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Biochemical Pharmacology 65 (2003) 143–151

Biochemical Pharmacology

Pharmacology of vanilloids at recombinant and endogenous rat vanilloid receptors

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Received 5 July 2002; accepted 23 August 2002

Abstract

This study compared the actions of members of five different chemical classes of vanilloid agonists at the recombinant rat vanilloid VR1 receptor expressed in HEK293 cells, and at endogenous vanilloid receptors on dorsal root ganglion cells and sensory nerves in the rat isolated mesenteric arterial bed. In mesenteric beds, vanilloids elicited dose-dependent vasorelaxation with the rank order of potency: resiniferatoxin ⩾ capsaicin = olvanil > phorbol 12-phenyl-acetate 13-acetate 20-homovanillate (PPAHV) > isovelleral. Scutigeral was inactive. Responses were abolished by capsaicin pretreatment and inhibited by ruthenium red. In VR1-HEK293 cells and dorsal root ganglion neurones, Ca^{2+} responses were induced by resiniferatoxin > capsaicin = olvanil > PPAHV; all four were full agonists. Isovelleral and scutigeral were inactive. The resiniferatoxin-induced Ca^{2+} response had a distinct kinetic profile. Olvanil had a Hill coefficient of ~1 whilst capsaicin, resiniferatoxin and PPAHV had Hill coefficients of ~2 in VR1-HEK293 cells. The capsaicin-induced Ca^{2+} response was inhibited in a concentration-dependent manner by ruthenium red > capsazepine > isovelleral. These data show that resiniferatoxin, capsaicin, olvanil and PPAHV, but not scutigeral and isovelleral, are agonists at recombinant rat VR1 receptors and endogenous vanilloid receptors on dorsal root ganglion neurones and in the rat mesenteric arterial bed. The vanilloids display the same relative potencies (resiniferatoxin > capsaicin = olvanil > PPAHV) in all of the bioassays.

Keywords: Isovelleral; Olvanil; PPAHV; Resiniferatoxin; Scutigeral; Vanilloid VR1 receptor

1. Introduction

There is renewed interest in the pharmacology of vanilloid receptors (see reference [1]) and the cloning of the vanilloid VR1 receptor, a non-selective cation channel [2], has opened new avenues for research in this field. More recently, vanilloid receptor homologues have been cloned [3,4]. Vanilloid receptor-1 is expressed almost exclusively on sensory nerves, characterised by their sensitivity to capsaicin. Sensory nerves extend throughout the body, including the cardiovascular system [5–7], but recent reports have also indicated low levels of vanilloid VR1 receptor in many brain regions [8,9].

To date, there are seven reported chemical classes of agonists at vanilloid receptors. These include capsaicinoids (e.g. capsaicin, olvanil), phorboid vanilloids (e.g. phorbol 12-phenyl-acetate 13-acetate 20-homovanillate (PPAHV), resiniferanoids (e.g. resiniferatoxin), unsaturated dialdehydes (e.g. isovelleral) and triprenyl phenols (e.g. scutigeral) [1]. The sixth and seventh classes, identified only recently, are represented by anandamide, an endogenous cannabinoid that is structurally related to capsaicin [10,11], and eicosanoids, products of lipoxygenases and also structurally similar to capsaicin [12].

The present study investigated the pharmacology of five different chemical classes of vanilloid agonists, namely resiniferatoxin, scutigeral, isovelleral, olvanil and PPAHV, at the recombinant rat vanilloid VR1 receptor expressed in HEK293 cells and at endogenous vanilloid receptors in dorsal root ganglion neurones and the rat isolated mesenteric arterial bed. Our objective was to provide a systematic

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Abbreviations: PPAHV, phorbol 12-phenyl-acetate 13-acetate 20-homovanillate.

0006-2952/02/$ – see front matter
PII: S0006-2952(02)01451-X

comparison of major classes of vanilloid ligands across three models of increasing complexity. Rat mesenteric arteries are richly innervated by capsaicin-sensitive sensory nerves containing predominantly the neuropeptides calcitonin gene-related peptide and substance P [5,6]. It is calcitonin gene-related peptide, however, that mediates mesenteric vasorelaxation after being released by electrical or chemical stimulation of the sensory nerves [13]. In the different bioassays we used capsazepine, a vanilloid receptor antagonist, ruthenium red, a blocker of vanilloid responses, and/or capsaicin pretreatment of the mesenteric arterial bed to cause desensitization and neurotransmitter depletion from sensory nerves.

2. Materials and methods

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

A rat vanilloid VR1 receptor mammalian expression construct was prepared by amplifying cDNA from reverse transcribed adult rat dorsal root ganglion mRNA, as described previously [14]. Reaction products were cloned into pBSSKII$^+$ (Stratagene), confirmed by sequencing and then subcloned into the *Hin*dIII–*Xba*I sites of pcDNA3.1 (Invitrogen). HEK293 cells were transfected with rat vanilloid VR1-pcDNA3.1 using Lipofectamine Plus (Life Technologies), according to the manufacturer's instructions, and stably expressing clones selected [14].

2.2. Cell culture

Vanilloid VR1-HEK293 cells were grown as monolayers in minimum essential medium supplemented with non-essential amino acids, 10% foetal calf serum (FCS), and 0.2 mM L-glutamine, and maintained under 95%/5% air/CO_2 at 37°. Cells were passaged every 3–4 days and the highest passage number used was 24. After passage 30 or so the quality of the responses begins to decline, but there are no differences in the pharmacology dependent on passage number in the range tested. Dissociated rat neonatal dorsal root ganglion cultures were prepared as described by Skaper *et al.* [15]. In brief, 8-day-old rats were sacrificed by Schedule 1 and decapitated. DRG were removed from all spinal segments, cleaned of the meninges and roots and placed in 0.25% Trysin–EDTA for 15 min at 37°. An equal volume of DMEM plus 10% FCS was added to neutralise the trypsin and the ganglia collected by centrifugation before being transferred to 0.1% collagenase for 30 min at 37°. An equal volume of DMEM plus 10% FCS was added to neutralise the collagenase and the ganglia collected into 2 mL fresh DMEM plus 10% FCS. The ganglia were triturated 20 times using a long Pasteur pipette with a flame polished tip and the resulting cell suspension was made up to 10 mL in DMEM + 10% FCS and plated onto a 100 mm tissue culture dish for 1 hr at 37°. Media containing the unattached neuronal cells was collected and the cells pelleted by centrifugation. The cell pellet was resuspended in growth media (DMEM plus 50 ng mL^{-1} NGF, N2 supplement and 0.05% BSA) and plated onto 384-well black and clear plates, previously coated with 100 ug mL^{-1} poly-L-lysine and 5 ug mL^{-1} laminin, at a density of 10,000 cells per well in 50 uL growth media. Cell plates were incubated overnight at 37°, 5% CO_2 before being assayed.

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

This was done as described previously [14]. Briefly, rat vanilloid VR1-HEK293 cells seeded (25,000 cells per well) into 96-well plates were incubated with minimum essential medium containing the cytoplasmic calcium indicator, Fluo-3AM (4 μM; Teflabs) at 25° for 120 min. Alternatively, dorsal root ganglion neurones seeded (5000 cells per well) into laminin coated 384-well plates were incubated with minimum essential medium containing the cytoplasmic calcium indicator, Fluo-3AM (4 μM; Teflabs) at 37° for 60 min. In either case the cells were then washed three times with, and finally resuspended in, Tyrode's medium containing 0.2% bovine serum albumin, before being incubated for 30 min at 25° with either buffer alone (control) or buffer containing various antagonists. The plates were then placed into a FLIPR (Molecular Devices) to monitor cell fluorescence ($\lambda_{EX} = 488$ nm, $\lambda_{EM} = 540$ nm) [11] before and after the addition of various agonists.

2.4. Isolated perfused mesenteric arterial beds

Male Wistar rats (250–300 g) were killed by exposure to CO_2, which resulted in anaesthesia/death, followed by decapitation. Mesenteric beds were isolated and perfused as described previously [16]. In brief, the abdomen was opened and the superior mesenteric artery exposed and cannulated with a hypodermic needle. The superior mesenteric vein was cut, blood flushed from the preparation with 0.5 mL of Krebs' solution and the intestine dissected carefully away from the mesenteric vasculature. The preparation was set up in a humid chamber and perfused at a constant flow rate of 5 mL min^{-1} using a peristaltic pump (model 7554-30, Cole-Parmer Instrument Co). The perfusate was Krebs' solution of the following composition (mM): NaCl 133, KCl 4.7, NaH_2PO_4 1.35, $NaHCO_3$ 16.3, $MgSO_4$ 0.61, $CaCl_2$ 2.52, and glucose 7.8, gassed with 95% O_2–5% CO_2 and maintained at 37°. Changes in perfusion pressure (mmHg) were measured with a pressure transducer (model P23XL, Viggo-Spectramed) on a side arm of the perfusion cannula, and recorded on a polygraph (model 7D, Grass Instrument Co).

After 30 min equilibration, the preparations were submaximally preconstricted (to about 50–80 mmHg above baseline) with methoxamine, and when stable tone had been achieved, vasorelaxation to a single vanilloid per

mesenteric arterial bed was investigated. Vanilloids were applied by injection of bolus doses (50 μL) into norprene rubber tubing proximal to the preparation. The dose interval was dictated by the recovery of the response (up to 1 hr for long-lasting responses to olvanil). Capsazepine and ruthenium red were added during the period of equilibration, at least 30 min before challenge with vanilloid agonists. Capsaicin pretreatment, to desensitize and/or deplete neurotransmitter from sensory nerves, was by perfusion and superfusion with capsaicin (10 μM) for 2 hr, followed by 40 min washout, as described previously [16]. The endothelium was removed by 12 min perfusion with distilled water and the success of the treatment was confirmed using acetylcholine (50 nmol), which produced a relaxation of 10.8 ± 1.1% (this dose elicits 80–90% relaxation in endothelially-intact preparations) [17].

2.5. Materials

All vanilloids were obtained from Alexis Biochemicals. Capsaicin (8-methyl-*N*-vanillyl-6-nonenamide) was from Sigma. Capsazepine was from Tocris Cookson Ltd. Stock solutions of the vanilloids were prepared in ethanol and serial dilutions made in distilled water. The equivalent concentration of vehicle at the greatest dose of agonist used had no significant effect on the tone of the mesenteric arterial beds. Different batches of scutigeral were used for the studies in the mesenteric arterial bed and those in the dorsal root ganglion and HEK293 cells, so we have no reason to suppose that the lack of effect of this compound was due to, for example, drug decomposition.

3. Data analysis

3.1. FLIPR studies

Responses were measured as peak fluorescence intensity minus basal fluorescence intensity, and where appropriate, were expressed as a percentage of a maximum capsaicin-induced response. Data are expressed as mean ± SEM unless otherwise stated. Curve-fitting and parameter estimation were carried out using Graph Pad Prism 3.00 (GraphPad Software Inc.). The pK_B values were generated from IC_{50} curves for the antagonist vs. a fixed EC_{80} concentration of agonist using the Cheng and Prusoff equation [18].

3.2. Mesenteric arterial beds

Vasorelaxant responses were measured as changes in perfusion pressure (mmHg) and expressed as percentage relaxation of the methoxamine-induced increase in tone above baseline. Data were analysed by Student's paired *t*-test, or, for comparisons of more than two groups, by analysis of variance with Tukey's post hoc test. A value of $P < 0.05$ was taken to indicate a statistically significant difference. The pD_{30} and pD_{50} are the negative logarithm of the dose of agonist required to elicit vasorelaxation of 30 and 50%, respectively.

4. Results

4.1. FLIPR studies

Capsaicin caused an increase in $[Ca^{2+}]_i$ in rat vanilloid VR1-HEK293 cells, which was characterised as an initially rapid then slowing onset (peak ~30 s), followed by a gradually declining secondary phase (Fig. 1). PPAHV and olvanil also increased $[Ca^{2+}]_i$ with similar kinetics (Fig. 1). However, the resiniferatoxin-induced Ca^{2+} response had different kinetics, with a more gradual onset (peak ~90 s) followed by a similar slowly declining secondary phase (Fig. 1). These agonists also caused Ca^{2+} responses with similar kinetics in primary dorsal root ganglion cultures, although the delayed, gradual onset with

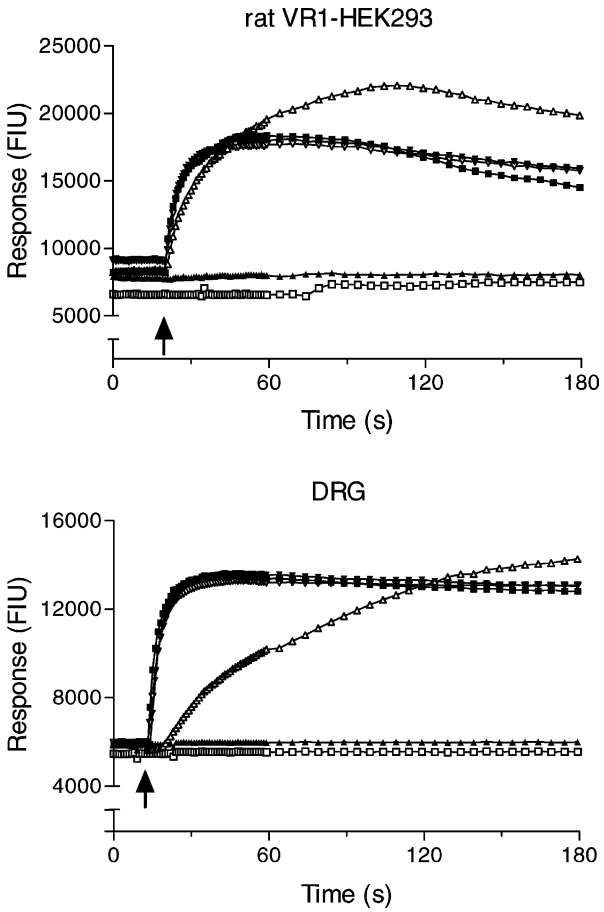

Fig. 1. Resiniferatoxin- and capsaicin-induced Ca^{2+} responses have different kinetics in both rat vanilloid VR1-HEK293 cells and dorsal root ganglion (DRG) neurones. $[Ca^{2+}]_i$ (as fluorescent intensity units) was monitored using Fluo-3AM in rat vanilloid VR1-HEK293 cells and Fluo-4AM in dorsal root ganglion neurones before and after the addition (at arrow) of resiniferatoxin (Δ, 10 nM), olvanil (▼, 100 nM), PPAHV (Δ, 10μM), isovelleral (▲, 10 μM), scutigeral (□, 10 μM), or capsaicin (■, 100 nM). Data shown are representative traces.

Table 1
Agonist pharmacology at the recombinant rat vanilloid VR1 receptor and the endogenous capsaicin receptor in dorsal root ganglion (DRG) neurones

	rVR1		DRG	
	pEC_{50}	Hill slope	pEC_{50}	Hill slope
Capsaicin	7.67 ± 0.04	1.76 ± 0.22	7.56 ± 0.03	2.26 ± 0.45
olvanil	7.53 ± 0.05	1.07 ± 0.12	7.33 ± 0.08	1.18 ± 0.17
RTX	8.12 ± 0.02	1.80 ± 0.15	7.93 ± 0.05	1.08 ± 0.12
PPAHV	6.02 ± 0.02	2.21 ± 0.20	6.52 ± 0.01	3.12 ± 0.57
Isovelleral	IA	IA	IA	IA
Scutigeral	IA	IA	IA	IA

Data are mean ± SEM, where N = 3–10. IA: inactive at all concentrations tested (100 pM–10 μM).

resiniferatoxin was more pronounced in these neurones (Fig. 1). Isovelleral and scutigeral had no effect on $[Ca^{2+}]_i$ in either rVR1-HEK293 cells or dorsal root ganglion neurones (Fig. 1; Table 1).

In both rat vanilloid VR1-HEK293 cells and dorsal root ganglion neurones all the agonist-induced Ca^{2+} responses were concentration-dependent (Fig. 2), with a rank order of potency (resiniferatoxin > capsaicin = olvanil > PPAHV) consistent with the established pharmacology of the vanilloid VR1 receptor (Table 1). Furthermore, all four were full agonists compared to capsaicin, although the resiniferatoxin-induced response obtained a greater maximum than the rest (Fig. 2). Interestingly, olvanil had a Hill coefficient of ~1 whilst capsaicin, resiniferatoxin and PPAHV had a Hill coefficient of ~2 in the rat vanilloid VR1-HEK293 cells (Table 1). However, resiniferatoxin, as well as olvanil, had a Hill coefficient of ~1 in the dorsal root ganglion neurones (Table 1).

The capsaicin (100 nM)-induced Ca^{2+} response was inhibited in a concentration-dependent manner by capsazepine (0.1 nM–10 μM), isovelleral (0.1 nM–10 μM) and ruthenium red (10 pM–1 μM) in both rat vanilloid VR1-HEK293 cells and dorsal root ganglion neurones, with a rank order of affinity of ruthenium red > capsazepine > isovelleral (Table 2). Interestingly, whilst the apparent affinity for ruthenium red was similar in the two systems, that of capsazepine and isovelleral were ~3-fold lower in the dorsal root ganglion neurones (Table 2). Capsazepine had no effects on basal Ca^{2+} in either cell type, and did not affect calcium mobilisation in response to carbachol or UTP (data not shown).

Table 2
Antagonist pharmacology at the recombinant rat vanilloid VR1 receptor and the endogenous capsaicin receptor in dorsal root ganglion (DRG) neurones

	pK_B at rVR1	pK_B in DRG
Capsazepine	6.91 ± 0.08	6.42 ± 0.02
Ruthenium red	8.54 ± 0.08	8.60 ± 0.06
Isovelleral	6.53 ± 0.07	6.08 ± 0.03

Data are mean ± SEM, where N = 4–6. All conducted vs. 100 nM capsaicin.

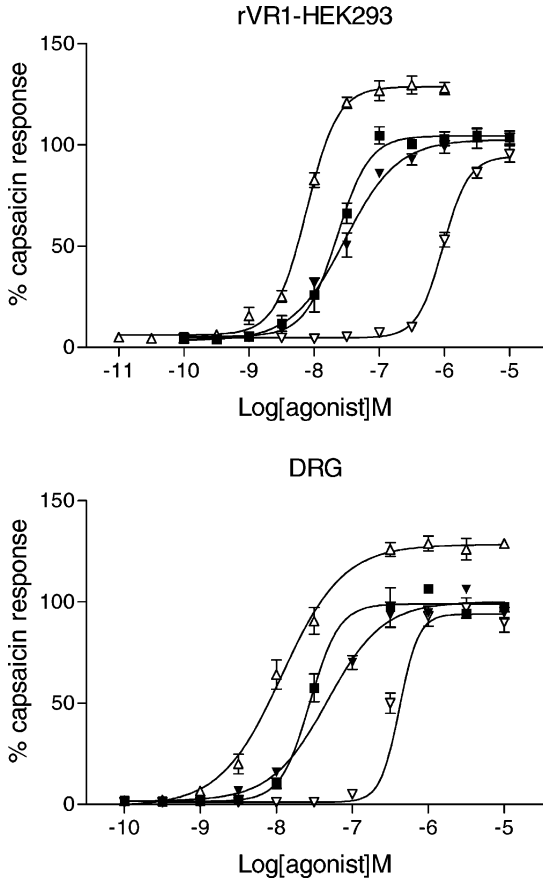

Fig. 2. The agonist-induced Ca^{2+} responses are concentration-dependent in both rat vanilloid VR1-HEK293 cells and dorsal root ganglion (DRG) neurones. $[Ca^{2+}]_i$ was monitored using Fluo-3AM in rat vanilloid VR1-HEK293 cells and Fluo-4AM in DRG neurones before and after the addition of resiniferatoxin (Δ, 10 pM–10 μM), capsaicin (■, 100 pM–10 μM), olvanil (▼, 100 pM–10 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 ± SEM, where N = 3–10.

4.2. Effect of vanilloids in the mesenteric arterial bed

The vanilloids relaxed the mesenteric arterial beds with the following order of agonist potency, based on pD_{50} values: resiniferatoxin ⩾ capsaicin = olvanil > PPAHV > isovelleral (Fig. 3). Scutigeral was inactive (N = 3). The pD_{50} values were: resiniferatoxin 18.2 ± 0.2 (N = 8); capsaicin 10.4 ± 0.1 (N = 7); olvanil 10.4 ± 0.2 (N = 8); PPAHV 9.7 ± 0.1 (N = 5). Maximal relaxation values were: resiniferatoxin 80.7 ± 5.0% (N = 8); capsaicin 96.4 ± 1.9% (N = 7); olvanil 58.0 ± 6.6% (N = 8); PPAHV 88.6 ± 5.2% (N = 5). It is likely that for resiniferatoxin and olvanil the maximal relaxations are underestimated due to the pronounced desensitization that was observed during the generation of dose-response relationships with these agonists. Indeed, for resiniferatoxin a dose interval of 1 hr was not sufficient to avoid some desensitization. Maximal relaxation was not obtained with isovelleral (N = 6).

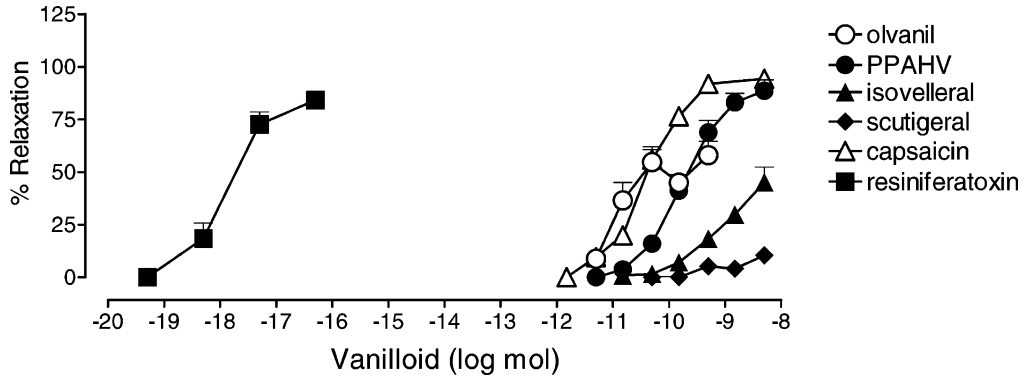

Fig. 3. Structure–activity relations of vanilloids in the rat isolated mesenteric arterial bed. Vasorelaxation was elicited by olvanil (N = 8), resiniferatoxin (N = 8), PPAHV (N = 5) and isovelleral (N = 6). Scutigeral was inactive (N = 3). Data for capsaicin (N = 7), which was equipotent with olvanil, has been submitted in another manuscript [16].

Interestingly, responses to resiniferatoxin and olvanil were slow in onset and in the time taken to reach the peak amplitude of responses compared to the other vanilloids. Responses of the vanilloids were compared at doses producing approximately 50% vasorelaxation. The time to the maximum amplitude of the response to resiniferatoxin was 408 ± 378 s (N = 8) and olvanil was 330 ± 24 s (N = 8). This was significantly greater ($P < 0.001$) than the time to maximum response of similarly efficacious doses of capsaicin (138 ± 12 s, N = 7), PPAHV (168 ± 18 s, N = 5) and isovelleral (156 ± 24 s, N = 6) (Fig. 4).

Endothelium removal had no significant effect on capsaicin-induced relaxation in the mesenteric arterial bed: pEC_{50} 10.25 ± 0.11; R_{max} $80.76 \pm 11.14\%$ (N = 5).

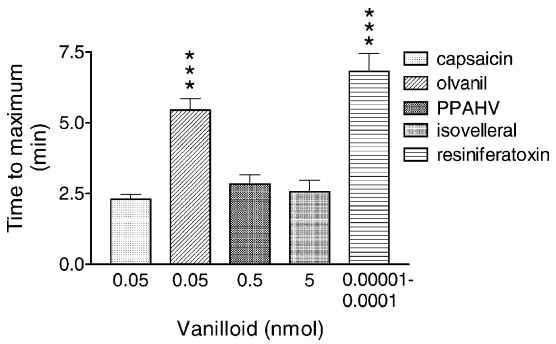

Fig. 4. Time to maximal amplitude of the relaxation response to vanilloids in the rat isolated mesenteric arterial bed. The dose of each vanilloid used in the comparison was that which elicited approximately 50% vasorelaxation of preconstricted mesenteric beds. Olvanil (N = 8) took significantly longer ($P < 0.001$) to reach a maximum relaxation response (at the doses indicated) than did capsaicin, PPAHV (N = 5) and isovelleral (N = 6). $^{***}P < 0.001$, denotes difference compared to capsaicin, PPAHV and isovelleral.

4.3. Effect of capsaicin pretreatment on responses to vanilloids in the mesenteric arterial bed

Capsaicin pretreatment (10 μM, 2 hr) abolished vasorelaxant responses to resiniferatoxin, PPAHV, olvanil (N = 3–4), and attenuated vasorelaxation to isovelleral (N = 4) (Fig. 5).

4.4. Effect of ruthenium red on responses to vanilloids in the mesenteric arterial bed

Ruthenium red (1 μM) attenuated responses to capsaicin (pEC_{50} 8.8 ± 0.2, N = 5) and PPAHV (Fig. 6a and b).

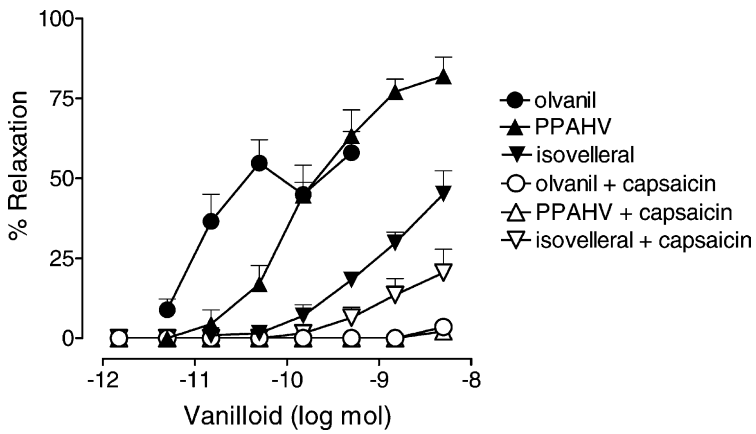

Fig. 5. Effect of capsaicin pretreatment (10 μM, for 2 hr) on vanilloid-evoked vasorelaxation in the rat isolated mesenteric arterial bed. Capsaicin pretreatment abolished responses to olvanil (N = 4) and PPAHV (N = 4) and attenuated responses to isovelleral (N = 4) ($P < 0.001$).

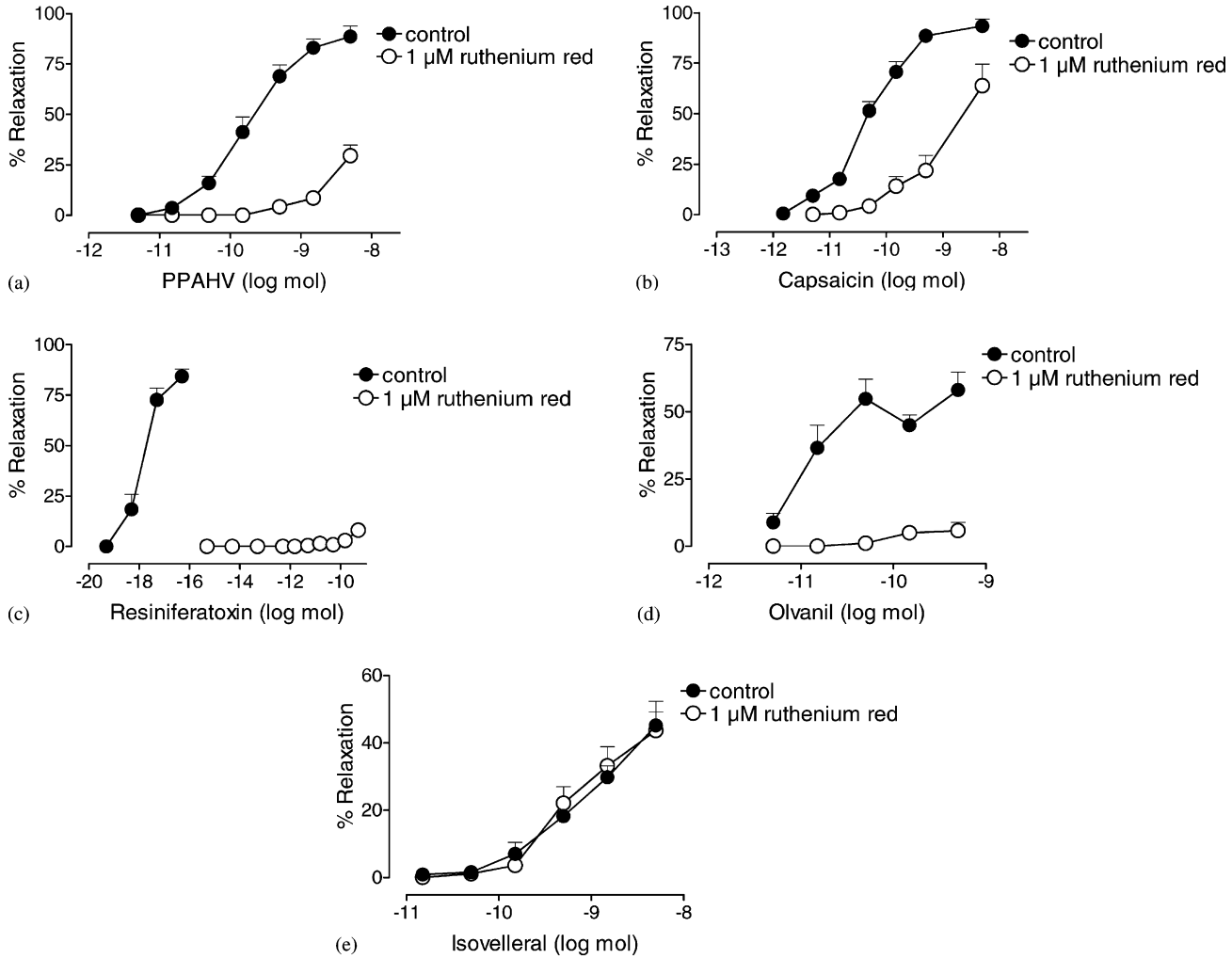

Fig. 6. Effect of ruthenium red (1 μM) on responses to vanilloids in the rat isolated mesenteric arterial bed. Ruthenium red significantly attenuated vasorelaxation elicited by: (a) PPAHV (N = 5); (b) capsaicin (N = 7); (c) resiniferatoxin (N = 8); and (d) olvanil (N = 8) ($P < 0.001$), but had no effect on the isovelleral-induced relaxation (e, N = 5).

Ruthenium red (1 μM) virtually abolished responses to resiniferatoxin and olvanil (Fig. 6c and d) but had no significant effect on responses to isovelleral (Fig. 6e).

4.5. *Effect of capsazepine on responses to vanilloids in the mesenteric arterial bed*

Capsazepine (3 μM) significantly attenuated responses to the lowest doses of the vanilloids, but did not consistently have an effect on the pD_{50} (data not shown). The pD_{30} values for PPAHV in the absence and presence of capsazepine were 10.0 ± 0.1 (N = 5) and 9.7 ± 0.1 (N = 5; $P < 0.05$), respectively. The pD_{30} values for isovelleral in the absence and presence of capsazepine were 8.8 ± 0.1 (N = 6) and 8.4 ± 0.01 (N = 3; $P < 0.05$), respectively. The pD_{30} values for capsaicin in the absence and presence of capsazepine were 10.6 ± 0.1 (N = 7) and 10.2 ± 0.1 (N = 6; $P < 0.01$), respectively. Responses to olvanil were also attenuated by capsazepine (N = 8). There was no significant effect of capsazepine on vasorelaxation to resiniferatoxin, likely because of the great potency of the agonist. It is noteworthy that higher concentrations of methoxamine had to be used to raise and maintain tone in preparations with capsazepine, indicating that capsazepine was likely causing vasorelaxation.

5. Discussion

This study has demonstrated a rank order of agonist potency of resiniferatoxin > capsaicin = olvanil > PPAHV at the recombinant rat vanilloid VR1 receptor and endogenous vanilloid receptors on dorsal root ganglion cells and sensory nerves in the rat isolated mesenteric arterial bed. However, whilst resiniferatoxin was only ~5-fold more potent than capsaicin at the rat vanilloid VR1 receptor and in dorsal root ganglion cells, it was far more (>1000-fold) potent than capsaicin in the rat isolated mesenteric arterial bed. Isovelleral and scutigeral were devoid of agonist activity at vanilloid receptors in all systems, although isovelleral evoked vasorelaxation independently of vanilloid receptors in the rat mesenteric arterial bed.

The potency order of the vanilloids was the same in all three bioassays, consistent with the established pharmacology of the vanilloid VR1 receptor [14]. However, there was a marked difference in the relative potencies of resiniferatoxin in the mesenteric arterial bed compared to the other two models. In the mesenteric arterial bed, actions of resiniferatoxin (as well as those to capsaicin, olvanil and PPAHV) were clearly mediated *via* sensory nerves because capsaicin pretreatment to cause desensitization and/or depletion of sensory neurotransmitters abolished vasorelaxation. Moreover, responses to resiniferatoxin in the mesenteric arterial bed were blocked by ruthenium red, consistent with resiniferatoxin acting at vanilloid receptors. Different binding sites on the vanilloid receptor for resiniferatoxin and capsaicin have been proposed [19], although there is some evidence which suggests that the resiniferatoxin-binding site is relatively weakly coupled to the activation of the cation channel [1]. Moreover, a recent study has identified both these binding sites on recombinant rVR1 [20]. Consistent with this and the present findings, it has previously been reported that at the recombinant rat vanilloid VR1 receptor [2,14], resiniferatoxin was only ~20-fold more potent than capsaicin, contrary to the several thousand fold higher affinity resiniferatoxin displayed in binding studies in native tissues [19,21]. Interestingly, the resiniferatoxin-induced relaxations in the mesenteric arterial bed were typically all or nothing responses. This may indicate pseudoirreversible binding, as has been suggested for resiniferatoxin previously [1], which could account for the relatively high potency of resiniferatoxin compared to capsaicin in this model. Indeed this model could highlight such differences in dissociation properties as the agonists are applied as bolus doses into a non-recirculating perfusate and thus only have a limited duration of exposure to the vanilloid receptors. In contrast, such differences in dissociation rate would not be apparent in the static systems used to study the recombinant rat vanilloid VR1 receptor and dorsal root ganglion neurones.

Resiniferatoxin showed different Hill coefficients in HEK cells and dorsal root ganglion neurones, with the values indicating co-operative binding in the HEK cells, but not in dorsal root ganglion neurones. This could reflect different expression levels or post-translational modifications of the vanilloid VR1 receptor.

In contrast to previous reports [22,23], neither isovelleral, a fungal terpenoid, nor scutigeral, a fungal triprenyl phenol, were active at vanilloid receptors in the present study. Isovelleral has been reported to bind to native [22] and recombinant [20] vanilloid receptors and to promote calcium uptake in native tissues [22]. In addition, scutigeral has been reported to mediate calcium uptake in rat dorsal root ganglion neurones in a capsazepine- and ruthenium red-sensitive manner [23]. In the mesenteric arterial bed, ruthenium red did not affect responses to isovelleral, indicating a non-vanilloid receptor mechanism was involved. Whilst capsaicin pretreatment caused a small attenuation of responses to isovelleral, this is likely to be due to functional antagonism of responses to this weak vasodilator, as tone of the mesenteric arterial beds is generally more robust after the capsaicin pretreatment protocol. This vanilloid-independent action of isovelleral in the mesenteric arterial bed is in itself of interest. Moreover, isovelleral acted as a competitive antagonist of capsaicin responses in both rat vanilloid VR1-HEK293 cells, as reported previously [14], and dorsal root ganglion neurones. Unfortunately, it was not possible to test isovelleral as a possible antagonist of capsaicin responses in the mesenteric arterial bed as at an effective concentration isovelleral evoked vasorelaxation.

Interestingly, responses to resiniferatoxin in the bioassays and to olvanil in the mesenteric arterial bed were significantly different to those mediated by the other vanilloids. In the mesenteric arterial bed relaxations evoked by resiniferatoxin and olvanil were slow in onset and time to maximal amplitude (~300 s), whereas the responses elicited by capsaicin, PPAHV and isovelleral were relatively rapid in onset and the peak of the response was reached in less than half the time (~120 s). These responses, with the exception of those induced by isovelleral which were not vanilloid VR1 receptor mediated (see above), may be due to differential activation of rapid and slow currents by the vanilloids. A considerably slower time course of action of resiniferatoxin compared to capsaicin at vanilloid receptors in rat vas deferens [24], and slower activation kinetics of olvanil compared to capsaicin at vanilloid receptors in rat trigeminal ganglion neurones [25] have been reported. It has been suggested that the lack of pungency of olvanil may be explained by activation kinetics that are slower than the rate at which it inhibits action potentials of polymodal nociceptors [26]. We observed no difference in kinetics of the response to olvanil vs. the other vanilloids at vanilloid VR1 receptors expressed in HEK293 cells or at dorsal root ganglion cells, but the response to resiniferatoxin was considerably slower. Different kinetics of responses, to capsaicin, PPAHV and olvanil, in rat trigeminal ganglion neurones has been taken as a possible indication of the existence of pharmacologically distinct subtypes of vanilloid receptor [25,26]. Alternatively, the differences between the cellular and complex systems may reflect the physio-chemical properties of the compounds leading to differential rates of penetration to the intracellular binding site [27].

Mesenteric arterial relaxation responses to capsaicin, PPAHV and isovelleral were additionally observed to be relatively short-lived (they terminated within about 15 min), whilst relaxation mediated by resiniferatoxin and olvanil lasted for up to 1 hr. Moreover, pronounced desensitisation of the responses to olvanil was observed. Consistent with this, in rat trigeminal ganglion neurones the response to repeated application of olvanil undergoes rapid tachyphylaxis, whilst tachyphylaxis to capsaicin develops only gradually [28]. Slow kinetics and desensitization of the response to olvanil could account for its

apparent partial agonism and explain why olvanil produces only a small release of calcitonin gene-related peptide-like immunoreactivity compared to capsaicin in the rat dorsal spinal cord [29,30].

Capsazepine blocked vanilloid-induced responses effectively in rat vanilloid VR1-HEK293 cells and dorsal root ganglion neurones, but was a relatively weak antagonist of vanilloid-induced responses in the rat mesenteric arterial bed. Indeed, capsazepine appeared to be relatively more potent as an inhibitor of responses to olvanil compared to the other vanilloids in the latter preparation. However, these results with capsazepine have to be viewed with caution, as capsazepine caused a decrease in tone of the mesenteric arterial bed in its own right, and partially inhibited the non-vanilloid receptor-mediated responses to isovelleral. Indeed capsazepine has been reported to have non-vanilloid receptor-mediated actions in other systems, e.g. the blockade of mitochondrial function [31].

Taken collectively these data offer little evidence to support the existence of multiple vanilloid receptor subtypes in either dorsal root ganglion neurones or the rat mesenteric arterial bed. It is clear, however, that the pharmacological properties of native and heterologously expressed vanilloid receptors whilst similar, are not identical, and the differences may lie in post-translational properties. Mechanisms that may contribute to the observed differences include modulation of vanilloid VR1 receptor activity *via* protein phosphorylation catalysed by both protein kinase A [32] and protein kinase C [33], as well as receptor polymerization [34].

6. Conclusion

In conclusion, the present study has provided a systematic comparison of all major classes of vanilloid ligands across three models of increasing complexity; it has demonstrated a potency order of resiniferatoxin > capsaicin = olvanil > PPAHV at the rat vanilloid VR1 receptor, and at endogenous vanilloid receptors on dorsal root ganglion cells and sensory nerves in the rat isolated mesenteric arterial bed. Interestingly, resiniferatoxin is only 5-fold more potent than capsaicin at the rat vanilloid VR1 receptor and dorsal root ganglion cells, but is far (>1000-fold) more potent than capsaicin in the rat mesenteric arterial bed. In addition, we have shown that isovelleral and scutigeral are inactive at vanilloid receptors in a range of bioassays, but isovelleral mediates vanilloid receptor-independent vasorelaxation in the rat mesenteric arterial bed.

Acknowledgments

V.R. is a Royal Society University Research Fellow. Thanks to V. Mitchel and J. Ranson for provision of recombinant cells.

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