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Effect of capsaicin and analogues on potassium and calcium currents and vanilloid receptors in *Xenopus* embryo spinal neurones

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1 The potassium current in embryo spinal neurones of *Xenopus* consists of at least two kinetically distinct components with overlapping voltage-dependencies of activation. We investigated whether capsaicin might specifically block these components in acutely dissociated neurones from stage 37/38 embryos by use of standard patch clamp techniques.

2 Capsaicin caused a time-dependent block of both the slow and fast components of the potassium current. The concentration-dependence was described by the Hill equation with a K_D of 21 μM and a coefficient of 1.5 ($n=9-11$ at each concentration). Differences between the observed and fitted values were not significant at the 5% level ($\chi^2=2.80$, 6 degrees of freedom).

3 Capsaicin did not affect the time course or voltage-sensitivity of activation, but the steady-state block was voltage-dependent. The block could be relieved by hyperpolarization, and the rate of the removal of block was voltage- and time-dependent. The time constant for the blocking reaction was also voltage-dependent for voltage steps below +30 mV, but above this level it was voltage-independent. These results suggest that capsaicin blocks potassium channels by an open channel mechanism.

4 Other derivatives of vanillin, such as capsazepine, resiniferatoxin, and piperine also blocked potassium channels. Capsazepine and resiniferatoxin caused a greater block than similar concentrations of capsaicin, and in the case of capsazepine, the block was also clearly time-dependent.

5 Capsaicin and capsazepine also blocked calcium currents in a time-dependent manner. Fitting the Hill equation to the averaged data gave a K_D of 43.5 μM, and a coefficient of 1.35 ($n=11$ at each concentration). The fitted values were not significantly different from the observed means at the 5% level ($\chi^2=12.1$, 6 degrees of freedom).

6 Six out of 29 Rohon-Beard sensory neurones responded to capsaicin with an inward current that appeared to be similar to the capsaicin activation of mammalian C sensory neurones. This response saturated at 10 μM capsaicin.

Keywords: *Xenopus*; capsaicin; capsazepine; resiniferatoxin; potassium channel; calcium channel; vanilloid receptor; open channel block

Introduction

The electrical properties of central neurones play a critical role in determining the activity of neural circuits. In the *Xenopus* embryo the spinal neural circuit that controls swimming has been well described in terms of its neuroanatomy, and synaptic physiology and connectivity (Roberts *et al.*, 1986). The biophysical properties of the neurones in this circuit have recently been described and reproduced in a quantitative model (Dale, 1995a). This study showed that the delayed rectifier potassium current includes two kinetically distinct components, and modelling studies of a circuit of neurones suggest that each component plays a distinct role in governing neuronal spiking activity and the swin motor pattern (Wall & Dale, 1994; Dale, 1995b). To test hypotheses suggested by the model we have searched for pharmacological agents that specifically block one of the components.

Capsaicin, a plant alkaloid from the hot pepper, *Capsicum*, is well known as a useful tool in the study of pain and analgesia because it specifically excites subsets of C-type sensory neurones in mammals. This effect is mediated by a receptor in the cell membrane which is coupled to a non-specific cation channel (Marsh, 1987; Wood *et al.*, 1988; Akerman & Gronblad, 1992). However, capsaicin also blocks voltage gated ion channels, and in the frog node of Ranvier it has been shown preferentially to block one of the two fast potassium channels (Dubois, 1982). In our initial survey of the potassium current in *Xenopus* spinal neurones, low concentrations of capsaicin changed the kinetics in a way that was consistent with a specific block of the slow component. In other excitable cells, including dorsal root ganglion neurones, capsaicin also changes the inactivation kinetics of potassium channels and blocks calcium channels (Peterson *et al.*, 1987; Bleakman *et al.*, 1990; Castle, 1992; Baker & Ritchie, 1994; Kehl, 1994). We found that high concentrations of capsaicin (150-200 μM) completely blocked both potassium currents and the calcium current, and in all cases the block appeared to be through an open channel blocking mechanism. Analogues of capsaicin, including those that are potent agonists and antagonists of the receptor in sensory neurones, either had no effect, or caused a similar block. In addition, we found that a subset of *Xenopus* neurones responded to capsaicin with an inward current, which may be similar to the response of mammalian sensory neurones. A part of this work has appeared in abstract form (Kuenzi & Dale, 1995).

Methods

Spinal neurones were dissociated from *Xenopus* embryos at stage 37/38 of development (Nieuwkoop & Faber, 1956) by use of the method of Dale (1991). Briefly, embryos were anaesthetized with Tricaine (Sigma) in Ringer solution (see below), and a section of the spinal cord, from the level of the anus to the obex of the brain stem, was removed. Cells were dissociated by mild trituration (for details see Dale, 1991; Wall &

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Dale, 1995). The naked cells were allowed to settle on poly-D-lysine coated culture dishes in normal Ringer saline. At the start of an experiment the Ringer was replaced with control saline (see below), which also perfused the recording chamber throughout the experiment. Drugs were applied through a multibarrelled microperfusion pipette that was positioned within 1 mm of the cell. All experiments were performed at room temperature, 18–22°C.

Solutions

Ringer solution contained the following (in mM): NaCl 115, KCl 3, $MgCl_2$ 1, $CaCl_2$ 1, $Ca(NO_3)_2$ 1, $NaHCO_3$ 2.4, HEPES 10 and glucose 10. The pH was adjusted to 7.6 and the osmolarity was 255 mOsm. For patch clamp recording calcium nitrate was omitted from the control saline and the $CaCl_2$ concentration was increased to 10 mM to increase the stability of recordings. The pH was adjusted to 7.4 and the osmolarity was 260 mOsm without additional glucose.

For recording potassium currents the pipette solution contained (mM): KOH 100, KCl 5, $MgCl_2$ 6, methanesulphonic acid 100, BAPTA 2, ATP 5 and HEPES 20. The pH was adjusted to 7.4 with KOH and the osmolarity was 240 mOsm. In addition, tetrodotoxin (TTX, 0.14 μM, Sigma) and $CdCl_2$ (100 μM) were added to the external solution to block sodium and calcium currents, respectively.

Calcium currents were recorded with a pipette solution containing (mM): CsOH 100, $CaCl_2$ 1, methanesulphonic acid 100, EGTA 10, $MgCl_2$ 6, ATP 5, HEPES 20, pH 7.4 and osmolarity, 240 mOsm. The external solution was modified by replacing one half of the NaCl with tetraethylammonium chloride (TEA, Sigma) and adding 1 mM 4-aminopyridine (4-AP, Aldrich) to block potassium currents. Tetrodotoxin (0.14 μM) was added as well to block sodium currents.

Capsaicin (Research Biochemicals International (RBI) or Sigma) was dissolved to give a stock solution of 100 mM in either absolute ethanol or dimethylsulphoxide (DMSO) and stored frozen as 25 μl aliquots in 10 ml stoppered centrifuge tubes. One aliquot was dissolved in 5 ml control saline (room temperature or warmer during solubilisation) and diluted with control saline to the final concentration. A similar procedure was used to dissolve capsaicin synthetic analogue (N-[4-hydroxy-3-methoxybenzyl]nonanamide from RBI), capsazepine (RBI), and resiniferatoxin (Sigma). In early experiments an equal quantity of the solvent was added to the control saline, with the highest concentration of DMSO being 0.2%. Most experiments were conducted with 0.025% DMSO, and as the highest concentration caused no discernible change in the amplitude or kinetics of the potassium or calcium currents, no solvent was added to the control saline in later experiments.

Data acquisition and analysis

Membrane currents were voltage clamped in the whole cell configuration by a List EPC7 patch clamp. Voltage steps were generated by a 486, 33 MHz computer, with software developed by Dale (1991) and a Data Translation DT2831 A/D converter. The same software displayed the results and stored

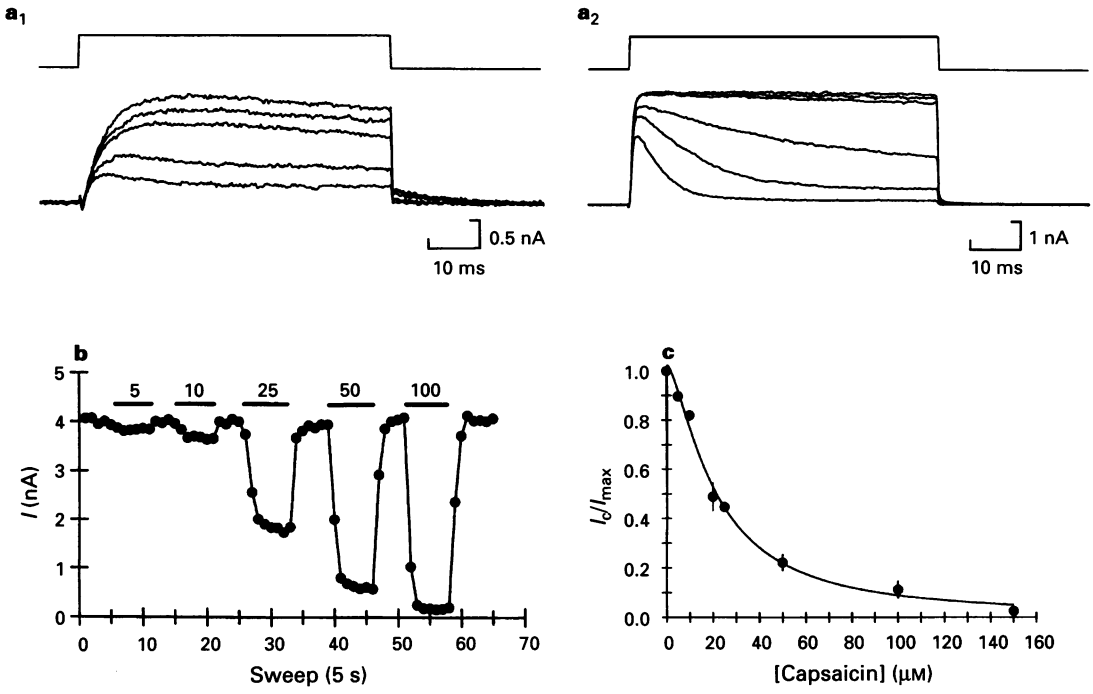

Figure 1 Both slow and fast potassium currents were blocked by capsaicin. (a_1) A voltage-clamp recording from a cell with a predominately slow component. From the top trace down the cell is superfused with control, 5, 10, 25 and 50 μM capsaicin. (a_2) An example from a cell in which the fast component predominated (control, 5, 10, 25, 50 and 100 μM capsaicin). For each trace the membrane potential was held at −50 mV and stepped to +20 mV. (b) The effect of capsaicin was readily reversible, even at concentrations causing maximum block. The current at the end of the pulse was plotted against time. Horizontal bars indicate superfusion with capsaicin at the indicated concentrations (μM). (c) Concentration-dependence of block of potassium current by capsaicin. The current measured at the end of the 60 ms pulse was normalised to either the current in the immediately preceding control, or, when there was significant rundown of current during the trial, to an average of the control and recovery currents. The solid line is the best fit of the Hill equation:

$$\frac{I_{Capsaicin}}{I_{Control}} = \frac{1}{1 + ([Capsaicin]/K_D)^{n_H}}$$

to the points ($n = 9-11$ cells for each point). The Hill coefficient (n_H) is 1.5 and the K_D is 21 μM. Vertical lines in this and subsequent figures represent s.e.

them on the computer's hard disc for later analysis. Current records and summary results were fitted to model functions by the maximum likelihood method and the simplex algorithm for optimisation (Press *et al.*, 1988).

All results are given as a mean ± s.e. and comparisons between means were by Student's *t* test. The goodness of fit for the Hill equation (Figures 1c and 7c) was assessed by the χ^2 test.

Results

Potassium currents

Bathing a cell in saline containing TTX and Cd^{2+} eliminated all inward current and blocked entry of sodium and calcium, which are known to elicit potassium currents in these cells (Dale, 1993; Wall & Dale, 1995). The remaining voltage-dependent potassium currents could be divided into slow and fast components, based on their rates of activation and deactivation (Dale, 1995a). For any given cell the ratio of slow to fast current was variable, with some cells exhibiting almost pure slow current (Figure $1a_1$, top trace of current record) and others showing a large fast component (Figure $1a_2$, top trace of current record). Cells also showed varying amounts of intrinsic inactivation, with a time constant of ~100 ms, when depolarized to +50 mV. Capsaicin changed the total potassium current of all cells ($n=108$) in two ways: (1) it decreased the maximum current, and (2) it changed the time course, causing an inactivation-like decay in the current during the step. The time constant of the latter effect was markedly shorter than the intrinsic inactivation. Cells possessing mostly slow current (Figure $1a_1$) showed a greater decrease in peak amplitude and less 'inactivation' than those with more fast current (Figure $1a_2$). Except in a few cells identified as Rohon-Beard cells (see below), capsaicin had no effect on the holding current.

At concentrations of capsaicin greater than 150 μM the total potassium current was completely blocked by the end of a 60 ms pulse. At lower concentrations the current decayed to a steady state during the pulse, the amplitude of which depended on concentration (Figure $1a_{1,2}$). The block developed rapidly and was rapidly reversible (Figure 1b), with the current at the end of the pulse reaching equilibrium within 20–30 s. This effect washed out with a similar, though slightly more rapid, time course. The dose-response relationship for the current remaining at the end of the pulse (including both fast and slow components) is shown in Figure 1b. When the Hill equation was fitted to the means at each concentration, an apparent K_D of 21 μM and a Hill coefficient of 1.5 gave the best fit (Figure 1c). Differences between the observed and fitted values were not significant at the 5% level ($\chi^2=2.80$, 6 degrees of freedom).

Capsaicin could block the current by reducing the number of channels passing current at any potential or by shifting the voltage-dependence of activation to more depolarized potentials. To determine the effect of capsaicin on activation we measured the amplitude of the tail currents after the membrane was stepped from a test potential back to the holding potential of −50 mV (Figure $2a_1$). The measurement was taken at a point immediately after the capacitance artefact so that both the fast and the slow components were included. We assumed that the unblocking reaction was so slow that at this point the current was due to the net conductance at the end of the step (see below). At each test potential above the threshold for activation, capsaicin reduced the tail current. Fifty micromolar capsaicin reduced it so much that it was difficult to measure above noise, but at 25 μM enough current remained to measure reliably (Figure $2a_2$). The results were leak subtracted and normalised to give the activation curves (G/G_{max} vs test potential) for both conditions (Figure 2b). When the Boltzmann equation was fitted to plots from individual cells, neither the difference in the membrane potential for half activation (−5.6 ± 4.7 mV in control and −8.7 ± 4.8 mV in 20–25 μM capsaicin) nor the difference in slope (−9.1 ± 1.8 and −6.2 ± 1.6 mV for e-fold increase in conductance respectively) were significant at the 5% level (paired samples, $n=4$). The corresponding parameters resulting from the fit to the averaged data at each voltage are presented in the legend of Figure 2. These results indicate that capsaicin does not affect the voltage-dependence of channel opening, so it must block by interfering with conduction through the channels.

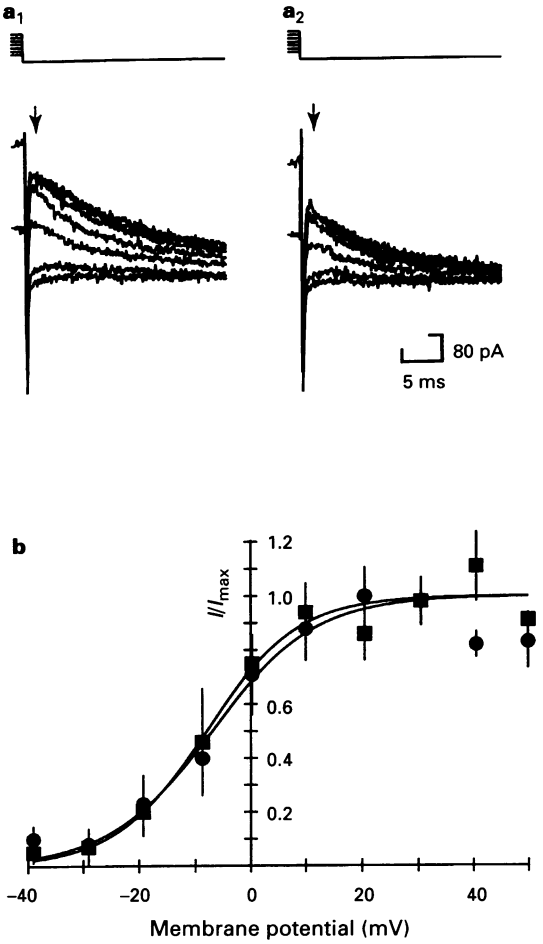

Figure 2 Capsaicin did not change the voltage-dependence of activation of the potassium currents. (a) Tail current records in control (a_1) and 25 μM capsaicin (a_2). (b) Tail currents were measured at the time indicated by the arrow in (a) in control saline (●, $n=6$) and in 25 μM capsaicin (■, $n=5$). These were normalised and leak subtracted to give the activation curve (mean ± s.e.). The means at each voltage were fitted to the Boltzmann equation (solid lines):

$$\frac{G}{G_{max}} = \frac{1}{\exp\left(\frac{V_m - A}{B}\right) + 1}$$

with A = −7.0 mV, B = −8.9 mV for control (●) and A = −8.0 mV, B = −8.3 mV for capsaicin (■).

The time-dependent block by capsaicin was similar to the inactivation of transient currents, such as I_{Na} and the A-type potassium current. Like the inactivation of these currents the steady-state block by capsaicin had an apparent voltage-dependence. This became evident in 50 μM capsaicin, where cells with both predominantly slow and predominantly fast currents showed a marked decay in current during a 100 ms step. To derive an empirical model of the blocking process we studied the voltage- and time-dependence of block in this concentration in more detail by use of the Hodgkin and Huxley (1952) formalism for inactivation.

The voltage-dependence of block was measured with a 'pre-

pulse' protocol; a conditioning pre-pulse to different voltages was followed by a test pulse to a fixed voltage. The conditioning pulses were long enough for the block to reach a steady state in 50 μM capsaicin. In control saline this protocol caused a slow inactivation in most cells. (Figure 3a). Capsaicin accelerated the decay in peak current over the range of voltage corresponding to current activation (Figure 3b). When the steady state current during the test pulse following the highest prepulse is subtracted from the peak current during each test, the points describe the steady state 'inactivation', or blocking, curve. The results from 5 cells that contained both slow and fast currents are summarised in Figure 3b.

The rate of capsaicin block depended on both membrane potential and concentration (Figure 4a). When the membrane was stepped to voltages greater than 0 mV, the decay in current caused by capsaicin followed an exponential time course that was well fitted by a single time constant. This was approximately 1/10th the magnitude of the time constant for slow inactivation that some cells showed in control conditions (Figure 3a). Increasing the concentration of capsaicin decreased the time constant at all voltages. At all concentrations it also decreased as the test potential increased up to +30 mV, but higher steps did not markedly change the time constant.

At lower voltages, where potassium currents are not active, the kinetics of the blocking reaction were studied by measuring the rate of recovery from block by use of a twin pulse protocol (Figure 4b). The peak current in 50 μM capsaicin recovered as an exponential function of the interval between pulses, as the example in the inset of Figure 4b shows. The second response usually did not recover to 100% due to the normal slow inactivation of the current. In cells with both slow and fast currents the time constant of recovery decreased with more negative holding potentials during the interval. These data, together with the time constants of block (e.g. Figure 4a), describe the voltage-dependence of the time constant for the blocking reaction (Figure 4c). The relationship is a bell-shaped curve at potentials below 20 mV, with a peak between −10 and −30 mV. The rate constants of the forward and backward 'inactivation' block can be estimated by combining this data with the voltage-dependence of steady state block (Figure 3b). This is shown in Figure 4d.

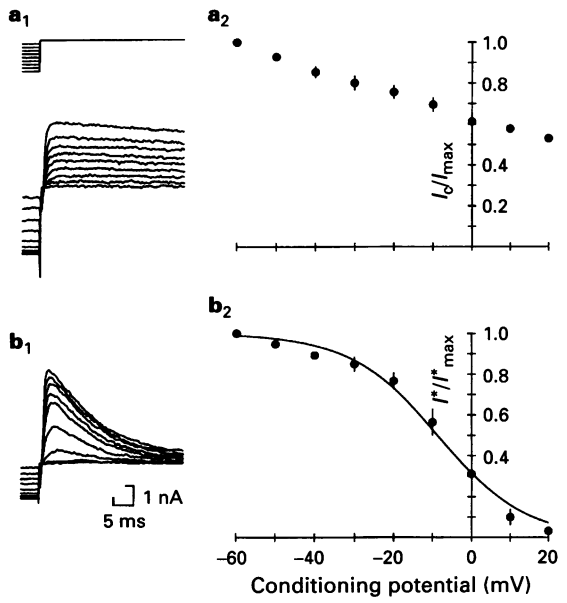

Figure 3 The steady state block by capsaicin was voltage-dependent. (a_1) In control saline the potassium current had a slow inactivation when the pulse protocol was given at 5 s intervals. The cell was held at −50 mV, stepped to a prepulse ranging from −60 – +20 mV for 145 ms, and then stepped to the test potential of +30 mV. (a_2) Summary of the peak normalised current during the test pulse (mean ± s.e., $n=5$). (b_1) While superfused with 50 μM capsaicin, the peak current during the test decreased as the amplitude of a conditioning prepulse was increased. (b_2) The steady-state block at each test potential was computed by subtracting the steady state current at +30 mV from the peak current during the test pulse (I^* and I_{max}^*). The normalised currents are plotted against the conditioning potential ($n=5$). The solid curve is a model of block based on the 'h' parameter used by Hodgkin and Huxley (1952) to model the sodium current of squid axons: $h=\alpha/\alpha+\beta$. The parameters for the forward and backward rate constant models are computed in Figure 4d. The data can also be fitted by the Boltzmann equation with $V_{1/2}=-4.9$ mV and a slope of 10.26 mV for an e-fold change in amplitude (not shown).

Effect of capsaicin on tail currents

The time course of the tail currents depends on the net rate of channel closing. In control saline this process is described as the sum of two exponential functions, suggesting the presence of two kinetically distinct types of channels (Dale, 1995a). In these experiments we found that the tail current after a step from +30 mV back to the holding potential, −50 mV, was often too small to be fitted reliably with a double exponential. Therefore we evoked tail currents by stepping to a post-pulse of −30 mV, which was sufficiently low to cause deactivation, but far enough from the reversal potential (taken to be −88 mV) to maintain a large driving potential on the current.

If capsaicin acts by blocking open channels, then the decay of current would depend on both the closing of open channels, and the continued blocking of open channels. This would act to decrease the time constants of the two currents. Furthermore, if blocked channels must unblock before closing, i.e. there is no closing from the blocked state, then capsaicin should add an additional slow component to the two currents. In 50 μM capsaicin, however, the tail currents continued to be well fitted by a dual exponential model. In some cells the fast time constant became markedly shorter, but overall the difference was not significant (control, 3.42 ± 0.65; capsaicin, 2.13 ± 0.42 ms; $P>0.05$). The slow time constant became significantly longer (control, 24.24 ± 2.75; capsaicin, 33.29 ± 3.42 ms; $P<0.05$, $n=8$).

Effect of capsaicin analogues on potassium currents

Capsaicin is a derivative of homovanillic acid which activates the capsaicin, or vanilloid, receptor on sensory fibres. Resiniferitoxin and capsazepine are also derivatives and are well known agonists and antagonists, respectively, of this receptor (Bevan *et al.*, 1992; Szallasi & Blumberg, 1989). If capsaicin blocks voltage gated channels of all cells by a direct effect, then this action may be mechanistically distinct from its effect on the highly cell specific receptor. The pharmacological profile could also be very different, and could provide important information on the structure-activity of channel block.

Vanillin and its acid (4-hydroxy-3-methoxy benzoic acid) lack the acetylamide bond and hydrophobic tail of capsaicin, and these were without effect on potassium currents at a concentration of 100 μM. Piperine is a compound derived from the pepper, *Piper*, and it shares the methoxy substituted phenol group and a hydrophobic tail in common with capsaicin. Piperine ($n=4$ cells) also inhibited potassium currents, but it did not produce the same 'inactivation' as capsaicin (Figure $5a_1$) and it was less potent than capsaicin (Figure $5a_2$). N-[4-hydroxy-3-methoxybenzyl]nonanamide (CSA) is a synthetic analogue of capsaicin in which the unsaturated, hydrophobic branched side chain is replaced by a saturated, unbranched side chain of equal length (Janusz *et al.*, 1993). This caused the same 'inactivation' (Figure $5a_1$) and was nearly as effective as natural capsaicin (Figure $5a_2$). These effects were observed in four cells. Finally, two drugs are widely used in studies of the capsaicin receptor: resiniferatoxin is a capsaicin agonist and capsazepine is an antagonist of capsaicin effects on C-fibres (Bevan *et al.*, 1992; Walpole *et al.*, 1994). However, both of

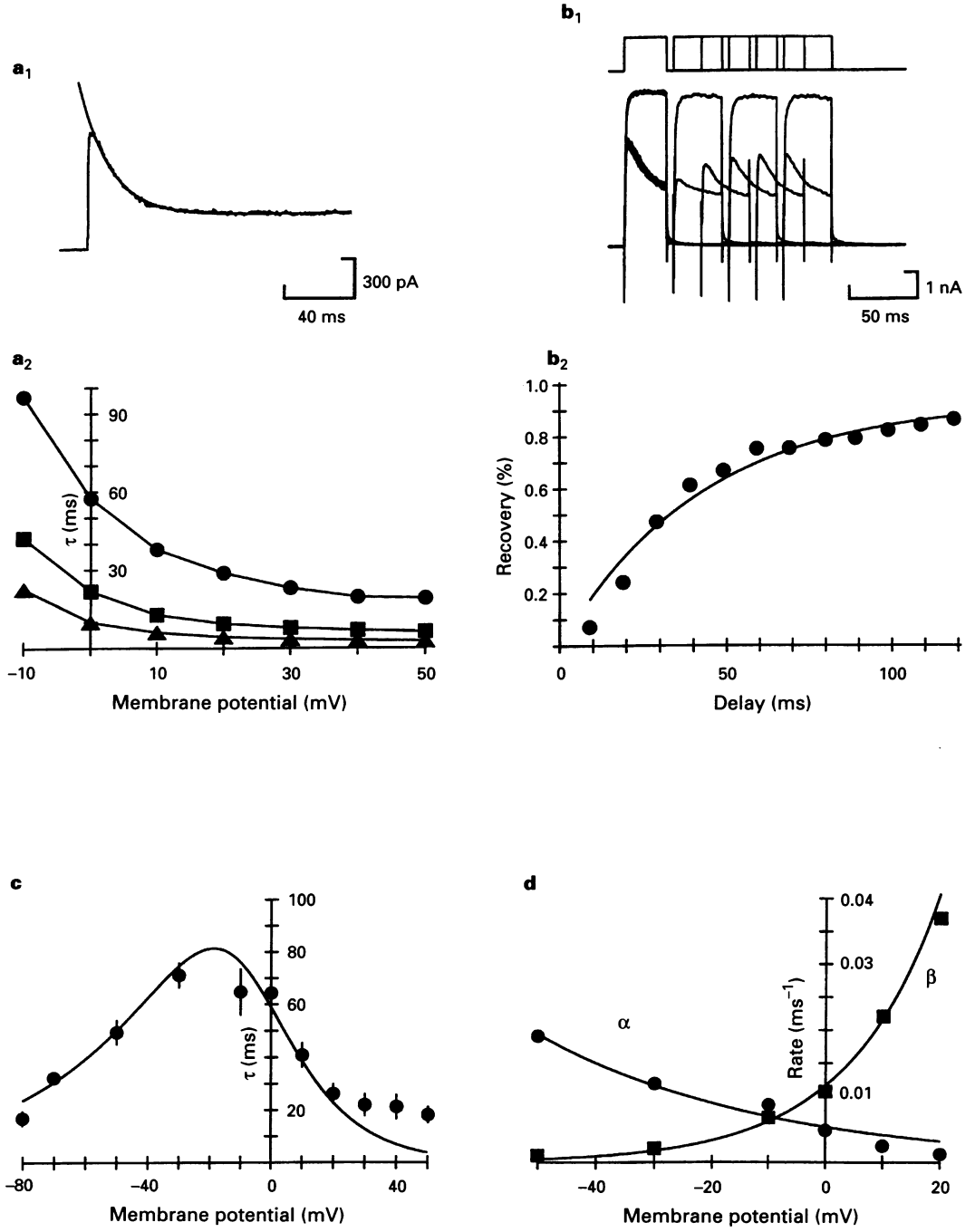

Figure 4 The rate of the capsaicin block was voltage-and concentration-dependent. (a_1) The time constant for the blocking reaction (τ) was measured by fitting the decay in current during a step with a single exponential. (a_2) This decreased with either an increase in capsaicin concentration from 25 μM (●) to 50 μM (■) and 100 μM (▲) or an increase in test potential (abscissa scale). Holding potential was −50 mV. (b_1) Time constant of recovery from block was measured by a twin pulse protocol. In control saline the amplitude of the second response either remained constant regardless of the interval, as in this example, or showed slow inactivation throughout the range of intervals. In the presence of 50 μM capsaicin (lower series of traces) the peak current partially recovered during the interval between pulses. (b_2) The recovery, R, was calculated by use of the equation:

$$R = \frac{p2 - e1}{p1 - e1}$$

where p1 and p2 are the peaks of pulses 1 and 2 and e1 is the end of pulse 1. The points were fitted to an exponential function of the interval, t ($R = 1 - \exp(-t/\tau)$). In the example shown the potential during the interval was −50 mV and the time constant of the model (solid line) was 43 ms. (c) The voltage-dependence of the time constant for block in 50 μM capsaicin was plotted by combining results from experiments such as (a) and (b). Each point is the mean of 3–10 cells (average $n = 6$). The solid line is the model: $\tau = 1/(\alpha + \beta)$, using models of the unblocking (α) and blocking (β) rates derived in (d). (d) The rate constants for the forward and backward blocking reaction were computed at each membrane potential by use of the equations: $\alpha = h/\tau$ and $\beta = (1 - h)/\tau$, where β is the forward rate (open-to-blocked), α is the reverse rate, h is the steady state block from Figure 3b and τ is the time constant for block (c). The fitted model is shown by solid lines in (c) and (d), where α and β are given by the equations: $\alpha = 0.0056*\exp(-(V + 1.87)(38.8)^{-1})$ and $\beta = 0.11*\exp((V - 36.0)(16.0)^{-1})$.

these compounds inhibited the potassium current. Unlike capsaicin, resiniferatoxin ($n=14$ cells) did not affect the kinetics of activation or inactivation (Figure $5b_1$); however, it did cause a steady decay in potassium current which did not recover during washout (Figure $5b_2$). The effects of capsazepine were similar to those of capsaicin in that the current showed more inactivation during the pulse in the presence of the drug than in control (Figure $5c_1$). However, the block took longer to develop and was slow to wash out (Figure $5c_2$). During washout, the current continued to show the inactivating time course, suggesting that the voltage-dependent blocking and unblocking remained intact even in the absence of superfused drug. These effects were observed in 15 cells. The slowness and irreversibility of the capsazepine and resiniferatoxin effects made dose-response relationships difficult to measure, but in both cases the concentrations used caused greater block than would be expected from the capsaicin dose-response curve, suggesting that they are more potent.

Calcium currents

Capsaicin blocked the calcium current in all the cells ($n=34$) where this current was measured. As with the block of potassium currents, calcium currents showed an increase in inactivation during the block (Figure 6a), but no change in the kinetics of activation. Capsaicin also did not change the voltage-dependence of activation, as measured by the tail currents (Figure 6b). When the tail currents in 25 and 50 μM capsaicin were scaled according to the control response evoked at +30 mV, the activation curves overlapped one another throughout the range of voltages up to complete activation. Increasing the concentration of capsaicin increased the amount of inactivation (Figure 7a), and at all concentrations the block developed quickly and was rapidly reversible (Figure 7b). Capsaicin has a slightly lower affinity for the calcium channels than potassium, as the best fit of the Hill equation to the dose-response curve gave a K_D of 43.5 μM and a Hill coefficient = 1.35 (Figure 7c). The fitted values were not significantly different from the observed means at the 5% level ($\chi^2=12.1$, 6 degrees of freedom). Capsazepine also blocked calcium channels, and although the decay in current was less marked, the

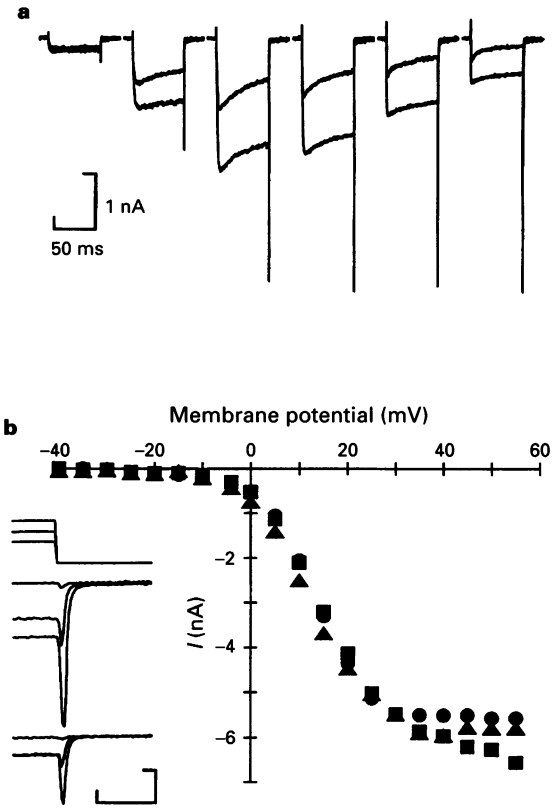

Figure 6 Effects of capsaicin on the calcium current in *Xenopus* neurones. (a) Responses to voltage steps from a holding potential of −50 mV to test potentials from 0 to +50 mV in 10 mV increments. The bottom series is in control saline and the top series is in 50 μM capsaicin. Capsaicin caused a time-dependent block of the calcium current. With large steps the peak of the tail current was truncated. (b) Voltage-activation curves, measured from tail currents and scaled to the control, were unchanged in 25 μM (■) and 50 μM (▲) compared to control medium (●). Inset shows tail currents evoked at −10, 10, and 30 mV in control (top series of traces) and 50 μM capsaicin (bottom traces). Scale bars: 2 ms, 1 nA, 60 mV.

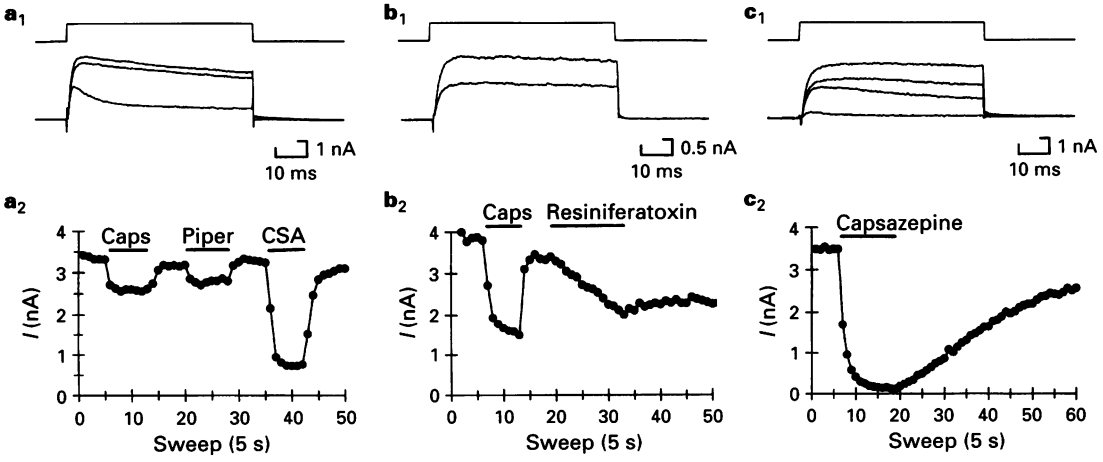

Figure 5 Effects of other vanilloids on potassium currents. (a_1) Traces, from the top down, are control, 100 μM piperine, 100 μM synthetic analogue of capsaicin (CSA). The cell was stepped to +20 mV for 60 ms. The synthetic analogue also caused a similar inactivation of the potassium current, but piperine did not noticeably change the kinetics. (a_2) The current at the end of the pulse is shown in control saline, 10 μM capsaicin (Caps), 100 μM piperine (Piper) and 100 μM CSA. (b_1) The block by resiniferatoxin (20 μM) did not affect the kinetics of activation and inactivation. (b_2) Compared to capsaicin (50 μM) the block developed slowly and only partially reversed during washout. (c_1) Potassium currents are also blocked by capsazepine. The top trace is the control, and from the bottom up, the other traces are in the presence of 50 μM capsazepine and at two points during washout (see points labelled 1 and 2 in C_2). During washout the inactivation persisted whilst the peak recovered. (C_2) The capsazepine block developed and reversed more slowly than that to capsaicin.

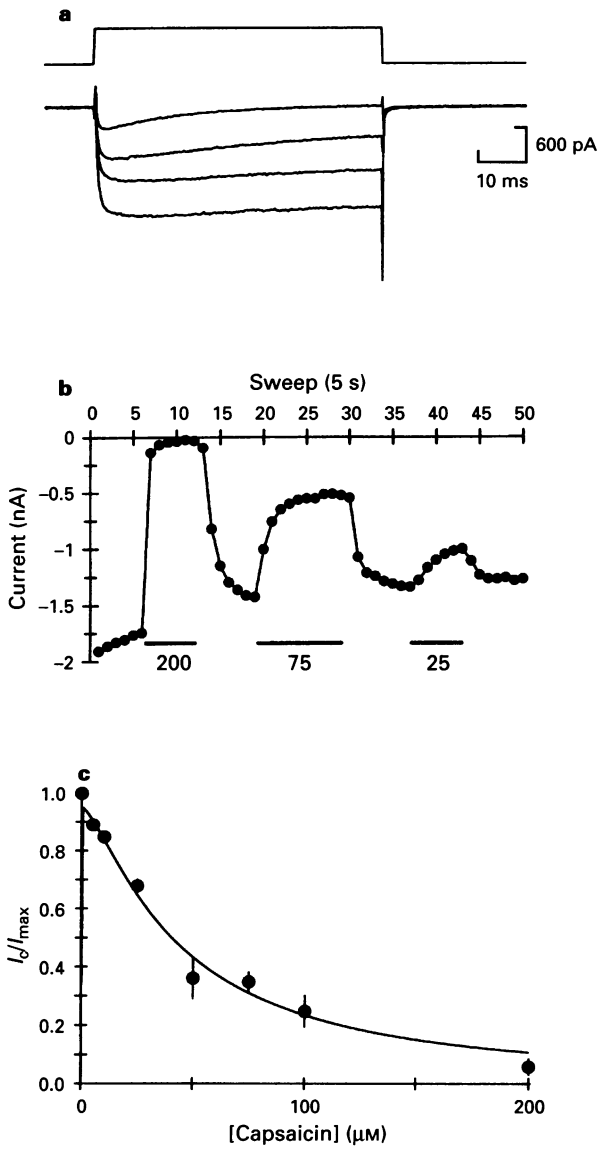

Figure 7 The block of calcium currents by capsaicin was dose-dependent and reversible. (a) Increasing concentrations of capsaicin decreased the current at the end of the pulse more than the peak current. The records are in response to a depolarizing pulse from the holding potential of −50 mV to +30 mV in (from the bottom trace upwards) control saline, 25 μM, 75 μM, and 200 μM capsaicin. These records have not been leak subtracted. (b) The calcium current, measured at the end of a 60 ms pulse, was reversibly blocked by capsaicin. The time of drug application and concentration are indicated by the labelled horizontal bar (μM). (c) The dose-response curve for the capsaicin block of calcium currents was measured as described in Figure 1c and the Hill equation was fitted to the points. The solid line is the line of best fit, using $K_D = 43.5$ μM and a Hill coefficient of 1.35 ($n = 11$).

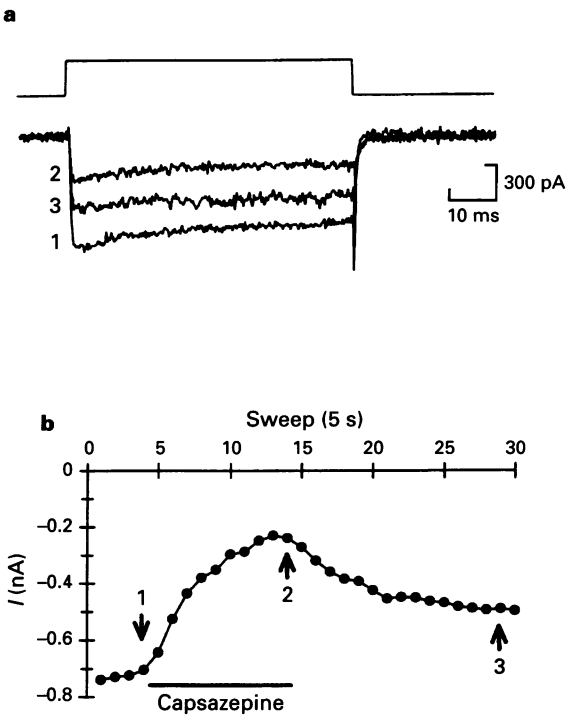

Figure 8 Calcium currents were also blocked by capsazepine. (a) In 50 μM capsazepine (trace 2) the current at the end of the pulse was reduced to approximately 30% of its value in control saline (trace 1). Cells were held at −50 mV and stepped to +30 mV. (b) The block developed and washed out much slower than the block by capsaicin (Figure 7b). The traces in (a) are from the correspondingly labelled points in (b).

slow time course of block and washout were similar to its block of potassium channels (Figure 8).

Capsaicin induced inward current

In a small set of cells capsaicin caused an inward current. These cells all had the morphology of Rohon-Beard neurones in culture; they were large in diameter, had a large nucleus with a prominent nucleolus and had a resting membrane potential of −70 to −80 mV (Dale, 1991). Not all neurones fitting this description (6 out of 29 cells) showed the inward current. The inward current developed in two stages, an initial fast stage, followed by a slower increase (Figure 9a). This response was much more sensitive to capsaicin than the block of voltage-dependent currents, as 5 μM caused a marked effect, and the response apparently saturated in 10 μM capsaicin (Figure 9b). Higher concentrations increased the rate of current development without increasing the steady state current.

Discussion

The potassium currents in embryonic *Xenopus* spinal neurones have both voltage-dependent and ion-dependent components. Blocking sodium entry with tetrodotoxin and calcium entry with extracellular cadmium removes inward current and isolates the voltage-dependent channels (Wall & Dale, 1995). In most cells this potassium current consists of two components which are kinetically distinct but which cannot be separated by their voltage-dependence of activation or inactivation (Dale, 1995a). We found that capsaicin did not selectively block either of these. In cells that expressed predominantly the fast component, capsaicin blocked the same relative amount at each concentration as in cells where the slow component predominated (Figure 1a). Secondly, when the results from all cells were pooled, the concentration-dependence curve was well fitted by a Hill equation which assumes one component (Figure 1c). In pituitary neurones (Kehl, 1994) and ventricular myocytes (Castle, 1992) capsaicin is also non-selective, blocking both transient and sustained outward potassium currents. In the latter it showed some specificity for outward currents against the inward rectifying potassium current, with the concentration for half block for the inward rectifier being 5 to 8 fold higher (Castle, 1992). In contrast Dubois (1982) found that in frog myelinated axons, 20 μM capsaicin specifically blocked the high threshold, fast current whilst having little or no effect on the low-threshold, fast and the slow components of the tail current. However, if capsaicin is acting on the node of Ranvier by the same open-channel blocking mechanism as shown in more recent work, then in this case too, the specificity may not be as great as previously thought (see below).

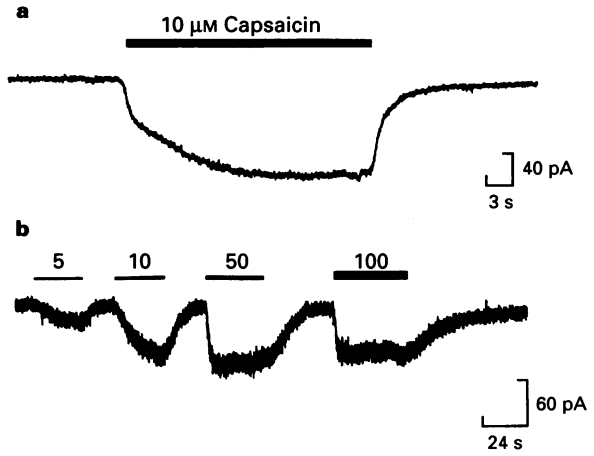

Figure 9 A few cells responded to capsaicin with an inward current at the holding potential of −50 mV. In (a) the cell was superfused with 10 μM capsaicin during the time indicated by the horizontal bar. The inward current developed in two stages, an initial fast stage, followed by a slower increase in inward current. In (b) the cell was superfused with saline containing 0, 5, 10, 50, or 100 μM capsaicin as indicated by the horizontal bar. The response appeared to saturate above 10 μM, with higher concentrations increasing the rate of current development without increasing the steady state. A slow desensitization was apparent in 50 μM and 100 μM capsaicin.

Quantitatively, the block of potassium currents in *Xenopus* neurones is comparable to the low micromolar block obtained for other excitable cells ($K_D = 8-17$ μM; Castle, 1992; Akins & McCleskey, 1993; Baker & Ritchie, 1994; Kehl, 1994).

In addition to blocking both slow and fast potassium currents, we found that in *Xenopus* spinal neurones calcium currents were also sensitive to block by capsaicin. The effect was very similar to the potassium current block in that it was time-dependent, it reversed readily on washout, and the K_D was in the low micromolar range. These effects suggest a similar, probably direct, interaction with the channel. Such a direct action on calcium channels has not been obtained previously. In the dorsal root ganglion neurones, capsaicin also blocks calcium currents, but the time course is more complex; some studies have shown a transient enhancement of the calcium current before inhibition (Petersen *et al.*, 1989; Kusano & Gainer, 1993; Urban & Dray, 1993). Docherty *et al.* (1991) found that in dissociated dorsal root ganglion cells concentrations of capsaicin in the 1–10 μM range inhibited calcium currents, but only in those cells that also responded to capsaicin with an inward current through the vanilloid receptor. The effects of capsaicin on calcium channels in these cells may be secondary to calcium influx though the vanilloid receptor rather than a direct action on the channel (Bleakman *et al.*, 1990; Docherty *et al.*, 1991).

Capsaicin as an open channel blocker

In previous work the block of potassium currents by capsaicin has been interpreted and modelled as an open-channel block (Baker & Ritchie, 1994; Kehl, 1994), analogous to the block of potassium channels by internally perfused tetraethylammonium (Armstrong, 1969). In an open channel blocking scheme, channels can only be blocked after they have been opened by a depolarizing voltage pulse. They may be unblocked by repolarizing the cell to a voltage that favours the closed state; as blocked channels unblock (opening briefly) they close, becoming inaccessible to capsaicin. This is summarized by the reaction scheme:

$$C \rightleftarrows O$$
$$O + Caps \rightleftarrows OB$$

where the closed channel (C) is opened (O) by the voltage step and capsaicin (Caps) binds to the open channel to produce the open, but blocked channel (OB). The time-dependent decay of both the calcium and potassium currents in *Xenopus* neurones, especially at higher concentrations (Figures 1a, 6a and 7a), is a hallmark of an open-channel block. Our further analysis of potassium channels supports this model. Firstly, as shown in Figure 3b, depolarizing pre-potentials reduced the peak current that could be elicited by the test pulse. This would be expected if at the end of the pre-pulse a fraction of the channels had opened and a fraction of these had been blocked by capsaicin. With higher depolarizing pre-pulses more channels would be in the blocked state and fewer would be available for opening by the test pulse. Secondly, the rate of the decay of the current during the step depended on both the amplitude of the step potential and the concentration of capsaicin (Figure 4a). At low test potentials, the rate of channel opening for both the slow and fast potassium channels is closer to the rate of block by capsaicin, so the opening and blocking reaction proceed in parallel; there is little additional steady-state block after the peak current. At higher test potentials, e.g. above +20 mV, the rate of channel opening is faster than the capsaicin block. A peak of current develops, which is followed by a decay as open channels become blocked. The rate of this decay increases with the capsaicin concentration. Thirdly, the capsaicin block could be relieved by repolarization to negative holding potentials (Figure 4b). Although the unbinding of capsaicin is not voltage-dependent, channel closure is, making the reverse reaction voltage-dependent. The longer the negative potential is maintained, the more channels move to the closed, unblocked state.

The open-channel blocking model predicts that capsaicin will change the kinetics of tail current by adding a second path away from the open, conducting channel. When the membrane is repolarized at the end of a voltage pulse, open channels become non-conducting both by closing and by binding capsaicin to produce open-blocked channels. The continued binding of capsaicin with the open channel should increase the initial rate of tail-current decay, but, because channels would open transiently on their way from the blocked to the closed state, a slow component would be added during the decay of OB state. This phenomenon has been observed in pituitary neurones by Kehl (1994), but in *Xenopus* neurones the changes in tail currents were not consistent; some cells responded as predicted, but others showed no change. There are two possible explanations for the apparent lack of effect. First, the control tail current has fast and slow components, so under the model capsaicin would divide each into fast and slow components, making the tail current the sum of four exponential decays. The tail currents were, however, well fitted by two exponentials. If such a transition occurred, then it is possible that the new fast component of the slow current masked the change in the fast current, and the new slow component of the fast current masked the change in the slow current. A similar argument might explain the apparent lack of effect of capsaicin on the slow and f_1 tail currents in frog node of Ranvier that was observed by Dubois (1982). An alternative explanation may be that the open-blocked channel is able to close without returning to the open conformation. If the OB-to-CB (closed, blocked) rate is similar to the O-to-C rate, then capsiacin would reduce the amplitude of the tail current without affecting its kinetics.

Cross-reactivity of calcium and potassium channel blocking agents

In addition to capsaicin, several other derivatives of vanillin block potassium and calcium channels. The capsaicin molecule consists of a methoxy, hydroxy substituted benzene ring joined to a hydrophobic alkyl tail by an amide bond (Walpole *et al.*, 1994). We found that the small molecules, such as vanillin and vanillic acid, which have a methone or methanoic group in place of the amide bond and alkyl tail, had no effect. On the other hand, zingerone, which has a butone in place of vanillin's methone group, blocks the transient and delayed rectifier po-

tassium channels in cardiac muscle (Castle, 1992). Capsazepine and resiniferatoxin have hydrophobic tails which are more sterically restrained or much more bulky than in capsaicin. Capsazepine blocked potassium and calcium channels, but the effect reversed slowly (Figures 5 and 8). Resiniferatoxin does not block cardiac potassium channels (Castle, 1992), but it did block potassium channels in *Xenopus* neurones. In this case the block developed much more slowly and did not cause the same change in kinetics as capsaicin. By contrast several types of cloned mouse and rat potassium channels are blocked by resiniferatoxin with the same time-dependency that we observed for capsaicin (Grissmer *et al.*, 1994). This suggests that it can act by the same blocking mechanism as capsaicin. Differences may be due to access to the binding site, which may be either intracellular or in a hydrophobic moiety of the channel. Finally, the calcium channel blocker, verapamil, is structurally similar to capsaicin, having two substituted aromatic rings linked by a hydrocarbon-amine chain. It also causes a time-dependent block of both calcium (Lee & Tsien, 1983) and potassium currents (Rampe *et al.*, 1993). Taken together, these results suggest that both a substituted benzene ring and a hydrophobic tail are necessary to cause open channel block of potassium and calcium channels. Other molecules with this basic structure may also be non-specific blockers of voltage-gated channels.

Mechanism of block

There are two general mechanisms by which capsaicin can produce an open channel block. First, it may occlude the pore by binding to a site that is only exposed when the channel is in the open conformation. Another hypothesis is that it binds to the channel and allosterically promotes a native inactivation process. Our results alone do not allow us to differentiate between these two mechanisms, but they and other work on the molecular pharmacology of potassium and calcium channels support an inactivation-promoting mechanism. Many delayed rectifier potassium channels undergo a slow inactivation process during prolonged or repetitive stimulation. The mechanism underlying this is localised to the membrane spanning, S6, region of the molecule (Hoshi *et al.*, 1991). In dihydropyridine sensitive calcium channels a homologous S6 region of the molecule is also involved in the inactivation process, as shown by the effects of mutations in and around this region on the rate of inactivation (Zhang *et al.*, 1994). This suggests that potassium and calcium channels may share a common mechanism of inactivation. The cross reactivity we have observed between capsaicin-like molecules and different potassium and calcium channels suggests that they are binding to a site on the channel molecule that is highly conserved in the family of voltage-dependent ion channels. We propose that this may be in or near the S6 region, where binding may cause an increase in the rate of inactivation. Interestingly, verapamil, which is structurally similar to capsaicin, blocks calcium channels by binding to a site that includes the S6 region, and it also blocks potassium currents through an open channel mechanism (Rampe *et al.*, 1993). If capsaicin were to act by stabilising the inactive state of the channel, then it should shift the steady-state inactivation curve in a hyperpolarized direction. This is difficult to judge in the slowly inactivating channels of the *Xenopus* neurones studied here, as the control current inactivated on a much slower time scale than the capsaicin block, but in rat heart cells, which have a transient potassium current, the block by capsaicin is accompanied by such a shift (Castle, 1992).

Vanilloid receptor in amphibians

In mammals low concentrations of capsaicin applied to the skin cause irritation and pain and activate C type sensory neurones *in vivo* and *in vitro*. The cellular effects are mediated by the vanilloid receptor on the sensory neurone, which is a non-specific cation channel gated by capsaicin and resiniferatoxin. These effects have not been observed in chickens (Szolcsáni *et al.*, 1985; Winter *et al.*, 1990), and there has been some doubt whether this receptor is present in other vertebrate classes or if it is specific to mammals. The inward current we observed in a subset of *Xenopus* neurones suggests that such a receptor is present in primitive tetrapods, and it may be phylogenetically very old. The isolated neurones that responded resembled the Rohon-Beard sensory neurones. Like the sensory C-type neurones in mammals, these cells in the *Xenopus* embryo are also immunoreactive for substance-P (Clarke *et al.*, 1984). As in mammalian neurones, capsaicin is more potent in eliciting the inward current than it is in blocking voltage-gated channels, although from the limited data available the *Xenopus* receptor may be less sensitive to capsaicin than the mammalian vanilloid receptor. The difference in potency, the agonist effect of capsazepine on voltage gated channels, and the relative rarity of the inward current in our sample suggest that the inward current and the block of voltage gated channels are mechanistically distinct actions of capsaicin. The function of vanilloid receptors in the *Xenopus* embryo is unknown at present. We have been unable to show a clear effect on the swimming motor pattern with low doses of capsaicin (unpublished results), but it would be premature to rule out a more subtle modulation of swimming or sensory responses by stimulation of this receptor.

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