Calcium-mediated Inactivation of the Calcium Conductance in Cesium-loaded Frog Heart Cells

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ABSTRACT Ca current inactivation was investigated in frog atrial muscle under voltage-clamp conditions. To inhibit the outward currents, experiments were performed on Cs-loaded fibers and in 20 mM Ca (K-free) Ringer with 4-AP added. Inactivation, produced by a conditioning pulse, was measured by reducing the current during a subsequent test pulse. The extent of inactivation increased initially with prepulse amplitude and then decreased as the prepulse potential became progressively positive. Relative inactivation follows a U-shaped curve. When Sr was substituted for Ca, both the degree and the rate of inactivation decreased. Relative inactivation appeared to be linearly related to the amount of divalent cations (Ca and Sr) carried into the cell during the prepulse. Elevating Ca enhanced peak current and accelerated its decline. Elevating Mg decreased peak current and slowed its decline. An application of Na-free (LiCl) solution resulted in a somewhat smaller but faster inactivating current. Adrenaline increased and D600 decreased the maximal Ca conductance with little alteration in the inactivation rate; Co decreased both peak current and the rate of inactivation. Enhancement of the outward currents, reduced driving force, and intracellular surface charge screening do not adequately account for the above results. Evidence was considered that Ca entry mediates most of Ca current inactivation in frog atrial fibers. Removal from inactivation was also investigated in normal-Ca, Ca-rich, and Sr solutions. Recovery after partial inactivation by high depolarization was biphasic. Recovery was slowed by 10 Ca and accelerated by 1.8 Sr, whereas opposite effects have been shown on activation.

INTRODUCTION

The existence of a Ca conductance in heart, originally suggested by Hagiwara and Nakajima (1966), is now well supported by experimental evidence obtained by several groups. However, the characteristics of this conductance have not been accurately determined, especially the voltage and time dependence of its activation and inactivation parameters. Ca channels were generally reported to undergo inactivation under maintained depolarizations in a manner qualitatively similar to that originally described by Hodgkin and Huxley (1952) for the Na current.
channels in squid axon (cardiac muscle: Beeler and Reuter, 1970; New and Trautwein, 1972; Horackova and Vassort, 1976; skeletal muscle: Sanchez and Stefani, 1983). However, it has been suggested recently that inactivation of Ca channels may be mediated by Ca entry in molluscan neurones (Tillotson, 1979; Eckert and Tillotson, 1981; Plant and Standen, 1981), in Paramecium (Brehm et al., 1980), and in insect skeletal muscle (Ashcroft and Stanfield, 1982). As another possibility, Almers et al. (1981) suggested that in frog skeletal muscle, the decline of Ca current under maintained depolarization could result from Ca depletion in a restricted extracellular space.

A few reports have indicated that in heart cells, Ca current is sensitive to intracellular Ca concentration. A decrease in Ca current was suggested in cat ventricular muscle treated with cardiac glycosides (McDonald et al., 1975), in sheep Purkinje fibers in the presence of caffeine (Eisner et al., 1979), in atria of different species after sodium removal (Linden and Brooker, 1980), and during aftercontraction induced in guinea pig papillary muscle (Bogdanov et al., 1981). It has also been shown that during injection of CaCl₂, the slow inward (Ca) current inactivates with a smaller time constant (Isenberg, 1977). Preliminary investigations in calf Purkinje (Marban and Tsien, 1981) and frog atrial fibers (Fischmeister et al., 1981; Hume and Giles, 1982) introduce strong arguments against a simple voltage dependence of Ca inactivation in heart.

In the present study, we put forward further considerations for a Ca-dependent Ca inactivation. They result mostly from an improvement of Ca current estimation following strong inhibition of the outward currents by cellular Ca loading. Thus, it is assumed that Ca ions accumulating intracellularly could shut off Ca channels by combining with a control site.

METHODS

Experiments were performed under voltage-clamp conditions in frog atrial trabeculae (Rana esculenta) 70–150 µm in diameter by the use of the double sucrose-gap method (Rougier et al., 1968). The voltage-clamp pulses and membrane currents were displayed on an oscilloscope (565; Tektronix, Inc., Beaverton, OR) and simultaneously digitized (at 1 kHz, 8 bits) after amplification and stored on a 10 M octets disk for subsequent analysis by a minicomputer (Plurimat S; Intertechnique, Plaisir, France). In most experiments, a conventional voltage clamp was applied. Under these conditions the initial surge of current showed two components on a semilogarithmic plot. The faster one had a time constant of 1.3 ms on the average; the slower one, which represented at most 5% of the total capacitive current, had a time constant of up to 20 ms. This long capacitive component could result from charging the membrane in the sucrose-gap regions where the Ca channels are largely deprived of current carriers. Its amplitude varied from fiber to fiber and increased during the experiment together with the longitudinal resistance of the preparation. In a few recent experiments a chopped-clamp amplifier was used. Membrane potential was periodically measured while current injection was discontinued and stored on an analogic sample and hold circuit. The difference between this signal and the stimulation signal was converted in a current that was injected during a time 3/10 of the chop period. At optimal adjustments, the percentage of error on the voltage control was nearly equal to the ratio of the chop period to the membrane time constant. The chop period was adjusted to 100 µs in these experiments; only the initial square-pulse injection of current lasted for 530 µs to charge briefly the membrane capacitance but not damage the tissue by too high an
instantaneous surge of current. This device made it possible to charge the membrane capacitance more rapidly despite the series resistance (Suchaud, 1982). In these experiments, by switching after each pulse from the continuous voltage-clamp mode to the chopped-clamp mode, it was verified that the traces of current elicited by different depolarizations were superimposed at any potential except for the first 5 ms. This suggested that in our normal clamp conditions the voltage drop on the series resistance (up to 6 kΩ) did not significantly alter the applied membrane potential and that the capacitive current almost vanished after 5 ms.

Ca current amplitude and time course were estimated by the difference at any time (every millisecond) between the current traces recorded in the standard solution for a series of applied depolarizations and the ones obtained during the same series of potentials in the presence of 3 mM Co. This procedure allowed us to take into account the slow capacitive surge of current. The amount of charge transferred during the course of a pulse is proportional to the time integral of the calcium current during that pulse. The area between current trajectories produced by conditioning pulses before and after Co addition was computed from the digitized currents.

Solutions

Normal Ringer solutions contained: 110.5 mM NaCl; 2.4 mM NaHCO₃; 1.8 mM MgCl₂; 1.8 mM CaCl₂; 2.5 mM KCl. In order to reduce the outward current, frog atria were bathed for 2–3 h in a K-free, 40 mM Cs Ringer solution, taking advantage of the large surface/volume ratio in frog heart. Atrial bundles were then dissected in the same solution and set in the double sucrose-gap bath. Substitution of Cs for intra- and extracellular K was accompanied by a slight depolarization. Preparations were held at their original resting potential by hyperpolarizing them. The standard solution, derived from the Ringer solution, contained 20 mM Cs but no KCl, 3 mM 4-aminopyridine (4-AP; Sigma Chemical Co., St. Louis, MO), and 5 × 10⁻⁶ g/liter tetrodotoxin (TTX; Sigma Chemical Co.); pH was adjusted (with HCl) to 7.5 in all solutions and the temperature was 18 ± 2°C. In some cases, Sr was substituted in the standard solution for Ca in an equimolar amount. Ca-rich or Mg-rich solutions were obtained by the addition of CaCl₂ or MgCl₂, respectively, to the standard solution at a final concentration of 10 mM. In some experiments [Ca²⁺]₀ was buffered to 10 mM by the organic anion malate and the solution then contained 63 mM Ca malate according to Almers et al. (1981); the change in osmolarity was not compensated. Na-free medium was obtained by substituting Li for Na ions. Cobalt (3 mM) and D600 (2.2 × 10⁻⁶ M; Knoll, Ludwigshafen, FRG) were used to inhibit the Ca current, and in other experiments, adrenaline (5 × 10⁻⁷ M) was added to increase it.

Nomenclature

The variations V (mV) in membrane potential Eₘ (mV) were imposed from the resting potential E_R (mV), which was estimated to be –80 mV. Thus, Eₘ = E_R + V. Inward current was downward and was considered as pure Ca current I_Ca (μA). The area of the membrane under clamp in the test gap was ~0.1–0.3 cm² according to the capacitive current elicited by small variations in membrane polarization. I_Ca was the difference between currents recorded before and after its blockage by Co. For a given depolarization, I_Ca is a product of a time-varying conductance and a driving force: I_Ca = g_Ca (Eₘ – E_Ca), where E_Ca represents the reversal potential (mV). By analogy with the voltage-dependent conductance exhibited by the Na system (Hodgkin and Huxley, 1952), the Ca conductance (g_Ca) was the product of a maximal conductance g_Ca with two activation and inactivation parameters. Although the mechanisms underlying them might be different, we chose to use this terminology and thus extend its original meaning.
RESULTS

General Effects of Cs Loading on Frog Heart Cells

The existence of time- and voltage-dependent outward currents, which partially superimpose the Ca current, complicates the analysis of the Ca conductance, particularly its inactivation under maintained depolarization. Effective inhibition of the outward currents was therefore essential. Cs ions have been shown to reduce strongly $I_{K1}$ and $I_{K2}$ in Purkinje fibers (Isenberg, 1976; Carmeliet, 1980) and to inhibit $I_K$ (DiFrancesco, 1981). Marban and Tsien (1982) reported that intracellular K substitution by Cs with the use of the ionophore nystatin inhibits the transient outward current and unmasks a large slow inward current. In the present experiments, K outward currents were tentatively suppressed by substituting Cs for intra- and extracellular K (see Methods). Under these conditions, estimation of the intracellular K concentration by atomic absorption and protein assay on six whole hearts revealed that after 2.5 h, $K_i$ was decreased to 25% compared with the K content of six other hearts left for the same time in Ringer solution. Intracellular K was supposed to be replaced by Cs since Cs can substitute for K on the Na-K pump (see Guérin and Wallon, 1979). Separate microelectrode impalements during the Cs loading procedure revealed a slow progressive decrease in the resting membrane potential, which was initially associated with an increased frequency of beating. Measurements often reached -60 mV and sometimes -50 mV after 2.5 h compared with -75 to -80 mV in the Ringer solution. Although the whole atria resumed beating after >1 h, action potentials, slightly prolonged, could still be elicited in the double sucrose-gap bath when the normal resting potential had been re-established by hyperpolarizing the fibers. The effectiveness of cellular Cs loading in reducing K currents is illustrated by the flattening of the current traces at membrane potentials ranging from $E_R$ -80 mV to $E_R$ +80 mV after blockage of the channel by Co (Fig. 1A). Only a small time and voltage dependence was revealed for depolarizations above $E_R$ +90 mV. The sum of the currents elicited by depolarizations and hyperpolarizations of equal amplitude was null up to $E_R$ ±40 mV or showed only a small steady positive difference at $E_R$ ±80 mV (Fig. 1B). The current-voltage ($I$-$V$) relationship, established at 500 ms, was linear for $E_m$ ranging from $E_R$ -70 mV to $E_R$ +90 mV and was slightly curved at potentials higher than $E_R$ +90 mV (Fig. 1B). This current was thus considered to be mostly a leakage current. It was also verified that Co addition did not significantly reduce the current in the hyperpolarizing range or change its time course.

When compared with other experiments in Ringer, the membrane resistance around the resting potential appeared very slightly increased, which agrees with the failure of Cs to carry a current through the inward and the outward rectifiers. Furthermore, it is very improbable that Ca entry in the absence of Co triggered an increase in K conductance because outward tails were never observed even when clamping back to $E_R$ +40 mV after 80-mV depolarizations of 500 or 1,000 ms in duration, a depolarization thought to activate Ca entry maximally while the driving force for outward K has been increased. Actually, an inward tail was always observed whose amplitude increased with the amplitude of the Ca current.
at the end of the pulses (e.g., Fig. 2). Application of 3 mM 4-AP, which is known to block the Ca-activated K currents, had no significant effect after Cs loading but was added to ensure the lack of such K currents.

The Ca current magnitude (and its time course in subsequent experiments)
was estimated as the difference between the total current and the current recorded in Co solution at the same potential (see Fig. 2). It should be noted that the time-to-peak current was ≤8 ms for an 80-mV depolarization. Fig. 1B illustrates the I-V relationship of the peak Ca current thus determined. The threshold of the Ca current appears at $E_R +20$ mV, and a maximal current was observed with depolarizations at $E_R +70$ or +80 mV. At larger positive potentials, a very small inward current subsided and vanished for an applied depolarization of +145 mV (or up to 155 mV in other experiments). The I-V relation bends at these high potentials.

**Ca Current Inactivation**

Ca current inactivation was measured with a double-pulse method: a constant test pulse of sufficient amplitude (+80 mV) to evoke maximal Ca current was preceded by a conditioning pulse of variable amplitude and duration. An interpulse of 10 ms enabled us to estimate the test pulse current without interference with the tail capacitive current of the prepulse, particularly for the large hyper- and depolarizations. The same series of pulses was repeated in standard and in Co solution. Fig. 2 shows the effect of a 200-ms conditioning pulse on the amplitude and the time course of the test pulse current. A 60-mV conditioning pulse was accompanied by a significant reduction in the test pulse current amplitude compared with the Ca current elicited without prepulse; the reduction was maximal with an 80-mV depolarizing prepulse, which induced the largest Ca current. Further increase in the prepulse potential to +120 mV led to a lower reduction in the test pulse current. The diminution of the test pulse current was taken as an index of the Ca current inactivation. Relative inactivation was shown by plotting the ratio of the test pulse current ($I$) after a conditioning pulse on the
maximum current ($I_{\text{max}}$) with no conditioning pulse as a function of prepulse potentials for various prepulse durations. Fig. 3 shows that the relative inactivation was progressively enhanced as the potential of the conditioning pulses approached +80 or +90 mV. However, a further increase in the conditioning pulse amplitude induced progressively less inactivation. For these large depolarizations, inactivation was more pronounced as the conditioning pulse duration was increased from 50 to 400 ms.

Inactivation Is Quantitatively Related to the Amount of Calcium

The above results indicate that Ca inactivation was not directly dependent on membrane potential. It may depend upon the number of Ca ions entering the cell during the conditioning pulse since the Ca inactivation curve (Fig. 3) mimics the $I$-$V$ relationship (Fig. 1B). Inactivation of the Ca channel related to previous

![Figure 3. Relative inactivation of the Ca current by 50-, 200-, and 400-ms conditioning pulses in control conditions, established with the protocol shown in the inset. The test pulse was an 80-mV, 400-ms depolarization.](image-url)

Ca entry might thus result from the persistence near the channel, at the inner side of the membrane, of Ca ions that entered and accumulated during the prepulse. Under these conditions, the degree of inactivation should be closely related to the total amount of Ca entering the cell during the conditioning pulse (Eckert and Tillotson, 1981).

This hypothesis was tested by varying the amplitude and the duration of the conditioning pulse. Estimation of the amount of Ca ion entering the cells during the prepulse was obtained from the surface area delimited by the inward and leakage currents recorded at the same potential in the absence or presence of Co (Fig. 2). The amount of Ca ion transferred during the prepulse was varied in this case by altering the prepulse amplitude to +140 mV and using two durations, 100 and 400 ms. The relation between the degree of inactivation ($I/I_{\text{max}}$) of the Ca current and the time integral ($Q_{\text{Ca}}$) of the conditioning pulse current was
roughly linear over the range investigated (Fig. 4). Similar results were observed in six out of nine preparations.

**Time Constant of Inactivation**

A semilogarithmic plot of the Ca current during 300-ms pulses revealed that in first approximation its amplitude decreased exponentially with time. In addition, some preparations showed an initial fast decline that was more apparent with currents of large amplitude (as in Fig. 2). As a rule, the time constant was estimated by avoiding the first 30 ms of the Ca current. The time constant of inactivation, \( \tau \), was plotted against membrane potential (Fig. 5). A slow decay was found for potentials close to the threshold \( (E_R +30 \text{ mV}) \) and for large depolarization (up to \( E_R +120 \text{ mV} \)) with a time constant of \( \sim 130 \text{ ms} \); the decay was the fastest \( (\tau = 70 \text{ ms}) \) for a depolarization around \( E_R +70 \text{ mV} \). When related to peak amplitude (see Fig. 1), the larger the current was, the faster was its decrease. These results were obtained in six experiments and \( \tau \) could be fitted, using the least-squares method, by the equation \( \tau = 543 e^{-0.045 \times V} + 10.4 e^{0.020 \times V} \).

The magnitude and time course of the Ca current varied from trabeculum to trabeculum, with relatively faster inactivation kinetics usually found in preparations exhibiting larger currents per unit membrane area.

The rate of decrease of Ca current elicited by a given depolarization can be significantly altered by a prior depolarization. This is emphasized in Fig. 2, where the test pulse current elicited by an 80-mV depolarization declined more slowly \( (\tau = 165 \text{ ms}) \) when its amplitude had been greatly reduced by a conditioning pulse of 80 mV, which triggered a large influx of Ca ions. Time constants of inactivation were 81 and 77 ms after applying conditioning pulses of 60 and 120
mV, respectively, which reduced the test pulse current amplitude equally. In the absence of a conditioning pulse, \( \tau \) was 60 ms.

**Inward Current Inactivation in Sr Solution**

Strontium, a divalent cation, was used as an effective substitute for Ca because of its ability to act as a charge carrier. An equimolar replacement of Sr for Ca resulted in larger but more slowly relaxing currents (Fig. 6A). Sr tails were also much slower. \( \tau \) deactivation, estimated from the difference between current traces before and after Co addition, were of the order of 57–65 ms and were not significantly affected by the amount of cation flowing during the pulse; the Ca tail current declined with a time constant of \( \sim 17 \) ms. The current-voltage relationship (not illustrated) was found to be shifted by \( \sim 10–15 \) mV toward a more negative potential in Sr solution. Time constants of inactivation of the currents in Sr solution are shown in Fig. 10 for two experiments: the values ranged from 550 to 370 ms at +70 mV, the membrane potential at which the largest Sr currents were recorded. It might be argued that Sr reduced the outward currents in a manner similar to that described for Ba (Gorman and Hermann, 1979), but in the present experiments these currents have already been inhibited by Cs and 4-AP. Fig. 6A shows that a 200-ms conditioning pulse of +50 or +120 mV had only a slight effect on the test pulse current, whereas a 70-mV prepulse was accompanied by a minimum test pulse current. The relative inactivation of the Sr current was progressively increased to a maximum as the conditioning pulse potential approached +80 mV. A further increase in prepulse depolarization to +140 mV was accompanied, however, by a smaller inactivation.
of the Sr current, and no inactivation was found with conditioning pulses of \( \geq 140 \) mV (Fig. 6B; mean of three experiments). A comparison has been made of the relative inactivation of the inward current in Ca solution. Although the amount of cation carried by the current was greater in Sr solution, the relative inactivation was smaller for the Sr current than for the Ca current. Moreover, the extent of inactivation produced by a conditioning pulse varied less from fiber to fiber in Sr than in Ca solution.

![Figure 6](image)

**Figure 6.** Inactivation of the inward current in Sr solution. (A) Equimolar substitution of Sr for Ca alters the amplitude and time course of the inward current, and particularly its decline under maintained depolarizations. Inactivation was checked by double pulse: conditioning pulses were 200 ms in duration and 50, 70, or 120 mV in amplitude; the test pulse was a depolarization of 70 mV and 500 ms. As in standard solution, the reference was the outward current recorded in 3 mM Co solution. (B) Relative inactivation of the inward current in 1.8 mM Sr compared with that obtained in 1.8 mM Ca for the same 200-ms conditioning pulses. Mean and SEM of three experiments in Sr (error bar smaller than symbol size) and of six experiments in Ca solution.

The degree of inactivation of inward current in Sr solution was then related to the time integral of the current during a conditioning pulse. Fig. 7 shows the relation obtained on the same trabeculum between the degree of inactivation of the inward current in Ca or in Sr and the amount of divalent cations flowing through the membrane during the conditioning pulse. In Sr solution, the relation was linear over the whole range investigated (up to +140 mV, a potential at which nearly no inactivation was observed). However, in Ca solution, the degree of inactivation for high depolarizations did not appear to be related only to the amount of divalent cations entering the cells. This could happen for at least two
reasons: (a) in this preparation, reduction of the outward currents by Cs and 4-AP was not sufficient, and (b) at these membrane potentials, only small Ca currents could be recorded that might lead to an inaccurate evaluation of its time integral. Besides, the strength of the observations brought by the linear relation between the integral of current entry and the degree of inactivation is weakened by the fact that the Ca channel is not highly selective for divalent cations.

**Effects of Ca-rich or Mg-rich Solutions**

An increase in the extracellular Ca concentration from 1.8 to 10 mM led to an approximately twofold increase in the maximal amplitude of inward current elicited by a depolarization of 80 mV. In this Ca-rich medium, the Ca current amplitude was estimated by comparison with the current trace elicited by the

![Figure 7](image)

**Figure 7.** Relative inactivation is quantitatively related to the amount of divalent cations, either Sr or Ca ions, entering the cells during prepulses. However, a discrepancy occurred for high depolarizations in Ca solution for this fiber (see text).
accelerated its decay similarly, whether Ca was added in the form of CaCl₂ (10 mM) or in the form of Ca malate (63 mM) to prevent extracellular depletion.

Relative inactivation of the Ca channel was also investigated at different membrane potentials in these two Ca-rich solutions and compared with that obtained in standard solution (Fig. 8C). High conditioning pulses were similarly inefficient in fully inactivating the test pulse currents. Nevertheless, the relative inactivation was significantly larger at high potentials in Ca-rich solution than in normal-Ca solution. Longer prepulses, of 400 ms rather than 200 ms, significantly increased the relative inactivation observed at each potential in normal-Ca solution (see Figs. 3 and 11) but not in the Ca-rich solution (not shown).

**Figure 8.** Effects of increasing the extracellular Ca concentration on the amplitude and time course of the inward current. (A) The decline of current recorded for a voltage step of +80 mV is much faster in 10 Ca than in control conditions (1.8 Ca). (B) Buffering of the external Ca concentration with organic anion malate does not significantly alter the time course of the current. (C) Relative inactivation of the inward current in 1.8 Ca (△), 10 Ca (○), and 63 Ca malate (χ) solutions. The conditioning pulse was 200 ms in duration and the test pulse was an 80-mV, 300-ms depolarization.
Altering the Ca concentration or substituting Ca with Sr results in variations of surface charges (Hille et al., 1975), which might account for some of the reported changes. In an attempt to avoid these effects, the I-V relations and the time constants of relaxation were compared in standard (1.8 Ca, 1.8 Mg), in Ca-rich (10 Ca, 1.8 Mg), and in Mg-rich (1.8 Ca, 10 Mg) solutions. The results of such an experiment are summarized in Fig. 9. It shows that Ca-rich or Mg-rich solutions markedly increased or decreased, respectively, the peak current amplitudes that were obtained for depolarizations 10 or 8 mV higher than in standard solution. At a given applied potential, time constants of inactivation were markedly different; for example, at $E_R +80$ mV, they were 74, 44, and 95 ms in standard, Ca-rich, and Mg-rich solutions (and see Fig. 10). On the other hand, similar values were obtained in these three solutions at membrane potentials ranging from $E_R +30$ mV to $E_R +130$ mV when the peak values of the elicited current were nearly equal (inset Fig. 9). Notice, however, that the currents always inactivated slightly faster at the lowest potentials in each solution.

Fig. 10 summarizes the plots of inactivation time constants at different membrane potentials in the four solutions of altered ionic composition. The values are compared with those obtained in standard solution. Consistent with current knowledge on surface charge screening and the above reported observations on the I-V relations, there was a shift in the potential at which the lowest values of inactivation time constant were obtained: roughly, these values were $E_R +80$ mV in 1.8 mM Ca, $E_R +70$ mV in 1.8 mM Sr, and $E_R +90$ mV in 10 mM Ca or 1.8

**Figure 9.** Current-voltage relationships established in standard, Ca-rich, and Mg-rich solutions. In the inset are the applied membrane depolarizations required to elicit currents of a given amplitude (0.5 $\mu$A) in the three solutions and their time constants of inactivation.
mM Ca and 10 mM Mg. (Notice that all the other solutions contained 1.8 mM Mg.) In the Ca-rich solution at any membrane potential, current amplitude was larger and inactivation was faster than in standard solution. However, opposite results were obtained in the Mg-rich solution, a solution having the same ionic strength and inducing roughly the same potential shift as the Ca-rich solution. At a given potential, much slower current inactivations were observed together with a smaller current amplitude. Finally, in Sr solution (containing no Ca), very high values of time constant were determined.

**FIGURE 10.** The time constants of inactivation of the inward current elicited by various depolarizations in 10 Ca or 63 Ca malate. 10 Mg and 1.8 Sr solutions are compared with time constants obtained in control conditions (1.8 Ca). Each symbol represents a different fiber.

**Effects of Ca Loading by Na-free Solution**

In neurones, Ca current inactivation is slowed by injection of the Ca-chelator EGTA (Eckert and Tillotson, 1981). It can be expected that cellular Ca loading will have an opposite effect. In heart cells, Na-free solution induces an increase in the intracellular Ca content through the alteration of the Na-Ca exchange mechanism (Glitsch et al., 1970). After \( \geq 10 \) min, allowing for a steady state to be reached, the maximal amplitude of the Ca current in Na-free (Li-substituted) solution was reduced by \( \sim 20\% \), whereas its time constant of inactivation was
decreased from 74 to 57 ms during an 80-mV depolarization (Fig. 11A). The I-V relationship was shifted by ~10 mV toward a lower potential in the Li solution, and the apparent Ca reversal potential decreased slightly (Fig. 11B). Fig. 11C shows the relative inactivation of the Ca current in Li solution. Ca channel inactivation appeared somewhat similar, as in standard solution, although for the same conditioning pulse sequence more inactivation was obtained in Li solution.

**Effects of Ca Current Blocking Agents**

D600, a methoxy derivative of verapamil, is generally believed to affect the Ca-carrying system, possibly by reducing the maximal channel conductance $g_{Ca}$ without any significant effect on the inactivation kinetics of the Ca current (Kass and Tsien, 1975; Nawrath et al., 1977; Kohlhardt and Mnich, 1978). Fig. 12A shows the effect of $2.2 \times 10^{-6}$ M D600 on the amplitude and time course of Ca current in frog atrial muscle. Under control conditions in 1.8 mM Ca, the inward
current recorded in response to a voltage step of +80 mV was inactivated with a time constant of 68 ms. After 2 and 5 min of application of D600 at a stimulation frequency of 0.125 s⁻¹, the Ca current was decreased, respectively, by 50 and 75% in amplitude, whereas τ inactivation increased to only 71 and 74 ms, respectively, a change at the limit of confidence of our estimations. The upper trace was obtained in standard solution in the presence of 3 mM Co. These results in Cs-loaded cells confirm the previous observations.

Several divalent cations were reported to inhibit the Ca current. Added at a concentration of 3 or 5 mM, Mn ions markedly decreased the inward current, but a significant inward current larger than the outward leakage current was recorded after Cs treatment with very slow or no inactivation. In other experiments, Ca was removed and Mg was added at 1.8 or 15 mM. In both cases, weak

![Figure 12: Time courses of the inward current elicited by an 80-mV depolarization during partial inhibition by Ca-blocking agents. (A) Effects of D600 (2.2 × 10⁻⁶ M) in 1.8 Ca solution: the second and fifth (steady state) traces are at a stimulation frequency of 0.125 s⁻¹. The upper trace shows inhibition by 3 mM Co. (B) Partial inhibition by 3 mM Co of the inward current recorded in 10 mM Ca solution (the third and seventh traces are after adding Co). The upper trace was obtained in 5 mM Co solution.](image-url)

but significant inward currents were observed. As shown in Fig. 2, Co ions added at a concentration of 3 mM most effectively inhibited the inward current elicited by step depolarizations in standard solution. Fig. 12B illustrates the effects of Co on the inward current elicited by an 80-mV depolarization in 10 mM Ca solution. 3 mM Co reduced the current but, even at steady state, did not abolish it. Complete inhibition was only achieved after the addition of 5 mM Co. Together with the decrease in current amplitude, the rate of current inactivation decreased. As inhibition proceeded, the time constant of inactivation, which was 34 ms in 10 mM Ca solution, increased to 70 ms in 3 mM Co. Similar experiments performed in 1.8 mM Ca solution also showed that on switching to 3 mM Co-containing solution, Ca current inactivated more and more slowly, whereas its amplitude decreased to zero.
**Effects of Adrenaline**

Adrenaline, like other cAMP-stimulating agents, increases the maximal Ca current (Vassort et al., 1969; Reuter and Scholz, 1977). The latter authors showed that the limiting conductance was increased, whereas the activation parameters remained unchanged, but little is known about its effects on inactivation. Thus, we reinvestigated the effects of adrenaline ($5 \times 10^{-7}$ M) on Ca-loaded frog cells. The results of one out of three similar experiments are shown in Fig. 13 for an 80-mV depolarization. In the standard solution $\tau$ was 56 ms. A low-Ca solution (0.6 mM) reduced peak current by ~50% and increased $\tau$ to 98 ms. Adding adrenaline to this low-Ca solution produced a threefold increase in the current, which then became larger than in standard solution, but it increased its inactivation only slightly ($\tau = 92$ ms). This was also true at higher depolarizations.

**Recovery from Inactivation: Time Dependence**

The time course of recovery from inactivation was investigated by the double-pulse method, i.e., a conditioning pulse is followed by a test pulse after an interval of increasing duration at resting membrane potential. Generally, these two pulses are identical and their amplitude is adjusted to elicit a maximal inward current. This amplitude, generally around +80 mV, also induces maximal inactivation. Similar experiments were repeated in the standard solution with the first depolarization at +60, +80, and +120 mV; in each case, the degree of inactivation was tested by an 80-mV depolarizing pulse. Fig. 14A illustrates current traces elicited by 300-ms, 80-mV test pulses after recovery from inactivation by a 200-ms, 80-mV pulse when the interval between the two pulses was lengthened. Simultaneously, with the increase in amplitude, current inactivation was speeded up. After interpulses of 50, 200, and 400 ms, peak currents were 10, 70, and
90% of maximal current and the time constants of inactivation were 267, 166, and 136 ms, respectively (Fig. 14B). The time constant was 106 ms in the absence of a conditioning pulse. Fig. 14C illustrates the plot of the relative amplitude of the test pulse current against the interpulse duration in standard solution. Removal from inactivation by a conditioning pulse of 200 ms duration and 80 mV amplitude occurred with a sigmoidal time course; the current was completely restored when the interpulse duration approached 500 ms. A 60-mV conditioning pulse produced only partial reduction of the test pulse current (Fig. 2).

**Figure 14.** Time dependence of recovery from inactivation. (A) Current traces during test pulses of 80 mV and 300 ms after conditioning pulses of 80 mV and 200 ms when varying the duration of interpulse at which the membrane was held at its resting potential. (B) Semilogarithmic plots of the digitized currents labeled in A. (C) Time dependence of recovery from different levels of inactivation elicited by 200-ms pulses of 60, 80, and 120 mV and checked by a second pulse to 80 mV after different pulse intervals.

Increasing the interval allowed for complete availability with a time course similar to the one obtained with an 80-mV conditioning pulse. Although approximately half-availability was also observed just after a 120-mV prepulse (Fig. 2), the time dependence of recovery was markedly different. Increasing the pulse interval from 5 to 60 ms resulted first in a further reduction of inward current during the test pulse; then for a longer delay, the current increased with a sigmoidal time course. Such an initial decline in the relative amplitude of current could be related to a further entry of Ca ions during the tail of the prepulse.
current, since at this large potential Ca conductance was not completely inactivated.

After equimolar substitution of Sr for Ca ions, larger but more slowly inactivating inward currents were elicited (see Fig. 6A). Removal of inactivation was investigated under these conditions after 500-ms conditioning pulses to increase the degree of inactivation of Sr current. Recovery from inactivation of the Sr inward current revealed two major differences (Fig. 15A). First, it was faster than in Ca solution. With any depolarizing prepulse, half-recovery was observed after 80 ms and complete restoration required <300 ms. Second, no significant initial decline was observed after a 120-mV condition pulse. Although a large Sr tail current occurred, it should contribute to only a small enlargement of Sr entry. Similar results were obtained after a 120-mV conditioning pulse of 200 ms in other experiments.

Inactivation was accelerated by Ca-rich solutions (Fig. 10). However, the time course of recovery was slowed by a 10 mM Ca solution. This is illustrated in Fig. 15B. Half-recovery after complete inactivation by a 200-ms, -80-mV conditioning pulse required 310 ms and complete re-availability required 800 ms. Notice also that a significant further decrease in current amplitude was observed when the interpulse duration was ≤60 ms after a conditioning pulse of 120 mV. Times for half-recovery and complete recovery are the same as after applying an 80-mV pulse.

Recovery from Inactivation: Voltage Dependence

High depolarizations to approximatively the value of $E_{Ca}$ produced only a slight inactivation of the inward current (Fig. 3). Such large membrane potentials might
have no effect on the inactivation or might reverse it. A small removal from inactivation by large conditioning pulses has been reported for the Na conductance in squid axons (Chandler and Meves, 1970). We investigated the existence of a similar process for the Ca conductance. In the experiment illustrated in Fig. 16, inactivation was induced by a conditioning pulse to $E_R + 80$ mV; a test pulse applied 200 ms later confirmed that inactivation recovered by ~50% when the membrane potential during the interpulse was fixed to $E_R$. However, if the interpulse potential was $E_R + 130$ mV, the test pulse current was nearly abolished, as would be the case if there was no delay between the conditioning and test pulses. This suggests that such a high depolarization, which would have inactivated only slightly by itself, has maintained the inactivation induced by a previous depolarization. On the contrary, when the membrane potential during the interpulse was hyperpolarized by 40 mV, recovery was facilitated as shown by the increase in the test pulse current.

**Discussion**

It has been suggested, either from direct estimation of the current or its tail (Beeler and Reuter, 1970; Bassingthwaighte and Reuter, 1972; New and Trautwein, 1972) or from tension measurement (Horackova and Vassort, 1976) that the slow inward current in heart cells inactivates under maintained depolarizations in a way similar to that originally described by Hodgkin and Huxley (1952) for the Na current in squid axon. In other tissues exhibiting Ca-dependent spikes, it was found that EGTA injection facilitates the Ca current much more than expected from an increased driving force (Hagiwara and Nakajima, 1966), and it was suggested that Ca ions might exhibit a self-blocking action (Kostyuk and
Krishtal, 1977). Recently, Brown et al. (1981) described both a voltage-dependent and a Ca-dependent inactivation in snail neurones, and a pure Ca-dependent inactivation of the Ca conductance has been reported in molluscan neurones (Tillotson, 1979; Eckert and Tillotson, 1981; Plant and Standen, 1981), in *Paramecium* (Brehm et al., 1980), and in insect skeletal muscle (Aschroft and Stanfield, 1982). The present findings in frog heart favor the latter view, with little evidence for a direct effect of the membrane potential.

We first ensured that the decline in current reflected only the inactivation of the Ca current, and special care was taken to isolate this current from contamination by outward currents. Several arguments indicate that the depression of the test pulse current resulted from an actual decrease in the inward current and not from an increase in time- and voltage-dependent outward currents. The effectiveness of the suppression of the K currents by substituting Cs for intracellular K and by adding 4-AP to the external solution was demonstrated by the flattening of the current traces during long pulses. This was confirmed by measuring the currents that remained after the blockage of inward current by Co and by comparing them during depolarizations and hyperpolarizations. Under these conditions, a slight time and voltage dependence appeared only for depolarizations above +90 mV (Fig. 1B), so that these currents could be considered to be mostly nonspecific leakage currents, although some Cs outward current might take place during larger depolarizations. Incidentally, influences of activity-dependent block of K channels by Cs and 4-AP and of Na channels by TTX could be tentatively ruled out by the observation that the current traces in Co solution during 80-mV depolarizations were unchanged by predepolarizations of increasing amplitude (see Fig. 2). The possibility that some outward currents might be increased by Ca, as is the case for the Ca-activated K currents in neurones (Meech and Standen, 1975), was thought improbable because at a given depolarization the current traces obtained in the absence or presence of Co never crossed, even with long steps, and because an outward tail was never observed after repolarizing to a potential positive to $E_R$.

Cs loading, Cs, and 4-AP in the test solution, together with Co inhibition, should allow for a better estimation of the Ca reversal potential. With increasing depolarizations the slope conductance decreased, an observation that was previously reported in neurones and corresponds to the fact that Cs ions are not going out through this channel in the presence of Ca ions (Hagiwara and Byerly, 1981). The inward current vanished for depolarizations of $\geq 150$ mV; this corresponds to an effective membrane depolarization of at least 190 mV if one takes into account the limitations of the double sucrose-gap technique (Fischmeister et al., 1982) and corresponds to a reversal potential above 110 mV. Besides Ca, several divalent cations—Sr, Mn, and Mg, but not Co—are shown to enter through this channel. However, the role of monovalent cations in the regulation of the slow inward current is not clear. The decrease in peak current amplitude in Li solution (Fig. 11) could be due to the inability of Li to substitute for Na in going through the slow channel, but could as well be the result of the faster inactivation or of an incomplete availability of this channel in the high internal Ca concentration as induced by the Na-free solution.

A remarkably slow inactivation was found close to the threshold potential and
for large depolarizations, whereas for a membrane potential of approximately $E_R +90 \text{ mV}$ (at which the inward current was maximum), the decline of the current was much faster. These results are in a qualitative respect similar to previous observations of New and Trautwein (1972) and Kohlhardt et al. (1975). Several hypotheses consistent with current knowledge can be put forward to account for the decline in Ca current. The most simple one is that inactivation of current could arise from a reduction in the driving force by either external depletion or internal accumulation. Although the narrow extracellular spaces between frog heart cells might be compared to the transverse tubular system of skeletal muscle, no evidence for depletion was observed. Inactivation rate was the same in 63 mM Ca malate as in 10 mM CaCl$_2$ (Fig. 8B), which is contrary to the observations in skeletal muscle (Almers et al., 1981). A significant local increase in Ca$_i$ should occur (see below); however, the variation in driving force alone does not seem to account for the following observations.

Ca current amplitude, and therefore the quantity of ions entering the cells, is a logarithmic function of its concentration. The increase in internal Ca concentration should therefore result in an exponential increase in the degree of inactivation. Nevertheless, a roughly linear relation was observed (Fig. 4). Inactivation was faster in Ca-rich than in 1.8 Ca solution (Fig. 8); this would require a much larger current so that the relative increase in internal Ca surpasses the external enhancement of the solution in Ca. Experiments performed in Sr further contradict a significant effect of depletion or accumulation. Substitution of Sr for Ca results in less inactivated currents, whereas the amount of charges entering the cell is two to three times larger than in Ca (cf. Figs. 2 and 6). Divalent cations entering the cells would also change the screening by surface charges at the inner side of the membrane. This will decrease the potential difference across the membrane and shift $I-V$ relations to more negative potentials. As discussed by Eckert and Tillotson (1981), this would increase rather than decrease the calcium current. Furthermore, similar changes in surface charges on the external face of the membrane induced by Ca-rich and Mg-rich solutions have markedly different effects (Fig. 9). None of these mechanisms (overlap with K currents, decrease in driving force, or change in surface charge screening) is strictly ruled out, but none can account fully for the above results. Finally, in recent simulations of the variations of Ca$_i$ following Ca current in frog heart, Fischmeister and Horackova (1983) have shown that Ca binding to specific sites slows down the diffusion of Ca$_i$ and may generate accumulation or depletion of Ca$_i$ near the membrane. However, these authors showed that Ca binding also decreases free Ca$_i$ and thus does not significantly affect the kinetics of $I_{Ca}$ through a modification in the driving force for Ca ions across the membrane. Another mechanism should be suggested.

The inactivation voltage relationships established in different solutions (Figs. 3, 6, 8C, and 11) show a special U-shape. Partial restoration of the availability of the Na-carrying system at large depolarizations has already been reported in squid axon by Chandler and Meves (1970) and was described by two types of voltage-dependent Na inactivation. It would be expected that inactivation decreases with longer prepulses. However, in frog heart, prolongation of the
conditioning pulse from 50 to 400 ms increases the inactivation (Figs. 3 and 11). In the case of the two types of voltage-dependent inactivation, it is difficult to account for the effects of Sr ions. First, it should be assumed that the voltage dependence of inactivation is altered by Sr (Fig. 6), and second, that removal from inactivation is slow and biphasic in Ca (Figs. 14 and 15B) or monophasic and accelerated in Sr (Fig. 15A). This requires that the nature of the ion flowing through the channel alter its kinetics. Furthermore, in this solution there was no evidence for an intrinsic voltage dependence of the Ca channel inactivation, since \( I/I_{max} \) reaches unity for strong depolarizations (Fig. 6).

Recently, Saimi and Kung (1982) have shown that ionic species influenced the gating of calcium channels in Paramecium. Similar observations are made in frog heart (Figs. 2 and 6). In both tissues, deactivation was not significantly influenced by intracellular Ca accumulation. On the contrary, several findings suggest that in frog heart, as in other tissues (Brehm et al., 1980; Ashcroft and Stanfield, 1982), inactivation of the Ca-carrying system is mediated by Ca itself: these are (a) a linear relation between the degree of inactivation and the amount of divalent charges entering the cells (Figs. 4 and 7); (b) a time constant of inactivation that is related to the amplitude of the current, i.e., the larger the current is, the faster is the inactivation for a given divalent cation (Figs. 8-10); (c) a relative inactivation that illustrates the same influence of potential as the peak inward current elicited during prepulse (Figs. 2 and 3); (d) a relative inactivation that is larger when the current elicited by the same depolarization is greater (Fig. 8B); and (e) a current decline that is faster as peak current increases during recovery from inactivation (Fig. 14A), an effect previously described by Schulze (1981). Inactivation, or more particularly its recovery, also seems related to Ca removal by internal and trans-sarcolemmal mechanisms. Thus, recovery from inactivation is much slower in Ca-rich (Fig. 15B), in low-Na (Weiss et al., 1974), or in Na-free solutions (Shimoni, 1981), in which local removal of Ca requires more time because of cellular Ca loading and lack of Na-Ca exchange. This emphasizes that the inactivation rate and recovery rate of the slow channel are very different and could not be described by simple voltage dependence. Notice that we purposely used 10 mM Ca to load the cells, which may explain the discrepancy between our results and those of Kohlhardt et al. (1975) and Shimoni (1981), who reported a faster recovery of inactivation (with a 2.5-fold Ca increase), probably only as a consequence of an alteration in external surface charges. Furthermore, high depolarizations inhibit inactivation removal (Fig. 16) either because they decrease or even reverse Na-Ca exchange in frog heart (Horackova and Vassort, 1979), or because they change the affinity of the Ca-binding site (Gorman and Thomas, 1980). A slower removal of Ca in Na-free solutions may also account for the observations that although the peak amplitude of current was slightly decreased, its decline during applied depolarization was significantly faster and its inactivation by prepulses was larger (Fig. 11). This extends previous observations by Linden and Brooker (1980), who showed that after Na removal, the amplitudes of contractions and slow action potentials declined in parallel; they suggested that intracellular Ca accumulation decreases slow channel conductance.
To account for these observations, we suggest that inactivation of the Ca current is related to the local amount of divalent cations at the inner mouth of the channel, depending both on Ca entry through the channel itself and on Ca removal. We further suppose that some negatively charged groups stand at the inner side of the membrane near the channel, so that Ca will bind there and create an additive energy barrier that will prevent further divalent cations from entering. This results in a self-regulated entry of ions.

The results obtained in Sr solution are easily explained by the reasonable assumption that the affinity of these sites for Sr ions is smaller, as is generally admitted with most negatively charged groups (Sillen and Martell, 1964). Thus, although current density is greater, its ability to inactivate the channel is weakened and the decline of the current is slowed. On the other hand, recovery from inactivation was faster in Sr (Fig. 15A) because of its easier diffusion. A similar interpretation holds for Mn and Mg ions, which can support the inward current and show no or very slow inactivation. Na, a monovalent cation, seems to flow through this channel in low-Ca solutions, but like divalent cations (except Ca), it appears unable to trigger fast inactivation so that very long action potentials are observed (Garnier et al., 1969; Linden and Brooker, 1982). Thus, at least under these particular conditions, the action potential duration is regulated by the slow conductance.

Cobalt ions decrease the amplitude of Ca current and markedly slow down its decline (Fig. 12). Co ions, like other divalent cations, alter the quantity of Ca moving through any Ca channel and reduce local accumulation by competing with Ca. Consequently, they slow inactivation, as observed after prepulses. The large decrease in current amplitude induced by D600 with only a minor increase in the time constant of inactivation (Fig. 12; see also Kohlhardt and Mnich, 1978) requires further comment. A simple interpretation would be that D600 decreases the number of Ca channels, while single channel characteristics remain unaltered, which leads to a current with a smaller amplitude but a similar time constant of inactivation. Another interpretation would be that D600 exerts two opposite effects: speeding current decay because of a preferential interaction with open Ca channels (Lee and Tsien, 1983) and slowing inactivation because of the reduction of Ca entry. Adrenaline markedly increases the current amplitude, but it increases the inactivation rate by only 10% (Fig. 19). Such a small change was also recently reported for isolated ventricular myocytes (Isenberg and Klöckner, 1982). Similarly, it can be proposed that adrenaline increases the number of available channels, or, as suggested by recent single Ca channel studies (Reuter et al., 1982), that it increases the opening probability of individual channels; in this case, it speeds up inactivation. To discriminate between the two interpretations or to combine them, further experimental work is needed.

In conclusion, even if Hodgkin-Huxley terminology was used to describe our observations, the above results suggest that the inward movement of Ca ions differs significantly from the inward movement of Na ions. The membrane mechanism that controls the influx of Ca ions and other divalent cations is modulated mostly by these ions rather than by the membrane potential.
MENTRARD ET AL.  Ca-mediated Inactivation of Ca Conductance

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MENTRARD ET AL. Ca-mediated Inactivation of Ca Conductance


