Intracellular Cs$^+$ activates the PKA pathway, revealing a fast, reversible, Ca$^{2+}$-dependent inactivation of L-type Ca$^{2+}$ current

Fabien Brette, Alain Lacampagne, Laurent Sallé, Ian Findlay, and Jean-Yves Le Guennec
Centre National de la Recherche Scientifique Unité Mixte de Recherche 6542, Faculté des Sciences, Université de Tours, 37041 Tours Cedex, France

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Brette, Fabien, Alain Lacampagne, Laurent Sallé, Ian Findlay, and Jean-Yves Le Guennec. Intracellular Cs$^+$ activates the PKA pathway, revealing a fast, reversible, Ca$^{2+}$-dependent inactivation of L-type Ca$^{2+}$ current. Am J Physiol Cell Physiol 285: C310–C318, 2003.—Inactivation of the L-type Ca$^{2+}$ current ($I_{\text{CaL}}$) was studied in isolated guinea pig ventricular myocytes with different ionic solutions. Under basal conditions, $I_{\text{CaL}}$ of 82% of cells infused with Cs$^+$-based intracellular solutions showed enhanced amplitude with multiphasic decay and diastolic depolarization-induced facilitation. The characteristics of $I_{\text{CaL}}$ in this population of cells were not due to contamination by other currents or an artifact. These phenomena were reduced by ryanodine, caffeine, cyclopiazonic acid, the protein kinase A inhibitor H-89, and the PKA-dependent protein kinase inhibitor. Forskolin and isoproterenol increased $I_{\text{CaL}}$ by only ~60% in these cells. Cells infused with either N-methyl-D-glucamine or K$^+$-based intracellular solutions did not show multiphasic decay or facilitation under basal conditions. Isoproterenol increased $I_{\text{CaL}}$ by ~200% in these cells. In conclusion, we show that multiphasic inactivation of $I_{\text{CaL}}$ is due to Ca$^{2+}$-dependent inactivation that is reversible on a time scale of tens of milliseconds. Cs$^+$ seems to activate the cAMP-dependent protein kinase pathway when used as a substitute for K$^+$ in the pipette solution.

L-type calcium current; calcium-dependent inactivation; facilitation; phosphorylation; cesium

SINCE INTRACELLULAR CALCIUM IONS were first shown to participate in the inactivation of the L-type Ca$^{2+}$ current ($I_{\text{CaL}}$) in cardiac muscle cells (24), Ca$^{2+}$-dependent inactivation of $I_{\text{CaL}}$ has been extensively studied (1, 10, 13, 16, 30, 34). It is now considered that calcium ions exert their effect via calmodulin linked to an IQ motif of the α1C-subunit (28). In cardiac muscle the Ca$^{2+}$ responsible for inactivation may enter the cell via sarcoplasmal Ca$^{2+}$ channels (13) and/or be released from the sarcoplasmic reticulum (SR) (34). There is close cross talk between $I_{\text{CaL}}$ (the dihydropyridine receptor, DHPr) and the SR Ca$^{2+}$ release channel sensitive to Ca$^{2+}$ (the ryanodine receptor, RyR) (7). This association has been directly observed in rabbit ventricular myocytes with the use of double-labeled immunofluorescence and confocal microscopy (8), and functional coupling between DHPr and RyR has been described in rat ventricular myocytes (32, 33). Thus the DHPr, by their close association with RyR in microdomains, regulate the release of Ca$^{2+}$ into the cytosol, which in turn regulates $I_{\text{CaL}}$ through Ca$^{2+}$-dependent inactivation. Ca$^{2+}$ current can be upregulated through different mechanisms. The best known of these is phosphorylation of the channel by a cAMP-dependent protein kinase (PKA) following stimulation of β-adrenergic receptors (9). During an action potential, increased entry of Ca$^{2+}$ following the phosphorylation of DHPrs has to be regulated to avoid Ca$^{2+}$ overload. This is done through different mechanisms such as increased K$^+$ current and Ca$^{2+}$-dependent inactivation of the DHPr (5). Another mechanism that leads to Ca$^{2+}$ current upregulation is facilitation. In cardiac cells an increase in the frequency of activation of Ca$^{2+}$ channels over a physiological range (0.5–5 Hz) induces an increase of the Ca$^{2+}$ current (11, 21, 29). Recently it was also shown (3) that moderate depolarization of the diastolic membrane potential also promotes facilitation of $I_{\text{CaL}}$, which is enhanced by intracellular cAMP.

The aim of the present study was to investigate the mechanism that underlies multiphasic inactivation of $I_{\text{CaL}}$, that was recorded in our experiments. In vitro patch-clamp studies of cardiac current usually seek to avoid contamination by other currents by substituting physiological Na$^+$ and K$^+$ by impermeant ions like Cs$^+$. We show that intracellular Cs$^+$ activates the PKA-dependent pathway leading to the phosphorylation of the DHPr and/or the RyR and/or the regulatory protein of the SR Ca$^{2+}$-ATPase (SERCA), phospholamban (PLB). Such phosphorylation underlies very fast and reversible Ca$^{2+}$-dependent inactivation of $I_{\text{CaL}}$.

MATERIALS AND METHODS

Cell isolation. All studies complied with the French Home Office Regulations Governing the Care and Use of Laboratory Animals. Ventricular myocytes were enzymatically dissociated from adult guinea pig hearts as described previously (20). Isolated cells were placed in a 1.5-ml Perspex chamber on the stage of an inverted microscope (Nikon Diaphot) and used between 2 and 8 h after cell isolation. The chamber was continuously perfused at a rate of 1–2 ml/min with a Tyrode

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Address for reprint requests and other correspondence: F. Brette, School of Biomedical Sciences, Univ. of Leeds, Leeds LS2 9NQ, UK (E-mail: bmsfpl@bms.leeds.ac.uk).

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solution (see Table 1). All experiments were performed at room temperature (~23°C).

**Solutions.** Experimental solutions are shown in Table 1. Extracellular test solutions were applied by using a multiple microcapillary perfusion system (19) placed in close proximity to the cell (0.5 mm). All salts and drugs were purchased from Sigma Chemicals, except H-89, which was obtained from Calbiochem. CaCl₂, 4-aminopyridine (4-AP), and caffeine were added to the external solutions at the concentrations given. Stock solutions of tetrodotoxin (TTX; 1 mmol/l), cyclopiazonic acid (CPA; 10 mmol/l), ryanodine (10 mmol/l), H-89 (1 mmol/l), forskolin (1 mmol/l), and isoproterenol (1 mmol/l) were prepared in distilled water and added to the external solutions to provide the concentrations indicated. Experiments involving the use of nifedipine (100 μmol/l, stock solution 10 mmol/l in ethanol) were performed in the dark. Protein kinase inhibitor (PKI; 200 mmol/l, stock solution 1 mmol/l in distilled water) was included in the internal solution of one set of experiments (see Fig. 5).

**Cell current measurements.** Ca²⁺ currents were recorded using the whole cell configuration of the patch-clamp technique. In most of the experiments, conditions were optimized to eliminate currents other than I_{CaL} by using Na⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻㈣

**Table 1. Composition of experimental solutions**

<table>
<thead>
<tr>
<th>Extracellular Solutions</th>
<th>Intracellular Solutions</th>
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| Tyrode                   | Na⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻㈣
| NaCl                    | 140                     | 10                        |
| KCl                     | 5                       | 140                       |
| TEACl                   | 140                     | 30                       |
| CsCl                    | 6                       | 110                       |
| NMDG                    | 130                     |                           |
| MgCl₂                   | 1                       | 1                         |
| CaCl₂                   | 1.8                     | 1.8                       |
| HEPES                   | 10                      | 10                        |
| Glucose                 | 10                      | 10                        |
| EGTA                    | 10                      | 10                        |
| ATP-Mg                  | 5                       | 5                         |
| GTP                     | 0.1                     | 0.1                       |
| pH                      | 7.3                     | 7.3                       |
| (NaOH)                  | (TEAOH)                 | (CsOH)                    |
| (HCl)                   | (KOH)                   |                           |

Concentrations are in mmol/l. The osmolarity of solutions ranged between 290 and 310 mosmol/l. NMDG, N-methyl-d-glucamine.

**RESULTS**

Different behavior in the time course of decay of I_{CaL}.

Figure 1, A and B, shows records of Ca²⁺ currents evoked at three voltages (−20, −10, and 0 mV) in two different myocytes that exhibit totally different kinetic patterns of inactivation. The records shown in Fig. 1A are typical of 18% of the cells (n = 90), where the currents showed classic exponential decay of I_{CaL}. The records shown in Fig. 1B are typical of 82% of the cells, which had more complex current inactivation, with a presence of a notch indicating the separation of two distinct phases of decay. All of these experiments were conducted with the Na⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻㈣

The voltage clamp circuit was provided by an RK 400 patch-clamp amplifier (Biologic, Grenoble, France). Voltage parameters were controlled by a Pentium PC connected through a DIGIDATA 1200 analog interface (Axon Instruments). Data acquisition and analysis were accomplished using pCLAMP software (Axon Instruments). Sampling frequency was 10 kHz, and signals were filtered at 3 kHz with the use of an 8-pole Butterworth filter before acquisition.

I_{CaL} was elicited with 300- or 500-ms voltage steps to 0 mV from a holding potential of −80 mV at a frequency of 0.125 Hz. I_{CaL} was measured as the difference between the peak inward current and the current at the end of the depolarizing pulse. Currents are expressed in current density (pA/pF). Current-voltage (I-V) relationships and availability (steady-state inactivation) curves were obtained with a double pulse protocol that consisted of 500-ms-duration pre pulses from −80 to +60 mV in 10-mV steps, a 5-ms interpulse interval at −80 mV, and a test pulse to 0 mV for 500 ms. The stimulus frequency was 0.125 Hz. Availability curves were obtained by normalization of the amplitude of the test pulse current to its amplitude recorded in the absence of a prepulse.

Results are expressed as means ± SE (n = no. of cells). Statistical differences were assayed by using one- or two-way ANOVA as appropriate, followed by the post hoc Dunnett’s test. Statistics were performed by using SigmaStat version 1.0 software.
larity would be more prominent at positive voltages where the current was larger. It was verified that series resistance and cell capacitance compensation up to 70% did not affect the shape of the current (n = 10).

Despite the lack of a shoulder in the I-V relationships at negative potentials, we looked for possible contamination of ICaL by the fast Na+ current (INa, notwithstanding that the external solution was Na+-free), the TTX-sensitive Ca2+ current (14), and the T-type Ca2+ current (ICaT). The addition of 30 μM TTX to the external solution had no effect on the shape of recorded ICaL (n = 6). The addition of 40 μM Ni2+, a concentration known to block ICaT without affecting ICaL (26), had no effect (n = 6; Fig. 2A). Transient outward K+ currents (IKO), which are present in cardiac cells from other species, are sensitive to 4-AP and/or to intracellular Ca2+. Although our experiments were conducted with a Ca2+-rich intracellular solution that did not contain K+, we tested the effect of the addition of 2 mM 4-AP, which blocks Ca2+-insensitive transient outward currents. This had no effect (n = 9; Fig. 2B). Also a transient outward current was not revealed when either external Ca2+ was removed from the medium (n = 2) or when ICaL was blocked by an inorganic blocker (200 μM Cd2+, n = 5; data not shown) or by an organic blocker (100 μM nifedipine, n = 8; Fig. 2C). We next examined whether calcium ions had to flow through the Ca2+ channels to induce multiphasic inactivation. Figure 2D shows that, after substitution of Ca2+ by Ba2+, notched decay of the current was no longer visible (n = 6). Also, complex inactivation of ICaL was not observed in experiments where BAPTA replaced EGTA in the pipette solution (n = 10; Fig. 2E).

These last results suggest that multiphasic decay of the current resulted from Ca2+ influx and accumulation beneath the membrane.

Origin of the Ca2+ dependence of complex decay of ICaL. In the following experiments, the involvement of the SR in multiphasic inactivation of the L-type Ca2+ current in Cs+-loaded myocytes due to Ca2+-induced Ca2+ release (CICR) was tested. In the presence of 100 μM ryanodine, the Ca2+ release channel (RyR) of the SR is unable to release Ca2+ stored in the SR, whereas sarcolemmal L-type Ca2+ channels are unaffected (18, 23). Figure 3A shows that the application of 100 μM of ryanodine induced the disappearance of notched decay, a slowing down of the inactivation phase of the current, and an increase of the current amplitude. In addition, prepulse voltage facilitation disappeared with ryanodine (Fig. 3B). Similar results were obtained after the application of either 100 μM CPA (n = 3), which prevents Ca2+ loading of the SR (37), or 10 mM caffeine (n = 5), which causes emptying of the SR. The notched decay of ICaL was therefore a direct consequence of Ca2+ released from the SR.

The notched and multiphasic decay of ICaL were more prominent at negative voltages (Fig. 1A). To further characterize this relationship, we evaluated the CICR-sensitive part of the current by subtracting control currents from those recorded after the application of 100 μM ryanodine (Fig. 4A). Interestingly, the form of these difference currents exhibited kinetics similar to estimated SR Ca2+ release fluxes from groups of RyR (36). The mean τ (time constant) for relaxation of the SR-sensitive current was 14 ± 1 ms (single exponential fitting, n = 6). The amplitude of the
SR-sensitive current decreased with depolarization (Fig. 4).

The dependence of multiphasic decay of $I_{Ca,L}$ on intracellular Ca$^{2+}$ and CICR could arise in one of two ways. First, the increase in intracellular Ca$^{2+}$ could activate a Ca$^{2+}$-dependent Cl$^{-}$ current [$I_{Cl(Ca)}$], which would distort the recording of $I_{Ca,L}$ and provoke an apparently complex decay resulting from the superposition of two different currents. Second, the increase in intracellular Ca$^{2+}$ could provoke the decay of $I_{Ca,L}$ via Ca$^{2+}$-induced inactivation, which would therefore represent a rapid and transient process that could be reversible on a time scale of tens of milliseconds.

AC a2$^{-}$-activated Cl$^{-}$ current has been characterized in rabbit, dog, and mouse cardiac cells (41–44). In our experimental conditions with the Na$^{+}$-H$^{+}$-K$^{+}$-free extracellular solution and the Cs$^{+}$-based intracellular solution described in Table 1, the equilibrium potential for chloride ($E_{Cl}$) would be about −2 mV. In these circumstances a Cl$^{-}$ current would appear as an inward current at negative membrane potentials and as an outward current at positive membrane potentials. The ryanodine-difference currents shown in Fig. 4 did not reverse direction at or near to 0 mV. They were outwardly directed at all voltages with no signs of reversing; instead, they approached zero asymptotically with depolarization. If the influx of Ca$^{2+}$ via $I_{Ca,L}$ and CICR were to have evoked $I_{Cl(Ca)}$, this would have appeared during voltage steps to negative membrane potentials as a reduction of the decay of $I_{Ca,L}$, which would then be accelerated when ryanodine blocked CICR. In the recordings shown in Fig. 3A, the opposite

Fig. 2. Multiphasic decay of $I_{Ca,L}$ is not due to contamination by another current. To clearly illustrate multiphasic decay of $I_{Ca,L}$, currents were evoked by voltage steps to −20 mV. A: effects of 40 μM Ni$^{2+}$ on Ca$^{2+}$ current. Currents recorded from 1 myocyte in the presence and absence of Ni$^{2+}$ are superimposed. Cell capacitance was 120 pF. B: effects of 2 mM 4-aminopyridine (4-AP) on Ca$^{2+}$ current. Currents recorded from 1 myocyte in the presence and absence of 4-AP were superimposed. Cell capacitance was 125 pF. C: effects of 100 μM nifedipine and 50 μM Ni$^{2+}$ on Ca$^{2+}$ current. Currents recorded from 1 myocyte in the presence and absence of nifedipine and Ni$^{2+}$ are superimposed. Cell capacitance was 110 pF. D: effects of substituting 1.8 mM external Ca$^{2+}$ with 1.8 mM external Ba$^{2+}$. Currents were normalized to the peak current amplitude and superimposed to emphasize the reduction of inactivation in Ba$^{2+}$. Cell capacitance was 125 pF. E: effects of using BAPTA instead of EGTA in the pipette solution. Cell capacitance was 67 pF.

Fig. 3. Notched decay of $I_{Ca,L}$ was due to Ca$^{2+}$ released from the sarcoplasmic reticulum (SR). A: effects of acute application of 100 μM ryanodine. Ca$^{2+}$ currents were elicited by steps to the voltages indicated to the right of the traces. Currents recorded from 1 myocyte in the presence and absence of ryanodine have been superimposed. B: availability curves of currents obtained in control conditions (■) and in the presence of ryanodine (○) (n = 6).
was true. Sipido et al. (34) also rejected contamination of the recording of $I_{\text{CaL}}$ by $I_{\text{Cl(Ca)}}$ in guinea pig ventricular myocytes. We conclude that multiphasic or notched decay of $I_{\text{CaL}}$ resulted from Ca\(^{2+}\)/H\(^{11001}\)-induced inactivation of the current that was provoked by CICR.

Modulation of $I_{\text{CaL}}$ inactivation by a phosphorylation pathway. The next question that we addressed was why, irrespective of identical basal experimental conditions with Na\(^{-}/\)K\(^{-}/\)H\(^{11001}\)-free external and Cs\(^{-}/\)H\(^{11001}\)-rich internal solutions, some myocytes showed an enlarged $I_{\text{CaL}}$ amplitude, multiphasic $I_{\text{CaL}}$ decay, and prepulse diastolic facilitation, whereas others showed apparently normal control $I_{\text{CaL}}$? Ca\(^{2+}\) cycling in cardiac cells is known to be very sensitive to phosphorylation, and Barrere-Lemaire et al. (3) showed that the diastolic depolarization-induced facilitation is enhanced by cAMP-dependent phosphorylation. We therefore postulated that the PKA pathway might be active in basal conditions in some cells in the absence of external agonist stimulation. To test this, we superfused cells that showed notched decay of $I_{\text{CaL}}$ with a solution that contained 5\(^{-}/\)H\(^{9262}\)M of the PKA inhibitor H-89. Figure 5A shows that this reduced current amplitude, slowed decay (Fig. 5A, inset), and abolished prepulse voltage-dependent facilitation (Fig. 5B, n = 7). To confirm these results, we used a more specific PKA inhibitor, the PKI peptide. This peptide was included in the pipette solution, and a representative result is presented in Fig. 5C. The amplitude of $I_{\text{CaL}}$ gradually decreased with time, and this was associated with the disappearance of the notch (n = 3). Interestingly, the effect on the inactivation phase was more marked with PKI than H-89. This result could be explained by the fact that phosphorylation of PLB may be less sensitive to the effect of H-89 (15). To check that this decrease of $I_{\text{CaL}}$ over time is not due to rundown, we performed the same experiments without PKI in the pipette solution, Fig. 4. Voltage dependence of the fraction of $I_{\text{CaL}}$ sensitive to inactivation by Ca\(^{2+}\) released from the SR. A: difference currents were obtained by subtracting currents that had been recorded under control conditions from those recorded during the acute application of ryanodine in a representative cell. See text for further details. The voltage at which each pair of currents was recorded is indicated to the right of the traces. Inset: absolute value of currents (horizontal bar, 25 ms; vertical bar, 2 pA/pF). B: relationship between the amplitude of the difference current and voltage (n = 6). Difference current amplitude was normalized to that measured at −20 mV.

Fig. 5. Complex decay of $I_{\text{CaL}}$ in Cs\(^{-}/\)loaded cells was due to activation of the PKA pathway. A: effect of 5 \(\mu\)M H-89, a PKA inhibitor, on cell currents evoked by a voltage step to −20 mV. Cell currents recorded from 1 myocyte in the presence and absence of H-89 have been superimposed. Arrow at left indicates the amplitude of the current during the application of H-89. Inset: currents were normalized to their peak amplitude to show that H-89 induced a slowing down of the decay of $I_{\text{CaL}}$. B: availability curves recorded in control conditions (●) and in the presence of H-89 (○) (n = 7). C: effect of inclusion of 200 nM PKA peptide inhibitor (PKI) in the pipette solution on cell currents evoked by a voltage step to −20 mV. Cell current was normalized to the value just after patch rupture, and the original traces from the time course are shown at top (a–d). Note the decrease in the amplitude of $I_{\text{CaL}}$ and the disappearance of the notch. Data points with error bars in the time course plot are from 7 cells recorded without PKI and show that the PKI effect is not due to “rundown.”
responses of cells with these different intracellular currents recorded with Cs
loaded with NMDG-rich intracellular solution (Fig.
notched or multiphasic decay when the myocytes were
(Fig.
activities of I_{CaL} consecutive to activation of the PKA pathway, we investigated the effects of isoproterenol on I_{CaL} in more physiological conditions with a Na+-rich extracellular solution and a K+-rich intracellular solution (Table 1). Figure 7A shows Ca^{2+} currents elicited by voltage steps to −40, −20, and 0 mV in a representative cell. In control conditions the decay of the current was always simple (Fig. 7Aa). The application of isoproterenol induced a large increase of Ca^{2+} current amplitude (Fig. 7B) and notched or multiphasic decay (Fig. 7Bb). As found in the previous experiments with Na+-K+ free extracellular and Cs+-rich intracellular solutions (Fig. 3), multiphasic decay of I_{CaL} in cells with a K+-rich intracellular solution was due to Ca^{2+} released from the SR, because the addition of ryanodine to the external solution slowed inactivation, removed multiphasic decay (Fig. 7Ac), and further inhibited the role of the PKA pathway.

Figure 6. Effect of β-adrenergic stimulation. Superimposed cell currents were evoked by voltage steps to −10 mV in control conditions (C) and after the application of 1 μM isoproterenol (I). A: a cell recorded with a Cs+-rich pipette solution. Cell capacitance was 89 pF. B: a cell recorded with an N-methyl-D-glucamine (NMDG)-rich pipette solution. Cell capacitance was 125 pF. C: influence of intracellular solutions on I_{CaL} evoked at 0 mV. Data are means ± SE from cell currents recorded with Cs+-rich solutions that showed notched decay (n = 11, Cs+ with notch) and those that showed normal decay (n = 14, Cs+ w/o notch) of I_{CaL}. NMDG indicates cell currents recorded with NMDG-rich intracellular solution (n = 6). Open bars indicate control conditions; filled bars indicate application of 1 μM isoproterenol. *P < 0.05, paired values for control vs. isoproterenol. #P < 0.05 vs. Cs+ with notch.

Table 2. Amplitude of I_{CaL} elicited with Cs+- or NMDG-based pipette solutions

<table>
<thead>
<tr>
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<th>Cs+ With Notch</th>
<th>Cs+ Without Notch</th>
<th>NMDG</th>
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<tbody>
<tr>
<td>Current amplitude, pA/pF</td>
<td>11.2 ± 0.6</td>
<td>5.0 ± 0.4*</td>
<td>6.7 ± 0.9*</td>
</tr>
<tr>
<td>n</td>
<td>65</td>
<td>25</td>
<td>13</td>
</tr>
</tbody>
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Amplitude of L-type Ca^{2+} current (I_{CaL}) elicited at 0 mV from a holding potential of −80 mV with Cs+- or NMDG-based pipette solutions was measured (means ± SE); n = no. of cells studied. With Cs+- pipette solutions, I_{CaL} displayed 2 kinds of behavior, either the presence or absence of notched or multiphasic decay. *P < 0.05 vs. Cs+ with notch.

We conclude from these experiments that the PKA-dependent phosphorylation pathway was active in basal conditions, leading to phosphorylation of the L-type Ca^{2+} channel and/or of the RyR and/or PLB.

Influence of the intracellular medium on inactivation of I_{CaL}. The next series of experiments was designed to elucidate the mechanism responsible for the apparently high level of phosphorylation in our basal experimental conditions. Vargas et al. (39) showed that Cs+ increased PKA activity in vitro. We first compared results from cells in which N-methyl-D-glucamine (NMDG) rather than Cs+ replaced intracellular K+ (Table 1). In basal conditions I_{CaL} never showed notched or multiphasic decay when the myocytes were loaded with NMDG-rich intracellular solution (Fig. 6B). Table 2 shows that under basal conditions, I_{CaL} in Cs+-loaded cells with notched decay was significantly larger than in those without notched decay, whether they were loaded with Cs+ or NMDG (P < 0.05). The responses of cells with these different intracellular media to β-adrenergic stimulation were tested. Figure 6A shows a Ca^{2+} current elicited at −10 mV in a cell with Cs+-based pipette solution. This cell clearly showed a notched decay under basal conditions, and the addition of 1 μM isoproterenol had only a weak effect on I_{CaL} amplitude. In 11 similar cells, the amplitude of the current was increased significantly by 55 ± 18% by addition of 1 μM isoproterenol. Figure 6B shows a Ca^{2+} current evoked in a cell with an NMDG-rich pipette solution. In basal conditions the current did not show notched decay, and the amplitude of the current was strongly increased by addition of 1 μM isoproterenol (168 ± 23%, n = 6, P < 0.05 compared with notch I_{CaL}). The response of I_{CaL} to isoproterenol in cells with either Cs+- or NMDG-rich intracellular solutions is summarized in Fig. 6C. Cells with multiphasic inactivation of I_{CaL}, although larger under control conditions than in their counterparts with simple decay of I_{CaL}, responded poorly to isoproterenol. Interestingly, cells with monophasic inactivation of I_{CaL} responded to isoproterenol stimulation with an increase in I_{CaL} amplitude of 207 ± 36% (n = 14). As found with NMDG-rich pipette solution, after isoproterenol stimulation I_{CaL} inactivation showed a multiphasic inactivation. To confirm the role of the PKA pathway, we used forskolin to directly stimulate the adenylyl cyclase. We found that the amplitude of I_{CaL} showing multiphasic inactivation increased by only 58 ± 8% (n = 6) with forskolin, whereas in cells presenting I_{CaL} with monophasic decay, I_{CaL} increased by 178 ± 18% (n = 15; data not shown). Forskolin also induced the appearance of the notch during the inactivation phase (n = 14 of 15 cells).

To further investigate the incidence of complex decay of I_{CaL} consecutive to activation of the PKA pathway, we investigated the effects of isoproterenol on I_{CaL} in more physiological conditions with a Na+-rich extracellular solution and a K+-rich intracellular solution (Table 1). Figure 7A shows Ca^{2+} currents elicited by voltage steps to −40, −20, and 0 mV in a representative cell. In control conditions the decay of the current was always simple (Fig. 7Aa). The application of isoproterenol induced a large increase of Ca^{2+} current amplitude (Fig. 7B) and notched or multiphasic decay (Fig. 7Bb). As found in the previous experiments with Na+-K+ free extracellular and Cs+-rich intracellular solutions (Fig. 3), multiphasic decay of I_{CaL} in cells with a K+-rich intracellular solution was due to Ca^{2+} released from the SR, because the addition of ryanodine to the external solution slowed inactivation, removed multiphasic decay (Fig. 7Ac), and further inhibited the role of the PKA pathway.
increased current amplitude (Fig. 7B). In basal conditions, ryanodine had no effect on current amplitude (Fig. 7B).

**DISCUSSION**

The objective of this study was to elucidate the mechanism that underlies multiphasic inactivation of ICaL, recorded in our experimental conditions. The major findings were that in the whole cell configuration of the ruptured patch-clamp technique, Cs⁺ was able to activate the PKA pathway, leading to the phosphorylation of targets such as DHPr and/or RyR and/or PLB that enhanced fast and rapidly reversible inactivation of ICaL by Ca²⁺ released by the SR.

**Intracellular Cs⁺ and excitation-contraction studies.** Most of the observations here correspond to a particular, but widely used, experimental condition where ICaL is isolated by replacing intracellular K⁺ by Cs⁺ (1, 2, 26). Recently, however, Cs⁺ has been shown to significantly increase the activity of the catalytic subunit of PKA (39). Though our experiments did not directly measure PKA activity, we recorded the activity of one of the effectors of this pathway, the L-type Ca²⁺ current. In the majority of cells infused with a Cs⁺-rich pipette solution under otherwise basal conditions, the application of PKA inhibitors (H-89, PKI) reduced current amplitude and slowed inactivation, causing the disappearance of notched or multiphasic decay. Also, in these cells isoproterenol had only a small effect on the amplitude of ICaL. It was as if the current had already been activated by this signaling pathway, which concords with its greater amplitude under basal conditions. Evidence that these results are due to Cs⁺ and not to the replacement of intracellular K⁺ was shown when cells were infused with NMDG-rich pipette solution, where ICaL was smaller, with normal current decay under basal conditions and greater responsiveness to isoproterenol. Similar results were obtained when quasi-physiological solutions were used in the recording of ICaL. Together, these results strongly suggest that intracellular Cs⁺ activates the PKA pathway.

Furthermore, it has been proposed that intracellular Cs⁺ blocks K⁺ channels present on the SR membrane and thereby alters Ca²⁺-cycling properties of cardiac cells (17, 22). Our finding and these findings indicate that care must be taken when Cs⁺ is used instead of K⁺ in the intracellular solution to study excitation-contraction (EC) coupling in cardiac myocytes.

**Fast and reversible Ca²⁺-dependent inactivation of ICaL.** Another result of this study was that in conditions where the PKA pathway was activated, the local release of Ca²⁺ by the SR (32) was able to rapidly and reversibly inactivate ICaL, which was responsible for the appearance of either multiphasic or notched decay of ICaL. The increase in the initial phase of decay was probably due to an increased Ca²⁺ concentration underneath the membrane after enhanced SR Ca²⁺ release. The recovery of ICaL during the maintained voltage step that gave rise to multiphasic decay was probably due to the reduction of Ca²⁺ concentration in the subsarcolemmal microdomain by diffusion and uptake by the SR. The channel then came back from the Ca²⁺-inactivated state to the open state. The Ca²⁺ uptake occurs via the SERCA, which must belong to the microdomain. Therefore, DHPr, RyR, and SERCA belong to the same Ca²⁺ microdomain, not influenced by the presence of the Ca²⁺ chelator EGTA. Calcium microdomains are known to exist in the rat ventricular cell, but our observations are interesting because the guinea pig myocyte bears more similarity to the human cardiac myocyte (5). With BAPTA, the notch was never observed, suggesting that the accessibility of EGTA is not steric but, more likely, limited to the speed necessary to chelate Ca²⁺ (25). Further studies are required to determine which of the key proteins was phosphorylated by Cs⁺ activation of PKA: the DHPr, the RyR, or the regulatory protein PLB (6).

If reversible inactivation were directly linked to the local control of Ca²⁺ release, this would assume a local release of Ca²⁺ from the SR that was tightly synchronized to the peak ICaL, with a duration of the local SR Ca²⁺ release event between 7 and 30 ms (5, 36). The fact that notched or multiphasic decay was more prominent at negative membrane potentials agrees with the gain of CICR (40), which increases with the level of phosphorylation (12). Thus these experiments provide new evidence that fast negative feedback from the release of Ca²⁺ from the SR to ICaL via Ca²⁺-dependent inactivation occurs proportionally to the amplitude of the triggering signal. In this manner, the increase of ICaL upon activation of the PKA phosphorylation pathway would permit the fast and reversible control of EC coupling. This mechanism would play an important role in the regulation of SR Ca²⁺ release, leading to the appearance of notched or multiphasic decay.
role during β-adrenergic stimulation of the heart. Finally, it is important to note that those cells that lacked complex inactivation of \( I_{\text{Ca,L}} \) under basal conditions acquired it upon the application of isoproterenol. This corroborates the hypothesis that fast inactivation of \( I_{\text{Ca,L}} \) mediated by Ca\(^{2+} \) release from the SR requires PKA-dependent phosphorylation.

**Low diastolic depolarization facilitation.** Diastolic potential-induced facilitation of the \( I_{\text{Ca,L}} \) was only observed under basal conditions in this study when Cs\(^+ \) was used to substitute for K\(^+ \), Barrere-Lemaire et al. (3), who also used Cs\(^+ \) in their pipette solution, suggested that this phenomenon was linked to CICR Ca\(^{2+} \) release from the SR. In the great majority of the studies in which investigators missed this point by using a depolarizing holding potential, either a release of Ca\(^{2+} \) during the subsequent test pulse, or the channel change to a Ca\(^{2+} \)-insensitive conformation. Whatever the mechanism, it required the PKA phosphorylation of the L-type Ca\(^{2+} \) channel and/or the RyR and/or PLB. It is interesting to note that similar properties have been described for frequency-dependent facilitation, e.g., increase in the amplitude and slowing of the inactivation phase of \( I_{\text{Ca,L}} \) (11).

Some questions remain to be elucidated. Why would only certain cardiomyocytes, under basal conditions, show fast inactivation upregulated by the PKA phosphorylation pathway? Such a question probably finds an answer in the regulatory mechanism proposed by DuBell et al. (12), who suggested that EC coupling, including \( I_{\text{Ca,L}} \), was a dynamic balance between phosphorylation and dephosphorylation. The two kinds of cells described here could therefore correspond to a difference in the equilibrium of such a dynamic process, exacerbated by the presence of Cs\(^+ \) in the internal medium. Populations of cells with monophasic inactivation of \( I_{\text{Ca,L}} \) could have a higher basal level of phosphatase activity, as indicated by the lower effect of isoproterenol and forskolin stimulation.

Why have other studies not reported this notched inactivation of \( I_{\text{Ca,L}} \) in the same species? Some investigators used NMDG-rich intracellular solution (e.g., see Ref. 35). In the great majority of the studies in which Cs\(^+ \) was used in the pipette solution, it is likely that investigators missed this point by using a depolarizing prepulse or holding potential (−40 mV) to inactivate \( I_{\text{Na}} \) and \( I_{\text{CaT}} \) (e.g., see Refs. 4, 27, and 31). It is known that a nonphysiological depolarized holding potential interferes with the functioning of the L-type Ca\(^{2+} \) channels, inducing a slowing of the inactivation phase of \( I_{\text{Ca,L}} \) (3, 38). Finally, many investigators have used depolarized test pulses of 0 or +10 mV at which the notch is less pronounced (Fig. 1B).

In conclusion, we have shown that intracellular Cs\(^+ \) is able to activate the PKA pathway, modifying in consequence the activity of the targets of this kinase. We have shown that PKA activation enhances Ca\(^{2+} \)-dependent inactivation of \( I_{\text{Ca,L}} \), which then closely follows the kinetics of localized variation in [Ca\(^{2+} \)]. We have shown that diastolic potential-induced facilitation of \( I_{\text{Ca,L}} \) is a phenomenon dependent on Ca\(^{2+} \)-dependent inactivation, which also requires activation of the PKA pathway.

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