Dual effect of external Ca^{2+} on noradrenaline-activated cation current in rabbit portal vein smooth muscle cells

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1. The influence of divalent cations was investigated on the noradrenaline-activated non-selective cation current (I_{cat}) in freshly dispersed smooth muscle cells of rabbit portal vein.

2. Reduction of external calcium concentration ($[Ca^{2+}]_o$) from 1·5 mM to 'test' levels of 100–500 μM during stimulation of I_{cat} produced a sustained potentiation of I_{cat}, which indicates an inhibitory action of Ca_o^{2+}. With 'test' concentrations lower than 100 μM Ca_o^{2+} the amplitude of I_{cat} was initially potentiated and then the current subsequently declined. Under these conditions re-addition of 1·5 mM Ca_o^{2+} transiently activated I_{cat} which demonstrates an additional facilitatory action of $[Ca^{2+}]_o$. The half-maximal $[Ca^{2+}]_o$ for the facilitatory and inhibitory action on I_{cat} was 6 and 400 μM, respectively.

3. When $[Ca^{2+}]_o$ was decreased to very low levels (< 10 nM) by inclusion of 1 mM BAPTA in nominally Ca^{2+}-free external solution, I_{cat} could still be evoked by noradrenaline. This suggested that Ca^{2+} is not obligatory for activation of I_{cat} but merely modulatory.

4. In 1·5 mM Ca_o^{2+} the current–voltage (I–V) relationship of I_{cat} was S-shaped with a reversal potential (E_r) of about +9 mV. The I–V characteristics and E_r of the potentiated current in 200 μM Ca_o^{2+} were not markedly different from those in 1·5 mM Ca_o^{2+}.

5. During the noradrenaline-induced I_{cat}, removal of external Mg^{2+} caused a small increase in the amplitude of I_{cat} which was not as great as that seen on reduction of $[Ca^{2+}]_o$ and there was no facilitatory effect of Mg_o^{2+}.

6. These results indicate that external Ca^{2+} has a dual action on I_{cat}, both facilitating and inhibiting the current. This dual action is not shared with external Mg^{2+}.

Conventional whole-cell recording with patch pipettes has provided evidence that noradrenaline evokes three types of membrane current in rabbit portal vein cells. These are calcium-activated potassium ($I_{K(Ca)}$) and chloride ($I_{Cl(Ca)}$) currents and a non-selective cation current (I_{cat}; Byrne & Large, 1988). It has been suggested that $I_{Cl(Ca)}$ and I_{cat} may produce depolarization in vascular smooth muscle and hence have an important physiological role (Amédée & Large, 1989). I_{cat} is carried principally by monovalent cations but since this conductance also has an appreciable permeability to divalent cations (Byrne & Large, 1988; Wang & Large, 1991; Inoue & Kuriyama, 1993) it is possible that I_{cat} may carry sufficient Ca^{2+} into the cell to produce contraction directly. It was observed that agents, such as caffeine and ionomycin, which are thought to increase the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$), did not evoke I_{cat} even in cells where caffeine induced $I_{Cl(Ca)}$ and noradrenaline activated I_{cat} (Byrne & Large, 1988; Wang & Large, 1991). Therefore it was concluded that I_{cat} was not triggered by a rise in $[Ca^{2+}]_i$ but linked more directly to the α_1-adrenoceptor. However, it was shown that removal of external calcium (Ca_o^{2+}), and addition of EGTA, initially potentiated I_{cat} in some cells and then after prolonged (10 min) exposure to Ca^{2+}-free conditions I_{cat} was blocked (Wang & Large, 1991). These data indicate that Ca_o^{2+} has a modulatory role on I_{cat} and the present experiments were undertaken to investigate this property more rigorously. Experiments will be described that show that Ca_o^{2+} has both a facilitatory and inhibitory action on I_{cat}.

METHODS

New Zealand White rabbits (2–2·5 kg) were killed by I.V. overdose of sodium pentobarbitone (120 mg kg^{-1}). The portal vein was removed and then dissected free of connective tissue and fat in normal physiological external solution (for composition see below) before being cut into strips and washed in nominally 'Ca^{2+}-free' external solution (for composition see below) for 10 min. The tissue was placed in 0·2–0·4 mg ml^{-1} protease type I or type XIV (in physiological saline) for 5 min, washed again and then placed in 0·5–1 mg ml^{-1} collagenase type XI (in physiological saline) for 10 min. All the enzyme and wash steps were performed at 37 °C in nominally Ca^{2+}-free external solution. The tissue then received a final wash before being mechanically agitated using a wide-bore

Pasteur pipette to release the cells. Cells were harvested in nominally Ca^{2+}-free external solution, centrifuged (100 *g*) to form a loose pellet and resuspended in 0·75 mM Ca_o^{2+}. The cells were plated onto glass coverslips and stored at 4 °C before use (1–8 h). The normal physiological solution contained (mM): NaCl, 126; KCl, 6; $CaCl_2$, 1·5; $MgCl_2$, 1·2; glucose, 10; Hepes, 11; pH was adjusted to 7·2 with 3 M NaOH. Nominally Ca^{2+}-free and 0·75 mM calcium external solution had the same composition except that Ca^{2+} was omitted and 0·75 mM $CaCl_2$ was added, respectively.

For electrophysiogical recording the cells were placed in the main bath (vol. *ca* 150–200 μl) and continuously perfused with solution by gravity feed at the rate of 5 ml min^{-1} from a number of reservoirs. These reservoirs could be switched manually to allow addition/removal of different solutions as required. Cell membrane currents were recorded with a List EPC-7 patch-clamp amplifier (List Electronic, Darmstadt, Germany) at room temperature (20–25 °C) using the standard whole-cell recording configuration. Patch pipettes were fabricated from borosilicate glass and had resistances of approximately 6 MΩ. The headstage ground was connected to an Ag–AgCl pellet which was placed in a side bath containing the pipette solution. This was then connected to the main bath via an agar bridge containing 150 mM KCl. The junction potential between the pipette solution and the external solution was assessed by measuring the change in voltage on replacement of the standard external solution in the bath with the pipette solution. This was found to be <3 mV and was not compensated in the final records. In the whole-cell recording configuration, series resistance (R_s) was calculated from $R_s = \tau/C_m$, where τ (time constant) was measured by fitting exponentials to the decay of the capacity current transient in response to 10 mV hyperpolarizing voltage clamp steps (V) from a holding potential (V_h) of −50 mV. C_m (membrane capacitance) was calculated by integrating the capacity current transient (q) and applying $C_m = q/V$. This procedure was routinely performed using the compensation controls, C-slow and G-series on the patch clamp amplifier so that series resistance compensation could be used. Uncompensated R_s was between 12 and 16 MΩ and in all situations at least 50% could be compensated. The voltage protocol used to evaluate the current–voltage (I–V) characteristics of the noradrenaline-evoked cation current involved clamping the membrane at $V_h = -50$ mV and then stepping to −120 mV for 50 ms before imposing a voltage ramp (0·3 V s^{-1}) to +40 mV. Leak currents were subtracted by applying a number of voltage ramps prior to activation of I_{cat} and subtracting the mean current from ramps applied during I_{cat}. It appeared that this relatively slow ramp approximated to the steady-state current since the current measured at a V_h of −50 mV immediately prior to a voltage ramp was the same as that recorded within the voltage ramp protocol at −50 mV. This was further substantiated by the identical I–V relationships produced for I_{cat} when voltage ramp current was compared with the current measured at the end of 100 ms voltage steps (R. M. Helliwell & W. A. Large, unpublished data). The voltage ramps were generated and the data captured with an IBM-compatable 486DX personal computer (Viglen, Alperton, Middlesex, UK) using a CED 1401 interface and software (Cambridge Electronic Design Ltd, Cambridge, UK). Data were filtered at 1 kHz and sampled at 5 kHz. Long-term records were played back from magnetic tape (Racal, Southampton, UK), filtered at 10 Hz and sampled at 25 Hz using the 1401 and CED Sigavg software. Data were plotted for both analysis and figure preparation using MicroCal Origin software (MicroCal Software Inc, Northampton, MA, USA).

The standard K^+-free external solution used for experimentation typically contained (mM): NaCl, 126; $CaCl_2$, 1·5; glucose, 10; Hepes, 11; pH was adjusted to 7·2 with 3 M NaOH, which added a further 5 mM Na^+. The external solution also contained 1·2 mM Mg^{2+} in some experiments as indicated. In the initial experiments where the effect of the external Ca^{2+} concentration ($[Ca^{2+}]_o$) was examined, Ca^{2+} was absent from the external solution (nominally Ca^{2+} free) and in some cases 1 mM BAPTA was added to nominally Ca^{2+}-free external solution to reduce $[Ca^{2+}]_o$ to <10 nM. This $[Ca^{2+}]$ was predicted using EQCAL software (Biosoft, Ferguson, MO, USA) where the assumed $[Ca^{2+}]$ in nominally Ca^{2+}-free solutions was taken to be approximately 50 μM. In later experiments $[Ca^{2+}]_o$ was buffered to concentrations in the range <10 nM to 500 μM by adding the appropriate amount of $CaCl_2$ to standard K^+-free external solutions where 1 mM BAPTA was added. In these solutions the concentration of NaOH required to change the pH to 7·2 added a further 8 mM Na^+ to the external solution. Nicardipine (3–5 μM) was also present in external solutions to block voltage-dependent calcium currents activated by depolarizing ramps. The standard internal pipette solution (CsCl–caesium aspartate) contained (mM): CsCl, 18; caesium aspartate, 108; $MgCl_2$, 1·2; Hepes, 10; glucose, 11; BAPTA, 10; $CaCl_2$, 1 (free $[Ca^{2+}]$ predicted by EQCAL was approximately 14 nM); pH was adjusted to 7·2 using Trizma base.

All drugs and enzymes were obtained from Sigma.

Values given in the text are means ± S.E.M. and the test for significance was Student's t test.

RESULTS

Isolation and characterization of the noradrenaline-activated cation current (I_{cat})

Noradrenaline activates a number of conductances in vascular smooth muscle (Byrne & Large, 1988; Amédée, Benham, Bolton, Byrne & Large, 1990) and, therefore, to examine the properties of noradrenaline-induced I_{cat} in isolation the following procedures were employed. The use of a pipette solution containing CsCl, rather than KCl, and removal of K^+ from the extracellular solution appeared to block all K^+ conductances. To prevent activation of calcium-activated conductances via Ca^{2+} release from the sarcoplasmic reticulum it was also necessary to buffer $[Ca^{2+}]_i$ at relatively low levels. This point is illustrated in Fig. 1*A* where it can be seen that with low concentrations of BAPTA (0·1 mM) in the pipette solution noradrenaline (100 μM) evoked a fast transient inward current followed by a 'noisy' sustained current (left-hand trace). With a higher concentration of BAPTA (10 mM) the rapid component was blocked leaving only the 'noisy' but relatively sustained inward current (Fig. 1*A*, right-hand trace). The fast component has been characterized extensively and has been shown to be a calcium-activated chloride conductance, whereas the slow 'noisy' current is I_{cat} (Byrne & Large, 1988; Inoue & Kuriyama, 1993). As illustrated in Fig. 1*B* a low concentration of noradrenaline (1 μM) evoked I_{cat} but since these currents were generally too small for analysis, a maximal concentration of 100 μM noradrenaline, which produced significantly larger responses (Fig. 1*B*), was used throughout this study.

In preliminary experiments we investigated the influence of the $[Ca^{2+}]$ of the pipette solution on the amplitude of I_{cat}. It was found that the amplitude of I_{cat} was increased if the pipette $[Ca^{2+}]$, which presumably approximates to $[Ca^{2+}]_i$, was buffered to relatively low concentrations (14 nM) compared with higher concentrations (100 nM, Fig. 2). Thus, comparing different cells from the same population, the amplitude of the responses produced using 14 nM Ca_i^{2+}, 21 ± 5 pA ($n = 7$), tended to be larger than those using 100 nM Ca_i^{2+}, 10 ± 3 pA ($n = 7$), although this difference was not statistically significant. Furthermore the amplitude of I_{cat} was more sustained with low $[Ca^{2+}]_i$ as after 6 min in noradrenaline the amplitude was significantly greater using 14 nM Ca_i^{2+} than using 100 nM Ca_i^{2+}: $5 \pm 1{\cdot}2$ and $1 \pm 0{\cdot}9$ pA ($P < 0{\cdot}05$), respectively. Therefore the pipette solution contained 14 nM Ca^{2+} for the remainder of the study.

Figure 3 illustrates the current–voltage relationship (I–V) of I_{cat} using slow voltage ramps as described in Methods. With a CsCl–caesium aspartate pipette solution in which the chloride equilibrium potential (E_{Cl}) was about −50 mV, I_{cat} displayed pronounced rectification (Fig. 3*A a*). Changing E_{Cl} to 0 mV using a CsCl pipette solution (no aspartate) produced similar I–V characteristics and reversal potential (Fig. 3*A a* and *A b*). This is evidence that Cl^- did not contribute to the noradrenaline-activated current at any voltage and that the calcium-activated chloride current had been sufficiently suppressed. Completely replacing Cs^+ with Na^+ in the pipette solution also had no effect on the I–V characteristics and the reversal potential (Fig. 3*A b* and *A c*) suggesting similar permeabilities for Na^+ and Cs^+. Figure 3*B* shows pooled data on the effect of voltage on noradrenaline-evoked I_{cat} using subtracted ramps ($n = 7$) with a pipette solution containing a mixture of CsCl and caesium aspartate (E_{Cl}, *ca* −50 mV) which was used in all subsequent experiments. The current in Fig. 3*B* was measured directly from the ramp data at 20 mV intervals from −120 to +40 mV and normalized in each case to the current evoked at −40 mV. At voltages between −40 mV and the reversal potential (E_r) the I–V was essentially linear but exhibited strong inward rectification at all voltages positive to E_r. Since very little current flowed in the outward direction, estimation of E_r was difficult. The E_r of the noradrenaline-evoked I_{cat} was $+9 \pm 1$ mV ($n = 12$), which is similar to the values reported previously for this conductance recorded under similar conditions (Wang & Large, 1991, Inoue & Kuriyama, 1993). At voltages negative to −40 mV the current became increasingly 'noisy' and deviated from linearity such that the rectifying properties of I_{cat} produced an S-shaped I–V relationship (Fig. 3). In other experiments I–V curves were constructed from 100 ms voltage steps applied to test potentials from $V_h = -50$ mV.

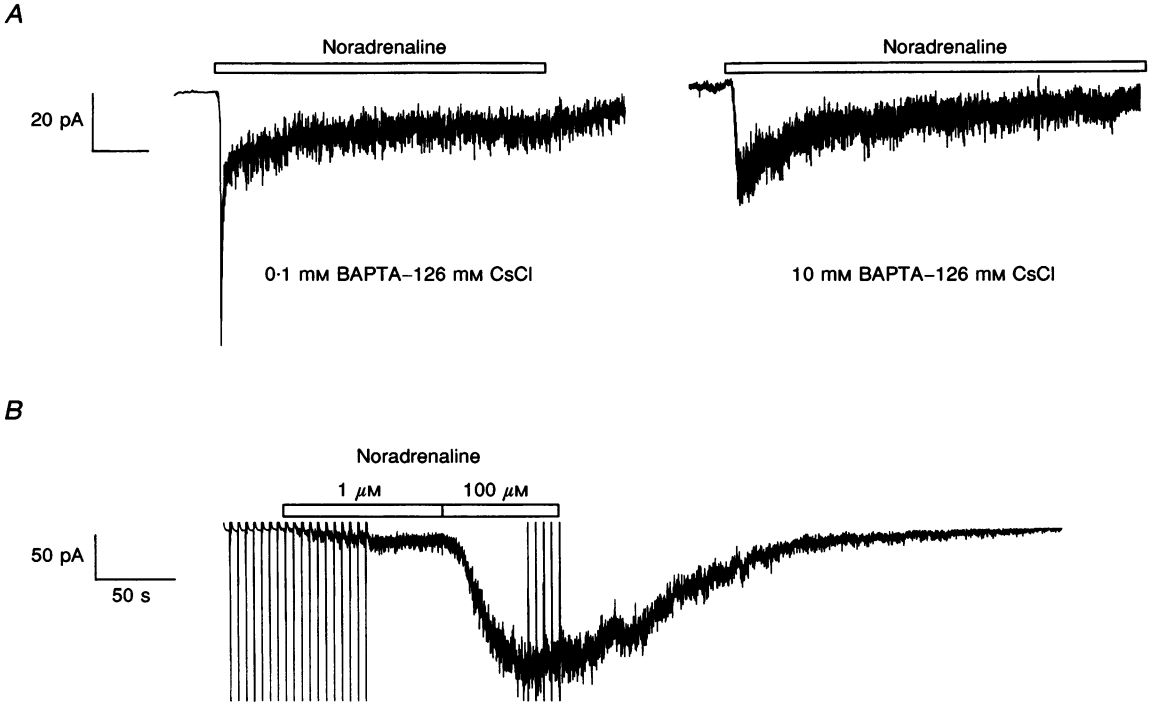

Figure 1. The effect of different buffered $[Ca^{2+}]_i$ on noradrenaline-evoked inward current

A, the pipette solution contained either 0·1 mM (left-hand trace) and 10 mM (right-hand trace) BAPTA in the pipette solution. Noradrenaline (100 μM) was applied for the period denoted by the horizontal bars and the time calibration is 15 s for the left-hand trace and 50 s for the right-hand trace. *B*, the activation of I_{cat} using 1 and 100 μM bath-applied noradrenaline. These experiments were performed in the standard K^+-free external solution containing 1·2 mM Mg^{2+} and V_h was −50 mV. The vertical spikes are indicative of voltage ramps used to construct I–V curves.

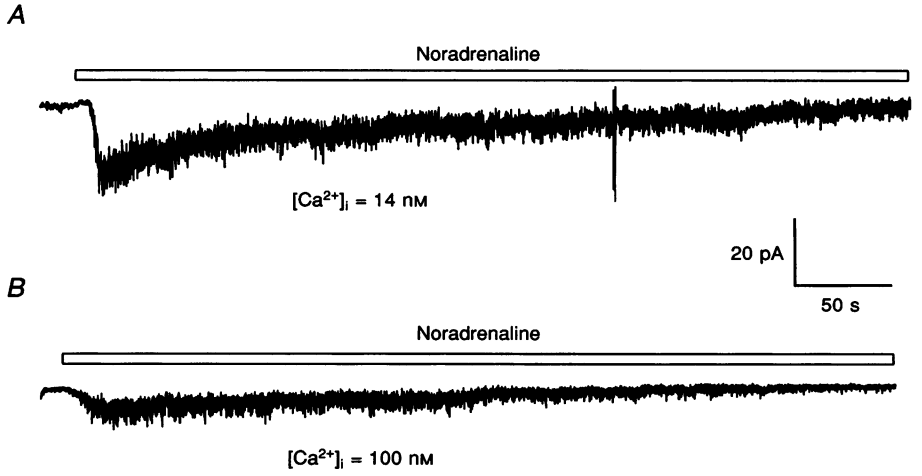

Figure 2. The effect of different buffered $[Ca^{2+}]_i$, using 10 mM BAPTA, on the amplitude of I_{cat}
The predicted free $[Ca^{2+}]_i$ was buffered to 14 nM (*A*) and to 100 nM (*B*) in 2 different cells. Noradrenaline (100 μM) was bath applied for the period denoted by the horizontal bars. The external solution was standard K^+-free solution containing 1·2 mM Mg^{2+} and V_h was −50 mV.

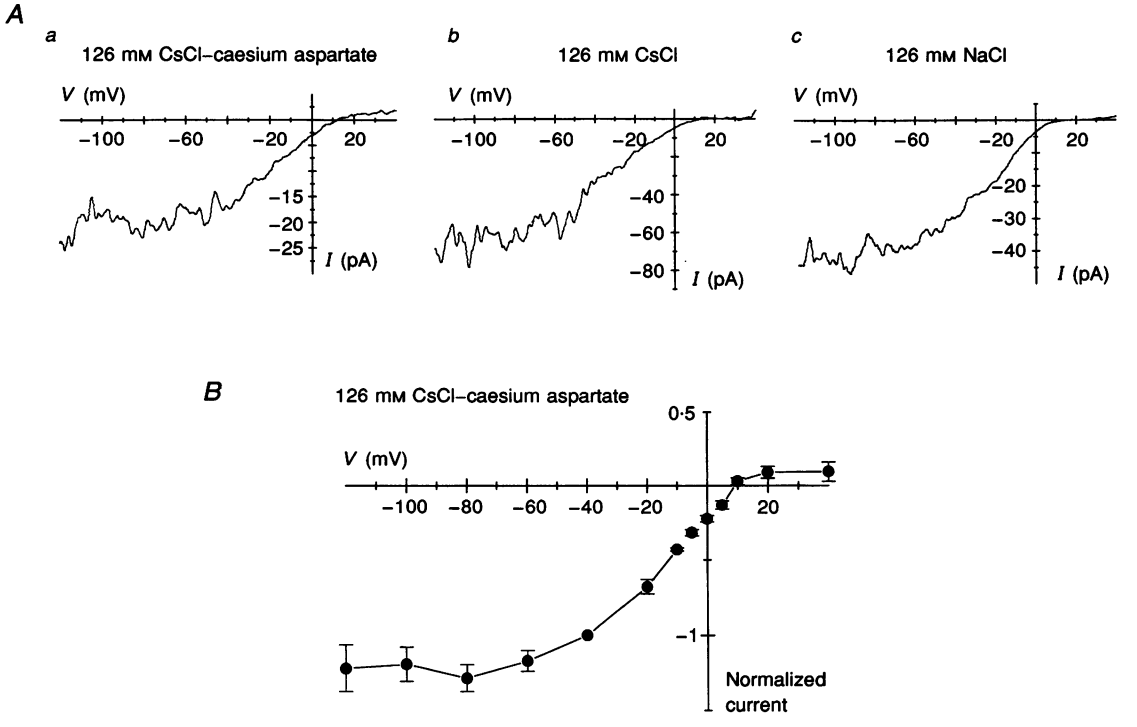

Figure 3. The effect of monovalent ion composition of the pipette solution on the current–voltage relationship of I_{cat} investigated using voltage ramps
A a, pipette solution 126 mM CsCl–caesium aspartate, $E_{Cl} = -50$ mV; *b*, pipette solution 126 mM CsCl, $E_{Cl} = 0$ mV; *c*, pipette solution 126 mM NaCl, $E_{Cl} = 0$ mV. *B*, pooled *I–V* of I_{cat} from 7 cells using the standard CsCl–caesium aspartate pipette solution ($E_{Cl} \approx -50$ mV), where current was normalized to the current recorded at −40 mV. The external solution was standard K^+-free solution containing 1·2 mM Mg^{2+} and V_h was −50 mV.

The $I-V$ curves obtained using this protocol showed similar characteristics to those obtained with the ramp experiments. Moreover, following the instantaneous command pulses to the test potentials there were no obvious current relaxations that could account for the non-linear $I-V$ relationship.

Effect of $[Ca^{2+}]_o$ on I_{cat}

The main purpose of these experiments was to investigate the effect of $[Ca^{2+}]_o$ on I_{cat}. The initial experiments contained 1·2 mM Mg^{2+} in the bathing solution and simply involved the removal of Ca_o^{2+} during I_{cat} evoked by noradrenaline. Figure 4*A* shows a typical experiment where I_{cat} was first evoked by noradrenaline in an external solution containing 1·5 mM Ca_o^{2+}. The 'noisy' current reached a peak amplitude of 35 ± 4 pA ($n = 7$) and then slowly decayed (half-decay = 64 ± 8 s) to an amplitude of 22 ± 3 pA. Then Ca^{2+} was removed from the bathing solution in the continued presence of noradrenaline (nominally Ca_o^{2+} free, '0 Ca^{2+}'), and on its removal I_{cat} was initially potentiated to an amplitude of 72 ± 11 pA but then decayed much more rapidly (half-decay in 0 Ca_o^{2+} was 26 ± 4 s, $n = 7$) than in 1·5 mM Ca_o^{2+} (64 ± 8 s, see above) until very little evoked current remained (Fig. 4*A*). On re-addition of 1·5 mM Ca_o^{2+} there was a small and transient activation of I_{cat} (Fig. 4*A*). It was suspected that micromolar amounts of Ca^{2+} were present in the nominally Ca^{2+}-free solution and, therefore, identical experiments were carried out but adding 1 mM BAPTA to the bathing solution to reduce $[Ca^{2+}]_o$ to <10 nM. When this solution was applied to the cell during stimulation of I_{cat} the current was first potentiated (Fig. 4*B*) and then rapidly decayed, i.e. qualitatively similar to that seen in 0 Ca_o^{2+}. The decay of I_{cat} in <10 nM Ca_o^{2+} was much more rapid (half-decay, $1·9 \pm 0·2$ s, $n = 4$) than the decay in 1·5 mM Ca_o^{2+} solution (half-decay, 60 ± 10 s) and, furthermore, decayed more rapidly than in nominally Ca^{2+}-free (half-decay, 26 s, see above). In the cell illustrated in Fig. 4*B* the current decayed in <10 nM Ca_o^{2+} to a reduced steady-state level and was accompanied by abolition of the membrane 'noise' normally associated with this conductance. This small residual component appeared to represent a non-specific leak change of the membrane due to the change from 1·5 mM to <10 nM Ca_o^{2+} as assessed by carrying out

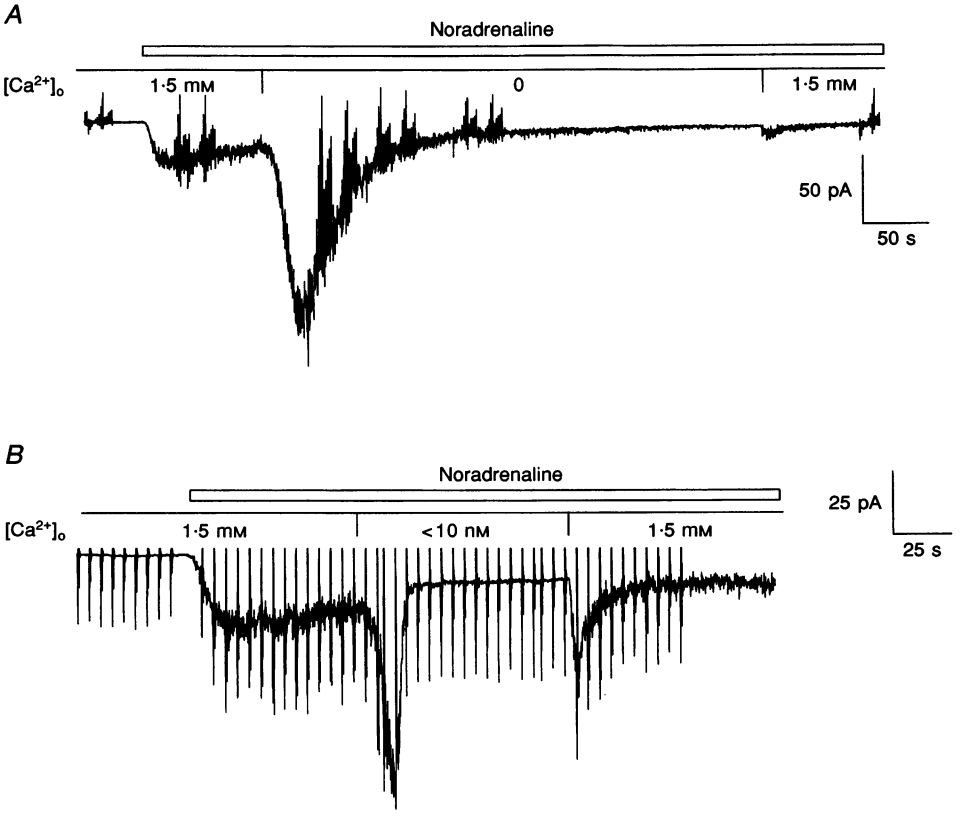

Figure 4. The effect of $[Ca^{2+}]_o$ on noradrenaline-evoked I_{cat} activated in 1·5 mM Ca^{2+}

A, I_{cat} was first activated in 1·5 mM Ca_o^{2+} and then subsequently exposed to nominally Ca_o^{2+}-free solution (0 Ca^{2+}). Once the potentiated current in 0 Ca^{2+} had decayed, 1·5 mM Ca_o^{2+} was reapplied. *B*, similar experiment in another cell except in this case 1 mM BAPTA was added to the 0 Ca^{2+} solution to reduce $[Ca^{2+}]_o$ to <10 nM. Noradrenaline (100 μM) was bath applied for the period denoted by the horizontal bars. The external solution was standard K^+-free solution containing 1·2 mM Mg^{2+} and V_h was −50 mV.

this solution change in the absence of noradrenaline. Thus I_{cat} had apparently been suppressed completely in external solutions with very low $[Ca^{2+}]_o$ (7 out of 7 cells). However, this complete suppression of I_{cat} after the initial potentiation on reduction of $[Ca^{2+}]_o$ did not occur when similar experiments were performed in Mg^{2+}-free external solutions. Furthermore, in external solutions where all divalent cations had been removed it was still possible to activate I_{cat} with noradrenaline (see later). On re-addition of 1·5 mM Ca_o^{2+} the current was again transiently activated and associated with the return of current 'noise' typical of this conductance (Fig. 4*B*). Therefore, removal of Ca_o^{2+} during noradrenaline-evoked I_{cat} first potentiated the current, although this potentiated current was not sustained, and then decayed more rapidly than in 1·5 mM Ca_o^{2+}. These results suggest Ca_o^{2+} may have an inhibitory and facilitatory action on I_{cat}.

These apparently paradoxical effects of $[Ca^{2+}]_o$ on I_{cat} are illustrated more clearly in Fig. 5. As above, 1·2 mM Mg_o^{2+} was present throughout and in these experiments I_{cat} was initially activated by noradrenaline in nominally Ca^{2+}-free external solution. On addition of 1·5 mM Ca_o^{2+} in the presence of noradrenaline there was a transient potentiation of the current and on subsequent removal of Ca_o^{2+} (nominally Ca^{2+}-free solution) a transient potentiation was again observed (Fig. 5). These effects were reproducible for as long as there was sufficient noradrenaline-activated current ($n = 4$). From these initial observations it appeared that Ca_o^{2+} had two effects. The initial effect on reducing $[Ca^{2+}]_o$, which potentiated I_{cat} (e.g. Fig. 4*A*), can be described as an inhibitory action of Ca^{2+} since the 'blocking effect' of this cation was relieved on its removal from the bathing solution. However, the potentiation was short lived and the current decayed much more rapidly in low $[Ca^{2+}]_o$ than in 1·5 mM Ca_o^{2+}. This suggests that Ca_o^{2+} is necessary to maintain I_{cat} and, therefore, this effect can be described as a facilitatory action of Ca_o^{2+}. Similarly re-addition of 1·5 mM Ca_o^{2+} after I_{cat} had decreased in low-Ca^{2+} solution led to transient activation of the current, which is also indicative of the facilitatory effect of Ca_o^{2+}. Qualitatively similar effects resulting from a reduction in $[Ca^{2+}]_o$ were also observed when 0·1 μM (rather than 100 μM) noradrenaline was used to evoke I_{cat}.

Quantitative relationship between $[Ca^{2+}]_o$ and the inhibitory and facilitatory effects of Ca_o^{2+} on I_{cat}

The following experiments were undertaken to establish whether these two effects, inhibitory and facilitatory, could be quantified in terms of a different concentration dependence on Ca_o^{2+}. In these experiments Mg^{2+} was not added to the bathing solution so that Ca^{2+} was the only extracellular divalent cation present. The procedure involved clamping the cell at −50 mV in 1·5 mM Ca^{2+}-containing bathing solution and then switching to various Ca_o^{2+} concentrations under test ('test' $[Ca^{2+}]_o$), before switching back again to 1·5 mM Ca_o^{2+} in the absence of noradrenaline to establish the extent of any non-specific leak changes produced by different Ca^{2+} concentrations (see Fig. 6). Noradrenaline was then applied at the bars and I_{cat} was evoked in 1·5 mM Ca_o^{2+} (Fig. 6) and once a reasonably stable response was achieved $[Ca^{2+}]_o$ was changed to 'test' concentrations ranging from 500 μM to <10 nM (Fig. 6). As illustrated in Fig. 6*A*, reducing $[Ca^{2+}]_o$ from 1·5 mM to 500 μM during activation of I_{cat} produced a small relatively sustained potentiation of the current but on return to 1·5 mM Ca_o^{2+} I_{cat} simply declined to approximately the control level in 1·5 mM Ca_o^{2+}. Changing from 1·5 mM to 200 μM Ca_o^{2+} produced a greater potentiation of I_{cat} but similarly on return to 1·5 mM Ca_o^{2+} I_{cat} decreased to the control value (Fig. 6*B*). Consequently at these concentrations only the inhibitory effect of Ca_o^{2+} was revealed. However when the 'test' $[Ca^{2+}]_o$ was reduced to 10 μM or less during I_{cat} another component of the response was seen (Fig. 6*C–E*). As above, a similar initial potentiation of the current was observed on reduction of $[Ca^{2+}]_o$ from 1·5 mM

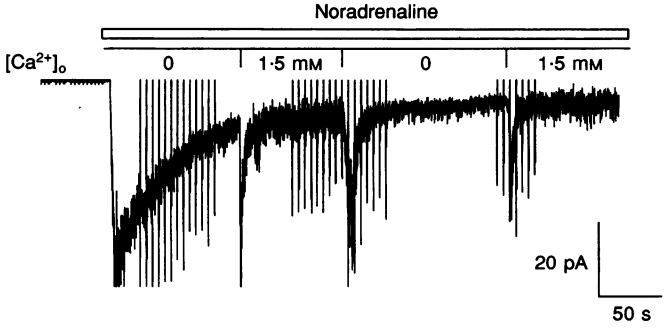

Figure 5. The effect of $[Ca^{2+}]_o$ on noradrenaline-evoked I_{cat} activated in nominally Ca^{2+}-free external solution

I_{cat} was first activated in nominally Ca^{2+}-free external solution (0 Ca^{2+}) and then 1·5 mM Ca_o^{2+} was added as indicated. Two further applications of 0 Ca_o^{2+} and 1·5 mM Ca_o^{2+} are also illustrated. Noradrenaline (100 μM) was bath applied for the period denoted by the horizontal bar. The external solution was standard K^+-free solution containing 1·2 mM Mg^{2+} and V_h was −50 mV. Note that I_{cat} was transiently potentiated whether in the presence or absence of 1·5 mM Ca_o^{2+}.

to 10 μM but on return to 1·5 mM Ca_o^{2+} there was a transient increase of the current (Fig. 6*C*, arrow), which was not seen with 500 and 200 μM Ca_o^{2+}, before I_{cat} declined to control levels. This transient activation of I_{cat} on re-addition of 1·5 mM Ca_o^{2+} became progressively larger and faster as the 'test' $[Ca^{2+}]_o$ was reduced even further (Fig. 6*D* and *E*, arrows). This represents the facilitatory effect of $[Ca^{2+}]_o$. Furthermore, although the extent of the initial potentiation on changing to the concentration of Ca^{2+} in the 'test' $[Ca^{2+}]_o$ was similar at all concentrations

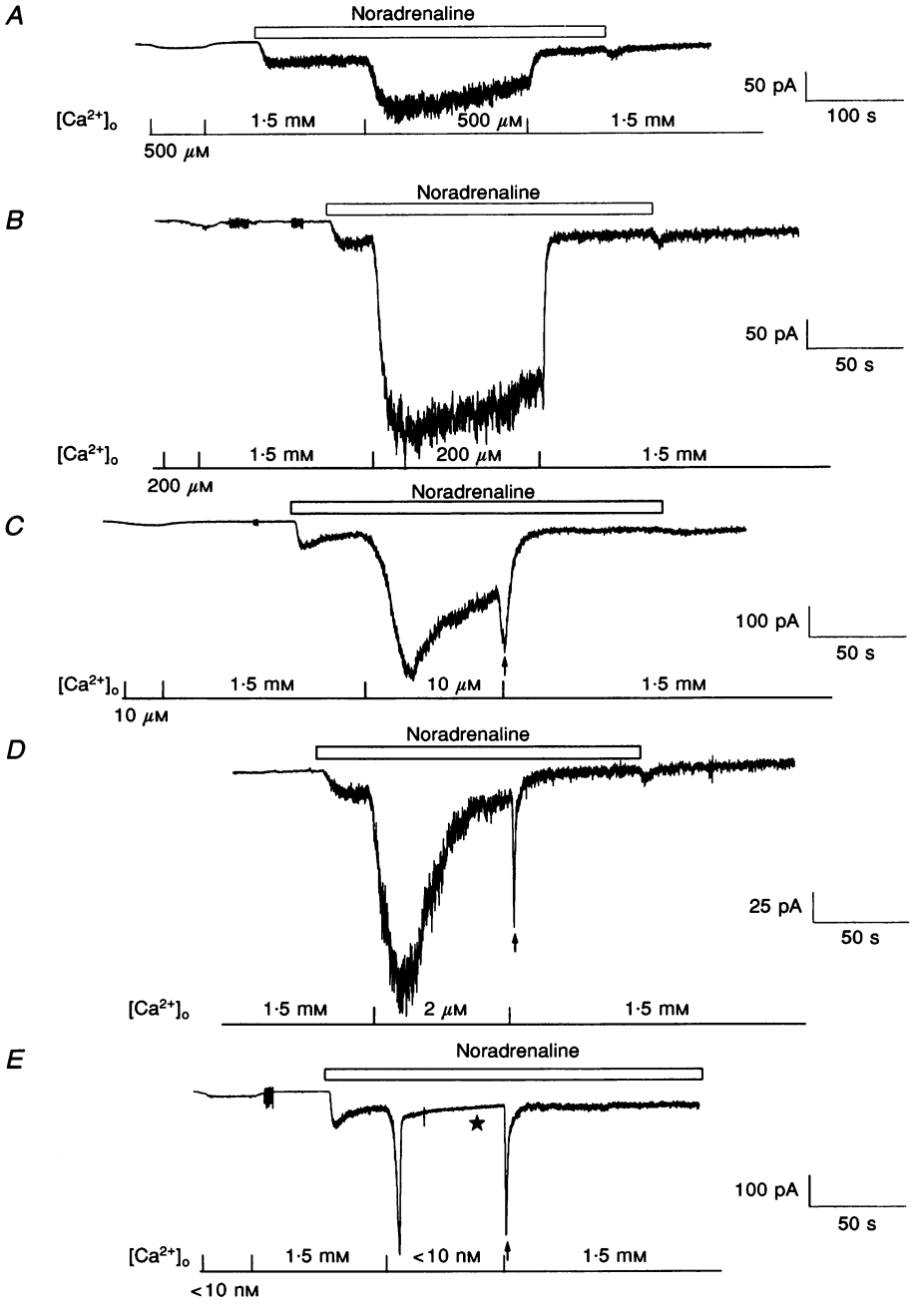

Figure 6. The concentration dependence of the facilitatory and inhibitory effects of $[Ca^{2+}]_o$ on I_{cat}

I_{cat} was first activated by bath application of noradrenaline (100 μM) in 1·5 mM Ca_o^{2+} (*A–E*) and then $[Ca^{2+}]_o$ was changed to 500 μM (*A*), 200 μM (*B*), 10 μM (*C*), 2 μM (*D*) and <10 nM (*E*) before re-application of 1·5 mM Ca_o^{2+} as indicated (*A–E*). In every case except *D* (not illustrated) non-specific leak changes due to the addition of 'test' $[Ca^{2+}]_o$ in the absence of I_{cat} are shown at the beginning of each trace. In *C–E* the arrows indicate the potentiation of I_{cat} on re-addition of 1·5 mM Ca_o^{2+}. In *E* the star represents sustained I_{cat} in <10 nM Ca_o^{2+}. Noradrenaline (100 μM) was bath applied for the period denoted by the horizontal bars. The experiments were performed in standard K^+-free conditions in the absence of Mg_o^{2+} and V_h was −50 mV.

from 200 μM to <10 nM, the decay of I_{cat} in the presence of 'test' $[Ca^{2+}]_o$ varied. The decay became progressively more rapid as the concentration of Ca_o^{2+} was reduced. This was consistent with the observations described above (Fig. 4) where it was proposed that a certain amount of Ca_o^{2+} was required to maintain the potentiated current and is therefore also consistent with the facilitatory action of Ca_o^{2+}. As noted above, when the 'test' concentration of Ca_o^{2+} was reduced to <10 nM, the initial potentiation of I_{cat} was followed by a rapid decay of the current to a steady-state level (Fig. 6*E*, star). This residual current could not simply be ascribed to a leak change as there was an appreciable amount of noradrenaline-activated current (observed in 6 out of 6 cells). Since residual I_{cat} was not observed when 1·2 mM Mg_o^{2+} was present (Fig. 4*B*) it is likely that Mg^{2+} has an inhibitory action on I_{cat}. The *I–V* characteristics of this residual current in <10 nM Ca_o^{2+} and the inhibitory effect of $[Mg^{2+}]_o$ are described later.

It can be seen in Fig. 6 (and other figures) that removal of noradrenaline from the external solution was accompanied by a small transient increase in the amplitude of I_{cat}. This phenomenon is not an artefact of solution change but has not been examined further.

The inhibitory effect of $[Ca^{2+}]_o$ on I_{cat} was estimated by quantifying the potentiation of the current on changing $[Ca^{2+}]_o$ from 1·5 mM to the 'test' concentrations by measuring the maximal amplitude of I_{cat} on changing to a given Ca_o^{2+} concentration (*b* in Fig. 7) and dividing this by the current in 1·5 mM Ca_o^{2+} immediately prior to addition (*a* in Fig. 7*A*), to obtain a ratio. Note that the measured amplitude of *b* allowed for the leak change when the Ca_o^{2+} concentration was changed in the absence of noradrenaline (dotted lines in Fig. 7*A*). The ratio *b*/*a* was calculated for various $[Ca^{2+}]_o$ and is plotted in Fig. 7*B* against $[Ca^{2+}]_o$ (open circles). From this graph it was evident that the potentiation of I_{cat} produced by reduction of $[Ca^{2+}]_o$ was maximal around 200 μM such that a further decrease in $[Ca^{2+}]_o$ did not lead to further potentiation of the noradrenaline-evoked current. The half-maximal $[Ca^{2+}]_o$ for this inhibitory effect was around 400 μM at a V_h of −50 mV (Fig. 7*B*).

The facilitatory action of Ca^{2+} on I_{cat} could be most easily quantified by dividing the maximum amplitude of the current on re-addition of 1·5 mM Ca_o^{2+} in the presence of noradrenaline (*d* in Fig. 7*A*) by the current in the 'test' $[Ca^{2+}]_o$ immediately prior to re-addition of 1·5 mM Ca_o^{2+} (*c* in Fig. 7*A*) to obtain a ratio. In this case no allowance was made for leak changes (dotted lines in Fig. 7*A*) since the latter changed more slowly than the transient activation of the current. This ratio (*d*/*c*) was plotted against $[Ca^{2+}]_o$ and is shown as filled circles in Fig. 7*B*. This effect only manifested itself below 100 μM Ca_o^{2+} and was maximal in the low micromolar range where the half-maximum $[Ca^{2+}]_o$ for this facilitatory effect was around 6 μM at $V_h = -50$ mV (Fig. 7*B*). As is evident from Fig. 6 (and described above) this facilitatory action of $[Ca^{2+}]_o$ was also manifest as a decline of I_{cat} after the initial potentiation on reduction of $[Ca^{2+}]_o$. For example after the initial potentiation on

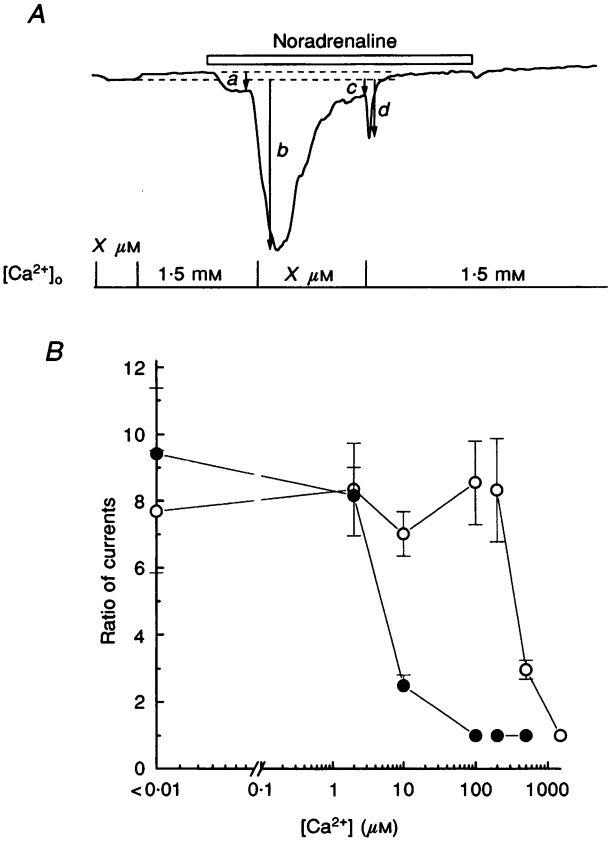

Figure 7. Quantification of the inhibitory and facilitatory effects of $[Ca^{2+}]_o$ on I_{cat}

A, diagram showing the method used to quantify the two effects. Quantification of the inhibitory effect of $[Ca^{2+}]_o$ was taken as the ratio of the maximum current amplitude, *b*, in the 'test' $[Ca^{2+}]_o$ (*X* μM) and the current amplitude, *a*, in 1·5 mM Ca_o^{2+} immediately prior to the change to *X* μM Ca_o^{2+}. The data were corrected for non-specific membrane leak changes on switching from 1·5 to *X* μM Ca_o^{2+} (dotted lines in *A*). *X* μM Ca_o^{2+} was either 500 μM ($n = 7$), 200 μM ($n = 6$), 100 μM ($n = 5$), 10 μM ($n = 6$), 2 μM ($n = 6$) or <10 nM ($n = 6$). Quantification of the facilitatory effect of $[Ca^{2+}]_o$ was taken as the ratio of the maximum current amplitude, *d*, on re-addition of 1·5 mM Ca_o^{2+} and the current amplitude, *c*, in *X* μM Ca_o^{2+} immediately prior to re-addition of 1·5 mM Ca_o^{2+}. Noradrenaline (100 μM) was applied for the period denoted by the horizontal bar. *B*, the ratios *b*/*a* (○, inhibitory effect) and *d*/*c* (●, facilitatory effect) of $[Ca^{2+}]_o$ are plotted against $[Ca^{2+}]$.

reduction of $[Ca^{2+}]_o$, I_{cat} decayed more rapidly in a concentration-dependent manner as $[Ca^{2+}]_o$ was decreased from 10 μM to <10 nM (compare the decay of I_{cat} in various 'test' $[Ca^{2+}]_o$; Fig. 6*A–E*). Moreover, the rate of inactivation of I_{cat} in low $[Ca^{2+}]_o$ seemed to parallel the extent of the transient activation of I_{cat} on re-addition of 1·5 mM Ca_o^{2+} (arrows in Fig. 6*C–E*). However, even though the facilitatory effect could have been quantified in this way, i.e. in terms of the decay after the initial potentiation, the decay of I_{cat} is also likely to be dependent on other factors (e.g. desensitization processes) and therefore independent of $[Ca^{2+}]_o$. Therefore, the method in Fig. 7 was used to assess the facilitatory effect since this was solely dependent on changes in $[Ca^{2+}]_o$. Consequently the facilitatory effect of $[Ca^{2+}]_o$ on I_{cat} occurs over a lower concentration range than the inhibitory action and therefore the effects are probably mediated by Ca^{2+} acting at different binding sites. Increasing $[Ca^{2+}]_o$ from 1·5 to 5 mM during the noradrenaline-activated current decreased the amplitude of I_{cat} from 15·8 ± 4·8 to 3·4 ± 4·1 pA ($n = 5$). Since the current in 5 mM Ca_o^{2+} was very small, in some cases negligible, and is outside the $[Ca^{2+}]_o$ range for observation of the facilitatory effect, the effects of Ca_o^{2+} at this concentration were not examined further.

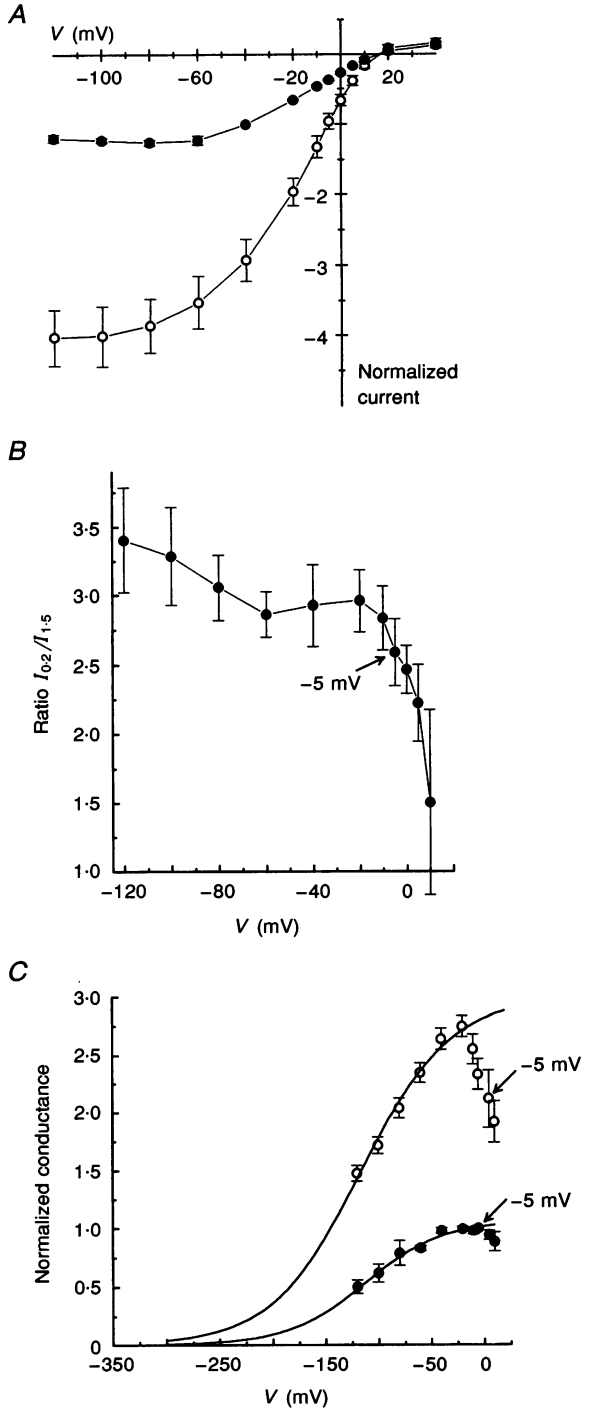

Figure 8. Voltage dependence of I_{cat} in 1·5 mM or 200 μM Ca_o^{2+}

A, current–voltage relationship of I_{cat} in 1·5 mM (●) and 200 μM Ca_o^{2+} (○) was established using voltage ramps. Currents were normalized to the amplitude of the current at −40 mV in 1·5 mM Ca_o^{2+} ($n = 5$). *B*, data from *A* where the ratio of the current recorded in 200 μM Ca_o^{2+} and the current in 1·5 mM Ca_o^{2+} ($I_{0\cdot2/1\cdot5}$) was plotted against membrane voltage. *C*, conductance plot of the data from *A* where conductance (G) was calculated from $G = I/(V - E_r)$, where I is the current recorded at a given membrane potential (V) and E_r is the estimated reversal potential. The conductances in both 1·5 mM Ca_o^{2+} (●) and 200 μM Ca_o^{2+} (○) were normalized to the maximum conductance in 1·5 mM Ca_o^{2+} obtained at −5 mV, which was 0·47 ± 0·09 nS ($n = 5$), and plotted against membrane voltage. The data could be described by a Boltzmann distribution (continuous curves), $G = G_{max}/(1 + \exp((V_m - V_h)/k))$, where G_{max} is the maximal normalized chord conductance and V_m, V_h and k are the membrane potential, the potential for half-maximal activation and the slope factor, respectively. Note that the maximum conductance in 200 μM Ca_o^{2+} was recorded at −20 mV which was 1·27 ± 0·25 nS ($n = 5$). The experiments were performed in standard K^+-free conditions in the absence of Mg_o^{2+} and V_h was −50 mV.

Current–voltage relationship of I_{cat} in various $[Ca^{2+}]_o$ *I–V* **characteristics in 200 μM Ca_o^{2+}.** An initial hypothesis which might account for the inhibitory action was that Ca^{2+} binds to a site or sites within the pore, and therefore the membrane electrical field, reducing the current carried by Na^+. In this scheme Ca^{2+} would have a greater blocking action at more negative potentials since it would be more effectively driven into the pore (i.e. the blocking action of Ca^{2+} would be voltage dependent). If this were the case it would be predicted that a reduction in $[Ca^{2+}]_o$ from 1·5 mM would produce a greater relief from block at increasingly negative potentials. This would be manifest by a progressive reduction in the degree of rectification at negative voltages compared with that observed in 1·5 mM Ca_o^{2+} (Fig. 3). To test this hypothesis voltage ramps were applied at the peak of the noradrenaline current in 1·5 mM Ca_o^{2+} and again after the change to 200 μM Ca_o^{2+}. This latter concentration of Ca_o^{2+} was selected since the inhibitory action of Ca^{2+} is significantly reduced and the facilitatory action is maximal (Fig. 7*B*). The amplitude of I_{cat} was increased at all potentials negative to the reversal potential in 200 μM Ca_o^{2+} (open circles, Fig. 8*A*) although the degree of rectification of the current at potentials negative to −40 mV and positive to the reversal potential was unchanged. Furthermore, the conductance of I_{cat} in 1·5 mM and 200 μM Ca_o^{2+} at voltages negative to −20 mV could be described by similar Boltzmann distributions (see Fig. 8 legend for details), where V_h and k in 1·5 mM and 200 μM Ca^{2+} were −112 and −37 mV, and −115 and −42 mV, respectively. Consequently even though the conductance increased markedly in 200 μM Ca_o^{2+} compared with 1·5 mM Ca_o^{2+}, the voltage dependence of the conductance at voltages negative to −20 mV remained the same. This is also indicated in Fig. 8*B* where the ratio of the current in 200 μM Ca_o^{2+} compared with that in 1·5 mM Ca_o^{2+} ($I_{0·2}/I_{1·5}$) is very similar at all voltages negative to −20 mV. However, there appeared to be a voltage-dependent effect over a very narrow voltage range between approximately −10 and 0 mV, typified by a progressive deviation from that predicted by the Boltzmann distribution in 200 μM Ca_o^{2+} and a progressive reduction in the ratio $I_{0·2}/I_{1·5}$. Even though it appeared that in this voltage range the inhibitory action of Ca_o^{2+} had a degree of voltage dependence and was therefore consistent with Ca^{2+} blocking a site within the pore, the voltage-dependent effect did not apply to potentials negative to −20 mV. Clearly our initial hypothesis would predict an even greater relief from block at progressively more hyperpolarized potentials in 200 μM than in 1·5 mM Ca_o^{2+}. Since this is not the case it is unlikely that the inhibitory effect of $[Ca^{2+}]_o$ results solely from a blocking action within the channel pore.

***I–V* characteristics in <10 nM Ca_o^{2+}.** As stated above when $[Ca^{2+}]_o$ was reduced to <10 nM in the presence of noradrenaline there was an initial potentiation of I_{cat} followed by a rapid decay to a steady-state level. In Mg^{2+}-free external conditions this steady-state level corresponded to an appreciable residual noradrenaline-activated current (6 out of 6 cells). It was conceivable that under these conditions that I_{cat} might have a different voltage dependence and to examine this possibility voltage ramps were applied during I_{cat} in 1·5 mM Ca_o^{2+} immediately prior to changing to a solution containing less than 10 nM Ca_o^{2+} (Fig. 9*A*, filled circle), and after the initial potentiation in very low $[Ca^{2+}]_o$ (Fig. 9*A*, open circle). These ramps were corrected for leak by subtraction of ramps prior to

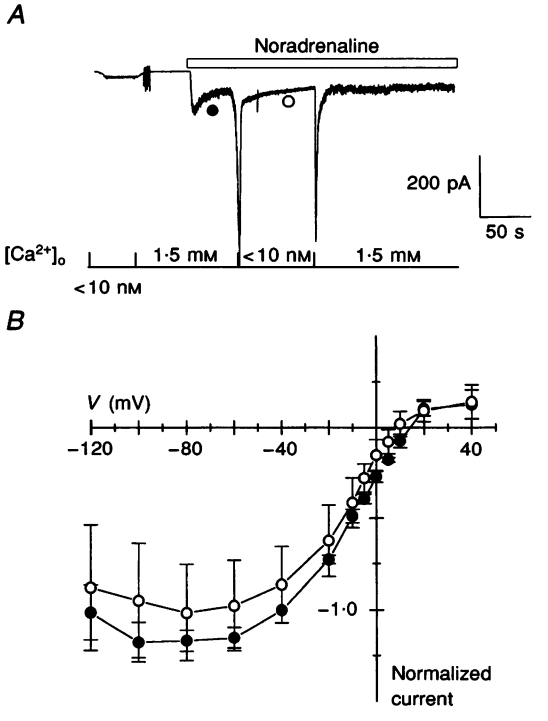

Figure 9. Comparison of the current–voltage relationships of I_{cat} in 1·5 mM Ca_o^{2+} and at steady state in <10 nM Ca_o^{2+}

A, voltage ramps were applied in 1·5 mM Ca_o^{2+} during I_{cat} (●) immediately prior to reducing $[Ca^{2+}]_o$ to <10 nM and again after the potentiated I_{cat} had decayed to a steady-state residual level in <10 nM Ca_o^{2+} (○). Noradrenaline (100 μM) was bath applied for the period denoted by the horizontal bar. The resulting *I–V* relationships in 1·5 mM Ca_o^{2+} (●) and <10 nM Ca_o^{2+} (○) are depicted in *B* and are normalized to the current recorded at −40 mV in 1·5 mM Ca_o^{2+} ($n = 6$). The experiments were performed in standard K^+-free conditions in the absence of Mg_o^{2+} and V_h was −50 mV.

noradrenaline addition in the appropriate $[Ca^{2+}]_o$. The resulting I–V curves were constructed as before and are shown in Fig. 9*B* where it can be seen that the I–V characteristics in 1·5 mM Ca_o^{2+} (filled circles) were very similar to those of the residual current in <10 nM Ca_o^{2+} (open circles). There was, however, a negative shift in E_r in four out of six cells in <10 nM Ca_o^{2+} compared with 1·5 mM Ca_o^{2+}. The mean shift was $-6 \pm 1{\cdot}4$ mV ($n = 4$) although in two remaining cells a change in E_r could not be detected. This negative shift in E_r may indicate a degree of Ca^{2+} permeability through the channel although estimation of E_r was difficult due to strong inward rectification at positive potentials. Interestingly the amplitude of the residual current in <10 nM Ca_o^{2+} is similar to the amplitude in 1·5 mM Ca_o^{2+} immediately prior to the change in $[Ca^{2+}]_o$. Therefore, it appeared that Ca_o^{2+} was not obligatory for the occurrence of I_{cat} although low micromolar $[Ca^{2+}]_o$ has a positive modulatory role on I_{cat} (see Fig. 6). This is clearly illustrated in Fig. 10 where significant I_{cat} could be evoked by bath application of noradrenaline in the presence of <10 nM Ca_o^{2+} (60 ± 21 pA, $n = 8$, Fig. 10). Furthermore, subsequent addition of 1·5 mM Ca_o^{2+} caused a transient potentiation of the noradrenaline-activated current before it decayed to a new steady-state level where the amplitude of I_{cat} was less in 1·5 mM Ca_o^{2+} than in <10 nM Ca_o^{2+} ($n = 4$, Fig. 10*A*). This observation is consistent with the hypothesis that as the $[Ca^{2+}]_o$ is changed from <10 nM to 1·5 mM, I_{cat} is first potentiated as a result of the facilitatory effect of Ca_o^{2+} (half-activation $[Ca^{2+}]_o$ approximately 6 μM) before the inhibitory effect is significantly involved (i.e. significant blocking effect occurs at $[Ca^{2+}]_o \geqslant 200$ μM). The current then decays rapidly to a much reduced steady-state level in 1·5 mM Ca_o^{2+} due to subsequent significant involvement of its inhibitory action. When $[Ca^{2+}]_o$ was increased from <10 nM to 200 μM, I_{cat} was greatly increased and then declined to a sustained level that was much greater than the amplitude of I_{cat} in low $[Ca^{2+}]_o$ ($n = 4$, Fig. 10*B*). With 200 μM Ca_o^{2+} the facilitatory effect of Ca_o^{2+} was dominant, whereas with 1·5 mM Ca_o^{2+} the inhibitory effect of Ca_o^{2+} became more pronounced (cf. Fig. 10*A* and *B*).

Effect of $[Mg^{2+}]_o$. Since there were indications that Mg_o^{2+} also had an inhibitory action on noradrenaline-evoked I_{cat} (see above), experiments were designed to examine these effects. These studies were performed in 200 μM Ca_o^{2+} where the inhibitory action of Ca_o^{2+} is greatly reduced (cf. Fig. 6*B*). Therefore, if Mg^{2+} interacts with the same inhibitory site as Ca^{2+}, removal of 1·2 mM Mg_o^{2+} during I_{cat} should lead to marked potentiation of the current. I_{cat} was first evoked in an external solution containing 1·2 mM Mg^{2+} which was

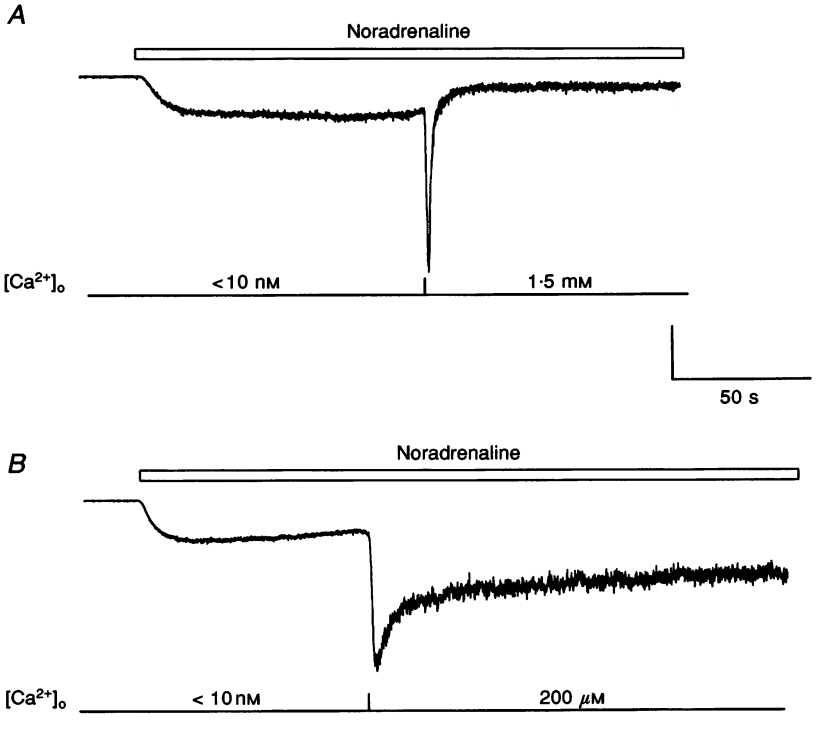

Figure 10. Effect of increasing $[Ca^{2+}]_o$ on noradrenaline-evoked I_{cat} activated in <10 nM Ca_o^{2+}

Noradrenaline (100 μM) was applied for the period denoted by the horizontal bar in < 10 nM Ca_o^{2+} and either 1·5 mM (*A*) or 200 μM (*B*) Ca_o^{2+} was added as indicated during activation of I_{cat}. The experiments were performed in standard K^+-free conditions in the absence of Mg_o^{2+} and V_h was −50 mV. Vertical calibration in *A* is 100 pA and in *B* is 200 pA. Note that when 1·5 mM Ca_o^{2+} was added I_{cat} was initially potentiated but then declined to a level lower than in <10 nM Ca_o^{2+} but with 200 μM Ca_o^{2+} I_{cat} was sustained at a potentiated level.

subsequently replaced by one with no added Mg^{2+} and then Mg^{2+} was re-added as shown in Fig. 11*A*. Voltage ramps were applied in 0 and in 1·2 mM Mg_o^{2+} when I_{cat} had reached a steady current level at the holding potential (Fig. 11*A*, stars). The *I–V* relationship of I_{cat} indicated a very small but significant sustained potentiation of the current on removal of Mg_o^{2+} (Fig. 11*B*). However, this slight inhibitory effect of Mg_o^{2+} was not comparable to the much greater inhibitory effect of Ca_o^{2+} even though both divalent cations were used at approximately the same molarity in the external solution. In addition there was no evidence for the involvement of Mg_o^{2+} in the facilitatory effect produced by Ca_o^{2+}. For example, experiments were performed in a similar manner to those described in Fig. 10, where I_{cat} was evoked by noradrenaline in <10 nM Ca_o^{2+}, in Mg_o^{2+}-free conditions, and addition of 1·2 mM Mg_o^{2+} simply lead to inhibition of I_{cat} and there was no transient activation of the current, indicative of the facilitatory effect (data not shown). Furthermore comparison of the *I–V* characteristics in 1·2 mM and 0 Mg_o^{2+} (Fig. 11*B*) indicated no marked differences in the voltage dependence of I_{cat}.

DISCUSSION

The major finding of this study was that Ca_o^{2+} had two effects on noradrenaline-activated I_{cat}. The first effect has been described as an inhibitory action of Ca_o^{2+} since the amplitude of I_{cat} was either increased or decreased when $[Ca^{2+}]_o$ was respectively reduced or increased from 1·5 mM Ca_o^{2+}. Under our experimental conditions the half-maximal $[Ca^{2+}]_o$ for this effect was 400 μM. In addition there was a significant facilitatory action which was dependent on low micromolar levels of Ca_o^{2+} where the $[Ca^{2+}]_o$ for half-maximal action was approximately 6 μM. For example, when $[Ca^{2+}]_o$ was reduced from 1·5 mM to <50 μM there was an initial potentiation followed by a rapid decline of the current and this latter effect was not observed in $[Ca^{2+}]_o \geqslant 100$ μM. Furthermore a subsequent increase in $[Ca^{2+}]_o$ from <50 μM to 1·5 mM in the presence of noradrenaline caused a transient potentiation of the current which was not observed when $[Ca^{2+}]_o$ was increased from ⩾100 μM to 1·5 mM (e.g. Fig. 6). These data indicate that there is a facilitatory effect of Ca_o^{2+} in addition to an inhibitory effect. This was confirmed in experiments where

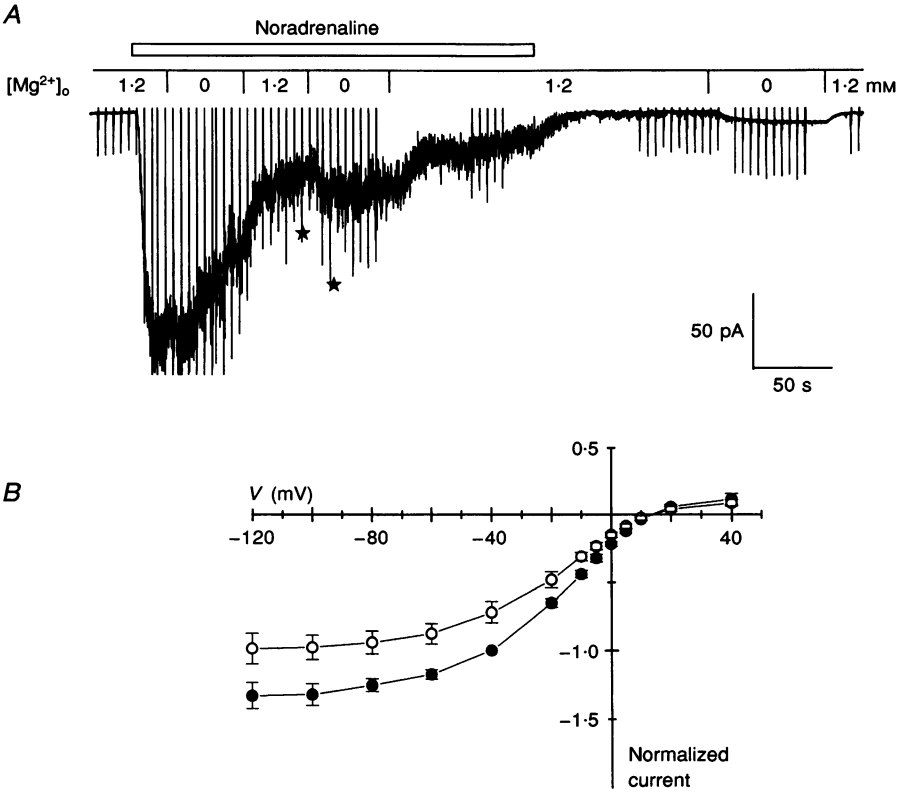

Figure 11. The effect of $[Mg^{2+}]_o$ on noradrenaline-evoked I_{cat}

A, I_{cat} was activated in 1·2 mM Mg_o^{2+} which was removed and re-applied during continued activation of I_{cat}. Voltage ramps were applied in 1·2 and 0 mM Mg_o^{2+} during I_{cat} and resulting *I–V* curves, taken when I_{cat} had reached a steady level at the holding potential (stars in *A*), are shown in *B*. Currents in both 1·2 mM (○) and 0 Mg_o^{2+} (●) were normalized to the current recorded in 0 Mg_o^{2+} at −40 mV($n = 5$) in *B*. Noradrenaline (100 μM) was applied for the period denoted by the horizontal bar. The experiments were performed in standard K^+-free conditions in the presence of 200 μM Ca_o^{2+} and V_h was −50 mV.

the cation current was first evoked in <10 nM Ca_o^{2+} and a subsequent change to 200 μM Ca_o^{2+} led to a sustained potentiation of the current (Fig. 10). However, since appreciable I_{cat} could be evoked by noradrenaline in the presence of <10 nM Ca^{2+}, it appears that Ca^{2+} is not obligatory for the activation of this conductance but merely has a facilitatory action. The inhibitory and facilitatory effects may be specific for Ca_o^{2+} since decreasing Mg_o^{2+} had a much reduced action in potentiating I_{cat} and Mg_o^{2+} appeared to have no facilitatory effect.

It is unlikely that any of the $[Ca^{2+}]_o$ effects described in this study resulted from indirect changes in $[Ca^{2+}]_i$ as the effects were rapid and occurred within 2 s of changing the bathing solution. It has been reported in other smooth muscle that changes in $[Ca^{2+}]_o$ take at least 30–60 s to effect a change in $[Ca^{2+}]_i$ (Inoue & Isenberg, 1990*a*). Moreover, we used 10 mM BAPTA in the pipette solution to 'clamp' $[Ca^{2+}]_i$ at low levels. However, we cannot rule out the possibility that under more physiological conditions, where $[Ca^{2+}]_i$ is not buffered with BAPTA, $[Ca^{2+}]_i$ may interact with the proposed Ca^{2+} binding sites (see below). Interestingly the effects of steady-state $[Ca^{2+}]_i$ on the amplitude of noradrenaline-evoked I_{cat} reported here (Fig. 2) are the opposite to those reported for acetylcholine-evoked non-selective cation current in guinea-pig ileum (Inoue & Isenberg, 1990*a*, *b*). In this latter study the authors reported a dose-dependent increase in the amplitude of the cation current as $[Ca^{2+}]_i$ was increased from 1 nM to 1 μM, with a half-maximal $[Ca^{2+}]_i$ of 100–300 nM for this effect. In contrast, in the present study the currents tended to be larger and were significantly more sustained with 14 nM rather than 100 nM Ca_i^{2+} in the pipette solution. This may reflect genuine differences in the way these two conductances are modulated by $[Ca^{2+}]_i$, although in a recent review it has been reported that increased $[Ca^{2+}]_i$ initially potentiated but then profoundly inhibited the amplitude of the acetylcholine-evoked cation current in guinea-pig ileum (Inoue & Chen, 1993).

Since the facilitatory and inhibitory effects have very different dependencies on $[Ca^{2+}]_o$ (see above) it is likely that they result from an interaction of Ca^{2+} with two distinct binding sites with different affinities for Ca^{2+}. This assumption can be incorporated into a hypothesis which may explain many of the effects on the characteristics of I_{cat} (at $V_h = -50$ mV) when $[Ca^{2+}]_o$ is changed during activation of the current. For example, when noradrenaline-induced I_{cat} was recorded in <10 nM Ca_o^{2+}, re-addition of 1·5 mM Ca_o^{2+} first led to a rapid facilitation of the current (Fig. 10*A*) which might reflect significant occupancy of the facilitatory site due to the higher affinity of this site for Ca^{2+} (half-maximal $[Ca^{2+}]_o \approx 6$ μM). The current then rapidly decayed which might reflect subsequent significant occupancy of the inhibitory site (half-maximal $[Ca^{2+}]_o \approx 400$ μM). In other experiments where $[Ca^{2+}]_o$ was reduced from 1·5 mM to 200 μM during I_{cat} a relatively sustained potentiated current was observed (e.g. Fig. 6). This potentiation might reflect a significant reduction in Ca^{2+} occupancy of the inhibitory site while at this $[Ca^{2+}]_o$ (200 μM), occupancy of the facilitatory site is near maximal and, hence the potentiated current is relatively sustained. In these experiments re-addition of 1·5 mM Ca^{2+} simply led to a reduction in the amplitude of I_{cat}, perhaps resulting from a subsequent significant increase in Ca^{2+} occupancy of the inhibitory site. Similarly when $[Ca^{2+}]_o$ was reduced from 1·5 mM to ⩽50 μM during I_{cat} the amplitude of the current was first potentiated, due to the removal of the inhibitory action of Ca^{2+}, and then the current declined because of reduced occupancy of Ca^{2+} at the facilitatory sites (Fig. 6). In these conditions re-addition of 1·5 mM Ca_o^{2+} produced an initial potentiation, due to an action at the facilitatory site, before I_{cat} was reduced due to the inhibitory action of Ca^{2+} (arrows in Fig. 6).

It was of interest to examine how a reduction in $[Ca^{2+}]_o$ affected the I–V characteristics of I_{cat} as this approach might reveal the blocking mechanism of Ca_o^{2+}. When $[Ca^{2+}]_o$ was reduced from 1·5 mM to 200 μM, marked potentiation of I_{cat} was observed at $V_h = -50$ mV. If Ca_o^{2+} behaved simply as a blocker of Na^+ flux (the main charge carrier) within the channel pore, and hence within the membrane electrical field, it might be predicted that there would be a greater block of the current at more hyperpolarized than depolarized potentials since Ca^{2+} would be more effectively driven into the channel at the more hyperpolarized potentials. This prediction holds both for models for which there is proposed to be only a single site for ion occupancy within the pore or whether there are multiple sites (see Hille, 1992). However, when $[Ca^{2+}]_o$ was reduced from 1·5 mM to 200 μM, a voltage-dependent effect was observed only over a narrow range of potentials, approximately between 0 and −10 mV. At potentials more negative than this there was no change in the voltage dependence of I_{cat}, and the rectification observed between −50 and −120 mV in 1·5 mM Ca_o^{2+} was almost identical to that in 200 μM Ca_o^{2+}. Therefore, the decrease in conductance seen in 1·5 mM Ca_o^{2+} relative to 200 μM Ca_o^{2+} cannot be explained simply in terms of a blocking effect of Ca^{2+} within the channel. Also, it can be concluded that the rectifying properties of the non-selective cation current are not dependent on divalent cations and, therefore, may be an intrinsic property of the channel.

Experiments concerning Ca^{2+} and other non-selective cation conductances where it has been possible to record single channel events (Benham & Tsien, 1987; Decker & Dani, 1990) have led to the hypothesis that Ca^{2+} competes with Na^+ for a binding site within the channel pore but then permeates more slowly than Na^+ thereby reducing the single channel conductance. From our whole-cell data it is not possible to conclude unequivocally that this is also the

case for noradrenaline-activated I_{cat}. However, there is some indirect evidence to suggest that Ca^{2+} may indeed permeate the channel. Two previous reports in rabbit portal vein have shown that Ba^{2+} can significantly permeate the channel (Wang & Large, 1991; Inoue & Kuriyama, 1993). Furthermore, in the present study a negative shift in E_r of I_{cat} was observed in most cells on reduction of $[Ca^{2+}]_o$ from 1·5 mM to <10 nM, which could indicate that I_{cat} has a significant permeability to Ca^{2+}. However, the fact that the voltage dependence of I_{cat} between −40 and −120 mV was unaffected by a reduction of Ca^{2+} from 1·5 mM to 200 μM in spite of the marked increase in conductance may indicate that Ca_o^{2+} inhibits I_{cat} by affecting P_{open} (probability of channel opening) and/or N (number of channels) in a voltage-independent manner. Clearly there is a need to examine the effects of Ca^{2+} on this conductance at the single channel level to determine the most likely mechanism for its inhibitory effect.

The dual effect of $[Ca^{2+}]_o$ on noradrenaline-activated I_{cat} is novel since there are no similar reports describing more than one action of Ca_o^{2+} on any other type of ligand-gated cation conductance (see reviews in Siemen & Hescheler, 1993). In intestinal smooth muscle increasing $[Ca^{2+}]_o$ in the millimolar range simply had a facilitatory effect on acetylcholine-evoked cation current, where an increase in $[Ca^{2+}]_o$ from 1 to 10 mM approximately doubled the amplitude of the current (Inoue, 1991). This is in contrast to the results reported here where increasing $[Ca^{2+}]_o$ in the millimolar range reduced the amplitude of I_{cat} which was almost blocked in 5 mM Ca_o^{2+}. Furthermore, the effect in intestinal smooth muscle is not comparable to the facilitatory effect of Ca^{2+} on noradrenaline-activated I_{cat} in portal vein since the latter occurs in the low micromolar range. Similarly, although the ATP-activated cation conductance in rabbit ear artery is significantly reduced as $[Ca^{2+}]_o$ is increased in the millimolar range (Benham & Tsien, 1987), there was no evidence for an additional facilitatory action. It is clear that these modulatory actions of Ca_o^{2+} on the noradrenaline-activated cation conductance could have profound physiological consequencies. Under our experimental conditions, reduction of Ca^{2+} from 1·5 mM to 500 μM doubled the amplitude of I_{cat}, whereas further reduction to 200 μM could increase the amplitude by up to 8 times. Consequently $[Ca^{2+}]_o$ could conceivably have a role in regulating the flux of Na^+ through the cation channel and therefore the extent of depolarization. It is also possible that the inhibitory effect of $[Ca^{2+}]_o$ on I_{cat} results from the relatively slow permeation of Ca^{2+} through the channel. Therefore, a major physiological role of I_{cat} could be in providing a Ca^{2+} influx pathway as suggested by others (Bolton, 1979; Byrne & Large, 1988; Wang & Large, 1991).

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