ON THE NATURE OF THE L-TYPE Ca^{2+} CURRENT INACTIVATION IN RAT VENTRICULAR CARDIOMYOCYTES.

ACERCA DE LA NATURALEZA DE LA INACTIVACION DE LA CORRIENTE DE Ca^{2+} TIPO L EN CARDIOMIOCITOS VENTRICULARES DE RATA

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Facilitation of cardiac L-type Ca^{2+} current (I_{CaL}) by high stimulation rates or by depolarizing prepulses is characterized by an increase in the fast inactivation time constant (τ_{fast}) of I_{CaL}. To explain this phenomenon, mechanisms related to calcium-dependent inactivation (CDI) have been considered. Here we studied in more detail the mechanism of I_{CaL} facilitation by depolarizing prepulses using isolated rat ventricular cardiomyocytes. Increases in τ_{fast} at a test pulse to 0 mV by low voltage (not activating I_{CaL}) prepulses were associated with hyperpolarizing shifts in I_{CaL} kinetics. Experimental conditions aimed to decrease CDI, by blocking the Ca^{2+} release from or its reload to the sarcoplasmic reticulum, did not abolish the increase in τ_{fast} by prepulses. Our results suggest that this kind of I_{CaL} facilitation is also associated to a strong voltage-dependent mechanism. A model is proposed in which depolarizing prepulses allow the Ca^{2+} channel to dwell longer times in the open state.

La facilitación de la corriente de Ca^{2+} tipo L cardíaca (I_{CaL}) por alta frecuencia de estimulación o prepulsos despolarizantes (PP), consiste en un aumento en la constante de tiempo de inactivación rápida (τ_{fast}) de I_{CaL}. Para explicar este fenómeno se han considerado mecanismos relacionados a la inactivación dependiente de calcio (IDC). Nosotros estudiamos más detalladamente la facilitación de I_{CaL} por PP utilizando cardiomiocitos ventriculares de rata. El incremento en τ_{fast} en un pulso test a 0 mV por PP que no activan I_{CaL} se asoció a un desplazamiento hiperpolarizante de las cinéticas de I_{CaL}. Diferentes condiciones experimentales en las que la IDC estaba deprimida, por bloqueo de la liberación de Ca^{2+} desde o su recaptación hacia el retículo sarcoplasmático, no previnieron el incremento en τ_{fast} por PP. Nuestros resultados sugieren que este tipo de facilitación de I_{CaL} está también asociado a un mecanismo dependiente de voltaje. Se propone un modelo en el que los PP permiten al canal de Ca^{2+} estar más tiempo en el estado abierto.

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I INTRODUCTION

The L-type Ca^{2+} channels in the sarcolemma of cardiomyocytes provide the main influx pathway for Ca^{2+} which is an essential intracellular messenger that plays pivotal roles in many processes from electrogenicity to excitation-contraction coupling, as well as biochemical and gene regulation [1, 2]. This pore-forming protein, encoded by the Ca_V 1.2 gene (CACNA1C or α_{1C}), undergoes conformation changes upon membrane depolarization and, due to the huge electrochemical gradient for Ca^{2+} ion across the sarcolemma (~20,000 fold concentration gradient and a negative resting membrane potential), gives rise to a voltage- and time-dependent gated calcium current (I_{CaL}), which quickly turns on (activation) to reach a peak, then slowly decays (inactivation) and returns to rest (deactivation) upon repolarization.

One of the most complex and intriguing phenomena of the biophysical properties of the L-type Ca^{2+} channel is the inactivation process, i.e. the decay of I_{CaL} during depolarization. I_{CaL} is inactivated by two mechanisms: a voltage-dependent inactivation (VDI) and a calcium-dependent inactivation (CDI) mechanisms. The first mechanism is common to many voltage-gated ion channels and was first described in the pioneering work of Hodgkin and Huxley on the sodium channel of the squid giant axon [3]. The second mechanism is exclusive of L-type Ca^{2+} channels. The molecular determinants of VDI are the cytosolic ends of the S6 segments (the I-II linker, considered to be the inactivation gate); the NH_2 and COOH termini of $Ca_V1.2$ are also involved in this process [2, 4, 5]. As well, the auxiliary β-subunit of the Ca^{2+} channel plays a role in VDI (see references in [2]). The CDI relies first, on local Ca^{2+} that enters via the L-type Ca^{2+} channels to the restricted subsarcolemmal space and second, on Ca^{2+} released from the sarcoplasmic reticulum [6, 7]. Ca^{2+} binds to calmodulin (CaM) tethered to an IQ domain in the C-terminus of the channel [8] causing a conformational change in the channel that prevents the EF-hand in the C-terminus from interacting with the cytosolic I-II linker, which then occludes the channel pore, thus accelerating inactivation [9]. A second CaM-interacting domain in the N-terminal domain of $Ca_V1.2$ channels can also mediate CDI in response to local rather than global Ca^{2+} [10, 11]. Based on the conserved regulation of both VDI and CDI by the auxiliary β-subunit, CDI has been envisioned as a Ca^{2+}-dependent brake for a pre-existing VDI [9].

The time course of I_{CaL} inactivation has been usually fitted to two exponentials and the fast and slow time constants have been usually "assigned" to CDI and VDI, respectively *e.g.* [12, 13]. Analyses of the relative contribution of CDI

and VDI to I_{CaL} inactivation have been based on this and are still under dispute. It has been suggested that the relative contribution of CDI to total inactivation of I_{CaL} is greater at negative potentials when VDI (which typically exhibits a U-shaped availability curve), is weak [6, 14, 15]. Under extreme non-physiological conditions, it has been suggested that VDI has a central role but, after β-adrenergic stimulation, CDI becomes the main inactivation mechanism due to a slow-down of VDI [14]. Nevertheless, using more physiological conditions, we have shown that the fast inactivation time constant of I_{CaL} of rat ventricular cardiomyocytes was slowed down after I_{CaL} was increased by β-adrenergic stimulation, as well as after manipulations not involving CDI [16, 17].

One striking property of L-type Ca^{2+} channels is that under certain conditions, voltage clamp steps after a rest period or prepulses before a test pulse can facilitate I_{CaL}, *i.e.* its fast inactivation phase is markedly slowed-down and even its amplitude could be slightly increased *e.g.* [13, 18]. However, although both CDI and VDI are involved in I_{CaL} inactivation, only mechanisms related to CDI (and CaM-CaM kinase II) have been considered to explain this facilitation. The presented arguments link the increase in the fast time constant of inactivation of I_{CaL} to a weakened CDI due to a decrease in sarcoplasmic reticulum Ca^{2+} load [13, 18]; see [19] for a recent review]. Taking into account that, as previously described, the fast inactivation phase of I_{CaL} could be slowed down after manipulations not involving CDI, the aim of this study was to further investigate whether the prepulse-induced facilitation of I_{CaL} in rat ventricular cardiomyocytes is only dependent of CDI-related mechanisms or has a voltage-dependent component.

II METHODS

Experiments were performed using male adult Wistar rats (180-200 g), heparinized (5000 UI/100 g weight) and anaesthetized with sodium thiopental (30 mg/100 g weight). Hearts were quickly removed and placed in cold Ca^{2+}-free Tyrode solution (~10°C). The enzymatic dissociation protocol to obtain single ventricular cardiomyocytes has been described elsewhere [20]. Myocytes thus obtained were kept at room temperature (25°C) and used for experiments for 6-8 hours.

L-type Ca^{2+} current (I_{CaL}) was recorded using the whole cell patch-clamp technique [21] standardized in our laboratory. Liquid junction potential was compensated before establishing the gigaseal. No capacitance or leakage compensations were performed. The sampling interval was 50 μs and the recording bandwidth was 3 KHz. The sodium current was blocked with tetrodotoxin (TTX, 50 μM; Alomone, Israel) and potassium currents were blocked by substituting all potassium by cesium in extra- and intracellular solutions (see below). Pipette resistance was 1.0 - 1.2 MΩ once filled with the intracellular solution. Membrane capacitance (C_M) and series resistance (R_S) were calculated on voltage-clamped cardiomyocytes as previously reported [22].

Current data of each cell was normalized to cell capacitance (current density in pA/pF). Average C_M was 176.8 ± 9.5 pF (N = 45). The uncompensated R_S was 4.2 ± 0.3 MΩ. R_S could be electronically compensated up to 50% without ringing.

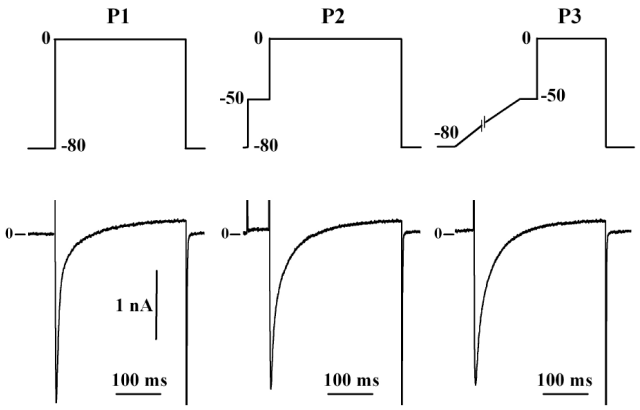

Figure 1. Voltage-clamp protocols. Top: Schemes of the three voltage-clamp protocols used in the present experiments. In all protocols the holding potential was -80 mV. P1: Is the "control" voltage-clamp protocol. From the holding potential, the cardiomyocytes were clamped to 0 mV for 300 ms. In P2, a 50-ms prepulse to -50 mV was applied before clamping the membrane to 0 mV. P3 consisted of a 500-ms ramp from -80 to -50 mV; a 50-ms step to -50 mV and then a 300-ms step to 0 mV. All protocols were applied at 0.25 Hz. Bottom: I_{CaL} currents, activated by the corresponding protocols, in a single cardiomyocyte. Note the significant increase in inactivation time course of I_{CaL} with the prepulses, specially with the P3 protocol.

I_{CaL} was routinely monitored (protocol P1; Figure 1) at 0 mV using 300-ms pulses from a holding potential (HP) of-80 mV and measured as the difference between peak inward current and the current level at the end of the 300-ms pulse. To study the effects of prepulses on I_{CaL} inactivation time course we used two different prepulse protocols to evoke I_{CaL} and compared the results with the routinely applied "P1" protocol already described. With the first prepulse protocol (protocol "P2"; Figure 1), the cell was clamped at a HP of -80 mV, depolarized to -50 mV for 50 ms and then to 0 mV for 300 ms. The second prepulse protocol ("P3"; Figure 1) consisted of a 500-ms ramp from the -80 mV HP to -50 mV, a 100-ms step at this voltage and then a 300-ms step to 0 mV. The three protocols were applied at 0.25 Hz. Values of I_{CaL} density and fast and slow components of I_{CaL} inactivation (τ_{fast} and τ_{slow}, respectively) were obtained after at least ten pulses in each case. Current-to-voltage (I-V) and availability curves were constructed using standard voltage-clamp protocols [22]. Potentials for half availability ($V_{0.5}$) and the corresponding slope factors (s) were obtained after fitting the experimental data to a Boltzmann function:

$$f_\infty = (1 + \exp[(V_m - V_{0.5})])^{-1}, \quad (1)$$

using the routines of SciDAVis (GNU/Linux version 0.2.4). In order to estimate how the Ca^{2+} influx into the cell increased with time during a voltage-clamp pulse, I_{CaL} tracings were integrated without taking into account the capacitive transients. This gave us the amount of charge (in pC) entering the cell during the flow of I_{CaL} and was

normalized to C_M. All recordings were done at room temperature (25°C).

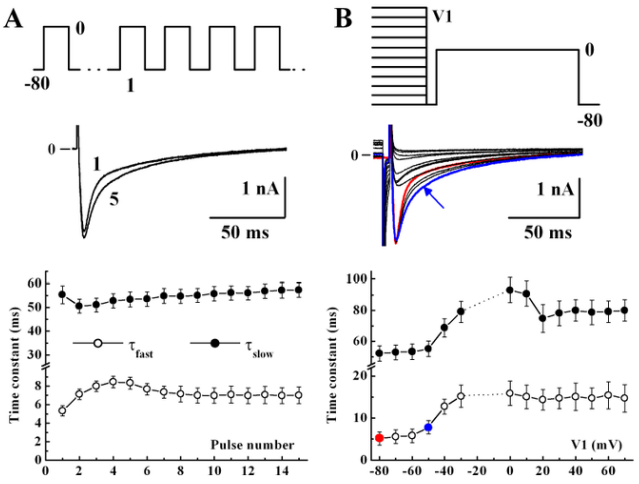

Figure 2. Changes in inactivation time constants of I_{CaL} using different voltage-clamp protocols. A: Pulse-dependent facilitation of I_{CaL}. Top: Scheme of voltage-clamp protocol. The cardiomyocyte was clamped at a holding potential of -80 mV and I_{CaL} was evoked with 300-ms voltage-clamp pulses to 0 mV applied at 0.25 Hz. Stimulation was stopped for 1 min and then restarted at 1 Hz with the same voltage-clamp pulses. Middle: The first and fifth I_{CaL} traces after re-initiation of stimulation are superimposed showing a clear delay in inactivation time course. Bottom: Fast (τ_{fast}) and slow (τ_{slow}) time constants of I_{CaL} inactivation plotted as a function of pulse number after re-initiation of stimulation. Note that while τ_{slow} is barely affected, τ_{fast} is significantly increased between 2nd and 6th pulses over both its value at the first pulse and its steady state value. B: Changes in inactivation time constants of I_{CaL} by prepulses. Top: Scheme of voltage-clamp protocol. The cardiomyocyte was clamped at a holding potential of -80 mV and voltage-clamped with a double pulse (300 ms) voltage protocol. The first pulse (V1) was increased (in 10 mV steps) from -80 to +70 mV and after a 5 ms step at the holding potential, the membrane was clamped to a fixed potential of 0 mV (300ms). Double pulses were applied at 0.125 Hz. Middle: Superimposed I_{CaL} traces recorded at the fixed pulse to 0 mV after the prepulses. I_{CaL} without prepulse and I_{CaL} after a prepulse to -50 mV are marked in red and blue, respectively. Bottom: τ_{fast} and τ_{slow} plotted as a function of prepulse potential (V1). Note that prepulses at -50 and -40 mV which do not activate I_{CaL} induced a marked increase in τ_{fast} .

In order to estimate the number of functional channels (N_f), nonstationary fluctuation analysis of Ca^{2+} current was implemented according to Sigworth [23]. Since I_{CaL} usually shows some rundown (decrease) during the experiment [24] we were forced to select myocytes in which at least 10 consecutive I_{CaL} tracings could be recorded without rundown. The maximal background current variance (σ_B^2) due to a resistor (membrane resistance) was estimated according to:

$$\sigma_B^2 = 4kTB/R_M, \quad (2)$$

where k is the Boltzmann constant, T the temperature, B the bandwidth and R_M the membrane resistance. In our analysis, the maximal background current variance σ_B^2, was 1.21 x 10^{-24} A^2 (σ ~1 pA) taking into account that T = 25°C, B = 3000 Hz and R_M, estimated from the chord conductance at 0 mV, was not less than 50 MΩ. σ_B^2 was subtracted from measurements before fitting.

The extracellular solution contained (mM): 117 NaCl, 20 CsCl, 10 HEPES, 2 $CaCl_2$, 1.8 $MgCl_2$ and 10 glucose (pH = 7.4). The standard pipette (intracellular) solution contained (mM): 130 CsCl, 0.4 Na_2GTP, 5 Na_2ATP, 5 Na_2-creatine phosphate, 11 EGTA, 4.7 $CaCl_2$ (free Ca^{2+} ~120 nM) and 10 HEPES, with pH adjusted to 7.2 with CsOH. In the experiments cells were first let to lie in Petri dishes filled with K^+-Tyrode solution with 1 mM Ca^{2+}. Cells attached to the micropipette could be positioned on the extremity of each of six microcapillaries (i.d. 250 μm) through which the different extracellular Cs^+-containing solutions were perfused by gravity (~15 μL/min) allowing rapid changes (~1 s) of the extracellular medium. Except TTX, all chemicals were purchased from Sigma-Aldrich Chemical Co (USA).

Results were analyzed using the statistical routines of Gnumeric Spreadsheet for GNU/Linux (Gnome Project, version 1.10.17) and are expressed as means and standard errors of means. Statistical significance was evaluated by means of paired or unpaired Students t test according to the experimental situation. Differences were considered statistically significant for $p < 0.05$.

III RESULTS

In our experimental control conditions, I_{CaL} had a current density of 11.8 ± 0.3 pA/pF and its inactivation time course could be well-fitted to a double exponential with time constants τ_{fast} of 5.1 ± 0.2 ms and τ_{slow} of 55.7 ± 2.2 ms (see also Table 1). The amplitude of fast component comprised 80.6 ± 2.5 % of total I_{CaL} amplitude. Since I_{CaL} facilitation has been related to CDI we first addressed the problem of I_{CaL} inactivation by reproducing two classic experimental protocols in order to demonstrate I_{CaL} facilitation in our experimental conditions (Figure 2 A and B). In the first approach (Figure 2 A), stimulation with protocol P1 (0.25 Hz) was stopped for at least 1 min, it was then reinitiated at a higher rate (1 Hz) and I_{CaL} was recorded until it reached a steady state (~ 10 - 15 pulses). In the second approach (Figure 2 B) I_{CaL} was recorded a stimulation protocol that is commonly used [22, 25] to construct current-to-voltage relationships and availability curves: a fixed pulse to 0 mV was preceded, in 10-mV steps, by prepulses from -80 to +70 mV (0.125 Hz). Figure 2 A clearly shows a frequency-dependent facilitation of I_{CaL} since τ_{fast} was significantly increased at the higher stimulation rate in the first 2 - 6 pulses. However, similar increases in τ_{fast} could be achieved with the second protocol (Figure 2 B). Prepulse potentials at -50 and -40 mV (that did not trigger I_{CaL}) and even prepulses to -30 mV, just at the threshold potential for a tiny I_{CaL}, provoked large increases in τ_{fast}. Contrary to frequency-dependent facilitation, this pulsing protocol also produced large increases in τ_{slow}, however, since previous studies have related τ_{fast} to CDI, we limited our analysis to this fast phase.

We then studied how τ_{fast} could change when prepulses are applied according to the protocols already described in Methods section. In a large number of cells in which the three voltage-clamp protocols could be successively applied,

prepulse protocols P2 and P3 provoked significant increases in τ_{fast} at the fixed test pulse to 0 mV without variations in τ_{slow}. However, the increase in τ_{fast} was significantly greater with the P3 protocol (~43% increase vs ~28% with the P2 protocol). Additionally, the P3 protocol also provoked a small but significant decrease in I_{CaL} density (Table 1). Since neither P2 or P3 protocol produced significant changes in τ_{slow} and the effects of protocol P3 on τ_{fast} were more marked, we focused our subsequent analysis on the effects of this protocol on fast inactivation of I_{CaL}. The increase in τ_{fast} by the ramp protocol P3 was so important that it implied an increase in transported charge of ~30% through the Ca^{2+} channel, from 0.208 ± 0.015 pC/pF to 0.285 ± 0.02 pC/pF ($p < 0.05$), even if peak I_{CaL} density was slightly decreased.

Table 1. Characteristics of I_{CaL} using different voltage-clamp protocols.
* $p < 0.05$ with respect to values obtained with protocol P1. N = 45

Protocol	dI_{Cal} (pA/pF)	τ_{fast} (ms)	τ_{fast} (ms)
P1	11.8 ± 0.3	5.1 ± 0.2	55.7 ± 2.2
P2	11.2 ± 0.4	6.1 ± 0.3*	52.8 ± 2.1
P3	10.1 ± 0.5	6.8* ± 0.5*	56.2 ± 3.2

The next step was to study the behavior of τ_{fast} at different test potentials without and with the ramp prepulse as well as the effects of a ramp prepulse on the availability of I_{CaL}. The results are shown in Figure 3 (A and B) and are suggestive of a leftward shift in the voltage dependency of I_{CaL} by the ramp prepulse. This could be confirmed by the leftward shift of the availability curve ($V_{0.5}$) from -21.1 ± 4.3 mV with protocol P1 (no prepulse) to -27.7 ± 3.1 mV with the protocol P3 (ramp prepulse), but without any change in the slope factor s (6.25 ± 3.8 mV vs 6.3 ± 3.1 mV). We next explored whether the ramp prepulse could increase the number of functional Ca^{2+} channels. Although limited to 6 cells in which at least 10 consecutive traces of I_{CaL} showed no rundown, the non stationary fluctuation analysis of I_{CaL} indicated that in these cardiomyocytes the number of Ca^{2+} channels (N_f) activated during I_{CaL} (at 0 mV) was 4867 ± 249 and 4932 ± 222 without and with the ramp prepulse, respectively (not statistically significant; C_M ranged from 165 to 220 pF). These results suggest that prepulses did not increase the number of functional Ca^{2+} channels.

These evidences, together with our previous results showing that τ_{fast} can be "unexpectedly" slowed down by β-adrenergic stimulation, as well as after manipulations not involving CDI (see Introduction), prompted us to study τ_{fast} under different experimental conditions aimed to modify CDI. Table 2 summarizes the steady state values for I_{CaL} density and τ_{fast} in each experimental condition and the results are as follows. The use of Ba^{2+} instead of Ca^{2+} as charge carrier through Ca_v 1.2 channels is known to significantly decrease CDI since high affinity sites involved in CDI display a much lower affinity for Ba^{2+} and τ_{fast} is then significantly increased. However, even after this large increase in τ_{fast} ramp prepulses were still able to further increase τ_{fast}. Ryanodine (100 μM) is a well-known blocker of the sarcoplasmic reticulum (SR) Ca^{2+} channel, so less Ca^{2+} is released after each voltage-clamp pulse and less Ca^{2+} will be available for CDI mechanisms. This provokes an increase in τ_{fast} which nevertheless could be still increased by the ramp prepulses. Another approach to study this problem is to block the SR Ca^{2+}-ATPase with cyclopiazonic acid (CPA) in an attempt to empty the SR in a pulse-to-pulse manner in order to have less Ca^{2+} from this source available for CDI. After a steady state is reached τ_{fast} is increased but to a lesser extent than with Ba^{2+} or Ryanodine. Again, ramp prepulses were still able to significantly increase τ_{fast}. We then proceeded inversely and in an attempt to increase Ca^{2+} influx and CDI we used the well-known Ca^{2+} channel agonist BayK8644. As expected, BayK8644 markedly increased I_{CaL} density but unexpectedly it showed a tendency to increase τ_{fast}. However, after a ramp prepulse, τ_{fast} was further increased in this experimental condition. Finally, it is well-known that most of the sarcolemmal Ca^{2+} channels lie in the transverse T-tubular system in close association with SR Ca^{2+} channels in the dyads where, due to restricted diffusion, Ca^{2+} concentration can be markedly increased during activation of I_{CaL}. A way to decrease or limit CDI is to break the T-tubules by osmotic shock using formamide leaving intact the Ca^{2+} channels on the surface sarcolemma. As can be seen in Table 2, after formamide treatment I_{CaL} density is markedly decreased and τ_{fast} is significantly increased. In this condition, also, ramp prepulses were able to significantly increase τ_{fast}.

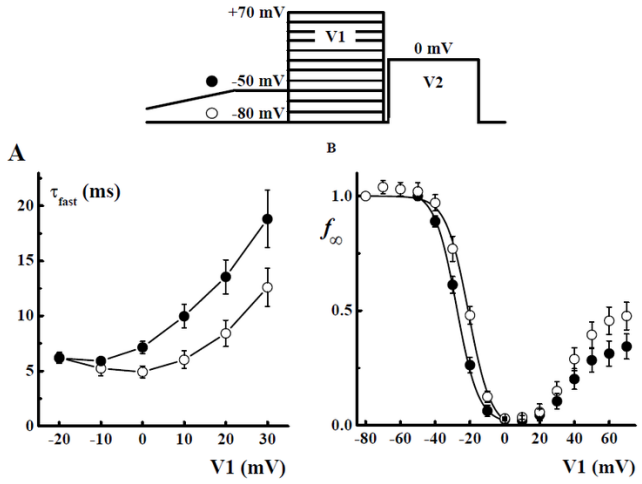

Figure 3. Effects of a ramp prepulse on the inactivation kinetics of I_{CaL}. Top: Scheme of the voltage-clamp protocols used to record I_{CaL}. In a first protocol (○) from a holding potential of -80 mV, 300-ms prepulses (V1) were applied to membrane potentials from -80 to +70 mV in 10 mV steps. After a gap of 5 ms at the holding potential, the membrane was clamped to 0 mV for 300 ms. The second protocol (●) was similar but with a ramp prepulse as defined before. Double pulse rate was 0.125 Hz. A: Voltage-dependency of τ_{fast} of I_{CaL} (at V1). I_{CaL} was recorded at each V1 and its inactivation was fitted to a double exponential. Since, the relationship τ_{slow} vs V1 was not affected by the ramp prepulse, only τ_{fast} is presented. Only recordings between -20 and +30 mV, where I_{CaL} inactivation can be fitted without errors, were used in the analysis. Values were statistically different ($p < 0.05$) between -10 and +30 mV. B: Availability (f_∞) curves of I_{CaL} obtained with the two protocols. f_∞ was obtained by normalizing the I_{CaL} obtained at the fixed V2 (0 mV) after each prepulse (V1) to the maximal (without prepulse) I_{CaL} recorded. Experimental data between -80 and 0 mV were fitted to a Boltzmann function.

Table 2. Effects of a ramp prepulse on I_{CaL} density and fast inactivation time constant (τ_f) under different experimental conditions.

P1 P3	Control	Ba^{2+}	Control	Ryanodine	Control	CPA	Control	BayK8644	Control	Formamide
dI_{CaL} (pA/pF) τ_f (ms)	11.8±0.8 4.8±0.3	14.9±1.1 † 16.0±0.6 †	10.5±0.4 5.0±0.4	12.3±0.3 † 16.2±1.5 **	11.1±0.6 4.7±0.2	9.8±0.8 5.9±0.5 †	12.1±1.3 5.7±1.0	24.5±2.5 † 6.5±0.3	10.5±0.6 4.6±0.2	5.6±0.5 † 8.5±1.7 †
dI_{CaI111} (pA/pF) τ_f (ms)	10.2±0.7 * 6.3±0.5 *	11.9±0.7 *† 21.0±0.6 *†	8.9±0.3 * 6.5±0.3 *	10.9±0.4 *† 20.5±2.0 *†	10.0±0.6 * 6.7±0.6 *	8.2±0.4 * 8.2±0.8 *†	10.1±1.0 * 7.4±1.0 *	18.0±2.2 *† 9.2±1.5 *	9.1±0.5 * 6.5±0.3 *	4.6±0.1 *† 11.0±1.3 *†
N	10	10	10	10	6	6	6	6	5	5

* $p < 0.05$ with respect to values with protocol P1. † $p < 0.05$ with respect to its own control.
Note: To record Ba^{2+} currents, extracellular Ca^{2+} was equimolarly substituted by Ba^{2+} (2 mM). Ryanodine: 100 μM; BayK-8644: 1 μM; CPA: 30 μM (cells were incubated for 30 min). Formamide: cardiomyocytes were incubated in 1.5 M formamide for 15 min. For CPA and formamide, controls refer to non-incubated cardiomyocytes from the same dissociation. Control intrapipette solution contained ~120 nM Ca^{2+}.

IV DISCUSSION

The most important finding of our study is that variations in membrane potential, within the "diastolic" physiological range, slow down the fast phase of I_{CaL} inactivation by a mechanism that is not related to calcium-induced inactivation. This kind of I_{CaL} facilitation by conditioning depolarizations imply an increase in Ca^{2+} entry to the cardiomyocytes and could then contribute to the feedback mechanisms that modulate intracellular Ca^{2+} concentration and signaling. The phenomenon we studied here has been previously described [18] but its underlying mechanism has been considered to be similar to that of the frequency-dependent I_{CaL} facilitation, *i. e.* a decreased CDI due to a reduced SR Ca^{2+} load. However, this interpretation relies on the misconception that the fast phase of I_{CaL} inactivation is solely dependent on a Ca^{2+} release-induced inactivation in a microdomain inaccessible to Ca^{2+} chelators [13,18,26,27] and that this local Ca^{2+} signaling can contribute to 65-75% of inactivation [27, 28]. Our results show that, as expected, manipulations aimed to abolish or to strongly decrease CDI increased τ_{fast} but failed to prevent its further increase by depolarizing prepulses. This result suggests that I_{CaL} facilitation by depolarizing prepulses could also have a pure voltage-dependent component in addition to a CDI-related component. However, it is hard to imagine how low depolarizing prepulses could release Ca^{2+} to account for the CDI mechanism. So far, in cardiomyocytes it has never been shown conclusively a voltage-dependent calcium release mechanism. In this regard, the suggestions made by Barrre-Lemaire et al., [18] are still waiting for a valid demonstration of a voltage-driven Ca^{2+} release from the SR and the conclusion that this pulse-dependent I_{CaL} and rate-dependent facilitations of I_{CaL} share common mechanisms is no longer valid. The present results are in the same line as those previously published by our group indicating that the fast phase of inactivation of I_{CaL} is not exclusively dependent on CDI.

In the present experiments we made use of several approaches in order to drastically decrease the CDI mechanism at different levels. The use of Ba^{2+} instead of Ca^{2+} as charge carrier is expected to decrease CDI since it is a poor substitute for Ca^{2+} in the CaM site [29]. Additionally, Ba^{2+} permeation should not induce a large Ca^{2+}-induced Ca^{2+} release from the SR. Blocking the Ca^{2+} release channel of the SR by Ryanodine should prevent large increases in Ca^{2+} concentration in the restricted junctional spaces in the vicinity of the Ca^{2+} channel and decrease CDI [30]. On the other hand CPA, a blocker of the SR Ca^{2+} ATPase [30] would promote emptying of the SR with subsequent stimulations and decrease CDI. Finally, T-tubule disruption by formamide, although imperfect, will leave the cardiomyocytes with a population of Ca^{2+} channels essentially expressed in surface sarcolemma where restricted spaces in the vicinity of the channels are less well-developed and thus, with a decreased influence of the CDI mechanism [31]. Although none of these procedures is expected to abolish CDI they could effectively decrease its influence on I_{CaL} inactivation. As could be seen in Table 2, in all these conditions τ_{fast} was significantly increased, as expected from a decreased CDI. However, also in all cases the ramp prepulse protocol could effectively increase τ_{fast} and in magnitudes that were fully comparable to those obtained in control condition where CDI was fully functional. This result strongly suggest that the increase in τ_{fast} by conditioning a prepulse could be more related to a voltage-dependent modulation of Ca_V 1.2 channel state. The experimental series with BayK8644 seems to confirm this idea. BayK8644 significantly increased I_{CaL} density. This predicts a decrease in τ_{fast} if CDI were the dominant mechanism. However, contrary to this expectation, we found that τ_{fast} was increased. Additionally, ramp prepulses further increased τ_{fast}. The observed leftward shift in $V_{0.5}$ of inactivation by ramp prepulses adds another piece of evidence in favour of a voltage-dependent effect since this shift occurs at membrane potentials where I_{CaL} is not activated by the prepulses. Asuming that the chemical free energy difference between the open and the closed (inactivated or unavailable) is characterized by two parameters: s, the slope factor, and $V_{0.5}$, we might estimate the variation in free energy by introducing a ramp prepulse as follows (see [32]):

$$\Delta G = RT\left[(V_{0.5}/s)_{control} - (V_{0.5}/s)_{prepulse}\right], \tag{3}$$

where R is the gas constant and T the temperature in Kelvin. In our experimental conditions ΔG due to the prepulse was estimated to be 0.995 kcal / mol, which is well in the range of changes in free energy for variations in gating behaviour of Ca^{2+} channels [33]. However, this estimate has the limitation that parameters s and $V_{0.5}$ were obtanied from the availability curves and not from the charge (Q) vs membrane potential relationships (see [32]).

Overall, our data suggest that following a prepulse, not activating I_{CaL}, the Ca^{2+} channel delays its inactivation by dwelling a longer time in the open state. A simplified

three-state Markovian model of the fast inactivation phase that considers only voltage-dependent transitions between states could account for our results (Figure 4). Taking advantage of the fitting procedures of the Winascd program we adjusted a three-state model to two selected experimental I_{CaL} traces, one without prepulse and the corresponding trace after a ramp prepulse. The model consisted of a C1 closed state (resting) an open state (O) and a C2 closed state that corresponds to the inactivated state. Values obtained for rate constants between states and steady state probabilities are summarized in Table 3. Best fit results of the fast inactivation phase of current traces to the proposed kinetic scheme suggest that following a prepulse the probability for the channel being open is increased (2.4 times) and even if the probability of the inactivated state C2 is slightly increased (1.24 times), the channel will stay longer in the open state because the rate constant from open to inactivated state is decreased while the rate constant of the inverse transition remains unchanged. The increased probability of the inactivated state is due to an increased rate constant from the closed (C1) state (1.5 x) and a decreased reverse (to C1) rate constant (0.82 x) but with a reduced probability of the channel to be in the closed state. This, however, also favours transition of the channel from the inactivated to open states. Also, the transition from closed to open states is favored since the forward rate constant is increased while the backward rate constant is decreased. As a result, the channel will stay longer in the open state after a prepulse.

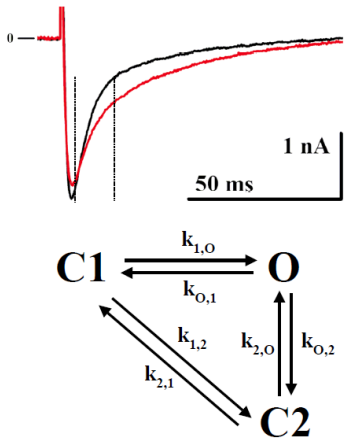

Figure 4. Kinetic scheme of I_{CaL} fast inactivation phase. Top: I_{CaL} traces recorded from the same cardiomyocyte without (black) and with a ramp prepulse (red). The dashed vertical lines indicate the fitted region. In this myocyte τ_{fast} and τ_{slow} were 5.1 and 60 ms without prepulse and 6.8 and 58 ms with the ramp prepulse. Bottom: Markovian three-state kinetic scheme. C1 is the closed (resting) state; O is the open state and C2 corresponds to a closed (inactivated) state of the channel. $k_{x,y}$ are the rate constants (1/s) for the forward and backward transitions between states.

V CONCLUSION

Facilitation of I_{CaL} by depolarizing prepulses below its activation threshold is due to a voltage-dependent mechanism. The increase in τ_{fast} could be explained by a voltage-dependent change in channel transitions that allow the channel to dwell longer in tis open state.

Table 3. Results of the kinetic scheme. k Rate Constants (1/s) and P steady state probabilities. The corresponding inactivation time constants of the selected traces were: No PP: τ_{fast} = 5.1 ms and τ_{slow} = 60 ms. PP: τ_{fast} = 6.8 ms and τ_{slow} = 58 ms.

	$k_{0,1}$	$k_{0,2}$	$k_{1,0}$	$k_{1,2}$	$k_{2,0}$	$k_{2,1}$
No PP	0.206	0.261	0.001	0.097	0.134	0.218
PP	0.134	0.144	0.012	0.114	0.137	0.146

	$P0$	$P1$	$P2$
No PP	0.071	0.687	0.242
PP	0.172	0.528	0.300

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