Calcium Current Restitution in Mammalian Ventricular Myocytes is Modulated by Intracellular Calcium

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Restitution of the conventional L-type calcium current (I_{Ca}) was studied in dog or guinea pig ventricular myocytes to understand its time course and regulation. Whole-cell I_{Ca} free of other overlapping currents was recorded with a suction pipette. The intracellular environment was varied by intracellular dialysis. The properties of I_{Ca} were similar in dog and guinea pig ventricular myocytes, except that the amplitude of I_{Ca} was larger in the latter (2.2 ± 0.5 nA in guinea pig cells and 0.9 ± 0.2 nA in dog cells, $n = 8$ for both). In both types of cells during restitution a holding voltage (V_h) negative to -50 mV induced a transient increase in I_{Ca} above the control level (I_{Ca} overshoot). This overshoot was inhibited by substituting barium for calcium, lowering $[Ca]_o$, increasing intracellular calcium buffering capacity, ryanodine (1–2 μM), or caffeine (10 mM). The overshoot peaked 30–100 msec after repolarization from the conditioning depolarization and gradually declined over the following 2–3 seconds. During the overshoot, although the amplitude of I_{Ca} was larger its half-time of decay was longer than the control. The maximum overshoot occurred following a conditioning step to plateau voltages and it was decreased by prolonging the conditioning step from 50 to 100 or 500 msec. It is concluded that intracellular calcium regulates restitution of the L-type calcium channels in mammalian ventricular myocytes and that the sarcoplasmic reticulum is involved in this process. (*Circulation Research* 1988;63:468–482)

In cardiac tissues, the rate of restitution of the conventional L-type calcium current (I_{Ca}) is important in determining the duration of premature action potentials[1,2] and the conduction of impulses in partially depolarized tissues.[3] Both action potential duration (and thus the duration of refractoriness) and the speed and certainty of impulse conduction are important determinants with respect to the initiation and perpetuation of reentrant arrhythmias, such as those observed during myocardial ischemia and infarction.[4] Therefore, it is of interest to study the time course and regulation of restitution of this type of calcium current.

The inactivation of I_{Ca} during depolarization has been studied extensively. This process follows a double-exponential time course and is regulated by both voltage and intracellular calcium.[5,6] The calcium-mediated inactivation probably results from dephosphorylation of the channel, at least in snail neurons.[7] However, our understanding of the restitution of I_{Ca} following depolarization is less complete. The time course of restitution of I_{Ca} has been described as following a single-exponential,[8] single- or double-exponential,[9] oscillatory,[10,11] or sigmoidal[6] time course. The regulation of restitution has been attributed either to a mainly voltage-dependent process[9] or to a decrease in cytoplasmic free calcium.[6] These different findings and interpretations concerning restitution of I_{Ca} may arise from the use of different voltage-clamp protocols by various investigators. The restitution of I_{Ca} is affected by the holding voltage,[2,12] the voltage and duration of the conditioning pulse,[12] the frequency of the double pulses,[12] and the extracellular ionic milieu.[12–14] Another reason for the differences in opinions about the restitution of I_{Ca} may be the dual actions of intracellular calcium on I_{Ca}.[13] Although it is established that during depolarization a fast and significant rise in intracellular calcium activity inactivates the calcium channels, the actions of intracellular calcium on the calcium channels when the membrane is normally polarized are uncertain. On the one hand, a persistent elevation of intracellular calcium activity

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resulting from dialysis with pipette solutions containing high [Ca] decreases I_{Ca}.[15] On the other, there have been studies showing that a transient increase in intracellular calcium can augment I_{Ca}.[11,16] It is possible that a rise in intracellular calcium activity can either enhance or inactivate I_{Ca} depending on the level of intracellular calcium activity and/or membrane voltage. Indeed, it has been suggested that a moderately elevated level of intracellular calcium can augment I_{Ca} and that a higher level inactivates I_{Ca}.[16] A third reason for the different observations of restitution of I_{Ca} may be due to the potentiation of I_{Ca}, that is, an increase in the amplitude of I_{Ca} caused by depolarization. This has been observed in cardiac tissues,[12,13,17,18] chromaffin cell,[19] and neurons.[20] This is opposite to the down-regulation of I_{Ca} caused by inactivation. The mechanism for this phenomenon is not well defined.

The present article describes the results of studies on the restitution of I_{Ca} in mammalian ventricular myocytes under conditions when other overlapping currents were largely eliminated and the intracellular milieu was directly manipulated. Two hypothetical schemes are proposed to explain the observations and the implications for conditions such as ischemia and infarction are discussed.

Materials and Methods

Cell Preparation

Single myocytes were isolated from the canine left ventricle by an enzymatic method adapted from that of Powell and Twist[21] and described previously.[22] Briefly, a wedge of left ventricular free wall was perfused through the first branch of left circumflex artery with calcium-free Tyrode's solution containing collagenase (Worthington type II, 190 units/mg, 0.5 mg/ml). The deep myocardium was then minced and triturated in the same medium. Isolated cells were washed twice with Tyrode's solution containing albumin (0.1%); stored in Tyrode's solution containing albumin (0.1%), insulin (1 unit/ml), mannitol (5 mM), and pyruvate (5 mM) at 4° C; and used for up to 6–10 hours. In some experiments, ventricular myocytes from guinea pig were used. The method of cell preparation has been described.[23]

Electrophysiological Experiments

The whole-cell calcium current was recorded using the continuous voltage clamp method with a suction pipette.[24] The pipette was made of Pyrex glass (Mercer Glass, New York, New York; ID: 1 mm) and pulled by a two-stage puller (PE-2 puller, Narishige, Tokyo, Japan). The pipette was heat-polished before use and filled with Tyrode's solution. Tip resistance was 2–3 MΩ. With the pipette in the bath solution, pipette potential was adjusted to give zero current; this was the reference potential for later measurements. After a "gigaseal" was formed, the pipette solution was switched to the internal solution using a pipette perfusion device.[25] The patch membrane was then ruptured by a sudden increase in suction pressure. At least 10 minutes was allowed for the intracellular dialysis to reach equilibrium. For experiments on dog ventricular cells, a List EPC-7 amplifier (List Medical, Eberstadt, FRG) was used for voltage clamping. The capacitance of the dog cells was 147.1 ± 22.4 pF ($n = 7$). The total series resistance was 5.8 ± 2.4 MΩ ($n = 7$). After capacitance cancellation and series resistance compensation, the capacitance transient decayed with a time constant of 0.3 ± 0.2 msec ($n = 7$). The residual series resistance was 2.9 ± 1.2 MΩ ($n = 7$). With a calcium current amplitude of 1 nA in dog ventricular cells, the voltage error was estimated to be 3 mV. For experiments on guinea pig ventricular cells, an amplifier made in the National Institute for Physiological Sciences, Okazaki, Japan, was used.[26] The size and capacitance of the guinea pig cells[23] were similar to those of the dog cells, and the same type of pipettes was used. Series resistance was partially compensated; the residual series resistance was estimated to be about 1–2 MΩ, giving a voltage error of 2–4 mV for an I_{Ca} amplitude of 2 nA in guinea pig cells. The current and voltage were recorded on an FM tape recorder (model B, Vetter, Rebersburg, Pennsylvania) at a speed of 7.5 inch/sec (3 dB bandwidth: 0–2.2 kHz). Data analysis was performed on an IBM-AT using pClamp software (Axon Instruments, Burlingame, California); current was sampled with a 12 bit analog/digital converter (Axon Instruments) at a sampling interval of 0.2 msec. Curve fitting was done with a simplex algorithm.[27]

Solutions and Chemicals

The Tyrode's solution had the following millimolar composition: NaCl 137, $NaHCO_3$ 12, dextrose 5.5, NaH_2PO_4 1.8, $MgCl_2$ 0.5, KCl 4, and $CaCl_2$ 2. The solution was equilibrated with 5% CO_2-95% O_2 and maintained at pH 7.1. The sodium- and potassium-free extracellular solution had the following millimolar composition: choline Cl 145, dextrose 5.5, $MgCl_2$ 0.5, $CaCl_2$ 5, and HEPES 5. The pH was adjusted to 7.4 with CsOH. This solution also contained 4-aminopyridine (4-AP), 2 mM. The internal solution had the following millimolar composition: Cs-aspartate 125, tetraethylammonium (TEA) Cl 20, HEPES 5, ATP (Mg salt) 5, and EGTA 10 mM. The pH was adjusted to 7.3 with CsOH. In some experiments, the calcium buffering capacity of the pipette solution was increased by increasing the EGTA concentration to 40 mM or by using 10 mM BAPTA.[28,29] When using 40 mM EGTA, the concentration of Cs-aspartate was lowered to 95 mM. As stated above, the reference potential for the membrane potential measurements was set when the pipette was filled with Tyrode's solution and positioned in the Tyrode's bath solution. Therefore, the reference potential should be very close to the true zero potential. Although there might have been junction potentials developing when

the pipette and bath solutions were changed, the reference potential was not likely to be in error since intervals of at least 20 and 10 minutes separated the pipette and bath solution changes, respectively, from the start of recording and since by that time the holding current at −30 or −80 mV was stable.

Ryanodine (1 mM) in distilled water was added to the superfusate to give a final concentration of 1–2 μM. When caffeine (10 mM; Sigma Chemical, St. Louis, Missouri) was used, it was necessary to treat the cells with caffeine in Tyrode's solution first to allow the Na/Ca exchanger to extrude the calcium released from the sarcoplasmic reticulum. Caffeine was added as a dry powder. After 10 minutes in caffeine-containing Tyrode's solution, the bath solution was then switched to the sodium- and potassium-free solution containing the same concentration of caffeine. After performing the voltage clamp protocol in this caffeine-containing solution, the bath solution was then switched to caffeine-free sodium- and potassium-free solution. After a period of 10–15 minutes the calcium current activated at a cycle length of 7 seconds was stable and the protocol was repeated to obtain data under caffeine-free conditions.

Intracellular Dialysis

Intracellular dialysis was achieved by using a pipette perfusion device.[25] Briefly, a fine polyethylene tubing was inserted into the pipette with the other end connected to a pipette solution reservoir. As suction was applied to the interior of the pipette, new pipette solution was drawn from the reservoir through the fine tubing to replace the original pipette solution. To obtain efficient intracellular dialysis, the pipette tip had to be as large as possible; typically the outer diameter was 3–5 μm as seen under a microscope of a magnification ×645. The distance between the inner tubing and the pipette tip was 200–300 μm. The time course and completeness of intracellular dialysis were estimated by monitoring the change either in holding current at a holding voltage of −60 mV or in membrane voltage on changing the pipette solution from a potassium-rich solution (having the same composition as the cesium-rich internal solution except Cs-aspartate and TEA-Cl were replaced by K-aspartate and KCl, respectively) to the cesium-rich internal solution (data not shown). The change in holding current or membrane voltage ended within 10 to 12 minutes. By that time, the resting membrane potential decreased from −76±6 mV with potassium inside to −32±23 mV with cesium inside ($n=3$, mean±SD), indicating 50–75% replacement of potassium by cesium.

Protocol

The cells were allowed to adhere to a poly-*l*-lysine-coated glass coverslip placed on the bottom of a 0.7-ml Lucite tissue chamber mounted on the stage of a Nikon inverted microscope and superfused continuously with Tyrode's solution. After the whole-cell recording configuration was achieved, at least 10 minutes was allowed for the intracellular dialysis to come to equilibrium. The bath solution was then changed to the sodium- and potassium-free solution. A period of 10 minutes of continuous superfusion before the start of recording was allowed to make sure that the extracellular sodium and potassium were totally washed out. The experiment was performed during continuous superfusion with the sodium- and potassium-free solution at 35–37° C.

To study calcium current restitution, a double-pulse protocol was used (upper part of Figure 2A). From a holding voltage (V_h) ranging from −30 to −80 mV, a pair of depolarizing pulses (V_1 and V_2), each lasting 100 msec, were applied every 7–10 seconds. The interpulse interval was varied between 10 and 5,000 msec. The depolarization voltage was the voltage giving the maximum peak I_{Ca} (+20 mV at 5 mM $[Ca]_o$) unless otherwise stated. The peak I_{Ca} amplitude was measured as the difference between the inward peak and the current at 100 msec. The degree of restitution was taken as the ratio of the peak I_{Ca} during the second pulse to that during the first pulse (I_2/I_1). The I_{Ca} "staircase" was measured by dividing the peak I_{Ca} during each first pulse by that during the first pulse in a run. During the course of the experiments, I_{Ca} amplitude could vary due to either rundown or the staircase; the effect of this on the determination of I_{Ca} restitution was minimized by taking the ratio of I_2 to I_1.

Results

In the present study, the time course of restitution of the conventional L-type calcium current (I_{Ca}) in ventricular myocytes isolated from dog or guinea pig was investigated. Although the two types of cells may differ with respect to some membrane currents (e.g., transient outward currents[30,31]) the calcium currents seemed very similar. Both had L and T types of calcium current (G.-N. Tseng, unpublished data and Mitra and Morad[32]), and the L-type calcium current in the two cell types was similar in terms of the peak current-voltage relation and the dependence of inactivation on intracellular calcium.[15,31] The amplitude of the maximum L-type calcium current was higher in guinea pig cells (2.2±0.5 nA, $n=8$) than in dog cells (0.9±0.2 nA, $n=8$). The "overshoot" during restitution of the L-type calcium current and the effects of interventions on the restitution time course (see below) were similar for these two cell types. In the following sections, the origin of the data, dog or guinea pig, will be specified. Figure 1 shows inactivation of I_{Ca} in a dog cell dialyzed with the pipette solution containing 10 mM EGTA. I_{Ca} decreased after more positive conditioning pulses between −35 and +10 mV. For still more positive conditioning pulses, the I_{Ca} during the conditioning pulse (I_1) decreased due to a decrease in driving force. At the same time, I_{Ca} during the test pulse (I_2) increased slightly. This

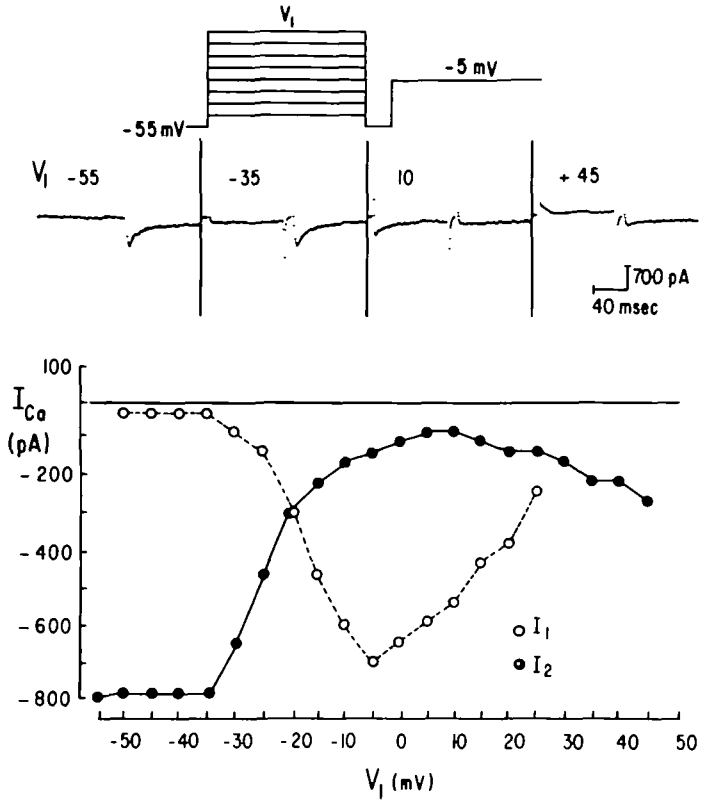

FIGURE 1. *Evidence for inactivation of the L-type calcium current mediated by intracellular calcium in a dog ventricular cell dialyzed with pipette solution containing 10 mM EGTA. $[Ca]_o$ was 2 mM. Voltage clamp protocol is illustrated schematically above original current traces. Double pulses each for 100 msec with an interpulse interval of 10 msec were applied every 10 seconds from a holding voltage of −55 mV. The voltage of the first pulse varied between −50 and +45 mV, and the voltage of the second pulse was −5 mV. The calcium current amplitudes during the first pulses (I_1, ○) and the second pulses (I_2, ●) are plotted against the voltages of the first pulses (V_1) and selected original traces are illustrated above, with the holding voltage or V_1 voltages marked above. The I_2 gradually decreased following V_1 from −35 to +10 mV; but following more positive V_1, I_2 increased again.*

increase in I_{Ca} following very positive conditioning pulses was due to a decline in the inactivation mediated by intracellular calcium.[5,6] Similar observations were obtained in two other dog cells and four guinea pig cells. Thus, with 10 mM EGTA in the pipette solution, even though there was no visible mechanical activity of the cell, there was sufficient intracellular calcium activity, perhaps in the subsarcolemmal compartment, to cause inactivation of I_{Ca}. This is consistent with the observation made by other investigators that 10 mM EGTA does not totally buffer the calcium ions in the vicinity of the cell membrane.[29]

Voltage-Dependent Restitution of L-Type Calcium Current

The upper row of Figure 2A illustrates the voltage clamp protocol used to study restitution of I_{Ca}. The time required for full recovery from inactivation was voltage-dependent; in dog cells, this was 43.4 ± 19.4, 41.9 ± 9.0, 114.5 ± 91.1, and 337.5 ± 249.6 msec at −80, −70, −50, and −30 mV. At holding voltages positive to −40 or −50 mV, the time course of restitution was monophasic. One example is provided by the middle set of traces in Figure 2A, which shows I_{Ca} restitution at a V_h of −30 mV in a guinea pig cell. The time constant of restitution was 170 msec, as estimated by fitting the time course of change in the fraction inactivated $(1-(I_2/I_1))$ with a single exponential. In six dog cells, the time constant of restitution at −30 mV was 100 ± 58 msec (ranging from 46 to 188 msec). At holding voltages negative to −40 or −50 mV, restitution was accelerated and became biphasic; peak current rose to a value higher than the control before gradually declining to the control level. One example is shown by the lower set of traces in Figure 2A, which illustrates restitution at $V_h = -80$ mV in a guinea pig cell (the same cell as in the middle traces). At a V_h of −80 mV, I_{Ca} during the second pulse exceeded the control by 18% at 90–110 msec and then gradually declined to the control level at 3 seconds. This is called an "overshoot" of I_{Ca} during restitution. Note also that at V_h of −30 mV during the restitution of I_{Ca}, as the amplitude of I_{Ca} increased its rate of decay was accelerated, consistent with enhanced inactivation by intracellular calcium.[5,14] At a V_h of −80 mV, however, although the amplitude of I_{Ca} during the overshoot was larger than the control, the decay was slower than the control. More discussion of the relation between I_{Ca} amplitude and rate of decay will be presented later (Figure 9). In Figure 2B the restitution time courses at four different holding voltages are shown. In these experiments, the sequence of the holding voltages at which the I_{Ca} restitution was studied was random. At −30 mV, the restitution was gradual and monophasic. As the holding voltage was made more negative, the initial rate of restitution was increased, the overshoot became more prominent, and the peak overshoot was reached earlier.

The magnitude and time course of the calcium current overshoot varied among cells. In dog cells, at −80 mV, 80% showed an overshoot of ≥10%

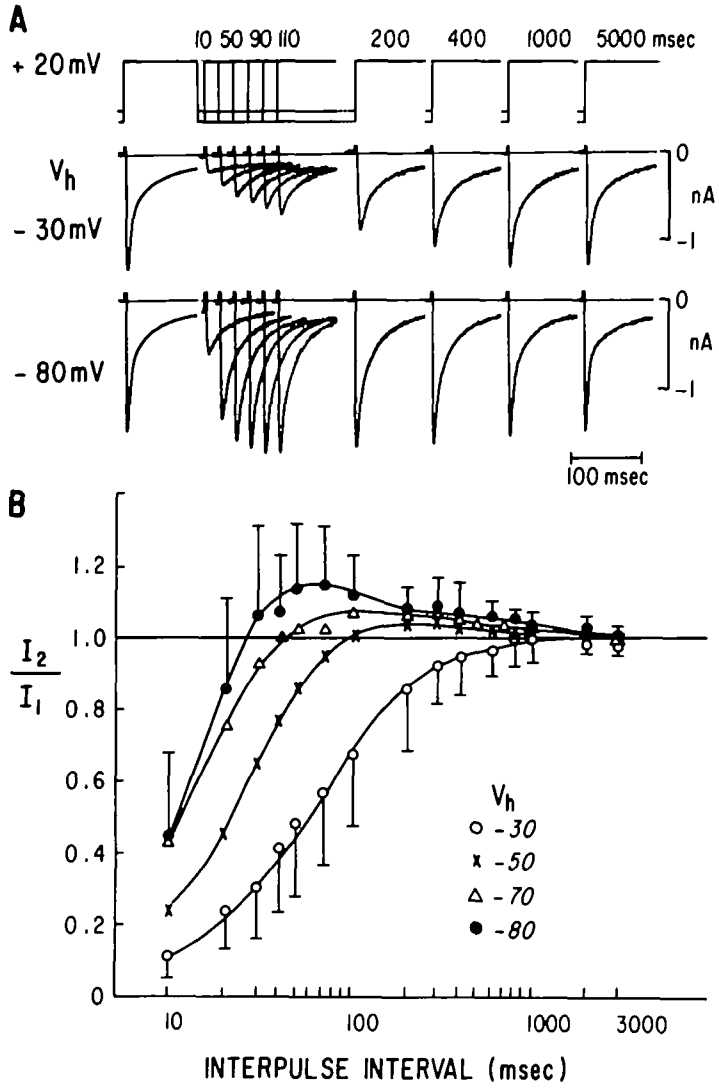

FIGURE 2. *Voltage-dependent restitution of the L-type calcium current.* Panel A: *Upper row illustrates the voltage clamp protocol. Middle and lower rows are original current traces at V_h of −30 mV and −80 mV, respectively, recorded from a guinea pig cell. The voltage of the first and the second pulses was +20 mV. For each row, the calcium current during the first pulses is shown on the left and calcium currents during selected second pulses are shown on the right with interpulse intervals marked above the upper row. Solid lines denote the zero current level.* Panel B: *Time courses of calcium current restitution at four different holding voltages; data are summarized from a total of six dog cells. x-Axis: interpulse interval on a logarithmic scale. y-Axis: degree of restitution measured as the ratio of I_{Ca} during the second pulse (I_2) to that during the first pulse (I_1). Curves are drawn by eye. The standard deviation bars are shown for the data at V_h −30 and −80 mV, but omitted for V_h −50 and −70 mV for the sake of clarity.*

over the control (average 27 ± 17%, range 10% to 50%, $n = 8$). Time to peak overshoot was 30–100 msec, and the calcium current declined to the control level by 2 to 3 seconds.

The overshoot could have resulted from a transient increase in I_{Ca} above the control level, slower restitution of overlapping outward potassium currents than of calcium current, or both. Under the experimental conditions, the potassium currents were largely, but perhaps not totally, eliminated. To exclude the possibility of contamination by potassium currents, the effect of D-600 on I_{Ca} restitution was tested. The voltage clamp protocol was performed before and after the addition of 20 μM D-600. The D-600–sensitive current was obtained by subtracting the current in the presence of D-600 from that in its absence (Figure 3A). Figure 3B illustrates the time course of restitution of the D-600–sensitive current. It is clear that it was the D-600–sensitive current, that is, I_{Ca}, that was responsible for the overshoot.

In these experiments, the amplitude of I_{Ca} during the first depolarizing pulse (I_1) sometimes was not constant but showed a gradual change: with a depolarizing voltage of +20 mV and at V_h negative to −40 or −50 mV it gradually increased (positive staircase), whereas at V_h positive to −40 or −50 mV it gradually decreased (negative staircase). Among different cells there was a positive relation between the magnitude of the I_{Ca} overshoot and the magnitude of the positive staircase (data not shown). This correlation suggests that these two phenomena may have the same cellular mechanism (see "Discussion"), and the variation in the magnitudes of the two may reflect an intrinsic heterogeneity of the properties or conditions among cells.

Overshoot Is Changed by Altering Extracellular Divalent Cations

The effect of substituting barium for calcium on the time course of calcium channel restitution was tested by recording the current in an external solu-

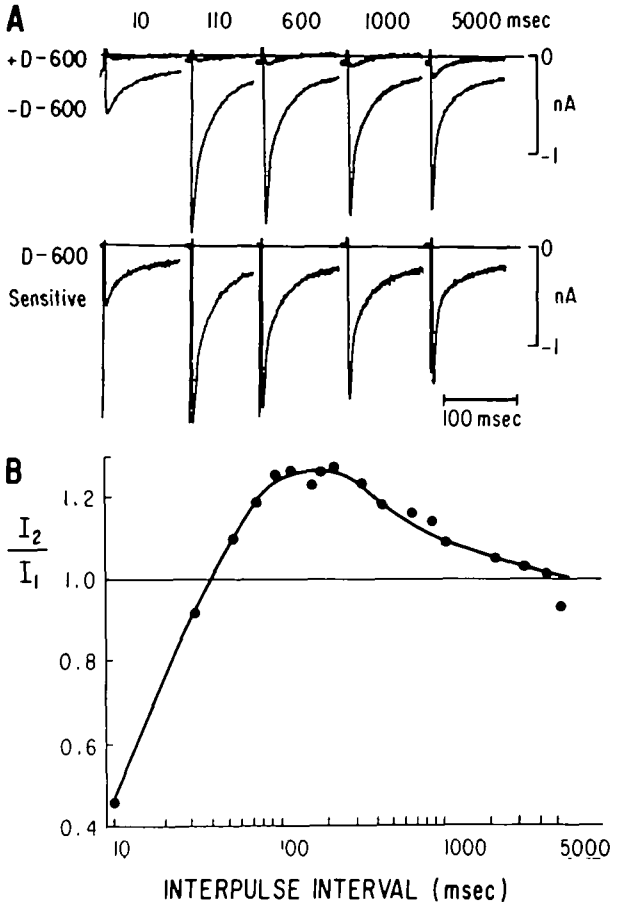

FIGURE 3. *Overshoot is caused by a D-600–sensitive current.* Panel A: *Original current traces recorded from a guinea pig cell. From a V_h of −80 mV a pair of depolarizing pulses each to +20 mV for 100 msec were applied every 10 seconds with the interpulse interval varied from 10 to 5,000 msec. Only calcium currents during the second pulses are shown and the intervals are marked above. Before the addition of D-600 (−D-600) there was a sizable calcium current 10 msec after the first pulse and calcium current increased rapidly to a level higher than the control before returning to the control level. In the presence of 20 μM D-600 (+D-600) the calcium current during the first pulses was greatly reduced (not shown). Calcium current during the second pulses after short intervals (e.g., 10 and 110 msec) was very small and increased slightly after longer intervals due to removal of use-dependent block. The D-600–sensitive current (lower trace) was the difference current obtained by subtracting the current in the presence of D-600 from that in its absence. Overshoot phenomenon is very marked in the D-600–sensitive current.* Panel B: *Time course of restitution of the D-600–sensitive current. Data are from the experiment shown in Panel A. Axes are the same as in Figure 2B.*

tion containing 5 mM Ca and then equimolar barium. Figure 4 illustrates the results of a representative experiment on a guinea pig cell. Substitution of barium increased the amplitude of current through calcium channels by 130% (from 1.8 nA to 4.1 nA) and slowed the rate of current decay (Figure 4A). Due to the extremely slow decay of the barium current, for this experiment the peak current amplitude was determined as the difference between the inward peak and the holding current. The plot of Figure 4B compares the time courses of restitution of calcium and barium currents. Barium dramatically altered the restitution time course. For interpulse intervals between 50 msec and 2 seconds, calcium current showed an overshoot with a peak 58% over the control at 100 msec. On the other hand, barium current showed a late overshoot of only 4% over the control between 400 msec and 1 second. Thus, when barium was the charge carrier, even though the current was larger the overshoot was largely suppressed. This indicates that the overshoot was calcium-dependent but not current-dependent. At short interpulse intervals (e.g., 10 msec), there was less inactivation of barium current than of calcium current due to the decrease in inactivation mediated by intracellular calcium.[5,9]

Decreasing $[Ca]_o$ from 5 to 2 mM abolished the overshoot observed in $[Ca]_o$ 5 mM at −80 mV and reduced the rate of I_{Ca} restitution slightly. Figure 5 depicts the results from a representative experiment on a dog cell. The slowing was apparent at a V_h of −80 and −30 mV, but the effect was more prominent at −80 mV. Similar findings were obtained in two other dog cells.

Overshoot Is Dependent on Intracellular Calcium

The change in the restitution time course caused by substitution of barium for calcium or by lowering $[Ca]_o$ might be due either to a change in the external surface charge,[33] or to a change in intracellular calcium activity. The possibility that intracellular calcium might be involved in the overshoot phenomenon was tested by changing the cytoplasmic calcium buffering capacity by dialyzing the cell with different calcium buffer solutions. As already shown (Figure 1) when 10 mM EGTA is used for dialysis only the free calcium in the bulk phase of the cytoplasm, but not the calcium transients in the vicinity of the cell membrane, is well buffered. Higher concentrations of EGTA can suppress intracellular calcium transients more completely. Figure 6A shows the effects on restitution of I_{Ca} of changing pipette [EGTA] from 10 mM to 40 mM. With 10 mM EGTA, I_{Ca} showed a prominent overshoot. Forty millimolars EGTA slowed the restitution of I_{Ca} and decreased or abolished the overshoot. BAPTA is a calcium chelator with a binding affinity 5–10 times higher than that of EGTA and with a much faster binding rate.[28,29] The pipette solution was changed from one containing 10 mM EGTA to one containing 10 mM BAPTA and the restitution of I_{Ca} under the two conditions was compared (Figure 6B). When BAPTA was the intracellular calcium buffer restitution of I_{Ca} was slowed and the overshoot abolished. From the similar effects of 40 mM EGTA and 10 mM BAPTA on restitution of I_{Ca}, it is concluded that the

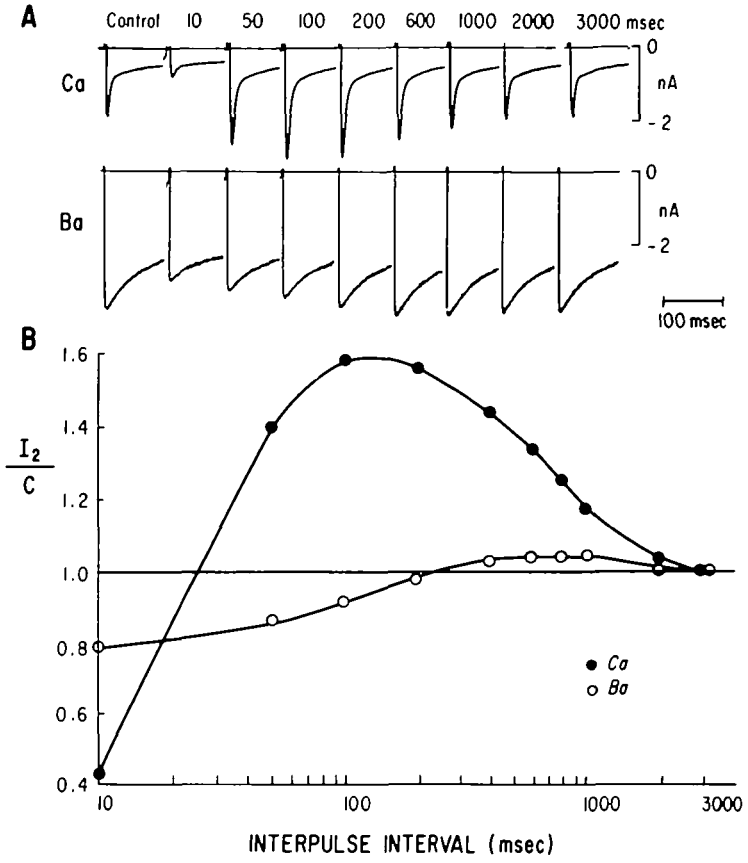

FIGURE 4. *Effects on overshoot of substituting barium for calcium. The experiment was done on a guinea pig cell. The V_h was −80 mV; the first pulse was to +10 mV and the second pulse to 0 mV. Both were 100 msec in duration. Only the current traces during the second pulses are shown* (Panel A) *and the control trace is the current at an interpulse interval of 7 seconds, when restitution was complete. Barium substitution increased current amplitude and slowed current decay during depolarization.* Panel B: *Different time courses of current restitution. The ordinate is the ratio of current during the second pulse to the control (I_2/C). Barium substitution both slowed time to peak and decreased the degree of overshoot during current restitution.*

overshoot phenomenon during restitution of I_{Ca} is dependent on intracellular calcium.

Increasing pipette [EGTA] from 10 to 40 mM or using BAPTA as the intracellular calcium buffer also exerted other effects on membrane currents. With 40 mM EGTA, the amplitude of I_{Ca} was augmented both during the first pulses (not shown) and when the restitution was complete (compare the I_{Ca} traces at an interpulse interval of 3,000 msec in Figure 6A). With the increase in I_{Ca} amplitude in 40 mM [EGTA], the decay of the current was accelerated. BAPTA slowed the decay of I_{Ca} during depolarization and increased the membrane conductance (current during depolarization shifted outward). With BAPTA in the pipette solution, the amplitude of I_{Ca}, measured between the inward peak and current level at 100 msec, was not changed or slightly decreased.

Function of Sarcoplasmic Reticulum Is Involved in Overshoot

Under the experimental conditions, ion transport by the Na/Ca exchanger was largely suppressed by removing sodium from both sides of the cell membrane. Therefore, the increase in intracellular calcium activity due to calcium influx via calcium channels during depolarization was dissipated by sequestration by the sarcoplasmic reticulum and extrusion by the sarcolemmal calcium pump.[34] Whether modifying saroplasmic reticulum function would affect the restitution of I_{Ca} was tested by studying the effects of caffeine and ryanodine. Caffeine enhances calcium release from and inhibits calcium uptake by the saroplasmic reticulum.[35] There is some disagreement over the actions of ryanodine on the saroplasmic reticulum. Ryanodine inhibits calcium release by the saroplasmic reticulum during depolarization,[36] and/or accelerates calcium depletion from the saroplasmic reticulum during rest.[37,38] Figure 7A shows the effects of 10 mM caffeine on restitution of I_{Ca} in a guinea pig cell. Under control conditions there was a clear overshoot. With caffeine in the external solution the restitution was slowed and the overshoot abolished. Caffeine also increased the amplitude of I_{Ca} and slowed its rate of decay during depolarization. Figure 7B shows the effects of 1 μM ryanodine on restitution of I_{Ca} in a dog cell. Like caffeine, ryanodine slowed restitution of I_{Ca} and abolished the overshoot. Ryanodine also slowed the rate of I_{Ca} decay during depolarization. However, unlike caffeine, ryanodine decreased the amplitude of I_{Ca}. Similar results were obtained in two other dog cells. From the similar effects of caffeine and ryanodine on the I_{Ca} decay during depolarization and its restitution following depolarization, it is concluded that the function of the sarcoplasmic reticulum is important not only for the inactivation of I_{Ca} during depolarization but also for the restitution of this current following depolarization.

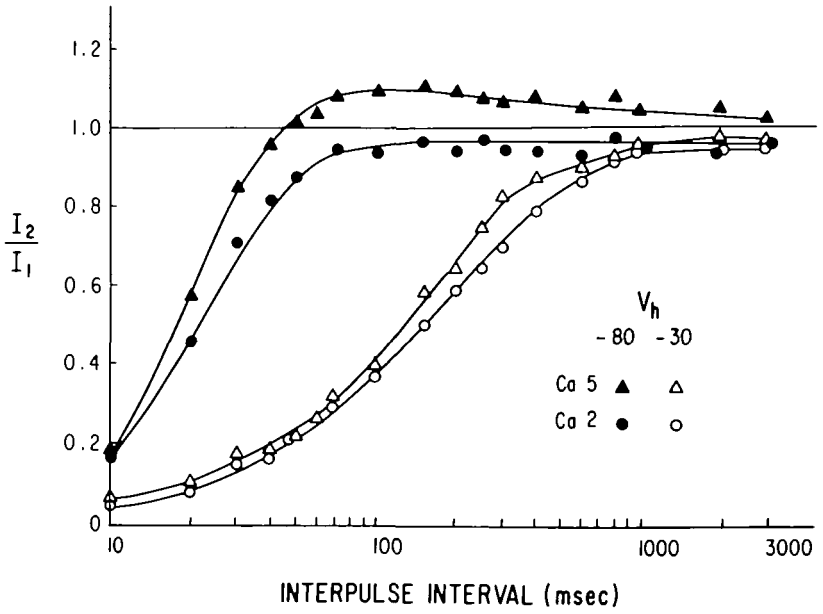

FIGURE 5. *Effects of lowering $[Ca]_o$ from 5 to 2 mM on the time course of calcium current restitution. The experiment was done on a dog ventricular cell. The V_h was −30 or −80 mV, and the voltage of both the first and the second pulses was +10 mV. Lowering $[Ca]_o$ abolished the overshoot at V_h −80 mV and slightly slowed the current restitution at both −30 and −80 mV.*

Kinetic Properties of Overshoot

After establishing that the restitution of I_{Ca} was modulated by intracellular calcium transients involving the function of sarcoplasmic reticulum, experiments designed to explore the cellular mechanism underlying the overshoot were performed. Based on the above results, it was assumed that the time course of restitution of I_{Ca} was determined by two processes, one dependent on voltage and the other dependent on intracellular calcium and responsible for the overshoot. When the intracellular calcium transients were largely suppressed by increasing [EGTA] or using BAPTA in the pipette solution, the time course of I_{Ca} restitution was mainly determined by the voltage-dependent process. The time course of the intracellular calcium–dependent process could then be determined by measuring the difference in the restitution rate between control and conditions when intracellular calcium transients were largely suppressed. Figure 8 shows the time course of the intracellular calcium–dependent process, summarized from six experiments on guinea pig cells. This process reached a peak at 30–100 msec and declined afterwards. Thus, the intracellular calcium–dependent process takes a finite time to develop and declines gradually.

It has been shown repeatedly[5,14] that there is an inverse relation between I_{Ca} amplitude and its rate of decay, that is, with an augmentation of I_{Ca}, its decay was accelerated due to an increase in intracellular calcium in the vicinity of the channel. During overshoot, the calcium channel conductance is augmented by some yet unknown mechanism. The relation between I_{Ca} amplitude and its rate of decay may then be changed. This was tested by studying the relation between I_{Ca} amplitude and its rate of decay during restitution, as shown in Figure 9. Under the control conditions overshoot occurred during the restitution of I_{Ca}. During the overshoot though the I_{Ca} was larger than the control its rate of decay was slower. Such a relation was not seen when overshoot was abolished. Making V_h less negative (from −80 to −30 mV, Figure 9A) or increasing the [EGTA] in the pipette solution (from 10 to 40 mM, Figure 9B) abolished the overshoot and converted the relation between I_{Ca} amplitude and its rate of decay to the simple, inverse one. Changing the calcium buffer in pipette solution from EGTA to BAPTA abolished overshoot and eliminated any sign for dependence of I_{Ca} on intracellular calcium, that is, decay half-time stayed the same regardless of the I_{Ca} amplitude (Figure 9C). Ryanodine abolished the overshoot and complicated the relation between I_{Ca} amplitude and its rate of decay: a smaller I_{Ca} during restitution was coupled with a shorter half-time of decay (Figure 9D).

Effects on Overshoot of Changing Voltage and Duration of the First Pulse

Since the I_{Ca} overshoot depended in some way on intracellular calcium, changing the calcium influx during the first pulse, by changing pulse voltage or duration, should modify the overshoot. Figure 10 shows results from five experiments on guinea pig cells depicting the effects on overshoot of changing the voltage of the first pulse (V_1 voltage). In each cell, the magnitude of the overshoot displayed a dependence on the V_1 voltage. In most cases (seven out of 10 observations), increasing I_{Ca} amplitude during the first pulse by making V_1 voltage more positive enhanced the overshoot, and still more positive V_1 voltages decreased both I_{Ca} amplitude during the first pulse and overshoot, indicating that a larger calcium influx exerted an enhancing effect on the following I_{Ca}. However, in some cases (three out of 10 observations), a larger I_{Ca} during the first

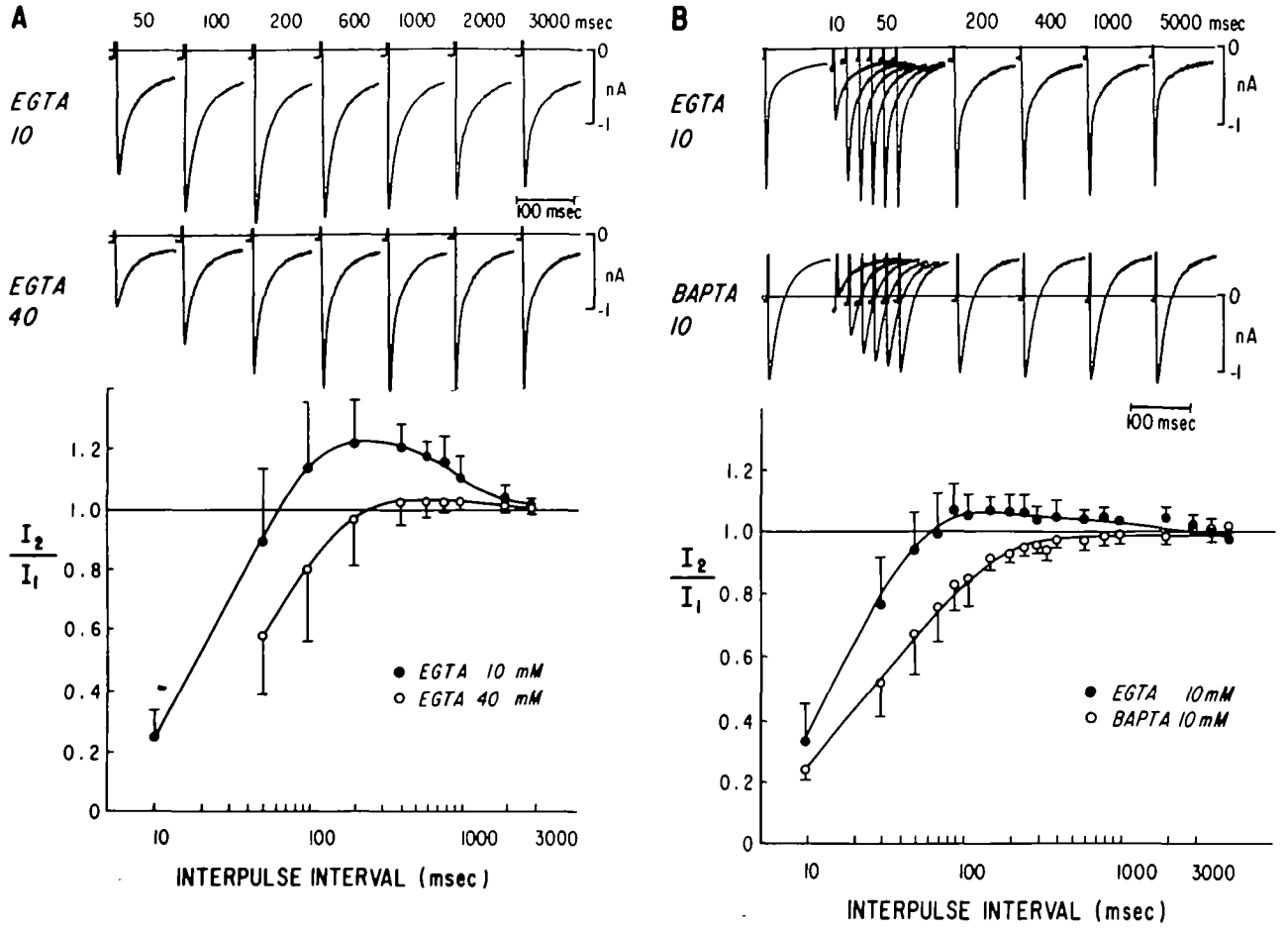

FIGURE 6. *Effects of increasing intracellular calcium buffering capacity on calcium current restitution.* Panel A: *Effects of increasing pipette [EGTA] from 10 to 40 mM. The V_h was −80 mV, and the voltage of both the first and the second pulses was +20 mV. Upper two traces show selected original current recordings during the second pulses with the interpulse intervals marked above. The lower plot shows the data summarized from four similar experiments.* Panel B: *Effects of changing pipette solution from one containing 10 mM EGTA to one containing 10 mM BAPTA. Voltage clamp protocol was the same as that in Panel A. Upper traces show selected original current recordings during the first and second pulses, with the interpulse intervals marked above. Lower plot shows the time course of calcium current restitution under the two conditions; data are summarized from three similar experiments. All experiments were done on guinea pig cells. Increasing pipette [EGTA] from 10 to 40 mM or using BAPTA in the pipette solution reduced or abolished the overshoot and slowed the restitution of I_{Ca}.*

pulse did not cause a larger overshoot (the data denoted by crosses and the data denoted by dots at +20 mV). Figure 11A shows that prolonging the first pulse from 50 msec to 4,000 msec caused a monotonic decrease in the amplitude of I_{Ca} elicited by a second pulse at a fixed interpulse interval of 100 msec. The inset illustrates that at the depolarization voltage used (+10 mV), the current level was still inward at the end of 600 msec. So there was probably a calcium influx throughout this period, due to "window" I_{Ca}[39] or slowly inactivating I_{Ca}.[40] Figure 11B shows that prolonging the first pulse from 50 msec to 100 or 500 msec decreased the degree of overshoot during restitution of I_{Ca} in a dog cell. Thus, increasing the amount of calcium influx by prolonging the V_1 duration will decrease the following I_{Ca} or reduce the degree of overshoot. These observations and the discrepancy in the relation between I_{Ca} amplitude during the first pulse and the magnitude of overshoot when changing the V_1 voltage (Figure 10) might be due to the dual actions of intracellular calcium on calcium channels.[5,13,16] It is possible that there is an optimal range of intracellular calcium level over which I_{Ca} will be augmented. Exceeding this range by prolonging the depolarization pulse or using some depolarization voltage will decrease I_{Ca}.

Discussion

The experiments reported here studied the restitution of the L-type calcium current (I_{Ca}) when overlapping currents were largely eliminated and the intracellular environment was directly manipulated. The results showed that at V_h negative to −40 or

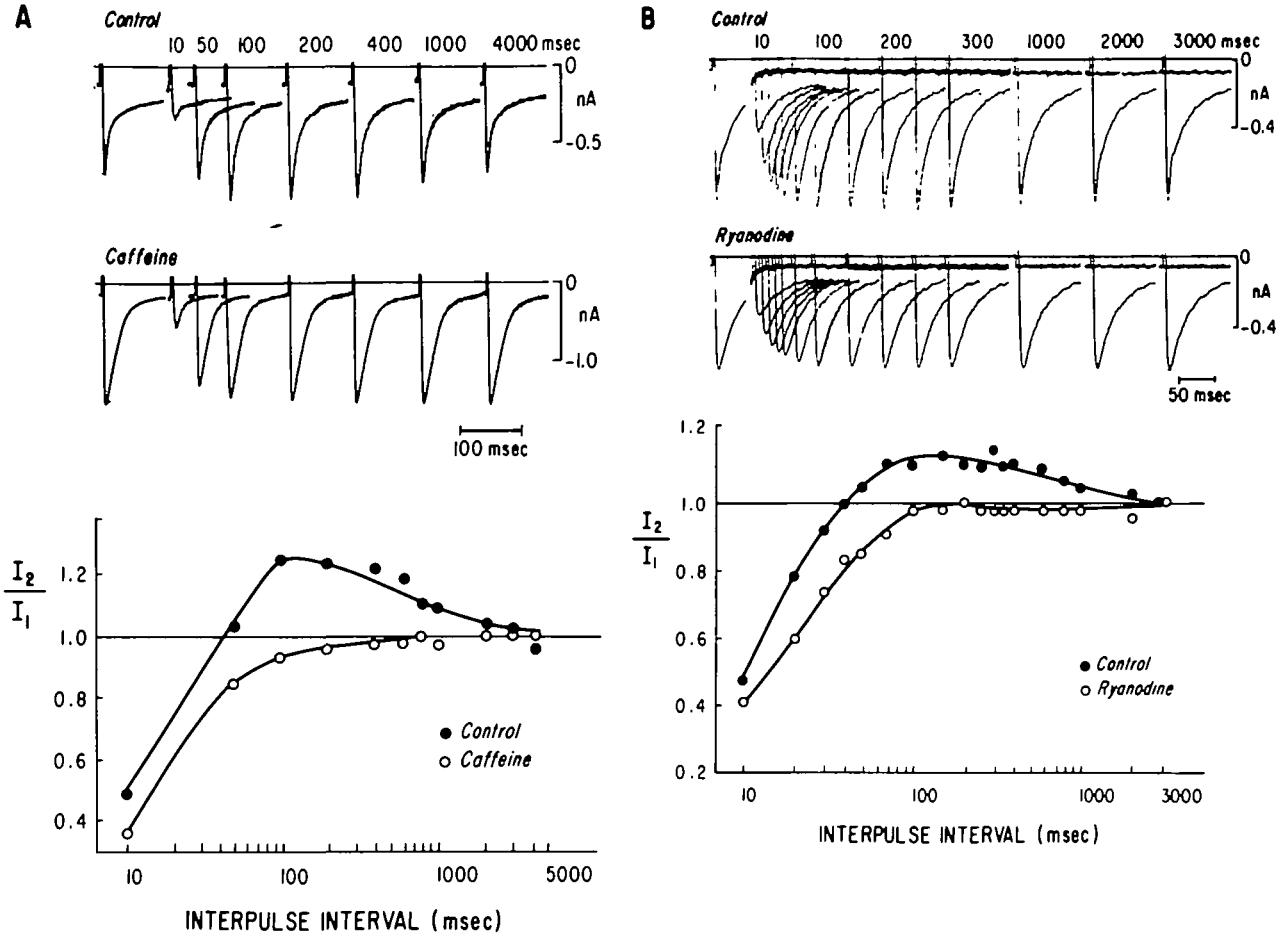

FIGURE 7. *Effects of caffeine and ryanodine on calcium current restitution.* Panel A: *Effects of caffeine, 10 mM, on I_{Ca} restitution in a guinea pig cell. V_h was −80 mV and the depolarizing voltage of both the first and the second pulses was +10 mV. Upper two traces are selected original current recordings during the first (left) and second pulses, with the interpulse intervals marked above. Note the difference in the current calibrations. Lower plot displays data from the experiment shown above.* Panel B: *Effects of ryanodine, 1 μM, on I_{Ca} restitution in a dog cell. Voltage clamp protocol was similar to that of Panel A except that the duration of the first pulse was 50 msec. Both caffeine and ryanodine abolished the overshoot during restitution of I_{Ca}.*

−50 mV, I_{Ca} recovers from inactivation with a transient increase in amplitude to a level higher than the control between 30–100 msec and 2–3 seconds, the overshoot phenomenon. The inhibitory effects on the overshoot of substituting barium for calcium or lowering $[Ca]_o$ indicate the involvement of calcium in this process. The inhibitory effects on overshoot of enhancing intracellular calcium buffering capacity, with a high concentration of EGTA or substituting BAPTA for EGTA in the pipette solution, indicate the involvement of intracellular calcium in mediating this process. Agents that inhibit the functions of sarcoplasmic reticulum, caffeine and ryanodine, abolish the overshoot. Although I_{Ca} is larger than the control during overshoot, it decays more slowly than the control. The most prominent overshoot occurs after a short conditioning pulse to an intermediate depolarization voltage.

Recording Conditions

In previous studies on the restitution of I_{Ca}, the data may have been distorted by other currents overlapping I_{Ca} and by effects of extracellular ion accumulation or depletion.[2,12-14] The methods employed in the present study largely overcame these problems. The use of sodium- and potassium-free external and internal solutions coupled with cesium and TEA in the internal solution and 4-AP in the external solution eliminated the sodium current, 4-AP–sensitive transient outward current, delayed rectifier current, and inward rectifier current. These conditions also should have eliminated currents generated by the Na/Ca exchanger[41] and through the nonselective cation channels.[42] The observation that the D-600–sensitive current showed a prominent overshoot confirms that I_{Ca} is largely responsible for the overshoot. In dog ventricular myocytes, there is an additional transient outward current that is activated by intracellular calcium.[31] This current probably can be carried by intracellular cesium (G.-N. Tseng, unpublished observation). As shown in Figure 1, under the experimental conditions with 10 mM EGTA in the pipette solution, there was still

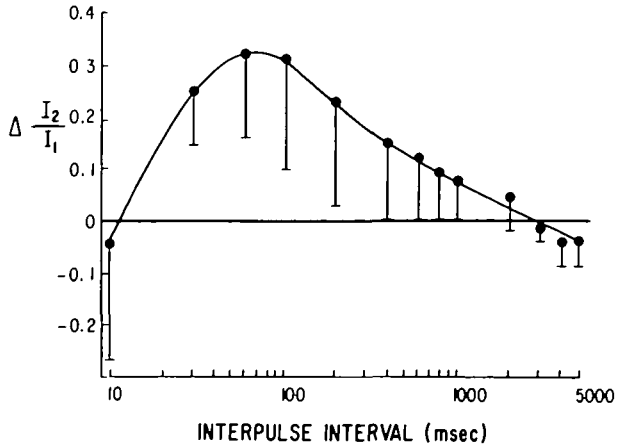

FIGURE 8. *Time course of the intracellular calcium–dependent process during restitution of I_{Ca}. Data are summarized from experiments on a total of six guinea pig cells. In each cell I_{Ca} restitution was measured at a V_h of −80 mV first under the control conditions with 10 mM EGTA in the pipette solution, and then with 40 mM EGTA (n=3) or 10 mM BAPTA (n=3) in the pipette solution. At each interpulse interval, the value of I_2/I_1 under the latter conditions was subtracted from that under the control conditions ($\Delta I_2/I_1$). This was averaged and plotted against interpulse interval. Circles represent means and bars standard deviations of means.*

intracellular calcium–mediated inactivation of I_{Ca}, indicating that the calcium transients in the subsarcolemmal compartment were not totally suppressed.[29] However, these calcium transients probably were not sufficient to activate the transient outward current because the decay time course of I_{Ca} was smooth; there was no upward hump suggesting a superimposed transient outward current. The experiments from this laboratory have shown that no transient outward current could be discerned until the [EGTA] in the pipette solution was lowered to 1 mM or less or catecholamines were added to the superfusate (G.-N. Tseng, unpublished observations). Therefore, there was negligible interference caused by the transient outward current in measuring the restitution of I_{Ca}. There may have been calcium depletion in a restricted extracellular space such as the T system or cell surface caeveolae during and following depolarization,[43] but this would not explain the overshoot because decreasing $[Ca]_o$, as shown in Figure 5, retarded the restitution of I_{Ca} and abolished the overshoot. In both dog and guinea pig ventricular cells, a T-type calcium current has been identified at a V_h negative to −50 mV.[32,44] It is unlikely that this current was responsible for the overshoot during the restitution of I_{Ca} for the following reasons. First, in these cells at 5 mM $[Ca]_o$, the maximal T-type current occurred at −40 to −30 mV, with an amplitude only 5–30% of the maximal I_{Ca}. Moreover, at more positive voltages, the T-type current decreased. Thus, the T-type current was too small to account for the overshoot. Furthermore, preliminary experiments on dog ventricular myocytes show that at a V_h of −80 mV the T-type current displays a monophasic time course of restitution that is slower than that of I_{Ca} (G.-N. Tseng, unpublished observation).

Cellular Mechanism for Overshoot During Restitution of I_{Ca}

Enhancement of I_{Ca} by repetitive depolarizations has been observed in frog atrial muscle.[12,13,18] This potentiation or up-regulation of I_{Ca} is opposite to the down-regulation of I_{Ca} by inactivation. One possible explanation for this phenomenon is that depolarization causes an increase in intracellular calcium–activated inward current, probably generated by the Na/Ca exchanger.[41] Such a current during depolarization has been reported for single frog atrial cells.[45] This current is dependent on intracellular calcium and extracellular sodium, as expected for the Na/Ca exchanger current.[46] However, the overshoot reported here was not caused by this mechanism because the sodium-free external and internal solutions eliminated the operation of the Na/Ca exchanger.[46]

To explain the observations of the present study, two hypothetical schemes are put forward. In the first scheme, it is assumed that intracellular calcium exerts dual actions on the L-type calcium channels: enhancement at moderately elevated levels and inactivation at high levels, as also suggested by other investigators.[5,13,16] According to this scheme, during depolarization calcium influx through the calcium channels causes a large increase in intracellular calcium level in the vicinity of the channels. This high calcium level inactivates the channels. After repolarization the calcium level in the vicinity of the channels gradually declines, due to sequestration by the sarcoplasmic reticulum and extrusion by the sarcolemmal calcium pump.[34] As the calcium level declines to within the range for enhancement, the I_{Ca} induced at this time will be larger than the control, causing the overshoot. As the calcium level continues to decrease the enhancement of calcium channels wanes and the overshoot declines. In the second scheme, it is assumed that the main action of intracellular calcium on the L-type channels is to inactivate the channels during depolarization and that most of the intracellular calcium is derived from the sarcoplasmic reticulum. According to this scheme following depolarization, the ability of the sarcoplasmic reticulum to release calcium transiently decreases due either to an inactivation of the sarcoplasmic reticulum releasing mechanism[47] or to a depletion of calcium at the sarcoplasmic reticulum's releasable site. I_{Ca} induced at this time will be larger than the control. As the ability of sarcoplasmic reticulum to release calcium recovers, the overshoot wanes. It has been suggested that in mammalian myocardium during depolarization the calcium that inactivates the L channels is mainly derived from the sarcoplasmic reticulum.[48,49] Consistent with this are

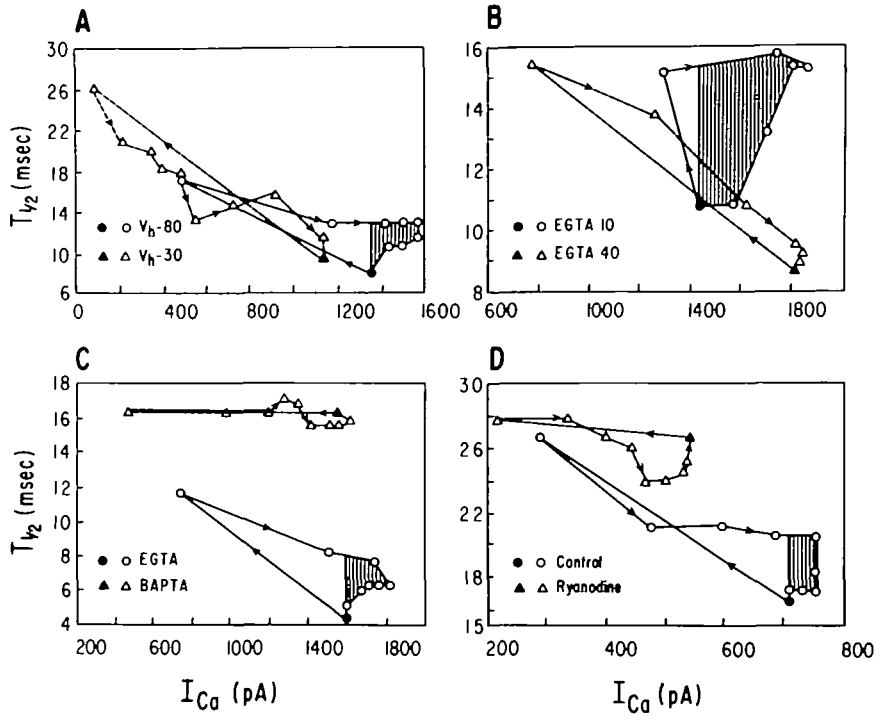

FIGURE 9. *Relation between the I_{Ca} amplitude and its rate of decay in the presence of overshoot differs from that in its absence. In each panel, the abscissa is peak I_{Ca} amplitude and the ordinate is the half-time ($T_{1/2}$) of I_{Ca} decay. Solid symbols represent the I_{Ca} amplitude and its half-time of decay during the first pulse. Open symbols represent data obtained during the restitution of I_{Ca} with the arrows pointing in the direction of restitution. Circles represent data obtained in the presence of overshoot, and triangles represent data obtained in its absence caused by changing V_h from −80 to −30 mV* (A), *changing pipette [EGTA] from 10 to 40 mM* (B), *changing pipette EGTA to BAPTA* (C), *and adding ryanodine* (D). *In the presence of overshoot, I_{Ca} during restitution rose to a level higher than control but decayed more slowly ($T_{1/2}$ longer), as denoted by the striped area in each panel. Such a relation is not seen in the absence of overshoot. All experiments were done on guinea pig cells, and in each panel data were obtained from the same cell.*

the present observations that caffeine and ryanodine slowed the decay of I_{Ca} during depolarization.

Both hypothetical schemes are compatible with the experimental observations that overshoot could be abolished by suppressing intracellular calcium transients (by substituting barium for calcium, lowering $[Ca]_o$, or increasing the intracellular calcium buffering capacity). According to the first scheme, caffeine and ryanodine inhibit the overshoot by preventing the uptake of calcium by the sarcoplasmic reticulum during rest.[35,37,38] According to the second scheme, these two agents inhibit the overshoot by decreasing calcium release from the sarcoplasmic reticulum during depolarization.[35,36] The observation that overshoot is not seen at V_h positive to −40 or −50 mV may be due to the much slower voltage-dependent restitution of I_{Ca} at these holding voltages,[2,12,14] masking the enhancement of L-type calcium channels explained by either scheme during rest. The first scheme can better explain the observation that overshoot took a finite time to develop (Figure 8): it takes time for the intracellular calcium level to decline to within the range for enhancement of the L channels. The second scheme can better explain the observation that during overshoot a larger I_{Ca} decays more slowly than the control (Figure 9): less calcium release from the sarcoplasmic reticulum will lead to a slower I_{Ca} decay. There is no straightforward explanation for the effects on overshoot of changing the voltage or duration of the conditioning pulse based on either scheme. It is possible that an optimal range of calcium influx during the conditioning pulse is needed for the overshoot to occur, and calcium influx over this range will decrease the overshoot.[13,16] Thus with the present experimental results, it is not possible to prove or disprove either scheme. New techniques that allow rapid, precise and reproducible changes in intracellular calcium activity to different levels are needed to differentiate the mechanism for overshoot of I_{Ca} during restitution.

In these experiments, I_1 sometimes gradually increased (positive staircase). There was a positive relation between the magnitude of overshoot and the magnitude of positive staircase among cells, although the time courses of the two differed: the overshoot reached a peak 30–100 msec following the depolarization pulse and declined to null by 2–3 seconds, whereas the positive staircase developed gradually over several pulses at an interpulse interval of 7–10 seconds. The close correlation between the two phenomena suggests that the two may share

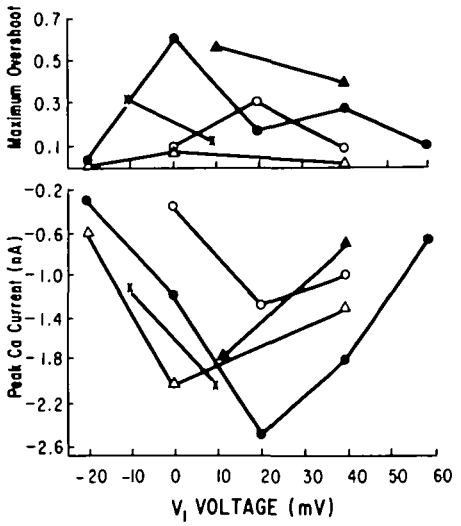

FIGURE 10. *Effects of changing the voltage of the first depolarizing pulse on the overshoot. Experiments were done on five guinea pig cells, represented by different symbols. In each cell, I_{Ca} restitution was studied at V_h of −80 mV with the voltage of the first pulse (V_1 voltage) varied. The voltage of the second pulse was fixed at +10 or +20 mV. The maximum degrees of I_{Ca} overshoot during restitution* (upper) *and the amplitudes of peak I_{Ca} during the first pulses* (lower) *are plotted against V_1 voltage.*

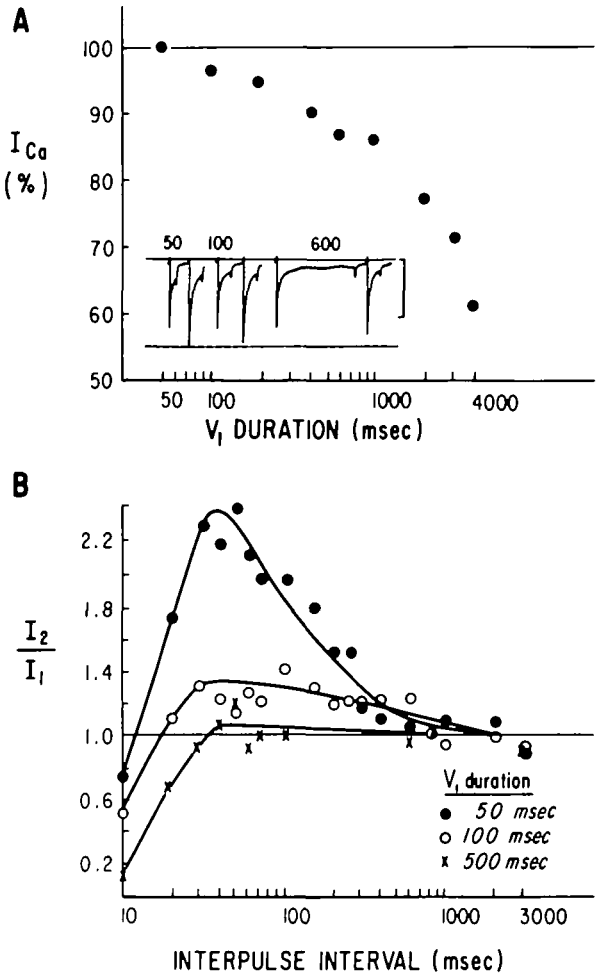

FIGURE 11. *Effects of changing the duration of the first pulse (V_1 duration) on the following I_{Ca} and the overshoot.* Panel A: *Amplitude of I_{Ca} elicited by a pulse to 0 mV after a depolarizing pulse to +10 mV for durations varied from 50 to 4000 msec. V_h was −80 mV and the interpulse interval was 100 msec. I_{Ca} amplitude was normalized to that after a V_1 of 50 msec.* Inset: *Three selected original current traces, with the V_1 durations marked above. Solid line denotes the zero current level, and dotted line denotes the peak I_{Ca} level following a V_1 of 50 msec. Calibration on the left denotes 1 nA. Note that current during V_1 at the end of 600 msec was still inward. The experiment was done on a guinea pig cell.* Panel B: *Time course of restitution of I_{Ca} with three different V_1 durations. V_h was −80 mV and both the first and the second pulses were to +20 mV. Data were from a dog cell.*

the same cellular mechanism. It is possible that although the overshoot apparently subsided by 2–3 seconds following depolarization, there was residual potentiation of I_{Ca} that accumulated over several pulses, causing a positive staircase.

Comparisons With Previous Observations

It has been shown for dog ventricular myocardium using the sucrose-gap voltage clamp technique that there is a transient increase in I_{Ca} to a level higher than control during the restitution of this current.[10] Moreover, in dog ventricular myocardium a premature depolarization induces an action potential with a more positive and longer plateau ("supernormal" action potential) that probably is due to a transient increase in I_{Ca} to higher than control level.[1] The present study confirms the previous observations and also shows that in guinea pig ventricular muscle there is a similar transient increase in I_{Ca} during its restitution. Similar observations have been obtained in other types of cardiac tissues.[11,12] Recently, potentiation of I_{Ca} by depolarization was also seen in guinea pig ventricular myocytes.[17] This potentiation is attributed by the author to a mechanism dependent solely on membrane voltage, based on the lack of effects when substituting barium for calcium. The reason for the discrepancy between that study and the present one is not clear.

Implications of I_{Ca} Overshoot Under Physiological and Pathological Conditions

Under the present experimental conditions, intracellular calcium transients are greatly reduced by intracellular dialysis. It is possible that in nondialyzed cells, since the intracellular calcium transients are intact, the overshoot of I_{Ca} during its restitution will be more prominent. Such a transient increase in I_{Ca} amplitude coupled with a slower decay time course will elevate the plateau voltage and prolong plateau duration in premature excitations, leading to the "supernormal" action potentials, as reported for dog and guinea pig ventricular myocardium.[1,8] The supernormal action potential

under physiological conditions can probably serve to protect the ventricular myocardium from reexcitation by a subsequent premature depolarization. During abnormal conditions such as myocardial ischemia and infarction, I_{Ca} can support impulse conduction in partially depolarized tissues. According to the observations reported in the present study, depending on the level of intracellular calcium, the resting membrane potential, the voltage and duration of the action potential plateau, and the proximity of premature depolarizations, the overshoot of I_{Ca} may occur to different degrees in the myocardium. Such a heterogeneity probably helps set the stage for reentrant arrhythmias.[4]

Acknowledgments

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KEY WORDS • calcium current • sarcoplasmic reticulum • heart • intracellular calcium