Fractional Ca^{2+} currents through capsaicin- and proton-activated ion channels in rat dorsal root ganglion neurones

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1. Capsaicin and protons cause excitation and sensitization of primary nociceptive afferents. In a subset of dorsal root ganglion (DRG) neurones, which probably represent nociceptive neurones, both capsaicin and protons induce slowly inactivating non-selective cation currents. Whole-cell as well as single channel currents activated by these two stimuli share many biophysical and physiological properties in these neurones. This has lead to the suggestion that protons and capsaicin might activate the same ion channels.

2. In this study we simultaneously measured fluorescence signals and whole-cell currents activated by capsaicin or protons in acutely isolated DRG neurones filled with a high concentration (1 mM) of the Ca^{2+} indicator dye fura-2. From these measurements the fractional contribution of Ca^{2+} (P_f; the portion of the whole-cell current carried by Ca^{2+}) to capsaicin- and two types of proton-induced (fast and slowly inactivating) membrane currents was determined.

3. Capsaicin- and slowly inactivating proton-induced currents were accompanied by a change in fluorescence that was dependent on the presence of extracellular Ca^{2+}. With 1·6 mM extracellular Ca^{2+} and at a holding potential of −80 mV P_f of capsaicin-induced currents (at pH 7·3) was 4·30 ± 0·17% (mean ± S.E.M.; no. of experiments, $n = 16$) and of slowly inactivating proton-induced currents (at pH 5·1) was 1·65 ± 0·11% ($n = 17$). P_f of fast inactivating proton-induced currents was negligible.

4. P_f of capsaicin- and slowly inactivating proton-induced currents increased with increasing extracellular Ca^{2+} concentration (0·5–4·8 mM).

5. P_f of both current types decreased linearly with decreasing extracellular pH by about 0·7% per pH unit over the pH range investigated. When determined at the same extracellular pH P_f values were significantly different for the two current types at all pH values tested.

6. In summary, our results provide evidence that capsaicin and protons activate ion channels which are markedly permeable to Ca^{2+}. The fractional contribution of Ca^{2+}, however, was significantly different for capsaicin- and slowly inactivating proton-induced currents. This strongly suggests that the two stimuli activate different populations of ion channels and supports the possibility that Ca^{2+} influx through these channels may be important for Ca^{2+}-dependent sensitization of primary nociceptive neurones.

Capsaicin (8-methyl-*N*-vanillyl-6-nonenamide), a pungent tasting ingredient of hot peppers, has long been known as a potent algogen (for reviews see Holzer, 1991; Kress & Reeh, 1996). Short exposure to capsaicin leads to selective excitation and pronounced sensitization of primary nociceptive afferents (Culp, Ochoa, Cline & Dotson, 1989; Simone & Ochoa, 1991), whereas prolonged exposure causes desensitization to nociceptive stimuli (e.g. Maggi & Meli, 1988) and a specific degeneration of small diameter nerve fibres and neurones (Szolcsanyi, 1987; Lang, Novak & Handwerker, 1990). Because of its specificity capsaicin has become a valuable tool for the identification of those dorsal root ganglion (DRG) neurones from which polymodal nociceptive C fibres originate (for review see Holzer, 1991). In these DRG neurones, which we hereafter refer to as 'nociceptive DRG neurones', capsaicin elicits a slowly inactivating cation current. Single channel activity underlying this current has been recorded in attached and excised patches of rat DRG neurones (Oh, Hwang & Kim, 1996) suggesting that capsaicin probably activates the

current by directly gating the ion channel via binding to its vanilloid receptor site.

In rat DRG neurones, protons activate two ionic conductances which differ in kinetics, pH dependence of activation and inactivation, and prevalence among different subtypes of DRG neurones. The first type exhibits slow activation and inactivation upon extracellular acidification to less than pH 6·6 and is restricted to small oval-shaped neurones, which constitute about 40% of the total population of DRG neurones (Bevan & Yeats, 1991). Most of these neurones also respond to capsaicin. This slowly inactivating proton-induced current shares many biophysical properties with the capsaicin-induced current. Both are conducted by rather non-selective cation channels with similar permeabilities to monovalent cations and with similar unitary conductances (Bevan & Geppetti, 1994). These similarities have led to the suggestion that protons and capsaicin might activate the same ion channel molecules (Bevan & Yeats, 1991; Bevan & Geppetti, 1994; Liu & Simon, 1994; but see also Oh *et al.* 1996). Protons might, therefore, function as endogenous activators of these ion channels leading to proton-induced excitation of primary nociceptive neurones and pain during inflammation and ischaemia, which are accompanied by substantial tissue acidosis (Steen & Reeh, 1993; Bevan & Geppetti, 1994). A second proton-activated current type is expressed in the vast majority (80%) of DRG rat neurones, as well as in many central nervous system neurones. This current exhibits rapid and transient activation at less than pH 7·0 and is mainly carried by Na^+ ions. It has been suggested to arise from proton-modified high voltage-activated Ca^{2+} channels (Konnerth, Lux & Morad, 1987).

Many ligand-gated cation channels exhibit a substantial permeability to Ca^{2+}. This is an important characteristic for the various channel types and has far-reaching functional implications. Elevations in the intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) upon exposure to capsaicin and acidic solutions have been demonstrated in rat trigeminal and DRG neurones (Cholewinski, Burgess & Bevan, 1993; García-Hirschfeld, López-Briones, Belmonte & Valdeolmillos, 1995; Zeilhofer, Swandulla, Reeh & Kress, 1996) and permeability ratios for Ca^{2+} *versus* monovalent cations (P_{Ca}/P_M) have been determined from reversal potential measurements (Kovalchuk, Krishtal & Nowycky, 1990; Koplas, Oxford & Rosenberg, 1995; Oh *et al.* 1996; Zeilhofer *et al.* 1996). These values, however, vary considerably among the different studies, ranging from 0·24 to 4·0. Here, we have determined relative permeabilities from fractional Ca^{2+} currents (Schneggenburger, Zhou, Konnerth & Neher, 1993; Neher, 1995) through capsaicin- and proton-activated ion channels in rat nociceptive DRG neurones. This method allows for the determination of Ca^{2+} permeability at physiological concentrations of extracellular Ca^{2+} ($[Ca^{2+}]_o$) and at non-zero electrochemical driving forces (Zhou & Neher, 1993; Neher, 1995).

In adult rat DRG neurones we have found fast and slowly inactivating proton-induced currents and slowly inactivating capsaicin-induced currents. We provide evidence that the ion channels underlying capsaicin- and slowly inactivating proton-induced currents are markedly permeable to Ca^{2+}. However, the fractional Ca^{2+} currents and the permeability ratios derived therefrom are significantly different for the two currents. These results strongly suggest that capsaicin- and proton-induced cation currents flow through different populations of ion channels and challenge previous views that protons and capsaicin activate the same channel protein.

METHODS

Cell preparation

Acutely dissociated DRG neurones were prepared from adult female Wistar rats weighing 110–160 g that had been killed by breathing 100% CO_2. Ganglia harvested from level T13 to L5 were transferred into Dulbecco's modified Eagle's medium (DMEM) supplemented with gentamicin (50 μg ml^{-1}). After removal of the connective tissue, ganglia were incubated in 0·28 U ml^{-1} collagenase for 75 min, washed twice in phosphate-buffered saline (containing (mM): 137 NaCl, 20 NaH_2PO_4, 2·5 K_2HOPO_4; pH 7·4, adjusted with HCl) and transferred into 25000 U ml^{-1} trypsin for 12 min. After three washes in supplemented DMEM ganglia were dissociated using fire-polished Pasteur pipettes, centrifuged at 2000 *g* and resuspended in F12 medium supplemented with 10% horse serum, 20 mM L-glutamine, 0·8 mM D-glucose and 10 μg (100 ml)$^{-1}$ nerve growth factor 7S and plated on poly-L-lysine (200 μg ml^{-1})-coated glass coverslips. Cultures were kept in a humid 5% CO_2–95% air atmosphere at 37 °C and used for recordings 2–36 h after plating.

Electrophysiological recordings

Recordings were made from small oval-shaped cells, presumed to be nociceptive neurones, which were free of visible processes. Proton- and capsaicin-induced currents were recorded in the whole-cell configuration of the patch-clamp technique using an EPC-7 patch-clamp amplifier (List Electronics, Darmstadt, Germany) and the pulse program (HEKA Electronics, Lamprecht, Germany) running on a Mackintosh Quadra 800 computer. The standard extracellular recording solution contained (mM): 145 NaCl, 10 TEA-Cl, 2·5 KCl, 1·6 $CaCl_2$, 1·0 $MgCl_2$, 10 Hepes; pH was adjusted to 7·3 or 7·5 using NaOH, osmolarity was 315 mosmol l^{-1}. In five separate experiments 145 mM NaCl was replaced by 154 mM NMDG-Cl to record voltage-gated Ca^{2+} currents in isolation (see Fig. 2). This was necessary since voltage-gated Na^+ currents are largely insensitive to TTX in these neurones. Acidic solutions (pH 5·1, 5·6, 6·1 and 6·6) were prepared using 10 mM Mes instead of 10 mM Hepes and using HCl to adjust the pH. Patch pipettes pulled from KIMAX 51 glass typically had resistances of 1·5–2·0 MΩ and were filled with internal solution containing (mM): 130 CsCl, 20 TEA-Cl, 2 $MgCl_2$, 10 Hepes, 2 Na_2-ATP, 0·2 Na_2-GTP, 1 fura-2 (pentapotassium salt); pH was adjusted to 7·30 using CsOH, osmolarity was 290 mosmol l^{-1}. Series resistance, usually between 3 and 5 MΩ, was not compensated. Passive leakage and capacitive currents were subtracted electronically using the *P*/4 method. Capsaicin-containing and acidic solutions were applied from a three-barrelled application pipette with a tip diameter of about 100 μm positioned about 70 μm from the cell soma being recorded from. All neurones were either stimulated with capsaicin-containing solution adjusted to pH 7·3 or 7·5 or with acidic solutions. Repeated stimulations of cells with capsaicin-containing or acidic solutions were performed at

frequencies of less than 0·3 min^{-1}. Under these conditions no reduction in amplitudes was observed with repeated applications, and P_f values did not change significantly.

Measurement of Ca^{2+} influx

Fractional Ca^{2+} currents through proton- or capsaicin-activated channels were measured by loading the cells with high concentrations (1 mM) of the Ca^{2+} indicator dye fura-2 (Grynkiewicz, Poenie & Tsien, 1985). A detailed description of this method is given in Zhou & Neher (1993) and Neher (1995). Briefly, the cells were loaded via the recording pipette with fura-2 at a concentration high enough to overcome endogenous Ca^{2+} buffers so that all incoming Ca^{2+} ions bind to fura-2 and induce a decrease in the fluorescence signal excited at 380 nm. The wavelengths (λ) at which fura-2 was excited were 350 and 380 nm and fluorescence was collected at $\lambda \geqslant 420$ nm at a frequency of 1 Hz using a slow scan CCD camera system coupled to a monochromator (TILL photonics, Planegg, Germany). Fluorescence signal intensities were standardized by comparing their intensity to that of standard fluorescent beads (carboxy Bright Blue 4·5 μM microspheres; Polysciences Europe, Eppelheim, Germany). Bead fluorescence was always measured in double-distilled water. From the background-corrected fluorescence intensities of fura-2 excited at 350 (F_{350}) and 380 nm (F_{380}), $[Ca^{2+}]_i$ and intracellular fura-2 concentration ($[\text{fura-2}]_i$) were calculated:

$$[Ca^{2+}]_i = K_{eff}(R - R_{min})/(R_{max} - R), \quad (1)$$

where K_{eff} is the effective K_D, R is the fluorescence ratio, R_{min} is the fluorescence ratio in the absence of Ca^{2+} and R_{max} is the fluorescence ratio when fura-2 is saturated with Ca^{2+} (Grynkiewicz *et al.* 1985). The calibration was performed as described by Neher (1988) using the following values: R_{min}, 0·45; R_{max}, 5·2; and K_{eff}, 2·0 μM.

$$[\text{fura-2}]_i = [\text{fura-2}]_{pip} \times (F_{350} + \alpha F_{380}), \quad (2)$$

where α is the so-called isocoefficient which makes the term $F_{350} + \alpha F_{380}$ independent from the actual $[Ca^{2+}]_i$ (Neher, 1995) and $[\text{fura-2}]_{pip}$ is the fura-2 concentration in the recording pipette (Fig. 1*C* and *D*).

From $[\text{fura-2}]_i$ and $[Ca^{2+}]_i$ the actual Ca^{2+} buffering capacity of fura-2 can be calculated (Neher, 1995):

$$\kappa B' = \frac{\Delta[\text{Ca–fura-2}]}{\Delta[Ca^{2+}]} = \frac{[\text{fura-2}]/K_D}{(1 + [Ca^{2+}]_{i,a}/K_D)(1 + [Ca^{2+}]_{i,b}/K_D)}, \quad (3)$$

where $[Ca^{2+}]_{i,a}$ and $[Ca^{2+}]_{i,b}$ are values of intracellular free Ca^{2+} concentration before (a) and after (b) the pulse and K_D is the dissociation constant of Ca–fura-2.

The ratio f of the change in F_{380} (ΔF_{380}) and the integral of any membrane current over time (Q) yields a relative measurement for the fractional contribution of Ca^{2+} to the total current flowing through the ion channels investigated. By dividing the fluorescence change/charge ratio $f = \Delta F_{380}/Q$ of the current under study by the maximum ratio value (f_{max}) of voltage-gated Ca^{2+} currents, which we assume to be exclusively carried by Ca^{2+}, an absolute measure for the fractional Ca^{2+} contribution can be obtained: $P_f = f/f_{max}$.

Estimates for P_{Ca}/P_M were calculated from P_f values using the following equation (Schneggenburger *et al.* 1993):

$$1/P_f = 1 + \frac{[M^+]}{[Ca^{2+}]_o} \times \left(\frac{1 - \exp(2FV_m/RT)}{4P_{Ca}/P_M}\right), \quad (4)$$

where $[M^+]$ is the total concentration of monovalent cations and $[Ca^{2+}]_o$ is the total external Ca^{2+} concentration. Both were corrected for ionic activities using the coefficient 0·76 for monovalent cations and 0·58 for Ca^{2+}. F, R and T have their usual thermodynamic meanings and V_m is the membrane potential. This equation was also used to predict the dependence of P_f on different $[Ca^{2+}]_o$ (see Fig. 4). As discussed by Spruston, Jonas & Sakmann (1995) this equation assumes equal permeabilities to all monovalent cations and that currents can be predicted from the Goldman–Hodgkin–Katz (GHK) equation.

Correction of ΔF_{380} values for Ca^{2+} sequestration and/or extrusion

ΔF_{380} values were corrected for Ca^{2+} extrusion and/or sequestration processes using the following equation, which assumes that the recovery of the ΔF_{380} signal can be described by a monoexponential function (see Fig. 2*B*):

$$\Delta F_{380}[(n + 1)\Delta t]_{corr} = \Delta F_{380}(n\Delta t)_{corr} + \{\Delta F_{380}[(n + 1)\Delta t]\Delta t/\tau\}, \quad (5)$$

where $\Delta F_{380}(n\Delta t)_{corr}$ is the corrected value of the ΔF_{380} signal n sample intervals after the beginning of the stimulation with a given agonist, Δt is the sample interval, n is the nth sample point after the beginning of the stimulation and τ is the time constant of the recovery of the ΔF_{380} signal. τ was determined for each stimulus and each cell individually from the time course of the recovery of the ΔF_{380} signal after complete recovery of the agonist-activated current. $\Delta F_{380}(n = 0)$ and $\Delta F_{380}(n = 0)_{corr}$, which are ΔF_{380} values before stimulation, were averaged for appropriate time intervals like 10 s, and set to 0. This procedure was used to demonstrate that the ΔF_{380} signal closely followed the current integral over time. During the first 5–10 s of agonist application no significant difference was found between corrected and uncorrected ΔF_{380} values. P_f was calculated from ΔF_{380} values taken 5 or 6 s after the beginning of the stimulation with either protons or capsaicin.

Measurement of intracellular pH

Intracellular pH (pH_i) was measured using the indicator dye 2′,7′-bis (2-carboxyethyl)-5(6)-carboxyfluorescein (BCECF) (100 μM), which was added to the standard pipette filling solution except that 1 mM fura-2 was replaced by 1 mM BAPTA to avoid errors arising from fura-2 fluorescence at 440 and 495 nm. pH_i was calculated from ratios of background-corrected fluorescent images excited at 440 and 495 nm.

Calibration was performed as described by Thomas, Buchsbaum, Zimniak & Racker (1979). Briefly, DRG neurones were incubated in 10 μM BCECF-acetoxymethylester (BCECF-AM, dissolved in DMSO; final DMSO concentration $\leqslant$ 0·1 %) for 30 min at 37 °C and thoroughly washed to remove unhydrolysed BCECF-AM. Extracellular solutions containing high potassium (135 mM) and nigericine (10 μM, dissolved in methanol; final methanol concentration $\leqslant$ 0·1 %), an activator of a plasma membrane H^+–K^+ exchanger, were used to facilitate equilibration of extracellular pH (pH_o) and pH_i. High potassium solutions contained (mM): 135 KCl, 1·6 $CaCl_2$, 1 $MgCl_2$ and 10 Hepes (pH 7·4 and 7·1, adjusted with NaOH), or 10 Mes (pH 6·8 and 6·5, adjusted with HCl). The calibration is shown in Fig. 6.

Chemicals

All inorganic chemicals were obtained from Merck. ATP, BAPTA, capsaicin, gentamicin, D-glucose, GTP, Hepes, Mes, nigericine, poly-L-lysine and trypsin were from Sigma. BCECF, fura-2 and BCECF-AM were from Molecular Probes Europe. DMEM, F12 medium and L-glutamine were from Gibco. Collagenase was from Boehringer Mannheim. Nerve growth factor 7S was from Calbiochem.

Statistics

All results are given as means ± S.E.M. Statistical significances were evaluated using Student's two-tailed unpaired t test.

RESULTS

Measurement of fractional Ca^{2+} currents in DRG neurones

The fractional contribution of Ca^{2+} to the total inward current (proportion of the whole-cell current carried by Ca^{2+}; $P_f = I_{Ca}/I_{total}$) through capsaicin- and proton-activated ion channels was measured at physiological $[Ca^{2+}]_o$ (1·6 mM) using a modification of the method originally described by Schneggenburger *et al.* (1993) and Zhou & Neher (1993). Acutely isolated DRG neurones were filled with a high concentration (1 mM) of the Ca^{2+} indicator dye fura-2 via the recording pipette and whole-cell membrane currents and fura-2 fluorescence were measured simultaneously. To optimize clamp conditions only cells free of processes and with agonist-activated current amplitudes of less than 2 nA were analysed. The ratio between the Ca^{2+} influx, which can be calculated from changes in Ca^{2+}-sensitive fluorescence, and the integral of the total inward current over time (charge, $Q = \int I(t)dt$) yields a reliable measure of the contribution of Ca^{2+} to the total current as long as nearly all incoming Ca^{2+} binds to fura-2. Under this condition ΔF_{380} is proportional to the amount of incoming Ca^{2+}. The proportionality coefficient was determined from voltage-activated Ca^{2+} currents, which we assume to be exclusively carried by Ca^{2+} ions.

Figure 1*A* and *B* shows an example of the fluorescence signals excited at 350 and 380 nm during loading of a DRG neurone with fura-2. Ca^{2+} influx through voltage-activated Ca^{2+} channels was elicited at a frequency of about 0·5 min^{-1} by step depolarizations from a holding potential of −80 to 0 mV for 50 ms (for details see Fig. 2*A*). This caused a slight increase in F_{350} and a decrease in F_{380}. From these measurements $[Ca^{2+}]_i$ and $[fura\text{-}2]_i$ were calculated (Fig. 1*C* and *D*; see Methods). Concentrations of fura-2 inside the cell

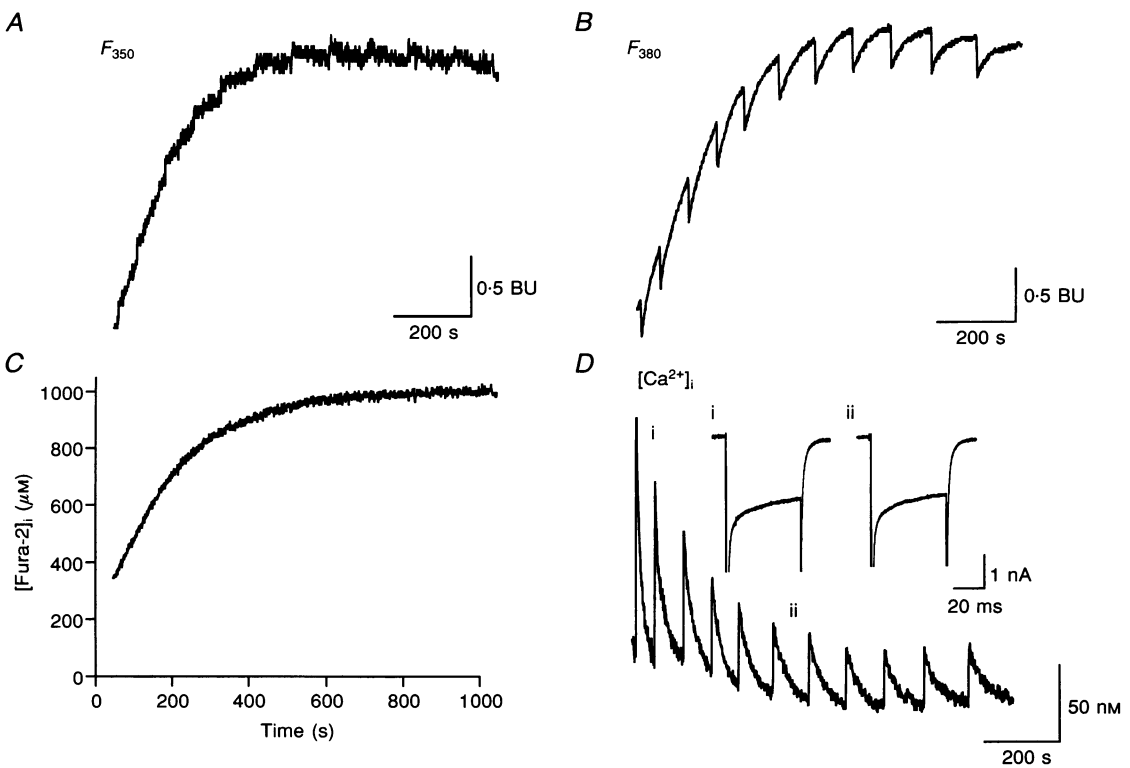

Figure 1. Combined patch-clamp and fura-2 fluorescence recordings from a rat DRG neurone

F_{350} (*A*) and F_{380} (*B*) *versus* time during dye loading of a DRG neurone. Fluorescence intensities (given as units of standard bead fluorescence, BU) increased during loading of the cell. Depolarizing voltage steps (50 ms duration) from a holding potential of −80 to 0 mV were applied at a frequency of about 0·5 min^{-1}. Ca^{2+} influx during these short depolarizations caused decreases in F_{380} and increases in F_{350}. From the two fluorescence signals the total concentration of fura-2 ($[fura\text{-}2]_i$) (*C*) and the actual free Ca^{2+} concentration inside the cell ($[Ca^{2+}]_i$) (*D*) can be calculated; for details see Methods. [Fura-2] equilibrated between the pipette and the cell interior within about 10 min. Increases in $[Ca^{2+}]_i$ caused by the depolarizing voltage steps became progressively smaller with increasing $[fura\text{-}2]_i$ while inward currents and, as a consequence, the amount of Ca^{2+} entering the cell per pulse remained remarkably constant. i and ii denote corresponding current traces (insets) and Ca^{2+} signals. Note that $[fura\text{-}2]_i$ had already reached 300 μM when recording of the fluorescence signal started (*C*).

and the pipette usually equilibrated within 10 min. Although inward currents remained remarkably constant the rises in $[Ca^{2+}]_i$ during filling of the cells with fura-2 became progressively smaller due to the increasing Ca^{2+} buffer capacity of fura-2.

As shown in Fig. 2*A*, voltage-activated inward currents evoked by depolarizing voltage pulses from a holding potential of −80 to 0 mV consisted of a fast and a slowly inactivating current component. The transient component was suppressed when extracellular Na^+ was replaced with impermeant *N*-methyl-D-glucamine ($NMDG^+$). Inward current components, which remained under this condition, were slowly inactivating and carried by Ca^{2+} ions flowing through voltage-activated Ca^{2+} channels. The Ca^{2+} component of the inward current was approximated by linearly extrapolating the last 20 ms of the current trace back to the beginning of the voltage step. This procedure was chosen since small (presumably nociceptive) rat DRG neurones possess voltage-activated Na^+ currents largely insensitive to TTX and substitution of Na^+ with $NMDG^+$ seemed to interfere with the recovery from the Ca^{2+} load. Figure 2*A* shows a comparison of this estimate (averaged current traces from five capsaicin-sensitive cells) and the Ca^{2+}

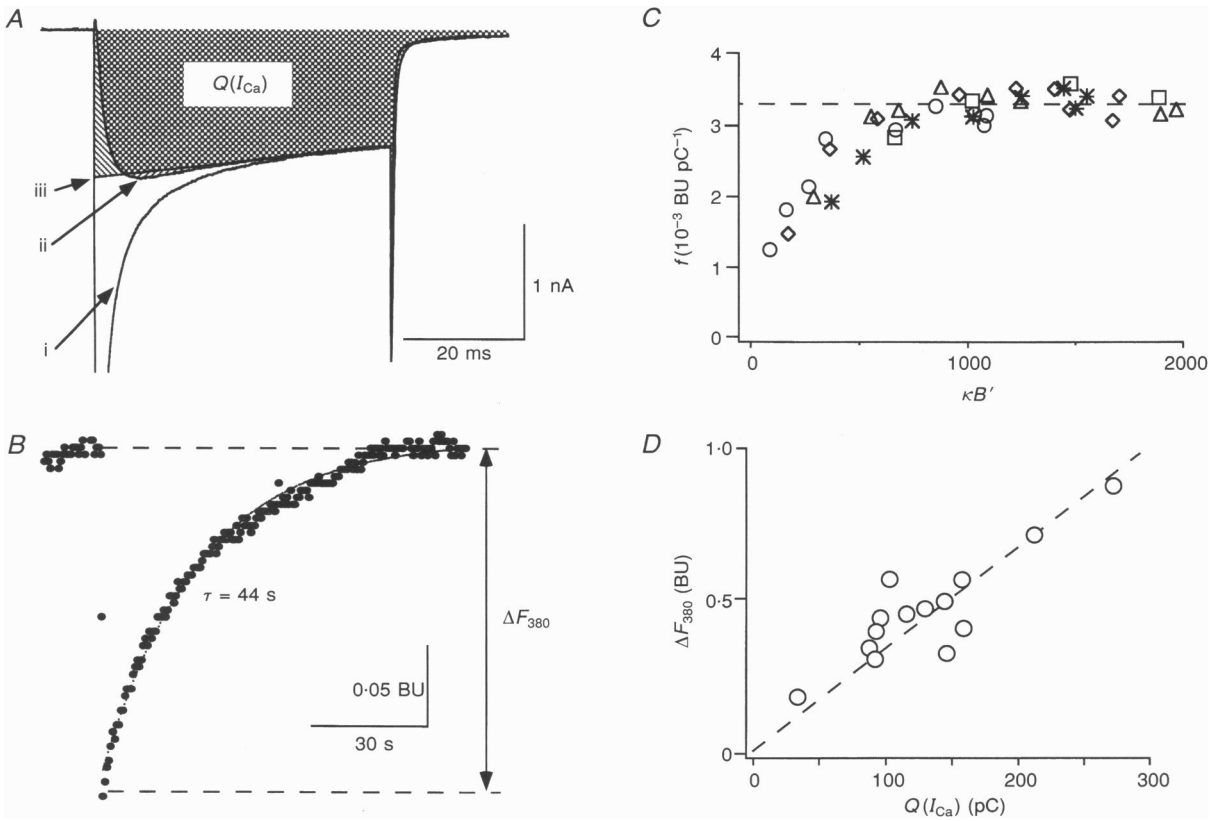

Figure 2. Saturation of f and determination of f_{max}

To determine f_{max}, Ca^{2+} influx through voltage-activated Ca^{2+} channels was elicited by step depolarizations from a holding potential of −80 to 0 mV for 50 ms and the concomitant ΔF_{380} recorded. *A*, digitally averaged current traces recorded from five capsaicin-sensitive cells. The inward currents consisted of a Na^+ current component, which inactivated within about 30 ms, and a slowly inactivating Ca^{2+} current component, which persisted during voltage step i. When Na^+ was replaced by impermeant $NMDG^+$ Ca^{2+} currents could be recorded in isolation (ii). The charge transferred by Ca^{2+} entering the cell per pulse ($Q(I_{Ca})$) was estimated from a linear extrapolation of the slowly inactivating part of the inward current back to the beginning of voltage step iii. The areas under curves ii and iii are almost identical, indicating that linear extrapolation provides a good measure for the total ($Q(I_{Ca})$). *B*, an example of a concomitant ΔF_{380}. Dashed lines represent baseline and minimum F_{380}. *C*, f *versus* $\kappa B'$ for five representative cells (each represented by a different symbol), the $\kappa B'$ values of which cover a wide range. $\kappa B'$ was calculated from $[\text{fura-2}]_i$ and $[Ca^{2+}]_i$ (for details see Methods). f approaches a maximum value (f_{max}, dashed line) at $3{\cdot}3 \times 10^{-3}$ BU pC^{-1} Ca^{2+} for $\kappa B' > 670$ ($f/f_{max} > 0{\cdot}9$). This f_{max} is identical to the proportionality coefficient of ΔF_{380} and Ca^{2+} influx into the cell. Note that this value differs from that reported by two other groups (Schneggenburger *et al.* 1993; Zhou & Neher, 1993; Burnashev *et al.* 1995) by a factor of three. This is due to differences in the experimental set-up, especially in the emission filters used (420 nm in our set-up *versus* 510 nm). *D* demonstrates that ΔF_{380} was proportional to the $Q(I_{Ca})$ for $\kappa B' > 700$. The ΔF_{380} signals are plotted *versus* $Q(I_{Ca})$. Data are pooled from seven representative cells. Dashed line represents a linear fit to the data points.

Table 1. Summary of P_f, P_{Ca}/P_M and current amplitudes

Stimulus	Resting pH_o	$[Ca^{2+}]_o$ (mM)	Amplitude (pA)	P_f (%)	Calculated P_{Ca}/P_M	n
pH_o 5·1	7·3	1·6	889 ± 141	1·65 ± 0·11	0·55	17
pH_o 5·6	7·3	1·6	820 ± 275	2·12 ± 0·19	0·70	6
pH_o 6·1	7·3	1·6	1298 ± 220	2·38 ± 0·12	0·81	6
Caps, pH_o 7·3	7·3	1·6	680 ± 126	4·30 ± 0·17	1·48	16
Caps, pH_o 6·6	7·3	1·6	840 ± 386	3·78 ± 0·09	1·29	4
Caps, pH_o 6·1	7·3	1·6	720 ± 123	3·24 ± 0·17	1·10	7
Caps, pH_o 5·6	7·3	1·6	1106 ± 232	3·11 ± 0·21	1·05	10
Caps, pH_o 5·1	7·3	1·6	1695 ± 162	2·98 ± 0·16	1·01	5
pH_o 5·1	7·3	0	629 ± 174	< 0·35	—	3
pH_o 5·1	7·3	0·5	987 ± 470	0·80 ± 0·21	0·84	3
pH_o 5·1	7·3	1·6	889 ± 141	1·65 ± 0·11	0·55	17
pH_o 5·1	7·3	4·8	1124 ± 383	5·4 ± 0·96	0·62	4
Caps, pH_o 7·3	7·3	0	538 ± 237	< 0·3	—	3
Caps, pH_o 7·3	7·3	0·5	1085 ± 453	1·50 ± 0·32	1·58	3
Caps, pH_o 7·3	7·3	1·6	680 ± 126	4·30 ± 0·17	1·48	16
Caps, pH_o 7·3	7·3	4·8	341 ± 207	11·3 ± 0·92	1·38	3
pH_o 6·6	7·5	1·6	970 ± 285	< 0·4	< 0·13	6

10 mM Ca_o^{2+} was achieved using 1 mM BAPTA. Note that only currents with amplitudes of less than 2 nA were included in the analysis and that the values given for the current amplitudes refer only to these currents. n, number of experiments; Caps, capsaicin.

current recorded in the same cells after substitution of Na^+ with impermeant $NMDG^+$. The calculated integrals of the currents over time differ only by less than 4%. Therefore, the charge transferred by Ca^{2+} entering the cell with each depolarization ($Q(I_{Ca}) = \int I_{Ca} dt$) could be well estimated from linear extrapolation. The corresponding ΔF_{380} signal is shown in Fig. 2*B*. As illustrated in Fig. 2*C* f increased with increasing $\kappa B'$ and approached a limit, f_{max}. This limit represents the maximum decrease in F_{380} per incoming pico-coulomb of Ca^{2+} and describes the relationship between the amount of incoming Ca^{2+} and ΔF_{380}; this must be determined individually for each experimental set-up and was $3·3 \times 10^{-3}$ BU pC^{-1} in the present case. This value was used for all P_f calculations. The requirement that nearly all incoming Ca^{2+} binds to fura-2 is fulfilled as soon as f approaches f_{max}. In rat DRG neurones this is achieved when $\kappa B'$ exceeds about 670. Although this was usually reached during the first minute of whole-cell recording, agonist-induced currents were elicited only after the fura-2 concentration had equilibrated between the patch pipette and the cell. Under this condition ΔF_{380} was proportional to $Q(I_{Ca})$ (Fig. 2*D*). f can be determined for any cation current and, under conditions where all incoming Ca^{2+} binds to fura-2, f/f_{max} provides an absolute measure for P_f to the current flowing through the ion channel investigated (Schneggenburger *et al.* 1993; Neher, 1995).

Fractional Ca^{2+} currents through capsaicin-activated channels

Exposure to capsaicin (3 μM) evoked an inward current in twenty-four of twenty-nine small oval-shaped DRG neurones. Only those cells that responded to capsaicin with an inward current showed a decrease in the F_{380} signal. When capsaicin was applied for 5 s inward currents lasting for 15–20 s were induced. During this time interval a considerable amount of the incoming Ca^{2+} may be extruded from the cell or sequestered into Ca^{2+} storage organelles. Indeed, the curves of the ΔF_{380} signal followed the integral of the capsaicin currents for the first 5–10 s of the current but then progressively deviated (filled circles in Fig. 3*Ab* and *Bb*). From the ΔF_{380} signals taken 5 or 6 s after the beginning of the stimulation with capsaicin a P_f of the capsaicin-activated current of 4·30 ± 0·17% ($n = 16$) was calculated at a holding potential of −80 mV (Fig. 3*Ab* and Table 1). From P_f values P_{Ca}/P_M can be derived (see Methods). A P_f value of 4·30, as determined for capsaicin, corresponds to a P_{Ca}/P_M of 1·48. The time course of the recovery of the ΔF_{380} signal, which reflects the kinetics of Ca^{2+} extrusion and/or sequestration processes, could be well described by a monoexponential function under our experimental conditions (see Fig. 2*B*). The time constant of this function was used to correct ΔF_{380} values for Ca^{2+} extrusion and/or sequestration (see Methods). ΔF_{380} values corrected in this

way closely followed the current integral over time. As shown in Fig. 3*Ab* the curves of ΔF_{380} (open circles) and (scaled) $\int I(t)\mathrm{d}t$ (continuous line) take a parallel course. This strongly indicates that no secondarily activated Ca^{2+} influx or release mechanism contributed to the ΔF_{380} signal. A rapid capsaicin-activated current response comparable to the fast inactivating proton-induced current (see below) was not found in any of the neurones tested ($n = 29$).

Fractional Ca^{2+} currents through proton-activated channels

Exposure to acidic extracellular solutions elicited two types of ionic current in the neurones investigated. These currents differed in respect to the pH dependence of activation and kinetics. One type could be evoked by lowering the pH_o from 7·3 to 6·6 or less. This current activated rather slowly and showed no or only little inactivation during application times as long as 10 s. It is therefore termed slowly inactivating proton-induced current (Fig. 3*Ba*).

The other current type could only be activated from a resting pH of greater than 7·4 and required less acidification for activation. This current type exhibited faster activation and complete inactivation within 5 s of proton application and was therefore termed fast inactivating. We separated these two currents by lowering the pH either from 7·5 to 6·6 (fast inactivating current), or from 7·3 to 6·1 or less (slowly inactivating current).

Slowly inactivating proton-induced currents and concomitant ΔF_{380} could be evoked in twenty-nine of thirty-seven small oval-shaped DRG neurones. Again, as with capsaicin, only those cells that responded with a slowly inactivating inward current upon exposure to pH_o 5·1 exhibited a decrease in

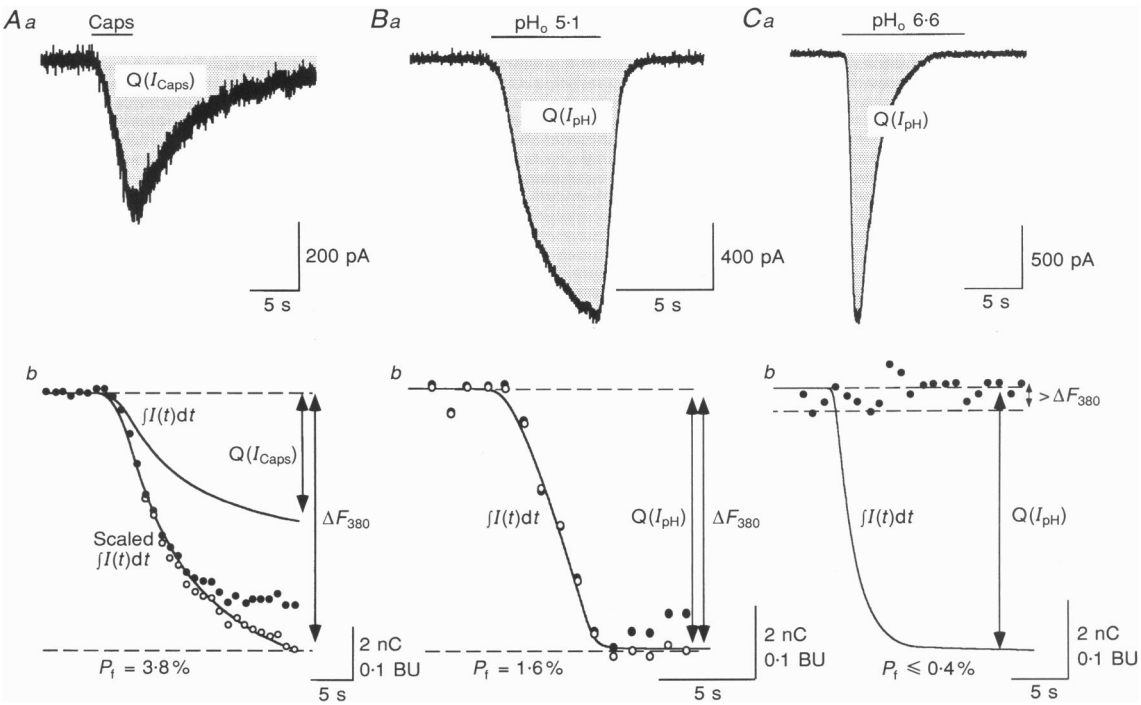

Figure 3. P_f of currents evoked by capsaicin (Caps, 3 μM) and protons

Upper traces, agonist-induced currents at −80 mV. Lower traces, concomitant ΔF_{380}. The uncorrected ΔF_{380} signal (●) and the ΔF_{380} signal after correction (○) for Ca^{2+} extrusion and/or sequestration are shown (for details see Methods). Continuous lines labelled $\int I(t)\mathrm{d}t$ are the current integrals. *A*, capsaicin-induced inward current (*a*) and concomitant ΔF_{380} (*b*). After correction for Ca^{2+} extrusion and/or sequestration (see Results) the time course of ΔF_{380} closely followed that of the total inward charge transferred through capsaicin-activated ion channels. Both curves are nearly congruent when the current integral is scaled to the amplitude of the ΔF_{380} signal. From the ratio of ΔF_{380} and the integral of the current over time, both taken 5 s after the beginning of the application of capsaicin, a P_f of the total capsaicin-induced current of 3·8% was calculated for this cell. *B*, slowly inactivating proton-induced inward current (*a*) and concomitant ΔF_{380} (*b*). The slowly inactivating proton-induced inward current was elicited by extracellular application of protons (pH 5·1) from a resting pH of 7·3. P_f was 1·6% in this cell. *C*, fast inactivating proton-induced inward current (*a*) and concomitant ΔF_{380} (*b*). When pH_o of 6·6 was applied from a less acidic resting pH of 7·5, a fast- and completely inactivating proton-induced inward current could be evoked. This fast-inactivating current was not accompanied by a decrease in ΔF_{380}, indicating that P_f for this current is negligible. In panel *Ab* the calibration for charge (nC) refers to the unscaled current integral.

F_{380}. We found a P_f of slowly inactivating proton-induced currents of $1{\cdot}65 \pm 0{\cdot}11\%$ ($n = 17$) at pH_o 5·1 and at a holding potential of −80 mV (Fig. 3*Bb* and Table 1). This corresponds to a P_{Ca}/P_M of 0·55. In all of six experiments in which an acidic solution of pH 6·6 was applied from a resting pH of 7·5 for several seconds fast- and completely inactivating proton-induced current responses could be elicited (Fig. 3*Ca*). In all these experiments ΔF_{380} remained below the threshold of detection (~0·4%) (Fig. 3*Cb* and Table 1) suggesting a P_{Ca}/P_M of less than 0·13.

In contrast to what has been described by Liu & Simon (1994) capsaicin- and proton-induced currents could be elicited in the absence of extracellular Ca^{2+} (0 mM Ca^{2+}, 1 mM BAPTA). F_{380}, however, remained constant under this condition indicating that neither protons nor capsaicin induced intracellular Ca^{2+} release. Increasing or decreasing $[Ca^{2+}]_o$ by a factor of three caused increases and decreases in P_f. These shifts were in good agreement with the predictions of the GHK equation (Fig. 4). This finding, together with the observation that the ΔF_{380} signals closely followed the current integrals over time (see Fig. 3), argues against a relevant contribution of Ca^{2+}-induced Ca^{2+} release to the fluorescence signals (Schneggenburger *et al.* 1993). Regulation of $[Ca^{2+}]_i$ in rat DRG neurones has been carefully studied by Thayer & Miller (1990). Even in the presence of only 0·1 mM fura-2, which is 10-fold less than that used in our experiments, they found that Ca^{2+}-induced Ca^{2+} release made no significant contribution to rises in $[Ca^{2+}]_i$ elicited by step depolarizations.

The contribution of Ca^{2+} was different for capsaicin-induced and slowly inactivating proton-induced currents. A possible explanation for these findings is that capsaicin- and slowly inactivating proton-induced currents flow through different ion channels with different permeabilities to Ca^{2+}. Another possibility might be that an increase in the extracellular proton concentration not only induces channel openings but

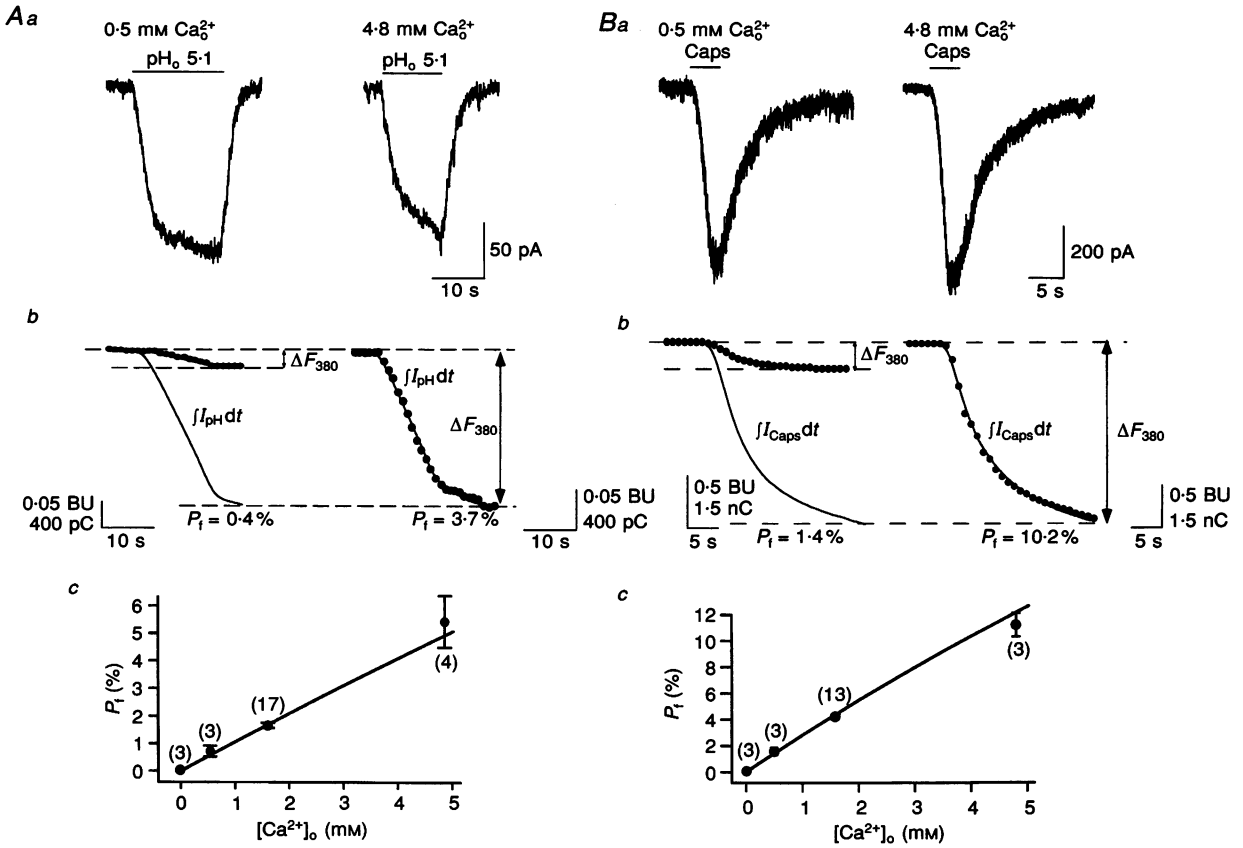

Figure 4. Dependence of P_f on $[Ca^{2+}]_o$

Ionic currents (*Aa* and *Ba*) and ΔF_{380} (*Ab* and *Bb*) were elicited through application of protons (pH_o 5·1) (*A*) or 3 μM capsaicin (Caps, *B*) in the presence of either 0·5 (left panels of *A* and *B*) or 4·8 mM (right panels of *A* and *B*) external Ca^{2+} (Ca_o^{2+}). The continuous lines in *Ab* and *Bb* represent the current integrals over time ($\int I(t)dt$). The time course of the ΔF_{380} closely followed these integrals for both concentrations and for both stimuli. As shown in *Ac* and *Bc* P_f increases with increasing $[Ca^{2+}]_o$. The continuous lines in *Ac* and *Bc* show P_f as a function of $[Ca^{2+}]_o$ predicted from the GHK equation assuming that P_{Ca}/P_M is independent of $[Ca^{2+}]_o$. P_{Ca}/P_M was calculated from P_f at 1·6 mM Ca_o^{2+} using eqn (4) (see Methods). All neurones to which protons or capsaicin were applied in the absence of extracellular Ca^{2+} were afterwards stimulated in the presence of Ca^{2+}; all cells then responded with a decrease in F_{380}. Number of experiments are given in parentheses in *Ac* and *Bc*.

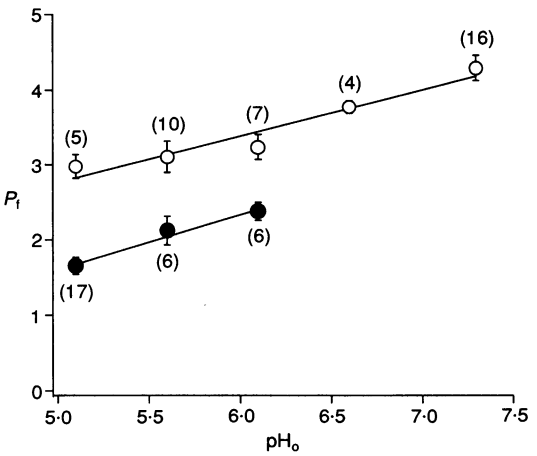

Figure 5. P_f of capsaicin- (○) and slowly inactivating proton- (●) induced currents at different pH_o values

P_f values (means ± S.E.M.) *versus* pH_o. Slowly inactivating proton-induced currents could be elicited by application of acidic solutions of pH 5·1, 5·6 and 6·1. The data points for the capsaicin-induced currents at pH_o 5·1, 5·6 and 6·1 are from cells which exhibited only small proton-induced currents (for details see text). P_f for both stimuli depended approximately linearly on pH_o over the range investigated. For capsaicin- and slowly inactivating proton-induced currents a shift in P_f of 0·68 and 0·73% per pH unit was determined, respectively. The differences between the P_f values for capsaicin- and slowly inactivating proton-induced currents is statistically significant with $p < 0·01$ at pH_o 5·6 and 6·1, and with $p < 0·001$ at pH_o 5·1. Number of experiments in parentheses.

also influences ionic selectivity of these channels, e.g. by surface charge screening effects (see Hille, 1992).

We have therefore investigated the influence of extracellular acidification on P_f values of capsaicin and slowly inactivating proton-induced currents (Fig. 5 and Table 1). P_f of the capsaicin- as well as slowly inactivating proton-induced currents significantly increased with decreasing proton concentrations. Application of acidic solutions of pH 5·1, 5·6 and 6·1 induced similar current responses but P_f of the currents increased from 1·65 ± 0·11% ($n = 17$) at pH_o 5·1

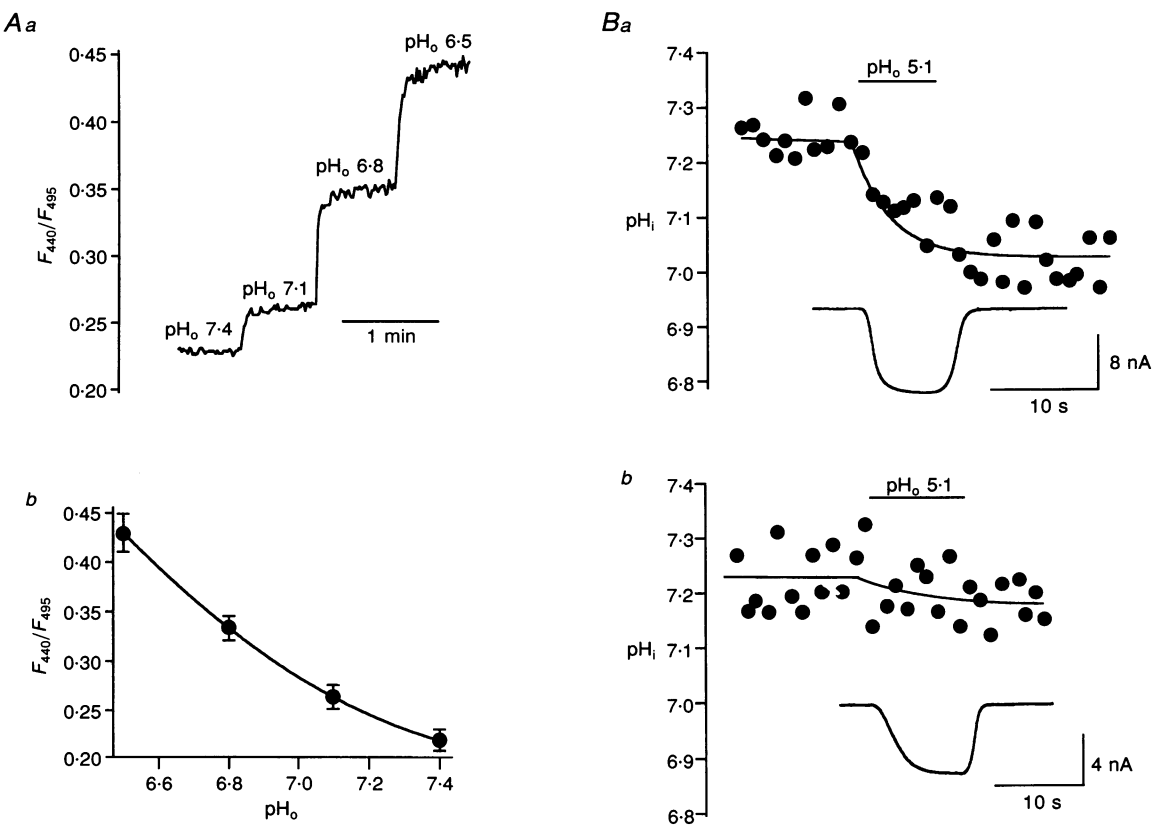

Figure 6. pH_i responses upon extracellular acidification

Changes in pH_i were measured using BCECF. Calibration was performed according to the method described by Thomas *et al.* (1979). High potassium extracellular solutions and nigericine were used to facilitate equilibration of pH_o and pH_i (for details see Methods). F_{440} and F_{495} values were taken at pH_o 7·4, 7·1, 6·8 and 6·5 in four cells. *Aa* shows such an experiment. The resulting calibration curve is shown in *Ab*. The continuous line represents a fit of the data points to the monoexponential function. pH_i responses upon extracellular acidification were recorded in a total of twenty-two DRG neurones. The maximum and an average pH_i response upon extracellular acidification to pH 5·1 are depicted in *Ba* and *Bb*, respectively. The continuous lines represent arbitrary fits to the data points and the insets show the corresponding current responses in these cells.

to 2·12 ± 0·19% ($n = 6$) at pH_o 5·6 and 2·38 ± 0·12% ($n = 6$) at pH_o 6·1. P_f of capsaicin-induced currents behaved similarly. In these experiments only cells with small proton-induced currents (< 10% in amplitude) compared with capsaicin-induced currents were selected. Interestingly, although proton-induced currents were very small in these cells (< 100 pA) extracellular acidification caused a significant potentiation of the capsaicin-induced currents by a factor of about 2–3 at pH 5·6 (see also Petersen & LaMotte, 1993). Application of capsaicin at pH_o 5·1, 5·6 or 6·1 yielded P_f values of 2·98 ± 0·16% ($n = 5$), 3·11 ± 0·21% ($n = 10$) and 3·24 ± 0·17% ($n = 7$) compared with 4·30 ± 0·17% ($n = 16$) at pH 7·3. These findings demonstrate that P_f values for capsaicin as well as proton-induced currents were indeed dependent on pH_o. However, the differences in P_f values of capsaicin and proton-induced currents were statistically significant ($p < 0·001$ at pH_o 5·1 and $p < 0·01$ at pH_o 5·6 and 6·1), when obtained in the presence of the same pH_o. This supports the conclusion that capsaicin- and slowly inactivating proton-induced currents were flowing through different ion channels. Table 1 summarizes P_f and P_{Ca}/P_M values under the various experimental conditions.

Since Ca^{2+} binding to fura-2 can be affected by intracellular acidification (White, Doeller, Verselis & Wittenberg, 1990), the shift in P_f might be explained by a decrease in the pH_i following application of acidic extracellular solutions. To rule out this possibility we monitored pH_i using the pH indicator dye BCECF. pH_i, which was buffered with 10 mM Hepes in all our experiments, remained remarkably constant when cells were exposed to acidic extracellular solutions. In seven cells loaded with BCECF (100 μM) the maximum decrease in pH_i was 0·25 pH units during application of pH_o 5·1 for 10 s, which was twice as long as in most of the Ca^{2+} flux measurements. The mean decrease in pH_i was 0·10 ± 0·02 ($n = 15$) and 0·08 ± 0·03 ($n = 7$), for pH_o 5·1 and 6·1, respectively. Figure 6 shows the maximum and a typical pH_i response upon exposure to pH_o 5·1 and the corresponding calibration curve (for details see Methods). This intracellular acidification remained well below the value, where protons significantly interfere with Ca^{2+} binding to fura-2 (Grynkiewicz *et al.* 1985; Lattanzio, 1990). From these findings it is likely that the observed dependence of P_f values on pH_o is due to a proton-mediated effect on the ion channels rather than on the Ca^{2+} indicator used.

DISCUSSION

By simultaneously measuring whole-cell currents and Ca^{2+} fluxes, we have shown that the ion channels underlying capsaicin- and slowly inactivating proton-induced currents in rat DRG neurones are permeable to Ca^{2+}. However, the fractional Ca^{2+} currents were significantly different for the two current types. Using a modification of the GHK equation (Schneggenburger *et al.* 1993) P_{Ca}/P_M could be calculated from these values. If we assume a constant electrical field across the membrane and that the ion channels underlying slowly inactivating proton- and capsaicin-induced currents discriminate only very slightly between different monovalent cations (Bevan & Yeats, 1991; Oh *et al.* 1996) these P_f values correspond to permeability ratios of 1·48 for capsaicin-induced currents (at pH_o 7·3) and of 0·55 for slowly inactivating proton-induced currents (at pH_o 5·1).

Alternatively, permeability ratios can be determined from reversal potential measurements. These measurements, however, require very high concentrations of Ca^{2+} (about 100 mM), which may alter ionic selectivity, e.g. by screening surface charges. Indeed, significant differences between permeability ratios determined from reversal potentials and from fractional Ca^{2+} currents have been reported previously (Burnashev, Zhou, Neher & Sakmann, 1995; Frings, Seifert, Godde & Kaupp, 1995). P_{Ca}/P_M values for capsaicin-induced currents given in the literature range from 0·24 (Oh *et al.* 1996) to 4·0 (Koplas *et al.* 1995). For slowly inactivating proton-induced currents a relative permeability of Ca^{2+} *versus* Cs^+ of 0·40 has been determined from reversal potential measurements (Zeilhofer *et al.* 1996).

Fractional Ca^{2+} currents reveal diversity of capsaicin- and proton-induced ion channels

The similarities of capsaicin- and slowly inactivating proton-induced currents led to the suggestion that capsaicin and protons activate the same ion channels (e.g. Bevan & Geppetti, 1994). In this case our finding that capsaicin- and slowly inactivating proton-induced currents differ significantly in their P_f values can be explained if one assumes that protons not only induce channel opening, but also affect the permeability of the channel to Ca^{2+}. Indeed with decreasing extracellular acidity an increase in P_f was observed for both slowly inactivating proton- and capsaicin-induced currents (Fig. 5). A possible explanation for this observation is the surface charge screening effect of protons. By neutralizing negative charges near the channel mouth protons may change the relative concentration of divalent and monovalent cations at the extracellular side of the ion channel (Hille, 1992) and thereby affect P_f. However, when capsaicin- and proton-induced currents were evoked at the same pH_o P_f values of slowly inactivating capsaicin- and proton-induced currents were still significantly different. This challenges the hypothesis that protons and capsaicin activate the same population of ion channels and favours the possibility that capsaicin and protons activate distinct ion channel populations with capsaicin-activated ion channels being significantly more permeable to Ca^{2+} than proton-activated channels. These findings are in line with the observation that capsaicin-induced ionic currents inactivate/desensitize (Koplas *et al.* 1995), whereas slowly inactivating proton-induced currents show little inactivation even with long pH stimuli. Inactivation of capsaicin-induced currents seems to depend on the external Ca^{2+} concentration and may be mediated by the substantial Ca^{2+} influx through capsaicin-sensitive channels (Koplas *et al.* 1995; Docherty, Yeats, Bevan & Boddeke, 1996; see also Cholewinski *et al.* 1993).

The possibility exists that more than two channel types may contribute to capsaicin- and slowly inactivating proton-induced currents and that these ion channel populations may somehow overlap. In this case, however, the relative expression of the different channel types must be very constant in the neurones investigated, since P_f values scattered only very little around the respective average value. Unfortunately, there are so far no specific ion channel blockers available for either slowly inactivating proton-induced or capsaicin-induced channels. Moreover, because of the known potentiating effect of protons on capsaicin-activated currents (Petersen & LaMotte, 1993) occlusion experiments, in which one agonist is applied in the continuous presence of the other one, are not applicable.

Functional implications of the Ca^{2+} influx through capsaicin- and proton-induced ion channels

The fractional Ca^{2+} current through capsaicin-activated channels (~4·3%) is comparable to that reported for native NMDA receptor channels e.g. in forebrain neurones of the medial septum (~6·8%; Schneggenburger *et al.* 1993). Important functional consequences of the Ca^{2+} influx through these ion channels may include Ca^{2+}-dependent inactivation of capsaicin-induced currents in rat DRG neurones (Cholewinski *et al.* 1993) and the release of neuropeptides from sensory nerve endings (Santicioli, del Bianco, Figini, Bevan & Maggi, 1993) as well as capsaicin-induced neurotoxic effects (Petsche, Fleischer, Lembeck & Handwerker, 1983). Indeed, the latter two effects depend on the presence of extracellular Ca^{2+} ions and are unaffected or only partially inhibited by blockers of voltage-activated Ca^{2+} channels (Santicioli *et al.* 1993). Furthermore, intracellular events induced by capsaicin like translocation of protein kinase C (Harvey, Davis, James & Burgess, 1995), increased expression of nitric oxide (NO) synthase (Vizzard, Erdmann & deGroat, 1995) and Ca^{2+}-dependent NO-mediated stimulation of cyclic GMP production in rat dorsal root ganglia (Bauer, Simmons, Murphy & Gebhart, 1993) are likely to be triggered by Ca^{2+} entering the cell through capsaicin-induced ion channels. NO can be algogenic by itself (Kress, Riedl, Rödl & Reeh, 1994) and production of NO and activation of protein kinase C have been related to the induction of sensitization to heat and mechanical stimuli (Schepelmann, Messlinger & Schmidt, 1993; Kress *et al.* 1994). These processes might therefore be closely related to the well-known sensitizing action of capsaicin to heat in polymodal nociceptive nerve fibres (e.g. Szolcsányi, 1987).

Comparable investigations with acidic solutions are not available. However, sensitizing effects of low pH_o in nociceptive nerve fibres have been described in experimental acidosis (Steen, Reeh, Anton & Handwerker, 1992) and Ca^{2+} influx through slowly inactivating proton-induced currents may also contribute to the sensitization of nociceptive afferents during inflammatory and ischaemic disease states, which can cause substantial tissue acidosis (Häbler, 1929; Jacobus, Taylor, Hollis & Nunnally, 1977).

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