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Neurogenic responses mediated by vanilloid receptor-1 (TRPV1) are blocked by the high affinity antagonist, iodo-resiniferatoxin

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1 Stimulation of the vanilloid receptor-1 (TRPV1) results in the activation of nociceptive and neurogenic inflammatory responses. Poor specificity and potency of TRPV1 antagonists has, however, limited the clarification of the physiological role of TRPV1.

2 Recently, iodo-resiniferatoxin (I-RTX) has been reported to bind as a high affinity antagonist at the native and heterologously expressed rat TRPV1. Here we have studied the ability of I-RTX to block a series of TRPV1 mediated nociceptive and neurogenic inflammatory responses in different species (including transfected human TRPV1).

3 We have demonstrated that I-RTX inhibited capsaicin-induced mobilization of intracellular Ca^{2+} in rat trigeminal neurons (IC_{50} 0.87 nM) and in HEK293 cells transfected with the human TRPV1 (IC_{50} 0.071 nM).

4 Furthermore, I-RTX significantly inhibited both capsaicin-induced CGRP release from slices of rat dorsal spinal cord (IC_{50} 0.27 nM) and contraction of isolated guinea-pig and rat urinary bladder (pK_B of 10.68 and 9.63, respectively), whilst I-RTX failed to alter the response to high KCl or SP.

5 Finally, *in vivo* I-RTX significantly inhibited acetic acid-induced writhing in mice (ED_{50} 0.42 μmol kg^{-1}) and plasma extravasation in mouse urinary bladder (ED_{50} 0.41 μmol kg^{-1}).

6 In *in vitro* and *in vivo* TRPV1 activated responses I-RTX was $\sim$3 log units and $\sim$20 times more potent than capsazepine, respectively. This high affinity antagonist, I-RTX, may be an important tool for future studies in pain and neurogenic inflammatory models.

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Keywords: Capsaicin; capsazepine; iodo-resiniferatoxin; resiniferatoxin; vanilloid receptor-1

Abbreviations: HEK, human embryonic kidney; I-RTX, iodo-resiniferatoxin; RTX, resiniferatoxin; TRPV1, vanilloid receptor-1

Introduction

A subset of primary sensory neurons found within dorsal root (DRG), trigeminal and vagal ganglia, which include both C and Aδ fibres, contain the neuropeptides, substance P (SP), neurokinin A (NKA) and calcitonin gene-related peptide (CGRP) (Holzer, 1991). These neuropeptides are released from central and peripheral terminals of these neurons upon activation of a recently cloned (Caterina *et al*., 1997), non-selective cation channel that belongs to the transient receptor potential (TRP) family of channels (Gunthorpe *et al*., 2002). Because of the ability of this channel to be gated by capsaicin, the pungent ingredient found in chilli peppers, and by other molecules with a vanilloid moiety (Szallasi & Blumberg, 1999) it has been termed vanilloid receptor-1 (TRPV1) (Caterina *et al*., 1997). An heterogeneous series of physical and chemical agents has been shown to activate and/or potentiate the activity of TRPV1. These agents include noxious heat (43–52°C) (Caterina *et al*., 1997), protons (pH 6-5) (Bevan & Geppetti, 1994; Tominaga *et al*., 1998), anandamide (Zygmunt *et al*., 1999), 12-HPETE and other lipid derivatives (Hwang *et al*., 2000) and ethanol (Trevisani *et al*., 2002). Two different studies performed in TRPV1 null mice indicate that TRPV1 mediates thermal hyperalgesia (Caterina *et al*., 2000; Davis *et al*., 2000). Recent findings that TRPV1 immunoreactivity is higher in gastrointestinal tissues taken from patients with ulcerative colitis and Crohn's disease (Yiangou *et al*., 2001), suggest that TRPV1 may play a role in the mechanism of these inflammatory conditions. However, the role of TRPV1 in the pathophysiology of such diseases is still elusive, and one of the reasons for these uncertainties is the absence of high affinity and selective TRPV1 antagonists.

Ruthenium red, a dye that exhibits properties of non-competitive antagonism for the TRPV1 (Amann & Maggi, 1991), has a poorly defined mechanism of action and its selectivity, if any, is restricted to a very narrow range of concentrations (Szallasi & Blumberg, 1999). The search for a selective TRPV1 blocker, and potential novel analgesic, led to the discovery of capsazepine (Bevan *et al*., 1992; Dickenson & Dray, 1991; Urban & Dray, 1991). Capsazepine was characterized as a competitive TRPV1 antagonist but is hindered by moderate potency (EC_{50} values ranging from 0.2 to 5 μM) (Acs *et al*., 1997; Bevan *et al*., 1992; Caterina *et al*.,

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1997; Liu & Simon, 1997; Szallasi *et al*., 1993; Wardle *et al*., 1996). In addition, at the concentrations ($\sim$10 μM) often required for antagonistic activity, capsazepine has shown non-specific effects, including blockage of voltage-gated calcium channels (Docherty *et al*., 1997) and nicotinic receptors (Liu & Simon, 1997; Wardle *et al*., 1997).

The ultrapotent TRPV1 agonist, resiniferatoxin (RTX), a toxin isolated from the cactus *Euphorbia resinifera* (Szallasi & Blumberg, 1999) has been successfully used to label the TRPV1 in radioreceptor binding assays by using its [^{3}H] and [^{125}I] derivatives. Recently, it has been reported (Wahl *et al*., 2001) that the iodinated form of resiniferatoxin (iodo-resiniferatoxin (I-RTX): 6,7-Deepoxy-6,7-didehydro-5-deoxy-21-dephenyl-21-(phenylmethyl)-daphnetoxin,20-(4-hydroxy-5-iodo-3-methoxybenzeneacetate) inhibited the binding of [^{125}I]-RTX to rat spinal cord membranes and HEK293 cells transfected with the rat TRPV1 with an affinity much higher than capsazepine (K_i, $\sim$5 nM and $\sim$5 μM, respectively). Because I-RTX did not show any agonistic activity, blocked capsaicin-induced currents in *Xenopus leavis* oocytes expressing the rat TRPV1 (IC_{50} 3.9 nM) and reduced the nociceptive response induced by intrathecal capsaicin injection in mice, it was proposed that I-RTX behaves as a high affinity TRPV1 antagonist (Wahl *et al*., 2001).

A high affinity and selective TRPV1 antagonist, in addition to being a lead for developing novel analgesics, may be of unprecedented value for discovering endogenous TRPV1 agonists and exploring the physiological role of TRPV1. TRPV1 stimulation results in the activation of a series of nociceptive and neurogenic inflammatory responses (Holzer, 1991; Szallasi & Blumberg, 1999; Szallasi & Di Marzo, 2000). Thus, the present study was undertaken to define the ability of I-RTX to inhibit 'typical' TRPV1 mediated responses in different mammalian species. These responses include capsaicin-induced increases in intracellular Ca^{2+} ($[Ca^{2+}]_i$) in cultured primary sensory neurones or HEK293 cells transfected with the human TRPV1 (hTRPV1 HEK293), release of CGRP and SP from slices of rat dorsal spinal cord, contraction of isolated guinea-pig bronchus or urinary bladder and of rat urinary bladder, neurogenic plasma protein extravasation in the mouse urinary bladder and acetic acid-induced writhing response in mice.

Methods

Animals and tissues

Male albino Dunkin-Hartley guinea-pigs ($\sim$ 250 g), Sprague-Dawley rats ($\sim$300 g) and Swiss mice ($\sim$30 g) were used (Morini, Italy). All experiments complied with the national guidelines and were approved by the regional ethics committee.

Ca^{2+} fluorescence measurements in cultured rat trigeminal ganglia and hTRPV1 HEK293 cells

Newborn rats (2–3 days old) were terminally anaesthetized and decapitated. The trigeminal ganglia were removed and rapidly placed in cold phosphate buffered solution (PBS) before being transferred to collagenase/dispase (1 mg ml^{-1} dissolved in Ca^{2+}-Mg^{2+}-free PBS) for 35 min at 37°C. Enrichment of the fraction of nociceptive neurons was obtained following the methods reported previously (Gilabert & McNaughton, 1997). After the enzymatic treatment ganglia were rinsed three times with Ca^{2+}-Mg^{2+}-free PBS and then placed in 2 ml of cold DMEM supplemented with 10% foetal bovine serum (FBS, heat inactivated), 2 mM L-glutamine, 100 u ml^{-1} penicillin and 100 μg ml^{-1} streptomycin. The ganglia were then dissociated into single cells by several passages through a series of syringe needles (23G down to 25G). Finally, the complex of medium and ganglia cells were sieved through a 40 μm filter to remove debris and topped up with 8 ml of DMEM medium and centrifuged (200 $\times$ *g* for 5 min). The final cell pellet was re-suspended in DMEM medium (supplemented with 100 ng ml^{-1} mouse Nerve Growth Factor (mouse-NGF-7S) and cytosine-b-D-arabino-furanoside free base (ARA-C) 2.5 μM). Cells were plated on poly-L-lysine (8.3 μM) and laminin (5 μM) coated 25 mm glass cover slips and kept for 2 to 5 days at 37°C in a humidified incubator gassed with 5% CO_2 and air. Cells were fed on the second day (and subsequent alternate days) with DMEM medium (with 1% FBS instead of 10% FBS). In another set of experiments hTRPV1 HEK293 were also used.

Experiments were performed as previously reported in rat DRG neurons (Tognetto *et al*., 2001). Briefly, plated neurons and hTRPV1 HEK293 cells (2 to 5 days) were loaded with Fura-2-AM-ester (3 μM) in Ca^{2+} buffer solution of the following composition (mM): $CaCl_2$ 1.4, KCl 5.4, $MgSO_4$ 0.4, NaCl 135, D-glucose 5, HEPES 10 with BSA 0.1%, at pH 7.4, for 40 min at 37°C, washed twice with the Ca^{2+} buffer solution and transferred to a chamber on the stage of Nikon eclipse TE300 microscope. The dye was excited at 340 and 380 nm to indicate relative $[Ca^{2+}]_i$ changes by the F_{340}/F_{380} ratio recorded with a dynamic image analysis system (Laboratory Automation 2.0, RCS, Florence, Italy). Capsaicin (0.1 μM), I-RTX (0.001–1 nM), capsazepine (0.01–10 μM) or their respective vehicles were added to the chamber. A calibration curve using a buffer containing Fura-2-AM-ester and determinant concentrations of free Ca^{2+} (Kudo *et al*., 1986) was used to convert the data obtained from F_{340}/F_{380} ratio to $[Ca^{2+}]_i$ (nM). Furthermore, I-RTX (10 nM) did not affect the response produced by ionomycin (5 μM) (data not shown).

CGRP-LI and SP-LI release from rat spinal cord

Rats were terminally anaesthetized and decapitated. The dorsal spinal cord was prepared at 4°C using a tissue slicer (McIlwain Tissue Chopper, U.K.). Slices ($\sim$100 mg) were placed in 2 ml chambers and superfused at 0.4 ml ml^{-1} with a Krebs solution of the following composition (mM) NaCl 119, $NaHCO_3$ 25, KH_2PO_4 1.2, $MgSO_4$ 1.5, $CaCl_2$ 2.5, KCl 4.7 and D-glucose 11. To the basic Krebs solution the following agents were added: 0.1% bovine serum albumin (BSA), 1 μM phosphoramidon and 1 μM captopril, and maintained at 37°C (gassed with 95% O_2 and 5% CO_2). High KCl solution was prepared by changing isoosmotically NaCl with KCl. After a 90 min stabilization period, 10 min fractions were collected into acetic acid (final solution 2 N). Two pre-stimuli samples were taken at 10 min intervals followed by a third set of samples during stimulation. A final post-stimulus 10 min sample was also collected. At the end of the experiment tissues were blotted and weighed. Fractions

were freeze-dried, re-constituted with assay buffer, and analysed by enzyme-immunoassays for CGRP and SP like immunoreactivities (LI) (CGRP-LI and SP-LI, respectively) according to the methods reported previously (Frobert *et al*., 1999; Ricciardolo *et al*., 2000). The detection limits of the assays were 5 pg ml^{-1} for CGRP and 2 pg ml^{-1} for SP. The level of release of CGRP-LI and SP-LI were calculated by subtracting the mean pre-stimulus value from those values obtained during- and post-stimulation. The results are expressed as fmol of peptide g^{-1} tissue 20 min. The highest concentration of capsaicin (10 μM), I-RTX (1 nM) and capsazepine (10 μM) did not show any significant cross-reactivity with CGRP and SP antisera.

Isolated tissues in organ baths

Animals were sacrificed by cervical dislocation and the airways and urinary bladder of the guinea-pig, and urinary bladder of the rat were removed. In the guinea-pig, rings from main bronchi (approximately 2 mm in width) were suspended with a resting tension of 1.5 g. In both the guinea-pig and rat urinary bladders vertical halves were suspended with a resting tension of 1 g. The tissues were bathed and aerated (95% O_2 and 5% CO_2) with Krebs solution (described above) which was maintained at 37°C, and contained phosphoramidon (1 μM) and captopril (1 μM) to minimize peptide degradation. Tissues were allowed to equilibrate for 60 min prior to the beginning and between each set of experiments (washed every 5 min). In all experiments the tissues were first contracted with carbachol (CCh, 1 μM).

Cumulative concentration-response curves were performed with capsaicin (0.1 nM – 100 μM) and SP (0.1 nM – 1 μM) either in the presence of the TRPV1 antagonists, I-RTX (0.01 – 1 μM in bronchi and 0.1 – 10 nM in urinary bladders) and capsazepine (0.1 – 10 μM in all tissues) or their respective vehicles. In a subset of experiments cumulative concentration-response curves were also performed with resiniferatoxin (0.1 nM – 1 μM).

Plasma extravasation

Swiss mice were anaesthetized with xylazine (100 mg kg^{-1}, i.m.) and ketamine (50 mg kg^{-1}, i.m.). Evans Blue (30 mg kg^{-1}) was injected into the femoral vein and 1 min later the administration of capsaicin (1 μmol kg^{-1}, i.v.) was performed. After five additional min animals were transcardially perfused. Pre-treatments with I-RTX (0.1 – 0.5 μmol kg^{-1}, i.v.), capsazepine (0.5 – 10 μmol kg^{-1}, i.v.), or their respective vehicles were given 20 and 15 min prior to the injection of the dye, respectively. Urinary bladders were removed, weighed and incubated in 1 ml of formamide for 24 h in the dark, at room temperature. The amount of extravasated Evans Blue was measured spectrophotometrically at 620 nm.

Writhing test

Swiss mice (~25 g) were intraperitoneally (i.p.) injected with acetic acid (0.9%, 5 ml kg^{-1}) after pretreatment with I-RTX (0.1 – 1 μmol kg^{-1}, i.p. 20 min prior to the stimulus), capsazepine (1 – 10 μmol kg^{-1}, i.p. 60 min prior to the stimulus) or their vehicles (0.01 – 0.1% DMSO and 0.01 – 0.1% ethanol, respectively).

Materials

Drugs and reagents were obtained from the indicated companies: acetic acid, capsaicin, capsazepine, captopril, ionomycin, laminin, phosphoramidon, poly-L-lysine, resiniferatoxin, substance P (Sigma, Italy); iodo-resiniferatoxin (Tocris, U.K.); mouse NGF-7S and collagenase/dispase (Roche Diagnostics, Italy); Dulbecco's Modified Eagle's medium (DMEM), foetal bovine serum (FBS) heat inactivated, L-glutamine (200 mM), penicillin/streptomycin (10,000 IU ml^{-1} – 10,000 UG ml^{-1}), Ca^{2+}-Mg^{2+}-free phosphate buffered solution (PBS) (Gibco, Italy); Fura-2-AM-ester (Societa' Italiana Chimici, Italy). SLIGKV-NH_2 was synthesized at the Laboratory of Pharmaceutical Sciences of the University of Ferrara. The stock concentrations of capsaicin (10 mM), capsazepine (10 mM) and resiniferatoxin (10 mM) were prepared in 100% ethanol. Fura-2-AM-ester and ionomycin were dissolved in 100% DMSO. All other drugs were dissolved in distilled water. The appropriate dilutions were then made in Krebs buffer solution.

Statistical analysis

The arithmetic mean and standard error of the mean were calculated throughout. Contractile responses are expressed as a percentage (%) of the response to CCh (1 μM). The concentration/dose of an agonist or antagonist that produced 50% of the maximal effect (EC_{50}, IC_{50} and ED_{50}) were obtained with an iterative curve fitting package (GraphPad Prism Software, San Diego, CA, U.S.A.; Origin Software, Microcal Software, Northampton, MA, U.S.A.). Values for pK_B, as a measure of the antagonist affinity were calculated according to Kenakin (1993). The pK_B value was calculated for each tissue, with the final value given as mean ± s.e.mean. Statistical analysis was performed by means of the Student's *t*-test or analysis of variance (ANOVA) and the Dunnett's test when required. If $P<0.05$ the results were considered significant.

Results

Ca^{2+} fluorescence measurements in cultured rat trigeminal ganglia and hTRPV1 HEK293 cells

Capsaicin (0.1 μM) caused an increase in $[Ca^{2+}]_i$ in the vast majority (>95%) of rat trigeminal neuronal cells (64 ± 9% of ionomycin, $n=85$), that therefore were identified as TRPV1 expressing neurons. The threshold concentration of I-RTX that produced an inhibitory effect was 0.1 nM and complete inhibition of the response to capsaicin was obtained with 10 nM I-RTX. IC_{50} of I-RTX to inhibit capsaicin-evoked $[Ca^{2+}]_i$ mobilization was 0.87 nM (Figures 1 and 2 and Table 1). For capsazepine (IC_{50}, 2344 nM) threshold and blocking concentrations were 1 μM and 100 μM, respectively (Figures 1 and 2 and Table 1). Mobilization of $[Ca^{2+}]_i$ evoked by 5 mM KCl (94 ± 13% of ionomycin, $n=44$) was not affected by I-RTX (1 μM) (90 ± 4% of ionomycin, $n=38$).

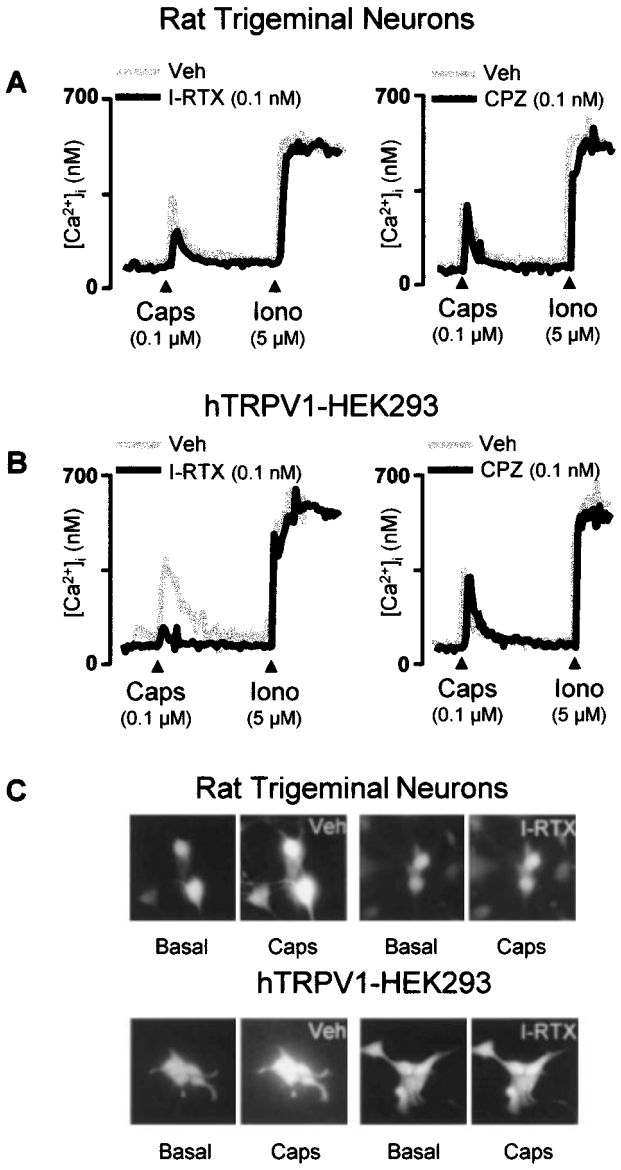

Figure 1 Typical tracings and microscopic images showing the inhibitory effect of iodo-resiniferatoxin (I-RTX, 0.1 nM) or capsazepine (CPZ, 0.1 nM) on capsaicin (0.1 μM) induced Ca^{2+} mobilization in rat trigeminal neurons (A, C) and HEK293 cells transfected with the human TRPV1 (hTRPV1 HEK293; B, C).

Practically all the hTRPV1 HEK293 cells responded to 0.1 μM capsaicin evoking a remarkable increase in $[Ca^{2+}]_i$ (54$\pm$3% of ionomycin, $n=57$). The threshold concentration of I-RTX to show an inhibitory effect was 0.03 nM and complete inhibition of the response to capsaicin was obtained with 3 nM I-RTX. IC_{50} of I-RTX to inhibit capsaicin-evoked $[Ca^{2+}]_i$ mobilization was 0.071 nM (Figure 1 and Table 1). For capsazepine (IC_{50} was 912 nM) the threshold and blocking concentrations were 1 μM and 10 μM, respectively (Table 1). Mobilization of $[Ca^{2+}]_i$ evoked by the proteinase activated receptor-2 (PAR2) agonist, SLIGKV-NH_2 (10 μM) (87$\pm$6% of ionomycin, $n=98$) was not affected by I-RTX (1 μM) (97$\pm$12% of ionomycin, $n=60$). I-RTX (1 μM), in both rat trigeminal neurons and hTRPV1 HEK293 cells did not cause any visible increase in $[Ca^{2+}]_i$.

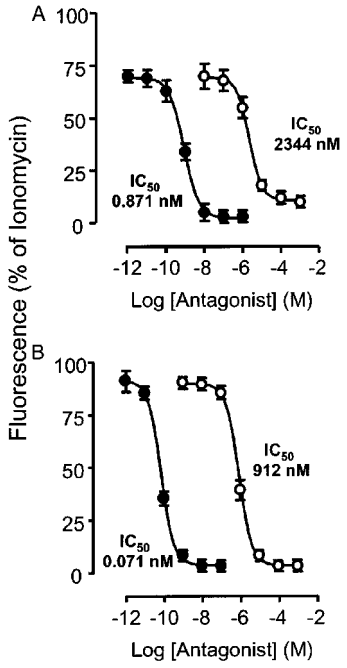

Figure 2 The inhibitory effect of iodo-resiniferatoxin (I-RTX, filled circles), capsazepine (CPZ, open circles) on capsaicin (0.1 μM) -induced increases in cytoplasmic Ca^{2+} ion concentration in rat trigeminal neurons (A) and HEK293 cells transfected with the human TRPV1 (hTRPV1 HEK293; B). Each entry is the mean$\pm$s.e.mean of at least 80 cells; $*P<0.05$.

Table 1 Affinities of capsazepine and iodo-resiniferatoxin for the TRPV1 in *in vitro* and *in vivo* in a number of responses activated by capsaicin

	Capsazepine	*Iodo-Resiniferatoxin*
In vitro		
Guinea-pig	pK_B	pK_B
Urinary bladder	6.56$\pm$0.20	10.68$\pm$0.24
Bronchus	6.29$\pm$0.31	7.26$\pm$0.40
Rat	pK_B	pK_B
Urinary bladder	6.22$\pm$0.40	9.63$\pm$0.30
Rat	IC_{50} (nM)	IC_{50} (nM)
TG neurons (Ca^{2+})	2344$\pm$53.9	0.871$\pm$0.009
CGRP release (dorsal spinal cord)	3802$\pm$0.04	0.269$\pm$0.06
Human	IC_{50} (nM)	IC_{50} (nM)
TRPV1 HEK cells	912.0$\pm$10.5	0.071$\pm$0.001
In vivo		
Mouse	ED_{50} (μmol kg^{-1})	ED_{50} (μmol kg^{-1})
Plasma extravasation	9.03$\pm$1.02	0.41$\pm$0.08
Writhing (acetic acid)	7.90$\pm$1.13	0.42$\pm$0.05

CGRP-LI and SP-LI release from rat spinal cord

Next we examined the effect of TRPV1 antagonists on capsaicin-induced release of CGRP-LI and SP-LI from slices of rat spinal cord. Capsaicin (0.1 μM) increased CGRP-LI and SP-LI outflow (488$\pm$90 fmol g^{-1} 20 min, $n=5$; and 31.5$\pm$3.5 fmol g^{-1} 20 min, $n=5$, respectively). Exposure of spinal cord slices to either I-RTX (1 nM) or capsazepine

(10 μM) did not produce any significant increase in CGRP-LI or SP-LI (data not shown) outflow. Pre-treatment with I-RTX (0.01 – 1 nM) and capsazepine (1 – 10 μM, Figure 3) reduced the capsaicin-induced increase in CGRP-LI outflow in a concentration-dependent manner, with IC_{50} that were 0.269 nM and 3802 nM, respectively (Table 1). I-RTX (1 mM) did not alter the release induced by a high KCl (80 mM) concentration (Figure 3).

Contractile responses in isolated guinea-pig bronchus and urinary bladder, and rat urinary bladder

Capsaicin evoked a robust concentration-related contraction of guinea-pig isolated bronchi and urinary bladder, and rat urinary bladder. Threshold concentration of capsaicin to cause a visible contractile effect was 1 nM in all tissues tested, and maximum response (% of CCh 1 μM, E_{max}) obtained with 1 μM of capsaicin was 107 $\pm$ 9% (n = 20) in guinea-pig bronchus, 58 $\pm$ 12% (n = 15) in guinea-pig urinary bladder and 39 $\pm$ 14% (n = 12) in rat urinary bladder. Exposure to I-RTX (1 μM) did neither affect the baseline tension in all the three tissues examined nor change the response to CCh 1 μM (data not shown). I-RTX (1 nM – 1 μM) caused a concentration-dependent and surmountable antagonism of capsaicin-induced contraction in all the tissues tested. Schild plot analysis of the results revealed pK_B values in the guinea-pig bronchi and urinary bladder of 7.26 $\pm$ 0.40 (n = 12, Figure 4) and 10.68 $\pm$ 0.24 (n = 10, Figure 5) respectively, and in rat urinary bladder of 9.63 $\pm$ 0.30 (n = 12, Figure 6) (Table 1). A similar study was performed with capsazepine (0.1 – 10 μM) against capsaicin-induced contraction: pK_B values in the guinea-pig bronchus and urinary bladder were 6.29 $\pm$ 0.31 (n = 8, Figure 4) and 6.56 $\pm$ 0.20 (n = 8, Figure 5) respectively, and in the rat urinary bladder was 6.22 $\pm$ 0.40 (n = 9, Figure 6) (Table 1). I-RTX (0.1 nM – 1 μM) did not affect the cumulative concentration response curve produced by SP in isolated guinea-pig bronchi (Figure 4) and urinary bladder (Figure 5), and rat urinary bladder (Figure 6).

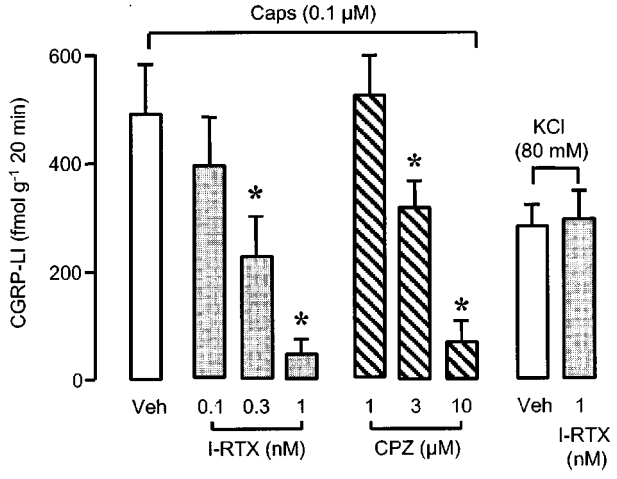

Figure 3 The inhibitory effect of iodo-resiniferatoxin (I-RTX, closed bars), capsazepine (CPZ, hatched bars) or the combination of their respective vehicles (empty bar) on the release of calcitonin gene-related peptide-like immunoreactivity (CGRP-LI) induced by capsaicin (0.1 μM) or KCl (80 mM) from slices of rat dorsal spinal cord. Each entry is the mean $\pm$ s.e.mean of at least five experiments; *P < 0.05.

I-RTX had no effect on baseline tone at any of the concentrations tested (1 nM, 10 nM, 100 nM and 1 μM) and in any of the isolated tissues (data not shown). In contrast, RTX (0.1 – 100 nM) caused a robust contractile response starting at 0.3 nM producing a maximum contraction similar to that of capsaicin (data not shown).

Plasma protein extravasation in mouse urinary bladder

Intravenous (i.v.) injection of capsaicin (1 μmol kg^{-1}) induced a significant increase in plasma extravasation in mouse urinary bladder (11.2 $\pm$ 1.3 ng EB/g of tissue, n = 6) as compared to the extravasation produced by the vehicle of capsaicin (4.1 $\pm$ 0.7 ng EB/g of tissue, n = 6, P < 0.01). Pretreatment with I-RTX (0.1 – 0.5 μmol kg^{-1}, i.v.) caused a dose-related inhibition (ED_{50} 0.41 $\pm$ 0.08 μmol kg^{-1}, Figure 7 and Table 1). Capsazepine (0.5 – 10 μmol kg^{-1}, i.v.) pretreatment also inhibited capsaicin-induced plasma extravasation in the urinary bladder with an estimated ED_{50} of 9.03 $\pm$ 1.02 μmol kg^{-1} (Figure 7 and Table 1). At the maximum dose used, I-RTX (0.5 μmol kg^{-1}, i.v.) alone neither produced any measurable increase in PE (data not shown) nor affected SP induced plasma extravasation in the mouse urinary bladder (Figure 7).

Writhing in mice

Intraperitoneal injections of acetic acid (0.9%, 5 ml kg^{-1}) evoked a significant increase in stereotypical writhing

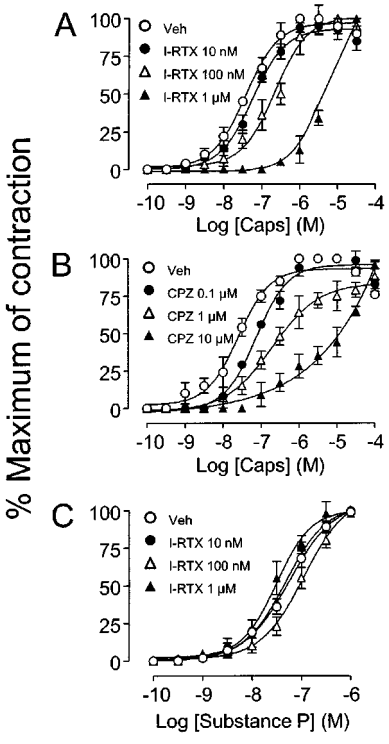

Figure 4 The effect of iodo-resiniferatoxin (I-RTX), capsazepine (CPZ) or their respective vehicles (empty circles) on the contraction produced by increasing concentrations of capsaicin (A, B) or substance P (C) (both given in a cumulative manner) in isolated guinea-pig bronchial rings. Each entry is the mean $\pm$ s.e.mean of at least six experiments.

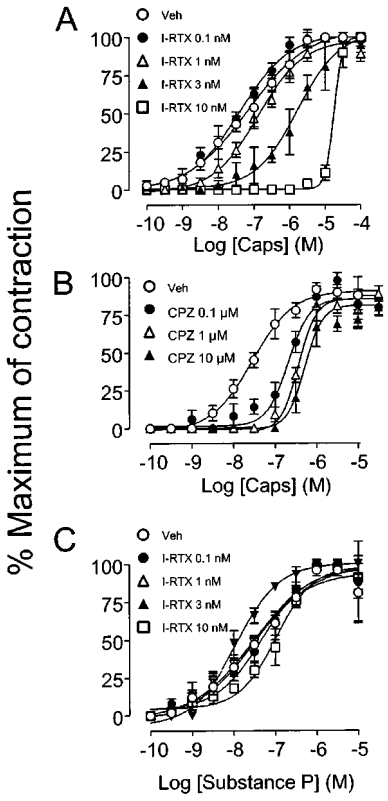

Figure 5 The effect of iodo-resiniferatoxin (I-RTX), capsazepine (CPZ) or their respective vehicles (empty circles) on the contraction produced by increasing concentrations of capsaicin (A, B) or substance P (C) (given in a cumulative manner) in isolated strips of guinea-pig urinary bladder. Each entry is the mean ± s.e.mean of at least six experiments.

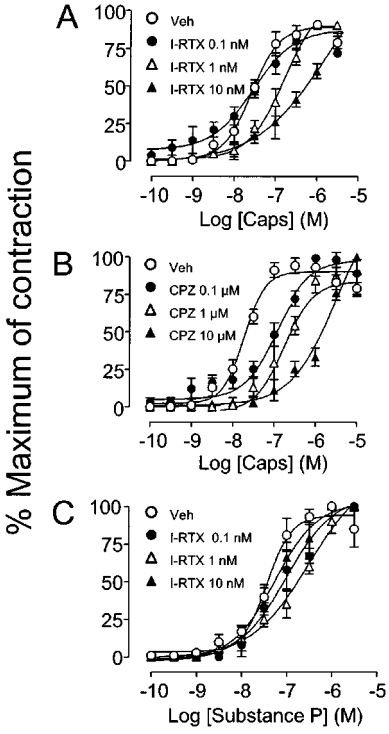

Figure 6 The effect of iodo-resiniferatoxin (I-RTX), capsazepine (CPZ) or their respective vehicles (empty circles) on the contraction produced by increasing concentrations of capsaicin (A, B) or substance P (C) (given in a cumulative manner) in isolated strips of rat urinary bladder. Each entry is the mean ± s.e.mean of at least six experiments.

responses over 20 min in mice. These writhing episodes were significantly reduced by pre-treatment with I-RTX (0.1–1 μmol kg^{-1}, i.p.) in a dose-related manner (ED_{50}: 0.42 ± 0.05 μmol kg^{-1}, Figure 8). Capsazepine (1–10 μmol kg^{-1}, i.p.) also inhibited the acetic acid induced writhing responses in mice with an ED_{50} of 7.90 ± 1.13 μmol kg^{-1} (Figure 8). I-RTX (1 μmol kg^{-1}, i.p.) caused in a few instances (two out of six mice tested) a few writhing responses (< 10/20 min).

Discussion

In the present study, we have investigated the ability of I-RTX to inhibit 'typical' neurogenic inflammatory and nociceptive responses produced by activation of TRPV1 in experimental preparations from different mammalian species. The main finding of this study is that I-RTX is a highly potent TRPV1 antagonist in all the paradigms and species examined, with the exception of the guinea-pig bronchus. First, we examined the ability of I-RTX to reduce the capsaicin-induced increase in $[Ca^{2+}]_i$ in cultured trigeminal neurons. In this preparation IC_{50} value of I-RTX was in the subnanomolar range, thus showing that I-RTX was about three log units more potent than capsazepine. These data are in complete agreement with the study reported by Wahl *et al.* (2001) where they estimated that the K_i of I-RTX for displacement of [^{125}I]-RTX binding from membranes of native (rat spinal cord) or recombinant (rat TRPV1 HEK293 cells) TRPV1 was approximately 1000 fold lower than that of capsazepine. In the present study I-RTX showed selectivity because at the highest concentration used it did not affect the increase in $[Ca^{2+}]_i$ produced by a high K^+ medium. Because KCl-induced $[Ca^{2+}]_i$ mobilization in sensory neurons is mediated by the influx of extracellular Ca^{2+} mainly through N and L type voltage sensitive Ca^{2+} channels, these findings indicate that I-RTX does not inhibit these type of channels. Furthermore, I-RTX showed a very high affinity for the human TRPV1 expressed in HEK293 cells, where I-RTX inhibited the capsaicin-induced increase in $[Ca^{2+}]_i$ with an IC_{50} of 0.071 nM confirming a similar previous observation (McDonnell *et al.*, 2002). The inhibitory response of I-RTX was more than four log units lower than that of capsazepine and 10 fold lower than that observed for I-RTX at the rat TRPV1 which is consistent with recent finding that antagonism at TRPV1, in this case by capsazepine, is greater in cells expressing human TRPV1 as compared to the rat (McIntyre *et al.*, 2001; Wahl *et al.*, 2001). HEK293 cells express constitutively the proteinase activated receptor-2 (PAR2) (Hollenberg *et al.*, 1997). The observation that I-RTX did not affect PAR2 agonist-induced increases in $[Ca^{2+}]_i$ indicates a degree of selectivity for the human TRPV1.

Increases in $[Ca^{2+}]_i$ produced by TRPV1 activation result in a series of intracellular events that lead to secretion of neuropeptides from peripheral and central endings of primary

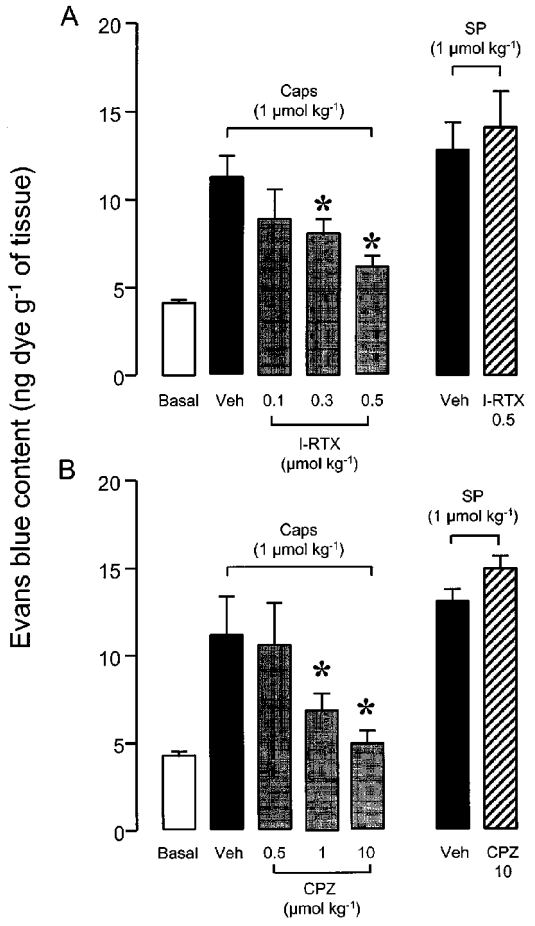

Figure 7 The effect of iodo-resiniferatoxin (I-RTX), capsazepine (CPZ) or the combination of their respective vehicles (black bars) on the Evans blue extravasation induced by the intravenous injection of capsaicin (Caps) or substance P (SP) in the mouse urinary bladder. Each entry is the mean ± s.e.mean of at least six experiments; $^*P<0.05$.

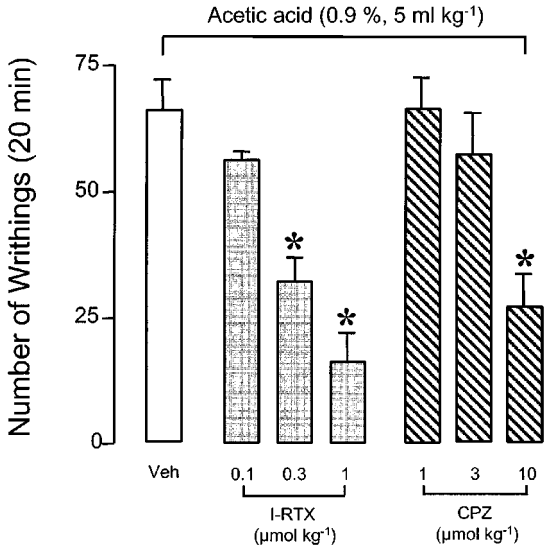

Figure 8 The inhibitory effect of iodo-resiniferatoxin (I-RTX), capsazepine (CPZ) or the combination of their respective vehicles (empty bar) on the number of writhing responses produced by intraperitoneal administration of acetic acid in mice. Each entry is the mean ± s.e.mean of at least seven experiments; $^*P<0.05$.

sensory neurons. SP and CGRP release from central terminals of primary afferents is considered to be a major function of nociceptors because it has been associated with pain transmission (Otsuka & Yanagisawa, 1990). In this important assay I-RTX also proved to be a high affinity antagonist of the rat TRPV1, being more than 1000 fold more potent than capsazepine. Again, the observation that 1 μM I-RTX did not affect the ability of high K^+ ion concentrations to release CGRP/SP indicates selectivity for TRPV1 over voltage-gated mechanisms.

Release of neuropeptides from peripheral terminals of primary sensory neurons that ensues from TRPV1 activation causes a series of responses collectively referred to as 'neurogenic inflammation' (Geppetti & Holzer, 1996). In the airways and urinary bladder neurogenic inflammatory responses include contraction of the bronchial and urinary smooth muscle and extravasation of plasma proteins in postcapillary venules. I-RTX was extremely potent in inhibiting capsaicin-induced contractions of the guinea-pig and rat urinary bladder. As in other assays, the pK_B of I-RTX was in both these cases more than three log units higher than that of capsazepine. At variance with all these observations, I-RTX potency to reduce capsaicin-induced contractions of guinea-pig bronchi was much lower ($\sim$100x) than that found in all the other preparations studied, and only slightly higher than that of capsazepine. This finding is consistent with previous reports that showed a low affinity constant of RTX (either iodinated or not) in binding assays using guinea-pig bronchial tissues (Szallasi *et al*., 1993). The observation that in the guinea-pig urinary bladder I-RTX shows a potency similar to that observed at the rat and human TRPV1, excludes that I-RTX has a low affinity for the guinea-pig TRPV1, but rather suggests that some hitherto unknown mechanism(s), specific for the guinea-pig bronchus is responsible for the low capability of RTX (Szallasi *et al*., 1993) and I-RTX to bind and (I-RTX) inhibit the TRPV1 in this tissue.

One possible explanation for the remarkable potency of I-RTX to inhibit the TRPV1 in different mammal species is that this molecule retains some agonistic activity from the parent drug, RTX, that causes desensitization of TRPV1 and of downstream mechanisms resulting from TRPV1 activation (Szallasi *et al*., 1993). However, the absolute absence of any visible agonist effect of low and even high I-RTX concentrations, in all the preparations tested, suggests that I-RTX does not act *via* first activating and then desensitizing the TRPV1/sensory nerve terminal, but most likely by its ability to antagonize the channel.

The low potency and poor selectivity for TRPV1 of ruthenium red and capsazepine has limited their use, particularly in *in vivo* studies. The present investigation, using a classical TRPV1 mediated *in vivo* response in the form of the capsaicin-induced plasma extravasation in the urinary bladder, shows that I-RTX is much more potent than capsazepine. The lack of effect of high doses of I-RTX upon SP-induced plasma extravasation indicates that TRPV1 and I-RTX act upstream of the release of tachykinins which stimulates NK_1 receptors on endothelial cells of the postcapillary venule and it provides further support for the

selectivity of I-RTX both *in vivo* as well as *in vitro*. An important point for the use of I-RTX for pathophysiological investigations is to demonstrate that this compound may block responses induced by endogenous stimulants of TRPV1. Acidic media have been proposed to be putative endogenous agonists of TRPV1 (Bevan & Geppetti, 1994) and the cloning of the rat TRPV1 has confirmed that protons stimulate this receptor (Tominaga *et al*., 1998). A recent study, showing that capsazepine inhibits the acetic acid induced writhing behaviour in mice, suggested that this complex nociceptive response involves an afferent arm in which the TRPV1 has a role (Ikeda *et al*., 2001). We found that I-RTX was about 20 fold more potent than capsazepine in reducing the acetic acid-induced writhing, which has two implications: firstly, it confirms the data obtained using capsazepine using an additional antagonist, and secondly, that I-RTX may be used to test the ability of putative endogenous agonists to stimulate nociceptive responses activated by the TRPV1.

In conclusion the present investigation confirms the early observation (Wahl *et al*., 2001) that I-RTX is a potent antagonist at the rat TRPV1. The study, for the first time shows that I-RTX exhibits high affinity for the TRPV1 in additional mammal species including the guinea-pig and human TRPV1. The fact that I-RTX at subnanomolar concentrations and with high selectivity blocks TRPV1-mediated responses indicates that this molecule may be an invaluable tool for the restless search of the physiological role(s) and of the putative endogenous pain-producing agonist of TRPV1.

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