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Involvement of Calcium in Calcium-Current Inactivation in Smooth Muscle Cells from Rat Vas Deferens

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Summary. Ca-channel currents were recorded in Cs-loaded single smooth muscle cells from rat vas deferens to define the dependence of the inactivation time course on Ca concentration. The decay of Ca-channel current obtained in a Ba^{2+}- or Sr^{2+}-containing external solution during long voltage-clamp pulses was much slower than that in a Ca-containing solution. The difference was not due to a change in the surface potential of the membrane as judged from the steady-state activation and inactivation curves. When Ca was the charge carrier, increasing external Ca concentration slightly accelerated the rate of inactivation. In addition, the rate of inactivation of Ca-channel current in 10.8 mM Ba was also accelerated by adding Ca to the external solution in a concentration-dependent manner. The time course of Ca-current inactivation was slowed when the cells were dialyzed with a high concentration of citrate, a Ca-chelating agent. From these results, we concluded that a mechanism regulated by intracellular Ca activity plays a role in the inactivation of Ca channels in smooth muscle. The Ca-dependent process may protect against Ca overload by regulating Ca entry in smooth muscle cells.

Key words smooth muscle cells · Ca channel · whole cell recording · inactivation · Ca dependency

Introduction

In smooth muscle cells depolarization can activate a Ca-channel current which, when depolarization is sustained, will subsequently inactivate. The inactivation rate has been found to depend on membrane potential (e.g., Klöckner & Isenberg, 1985*b*; Droogmans & Callewaert, 1986). In addition, it has been reported that a Ca-entry-dependent mechanism plays a role in the inactivation of Ca current in excitable membranes such as neurons (Eckert & Tillotson, 1981), paramecium (Brehm, Eckert & Tillotson, 1980), skeletal muscles (Ashcroft & Stanfield, 1981), cardiac cells (Brown et al., 1984; Josephson, Sanchez-Chapula & Brown, 1984; Kass & Sanguinetti, 1984; Mentrard, Vassort & Fischmeister, 1984; Bechem & Pott, 1985; Lee, Marban & Tsien, 1985).

Ca-dependent inactivation of the Ca current has also been reported to occur in the smooth muscle of multicellular preparation of rat myometrium using a double sucrose gap technique (Jmari, Mironneau & Mironneau, 1986). However, the inactivation process in smooth muscle can be studied with better reliability in isolated cells because better space-clamp during measurement of membrane current is achieved, and the intracellular milieu can be controlled when the tight-seal suction pipette technique is used (Klöckner & Isenberg, 1985*a*,*b*).

For the present study, the inactivation process of Ca-channel current was investigated using isolated smooth muscle cells from rat vas deferens. We found that Ca current exhibited much faster inactivation kinetics than either Ba or Sr current and that the rate of inactivation was accelerated by extracellular Ca in a concentration-dependent manner. The effects of the Ca-chelators, EGTA and citrate, on the Ca current were also compared by dialyzing cells with pipettes loaded with these compounds.

Materials and Methods

Single smooth muscle cells were prepared from rat vas deferens with collagenase and elastase according to Nakazawa et al. (1987). The isolated cells were superfused with the extracellular solution containing (in mM) 135 NaCl, 1.2 $MgCl_2$, 11.1 dextrose, 10 HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid) and a salt of a desired concentration of divalent cation, $CaCl_2$, $BaCl_2$ or $SrCl_2$ (adjusted with NaOH to pH 7.4). The artificial intracellular solution was as follows (in mM): 130 CsCl, 5 succinic acid, 5 pyruvic acid, 5 oxalacetic acid, 10 HEPES, 1.0 $MgCl_2$, 0.18 $CaCl_2$, 2 EGTA (ethyleneglycol-*bis*(2-aminoethylether)-N,N,N',N-tetraacetic acid), adjusted with CsOH to pH 7.2. Free Ca^{2+} concentration of the internal solution is about 10^{-7} M, assuming the apparent dissociation constant of Ca · EGTA is 10^{-6} M (Harafuji & Ogawa, 1980). When using solutions containing 20 mM EGTA or 50 mM citric acid, $CaCl_2$ was omitted and CsCl was replaced with equiosmolar of these compounds.

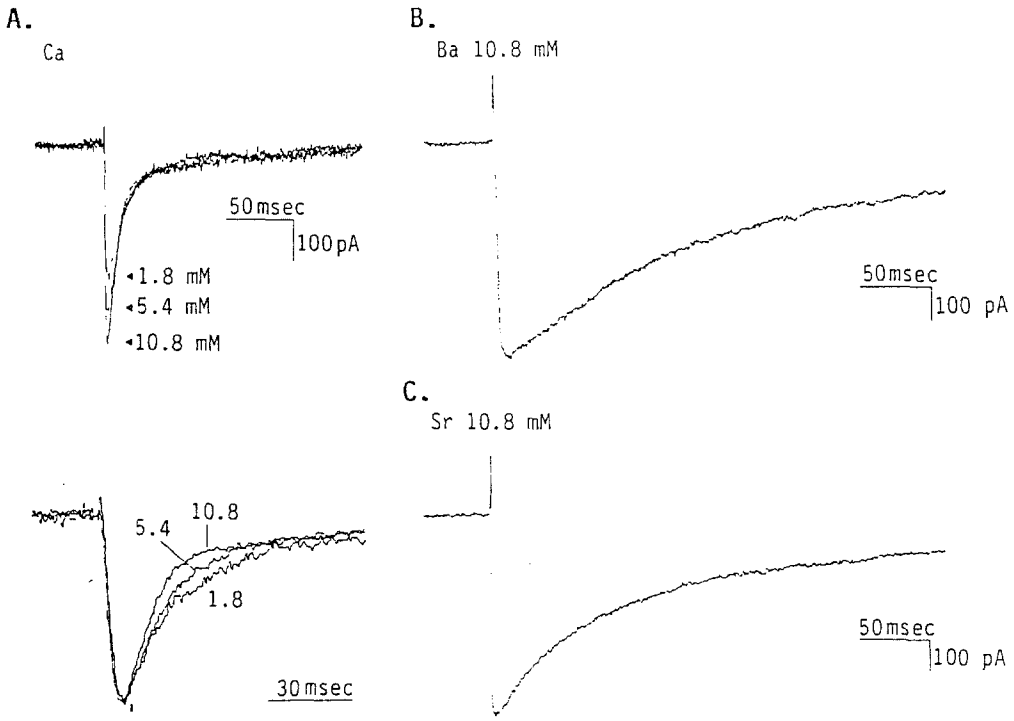

Fig. 1. (*A*) *Upper:* Ca-channel current recorded in the extracellular solution containing 1.8, 5.4 or 10.8 mM Ca. The Ca concentration was successively increased in steps from 1.8 mM and the current in 5.4 or 10.8 mM was measured at 3 min after the onset of the superfusion. *Lower:* The traces of the same currents were scaled up in order to compare the time courses. The inactivation was slightly accelerated with the increment in extracellular Ca concentration. (B, C) Ca-channel current in 10.8 mM Ba (*B*) or Sr (*C*). These currents showed much slower inactivation kinetics than Ca current (*A*). (*A*), (*B*), (*C*) were obtained from three different cells. All currents were activated by test pulses to 0 mV from a holding potential of −60 mV

The membrane currents were measured using the tight-seal suction pipette technique (whole-cell recording configuration: Hamill et al., 1981; Marty & Neher, 1983). Heat-polished patch pipettes filled with the intracellular solution had tip resistance of 2 to 5 MΩ. The currents were measured with a patch-clamp amplifier (Nihon Kohden, S-3666) and filtered through a 6-pole Bessel filter. Series resistance compensation was employed. All the data were digitized and the linear component of the leak current was subtracted using the traces obtained with 30-mV depolarizing steps from −90 mV. When comparing the inactivation time courses of the Ca-channel currents evoked in the several types of solutions used for this study, current was activated by test pulses to 0 mV from a holding potential of −60 mV. All the experiments were done at room temperature (25 to 27°C).

Results

Ca-Channel Currents Carried by Ca, Ba and Sr

Figure 1 illustrates the Ca-channel currents recorded in extracellular solutions containing Ca, Ba and Sr. The currents in both Ba- and Sr-containing solutions showed much slower inactivation time courses than that in Ca-containing solution; half-decay times ($t_{1/2}$) of the current at 0 mV were 27.3 ± 10.0 msec in 1.8 mM Ca (mean ± SEM, $n = 4$), 112.0 ± 46.6 msec in 10.8 mM Ba ($n = 4$) and 72.7 msec in 10.8 mM Sr ($n = 2$). When the extracellular Ca concentration was raised from 1.8 mM to 5.4 or 10.8 mM, the amplitude was markedly increased and $t_{1/2}$ was slightly decreased (21.0 msec in 1.8 mM, 18.5 msec in 5.4 mM, 16.5 msec in 10.8 mM; $n = 2$).

One possible explanation for the observed difference in Ca, Ba and Sr current inactivation rates is that inactivation is purely voltage dependent, and these divalent ions changed the potential difference that falls across the plasma membrane. Therefore, we studied surface charge effects of the divalent cations on steady-state activation and inactivation. Figure 2 shows the voltage dependencies of steady-state activation and inactivation of the Ca, Ba and Sr currents. The curves for 1.8 mM Ca and 10.8 mM Ba were very similar while the curve for 10.8 mM Sr was shifted about 20 mV more negative relative to the other two curves. The differences in the voltage-dependencies might reflect differences in the ability of the several divalent cations to screen membrane surface charge. Such an effect has been reported in other excitable membranes (Ohmori & Yoshii, 1977; Kass & Sanguinetti, 1984; McDonald

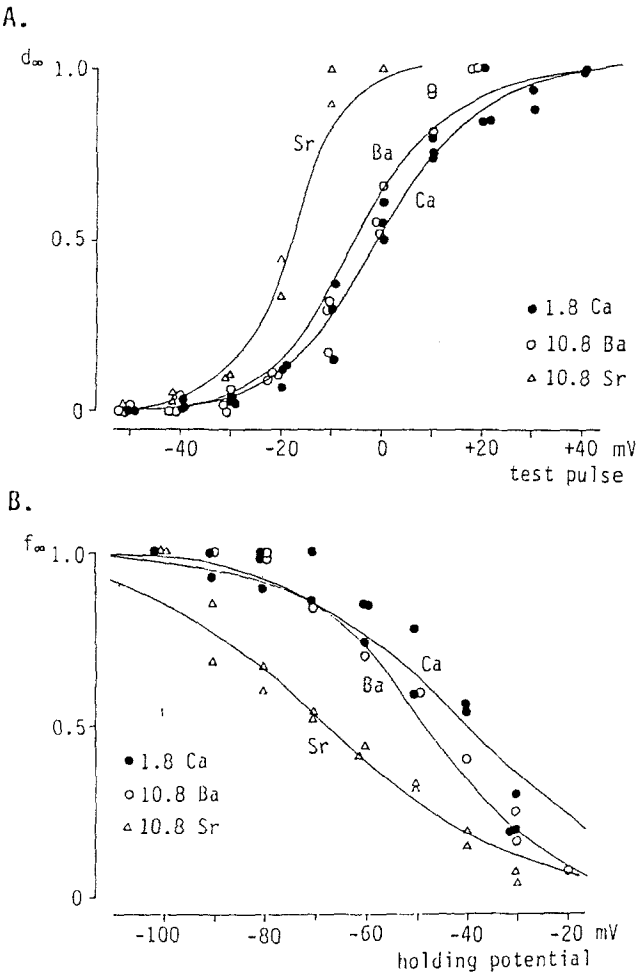

Fig. 2. Steady-state activation (*A*) and inactivation (*B*) of the Ca-channel current recorded in 1.8 mM Ca (filled circles), 10.8 mM Ba (open circles) or 10.8 mM Sr (open triangles). (*A*) Steady-state activation (d_∞) was calculated from the peak amplitude (I) of the current activated by 800-msec test pulses to various potentials (V) from −60 mV at intervals of 10 to 20 sec, using the formulas: $d_\infty = g/g_{max}$, $g = I/(V - V_r)$; (V_r: measured reversal potential of the current). Smooth curves were drawn according to: $d_\infty = 1/[1 + \exp(V_{1/2} - V)/k]$; ($V_{1/2}$: the voltage at the half-maximal activation). The data were best fitted by linear regression. The calculated parameters were as follows: $V_{1/2}$, −0.2 mV (Ca), −4.8 mV (Ba), −19.3 mV (Sr); k, 9.8 mV (Ca), 8.8 mV (Ba), 5.8 mV (Sr). The results obtained from three cells (Ca and Ba) or two cells (Sr). (*B*) Steady-state inactivation (f_∞) was calculated from the current activated by a test pulse to +10 mV from various holding potentials using the formula: $f_\infty = I/I_{max}$; (I_{max}: the maximal current in each cell was usually obtained by pulses to 10 mV from −100 mV). The best-fit curves were drawn using a similar formula for steady-state activation. $V_{1/2}$: −38.4 mV (Ca), −47.9 mV (Ba), −67.8 mV (Sr); k: −17.0 mV (Ca), −12.3 mV (Ba), −17.5 mV (Sr). The results were obtained from three (Ca) or two cells (Ba or Sr)

et al., 1986). The order of the potency to neutralize surface charge is expected to be Ca > Ba > Sr when compared at the same concentration. Therefore, the difference in the inactivation kinetics between the current in 1.8 mM Ca and that in 10.8 mM Ba cannot be attributed to a change in the surface charge because the curves for 1.8 mM Ca and 10.8 mM Ba showed similar voltage dependencies. In case of the Sr current, $t_{1/2}$ at −20 mV was 118.0 msec ($n = 2$), a value which is much larger than $t_{1/2}$ of Ca current at 0 mV. If Ca-channel current underwent conventional voltage-dependent inactivation, Sr current would show faster inactivation kinetics. Thus, the surface charge effects of Ba and Sr cannot account for the slowed inactivation kinetics.

Figure 3 illustrates the change in Ca-channel current when the extracellular solution containing 10.8 mM Ba was replaced with that containing 10.8 mM Ca. The peak current decreased when the solution change-over was about half accomplished (1-min trace). However, it rose to an increased level when the exchange was completed (3-min trace). The transient decrease in amplitude, which is not predicted from a simple competition between Ca and Ba, may reflect the anomalous mole fraction effect previously reported for the Ca channel in the heart cells (Hess & Tsien, 1984; Lansman, Hess & Tsien, 1986; McDonald et al., 1986).

Ca-Dependency of the Inactivation Time Course of Ca-Channel Current

In the early experiments, Ca-channel current was recorded from cells superfused with extracellular solution containing 10.8 mM Ba^{2+} and 0.45 and 0.9 mM Ca^{2+}. Inactivation was much faster in the presence of extracellular Ca ions. The half-decay time was decreased to 32.0 ± 4.4 msec (at 0 mV; mean ± SEM, $n = 4$) in the presence of 0.9 mM Ca as compared to 112.0 msec in Ca-free solution.

The sensitivity of the inactivation time course of Ca-channel current to the extracellular Ca is depicted in Fig. 4. The cell was superfused continuously with the 10.8 mM Ba-containing solution and the Ca^{2+} concentration was raised stepwise. Extracellular Na^+ was replaced with tetraethylammonium (TEA). Inactivation was accelerated by Ca in a concentration-dependent manner. When plotted against the time, the current obtained during the first 400 msec could be fitted to a single exponential (Fig. 4*B*). The inactivation rate of the Ba current during the first 400 msec was also accelerated by increasing the amplitude of the depolarizing steps (−30 to +30 mV: Nakazawa et al., *unpublished*). Plotting the time courses of currents evoked by pulses much longer than 400 msec revealed a more slowly inactivating component. This slower inactivation suggests the existence of more than one closed or inactivated state for Ca channels in

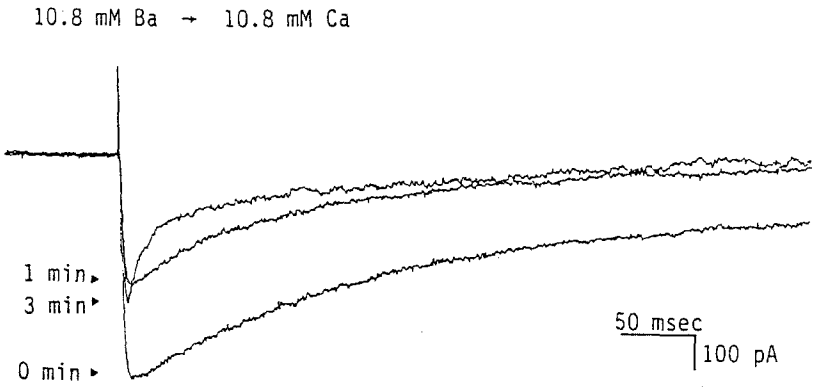

Fig. 3. Change in Ca-channel current when the extracellular Ba (10.8 mM) was replaced with Ca (10.8 mM). The currents before (0 min), during (1 min) and after (3 min) the replacement were superimposed. Note that the peak amplitude of the current at 1 min was the smallest

smooth muscles similar to those suggested in cardiac cells (Hess, Lansman & Tsien, 1984; Cavalié, Pelzer & Trautwein, 1986). The slower component of inactivation also seemed to become faster with more depolarizing steps. However, it was difficult to determine the time constant of the slower component precisely, especially in the cases of the current obtained in both Ba- and Ca-containing solutions, where the amplitudes became relatively small. Additional complications disturbing the accurate measurement of the slow component will arise if there are incomplete suppression of outward current and a nonlinear component of the leak current. Therefore, we employed only the time constants of the fast component to evaluate the effect of extracellular Ca on the inactivation kinetics.

Evaluation of the accelerating effect of Ca was made on the assumption that the inactivation of Ca-channel current has two components, a voltage-dependent component and a Ca-dependent component (Kass & Sanguinetti, 1984; Lee et al., 1985; Jmari et al., 1986). The two components can be mathematically represented as follows:

$$O \xrightarrow{\alpha} I, \text{ where } \alpha = \alpha(V) + \alpha(\text{Ca}).$$

O and I are open and inactivated states, respectively. $\alpha(V)$ is the rate constant of voltage-dependent inactivation and α(Ca) is that for Ca-dependent inactivation. α is the rate constant of total inactivation and is equal to the reciprocal of the measured inactivation time constant ($1/\tau$). The rate constant of voltage-dependent inactivation was calculated from the time course of Ba current in the absence of extracellular Ca^{2+}.

As shown in Fig. 4(*C*), the inactivation rate constant depended on the Ca concentration with inactivation being accelerated by increasing extracellular Ca^{2+} concentration. A sigmoidal shape of the concentration-effect curve indicates that the acceleration of inactivation induced by Ca is saturable and apparently depends log-linearly on extracellular Ca. The concentration which produced a half-maximal effect was about 2 mM. Hess and Tsien (1984) reported Ca^{2+} ions may interfere with the permeation of Ba^{2+} ions through Ca channels in cardiac cells, which was also suggested in the present study (Fig. 2). If considering this ion permeation mechanism of the anomalous mole fraction, the half-maximal concentration in the solution containing only Ca as a charge carrier will become larger.

Ca Current in the Cells Loaded with High Concentration of Ca-Chelators

In isolated cardiac cells, the inactivation of Ca current was slowed by the pressure injection of EGTA (Josephson et al., 1984) or loading the cells with 50 mM citrate (Bechem & Pott, 1985). We investigated the effect of high concentrations of these chelators on Ca current in smooth muscle cells. The current was recorded in extracellular solution containing 1.8 mM Ca.

The inactivation time course of Ca current measured with the 20 mM EGTA, Ca-free internal solution was not much different from control, i.e., that obtained with the 2 mM EGTA and 0.18 mM Ca solution in the pipette (Fig. 5*A*, *see also* Fig. 1*A*). $t_{1/2}$ at 0 mV was increased only slightly to 33.9 ± 2.4 msec (mean ± SEM, $n = 3$) from the control value of 27.3 msec. The relatively fast inactivation kinetics were not changed even after 20 min of dialysis.

In contrast to the results in 20 mM EGTA, the Ca current in 50 mM citrate-loaded cells exhibited a much slower time course of inactivation (Fig. 5*B*). The slowing of the inactivation rate became apparent within 1 min and reached a steady state about 3 min after the start of citrate loading, or rupture of the membrane. $t_{1/2}$ was 155.5 ± 8.0 msec (at 0 mV: $n = 4$); this was comparable to the values obtained for Ba or Sr currents (*see* Fig. 1*B*,*C*).

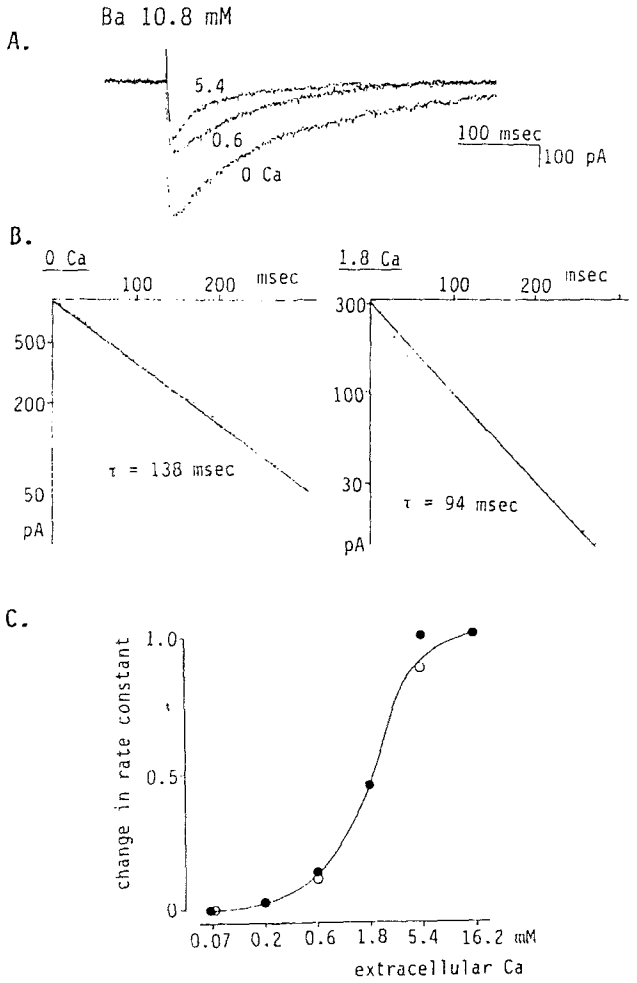

Fig. 4. Extracellular Ca-dependency of the inactivation time course. The Ca-channel current was activated by a test pulse to 0 mV from a holding potential of −60 mV. The external NaCl was omitted by replacement with TEA-Cl in this case. (*A*) Currents in 0, 0.6 or 5.4 mM Ca-containing solutions (10.8 mM Ba was continuously present). The Ca concentration was increased stepwise and the currents in Ca-containing solutions were measured 3 min after onset of the superfusion. (*B*) Inactivation time course of Ca-channel current recorded in 10.8 mM Ba and 0 mM Ca (left) and 10.8 mM Ba and 1.8 mM Ca (right). The logarithm of the difference in current amplitude from that at 400 msec was plotted against time. (*C*) Concentration-dependent effect of Ca on inactivation. The ordinate indicates the normalized values for the difference between the inactivation rate constant at the given Ca concentration and that in Ca-free, 10.8 mM Ba solution. The rate constants were calculated from the inactivation kinetics as shown in (*B*) (*see* text for details). The extracellular Ca was increased (filled circles) as shown in (*A*) and then decreased (open circles) stepwise. (*A*), (*B*), (*C*) are from the same cell

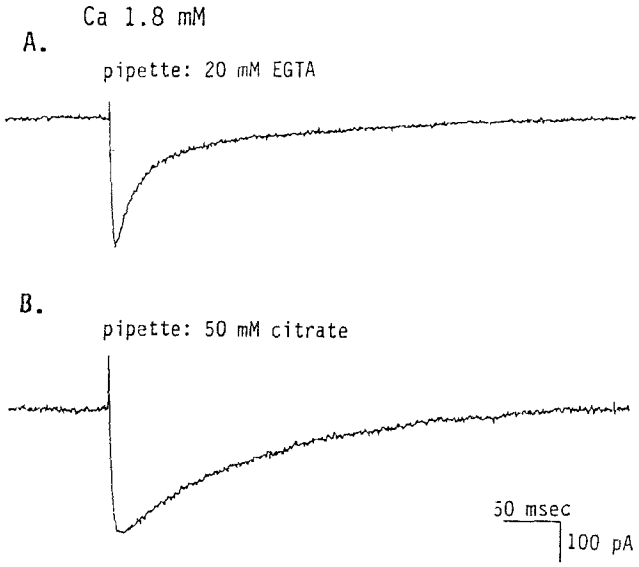

Fig. 5. (*A*, *B*) Ca current obtained with the pipette containing 20 mM EGTA (*A*) or 50 mM citrate (*B*). The inactivation kinetics of the current in the cell loaded with 20 mM EGTA were not much different from that loaded with 0.18 mM Ca and 2 mM EGTA (*see* Fig. 1*A*). However, the current in 50 mM citrate-loaded cells exhibited much slower kinetics. The current at 0 mV activated from −60 mV was measured 10 min (*A*) or 3 min (*B*) after gaining intracellular access

Discussion

The inactivation kinetics of Ca-channel current in isolated smooth muscle cells from rat vas deferens were accelerated by the elevation of the extracellular Ca concentration and slowed when the cells were loaded with 50 mM citrate. These results indicate that a mechanism involving Ca underlies at least part of the inactivation process in Ca channels in smooth muscle cells. This notion has already been suggested for isolated cardiac cells (Hess & Tsien, 1984; Josephson et al., 1984; Bechem & Pott, 1985; Lee et al., 1985). Recently, a Ca-dependent mechanism was suggested by Jmari et al. (1986) from results obtained using a double sucrose-gap to voltage clamp uterine smooth muscles. The present study demonstrates the Ca dependence more directly because the intracellular milieu was controlled. Therefore, Ca-mediated inactivation may be a common mechanism for regulating Ca-channel current inactivation rate in smooth muscle.

Standen and Stanfield (1982) proposed a binding site model for Ca-dependent inactivation of Ca current in skeletal muscles. They assumed that at least two areas involved with intracellular Ca diffusion exist in cells, and that the time course of inactivation depends on the concentration of Ca^{2+} in the area just beneath the cell membrane (about 20% of total volume). However, the diameter of the skeletal muscle preparations they used was about 70 μm, and this model cannot be readily applied to much smaller preparations such as isolated smooth muscle cells from rat vas deferens (the diameter was about 10 μm: Nakazawa et al., 1987). Thus, it seems adequate, at present, to analyze this Ca-de-

pendent inactivation mechanism with a simpler model such as we employ.

The acceleration of Ca-channel current depended log-linearly on the concentration of the extracellular Ca when assuming our simple kinetic model of inactivation. The apparent half-maximal value of 2 mM does not necessarily reflect the affinity of the presumed Ca-binding sites responsible for the Ca-dependent inactivation which may exist either within the lumen or on intracellular surface of the Ca channel. The concentration around the site cannot be determined precisely. The precise Ca dependency of the inactivation rate is expected to be determined by the intracellular free-Ca concentration. However, in preliminary experiments with cells which were dialyzed with an internal solution having a higher free-Ca concentration (ca. 10^{-5} M), it was difficult to measure amplitude and time course of Ca-channel current because of large oscillatory currents (about 100 to 300 pA).

Hess and Tsien (1984) modelled the cardiac Ca channel as a single-file pore with two ion-binding sites and three energy barriers. The model could predict an anomalous mole fraction effect. The present results may indicate that their model can also be applied to the smooth muscle.

The inactivation of Ca current in 1.8 mM Ca was not changed by the dialysis of the cell with 20 mM EGTA but was significantly slowed with 50 mM citrate. Similar results were reported in isolated cardiac cells (Bechem & Pott, 1985). The free-Ca concentration of 20 mM EGTA solution is not expected to be less than 50 mM citrate solution because the affinity of EGTA to Ca is higher than that of citrate: the dissociation constants were 10^{-6} M for EGTA (Harafuji & Ogawa, 1980) and 4×10^{-4} M for citrate (Sillen & Martell, 1971). However, the difference in the blocking effects of these chelators on the Ca-dependent inactivation may indicate that their intracellular chelating ability differs from that estimated in free solution. Bechem and Pott (1985) suggested that the capacity of Ca chelators near the inner surface of the cell membrane may change depending on the level of membrane potential or on local pH. It is also possible that the relatively large molecular size of EGTA may hinder free access to the putative Ca-binding site responsible for Ca-dependent inactivation. Another possibility that cannot be denied at present is that citrate delayed the inactivation by some unknown mechanism other than its Ca-chelating action.

The present results indicate the existence of Ca-dependent inactivation of Ca current in the smooth muscle using the isolated cells. This Ca-dependent inactivation may serve to provide negative feedback to protect against Ca overload in smooth muscle cells. Further studies are necessary to clarify the precise mechanism of Ca-induced inactivation, especially its relation to voltage-dependent mechanism.

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