0026-895X/01/5901-9–15$3.00
MOLECULAR PHARMACOLOGY
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Mol Pharmacol 59:9–15, 2001

Vol. 59, No. 1
361/875315
Printed in U.S.A.

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ACCELERATED COMMUNICATION

Iodo-Resiniferatoxin, a New Potent Vanilloid Receptor Antagonist

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Received June 14, 2000; accepted September 28, 2000

This paper is available online at http://molpharm.aspetjournals.org

ABSTRACT

The highly potent vanilloid receptor (VR) agonist resiniferatoxin has been radiolabeled with ^{125}I, and the pharmacology to the cloned rodent VR, VR1, and the endogenous VR in rat spinal cord membranes has been characterized. [^{125}I]RTX binding to human embryonic kidney 293 cells expressing VR1 was reversible and with high affinity (K_d = 4.3 nM) in an apparent monophasic manner. In rat spinal cord membranes, [^{125}I]RTX bound with a similar high affinity (K_d = 4.2 nM) to a limited number of binding sites (B_{max} = 51 ± 8 fmol/mg of protein). The pharmacology of recombinant rodent VR1 and the endogenous rat VR1 was indistinguishable when measuring displacement of [^{125}I]RTX binding (i.e., the following rank order of affinity was observed: RTX > I-RTX > olvanil > capsaicin > capsazepine). Capsaicin and RTX induced large nondesensitizing currents in *Xenopus laevis* oocytes expressing VR1 (EC_{50} values were 1300 nM and 0.2 nM, respectively), whereas I-RTX induced no current per se at concentrations up to 10 μM. However, I-RTX completely blocked capsaicin-induced currents (IC_{50} = 3.9 nM). In vivo, I-RTX effectively blocked the pain responses elicited by capsaicin (ED_{50} = 16 ng/mouse, intrathecally). The present study showed that I-RTX is at least 40-fold more potent than the previously known VR antagonist, capsazepine. Thus, I-RTX as well as its radiolabeled form, should be highly useful for further exploring the physiological roles of VRs in the brain and periphery.

Vanilloid receptors (VRs) are activated by capsaicin, the pungent ingredient in chilli peppers and more potently by resiniferatoxin (RTX), a toxin isolated from the cactus *Euphorbia resinifera* (Szallasi and Blumberg, 1999). VRs have been shown to be expressed on unmyelinated pain-sensing nerve fibers (C-fibers) and small Aδ fibers in the dorsal root, trigeminal, and nodose ganglia (Holzer, 1991; Guo et al., 1999; Szallasi and Blumberg, 1999). Initially, activation of VRs by pungent agonists such as capsaicin leads to excitation of primary sensory neurons gating nociceptive inputs to the central nervous system (Holzer, 1991). Subsequently, however, these fibers become desensitized, and this forms the basis for the therapeutic use of VR agonists in chronic pain states such as spinal cord injury, diabetic neuropathy, or arthritis (Holzer, 1991; Szallasi and Blumberg, 1999). The target of capsaicin and RTX has been identified with the molecular cloning of a capsaicin-sensitive VR, termed VR1 (Caterina et al., 1997). VR1 encodes a protein of 838 amino acids forming a calcium-permeable channel that is activated by capsaicin but also by noxious heat and low extracellular pH (Caterina et al., 1997; Tominaga et al., 1998). Indeed, VR1 is currently believed to serve as an integrator of painful stimuli resulting from noxious heat and acidosis (as is frequently occurring under inflammatory conditions) leading to a lowered threshold for pain (Caterina et al., 1997; Tominaga et al., 1998). This has recently been confirmed using mice genetically deficient in VR1 (Caterina et al., 2000). Several new agonists acting at the VR have been extracted or synthesized that differ in their ability to excite versus desensitize dorsal root ganglion neurons (Acs et al., 1995; 1996; Liu et al., 1997). Olvanil is an example of such a VR agonist that has an affinity almost similar to capsaicin but is less pungent (Liu et al., 1997). However, only a few VR antagonists are available: 1) capsazepine is a relatively weak competitive antagonist with a potency ranging from 0.2 to 4 μM (Bevan et al., 1992; Szallasi et al., 1993; Acs et al., 1996; Caterina et al.,

ABBREVIATIONS: VR, vanilloid receptor; RTX, resiniferatoxin; I-RTX, 6,7-deepoxy-6,7-didehydro-5-deoxy-21-dephenyl-21-(phenylmethyl)-daphnetoxin,20-4-hydroxy-5-iodo-3-ethoxybenzeneacetate; HPLC, high-performance liquid chromatography; HEK, human embryonic kidney; I-V, current-voltage; PdBu, phorbol-12,13-bibutyrate.

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1997; Liu et al., 1997; Wardle et al., 1997), which unfortunately has nonspecific effects at the concentrations (10 μM) often required for antagonist activity (Docherty et al., 1997; Liu and Simon, 1997; Wardle et al., 1997) and, 2) ruthenium red, which is a weak and noncompetitive antagonist with a poorly defined mechanism of action (Szallasi and Blumberg, 1999). Thus, there is clearly room for improvement regarding the development of potent and specific VR1 antagonists.

In the present study, the preparation and pharmacological characterization of I-RTX, a novel specific VR1 antagonist, is described in its ^{125}I-labeled form as well as its nonradioactive form. This compound is shown to be much more potent than capsazepine and represents, to our knowledge, the most potent VR antagonist yet described.

Materials and Methods

Preparation of I-RTX and [^{125}I]RTX. Nonradioactive I-RTX (Fig. 1) was prepared from RTX (RBI, Natick, MA) by electrophilic aromatic substitution using sodium iodide and the chloramine-T method (Hunter and Greenwood, 1962). Because there is one highly activated position available for iodination in the phenol moiety of RTX (ortho to the hydroxy group) it was expected to achieve only one product. HPLC proved this to be the case and the product could be separated from nonreacted RTX and impurities in the reaction mixture using reverse-phase HPLC. The yield was 30 to 45% and the purity determined by HPLC was high (>97%). NMR and mass spectroscopy confirmed the identity of 6,7-deepoxy-6,7-didehydro-5-deoxy-21-dephenyl-21-(phenylmethyl)-daphnetoxin,20-(4-hydroxy-5-iodo-3-ethoxybenzeneacetate) (I-RTX) (for chemical structure, see Fig. 1).

[^{125}I]RTX was prepared by the lactoperoxidase method as described below, which resulted in high incorporation of radioactivity and only small amounts of radioiodinated by-products. In brief, carrier-free Na^{125}I (2100 Ci/mmol; Amersham Pharmacia Biotech, Buckinghamshire, UK) was added to RTX in 0.4 M sodium phosphate, pH 7.2, and the mixture was stirred while adding 0.01% H_2O_2 and 0.1 mg/ml lactoperoxidase (EC 1.11.1.7). After 5 min at room temperature, trifluoracetic acid was added and the labeled product was separated from nonlabeled RTX and by-products by reverse-phase HPLC. The ^{125}I-labeled product was coeluted with a reference sample of the nonradioactive I-RTX. The analysis showed a radiochemical purity of >98% and a specific radioactivity of 2100 Ci/mmol. Capsaicin, ruthenium red, and capsazepine was purchased from RBI and olvanil was from Tocris (Bristol, UK).

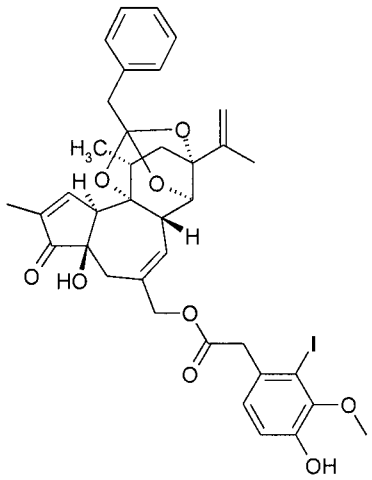

Fig. 1. The chemical structure of I-RTX.

Cloning of VR1, Cell Cultures, and Membrane Preparations. VR1 was cloned using polymerase chain reaction primers based on the published VR1 sequence (Caterina et al., 1997; GenBank accession no. AF029310) from a rat dorsal root ganglion cell library and HEK 293 cells were transfected with VR1-containing plasmid (pcDNA3; InVitrogen, Carlsbad, CA). Cells were subcloned and cultured in Dulbecco's modified Eagle's medium supplemented with 5% fetal bovine serum, 2 mM glutamine, 0.05 mg/ml gentamycin, and 0.5 mg/ml G418 in 95% air/5% CO_2 at 37°C. Confluent HEK 293 cells were harvested using phosphate-buffered saline/EDTA (Mg^{2+}- and Ca^{2+}-free, pH 7.4), centrifuged (1000*g*, 10 min, 25°C) and pellets were frozen at −80°C. Spinal cords were dissected from Sprague-Dawley rats (weighing 200–250 g) and frozen at −80°C. Membranes from HEK 293 cells expressing VR1 or spinal cord were prepared as follows. The tissue was placed in ice-cold assay buffer (5.8 mM NaCl, 5 mM KCl, 2 mM $MgCl_2$, 0.75 mM $CaCl_2$, 0.25 mg/ml bovine serum albumin, 137 mM sucrose, and 10 mM HEPES, pH 7.8) and homogenized with an Ultra-Torrax homogenizer (Janke & Kunkel, Germany) for 30 s. After a brief centrifugation (1000*g*, 10 min, 4°C) the pellet was discarded and the supernatant centrifuged again (40000*g*, 30 min, 4°C). The resulting pellet was resuspended in assay buffer and frozen in aliquots at −80°C.

[^{125}I]RTX Binding Experiments. Receptor binding experiments using [^{125}I]RTX were performed essentially as described for [^{3}H]RTX experiments (Szallasi et al., 1995). In brief, tissue (0.5 mg/tube of rat spinal cord or 0.2 mg/tube of HEK 293 cells expressing VR1), buffer, test compounds, and [^{125}I]RTX were added to microcentrifuge tubes and the incubation was carried out for 60 min at 37°C (unless otherwise indicated). The samples were placed on an ice-bath and 50 μl of α_1-acid glycoprotein (2 mg/ml) was added to reduce nonspecific binding. Bound and free radioactivity was separated by centrifugation (40,000*g*, 10 min, 4°C) and the pellets were counted in a gamma counter (Cobra II; Packard Instruments, Meriden, CT). In saturation binding experiments, the concentration of radioligand varied from 0.01 nM to 30 nM, whereas a concentration of 0.2 nM [^{125}I]RTX (corresponding to ~150.000 dpm/assay) was used in the remaining receptor binding experiments. Nonspecific binding was defined as binding in the presence of 100 nM RTX.

Functional Expression of VR1 in *Xenopus laevis* Oocytes. In vitro transcripts from VR1 was made using a mRNA capping kit (Strategene, La Jolla, CA) and electrophysiological studies were performed with oocytes from *X. laevis* exactly as described previously (Wahl et al., 1998). Oocytes were injected with 1 to 10 ng of VR1 cRNA and recordings were performed using a two-electrode voltage-clamp (Warner Instrument Corp., Hamden, CT), over periods ranging between 3 and 8 days after injection, as described previously (Wahl et al., 1998). Drugs were applied through the bath solution and currents were typically elicited from a holding potential of −50 mV. Current-voltage (I-V) relationships were obtained from 3-s voltage ramps digitized at 1 kHz. Pulse and PulseFit software (HEKA Electronik, Darmstadt, Germany) was used for data acquisition and analysis.

[^{3}H]Phorbol-12,13-Dibutyrate (PdBu) Binding Experiments. [^{3}H]PdBu (Amersham Pharmacia Biotech; specific activity, 17.5 Ci/mmol) was used to label protein kinase C in both particulate and cytosolic fractions of rat spinal cord membranes exactly as described previously (Mortensen et al., 1995). The fractions were incubated with 5 nM [^{3}H]PdBu and together with the displacing agent for 90 min at 4°C. Nonspecific binding was defined as binding in the presence of 1 μM PdBu which accounted for 30 to 35% of total binding.

In Vivo Activity of I-RTX. The so-called "capsaicin pain test" was performed exactly as described previously (Sakurada et al., 1992) using male NMRI mice weighing 19 to 22 g. In brief, test compounds (5 μl in 50% dimethyl sulfoxide) or vehicle were administered intrathecally 5 min before injection of 20 μl of capsaicin (0.1% w/v) and the time mice spent licking their paws was recorded. The procedures used in this study were in accordance with the guidelines

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of the European Communities Council directive of 24 November 1986 (86/609/EEC) and the Danish State Animal Inspectorate approved the protocols.

Results

Characterization of [^{125}I]RTX Receptor Binding. High levels of specific [^{125}I]RTX binding to HEK 293 cells expressing VR1 were observed that were dependent upon temperature and pH. As shown in Fig. 2A, the optimal pH was around pH 7.8 to 8.0 and binding increased markedly with temperature up to 37°C and then decreased at higher temperatures (Fig. 2B). In the following experiments, pH was set to 7.8 and the temperature to 37°C. A time-course study revealed that maximal specific [^{125}I]RTX binding to membranes from HEK 293/VR1 cells was achieved after 30 to 60 min (Fig. 3A). Specific [^{125}I]RTX binding was reversible (Fig. 3A) and half of the specific binding was dissociated after about 4 min. HEK 293 cells expressing vector only did not show any specific binding, as defined by [^{125}I]RTX binding displaced by 100 nM RTX, and the levels in these control membranes were comparable with the nondisplaceable component of the dissociation curve with VR1/HEK 293 mem-

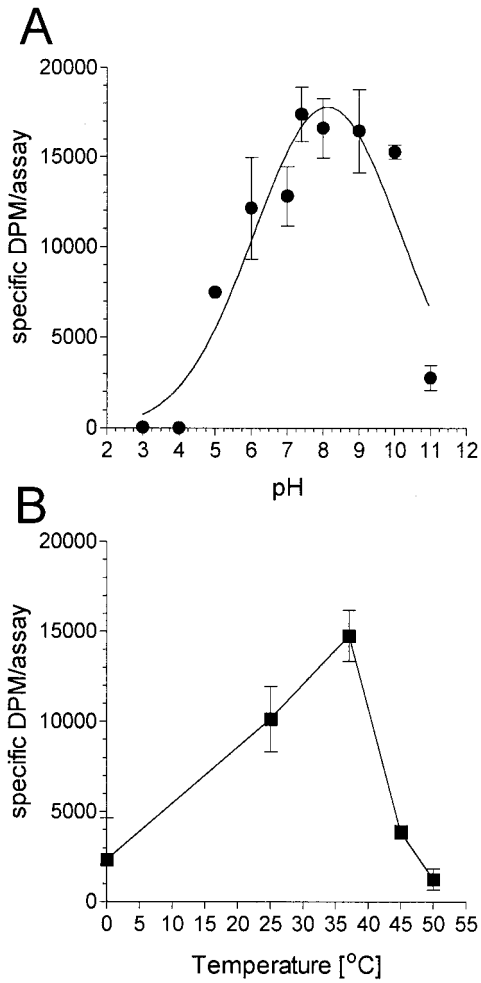

Fig. 2. Temperature and pH dependence of [^{125}I]RTX binding to VR1. A and B show the effects of varying pH and temperature, respectively, on [^{125}I]RTX binding to VR1. When the temperature was varied, the pH was held constant (7.8), whereas the temperature was 37°C in pH-sensitivity experiments. The samples were incubated for 60 min with 0.2 nM [^{125}I]RTX and nonspecific binding was defined by the binding observed in the presence of 100 nM RTX. Results are shown as specific [^{125}I]RTX binding and are mean ± S.E.M. of three to four experiments, which were performed in triplicate.

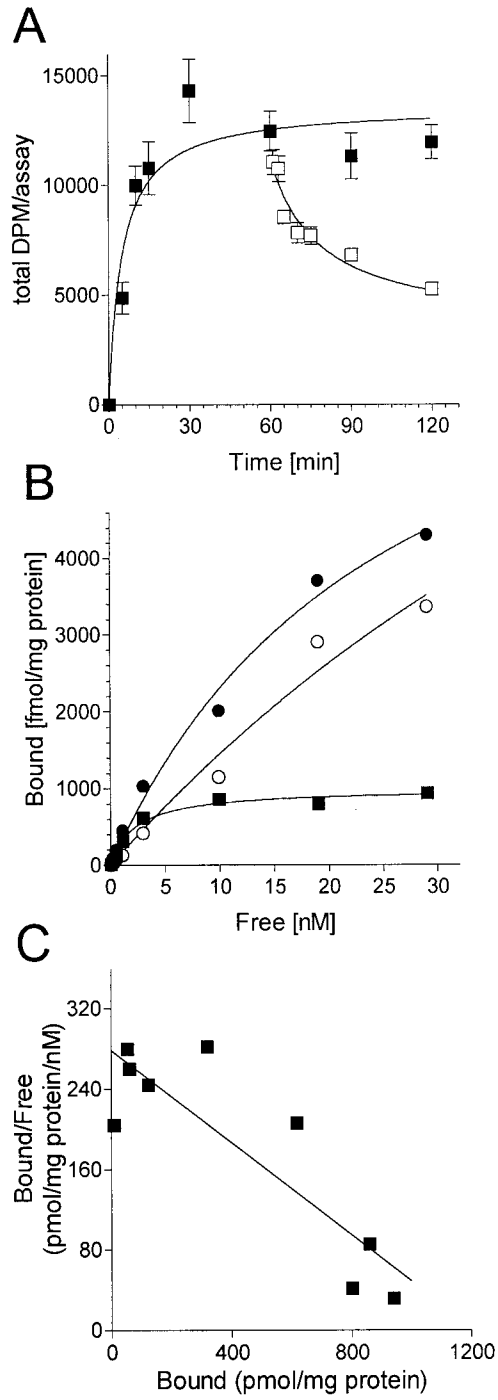

Fig. 3. Receptor binding kinetics of [^{125}I]RTX binding to VR1: A, the time course for association (■) and dissociation (□) of [^{125}I]RTX binding to VR1 at 37°C and pH 7.8. The data is presented as total [^{125}I]RTX binding and are means ± S.E.M. of three experiments that were performed in triplicate. In dissociation experiments 100 nM RTX was added and the residual amount of binding was determined at the time points indicated. B, a representative saturation binding experiment with [^{125}I]RTX showing total (●), nonspecific (○), and specific binding (■) which was performed as described under *Materials and Methods*. The results are shown as total [^{125}I]RTX binding and are means ± S.E.M. of four experiments that were performed in triplicate. C, Scatchard transformation of the saturation binding data which was fitted to an one-site receptor binding model.

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branes (data not shown). From the kinetic experiments, the calculated association constant, k_+ was 0.077 ± 0.006 nM/min and the apparent dissociation constant, k_- was 0.17 ± 0.02 nM/min. Saturation binding experiments showed that the binding was saturable (Fig. 3B) and a Scatchard transformation of the data indicated that [^{125}I]RTX binding to HEK 293/VR1 (K_d = 4.3 ± 0.9 nM) was in an apparent monophasic manner (Hill coefficient, 0.97 ± 0.02) with a relatively high capacity (B_{max}, 1.32 ± 0.26 pmol/mg of protein) (Fig. 3C). The affinity derived from the saturation binding experiments is in the range of the K_d value, which can be calculated from the time course experiments ($K_d = k_-/k_+$ = 2.2 nM). In rat spinal cord membranes, [^{125}I]RTX bound with a similar high affinity (K_d = 4.2 ± 1.0 nM) to a limited number of binding sites (B_{max} = 51 ± 8 fmol/mg of protein) also in an apparent monophasic manner (Hill coefficient, 0.93 ± 0.05) (data not shown). The pharmacology of [^{125}I]RTX binding to HEK 293/VR1 was compared with the endogenous rat VR in spinal cord membranes and the same rank order of affinity was observed: RTX > I-RTX > olvanil > capsaicin > capsazepine (Fig. 4; Table 1). In these experiments, I-RTX was more than 300-fold more potent compared with the standard VR1 antagonist, capsazepine. Ruthenium red did not displace [^{125}I]RTX binding to either HEK 293/VR1 membranes or rat spinal cord membranes at concentrations up to 30 μM (data not shown), in line with its noncompetitive nature.

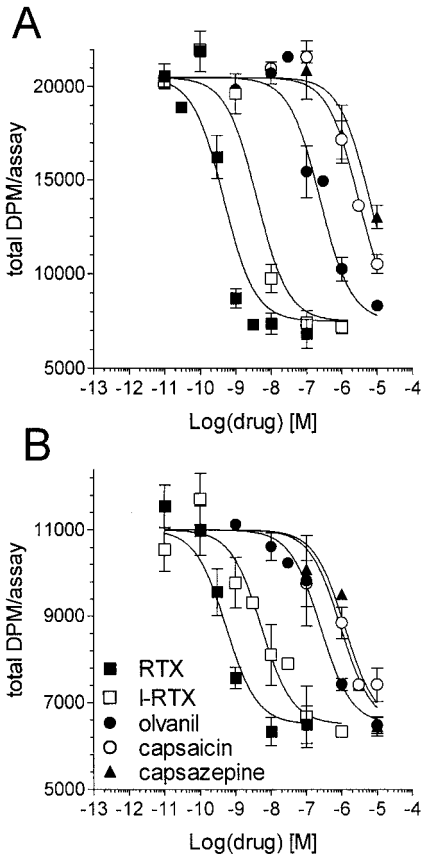

Fig. 4. Displacement by VR ligands of [^{125}I]RTX binding to membranes from (A) HEK 293 cells expressing VR1 and (B) rat spinal cord. For further information see Table 1. The data are expressed as total dpm and are means ± S.E.M. of three to five experiments that were performed in triplicate. Specific binding accounted for 55 to 65% (A) and 45 to 50% (B) of total binding in these experiments.

Selectivity of I-RTX for VR1. The presence of high levels of specific [^{125}I]RTX binding in HEK 293/VR1 cells and the absence of binding to control HEK 293 cells demonstrated that this isotope labels VR1 and not a secondary protein in these cells, such as protein kinase C. In rat spinal cord membranes, the complete displacement of [^{125}I]RTX binding by RTX, which is a specific VR1 agonist, also suggested that VR1 is specifically labeled. Nevertheless, because some RTX analogs (but not RTX) have been showed to have moderate affinity for protein kinase C, as measured by displacement of [^{3}H]PdBu binding (Acs et al., 1995), such experiments were performed to examine the specificity of I-RTX. [^{3}H]PdBu bound with high affinity to cytosolic (K_i = 5 ± 1 nM) and particulate (K_i = 7 ± 2 nM) spinal cord membrane fractions and in parallel experiments I-RTX showed no displacement of [^{3}H]PdBu binding to either fractions at concentrations up to 10 μM suggesting that I-RTX has no affinity for either the activated or nonactivated form of protein kinase C (data not shown). Furthermore, PdBu did not displace [^{125}I]RTX binding to VR1 at concentrations up to 10 μM (data not shown).

I-RTX Inhibits Capsaicin-Elicited Currents in VR1 Expressing *X. laevis* Oocytes. *X. laevis* oocytes expressing VR1 bath application of capsaicin or RTX in the absence of extracellular calcium at a holding potential of −50 mV elicited inward membrane currents, which showed little desensitization (Fig. 5). Noninjected oocytes showed no response to the highest concentration of agonist tested (data not shown). Capsaicin-evoked currents were fully reversible and could be repeated upon reapplication of capsaicin after an 8-min wash interval (Fig. 5). In contrast, RTX-induced currents did not return to baseline level even after a prolonged washout period (data not shown). Concentration-response curves were constructed by exposing individual oocytes sequentially to increasing concentrations of agonist with no intervening periods of wash. The EC_{50} values for capsaicin (Fig. 6B) were 1.3 μM and 0.18 nM for RTX (data not shown). Superfusion of VR1-expressing oocytes with I-RTX (1–10000 nM) elicited no detectable currents (see Fig. 5 for trace after application of 3000 nM). In contrast, when I-RTX was coapplied with 1 μM capsaicin, a dose-dependent inhibition of the capsaicin-evoked current was observed (IC_{50} = 3.8 nM) (Fig. 6A). The inhibition produced by I-RTX was not reversible within the time frame of the experiment (about 30 min), whereas capsaicin-evoked currents were reversibly inhibited by capsazepine (IC_{50} = 152 nM) (Fig. 6A). The mechanism of I-RTX-

TABLE 1

Binding affinities for [^{125}I]RTX binding to membranes from native rat VR1 and recombinant rodent VR1

IC_{50} values were calculated from competition binding experiments by a nonlinear regression analysis fitted to an one-site model using the GraphPad Prism program (GraphPad Software, San Diego, CA) and converted to K_i values using the equation $K_i = IC_{50}/(1 + [L]/K_d)$, where [L] is the concentration of radioligand (0.2 nM).

	Rat spinal cord K_i	HEK293/VR1 K_i
	nM	
Agonists		
RTX	0.36 ± 0.07	0.31 ± 0.06
Capsaicin	1995 ± 360	2030 ± 270
Olvanil	202 ± 35	397 ± 80
Antagonists		
Capsazepine	7880 ± 1570	2150 ± 850
Ruthenium red	>30.000	>30.000
I-RTX	4.8 ± 0.6	5.8 ± 1.1

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induced inhibition was investigated by constructing concentration-response curves for capsaicin in the absence and presence of I-RTX (Fig. 6B). The marked reduction of I-RTX on I_{max} and no shift in the EC_{50} value for the capsaicin concentration-response curve may suggest that I-RTX exert its action via a noncompetitive mechanism. Furthermore, a separate set of experiments showed that when I-RTX (3 nM) and capsaicin (100 μM) were coapplied for 12 min after an initial capsaicin (10 μM) application, the inhibitory effect of I-RTX could not be surmounted during prolonged capsaicin application (Fig. 6C). Finally, current-voltage relationships for capsaicin-induced currents in the absence and presence of 1 and 3 nM I-RTX are shown in Fig. 6D. In each case, capsaicin elicited outwardly rectifying cationic currents characteristic of VR1. These results demonstrate that inhibition of VR1-mediated ion currents by I-RTX is largely voltage independent, which is also expected from the neutral charge distribution of I-RTX (Fig. 1).

In Vivo Activity of I-RTX in the Capsaicin Pain Test. As shown in Fig. 7, intrathecal administration of I-RTX (ED_{50} = 16 ng/mouse), morphine (ED_{50} = 56 ng/mouse), and nociceptin (ED_{50} = 44 ng/mouse) effectively blocked pain responses induced by injection of capsaicin into the paw of mice. Capsazepine in doses up to 3 μg/mouse was ineffective in this test (data not shown).

Discussion

Research in the field of VRs has taken a major step forward with the recent molecular cloning of a capsaicin-sensitive VR, VR1. However, more potent and selective VR antagonists are a prerequisite for obtaining a better understanding of the physiology of these receptors. In the present study, we describe the synthesis and pharmacological characterization of a new potent antagonist that is specific to the VR. This antagonist, I-RTX is at least 40-fold more potent than capsazepine, which was previously known as the most potent VR antagonist. Moreover, I-RTX can readily be radiolabeled to a high specific activity with ^{125}I; this isotope has been shown to be a highly useful probe for labeling VR1. Because of the structural similarity of RTX with activators of protein kinase C (e.g., PdBu) the selectivity of RTX for VRs over protein kinase C has been thoroughly investigated [see Szallasi and Blumberg (1999) for review]. However, RTX showed no ap-

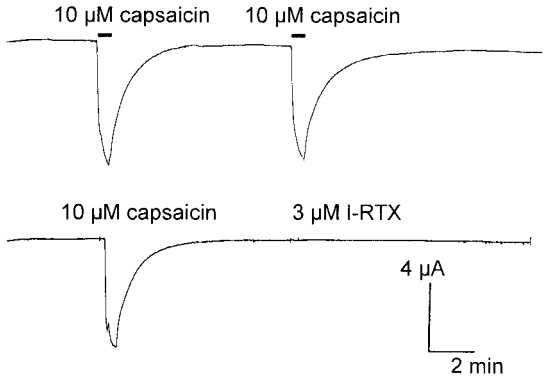

Fig. 5. Nondesensitizing VR1-mediated currents evoked by capsaicin but not by I-RTX. Recordings were performed at a holding potential of −50 mV and in the absence of extracellular calcium, where responses were nondesensitizing and reproducible after 8-min wash interval. Bars indicate the duration of drug application.

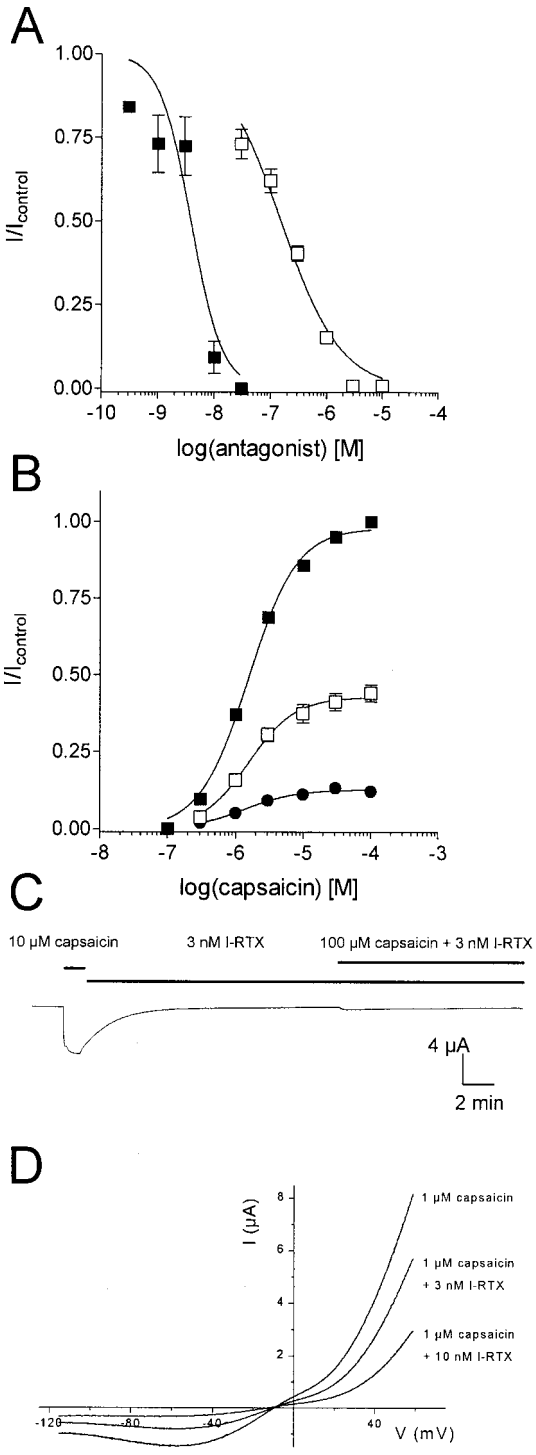

Fig. 6. Apparent noncompetitive inhibition by I-RTX of VR1 expressed in *X. laevis* oocytes. A, concentration-inhibition curves comparing potencies of capsazepine (□) and I-RTX (■) at VR1 expressed as a fraction of control responses (mean ± S.D., n = 3). B, concentration-response curves of capsaicin in the absence (■) and presence of 3 nM (□) or 10 nM I-RTX (●). C, sample record illustrating currents elicited by capsaicin (10 μM) and capsaicin (100 μM) plus I-RTX (3 nM) from an oocyte expressing VR1. Horizontal bars, time of drug application. D, current-voltage relationships for currents obtained from a VR1-expressing oocyte elicited by capsaicin in the absence or presence of 10 and 100 nM I-RTX. A voltage ramp was applied from −140 mV to + 60 mV in 3 s during the steady-state current elicited by 1 μM capsaicin, 1 μM capsaicin + 10 nM I-RTX, and 1 μM capsaicin + 100 nM I-RTX. I-V curves of responses to capsaicin were constructed by subtracting I-V curves obtained in the control solution.

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preciable affinity for protein kinase C as measured by displacement of [^{3}H]PdBu binding (Szallasi and Blumberg, 1990; Acs et al., 1995) and this selectivity did not seem to be altered by placing an iodine atom in the phenol moiety of RTX (present study). Thus, whereas I-RTX is less potent than the ultrapotent agonist RTX, it seems to maintain the selectivity for VRs.

The general binding characteristics of [^{125}I]RTX to VR1 are very similar compared with previous studies using [^{3}H]RTX binding to rat spinal cord membranes (Szallasi and Blumberg, 1990; Szallasi et al., 1995) in terms of temperature optimum, kinetics, and pH dependence. However, whereas [^{125}I]RTX showed an apparent monophasic pattern of binding to both the recombinant VR1 and the endogenous VR in rat spinal cord membranes, the binding properties of [^{3}H]RTX to rat spinal cord membranes or VR1 is more complex, showing a high degree of positive cooperativity (Szallasi et al., 1995; Acs et al., 1996, 1999). The above differences seem likely be related to the fact that [^{3}H]RTX is an agonist radioligand, whereas [^{125}I]RTX is an antagonist that causes no conformational changes in the receptor complex (Colquhoun, 1998). The receptor density of VRs in the rat spinal cord as determined by [^{125}I]RTX saturation binding experiments (B_{max} = 51 ± 8 fmol/mg protein) is reminiscent of the values reported using [^{3}H]RTX (B_{max} = 43 ± 3 fmol/mg of protein) using a similar preparation (Acs and Blumberg, 1994). The affinity (K_d) of [^{3}H]RTX for rat spinal cord or dorsal root ganglion membranes is generally reported to be very high (K_d = 15–30 pM) (Szallasi et al., 1995), although lower values have also been observed (K_d = 0.3–0.6 nM) (Szallasi and Blumberg, 1990). When measuring displacement of [^{125}I]RTX binding from spinal cord membranes, RTX showed an affinity (K_i) of 0.3 nM, which is consistent with the latter studies. In the case of the additional VR-active compounds examined in the present study, the K_i values obtained for displacement of [^{125}I]RTX binding to spinal cord membranes (see Table 1) are in very good agreement with previous affinities obtained using [^{3}H]RTX (Szallasi et al., 1993). Overall, these data strongly suggest that [^{3}H]RTX and [^{125}I]RTX label the same receptor in the rat spinal cord; this receptor is likely to be VR1 given the identical pharmacology of [^{125}I]RTX binding observed in rat spinal cord membranes and HEK 293/VR1 membranes (Table 1).

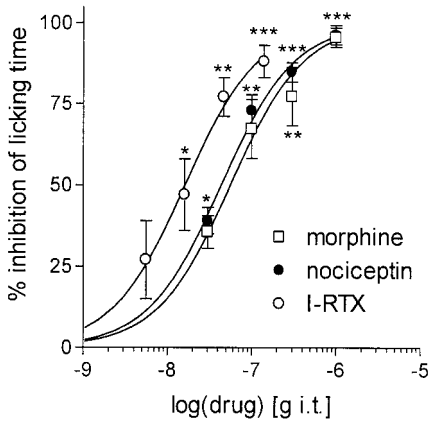

Fig. 7. Effects of intrathecal I-RTX on licking time (mean ± S.E.M.) induced by injection of capsaicin under the skin in the paw of mice. The effect of morphine and nociceptin is shown for comparison. Asterisks indicate a significant effect of the compound compared with saline injected mice (*$P < 0.05$, **$P < 0.01$, ***$P < .001$, nonpaired two-tailed t test, n = 12–16).

Capsazepine is used as the standard competitive antagonist to block VR-mediated responses in various preparations. However, the observed potency of capsazepine is modest, with ED_{50} values ranging from 0.2 μM and up to 5 μM; most frequently, 10 μM is used to block VR responses (Bevan et al., 1992; Szallasi et al., 1993; Acs et al., 1996; Caterina et al., 1997; Liu et al., 1997; Wardle et al., 1997) which has also been shown to result in nonspecific effects on ion-channels (e.g., nicotinic and calcium channels) (Docherty et al., 1997; Liu and Simon, 1997). The introduction of iodine in RTX completely removed the agonist properties of RTX because no currents were induced at concentrations of I-RTX up to 3 μM (Fig. 5). The potency of I-RTX to block capsaicin-induced currents in oocytes expressing VR1 (IC_{50} = 3.9 nM) is in good agreement with its affinity for inhibiting [^{125}I]RTX binding (Table 1). However, the apparently noncompetitive mode of action of I-RTX is in contradiction to the receptor binding data in the present study. Accordingly, the complete displacement of [^{125}I]RTX binding by VR agonists such as capsaicin, olvanil, and RTX and also by the competitive antagonist capsazepine strongly suggested that I-RTX recognizes the agonist binding domain of VR1 and thus is a competitive antagonist. These data may suggest that the binding site for I-RTX is distinct from the agonist binding site for capsaicin and RTX and that the effect on binding is caused by an allosteric interaction. However, considering the close structural similarity between RTX and I-RTX, a more likely explanation may be that the off-rate of I-RTX in functional measurements is too slow to allow for measuring competition by capsaicin within the time frame studied. In such functional measurements, the VR agonist RTX is very difficult to wash out, and I-RTX seems to have similar properties. Indeed, prolonging the time of exposure to capsaicin up to 15 min, even at a 100 μM concentration, did not result in functional responses to capsaicin when measured in the presence of 5 nM I-RTX (Fig. 6C). Because the ligand binding domain of the VR1 channel is believed to be located intracellularly (Jung et al., 1999), this ligand may be "trapped" inside the cell; this is not the case in receptor binding experiments to disrupted membrane fractions.

Finally, specific VR antagonists are likely to have therapeutic potential for alleviating chronic pain, analogous to the use of capsaicin and RTX for such conditions (Szallasi and Blumberg, 1999). In fact, I-RTX alleviated capsaicin-induced licking responses (Fig. 7) in the "capsaicin test" (Sakurada et al., 1992) more potently than morphine and nociceptin. In contrast, capsazepine in doses up to 3 μg/mouse was ineffective in relieving the capsaicin-evoked pain responses but this may be related to its poor affinity for the VR and its nonspecific effects at higher concentrations (Docherty et al., 1997; Liu and Simon, 1997; Wardle et al., 1997). Finally, the therapeutic potential of VR antagonists seems quite favorable in the sense that they may block nociceptive transmission mediated by VR without causing any initial activation and concomitant pain.

Acknowledgments

We acknowledge the skilled technical assistance of Lisbeth Eriksen, Lone Sørensen, Anne-Mette Dall, Thokil F. Emdal, Tinna Larsen, and Dorte D. Andersen.

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