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Selective antagonism of capsaicin by capsazepine: evidence for a spinal receptor site in capsaicin-induced antinociception

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1 Capsazepine has recently been described as a competitive capsaicin antagonist. We have used this compound to test the hypotheses that the *in vitro* and *in vivo* effects of capsaicin are due to interactions with a specific receptor.

2 In an *in vitro* preparation of the neonatal rat spinal cord with functionally connected tail, the activation of nociceptive afferent fibres by the application of capsaicin, bradykinin or noxious heat (48°C) to the tail could be measured by recording a depolarizing response from a spinal ventral root. Application of capsaicin or substance P to the spinal cord also evoked a depolarizing response which was recorded in a ventral root.

3 When capsazepine (50 nM–20 μM) was administered to the tail or spinal cord it did not evoke any measurable response. However on the tail, capsazepine reversibly antagonized ($IC_{50} = 254 \pm 28$ nM) the responses to capsaicin but not to heat or bradykinin administered to the same site. Similarly capsazepine administration to the spinal cord antagonized the responses evoked by capsaicin ($IC_{50} = 230 \pm 20$ nM) applied to the cord but not responses evoked by substance P on the cord or by noxious heat and capsaicin on the tail.

4 In halothane anaesthetized rats, C-fibre responses evoked by transcutaneous electrical stimulation of the receptive field were recorded from single wide dynamic range neurones located in the spinal dorsal horn. C-fibre evoked discharges were consistently reduced by the systemic administration of capsaicin (20 μmol kg^{-1}, s.c.) and this action of capsaicin was antagonized by capsazepine (100 μmol kg^{-1}) administered by the same route. In addition the systemic effect of capsaicin was antagonized by a spinal intrathecal administration of capsazepine (5–50 nmol).

5 Intradermal injections of capsaicin, localized to the peripheral receptive field, usually one toe of the ipsilateral hind-paw, produced a transient increase in C-fibre-evoked activity followed by a prolonged period of localized insensitivity to transcutaneous C-fibre stimulation. These effects of capsaicin were significantly reduced by the concommitant administration of capsazepine to the same site.

6 These data demonstrate that capsazepine is a selective antagonist of capsaicin on nociceptive neurones *in vitro* and *in vivo* and suggest that the effects of capsaicin were mediated by activation of a specific receptor. Since the antinociceptive effect produced by systemically administered capsaicin was antagonised by spinal intrathecal capsazepine this further supports the hypothesis that capsaicin exerts its antinociceptive effect by acting on specific receptors localized to sensory nerve fibres in the spinal cord.

Keywords: Capsaicin; capsazepine; peripheral nociceptors; spinal cord; antagonist

Introduction

Capsaicin (8-methyl N-vanillyl-6-nonenamide) produces a number of effects by selective actions on mammalian polymodal nociceptive neurones and warm thermoceptors (Fitzgerald, 1983; Szolcsanyi, 1985; 1990; Buck & Burks, 1986; Bevan *et al.*, 1987). In man, local applications of capsaicin produce a sensation of burning-pain due to the activation of primary afferent C- and probably Aδ-neurones. However, with repeated exposure to capsaicin both the C-fibre activation and the algesic effect desensitize (Petsche *et al.*, 1983; Marsh *et al.*, 1987). On the other hand acute systemic administration of capsaicin produces antinociception which has been shown by several conventional tests (Hayes & Tyers, 1980; Hayes *et al.*, 1984; Campbell *et al.*, 1989). The basis for the reversible antinociceptive effect of capsaicin is unclear though recent studies (Dickenson *et al.*, 1990a,b) have indicated that sensory nerve terminals in the spinal cord are important.

It has been suggested that the effects of capsaicin on mammalian sensory neurones are mediated through an interaction with a specific membrane receptor. However, the evidence for this has been rather indirect. Thus a capsaicin recognition site has been proposed from structure-activity studies (Szolcsanyi & Jancso-Gabor, 1975; 1976; Hayes *et al.*, 1984) and by the use of a capsaicin-like photoaffinity probe (James *et al.*, 1988). Somewhat more directly, capsaicin has been shown to displace the binding of [^{3}H]-resiniferatoxin (Szallasi & Blumberg, 1990), a highly potent, naturally occurring capsaicin analogue (Szallasi & Blumberg, 1989; Winter *et al.*, 1990) with a similar mechanism of action (Winter *et al.*, 1990). Recently however, a selective capsaicin antagonist, capsazepine, has been described (Bevan *et al.*, 1991; Dray *et al.*, 1991) which has provided direct pharmacological evidence for a capsaicin receptor localized on the mammalian sensory neural membrane.

In the present experiments we have used capsazepine to indicate whether the capsaicin-mediated activation of nociceptors *in vitro* or *in vivo* is a consequence of specific receptor interactions. Furthermore we have sought additional evidence for sensory elements in the spinal cord as primary targets for the acute analgesic action of systemic capsaicin. Parts of this study have been published as abstracts (Dickenson *et al.*, 1991; Dray *et al.*, 1991).

Methods

The in vitro *preparation*

The intact spinal cord and the functionally connected tail were removed from 1–2 day old rats following decapitation

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and prepared by the method described by Otsuka & Yanagisawa (1988). The skin was carefully removed from the distal four fifths of the tail. This procedure was thought to expose cutaneous fibres and their endings to facilitate activation of nociceptors by capsaicin and other algesic agents (Dray *et al.*, 1990a,b). Damage to the underlying tissue was avoided as this severely compromised responsiveness to peripheral stimuli. We cannot be certain however that our tests were made in an environment entirely free of tissue damage. Histology was not routinely performed. Indeed electron microscopy would have been necessary to confirm the structural integrity of fine afferent nerve endings and quantification of this would have presented considerable difficulty. However bradykinin, which was used routinely as an algesic chemical stimulus, is not known to activate axons of nociceptors. It is more likely to stimulate nociceptors via the signal transducing elements localized to the terminations of primary afferent fibres. The efficacy of bradykinin thus suggested that nociceptors were preserved in our viable preparations. This would strongly indicate minimal nerve fibre damage. In addition the effects of our peripheral stimuli were robust and reproducible over many hours. This also would be unlikely in the face of significant tissue damage.

The preparation was placed in a chamber such that the cord and tail could be separately superfused (2–4 ml min^{-1}) with a physiological salt solution (composition mM: NaCl 138.6, KCl 3.35, $CaCl_2$ 1.26, $MgCl_2$ 1.16, $NaHCO_3$ 21.0, $NaHPO_4$ 0.58, glucose 10) at 24°C and gassed with 95% O_2/5% CO_2. Peripheral nociceptive fibres were activated by superfusion of the tail with noxious chemicals (capsaicin, bradykinin) and by superfusate heated to 48°C. Each stimulus was applied for 10 s with an intervening period of 15 min between stimuli. Bradykinin doses were separated by at least 40–60 min to avoid tachyphylaxis.

The activation of peripheral fibres was assessed by measuring the depolarization produced in a spinal ventral root (L_3–L_5). The ventral root depolarization was recorded d.c. (with respect to the spinal cord which was earthed) with a low impedence glass pipette. This was placed in an electrolyte-filled well which contained the selected ventral root. The signals were amplified by conventional means and displayed simultaneously on an oscilloscope and on a rectilinear chart recorder.

Reproducible ventral root responses were obtained to peripheral stimuli including noxious heat and submaximal concentrations of capsaicin and bradykinin. Following this capsazepine was administered in the tail superfusate for 15 min prior to retesting capsaicin and other stimuli. The antagonist potency (IC_{50}) of capsazepine was estimated by cumulatively increasing the concentration to produce an incremental reduction of the response to capsaicin. Three or more submaximal concentrations of capsazepine were used in each experiment to determine the IC_{50} concentration. Ventral root responses were also used to measure the activation of afferent fibres and postsynaptic elements in the spinal cord by spinal administrations of capsaicin and substance P respectively. The effect of capsazepine administered to the spinal cord was determined as described above against responses evoked by administration of substance P and capsaicin to the spinal cord and to responses evoked by noxious heat and capsaicin administration to the tail.

In vivo *preparation*

Single unit recordings were made from dorsal horn neurones in the intact halothane-anaesthetized rat (Dickenson & Sullivan, 1987). Following induction of anaesthesia (3% halothane in O_2/N_2O) a tracheal cannula was inserted and a laminectomy was made to expose the spinal L_1–L_3 area. The animal was then mounted in a frame so that the vertebrae rostral and caudal to the recording site were securely clamped. Anaesthesia was then maintained by 1–1.5% halothane. A tungsten-glass microelectrode was inserted into the dorsal horn and extracellular recordings were made, by conventional techniques, from single neurones located in the superficial and deep laminae. All cells responded to innocuous peripheral stimuli (touch/brush/pressure) and to noxious thermal and mechanical (pinch) stimuli. Cells were characterized by their responses to the natural peripheral stimuli mentioned above and then two fine needles were placed in the center of the hindpaw receptive field for transcutaneous stimulation. Both Aβ and C-fibre responses, based on latency and threshold could always be elicited in the neurones, and on occasion Aδ fibre responses were observed. Following at least two stable control responses (3 times threshold for the C-fibre and then Aβ-fibre responses), capsaicin or the vehicle (10% Tween/10% ethanol (10%); physiological saline) were administered at the following sites: (1) subcutaneously into the scruff of the neck, (2) intrathecally in a volume of 50 μl onto the exposed surface of the spinal cord and (3) locally, in a 10 μl volume into a discrete region of the receptive field. Administration at these sites was repeated with the co-administration of capsazepine or with capsazepine alone. The effect of intrathecal capsazepine was also tested against the effect of systemic capsaicin. Responses to transcutaneous stimulation were measured at 5 min post injection and then at 10 min intervals for between 45 and 180 min. Responses were quantified by use of post stimulus histograms, analysed and built with a CED 1401 interface and software (Dickenson & Sullivan, 1990). Only one cell was used per animal for each systemic drug study. Data were statistically evaluated by the unpaired *t* test (95% confidence limits).

The following drugs were used: capsaicin (Sigma), capsazepine (2-[2-4-chlorphenyl)ethylamino-thiocarbonyl]-7,8-dihydroxy-2,3,4,5-tetrahydro- 1H-2-benzazepine; bradykinin (Bachem, CRB).

Results

In vitro *study*

As in previous studies (Dickenson *et al.*, 1990; Dray *et al.*, 1990a,b), a brief administration of capsaicin at a submaximal concentration (usually 0.5–0.7 μM), in the tail superfusate, evoked a short lived (45–90 s) depolarizing response in a ventral root. These responses could be reproduced over several hours. In addition stable and reproducible responses could be evoked by brief noxious heat stimulation of the tail. In a number of experiments responses evoked by bradykinin (100–350 nM) were used to test further the selectivity of the antagonist action of capsazepine.

Continuous superfusion of the tail with capsazepine (50 nM–20 μM) for 15 min preceding and during the administration of capsaicin or other stimuli did not itself evoke any response. However capsazepine consistently and reversibly (within 30–60 min) attenuated the effect of capsaicin in a concentration-related manner (Figure 1). The capsazepine IC_{50} concentration was 254 $\pm$ 28 nM (n = 8). However, even at the highest concentration tested (20 μM), capsazepine did not affect the responses evoked by heat (n = 8) or bradykinin (n = 5) (Figure 1).

In a separate series of experiments, brief administration (10 s) of a submaximal concentration of capsaicin (200 nM), in the spinal cord superfusate, evoked reproducible depolarizing responses following the activation of nociceptive afferent nerve terminals. A selective effect of capsaicin on afferent terminals had been indicated from previous studies showing (a) capsaicin-evoked release of neuropeptide specifically located in fine afferent fibres (Theriault *et al.*, 1976; Dickenson *et al.*, 1990b), (b) lack of effect of capsaicin on spinal neurones following neurotoxic doses (Yaksh *et al.*, 1979) and (c) lack of a direct effect of capsaicin on spinal dorsal horn neurones measured electrophysiologically (Urban & Dray, 1990). Responses were also evoked by administration of a submaximal concentration of substance P (25 nM), presumably through activation of receptors on postsynaptic spinal elements (Akagi *et al.*,

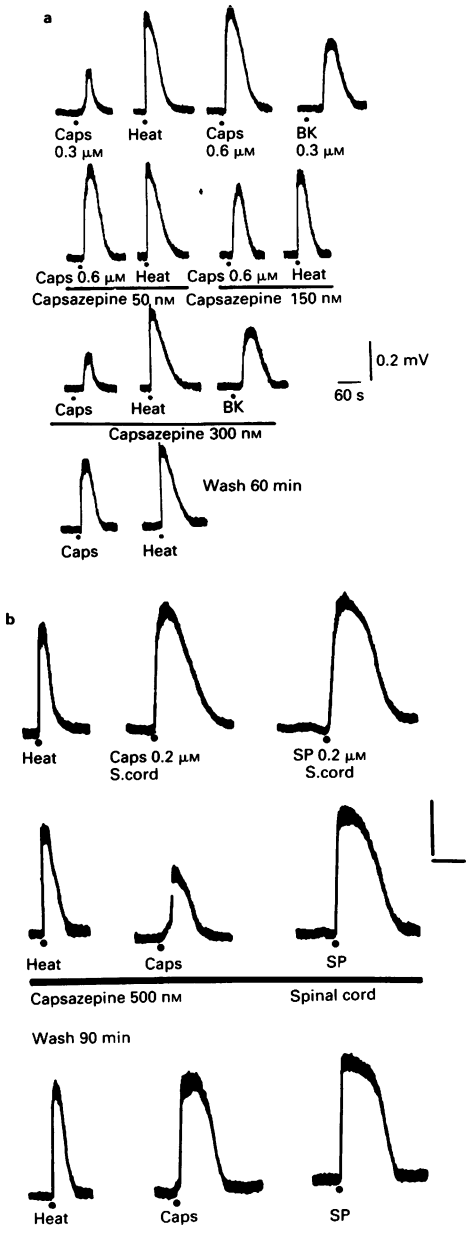

Figure 1 (a) Concentration-related antagonism of capsaicin (Caps) by capsazepine in the spinal cord/tail *in vitro*. The top traces show ventral root responses produced by application of capsaicin (0.3 and 0.6 μM), heat (48°C) and bradykinin (BK, 0.3 μM) to the tail. The second and third row of traces show concentration-related reduction of the response to capsaicin (0.6 μM) by capsazepine (50, 150 and 300 nM) but not the responses to heat or bradykinin (0.3 μM). The application of capsazepine was started 15 min before capsaicin was tested and the concentration was increased cumulatively. The response to capsaicin recovered following a 60 min wash of the tissue (bottom traces). The calibration bars of 60 s and 0.2 mV are indicated in (a) and (b). (b) Selective antagonism of capsaicin on the spinal cord by capsazepine. The top traces show control responses to heat administered to the tail and to capsaicin (0.2 μM) and substance P (SP, 0.2 μM) administered in the spinal cord superfusate. During the continuous administration of capsazepine to the spinal cord (500 nM), begun 15 min before the agonists were retested, the effect of capsaicin was selectively reduced. The bottom traces show complete recovery of capsaicin responses after washing the tissue for 90 min.

1985). Such responses, together with the ventral root responses evoked by peripheral application of heat or capsaicin, served as controls to check for selectivity of the effect of capsazepine.

As before, prolonged superfusions of the spinal cord with capsazepine (50 nM–5 μM) did not produce any response or change the responses evoked by spinal administration of substance P. In addition, responses evoked by peripheral noxious heat or capsaicin applications were unaffected (Figure 2). However, the responses evoked by capsaicin administration to the spinal cord were consistently attenuated in a concentration-related manner (IC_{50} = 230 ± 20 nM, n = 5). In each case recovery from the effect of capsazepine occurred within 30–60 min.

In vivo *study*

Administration into the receptive field Eight dorsal horn neurones were studied. All had receptive fields in two or more toes. Each neurone served as its own control. Thus the effect of capsaicin alone (200 pmol) and capsaicin plus capsazepine (1 nmol) was tested on each cell. Each cell responded to C-fibre stimulation (3 × threshold) with a mean response of 20 ± 6 spikes per stimulus. After capsaicin, none of the cells responded to C-fibre stimulation for at least 1 h afterwards. Each cell was activated by Aβ-fibre stimulation but these responses were unchanged by capsaicin. After the co-administration of capsazepine with capsaicin the effect of capsaicin was completely abolished. Thus the mean C-fibre evoked responses of the population was 17 ± 5 spikes per stimulus and this was not significantly different from values obtained following vehicle injections. The same concentration of capsazepine also prevented the activation of the neurones by capsaicin (Figure 2). Capsazepine alone (n = 4), had no effect on cell firing or on C-fibre evoked responses.

Systemic capsazepine versus systemic capsaicin Capsaicin (20 μmol kg^{-1}) injected subcutaneously into the scruff of the neck at a dose found to produce antinociception both in behavioural and electrophysiological studies (Campbell *et al.*, 1989; Dickenson *et al.*, 1990a), produced a maximal 66% inhibition of the C-fibre evoked responses of dorsal horn cells at 40 min post injection (n = 7). In three other animals the administration of capsaicin was immediately preceded by a s.c. injection of 100 μmol kg^{-1} capsazepine into the same or another s.c. site. In each of these studies the inhibitory effect of

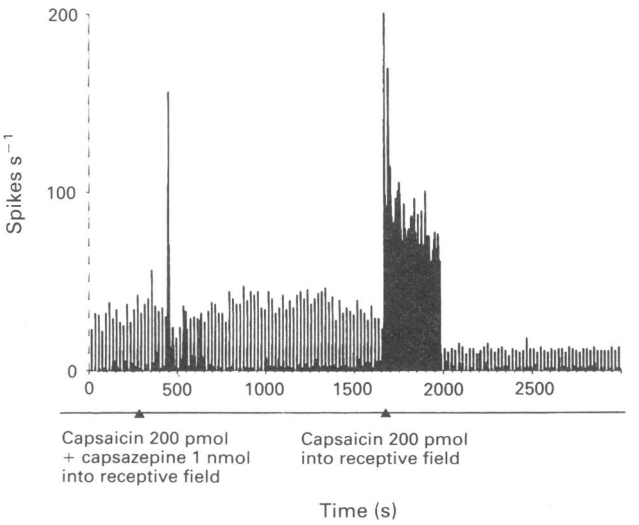

Figure 2 Example of a ratemeter recording of a dorsal horn nociceptive neurone in response to transcutaneous electrical stimulation of the receptive field (two toes on the ipsilateral hindpaw). The combined Aβ and C-fibre evoked response is plotted as spikes s^{-1} (vertical axis) against time (s). The combined intradermal injection of capsaicin (200 pmol) and capsazepine (1 nmol) produced minimal C-fibre activation and little evoked effect on the neurone (injected into toe 1), whereas capsaicin alone (injected into toe 2) activated the cell and then abolished the C-fibre evoked response. The residual activity seen in the post-stimulus histogram after capsaicin was due entirely to the Aβ-fibre evoked activity.

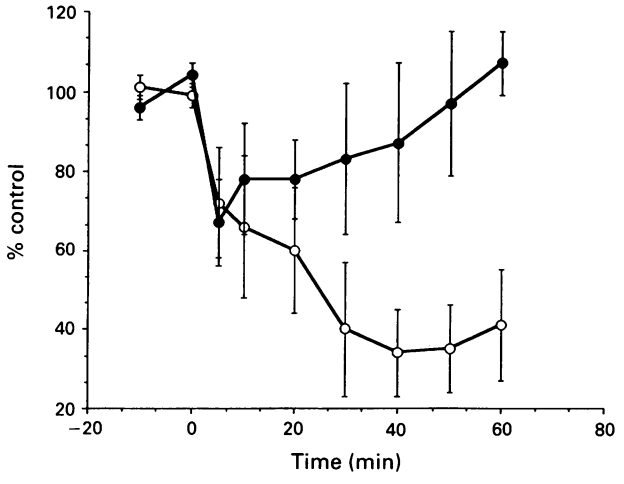

Figure 3 The C-fibre-evoked responses of dorsal horn neurones, expressed as percentage (with s.e.mean shown by vertical bars) of the pre-injection control responses, were inhibited by s.c. capsaicin ($20\,\mu mol\,kg^{-1}$, $n = 7$, ○). Co-administration of capsazepine ($100\,\mu mol\,kg^{-1}$, s.c., $n = 3$, ●) together with s.c. capsaicin produced a significant reduction ($P < 0.05$), at the 30, 40, 50 and 60 min time points. The control readings prior to the application of the agents at time 0 are shown.

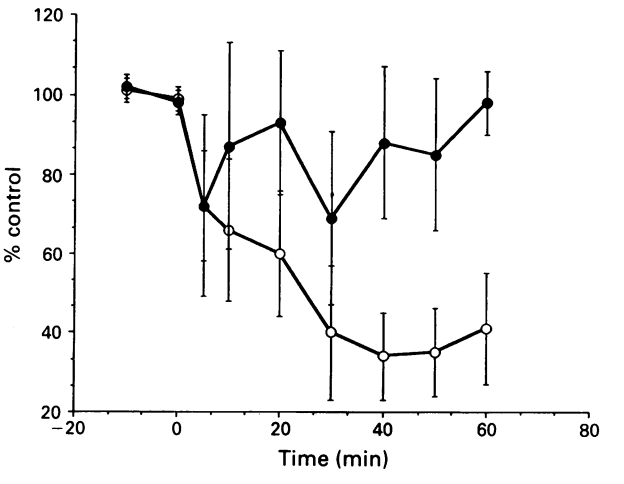

Figure 4 The inhibition of the C-fibre responses of dorsal horn nociceptive neurones produced by s.c. capsaicin ($20\,\mu mol\,kg^{-1}$, s.c., $n = 7$, ○) was attenuated by intrathecal capsazepine (50 nmol) applied just prior to the capsaicin ($n = 5$, ●). The capsaicin response was significantly reduced ($P < 0.01$) at the 40, 50 and 60 min time points. Applicatoin of the drugs was at time zero.

capsaicin was now transient and at 30 min post injection C-fibre evoked activity was depressed by only 13% (Figure 3). The response of cells to Aβ fibre stimulation was unaffected by capsaicin, capsazepine or by the combination of these substances.

Intrathecal capsazepine and systemic capsaicin Neuronal activity was recorded for at least 60 min following the intrathecal administrations of capsazepine. No significant change in spontaneous firing of dorsal horn cells was observed and no effects on responses evoked by C- or Aβ-fibre stimulation were produced by 5 nmol, (4 cells) or 50 nmol (3 cells) capsazepine.

In control animals ($n = 7$), s.c. capsaicin ($20\,\mu mol\,kg^{-1}$) produced a gradual reduction of the discharges evoked by C-fibre stimulation. This effect plateaued some 40 min after the injection and reached a maximum of 66% inhibition. The Aβ-fibre evoked responses of the same cells were maximally reduced by only 25% at 30 min after the adminstration of capsaicin. Intrathecal capsazepine (50 nmol; $n = 5$) significantly ($P < 0.05$) reduced (at the 40, 50 and 60 min time points) the inhibitory effect of systemic capsaicin (Figure 4). Following capsazepine the responses of these cells to Aβ fibre input was maximally reduced by capsaicin by only 8%. When the C-fibre evoked responses were analysed, taking into account the firing frequency and duration of the response, the intrathecal capsazepine-induced reduction of capsaicin (by 58%, $P < 0.01$, Figure 4) was highly significant. Also the antagonism of capsaicin by capsazepine appeared to be dose-related since the inhibition of C-fibre responses produced by capsaicin ($20\,\mu mol\,kg^{-1}$, s.c.) was unaffected by a lower dose (5 nmol, i.t.) of capsazepine ($n = 4$).

Discussion

Indirect evidence obtained from a number of previous studies has supported the suggestion that the unique effects of capsaicin on mammalian sensory neurones was due to an interaction with a specific membrane receptor (Szolcsanyi & Jancso-Gabor, 1975; 1976; Hayes *et al.*, 1984; James *et al.*, 1988; Szallasi & Blumberg, 1989; 1990). This hypothesis now receives direct support following the discovery of capsazepine, a capsaicin analogue with selective antagonist properties. Capsazepine antagonized a number of the effects of capsaicin *in vitro*, including the evoked whole cell membrane currents and the flux of a number of cations (Bevan *et al.*, 1991). The antagonistic effect was both selective and competitive.

The present *in vitro* experiments further extend these observations. We have thus shown, as in previous studies (Yanagisawa & Otsuka, 1984; Dray *et al.*, 1990a,b) that physiological stimulation of peripheral nociceptive fibres by capsaicin and a number of other noxious stimuli, can evoke reproducible responses in the spinal cord, maintained *in vitro* with the functionally connected tail. Capsazepine, at concentrations which itself produced no discernible effect on peripheral nerves or on the excitability of the spinal cord, consistently and reversibly antagonized the effects of capsaicin when both substances were administered to the same peripheral or central elements of sensory neurones. Capsazepine did not reduce the responses evoked by capsaicin when each substance was administered to different regions (i.e. spinal vs. peripheral) of sensory neurones.

Although the present experiments did not test whether capsazepine was a competitive antagonist, they clearly demonstrated that capsazepine was selective, since responses produced by noxious heat, bradykinin or substance P administered to the periphery or spinal cord respectively, were unaffected. The potency of capsazepine, under the present conditions, was similar to that measured in dorsal root ganglion neurones or vagal afferent fibres maintained in vitro (Bevan *et al.*, 1991) where competitive interactions were also demonstrated. This supports the possibility that the capsaicin receptive site is similar in a variety of *in vitro* preparations of sensory neurones.

In addition we have tested capsazepine against a number of effects mediated by capsaicin *in vivo*: specifically against the effects produced by local and systemic capsaicin administrations. Thus capsazepine antagonized the initial capsaicin-induced C-fibre activation and the subsequent capsaicin-induced inactivation of C-fibres following the administration of capsaicin into the peripheral receptive field, (usually a single toe) of dorsal horn neurones. These effects were clearly selective and well localized as a subsequent administration of capsaicin into another part of the receptive field (another toe), evoked both an initial activation and a subsequent blockade of C-fibre evoked activity. In keeping with our previous observations (Dickenson *et al.*, 1990a,b), systemic capsaicin also reduced C-fibre evoked responses of dorsal horn nociceptive neurones. This antinociceptive effect of capsaicin was also reduced by the concomitant administration of capsazepine by the same route. Finally and significantly, spinal intrathecal administration of capsazepine prevented the reduction of the C-fibre evoked input produced

by systemic capsaicin. This was considered to be due to an antagonism of a capsaicin-induced effect exerted at the level of the spinal cord since (a) capsazepine itself did not produce a response or alter nerve excitability but acted only as a selective capsaicin antagonist and (b) the amount of capsazepine administered i.t. was insufficient to antagonize the systemic effect of capsaicin should diffusion out of the spinal cord have occurred.

In summary, the present experiments have demonstrated that capsazepine is a selective antagonist against a number of capsaicin-induced effects on nociceptive neurones *in vitro* and *in vivo*. In addition, the effectiveness of capsazepine following spinal intrathecal administration further supports the likelihood that the systemic antinociceptive effect of capsaicin is produced by an action within the spinal cord as we have previously suggested (Dickenson *et al.*, 1990a,b).

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