Characterization of the Vanilloid Receptor 1 Antagonist Iodo-Resiniferatoxin on the Afferent and Efferent Function of Vagal Sensory C-Fibers

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ABSTRACT
The effect of iodo-resiniferatoxin (I-RTX) on efferent function (tachykinergic contractions of airway smooth muscle) and afferent function (action potential discharge) of vagal C-fibers mediated by vanilloid receptor 1 (VR1) activation was studied in an isolated guinea pig airway preparation. I-RTX (1 µM) had no VR1 agonist activity in either the afferent or efferent assays. I-RTX (30 nM–1 µM) shifted the resiniferatoxin and capsaicin concentration-response curves for neurokinin-mediated contractions rightward but did not inhibit the maximum response. The pKᵢ value calculated from 0.3 µM I-RTX against resiniferatoxin and capsaicin was 7.3 ± 0.2 and 6.8 ± 0.2, respectively, showing 10 to 30 times higher potency compared with capsazepine. The slope of Schild plot from the resiniferatoxin efferent studies deviated from unity (~0.6), suggesting complex interactions at VR1 binding site(s). This notion was further supported by lack of additional inhibitory effect of 1 µM I-RTX on capsaicin-evoked contractions compared with 0.3 µM I-RTX. Concentrations of I-RTX up to 1 µM had no effect on trypsin-induced neurokinin-mediated contractions, nor neurokinin A-induced contractions of guinea pig trachea. However, nonselective effects on airway smooth muscle contractions were noted with 10 µM I-RTX. In both afferent and efferent studies I-RTX (30 nM–10 µM) caused a substantial delay of the response to capsazepine. This led to an apparent increase in potency in experiments where the agonist was applied transiently, with insufficient time to reach equilibrium. I-RTX inhibited contractions induced by anandamide and action potential discharge induced by low pH, showing that the I-RTX-antagonism of VR1 does not strictly depend on the vanilloid nature of the agonist.

A defining feature of many nociceptive sensory fibers is their sensitivity to capsaicin (Maggi and Meli, 1988; Julius and Basbaum, 2001). Capsaicin and related compounds such as resiniferatoxin have therefore been used to great advantage as pharmacological tools with which to probe sensations and reflexes associated with nociceptive nerve activation. A major breakthrough in capsaicin pharmacology was the molecular characterization of the capsaicin receptor (Caterina et al., 1997). This receptor, referred to as vanilloid receptor 1 (VR1), is an ionotropic receptor. When capsaicin binds to the receptor the ion channel opens and cations cross the membrane, resulting in membrane depolarization and calcium influx. The membrane depolarization can lead to action potential discharge (afferent function), and the calcium flux can lead to local release of neurokinins and other transmitters stored in vesicles within the peripheral nerve terminals (afferent function) (Maggi and Meli, 1988).

In recent years, several endogenous agonists of VR1 have been identified, including anandamide, hydrogen ions, and certain lipoygenase products of arachidonic acid (Tominaga et al., 1998; Zygmunet et al., 1999; Hwang et al., 2000). In addition, it has been shown that heat can gate the VR1 ion channel (Caterina et al., 1997). The presence of endogenous agonists supports the hypothesis that VR1 may not only be involved in the pharmacological activation of nociceptors but also play a role in the physiological activation of these fibers. To ascertain the role of VR1 in various physiological processes requires either the use of animals in which the VR1 gene has been deleted (Caterina et al., 2000), selective desensitization strategies, or the use of specific inhibitors of VR1.

Until recently, pharmacological inhibitors of VR1 have been limited to capsazepine and ruthenium red. Capsazepine is a competitive antagonist of vanilloid binding, whereas ruthenium red noncompetitively acts as a VR1 channel.

ABBREVIATIONS: VR1, vanilloid receptor 1; I-RTX, iodo-resiniferatoxin; KBS, Krebs bicarbonate solution; NKA, neurokinin A; NK1, neurokinin 1 receptor; NK2, neurokinin 2 receptor; SR48968, (S)-N-methyl-N-[4-acetylamino-4-phenylpiperidino]-2-[3,4-dichlorophenyl]butylbenzamide; SR140333, (S)-1-[2-[3-[3-(4,4-dichlorophenyl)]-1-(3-isoproxyphenyl)acetyl]piperidin-3-yl]ethy]-4-phenyl-1-azoniabicyclo[2.2.2]octane chloride.
blocker (Amann and Maggi, 1991; Dickenson and Dray, 1991). A drawback of these two inhibitors is their nonselectivity in action. Both capsazepine and ruthenium red at concentrations only slightly greater than those needed to inhibit capsaicin responses are known to inhibit a variety of ion channels (Docherty et al., 1997; Liu and Simon, 1997). The recent discovery of a novel and very potent inhibitor of VR1 was therefore welcomed by those interested in VR1 pharmacology. The potent antagonist was discovered when it was noted that an iodinated form of the VR1 agonist resiniferatoxin competed with vanilloids for VR1 binding, but had no intrinsic efficacy (Wahl et al., 2001). Binding studies carried out in rat spinal cord membrane preparations showed that iodo-resiniferatoxin (I-RTX) was a competitive antagonist of vanilloids at VR1 with a $K_i$ value of about 5 nM. In this preparation, I-RTX had an affinity for VR1 approximately 8000 times greater than capsazepine (Wahl et al., 2001).

Functional evidence that I-RTX acts as an antagonist for VR1 was limited to whole-cell patch-clamp studies of membrane currents in oocytes transfected to express VR1. Again, the data were impressive with a concentration of I-RTX as low as 3 nM effectively blocking the inward current evoked by 100 µM capsaicin. In this functional assay, in contrast to the binding studies, the antagonism by capsaicin was insurmountable, arguing against a purely competitive antagonist (Wahl et al., 2001).

The pharmacology of VR1 expressed in oocytes, however, may be different than that expressed in nociceptive afferents within tissues. The types and extent of glycosylation of VR1 as well as the manner with which the VR1 peptides form a multimeric ion channel may be different in different expression systems (Kedei et al., 2001). Inasmuch as I-RTX will likely become an invaluable research tool for the study of VR1 biology, experiments were designed with the purpose of more fully characterizing the pharmacology of I-RTX in inhibiting vanilloid activation of C-fiber afferent nerve endings in the guinea pig isolated airway preparation. The guinea pig isolated airway preparation is an ideal model to study both the efferent and afferent activity of vagal C-fibers. The guinea pig airway receives a relatively dense neurokinin-containing C-fiber innervation, and neurokinins are potent and effective contractile agonists of guinea pig airway smooth muscle (Ellis and Undem, 1994). Therefore, airway smooth muscle contraction in response to VR1 activation is a convenient assay for neurokinin secretion from airway sensory nerves. Action potential discharge in identified single vagal C-fibers can also be readily quantified in this model using standard electrophysiological approaches (Fox et al., 1995; Riccio et al., 1996a).

**Materials and Methods**

**Effferent Function of C-Fibers.** Male Hartley guinea pigs (200–400 g) were killed by asphyxiation with CO$_2$ and exsanguinated. The trachea was removed and divided into consecutive rings, each roughly two to three cartilage rings in width. Tracheal rings were placed in tissue baths and tied with silk surgical suture to force displacement transducers (PT03C; Grass Instruments, Quincy, MA) for recording of isometric tension on a polygraph (Grass Instruments). Resting tension was set at 1 g. Tissue baths contained 30 ml of Krebs bicarbonate solution (KBS; 118 mM NaCl, 5.4 mM KCl, 1.0 mM NaH$_2$PO$_4$, 1.2 mM MgSO$_4$, 1.9 mM CaCl$_2$, 25.0 mM NaHCO$_3$, and 11.1 mM dextrose), which was maintained at 37°C and gassed with 95% O$_2$, 5% CO$_2$ and replaced every 15 min during a 60-min equilibration period. Indomethacin (3 µM) was added to KBS to suppress prostaglandin release from the tissue. Thiorphan (1 µM) was added to the tissue bath 15 min before the completion of equilibration period to suppress the neutral endopeptidase activity (Ellis and Undem, 1990). This method has been described elsewhere (Carr et al., 2000). The experiments were approved by the Johns Hopkins Animal Care and Use Committee.

After the equilibration period, I-RTX was added 30 min before the addition of agonist. In preliminary experiments, incubation with I-RTX for 30 min was found to have greater effect than incubation for 15 min. Prolonging the incubation for 60 min, however, did not further increase the effect of I-RTX, suggesting that antagonist has reached equilibrium at the receptor within the 30-min period. Cumulative concentration-response curves for resiniferatoxin (0.1 nM–1 µM), capsaicin (3 nM–3 µM), and anandamide (1 µM–3 mM) were carried out in the presence of I-RTX (30 nM; 0.3 µM, and 1 µM for resiniferatoxin and capsaicin; 1 µM for anandamide) or vehicle dimethyl sulfoxide (1 µM). In paired experiments, resiniferatoxin concentration-response curves were carried out in the presence and absence of capsazepine (1 µM). After the final concentration of agonist was added, the tissues were maximally contracted with 30 mM BaCl$_2$. The time delay between the addition of agonist and the onset of contraction was also recorded for analysis. To assess the nonspecific effects of I-RTX on the system, cumulative concentration-response curves for trypsin (1–100 µg/ml) and neurokinin A (NKA) were obtained in the presence of I-RTX (1 µM for trypsin; 1 µM and 10 µM for NKA) or vehicle.

**Afferent Function Studies.** Male Hartley guinea pigs (200–400 g) were killed as described above. The airways with intact right-side vagal innervation were removed and placed in a dissecting dish containing KBS. Connective tissue was trimmed away, leaving the trachea, larynx, and right bronchi with intact nerves (vagus, superior laryngeal, and recurrent) and nodose and jugular ganglia. A longitudinal cut was made along the ventral surface to open the larynx, trachea, and bronchi. Airways were then pinned to a Sylgard-lined Perspex chamber. The right nodose and jugular ganglia, along with the rostral-most vagus and superior laryngeal nerves, were pulled through a small hole into an adjacent compartment of the same chamber for extracellular recording. Both compartments were separately superfused with the KBS containing 3 µM indomethacin. The KBS was gassed with 95% O$_2$, 5% CO$_2$, the temperature was maintained at 37°C, and the flow rate was 6 to 8 ml min$^{-1}$. This method has been described previously (Riccio et al., 1996b). The experiments were approved by the Johns Hopkins Animal Care and Use Committee.

Extracellular recordings were performed with a fine alumino-silicate glass electrode placed near cell bodies in the jugular ganglion. The microelectrodes were pulled using a Flaming Brown micropipette puller (Sutter Instrument, Novato, CA) and filled with 3 M sodium chloride. The electrode was placed into an electrode holder connected directly to a headstage (A-M Systems, Everett, WA). A return electrode of silver-silver chloride wire and an earthed silver-silver chloride pellet were placed in the perfusion fluid of the recording chamber and attached to the headstage. The recorded signal was amplified (A-M Systems) and filtered (low cutoff, 0.3 kHz; high cutoff, 1 kHz), and the resultant activity was displayed on an oscilloscope (TDS 340; Tektronix, Beaverton, OR) and a chart recorder (model TA240S; Gould, Valley View, OH). The data were stored on digital tape (DTC-59ES; Sony, Tokyo, Japan) for off-line waveform analysis on a Macintosh computer using the software program TheNerveOfft (PHOCIS, Baltimore, MD).

Single fiber activity was discriminated by placing a concentric electrical stimulating electrode on the recurrent laryngeal nerve, through which the majority of fibers enter the trachea. The recording electrode was placed within the ganglion and manipulated until single unit activity was detected. When electrically evoked action potentials were seen, the stimulator was switched off, and the tra-
Mechanically sensitive receptive fields were revealed when a burst of action potentials was elicited in response to mechanical stimulation. Mechanical thresholds were determined for each nerve ending by using von Frey filaments (Stoelting, Wood Dale, IL) calibrated to give fixed amounts of force ranging from 0.078 to 2.738 mN. Beginning with the lowest force von Frey filament, nerve endings were gently probed with filaments of increasing force until a threshold mechanical sensitivity was determined. This was achieved when touching of the receptive field evoked a burst of action potentials. Confirmation of threshold sensitivity was established by probing the nerve ending with the subthreshold filament. Conduction velocities were calculated by electrically stimulating the receptive field and measuring the distance traveled along the nerve pathway divided by the time between the shock artifact and the recorded action potential. Conduction velocity and amplitude of the action potential were then compared with responses elicited by electrical stimulation of either the superior laryngeal, recurrent laryngeal, or vagus nerve trunks to determine the trunk that supplied the fiber. In the majority of experiments, one fiber per animal was recorded. Occasionally, the activity of two fibers projecting to the airways was recorded simultaneously.

In this study, capsaicin-sensitive C-fiber nociceptors with cell bodies in jugular ganglion were used. Because in preliminary experiments airway nociceptors desensitized in response to repeated capsaicin exposure, each tissue was treated with capsaicin only once. Transient administration of capsaicin was achieved by administering a 500-µl aliquot of capsaicin (0.1 or 0.3 µM) into superfusion over the mechanically sensitive receptive field of the airway C-fiber in ~3 s. Continuous administration of capsaicin was carried out by superfusing the compartment with airway tissue with KBS containing capsaicin (0.3 or 1 µM) for 20 min. Because of desensitization, action potential discharge induced by continuous administration of capsaicin did not follow any regular pattern of activation. Some fibers responded only for a relatively brief period of time (a few minutes), whereas other fibers responded throughout the 20-min exposure. We therefore limited our quantitation of action potential discharge to the 3-min period after the onset of activation. In the studies examining the effect of I-RTX on capsaicin response, the airway tissue was superfused with KBS containing I-RTX (30 nM or 1 µM) for 30 min before the capsaicin administration, and I-RTX was continuously present in the superfusion during the capsaicin treatment. We noted no obvious difference in the pattern of response in control and I-RTX-treated tissues.

Citric acid (10 mM) challenge was preformed by administration a 500-µl aliquot into superfusion over the mechanically sensitive receptive field in ~3 s. Preliminary experiments showed that the response to citric acid was reproducible up to four times in 15-min intervals. In paired experiments, airway C-fibers were treated with citric acid before and after 30 min of superfusion with 1 µM I-RTX.

Drugs. BaC12, capsaicin, citric acid, indomethacin, resiniferatoxin, and thiorphan were obtained from Sigma-Aldrich (St. Louis, MO). Bovine trypsin was obtained from Worthington Biochemicals (Freehold, NJ). Iodo-resiniferatoxin was purchased from Tocris Cookson (Ellisville, MO). Neurokinin A was obtained from Cambridge Biochemical (Wilmington, DE). SR140333 and SR48968 were used to block NK1 and NK2 receptors, respectively (kindly provided by Zeneca Pharmaceuticals, Wilmington, DE). BaC12, NKA, trypsin, and citric acid were dissolved in distilled water. Capsaicin, indomethacin, and thiorphan were dissolved in absolute ethanol. Resiniferatoxin and iodo-resiniferatoxin were dissolved in dimethyl sulfoxide. Subsequent dilution of all drugs except citric acid was in KBS. Citric acid was diluted in saline.

Data Analysis. Contraction in guinea pig isolated tracheal and bronchial preparations was expressed as a percentage of maximal contraction induced by 30 mM BaC12. Apparent dissociation constants (KB) were calculated separately for each agonist and each concentration of I-RTX using the standard equation of (antagonist)/[(dose ratio – 1), converted to the negative logarithm, and expressed as pKB. Schild plot was constructed from the series of curves using Microsoft Excel, and pA2 and slope of the Schild plot were obtained from the line of best fit. The efferent function data were expressed as arithmetic mean ± S.E.M. and compared using Student’s nonpaired t test.

Activation of airway C-fibers was expressed as the number of action potentials elicited by agonist treatment. To quantify the response to transient exposure to capsaicin total number of action potentials was used. For prolonged exposure to capsaicin, the number of action potentials was counted in 3-min interval after the onset of the response. In addition, the delay to the onset of the response was measured. The response to citric acid was expressed as a total number of action potentials. The afferent function data were compared using Student’s paired and nonpaired t test as appropriate.

Results

Efferent Function Studies. As we and other have noted previously, capsaicin and resiniferatoxin stimulate the release of neurokinins from sensory nerves in the isolated guinea pig airway preparation, leading to smooth muscle contractions that can be abolished by blocking NK1 and NK2 receptors (Belvisi et al., 1992; Ellis and Undem, 1994). This is therefore a useful assay for the quantitative study of the efferent function of airway C-fibers. In our initial series of experiments we assessed I-RTX for any nonspecific effects on the guinea pig airway smooth muscle response to exogenously applied NKA. I-RTX (1 µM) had no effect on NKA-induced contractions of guinea pig airway smooth muscle. In five experiments the NKA concentration-response curves for tracheal contractions in the absence and presence of I-RTX were superimposable (data not shown). The –log (M) ED50 for NKA was 11.1 ± 0.3 and 11.3 ± 0.5 in the presence and the absence of I-RTX, respectively (P > 0.1). However, at a concentration of 10 µM, I-RTX caused a significant rightward shift in the NKA concentration-response curve that averaged 4 ± 0.2-fold (n = 5, P < 0.01; data not shown), indicating a nonspecific inhibitory effect of this large concentrations of I-RTX on airway smooth muscle contraction. Therefore, in all studies the maximum concentration of I-RTX evaluated was 1 µM. I-RTX alone, even at 10 µM, had no direct effect on airway smooth muscle tone (i.e., it revealed no partial agonist activity at VR1 in this assay).

The effect of different concentrations of I-RTX on the concentration-response curve for resiniferatoxin-induced airway contraction is shown in Fig. 1. All concentrations of I-RTX from 30 nM to 1 µM caused rightward shifts in the resiniferatoxin concentration-response curves without affecting the maximum response. When results from this series of curves were plotted as a Schild plot (Fig. 1, inset), a pA2 value of 7.8 was obtained from a line of best fit. The slope of the Schild plot, however, obviously deviated from unity, suggesting something other than simple competition at a single binding site was occurring. For comparison purposes, in paired experiments, capsaizepine (10 µM) shifted the resiniferatoxin curve to the right in a parallel manner. Consistent with our previous study, the calculated pKB for capsaizepine was 5.8 ± 0.2 (n = 6) (Ellis and Undem, 1994). The pA2 value calculated from 0.3 µM I-RTX against resiniferatoxin was 7.3 ± 0.2. Thus, I-RTX was approximately 30 times more potent than capsaizepine, although this conclusion must be cautiously interpreted because of the shallow slope in the Schild plot.
I-RTX. In other words, at these concentrations the capsaicin concentration-response curve became stationary (Fig. 2). The capsaicin-induced contraction obtained in the absence or presence of 1 μM I-RTX were abolished by the combination of NK1 and NK2 receptor antagonists (SR140333 and SR48968, 1 μM each, n = 2; data not shown). An estimated pKᵢ value of 6.8 ± 0.2 (n = 8) was calculated from results obtained 0.3 μM I-RTX against capsaicin. This value was not significantly different than that obtained when the data used to calculate the pKᵢ was with 0.3 μM I-RTX against resiniferatoxin (7.3 ± 0.2, n = 7).

In addition to shifting the capsaicin concentration-response curve to the right, I-RTX caused a substantial delay in the onset of the capsaicin-induced response. The onset of the contractile response to a concentration of capsaicin that caused a 20 to 50% contraction (first effective concentration in the concentration-response curve) averaged less than 1 min in control tissues. This time was increased by about 4- to 5-fold in tissues treated with I-RTX. The delay in onset caused by 0.3 μM was not significantly different than that caused by 1 μM resiniferatoxin. The delay in onset is illustrated and quantified in Fig. 2.

The inhibitory effect of I-RTX did not depend on the agonist being a vanilloid compound. Others have reported that VR1 can be activated by anandamide to evoke tachykinergic contractions of airway smooth muscle (Tucker et al., 2001). Consistent with these findings we found that anandamide (1–100 μM) caused contractions of tracheal rings that were abolished by NK1 and NK2 receptor blockade with SR140333 and SR48968 (1 μM each, n = 2; data not shown). The contractions obtained when anandamide was used as the VR1 agonist were effectively antagonized by I-RTX (1 μM). The unimpressive potency of anandamide precluded a critical analysis of complete concentration-response curves (Fig. 3A).

Fig. 1. Concentration-response curves for resiniferatoxin-induced tachykinergic contractions of guinea pig tracheal rings in the absence and presence of different concentrations of I-RTX. Each point represents the mean ± S.E.M. of eight experiments. Inset, Schild plot of the effects of I-RTX on resiniferatoxin response, pA₂ = 7.8 obtained from the line of best fit.

The assumption that I-RTX is acting purely as a competitive antagonist at a single vanilloid receptor binding site is more obviously violated when capsaicin was used as the agonist (Fig. 2). In this case, the I-RTX again caused a rightward shift in the concentration-response curve without affecting the maximum response, but the shift in the presence of 1 μM I-RTX was no larger than that observed with 0.3 μM I-RTX. In other words, these concentrations the capsaicin concentration-response curve became stationary (Fig. 2). The capsaicin-induced contraction obtained in the absence or presence of 1 μM I-RTX were abolished by the combination of NK1 and NK2 receptor antagonists (SR140333 and SR48968, 1 μM each, n = 2; data not shown). An estimated pKᵢ value of 6.8 ± 0.2 (n = 8) was calculated from results obtained 0.3 μM I-RTX against capsaicin. This value was not significantly different than that obtained when the data used to calculate the pKᵢ was with 0.3 μM I-RTX against resiniferatoxin (7.3 ± 0.2, n = 7).

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Trypsin, like capsaicin, induces guinea pig airway smooth muscle contraction by evoking the release of tachykinins (Carr et al., 2000). Unlike capsaicin, however, trypsin does not evoke action potential discharge in airway C-fibers. I-RTX (1 μM) had no effect on the trypsin-induced contractions of the tissue (Fig. 3B). Consistent with our previous findings (Carr et al., 2000), the contractions evoked by 100 μg/ml were immediately and completely reversed by SR140333 and SR4896 (1 μM each; data not shown). In addition to indicating that trypsin may not act on VR1 to evoke neurokinin release, these data provide additional evidence that I-RTX (1 μM) is non-selectively affecting the assay (i.e., affecting the smooth muscle or directly affecting neurokinin secretion).

Afferent Function Studies. In 10 of 10 experiments, a 500-μl aliquot of capsaicin (0.3 μM) applied to the superfusion solution over the receptive field of C-fibers innervating the isolated trachea/bronchus preparation caused a robust action potential discharge (Fig. 4A, left; Table 1). Capsaicin desensitizes VR1 so the effect of I-RTX was studied in separate C-fibers. I-RTX (1 μM) did not evoke action potential discharge in any fiber studied. When the airway was superfused with buffer containing 30 nM I-RTX for 30 min, a 500-μl aliquot of 0.3 μM capsaicin evoked only about seven action potentials (Fig. 4A, right; Table 1). This potent inhibitory effect of I-RTX was likely due to the delay in onset of the capsaicin response as described in the efferent studies above. When 0.3 μM capsaicin was continuously applied (superfused over the receptive field) for a more extended period (>10-min) so that it had time to equilibrate with VR1, it was capable of surmounting the inhibition of I-RTX. An example of this is shown in Fig. 4B.

The role of the time delay in the high apparent potency of I-RTX against a transient capsaicin treatment was even more suggestive when a relatively large concentration of I-RTX (1 μM) was used. At this concentration, I-RTX completely abolished the response of airway C-fibers to transient exposure to 1 μM capsaicin (n = 4). In contrast, the number of action potentials discharged by prolonged exposure to 1 μM capsaicin was quantitatively unaffected, although there was a significant delay in the onset of the response (Table 1). The onset of action potential discharge caused by 1 μM capsaicin in the absence and presence of I-RTX was 76 ± 18 and 420 ± 98 s, respectively (P < 0.05). These findings are consistent with data obtained in efferent studies discussed above in which 1 μM I-RTX had no effect on the magnitude of tracheal contraction induced by 1 μM capsaicin, but substantially delayed its onset.

We evaluated whether the inhibitory effect of I-RTX on VR1-mediated action potential discharge was specific for vanilloid agonists. When studied at 35°C, reduction of the pH at the receptive field of airway C-fibers results in action potential discharge. This is thought to be, at least in part, due to activation of VR1 (Fox et al., 1995). Applying 500 μl of citric acid (10 mM) to the receptive field of evoked action potential discharge that did not desensitize over repeated application. The response of C-fibers to 3-s exposure of citric acid (10 mM) was significantly inhibited by more than 50% by 30-min treatment with 1 μM I-RTX (203 ± 54 versus 96 ± 38, n = 5, P < 0.05).

**Discussion**

I-RTX has recently been reported to reversibly bind to VR1 in rat spinal cord membrane preparations or human embryonic kidney 293 cells stably expressing human VR1 with an affinity constant (K_i of ~1–5 nM) more than 1000-fold lower than that of capsazepine (K_i of ~8 μM) (McDonnell et al., 2002; Wahl et al., 2001). These observations, considered with the finding that I-RTX had no intrinsic efficacy, predicted that I-RTX would be a potent competitive antagonist at VR1. Functional studies in which the I-RTX was used to inhibit capsaicin-induced inward current in VR1-expressing oocytes.

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**Table 1**

<table>
<thead>
<tr>
<th>Antagonist Treatment</th>
<th>Capsaicin&lt;sup&gt;a&lt;/sup&gt; Transient (3 s) Application</th>
<th>Capsaicin&lt;sup&gt;a&lt;/sup&gt; Continuous Application</th>
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<tr>
<td>Control (n)</td>
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<td>Treated (n)</td>
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<tr>
<td>I-RTX (30 nM)</td>
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<td>N.D.</td>
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<tr>
<td>I-RTX (1 μM)</td>
<td>7 ± 2 (4)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>199 ± 57 (12)</td>
</tr>
<tr>
<td>I-RTX (5 μM)</td>
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<td>191 ± 98 (4)</td>
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N.D., experiment not done.

<sup>a</sup>Maximally effective concentration of capsaicin (0.3 μM) was applied directly to the receptive field over a period of 3 s. Capsaicin was applied only once to each tissue; therefore, the data were analyzed using a nonpaired t test. An asterisk denotes a difference between the mean value in the control and treated group (P < 0.05). See Fig. 4 for representative recordings.

<sup>b</sup>Supramaximal effective concentration of capsaicin (1 μM) was continuously superfused over the tissue to evaluate whether the inhibition by I-RTX of the capsaicin-induced action potential discharge was surmountable. In these experiments the delay in onset of the capsaicin-induced action potential discharge was 76 ± 18 and 420 ± 98 s in the absence and presence of I-RTX, respectively (P < 0.05). However the number of action potential in a 3-min period after the action potential discharge had commenced were not different between the two groups. In four additional studies, I-RTX (1 μM) abolished the response to a 3-s application of capsaicin (1 μM).
revealed that I-RTX is an extremely potent antagonist in this system. Concentrations of I-RTX as low as 10 nM nearly abolished capsaicin-induced inward current in the voltage-clamped oocytes (Wahl et al., 2001). The results presented herein are in agreement with the conclusion that I-RTX is a selective VR1 antagonist. As was observed in the oocyte preparation, we found no evidence of partial agonist activity with I-RTX in either the efferent or afferent assays of airway C-fiber function. In studies on airway C-fibers, however, the potency of I-RTX at inhibiting vanilloid-induced responses is significantly lower than that observed in VR1-expressing oocytes or VR1-expressing human embryonic kidney 293 cells. In the isolated airway preparation, I-RTX had a $K_B$ value of about 0.1 $\mu$M that in paired experiments was only about 30-fold more potent than capsazepine.

In binding studies, I-RTX behaved as a competitive antagonist (Wahl et al., 2001; McDonnell et al., 2002). Consistent with competitive antagonism, we noted that the I-RTX shifted the vanilloid agonist concentration-response curves to the right but did not inhibit the maximum response. Thus, the antagonism by even large concentrations of I-RTX (1 $\mu$M) was completely surmountable by increasing the agonist concentration in both the afferent and efferent assays. This is in contrast to the functional studies in VR1-expressing oocytes. In the oocyte system, the characteristics of inhibition were consistent with a noncompetitive mode of action. For example, 3 nM I-RTX had apparently no effect on the EC$_{50}$ for capsaicin, but inhibited the maximum response by more than 50% (Wahl et al., 2001). The explanation for this discrepancy between the effect of I-RTX on C-fiber terminals in guinea pig airway tissue and that observed in transfected cells is not clear but may be related to the kinetics of I-RTX. We found that in addition to causing a parallel rightward shift in the capsaicin concentration-response curves, I-RTX caused an obvious and profound increase in the latency of the agonist response. This was observed when the effects of capsaicin on either the afferent function (action potential discharge) or efferent function (contractions) were studied. The mechanism underlying this delay is not known but may involve the relatively slow rate at which I-RTX dissociates from the receptor (Wahl et al., 2001). Regardless of the mechanism, this effect of I-RTX needs to be considered when interpreting concentration-response data. For example, if capsaicin is not allowed time to equilibrate with the receptor (i.e., when the agonist is given in a transient manner, as is often the case in electrophysiological experiments), this effect of I-RTX will lead to an apparent noncompetitive antagonism, as well as an overestimation of the antagonist affinity. This was observed in the action potential discharge study, when it was noted that when capsaicin was applied over a 3-s period, a concentration of I-RTX as low as 30 nM was capable of essentially abolishing the response of a maximally effective concentration of capsaicin. When the same concentration of capsaicin was allowed to superpose over the C-fiber receptive field for $>10$ min, the antagonism afforded by I-RTX was less apparent.

A more critical analysis of the vanilloid agonist concentration-response curves in the absence and presence of different concentrations of I-RTX would seem to indicate that something more than simple competitive antagonism at a single binding site is occurring between I-RTX and the agonists. The slope of the Schild plot was significantly less than unity when resiniferatoxin was used as the agonist. That something other than simple competitive antagonism was occurring was even more evident when capsaicin was used as the agonist. In fact, the capsaicin concentration-response curves essentially became stationary in the presence of 0.3 and 1 $\mu$M I-RTX. These results are consistent with the speculation that capsaicin may be acting on at least two receptors (or two isoforms of the same receptor), one with high affinity the other with a lower affinity for capsaicin, to evoke tachykinin release from the C-fibers. When the concentration-response curve is shifted sufficiently to the right (e.g., by 0.3 $\mu$M I-RTX) capsaicin may act on the lower affinity form of the receptor to evoke the measured response. In this scenario, I-RTX has an affinity for the high-affinity capsaicin binding site of about 0.1 $\mu$M ($K_B$ value estimated with 30 nM I-RTX) but not for the lower affinity binding state. A more reduced experimental design than the isolated tissue is required to directly address this hypothesis.

We have previously shown that trypsin contracts guinea pig airways by evoking the release of tachykinins from C-fibers (Carr et al., 2000). Although trypsin induces the efferent activity in C-fibers, it does not evoke action potential discharge. The mechanism by which trypsin causes tachykinin release is not known but likely involves some type of protease-activated receptors. That I-RTX (1 $\mu$M) had no effect on the trypsin-induced tachykininergic responses indicates that trypsin may release tachykinins from C-fibers independently of VR1 (or at least independently of the vanilloid binding sites). These data also serve as a testament to the selectivity of I-RTX as a VR1 antagonist. A lack of effect on the trypsin-induced tachykinergic contractions means that I-RTX (1 $\mu$M) did not nonselectively interfere with tachykinin secretion, or airway smooth muscle contraction in response to the endogenously released tachykinins. This is consistent with the lack of effect of I-RTX (1 $\mu$M) on NKA concentration-response curve. However, the observation that 10 $\mu$M I-RTX had significant inhibitory effects on the NKA-mediated tracheal contractions indicates that at this concentration of I-RTX acts on cellular processes other than VR1.

The antagonism by I-RTX of VR1 activation was not selective for vanilloid agonists. Anandamide is known to activate VR1 in isolated neurons and in VR1 expression systems (Zygmunet et al., 1999). Anandamide stimulates the release of tachykinins from guinea pig airway C-fibers by a mechanism that can be inhibited with capsazepine (Tucker et al., 2001). The rightward shift caused by I-RTX in the anandamide concentration-response curve is consistent with the hypothesis that anandamide activates VR1 by binding to the vanilloid binding site(s).

Acid is another nonvanilloid that can activate VR1. The finding that I-RTX inhibited acid-induced action potential discharge is similar what has been reported with capsazepine (Fox et al., 1995). How this occurs is not clear. Protons have been shown to bind to VR1 and increase the channel's open probability (Tominaga et al., 1998). This, however, is not thought to occur by directly affecting the vanilloid binding site. It may be argued that upon specific binding to the vanilloid binding site, I-RTX (or capsazepine) nonspecifically blocks the channel pore. However, capsazepine is relatively ineffective in inhibiting inward current or calcium entry through VR1 that is induced by heat (Liu and Simon, 2000; Savidge et al., 2001). A possibility remains that acid appli-
cation leads to production of an endogenous agonist that acts at the vanilloid binding site. In this light it is interesting to note that there are several endogenous molecules that can act as VR1 agonists, including anandamide, and lipoxigenase products of arachidonic acid (Zygumn et al., 1999; Hwang et al., 2000). The question of whether acid leads to VR1 activation in airway C-fibers by a direct or indirect mechanism cannot be further resolved within the framework of a complex tissue. This issue requires a determination of whether I-RTX can inhibit acid-induced VR1 channel activity in excised membranes, at the single channel level.

In summary, I-RTX is a selective VR1 antagonist that can be used to inhibit VR1-mediated afferent and efferent activity in airway C-fiber terminals. Under conditions in which the antagonists and agonist are allowed time to equilibrate with the receptor, I-RTX provides a moderately potent, surmountable antagonism of VR1 with a $K_B$ value of around 0.1 $\mu$M (about 30 time more potent than capsazeine in this system). Concentrations of I-RTX as low as 30 nM are effective at abolishing the response to relatively large concentrations of capsaicin, however, if capsaicin is added to the tissue in a transient manner. This is because I-RTX effectively and potently delays the onset of capsaicin-induced responses.

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References


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