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Capsazepine block of voltage-activated calcium channels in adult rat dorsal root ganglion neurones in culture

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1 We have found that capsazepine, a competitive antagonist at the vanilloid (capsaicin) receptor, blocks voltage-activated calcium currents in sensory neurones.

2 The block of calcium current was slow to develop with a half time of about one minute at 100 μM and lasted for the duration of the experiment. The rate of block of calcium current was strongly concentration-dependent.

3 The EC_{50} for the blocking effect at 0 mV was 7.7 ± 1.4 μM after 6 min exposure to capsazepine. The EC_{50} at equilibrium was estimated to be 1.4 ± 0.2 μM.

4 The block of calcium current showed some voltage-dependence but there was no indication of any selectivity of action for a calcium channel subtype. The characteristics of the blocking action of capsazepine on the residual current of cells which were pretreated with either ω-conotoxin or nimodipine were similar to control.

5 The data suggest that capsazepine, in addition to its competitive antagonism of vanilloid receptors, has a non-specific blocking action on voltage-activated calcium channels which should be taken into account when interpreting the effects of this substance on intact preparations *in vitro* or *in vivo*.

Keywords: Capsaicin; capsazepine; sensory neurone; calcium current; calcium channel; calcium antagonist

Introduction

Capsaicin, from hot peppers of the *capsicum* family and resiniferatoxin from the flowering cactus *euphorbia resinifera* are naturally occurring agonist ligands of the vanilloid receptor (see Holzer, 1991; Szallasi, 1994). Several structurally related compounds, including synthetic compounds, have been found which have agonist activity at the receptor (Szolcsányi & Jancsó-Gàbor, 1975a,b; Brand *et al*., 1986; Walpole & Wrigglesworth, 1993). In addition a synthetic antagonist, capsazepine, has been developed (Bevan *et al*., 1992a).

The vanilloid receptor is expressed in a subpopulation of peripheral c- and Aδ-fibre sensory neurones (reviewed by Holzer, 1991) where it ti is linked to a cation-specific ion channel (Marsh *et al*., 1987; Wood *et al*., 1988; Bevan & Szolcsányi, 1990; Bevan & Docherty, 1993; Wood & Docherty, 1997). Vanilloid agonists such as capsaicin activate the ion channel causing depolarization and excitation (Heyman & Rang, 1985; Marsh *et al*., 1987; Bevan & Docherty, 1993), effects that are blocked competitively by the antagonist capsazepine (Bevan *et al*., 1992a).

The physiological function of the sensory neurone vanilloid receptor is not known but there is evidence that it may be involved in mediating the antinociceptive effects of vanilloid compounds (Dray, 1992; Perkins & Campbell, 1992). No molecule has yet been identified as an endogenous ligand for the receptor, though it has been proposed that protons are endogenous activators of the ion channel associated with the receptor (Bevan & Yeats, 1991; Bevan *et al*., 1993; Bevan & Geppetti, 1994). Since capsazepine blocks the vanilloid receptor but not the ion channel (Bevan *et al*., 1992a), it is potentially useful as a chemical probe for investigating possible physiological roles for the receptor.

The general pharmacology of capsazepine is largely unknown. In the present paper we show that capsazepine may have a non-specific action in addition to its effects on vanilloid receptors. The drug blocks voltage-dependent calcium channels in sensory neurones. This raises the possibility that the compound may produce effects in intact preparations or *in vivo* which are independent of its effects on the vanilloid receptor. Consideration should be given to such non-specific effects in the interpretation of data obtained from complex physiological models *in vitro* or *in vivo*.

Methods

Experiments were performed on cultured dorsal root ganglion (DRG) neurones taken from adult Wistar or Sprague-Dawley rats (>200 g, of either sex). Animals were killed by asphyxiation in a chamber filled with a slowly rising concentration of CO_2 gas. Details of culture methods and recording conditions have been described previously (Docherty *et al*., 1991). Briefly, neurones were maintained in culture in the presence of 200 ng ml^{-1} nerve growth factor for one to seven days and replated on *p*-ornithine and laminin coated glass coverslips 2–6 h before an experiment. The replating procedure provided a preparation of spherical neuronal somata which were free of processes. All recordings were made at room temperature (18–20°C) from neuronal somata by use of the whole-cell patch-clamp technique with either a List EP7 (List Electronics) or Axopatch 1C or 200B (Axon instruments) amplifiers. The holding potential for all experiments was −90 mV and calcium currents were evoked during square voltage steps of 50 to 100 ms duration and were applied at 20 s intervals unless otherwise indicated. The test potential was 0 mV unless otherwise indicated (e.g. during construction of current-voltage curves). Cells had a mean capacitance of 30.1 ± 1.1 pF and series resistance of 9.2 ± 0.3 MΩ (mean ± s.e.mean, $n = 110$). Compensation for cell capacitance was made routinely with the analogue circuitry available on the amplifier and any residual capacitance transients and linear leak currents were subtracted by use of a P/n protocol with $n = 8$ or 10 (positive going steps). Series resistance compensation was not performed routinely except during construction of current-voltage curves (see legends to Figures 4 and 5). The superfusate contained (in mM): choline Cl 140, $CaCl_2$ 2.5, $MgCl_2$ 1, CsCl 3, HEPES 5, glucose 11 and 1% dimethylsulphoxide (DMSO); pH 7.4. The pipette solution was made up as follows (in mM): CsCl 110, $MgCl_2$ 3, EGTA 5, HEPES 40; pH 7.4.

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Final adjustment of pH 7.4 was achieved by addition of CsOH to the extracellular (additional 1.5 mEq Cs) and pipette (additional 4 mEq Cs) solutions. Drugs were applied to cells by use of a U-tube which allowed a complete solution change in the vicinity of the cell in 0.70 ± 0.10 s (measured by applying 40 mM KCl, $n = 9$).

Data were analysed by pClamp software (v.5 and v.6, Axon Instruments) and further analysis and statistical tests, as identified in the text, were applied with Microsoft Excel software. Curve fitting to data was performed by use of MicroCal Origin (v 3.7) software.

Chemicals were prepared as concentrated stock solutions in either distilled water or DMSO and diluted to the final concentration with superfusate solution. Capsazepine was synthesized by Sandoz Chemical laboratories or by Maybridge Chemical Co. ω-Conotoxin GVIA was supplied by Calbiochem. Other drugs were supplied by Sigma Chemical Co. except nerve growth factor which was a gift from Dr J. Winter (Sandoz Institute for Medical Research, London).

Results

Capsazepine (1 – 100 μM) inhibited voltage-dependent calcium currents in sensory neurones from DRG. Unlike capsaicin, which evokes an inward current in a sub-population of DRG sensory neurones, capsazepine had no consistent effect on membrane holding current. The inhibitory effect on calcium current was not restricted to any particular sub-population of DRG neurones. Capsazepine ($\geqslant 1$ μM), applied in the absence of other drugs, inhibited calcium current in 81 out of 91 neurones studied. In the ten neurones where capsazepine was ineffective the drug application was brief (30 s) and the apparent lack of effect reflects the slow blocking kinetics of the drug (see below) rather than a resistant sub-population of cells. It was possible to reverse the inhibitory effect of capsazepine partially if the drug was applied only briefly ($\leqslant 60$ s), but for longer applications (see below) there was no reversal even after washing the preparation in drug-free solution for at least

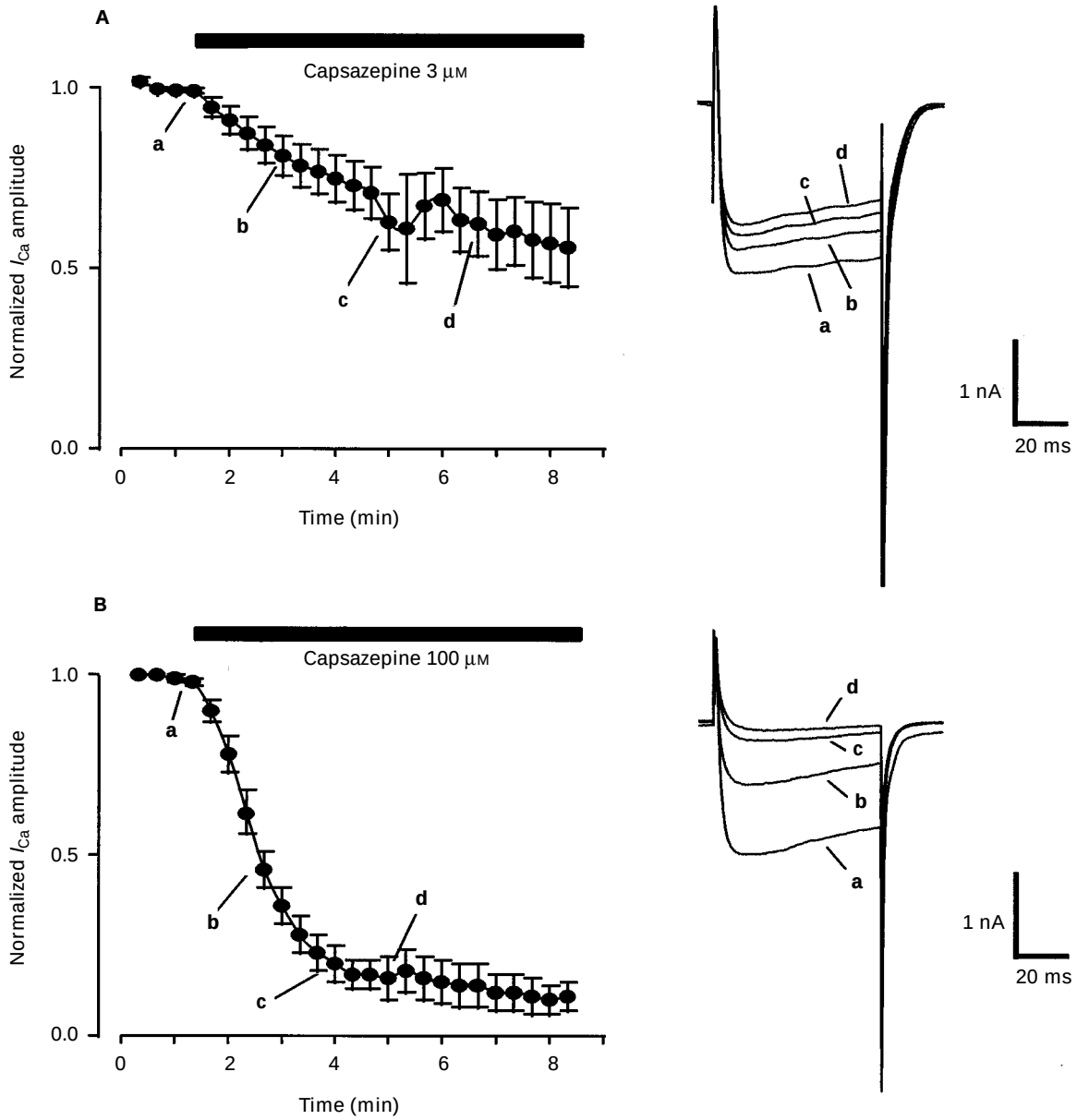

Figure 1 Capsazepine inhibited voltage-activated calcium currents. Voltage-dependent calcium currents were evoked by stepping from a holding potential of -90 mV to a test potential of 0 mV for 70 ms. The data shown in (A) are the mean $\pm$ s.e.mean of normalized data from 10 experiments and in (B) from 8 experiments. Examples of current traces from individual experiments, sampled at points a – d as indicated, are shown to the right of the averaged data. The peak amplitude of each current was normalized to the amplitude of the first current evoked in the series. Capsazepine (3 μM in (A) and 100 μM in (B)) was applied during the period indicated by the horizontal black bars.

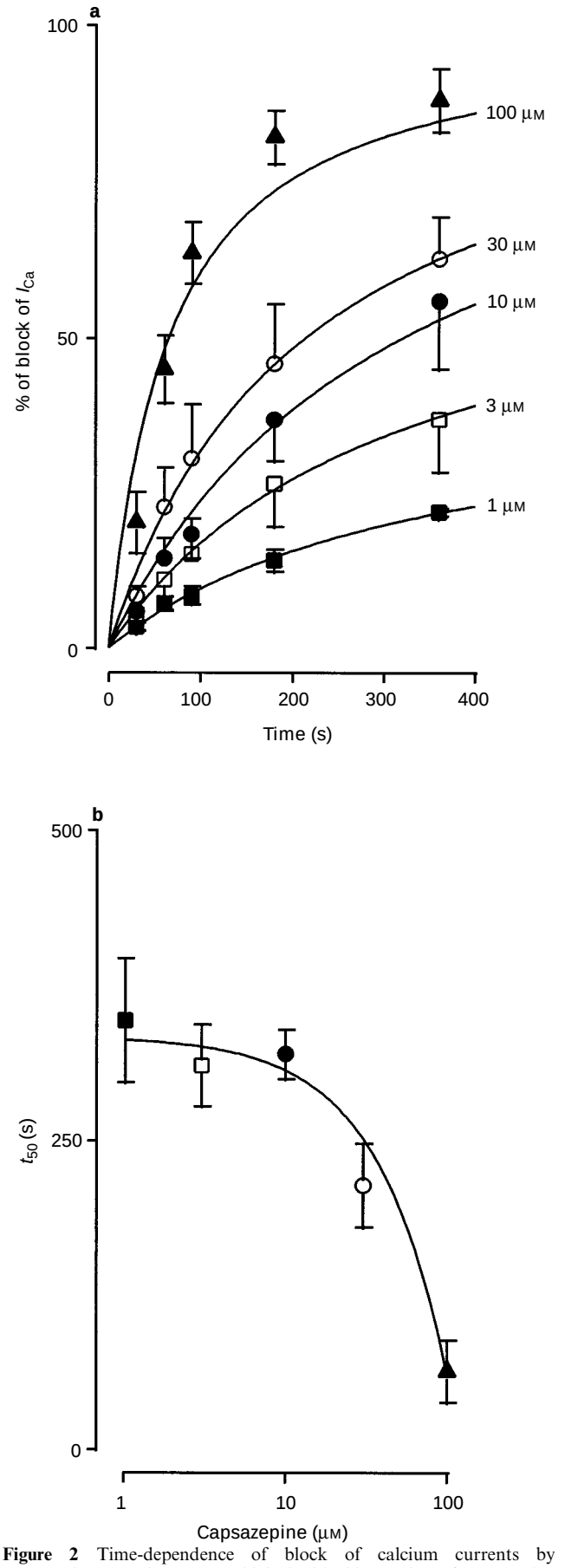

Figure 2 Time-dependence of block of calcium currents by capsazepine. (a) The rate at which the blocking action of capsazepine increased with time for a range of concentrations of capsazepine. Each point is the mean for 4–9 different experiments; vertical lines show s.e.mean. Solid lines are the best fit of a rectangular hyberbola such that:

$$f(x) = \frac{A \times x}{t_{50} + x}$$

where f(x) = % block of I_{Ca}, x = time, A = extrapolated maximum effect, t_{50} = time to reach half-maximum effect. (b) A plot of the t_{50} values, derived from the data plotted in (a), against concentration of capsazepine. The solid line indicates a linear relationship (note log scale).

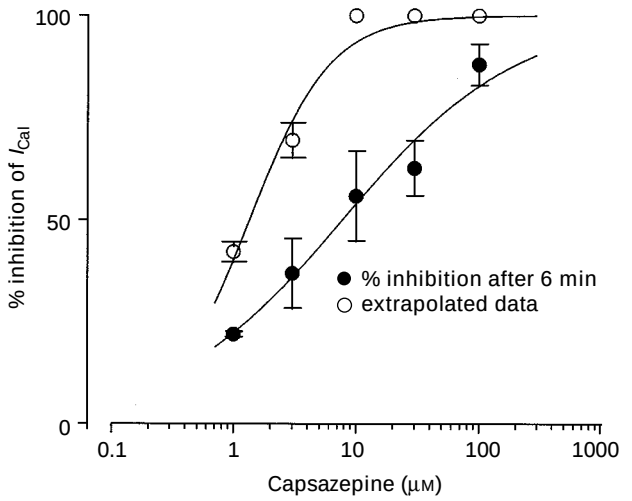

Figure 3 Dose-response curves for inhibition of voltage-activated calcium currents by capsazepine. Each point is the mean of the percentage inhibition of calcium current from 4–9 different experiments; vertical lines show s.e.mean. Calcium currents were evoked at 20 s intervals as shown in Figure 1. The data shown are for the percentage inhibition of calcium currents after 6 min exposure to capsazepine and for the extrapolated maximum effect at equilibrium (the parameter 'A' from the fits in Figure 2a). The solid lines were calculated as the best fit of a logistic equation:

$$f(x) = 100 \times \left(1 - \frac{1}{1 + \left(\frac{x}{EC_{50}}\right)^{p}}\right)$$

where f(x) = % inhibition of I_{Ca}, x = concentration of capsazepine, EC_{50} = half-maximum concentration and p = slope factor. The EC_{50} values calculated from the fitted data were $7.7 \pm 1.4\,\mu$M (6 min exposure) and $1.4 \pm 0.2\,\mu$M (extrapolated data) and the slope factors were 0.6 ± 0.1 and 1.3 ± 0.2, respectively.

30 min. Figure 1 shows examples of the effect of capsazepine at two different concentrations.

It was a characteristic of the effect of capsazepine that the block of calcium current developed slowly (Figure 1). Quantitative data concerning the rate of development of the effect at different concentrations of capsazepine is shown in Figure 2. The rate of development of block of calcium current was described well by a simple rectangular hyperbola. The half-time for the level of block to reach an equilibrium value for a given concentration of capsazepine was estimated by fitting a hyperbolic function to the data (Figure 2a). With this procedure it was found that the half-time for development of the blocking effect was strongly concentration-dependent (Figure 2b). The half-time was inversely related to the concentration of drug applied and the relationship was approximately linear. At 100 μM, which was the highest concentration studied, the half time was nearly one minute.

Figure 3 shows data from experiments designed to establish the concentration-dependence of the effect of capsazepine. Concentration-effect curves were constructed from data obtained after 6 min exposure to capsazepine (Figure 3). The EC_{50} value obtained from the best-fit of a logistic curve (see legend to Figure 3 and Methods) to this data was $7.7 \pm 1.4\,\mu$M. It is clear from the data in Figure 2a that the effect of capsa-

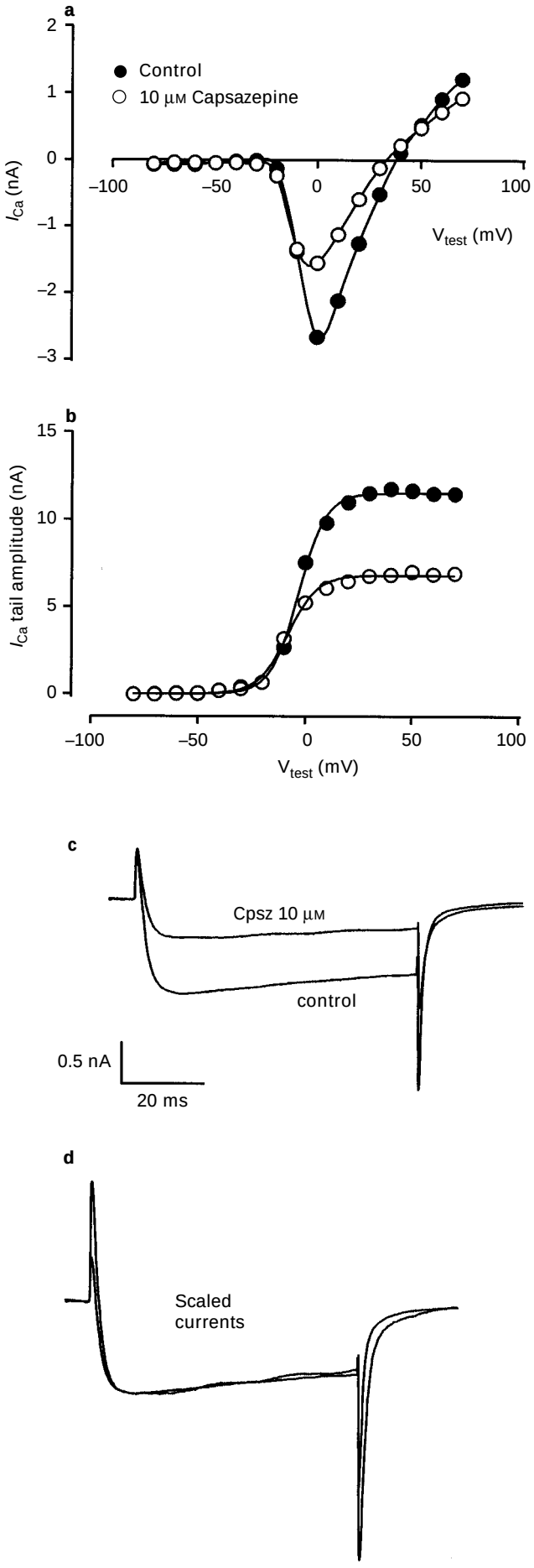

Figure 4 Effect of capsazepine (Cpsz) on voltage-dependence and kinetics of the calcium current. (a) An *I*-V curve for voltage-activated calcium current obtained in the absence (closed circles) and presence (open circles) of 10 μM capsazepine and (b) shows data for tail current amplitudes from the same experiment. In (c), calcium currents recorded during a step from −90 mV to 0 mV from 6 different experiments have been averaged. The traces shown in (c) are the average of the control currents and after 6 min in the presence of capsazepine. The data in (d) are the same as the data in (c) after rescaling to allow comparison of the kinetics of the current in the absence and presence of capsazepine.

zepine had not yet reached equilibrium by 6 min. For example at 10 μM, the data suggest that it would take about 30 min to reach 90% of the full response and still longer for the lower concentrations. Therefore, in order to estimate the EC_{50} at equilibrium, the full response for each concentration at equilibrium was estimated from the asymptotes to the fitted hyperbolic functions in Figure 2a and plotted as a concentration-effect curve in Figure 3. The EC_{50} value obtained from the best-fit of a logistic curve to this data was 1.4 ± 0.2 μM.

Figure 4a and b show an *I*-V curve and an activation curve for calcium current before and after administration of capsazepine. Capsazepine was most effective at positive potentials and had less effect at potentials close to the voltage threshold for activation of calcium current. Similar data were obtained in cells pretreated with 100 nM ω-conotoxin GVIA (Figure 5a) or 500 nM nimodipine (Figure 5b).

Although the calcium current was reduced in size by capsazepine there was no obvious change in the kinetics of the current. Figure 4c shows averaged data traces which have been re-scaled in Figure 4d to allow comparison of current kinetics before and after applying the drug. The slowing of the decay rate of the tail current, which is apparent in Figure 4d, was not statistically significant–current tails decayed (at −90 mV) with time constants of 0.9 ± 0.3 ms in control and 1.3 ± 0.3 ms after 6 min exposure to 10 μM capsazepine ($n = 6$, $P = 0.42$ by paired 2-tailed Student's *t* test).

The effect of capsazepine did not show use-dependence. When calcium currents were evoked at intervals of 3 min, 30 μM capsazepine caused a reduction of current amplitude of 49.2 ± 14.1% after 3 min and 69.9 ± 20.6% after 6 min ($n = 3$). These compare to values of $45.8 + 9.6$% and $62.8 + 6.7$% ($n = 6$), respectively, when currents were evoked at 20 s intervals.

Previous studies have suggested that voltage-dependent calcium current in dorsal root ganglion neurones is a composite of current through three or more calcium channel subtypes (Fox *et al.*, 1987; Regan *et al.*, 1991; Mintz *et al.*, 1992). At high concentrations capsazepine blocked almost all of the calcium current (see Figure 1) which suggests that the drug is not specific for a particular channel type. Further, since the voltage-dependence of the effect was still evident even when cells were pretreated with ω-CTX or nimodipine (Figure 5) it is not likely to be due to selective block of N- or L type calcium channels. To test this quantitatively we compared the effect of capsazepine before and after administration of a high concentration of ω-CTX, which should be sufficient to block both N- and L-type calcium channels (Fox *et al.*, 1987). The data are summarized in Figure 6.

We found that 40.5 ± 6.0% ($n = 10$) of the current in DRG neurones was blocked by ω-CTX (5 μM) which is close to the value obtained by others (Regan *et al.*, 1991; Mintz *et al.*, 1992). When capsazepine (30 μM) was added to cells pretreated with ω-CTX there was a further decrease of 29.0 ± 9.9% ($n = 10$) in the size of the current (Figure 6a). There was no significant difference ($P = 0.22$ by unpaired, 2-tailed Student's *t* test) between the sensitivity of the residual, ω-CTX-insensitive current and the control current to capsazepine (Figure 6b).

Discussion

It is unlikely that the effect of capsazepine on calcium currents is due to an interaction with the vanilloid receptor. Capsaicin, by activating vanilloid receptors, induces a non-

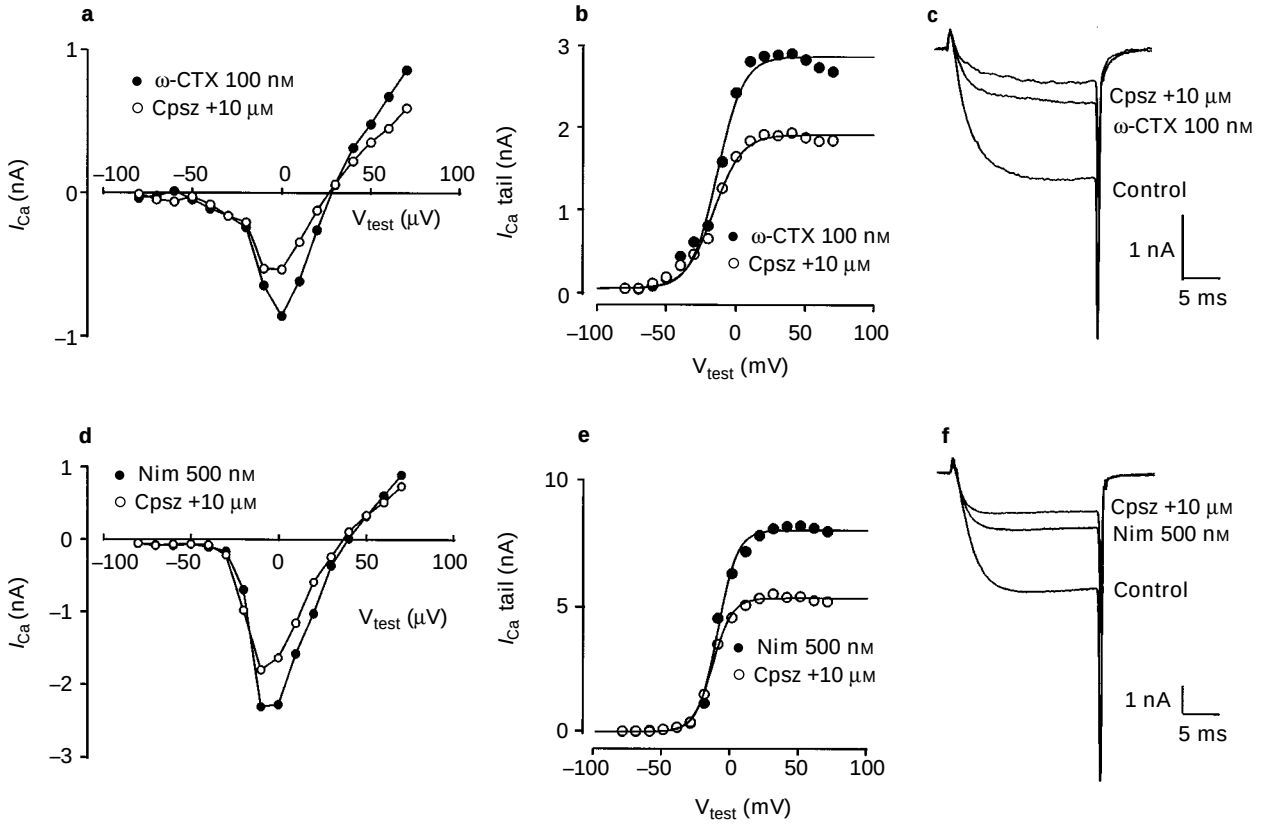

Figure 5 Effect of capsazepine in the presence of ω-conotoxin and nimodipine. (a) and (d) *I*-V curves for voltage-activated calcium currents obtained in the presence of 100 nM ω-conotoxin (ω-CTX) or 500 nM nimodipine (Nim), respectively (closed circles) and after further addition of 10 μM capsazepine (open circles). (b) and (e). The corresponding data for tail current amplitudes from the same experiments as in (a) and (d). The *I*-V curves and corresponding activation curves obtained in the absence of any drugs have been omitted for clarity. Example current traces, including data obtained in the absence of any drugs, are shown in (c) and (f). Data in (c) and (f) are calcium currents recorded during a step from −90 mV to 0 mV.

specific cation current in a sub-population of sensory neurones and inhibits voltage-dependent calcium currents (Bleakman *et al*., 1990; Docherty *et al*., 1991). Inhibition of calcium current is dependent on calcium entry via the capsaicin-activated ion channels and presumably depends on an interaction of capsaicin with vanilloid receptors. Given that capsazepine is also a ligand for the vanilloid receptor (Szallasi, 1994), albeit as an antagonist of capsaicin (Bevan *et al*., 1992a), it is important to establish whether its effects on calcium current are a consequence of this interaction or due to some other, non-specific effect. Several observations suggest that the effect of capsazepine on calcium current is a non-specific effect. Firstly, the IC_{50} for reversible block by capsazepine of capsaicin-induced responses in a variety of sensory nerve preparations is about 100–700 nM (Bevan *et al*., 1992a) i.e. capsazepine is about 10 fold more potent as a capsaicin antagonist than as a calcium current blocker. Secondly, block of capsaicin-induced responses in DRG neurones is rapidly developing and reversible (Bevan *et al*., 1992a), whereas block of calcium currents is slowly developing and irreversible, at least within the washing time allowed during the present experiments. Also, capsazepine (if applied for $\geqslant$90 s) inhibited calcium current in all of the neurones, whereas only about 40% of the neurones respond to capsaicin (see Holzer, 1991). For these reasons it is unlikely that the effects on calcium currents are due to an interaction of capsazepine with vanilloid receptors. Therefore, they are a non-specific effect of the drug.

It is unlikely that the effects of capsazepine on voltage-dependent calcium channels described above are due to a general neurotoxicity or to a non-specific block of ion channels. For example, responses of ligand-gated ion-channels in DRG such as those gated by adenosine 5′-triphosphate (ATP) and γ-aminobutyric acid (GABA) are not blocked by capsazepine although capsazepine (10 μM) was applied for only 3 min in these experiments (Bevan *et al*., 1992a). It has also been shown that capsazepine has no effect on [^{14}C]-guanidinium flux evoked by high extracellular K in isolated preparations, which suggests that voltage-dependent sodium channels are not much affected (Bevan *et al*., 1992a). However, the effects of capsazepine on sodium channel activity have not been studied directly. It was shown recently that vanilloid compounds and also capsazepine inhibit voltage-activated calcium channels and potassium channels in spinal neurones from *Xenopus* embryos (Kuenzi & Dale, 1996).

The experiments described in this paper were all performed on isolated dorsal root ganglion neurones where there are no barriers to drug diffusion other than the plasma membrane and intracellular membranes of the neurones. Capsazepine is a very lipophilic molecule so the slow rate of block of calcium currents is not likely to be due to slow diffusion to an intracellular binding site. The rate of block was strongly concentration-dependent which also suggests that diffusion is not a rate-limiting factor. It is more likely that capsazepine associates slowly with a binding site which is expressed on calcium channels. Whether the binding site is extracellular, intracellular or intramembranous cannot be discerned from the available data. Kuenzi & Dale (1996) have also found that the blocking effect of capsazepine on calcium channels is slow to develop and they favoured a mechanism whereby the vanilloids (and presumably capsazepine) promote a native inactivation process rather than acting as open channel blockers. In our experiments capsazepine had very little effect on the kinetics of the calcium current so it is not likely to act as an open channel blocker. Whether the drug stabilizes a native inactivated state of the channel as Kuenzi and Dale suggest or simply binds to

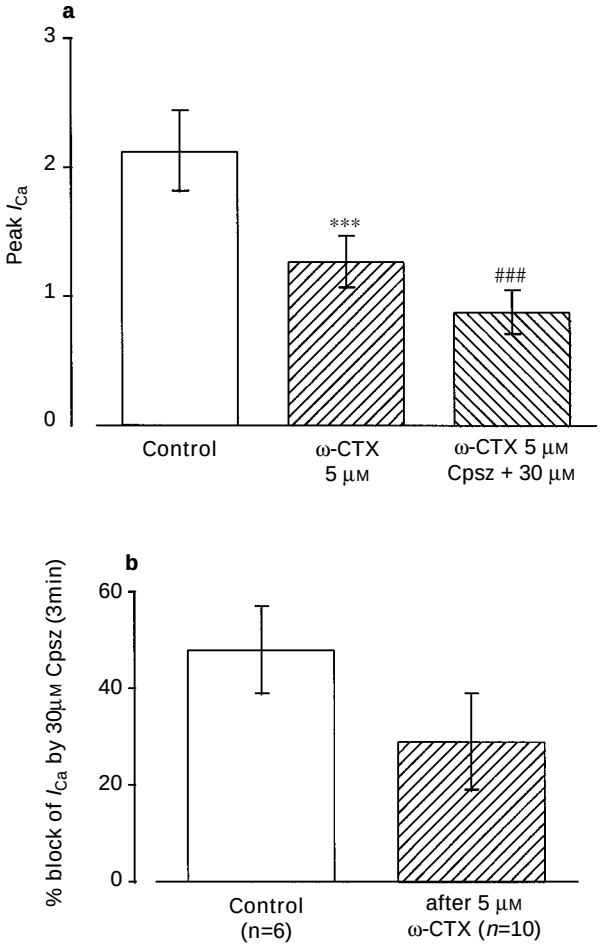

Figure 6 (a) A comparison of the amplitude of calcium currents recorded during a step from −90 mV to 0 mV in control neurones, after applying 5 μM ω-conotoxin (ω-CTX) and after additional 30 μM capsazepine (Cpsz). Data are the mean ± s.e.mean from 10 experiments as indicated (*** indicates $P<0.005$ comparing data in the left-hand and middle columns and ### indicates $P<0.005$ comparing data in the middle and right-hand columns by 2-tailed, paired Student's t test). (b) A comparison of the effect of 30 μM capsazepine applied to control cells with the effect when applied to cells pretreated with 5 μM ω-CTX at an equivalent time point.

the channel independently of its state cannot be discerned from the available data.

The block of calcium current by capsazepine was greater at positive than at negative activation potentials. For many reasons it is very unlikely that this is a reflection of a selective effect of capsazepine on any particular subpopulation of calcium channels. Firstly, at sufficiently high concentrations capsazepine blocked all of the calcium current which is not consistent with a selective blocking action. Secondly, the voltage-dependence of the blocking effect remained even when N- or L-type calcium channel subtypes were already blocked with ω-CTX or nimodipine, respectively. Thirdly there was no significant difference in the sensitivity of normal calcium currents with the residual currents left after N- and L-type currents had been blocked with a high concentration of ω-CTX (Fox *et al*., 1987). These data suggest that capsazepine is a non-specific blocker of voltage-dependent calcium channels of moderate potency.

An obvious potential use for capsazepine is as a pharmacological probe for studying possible physiological mechanisms involving vanilloid receptors. At present it is not known whether any endogenous compound(s) are important physiologically as ligands for the vanilloid receptor or what role, if any, is played by the vanilloid receptor in normal sensory function (Wood & Docherty, 1997). There is evidence from studies on single sensory neurones and single-channels that protons open the same species of ion channel as vanilloid agonists (Bevan & Yeats, 1991; Bevan *et al*., 1993) but this is not blocked by capsazepine (Bevan *et al*., 1992a). Similar data have been obtained for the isolated vagus nerve preparation from rat (Bevan *et al*., 1992b) and guinea-pig (Fox *et al*., 1995). In contrast, Liu & Simon (1994) have shown that capsazepine blocks responses to both vanilloids and protons in single cells. In more intact preparations, capsazepine has usually been found to block responses of sensory neurones to both acidic stimuli and to vanilloid agonists (Lou & Lundberg, 1992; Bevan & Geppetti, 1994; Fox *et al*., 1995). In particular, neuropeptide release from sensory neurones evoked by a variety of stimuli including low pH is blocked by capsazepine (Franco-Cereceda & Lundberg, 1992; Santicioli *et al*., 1993; Franco-Cereceda *et al*., 1994). If nociceptive stimuli such as low pH release endogenous sensory excitants/inflammatory mediators from other tissue, then the fact that capsazepine blocks them suggests the important conclusion that endogenous vanilloids may contribute to nociception and neurogenic inflammation (Fox *et al*., 1995). If, however, the results are due to a non-specific effect of capsazepine on the release of mediators due to block of calcium channels (or any other unknown non-specific drug effect) then this conclusion cannot be made. Unless an effect of capsazepine on calcium channels can be ruled out in such studies the suggestion that release of an unidentified vanilloid is responsible must be viewed with some caution.

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