Journal of Physiology (1993), **471**, *pp.* 87–105
With 9 figures
Printed in Great Britain

EVIDENCE FOR MULTIPLE OPEN STATES OF THE Ca^{2+} CHANNELS IN SMOOTH MUSCLE CELLS ISOLATED FROM THE GUINEA-PIG DETRUSOR

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(*Received 2 December 1992*)

SUMMARY

1. Whole-cell voltage clamp techniques were used to examine the properties of voltage-dependent Ca^{2+} channel currents in single smooth muscle cells enzymatically dissociated from guinea-pig urinary bladder. Potassium currents were blocked with intracellular Cs^+. A holding potential of -60 mV was normally applied.

2. When the membrane potential was returned to the holding potential after a depolarizing step, tail currents were seen after depolarizations to positive potentials, and the size of the tail current increased with increasing positivity of the preceding depolarization.

3. After depolarization to $+80$ mV (a potential at which little inward current flowed through the Ca^{2+} channels) tail currents on returning to the holding potential increased in size as the duration of the depolarization was increased.

4. Investigation of the mechanism mediating the tail currents showed that they were not flowing through non-selective cation channels, and had no contribution from Ca^{2+}-activated Cl^- channels or Na^+–Ca^{2+} exchange.

5. The tail currents and the inward currents evoked by a simple depolarizing test potential were equally decreased by nifedipine in a dose-dependent manner. This suggests that L-type Ca^{2+} channels are responsible for both of the two types of inward currents. The inward currents were also inhibited in a similar manner when caffeine was applied.

6. Although the tail currents evoked on stepping from $+80$ mV to a holding potential of -60 mV increased in size with the duration of the conditioning potential, the total membrane Ca^{2+} conductance did not increase, since the inward currents evoked on stepping to $+20$ mV (a potential at which the Ca^{2+} channels are still fully activated) did not change with time.

7. The amplitude of the inward current evoked by a simple depolarizing test potential was similar to that evoked on stepping to the same test potential after preconditioning at $+80$ mV, if the test potential was higher than $+20$ mV. However, following repolarization to the holding potential, the amplitude of the subsequent tail current was larger and the deactivation time constant longer, after the conditioning depolarization. These results suggest that the voltage-dependent Ca^{2+} channels have at least two open states with different time constants, the tail current being the result of a long open channel state induced by large depolarizations.

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8. When variable repolarizing potentials were applied after n +80 mV depolarization (5 s), the current–voltage relationship of the tail current was nearly linear between −60 and +30 mV. The deactivation was faster when a larger repolarization step was applied.

9. The results are consistent with the voltage-dependent Ca^{2+} channels in bladder smooth muscle cells having at least two available open channel states, one rapidly deactivating and one slowly deactivating. The slowly deactivating state can be induced in a time-dependent manner by depolarization to positive potentials.

INTRODUCTION

Smooth muscle action potentials are commonly the result of Ca^{2+} influx through voltage-sensitive Ca^{2+} channels, and these channels have been extensively studied in isolated smooth muscle cells using the whole-cell configuration of the voltage clamp technique. Evidence suggests that in the majority of smooth muscles L-type Ca^{2+} channels are the predominant type, and that these channels display both voltage- and Ca^{2+}-dependent inactivation (Pelzer, Pelzer & McDonald, 1990).

In the urinary tract, the smooth muscles of the ureter, bladder and urethra show remarkable differences in their behaviour, particularly with respect to their spontaneous activity. The ureter normally only contracts in response to action potentials initiated in the pelvis of the kidney and propagated the length of the ureter. The dome of the bladder (detrusor) shows spontaneous electrical and mechanical activity, but the cells develop little sustained tone, whereas in the urethra the smooth muscles develop sustained tone in the absence of nervous activity. The properties of the voltage-sensitive Ca^{2+} channels are being investigated as part of a program examining these differences.

In this study we report the results of experiments carried out on the detrusor of guinea-pigs, a tissue in which both the action potential and voltage-dependent inward currents are carried by calcium ions (Creed, 1971; Klöckner & Isenberg, 1985). Our results are in agreement with other studies, but we also present evidence that the Ca^{2+} channels may be able to undergo a voltage-sensitive conversion from the normal open configuration to a configuration in which the gating kinetics are changed, resulting in delayed closing. This may be similar to the voltage-dependent long open state of Ca^{2+} channels in adrenal chromaffin cells described by Hoshi & Smith (1987) and in cardiac myocytes by Pietrobon & Hess (1990).

METHODS

Single smooth muscle cells were enzymatically dissociated from urinary bladders of male guinea-pigs (450–750 g), killed either by stunning, or by cervical dislocation, and exsanguinated. Muscle strips (total approximately 0·1 g wet weight), from which the epithelium had been removed, were incubated in a nominally Ca^{2+}-free solution for 60 min (at 35 °C). Subsequently, the strips were digested in an enzyme-containing (0·05% collagenase, Type 1, Sigma Chemical Co. UK and 0·1% pronase, Fulka, UK) Ca^{2+}-free solution for 10–15 min and then agitated with a glass pipette. Some of the cell suspension was stored at 5 °C and used for up to 6 h.

The equipment and procedures used to measure macroscopic currents were essentially the same as those previously described (Inoue & Brading, 1990). The single smooth muscle cells were allowed to settle in the recording bath and continuously superfused with physiological saline. The

resistance of the patch pipette was in the region of 5 MΩ. The membrane potential was clamped using a List amplifier (EPC 7, Germany). Most smooth muscle cells used had a membrane capacitance between 40–70 pF. The capacitative surge was electrically compensated. After rupture of the cell membrane, the series resistance was less than 10 MΩ. In most experiments, particularly when large inward tail currents were evoked by application of extremely high (+80 mV) conditioning potentials, the series resistance was partially compensated (by 50–70%). The speed of the voltage clamp (< 0·2 ms) was sufficiently fast to enable the large depolarization-induced tail currents (the decay time constant > 6 ms) to be accurately followed. A cut-off frequency of 10 kHz (3-pole Bessel filter) was applied to reduce the noise. All experiments were performed at room temperature (24–28 °C). Unless otherwise stated, the membrane potential was clamped at −60 mV (holding potential). An AD/DA converter (DT 2801A, Data Translation, UK) was used for voltage step generation and on-line data acquisition. Data were collected on an IBM compatible personal computer using the Quick Basic software package.

Curve fitting of the decay of the inward currents was done by fitting the curves iteratively with single or multiple exponential functions using a modified 'simplex' program. The total residual currents were used as a criterion for the convergence. At convergence, the mean residual currents were usually less than 8 pA or 2% of the peak inward current.

The normal bathing solution had the following composition (mM): NaCl, 125; KCl, 5·9; $CaCl_2$, 2·5; $MgCl_2$, 1·2; glucose, 11·8 and Hepes (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid), 11·8; pH adjusted to 7·4 with Tris base. In the nominally Ca^{2+}-free solution used for cell dispersion, Ca^{2+} was isosmotically substituted with Na^+. When Ca^{2+} was removed during voltage clamp experiments, Mg^{2+} was increased to 3·7 mM (Ca^{2+}-free, Ba^{2+}-free solution) to keep a constant divalent cation concentration. In Ba^{2+}-containing solution, 2·5 mM Ba^{2+} was added instead of Ca^{2+}. The composition of the pipette solution was (mM): CsCl, 141; $MgCl_2$, 1·4; EGTA (ethyleneglycol-bis-(β-aminoethylether) *N,N,N',N'*-tetraacetic acid), 2; Hepes/Tris, 10 (pH 7·2).

The numerical data are expressed as means ± S.D. Differences were evaluated by paired or unpaired *t* tests, and a probability of less than 0·05 was taken as a statistically significant difference. The data used for statistics were obtained from cells initially showing a peak inward current of more than 400 pA at a step potential to 0 mV.

RESULTS

To test voltage dependence of the Ca^{2+} channel current, simple depolarizations (200 ms duration) with variable amplitude were applied, when the cell membrane was held at −60 mV ($n = 6$). The peak inward current reached a maximum at a test potential of about −10 mV. Test potentials to more positive values resulted in a decrease in size of the inward current, and the membrane current reversed direction at potentials between +40 and +60 mV. These results agree well with the voltage dependence of the Ca^{2+} channel current reported previously in guinea-pig bladder smooth muscle cells (Klöckner & Isenberg, 1985; Ganitkevich & Isenberg, 1991). An interesting observation is that on repolarization from the test potential, tail currents are seen after test pulses to positive potentials, and the amplitude of these currents becomes larger as the test potentials become more positive (Table 1). We further investigated the properties of the tail current.

Effect of duration of depolarization on the tail current amplitude

The time dependence of the tail current activation is quantified in Table 2 and illustrated in Fig. 1. The cell membrane was transiently depolarized from −60 to +80 mV and the duration of the depolarization was changed. A potential of +80 mV was chosen since at this potential membrane current is almost constant (time independent). It is probable that so little Ca^{2+} enters at +80 mV that Ca^{2+}-dependent inactivation of the channels does not occur, and one can study the time

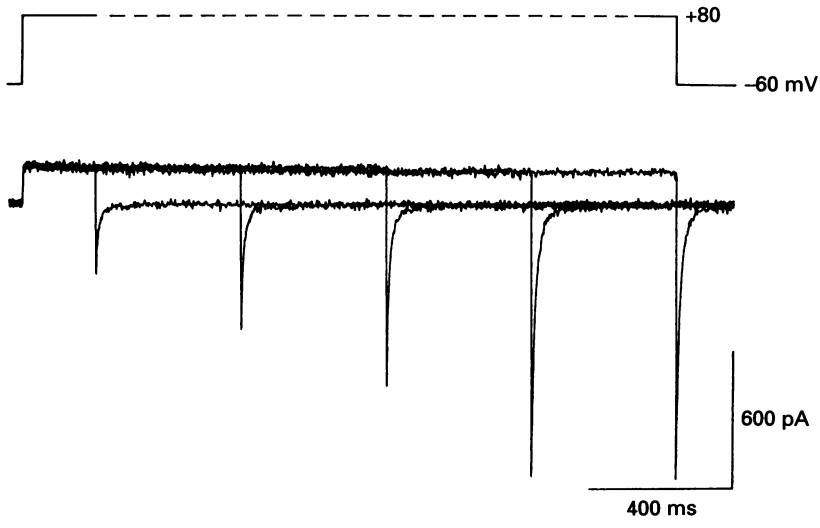

Fig. 1. The effect of the duration of the preconditioning potential on the tail current. The cell membrane was depolarized to +80 mV for variable durations, and then returned to −60 mV. The duration was increased from 200 to 1400 ms in 400 ms steps. The sequence was repeated at 90 s intervals.

TABLE 1. The tail currents observed after simple depolarizations (200 ms)

Depolarizing step (mV)	Amplitude	Decay time constant (ms)
+20	0·27 ± 0·12	6·5 ± 1·7
+40	0·53 ± 0·09	5·9 ± 0·8
+60	0·82 ± 0·09	6·9 ± 1·8
+80	1	8·0 ± 1·5

Four different potentials (+20, +40, +60 and +80 mV) were applied. Tail current decays were fitted by a single exponential. The amplitude of the inward current after depolarization to +80 mV was taken as 1. The increase in amplitude of the tail current was clear as depolarization increased. However, the decay time constant varied from cell to cell, presumably due to the limitation of the apparatus as described in the discussion. Means ± S.D. $n = 6$ (except at +20 mV where $n = 5$). The actual mean tail current at +80 mV was 262 ± 113 pA.

TABLE 2. The characteristics of the tail currents seen on repolarization to the holding potential (−60 mV) after different durations of a preconditioning depolarization to +80 mV

+80 mV depolarization duration (ms)	Amplitude	Decay time constant (ms)
200	0·34 ± 0·14	7·2 ± 1·6
600	0·61 ± 0·19	8·7 ± 1·3
1000	0·82 ± 0·14	9·3 ± 1·8
1400	0·95 ± 0·09	10·4 ± 1·7
1800	1	10·5 ± 1·3

Tail current decays were fitted by a single exponential. The amplitude of the inward current after 1800 ms depolarization to +80 mV was taken as 1 ($n = 9$). The actual mean current at this potential was 1545 ± 731 pA.

dependence of the development of the tail current without interference from this process. The amplitude and the decay time constant of the tail current evoked by returning the membrane potential to −60 mV were increased by increasing the duration of depolarization. If the tail currents were fitted by a single exponential, the means of the deactivation time constants were 7·2 ± 1·6 ms after a 200 ms depolarization and 10·5 ± 1·3 ms after 1800 ms ($n = 9$, Table 2). The mean amplitude after a 200 ms depolarization was 34 ± 14 % of that after 1800 ms, and the increase reached a maximum around 1400 ms.

What mechanism is responsible for the tail currents?

The large inward tail currents could theoretically be mediated by several different mechanisms. For instance in guinea-pig ileal smooth muscle a large inward tail current is evoked on repolarization when non-selective cation channels are activated by acetylcholine (Inoue & Isenberg, 1990); in several smooth muscles Ca^{2+}-activated Cl^- channels are present (Byrne & Large, 1988; Pacaud, Loirand, Mironneau & Mironneau, 1989; Amédée, Large & Wang, 1990; Klöckner & Isenberg, 1991) which could mediate the tail currents; in heart, Na^+–Ca^{2+} exchange can also evoke large inward tail currents upon repolarization (Chapman & Noble, 1989; Eisner & Lederer, 1989). The fourth possibility is that the tail currents are flowing through voltage-sensitive Ca^{2+} channels, the kinetics of which have been altered by the preceding depolarization. In order to determine what mediates the tail currents in the guinea-pig detrusor, experiments were performed to examine each of the possibilities.

Non-selective cation channels

Current flowing through these channels in guinea-pig ileum is eliminated in the presence of extracellular Ba^{2+} (Inoue & Isenberg, 1990; Loirand, Pacaud, Baron, Mironneau & Mironneau, 1991). Figure 2*A* and *B* shows the effects of substituting extracellular Ca^{2+} (2·5 mM) with equimolar Ba^{2+}, and *C* shows the effect of replacing the Ba^{2+} with Mg^{2+}. The cells were held for 5 s at +80 mV followed by 100 ms at 0 mV before repolarizing to the holding potential of −60 mV. In the presence of Ba^{2+}, the amplitude of the peak inward current evoked by repolarization to 0 mV was similar to that in normal solution. However, the inactivation time course was significantly slower in the presence of Ba^{2+}. The amplitude of the subsequent inward tail current (at −60 mV) was larger. The decay time constant of the tail current was slightly longer (by 2 ms) in the presence of Ba^{2+}. When extracellular Ba^{2+} was then replaced with Mg^{2+}, both the inward currents were nearly abolished. In more than ten experiments, similar results were obtained. These findings are inconsistent with non-selective cation channels mediating the tail currents, but consistent with the involvement of L-type Ca^{2+} channels. Application of atropine (10 μM) had little effect on either of the inward currents (at 0 and −60 mV), also supporting the negligible contribution of non-selective cation channels.

Ca^{2+}-activated Cl^- channels

The existence of these channels has been demonstrated in several smooth muscle cells, and it has been shown that they can be activated by large depolarizations in a time-dependent manner (Evans & Marty, 1986). However, in the bladder, as shown

in Fig. 1, the outward current observed at +80 mV is constant, suggesting that opening of Cl^- channels is not occurring (E_{Cl} (reversal potential for Cl^-) in our conditions is nearly 0 mV). Nevertheless, one may suspect that the inward tail current evoked by repolarization could activate Ca^{2+}-dependent Cl^- channels,

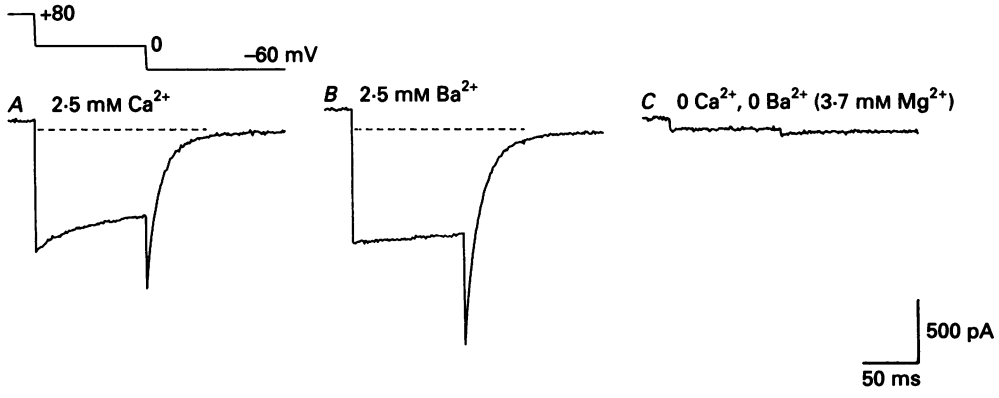

Fig. 2. Effects of substituting extracellular Ca^{2+} with Ba^{2+} or Mg^{2+}. After a 5 s conditioning depolarization at +80 mV, the potential was stepped to 0 mV for 100 ms and then returned to the holding potential of −60 mV. After observing a control current (*A*) in normal solution (2·5 mM Ca^{2+}), extracellular Ca^{2+} was substituted by equimolar Ba^{2+}, then Ba^{2+} was substituted by equimolar Mg^{2+} (total Mg^{2+}, 3·7 mM). *B* and *C* were obtained 4 and 8 min after the substitutions, respectively.

especially if the tail current is carried by Ca^{2+}. The current trace shown in Fig. 3, was obtained by a transient (5 ms) repolarization to −60 mV after a 5 s depolarization to +80 mV. While the inward tail current was decaying, the membrane potential was again clamped to +80 mV. Since the Cl^- concentrations in the bathing and pipette solutions are symmetrical, if there is a contribution of Ca^{2+}-activated Cl^- current to the inward tail current evoked at −60 mV, the outward current evoked by returning the membrane potential to +80 mV should also be larger than that observed during preconditioning. In fact the amplitudes of the outward currents were almost the same, suggesting that Ca^{2+}-dependent Cl^- channels were not activated. When caesium acetate was used instead of CsCl in the pipette solution, repolarization to the holding potential after a large depolarization still produced a large inward tail current ($n = 5$). This also supports the view that the inward tail current is Ca^{2+} movement through voltage-dependent Ca^{2+} channels and does not have a component of Cl^- current.

Na^+–Ca^{2+} exchange

This mechanism would be activated by an increase in intracellular Ca^{2+}, and as the stoichiometry of Na^+ and Ca^{2+} is 3:1, would generate an inward current which would extrude Ca^{2+} at the holding potential. At +80 mV the current would be reversed, and Ca^{2+} entry should occur. However, as shown in Fig. 8*B* and *C*, depolarization to +80 mV induced little inactivation, suggesting no significant increase in intracellular

Ca^{2+} concentration. Furthermore, the inward tail current evoked by returning the membrane potential from a conditioning depolarization of +80 mV (5 s) to the holding potential inactivates the subsequent inward current evoked by a test potential of 0 mV, as shown in the accompanying paper (Nakayama & Brading,

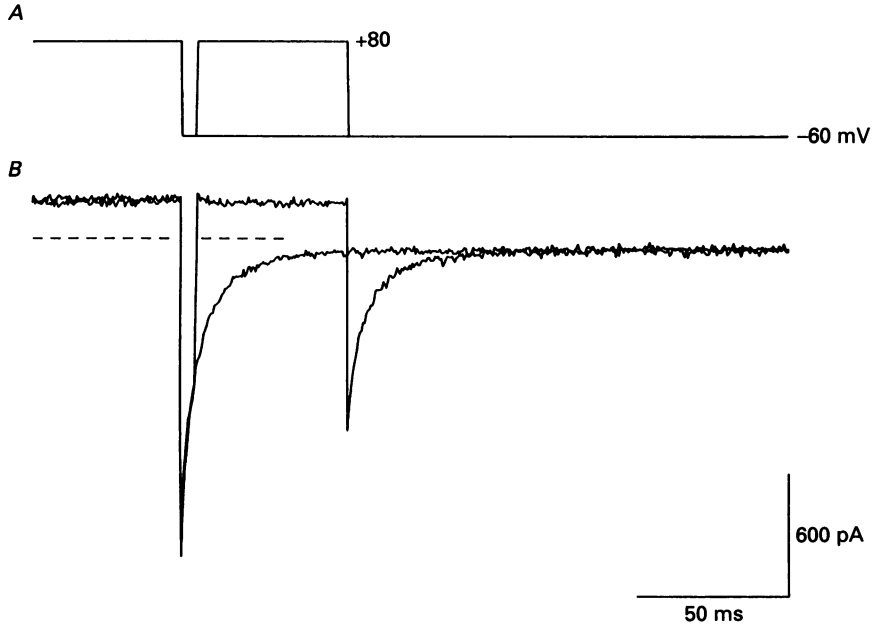

Fig. 3. Effects of transient repolarization. In normal solution, after a 5 s depolarization at +80 mV, the membrane potential was transiently (5 ms) repolarized to −60 mV, then again depolarized to +80 mV (*A*). The current trace, *B*, obtained by a simple repolarization to the holding potential is superimposed in order to show the whole decay of the inward tail current.

1993), suggesting that Ca^{2+} enters during the tail current, rather than being extruded.

L-type Ca^{2+} channels

In order to provide evidence that the tail current is using the same channels as the voltage-dependent inward current, the effects of nifedipine, an L-type Ca^{2+} channel blocker, and caffeine were investigated. The effects of nifedipine on inward membrane currents are shown in Fig. 4. Cells were preconditioned at +80 mV for 5 s, and then the membrane potential was stepped to 0 mV to elicit an inward current through the activated channels, and then after 100 ms returned to the holding potential (−60 mV) to elicit the tail current. This sequence was repeated at 2 min intervals. After control membrane currents were recorded in normal solution, nifedipine was applied cumulatively. The inward current recorded at 0 mV was decreased to 79 and 28% of the control after 6 min application of 10 and 100 nM nifedipine, respectively (Fig. 4*A*). After 8 min wash-out, the amplitude of the current had recovered to 49%. The time course of the inhibition and recovery is plotted in Fig. 5*A*. The amplitudes

of the inward current recorded at 0 and −60 mV (tail current) were affected to the same extent during the application and wash-out of nifedipine. Nifedipine had no effect on the outward current recorded at +80 mV. In Fig. 4*B* the results of another experiment are shown, in which the effect of nifedipine on the current elicited by a

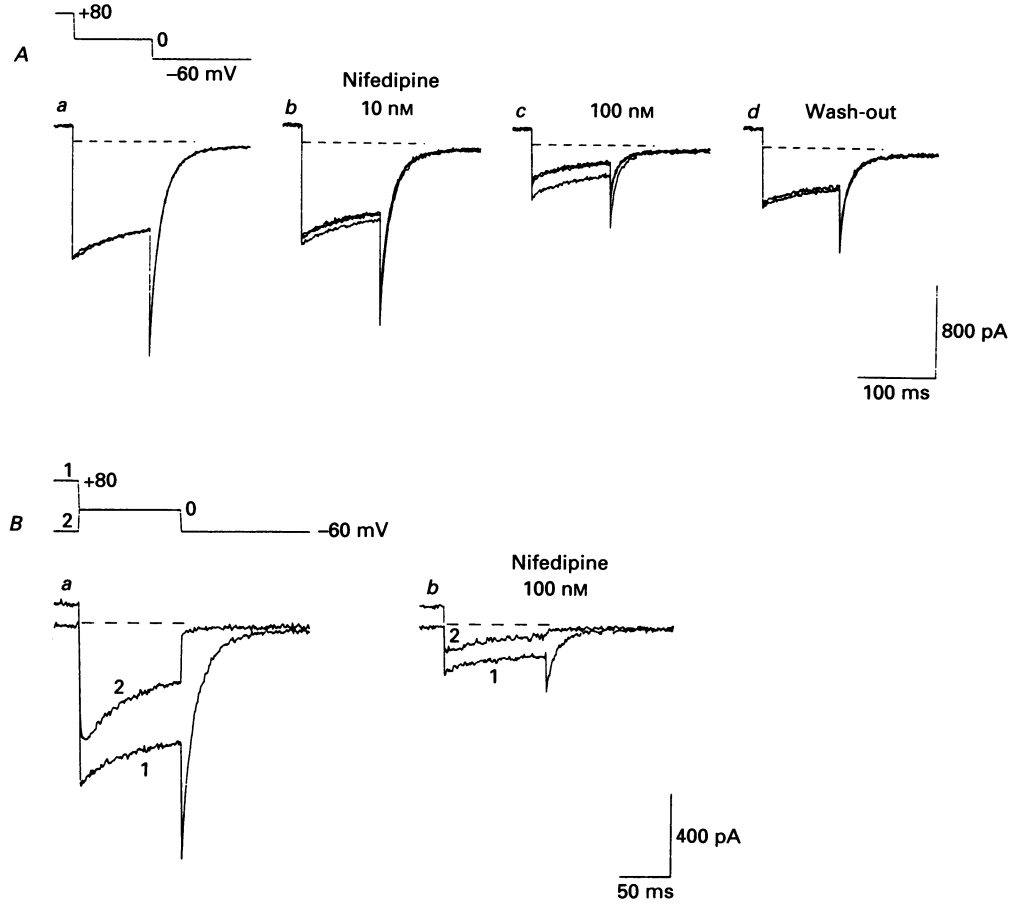

Fig. 4. Effects of nifedipine on membrane currents. *A*, the following voltage sequence was applied every 2 min: 5 s at +80 mV followed by 100 ms at 0 mV to look at the inward current, followed by repolarization to the holding potential to elicit the tail current. After two control current sequences were observed (*a*), 10 nM (*b*) and 100 nM nifedipine (*c*) were cumulatively applied for 6 min each. Nifedipine was washed out for 8 min. The first and fourth membrane currents following wash-out are shown in *d*. In each trace, the dotted line represents the 0 current level. *B*, the same voltage sequence shown in *A* and a simple depolarizing step to 0 mV (100 ms) were alternately repeated at 2 min intervals. After observing the control currents (*a*), the effect of nifedipine (100 nM) was examined on the resultant inward currents. The current traces shown in *b* were obtained 2 min (1) and 4 min (2) after an exposure to nifedipine.

simple depolarizing step to 0 mV is compared with the effect on the current after preconditioning at +80 mV. Again, both currents are affected to a similar extent (70% reduction, preconditioned; 76% simple). The correlation of the inhibition

ratios between the inward currents (at 0 and −60 mV) is shown in Fig. 5*B*. The results obtained from a different cell have also been plotted (open symbols). In this cell, 1 μM nifedipine reduced the inward current to 16% of its control amplitude and by the end of the 8 min wash-out it had recovered to 23% of the original amplitude.

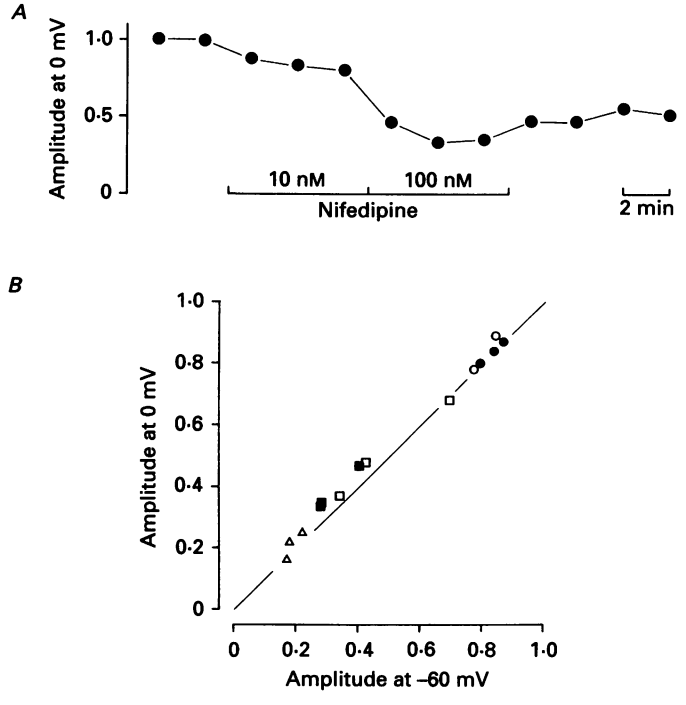

Fig. 5. Inhibitory ratios of nifedipine calculated from the experiments shown in Fig. 4*A*. *A*, the time course of the changes in the amplitude of inward current at 0 mV. *B*, the correlation between the inhibitory effect of nifedipine on the inward current amplitudes at 0 and −60 mV. The currents were normalized by each control inward current obtained in normal solution. The filled data points were obtained from the experiment shown in *A*. Open data points were taken from a different cell. ○, □ and △ represent the inhibition ratios with 10^{-8}, 10^{-7} and 10^{-6} M nifedipine, respectively. The continuous line has a correlation coefficient = 1.

It can be seen from Fig. 5*B* that all points lie close to a line with unity slope. The concentration of nifedipine required to produce half-maximal inhibition was between 10 and 100 nM for both of the inward currents. These results indicate that the inward currents are mainly due to Ca^{2+} influx through L-type Ca^{2+} channels.

In three cells, the effect of caffeine was examined using the same experimental protocol as for nifedipine (shown in Fig. 4*A*). The sequence of voltage steps was repeated at 2 min intervals, and 10 mM caffeine was applied for 4 min after observing the control currents. Figure 6 shows an example of the changes in membrane current which occurred following the addition of caffeine. Caffeine (10 mM) reduced the amplitudes of both of the inward currents recorded at 0 and −60 mV by similar ratios (22–24%). The outward current recorded at +80 mV was not significantly

changed. The amplitude of both inward currents recovered to control values within 6 min after wash-out. In two other cells, caffeine caused a larger reduction (by 30–60%). This reduction was not fully reversed by wash-out. The incomplete recovery of inward currents was presumably due to cell deterioration, because a

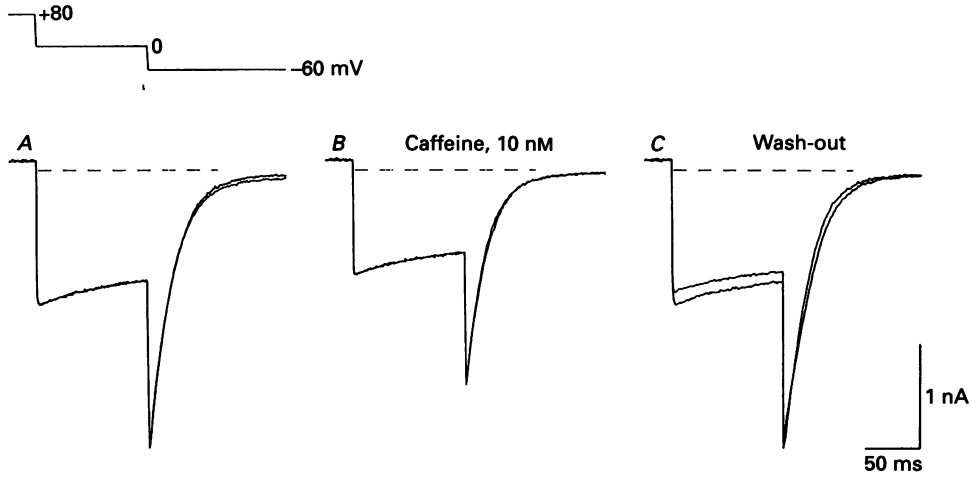

Fig. 6. Effects of caffeine. The same voltage sequence as Fig. 4*A* was repeated at 2 min intervals. After two control currents were observed (*A*), caffeine (10 mM) was applied for 4 min (*B*). Caffeine was washed out for 10 min. The first and the third membrane currents following wash-out are shown (*C*).

similar reduction in both inward currents was often observed after rupture of the cell membrane.

The effect of TTX (tetrodotoxin) was examined using the same voltage steps. There was no significant change recorded at the three membrane potentials (+80, 0 and −60 mV) after addition of 3·1 μM TTX, suggesting negligible contribution of voltage-dependent Na^+ channels.

The evidence thus suggests that the tail currents are in fact flowing through the L-type Ca^{2+} channels. The increase in size of the tail currents that occurs as the duration of the preconditioning depolarization is increased could either indicate that there was a progressive increase in the Ca^{2+} conductance with time at +80 mV, or that there was a progressive slowing of the deactivation of the channels at the holding potential. In the latter case, after short exposures to +80 mV most of the channels would deactivate so rapidly at −60 mV that there would not be time for the full size of the inward tail current to be recorded, as seen in Fig. 1. In Fig. 7 the membrane potential was stepped to +20 mV for 100 ms after various durations of conditioning step to +80 mV. On stepping to this potential, the channels should still be maximally activated, but there will be a driving force causing an inward Ca^{2+} current. It can be seen that the peak amplitude of the inward current recorded at +20 mV was little changed by increasing the duration of the +80 mV voltage step. This is consistent with the second alternative given above, i.e. that there is not a progressive increase in the Ca^{2+} conductance with time at +80 mV. At +20 mV

there is then some inactivation of the inward current, presumably caused by the entry of Ca^{2+} ions. The extent and time course of this inactivation is again not greatly affected by the duration of the conditioning pulse, but the size of the tail current on subsequently stepping back to the holding potential shows a clear dependence on the

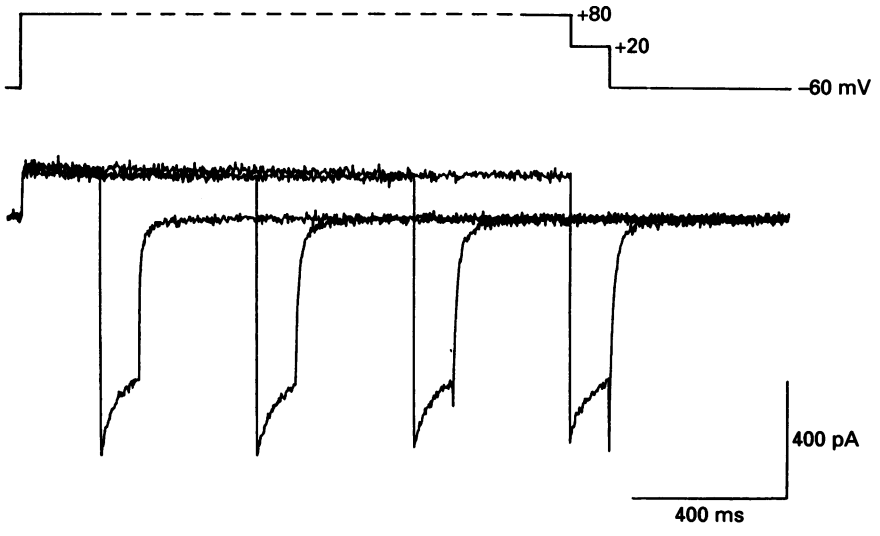

Fig. 7. The effect of the duration of the preconditioning potential on the inward current and tail current. The cell membrane was depolarized to +80 mV for variable durations, and then returned to +20 mV (100 ms duration) before returning to −60 mV. The duration was increased from 200 to 1400 ms by 400 ms steps. The sequence was repeated at 90 s intervals. Note that the inward current does not increase with time, but the tail current does.

duration of the +80 mV step, increasing in size with much the same time course as occurred for the tail current in Fig. 1. Similar results were obtained in another cell. These results again demonstrate that the voltage-dependent kinetics of the open channel change with time at +80 mV.

In Fig. 8, the inward currents and tail currents seen during and after a depolarizing test potential are compared in cells preconditioned for 5 s at +80 mV with those held at −60 mV. In both of these conditions, little Ca^{2+} should be entering before the test potential. At a test potential of 0 mV, the inward current evoked was larger in the preconditioned cell, probably because at 0 mV the current is not quite fully activated. However, at test potentials more positive than +20 mV, the inward currents evoked were very similar, both in size and rate of inactivation (during 100 ms), suggesting that there was little difference in either the conductance or Ca^{2+}-dependent inactivation of the Ca^{2+} channels open at the beginning of the test potential after preconditioning at +80 mV and those opened on simply stepping from the holding potential to the test potential. In some other experiments, however, the rate of inactivation was sometimes slightly slower after preconditioning at +80 mV. In contrast, the tail currents on returning to the holding potential were markedly different in the two instances, and this difference was always seen.

Although the amplitude of both the conditioned and unconditioned tail currents was enhanced by increasing the test potential, the conditioned tail currents were much larger, and their deactivation slower. The time constants of the unconditioned tail currents in this experiment were less than 2 ms (single exponential fitting), while

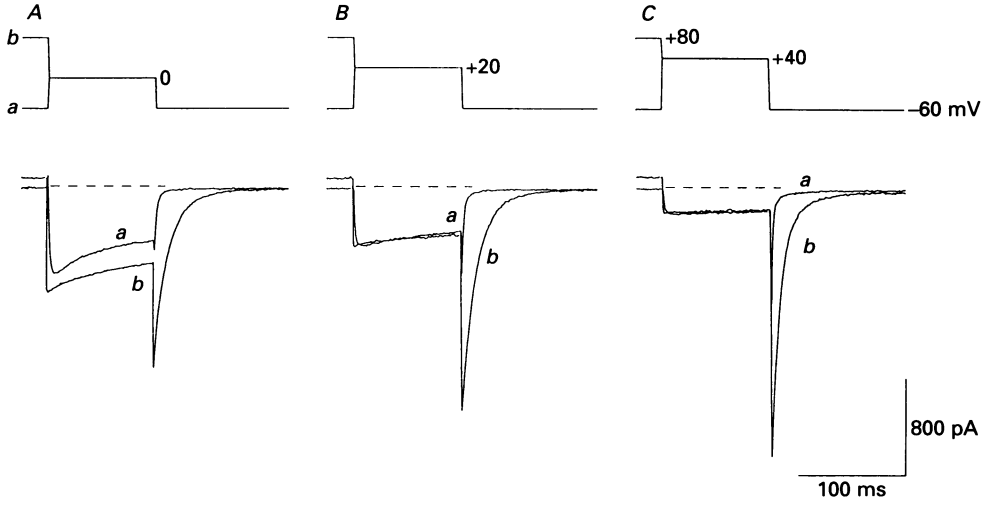

Fig. 8. The effects of changing the test potential on the inward and tail currents with and without a preceding 5 s conditioning depolarization to +80 mV. The test potential (100 ms) was elevated from 0 mV (*A*) to +20 mV (*B*) and +40 mV (*C*). The last 25 ms of the conditioning potential is shown. Dashed lines represent the 0 current level. Each sequence of voltage steps was applied at 90 s intervals, and the experiments were performed from (*C*) to (*A*).

those of the conditioned tail currents were approximately 10 ms. Similar results were obtained in three more experiments. The different characteristics of the tail currents indicate that two different processes are involved in the closure of voltage-dependent Ca^{2+} channels.

I–V relationship of the Ca^{2+} channels

Since a long depolarizing prepulse (+80 mV for 5 s) seems to put the Ca^{2+} channels into a steady state in which their kinetics of deactivation are slow, this is an excellent condition in which to measure the current–voltage relationship of the open channel, without the risk of losing some current because of the rapid deactivation that occurs repolarizing from less positive voltages. Figure 9*A* shows an example of the membrane currents evoked by various repolarizing voltage steps (100 ms) which were applied before returning the membrane potential to −60 mV. The amplitudes of the peak inward currents are plotted against potential in Fig. 9*B*. The *I–V* relationship was nearly linear between −60 and +30 mV. Similar results were obtained in other experiments ($n = 6$). Repolarization of the membrane to potentials between −80 and −100 mV did not produce a linear increase in amplitude of the inward current, probably because, as can be seen, deactivation is considerably speeded up at more negative potentials. To fit the *I–V* relationship of the currents

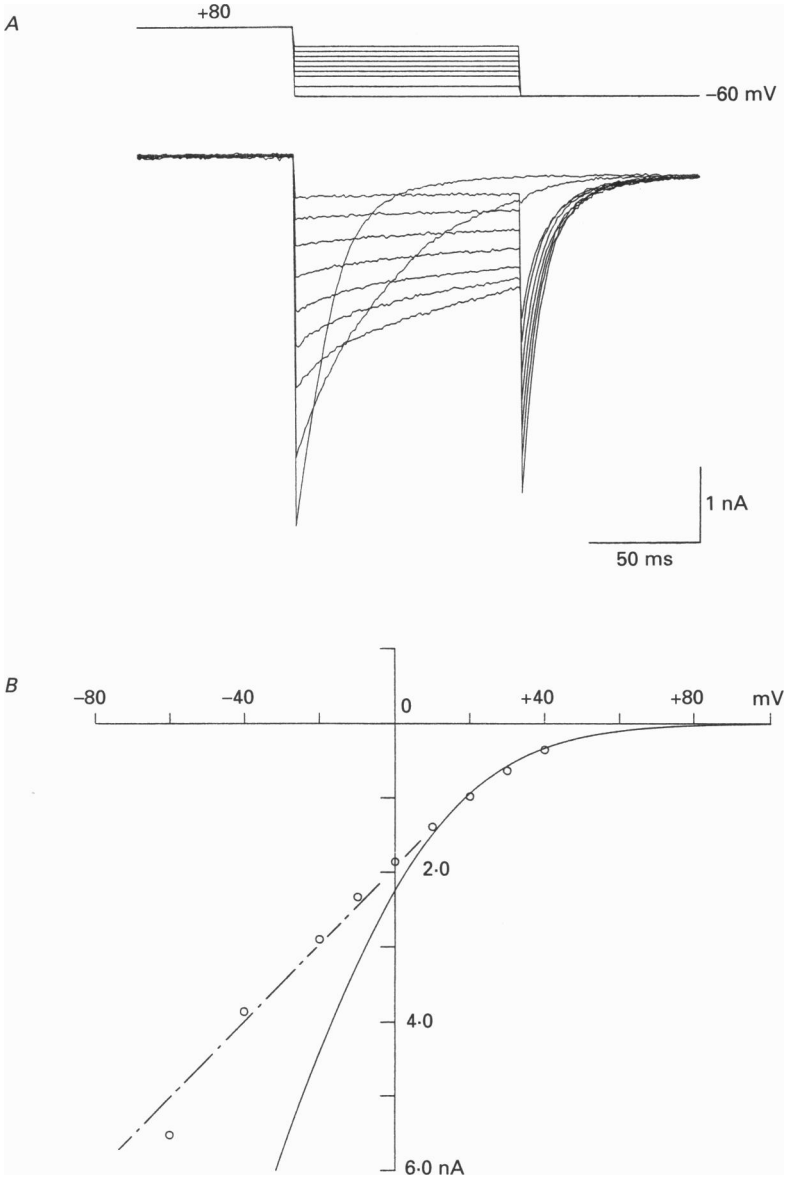

Fig. 9. Measurement of the instantaneous current–voltage relationship of the open channel. *A*, after a 5 s conditioning potential of +80 mV, the membrane potential was repolarized to variable potentials for 100 ms then returned to −60 mV. The variable potentials were applied in the following order: +40 to −20 in −10 mV steps followed by −40 and −60 mV. The sequence of potentials was repeated at 90 s intervals. The last 70 ms of the +80 mV depolarization is shown. *B*, the amplitude of the inward current is plotted against the potential. The peak amplitudes at −40 and −60 mV were estimated by single exponential fitting. The curve (continuous line) was drawn based on eqn (1) (see text), with $[Ca^{2+}]_i$, 100 nM; P_{max}, 4·7 pl/s. The dashed line was fitted by eye.

after large depolarization, we tried to apply a standard equation for the constant field theory:

$$I_{Ca} = P_{max} zF(E/G)([Ca^{2+}]_i - [Ca^{2+}]_o \exp(-E/G))/(1-\exp(-E/G)), \quad (1)$$

where (I_{Ca}) is the amplitude of the Ca^{2+} current, G represents RT/zF (z, F, R and T having their usual thermodynamic meanings), and $[Ca^{2+}]_i$ and $[Ca^{2+}]_o$ are intracellular and extracellular Ca^{2+} concentrations, respectively. P_{max} is the maximal Ca^{2+} permeability. All the channels should be maximally activated by the long prepulse to +80 mV. This relationship is plotted by the continuous line in Fig. 8*B*. In none of the experiments, using a reasonable set of parameters, did this produce a good fit for the data.

The decay time courses of the inward currents at −40 and −60 mV could be described by single exponentials and the time constants were 41 and 14 ms, respectively. The factors determining this time course are, however, likely to be more complicated than this suggests, since both calcium-dependent inactivation and voltage-dependent deactivation will occur.

DISCUSSION

In Cs^+-loaded single smooth muscle cells from guinea-pig urinary bladder, we have found that the membrane currents obtained by simple step depolarization showed a similar voltage dependence to that reported by Klöckner & Isenberg (1985). We also noted that on repolarizing from positive potentials, tail currents develop with amplitudes related to the size of the depolarizing step, and in this paper we have examined the properties of these tail currents.

We have ruled out the possibility that the tail currents are through non-selective cation channels of the type opened by agonists, through Ca^{2+}-activated Cl^- channels or through Na^+–Ca^{2+} exchange. Our results strongly suggest that the mechanism responsible for the inward tail current is Ca^{2+} entry through the same L-type Ca^{2+} channels that carry the inward currents in response to a simple depolarization. Both currents are dose-dependently blocked in an identical manner by the calcium antagonist drug nifedipine, and by caffeine. Caffeine, which induces Ca^{2+} release from sarcoplasmic reticulum (Endo, 1977), reversibly decreased the amplitudes of the currents by the same degree. This is consistent with the notion that both currents show Ca^{2+}-dependent inactivation. However, the direct blocking action of caffeine on voltage-dependent Ca^{2+} channels (Martin, Dacquet, Mironneau & Mironneau, 1989; Hughes, Hering & Bolton, 1990) may also have been involved in the inhibition. Either explanation supports identification of the inward tail current. The high inhibitory potency (10 nM < IC_{50} < 100 nM) of nifedipine on all the inward currents suggests that L-type Ca^{2+} channels are responsible for them (Bean, 1989). In ureteral and vascular single smooth muscle cells, only L-type Ca^{2+} channels have been reported to contribute to the voltage-activated inward currents (Lang, 1990; Matsuda, Volk & Shibata, 1990). It seems unlikely that T-type voltage-dependent Ca^{2+} channels make a significant contribution to the inward currents observed in the present experiments, because the T-type current shows rapid inactivation during large depolarizations (Benham, Hess & Tsien, 1987; Aaronson, Bolton, Lang & MacKenzie, 1988).

If both the inward currents are indeed through the same channels, then our results clearly demonstrate that when the cell membrane is subjected to large depolarizations, there is a voltage- and time-dependent change in the channels which results in the development of the tail currents on repolarization. This change takes between 1 and 2 s to complete at +80 mV. There seem to be several possible explanations: a second population of channels with slower kinetics may open; the channels opened initially may change their properties so as to increase their conductance; there may be a change in the kinetics such that the channels deactivate more slowly on repolarization, or there may be a combination of these changes. It has been possible to eliminate some of these possibilities by choosing the correct conditions. For instance, we can show that there is no significant increase in the membrane conductance with time at +80 mV after the initial channel activation even though the channel properties are clearly changing. We can examine this by a careful comparison of the peak inward currents flowing during steps to +20 and +40 mV (potentials at which maximum activation of the inward current is seen, and there is still an inward driving force for the current) with and without a long +80 mV conditioning potential (conditioned and unconditioned inward currents). Under these conditions the currents are virtually identical (Fig. 9), neither is the initial inward current changed in size if the duration of the conditioning potential is varied (Fig. 7). This suggests that there is not a progressive increase in the Ca^{2+} conductance of the membrane, and makes it unlikely that a second set of calcium channels is opening. It is also unlikely that there is a change in the calcium conductance of the individual channels, or that there is an increase in the number of channels open.

It appears therefore that we are dealing with L-type Ca^{2+} channels which change their kinetic properties but not their individual conductances during long depolarizations. To fit the results we need a model in which progressive exposure to positive potentials changes channel kinetics so that, on stepping out of the channel activation range, the rate of deactivation is slowed. A model which would have these properties would include more than one open state. The simplest, leaving out inactivation, would be:

$$C \underset{\beta_1}{\overset{\alpha_1}{\leftrightarrow}} O_1 \underset{\beta_2}{\overset{\alpha_2}{\leftrightarrow}} O_2,$$

where C is closed state; O_1 and O_2 are open states 1 and 2 and α_1, α_2, β_1 and β_2 are voltage-dependent rate constants. If the transitions between the three states are voltage sensitive and depolarization shifts the equilibrium to the right, there will be a time- and voltage-dependent increase in the number of channels in the O_2 configuration. If at the holding potential (−60 mV) the time constant of the $O_1 \rightarrow C$ transition is fast in comparison with the resolving time of the apparatus, then after depolarizations which either because of their size or duration, result in few channels in the O_2 configuration, very little tail current will be recorded. Larger and longer depolarizations will result in more channels in the O_2 configuration, and on repolarization the channels will stay open long enough because of the $O_2 \rightarrow O_1$ transition for sufficient inward current to flow to be seen as a tail current before deactivation occurs.

This model would suggest that the time course of deactivation of the tail current should be the sum of two exponential terms, and that the size of the slower

component should become relatively greater with time and size of the conditioning potential. In fact, most of the experimental curves can be adequately fitted with a single exponential, with an increase in time constant. There are several problems, however, with this type of analysis: firstly, curves which are the sum of two exponentials can only clearly be analysed graphically as the sum of two components if the time constants differ by severalfold, which may not be the case here, and secondly with our experimental set-up we are unable to adequately resolve fast components of deactivation because of limitations of the apparatus. This becomes particularly important with small tail currents, where the only resolvable part of the curve will be the small slow component, and fitting with a single exponential may give a spuriously long time constant. Table 2 shows the average effects of the size and duration of the conditioning potential on the single time constant fitted to the tail-current decay in a number of cells.

Although the simple model would suggest an increase in the number of channels open with time at depolarized potentials, the experiment shown in Fig. 7 suggests that at +80 mV, such a progressive increase in the number of channels open does not, in fact, occur. This is presumably because stepping to +80 mV produces almost maximal activation of the channels, so that any subsequent increase in the channel open probability would not be detectable. It is unfortunately not technically possible to look at the effects on the number of channels activated of the duration of conditioning potentials which initially produce less than maximal activation, since at these potentials Ca^{2+} entry and Ca^{2+}-dependent inactivation of the channels occurs.

Another rather different model which might explain our results is the allosteric model proposed by Marks & Jones (1992). This model is based on the knowledge that there are four repeated homologous domains of the calcium channel, and the suggestion that each domain possesses a voltage sensor with two states. Depolarizations result in shifting of the voltage sensors, and there are thus five closed and five open channel states, with voltage-independent transitions between the closed and open states theoretically possible.

The models also do not incorporate inactivation. We have shown (Fig. 8) that the conditioned and unconditioned inward currents in the fully activated potential range inactivate with a rather similar time course. Since inactivation is mainly Ca^{2+} dependent during a short depolarization (Nakayama & Brading, 1993), this suggests that the channels mediating the conditioned and unconditioned inward currents are affected similarly by Ca^{2+} ions. However, there is some indication that the conditioned inward current actually inactivates somewhat more slowly than the unconditioned one, and there is thus a possibility that there is some difference in either Ca^{2+}-dependent or voltage-dependent inactivation of the two currents.

In order to model the behaviour of the channels, it is necessary to have some information about the current–voltage relationship of the individual channels. We first attempted to fit the voltage dependence of the amplitude of the peak inward current elicited by simple depolarizations, using the constant field theory and the Boltzmann distribution, because of the lack of evidence about how single unit conductance is affected by changing the membrane potential at a physiological concentration (2·5 mM) of Ca^{2+}. The development of a slowly closing channel state

allowed us to measure the *I–V* relationship of the open Ca^{2+} channels directly. The relationship was close to linear over a wide voltage range, agreeing well with the ohmic conductance of single voltage-dependent (L-type) Ca^{2+} channels (Hess, Lansman & Tsien, 1986), measured at higher concentrations of Ca^{2+} or Ba^{2+}.

When the membrane potential was repolarized to -80 and -100 mV, the amplitude of the tail current did not increase linearly. The faster deactivation time course in this voltage range presumably prevented accurate estimation of the tail current amplitude at $t = 0$. An alternative explanation for this non-linear increase in amplitude of the tail current is that at more negative potentials there is a greater block by Mg^{2+} of the voltage-dependent Ca^{2+} channel conductance (Fukushima & Hagiwara, 1985; Lansman, Hess & Tsien, 1986).

If the channel behaviour follows the constant field theory, eqn (1) should predict the relationship. The results show that the data cannot be fitted in this manner (Fig. 9*B*), suggesting that the constant field theory does not fully apply to the voltage-dependent Ca^{2+} channels (Hagiwara & Ohmori, 1982) at least when the channels are in a long open state induced by a large depolarization. In our preliminary experiments, we obtained an *I–V* relationship using a simple depolarizing step and the peak amplitude of the resultant inward current. If the voltage dependence of the unit conductance for the normal open state is similar to that for the long open state, the following simple ohmic equation may provide a good fitting:

$$I_{Ca} = P_{Ca}(E - E_{rev}), \tag{2}$$

being combined with a Boltzmann equation. However, using this equation, the predicted degree of activation close to the reversal potential (E_{rev}) deviated from the observed results suggesting that the *I–V* relationship of single channel conductance is not fully linear and may follow constant field theory at higher potentials ($+10$ to $+40$ mV). This fact is also true for Fig. 9*B*. The best fit of the *I–V* relationship for the Ca^{2+} channel current may require a combination of linear (eqn (2)) and curved (eqn (1)) conductances.

In conclusion, in single smooth muscle cells isolated from guinea-pig urinary bladder, the inward currents evoked by step depolarizations from the holding potential of -60 mV, and the tail currents occurring on stepping back to the holding potential, can both be attributed to L-type Ca^{2+} channels. The channels show Ca^{2+}-dependent inactivation and have a high sensitivity for the dihydropyridine Ca^{2+} antagonist, nifedipine. The difference between conditioned and unconditioned tail currents predicts the presence of at least two open states of the voltage-dependent Ca^{2+} channels.

This work was carried out during the tenure of a grant from Bristol Myers Squibb. The authors are indebted to Dr Derek Terrar for stimulating discussions and also grateful to Drs Narelle Bramich and Ryuji Inoue for helpful comments on the manuscript.

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