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Pharmacological characterization of the vanilloid receptor in the rat dorsal spinal cord

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1 In the present study a novel 96-well plate assay system was used to characterize pharmacologically the vanilloid receptor in the dorsal spinal cord of the rat. When activated, this receptor stimulates release of calcitonin gene-related peptide (CGRP) from the central terminals of the afferent nerves.

2 Capsaicin, resiniferatoxin (RTX) and olvanil each evoked a concentration-dependent increase in CGRP release with pEC_{50} values of 6.55 ± 0.07, 7.90 ± 0.24 and 6.19 ± 0.15 respectively. RTX and olvanil were partial agonists with respect to capsaicin. All concentration-effect curves were bell-shaped.

3 The vanilloid receptor antagonist, capsazepine (10 μM) had no effect on basal peptide release but inhibited the CGRP release evoked by all 3 agonists to a similar extent. These results suggest that the antagonistic effects of capsazepine were agonist-independent.

4 The capsaicin-sensitive cation channel blocker, ruthenium red (10 μM) had no effect on basal CGRP release, but antagonized the peptide release evoked by capsaicin, olvanil and RTX.

5 The pharmacology of the vanilloid receptor in the rat dorsal spinal cord is not identical to that previously found in other systems. The reason for these differences is unclear, but the possibility of multiple classes of receptor cannot at this stage be ruled out.

Keywords: Capsaicin; vanilloid; spinal cord; olvanil; resiniferatoxin (RTX); capsazepine; ruthenium red

Introduction

A subpopulation of primary sensory neurones are stimulated and subsequently desensitized by capsaicin, the pungent agent contained in hot chilli peppers (Buck & Burks, 1986; Holzer, 1991). These neurones are, in general, peptidergic, of small to medium diameter, having unmyelinated C fibres or thinly myelinated Aδ fibres (Szallasi & Blumberg, 1993a). Functionally, such neurones are believed to transmit nociceptive and thermoceptive information to the spinal cord and are sites of release of proinflammatory mediators in the periphery (Szallasi & Blumberg, 1993a).

These actions of capsaicin are believed to be mediated via stimulation of a specific membrane-bound receptor, termed the vanilloid receptor (James *et al*., 1993). Vanilloid receptors have been visualized by [^{3}H]-resiniferatoxin ([^{3}H]-RTX) autoradiography in dorsal root ganglia and spinal cord of several species including man (Szallasi *et al*., 1994). Within the rat spinal cord, vanilloid receptors have been localized on primary afferent neurones in the cervical, thoracic and lumbar segments, with receptor density in the lumbar region being approximately twice that of the other two segments (Szallasi *et al*., 1995). Transmission of afferent impulses into the spinal cord is mediated, in part, by calcitonin gene-related peptide (CGRP, Wiesenfeld-Hallin *et al*., 1984). Unlike substance P, CGRP is not contained within intrinsic spinal neurones and hence is a relatively specific marker for primary sensory neurones (see Duggan, 1994).

Thus, in the present study, release of CGRP has been used to characterize pharmacologically the vanilloid receptor on the afferent nerve terminals projecting into the rat spinal cord. These data can then be used to compare 'pharmacological fingerprints' between different tissues. Our results generated in rat spinal cord (central terminal of sensory afferent neurones) are, for example, different from those obtained from rat tissues containing peripheral terminals (vas deferens, Wardle *et al*., 1996). These results are discussed.

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Methods

Experimental procedure

Experiments were carried out as previously described (Banner & Bowen, 1994). Briefly, male Sprague Dawley rats (250–350 g) were killed by asphyxiation. The spinal cord was rapidly removed and placed in cold oxygenated (5% CO_2 in O_2) Krebs solution and sectioned into dorsal and ventral halves. Krebs solution of the following composition was used (mM): NaCl 121.5, $CaCl_2$ 2.5, KH_2PO_4 1.2, KCl 4.7, $MgSO_4$ 1.2, $NaHCO_3$ 25.0 and glucose 5.6. Dorsal spinal cord was sliced transversely and longitudinally (300 μm $\times$ 300 μm) on a McIlwain tissue chopper. Slices were resuspended in 20 ml oxygenated Krebs solution containing 0.1% bovine serum albumin (BSA), washed 5 times in fresh oxygenated Krebs/BSA and 200 μl aliquots of the suspension added to each well of a 96-well Millipore filtration plate. Each animal produced enough tissue to fill one 96 well plate. Each plate contained spinal cord from a single animal.

Tissues were incubated with 50 μl aliquots of antagonist or vehicle for 10 min in a Wesbart plate incubator at 37°C. Following equilibration of antagonist, tissues were incubated for 10 min (again at 37°C) with the appropriate concentration of agonist (50 μl aliquots) before collection of the filtrate, under vacuum, into a second 96-well plate. Preliminary studies with agonist and antagonist incubation times of up to and including 30 min showed no greater effect with any of the agonists or antagonists used than that seen following 10 min incubation with each compound (data not shown). At the end of the experiment, 100 μl aliquots of filtrate were assayed for calcitonin gene-related peptide-like immunoreactivity (CGRP-LI) by radioimmunoassay (RIA).

To measure CGRP-LI, 100 μl aliquots of rat CGRP (Peninsula) or samples were incubated for 24 h at 4°C with 100 μl of 1/20 000 dilution anti-CGRP serum (raised in rabbits against rat CGRP, Peninsula); 100 μl of [^{125}I]-CGRP (Amersham) were added and incubated for a further 24 h at 4°C. Separation of free from bound antigen was achieved by use of the donkey anti-rabbit separation reagent, Amerlex-M (Amersham). The sensitivity of the RIA was 3 pg per tube.

Analysis of results

Within each 96-well plate, all agonist additions were performed in triplicate. These values were averaged to form a single value. Peptide release values were read off the standard curve in terms of pg CGRP per well. A control capsaicin concentration-effect curve was constructed for each plate, allowing all data to be expressed as a percentage of the maximum response to capsaicin in each animal. This method of normalizing the data minimalized variability due to the different amounts of tissue harvested from each animal and the activity of the [^{125}I]-CGRP, a radioligand with a short half-life. The ability of agonists to evoke CGRP release was then expressed in terms of a pEC_{50} value (relative to their own maxima) and in terms of a maximum response, relative to capsaicin (intrinsic activity, where intrinsic activity of capsaicin = 1.0).

pA_2 values for capsazepine were not possible to estimate in the present study due to the bell-shaped nature of the agonist-concentration-effect curves (see Results). Hence, the activity of the antagonist was estimated in terms of % reduction in maximum response to the agonist.

All results are expressed in terms of mean ± s.e.mean for a number (n) of observations. Statistical analysis was performed by means of a Student's t test for paired or unpaired data. A P value of less than 0.05 was taken to be significant.

Drugs used

The following drugs were used: capsaicin (Sigma), RTX (Sigma), olvanil (Procter and Gamble), capsazepine (Tocris Cookson), ruthenium red (RBI) and rat CGRP (Sigma). Ruthenium red and CGRP were dissolved in distilled water. Stock solutions (6 mM) of RTX, olvanil and capsazepine were made in absolute ethanol and further diluted in Krebs/BSA (RTX and capsazepine) or ethanol/Krebs/BSA mixture (olvanil) to give the required concentration. Stock solutions (6 mM) of capsaicin were dissolved in 10% ethanol, 10% Tween 80, 80% Krebs/BSA and further diluted in Krebs/BSA.

Results

The rat dorsal spinal cord had a basal CGRP release of 18 ± 2 pg per tube (n = 20). In the presence of 1 μM capsaicin, this value was increased around 10 fold (197 ± 21 pg per tube, n = 20). All solvents used in this study were without significant effect on either basal or agonist-evoked CGRP release (results not shown).

Capsaicin (0.3 – 1 μM, n = 14) evoked a concentration-dependent increase in CGRP release with a pEC_{50} of 6.55 ± 0.07. The concentration-effect curve to capsaicin was bell-shaped, with a maximum response at 0.8 μM. The vanilloid receptor agonist olvanil (0.3 – 10 μM, n = 7) evoked a concentration-dependent release of CGRP with a pEC_{50} of 6.19 ± 0.24 and an intrinsic activity (relative to capsaicin) of 0.63 ± 0.55. RTX (1 nM – 1 μM, n = 8) evoked an increase in CGRP release with a pEC_{50} of 7.90 ± 0.24 and an intrinsic activity of 0.57 ± 0.08. As with capsaicin, the concentration-effect curve to RTX was bell-shaped (Figure 1).

The ability of the competitive antagonist capsazepine (3 – 10 μM, n = 6 each) to inhibit the CGRP release evoked by capsaicin (Figure 2a), olvanil (Figure 2b) and RTX (Figure 2c) was investigated. At these concentrations, capsazepine had no significant effect on the basal release of CGRP from the spinal cord (n = 4, data not shown). At 3 μM, capsazepine produced no significant antagonism of peptide release, whereas at 10 μM, release evoked by all concentrations of capsaicin, olvanil and RTX was significantly antagonized. The variability in the response to RTX, relative to the other agonists, precluded any clear effect of capsazepine at the lower concentrations (0.3 μM) of RTX. Because of the bell-shaped nature of each of the agonist concentration-effect curves, it was not possible to overcome the effects of the competitive antagonist by increasing agonist concentration, hence pA_2 estimates were impossible to make. At 10 μM, capsazepine caused a 51 ± 7%, 49 ± 6% and 62 ± 8% reduction in the maximum responses to capsaicin, olvanil and RTX, respectively.

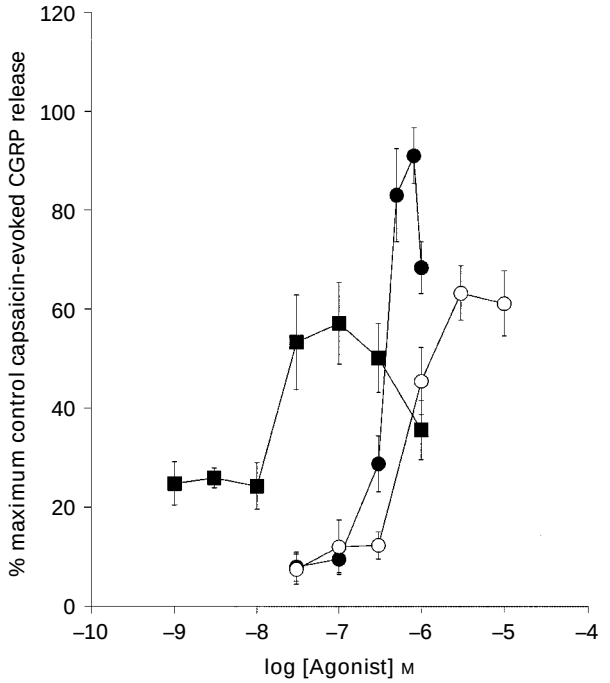

Figure 1 Concentration-effect curves to (●) capsaicin (n = 11), (○) olvanil (n = 7) and (■) resiniferatoxin (RTX, n = 8) in evoking calcitonin gene-related peptide (CGRP) release from rat dorsal spinal cord. Each agonist was incubated for 10 min. Each point represents the mean of a number (n) of observations; vertical lines show s.e.mean.

CGRP release evoked by capsaicin (Figure 3a), olvanil (Figure 3b) and RTX (Figure 3c) was also antagonized by the inorganic dye ruthenium red (3 – 10 μM, n = 6 each). At these concentrations, ruthenium red had no significant effect on the basal release of CGRP from the spinal cord (n = 4, data not shown). At 3 μM, ruthenium red produced a small, but in some cases significant, inhibition of peptide release, whereas at 10 μM, release evoked by all concentrations of capsaicin, olvanil and RTX was significantly antagonized. At 10 μM, ruthenium red caused a 67 ± 7%, 60 ± 8% and 53 ± 10% reduction in the maximum responses to capsaicin, olvanil and RTX, respectively.

Discussion

The vanilloid receptor has been localized on afferent neurones projecting into the rat dorsal spinal cord by use of a series of radioligand binding (Szallasi & Blumberg, 1993b; Szallasi *et al*., 1993) and autoradiographical (Szallasi *et al*., 1995) studies. Previous studies have utilized CGRP release as a means of functionally investigating the vanilloid receptor in the rat dorsal spinal cord (see, for example Maggi *et al*, 1990a, Santicioli *et al*., 1992). However, due to the slow throughput of these superfusion assays, limited pharmacological data have been obtained. By use of a new 96-well plate functional assay system (Banner & Bowen, 1994), the present investigation has generated previously unobtainable concentration-effect curves to a range of vanilloid receptor agonists in the rat dorsal spinal cord. Using this model, we have been able to begin the process of pharmacologically profiling the vanilloid receptor in this tissue with a view to obtaining greater insight into the characterization of the vanilloid receptor.

In the rat dorsal spinal cord, capsaicin, RTX and olvanil have each been shown to evoke a concentration-dependent

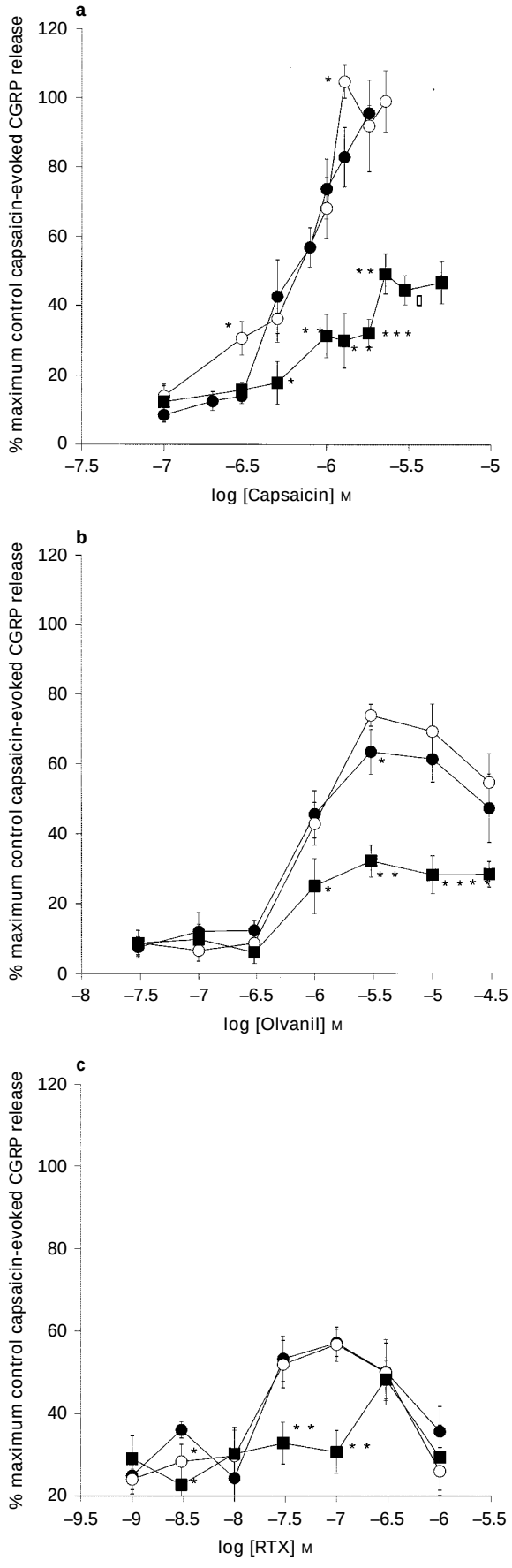

Figure 2 Concentration-effect curves to capsaicin (a), olvanil (b) and resiniferatoxin (RTX, c) in the absence (●) and presence of 3 μM (○) and 10 μM (■) capsazepine in evoking CGRP release from the rat dorsal spinal cord. All agonists and antagonists were incubated for 10 min. Each point represents the mean of 6 observations; vertical lines indicate s.e.mean. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$.

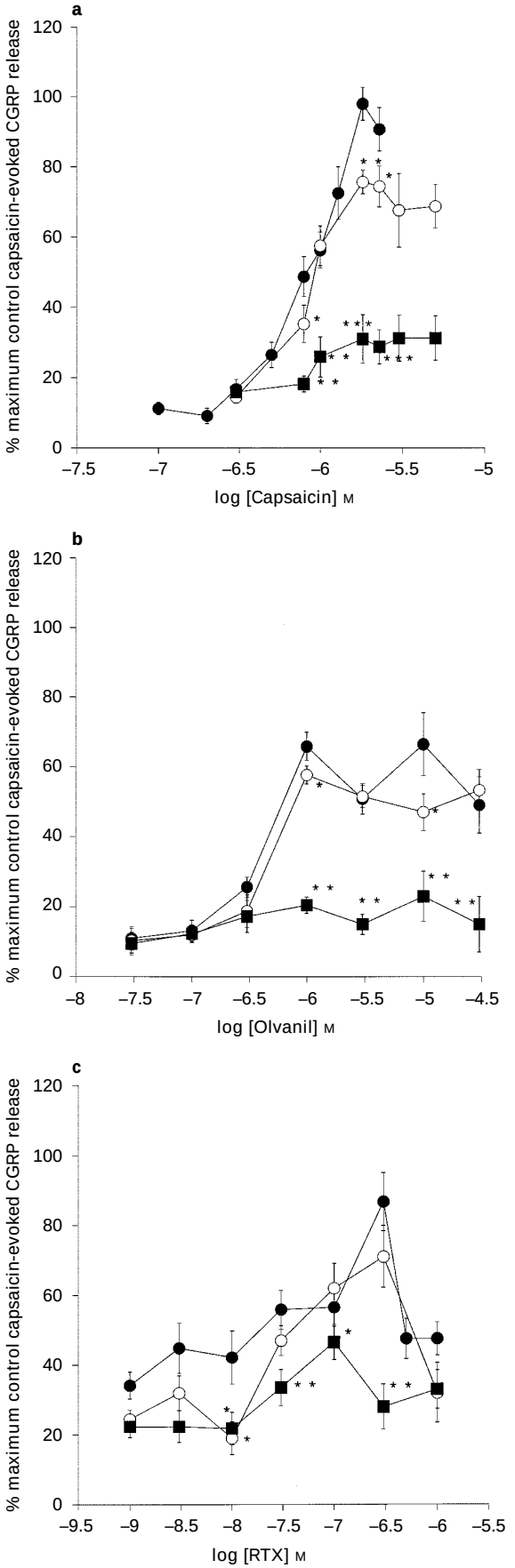

Figure 3 Concentration-effect curves to capsaicin (a), olvanil (b) and resiniferatoxin (RTX, c) in the absence (●) and presence of 3 μM (○) and 10 μM (■) ruthenium red in evoking CGRP release from the rat dorsal spinal cord. All agonists and antagonists were incubated for 10 min. Each point represents the mean of 6 observations; vertical lines indicate s.e.mean. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$.

release of CGRP, with a rank order of potency of RTX > capsaicin > olvanil. Both RTX and olvanil were partial agonists with respect to capsaicin and each agonist evoked a bell-shaped concentration-effect curve. The reason for the bell-shaped nature of the curves was unclear. One possible explanation is receptor desensitization, a well-known phenomenon of the vanilloid receptor (Buck & Burks, 1986; Holzer, 1991). Alternatively, this observation may be explained in terms of the existence of two receptor subclasses; a high affinity receptor which stimulates release and a lower affinity receptor which blocks release. Thus, in a recent study of capsaicin-induced oxygen uptake in the perfused rat hindlimb, this explanation has been put forward to explain the biphasic nature of the response (Griffiths *et al*., 1996). In contrast to the study of Griffiths *et al*., no evidence was found to suggest that low concentrations of capsazepine were able to antagonize selectively the first phase of the concentration effect curve. One final possibility is that the bell-shaped concentration-effect curve may be related to the cooperativity of vanilloid receptor binding, previously obtained by Szallasi (1994).

Capsaicin evoked CGRP release from the rat dorsal spinal cord with an EC_{50} of 0.3 μM, compared with an EC_{50} of around 12 nM for RTX. Responses to RTX appeared to be more variable and less concentration-dependent that those to capsaicin, an observation which may explain the elevated baseline of the RTX curve. This was not believed to be due to the failure of RTX to reach equilibrium, as increasing the agonist incubation time to 30 min had no significant effect on the concentration-effect curve to RTX (data not shown). However, the possibility cannot be ruled out that the potency of RTX has been slightly underestimated as a result of this elevated baseline.

The apparent 20 fold difference in potency between RTX and capsaicin observed in this study contrasts markedly with radioligand binding studies (Szallasi & Blumberg, 1993b; Szallasi *et al*., 1993) in rat spinal cord membranes in which RTX had somewhere in the region of a 45,000 fold greater affinity for the vanilloid receptor than capsaicin. This observation is in agreement with an earlier finding of Szallasi *et al*. (1991) in which the stimulating potency of vanilloids in rat dorsal root ganglia (DRG) failed to correspond with their binding affinities. While it is sometimes difficult to compare affinities from binding studies with potencies from functional studies, it can clearly be seen that while capsaicin has a similar 'activity' in both systems, RTX has a much greater ability to bind to the vanilloid receptor than it does to activate it. The reason for this apparent discrepancy is unclear, but it may represent a rapid desensitization and hence loss of activity in the functional system with no corresponding change in binding affinity in the desensitized state. In support of this theory is the observation that the membranes from DRG obtained from control rats and rats desensitized acutely with RTX displayed no difference in the RTX binding affinity (Szallasi & Blumberg, 1992).

There are many studies which highlight the fact that the potency of RTX varies between models (for review see Szallasi, 1994). In the present study in the rat dorsal spinal cord, RTX was around 20 fold more potent than capsaicin at stimulating the vanilloid receptor. This contrasts greatly with other studies (Maggi *et al*., 1990b; Wardle *et al*., 1996) in which RTX was found to be between 10 and 30 thousand fold more potent than capsaicin at releasing CGRP from afferent neurones projecting into rat vas deferens. Such data can be used to support the existence of vanilloid receptor subclasses (Holzer, 1991; see below).

The capsaicin analogue olvanil has previously been shown to display a similar antinociceptive potency to capsaicin *in vivo* (Sietsema *et al*., 1988; Campbell *et al*., 1989). In support of these *in vivo* findings, olvanil has, in the present study, been shown to be approximately equipotent with capsaicin at evoking CGRP release from the rat dorsal spinal cord. This markedly contrasts with earlier findings in the rat vas deferens (Wardle *et al*., 1996) in which olvanil failed to display any agonistic or antagonistic activity at the vanilloid receptor. It has been proposed (Liu & Simon, 1996) that the low pungency of olvanil in some tissues may be due to slower activation kinetics. While this possibility cannot be ruled out, it would be unlikely to explain the lack of observed effect in the vas deferens, since incubation times of up to 40 min failed to reveal any agonistic activity (Wardle, unpublished observation). Thus, while a pharmacokinetic explanation for the differences in the potency of RTX and the intrinsic activity of olvanil cannot be ruled out (Maggi *et al*., 1990b; Wardle *et al*., 1996), our results could also be explained in terms of a 'peripheral' and 'central' type of receptor, with RTX and olvanil distinguishing between these subtypes. Indeed, Szallasi has already proposed the existence of two basic receptor subtypes; a 'central type' which binds RTX with high affinity in a co-operative fashion and prefers capsaicin to capsazepine, and a 'peripheral type' which binds RTX with lower affinity in a non-cooperative manner and recognises capsazepine with higher affinity than capsaicin (for review see Szallasi, 1994). Unfortunately, the activity of olvanil was not obtained in these studies.

In the present study capsazepine antagonized the CGRP release evoked by capsaicin, olvanil and RTX. The potency of capsazepine was similar when tested against each of the three agonists, suggesting that antagonism was agonist-independent. In other systems (see, Bevan *et al*., 1991; 1992; Dickenson *et al*., 1991; Dray *et al*., 1991; Maggi *et al*., 1993; Wardle *et al*., 1996), capsazepine has been shown to cause a rightward displacement of the agonist concentration-effect curves with no reduction in the maximum response. However, in the rat spinal cord, the maximum response to all agonists was reduced, an effect which can probably be explained in terms of the bell-shaped nature of each of the agonist concentration-effect curves. Interestingly, in previous studies in the rat vas deferens (Wardle *et al*., 1996), capsazepine produced significant antagonism at concentrations as low as 1 and 3 μM. In the present study, no antagonism was seen with capsazepine until a concentration of 10 μM. This observation points towards a greater potency of capsazepine at peripheral as compared with central vanilloid receptors and may add support to Szallasi's 'peripheral'/'central' subtype hypothesis (see above).

Ruthenium red also antagonized CGRP release evoked by all 3 agonists (capsaicin, olvanil and RTX) to a similar extent, suggesting that antagonism was agonist independent. At these concentrations, ruthenium had no effect on basal CGRP release and failed to antagonize significantly potassium and 3,4-diaminopyridine-evoked peptide release (Wardle, unpublished observations).

In conclusion, the present study has utilized a new 96-well plate functional assay system to investigate the ability of a range of vanilloid receptor agonists and antagonists to modify CGRP release from the rat dorsal spinal cord. Using this model, we have begun the process of pharmacologically profiling this 'central' vanilloid receptor. Comparison of the results from the spinal cord with those from peripheral functional models clearly points towards differences in the pharmacology. While these effects may be due to pharmacokinetic differences, the existence of multiple classes of receptor cannot at present be excluded. Elucidation of this point awaits further investigation and the development of a range of selective pharmacological tools.

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