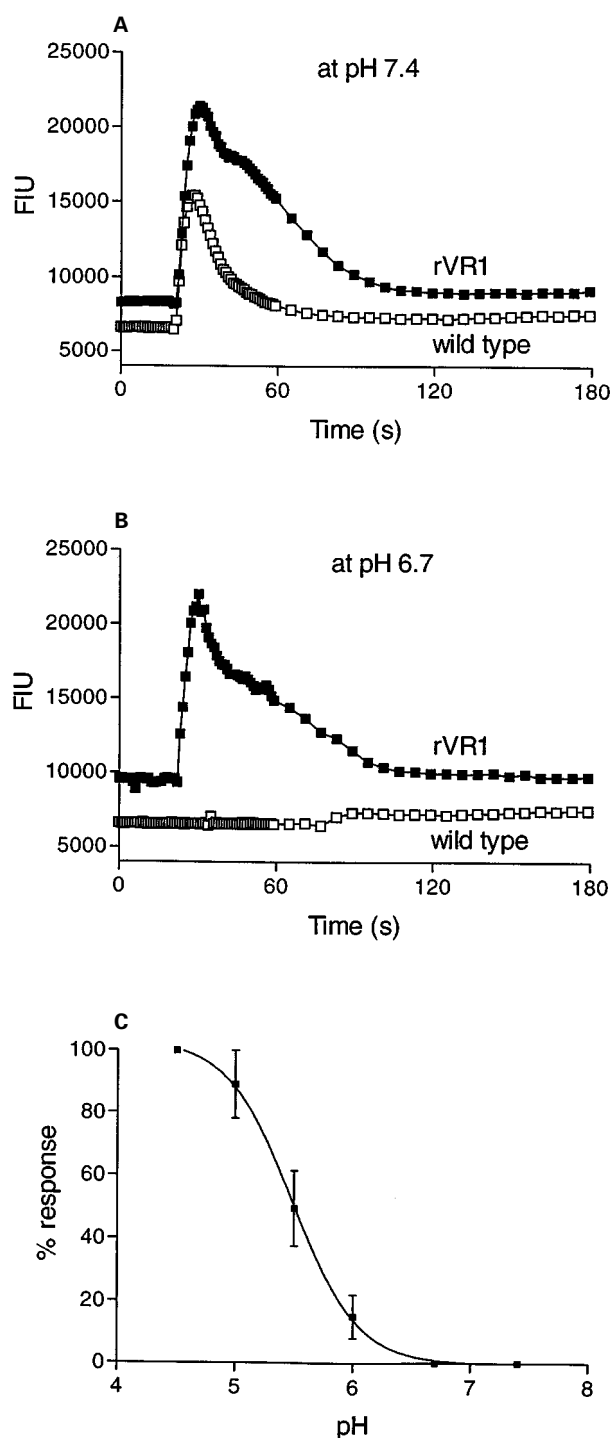Data are mean±s.e.mean, $n = 6–8$. *Denotes $P < 0.05$ increased compared to at pH 7.4.

Figure 3 Acidic conditions cause a rVR1-mediated Ca^{2+} response. $[Ca^{2+}]_i$ was measured (as fluorescent intensity units) using Fluo-3AM in wild type or rVR1-transfected HEK293 cells before and after the addition at 20 s of HCl (4.2 mM, A or 2.1 mM, B), and the associated change in pH from 7.4 to 5.5 (A) and 6.7 to 5.5 (B). Traces shown are typical of at least $n=20$. (C) Depicts the pH-dependency of the response in rVR1-expressing cells. Data are mean$\pm$s.e.mean, where $n=5$.

P= PPAHV (1µM), C= capsaicin (100nM),
O= olvanil (300nM), R= RTX (30nM)
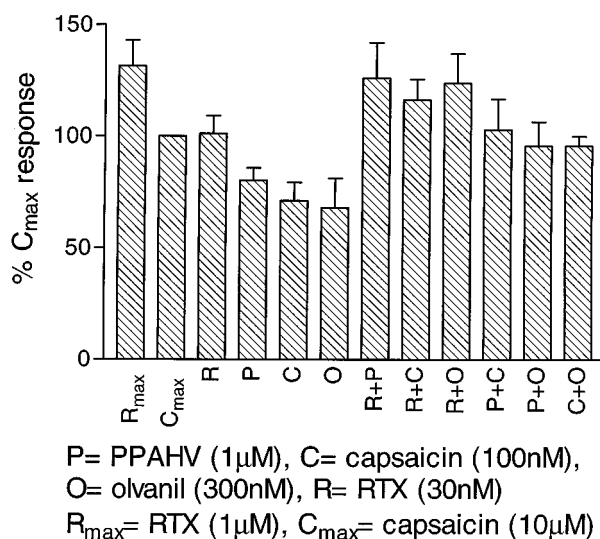R_{max}= RTX (1µM), C_{max}= capsaicin (10µM)

Figure 4 rVR1 agonists do not display cooperativity. $[Ca^{2+}]_i$ was monitored using Fluo-3AM in rVR1-HEK293 cells before and after the addition of RTX (30 nM or 1 µM), capsaicin (100 nM or 10 µM), olvanil (300 nM), or PPAHV (1 µM), alone or in combination. Responses were measured as peak increase in fluorescence minus basal, expressed relative to the maximum capsaicin response and are given as mean$\pm$s.e.mean, where $n=5$.

previously demonstrated in native tissues (Petersen *et al.*, 1996) and *Xenopus* oocytes expressing recombinant rVR1 (Caterina *et al.*, 1997). Furthermore, the magnitude of the maximum Ca^{2+} response induced by RTX was $\sim 20\%$ greater than that induced by capsaicin, again in agreement with previous reports (Petersen *et al.*, 1996; Caterina *et al.*, 1997). Surprisingly, isovelleral, which has been reported to be a vanilloid receptor agonist in native tissues (Szallasi *et al.*, 1996), had no agonist effect at any concentration tested.

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 (Szallasi & Blumberg, 1999). However, as previously reported for the recombinant rVR1 (Caterina *et al.*, 1997), RTX was only a few fold more potent than capsaicin, contrary to the several thousand fold higher affinity RTX displayed in binding studies in native tissues (Szallasi, 1994; Szallasi & Blumberg, 1996). Nevertheless, different binding sites on the vanilloid receptor for RTX and capsaicin have been proposed (Szallasi, 1997), and there is some evidence which suggests that the RTX-binding site is relatively unimportant in the activation of the cation channel (Szallasi & Blumberg, 1999). Moreover, a recent study has identified both these binding sites on cells expressing recombinant rVR1 (Szallasi *et al.*, 1999). It is worth noting however that RTX was more potent than capsaicin in a $^{45}Ca^{2+}$ efflux assay in DRG cells as well (Acs *et al.*, 1996). Another important feature of the vanilloid-induced responses in the present study was that all the agonists tested displayed Hill coefficients of ~ 2, indicative of positive cooperativity. Similar Hill coefficients have been obtained electrophysiologically for RTX and capsaicin in both recombinant (Caterina *et al.*, 1997) and endogenous (Szallasi *et al.*, 1993; Oh *et al.*, 1996) systems. Interestingly, in native tissues PPAHV does not display cooperativity, but does activate two distinct currents (Liu *et al.*, 1998). This apparent discrepancy most likely reflects differences in the experimental conditions. Indeed, the kinetics of the PPAHV-
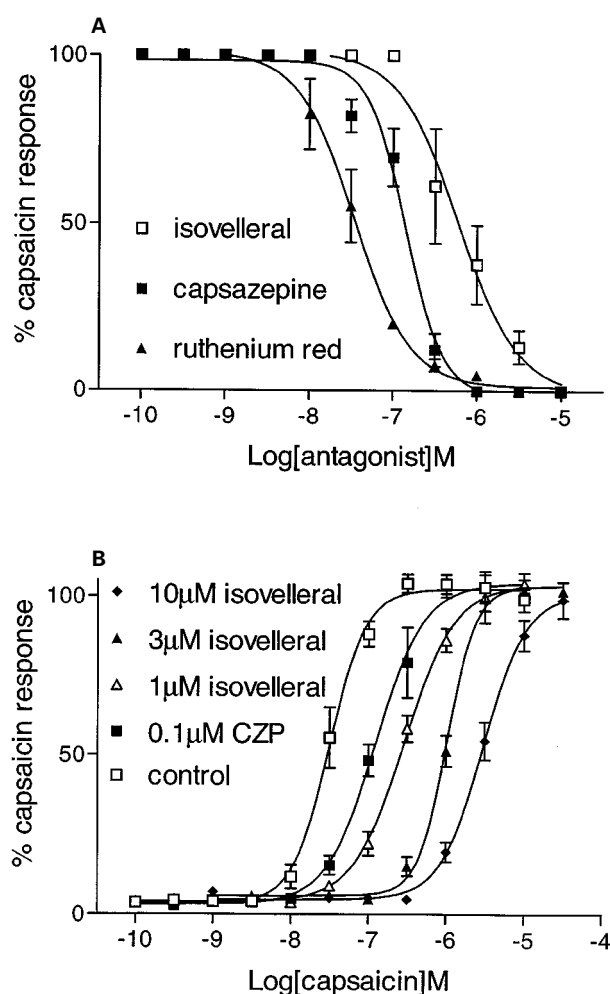
Blumberg, 1999). Olvanil and PPAHV also evoked similar responses, again consistent with previous reports from native tissues (Szallasi & Blumberg, 1999). However, the RTX-induced Ca^{2+} response displayed different kinetics, with a markedly slower onset, peaking at ~ 90 s, followed by a subsequent maintained phase. Similar differences in kinetics between RTX- and capsaicin-induced responses have been

Figure 6 The role of extracellular Ca^{2+} and PKC in the rVR1-mediated Ca^{2+} response. $[Ca^{2+}]_i$ was monitored (as fluorescent intensity units) using Fluo-3AM in rVR1-HEK293 cells, incubated in calcium-containing or calcium-free buffer, in the presence or absence of chelerythrine chloride (Ch. Cl, 10 μM), before and after a capsaicin (1 μM) challenge at 20 s. Data are representative traces, typical of at least $n = 5$.

Figure 5 VR1 antagonists inhibit the capsaicin-induced Ca^{2+} response. In (A) Fluo-3AM-loaded rVR1-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 37°C, and then $[Ca^{2+}]_i$ was monitored before and after the addition of capsaicin (100 nM). In (B), the concentration-response relationship for capsaicin (0.1 nM – 30 μM) was assessed in the presence or absence of capsazepine (CZP, 0.1 μM) or isovelleral (1 – 10 μM). Responses were measured as peak increase in fluorescence minus basal, expressed relative to the control capsaicin response and are given as mean $\pm$ s.e.mean, where $n = 6$.

Table 2 Antagonist affinities at recombinant rVR1 expressed in HEK cells

	pK$_B$ values		
	Capsazepine	*Isovelleral*	*Ruthenium red*
Vs capsaicin	7.52 $\pm$ 0.08	6.92 $\pm$ 0.11	8.09 $\pm$ 0.12
Vs RTX	7.48 $\pm$ 0.12	7.14 $\pm$ 0.14	8.58 $\pm$ 0.09
Vs olvanil	7.74 $\pm$ 0.14	7.46 $\pm$ 0.23	8.20 $\pm$ 0.06

Data are mean $\pm$ s.e.mean, $n = 6$.

induced response in the current investigation indicate that only one current was activated in rVR1-HEK293 cells.

It has been proposed that protons associated with low extracellular pH are the endogenous activators of the vanilloid receptor (for review see Kress & Zeilhofer, 1999), although others have suggested that they merely play a modulatory role (Szallasi & Blumberg, 1999). In keeping with this in the present study the potency of capsaicin was enhanced ~ 3 fold by lowering the pH to 6.4. Similar findings have been reported for
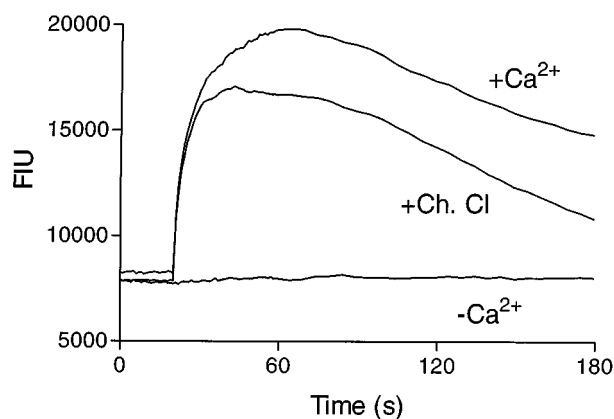
both the recombinant VR1 (Caterina *et al.*, 1997; Tominaga *et al.*, 1998) and endogenous vanilloid receptor (Petersen & LaMotte 1993; Baumann *et al.*, 1996). The effect of pH was specific to the vanilloid receptor as the muscarinic receptor-mediated Ca^{2+} response in the same cells was unaffected. However, although the response to olvanil was also enhanced at pH 6.4, the responses to RTX and PPAHV were not, indicating that the modulatory effects of pH were agonist-dependent. Indeed, as PPAHV and RTX are structurally similar to each other (Appendino *et al.*, 1996), and dissimilar to capsaicin and olvanil (Szallasi & Blumberg, 1999), this suggests that the structure-activity relationships of the vanilloid and pH-mediated sites are different. Moreover, and consistent with earlier studies of the cloned receptor (Tominaga *et al.*, 1998), lowering the pH further, to 5.5, activated the cation channel and so increased $[Ca^{2+}]_i$ in the rVR1-HEK293 cells, in the absence of capsaicin. However, more transient, Ca^{2+} responses of a similar magnitude were also found in the non-transfected HEK293 cells, which were not activated by capsaicin nor blocked by capsazepine, suggesting an endogenous acid-sensitive ion channel (ASIC) was present. Preincubation of the cells at pH 6.7 desensitized the ASIC, but did not effect the VR1-mediated response. Under these conditions the pH-induced response in the VR1-expressing cells was concentration-dependent, with a similar EC_{50} to that previously reported for VR1 by Tominaga & colleagues (1998), and capsazepine-sensitive.

As pH potentiated responses to some VR1 agonists, and others have suggested that different ligands may bind to different sites on the vanilloid receptor (Szallasi, 1997), the possibility of cooperativity between different agonists was tested. The responses to RTX, 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 RTX in DRG neurones (Szallasi *et al.*, 1996).

In the present study capsazepine inhibited the capsaicin-induced $[Ca^{2+}]_i$ response in a concentration-dependent manner, with an affinity similar to that reported for both the recombinant (Caterina *et al.*, 1997) and endogenous (Bevan *et al.*, 1992; Acs *et al.*, 1997) vanilloid receptors. Capsazepine also inhibited the RTX- and olvanil-induced Ca^{2+} responses in

rVR1-HEK293 cells with similar affinities. This is in agreement with the affinity obtained for capsazepine *versus* RTX in sensory neurones (Bevan *et al.*, 1992; Wardle *et al.*, 1997), but is contrary to the report that capsazepine was ineffective *versus* olvanil (Davey *et al.*, 1994). However, this apparent discrepancy may simply reflect non-vanilloid receptor mediated effects of olvanil (Beltramo & Piomelli, 1999). Capsazepine has also been reported to have agonist-like activity in rabbits (Wang & Hakanson, 1993), but had no such effects in rVR1-HEK293 cells. Ruthenium red also inhibited the capsaicin-induced Ca^{2+} response in the current investigation, with an affinity similar to that reported in native tissues (Acs *et al.*, 1997). Others have previously reported ruthenium red inhibited the capsaicin-induced response in rVR1 expressing cells, but did not examine the concentration-dependency of this effect (Caterina *et al.*, 1997; Tominaga *et al.*, 1998). Surprisingly, isovelleral also acted as a competitive antagonist at rVR1, inhibiting capsaicin-, RTX- and olvanil-induced Ca^{2+} responses at sub-micromolar concentrations. This suggests that the capsaicin-like agonist effects of isovelleral in rat sensory neurones (Szallasi *et al.*, 1996) may not be mediated by rVR1.

Both the recombinant rVR1 (Caterina *et al.*, 1997) and the endogenous vanilloid receptor (Wood *et al.*, 1988) have been reported to act as non-selective cation channels with a preference for Ca^{2+}. Consistent with this, the removal of extracellular Ca^{2+} abolished the capsaicin-induced Ca^{2+} response in rVR1-HEK293 cells, whilst thapsigargin and U73122 were without effect, indicating that activation of VR1 promoted Ca^{2+} influx, but did not mobilise Ca^{2+} from intracellular stores (Berridge 1993). Furthermore, although some have suggested that TTX-sensitive sodium channels and/or voltage-sensitive calcium channels are involved in the effects of capsaicin in sensory neurones (Maggi *et al.*, 1988), neither TTX, nimodipine nor ω-GVIA conotoxin had any effect on the capsaicin-induced response in the present study. Taken collectively these data conform rVR1 acts as a ligand-gated ion channel. Moreover, inhibition of PKC with chelerythrine chloride inhibited the capsaicin-induced Ca^{2+} response in rVR1-HEK293 cells, whilst inhibition of PKA with H-89 was without effect. This was somewhat surprising as both PKC and PKA modulate the capsaicin-induced response in sensory neurones (Sluka *et al.*, 1997; Lopshire & Nicol, 1998), and rVR1 has three PKA phosphorylation sites (Caterina *et al.*, 1997), but may reflect the differing tonic activity of these two kinases.

In conclusion, the present study has confirmed and extended the pharmacological characterization of rVR1, identifying for the first time in a homogenous, recombinant system, agonist-dependent aspects of the rVR1-mediated response. Furthermore, these data suggest the pharmacology of rVR1 can account for some, but not all, of the putative vanilloid receptor subtypes identified pharmacologically in native tissues.

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