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CALCIUM-DEPENDENT INACTIVATION OF POTENTIAL-DEPENDENT CALCIUM INWARD CURRENT IN AN ISOLATED GUINEA-PIG SMOOTH MUSCLE CELL

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SUMMARY

1. Calcium current (I_{Ca}) was studied in single isolated smooth muscle cells of a guinea-pig taenia caeci dialysed with Cs^+-containing solution to suppress K^+ outward current.

2. With increasing step depolarizations up to +10 mV, acceleration of I_{Ca} inactivation was observed. With further increase of step depolarization, I_{Ca} inactivation was slowed down. The largest I_{Ca} (observed at +10 mV) was characterized by the maximal speed of inactivation.

3. Comparison of I_{Ca} in different external concentrations of Ca^{2+} ions ($[Ca^{2+}]_o$) revealed that at the same membrane potential the time course of I_{Ca} inactivation was slower, the smaller the amplitude of I_{Ca}. Slowing down of I_{Ca} inactivation was observed also during its partial block by Co^{2+} ions.

4. Elevation of temperature increased I_{Ca} peak amplitude and accelerated its decay. The amplitude of I_{Ca} was increased by a factor of $1{\cdot}7 \pm 0{\cdot}14$ ($n = 6$) when the temperature was raised by 10 °C.

5. Calculations of Ca^{2+} entry during I_{Ca} as a time integral of Co^{2+}-sensitive current, and comparison with the degree of I_{Ca} inactivation, showed that inactivation was tightly related to Ca^{2+} entry in the membrane potential range −20 to +40 mV.

6. Ba^{2+} current through Ca^{2+} channels was larger than I_{Ca} and its inactivation was considerably slower.

7. Recovery of I_{Ca} from inactivation was found to be potential dependent. When the cell membrane was hyperpolarized, I_{Ca} recovery was accelerated.

8. It was concluded that inactivation and recovery of I_{Ca} in smooth muscle cells were influenced by both Ca^{2+} entry and membrane potential. It was also pointed out that the observed events are difficult to explain by the hypothesis that inactivation was produced simply by accumulation of Ca^{2+} ions near the inner side of the membrane, and that recovery was due to lowering of internal free Ca^{2+} ion concentration ($[Ca^{2+}]_i$).

INTRODUCTION

During the last few years some progress has been achieved in our knowledge of mechanisms controlling Ca^{2+} entry into smooth muscle cells during depolarization.

This has been mainly due to the application of the patch-clamp technique to single isolated smooth muscle cells, which has allowed quantitative measurement of potential-sensitive Ca^{2+} inward current (Ganitkevich, Smirnov & Shuba, 1985, 1986; Klöckner & Isenberg, 1985; Mitra & Morad, 1985; Droogmans & Callewaert, 1986).

There are some differences between the results of these studies, which are probably due to the different smooth muscle preparations used. For example, in taenia (Ganitkevich *et al.* 1985) and in gastric smooth muscle cells of the guinea-pig (Mitra & Morad, 1985) maximal Ca^{2+} current (I_{Ca}) was observed in the membrane potential range +10 to +20 mV, while in ileum smooth muscle cells (Droogmans & Callewaert, 1986) and in urinary bladder of the guinea-pig (Klöckner & Isenberg, 1985) it was observed at membrane potentials close to −10 mV. Also, it was shown that I_{Ca} inactivation in taenia was not complete in 10 s (Ganitkevich *et al.* 1986), while in other studies inactivation of inward current was suggested to be complete in 3 s (Droogmans & Callewaert, 1986) and in 2 s (Klöckner & Isenberg, 1985). In these studies I_{Ca} inactivation was characterized by a steady-state inactivation curve, as for a purely voltage-dependent conductance. At the same time it was suggested that a component of I_{Ca} inactivation was mediated via Ca^{2+} entry (Ganitkevich *et al.* 1986). It is doubtful if fundamental differences between I_{Ca} inactivation mechanisms in various smooth muscle preparations exist. Moreover, a U-shaped dependence of the second time constant of I_{Ca} inactivation on membrane potential (Klöckner & Isenberg, 1985) and a slowing down of inward current decay when Ca^{2+} ions were changed for Ba^{2+} ions in the external solution (Droogmans & Callewaert, 1986) were shown. These observations also may indicate the presence of a Ca^{2+}-dependent component in I_{Ca} inactivation in smooth muscle cells.

In this work we present evidence that at least a large part of I_{Ca} inactivation in taenia smooth muscle is associated with Ca^{2+} entry into the smooth muscle cell. Nevertheless, membrane potential exerts a significant effect on the recovery of I_{ca} from inactivation.

METHODS

The techniques of smooth muscle cell isolation and Ca^{2+} current recording did not differ from those already described (Ganitkevich *et al.* 1986). In some experiments, series-resistance compensation was used and series resistance was effectively decreased to 2 MΩ. Also, Sylgard coating of the pipettes was used to improve the frequency response.

A standard external solution of the following composition was used (mM): 110, NaCl; 2·5, $CaCl_2$; 10, HEPES; 20, CsCl; and 6, glucose. It was adjusted to pH = 7·2 with TEA-OH. In some experiments (shown in Figs 2, 3*B* and 5) external solution contained in addition 5 mM-$MgCl_2$.

Changing the external solutions was performed according to the technique of Krishtal & Pidoplichko (1980) as modified by Fenwick, Marty & Neher (1982). Briefly, a U-shaped glass tube with a hole of 50–100 μm in diameter on the tube base was placed at a distance of 100–200 μm from the cell. Test solution flowed through the tube. A flow rate was created by peristaltic pumps so that outlet flow was twice inlet flow. When the outlet flow was interrupted, the test solution was applied to a cell. Efficiency of the solution change was tested in two ways: (i) block of I_{Ca} during a continuous depolarizing step (5 s duration) by Co^{2+} ions (5 mM) was developed in 0·2–0·5 s and (ii) I_{Ca} disappeared in 0·5–1·5 s when Ca^{2+}-free solution containing 5 mM-$MgCl_2$ was applied. The time for exchange of the external solution was found to depend on the flow rate of the solution in the tube. Usually the records were taken 10 s after changing the external solution.

The temperature was changed by application to the cell of heated standard external solution. Temperature was measured by a microthermistor directly near the hole of the tube. When the heated solution was applied, I_{Ca} increased in amplitude and subsequently no detectable change of I_{Ca} was observed during at least 1 min. This indicates rapid equilibration of the cell with the heated solution.

The solutions containing different Ca^{2+} or Ba^{2+} concentrations were made by adding the appropriate amounts of $CaCl_2$ or $BaCl_2$ to the external Ca^{2+}-free solution.

Internal solution of the following composition was used (mM): 130, CsCl; 5, HEPES; 10, EGTA; 0·1, cyclic AMP; 5, Na_2ATP; and 2·25, $MgCl_2$. Its pH was adjusted to 7·2 with TEA-OH. ATP, cyclic AMP and Mg^{2+} ions were added to this solution since their effect on I_{Ca} stability in a variety of preparations has been shown (Fedulova, Kostyuk & Veselovsky, 1981; Doroshenko, Kostyuk & Martynyuk, 1982; Irisawa & Kokubun, 1983; Bechem & Pott, 1985; Forscher & Oxford, 1985; Byerly & Yazejian, 1986).

Holding potential was maintained at −50 mV throughout the experiments. The temperature was 22–24 °C, except where otherwise indicated.

RESULTS

Inactivation of I_{Ca} at different membrane potentials

The time course of I_{Ca} inactivation over a wide range of membrane potentials was analysed in smooth muscle cells which were characterized by a relatively small outward non-specific current (I_{ns}) observed after suppression of I_{Ca} by Co^{2+} ions (4 or 5 mM). The reason for this was the following: we suggest that if I_{ns} was small, the Ca^{2+}-dependent component of I_{ns} (sensitive to Co^{2+} ions) was probably also small, so I_{Ca} was minimally contaminated by this type of conductance. In such cells, the decay of inward current was probably due to decline of Ca^{2+} conductance.

The current–voltage relationship of a peak inward current and current traces before and after addition of 4 mM-Co^{2+} are presented in Fig. 1. As can be seen from the current–voltage relationship, significant deviation from linearity of transmembrane current in the presence of Co^{2+} ions was observed at potentials more positive than +50 mV. Note that leak current was not subtracted.

Step depolarization of the cell membrane to a level exceeding −30 mV elicited I_{Ca}. Increasing step depolarization was accompanied by an increase of I_{Ca} peak amplitude and decrease in the half-time of its decay (indicated by arrows, Fig. 1*A*) if the level to which the membrane was depolarized did not exceed +10 mV. Assuming a purely potential-dependent mechanism of I_{Ca} inactivation one can expect that further depolarization of the membrane (to levels above +10 mV) will be accompanied by acceleration of the inactivation process. However, as can be seen from the current traces (Fig. 1*A*), increasing the amplitude of the step depolarization resulted in slowing down the decay of I_{Ca} together with a decrease of its peak amplitude. Similar changes in time course of I_{Ca} inactivation over a wide range of membrane potentials were observed in cardiomyocytes by Lee, Marban & Tsien (1985).

It must be noted, however, that in some cells acceleration of inward current inactivation was observed at potentials more positive than +20 to +40 mV (for example see Ganitkevich *et al.* 1986; Fig. 6*A*, depolarization to +50 mV). Blocking of I_{Ca} by Co^{2+} ions revealed significant I_{ns} at these potentials. In our opinion, acceleration of inactivation of the inward current, observed in some cells at large positive potentials, was presumably due to a relatively large component of the Ca^{2+}-dependent outward current which distorted the time course of I_{Ca} inactivation.

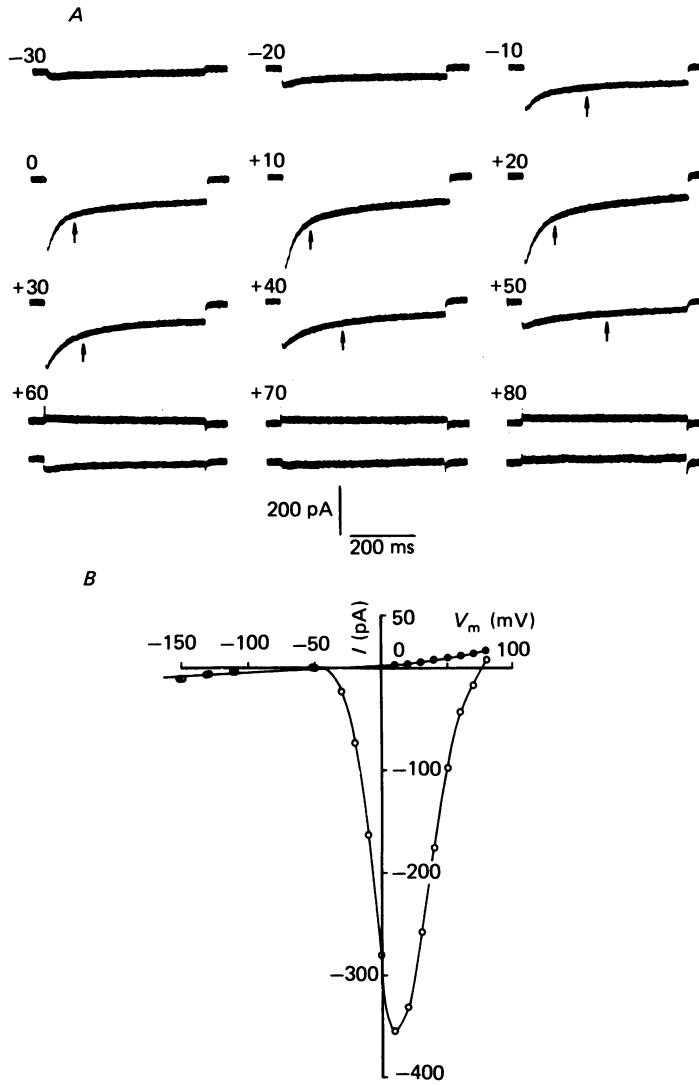

Fig. 1. *A*, current traces of I_{Ca} at different membrane potentials (indicated in millivolts near each current trace). Arrows indicate the amplitude of I_{Ca} equal to a half of its peak. For potentials +60, +70 and +80 mV upper and lower traces show currents after and before addition of 4 mM-Co^{2+}, respectively. *B*, current–voltage relation of the peak of I_{Ca} from the same cell as in *A*. Open and filled circles show the peak amplitude of current before and after addition of 4 mM-Co^{2+}, respectively. Leak current was not subtracted. V_m, membrane potential.

The probability of Ca^{2+}-dependent channels (in physiological conditions they are K^+ channels) being open at a constant $[Ca^{2+}]_i$ was greatly increased at large positive potentials (Bolton, Lang, Takewaki & Benham, 1985).

Thus, at potentials more positive than those corresponding to maximal I_{Ca}, inactivation of Ca^{2+} current was usually slowed down. One possible explanation of this

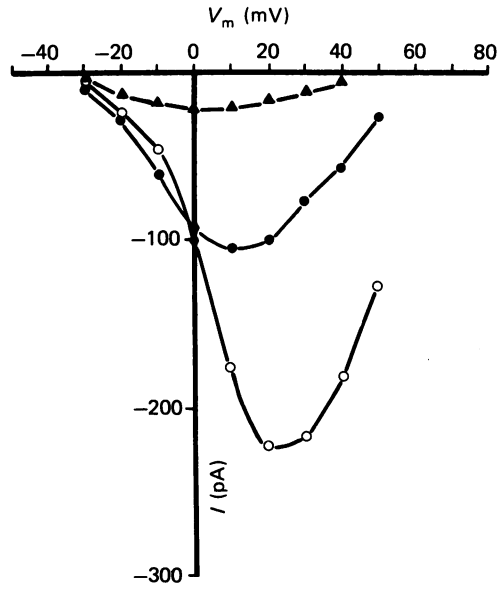

Fig. 2. Current–voltage relationship of the peak of I_{Ca} in different external Ca^{2+} ion concentrations: 0·5 (triangles), 2·5 (filled circles) and 20 mM (open circles). V_m, the membrane potential.

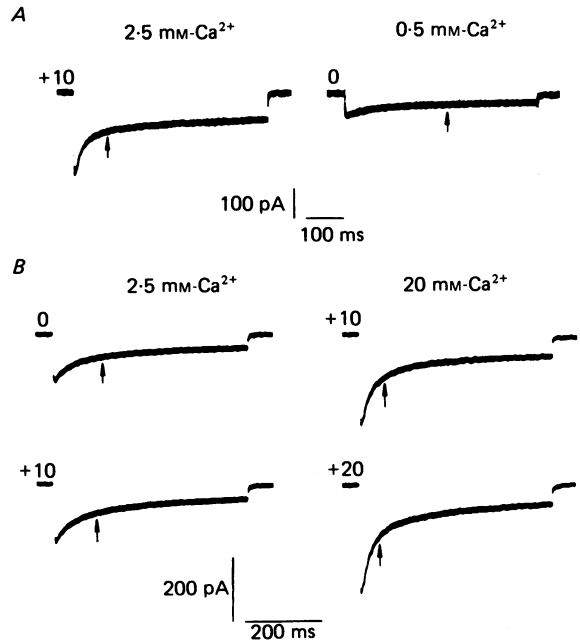

Fig. 3. Effect of external Ca^{2+} on I_{Ca} inactivation. *A*, current traces of I_{Ca} at membrane potentials (indicated in millivolts near each current trace) corresponding to the peak of current–voltage curves in 2·5 and 0·5 mM-external Ca^{2+}. *B*, current traces of I_{Ca} elicited by step depolarization to the membrane potential indicated near each current trace in millivolts in 2·5 and 20 mM-Ca^{2+} in the external solution. *A* and *B* from different cells. Half-amplitude of each I_{Ca} was indicated by arrow.

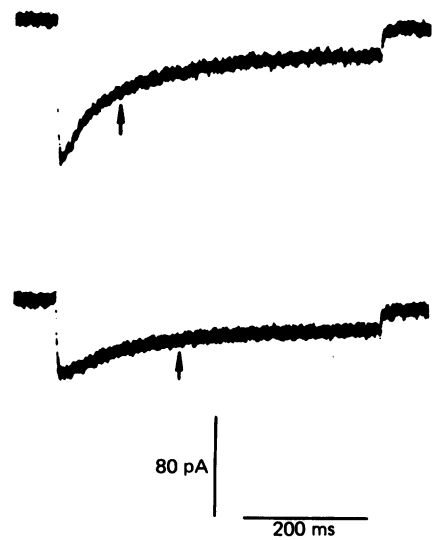

Fig. 4. Suppression of I_{Ca} by Co^{2+} ions. I_{Ca} at 0 mV before (upper trace) and after (lower trace) addition of 0·4 mM-Co^{2+}. Half-amplitude of each I_{Ca} was indicated by arrow.

phenomenon is that inactivation of I_{Ca} was related to Ca^{2+} entry, since the larger the amplitude of I_{Ca}, the faster was its inactivation (depolarization to +10 mV, Fig. 1*A*). Another way to evaluate this dependence is the change of external Ca^{2+} ion concentration.

Effect of changing $[Ca^{2+}]_o$ *on* I_{Ca} *and its inactivation*

In these experiments we used three external Ca^{2+} ion concentrations: normal (2·5 mM), low (0·5 mM) and high (20 mM). When $[Ca^{2+}]_o$ was changed, the maximal peak amplitude of I_{Ca} was changed and shifts of the current–voltage relationship of I_{Ca} (current–voltage curve) along the voltage axis were always observed (Fig. 2). It is now generally accepted that the voltage shift of the current–voltage curve of I_{Ca} observed when $[Ca^{2+}]_o$ is changed, is due to a change in surface membrane potential (Ohmori & Yoshii, 1977; Kostyuk, Mironov, Doroshenko & Ponomarev, 1982; Wilson, Morimoto, Tsuda & Brown, 1983; Cota & Stefani, 1984; Byerly, Chase & Stimers, 1985). Shifts of both steady-state activation and relative inactivation curves with changing $[Ca^{2+}]_o$ have been shown for Ca^{2+} channels (Wilson *et al.* 1983; Lee *et al.* 1985). It is generally assumed that voltage-sensitive gating of Ca^{2+} channels is not modified by changes in external Ca^{2+} concentration, so maximal I_{Ca} is observed at the same transmembrane potential and measured shifts are entirely related to the changes in surface membrane potential.

For these reasons we compared amplitude and inactivation kinetics, when $[Ca^{2+}]_o$ was 2·5 mM and when $[Ca^{2+}]_o$ was 0·5 mM at potentials of +10 and 0 mV respectively, to potentials where maximal inward current occurred in each solution. Also, when $[Ca^{2+}]_o$ was increased from 2·5 to 20 mM, maximal Ca^{2+} currents observed at +10 and +20 mV in corresponding solutions were compared.

To plot a peak of I_{Ca} against the membrane voltage (current–voltage curve as

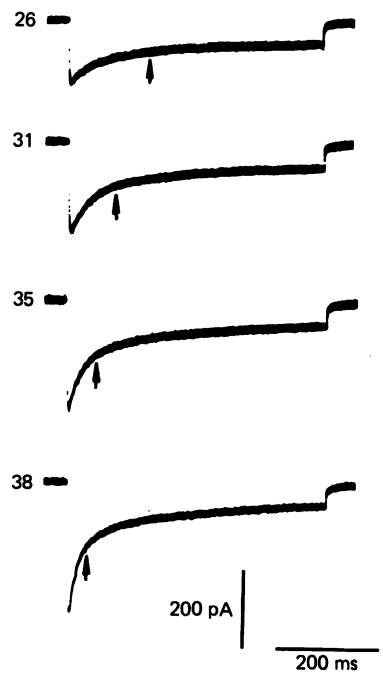

Fig. 5. Effect of temperature on I_{Ca}. Ca^{2+} currents were elicited by step depolarizations to 0 mV. Temperature in centigrade is indicated near each current trace. Half-amplitude of each I_{Ca} was indicated by arrow.

shown in Fig. 2) currents were recorded using 50 ms step depolarizations. Such an experimental protocol allowed an increase in the frequency of stimulation (with 500 ms pulses, frequency was usually 0·03 Hz) and minimized distortions connected with 'run-down' of I_{Ca}. This phenomenon was analysed in detail in a number of studies and factors affecting stability of I_{Ca} were determined (for review see Kostyuk, 1984). In smooth muscle cells, the speed of run-down was variable from cell to cell. Moreover, in some cells amplitude of I_{Ca} was nearly stable during 2–3 h of perfusion. In our experiments we used mainly such cells.

Step depolarization of 500 ms duration was used to evaluate I_{Ca} inactivation kinetics when external Ca^{2+} was decreased to 0·5 mM in eight cells. A typical result is presented in Fig. 3*A*. Decrease in $[Ca^{2+}]_o$ resulted in a significant decrease in I_{Ca} maximal amplitude. Also, the time course of inactivation was slowed down, as can be seen from an increase of the half-time of I_{Ca} decay.

With an increase of $[Ca^{2+}]_o$ to 20 mM (pulse duration was also 500 ms) an increase in maximal peak amplitude of I_{Ca} was observed (Fig. 3*B*). Decay of maximal I_{Ca}, observed when $[Ca^{2+}]_o$ was 20 mM, was significantly faster in comparison with the decay of maximal I_{Ca} in the presence of 2·5 mM-Ca^{2+} in the external solution. Inactivation of I_{Ca} recorded at the same membrane potential (+10 mV in 2·5 and 20 mM-Ca^{2+}) was also faster. This can be seen from a half-time of decay of currents shown in Fig. 3*B*.

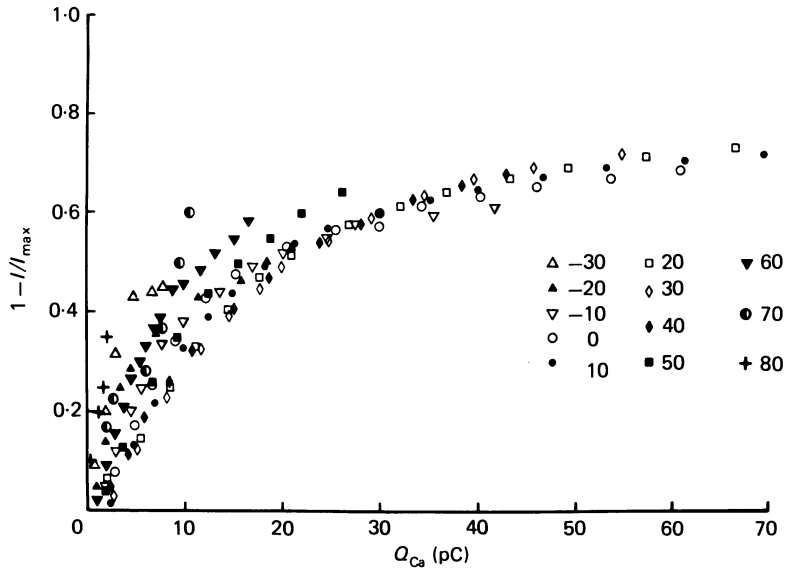

Fig. 6. I_{Ca} fractional inactivation plotted against Ca^{2+} entry at different membrane potentials. Fractional inactivation ($1-I/I_{max}$) and Ca^{2+} entry (Q_{Ca}) were calculated as described in the text. Different symbols indicate different membrane potentials which are shown in millivolts near symbols. Further explanations in the text. The same cell as in Fig. 1.

Inactivation of I_{Ca} during Co^{2+} block

Another way to decrease the peak amplitude of I_{Ca} is to block partially I_{Ca} by Co^{2+} ions. I_{Ca} elicited by a 500 ms step depolarization to 0 mV is shown in Fig. 4, upper trace. When Co^{2+} ions were applied (0·4 mM), the peak of I_{Ca} was decreased to about half (Fig. 4, lower trace). As can be seen, a decrease in I_{Ca} amplitude during Co^{2+} block was accompanied by a slowing down of its decay.

Effect of temperature on I_{Ca}

Elevation of temperature to 38 °C increased I_{Ca} amplitude and accelerated its inactivation (Fig. 5). The amplitude of I_{Ca} was increased by a factor of $1·7 \pm 0·14$ ($n = 6$) when temperature was increased by 10 °C. Sometimes increasing temperature accelerated the process of I_{Ca} run-down. That is why the detailed investigation of the temperature dependence of I_{Ca} was not performed.

Thus, when the peak amplitude of I_{Ca} was changed by changing $[Ca^{2+}]_o$ or by applying Co^{2+} ions, the time course of I_{Ca} inactivation was also changed. The results presented indicate that Ca^{2+} ions, entering the smooth muscle cell during the development of I_{Ca}, participate in inactivation of the Ca^{2+} conductance. That is why we analysed the relation between Ca^{2+} entry and I_{Ca} inactivation using an approach recently proposed (Chad, Eckert & Ewald, 1984).

Relation between Ca^{2+} entry and I_{Ca} inactivation

After reaching its peak value (I_{max}) I_{Ca} decayed. During the time t from the beginning of depolarization, I_{Ca} was partially inactivated and had an amplitude

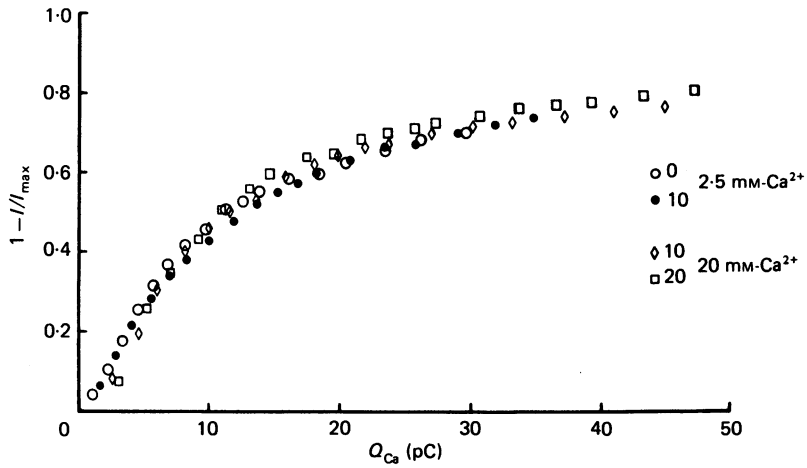

Fig. 7. I_{Ca} fractional inactivation plotted against Ca^{2+} entry in different external Ca^{2+} concentrations. Different symbols indicate different membrane potentials (indicated in millivolts near symbols) when $[Ca^{2+}]_o$ was 2·5 and 20 mM. The same cell as in Fig. 3*B*.

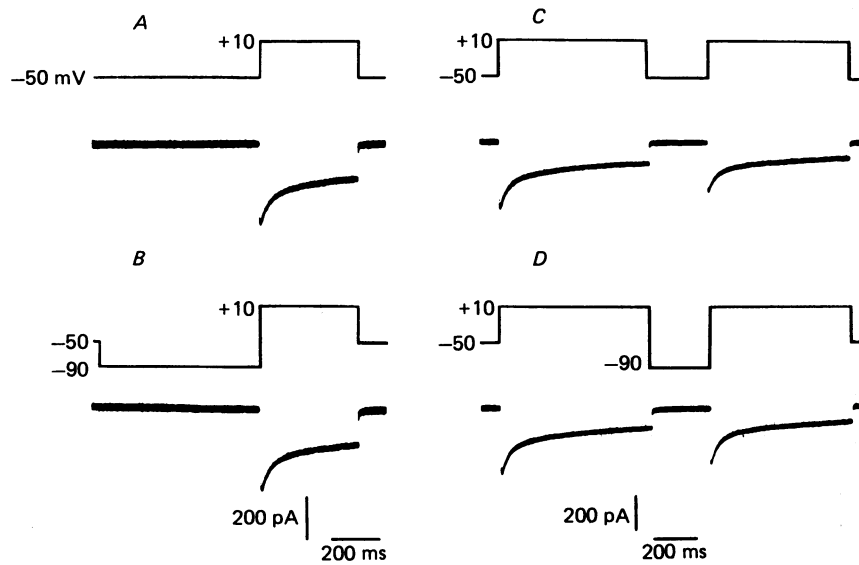

Fig. 8. Effect of conditioning hyperpolarization on I_{Ca} and on I_{Ca} recovery from inactivation. *A* and *B*, I_{Ca} at +10 mV without and with conditioning pre-pulse to −90 mV, respectively. *C* and *D*, effect of decreasing the membrane potential from −50 to −90 mV during interpulse interval on I_{Ca} elicited by the second pulse. *A*, *B*, *C* and *D* from the same cell. Note different calibration in *A* and *B* compared to *C* and *D*. The scheme of the experiment was shown above each current trace.

which we designated *I*. Thus, inactivation at time *t* was defined as $1-I/I_{max}$. It must be noted, however, that only decay of I_{Ca} was used for calculations since at times before peak due to additional activation of Ca^{2+} channels it was not possible to compare Ca^{2+} entry and inactivation. However, inactivation of Ca^{2+} channels at the peak of I_{Ca} seems to be insignificant since small amounts of Ca^{2+} enter the cell during

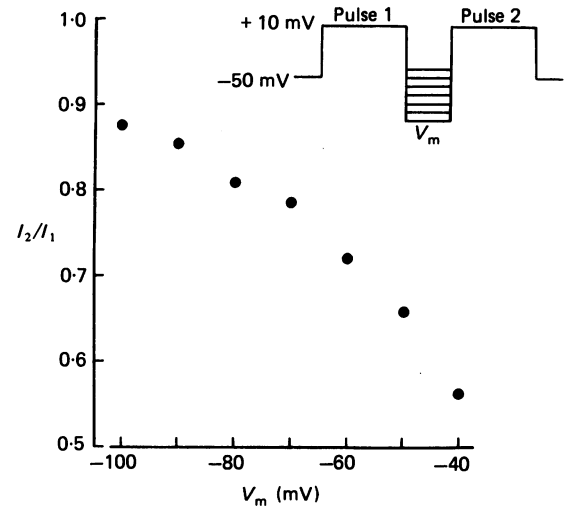

Fig. 9. Dependence of the relative inactivation of I_{Ca} (I_2/I_1) defined with two-pulse protocol (scheme shown in the inset) on interpulse membrane potential, V_m. Interpulse interval 240 ms.

activation of I_{Ca}. Thus, inactivation values obtained seem to be slightly underestimated. Ca^{2+} entry was defined as the area of inward current, sensitive to Co^{2+} ions, i.e. Ca^{2+} entry up to time t was $\int_0^t I_{Ca}\,dt$. Then inactivation of I_{Ca} at a given time from the beginning of depolarization was plotted against Ca^{2+} entry during the same time (Fig. 6). Calculations were made for a large number of Ca^{2+} currents recorded at different membrane potentials from −30 to +80 mV as shown in Fig. 1. As can be seen from Fig. 6: (i) dependence of inactivation on Ca^{2+} entry was non-linear; linearity was observed only when a small amount of inactivation occurs; (ii) a good correlation between inactivation of I_{Ca} and Ca^{2+} entry was observed in the potential range from −20 to +40 mV; and (iii) with increasing depolarization from +50 to +80 mV, inactivation of I_{Ca} corresponded to a considerably smaller Ca^{2+} entry.

It must be noted, however, that dependence of the degree of I_{Ca} inactivation on the amount of Ca^{2+} entry varied significantly from cell to cell. For I_{Ca} used for calculations shown in Fig. 6 half-inactivation corresponded to a Ca^{2+} entry of approximately 20 pC. For I_{Ca} shown in Fig. 3*B* (relation of inactivation to Ca^{2+} entry shown in Fig. 7) half-inactivation occurred when Ca^{2+} entry did not exceed 10 pC. At the same time in some cells half-inactivation of I_{Ca} corresponded to Ca^{2+} entry of 100 pC or even 200 pC. At present it is not clear what is the reason for such differences. One possible explanation is the different activity of intracellular Ca^{2+}-binding systems in different cells. Moreover, inactivation correlated with Ca^{2+} entry for I_{Ca} recorded up to +40 mV in the cell shown in Fig. 6. In other cells such a correlation was observed up to +30 mV or only up to +20 mV. Suppression of I_{Ca} in these cells by Co^{2+} ions revealed the existence of I_{ns} at these potentials.

In our opinion, results presented in Fig. 6 indicate that observed differences in the time course of I_{Ca} inactivation in the potential range −20 to +40 mV (Fig. 1) can be explained by different amounts of Ca^{2+} entering, i.e. by differences in the amplitude of I_{Ca}. A similar conclusion can be made from Fig. 7, where the relation between

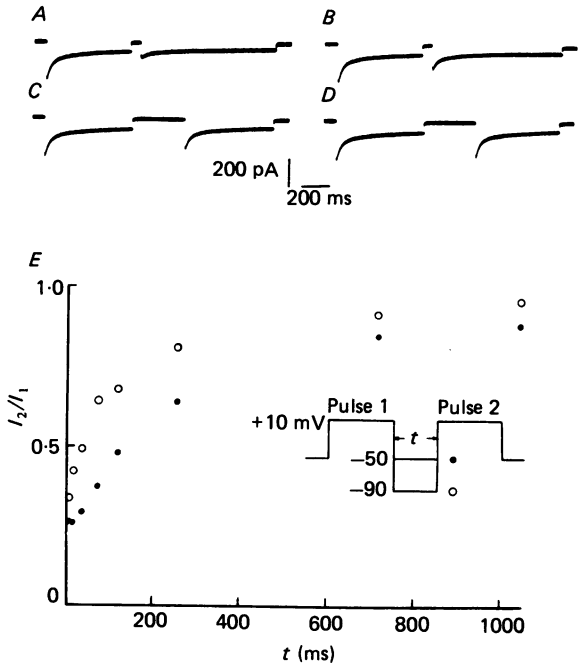

Fig. 10. Comparison of the time course of I_{Ca} recovery from inactivation at two membrane potentials (−50 and −90 mV). *A* and *B*, current traces showing recovery of I_{Ca} during 77 ms at potentials −50 (*A*) and −90 mV (*B*). *C* and *D*, current traces showing recovery of I_{Ca} during 370 ms at potentials −50 (*C*) and −90 mV (*D*). *E*, time course of I_{Ca} recovery at −50 mV (filled circles) and at −90 mV (open circles). The scheme is shown in the inset. *A*, *B*, *C* and *D* from one cell; *E* from another one.

I_{Ca} inactivation and Ca^{2+} entry with varying $[Ca^{2+}]_o$ is shown. As can be seen from the Figure, acceleration of I_{Ca} decay when $[Ca^{2+}]_o$ was increased can be explained by enhancement of Ca^{2+} entry, since the relation between inactivation and amount of Ca^{2+} entering was the same when $[Ca^{2+}]_o$ was 2·5 mM and when $[Ca^{2+}]_o$ was 20 mM.

The results presented provide good evidence of Ca^{2+}-dependent inactivation of Ca^+ inward current in smooth muscle cells. If inactivation was due to increase in $[Ca^{2+}]_i$, recovery of I_{Ca} from inactivation may be expected to reflect a lowering of $[Ca^{2+}]_i$.

Recovery of I_{Ca} from inactivation

As was reported earlier, recovery of I_{Ca} from inactivation is a biphasic process (Ganitkevich *et al.* 1986). In present experiments we did not try to give its full characterization. Our aim was to test the possibility that recovery of I_{Ca} from inactivation reflected a lowering of $[Ca^{2+}]_i$.

It was found that recovery from inactivation of I_{Ca} (as well as of I_{Ba}) was a potential-dependent process. The results of an experiment are shown in Fig. 8. Holding potential was, as usual, −50 mV. A test pulse to +10 mV was used to elicit I_{Ca} (Fig. 8*A*). A similar I_{Ca} was elicited by the test pulse following a conditioning hyperpolarization to −90 mV (Fig. 8*B*). In double-pulse experiments from the same

cell, I_{Ca} elicited by the first pulse to +10 mV (I_1) resulted in a significant inactivation of I_{Ca} elicited by the second pulse (I_2). When, during the interpulse interval, membrane potential was held at −50 mV, some recovery occurred. However, when the interpulse membrane potential was −90 mV, I_{Ca} recovered from inactivation to a greater extent (Fig. 8*C* and *D*). This result suggests that the membrane potential transition from −50 to −90 mV has no influence on I_{Ca}; however, when I_{Ca} was partially inactivated, hyperpolarization accelerated the process of I_{Ca} recovery.

For characterization of the dependence of the I_{Ca} recovery process on the membrane potential, a relative inactivation (I_2/I_1) was taken as a measure of recovery at a fixed interpulse interval. The dependence of I_2/I_1 on the interpulse membrane potential is shown in Fig. 9. Similar results were obtained from six cells. The membrane potential was in the range from −100 to −40 mV, as at more positive potentials Ca^{2+} entry with inward Ca^{2+} current can distort the recovery process. As can be seen from Fig. 9, there was significant dependence of I_{Ca} recovery on the membrane potential. For example, during the same interpulse interval, I_2/I_1 was 0·565 when membrane potential was −40 mV and I_2/I_1 was 0·875 when membrane potential was −100 mV. Note that the amplitude and the time course of I_1 were similar in both cases.

A similar effect on I_{Ca} recovery from inactivation was exerted by a change of the holding potential. Change of the holding potential from −50 to −100 mV resulted in an increase of I_2 peak amplitude, while I_1 peak amplitude was not changed. Complete removal of Na^+ ions from the extracellular solution had no effect on the dependence of I_{Ca} recovery on the membrane potential (not shown).

Comparison of the initial phase of I_{Ca} recovery for two different membrane potentials is shown in Fig. 10. As can be seen, for short interpulse intervals, a decrease of the membrane potential from −50 to −90 mV had a little effect on I_2. When the interpulse interval was increased, hyperpolarization resulted in acceleration of I_{Ca} recovery. When the interval between two pulses was sufficiently long to allow complete recovery, hyperpolarization had no effect on I_{Ca} (see also Fig. 8*B*).

Variability of I_{Ca} inactivation

I_{Ca} at +10 mV was always inactivated faster than I_{Ca} at more negative potentials, independently of I_{Ca} amplitude. If a Ca^{2+}-dependent mechanism of I_{Ca} inactivation is operating, it would be expected that there will be correlation between the inactivation kinetics and I_{Ca} amplitude in different cells. For example, if in one cell the maximal I_{Ca} amplitude was 100 pA while in another one the peak of I_{Ca} was 400 pA, inactivation of the smaller current would be expected to be slower. However, it was not so. No correlation was found between I_{Ca} amplitude and its inactivation time course in different cells. In some cells, the amplitude of I_{Ca} was large, and correspondingly, Ca^{2+} entry into the cell was also large, while inactivation was slow. The results presented in this paper were obtained mainly from freshly dissociated cells. In most cells which were studied 6–8 h after the isolation, slow inactivation of I_{Ca} was observed. The reason for this is not clear. That is why inactivation of I_{Ca} was not analysed in terms of the time constants in this paper.

We also tested the effect of changing the EGTA concentration in the intracellular solution on I_{Ca} inactivation. Such results, however, were hardly comparable since

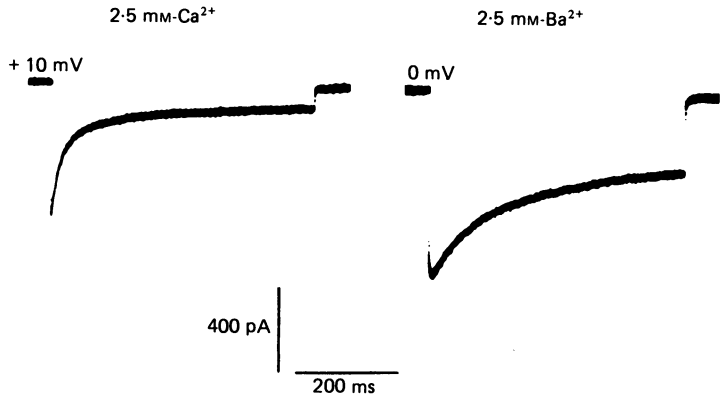

Fig. 11. Comparison of I_{Ca} and I_{Ba} in one cell. Records were taken at the peak of current–voltage curves in each solution (indicated in millivolts near each current trace). Holding potential was −60 mV. Temperature was 29 °C.

they were obtained from different cells. Observed differences in the I_{Ca} inactivation kinetics, when EGTA was lowered to 1 mM, were not large and we cannot separate the effect of internal EGTA on I_{Ca} inactivation from the variability of I_{Ca} inactivation observed in different cells.

Inactivation of Ba^{2+} current through Ca^{2+} channels

The ability of Ba^{2+} ions to carry inward current through the Ca^{2+} channels in smooth muscle is well established (Walsh & Singer, 1980; Bolton *et al.* 1985; Inomata & Kao, 1985; Droogmans & Callewaert, 1986). However, a detailed study of Ba^{2+} currents (I_{Ba}) was not performed since we were mainly interested in its inactivation kinetics.

Substitution of 2·5 mM-Ba^{2+} for 2·5 mM-Ca^{2+} ions in the external solution induced a shift of the current–voltage relation of I_{Ba} to negative membrane potentials when compared to those of I_{Ca} (not shown). At the same time I_{Ba} amplitude was larger than the amplitude of I_{Ca}, when maximal inward currents in each solution were compared (Fig. 11). Also, as can be seen from the figure, I_{Ba} was inactivated considerably slower than I_{Ca}.

It was found that inactivation of I_{Ba} (as well as I_{Ca}) varied from cell to cell. The reason for this is unclear and more detailed study is needed to resolve this problem.

DISCUSSION

The results presented in this paper indicate that Ca^{2+} ions entering the smooth muscle cell during I_{Ca} development participate in some way in the inactivation of the Ca^{2+} conductance. The existence of Ca^{2+}-dependent inactivation of the Ca^{2+} conductance was shown in a number of studies on different excitable cells (Brehm, Eckert & Tillotson, 1980; Brown, Morimoto, Tsuda & Wilson, 1981; Eckert & Tillotson, 1981, Ashcroft & Stanfield, 1982; Mentrard, Vassort & Fischmeister, 1984; Lee *et al.* 1985; for review see Eckert & Chad, 1984).

For I_{Ca}, three possible mechanisms of inactivation may be suggested: (i) purely potential dependent; (ii) purely Ca^{2+} dependent; and (iii) combined inactivation, i.e. both depolarization and Ca^{2+} entry results in inactivation of the Ca^{2+} conductance (Kass & Sanguinetti, 1984; Lee *et al.* 1985).

Purely potential-dependent inactivation is characterized by the following features: (i) monotonic acceleration of I_{Ca} inactivation with increasing depolarization; (ii) the existence of steady-state inactivation of I_{Ca} in the potential range where no Ca^{2+} entry occurs; and (iii) inactivation is not dependent on the species of permeant ion, i.e. inactivation of I_{Ba} is similar to inactivation of I_{Ca} (Fox, 1981; for review see Tsien, 1983); so the hypothesis of purely potential-dependent inactivation of I_{Ca} in smooth muscle cells failed to explain our experimental observations.

The following features may be expected for a purely Ca^{2+}-dependent mechanism of I_{Ca} inactivation: (i) at the membrane potential where maximal I_{Ca} is observed, inactivation will be faster than at other membrane potentials; (ii) at constant membrane potential, the time course of inactivation will depend on the I_{Ca} amplitude; (iii) the availability of I_{Ca} defined in double-pulse experiments will be U-shaped and its minimum will be observed at potentials corresponding to maximal I_{Ca}, i.e. maximal Ca^{2+} entry during the pre-pulse; (iv) inward-current inactivation kinetics will depend on the species of permeant cation. If Ba^{2+} ions are ineffective in producing inactivation, Ba^{2+} current will not inactivate at all; (v) inactivation of I_{Ca} must be tightly related to Ca^{2+} entry and independent of the membrane potential; and (vi) recovery from inactivation will reflect the process of removing Ca^{2+} ions, i.e. the process of lowering $[Ca^{2+}]_i$.

Some of our results are consistent with the hypothesis of purely Ca^{2+}-dependent inactivation of I_{Ca} in smooth muscle cells. For example, inactivation of I_{Ca} was slower at more positive potentials than at the potential inducing maximal I_{Ca} (Fig. 1). Availability was U-shaped (Ganitkevich *et al.* 1986). Inactivation was slowed down when $[Ca^{2+}]_o$ was decreased or when I_{Ca} was suppressed by Co^{2+} ions, and it was accelerated when $[Ca^{2+}]_o$ was increased. Inactivation was tightly related to Ca^{2+} entry into the cell (Fig. 6). It must be noted, however, that estimation of Ca^{2+} entry into the cell as $\int_0^t I_{Ca}\,dt$ is correct only when there is not any other conductance sensitive to Co^{2+} ions.

The possibility exists that Cs^+ ions (the main cation in the intracellular solution) may carry outward current though the Ca^{2+}-dependent K^+ channels of large conductance (it is the activity mainly of these channels which can disturb the time course of I_{Ca} due to their large elementary currents). This outward current may be blocked by Co^{2+} ions together with I_{Ca} and as a result Ca^{2+} entry may be underestimated. However, it was shown recently that this type of channel is effectively blocked by Cs^+ ions (Benham, Bolton, Lang & Takewaki, 1986). So, we suggest that in our experiments I_{Ca} was not significantly disturbed by this type of conductance. However, there still remains the question about disturbances due to other types of K^+ channels, since their selectivity to Cs^+ ions is not yet known (Benham & Bolton, 1983; Berger, Grygorcyk & Schwarz, 1984; Inoue, Kitamura & Kuriyama, 1985). At present, however, the existence of different types of K^+ channels in taenia smooth muscle has not been shown.

It is possible also that Co^{2+} ions directly blocked some I_{ns}, and as a result Ca^{2+}

entry was also underestimated. This may disturb the relation between I_{Ca} inactivation and Ca^{2+} entry observed in single-pulse experiments (Fig. 6) as with a double-pulse protocol (Ganitkevich *et al.* 1986, their Fig. 6*C*) in a potential range where residual I_{ns} was observed in the presence of Co^{2+} ions. Another possibility is that at these potentials, due to development of potential-dependent inactivation, the relation between inactivation and Ca^{2+} entry was changed (see also Lee *et al.* 1985).

Inactivation of Ba^{2+} current is considerably slower than I_{Ca} as was reported for a variety of tissues (Brown *et al.* 1981; Lee *et al.* 1985). Nevertheless, if Ba^{2+} was the only permeant cation, inactivation was still present (Fig. 11). It was suggested that inactivation of I_{Ba} is purely potential dependent (Lee *et al.* 1985). However, before such a conclusion can be made, further investigation of I_{Ba} in smooth muscle is needed.

The main difficulty with interpretation of I_{Ca} inactivation as a purely Ca^{2+}-mediated process is that recovery from inactivation is potential dependent. Similar phenomena have been observed in molluscan neuronal membrane (Yatani, Wilson & Brown, 1983) and in heart cells (Mentrard *et al.* 1984). Potential dependence of the recovery process is probably due to moving charged particle(s) in the membrane electrical field. If recovery of I_{Ca} reflects a lowering of $[Ca^{2+}]_i$ it seems to be doubtful that this process can be potential dependent if it was due to the intracellular Ca^{2+} binding by cell-buffering systems (and internal EGTA in our experiments). Involvement of the electrogenic Na^+–Ca^{2+} exchange in recovery of I_{Ca} can be excluded since potential dependence of recovery was similar in Na^+-free solution. Existence of a potential-dependent Ca^{2+} pump in the smooth muscle cell membrane is also doubtful. Our preliminary observations suggest that recovery of I_{Ba} is also potential dependent.

At present we cannot give a complete explanation of our results. Inactivation of I_{Ca} in the potential range studied agrees better with the possibility of its mediation by Ca^{2+} entry. It must be noted that a hyperbolic relation between I_{Ca} inactivation and Ca^{2+} entry (Fig. 6) is predicted from a binding-site model of Ca^{2+}-mediated inactivation (Standen & Stanfield, 1982; Plant, Standen & Ward, 1983; Eckert & Chad, 1984). At the same time the simplest explanation of the recovery process is a potential-dependent one.

Mechanisms involved in Ca^{2+}-dependent inactivation are still far from understood today (see Lee *et al.* 1985). Some experimental findings do not seem to be explained by the hypothesis that Ca^{2+}-dependent inactivation occurred simply due to Ca^{2+} ions accumulating near the inner side of the membrane. Recently it was shown that I_{Ca} inactivation in the neuronal membrane is tightly related to Ca^{2+} entry (Chad *et al.* 1984). Evidence for purity of I_{Ca} was presented. This means that estimation of Ca^{2+} entry as $\int_0^t I_{Ca}\,dt$ was correct, as well as a determination of I_{Ca} inactivation. The family of Ca^{2+} currents during progressive block by Cd^{2+} ions was used for calculations. As was shown in their Fig. 5*C*2, similar inactivation of I_{Ca} was observed when 40 nC of Ca^{2+} enter the cell for 200 ms (without Cd^{2+} added) and for 900 ms (during partial block by Cd^{2+} ions, indicated by open circles). It means that a similar amount of Ca^{2+} entry corresponded to a similar inactivation of I_{Ca}, despite the fact that this entry occurred over a different period. It is very doubtful that there is no binding of Ca^{2+} ions in the cell during 700 ms. Moreover, a significant recovery of I_{Ca} was usually

observed in the 700 ms after the end of a depolarizing pulse. This result is difficult to explain on the assumption that Ca^{2+} current inactivation is induced by accumulation of $[Ca^{2+}]_i$ and subsequent equilibrium binding of Ca^{2+} to its receptor (Eckert & Chad, 1984), while recovery reflects lowering of $[Ca^{2+}]_i$.

Similar phenomena were observed in smooth muscle cells (Fig. 6). It must be noted, however, that in the neuronal somatic membrane a dependence of I_{Ca} inactivation on Ca^{2+} entry varied with changes of the membrane potential: this was attributed to some potential dependence of the inactivation process (Chad *et al.* 1984).

It was suggested recently that Ca^{2+}-dependent inactivation of I_{Ca} may be mediated by some intracellular enzymes (Chad & Eckert, 1985; Kalman & Eckert, 1985). Thus, it is very attractive to speculate that, as well as in neurones, the inhibitory effect of Ca^{2+} ions entering the smooth muscle cell on Ca^{2+} conductance can be mediated by intracellular enzymes. However, much more study is needed to test this possibility.

Some findings also indicate that recovery of I_{Ca} from inactivation was not directly linked with a lowering of $[Ca^{2+}]_i$. In neurones it was found that $[Ca^{2+}]_i$ decreased faster than recovery of I_{Ca} occurred (Eckert & Chad, 1984). A similar observation was made in an aequorin-injected dog Purkinje fibre (Wier & Isenberg, 1982): $[Ca^{2+}]_i$ fell back close to its resting level while I_{Ca} inactivation persisted during a voltage-clamp step.

So, we conclude that I_{Ca} inactivation and recovery in smooth muscle cells were influenced by both Ca^{2+} entry and membrane potential. The mechanisms underlying these processes still remain unknown. It must be noted that little is known about the link between inactivation and recovery of I_{Ca}, despite the fact that the co-existence of Ca^{2+} dependence of I_{Ca} inactivation and potential dependence of I_{Ca} recovery was shown in neurones (Brown *et al.* 1981; Yatani *et al.* 1983) and in heart cells (Mentrard, Vassort & Fischmeister, 1984).

A reason for the variability of I_{Ca} is not known yet. In our opinion it was not due to different efficiency of intracellular perfusion. Two main facts confirmed this conclusion. Both cells with 'slow' inactivation and with 'fast' inactivation of I_{Ca} exhibited nearly similar outward currents after addition of Co^{2+} ions (Ganitkevich *et al.* 1986, their Fig. 2; this paper, Fig. 1). Variability of Ba^{2+} currents was also observed. Since Ba^{2+} ions are known to block K^+ channels, including Ca^{2+}-dependent K^+ channels (Benham, Bolton, Lang & Takewaki, 1985), it seems unlikely that a variability of I_{Ba} inactivation was due to the presence of the Ca^{2+}-dependent outward current disturbing the time course of inactivation.

It was shown recently that Ca^{2+}-dependent inactivation depends on the activity of some intracellular enzymes (Chad & Eckert, 1985; Kalman & Eckert, 1985). Also, significant slowing down of I_{Ca} inactivation was observed when dipicolin acid was used instead of EGTA in the internal solution for cardioball perfusion (Bechem & Pott, 1985). All these observations may indicate that the variability of I_{Ca} inactivation can be due to a different activity of intracellular enzyme systems, binding Ca^{2+} ions entering the smooth muscle cell and inducing inactivation of Ca^{2+} conductance. This question needs further investigation.

Ca^{2+}-dependent inactivation of a potential-dependent Ca^{2+} inward current seems to be important for function of smooth muscle. It may serve, probably, as a negative

feed-back system participating in the regulation of Ca^{2+} entry. Such a mechanism can restrict Ca^{2+} ion entry into smooth muscle cells during sustained depolarization.

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