British Journal of Pharmacology (1999) 128, 428–434
http://www.stockton-press.co.uk/bjp

Resiniferatoxin-type phorboid vanilloids display capsaicin-like selectivity at native vanilloid receptors on rat DRG neurons and at the cloned vanilloid receptor VR1

*,[1]A. Szallasi, [1]T. Szabó, [1]T. Bíró, [1]S. Modarres, [1]P.M. Blumberg, [2]J.E. Krause, [2]D.N. Cortright & [3]G. Appendino

[1]National Cancer Institute, Bethesda, Maryland, U.S.A.; [2]Neurogen Corporation, Branford, Connecticut, U.S.A. and [3]Dipartimento di Scienza e Tecnologia del Farmaco, Universitá di Torino, Torino, Italy

1 Although the cloned rat vanilloid receptor VR1 appears to account for both receptor binding and calcium uptake, the identification of vanilloids selective for one or the other response is of importance because these ligands may induce distinct patterns of biological activities.

2 Phorbol 12,13-didecanoate 20-homovanillate (PDDHV) evoked $^{45}Ca^{2+}$-uptake by rat dorsal root ganglion neurons (expressing native vanilloid receptors) in culture with an EC_{50} of 70 nM but inhibited [^{3}H]-resiniferatoxin (RTX) binding to rat dorsal root ganglion membranes with a much lower potency ($K_i > 10{,}000$ nM). This difference in potencies represents a more than 100 fold selectivity for capsaicin-type pharmacology.

3 $^{45}Ca^{2+}$ influx by PDDHV was fully inhibited by the competitive vanilloid receptor antagonist capsazepine, consistent with the calcium uptake occurring *via* vanilloid receptors.

4 PDDHV induced calcium mobilization in CHO cells transfected with the cloned rat vanilloid receptor VR1 with an EC_{50} of 125 nM and inhibited [^{3}H]-RTX binding to these cells with an estimated K_i of 10,000 nM. By contrast, PDDHV failed to evoke a measurable calcium response in non-transfected CHO cells, confirming its action through VR1.

5 We conclude that PDDHV is two orders of magnitude more potent for inducing calcium uptake than for inhibiting RTX binding at vanilloid receptors, making this novel vanilloid a ligand selective for capsaicin-type pharmacology. These results emphasize the importance of monitoring multiple endpoints for evaluation of vanilloid receptor structure-activity relations. Furthermore, PDDHV now provides a tool to explore the biological correlates of capsaicin-type vanilloid pharmacology.

Keywords: Phorbol 12,13-didecanoate 20-homovanillate; phorboid vanilloids; capsaicin-type vanilloid pharmacology; [^{3}H]-resiniferatoxin binding; capsaicin; native vanilloid receptors; vanilloid receptor subtype 1 (VR1); VR1-transfected CHO cells

Abbreviations: CHO cells, Chinese Hamster Ovary cells; CHO/VR1 cells, VR1-transfected Chinese Hamster Ovary cells; DMEM, Dulbecco's Modified Eagle's Medium; DRG, dorsal root ganglion; PDDHV, phorbol 12,13-didecanoate 20-homovanillate; RTX, resiniferatoxin; PDNHV, phorbol 12,13-dinonanoate 20-homovanillate; VR1, vanilloid receptor subtype 1

Introduction

In the late eighties, the analysis of capsaicin-induced ion currents in sensitive neurons led to the conclusion that the capsaicin receptor was a non-specific cation channel with a limited selectivity for calcium (Bevan *et al*., 1987; Marsh *et al*., 1987; Wood *et al*., 1988; Bevan & Szolcsányi, 1990). This conclusion was recently confirmed by the cloning of the first capsaicin receptor, termed VR1, from a rat cDNA library (Caterina *et al*., 1997). This VR1 is distantly related to the transient-release potential family of store-operated calcium channels and is similar to NMDA receptors in selectivity for divalent cations such as calcium (Caterina *et al*., 1997). A $^{45}Ca^{2+}$-uptake assay employing rat dorsal root ganglion (DRG) neurons was introduced in 1988 to measure the potency of capsaicin congeners (Wood *et al*., 1988) and has been used extensively since then to evaluate structure-activity relations at capsaicin receptors (*cf*. Walpole & Wrigglesworth, 1993; Szallasi & Blumberg, 1999).

*Author for correspondence at: Department of Pathology, Saint Louis University School of Medicine, 1402 South Grand Blvd., St. Louis, MO 63104, U.S.A. E-mail: arpads@thalamus.wustl.edu

In 1989, resiniferatoxin (RTX), a phorbol-related diterpene, was identified as an ultrapotent capsaicin analog (Szallasi & Blumberg, 1989). Since RTX and capsaicin are dissimilar in chemical structure but share a vanillyl substituent as a feature essential for bioactivity (Figure 1), the common recognition site for these two classes of irritant compounds was re-named as the vanilloid receptor (Szallasi & Blumberg, 1990a). Specific binding of [^{3}H]-RTX provided a new biochemical tool to study the pharmacology of vanilloid receptors (Szallasi & Blumberg, 1990b). Further analysis of vanilloids revealed that these compounds showed distinct structure-activity relations for receptor binding and $^{45}Ca^{2+}$-uptake (Ács *et al*., 1996). Capsaicin was approximately 20 fold more potent for inducing $^{45}Ca^{2+}$-influx than for binding (capsaicin-type pharmacology) whereas RTX showed the opposite behaviour (RTX-type structure-activity relations) (*cf*. Szallasi & Blumberg, 1996; Bíró *et al*., 1997). Other vanilloids ranged in selectivity between capsaicin-like and RTX-like behaviour in these two vanilloid receptor assays, some being non-selective such as RTX-amide (Ács *et al*., 1996) and olvanil (Szallasi & Blumberg, 1999). Although binding and calcium uptake were initially believed to be detecting independent vanilloid receptor subtypes (Szallasi

& Blumberg, 1996; Bíró *et al*., 1997), now it is clear that the cloned rat vanilloid receptor VR1 can account for both the binding and functional activity of vanilloid ligands which show RTX- or capsaicin-type structure-activity relations, respectively, in rat DRG neurons expressing native vanilloid receptors (Szallasi *et al*., 1999).

Since RTX and capsaicin share a vanillyl moiety (Figure 1), the diterpenoid core of RTX, resiniferonol 9,13,14-orthophenylacetate, seems to be responsible for the markedly enhanced potency and altered selectivity of RTX. Resiniferonol is similar to phorbol (Figure 1). As early as 1989, we showed that vanilloids of considerable activity could be synthesized by using phorbol as a diterpene core (Szallasi *et al*., 1989). Compared to RTX or resiniferonol 9,13,14-orthophenylacetate, phorbol is more readily available and its chemistry is better studied, therefore we relied on phorbol to prepare RTX mimetics as one strategy to explore the role of the diterpene core and its substitutions in determining RTX- and capsaicin-type structure activity relations. In 1996, we reported that phorbol 12-phenylacetate 13-acetate 20-homovanillate displayed poor discrimination between binding ($K_i = 600$ nM) and calcium uptake ($EC_{50} = 1800$ nM) (Szallasi *et al*., 1996). Subsequently, we have analysed a number of phorboid vanilloids in the vanilloid receptor binding and calcium uptake assays and identified several candidate molecules for being capsaicin-type selective. In the present study, we have characterized a number of these ligands in various assays using native and cloned vanilloid receptors. We report here that phorbol 12,13-didecanoate 20-homovanillate (PDDHV) as well as its closely related homologue, 12,13-dinonanoate 20-homovanillate (PDNHV), show at least 100 fold selectivity in assays for calcium influx relative to receptor binding.

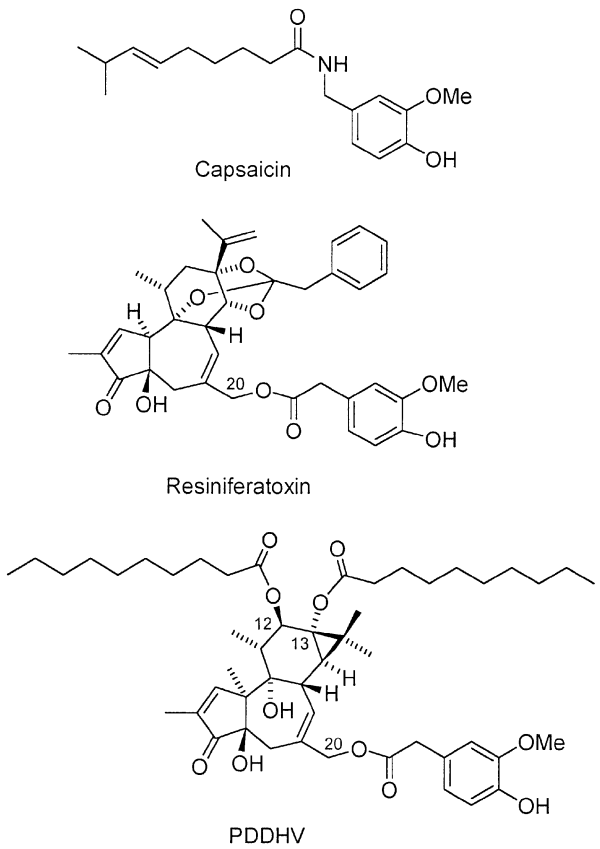

Figure 1 Vanilloid structures. Note the similarities and differences among capsaicin (upper panel), resiniferatoxin (resiniferonol 9,13,14-orthophenylacetate 20-homovanillate; middle panel), and phorbol 12,13-didecanoate 20-homovanillate (PDDHV; lower panel).

Methods

Cells or membranes expressing native vanilloid receptors or the cloned rat vanilloid receptor VR1

Dorsal root ganglia (DRGs) from all levels of the spinal column of Sprague-Dawley rats (females, weighing 200–250 g) euthanized with CO_2 were collected aseptically. To obtain a partially purified membrane preparation for vanilloid binding experiments (Szallasi *et al*., 1992, 1993), DRGs were disrupted with the aid of a Polytron tissue homogenizer in an ice-cold buffer (pH 7.4) containing (in mM) KCl 5, NaCl 5.8, $CaCl_2$ 0.75, $MgCl_2$ 2, sucrose 320, and HEPES 10. Tissue homogenates were centrifuged for 10 min at $1000 \times g$ (4°C), and the resulting supernatant was further centrifuged for 30 min at $35{,}000 \times g$ (4°C). The pellet from the second centrifugation was resuspended in the above buffer and stored at −80°C until assayed.

In order to obtain isolated neurons for $^{45}Ca^{2+}$-uptake experiments, DRGs were digested twice with 0.125% collagenase (Sigma, St. Louis, MO, U.S.A.) in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 4.5 mg ml^{-1} glucose, 0.5% heat-inactivated foetal bovine serum (Gibco BRL, Gaithersburg, MD, U.S.A.), 1 mM Na-pyruvate, 25 mM HEPES, and antibiotics as described previously (Wood *et al*., 1988; Ács *et al*., 1996). Briefly, DRGs were triturated through a flame-polished Pasteur pipette following collagenase digestion for 90 min at 37°C and then the cells were pelleted through a cushion of DMEM containing 15% fatty acid-free bovine serum albumin to separate the myelin debris. In other experiments, isolated DRG neurons were enriched for small- to medium-size neurons by layering the cell suspension on the top of 5 ml of 40% Ficoll (Sigma, St. Louis, MO, U.S.A.) and centrifuging for 15 min at $100 \times g$ (Gilabert & McNaughton, 1997). Small- to medium-size cells are retained at the Ficoll interface and can be recovered by collecting this band and pelleting the cells ($200 \times g$; 5 min; 4°C). Finally, DRG neurons were plated in MultiScreen-DV 96-well filtration plates (Millipore, Marlborough, MA, U.S.A.) at an approximate density of 5000 cells per well.

A cDNA encoding the vanilloid receptor VR1 was cloned from rat DRG total RNA by reverse-transcription-polymerase chain reaction using primers based on the published nucleotide sequence (Caterina *et al*., 1997). A 2.7 kb cDNA was isolated and the nucleotide sequence was verified to be identical to the published sequence. This cDNA was subcloned into pUHG102-3 (Clontech, Palo Alto, CA, U.S.A.) for expression in Chinese hamster ovary (CHO) cells carrying the pTet Off Regulator plasmid (Clontech, Palo Alto, CA, U.S.A.). In these cells, expression of the pUHG plasmid is repressed in the presence of tetracycline but is derepressed upon removal of the antibiotic. Stable clones were isolated in culture medium containing puromycin (10 μg ml^{-1}) and maintained in medium supplemented with tetracycline (1 μg ml^{-1}). Cells utilized for assay were grown in culture medium in the absence of tetracycline for 48–72 h prior to use. For radioligand binding experiments, cells were seeded in T175 cell culture flasks in medium without antibiotics and grown to approximately 90% confluency. The flasks were then washed with PBS and harvested in PBS containing 5 mM EDTA. The cells were pelleted by gentle centrifugation and stored at −80°C until assayed. For calcium-activated fluorescence measurement

assays, cells were seeded into 96-well plates and grown to 70–90% confluency.

Inhibition of [^{3}H]-resiniferatoxin binding to native vanilloid receptors in rat dorsal root ganglion membranes and to the cloned vanilloid receptor VR1 expressed in CHO cells

Binding assay mixtures were set up on ice and contained either 40 μg membrane protein or 5×10^4 VR1-transfected CHO cells, 0.25 mg ml^{-1} bovine serum albumin (Cohn fraction V, Sigma, St. Louis, MO, U.S.A.), [^{3}H]-resiniferatoxin (RTX; 37 Ci mmol^{-1}; Chemical Synthesis and Analysis Laboratory, NCI-FCRDC, Frederick, MD, U.S.A.), and non-radioactive ligands; the final volume was adjusted to 500 μl (membranes) or 1000 μl (CHO/VR1 cells) with the buffer described above. Non-specific binding was defined as that occurring in the presence of 1 μM non-radioactive RTX (Alexis Corp., San Diego, CA, U.S.A.). Binding was analysed in the presence of a fixed concentration of [^{3}H]-RTX (25 pM for DRG membranes and 60 pM for VR1-transfected cells) and various concentrations of competing ligands.

The binding reaction was initiated by transferring the assay tubes into a 37°C waterbath and was then terminated following a 60 min incubation period by cooling the assay mixtures on ice. Non-specific binding was reduced by adding 100 μg of bovine α_1-acid glycoprotein (Sigma, St. Louis, MO, U.S.A.) to each tube (Szallasi *et al*., 1992). α_1-Acid glycoprotein sequesters free [^{3}H]-RTX from the aqueous phase. Since at 0°C the dissociation of receptor-bound RTX is unmeasurable, whereas free and non-specifically bound RTX remain in equilibrium, α_1-acid glycoprotein is able to remove most of the non-specifically bound RTX from the membrane lipid phase by binding free RTX without compromising the specific binding (Szallasi *et al*., 1992, 1993). Membrane-bound RTX was separated from the free as well as the α_1-acid glycoprotein-bound RTX by pelleting the membranes in a Beckman 12 benchtop centrifuge (15 min; maximal velocity), and the radioactivity was determined by scintillation counting.

Competition experiments were analysed by the curvilinear regression program LIGAND (Biosoft, Ferguson, MO, U.S.A.). Equilibrium binding parameters in saturation binding experiments were determined by fitting the Hill equation to the measured values with the aid of the computer program collection FitP (Biosoft, Ferguson, MO, U.S.A.) as described before (Szallasi *et al*., 1993).

$^{45}Ca^{2+}$-uptake by adult rat dorsal root ganglion neurons in culture

DRG neurons were incubated for 30 min at 37°C with 1 μCi ml^{-1} $^{45}Ca^{2+}$ (23.55 mCi mg^{-1}; DuPont-New England Nuclear, Boston, MA, U.S.A.) in the presence of 1.8 mM $CaCl_2$, 0.25 mg ml^{-1} bovine serum albumin, and various concentrations of PDDHV (1–10,000 nM). Maximal calcium uptake by PDDHV was compared to that evoked by capsaicin (30–10,000 nM) and was also determined in the presence of the competitive vanilloid receptor antagonist capsazepine (RBI, Natick, MA, U.S.A.). Cells were washed six times with ice-cold serum-free DMEM with the aid of a MultiScreen Vacuum Manifold (Millipore, Marlborough, MA, U.S.A.). Filters were dried under a heat lamp and the radioactivity determined by scintillation counting. For each PDDHV concentration, at least 5 wells were analysed and the resulting dose-response curve was fitted to the Hill equation using the computer program MicroCal Origin 3.5 (MicroCal Software, Northampton, MA, U.S.A.). For statistical analysis of the curve fitting to the measured values, the χ^2 goodness of fit test was used.

Calcium-activated fluorescence assays using VR1-transfected CHO cells

Cells grown to 70–80% confluence were washed once with Krebs-Ringer's HEPES buffer (mM): HEPES 25, KCl 5, NaH_2PO_4 0.96, $MgSO_4$ 1, $CaCl_2$ 2, glucose 5, probenecid 1 (pH 7.4). After washing, cells were loaded with Fluo3-AM (2.5 μg ml^{-1}; Teflabs, Austin, TX, U.S.A.) at 37°C for 1–2 h. The wells were then washed twice with Krebs-Ringer's HEPES buffer. Vanilloid stock solutions made up in ethanol were diluted in Krebs-Ringer's HEPES buffer. As indicated in Results, in some experiments phorboid vanilloid stock solutions were diluted in Krebs-Ringer's HEPES buffer containing 0.1% bovine serum albumin to facilitate their solubility. Agonist-activated calcium mobilization was monitored with a FLIPR (Molecular Devices, Sunnyvale, CA, U.S.A.) instrument. Fluorescence data were collected for at least 12 min and the maximum fluorescence signal was determined. For capsaicin-induced calcium response, data obtained between 30 and 60 s after agonist application were used to generate an EC_{50} value. For all other VR1 agonists, concentration-response data were based on a 6 min timepoint. Kaleidagraph software (Synergy Software, Reading, PA, U.S.A.) was utilized to fit the data to the equation:

$$y = a(1/(1 + (b/c)^d))$$

where y = maximum fluorescence signal; $a = E_{max}$; $b = EC_{50}$; c = agonist concentration; and d = Hill coefficient.

Drugs and chemicals

Phorboid vanilloids (PDDHV; the constrained analogue of PDDHV in which the two decanoate groups are fused together; PDNHV; and the 4-O-methyl derivative of PDNHV) were synthesized using a modification of the procedure published previously (Appendino *et al*., 1996). Details will be described elsewhere. Stock solutions were made up in ethanol, aliquoted, and stored at −80°C until assayed. Chemicals were purchased from Sigma, St. Louis, MO, U.S.A., unless indicated otherwise, and were of the highest quality available.

Results

Inhibition by phorbol 12,13-didecanoate 20-homovanillate and related compounds of [^{3}H]-resiniferatoxin binding to rat dorsal root ganglion membranes and to VR1-transfected CHO cells

In competition experiments, the affinity of PDDHV for specific RTX binding sites on rat DRG membranes could only be estimated due to limited solubility: at PDDHV concentrations 30 μM or higher the assay mixture turned visibly cloudy, indicative of precipitation of the ligand. At 30 μM, PDDHV inhibited [^{3}H]-RTX binding to DRG membranes by 44 ± 6% (mean ± s.e.mean; three experiments; Figure 2), suggesting a K_i value of about 20 μM. In parallel experiments, capsaicin competed for specific RTX binding sites with a K_i value of 3.2 ± 0.6 μM (mean ± s.e.mean; three experiments; Figure 2). Using VR1-transfected CHO cells, PDDHV concentrations higher than 10 μM were not evaluated because the assay

mixture not only turned cloudy but the non-specific [^{3}H]-RTX binding also became elevated. Ten μM PDDHV inhibited specific [^{3}H]-RTX binding by 24±4% (mean±s.e.mean; three determinations; Figure 2) whilst capsaicin displayed a K_i of 1.3±0.4 μM (mean±range; two determinations; Figure 2). At a concentration of 10 μM, a PDDHV analogue, in which the two decanoate substituents are connected at the last carbon, and PDNHV inhibited [^{3}H]-RTX binding to CHO/VR1 cells by 38±6 and 26±5%, respectively (mean±range; two determinations; not shown). The 4-O-CH_3 derivative of PDNHV failed to inhibit [^{3}H]-RTX binding to CHO/VR1 cells up to the concentration of 10 μM (two determinations; not shown).

Stimulation of $^{45}Ca^{2+}$-uptake by phorbol 12,13-didecanoate 20-homovanillate and related phorboid vanilloids in intact rat dorsal root ganglion neurons and of calcium-activated fluorescence in CHO/VR1 cells

PDDHV induced $^{45}Ca^{2+}$-uptake by rat DRG neurons with an EC_{50} value of 70±31 nM (mean±s.e.mean; seven determinations; Figure 3), which was fully prevented by the competitive vanilloid receptor antagonist capsazepine (10 μM; five determinations; not shown). The capsaicin-evoked $^{45}Ca^{2+}$-influx response attained its half-maximal value at 300±40 nM (mean±s.e.mean; three determinations; Figure 3) and was similar in its peak value to the PDDHV response.

In the absence of bovine serum albumin, PDDHV acted as a weak agonist for VR1 expressed in CHO cells: PDDHV had to be evaluated in the micromolar concentration range in order to detect a calcium-activated fluorescence response. The inclusion of 1 mg ml^{-1} bovine serum albumin in the assay buffer resulted in a 100 fold improvement in the apparent potency of PDDHV: under these conditions the EC_{50} value was 125±9 nM (mean±s.e.mean; six determinations; Table 1).

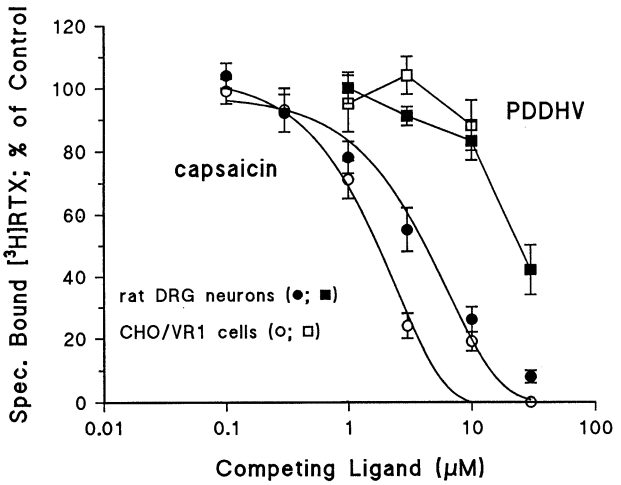

Figure 2 Inhibition of [^{3}H]-resiniferatoxin (RTX) binding by capsaicin and phorbol 12,13-didecanoate 20-homovanillate (PDDHV) to rat dorsal root ganglion (DRG) membranes expressing native vanilloid receptors and to Chinese Hamster Ovary (CHO) cells transfected with the cloned rat vanilloid receptor VR1 (CHO/VR1 cells). Observe that PDDHV is at least 20 fold less potent than capsaicin in both binding assays. At 30 μM or higher, PDDHV solutions turned cloudy, indicative of precipitation of the compound in the aqueous solution. In CHO/VR1 cells, 30 μM PDDHV markedly enhanced non-specific [^{3}H]-RTX binding, therefore this value is not plotted in the Figure. Points represent mean values of triplicate determinations in a single experiment; error bars indicate s.e.mean of triplicates. Additional experiments gave similar results; see Results for details.

With an EC_{50} value of 187±18 nM (mean±s.e.mean; twelve determinations; not shown), the PDDHV analogue with the fused decanoate moieties was slightly less potent than PDDHV. PDNHV was modestly more potent than PDDHV in the calcium mobilization assay (EC_{50}=87±6 nM; mean± s.e.mean; eleven determinations; not shown). By contrast, the substitution of the free −OH group at C4 of PDNHV by a methyl group resulted in an approximately 100 fold decrease in potency (EC_{50}>10,000 nM; three determinations; not shown), although this molecule's potency is only an estimate due to its low solubility in the assay buffer.

The time-course of the PDDHV-induced calcium mobilization response was slow in onset, in which its kinetics appeared to be intermediate between that of capsaicin and RTX (Figure 4). As with RTX, the onset of the PDDHV-evoked response was concentration-dependent: at higher doses, the onset was more rapid (not shown). The PDDHV-evoked response did

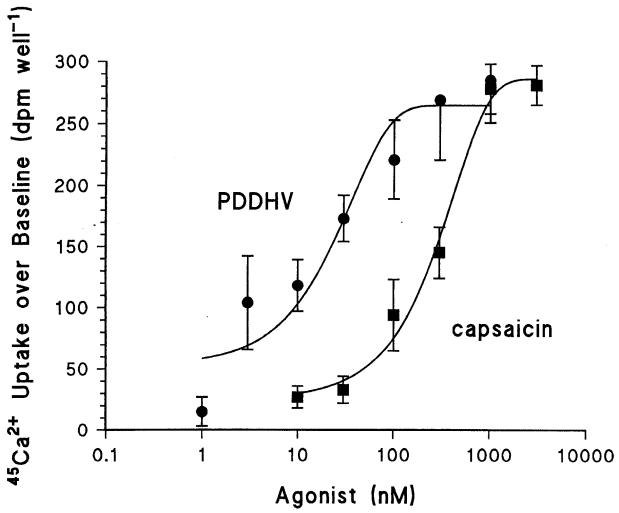

Figure 3 Stimulation of $^{45}Ca^{2+}$-uptake by capsaicin and phorbol 12,13-didecanoate 20-homovanillate (PDDHV) using isolated rat dorsal root ganglion neurons expressing native vanilloid receptors. Observe that PDDHV acts as a full capsaicin agonist. Also note that PDDHV is approximately 10 fold more potent than capsaicin to evoke calcium uptake. Points represent mean values of a single experiment done with quintuplicate wells; error bars indicate s.e.mean. As shown, EC_{50} values are 27 and 230 nM for PDDHV and capsaicin, respectively. Two (for capsaicin) to six (for PDDHV) additional experiments yielded similar results; see Results for mean±s.e.mean values.

Table 1 Selectivity of capsaicin, resiniferatoxin, and phorbol 12,13-didecanoate 20-homovanillate (PDDHV) for calcium uptake over receptor binding, denoted as capsaicin-type selectivity

	Resiniferatoxin	*Capsaicin*	*PDDHV**
DRG neurons			
Binding affinity	0.07 nM***	3200 nM	>10 000 nM
$^{45}Ca^{2+}$-uptake (EC_{50})	1.0 nM***	300 nM	70 nM
Relative potency**	0.07	11	>150
CHO/VR1 cells			
Binding affinity	0.13 nM****	1300 nM	>10 000 nM
Calcium mobilization (EC_{50})	0.3 nM****	30 nM****	125 nM
Relative potency**	0.4	43	>80

*PDDHV, phorbol 12,13-didecanoate 20-homovanillate; **relative potency=ratio of binding affinity (K_D for resiniferatoxin and K_i for capsaicin and PDDHV) and EC_{50} value in the calcium response assay; ***from Ács *et al.*, 1996; ****from Szallasi *et al.*, 1999.

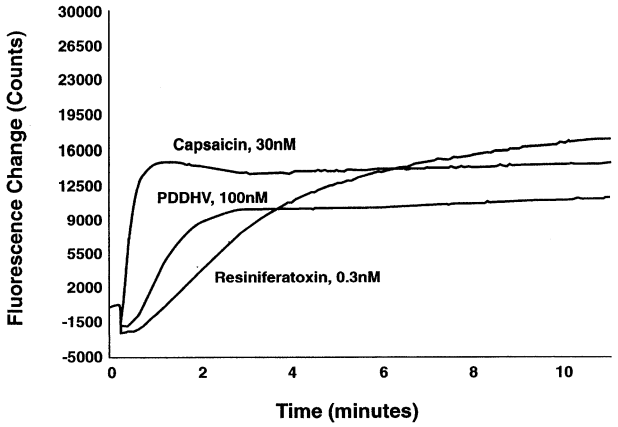

Figure 4 Time-dependence of capsaicin-, resiniferatoxin-, and phorbol 12,13-didecanoate 20-homovanillate-induced calcium mobilization in VR1-transfected CHO cells. Calcium mobilization was determined as calcium-activated fluorescence with the aid of Fluo3-AM dye. The concentrations used (30 nM for capsaicin, 0.3 nM for resiniferatoxin, and 100 nM for phorbol 12,13-didecanoate 20-homovanillate, respectively) correspond to the approximate EC_{50} values for these compounds as determined previously in concentration-response experiments assessed at 3 min after compound addition. Observe that the peak response by capsaicin develops rapidly in about 1 min. The resiniferatoxin-evoked response is slower in onset, and the response did not reach its maximum level yet at 12 min after agonist application. The onset of the calcium response to phorbol 12,13-didecanoate 20-homovanillate is intermediate to that of capsaicin and resiniferatoxin: it takes approximately 3–4 min for the peak value to occur. The curves shown in the figure are representative original recordings selected from nine independent experiments.

reach its maximum value between 3–5 min after agonist application (Figure 4).

The effect of PDDHV on calcium mobilization was specific for VR1 as 1 μM PDDHV had no effect on calcium-activated fluorescence in untransfected CHO cells (two independent experiments; not shown).

Discussion

Vanilloids show different potencies as measured by binding to native vanilloid receptors expressed on rat DRG neurons and as measured by induction of calcium uptake *via* these receptors (Ács *et al*., 1996; Szallasi & Blumberg, 1999). Generally speaking, capsaicin congeners are more potent for inducing calcium influx (capsaicin-type structure-activity relations) whereas RTX analogues display higher potency in the binding assay (RTX-type pharmacology) (Szallasi & Blumberg, 1996, 1999). Previously, the measurement of binding and calcium uptake were believed to reflect separate vanilloid receptor subtypes (Szallasi & Blumberg, 1996; Bíró *et al*., 1997), but now it is clear that capsaicin- and RTX-type pharmacologies reflect different measures of the same target, the cloned rat vanilloid receptor VR1 (Szallasi & Blumberg, 1999).

In 1996, we synthesized phorbol 12-phenylacetate 13-acetate 20-homovanillate (PPAHV) (Szallasi *et al*., 1996). This phorboid vanilloid, like RTX-amide (Ács *et al*., 1996), showed similar potencies for binding to DRG neurons and for inducing calcium uptake by these cells (Szallasi *et al*., 1996). Moreover, PPAHV abolished the positive cooperativity that characterizes RTX binding to vanilloid receptors (Szallasi *et al*., 1996), indicating that cooperative binding is a ligand-induced feature rather than an inherent property of vanilloid receptors. PPAHV revealed the existence of two distinct types of vanilloid-gated conductances in voltage-clamped rat trigeminal ganglion neurons, one which is sensitive to capsazepine and another one that cannot be blocked by this vanilloid receptor antagonist (Liu *et al*., 1998). PPAHV also showed novel pharmacology at the whole animal level: for example, PPAHV given s.c. failed to induce a measurable hypothermic response at doses at which this treatment desensitized against neurogenic inflammation (Appendino *et al*., 1996). These results suggest that the peculiar behaviour of phorboid vanilloids in vanilloid receptor assays might be associated with a distinct spectrum of biological activity. Driven by these observations, we have decided to explore further structure-activity relations for phorboid vanilloids.

This paper contains data accumulated over a period of several years. Until recently, we used a ^{45}Ca-uptake by isolated rat DRG neurons assay to determine functional vanilloid potencies at the cellular level, allowing us to compare our values directly to a previous, extensive structure-activity analysis of capsaicin congeners by Walpole, Wrigglesworth, and colleagues (*cf*. Walpole and Wrigglesworth, 1993). This assay, however, has several shortcomings: for example, it is tedious to perform and the measured potencies are difficult to reproduce due to a combination of marked intra- and interassay variability. With the recent availability of VR1-transfected mammalian cells (Caterina *et al*., 1997; Tominaga *et al*., 1998), we decided to discontinue evaluation of ^{45}Ca-uptake by DRG neurons and instead use the cloned rat VR1 receptor for our studies. Now we measure calcium-activated fluorescence in VR1-transfected CHO cells following vanilloid challenge. These cells, in which the expression of the VR1 is under tetracycline repression, are easy to maintain and give highly reproducible results when tetracycline is removed from the medium (Szallasi *et al*., 1999). Importantly, vanilloid potencies determined using native (^{45}Ca-uptake by DRG neurons) and cloned (calcium-activated fluorescence in CHO/VR1 cells) vanilloid receptors are very similar (Szallasi *et al*., 1999). Some phorboid vanilloids presented in this paper were originally tested in the DRG ^{45}Ca-uptake assay and then re-evaluated for inducing calcium-activated fluorescence in CHO/VR1 cells. The recently synthesized derivatives were, however, characterized in the latter assay only.

The main finding in this study is that phorbol 12,13-didecanoate 20-homovanillate (PDDHV), like capsaicin, is more potent for inducing calcium uptake than for binding using both native (rat DRG neurons) and cloned (VR1-transfected CHO cells) vanilloid receptors (compare potencies in Table 1). As a matter of fact, PDDHV shows an even higher (80–150 fold selectivity for cloned and native vanilloid receptors, respectively) selectivity for calcium uptake over binding than does capsaicin (10–40 fold selectivity, using DRG neurons and CHO/VR1 cells, respectively; Table 1). The conclusion that PDDHV-evoked calcium uptake was mediated by vanilloid receptors (and not, for instance, by the phorbol ester receptor protein kinase C) was confirmed by two lines of evidence: (1) PDDHV failed to induce a detectable calcium-activated fluorescence response in non-transfected CHO cells and (2) the PDDHV-evoked calcium uptake was fully inhibited by the vanilloid receptor antagonist capsazepine. This finding implies that a diterpene pharmacophore can yield both RTX- (resiniferonol derivatives) and capsaicin-type selective (phorbol derivatives) vanilloids. Since RTX-amide and PPAHV are similar in relative potencies, RTX- versus capsaicin-type selectivity is not determined solely by the diterpene core but rather the nature of the chemical bond (ester vs amide) between C20 and the vanillyl group as well as the substitutions on the 7-membered C-ring of the diterpene.

A second interesting observation of this study is that the apparent potency of PDDHV greatly depends on assay conditions: PDDHV is a weak agonist for VR1 expressed in CHO cells in the absence of bovine serum albumin but is a powerful agonist in the presence of bovine serum albumin. This observation is not unexpected. Some phorbol esters are highly lipophilic and it was noted long ago that these compounds lost activity in aqueous solutions (Delclos *et al*., 1980). It is likely that these compounds accumulate at the air-water interface, thus their actual free concentration in the aqueous phase is much lower than that calculated. By coating the air-water interphase with bovine serum albumin (or other proteins like IgG), the accumulation of ligands can be reduced and thus their free concentration in the aqueous solution may be maintained. Alternatively, these drugs may bind to bovine serum albumin, providing a new equilibrium for free and bound forms in the buffer. No matter how bovine serum albumin works, the omission/inclusion of surface-active proteins like bovine serum albumin may account for many of the conflicting reports in the literature with regard to vanilloid potencies. For instance, PDDHV itself was previously synthesized and found to be marginally active in the $^{45}Ca^{2+}$-uptake assay by Walpole and colleagues (Walpole *et al*., 1996). However, they assayed PDDHV in the absence of bovine serum albumin only and under similar conditions we, too, find this compound to be a weak agonist. Notably, the apparent K_i for capsazepine also depends on assay conditions: K_i values are 2430 and 330 nM in the absence or presence of bovine serum albumin, respectively (Szallasi *et al*., 1999).

Another intriguing observation is the time-course of the PDDHV-evoked calcium mobilization response in CHO/VR1 cells, which is intermediate between that of RTX and capsaicin. Since PDDHV, like RTX, is a bulky molecule (compare structures in Figure 1), it is not unlikely that its somewhat slow onset compared to capsaicin reflects this property of the molecule. RTX is more potent for desensitization than activation of the same biological endpoint in most assays (Szallasi & Blumberg, 1990a, 1996, 1999). This behaviour is consistent with the sustained channel opening by RTX (Winter *et al*., 1990; Liu & Simon, 1996): the slowly increasing intracellular calcium concentration may ultimately result in desensitization without causing impulse generation. Since PDDHV shows capsaicin-type pharmacology, it is not unexpected that this compound mimics capsaicin in that the calcium-activated fluorescence response achieves its peak value rapidly when compared to RTX.

Finally, the 100 fold drop in the potency of 4-O-methyl-PDNHV compared to PDNHV implies that the free hydroxyl group at C20 plays an important role in the capsaicin-type bioactivity of phorboid vanilloids. This is unexpected since the 4-O-methyl-RTX is only 4 fold less potent than RTX in the $^{45}Ca^{2+}$ uptake by rat DRG neurons assay (Walpole *et al*., 1996).

We conclude that phorboid vanilloids, which are more similar in structure to RTX than to capsaicin, can show capsaicin-type structure activity relations. Further structure-activity analysis of such compounds may help define structural requirements leading to capsaicin-type pharmacology. These studies may potentially lead to the synthesis of simplified RTX- and capsaicin-type selective vanilloids. Given the interest in vanilloids as novel analgesic-antiinflammatory drugs (Campbell *et al*., 1993; Winter *et al*., 1995; Szallasi & Blumberg, 1996, 1999), the synthesis of such selective compounds may be of great importance.

References

ÁCS, G., LEE, J., MARQUEZ, V.E. & BLUMBERG, P.M. (1996). Distinct structure-activity relations for stimulation of ^{45}Ca uptake and for high affinity binding in cultured rat dorsal root ganglion neurons and dorsal root ganglion membranes. *Mol. Brain Res.,* **35,** 173–182.

APPENDINO, G., CRAVOTTO, G., PALMISANO, G., ANNUNZIATA, R. & SZALLASI, A. (1996). Synthesis and evaluation of phorboid-20-homovanillates. Discovery of a class of ligands binding to the vanilloid receptor with different degrees of cooperativity. *J. Med. Chem.,* **39,** 3123–3131.

BEVAN, S., JAMES, I.F., RANG, H.P., WINTER, J. & WOOD, J.N. (1987). The mechanism of action of capsaicin–a sensory neurotoxin. In *Neurotoxins and their Pharmacological Applications*. ed. Jenner, P. pp. 261–277, New York: Raven Press.

BEVAN, S. & SZOLCSÁNYI, J. (1990). Sensory neuron-specific actions of capsaicin: mechanisms and applications. *Trends Pharmacol. Sci.,* **11,** 330–335.

BÍRÓ, T., ÁCS, G., ÁCS, P., MODARRES, S. & BLUMBERG, P.M. (1997). Recent advances in understanding of vanilloid receptors: a therapeutic target for treatment of pain and inflammation in skin. *J. Invest. Dermatol. Symp. Proc.,* **2,** 56–60.

CAMPBELL, E., BEVAN, S. & DRAY, A. (1993). Clinical applications of capsaicin and its analogues. In *Capsaicin in the Study of Pain*. ed. Wood, J.N. pp. 255–272. San Diego: Academic Press.

CATERINA, M.J., SCHUMACHER, M.A., TOMINAGA, M., ROSEN, T.A., LEVINE, J.D. & JULIUS, D. (1997). The capsaicin receptor: a heat-activated ion channel in the pain pathway. *Nature,* **389,** 816–824.

DELCLOS, K.B., NAGLE, D.S. & BLUMBERG, P.M. (1980). Specific binding of phorbol ester tumor promoters to mouse skin. *Cell,* **19,** 1025–1032.

GILABERT, R. & McNAUGHTON, P. (1997). Enrichment of the fraction of nociceptive neurones in cultures of primary sensory neurones. *J. Neurosci. Meth.,* **71,** 191–198.

LIU, L. & SIMON, S.A. (1996). Capsaicin-induced currents with distinct desensitization and Ca^{++} dependence in rat trigeminal ganglion cells. *J. Neurophysiol.,* **75,** 1503–1514.

LIU, L., SZALLASI, A. & SIMON, S.A. (1998). A non-pungent resiniferatoxin analogue, phorbol 12-phenylacetate 13-acetate 20-homovanillate, reveals vanilloid receptor subtypes on rat trigeminal ganglion neurons. *Neuroscience,* **84,** 569–581.

MARSH, S.J., STANSFELD, C.E., BROWN, D.A., DAVEY, R. & McCARTHY, D. (1987). The mechanism of action of capsaicin on sensory C-type neurons and their axons *in vitro*. *Neuroscience,* **23,** 275–289.

SZALLASI, A., ÁCS, G., CRAVOTTO, G., BLUMBERG, P.M., LUNDBERG, J.M. & APPENDINO, G. (1996). A novel agonist, phorbol 12-phenylacetate 13-acetate 20-homovanillate, abolishes positive cooperativity of binding by the vanilloid receptor. *Eur. J. Pharmacol.,* **299,** 221–228.

SZALLASI, A. & BLUMBERG, P.M. (1989). Resiniferatoxin, a phorbol-related diterpene, acts as an ultrapotent analog of capsaicin, the irritant constituent in red pepper. *Neuroscience,* **30,** 515–520.

SZALLASI, A. & BLUMBERG, P.M. (1990a). Minireview. Resiniferatoxin and its analogs provide novel insights into the pharmacology of the vanilloid (capsaicin) receptor. *Life Sci.,* **47,** 1399–1408.

SZALLASI, A. & BLUMBERG, P.M. (1990b). Specific binding of resiniferatoxin, an ultrapotent capsaicin analog, by dorsal root ganglion membranes. *Brain Res.,* **524,** 106–111.

SZALLASI, A. & BLUMBERG, P.M. (1996). Vanilloid receptors: new insights enhance potential as a therapeutic target. *Pain,* **68,** 195–208.

SZALLASI, A. & BLUMBERG, P.M. (1999). Vanilloid (capsaicin) receptors and mechanisms. *Pharmacol. Rev.,* **51,** 159–212.

SZALLASI, A., BLUMBERG, P.M., ANNICELLI, L.L., KRAUSE, J.E. & CORTRIGHT, D.N. (1999). The cloned rat vanilloid receptor VR1 mediates both R-type binding and C-type calcium response in dorsal root ganglion neurons. *Mol. Pharmacol.,* in press.

SZALLASI, A., LEWIN, N.E. & BLUMBERG, P.M. (1992). Identification of α_1-acid glycoprotein (orosomucoid) as a major vanilloid-binding protein in serum. *J. Pharmacol. Exp. Ther.,* **262,** 883–888.

SZALLASI, A., LEWIN, N.E. & BLUMBERG, P.M. (1993). Vanilloid (capsaicin) receptors in the rat: positive cooperativity of resiniferatoxin binding and its modulation by reduction and oxidation. *J. Pharmacol. Exp. Ther.,* **266,** 678–683.

SZALLASI, A., SHARKEY, N.E & BLUMBERG, P.M. (1989). Structure-activity analysis of resiniferatoxin analogs. *Phytother. Res.,* **3,** 253–257.

SZALLASI, A., LEWIN, N.E. & BLUMBERG, P.M. (1992). Identification of α_1-acid glycoprotein (orosomucoid) as a major vanilloid-binding protein in serum. *J. Pharmacol. Exp. Ther.,* **262,** 883–888.

TOMINAGA, M., CATERINA, M.J., MALMBERG, A.B., ROSEN, T.A. GILBERT, H., SKINNER, K., RAUMAN, B.E., BASBAUM, A.I. & JULIUS, D. (1998). The cloned capsaicin receptor integrates multiple pain-producing stimuli. *Neuron,* **21,** 531–543.

WALPOLE, C.S.J., BEVAN, S., BLOOMFIELD, G., BRECKENRIDGE, R., JAMES, I.F., RITCHIE, T., SZALLASI, A., WINTER, J. & WRIGGLESWORTH, R. (1996). Similarities and differences in the structure-activity relationships of capsaicin and resiniferatoxin analogues. *J. Med. Chem.,* **39,** 2939–2952.

WALPOLE, C.S.J. & WRIGGLESWORTH, R. (1993). Structural requirements for capsaicin agonists and antagonists. In *Capsaicin in the Study of Pain*. ed. Wood, J.N. pp. 63–82. San Diego: Academic Press.

WINTER, J., BEVAN, S. & CAMPBELL, E.A. (1995). Capsaicin and pain mechanisms. *Br. J. Anaesth.,* **75,** 157–168.

WINTER, J., DRAY, A., WOOD, J.N., YEATS, J.C. & BEVAN, S. (1990). Cellular mechanism of action of resiniferatoxin: a potent sensory neuron excitotoxin. *Brain Res.,* **520,** 131–140.

WOOD, J.N., WINTER, J., JAMES, I.F., RANG, H.P., YEATS, J. & BEVAN, S. (1988). Capsaicin-induced ion fluxes in dorsal root ganglion cells in culture. *J. Neurosci.,* **8,** 3208–3220.

(Received March 22, 1999
Revised June 15, 1999
Accepted June 30, 1999)