Regulation of the frequency-dependent facilitation of L-type Ca^{2+} currents in rat ventricular myocytes

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1. An increase in the rate of stimulation induces an augmentation of L-type Ca^{2+} currents (I_{Ca}) and concomitant slowing of current decay in rat ventricular cells. This facilitation is quasi immediate (1–3 s), graded with the rate of stimulation, and occurs only from negative holding potentials. We investigated this effect using trains of stimulation at 1 Hz and the whole-cell patch-clamp technique (18–22 °C).

2. The decay of I_{Ca} is normally bi-exponential and comprises fast and slow current components ($I_{Ca,fc}$ and $I_{Ca,sc}$, respectively). Facilitation of I_{Ca} was observed only when $I_{Ca,fc}$ was predominant.

3. Facilitation developed during the run-up of I_{Ca} with the interconversion of $I_{Ca,sc}$ into $I_{Ca,fc}$, and vanished during the run-down of I_{Ca} with the loss of $I_{Ca,fc}$. Ni^{2+} (300 μM) and nifedipine (1 μM) suppressed facilitation owing to the preferential inhibition of $I_{Ca,fc}$.

4. Facilitation of I_{Ca} was not altered (when present) or favoured (when absent) by the cAMP-dependent phosphorylation of Ca^{2+} channels promoted by isoprenaline or by intracellular application of cAMP or of the catalytic subunit of protein kinase A (C-sub). A similar effect was observed when the dihydropyridine agonist Bay K 8644 was applied. In both cases, facilitation was linked to a preferential increase of $I_{Ca,fc}$.

5. Following intracellular application of inhibitors of protein kinase A in combination with a non-hydrolysable ATP analogue, I_{Ca} consisted predominantly of $I_{Ca,sc}$ and no facilitation was observed. The calmodulin antagonist naphthalenesulphonamide had no effect on facilitation.

6. When Bay K 8644 was applied in combination with isoprenaline, cAMP or C-sub, the decay of I_{Ca} was slowed with the predominant development of $I_{Ca,sc}$, and facilitation of I_{Ca} was nearly abolished. Facilitation also depended on extracellular Ca^{2+}, and was suppressed when Ba^{2+} replaced Ca^{2+} as the permeating ion.

7. When no EGTA was included in the patch pipette, facilitation was not further enhanced but a use-dependent decrease of I_{Ca} frequently occurred. When BAPTA was used in place of EGTA, the rate of inactivation of I_{Ca} was reduced and facilitation was abolished.

8. In conclusion, the facilitation of I_{Ca} that reflects a voltage-driven interconversion of $I_{Ca,fc}$ into $I_{Ca,sc}$ is also regulated by Ca^{2+} and by cAMP-dependent phosphorylation. The presence of the gating pattern typified by $I_{Ca,fc}$ is required. Ca^{2+} may exert its effect near the inner pore of the Ca^{2+} channel protein and control the distribution between the closed states of the two gating pathways.

Voltage-gated dihydropyridine-sensitive (L-type) Ca^{2+} channels are multimeric transmembrane proteins crucially involved in the development and control of heart contractility (for review see Hartzell, 1988; Pelzer, Pelzer & McDonald, 1990). Their opening is promoted primarily by membrane depolarization but their regulation by neurotransmitters, hormones, drugs and intracellular second messengers is fundamental in cellular physiology (Trautwein & Kameyama, 1986; Tsien, Bean, Hess, Lansman, Nilius & Nowycky, 1986; Hartzell, 1988; Pelzer *et al.* 1990). L-type Ca^{2+} channels are also subject to modulation by a variety of exogenous synthetic ligands, termed Ca^{2+}channel antagonists/agonists, such as dihydropyridines (Tsien *et al.* 1986; Pelzer *et al.* 1990).

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One of the most interesting adaptive properties of cardiac Ca^{2+} channels is that, depending on the recording conditions, they can be down- or up-regulated upon an increase in the rate of cell stimulation. Repetitive depolarization, for example, produces channel inactivation when cells are stimulated from depolarized membrane holding potentials (Mitchell, Powell, Terrar & Twist, 1985; Fedida, Noble & Spindler, 1988*a*,*b*; Peineau, Garnier & Argibay, 1992; Richard, Charnet & Nerbonne, 1993). Ca^{2+}-dependent inactivation (Mitchell *et al.* 1985) or incomplete voltage-dependent reactivation (Hryshko & Bers, 1990; Peineau *et al.* 1992; Richard *et al.* 1993) of Ca^{2+} channels have been suggested to account for the decrease of I_{Ca}. Alternatively, repetitive depolarization can produce potentiation of Ca^{2+} channel activity (Noble & Schimoni, 1981; Mitra & Morad, 1986; Lee, 1987; Fedida *et al.* 1988*a*,*b*; Tseng, 1988; Schouten & Morad, 1989; Richard, Tiaho, Charnet, Nargeot & Nerbonne, 1990; Zygmunt & Maylie, 1990; Peineau *et al.* 1992) when I_{Ca} is evoked from negative holding potentials (< -50 mV). The molecular mechanisms underlying this potentiation, known as facilitation, are also a matter of debate. A voltage-dependent mechanism has been described using Ba^{2+} (instead of Ca^{2+}) as the charge carrier (Lee, 1987; Schouten & Morad, 1989). Possibilities suggesting a key role for Ca^{2+} ions include: (i) the relief of a Ca^{2+}-dependent inactivating process via extrusion of Ca^{2+} through the Na^+–Ca^{2+} exchanger (Argibay, Fischmeister & Hartzell, 1988) or a decrease in the release of Ca^{2+} from the sarcoplasmic reticulum (Tseng, 1988); (ii) intracellular modulation of a phosphorylation step activated following Ca^{2+} entry (Fedida *et al.* 1988*b*; Zygmunt & Maylie, 1990).

In isolated adult rat ventricular myocytes only L-type Ca^{2+} channels are present, i.e. no low threshold-activated T-type Ca^{2+} current has been recorded (Richard *et al.* 1990, 1993; Scamps, Mayoux, Charlemagne & Vassort, 1990; Xu & Best, 1990). However, as initially shown in bovine ventricular myocytes (Isenberg & Klöckner, 1982), the macroscopic current (I_{Ca}) waveform of the L-type Ca^{2+} current is comprised of fast ($I_{Ca,fc}$) and slow ($I_{Ca,sc}$) inactivating components which are differentially regulated by membrane potential, frequency of stimulation and physiological stimuli (Richard *et al.* 1990, 1993). An increase in the rate of stimulation from 0·1 to 1 Hz induces a moderate increase in the global I_{Ca} but a marked slowing of its decay, which can be accounted for entirely by the interconversion from $I_{Ca,fc}$ into $I_{Ca,sc}$ (Richard *et al.* 1993). This increase of Ca^{2+} influx is referred to here as a frequency-dependent facilitation of I_{Ca}. In this paper, manipulations of I_{Ca} waveforms, by means of pharmacological agents, demonstrate that facilitation of I_{Ca}, which is conditioned by the predominance of $I_{Ca,fc}$, is Ca^{2+} sensitive and is also regulated by cAMP-dependent phosphorylation. Ca^{2+} ions seem to play a key role in the regulation of Ca^{2+} channel gating modes at physiological Ca^{2+} concentrations.

METHODS

Experiments were performed on single ventricular myocytes freshly dissociated from 6- to 10-week-old rats after collagenase treatment, as previously described in detail (Richard *et al.* 1990, 1993). Animals were killed by rapid cervical dislocation, the hearts quickly removed and perfused retrogradely through the aorta with the enzymatic solution. Waveforms of currents through voltage-activated Ca^{2+} channels were measured at 18–22 °C 2–10 h after cell dispersion using the whole-cell patch-clamp technique (Hamill, Marty, Neher, Sakmann & Sigworth, 1981) under conditions optimized to eliminate contaminating voltage-gated inward Na^+ (I_{Na}) and outward K^+ (I_{out}) currents (Richard *et al.* 1990, 1993; Tiaho, Richard, Lory, Nerbonne & Nargeot, 1990). Ca^{2+} currents (I_{Ca}) were recorded in standard bath conditions (unless otherwise noted) containing (mM): TEACl, 140; $CaCl_2$, 1·5; $MgCl_2$, 2; Hepes, 10; glucose, 10; and tetrodotoxin, 0·001; adjusted to pH 7·2 with TEAOH. To measure the waveforms of Ba^{2+} currents (I_{Ba}) through Ca^{2+} channels in the same conditions, 1·5 mM $BaCl_2$ replaced $CaCl_2$.

Recording pipettes were constructed from micro-haematocrit tubes (Modulohm I/S, Herlev, Denmark) and filled with the following solution (mM): CsCl, 140; EGTA, 10; Hepes, 10; MgATP, 3; MgGTP, 0·4; adjusted to pH 7·2 with CsOH, unless otherwise noted. In some experiments 1,2-bis(*o*-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid (BAPTA; 20 mM) was used in place of EGTA. The pipette resistance ranged from 2 to 3 MΩ after filling with the recording solutions. Junction potentials between the intrapipette solution and the reference electrode were zeroed prior to obtaining the seals. After seal formation (resistance in the range 1–20 GΩ) and membrane rupture (whole-cell recording configuration: WCR), the series resistance (1·5–2 times the pipette resistance) was electronically compensated by 80 %, as previously described in detail (Tiaho *et al.* 1990). Because routinely recorded currents were < 5 nA, voltage errors resulting from the uncompensated series resistance (0·5–2·0 MΩ), estimated from the decay of the capacitive transients evoked by a ±10 mV test pulse, were ≤ 6 mV. Cell input resistances were in the range 0·4–4 GΩ. Currents were recorded using a Biologic RK300 amplifier (Claix, France). Data acquisition and analyses were performed using pCLAMP software (Axon Instruments Inc., Foster City, CA, USA). Sampling frequencies ranged from 40 to 1 kHz and, when needed, current signals were filtered at 10 kHz prior to digitization and storage.

Ca^{2+} currents were recorded at the earliest opportunity (unless otherwise noted) 5 min after attaining the whole-cell recording configuration, i.e. once a steady state was reached after the initial run-up of I_{Ca} (Tiaho, Nargeot & Richard, 1993). I_{Ca} was recorded at a test pulse (TP) of −10 mV delivered from a holding potential of −80 mV at a frequency of 0·1 Hz (unless otherwise noted in particular experiments). Frequency-dependent facilitation of I_{Ca} was routinely examined using a train of ten stimulations at a frequency of 1 Hz with rest periods ≥10 s. No change in I_{Ca} waveforms was observed when

the interpulse duration (or the rest period prior to applying trains of stimulations) was >5 s (see Richard *et al.* 1993). Inward peak currents were measured as the difference between the maximal inward current amplitude and the zero current level (Tiaho *et al.* 1990). Inactivation of I_{Ca} was best fitted by the sum of two sequential exponentials using the expression:

$$I(t) = I_{Ca,fc} \times \exp(-t/\tau_{fc}) + I_{Ca,sc} \times \exp(-t/\tau_{sc}),$$

where I is the current at time t; $I_{Ca,fc}$ and $I_{Ca,sc}$ are the amplitudes and τ_{fc} and τ_{sc} are the time constants of the fast and slow components of I_{Ca}. There was no evidence for a non-inactivating component of I_{Ca} (Richard *et al.* 1990, 1993). The quality of the fits was determined based on excellent visual superimposition with original traces and selection of the best correlation coefficients. These procedures have previously been described in detail (Richard *et al.* 1990). Results are expressed as means ± S.D.

Isoprenaline (Iso; Sigma Chemical Co., Saint Quentin Fallavier Cedex, France) was prepared daily as a 1 mM stock solution in H_2O. Bay K 8644 and nifedipine (Bayer AG, Wuppertal, Germany) were dissolved in 50 % ethanol to make a concentrated stock solution (10 mM), which was stored at −20 °C. Controls revealed that the solvent had no effect on Ca^{2+} channel currents at the final dilutions used here (< 0·005 % v/v). Extracellular superfusion of the cells was performed using 300 μM inner diameter capillary tubing (flow rate 0·2 ml min^{-1}) placed in the vicinity of the cell (< 0·5 mm). The diameter of the capillaries ensured that the extracellular environment of the cell could be easily and rapidly modified (within 1 s).

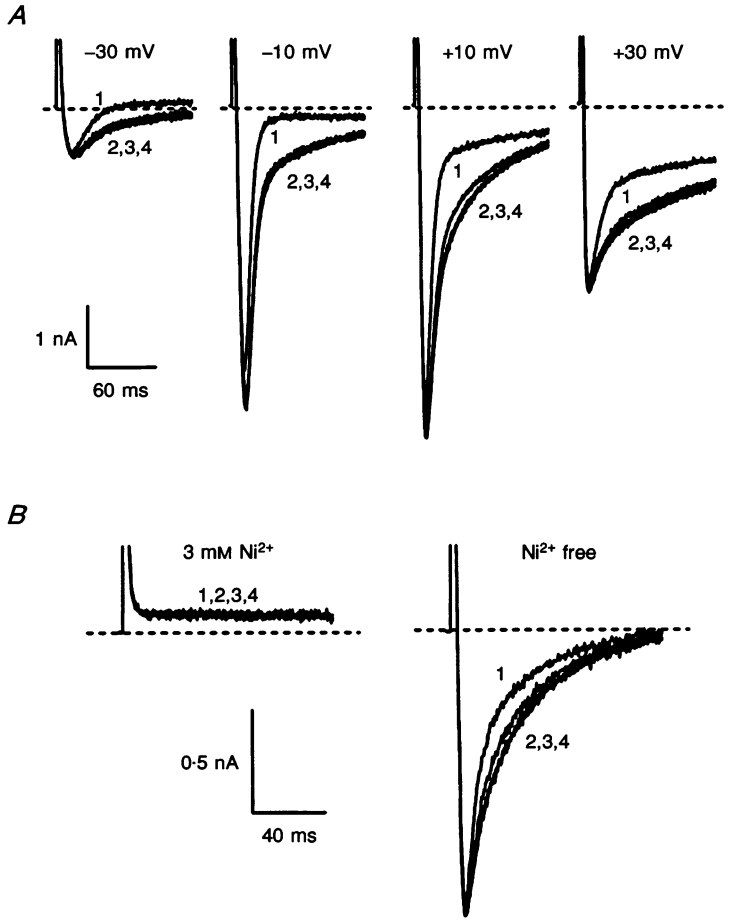


Figure 1. Post-rest facilitation of I_{Ca} at a stimulation frequency of 1 Hz in a rat ventricular myocyte

In all experiments, I_{Ca} was recorded at a test potential (TP) of −10 mV delivered from a holding potential (V_h) of −80 mV with a train of stimulations at a frequency of 1 Hz after a 10 s rest period (unless otherwise noted). *A*, I_{Ca}s were evoked at test depolarizations of −30, −10, +10 and +30 mV. The numbers on each trace represent the sequential order of their acquisition. The first four stimulations are shown. Note the moderate increase of peak I_{Ca} and the marked slowing of current decay. A steady state was attained after the second or, at the most, the third stimulation. *B*, complete inhibition of I_{Ca} by Ni^{2+} abolished the frequency-dependent change in current waveform (left panel). The train of stimulations was applied after the steady-state effect of Ni^{2+} (attained at 0·1 Hz). Facilitation of I_{Ca} was unmasked after the wash-out of Ni^{2+} (right panel) and restoration of I_{Ca}. The first four stimulations are shown in each case.

The catalytic subunit (C-sub) of cAMP-dependent protein kinase was purified from bovine heart as described elsewhere (Betchel, Beavo & Krebs, 1977) and was kindly provided for us by J.C. Labbé, (CRBM, Montpellier, France). The inhibitory peptide for protein kinase A (PKI) is the same as that used by Lory & Nargeot (1992) to block cAMP-dependent phosphorylation of cardiac Ca^{2+} channels expressed in *Xenopus laevis* oocytes. It is referred to as [PKI(m) (6–24)] and was synthesized in the laboratory and kindly provided by N. Lamb (CRBM, Montpellier). Similar to the commercially available A-PKI, PKI(m) has retained specific inhibitory activity against A-kinase but it has been stabilized in order to increase its lifetime in living cells (Fernandez, Mery, Vandromme, Basset, Cavadore & Lamb, 1991). The inhibitor of protein kinase C and protein kinase A H-7 (1-(5-iso-quinolinesulphonyl)-2-methylpiperazine) was purchased from RBI (Natick, MA, USA). The non-hydrolysable ATP analogue AMP-PNP (5′-adenylyl-imido-diphosphate) was purchased from Boehringer (Mannheim, Germany). The calmodulin antagonist W-13 (*N*-(4-aminobutyl)-5-chloro-1-naphthalene-sulphonamide; purchased from RBI) binds specifically to calmodulin and inhibits its activity (for review see Hidaka & Ishikawa, 1992). It has been shown to accelerate the run-up of Ca^{2+} currents by blocking the activity of a calmodulin-activated protein phosphatase in neurones (Mironov & Lux, 1991).

RESULTS

Frequency-dependent facilitation of I_{Ca}

We have previously reported in detail that waveforms of L-type I_{Ca} in rat ventricular cells vary as a function of the frequency of activation (Richard *et al.* 1990, 1993). As illustrated in Fig. 1*A*, when myocytes were repetitively depolarized using a train of stimulations at a frequency of 1 Hz, after a rest period of 10 s at a holding potential (V_h) of −80 mV, I_{Ca} peak amplitude was augmented and its decay was markedly slowed. Although the increase in I_{Ca} peak amplitude was moderate (< 15 % in 23 cells), the change in current decay substantially increased the amount of Ca^{2+} entering the cell (Fig. 1*A*). This facilitating effect occurred at all depolarizations activating an inward Ca^{2+} current in the *I–V* range (Fig. 1) and was independent of I_{Ca} peak amplitude. It was graded with the rate of stimulation, occurring generally in the range 0·2–2 Hz, and was reversible. Steady-state effects were reached immediately, i.e. by the second stimulation after the change in the rate of stimulation (Fig. 1*A*) in many cells or, at the most, after three to four stimulations. Then I_{Ca} waveforms remained stable for several minutes. Although a gradual and irreversible decrease of I_{Ca} peak amplitude occurred in two out of five cells, probably owing to run-down, decay kinetics of I_{Ca} remained slow (data not shown). Facilitation was suppressed when I_{Ca} was evoked from V_h values more positive than −50 mV (data not shown but for details see Richard *et al.* 1993). All the experiments described below were performed by routinely changing the rate of stimulation from rest to 1 Hz at a V_h of −80 mV and for a limited duration (see Methods).

The presence of millimolar concentrations of inorganic Ca^{2+} channel blockers, such as Ni^{2+} or Co^{2+}, in the extracellular bath provided complete blockade of I_{Ca} in its whole range of activation. This was consistent in all ten cells tested. As illustrated in Fig. 1*B*, no transient outward current is apparent in these conditions owing to the efficient blockade provided by the simultaneous presence of extracellular TEA^+ and intracellular Cs^+ (see Richard *et al.* 1990, 1993; Tiaho *et al.* 1993). Furthermore, no frequency-dependent change in current waveform was observed (Fig. 1*B*, left panel). In contrast, when Ni^{2+} was washed out, not only did I_{Ca} recover rapidly but frequency-dependent facilitation of the inward current was unmasked (Fig. 1*B*, right panel). We concluded, therefore, that the apparent change in I_{Ca} waveform following an increase in the rate of stimulation reflects a genuine facilitation of I_{Ca}. The following experiments were aimed at investigating the modulation of the facilitation by means of various electrophysiological protocols and pharmacological agents.

Facilitation is conditioned by I_{Ca} waveforms

The decay of macroscopic I_{Ca} in isolated adult rat ventricular myocytes is best described by the sum of two exponentials with time constants separated by an order of magnitude (4–10 ms and 40–100 ms, respectively; for details see Richard *et al.* 1990, 1993). This reflects the presence and

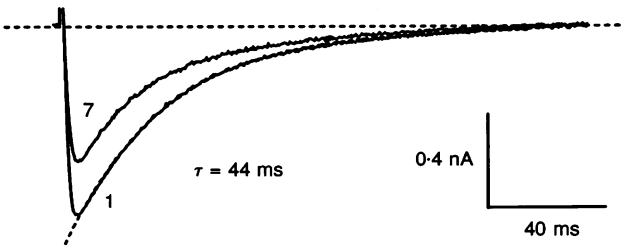


Figure 2. Waveform dependence of the facilitation of I_{Ca}
No facilitation but a use-dependent decrease of peak I_{Ca} was observed in this cell characterized by a slow decay. Superimposed are currents evoked at the 1st and 7th stimulations (steady-state effect). The best fit of I_{Ca} decay evoked by the first stimulation is represented. The decay of I_{Ca} was monoexponential with a time constant $\tau = 44$ ms. The decay of I_{Ca} was unchanged after the increase in the rate of stimulation. This type of current was observed very rarely (< 5 % of cells).

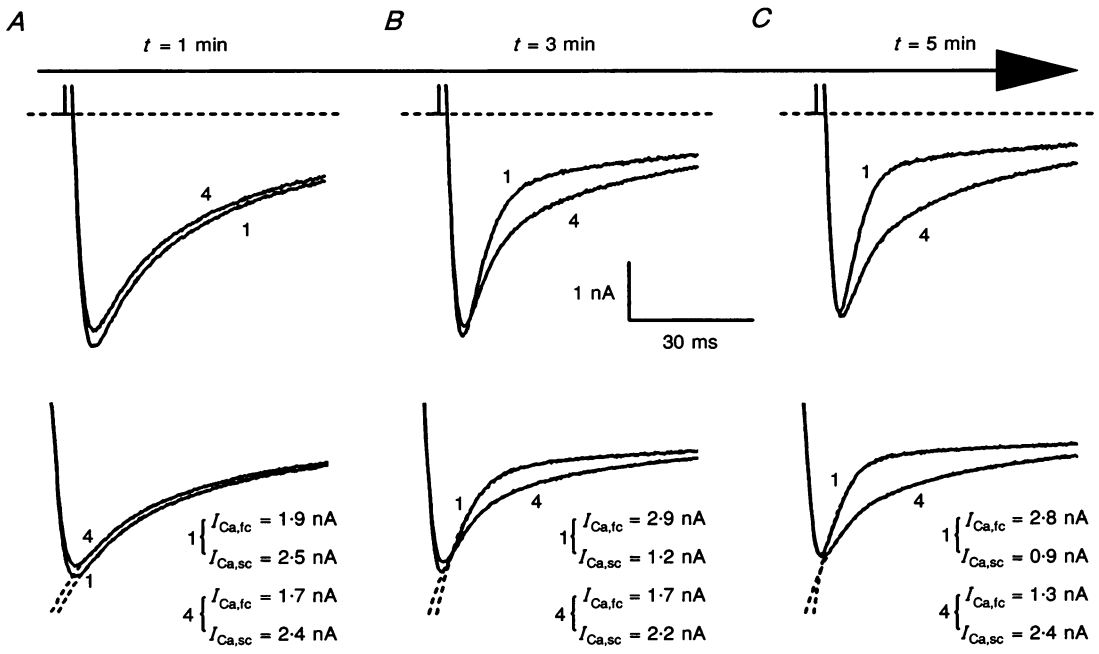


Figure 3. Facilitation during the run-up of I_{Ca}
Upper panel, original records of I_{Ca} evoked 1 min (*A*), 3 min (*B*) and 5 min (*C*) after establishing the WCR configuration in the same cell. Inactivation of early I_{Ca} was initially slow (left traces) but a dramatic acceleration occurred with the time of recording (middle and right traces). No facilitation of I_{Ca} (but a use-dependent decrease of peak current) is observed in *A*, whereas facilitation develops concomitant with the appearance of a fast inactivating component of I_{Ca} (*A* and *B*). Superimposed are currents evoked at the 1st and 4th stimulations (steady-state effects). Lower panel, best fits of I_{Ca} decay for the records shown in the upper panel and illustrating the dramatic change in the distribution of $I_{Ca,fc}$ and $I_{Ca,sc}$ during the run-up of I_{Ca}.

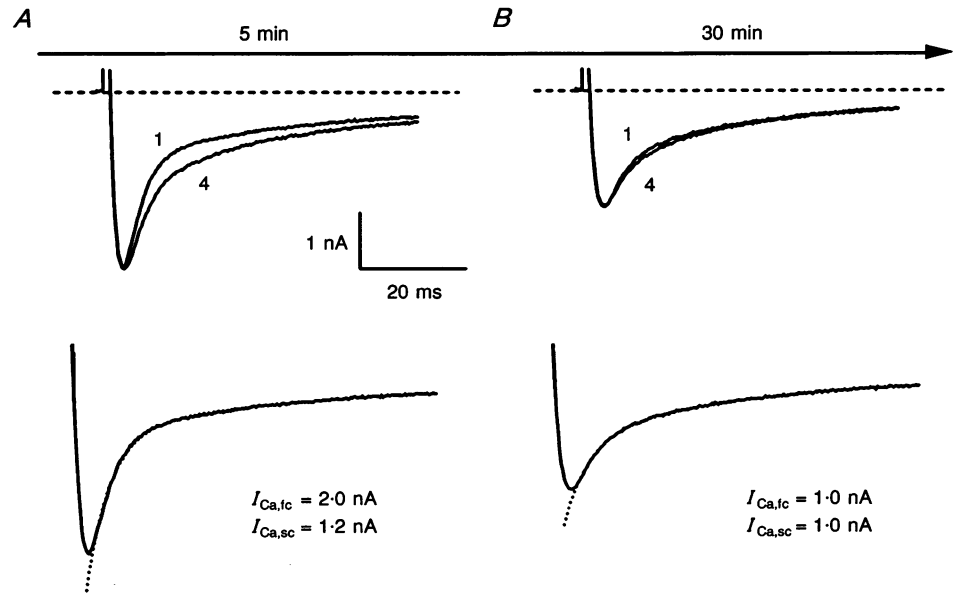


Figure 4. Facilitation of I_{Ca} during run-down
Upper panel, original records of I_{Ca} evoked 5 min (*A*) and 30 min (*B*) after establishing the WCR configuration in the same cell. Facilitation of I_{Ca} is observed in *A* but is markedly attenuated in *B*. Superimposed are currents evoked at the 1st and 4th stimulations (steady-state effects). Lower panel, best fits of I_{Ca} decay for the records shown in the upper panel and illustrating the greater decrease of $I_{Ca,fc}$ (*versus* $I_{Ca,sc}$) during the run-down of I_{Ca}.

activation of two kinetically distinct current components, referred to as $I_{Ca,fc}$ and $I_{Ca,sc}$ to denote the fast and the slow components. The relative contribution of each component to the total current determines the I_{Ca} waveform and varies among cells. However, Ca^{2+} currents exhibiting a monoexponential decay could sometimes be observed (Tiaho *et al.* 1993). The Ca^{2+} current illustrated in Fig. 2 had a slow monoexponential decay owing to the exclusive presence of $I_{Ca,sc}$, a situation that we have observed infrequently over the past four years. In these conditions, no facilitation of I_{Ca} occurred upon an increase in the rate of stimulation. Instead, a slight decrease in I_{Ca} peak amplitude (without modification of kinetics) was observed.

Figure 3 shows how, in the same cell, I_{Ca} can sequentially display the two distinct behaviours reported above in response to a change in the rate of stimulation. We have recently described the run-up of I_{Ca} which occurs during the first minutes after establishing the whole-cell clamp recording configuration (for details see Tiaho *et al.* 1993). Briefly, immediately after membrane disrupture (< 10 s) I_{Ca} was absent but gradually increased in amplitude with a concomitant dramatic change in decay kinetics, to reach its final waveform (amplitude and kinetics) 2–5 min later. As illustrated in Fig. 3, its rate of decay was initially slow and monoexponential (reflecting the presence and activation of $I_{Ca,sc}$) but this decay became biexponential owing to the appearance of a fast component of inactivation ($I_{Ca,fc}$). It was striking that facilitation of I_{Ca} occurred concomitant with the acceleration of current decay, i.e. with the interconversion of the slow into the fast inactivating current components (Fig. 3). It should be noted that the acceleration of I_{Ca} decay was not related to an increase in current amplitude. Furthermore the frequency-dependent facilitation was also independent of I_{Ca} amplitude (and amount of Ca^{2+} entering the cell). Facilitation developed with the slow conversion of $I_{Ca,sc}$ into $I_{Ca,fc}$, irrespective of

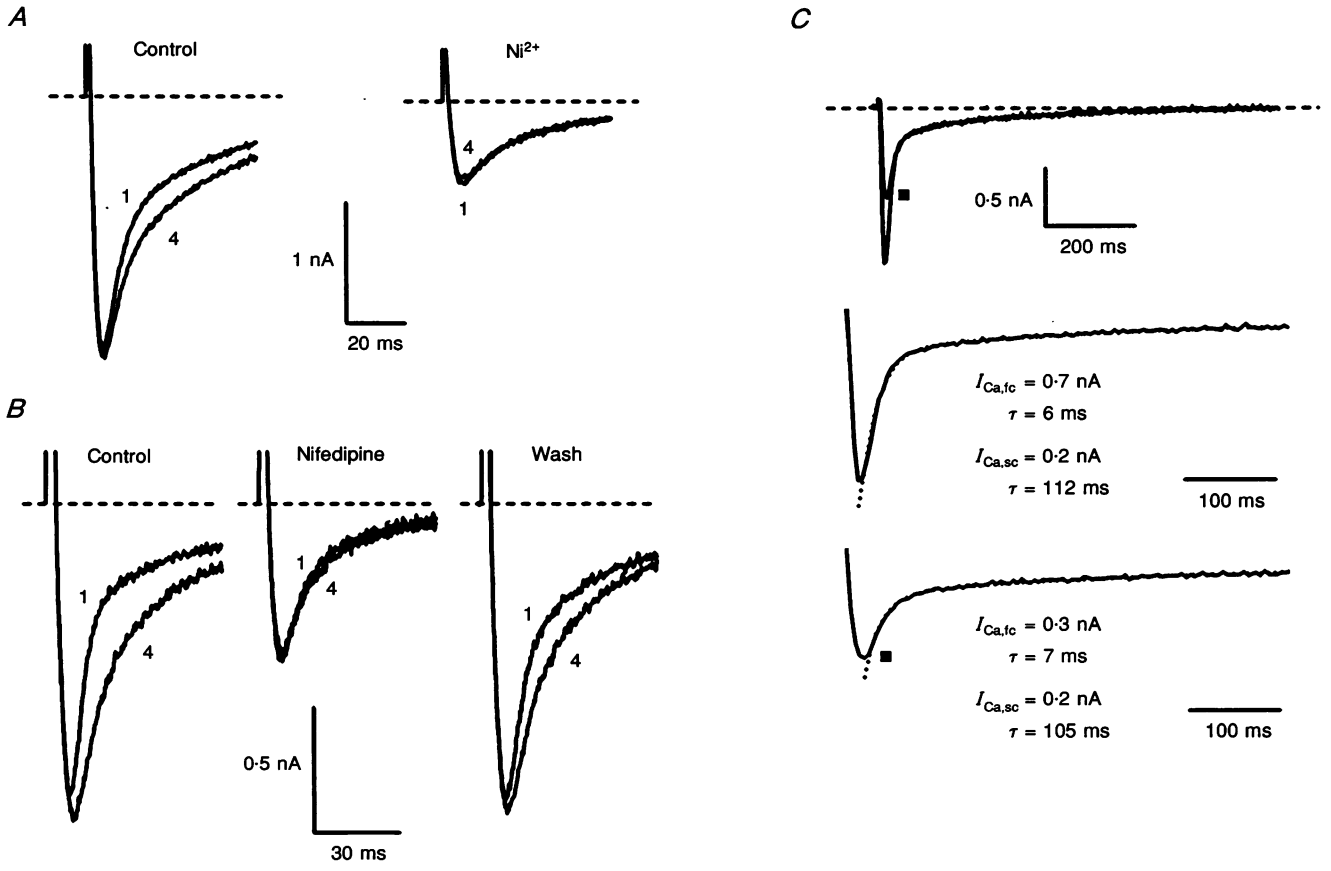


Figure 5. Suppression of the facilitation of I_{Ca} by Ca^{2+} channel blockers
A, effect of 300 μM Ni^{2+}. The train of stimulations was applied (see Methods) before (left traces) and after (right traces) the steady-state effect of Ni^{2+} (attained at 0·1 Hz). Superimposed are currents evoked at the 1st and the 4th stimulations (steady-state effect). *B*, effect of 1 μM nifedipine. The train of stimulations was applied (see Methods) before (left traces) and after (middle traces) the steady-state effect of nifedipine (attained at 0·1 Hz). Currents evoked at the 1st and 4th stimulations are superimposed. Facilitation of I_{Ca} was recovered after the wash-out of nifedipine (right traces) and restoration of I_{Ca}. *C*, preferential decrease of $I_{Ca,fc}$ in the presence of 300 μM Co^{2+} as determined visually and by best fits of the decay of I_{Ca} before and after (■) the effect of the blocker (in the upper panel, ■ refers to the upper trace). Steady-state effect of Co^{2+} was attained at 0·1 Hz.

the mechanisms involved (possibly phosphorylation: for detail see Tiaho *et al.* 1993). This behaviour was consistent in all cells tested ($n = 7$). As a corollary, the frequency-dependent facilitation of I_{Ca} disappeared during slow irreversible run-down which preferentially altered the amplitude of $I_{Ca,fc}$ over time during prolonged experiments. This is illustrated in Fig. 4 where kinetics analyses of I_{Ca} decay revealed that this suppression parallels the more rapid decrease of $I_{Ca,fc}$ as compared with that of $I_{Ca,sc}$. All these observations suggested therefore that the frequency-dependent facilitation of I_{Ca} is conditioned by the decay kinetics of macroscopic current, i.e. the presence of $I_{Ca,fc}$ is required.

Effects of Ca^{2+} channel inhibitors on facilitation of I_{Ca}

We next investigated whether facilitation of I_{Ca} could be modulated by pharmacological means. Figure 5*A* shows the effects of the inorganic Ca^{2+} channel blocker Ni^{2+} (300 μM). At this concentration, Ni^{2+} induced a partial blockade of I_{Ca} ($63 \pm 5\%$, $n = 4$) but consistently abolished the frequency-dependent facilitation of I_{Ca}. Co^{2+} applied at similar concentrations ($n = 2$, data not shown) and the organic Ca^{2+} channel antagonist nifedipine (1 μM), which also induced only a partial inhibition of I_{Ca} ($51 \pm 8\%$, $n = 5$), had the same suppressing effects (Fig. 5*B*). These effects were generally reversible and facilitation of I_{Ca} recovered after wash-out of the Ca^{2+} channel blocker (Fig. 5*B*). Interestingly, kinetics analyses revealed that both inorganic and organic Ca^{2+} channel blockers preferentially inhibited $I_{Ca,fc}$ (data not shown). This is illustrated in Fig. 5*C* where the preferential inhibition of $I_{Ca,fc}$ can be visually estimated.

Effects of cAMP-dependent phosphorylation on facilitation of I_{Ca}

We investigated the effects of the β-adrenergic agent isoprenaline (Iso) on facilitation of I_{Ca}. Iso was employed at its maximally effective concentration (1 μM). Usually a two- to threefold increase of I_{Ca} peak amplitude occurs at this concentration (Tiaho *et al.* 1990). Two sets of results were obtained: (i) in all cells ($n = 10$) where a substantial facilitation of I_{Ca} was observed in control conditions, this facilitation was conserved after application of Iso (Fig. 6*A*); (ii) in four other cells where facilitation of I_{Ca} was quasi-absent prior to application of Iso, the β-adrenergic agonist favoured the appearance of marked stimulation-dependent changes in I_{Ca} waveforms (Fig. 6*B*); the promotion of $I_{Ca,fc}$ by Iso, clearly observed for the example shown in Fig. 6*B* and determined by kinetics analyses (data not shown), was a determining factor. Similar results were obtained when a saturating concentration of cAMP (100 μM; Trautwein &

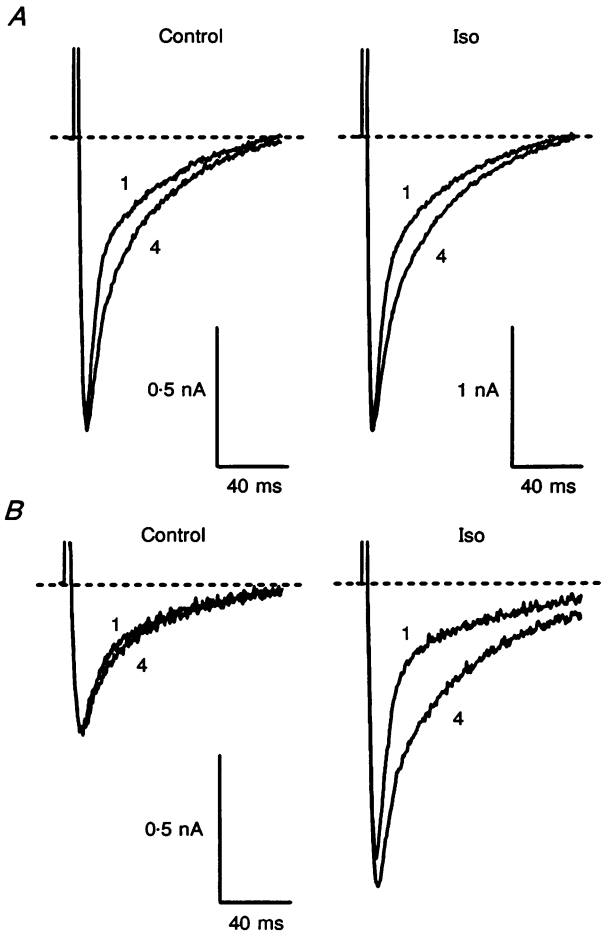


Figure 6. Modulation of the facilitation of I_{Ca} by isoprenaline
The numbers next to each trace represent the sequential order of their acquisition. *A*, effect of 1 μM Iso in a cell where facilitation of I_{Ca} existed prior to exposure to Iso (left panel). Iso promoted a twofold increase (right panel) in this cell (note the different vertical scale bars). Facilitation of I_{Ca} was not altered. *B*, effect of Iso in a cell where facilitation of I_{Ca} was absent prior to exposure to 1 μM Iso (left panel). Iso induced the appearance of the frequency-dependent facilitation of I_{Ca} (right panel). Note the promotion by Iso of a fast-inactivating component of I_{Ca} ($I_{Ca,fc}$).

Kameyama, 1986; Hartzell, 1988; Tiaho *et al.* 1990), was included in the recording patch pipette solution (data not shown). Thus it seems that elevating intracellular cAMP does not prevent enhancement of I_{Ca} by the rate of stimulation.

This hypothesis was further assessed by directly including in the recording pipette solution the catalytic subunit (C-sub) of cAMP-dependent kinase, or alternatively, two inhibitors (A-PKI and H-7) of this kinase employed in combination with a non-hydrolysable ATP analogue (AMP-PNP; see Methods). When 10 μM C-sub (a saturating concentration) was included in the patch pipette (Brum, Flockerzi, Hofmann, Osterrieder & Trautwein, 1983; Trautwein & Kameyama, 1986), large I_{Ca}s were recorded (3·6 $\pm$ 1·3 nA, $n = 5$) which consisted predominantly of $I_{Ca,fc}$ (Fig. 7*A*, left panel). In these conditions, I_{Ca} waveforms also varied as a function of the frequency of activation, in agreement with the above observations that Iso and cAMP favour facilitation of I_{Ca}. Double exponential fits to the decay phases of I_{Ca} revealed that increasing the rate of stimulation resulted in a reduction in the amplitude of $I_{Ca,fc}$ and a corresponding increase in the amplitude of $I_{Ca,sc}$. Alternatively, when 10 μM of the A-kinase inhibitor PKI(m) was intracellularly applied in combination with AMP-PNP (6 mM) and H-7 (50 μM) (see Methods), I_{Ca}s of moderate amplitude (0·4 $\pm$ 0·2 nA, $n = 5$) could be recorded (Fig. 7*B*). Iso (1 μM) had no effect on these currents (data not shown), which may suggest that the observed fraction of I_{Ca} reflects the presence and activation of dephosphorylated channels (Kameyama, Hescheler, Hofmann & Trautwein, 1986). In contrast to Ca^{2+} currents recorded in control conditions, I_{Ca} had a predominant slow component of inactivation. In these conditions, little or no facilitation of I_{Ca} was observed (Fig. 7*B*). We concluded, therefore, that cAMP-dependent phosphorylation favours facilitation, probably as a consequence of the preferential increase of $I_{Ca,fc}$. Indeed, Bay K 8644, which is able to increase the presumably dephosphorylated fraction of I_{Ca} (Tiaho *et al.* 1990) and increase preferentially $I_{Ca,fc}$ (Richard *et al.* 1990), also favoured facilitation of I_{Ca} (data not shown).

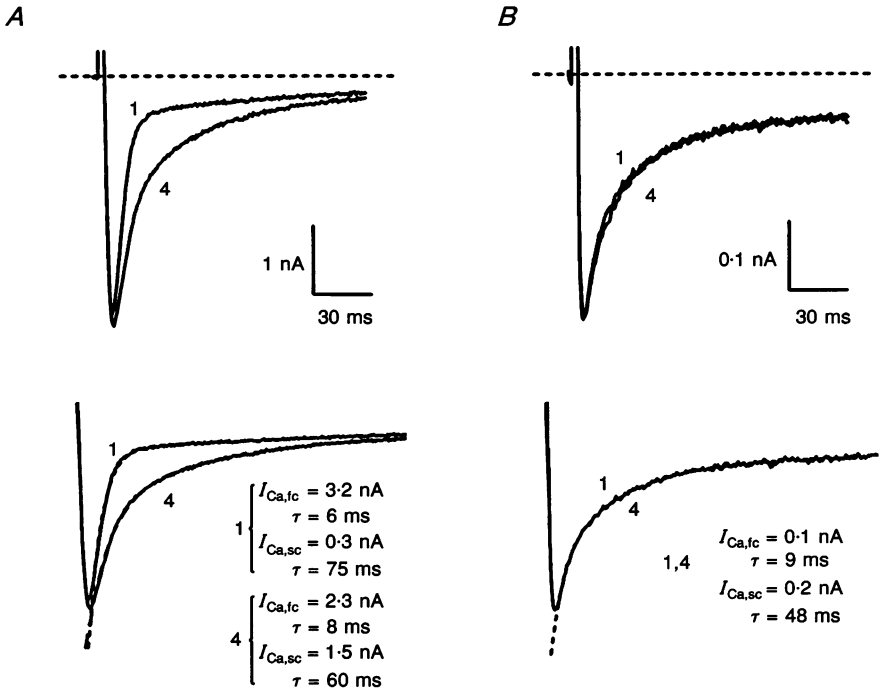


Figure 7. Modulation of the facilitation of I_{Ca} by protein kinase A
The numbers next to each trace represent the sequential order of their acquisition. *A*, facilitation of I_{Ca} recorded in the presence of 10 μM of the catalytic subunit (C-sub) of the protein kinase A included in the recording pipette solution (see Methods). I_{Ca} (peak) amplitude was increased and current decay was slowed at the higher stimulation frequency (trace 4). From double exponential fits to decay phases of currents, $I_{Ca,fc}$ and $I_{Ca,sc}$ were determined and are displayed (lower panel). $I_{Ca,fc}$ is the dominant component of I_{Ca}. A reduction in $I_{Ca,fc}$ and a corresponding increase in $I_{Ca,sc}$ were evidenced at the higher stimulation frequency. *B*, I_{Ca} was recorded in the presence of 10 μM of the A-kinase inhibitor PKI(m), AMP-PNP (6 mM) and H-7 (50 μM) in the recording pipette solution (see Methods). Current waveform was not altered by the increase in the rate of stimulation. Double exponential fits to decay phases of currents revealed that $I_{Ca,sc}$ is the dominant component of I_{Ca} (lower panel). Note the tenfold difference in the amplitude of I_{Ca} between *A* and *B*.

Effect of a calmodulin-dependent kinase inhibitor

A depolarization-dependent enhancement of I_{Ca}, mediated by activation of calmodulin-dependent protein kinase II, has been recently reported in smooth muscle cells (McCarron, McGeown, Reardon, Ikebe, Fay & Walsh, 1992). We used the calmodulin kinase inhibitor W-13 (Hidaka & Ishikawa, 1992) in order to investigate the possible involvement of a calmodulin-dependent protein kinase activated by Ca^{2+}. Figure 8*A* shows that, after a 10 min application of 10 μM W-13, no significant alteration of the frequency-dependent facilitation of I_{Ca} was observed. When myocytes were bathed for at least 1 h in the presence of W-13, prior to recording I_{Ca}, frequency-dependent facilitation of I_{Ca} was not altered (Fig. 8*B*). These experiments suggest that activation of a calmodulin-kinase by Ca^{2+} does not account for the frequency-dependent facilitation of I_{Ca}.

Promotion of $I_{Ca,sc}$ prevents facilitation

The above experiments have shown how inhibitors of Ca^{2+} channel activity can modulate I_{Ca} waveforms and block frequency-dependent modulation of these currents. This antagonism was correlated with the preferential block of the fast inactivating component of I_{Ca}. We have previously shown that dihydropyridine agonists and cAMP-dependent phosphorylating agents, when applied alone, accelerate I_{Ca} decay as a result of the preferential enhancement of $I_{Ca,fc}$ (Richard *et al.* 1990). However, when applied in combination these drugs induce a dramatic slowing of both I_{Ca} decay and deactivating tail current (Tiaho *et al.* 1990). This is illustrated in Fig. 9*A* where the dihydropyridine agonist Bay K 8644 was applied in combination with A-kinase phosphorylating agents (C-sub was used here). Kinetic analyses revealed that the conversion of $I_{Ca,fc}$ into $I_{Ca,sc}$ can account for the change in I_{Ca} waveform (Fig. 9*A*). $I_{Ca,sc}$ was the predominant current component which resulted in the suppression of the stimulation-dependent modulation of I_{Ca} waveform (Fig. 9*B*). Thus the two agents Iso and Bay K 8644, which could favour facilitation of I_{Ca} when they were applied separately, had opposite effects when they were applied in combination. We concluded, therefore, that the stimulation or the suppression of the use-dependent facilitation of I_{Ca} by these agents was linked to changes in waveforms of macroscopic currents.

Facilitation requires Ca^{2+}

L-type cardiac Ca^{2+} channels are permeable to Ba^{2+} ions when they are used in place of Ca^{2+}. In addition to a slight increase in Ca^{2+} channel current amplitude, replacement of Ca^{2+} as the permeating cation by Ba^{2+} induced a marked

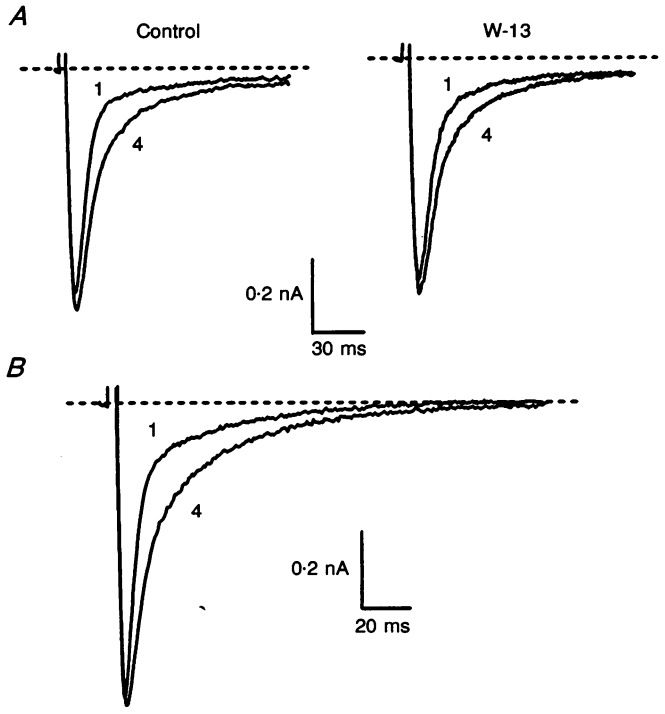


Figure 8. Effect of the calmodulin antagonist W-13 on the facilitation of I_{Ca}
The numbers next to each trace represent the sequential order of their acquisition. *A*, I_{Ca} was recorded prior to (control) and 10 min after application of 10 μM W-13 in the same cell. The frequency-dependent facilitation of I_{Ca} was not markedly altered. *B*, when the myocytes were bathed for at least 1 h in the presence of W-13, prior to recording, the frequency-dependent facilitation of I_{Ca} was still observed.

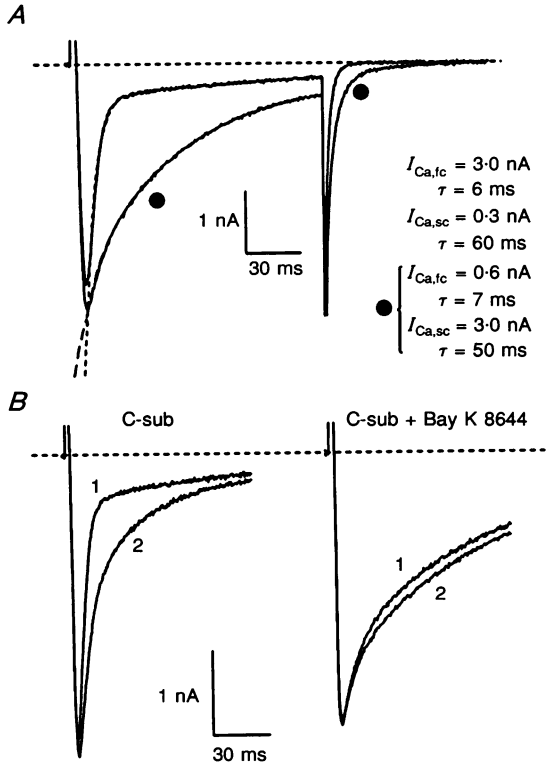


Figure 9. Combined effects of Bay K 8644 and C-sub on the facilitation of I_{Ca}
I_{Ca} was recorded in the presence of 10 μM of the catalytic subunit (C-sub) of protein kinase A (same cell as in Fig. 7*A*). *A*, the application of Bay K 8644 induced an increase in I_{Ca} (peak) amplitude and a marked slowing of both current decay and tail current (●). Steady-state effect was attained at 0·1 Hz. From double exponential fits to decay phases of currents, $I_{Ca,fc}$ and $I_{Ca,sc}$ were determined and are displayed. $I_{Ca,sc}$ was the dominant component of I_{Ca} after exposure to Bay K 8644. *B*, in the combined presence of C-sub and Bay K 8644, very little facilitation of I_{Ca} was observed upon application of a train of stimulations at a frequency of 1 Hz.

slowing of current (I_{Ba}) decay (for review, see Pelzer *et al.* 1990). Figure 10 shows that equimolar substitution of extracellular Ca^{2+} for Ba^{2+} suppressed the stimulation-dependent potentiation of Ca^{2+} channel currents. In contrast, little or no decrease (depending on pulse duration) of I_{Ba} peak amplitude was observed in all of seven cells in which facilitation of I_{Ca} was observed prior to replacing Ca^{2+} by Ba^{2+}. The facilitation of I_{Ca} was recovered when Ca^{2+} was reintroduced in place of Ba^{2+} in the extracellular perfusion (Fig. 10).

Next we recorded I_{Ca} under conditions where no EGTA was included in the recording pipette solution to test the effect of intracellular Ca^{2+} on facilitation of I_{Ca} (see Methods). In these conditions, I_{Ca} waveforms were not significantly different from those of currents recorded using EGTA in the whole I–V range (data not shown). Use-dependent facilitation of I_{Ca} could be recorded in EGTA-free conditions (Fig. 11), although it was often concealed by concomitant Ca^{2+}-dependent inactivation effects (Fedida *et al.* 1988*a*, *b*) on I_{Ca} peak amplitude as illustrated in Fig. 11*A*.

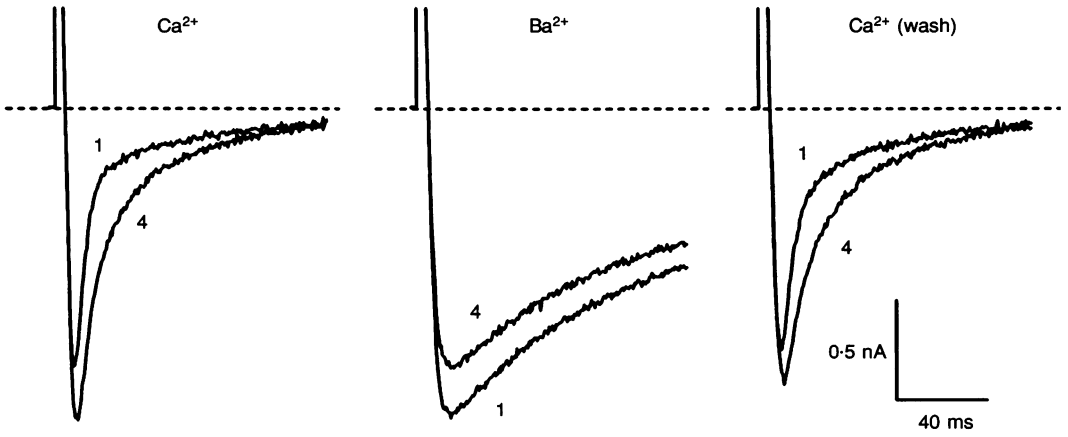


Figure 10. Ca^{2+} dependence of the facilitation of Ca^{2+} channel activity
Superimposed are currents evoked at the 1st and 4th stimulations in the presence of 1·5 mM Ca^{2+} (left trace), following exchange of external Ca^{2+} for 1·5 mM Ba^{2+} (middle trace) and after restoration of external Ca^{2+} (right trace). Note the absence of facilitation of I_{Ba}.

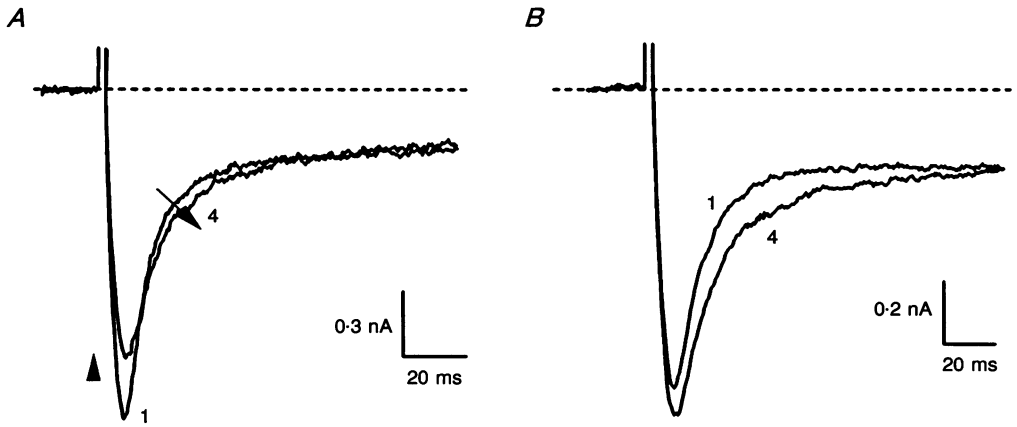


Figure 11. Facilitation of I_{Ca} using EGTA-free pipette solutions
Superimposed are currents evoked at the 1st and 4th stimulations. I_{Ca} was recorded using EGTA-free intrapipette solutions (see Methods). Use-dependent facilitation of I_{Ca} could be observed either with a concomitant decrease in I_{Ca} peak amplitude (*A*) or alone (*B*). The arrowhead signifies a decrease from 1 to 4; the arrow signifies a slowing from 1 to 4.

It is not clear whether this decrease reflected a Ca^{2+}-dependent inactivation of I_{Ca}. This result is consistent with the idea that facilitation of I_{Ca} is favoured by intracellular EGTA (Fedida *et al.* 1988*b*). Despite the fact that intracellular Ca^{2+} is certainly increased during each stimulation in EGTA-free cells, the development of facilitation was not altered in its time course. This suggests that Ca^{2+}-mediated phosphorylation is not involved.

From the above experiments, it is evident that EGTA (10 mM) helps to evidence facilitation of I_{Ca} but does not interfere with Ca^{2+}-mediated inactivation of Ca^{2+} channels as observed in current decay. This may be because the Ca^{2+} ions near the membrane are not properly buffered. BAPTA has been shown to be a faster and more efficient chelator than EGTA (Tsien, 1980; Argibay *et al.* 1988), and indeed including 20 mM BAPTA (in place of EGTA) in the

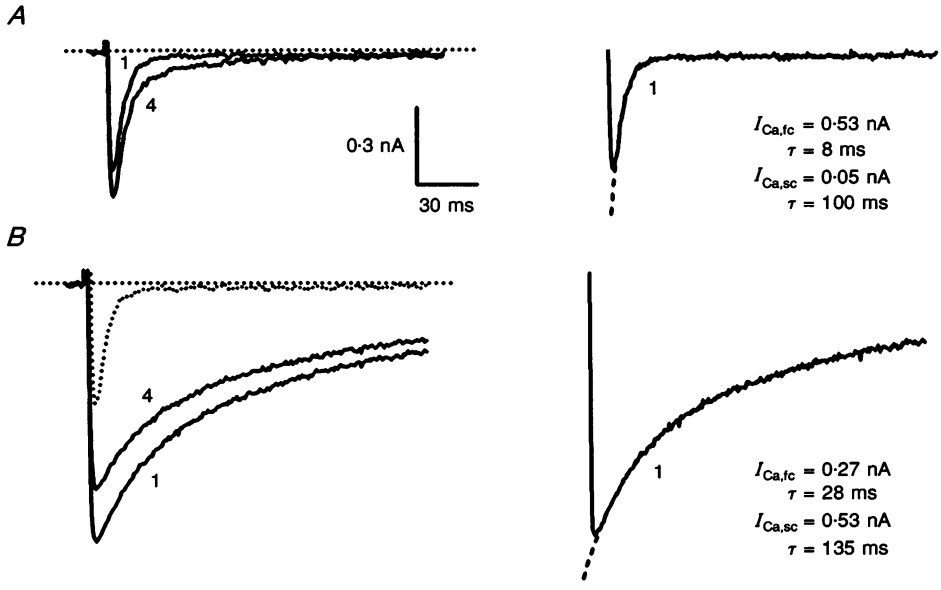


Figure 12. Facilitation of I_{Ca} using 20 mM BAPTA in the recording pipette solution
Superimposed are currents evoked at the 1st and 4th stimulations. In this cell facilitation of I_{Ca} was observed approximately 3 min after establishing the WCR configuration (*A*) but disappeared after 10 min. *B*, decay of early I_{Ca} was initially fast but a dramatic slowing occurred over the time of recording, probably due to the slow effect of BAPTA. In the same time, facilitation of I_{Ca} was suppressed. Right panel, best fits of I_{Ca} decay for the records (traces 1) shown in *A* and *B* (left panel) illustrating the change in the rate constants and in the distribution of $I_{Ca,fc}$ and $I_{Ca,sc}$.

recording pipette solution reduced the rate of inactivation and abolished facilitation of I_{Ca}. This is illustrated in Fig. 12 where, in the same cell, facilitation was detected early after establishing the whole-cell recording but abolished later, along with the marked slowing of I_{Ca} decay. BAPTA induced a marked slowing of current inactivation in all of twenty cells tested although the decay of I_{Ca} was still biexponential. Both rate constants of inactivation were altered (20 ± 6 and 111 ± 45 ms, respectively; $n = 14$) but the fast constant was more sensitive to Ca^{2+} buffering. In addition, the slow inactivating current component was predominant in ten out of fourteen cells, which indicates that there was also an alteration in the voltage-dependent distribution of $I_{Ca,fc}$ and $I_{Ca,sc}$ (for illustration see Fig. 12). Similar results were observed when [EGTA] was elevated to more than 50 mM ($n = 5$).

DISCUSSION

The frequency-dependent potentiation of I_{Ca} peak amplitude and concomitant slowing of its decay, observed at physiological Ca^{2+} concentrations in rat ventricular cells, is a voltage-dependent process and is regulated by Ca^{2+} ions and cAMP-dependent phosphorylation. Our experiments do not support the hypothesis that a Ca^{2+}-dependent phosphorylation step underlies the frequency-dependent change in the waveform of I_{Ca}.

Multiple types of facilitation?

Multiple types of use-dependent facilitation of Ca^{2+} channel activity have been described in cardiac cells. They all implicate Ca^{2+} and/or voltage and/or phosphorylation as the underlying factor(s). For example, many studies on frog (Argibay *et al.* 1988), guinea-pig (Fedida *et al.* 1988*a*, *b*; Zygmunt & Maylie, 1990; Peineau *et al.* 1992), canine (Tseng, 1988), rabbit (Hryshko & Bers, 1990) and rat (Richard *et al.* 1990, 1993) ventricular myocytes have concluded that the presence of Ca^{2+} as the charge carrier is a prerequisite. In contrast, it has also been concluded that facilitation is purely voltage dependent in guinea-pig (Lee, 1987) and in frog ventricular cells where facilitation can occur even when Ba^{2+} replaces Ca^{2+} as the charge carrier (Schouten & Morad, 1989). Facilitation in frog cardiac cells develops quite slowly indicating that a phosphorylation step may also be involved (Charnet, Richard, Gurney, Ouadid, Tiaho & Nargeot, 1991), presumably via cAMP-dependent phosphorylation (Schouten & Morad, 1989). A similar conclusion was reached in guinea-pig ventricular cells based on the observation that isoproterenol at high concentration abolishes the frequency-dependent facilitation of I_{Ca} (Fedida *et al.* 1988*b*; Zygmunt & Maylie, 1992). A Ca^{2+}-dependent phosphorylation step was proposed, consistent with the idea that Ca^{2+} may trigger a phosphorylation process in the same way as cAMP (Gurney, Charnet, Pye & Nargeot, 1989; Charnet *et al.* 1991). It has been suggested in chromaffin cells that phosphorylation is the mechanism underlying the high-prepulse- and high-frequency-induced facilitation of the L-type Ca^{2+} channel (Artelajo, Ariano, Perlman & Fox, 1990; Artalejo, Rossie, Perlman & Fox, 1992).

What is the facilitation described here?

The type of facilitation described here in rat ventricular cells is characterized by a moderate increase of peak I_{Ca} and a marked slowing of I_{Ca} decay, and reaches its steady state within 3 s. It is observed at physiological Ca^{2+} concentrations and reflects a genuine change of Ca^{2+} channel activity. The recording conditions employed limit the contribution of other ionic currents, such as voltage- or Ca^{2+}-activated K^+ currents, to the facilitation of I_{Ca} (for discussion see Richard *et al.* 1990, 1993). It is noteworthy that frequency-dependent facilitation of I_{Ca} varies with the membrane potential from which the currents are evoked, and occurs exclusively from negative V_h (Lee, 1987; Tseng, 1988; Schouten & Morad, 1989; Richard *et al.* 1990). The reason is that the rates of recovery of I_{Ca} are voltage dependent and decrease with increasing holding potentials (Hryshko & Bers, 1990; Peineau *et al.* 1992; Richard *et al.* 1993). As a result, an increase in the rate of stimulation leads to a use-dependent inactivation (i.e. a decrease in Ca^{2+} channel availability) when I_{Ca} is evoked from holding potentials more positive than -50 mV. There is wide agreement in concluding that facilitation reflects an overshoot in the reactivation of I_{Ca} (Argibay *et al.* 1988; Tseng, 1988; Peineau *et al.* 1992; Richard *et al.* 1993). However, two fundamentally divergent mechanisms have been proposed to explain this overshoot: (i) relief of the Ca^{2+}-dependent inactivation of Ca^{2+} channels through a transient decrease in $[Ca^{2+}]_i$ (Argibay *et al.* 1988; Tseng, 1988); (ii) direct activation of Ca^{2+} channel activity via a phosphorylation step (Fedida *et al.* 1988*b*; Zygmunt & Maylie, 1990) or through a voltage-operated change in gating (Lee, 1987; Richard *et al.* 1990, 1993).

Facilitation and calcium

Intracellular Ca^{2+} has multiple effects on Ca^{2+} channel activity including inactivation (Lee *et al.* 1985; Yue, Backx & Imredy, 1990*a*), inhibitory coupling between individual channels (Imredy & Yue, 1992), and potentiation in the submicromolar range (Gurney *et al.* 1989; Charnet *et al.* 1991). The frequency-dependent facilitation of I_{Ca} described here is clearly Ca^{2+} sensitive. The presence of the physiological ion Ca^{2+} is required (see also Fedida *et al.* 1988*b*; Zygmunt & Maylie, 1990; Richard *et al.* 1990, 1993; Peineau *et al.* 1992). Facilitation is altered by manipulations of both intra- and extracellular Ca^{2+} concentrations. However, the complex underlying mechanism is not just related to I_{Ca} amplitude and the amount of Ca^{2+} entry (see Fig. 3). Relief of the Ca^{2+}-dependent inactivation of Ca^{2+} channels via extrusion of intracellular Ca^{2+} ions through the Na^+–Ca^{2+} exchanger or a decrease in the release from the sarcoplasmic reticulum seems unlikely, owing to the

use of Na^+-free extracellular media and high [EGTA] in the recording pipette solution (see also Lee, 1987; Fedida *et al.* 1988; Zygmunt & Maylie, 1990; Richard *et al.* 1990, 1993). Facilitation of I_{Ca} was still observed in internal EGTA-free conditions despite possible contamination by other Ca^{2+}-dependent processes, such as Ca^{2+}-mediated inactivation, on peak current amplitude. This result is also inconsistent with the idea that facilitation is stimulated by an elevation of the cytosolic free Ca^{2+} concentration such as that originating from the sarcoplasmic reticulum. Nevertheless, we found good evidence that Ca^{2+} is poorly buffered in the microenvironment of the Ca^{2+} channels (see also Argibay *et al.* 1988; Stern, 1992). The presence of 10 mM EGTA in the recording pipette solution does not interfere with both the decay kinetics of I_{Ca} and the Ca^{2+}-sensitive facilitation of I_{Ca}. High concentrations of the fast chelating agent BAPTA ($\geqslant$ 20 mM) are required to slow the decay of I_{Ca} and abolish the frequency-dependent facilitation of I_{Ca}. These results are consistent with intracellular Ca^{2+}-dependent effects very close to, or in, the Ca^{2+} channel protein (Stern, 1992) which may promote channel phosphorylation (Fedida *et al.* 1988*b*; Zygmunt & Maylie, 1990) and/or change its gating (Lee, 1987; Yue, Herzig & Marban, 1990*b*; Richard *et al.* 1993).

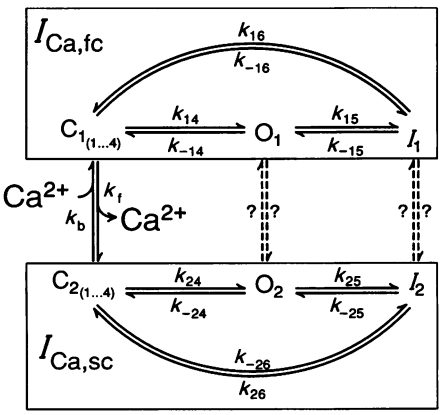
Facilitation and phosphorylation

It seems unlikely that the type of facilitation described here reflects the promotion of Ca^{2+} channel phosphorylation by Ca^{2+} for several reasons. Facilitation is not prevented by cAMP-dependent phosphorylation and is not inhibited by the Ca^{2+}-calmodulin kinase inhibitor W-13. Both the rapid change of I_{Ca} waveforms (< 3 s) and the slowing of I_{Ca} decay, observed even in EGTA-free cells, are clearly inconsistent with the slow kinetics of Ca^{2+}- or cAMP-dependent phosphorylation (Nargeot, Lester, Nerbonne & Engels, 1983; Gurney *et al.* 1989; Tiaho *et al.* 1990; Charnet *et al.* 1991) and with the effect of cAMP-dependent phosphorylation on I_{Ca} decay (Richard *et al.* 1990; Tiaho *et al.* 1990, 1993), respectively. Alternatively, our data suggest that, in addition to Ca^{2+}, cAMP-dependent phosphorylation is a prerequisite for facilitation of I_{Ca}. Facilitation is not observed in the presence of an inhibitor of A-kinase. An increase in the activity of the protein kinase A by the β-adrenergic agent Iso or inclusion in the recording pipette solution of the second messenger cAMP or its catalytic subunit (C-sub) directly could favour facilitation of I_{Ca}, indicating that phosphorylation can play a part in this mechanism via the preferential increase of $I_{Ca,fc}$.

Facilitation and I_{Ca} waveform

The presence of Ca^{2+} is required for the facilitation of Ca^{2+} channels but it is not sufficient on its own. Facilitation is also clearly conditioned by macroscopic decay kinetics of I_{Ca}. We have determined that $I_{Ca,fc}$ must be the predominant current component to allow the development of the frequency-dependent facilitation of I_{Ca}. All factors that alter the ratio $I_{Ca,fc}/I_{Ca,sc}$ markedly (i.e. the rates of interconversion between the two gating patterns) will influence the frequency-dependent facilitation of I_{Ca}. The preferential reduction of $I_{Ca,fc}$ by means of pharmacological agents such as organic and inorganic Ca^{2+} channel blockers (low concentrations), A-kinase inhibitor or after run-down prevents facilitation of I_{Ca}. When the ratio $I_{Ca,fc}/I_{Ca,sc}$ is decreased, e.g. when $I_{Ca,sc}$ is promoted by the combined presence of dihydropyridine agonists and phosphorylating agents, little or no facilitation is evident. Interestingly, the contribution of $I_{Ca,fc}$ and $I_{Ca,sc}$ to the global current can vary dramatically in the same cell. This occurs, for example, during the initial run-up of I_{Ca} (see Tiaho *et al.* 1993) where, independent of the amplitude of I_{Ca} (Fig. 3), the development of facilitation parallels the development of $I_{Ca,fc}$. Thus it seems that gating itself controls its own regulation by voltage and Ca^{2+}, with the implication that additional factors (other than Ca^{2+} and voltage) are involved which are able to influence the gating pattern of Ca^{2+} channels. There is little doubt that phosphorylation is one of them because Iso, cAMP and C-sub stimulated facilitation of I_{Ca}. This stimulation is indirect and, once again, occurs only as a consequence of the preferential increase of $I_{Ca,fc}$ (Richard *et al.* 1990; see also Fig. 7 in Zygmunt & Maylie, 1990).

Figure 13. Hypothetical Ca^{2+} channel gating scheme in rat ventricular myocytes
In this model (for details see Richard *et al.* 1993) interconversions between the two parallel gating pathways occur via the closed states of the channels ($C_{1_{(1...4)}}$ and $C_{2_{(1...4)}}$). A decrease in the voltage-dependent backward (k_b) rate constant, accounts entirely for the frequency-dependent facilitation which, in fact, reflects the over-recovery of I_{Ca} from steady-state inactivation linked to accumulation of the channels in the $I_{Ca,sc}$-related gating mode. In contrast, an increase in the voltage-dependent forward (k_f) rate constant accounts for the voltage-dependent facilitation which occurs when currents are evoked after conditioning prepulses (or at V_h values) between −90 and −50 mV. O_1 and O_2 denote open-state channels and I_1 and I_2 denote inactivated-state channels.



Facilitation and gating pathways

With regard to its Ca^{2+} dependence, the frequency-dependent facilitation of I_{Ca} here is probably different from the potentiation observed following strong prepulses, because this type of facilitation occurs even when Ba^{2+} is used as the permeating ion (Pietrobon & Hess, 1990) and it is not stimulated by β-adrenergic agents (Tiaho, Nargeot & Richard, 1991). We have previously shown in rat ventricular myocytes that $I_{Ca,fc}$ and $I_{Ca,sc}$ reflect two interconverted gating pathways operated from two different closed states (see gating scheme in Fig. 13) and leading to different inactivation rates of macroscopic I_{Ca} (Richard *et al.* 1990). These pathways have similar voltage-dependent activation and deactivation kinetics but distinct voltage-dependent reactivation rates (Richard *et al.* 1993). This gating scheme is reminiscent of, but distinct from, the observations at the single channel level that both the L-type Ca^{2+} current in guinea-pig ventricular myocytes (Hess, Lansman & Tsien, 1984; Yue *et al.* 1990*b*; Rose, Balke, Wier & Marban, 1992) and the N-type Ca^{2+} channel current in rat superior cervical ganglion neurons (Plummer & Hess, 1991) can carry two kinetically distinct components of current, one that is transient and one that is non-inactivating. However, our results were obtained at physiological Ca^{2+} concentrations and cannot be extrapolated to single channel data which require higher concentrations. In addition, we have no evidence for the presence of a non-inactivating current component. Nevertheless, in terms of slow voltage-driven interconversions between two gating pathways (Richard *et al.* 1993), our model shares some similarities with that of Plummer & Hess (1991).

The bimodal gating behaviour of voltage-dependent Ca^{2+} channels has been well established at the single channel level but little is known about their regulation (Hess *et al.* 1984; Yue *et al.* 1990*b*; Plummer & Hess, 1991; Rose, Balke, Wier & Marban, 1992). The present paper emphasizes the possibility that Ca^{2+} also plays a key role in the regulation of Ca^{2+} channel gating modes at physiological Ca^{2+} concentrations. The voltage-driven interconversion between the two gating pathways, $I_{Ca,fc}$ and $I_{Ca,sc}$, which is responsible for the facilitation of I_{Ca} (Richard *et al.* 1990, 1993), occurs via the closed states of the channels (Fig. 13). This implies that Ca^{2+} controls the switch between the closed states of the two gating pathways. This Ca^{2+}-dependent redistribution is different from the Ca^{2+}-mediated inactivation of I_{Ca} (see also the recent abstract by Imredy & Yue, 1993). The change of the inactivation time course of I_{Ca} upon an increase in the rate of stimulation is linked to accumulation of the channels in the $I_{Ca,sc}$-related gating mode (at the expense of $I_{Ca,fc}$), irrespective of the mechanims underlying current inactivation of both $I_{Ca,fc}$ and $I_{Ca,sc}$. The depolarization-driven interconversion between $I_{Ca,fc}$ and $I_{Ca,sc}$ could result from a voltage-sensitive dissociation/association of Ca^{2+} from a binding site located near the inner pore of the Ca^{2+} channel protein during depolarization and hyperpolarization, respectively. The slow transition rates of the switch between $I_{Ca,sc}$ and $I_{Ca,fc}$ (several seconds during reactivation for example: Richard *et al.* 1993) suggest a reversible, covalent modification of the Ca^{2+} channel protein. In particular, Ca^{2+} could favour a stabilizing effect on the closed states of the gating pattern corresponding to $I_{Ca,fc}$. We speculate that if phosphorylation adds a negative charge near this Ca^{2+} binding site, an increase in the affinity of Ca^{2+} for this site could explain the preferential gating of the channel in the mode related to $I_{Ca,fc}$. It will be important to design new experiments to investigate the mechanism by which Ca^{2+} controls the equilibrium between the two gating pathways of the cardiac Ca^{2+} channel.

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