Calcium-Induced and Voltage-Dependent Inactivation of Calcium Channels in Crab Muscle Fibres

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Abstract. Inactivation of Ca channels was examined in crab muscle fibres using the voltage-clamp method. A satisfactory suppression of outward currents was attempted by the use of K^+ blocking agents: TEA, 4AP and Cs ions instead of K^+ ions applied extracellularly. The inactivation of Ca current appeared as a bi-exponential process. The faster component had a mean value of the time constant of 50 ms while the second component inactivated at a tenfold slower rate. The extent of inactivation of the faster component increased as the Ca current itself increased in different experimental conditions. Inactivation decreased when I_{Ca} was reduced for large applied depolarizations. The time constant of the faster calcium component also depended on the calcium current. Thus the results suggested that Ca^{2+} entry leads to inactivation of one component of calcium current in crab muscle. Substitution of Ca^{2+} ions by Sr^{2+} or Ba^{2+} ruled out the hypothesis concerning an accumulation process which would explain the decrease of the inward current. The second slower component of Ca current was better described by a voltage-dependent mechanism and its rate was not modified in Ca^{2+} rich solution or when the inward current was carried by Sr^{2+} or Ba^{2+} ions. Thus in crab muscle fibres, inactivation is mediated by both calcium entry and a voltage-gated mechanism.

Key words: Calcium channel — Inactivation — Strontium — Barium — Crustacean muscle

Introduction

In many excitable cell membranes, an inward calcium current has been described. It has been found to decline under maintained depolarization in voltage-clamp conditions and this effect has been generally attributed to inactivation of Ca^{2+} channels. In crustacean muscles, inactivation has been reported as the

result of a voltage-and time-dependent mechanism which is similar in nature to the inactivation process described for Na currents by Hodgkin and Huxley (1952). Examples have been provided by Mounier and Vassort (1975 a) on crab muscle, and by Henček and Zachar (1977) on crayfish fibres. However, in barnacle fibres perfused with a Ca^{2+} chelator the Ca current did not appear to inactivate (Keynes et al. 1973).

In recent years various investigators have reported the existence of another mechanism of inactivation for the Ca channels, which is dependent on Ca^{2+} entry. This has been well described in paramecium (Brehm and Eckert 1978; Brehm et al. 1980), molluscan neurones (Tillotson 1979), insect muscle (Ashcroft and Stanfield 1981) and cardiac muscle (Mentrard et al. 1984). However with similar experimental set up Fox (1981) did not find any Ca^{2+}-dependent inactivation in eggs of the marine polychaete *Neanthes arenaceodentata*, and Brown et al. (1981) reported the existence of both Ca^{2+} and voltage inactivation mechanism in nerve cell bodies of *Helix aspersa*.

The fall of the inward current may have several other causes. First, there is the problem of contamination by an outward flux of current either voltage-dependent or triggered by Ca^{2+} entry for K^+ currents (Mounier and Vassort 1975 b; Standen 1975; Connor 1977; Meech 1978). Secondly, Almers et al. (1981) reported that the decline of calcium current in frog muscle may be due to Ca^{2+} depletion in the transverse tubules.

Recent reports have shown the existence of two calcium currents (Zahradník and Zachar 1982; Jdaiaa and Guilbault 1986, on crayfish) in conditions in which the outward currents were suppressed more strongly. This offered us the opportunity to re-examine Ca channel inactivation in crab muscle. Here we report results obtained with improved experimental techniques for I_{Ca} recording. Results show that the decline of I_{Ca} follows a double exponential time course. The inactivation of the faster component can be described by a Ca^{2+}-dependent mechanism while the inactivation of the slower one is related to a voltage-dependent process.

Materials and Methods

Muscle fibres and the clamp technique

Experiments were performed on single fibres of the extensor muscle isolated from the walking legs of the crab *Carcinus maenas*. The preparations were placed in sucrose-gap apparatus which allows both current and voltage clamping. The clamp method has been fully described in previous papers (Mounier and Vassort 1975a, 1979) and the reliability of the method has also been tested (Mounier and Vassort 1975b).

The leakage current is defined as the current that remains after blockage of I_{Ca} by Co^{2+} ions and as complete as possible suppression of I_{K1} and I_{K2} by K^+ blocking agents (see solutions). It has

linear and nonlinear components (see Fig. 1*C*). The linear current appears alone for applied hyperpolarizing or depolarizing pulses lower than 60 mV. For higher depolarizations the non-linear current is evident and outward rectification can be described.

Measurements of the peak calcium current amplitudes were made with two methods. The first consisted in measuring the difference between the two currents obtained for a given membrane polarization in control solution and then in Co^{2+} solution. The other is used in some long experiments with several pulses when Co^{2+} solution cannot be applied. It consists in measuring the difference between the peak value and the steady value of the current at the end of long duration pulses (1500 ms or more). The method used is indicated for each experiment. Both methods gave similar results. Treatments which reduce or suppress Ca currents such as Co^{2+}, Mn^{2+}, La^{3+}, verapamil, have been shown to reduce outward currents in a variety of membranes. In crab muscle a slight decrease of the late outward current has been observed using Mn^{2+} ions (20 mmol/l) (Mounier and Vassort 1975a, their Figure 5). A similar slight decrease for applied pulses higher than 65 mV or no effect were found with Co^{2+} ions (20 mmol/l) (Stal 1984).

The holding potential was set at — 70 mV in all our experiments. Experiments were done at room temperature (18—20°C).

Paired-pulse inactivation protocol

The two pulse method was applied to study the inactivation process. The first pulse, called prepulse or conditioning pulse, was of variable amplitude and duration. The second pulse or test pulse never exceeded 60 mV and was generally chosen equal to 50 or 55 mV in order to elicit large inward currents minimally contaminated, as mentioned above, by outward remaining K current or non-specific currents.

An interval of 20 ms was allowed between the pulses which served two purposes: first, it allowed the activation kinetics of Ca^{2+} permeability to return to the resting state; secondly, it allowed any remaining K^+ permeability activated during the first pulse to return to rest prior to the test pulse. The test pulse was thus always applied from the same holding potential ($E_m = -70$ mV) and the driving force as well as the capacitive component remained constant independently of the conditioning pulse. An interval of 20 ms between the two pulses was chosen since the capacitive current declines with a time constant of 1 to 2 ms and Ca current deactivates with a time constant of 9 ms after 50 mV depolarization. Steady-state inactivation was computed as the ratio of test pulse current following a prepulse to test pulse current preceeded by no prepulse.

Solutions

The control solution was derived from artificial sea water ASW (Fatt and Katz 1953) in order to obtain a solution which permits as complete a suppression of the outward currents as possible. It was a K^+-free saline solution containing (in mmol/l); 413 NaCl; 5 $CaCl_2$; 23.6 $MgCl_2$; 2.6 $NaHCO_3$; 12.9 CsCl; 50 tetraethylammonium (TEA) and 1 4-aminopyridine (4AP). Since it has been shown that Cs^+ ions exert their blocking effect on the inner membrane surface and that permeability of the membrane for Cs^+ is very low (Gainer and Grundfest 1968) the fibres were allowed to imbibe in the control solution during two hours in order to facilitate entry of Cs^+ ions in the cell. A solution in which Cs^+ replaced external K^+ was used with barnacle muscle without previous imbibition. Changes in resting potential were observed suggesting that Cs^+ ions are able to cross the membrane; however the permeability is much less to Cs^+ than to K^+ (Lakshminarayanaiah and Rojas 1973).

Recently, Zucker (1981) has reported that commercial TEA chloride contains an impurity which alkalinizes the cytoplasm of Aplysia neurones and reduces their Ca^{2+}-buffering capacity.

Since changes in myoplasmic pH affect muscle function (Mac Donald and Jobsis 1976; Robertson and Kerrick 1979) the effect of TEA treatment on intracellular pH (pH_i) was checked. Electrodes for measuring pH_i and membrane potentials (E_m) were inserted in intact muscle fibres. The electronically obtained difference between the pH and the E_m electrode signal was the measure of pH_i; that between the E_m and the external reference electrode signal was membrane voltage. The pH-sensitive electrodes were similar to those used by Ammann et al. (1981). Three pH electrodes were used for the experiments; their slope was 56.4; 60.8; and 64.6 mV per unit pH. With the ASW solution of Fatt and Katz (1953) (pH 7.80) intracellular pH values of 7.06 to 7.14 were obtained and were in good agreement with those proposed by Paillard (1972) for similar intact fibres in ASW. The addition of TEA (50 mmol/l) and 4AP (1 mmol/l) in ASW solution and keeping of the extracellular pH at 7.80 did not induce any significant change in intracellular pH (from 7.05 to 7.16) even after long imbibition times.

Co^{2+} solution used to block Ca currents (Hagiwara and Takahashi 1967) was prepared by adding Co^{2+} ions (20 mmol/l) to the control solution. The Ca^{2+}-rich solution was prepared by adding $CaCl_2$ to the control solution. Sr^{2+} or Ba^{2+} solutions were made by replacing $CaCl_2$ in the control solution by equivalent amounts of $SrCl_2$ or $BaCl_2$.

The osmotic pressure of all solutions was adjusted to that of ASW, by reducing NaCl concentration when necessary. The external pH was always kept at 7.80.

Results

Ca currents

In crab muscle fibres as well as in crayfish and insect muscles, a graded and small electrical activity has been reported. In voltage-clamp conditions depolarization elicits three ionic currents: an inward current, carried by calcium ions and two potassium currents, a fast outward I_{K_1} and a slow outward current I_{K_2} (Mounier and Vassort 1975a, b; Henček and Zachar 1977; Ashcroft and Stanfield 1982a). By recording electrical activity on isolated fibres exposed to K^+ blocking agents such as TEA (20 mmol/l) and Cs^+ ions (12 mmol/l instead of external K^+ ions) it is possible to avoid most outward currents that normally mask the Ca current. Thus, a large all or none action potential of 120 mV amplitude and 300 ms duration appears (Fig. 1*A*). In many fibres the amplitudes generally range between 120 and 140 mV while the durations are more variable and may reach up to 500 ms. Ionic currents elicited by long duration depolarizing pulses (Fig. 1*B*) show a peak inward current with a fast initial decrease followed by a second slower decreasing component. In previous experiments (Mounier and Vassort 1975a) K^+ currents were only blocked by TEA, and short duration pulses (below approx, 150 ms) were applied. In such conditions the decrease of ionic currents did not exhibit a similar complex time course and the falling phase was fit by a single exponential function. In our new conditions (TEA, 4AP, Cs^+) single exponential decay was rarely observed, generally in fibres which exhibited a small I_{Ca}.

Since Ca currents have been frequently suppressed by different divalent ions (Hagiwara and Takahashi 1967), Co^{2+} ions (20 mmol/l) were added to the extracellular control solution. The currents elicited by hyperpolarizing and depolarizing pulses of 1700 ms duration are shown (inset Fig. 1*C*). The two components of currents relaxation were suppressed. This confirms that the currents in Figure 1 (*B*) were carried by Ca^{2+}. For hyperpolarizations and depolarizations smaller than to about 60 mV steady leak currents were elicited and the leak current-voltage relationship was linear (Fig. 1*C*). At potentials higher than 60 mV a delayed outward rectification appeared. For this reason, as indicated in the Method section, test pulses in the paired-pulse inactivation

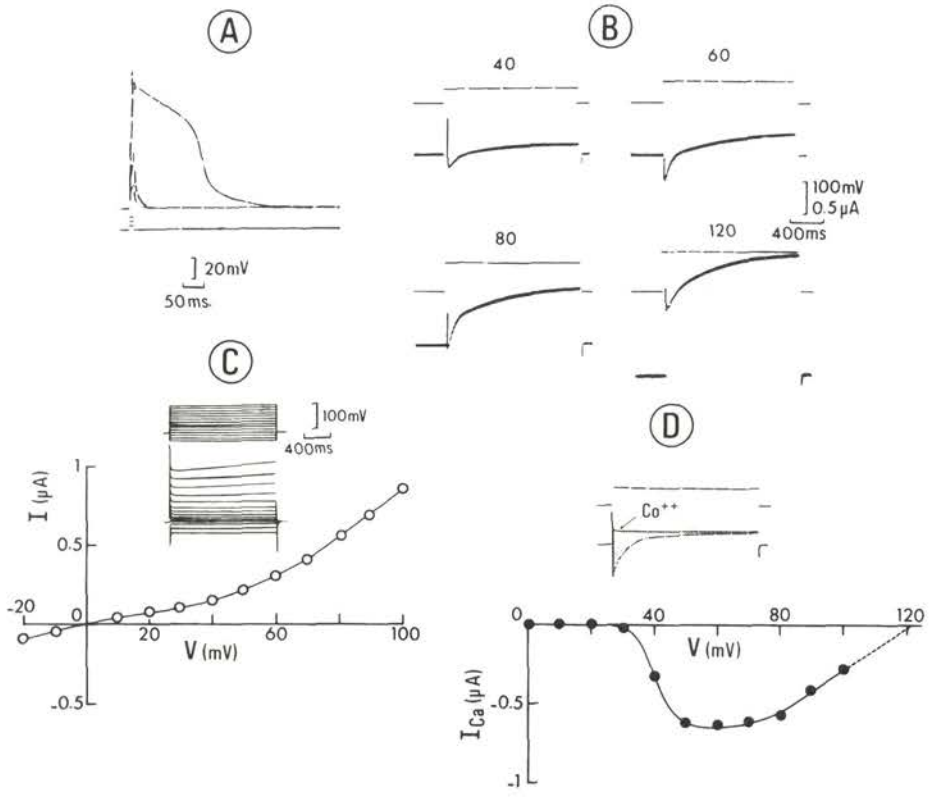


Fig. 1. Electrical activity in control solution (K^+-free ASW + TEA, 4AP, Cs^+). *A*: Regenerative action potential recorded in current clamp conditions; *B*: Ionic currents recorded in voltage clamp conditions for 4 depolarizing steps (values indicated on the records); *C*: Current-voltage relationship in Co^{2+} solution for current measured at the end of 1700 ms pulses (records are shown in inset); *D*: Relationship between maximal calcium current I_{Ca} and applied voltage. I_{Ca} is measured as the difference between the peak inward current in control solution and the current at the same time after application of the Co^{2+} solution as shown in the inset.

experiments were chosen equal to or lower than 60 mV. The inward current amplitudes were estimated either with reference to the same current in Co^{2+} solution (as illustrated in Fig. 1) or with reference to the steady current at the end of long duration pulses. The peak Ca current-voltage relationship could thus be established (Fig. 1*D*). In this experiment, the reversal potential estimated by extrapolation of the curve was for a 120 mV depolarization. It generally happens for 120 to 150 mV depolarizations, but these values are probably underestimated (see Discussion). No differences were observed between the methods used for measuring the Ca current.

Moreover we checked that the time-dependent current relaxation observed was due to a decreased conductance for inward current rather than a conductance increase for outward current. If one supposes that the second hypothesis (activation of outward current) is true, the instantaneous tail currents observed on repolarizing the membrane after a given step V_1 would become gradually larger as the duration of V_1 increased. The experiment is illustrated in Figure 2. The fibre is depolarized by 60 mV (V_1) for 60; 100; 160; 200 ms before stepping back to a level V_2 which was chosen in order to maximize outward tail current due to a previous outward current activation. So, a value of +15 mV was chosen for V_2 for the following reason. If outward current has been predominantly activated during V_1, a large component of this current would be carried by K^+ ions. Thus, a slight depolarization from the holding potential would enhance the outward direction of the tail since the equilibrium potential for K^+ ions is very near the holding potential (Fatt and Katz 1953; Hays et al. 1968). The records show that the tail currents at V_2 are always inward and decrease when the

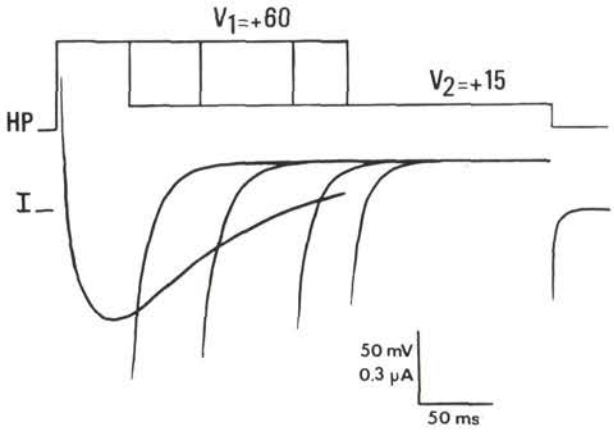


Fig. 2. Conductance decrease during calcium current inactivation. The inward current was elicited by a 60 mV depolarizing step of different durations (V_1); then the fibre was repolarized to a level V_2 (+15 mV from HP). Note the decrease of the tail currents.

duration of V_1 increases. A similar result is obtained with depolarizations up to 1000 ms. This is a clear indication of the conductance decreasing over time. Other experimental arguments will be shown in following experiments and summarised in the Discussion section.

The observation of two phases in the decrease of the currents elicited by long duration pulses can be attributed to the existence of two components of inward current. We will call them I_{Ca1} and I_{Ca2}. The decay of the Ca current follows a double exponential time course and we examined the two rates of inactivation. On 16 fibres, for 55 mV-1700 ms depolarizations the fast time constant ζ_1 was 50.5 $\pm$ 3.2 ms (mean + SEM) and the slow time constant ζ_2 was 401.8 + 35.9 ms (mean + SEM). The ratio ζ_2/ζ_1 was 8.2 + 0.7.

Effect of prepulses on Ca currents

To investigate this point we adopted the following protocol. We used an inactivating prepulse V_1 of different amplitudes followed by an interval of 20 ms at the holding potential. A test pulse V_2 of 50—55 mV then measured the amount of remainig Ca current. Steady-state inactivation of h_∞ measurements requires prepulses of sufficient duration to reach a new steady state. However long durations (1 or 2 s) limited the number of trials that could be done without deterioration of the fibre. We used prepulse durations of 25 to 300 ms (generally 100 or 200) as a measure of inactivation although the process was incomplete particularly with the shorter durations. However the relationships are similar to that obtained for 1500 ms prepulse duration (see Fig. 6).

Figure 3 shows that peak amplitude of the Ca current during the test pulse depended on the membrane potential during the prepulse V_1 of 100 ms duration. It decreased when V_1 was raised up to 60 mV, but when V_1 was further increased up to 150 mV, the Ca current reappeared during the test pulse. The semi-logarithmic plot of the different current in the test pulse (50 mV-1700 ms) obtained after 100 ms prepulses of different amplitudes is illustrated in Figure 4. The current amplitudes during the test pulse refer to the final steady level. An analysis of the time constants ζ_1 shows a feature which would be highly unusual for a voltage-dependent gating proces. ζ_1 is always measured during the test pulse but appears modified by the prepulse amplitude. Prepulses which induce larger inactivations increase the time constant of the decline of the first Ca component. ζ_1 is 64 ms for a 20 mV prepulse and reaches 112 ms for a 60 mV prepulse. Similar complete experiments were performed on 10 fibres and except the absolute values of time constants which differed from fibre to fibre, the maximal increase in ζ_1 appeared always when the current in the test pulse was smallest i. e. for a prepulse of about 60 mV. The ζ_1 increase ranged between

57 and 80 % of the value of ζ_1 measured in the test pulse without prepulse. For higher prepulses which induced smaller inactivation ($V_1 = +120$ or $+150$ mV) ζ_1 decreased again to its initial value. The time constant ζ_2 of the second component of Ca current was not significantly modified by the prepulse amplitude.

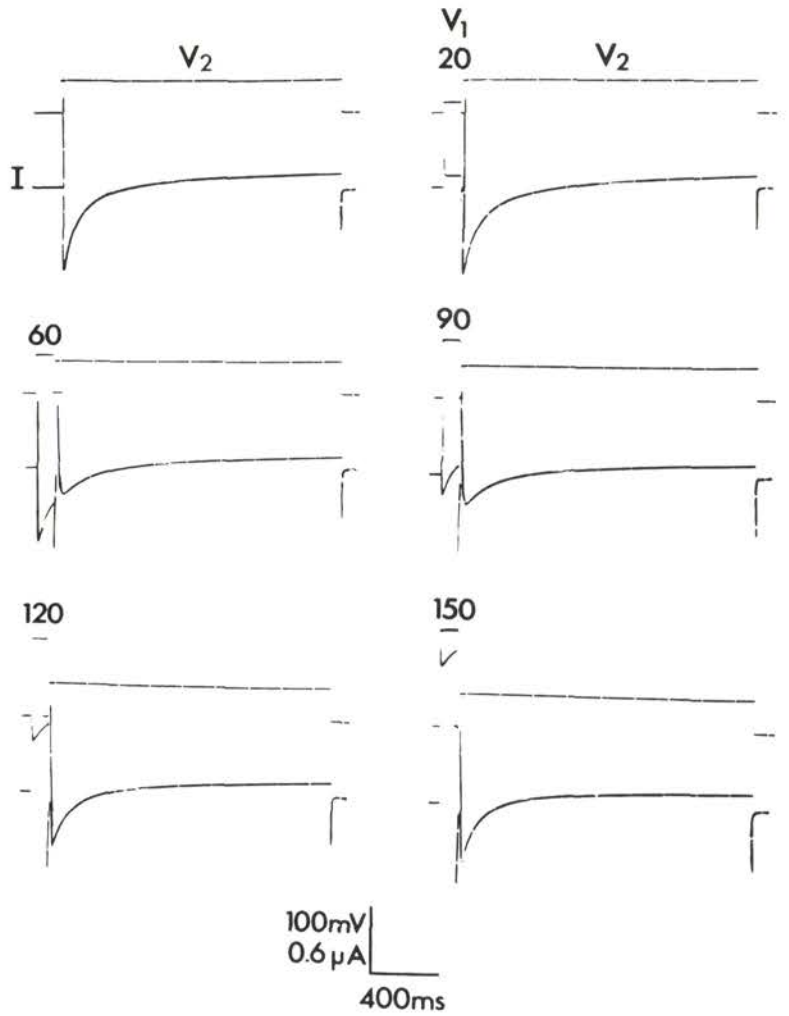
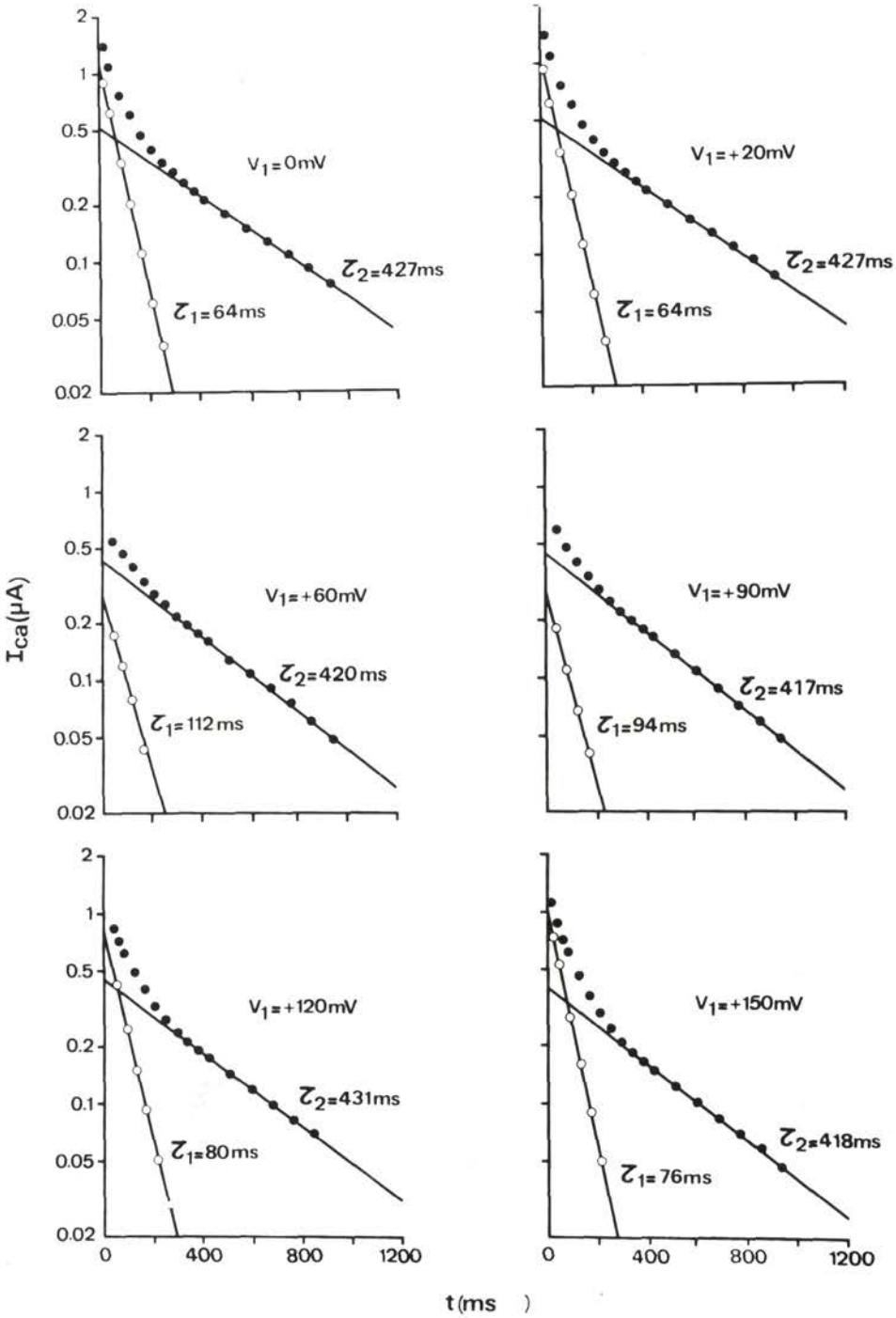


Fig. 3. Analysis of the inactivation process by a paired pulse experiment. A test pulse V_2 (50 mV-1700 ms) was preceeded by different depolarizing steps V_1 (amplitude shown at the records) of 100 ms duration.

Fig. 4. Semilogarithmic analysis of the inward current decrease measured during the test pulse V_2 after different prepulses V_1 (same fibre and protocol as in Fig. 3).


$I_{Ca}(\mu A)$
2
1
0.5
0.2
0.1
0.05
0.02
V_1=0mV
τ_2=427ms
τ_1=64ms
V_1=+20mV
τ_2=427ms
τ_1=64ms
V_1=+60mV
τ_2=420ms
τ_1=112ms
V_1=+90mV
τ_2=417ms
τ_1=94ms
V_1=+120mV
τ_2=431ms
τ_1=80ms
V_1=+150mV
τ_2=418ms
τ_1=76ms
0
400
800
1200
t(ms)

This semi-logarithmic analysis (Fig. 4) also permits to determine the peak amplitudes of I_{Ca1} and I_{Ca2}. Amplitudes of I_{Ca1} and I_{Ca2} related to maximal values versus the prepulse potential amplitude are shown in Figure 5. The I_{Ca1} inactivation is maximal for 60—70 mV prepulses and is smaller for both smaller pulses and positive potentials as E_{Ca} is approached. This I_{Ca1} voltage-dependence would suggest that inactivation of the first component of Ca current is dependent on Ca^{2+} entry. The plot of I_{Ca2} inactivation versus membrane potential shows that at potential higher than 60 mV the degree of inactivation remains unchanged. This is inconsistent with Ca^{2+}-induced inactivation and is better described by the Hodgkin-Huxley voltage dependent mechanism.

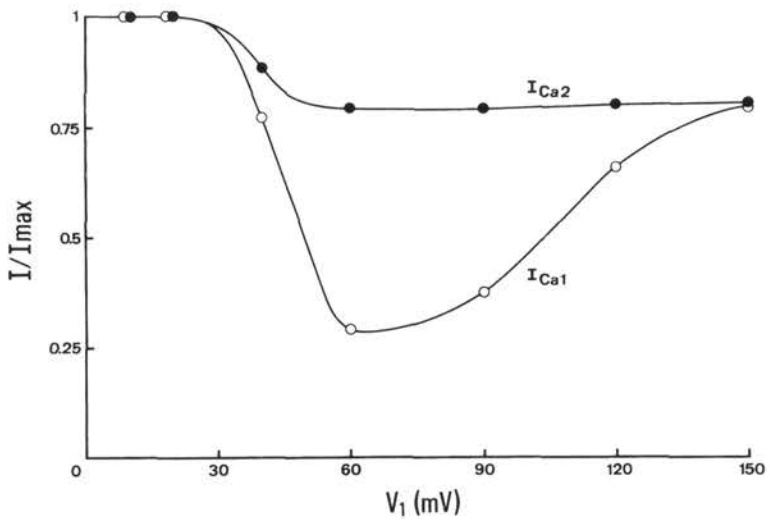


Fig. 5. Current amplitude (I) measured during the test pulse and expressed as percentage of the maximal value (Imax) versus the amplitude of the prepulse V_1. Open circles: amplitude of the first component (I_{Ca1}) of inward current. Filled circles: amplitude of the second component (I_{Ca2}) of inward current.

Influence of Ca^{2+} entry on I_{Ca1} inactivation

If I_{Ca1} inactivation depends on Ca^{2+} entry, the extent of inactivation would vary with the influx of Ca^{2+} ions during the prepulse. This is illustrated in Fig. 6 for different prepulse durations. Current amplitudes during the test pulse were measured either with reference to the steady current at the end of long duration test pulse or to the same currents in Co^{2+} solutions. Similar results were obtained with both methods.

For 1500 ms prepulse duration, i. e. steady-state conditions, inactivation was complete for 60 to 80 mV potentials and was partially overcome at larger membrane depolarizations. With shorter prepulses (200 ms) a similar result was obtained. With 100 ms prepulses, Ca^{2+} entry during the prepulse was reduced and the inactivation appeared incomplete for 60—80 mV potentials. However the whole curve has a shape similar to those described above for longer prepulses. This justifies the use of 100 to 200 ms prepulses in many experiments. With very short prepulses of 25 ms there was a smaller influx of Ca^{2+} ions and the maximal inactivation was small. It should be noted that although the degree of inactivation slightly varied from fibre to fibre the curves always had the same shape.

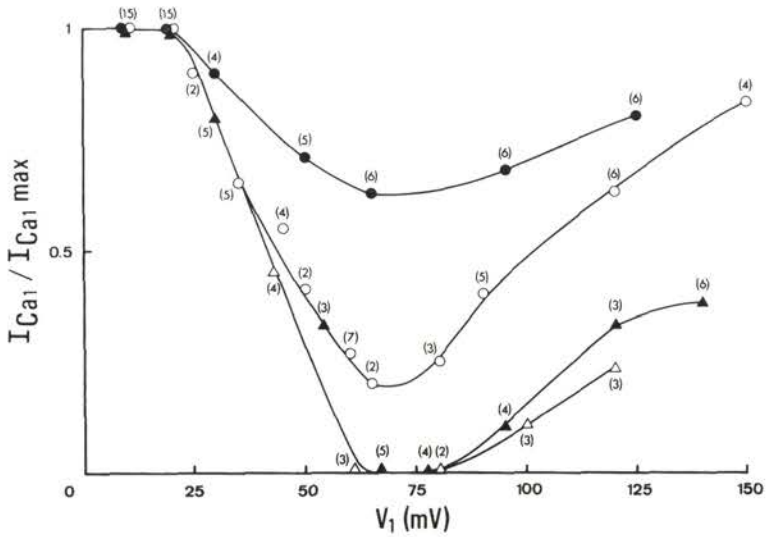


Fig. 6. Inactivation ratio of the first component of calcium current expressed as values of I_{Ca1}/I_{Ca1max} versus the amplitude of the prepulse V_1 of different durations: 1500 ms (open triangles), 200 ms (filled triangles), 100 ms (open circles), 25 ms (filled circles). Values in parentheses indicate the number of measurements.

Another indication of the existence of an inactivation process dependent on the current during the prepulse is a change of the time constant ζ_1 of the current decrease during the test pulse ($V_2 = +50$ mV) following a given prepulse V_1 of different duration ($V_1 = +60$ mV in the experiment described). When the duration of V_1 was 25 ms, ζ_1 was 50 ms. At a duration of V_1 increased to 100 ms ζ_1 was reduced to 33 ms. Similar changes were observed on two other fibres. This is another evidence for an inactivation process different from that described by

Hodgkin and Huxley. For the latter, ζ_1 must remain unchanged and independent of the prepulse duration.

A correlation between Ca^{2+} entry and inactivation is also obvious from Fig. 7. The peak amplitudes (I) of Ca current elicited during the 100 ms prepulses are shown with reference to the current in Co^{2+} solution. As I increases, I_{Ca1} inactivation increases and both parameters reach their maximal values at 60 mV depolarization. For higher membrane polarizations I_{Ca} decreases and the inac-

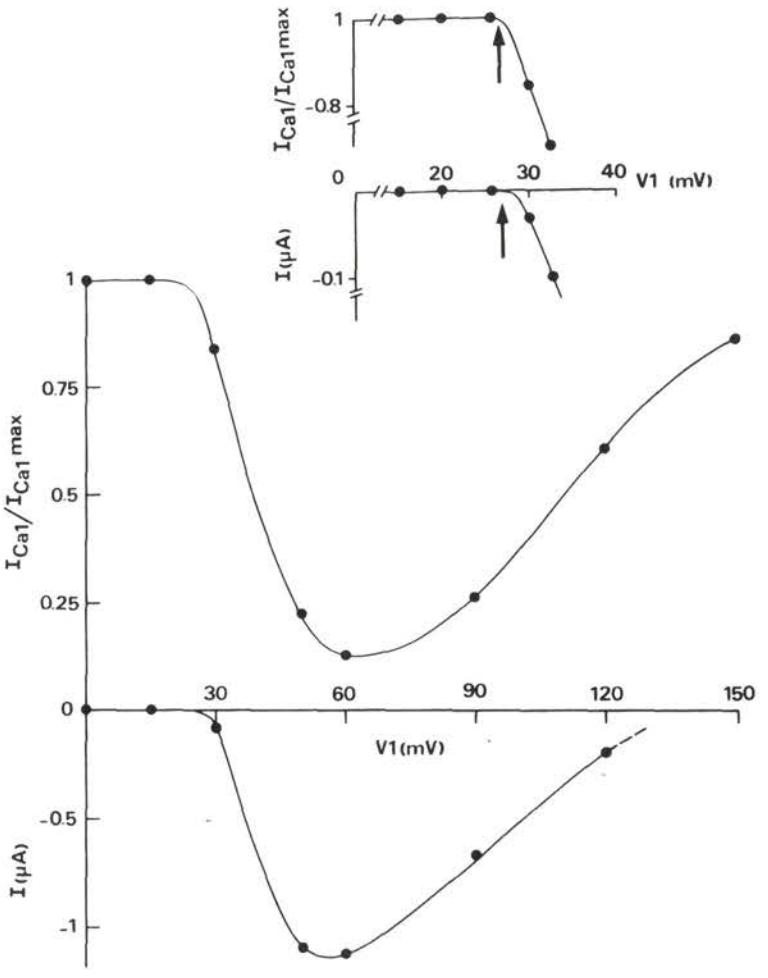


Fig. 7. Inactivation ratio of the first component of calcium current expressed as I_{Ca1}/I_{Ca1max} versus the amplitude of the prepulse V_1 of 100 ms duration (upper curve) and amplitude of the peak inward current measured during the prepulse versus the amplitude of the prepulse V_1 (lower curve). Inset: expanded scale for the results obtained for V_1 (+10 to +40 mV).

tivation also falls. The inset illustrates, on an expanded scale, the voltage region where inactivation starts; this is close to the threshold for the Ca current. Both Ca current and inactivation seem to be initiated at about the same potential $E_m = -43$ mV (27 mV depolarization from HP $= -70$ mV). This contrasts with the Ca current in some other structures where inactivation is voltage dependent and initiated at potentials about 20 mV more negative than the threshold of the Ca current (Cota et al. 1981; Fox 1981).

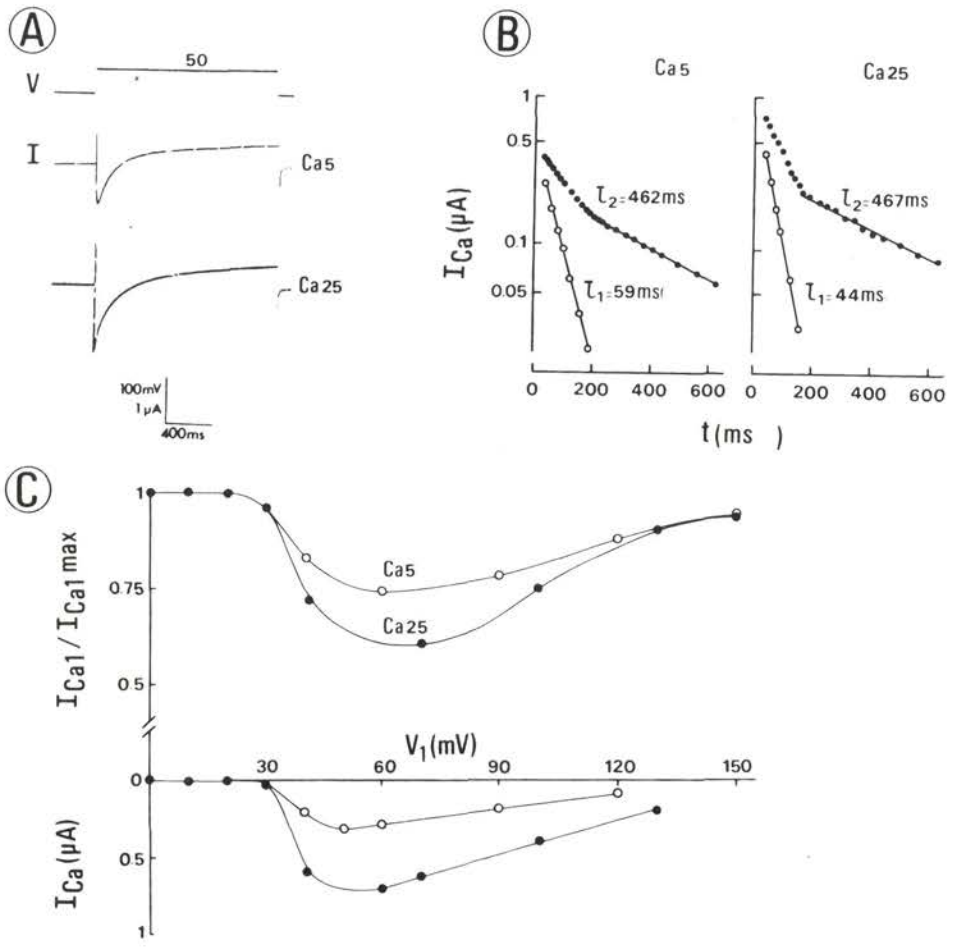


Fig. 8. Effect of the application of a Ca^{2+}-rich solution ($[Ca]_o = 25$ mmol/l) after control solution ($[Ca]_o = 5$ mmol/l). *A*: Ionic current (*I*) recorded for a 50 mV depolarization (*V*); *B*: Semi-logarithmic plot of the calcium current decrease in the control (Ca5) and Ca^{2+}-rich (Ca25) solution; *C*: Inactivation ratio of the first component of calcium current expressed as I_{Ca1}/I_{Ca1} max and maximal calcium current measured in the prepulse versus amplitude of the prepulse V_1 in the control (Ca5, open circles) and Ca^{2+}-rich (Ca 25, filled circles) solution.

If Ca current inactivation occurs due to Ca^{2+} entry, a change in Ca^{2+} entry may lead to a similar change in inactivation. Fig. 8 shows results upon a fivefold increase in $[Ca]_o$. The 50 mV-1500 ms test-pulse shows (Fig. 8*A*) an increase in the peak Ca current when $[Ca]_o$ is raised from 5 to 25 mmol/l. The Ca current is about 1.5 times larger at 25 mmol/l Ca^{2+}. The decay of the Ca current (Fig. 8*B*) is characterized by two exponentials in both solutions. The inactivation time constant ζ_1 of the first component is reduced when $[Ca]_o$ increases from 59 ms in normal solution to 44 ms in Ca^{2+} rich solution. It is interesting to note that the time constant ζ_2 remains unchanged when $[Ca]_o$ varies (respectively 462 and 467 ms in solution containing 5 and 25 mmol/l Ca^{2+}).

The degree of inactivation was checked with the two pulse protocol using the test pulse as above and 25 ms prepulses. This duration allows to more clearly demonstrate the expected increase in inactivation. In fact, increasing $[Ca]_o$ results in an increase in inactivation (Fig. 8*C*, upper part) which follows the increase in Ca current occurring during the prepulse (with reference to the same currents in Co^{2+} solution) (Fig. 8*C*, lower part). For low predepolarizations as well as for large depolarizations Ca currents are smaller and inactivation is reduced. A small shift in the depolarizing direction for the inactivation curve with 25 mmol/l Ca^{2+} may be related to similar shift in the current voltage relation of the Ca current. Thus, the effect of $[Ca]_o$ on inactivation suggests that the rate and extent of inactivation may be more closely related to the amount of Ca^{2+} which entered the fibre than to membrane potential.

Inactivation in Sr^{2+} and Ba^{2+} solutions

It is well known that in crustacean muscles Sr^{2+} and Ba^{2+} also permeate the Ca channel. Experiments were performed to study the degree and kinetics of the inactivation process with Ca^{2+} ions substituted by Sr^{2+} or Ba^{2+}. In the double pulse protocol, a 100 ms prepulse duration was chosen in order to detect either an increase in or a decrease of inactivation since this duration never induces complete inactivation in Ca^{2+} solution (see Fig. 6). Fig. 9 (*A*) shows Ca^{2+} and Sr^{2+} currents elicited by a 50 mV — 800 ms depolarization in Ca^{2+} and Sr^{2+} solutions (5 mmol/l Ca^{2+} or 5 mmol/l Sr^{2+}). The two currents have similar amplitudes and kinetics since the decay of Sr current follows a double exponential time course similarly as does that of Ca current (Fig. 9 *B*) with time constants ζ_1 of 44 and 43 ms and ζ_2 of 311 and 315 ms in Ca^{2+} and Sr^{2+} solutions respectively. The degree of inactivation of each component versus membrane potential in Ca^{2+} and Sr^{2+} solutions was measured as shown in Figs. 4 and 5*B* with reference to the final steady level for the amplitudes. The inactivation of the first component (called I_{i1} since the inward current is carried either by Ca^{2+} or

Sr^{2+}) is similar with Ca^{2+} or Sr^{2+} ions as charge carriers. Inactivation reaches a maximum for prepulses of about 60 mV and is overcome in a similar extent in both solutions for higher depolarizations. The inactivation of the second component (I_{i2}) is also maximal for a 60 mV potential and remains unchanged for higher potentials in Sr^{2+} solution as well as in Ca^{2+} solution. Similar results were obtained with 4 fibres.

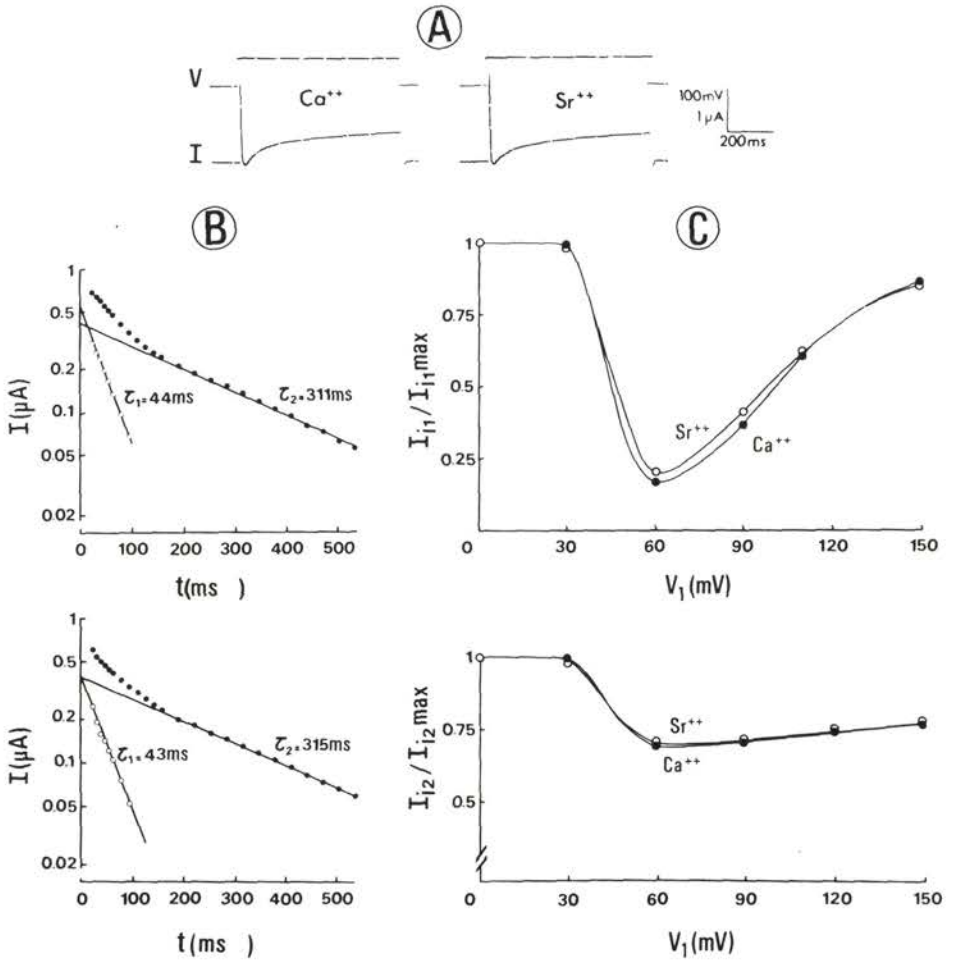


Fig. 9. Effect of the substitution of Sr^{2+} ions for Ca^{2+} ions in the external solution. *A*: Ionic current (*I*) recorded for a 50 mV-800 ms depolarization (V); *B*: Semilogarithmic plot of the inward current decrease in Ca^{2+} solution (upper graph) and Sr^{2+} solution (lower graph); *C*: Inactivation ratio of inward current for the first component (upper graph) and the second component (lower graph) versus amplitude of the prepulse V_1 of 100 ms duration.

Substitution od Ca^{2+} by Ba^{2+} (5 mmol/l), resulted in larger currents, as illustrated in Fig. 10 (*A*) for a 50 mV-1700 ms depolarization. The time course of current decay followed a double exponential in both Ca^{2+} and Ba^{2+} solutions. However, the first component inactivated slowly in Ba^{2+} solution since ζ_1 was 75 and 110 ms in Ca^{2+} and Ba^{2+} solutions respectively. For two other fibres the time constant increased by 40 to 50 %. The time constant ζ_2 appeared slightly

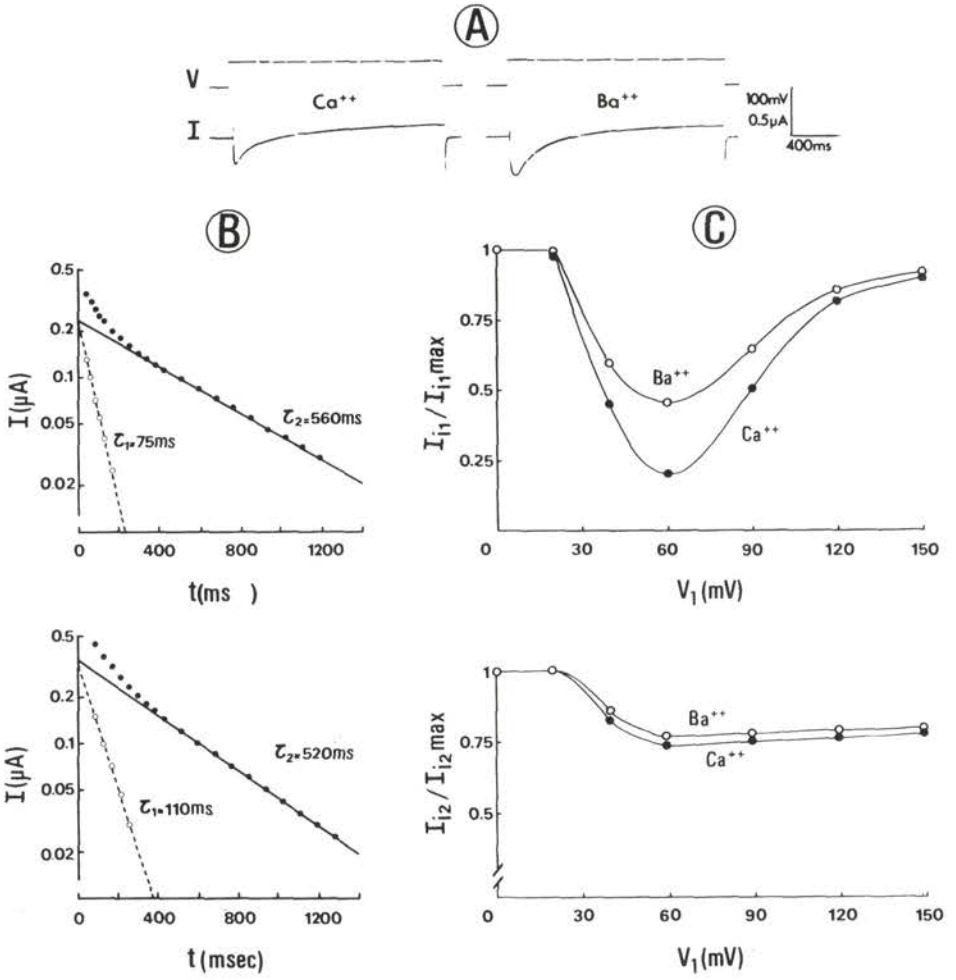


Fig. 10. Effect of the substitution of Ba^{2+} ions for Ca^{2+} ions in the external solution. *A*: Ionic current (*I*) recorded for a 50 mV-1700 ms depolarization (V); *B*: Semilogarithmic plot of the inward current decrease in Ca^{2+} solution (upper graph) and Ba^{2+} solution (lower graph); *C*: Inactivation ratio of inward current for the first component (upper graph) and the second component (lower graph) versus amplitude of the prepulse V_1 of 100 ms duration.

reduced in Ba^{2+} solution in this experiment, but a smaller occurred with the two other fibres (about 5 %). Measurements of the degree of inactivation for each component versus membrane potential (Fig. 10*C*) were made as in Sr^{2+} experiments. Less inactivation occurred as V_1 was raised up to + 60 mV for the fast component I_{i1} in Ba^{2+} solution compared to inactivation in Ca^{2+} solution. For higher depolarizations, inactivation decreased in Ba^{2+} solution as well as in Ca^{2+} solution. The second component exhibited similar inactivation curves when the current was carried by Ba^{2+}, or Ca^{2+} ions. The extent of inactivation remained maximal for potentials higher than 60 mV in both solutions.

Discussion

Experimental conditions

The results presented in this paper provide evidence that Ca current relaxation follows a double exponential time course. Particular precautions were taken to record the calcium alone and this was achieved over most of the voltage range necessary for our study by the use of TEA and 4AP applied extracellularly and by substituting Cs^+ ions for K^+ ions. In such conditions large all or none action potentials of long duration appear. The reversal potential of calcium current corresponds to 120 to 150 mV depolarizations applied from a holding resting level of — 70 mV. Thus the value of the equilibrium potential for Ca^{2+} ions estimated by the Nernst equation reaches + 50 to + 80 mV. It appears lower than the theoretical value of + 127 mV at $[Ca]_o = 5$ mmol/l and $[Ca]_i = 10^{-7}$ mol/l (Portzehl et al. 1964). The first explanation is that the isolation of I_{Ca} was incomplete and that K channels were still incompletely suppressed although more completely than in previous experiments (Mounier and Vassort 1975a). Another explanation is that the reversal potential was underestimated since for large depolarizations the leak current exhibits slight rectification and/or a non-specific outward current remains. However, for pulses lower than + 60 mV (similar to the amplitudes of test pulses used in the paired pulse experiments), with Co^{2+} ions, only a stationary current (leak current) remained. In these experiments the contamination by outward currents would be negligible.

Existence of two mechanisms of inactivation

In our new conditions, inactivation of Ca current occurs as a bi-exponential process as already described for some neurones (Kostyuk and Krishtal 1977; Brown et al. 1981); this process is dependent upon both Ca current and voltage.

even if a contribution of these mechanisms cannot be completely ruled out. Moreover an effect of surface charges change on the membrane field is also unlikely since we used Ca^{2+}, Ba^{2+} and Sr^{2+} ions at similar external concentrations (5 mmol/l).

To conclude, in crab muscle both Ca^{2+}-induced and voltage-dependent inactivation mechanisms exist. The phenomenon can be explained either by the presence of two inactivated states of one channel as proposed by Kostyuk and Krishtal (1977) or by the presence of two different channel populations. With the latter possibility, the channels could be similar but located at different places, e. g. in the surface membrane and in the tubular membrane; they also could have both different properties and localisations. An additional question arises as well: we do not know whether calcium or permeant ions bind to a site at the inner membrane surface to produce inactivation or if they induce inactivation during their passage through the membrane by interaction with a site in the channel.

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