Br. J. Pharmacol. (1993), **108**, 801–805

A comparison of capsazepine and ruthenium red as capsaicin antagonists in the rat isolated urinary bladder and vas deferens

[1] Carlo Alberto Maggi, *Stuart Bevan, *Christopher S.J. Walpole, *Humphrey P. Rang & Sandro Giuliani

Pharmacology Department, A. Menarini Pharmaceuticals, Via Sette Santi 3, 50131, Florence, Italy and *Sandoz Institute for Medical Research, London, England

1 The ability of capsazepine, a recently developed capsaicin receptor antagonist, to prevent the effects of capsaicin on the rat isolated urinary bladder (contraction) and vas deferens (inhibition of electrically-evoked twitches) was compared to that of ruthenium red, a dye which behaves as a functional antagonist of capsaicin.

2 In the rat bladder, capsazepine (3–30 μM) produced a concentration-dependent rightward shift of the curve to capsaicin without any significant depression of the maximal response to the agonist. By contrast, ruthenium red (10–30 μM) produced a non-competitive type of antagonism, characterized by marked depression of the maximal response attainable. Similar findings were obtained in the rat isolated vas deferens in which capsazepine (10 μM) produced a rightward shift of the curve to capsaicin while ruthenium red (3 μM) depressed the maximal response to the agonist.

3 At the concentrations used to block the effect of capsaicin, neither capsazepine nor ruthenium red affected the contractile response of the rat urinary bladder produced by either neurokinin A or electrical field stimulation or the twitch inhibition produced by rat α-calcitonin gene-related peptide (αCGRP) in the vas deferens.

4 These findings provide additional evidence that both capsazepine and ruthenium red are valuable tools for exploration of the function of capsaicin-sensitive primary afferent neurones. The antagonism of the action of capsaicin by capsazepine is entirely consistent with the proposed interaction of this substance with a vanilloid receptor located on primary afferents, while the action of ruthenium red apparently involves a more complex, non-competitive antagonism.

Keywords: Capsaicin, primary afferent neurones; efferent function of sensory nerves; capsazepine; ruthenium red

Introduction

In recent years, much interest has developed in the pharmacological modulation of sensory neurone function (Maggi & Meli, 1988; Holzer, 1988; 1991; Maggi, 1991). A specific receptor on the cell membrane of primary afferent neurones has been recently discovered, which recognizes capsaicin and other natural pungent principles, including the ultrapotent capsaicin analogue, resiniferatoxin (Szallasi & Blumberg, 1990). This capsaicin or 'vanilloid' receptor fulfils several criteria to be considered the molecular target through which capsaicin and its congeners produce their specific actions on primary afferent neurones, which are characterized by long-lasting desensitization. Capsaicin also possesses non-specific actions (i.e. not restricted to primary afferent neurones) on nerves and smooth muscle which do not exhibit desensitization (Maggi & Meli, 1988; Holzer, 1991 for reviews). Studies from various laboratories have indicated that stimulation of the 'vanilloid' receptor on the membrane of primary afferent neurones is followed by the opening of a novel type of receptor-operated ion channel, which admits both sodium and calcium ions (Bevan & Szolcsanyi, 1991). It has recently been proposed that two different substances act as capsaicin antagonists, the inorganic dye ruthenium red (see Amann & Maggi, 1991 for review) which does not interact with the vanilloid receptor (Szallasi & Blumberg, 1990) and the benzazepine derivative, capsazepine (Bevan *et al.*, 1991; 1992; Dray *et al.*, 1991; Dickenson *et al.*, 1991; Urban & Dray, 1991), which was proposed as the first competitive antagonist of the capsaicin or vanilloid receptor.

Owing to the different mechanisms by which capsazepine and ruthenium red are thought to act as capsaicin antagonists, a direct comparison of these two drugs appeared of interest. With this aim we have chosen two preparations, the rat isolated urinary bladder and vas deferens, in which the selective action of capsaicin has been well characterized. In both preparations ruthenium red has been shown to antagonize the action of capsaicin by acting at a prejunctional site to inhibit the release of sensory neuropeptides which mediate the response to capsaicin (Maggi *et al.*, 1988a,b). The resultant contraction of the rat isolated bladder is mediated through the release of endogenous tachykinins (Maggi *et al.*, 1991); inhibition of electrically-evoked (field stimulation) twitches of the rat isolated vas deferens is mediated by the release of calcitonin gene-related peptide (CGRP) (Maggi *et al.*, 1987; Santicioli *et al.*, 1988). In both preparations a cumulative concentration-response curve to capsaicin has been used in order to determine the nature of the antagonism produced by capsazepine and ruthenium red.

Methods

Male albino rats of Wistar strain (body wt. 340–360 g) were stunned and bled. The whole urinary bladder and vasa deferentia were excised and placed in oxygenated (96% O_2 and 4% CO_2, pH 7.4) Krebs solution of the following composition (mM): NaCl 119, KCl 4.7, $MgSO_4$ 1.5, KH_2PO_4 1.2, $CaCl_2$ 2.5, $NaHCO_3$ 25 and glucose 11.

Contractile responses of the urinary bladder and vas deferens (pars prostatica) were studied as described previously (Maggi *et al.*, 1987; 1991). Briefly, iosmetric tension

[1] Author for correspondence.

recording was made from 1 cm long longitudinal strips of rat detrusor muscle and segments of the vas deferens 1.5 cm in length under a load of 10 mN (bladder) and 5 mN (vas deferens). In both preparations, electrical field stimulation was carried out by means of two wire platinum electrodes placed at the top and the bottom of the organ bath which were connected to a GRASS S88 stimulator. Square wave pulses (pulse width 0.5 ms, 60 V) were automatically delivered at a frequency of 0.01 Hz (bladder) and 0.2 Hz (vas deferens).

Experiments started after a 60 min equilibration period. In the rat bladder the contractile response to KCl (80 mM) was determined at 15 min intervals until reproducible responses were obtained. This response to KCl was used as an internal standard to evaluate the maximal response to capsaicin.

The effects of capsazepine and ruthenium red were investigated on a concentration-response curve produced by addition of capsaicin (10 nM–10 μM). In order to avoid or minimize capsaicin desensitization (see Results), the capsaicin concentration was increased by a factor of 10 for each successive dose of the cumulative nerve. Each dose of the cumulative curve was added when the effect of the preceding one had reached its maximum. Only one concentration-response curve to capsaicin was obtained in each preparation. Several preparations were made from the same animal, one of which served as control while the others received capsazepine or ruthenium red before capsaicin application.

Contact time for capsazepine and ruthenium red was 30 min. To check whether capsazepine and ruthenium red may have affected the capsaicin response at the postjunctional level, their effect was studied on the contraction of the rat urinary bladder produced by neurokinin A (1–30 nM) and inhibition of twitch contractions of the vas deferens produced by rat αCGRP (1–300 nM).

Statistical analysis of the data was performed by means of the Student's *t* test for paired or unpaired data or by analysis of variance, when applicable. All values in the text, figures and table are means ± s.e.mean.

Stock solutions were prepared as follows: capsaicin to given a final concentration of 10–100 mM, was dissolved in absolute ethanol and then diluted in water. Ruthenium red (1–10 mM, Aldrich) was dissolved in water. Capsazepine (10–100 mM) was dissolved in dimethylsulphoxide. Rat αCGRP and neurokinin A (Peninsula) were dissolved in water. The vehicles used to dissolve capsaicin (0.01% ethanol) or capsazepine (0.03% dimethylsulphoxide) had no effect on the preparations.

Results

General

Cumulative addition of capsaicin (10 nM–10 μM) with a ten fold increase in concentration between doses produced a concentration-dependent contraction of the rat isolated bladder. The maximal effect was observed at 1 μM, after which tension declined and 10 μM capsaicin did not evoke a larger response. The maximal response to capsaicin obtained with this protocol of cumulative addition (48 ± 4% of the response to 80 mM KCl, mean ± s.e.mean, $n = 10$) was slightly less than the response (55 ± 3% of KCl response, $n = 8$) observed when 1 μM capsaicin was added to the bath as a single concentration.

In the rat isolated vas deferens, cumulative addition of capsaicin (10 nM–10 μM) produced concentration-dependent inhibition of electrically-evoked twitches. The maximal inhibitory effect was observed with 1 μM capsaicin and was followed by a gradual recovery of the twitches amplitude. Addition of 1 μM capsaicin to the bath as a single dose produced a transient inhibitory effect (58 ± 4%, $n = 6$, cf. Maggi *et al.*, 1987) which was not different from that obtained in response to 1 μM capsaicin using a cumulative protocol (54 ± 3% inhibition, $n = 8$).

In the vas deferens, the twitch inhibition is ascribable to a specific action of capsaicin on sensory nerves and release of CGRP (Maggi *et al.*, 1987). In this preparation, at a threshold concentration of 10 μM, capsaicin produces an opposite effect, twitch enhancement (not shown), as originally described by Moritoki *et al.* (1987). This effect, which is repeatedly observed upon consecutive application of capsaicin in the same preparation, is ascribable to a nonspecific effect of the drug (i.e. not involving activation of sensory nerves, Maggi & Meli, 1988) and limits the possibility of exploring the effects of capsazepine over a large range of concentrations.

Effect of capsazepine and ruthenium red in the rat urinary bladder

Neither capsazepine nor ruthenium red affected spontaneous motility of the rat urinary bladder.

Capsazepine (3–30 μM) produced a concentration-dependent rightward shift of the concentration-response curve to capsaicin in the rat bladder (Figure 1). In the presence of 3 μM capsazepine, the response to a threshold concentration of capsaicin (10 nM) was blocked but the maximal response to capsaicin was obtained at the same concentration (1 μM) as in controls. Ten and 30 μM capsazepine produced a further inhibition of the response to capsaicin and in this case the maximal response to the agonist was obtained at 10 μM capsaicin (Figure 1).

The maximal response to capsaicin in the presence of 3–30 μM capsazepine was not significantly different from that found in control experiments (Table 1).

Ruthenium red (3–10 μM) produced a concentration-dependent inhibition of the response to capsaicin in the rat bladder. As can be noted from curves presented in Figure 1b, the maximal response to capsaicin in the presence of ruthenium red was depressed as compared to controls.

Neither capsazepine (10–30 μM) nor ruthenium red (30 μM) significantly affected contractions produced by neurokinin A (Figure 1c and d) nor twitch contractions of the rat bladder produced by electrical field stimualtion at 0.01 Hz (Table 1).

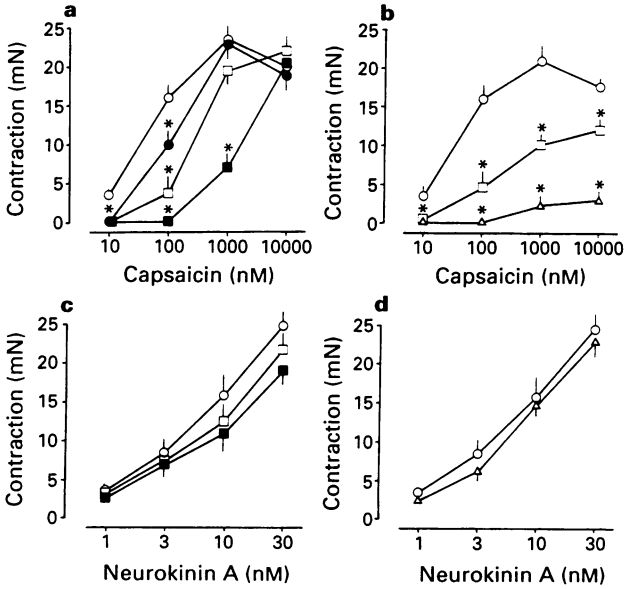

Figure 1 Effect of capsazepine (CPZ, a,c) or ruthenium red (RR, b,d) on concentration-response curve for capsaicin-induced (a,b) or neurokinin A-induced (c,d) contraction of the rat isolated urinary bladder. In (a) and (c): control (○); CPZ 3 μM (●); 10 μM (□); 30 μM (■). In (b) and (d): control (○); RR 10 μM (□); 30 μM (△). Each value is mean ± s.e.mean (vertical lines) of 4–12 experiments.

Table 1 Effect of capsazepine and ruthenium red on contractile responses of the rat isolated urinary bladder to various agents

	Response to EFS (mN	*Response to NKA* (30 nM) (mN)	*Maximal response to capsaicin* (mN)
Controls	13.8 ± 1.5	25 ± 2	23 ± 2
Capsazepine 3 μM	14.0 ± 2.5	NT	23 ± 6
Capsazepine 10 μM	13.2 ± 4.0	21 ± 2	22 ± 4
Capsazepine 30 μM	11.0 ± 1.2	19 ± 3	20 ± 4
Ruthenium red 10 μM	11.7 ± 3.0	NT	12 ± 2*
Ruthenium red 30 μM	12.5 ± 2.0	23 ± 2	3 ± 1*

Contractile response to electrical field stimulation (EFS, 0.01 Hz, 0.5 ms pulse width, maximal voltage), neurokinin A (NKA) or capsaicin are mean ± s.e.mean.
Number of experiments is 9–12 in the control group and 4–6 for each group with capsazepine or ruthenium red.
Significantly different from control: $*P<0.05$.

Effect of capsazepine and ruthenium red in the rat vas deferens

Capsazepine (10 μM) produced a slight (10–20%) enhancement of twitch contractions of the rat isolated vas deferens to electrical stimulation (0.2 Hz, 0.5 ms pulse width, maximal voltage). Capsazepine (10 μM) produced a parallel rightward shift of the concentration-response curve to capsaicin, with no apparent depression of maximal inhibitory response (Figure 2). In the presence of 10 μM capsazepine, the response to 10–100 nM capsaicin was virtually abolished and a concentration of 10 μM capsaicin was required to produce a transient maximal twitch inhibition. This effect was followed by a prompt recovery of twitch amplitude, possibly related to the nonspecific potentiating effect produced by capsaicin at 10 μM, as described above.

Ruthenium red (3 μM) inhibited twitch amplitude by 38 ± 5% ($n = 4$). In the presence of ruthenium red, the concentration-response curve to capsaicin was shifted to the right and maximum effect was depressed compared to controls (Figure 2). In Figure 2, the inhibitory effect of capsaicin in the presence of ruthenium red (and rat αCGRP, see below) is expressed as % reduction of the new baseline. The maximal twitch inhibition produced by 10 μM capsaicin in the presence of 3 μM ruthenium red (21 ± 3% inhibition) was significantly less than the maximal response obtained in either control preparations (55 ± 4% inhibition at 1 μM capsaicin) or in the presence of capsazepine (54 ± 3% inhibition at 10 μM capsaicin).

Rat αCGRP (10 nM–0.3 μM) produced a concentration-dependent inhibition of twitch amplitude in the rat isolated vas deferens, as described previouisly (Maggi *et al.*, 1987). The inhibitory effect of CGRP was unaffected by capsazepine (10 μM) or ruthenium red (3 μM) (Figure 2b).

Discussion

In this study we have used a cumulative protocol of capsaicin administration to investigate capsaicin antagonism by capsazepine and ruthenium red. It is well known that capsaicin produces a functional desensitization of primary afferent neurones. In descriptive terms, this means that, after application of the drug, the primary afferents become insensitive to further applications of capsaicin itself as well as to other agents which stimulate the primary afferent neurones. The capsaicin desensitization phenomenon is concentration-, time- and temperature-dependent (e.g. Amann, 1990) and probably involves different mechanisms such as a true desensitization (tachyphylaxis) of the vanilloid receptor (Dray *et al.*, 1989; Maggi *et al.*, 1990), blockade of transmitter release through the inactivation of voltage-sensitive calcium channels (Bleakman *et al.*, 1990; Docherty *et al.*, 1991) and degenerative changes in primary afferents owing to the marked influx of cations produced by capsaicin (Bevan & Szolcsanyi, 1990; Maggi, 1991; Holzer, 1991).

To overcome this problem, increasing concentrations of capsaicin were added to the bath with a ten fold increase in concentration at each step, while the effect of antagonists was compared to control curves obtained in preparations from

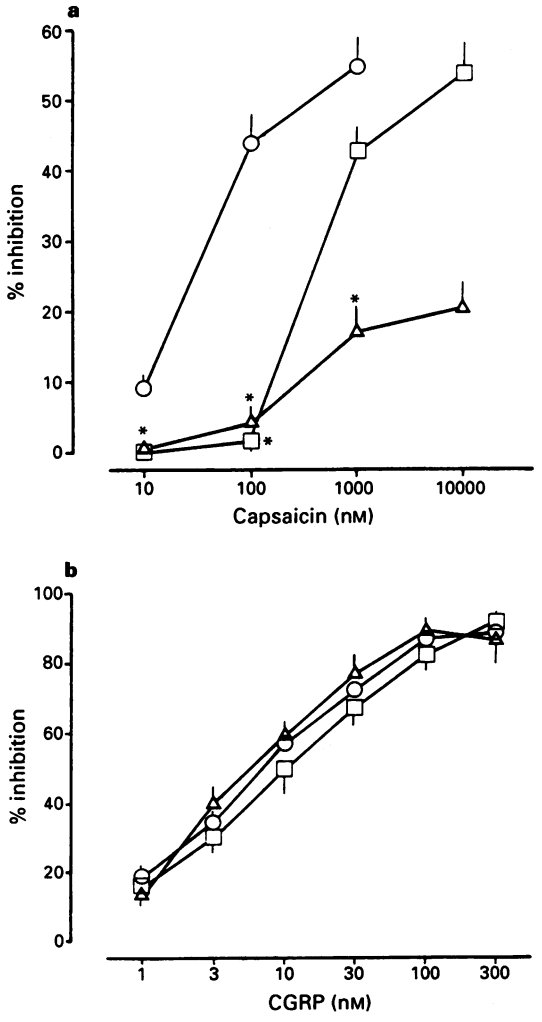

Figure 2 Effect of capsazepine (CPZ) or ruthenium red (RR) on concentration-response curve for capsaicin-induced (a) or rat α-calcitonin gene-related peptide (αCGRP)-induced (b) inhibition of twitch contractions of the rat isolated vas deferens: control (○); CPZ 10 μM (□); RR 3 μM (△). Each value is mean ± s.e.mean (vertical lines) of 4–12 experiments.

the same animal. The maximal response produced by cumulative application of capsaicin was only slightly less (bladder) or not significantly different (vas deferens) from that produced by the administration of a 1 μM single dose of capsaicin.

A drawback of the technique we have used is that the whole curve obtained with the agonist, either in the absence or presence of antagonists, is very steep and the few experimental points obtained in this way do not lend themselves to a conventional analysis for establishing whether competitive antagonism occurred (i.e. measurement of ED_{50}s, dose-ratios and Schild plots). In spite of this limitation, the different nature of antagonism by capsazepine and ruthenium red was very evident in both preparations investigated. Capsazepine essentially produced a rightward shift of the concentration-response curve to capsaicin without any significant depression of the maximum response. The failure of capsazepine to modify significantly the contractile response to neurokinin A in the bladder or to CGRP in the vas deferens ruled out a postjunctional action to inhibit the effect of endogenous neuropeptides released by capsaicin. Likewise, the failure of capsazepine to affect the amplitude of twitch contractions in both the bladder and the vas deferens excludes a nonspecific neuronal depressant action.

Overall, the present results are in good agreement with the competitive antagonist character of capsazepine action at the vanilloid receptor, as demonstrated in other test systems (Bevan *et al.*, 1991; 1992; Dray *et al.*, 1991).

In sharp contrast, ruthenium red displaced the curve to capsaicin in a manner that is indicative of non-competitive antagonism. The ability of ruthenium red to antagonize the action of capsaicin in the rat bladder and vas deferens at a prejunctional site of action was demonstrated in previous studies (Maggi *et al.*, 1988a,b) but the nature of antagonism (competitive vs. noncompetitive) had not been investigated. In contrast to capsazepine, ruthenium red at the concentration required to antagonize capsaicin, may have a neuronal depressant action not restricted to primary afferent neurones. This aspect of ruthenium red pharmacology, already noted in previous investigations (e.g. Chahl, 1989), is further confirmed here by its depressant action on twitch contractions of the isolated vas deferens. This effect of ruthenium red is however very variable from one preparation to another and cannot account for the ability of the dye to act as functional capsaicin antagonist (Amann & Maggi, 1991). Evidence has been presented (Dray *et al.*, 1990; Bleakman *et al.*, 1990) indicating that ruthenium red acts at the level of the cell membrane of primary afferent neurones to prevent the opening of the vanilloid receptor-operated cation channel. Although the molecular mechanism by which ruthenium red acts as a functional capsaicin antagonist has not yet been established, the non-competitive antagonism seen in this study is in keeping with its proposed site of action at a step beyond the occupation of the vanilloid receptor by the agonist.

The results of the present study, while confirming that both capsazepine and ruthenium red are useful tools to explore sensory neurone function, indicate that these two agents act at distinct steps in the sequence of events which is activated by capsaicin application. The different nature of the antagonism by capsazepine and ruthenium red is strongly reminiscent of the manner in which other drugs (e.g. bicuculline and picrotoxin at the $GABA_A$ receptor) act to inhibit receptor-operated ion channels and this parallel may be kept in mind in planning further experiments analysing the pharmacology of the vanilloid receptor and functional responses coupled to its activation.

References

AMANN, R. (1990). Desensitization of capsaicin-evoked neuropeptide release – influence of Ca^{2+} and temperature. *Naunyn Schmiedebergs Arch. Pharmacol.*, **342**, 671–676.

AMANN, R. & MAGGI, C.A. (1991). Ruthenium red as a capsaicin antagonist. *Life Sci.*, **49**, 849–856.

BEVAN, S.J., HOTHI, S., HUGHES, G., JAMES, I.F., RANG, H.P., SHAH, K., WALPOLE, C.S.J. & YEATS, J.C. (1992). Capsazepine: a competitive antagonist of the sensory neurone excitant capsaicin. *Br. J. Pharmacol.*, **107**, 544–552.

BEVAN, S.J., JAMES, I.F., RANG, H.P., SHAH, K. & YEATS, J.C. (1991). The development of a capsaicin antagonist for the sensory neurone excitant, capsaicin. *Br. J. Pharmacol.*, **102**, 77P.

BEVAN, S. & SZOLCSANYI, J. (1991). Sensory neuron-specific actions of capsaicin: mechanisms and applications. *Trends Pharmacol. Sci.*, **11**, 330–333.

BLEAKMAN, D., BRORSON, J.R. & MILLER, R.J. (1990). The effect of capsaicin on voltage-gated calcium currents and calcium signals in cultured dorsal root ganglion cells. *Br. J. Pharmacol.*, **101**, 423–431.

CHAHL, L.A. (1989). The effects of ruthenium red on the response of guinea-pig ileum to capsaicin. *Eur. J. Pharmacol.*, **169**, 241–247.

DICKENSON, A.H., DRAY, A., HUGHES, G.A. & WALPOLE, C.S.J. (1991). The selective antagonist capsazepine inhibits capsaicin induced antinociception: electrophysiological studies in rodents. *Br. J. Pharmacol.*, **102**, 79P.

DOCHERTY, R.J., ROBERTSON, B. & BEVAN, S. (1991). Capsaicin causes prolonged inhibition of voltage-activated calcium currents in adult rat dorsal root ganglion neurons in culture. *Neuroscience*, **40**, 513–521.

DRAY, A., BETTANEY, J. & FORSTER, P. (1989). Capsaicin desensitization of peripheral nociceptive fibers does not impair sensitivity to other noxious stimuli. *Neurosci. Lett.*, **99**, 50–54.

DRAY, A., FORBES, C.A. & BURGESS, G.M. (1990). Ruthenium red blocks the capsaicin-induced increase in intracellular calcium and activation of membrane currents in sensory nurones, as well as the activation of peripheral nociceptors in vitro. *Neurosci. Lett.*, **110**, 52–59.

DRAY, A., CAMPBELL, E.A., HUGHES, G.A., PATEL, I.A., PERKINS, M.N., RANG, H.P., RUEFF, A., SENO, N., URBAN, L. & WALPOLE, C.S.J. (1991). Antagonism of capsaicin-induced activation of C fibres by a selective capsaicin antagonist capsazepine. *Br. J. Pharmacol.*, **102**, 78P.

HOLZER, P. (1988). Local effector functions of capsaicin-sensitive sensory nerve endings: involvement of tachykinins, calcitonin gene-related peptide and other neuropeptides. *Neuroscience*, **24**, 739–768.

HOLZER, P. (1991). Capsaicin: cellular targets, mechanisms of action and selectivity for thin sensory neurons. *Pharmacol. Rev.*, **43**, 143–201.

MAGGI, C.A. (1991). Capsaicin and primary afferent neurons: from basic science to human therapy? *J. Autonom. Nerv. System*, **33**, 1–14.

MAGGI, C.A., ASTOLFI, M., DONNERER, J. & AMANN, R. (1990). Which mechanisms account for the sensory neuron blocking action of capsaicin on primary afferents in the rat urinary bladder? *Neurosci. Lett.*, **110**, 267–272.

MAGGI, C.A., GIULIANI, S., SANTICIOLI, P. & MELI, A. (1987). Capsaicin-induced inhibition of motility of the rat isolated vas deferens: do multiple neuropeptides mediate the visceromotor effects of capsaicin? *J. Autonom. Pharmacol.*, **7**, 243–255.

MAGGI, C.A. & MELI, A. (1988). The sensory-efferent function of capsaicin-sensitive sensory neurons. *Gen. Pharmacol.*, **19**, 1–43.

MAGGI, C.A., PATACCHINI, R., SANTICIOLI, P. & GIULIANI, S. (1991). Tachykinin antagonists and capsaicin-induced contraction of the rat isolated urinary bladder: evidence for tachykinin-mediated cotransmission. *Br. J. Pharmacol.*, **103**, 1535–1541.

MAGGI, C.A., PATACCHINI, R., SANTICIOLI, P., GIULIANI, S., GEPPETTI, P. & MELI, A. (1988a). Protective action of ruthenium red toward capsaicin desensitization of sensory fibers. *Neurosci. Lett.*, **88**, 201–205.

MAGGI, C.A., SANTICIOLI, P., GEPPETTI, P., PARLANI, M., ASTOLFI, M., PRADELLES, P., PATACCHINI, R. & MELI, A. (1988b). The antagonism induced by ruthenium red of the actions of capsaicin on the peripheral terminals of sensory neurons: further studies. *Eur. J. Pharmacol.*, **154**, 7–10.

MORITOKI, H., IWAMOTO, T., KANAYA, J., ISHIDA, Y., ANDO, K. & KITAGAWA, K. (1987). Capsaicin enhances the nonadrenergic twitch response of rat vas deferens. *Br. J. Pharmacol.*, **92**, 469–475.

SANTICIOLI, P., MAGGI, C.A., GEPPETTI, P., DEL BIANCO, E., THEODORSSON, E. & MELI, A. (1988). Release of calcitonin gene-related peptide-like immunoreactivity (CGRP-LI) from organs of the genitourinary tract in rats. *Neurosci. Lett.*, **92**, 197–201.

SZALLASI, A. & BLUMBERG, P.M. (1990). Specific binding of resiniferatoxin: an ultrapotent capsaicin analog by dorsal root ganglion membranes. *Brain Res.*, **524**, 106–111.

URBAN, L. & DRAY, A. (1991). Capsazepine, a novel capsaicin antagonist, selectively antagonises the effects of capsaicin in the mouse spinal cord in vitro. *Neurosci. Lett.*, **134**, 9–11.

(Received April 10, 1992
Revised November 2, 1992
Accepted November 4, 1992)