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The Cloned Rat Vanilloid Receptor VR1 Mediates Both R-Type Binding and C-Type Calcium Response in Dorsal Root Ganglion Neurons

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ABSTRACT

[^{3}H]Resiniferatoxin (RTX) binding and calcium uptake by rat dorsal root ganglion (DRG) neurons show distinct structure-activity relations, suggestive of independent vanilloid receptor (VR) subtypes. We have now characterized ligand binding to rat VR1 expressed in human embryonic kidney (HEK293) and Chinese hamster ovary (CHO) cells and compared the structure-activity relations with those for calcium mobilization. Human embryonic kidney cells (HEK293/VR1 cells) and Chinese hamster ovary cells transfected with VR1 (CHO/VR1 cells) bound [^{3}H]RTX with affinities of 84 and 103 pM, respectively, and positive cooperativity (Hill numbers were 2.1 and 1.8). These parameters are similar to those determined with rat DRG membranes expressing native VRs (a K_d of 70 pM and a Hill number of 1.7). The typical vanilloid agonists olvanil and capsaicin inhibited [^{3}H]RTX binding to HEK293/VR1 cells with K_i values of 0.4 and 4.0 μM, respectively. The corresponding values in DRG membranes were 0.3 and 2.5 μM. HEK293/VR1 cells and DRG membranes also recognized the novel vanilloids isovelleral and scutigeral with similar K_i values (18 and 20 μM in HEK293/VR1 cells; 24 and 21 μM in DRGs). The competitive vanilloid receptor antagonist capsazepine inhibited [^{3}H]RTX binding to HEK293/VR1 cells with a K_i value of 6.2 μM and binding to DRG membranes with a K_i value of 8.6 μM. RTX and capsaicin induced calcium mobilization in HEK293/VR1 cells with EC_{50} values of 4.1 and 82 nM, respectively. Thus, the relative potencies of RTX (more potent for binding) and capsaicin (more potent for calcium mobilization) are similar in DRG neurons and cells transfected with VR1. We conclude that VR1 can account for both the ligand binding and calcium uptake observed in rat DRG neurons.

The term vanilloid receptor was coined to describe the neuronal membrane recognition site for capsaicin and related irritant compounds (Szallasi and Blumberg, 1990a, 1999). It was postulated that the VR is a nonselective cation channel with a preference for calcium (Bevan et al., 1987; Bevan and Szolcsányi, 1990). Consequently, a $^{45}Ca^{2+}$-uptake assay performed with intact rat dorsal root ganglion (DRG) neurons has been used extensively to characterize structure-activity relations for vanilloids (Wood et al., 1988; Walpole and Wrigglesworth, 1993; Ács et al., 1996). In 1989, resiniferatoxin (RTX) was recognized as an ultrapotent vanilloid (Szallasi and Blumberg, 1989). Specific binding of [^{3}H]RTX provided the first unequivocal proof for the existence of a vanilloid receptor (Szallasi and Blumberg, 1990b) and has furnished a new, biochemical tool to study vanilloid receptor pharmacology (Szallasi and Blumberg, 1990a, 1999; Szallasi, 1994).

If binding and calcium uptake were mediated by the same receptor, a logical prediction would be that these two responses should display similar structure-activity relations. With regard to DRG neurons expressing native vanilloid receptors, this is clearly not the case: structure-activity analysis of different vanilloid derivatives revealed that the various compounds have distinct potencies for receptor binding and inducing $^{45}Ca^{2+}$-uptake (Ács et al., 1995, 1996; Walpole et al., 1996). One extreme is RTX, which is approximately 25-fold more potent for binding (K_d = 40 pM) than for inducing calcium uptake (EC_{50} = 1.0 nM) (Ács et al., 1996). The other extreme is capsaicin, which evokes calcium influx with an EC_{50} of 270 nM but inhibits [^{3}H]RTX binding with a 10-fold lower affinity of 3 μM (Ács et al., 1996). One model to account for the above discrepancies in vanilloid structure-activity relations was that RTX binding and calcium uptake detected two distinct classes of vanilloid receptors (Szallasi and Blumberg, 1996; Bíró et al., 1997). These putative receptors were referred to as R-type (preferentially labeled by RTX) and C-type (displaying a higher potency for capsaicin) vanilloid receptors, respectively. This model was further sup-

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ABBREVIATIONS: DRG, dorsal root ganglion; CHO/VR1 cells, Chinese hamster ovary cells transfected with VR1; HEK293/VR1 cells, human embryonic kidney cells transfected with VR1; RTX, resiniferatoxin; VR1, cloned rat vanilloid receptor subtype-1.

ported by the identification of non-neuronal cell lines that responded to vanilloids with calcium uptake (implying the presence of C-type vanilloid receptors) but lacked detectable specific RTX binding sites (Bíró et al., 1998a,b). An alternative model was based on the assumption that the vanilloid binding site may be within or at the inner face of the cell membrane (Walpole and Wrigglesworth, 1993). If this is so, then the calcium uptake assay may be subject to access constraints due to differences in cell membrane penetration, leading to distinct and independent structure-activity profiles from binding affinities for the compounds.

A functional rat vanilloid receptor, termed VR1, activated not only by vanilloids but also by noxious heat and low pH, has recently been cloned (Caterina et al., 1997; Tominaga et al., 1998). As predicted, this VR1 is a nonselective cation channel with a preference for calcium (Caterina et al., 1997). In *Xenopus* oocytes expressing VR1, vanilloids evoke inward currents, with RTX being approximately 20-fold more potent (EC_{50} = 39 nM) than capsaicin (EC_{50} = 710 nM) (Caterina et al., 1997). In VR1-transfected mammalian [human embryonic kidney (HEK293)] cells, capsaicin induces whole-cell currents with a potency of 110 nM (Tominaga et al., 1998). Taken together, these results suggest that VR1 corresponds to the site in DRG neurons that mediates calcium uptake.

To evaluate the hypothesis that binding and calcium uptake detect two distinct classes of vanilloid receptors in DRG neurons (Szallasi and Blumberg, 1996; Bíró et al., 1997), in the present study we have transfected mammalian [HEK293 and Chinese hamster ovary (CHO)] cells with a cDNA encoding the rat VR1 and used these cells for RTX binding and calcium mobilization experiments, respectively. In the binding experiments, both typical (capsaicin and olvanil) and novel (isovelleral and scutigeral) vanilloids were included, as well as the competitive vanilloid receptor antagonist capsazepine. Vanilloid binding to HEK293/VR1 cells was compared to that measured in rat DRG neurons expressing native vanilloid receptors. Calcium mobilization in HEK293/VR1 or CHO/VR1 cells was determined in response to RTX, olvanil, and capsaicin, by using a calcium-activated fluorescent dye method. In addition, capsaicin-induced calcium mobilization in the VR1-transfected cells was measured in the presence of capsazepine or the so-called functional vanilloid receptor antagonist, ruthenium red. Agonist and antagonist potencies determined in the calcium mobilization assays with HEK293/VR1 or CHO/VR1 cells were compared with values measured previously in this laboratory for vanilloid-induced $^{45}Ca^{2+}$-uptake by intact rat DRG neurons.

Experimental Procedures

Materials. [^{3}H]RTX (37 Ci/mmol) was synthesized by the Chemical Synthesis and Analysis Laboratory, National Cancer Institute-Frederick Cancer Research and Development Center (Frederick, MD). Nonradioactive RTX was purchased from Alexis Corp. (San Diego, CA), and capsazepine was from Research Biochemicals, Inc. (Natick, MA). Olvanil was a generous gift from Procter & Gamble Corp. (Cincinnati, OH). Isovelleral and scutigeral were donated by Olov Sterner (Lund University, Lund, Sweden). All the other chemicals used were purchased from Sigma (St. Louis, MO) unless indicated otherwise.

Molecular Biology. A cDNA encoding the vanilloid receptor VR1 was cloned from rat DRG total RNA by reverse transcription-polymerase chain reaction, by using primers based on the published nucleotide sequence (Caterina et al., 1997). A 2.7-kilobase cDNA was isolated, and the nucleotide sequence was verified to be identical with the published sequence. This cDNA was subcloned into pcDNA3.1 (Invitrogen, Carlsbad, CA) and pUHG102–3 (Clontech, Palo Alto, CA) for recombinant expression in mammalian cells.

Cell Culture. The pcDNA3.1 VR1 plasmid was transfected into HEK293 cells by standard methods. These transfected cells were selected for 2 weeks in media containing G418 (400 μg/ml) and then maintained as a pool of stably transfected cells. The pUHG102 VR1 plasmid was transfected into CHO cells containing the pTet Off Regulator plasmid (Clontech). In these cells, expression of the pUHG plasmid is repressed in the presence of tetracycline but is induced upon removal of the antibiotic. Stable clones were isolated in culture medium containing puromycin (10 μg/ml) and maintained in medium supplemented with tetracycline (1 μg/ml). Cells utilized for assays were grown in culture medium without antibiotic for 48 to 72 h before use. For radioligand binding experiments, cells were seeded in T175 cell culture flasks in media 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 mobilization assays, cells were seeded into 96-well plates and grown to 70 to 90% confluency.

Membrane Preparations. Female Sprague-Dawley rats weighing 200 to 250 g were euthanized under CO_2 anesthesia. The spinal columns were opened and DRGs were collected from all levels into ice-cold physiological saline. DRGs were disrupted with the aid of a tissue homogenizer in an ice-cold buffer (pH 7.4) containing 5 mM KCl, 5.8 mM NaCl, 0.75 mM $CaCl_2$, 2 mM $MgCl_2$, 320 mM sucrose, and 10 mM HEPES. Tissue homogenates were first centrifuged for 10 min at 1,000*g* (4°C) to remove the nuclear fraction and debris, and then the supernatant from the first centrifugation was again centrifuged for 30 min at 35,000*g* (4°C) to obtain a partially purified membrane fraction. Membranes resuspended in the homogenization buffer were stored at −80°C until assayed.

Radioligand Binding. Binding studies with [^{3}H]RTX were carried out according to a published protocol (Szallasi et al., 1992) in which nonspecific RTX binding is reduced by adding bovine α_1-acid glycoprotein (100 μg per tube) after the binding reaction has been terminated. Binding assay mixtures were set up on ice and contained [^{3}H]RTX, nonradioactive ligands, 0.25 mg/ml BSA (Cohn fraction V), and either 5×10^4 to 1×10^5 VR1-transfected cells or 40 μg of DRG membrane protein. The final volume was adjusted to 500 (competition binding assays) or 1000 μl (saturation binding assays) with the buffer described above. Nonspecific binding was defined as that occurring in the presence of 1 μM nonradioactive RTX. For saturation binding, [^{3}H]RTX was added in the concentration range of 7 to 1000 pM, with one to two dilutions. Competition binding assays were performed in the presence of 30 (for DRG membranes) or 60 pM (for VR1-transfected cells) [^{3}H]RTX and various concentrations of competing ligands. The binding reaction was initiated by transferring the assay mixtures into a 37°C water bath and was terminated after a 60-min incubation period by cooling the tubes on ice. 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. Equilibrium binding parameters were determined by fitting the Hill equation to the measured values with the aid of the computer program FitP (Biosoft, Ferguson, MO) as described previously (Szallasi et al., 1993).

Calcium Mobilization Assays. VR1-transfected cells were seeded into 96-well plates and grown to 70 to 90% confluency. The cells were then washed once with Krebs-Ringer HEPES buffer (25 mM HEPES, 5 mM KCl, 0.96 mM NaH_2PO_4, 1 mM $MgSO_4$, 2 mM $CaCl_2$, 5 mM glucose, 1 mM probenecid, pH 7.4) and incubated for 1 to 2 h in the above buffer supplemented with the calcium-sensitive fluorescent dye Fluo3-AM (2.5–10 μg/ml; Teflabs, Austin, TX) at 37°C in an environment containing 5% CO_2. In some experiments (as

indicated in *Results*), the Krebs-Ringer HEPES buffer was also supplemented with 1 mg/ml BSA (Cohn fraction V). The wells were then washed twice with Krebs-Ringer HEPES buffer. Addition of agonist (olvanil, capsaicin, or RTX) to the wells resulted in concentration-dependent changes in intracellular calcium levels and subsequent activation of Fluo3 fluorescence. Fluoroskan Ascent (Labsystems, Franklin, MA) or FLIPR (Molecular Devices, Sunnyvale, CA) instruments were used to monitor changes in fluorescence for up to 180 s and to determine the maximum fluorescence signal. Similarly, varying concentrations of the antagonists ruthenium red and capsazepine were added to cells concurrently with agonist (25–50 nM capsaicin). For the capsaicin- and olvanil-induced calcium responses, data obtained between 30 and 60 s after agonist application were used to generate the EC_{50} values. Because the time course of RTX-evoked responses was more prolonged than that of capsaicin (see *Results*), data obtained between 120 and 180 s were used to determine EC_{50} values. Kaleidagraph software (Synergy Software, Reading, PA) was utilized to fit the data to the equation $y = a \cdot (1/(1 + (b/x)^c))$ to determine the EC_{50} for the response. In this equation, y is the maximum fluorescence signal, x is the concentration of the agonist or antagonist, a is the E_{max}, b corresponds to the EC_{50} or IC_{50} value, and, finally, c is the Hill coefficient.

Results

VR1-Transfected Mammalian Cells (HEK293 and CHO) and Rat DRG Membranes Expressing Native Vanilloid Receptors Bind [^{3}H]RTX with Similar Parameters. The association of [^{3}H]RTX (60 pM) to VR1 expressed on HEK293 cells was rapid: within 10 min, the specific binding attained approximately 90% of its peak value, and it then remained on a plateau between 20 and 60 min of incubation (a single experiment; data not shown). When dissociation was initiated after a 60-min association, it could be fitted to a first order decay curve, yielding a dissociation constant of 0.12 ± 0.02 min^{-1} (two determinations; data not shown). Based on these preliminary experiments, an incubation period of 60 min was selected for the equilibrium binding studies.

[^{3}H]RTX (7–1000 pM) displayed saturable binding to HEK293/VR1 cells (Fig. 1A). The half-maximal binding occurred at 84 ± 11 pM (mean ± S.E.M.; four determinations); at the K_d, nonspecific binding represented approximately 20% of the total binding (not shown). The saturation binding curve was sigmoidal, indicating positive cooperativity (Fig. 1B). A fit to the Hill equation yielded a cooperativity index of 2.1 ± 0.1 (mean ± S.E.M.; four determinations). This binding behavior results in a convex Scatchard plot (Fig. 1C). The B_{max} value was 250 ± 24 fmol/10^6 cells (mean ± S.E.M.; four determinations), corresponding to a receptor density of 1.5 × 10^5 binding sites per cell. CHO/VR1 cells bound RTX with similar affinity (a K_d of 103 ± 13 pM; mean ± range; two determinations) and cooperativity values (a Hill number of 1.9 ± 0.1; mean ± range; two experiments). The maximal receptor density was, however, approximately 2-fold higher than in the HEK293/VR1 cells (470 ± 30 fmol/10^6 cells; mean ± range; two determinations) (Fig. 1C). The VR1-transfected cells lines bound RTX with parameters similar not only to each other but also to rat DRG membranes expressing native vanilloid receptors (Fig. 2). DRG membranes bound [^{3}H]RTX with a K_d of 70 ± 10 pM and a B_{max} of 290 ± 10 fmol/mg protein (mean ± range; two determinations); the cooperativity index was 1.7 ± 2 (mean ± range; two determinations).

Although the CHO/VR1 cells provided a higher level of specific binding, HEK293/VR1 cells were chosen for additional detailed analysis for a better comparison with the literature (Caterina et al., 1997; Tominaga et al., 1998).

Vanilloid Agonists and the Antagonist Capsazepine Inhibit [^{3}H]RTX Binding by VR1-Transfected Mammalian Cells and Rat DRG Membranes, Respectively, with Similar Affinities. For the pharmacological characterization of the RTX recognition site on rat VR1 expressed in HEK293 cells, four agonists (olvanil, capsaicin, isovelleral, and scutigeral) and an antagonist (capsazepine) were selected (Fig. 3). Apparent K_i values of the agonists were: olvanil, 0.4 ± 0.1 μM (n = 4); capsaicin, 4.0 ± 0.8 μM (n = 6); isovelleral, 20 ± 4 μM (n = 3); and scutigeral, 18 ± 3 μM (n = 3; all values are mean ± S.E.M.). The competitive antagonist capsazepine inhibited [^{3}H]RTX binding with a K_i of 6.2 ± 0.7 μM (mean ± S.E.M.; five experiments). These K_i values are similar to those determined with rat DRG membranes: olvanil, 0.3 ± 0.1 μM; capsaicin, 2.5 ± 1.1 μM; isovelleral, 24 ± 4 μM; scutigeral, 21 ± 3 μM; and capsazepine, 8.6 ± 3.5 μM (mean ± S.E.M.; three experiments; Table 1). The binding affinities of olvanil, capsaicin, and capsazepine were also determined with CHO/VR1 cells: K_i values were 0.26 ± 0.5, 1.7 ± 0.4, and 6.6 ± 1.4 μM, respectively (mean ± range; two determinations; Table 1).

Characterization of VR1-Transfected Mammalian Cells in the Calcium Mobilization Assay by Using Various Vanilloid Compounds. Capsaicin induced calcium mobilization in HEK293/VR1 cells and CHO/VR1 cells with EC_{50} values of 82 ± 17 nM (mean ± S.E.M.; n = 4) and 38 ± 13 nM (mean ± S.E.M.; n = 5), respectively. RTX was more than an order of magnitude more potent in both cell lines; EC_{50} values were 4.1 ± 1.3 nM in HEK293/VR1 cells (mean ± S.E.M.; n = 5), and 1.4 ± 0.8 nM in CHO/VR1 cells (mean ± S.E.M.; n = 4). Capsaicin and RTX differed not only in potency in the calcium mobilization assay, but also in the kinetics of the response (Fig. 4). Capsaicin administration resulted in a rapid response (Fig. 4A). By contrast, RTX-evoked calcium mobilization became detectable after an initial delay only (compare Fig. 4, A and B). By using 30 nM capsaicin, a concentration close to the EC_{50} in CHO/VR1 cells, the calcium mobilization response achieved its peak value within 1 min and then started to decline, suggesting the development of tachyphylaxis, or due to some other aspect of channel gating (Fig. 4A). By contrast, calcium mobilization in response to 1 nM RTX increased steadily over a 3-min period after challenge, approaching the maximal response evoked by 100 nM RTX (Fig. 4B). This difference between the kinetics of capsaicin- and RTX-induced responses, however, did disappear when high, supramaximal doses were used (30 μM capsaicin or 100 nM RTX; compare Fig. 4, A and B). Olvanil evoked the calcium response in CHO/VR1 cells with a potency of 22 ± 6 nM (mean ± S.E.M.; n = 7). The time course of the olvanil-induced calcium mobilization response was similar to that by capsaicin (not shown). Administering 25 nM capsaicin to evoke calcium mobilization, capsazepine inhibited this response with an IC_{50} value of 2.4 ± 0.5 μM (mean ± S.E.M.; n = 6). However, this value was shifted by almost an order of magnitude in the presence of 1 mg/ml BSA to yield an IC_{50} of 0.33 ± 0.03 μM (mean ± S.E.M.; n = 5). From the concentration of agonist (25 nM capsaicin) and the IC_{50} for antagonist, the following capsazepine K_i values can be calculated: 140 nM in the pres-

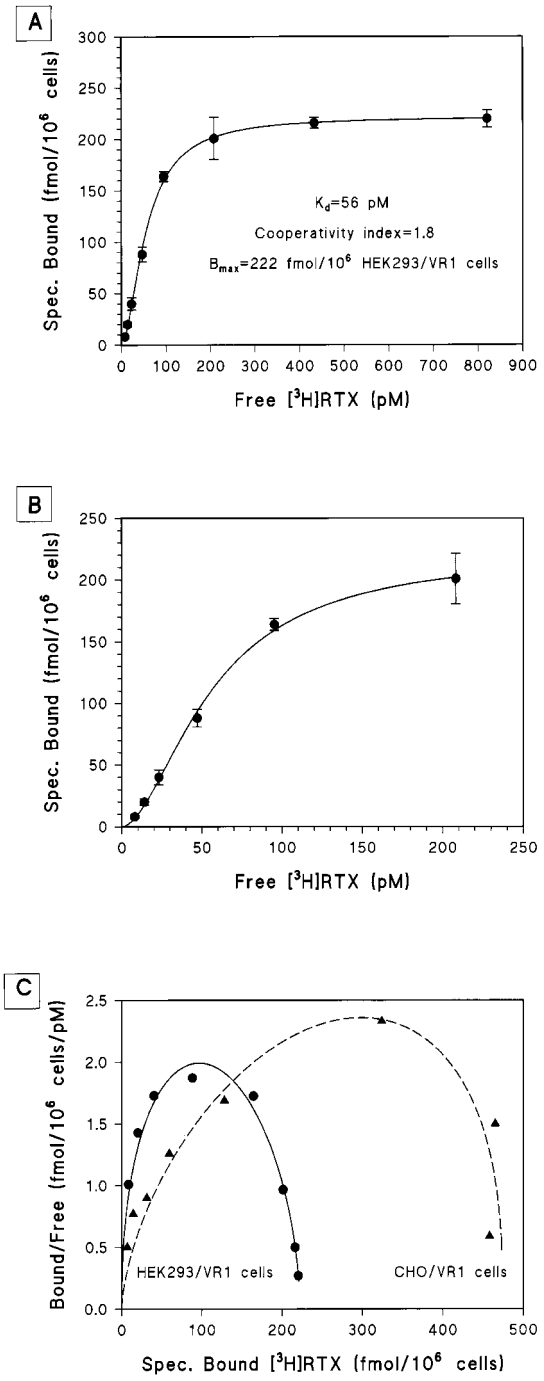

Fig. 1. Specific binding of [^{3}H]RTX to HEK293 cells transfected stably with a cDNA encoding the rat VR1 (HEK293/VR1 cells). A, [^{3}H]RTX displays saturable specific binding to HEK293/VR1 cells. Binding data are from a single experiment; points are mean values of triplicate determinations; error bars indicate S.E.M. The binding curve was generated by a computer fit of the measured values to the Hill equation. Half-maximal binding occurred at a concentration of 56 pM [^{3}H]RTX; the maximal receptor density was 222 fmol/10^6 cells. Note that the Hill coefficient (1.8) is indicative of positive binding cooperativity. Three additional experiments yielded similar results (see *Results* for mean ± S.E.M. binding parameters). B, as a result of the cooperativity index approaching 2, the specific binding curve is sigmoidal in the concentration range of 0 to 200 pM [^{3}H]RTX. Measured values are from A. The binding curve is hypothetical and was computer-generated by using the binding parameters from A. C, Scatchard plots of specific [^{3}H]RTX binding to HEK293/VR1 cells and CHO/VR1 cells. Observe that the Scatchard plots are convex due to the positive cooperativity of the binding. Also note that the B_{max} value is approximately twice as high in CHO/VR1 as in HEK203/VR1 cells.

ence and 2400 nM in the absence of BSA, respectively (Table 1). For the other vanilloids tested in this study, the presence or absence of BSA had no detectable influence on the calcium mobilization potency. With an IC_{50} of 210 ± 30 nM (mean ± S.E.M.; n = 7), the functional antagonist ruthenium red was approximately 1.5-fold more potent than capsazepine (IC_{50} = 330 nM in the presence of BSA) to prevent calcium mobilization by capsaicin.

Discussion

[^{3}H]RTX binding and $^{45}Ca^{2+}$-uptake are standard assays to characterize the pharmacology of native vanilloid receptors on primary sensory neurons. Structure-activity analysis of a number of vanilloid derivatives belonging to the capsaicinoid (Winter et al., 1993), resiniferanoid (Ács et al., 1995, 1996; Walpole et al., 1996), unsaturated dialdehyde (Szallasi et al., 1996, 1998), and triprenyl phenol (Szallasi et al., 1999a) classes revealed that these compounds have dissimilar potencies for receptor binding and inducing $^{45}Ca^{2+}$-uptake, respectively, in intact rat DRG neurons. The mechanistic basis for these differences has not been defined; however, the distinct rank order of vanilloid potencies for binding versus calcium uptake raised the interesting possibility that these responses might, in fact, detect two separate classes of vanilloid receptors. For example, RTX had been found to bind to intact rat DRG neurons with an affinity of 40 pM but only evoked calcium uptake in these cells with a 25-fold lower potency (Ács et al., 1996). Capsaicin showed the opposite behavior: it was more potent for inducing calcium uptake (270 nM) than for inhibiting RTX binding (3 μM) (Ács et al., 1996). A vanilloid receptor termed VR1 was recently cloned from a rat DRG cDNA library (Caterina et al., 1997). Therefore, the present study was designed to address two questions of great importance. First, we examined whether the cloned VR1 could bind RTX and, if so, whether it resembled the specific RTX binding site described on DRG neurons. Second, we studied whether or not VR1-transfected mammalian cells

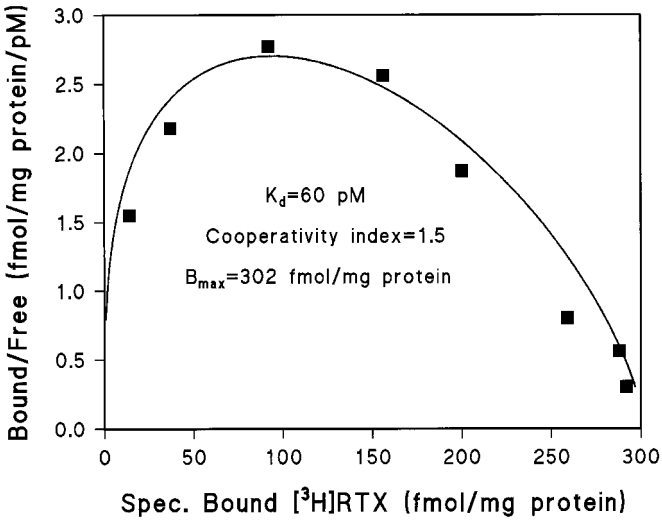

Fig. 2. Scatchard plot of specific [^{3}H]RTX binding to rat DRG membranes. Compare with Fig. 1C and note that [^{3}H]RTX binds to rat DRG membranes expressing native vanilloid receptors and to mammalian cells (HEK293 and CHO) transfected with the cloned rat vanilloid receptor VR1 with similar binding parameters. As shown, a single experiment; a second experiment yielded similar results.

can mimic the distinct affinities of selected vanilloids for receptor binding and calcium response in DRG neurons.

C-type vanilloid receptors were believed to mediate calcium uptake in sensory neurons, implying that these receptors were ion channels. Because RTX did not evoke any detectable calcium uptake at low concentrations, at which it labeled specific binding sites in DRG neurons (Ács et al., 1997), it was suggested that R-type vanilloid receptors may not be ionotropic (Bíró et al., 1997). Our clear finding is that VR1-transfected HEK293 or CHO cells bind vanilloids with parameters very similar to those determined in rat DRG neuron membranes in parallel experiments. Therefore, our conclusion is that VR1 displays the structure-activity characteristics of the putative R-type vanilloid receptor. Because VR1, a nonspecific cation channel (Caterina et al., 1997), should correspond to C-type vanilloid receptors, our finding indicates that R- and C-type vanilloid pharmacologies reflect different measures of the same target.

VR1 expression in various cellular systems shows discrepancies in agonist potencies that may be indicative of receptor

TABLE 1
Binding affinity and $^{45}Ca^{2+}$-uptake or Ca^{2+} mobilization of rat DRG neurons and CHO/VR1 cells by RTX and other ligands
Values are the mean of at least four independent determinations.

Ligand	Rat DRG Neurons		CHO/VR1 Cells	
	Binding affinity (K_d or K_i)	$^{45}Ca^{2+}$-uptake (EC_{50} or K_i)[a]	Binding affinity (K_d or K_i)	Ca^{2+} mobilization (EC_{50} or K_i)
	nM			
RTX	0.07	1	0.13	1.4
Capsaicin	2500	340	1700	38
Olvanil	300	170[b]	260	22
Capsazepine[c]	8600	271	6600	140 (1100)
Ruthenium red	no effect	790	no effect	210

[a] From Ács G et al. (1996).
[b] From Walpole and Wrigglesworth (1993).
[c] For capsazepine, K_i values are given; the value in parentheses (1100 nM) was obtained in the absence of bovine serum albumin, 1 mg/ml.

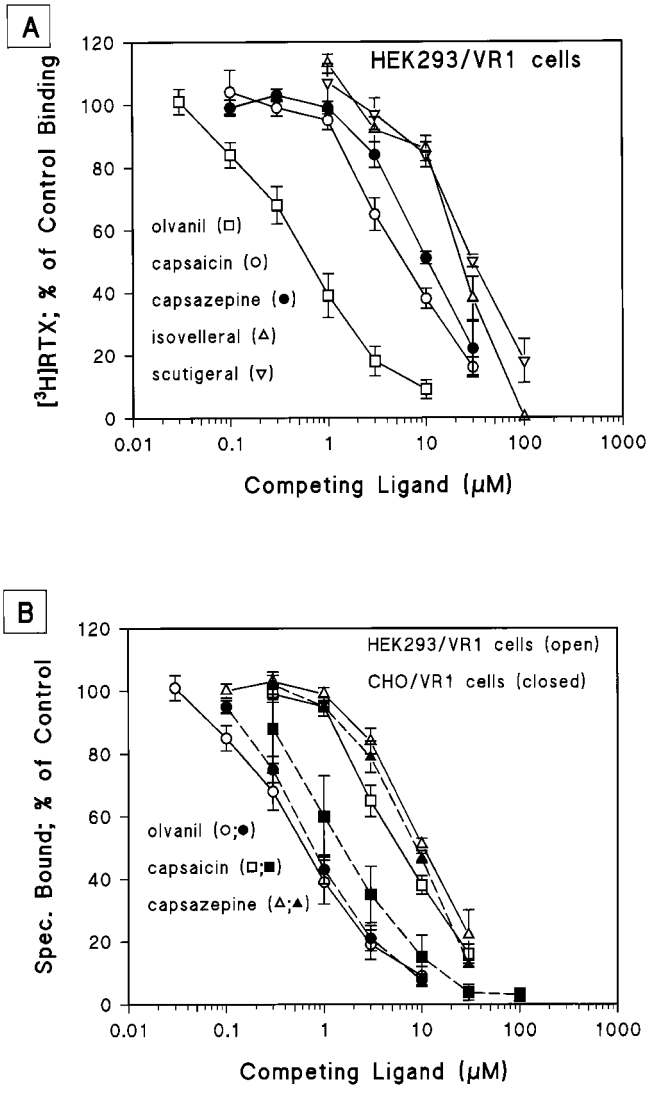

Fig. 3. A, inhibition of specific [^{3}H]RTX binding to HEK293/VR1 cells by the typical vanilloid agonists olvanil and capsaicin, the novel vanilloids isovelleral and scutigeral, and the competitive vanilloid receptor antagonist capsazepine. Points represent the mean values from three to six independent determinations; error bars indicate S.E.M. See *Results* for calculated K_i values. B, inhibition by olvanil, capsaicin, and capsazepine of specific [^{3}H]RTX binding to HEK293/VR1 cells and CHO/VR1 cells. HEK293/VR1 data are from A. For CHO/VR1 cells, points represent mean values of two determinations; error bars indicate range.

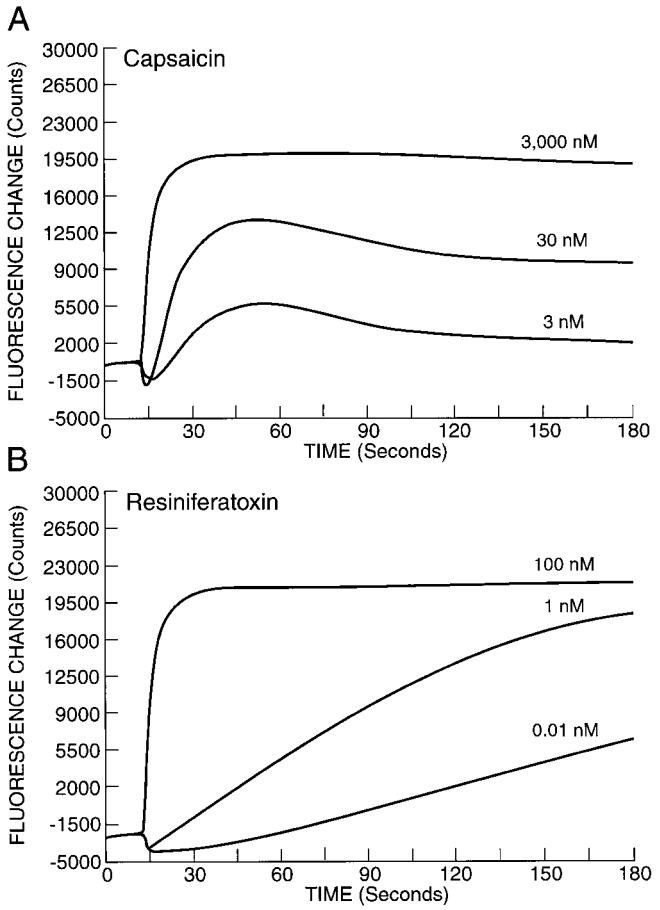

Fig. 4. Time dependence of capsaicin (A)- and RTX (B)-evoked calcium mobilization in CHO/VR1 cells. Note that these two typical vanilloid agonists differ substantially in the kinetics of the response they evoke. Capsaicin administration leads to rapid calcium mobilization responses: the maximal fluorescence change occurs within 30 (30 μM capsaicin) to 50 s (3 nM capsaicin; A). Unless high RTX concentrations are used, such as 100 nM, a value almost 100-fold higher than the EC_{50}, RTX application results in slowly developing, but persistent calcium currents (B). Traces from a representative side-by-side comparison of capsaicin and RTX effects are displayed.

biology not yet understood. For instance, Julius and coworkers (Caterina et al., 1997; Tominaga et al., 1998) have described a 7-fold difference in EC_{50} for capsaicin-evoked inward currents mediated by VR1 in oocytes (710 nM) and in HEK293 cells (110 nM). The latter value is in accord with the potency of capsaicin for mobilizing calcium in HEK293/VR1 cells (82 nM; this study). We find, however, a significant difference between capsaicin-evoked calcium mobilization in CHO/VR1 cells (EC_{50} = 38 nM) and capsaicin-induced $^{45}Ca^{2+}$-uptake by DRG neurons (270 nM). This difference in capsaicin potencies between CHO/VR1 cells and DRG neurons was surprising because RTX showed similar potencies (1.4 nM in CHO/VR1 cells and 1.0 nM in rat DRG neurons, respectively) in the two assays. RTX, however, was 3-fold more potent for mobilizing calcium in CHO/VR1 cells (EC_{50} = 1.4 nM) than in HEK293/VR1 cells (EC_{50} = 4.1 nM), although RTX was recognized by both VR1-transfected cell types with similar binding affinities (K_d values were 103 and 84 pM for CHO/VR1 and HEK293/VR1 cells, respectively). To a limited degree, the discrepancies in agonist potencies observed for VR1-transfected cells and DRG neurons may reflect methodological differences between the calcium-activated fluorescence and the $^{45}Ca^{2+}$-uptake assays. However, methodology cannot account for all the differences in vanilloid potencies, because capsaicin was found to be more active for opening VR1 in HEK293 cells than in oocytes with use of a similar (patch-clamping) procedure (Caterina et al., 1997; Tominaga et al., 1998). Possible explanations include the existence of VR1 in multimeric and/or spliced variant forms in neurons, leading to distortions in vanilloid potencies in the calcium influx measurements. This is in accord with the heterogeneity of vanilloid-gated conductances in voltage-clamped neurons (Liu and Simon, 1996; Petersen et al., 1996).

The mechanistic basis for the discrepancies for vanilloids between binding affinity and potency to mobilize calcium via VR1 also remains to be established. It may be of relevance that RTX and capsaicin differ not only in binding affinity and potency to evoke calcium uptake but also in channel-gating kinetics (Winter et al., 1990; Liu and Simon, 1996). Upon challenge, capsaicin opens the channel rapidly whereas RTX, a more bulky molecule, is delayed in action. Capsaicin-evoked inward currents desensitize rapidly. By contrast, RTX-induced currents are prolonged. This difference between capsaicin and RTX acting on native vanilloid receptors expressed on intact neurons (Liu and Simon, 1996) is replicated in this study by using VR1-transfected mammalian cells. Consequently, this behavior relates to the biology of the receptor rather than to its neural environment. Interestingly, the time course of the olvanil-evoked calcium response is similar to that by capsaicin (this study), although olvanil mimics RTX rather than capsaicin in its biological activities. For example, olvanil (Brand et al., 1990), like RTX (Szolcsányi et al., 1990; Ács et al., 1997), may desensitize neurons without causing a detectable prior excitation.

At the methodological level, VR1-transfected mammalian cells represent an attractive alternative to replace DRG neurons for pharmacological characterization of vanilloid receptors. VR1 is gated not only by vanilloids but also by noxious heat (>43°C) and protons (Tominaga et al., 1998). Slight acidification of the culture medium during cell maintenance may decrease the temperature threshold of VR1 to 37°C (Tominaga et al., 1998). Thus, cells that continuously express VR1 eventually die due to the rising intracellular calcium levels. The use of the pTet Off Regulator plasmid in the CHO/VR1 cells is important in that it makes the long-term maintenance of VR1-tranfected cells possible when tetracycline is included in the culture medium.

An interesting observation of this study is that the apparent potency of capsazepine greatly depends on assay conditions; capsazepine is almost 10-fold more potent to block capsaicin-induced calcium mobilization in the presence of BSA. As yet, the mechanism(s) underlying this phenomenon is unclear. Possibilities include the coating of the air-water interphase with BSA and/or capsazepine binding to this serum protein. Both mechanisms may provide a new equilibrium for free capsazepine at a higher concentration. The inclusion/omission of BSA (or any other factor that works in a similar fashion) may account for many of the conflicting reports in the literature with regard to apparent capsazepine potencies. For example, capsazepine was reported to antagonize capsaicin responses in the isolated rat spinal cord-tail preparation with an IC_{50} value of 254 nM (Dickenson and Dray, 1991) but was found to block the capsaicin-evoked contractions of the rat urinary bladder with an IC_{50} of 5000 nM (Maggi et al., 1993). Although in this study the presence or absence of BSA had no effect on the potency of any other vanilloid tested in the calcium mobilization assay, capsazepine is not the only vanilloid whose apparent potency may be enhanced by the inclusion of BSA. Some lipophilic phorboid vanilloids appear to behave in a similar fashion (Szallasi et al., 1999b).

Vanilloids are not only important tools to identify and study subsets of primary sensory neurons (Holzer, 1991) but have a clear therapeutic potential as well (Maggi, 1992; Szallasi and Blumberg, 1996; Hautkappe et al., 1998). Capsaicin-containing creams are already commercially available to relieve itch and pain. RTX is currently undergoing clinical trials for the indication of urinary bladder hyperreflexia with promising results; a single RTX treatment seems to result in a long-lasting improvement in patients without producing the barely tolerable initial pain that limits the clinical usefulness of capsaicin (Cruz et al., 1997; Lazzeri et al., 1998). RTX, however, has its own shortcomings. For example, it is difficult to obtain by chemical synthesis and cannot be given p.o. Therefore, the identification of simplified, orally active vanilloids is an ongoing objective. As discussed above, RTX is associated with R-type pharmacology (higher affinity for binding), whereas capsaicin is the prototype of compounds following C-type pharmacology (higher potency for calcium mobilization). Therefore, we conclude that the simultaneous evaluation of compounds for binding versus calcium mobilization in VR1-transfected mammalian cells could provide a rapid screen to identify novel vanilloids that follow R- and C-type pharmacologies, respectively. Biological evaluation of additional compounds selective for one or the other response might be of interest.

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