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REPRIMING OF L-TYPE CALCIUM CURRENTS REVEALED DURING EARLY WHOLE-CELL PATCH-CLAMP RECORDINGS IN RAT VENTRICULAR CELLS

BY FRANÇOIS TIAHO, JOËL NARGEOT AND SYLVAIN RICHARD*

From the Centre de Recherches de Biochimie Macromoléculaire, CNRS UPR 8402, INSERM U 249, Route de Mende, BP 5051, 34033 Montpellier Cedex, France

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SUMMARY

1. The establishment of the whole-cell patch-clamp recording configuration (WCR) revealed a type of inhibition to which L-type Ca^{2+} channels were subject in static rat ventricular myocytes before obtaining the WCR.

2. Immediately after membrane disruption (< 10 s), the Ca^{2+} current (I_{Ca}) was absent but gradually increased in amplitude to reach its final waveform (amplitude and kinetics) 2–3 min after the WCR was reached.

3. Three distinct phases (P) were identified. First, no inward but an outward current, blocked (1–2 min) by Cs^+ dialysing from the patch pipette (P1), was recorded. Second, overlapping with (P1), I_{Ca} increased dramatically to reach a maximum peak amplitude within 2–3 min (P2). Concomitantly, its rate of decay, initially monoexponential and slow, became biexponential owing to the appearance of a fast component of inactivation (P3). Complete interconversion between slow and fast components sometimes occurred.

4. Changes in current waveform were not related to voltage loss or series resistance variation, and suppression of an outward current (P1) was unlikely to account for P2 and P3.

5. The run-up of I_{Ca} was independent of the nature of the permeating ions, the membrane holding potential, depolarization, rate of stimulation, the intracellular Ca^{2+}, ATP, Mg^{2+}, Cs^+ and the pH of the pipette solution. Since large Ca^{2+} currents were recorded using the perforated patch technique, the run-up of I_{Ca} is not explained by the wash-out of an inhibitory endogenous macromolecule during cell–pipette exchanges.

6. Pharmacological manipulations, including the use of Ca^{2+}–Ba^{2+}–EGTA and exposure of the cells to isoprenaline and/or Bay K 8644 prior to recording, did not alter the mechanism primarily responsible for build-up. Unrepriming of channel activity was required before these modulations could be effective.

7. Currents could however be instantly augmented when cells were extracellularly superfused during the run-up step. The wash-out of an inhibitory agent originating in the cell itself (such as H^+, NH_4^+ and lactate) and accumulating in the extracellular microenvironment of the cells seems unlikely. Rather, we suggest that pressure-

* To whom correspondence should be addressed.

induced mechanostimulation may be involved in the restoration of Ca^{2+} channel activity.

INTRODUCTION

The patch-clamp techniques (Hamill, Marty, Neher, Sakmann & Sigworth, 1981) have been extensively developed to study ionic channels and their regulation by neurotransmitters, hormones, drugs and intracellular second messengers (for review see Marty & Neher, 1983; Sakmann & Neher, 1984; Neher, 1988). The 'tight-seal whole-cell recording configuration' or, in short WCR (Marty & Neher, 1983), was originally used on chromaffin cells (Hamill *et al.* 1981) but, with concomitant development of single-cell technology, the technique was rapidly applied to an increasingly larger variety of cell types. It provides several advantages, such as accurate measurement and facilitated statistical analysis of ionic currents, including rapidly settling and spatially uniform voltage clamp, manipulation of ionic solutions on both sides of the membrane, and improved elimination of contaminating currents. However, as a debt to these advantages, the major problem arising from pipette–cell exchanges seems to be the wash-out of diffusible intracellular factors which may be involved in survival and stability of channel activity (Pusch & Neher, 1988). For instance, the amplitude of the L-type Ca^{2+} current slightly, but irreversibly, decreases during long periods of recording. This loss of Ca^{2+} currents, termed 'run-down' (for review see Pelzer, Pelzer & McDonald, 1990) has still not been elucidated.

Voltage-gated dihydropyridine-sensitive (L-type) Ca^{2+} channels are present in a variety of cell types. In cardiac cells they play a pivotal role in the regulation of excitability and contractility (for review see Hartzell, 1988; Pelzer *et al.* 1990). As many other channels, Ca^{2+} channels respond primarily to membrane depolarization by opening in a steeply voltage-dependent manner. However, their modulation by phosphorylation is a fundamental regulatory mechanism which has profound physiological consequences. The best known example is the sympathetic stimulation, which produces dramatic increases in the rate and force of the heart beat. This modulation involves primarily a receptor-mediated elevation of intracellular cAMP level, resulting in the stimulation of protein kinase A and protein phosphorylation (for review see Tsien, Bean, Hess, Lansman, Nilius & Nowycky, 1986; Hartzell, 1988; Pelzer *et al.* 1990). L-type Ca^{2+} channels are also subject to regulation by a variety of exogenous synthetic ligands termed Ca^{2+} channel antagonists/agonists, of which dihydropyridines are the most specific.

In this paper, we show that Ca^{2+} channels in static cells are strongly reprimed as revealed immediately after establishment of the WCR configuration. Immediately after breaking into the cell, the Ca^{2+} current (I_{Ca}) was absent but gradually increased in amplitude to reach its final waveform within 2–3 min. Not only was the basal activity enhanced, but there was also a dramatic alteration of channel gating. This down-regulation of Ca^{2+} channel activity was not altered by exposure of the static cells to known modulators (voltage, Ca^{2+}, cAMP-dependent phosphorylation) prior to the WCR, and unrepriming was required before these modulations could be effective. Although the mechanism involved is a matter for speculation, we discovered that extracellular perfusion provides a unique means for a rapid

restoration of Ca^{2+} channel activity which may open the new perspective that mechanostimulation is a way of regulating Ca^{2+} channel activity.

Preliminary accounts of this work have appeared as an abstract (Richard, Tiaho & Nargeot, 1991).

METHODS

Experiments were performed on single ventricular myocytes freshly dissociated from 6- to 10-week-old rats after collagenase treatment. Animals were killed by cervical dislocation and the heart was quickly removed. Cells were isolated using a method similar to that described in detail by Wittenberg, White, Ginzberg & Spray (1986). Currents were recorded at 20–22 °C, 2–10 h after cell dispersion. The waveforms of the currents through voltage-activated Ca^{2+} channels were measured using the whole-cell clamp variation of the patch-clamp technique under conditions optimized to eliminate contaminating voltage-gated inward Na^+ (I_{Na}) and outward K^+ (I_K) currents (see Richard, Tiaho, Charnet, Nargeot & Nerbonne, 1990; Tiaho, Richard, Lory, Nerbonne & Nargeot, 1990). Ca^{2+} currents (I_{Ca}) were recorded in standard bath conditions containing (mM): TEACl 140, $CaCl_2$ 1·5, $MgCl_2$ 2, Hepes 10, glucose 10, and tetrodotoxin (TTX) 0·001 with pH adjusted to 7·2 using TEAOH, unless otherwise noted. To measure the waveforms of Ba^{2+} currents (I_{Ba}) through Ca^{2+} channels in the same conditions, 1·5 mM $BaCl_2$ replaced $CaCl_2$. Na^+ currents through Ca^+ channels ($I_{Na,ns}$) were recorded in the absence of divalent cations (Ca^{2+} and Mg^{2+}) and presence of EDTA (5 mM). K^+ currents were recorded using K^+ (instead of Cs^+) ions in the recording pipette and with NaCl instead of TEACl in the standard extracellular solution. They were isolated from Ca^{2+} currents in the presence of inorganic Ca^{2+} channel blockers (2 mM Co^{2+} or Ni^{2+}). 4-Aminopyridine (10 mM) was employed in some of the experiments in order to block the transient outward K^+ current.

Recording pipettes were constructed from the microhaematocrit tubes (modulohm I/S, Herlev, Denmark) and filled with the following standard solution (mM): CsCl 140, EGTA 10, Hepes 10, MgATP 3, MgGTP 0·4, adjusted to pH 7·2 with CsOH, unless otherwise noted. The pipette resistance ranged from 3–5 MΩ after filling with the recording solution. Junction potentials between the intrapipette solution and the reference electrode were zeroed before obtaining the WCR. Currents were recorded using a Biologic RK300 amplifier. After seal formation (resistance > 1 GΩ) and membrane disruption, series resistances were electronically compensated by 80% as previously described in detail (Tiaho *et al.* 1990) and the remaining series resistance was estimated from the decay of the capacitative transients evoked by a ±10 mV test pulse. Data acquisition and analyses were performed using the pCLAMP software (Axon Instruments, Burlingame, CA, USA). Sampling frequencies ranged from 1 to 40 kHz.

In order to study the changes occurring in current waveforms immediately after establishing the WCR, the holding potential (HP) was set before disrupting the cell membrane and the first stimulation was applied after a delay estimated to be less than 10 s. In the kinetic plots, this delay was ignored and the time of the first stimulation was taken as the origin of the time axis. The numbers quoted for each original data trace (or point in the *X–Y* curves) represent their sequential acquisition number (order) in a series of records. Ca^{2+} channels currents were routinely recorded at a test pulse (TP) of −10 mV delivered from a HP of −80 mV every 15 s (unless otherwise noted in particular experiments). Inward peak currents were measured as the difference between the maximal inward current amplitude and the zero current level (Tiaho *et al.* 1990). Current inactivation 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 $I(t)$ is the current at time t, $I_{Ca,fc}$ and $I_{Ca,sc}$ are the amplitudes and τ_{fc}, 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). The quality of the fits was determined based on excellent visual superimposition with original traces and selection of the best correlation coefficients (r). These procedures have been described in detail previously (Richard *et al.* 1990). Results are expressed as means ± S.D. Statistical analysis was performed using a binomial test based on the sign of the difference between paired samples.

For perforated patch experiments, 300 μg/ml amphotericin (A4888, Sigma) was added to the standard pipette solution and employed as previously described (for review, see Rae & Fernandez,

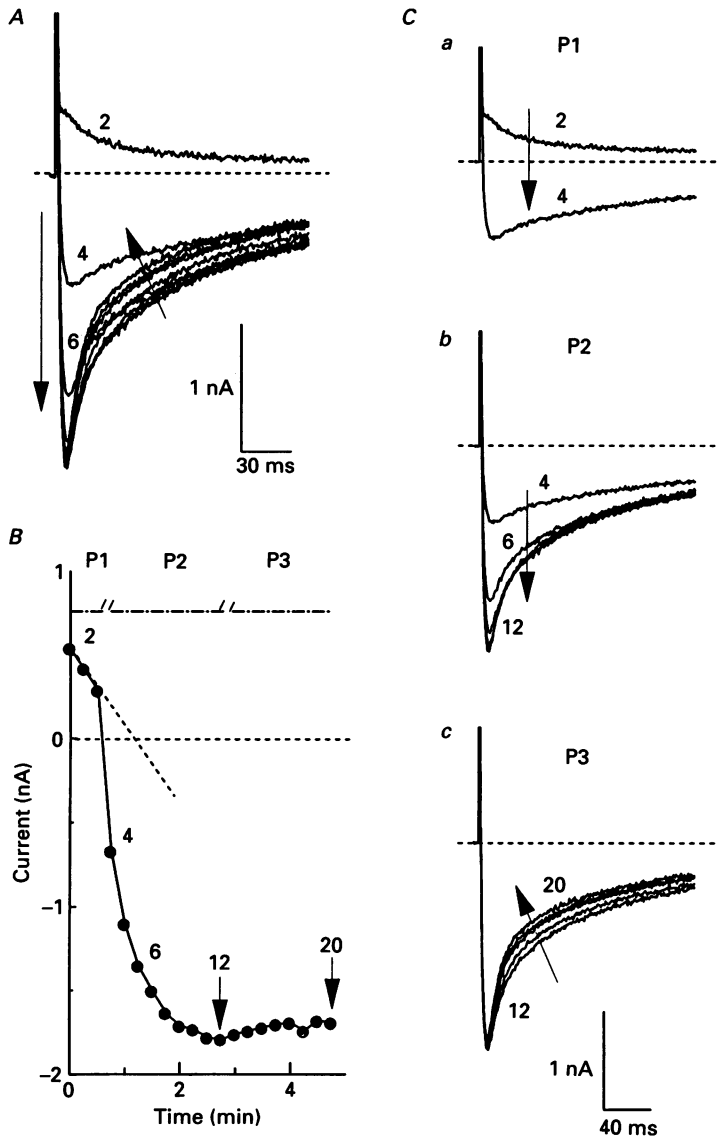

Fig. 1. Run-up of I_{Ca} in a rat ventricular myocyte. Currents were recorded every 15 s at a test depolarization of −10 mV from a HP of −80 mV. The numbers above each trace (or point in the curve) represent the sequential order of their acquisition in the series (see Methods). *A*, increase of I_{Ca} and changes in current waveform occurring during the first minutes after establishing the whole-cell recording configuration. First stimulation, applied at most 10 s after seal disruption, evoked an outward current (trace 2). Subsequent depolarizations evoked inward currents from which peak amplitudes increased and inactivation time course became faster with the time of recording. Currents displayed were evoked every 30 s. *B*, complete kinetic of the build-up of I_{Ca} peak amplitude plotted as a function of time. Three sequential phases have been distinguished: suppression of an outward current (P1), increase of I_{Ca} peak at steady state (P2) and acceleration of I_{Ca} decay determining final current waveform (P3). Note the small decrease in I_{Ca} peak amplitude during P3. *C*, original records corresponding to P1, P2 and P3. Note

1991). The pipette tip was back-filled with intracellular saline solution without amphotericin B before adding the amphotericin-containing solution. When a GΩ-seal had formed in the cell-attached configuration, the decrease in access resistance of the cell-patch assembly was monitored by recording the capacitive transients evoked by a +10 mV prepulse prior to recording I_{Ca}. The access resistance gradually decreased to less than 20 MΩ (16±3 MΩ, $n = 4$) within 2–5 min. Then series resistances were electronically compensated as described above.

Isoprenaline (Iso) (Sigma), was prepared daily as a 1 mM stock solution in H_2O. Bay K 8644 (Bayer AG, Wuppertal 1, FRG) was 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 effects on Ca^{2+} channel currents at the final dilutions used here (< 0·005%). L-(+)-lactic acid and NH_4OH (Sigma) were prepared directly in the extracellular solution and the pH was adjusted to 7·2. Extracellular superfusion of the cells with control and test solutions was performed using 300 μm inner diameter capillary tubing (flowing rate 0·2 ml/min) 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 a second).

RESULTS

Increase of Ca^{2+} current during early whole-cell recordings

Figure 1 illustrates the dramatic changes in the waveform of voltage-activated Ca^{2+} currents (I_{Ca}) evoked in a static isolated rat ventricular cell immediately after establishing the WCR (see Methods). I_{Ca} was evoked every 15 s at a test pulse of −10 mV from a HP of −80 mV. An outward current was observed following the first stimulations (1–3) but subsequent depolarizations evoked inward currents from which peak amplitudes increased apparently from nearly zero and inactivation time course became faster with time (Fig. 1*A*). I_{Ca} reached its maximum amplitude after 2 min (116±24 s, $n = 17$) as exemplified in Fig. 1*B*. In the same time, the time course of I_{Ca} decay, which was slow during initial stimulations, became faster (Fig. 1*A* and *C*). Although this second effect superimposed on the increase of I_{Ca} peak amplitude for a part, it required more time (2–5 min) to reach steady state (Fig. 1*B* and *C*). Thus, during the establishment of the final waveform of I_{Ca}, which we termed 'build-up' (Richard *et al.* 1991), three sequential phases can be distinguished (Fig. 1*B* and *C*): apparent suppression of an outward current (P1), increase of I_{Ca} peak steady state (P2) and acceleration of I_{Ca} decay (P3). Interestingly, I_{Ca} decay was slow and best fitted by a single exponential during P1–P2 but became faster and clearly biexponential, with time constants separated by approximately an order of magnitude, after it reached its final waveform (P3, Fig. 1*Cc*). In most cells final I_{Ca} waveforms comprise both the fast and the slow components of currents denoted by these time constants. However, an extreme situation was sometimes observed. As illustrated in Fig. 2*A*, best fits of I_{Ca} decay (see Methods) revealed that I_{Ca} can switch from an exclusive slow monoexponential ($\tau_{sc} = 75$ ms) to an exclusive fast monoexponential ($\tau_{fc} = 7{\cdot}5$ ms) time course (traces 5 and 15 in Fig. 2*B*). An intermediate step where I_{Ca} comprised the two current components (trace 9) was however observed between these two extreme situations. This behaviour was favoured when cAMP was included in the recording pipette or after pre-exposure to

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that the decay of I_{Ca}, which is initially monoexponential with a slow rate constant (τ_{sc} = 76 ms) at the beginning of P2, becomes clearly biexponential during P3 with the appearance of a fast rate constant ($\tau_{fc} = 7$ ms).

isoprenaline. After this step, I_{Ca} generally remained constant for several minutes at negative HPs and then decreased slowly but irreversibly with time (data not shown), as previously described in various tissues (run-down).

Run-up is independent of the nature of the permeating ion

Figure 3 shows the run-up of I_{Ba} and $I_{Na,ns}$ (non-specific Ca^{2+} channel current) which occurred immediately after establishing the WCR, when Ba^{2+} (Fig. 3*A*) and

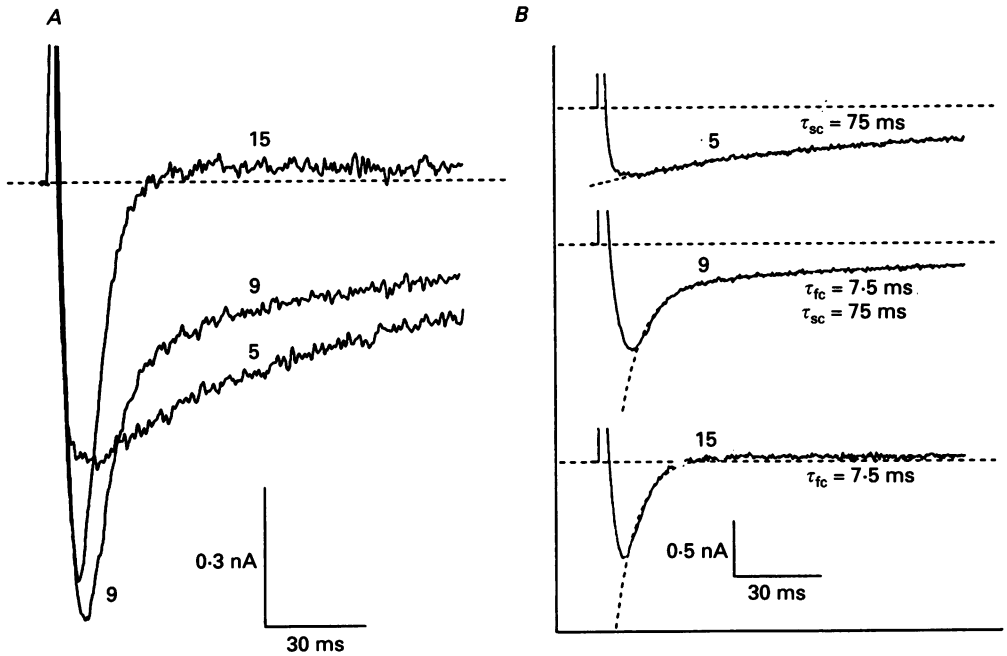

Fig. 2. Change in the distribution of two distinct components of I_{Ca} during current run-up. *A*, original records of I_{Ca} evoked at time $\approx$ 60, 120 and 210 s (traces 5, 9 and 15, respectively) after establishing the WCR configuration in the same recording conditions as in Fig. 1*A*. Note the early appearance of a slow component of I_{Ca} (trace 5, P2) which precedes a dramatic acceleration of current decay with the time of recording (traces 9 and 15, P3). *B*, best fits of I_{Ca} decay for the three records shown in *A*, illustrating a dramatic change in the distribution of the slow and fast current components of I_{Ca} during the build-up. τ_{sc} was 75 ms for both traces 5 and 9. τ_{fc} was 7·5 ms. In this example, I_{Ca} switched from exclusively slow (trace 5, P2) to exclusively fast (trace 15, P3) with an intermediate step (trace 9).

Na^+ (Fig. 3*B*) ions respectively were employed as charge carriers (see Methods). The first depolarization generally revealed an outward current of moderate amplitude (trace 1) but subsequent test pulses evoked inward currents during which peak amplitudes increased over time. I_{Ba} and $I_{Na,ns}$ reached their maximal amplitude after 126 ± 20 s ($n = 15$) and 174 ± 49 s ($n = 5$), respectively. The kinetics of increase were not significantly different between the two ions and with that of I_{Ca}. I_{Ba} and $I_{Na,ns}$ had phases P1 and P2, but P3 was apparently absent. No marked acceleration of

current decay was observed. Instead, a slight slowing was observed after currents had reached their maximal amplitude i.e. during a phase corresponding to P3 for I_{Ca}.

Run-up is not an artifact of unresolved membrane potential

It was crucial to determine whether the apparent build-up of Ca^{2+} channel currents was artifactual (change in voltage control or series resistance). Figure 4*A* shows the

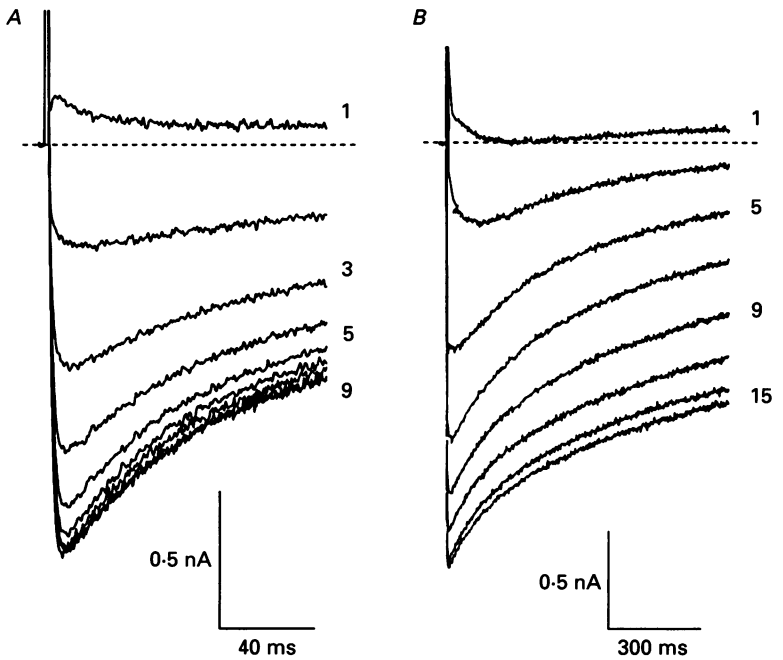

Fig. 3. Run-up of I_{Ba} and $I_{Na,ns}$. *A*, increase of I_{Ba} after establishing the WCR configuration. I_{Ba} was recorded in the same conditions as in Fig. 1*A*, except that Ba^{2+} replaced Ca^{2+} in the bath. First stimulation evoked an outward current (upper trace). Subsequent depolarizations evoked inward currents from which peak amplitudes increased with time. Steady-state I_{Ba} peak amplitude was obtained after 2 min. *B*, increase of $I_{Na,ns}$ after establishing the WCR configuration. $I_{Na,ns}$ was recorded every 15 s at a test depolarization of −30 mV from an HP of −80 mV with no Ca^{2+} or Mg^{2+} ions but with Na^+ and EDTA in the extracellular bath (see Methods). First stimulation evoked an outward current (upper trace). Subsequent depolarizations evoked inward currents from which peak amplitude increases with time. Steady state was obtained after 2 min.

variation of peak amplitude of an outward K^+ current (I_K) evoked at +60 mV from HP −80 mV during the first 3 min after breaking into the cell. The stimulation protocol employed was essentially the same as for I_{Ca} (or I_{Ba} and $I_{Na,ns}$) but I_K was recorded using K^+ instead of Cs^+ in the pipette solution, and with the Ca^{2+} blocker Ni^{2+} added to the extracellular solution (see Methods). In contrast to what has been described above, a large outward K^+ current was recorded quasi-instantly (Fig. 4*A a*), whose waveform remained relatively stable upon subsequent depolarizations

(Fig. 4*A b*). A similar behaviour was observed for five other cells. One implication is that I_K does not undergo a similar run-up to I_{Ca}, which was a property also shared by the fast inward I_{Na} (data not shown). Another implication is that the voltage clamp (HP, TP) of the cell membrane did not vary during the course of the experiments.

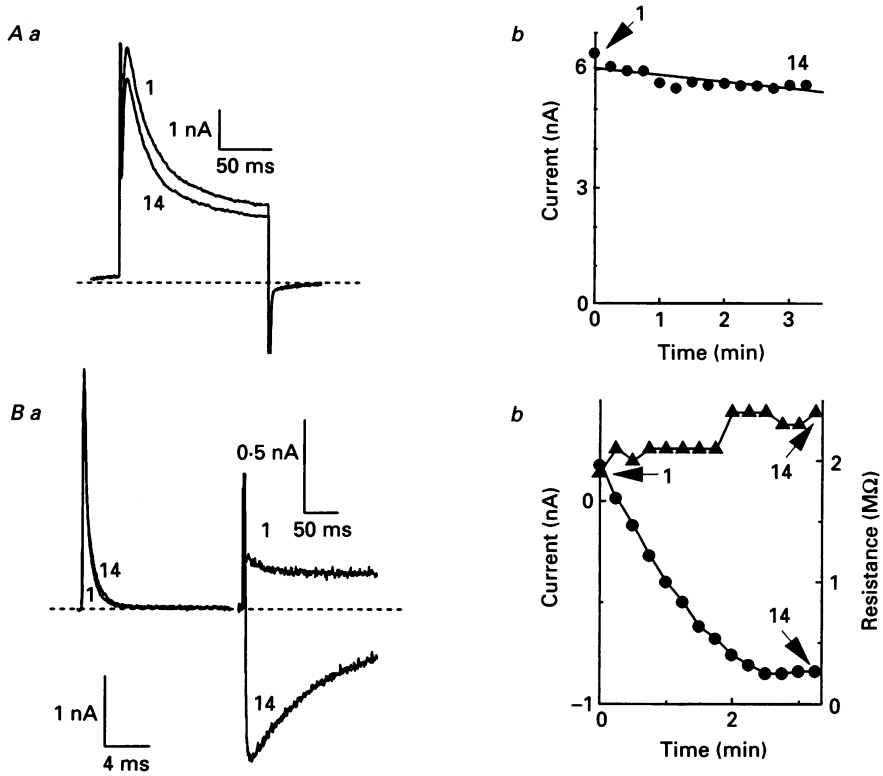

Fig. 4. Run-up is not related to unresolved membrane potential. *A*, maximal variation of I_K peak amplitude ($\approx 10\%$) after establishing the WCR configuration. I_K was recorded every 15 s at a test depolarization of +60 mV from a HP of −80 mV. The recording pipette contained KCl instead of CsCl. The bath contained the standard recording solution (see methods but with NaCl instead of TEACl) in which 2 mM Co^{2+} was added to block I_{Ca}. *A a*, original records. The upper trace shows I_K evoked by the first stimulation. The lower trace shows I_K recorded more than 3 min later (*14th* stimulation). *A b*, complete kinetic of the variation of I_K peak amplitude as a function of time after seal rupture. Note that in contrast to I_{Ca}, I_K is maximal at the first stimulation. Note also the minor decrease in peak current amplitude suggesting that the voltage clamp is stable. *B*, independence between series resistance variation and the build-up of I_{Ba}. I_{Ba} was recorded as described in Fig. 3*A*. *B a*, original records of the capacitive transients evoked by a +10 mV prepulse (left) delivered prior to recording I_{Ba} (right). Currents shown were recorded at the first stimulation (upper trace) and 3 min later (lower trace). No change was observed in the transient capacitive current during the 'build-up' of I_{Ba}. *B b*, complete kinetic of the variation of both series resistance and I_{Ba} peak amplitude during the first minutes after establishing the WCR configuration. ● represents I_{Ba} peak and ▲ represents the calculated series resistance.

We have also examined whether an error in current amplitude resulting from a variation in the uncompensated ohmic drop across the series resistance (see Methods) could account for the build-up of Ca^{2+} channel current. Figure 4*B* shows that the slight variations, which may occur during the course of an experiment, cannot explain the large variation in current amplitude. For instance in Fig. 4*Bb*, where the variation of the series resistance was less than 0·5 MΩ, the resulting voltage error for a current of 2 nA would be 1 mV. Similar results were found in six other cells. We also excluded the possibility that the run-up reflects a shift in the voltage-dependent activation of Ca^{2+} channels which might occur during intracellular dialysis of the myocytes owing to a slow gradual drift in junction potential between pipette solution and cell interior (see Marty & Neher, 1983). This drift is approximately $-2{\cdot}0 \pm 2{\cdot}2$ mV per 15 min in these cells (Tiaho *et al.* 1991). In addition, the 'build-up of I_{Ca} occurred for all test potentials activating I_{Ca} (data not shown).

Run-up is unrelated to Cs^+ block of K^+ currents

The first stimulation, applied less than 10 s after establishing the WCR, often reveals an outward current. We tested whether the apparent increase in I_{Ca} could alternatively reflect the slow inhibition of outward K^+ currents (I_K) following intracellular dialysis with Cs^+ ions. Figure 5*A* shows a typical family of K^+ currents recorded as described above (see also Methods) for increasing TPs between -30 to $+90$ mV from HP -80 mV. These currents activated above -20 mV and their peak amplitude increased monotonically with voltage. As evidenced in the figure, the TP of -10 mV, which was routinely used to evoke maximal I_{Ca}, nearly corresponds to the activation threshold of I_K. At this voltage, and in the most favourable conditions (K^+ in the pipette, Ca^{2+} channel blockers in the bath), the outward K^+ currents had only moderate amplitude: I_K peak ranged between 100–500 pA (mean 287 ± 114 pA, $n = 9$), which seemed unlikely to account exclusively for the large increase of I_{Ca} observed during the build-up (> 1 nA in most cells). Figure 5*B* shows the suppression by Cs^+ of an outward current recorded immediately after establishing the WCR in the presence of extracellular Ni^{2+} (Ca^{2+} channel blocker). In this cell, the current was half-blocked after 30–45 s, and suppressed after 75 s (Fig. 5*B* and *C*). The mean time needed to achieve suppression was 96 ± 17 s ($n = 5$). Complete block was faster (30 ± 18 s, $n = 5$) (with Ba^{2+} instead of Ca^{2+}. Thus suppression of outward currents (phase P1) apparently occurs prior to the increase of Ca^{2+} channel currents (phase P2). In addition, not only did P2 seem different from P1, but the acceleration of inward current (I_{Ca}) decay during P2–P3 was inconsistent with the suppression of a transient outward K^+ current.

The hypothesis that I_{Ca} increases because I_K decreases owing to Cs^+ block was finally excluded after considering the following results. (i) The run-up of I_{Ca} occurred even when KCl was used (instead of CsCl) in the pipette solution. An outward K^+ current was recorded instantly after obtaining the WCR but its waveform was gradually altered upon subsequent depolarizations owing to the development of a predominent inward current (Fig. 6*A*) which, based on the observation that I_K is not subject to build-up (see Fig. 4*A*), was identified as I_{Ca}. Similar behaviour was observed for five other cells. (ii) In the presence of 4-aminopyridine (10 mM), which is a potent blocker of the transient outward current at this concentration (Apkon &

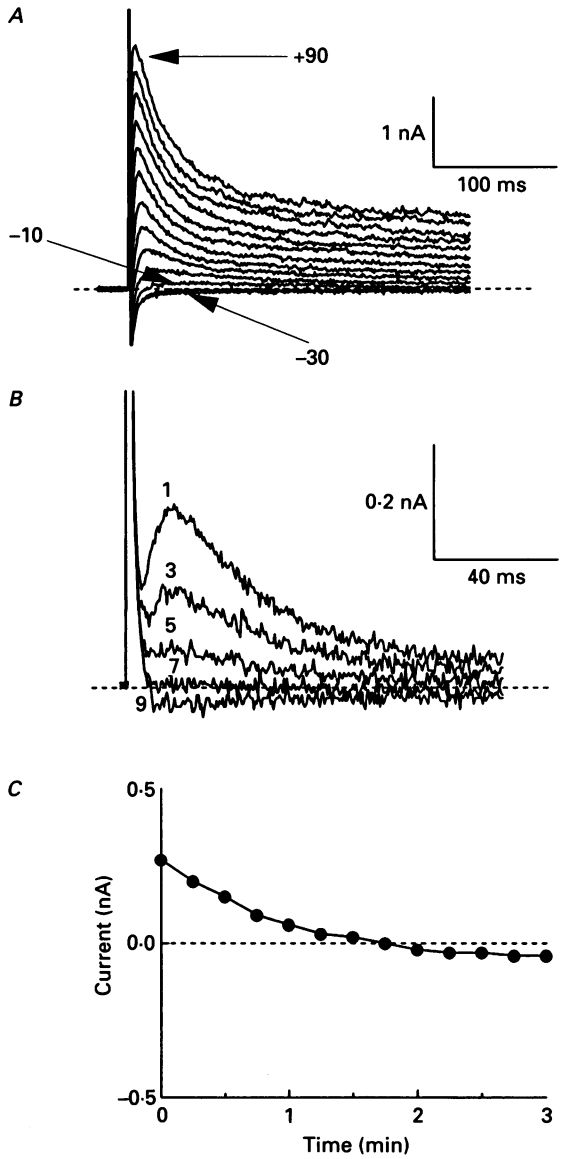

Fig. 5. Suppression of an outward current by Cs^+ during dialysis (P1). *A*, a family of outward K^+ currents (I_K) evoked between −30 and +90 mV from a HP of −80 mV. The recording pipette contained KCl instead of CsCl. The bath contained the standard recording solution (see Methods, but with NaCl instead of TEACl) in which 2 mM Co^{2+} was added to block I_{Ca}. Note that I_K begins to activate at ≈ -10 mV. The peak inward currents are Na^+ currents not blocked by TTX. *B*, suppression of an outward current after establishing the WCR configuration. Currents were evoked every 15 s at −10 mV from a HP of −80 mV as in Fig. 1*A* (standard conditions), i.e. with Cs^+ in the recording pipette but with 2 mM Ni^{2+} added in the extracellular bath to block I_{Ca}. Note that 50% of the current was blocked within ≈ 30 s (trace 3). *C*, kinetics of suppression of the early outward current. Current amplitudes are plotted as a function of the time of recording. Peak currents were measured between maximal outward current amplitude and current at the end of the 125 ms test pulse.

Nerbonne, 1991), build-up of I_{Ca} was still observed (Fig. 6*B*) A contribution of Na^+–Ca^{2+} exchange to I_{Ca} was unlikely owing to the use of extracellular TEACl (instead of NaCl). Involvement of Cl^- currents was also excluded (data not shown) using Cl^--free extra- and intracellular solutions (caesium aspartate instead of TEACl

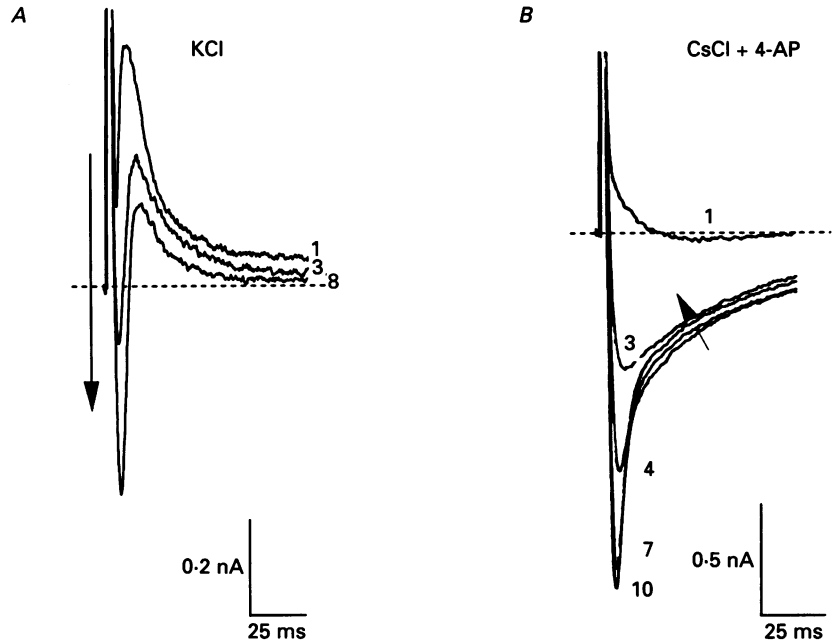

Fig. 6. Run-up is unrelated to Cs^+ block of K^+ current. *A*, increase of I_{Ca} and changes in current waveform occurring after establishing WCR in the same conditions as in Fig. 4*A* (KCl instead of CsCl in the solution filling the patch pipette and NaCl instead of TEACl in the extracellular bath). Currents were evoked every 15 s at −10 mV from a HP of −60 mV (to ensure suppression of Na^+ currents). First stimulation was applied at most 10 s after seal rupture (trace 1). Also displayed are currents recorded 30 s (trace 3) 1 min 30 s (trace 8) later. *B*, increase of I_{Ca} and changes in current waveform occurring during the first minutes after establishing the WCR configuration in the same conditions as in Fig. 1, but with 10 mM 4-AP added to the bath. Currents were recorded every 15 s at a test depolarization of −10 mV from a HP of −80 mV (see Methods). First stimulation was applied at most 10 s after seal rupture (trace 1). Currents displayed were evoked 30 s (trace 3), 45 s (trace 4), 1 min 30 s (trace 7) and 2 min 15 s (trace 10) later.

and CsCl, $Ba(OH)_2$ instead of $BaCl_2$, $Mg(OH)_2$ instead of $MgCl_2$). At this point in our study, we concluded, therefore, that the build up of I_{Ca} is related to the enhancement of Ca^{2+} channel activity during P2 and P3.

Run-up is independent of HP level and stimulation

We examined whether the build-up of I_{Ca} reflects a voltage-dependent recovery of inactivated Ca^{2+} channels, based on the possibility that the cell membrane could be depolarized prior to obtaining the WCR. Voltage-dependent inactivation normally occurs positive to −50 mV in these cells (Richard *et al.* 1990). Thus we tested the

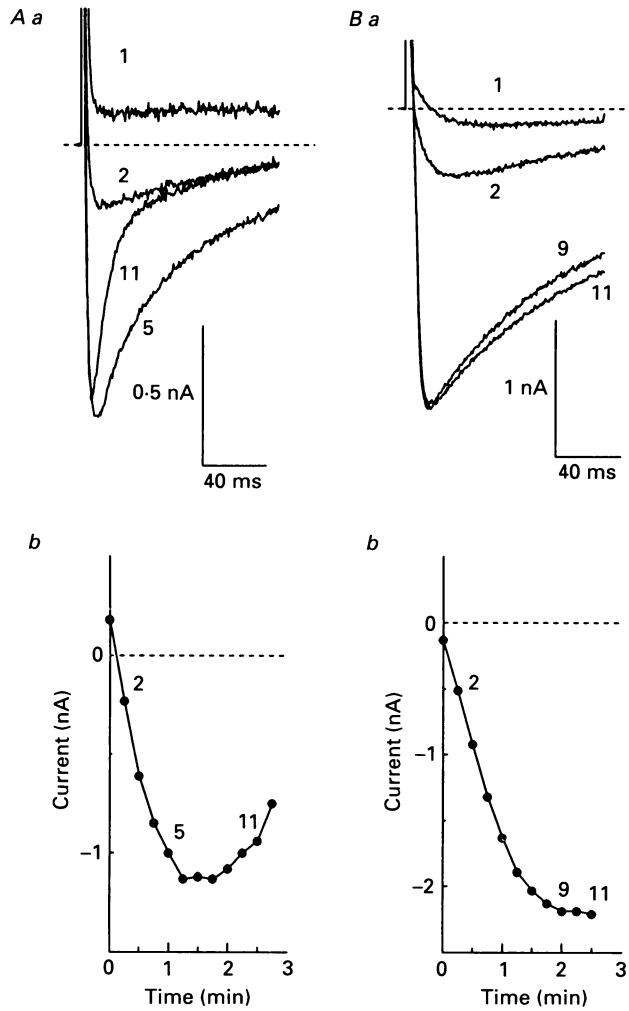

Fig. 7. Run-up is independent of the HP. *A*, build-up of I_{Ca} at HP −40 mV. The recording protocol was the same as in Fig. 1 except for the HP level. *Aa*, original records of I_{Ca} evoked ≈ 0, 15, 75 and 165 s (first, second, fifth and eleventh stimulations, respectively) after establishing the WCR configuration. Note the similarity with Fig. 1*A*, i.e. there is build-up of I_{Ca} from which peak amplitude increases with time and inactivation time course becomes faster with time. *Ab*, complete kinetic of the build-up of I_{Ca} peak. Note that I_{Ca} rapidly reached an apparent maximal amplitude (≈ 1 min; compare with Fig. 1*A*), due to the voltage-dependent run-down that overlaps with the build-up and leads to the rapid decrease of I_{Ca} peak amplitude. *B*, build-up of I_{Ba} at HP −40 mV. The recording protocol was the same as in Fig. 2*A* except for the HP level. *Ba*, original records of I_{Ba} evoked ≈ 0, 15, 135 and 165 s (first, second, fifth and eleventh stimulations, respectively) after establishing the WCR configuration. Note the similarity with Fig. 2*A*. Note the slight slowing of current decay between traces 9 and 11. *Bb*, complete kinetic of the build-up of I_{Ba} peak amplitude.

build-up of I_{Ca} recorded from depolarized HPs (> −50 mV). Figure 7*A* and *B* shows that the build-up of Ca^{2+} channel currents recorded from HP −40 mV with either Ca^{2+} or Ba^{2+} resembles those observed from HP −80 mV. I_{Ca} and I_{Ba} reached their

maximal amplitude within 131 ± 12 s ($n = 4$) and 148 ± 40 s ($n = 8$), respectively. Current waveforms were altered similarly as from HP -80 mV. For instance, the three phases P1, P2 and P3 distinguished for I_{Ca} were observed, despite the rapid decrease of the fast component of current that rapidly occurred at this HP. Build-

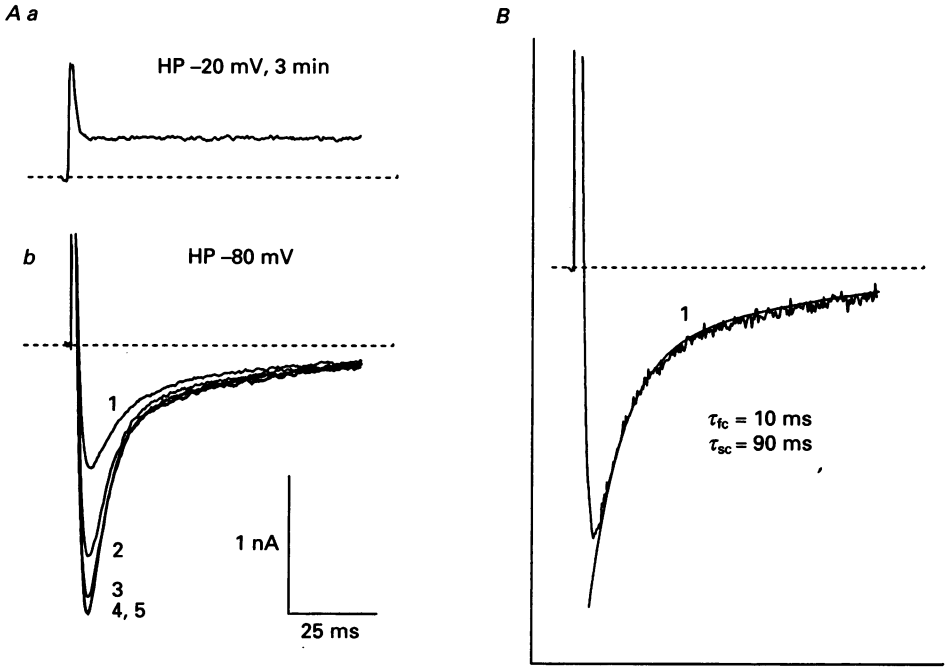

Fig. 8. Run-up of I_{Ca} at HP -20 mV. *A*, the WCR was established at the HP of -20 mV. No inward current was recorded 3 min after breaking into the cell (top panel). Bottom panel shows recovery of I_{Ca} immediately after the cell membrane was hyperpolarized to -80 mV. Currents displayed were evoked immediately (delay < 10 s, trace 1), 15 s (trace 2), 30 s (trace 3), 45 s (trace 4) and 1 min (trace 5) later. *B*, best fits of I_{Ca} decay, for I_{Ca} shown in trace 1. Current decay was biexponential. τ_{fc} and τ_{sc} were 10 ms and 90 ms, respectively.

up of I_{Ca} was also observed at HP -30 mV (only P2, data not shown) but not at -20 mV, owing to the complete unavailability of Ca^{2+} channels at this voltage (Richard *et al.* 1990). When the cell membrane was maintained at -20 mV for 3 min (which is long enough to achieve steady-state build-up) and then held at the negative HP of -80 mV, I_{Ca} generally recovered within less than 1 min (Fig. 8*A*) i.e. with a shorter delay than during build-up. In addition, current decay was immediately (< 10 s) biexponential (Fig. 8*B*) which suggests that the two components of inactivation were already potentially present, in contrast with the slow development of the fast component during build-up (see Fig. 1). These results along with the fact that these cells normally have a negative HP (79 ± 3 mV, $n = 12$), suggested that build-up was not related to voltage-dependent recovery of Ca^{2+} channels.

Next, we tested whether the build-up of I_{Ca} is use-dependent; i.e. is it necessary to stimulate the cell to generate channel activity? After the WCR was obtained, cells

were held at a HP of −80 mV for 3 min and no stimulation was applied during this time. Figure 9 shows the large I_{Ca} evoked immediately at the first stimulation following a resting period of 3 min in the WCR mode. No increase in the amplitude of I_{Ca} was observed at the subsequent stimulations in the cells tested ($n = 8$). Rather,

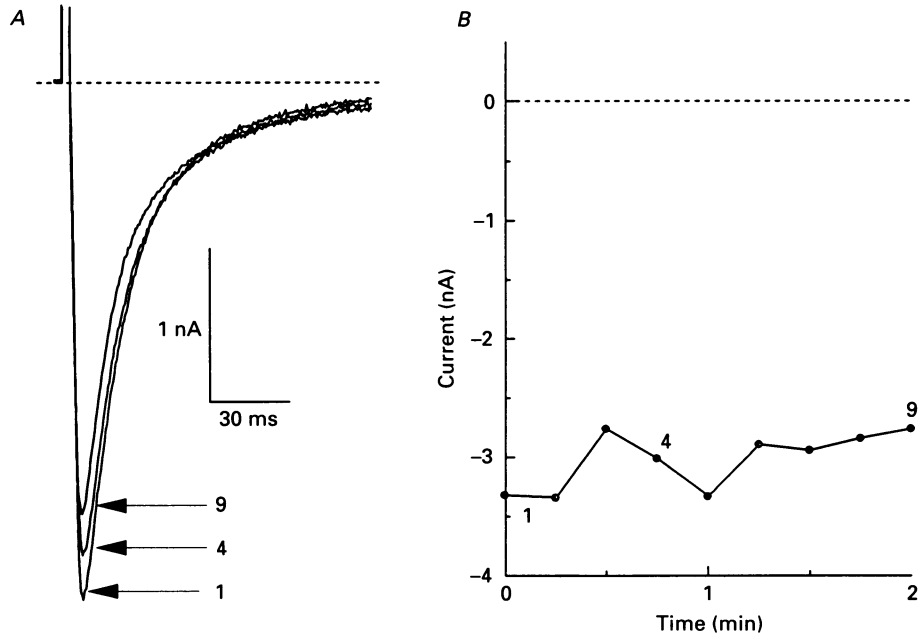

Fig. 9. Use-independence of the build-up of I_{Ca}. *A*, no stimulation was applied for 3 min after establishing the WCR configuration. The first stimulation evoked I_{Ca} at its maximal amplitude and final waveform. I_{Ca} was decreased rather than increased by further stimulations (traces 4 and 9 recorded 45 and 120 s later, respectively). *B*, complete kinetic plot showing that the build-up of I_{Ca} does not depend upon repetitive electrical simulations.

a small decrease was observed in some cells, possibility due to run-down. From these experiments, it was concluded that the build up of I_{Ca} does not depend upon repetitive electrical stimulations or cell membrane resting potential.

Run-up is independent of pipette constituents

Because the build-up of I_{Ca} could possibly be related to intracellular dialysis, we tested whether one of the pipette constituents might be involved. One possibility explored was that, prior to obtaining the WCR, the intracellular free Ca^{2+}level in the resting cell is high enough to inactivate Ca^{2+} channels according to the Ca^{2+}-dependent hypothesis (for review see Pelzer *et al.* 1990) and that buffering Ca^{2+} with dialysing EGTA relieves this inhibition. When intracellular EGTA was further elevated to 70 mM, the increase of I_{Ca} amplitude after the WCR was established (P2) was not noticeably altered (Fig. 10). Maximal amplitude of I_{Ca} was reached within 180 ± 35 s ($n = 7$). However, despite an acceleration of I_{Ca} decay (compare trace 6 and trace 16 in Fig. 10), the typical phase P3 was occulted owing to the

superimposition of a slowing effect of high EGTA on current decay (authors' unpublished results). Addition or removal of EGTA from the pipette solution had no effect on the build-up of I_{Ba}. We concluded, therefore, that Ca^{2+} is not involved in the repriming of Ca^{2+} channel activity prior to the build-up step. In addition, the

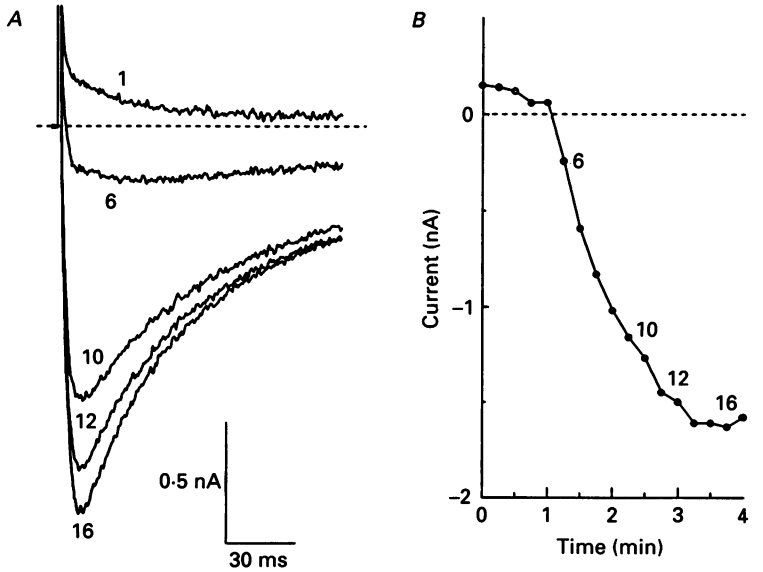

Fig. 10. Effects of intracellular Ca^{2+} buffering on the build-up of I_{Ca}. *A*, build-up of I_{Ca} recorded with high-EGTA (70 mM) in the pipette. The recording conditions were the same as those described in Fig. 1*A* except that EGTA in the pipette was raised from 10 to 70 mM. Note that there is no acceleration of I_{Ca} (P3, compare with Fig. 1*A*). *B*, complete kinetic of the build up of I_{Ca} peak amplitude plotted as a function of time.

acceleration of I_{Ca} during P3 (in standard conditions) occurring during dialysis of EGTA in cells could not be related to the Ca^{2+}-dependent hypothesis.

The role of other constituents in the recording solution was also explored. In particular, we investigated the possibility that increase in Ca^{2+} channel activity could be related to phosphorylation and crucially depend on pipette ingredients. Reconstitution of intracellular pools of substrates in depleted cells probably occurs upon cell dialysis of nucleotides (ATP, GTP) and Mg^{2+}. However, when these agents were simultaneously excluded from the patch pipette, no significant change was observed in the build-up of I_{Ca}. The maximal amplitude of I_{Ca} was reached after 129 ± 17 s ($n = 5$) and current waveform was similar to that observed in standard conditions.

We next examined whether build-up could result from a change in intracellular pH. For example, metal hypodermic needles employed to fill recording pipettes with solutions containing EGTA have been reported to acidify intracellular pH (Corey & Stevens, 1983), owing to release of metal ions. Changing the pH of the recording pipette solution to either pH 6 or 8 had no significant effect on current waveform during the period of build-up. I_{Ca} reached its maximal amplitude after 126 ± 30 s at pH 6 ($n = 3$) and after 150 ± 31 s at pH 8 ($n = 4$). Finally the build-up of Ca^{2+}

channel currents was not altered by the presence of intracellular K^+ instead of Cs^+ (steady state reached within 133 ± 41 s, $n = 3$ for I_{Ba}) and when both extra- and intracellular chloride were replaced by aspartate (data not shown).

Run-up is unrelated to the wash-out of a cytoplasmic macromolecule

We evaluated whether, during cell–pipette exchanges, the wash-out of some endogenous diffusible factor accumulated in the static cell could be involved in the run-up of I_{Ca}. We performed perforated patch recordings of I_{Ca} (using amphotericin B: see Methods), which allow the major cytoplasmic molecules in the cell to be retained (for review, see Rae & Fernandez, 1991). In good agreement with previous work (Kurachi, Asano, Takikawa & Sugimoto, 1989), large inward I_{Ca} were recorded in this configuration (amplitude 1010 ± 298 pA, $n = 4$). In addition, when breaking into the cell afterwards to establish the conventional whole-cell recording configuration, no run-up was observed (data not shown). These experiments therefore suggest that the wash-out of a diffusible cytoplasmic macromolecule is not involved in the run-up of I_{Ca}.

Run-up in the presence of agonists

The above results suggest that run-up of I_{Ca} does not reflect enhancement of channel phosphorylation subsequent to the addition of exogenous substrates. Alternatively, we examined the run-up in conditions where cAMP-dependent phosphorylation was promoted. Cells were continuously exposed to 1 μM isoprenaline (Iso) for several minutes (> 5 min) prior to obtaining the WCR. Figure 11*A* shows a representative example of the build up of I_{Ca} in these conditions. The main observation was that Iso did not significantly change the time required to reach the maximal amplitude of I_{Ca} (120 ± 29 s, $n = 7$). This indicates that the mechanism responsible for channel repriming is not influenced by phosphorylation. Phases P1, P2 and P3 were observed but P3 appeared early (P3 preceded P2) i.e. current inactivation became dramatically fast before I_{Ca} reached its maximal peak amplitude (compare Fig. 11*A* with Fig. 1). Iso obviously accelerated the recruitment of the fast component of I_{Ca} (P3) which normally required more than 2 min. This is related to the promotion of this fast component by Iso (Richard *et al.* 1990).

We have also studied the build-up of Ca^{2+} channel currents in the presence of agonists that are known to directly enhance channel activity upon binding to a channel receptor. Cells were exposed to 1 μM of either (−)-Bay K 8644 or (+)-202-791 applied extracellularly for several minutes (> 5 min) prior to recording. Figure 11*B* shows a representative illustration of the build-up of I_{Ca} in the presence of (+)-202-791. The presence of the dihydropyridine did not significantly alter the increase of I_{Ca} during the first minutes of recording. For example, in the presence of I_{Ca} during the first minutes of recording. For example, in the presence of Bay K 8644, I_{Ca} reached its maximum amplitude within 130 ± 29 s ($n = 10$). In addition, the three phases P1, P2 and P3 developed with the same sequence as in control. When the cells were bathed in the simultaneous presence of a dihydropyridine agonist and Iso, the build-up of I_{Ca} did not develop exactly as in the presence of each drug applied alone (Fig. 11*C*). The time required to reach maximal current amplitude was not substantially altered (154 ± 22 s, $n = 6$) but the time course of I_{Ca} decay, which was

fast during initial stimulations, became slower (Fig. 11*Ca*) in contrast with the observed effects in control conditions. This slowing is likely to reflect cAMP-dependent modulation of the effects of DHP agonists on cardiac Ca^{2+} channels (see Tiaho *et al.* 1990) which again indicates that Ca^{2+} channels are not phosphorylatable before they are rendered activatable during build-up.

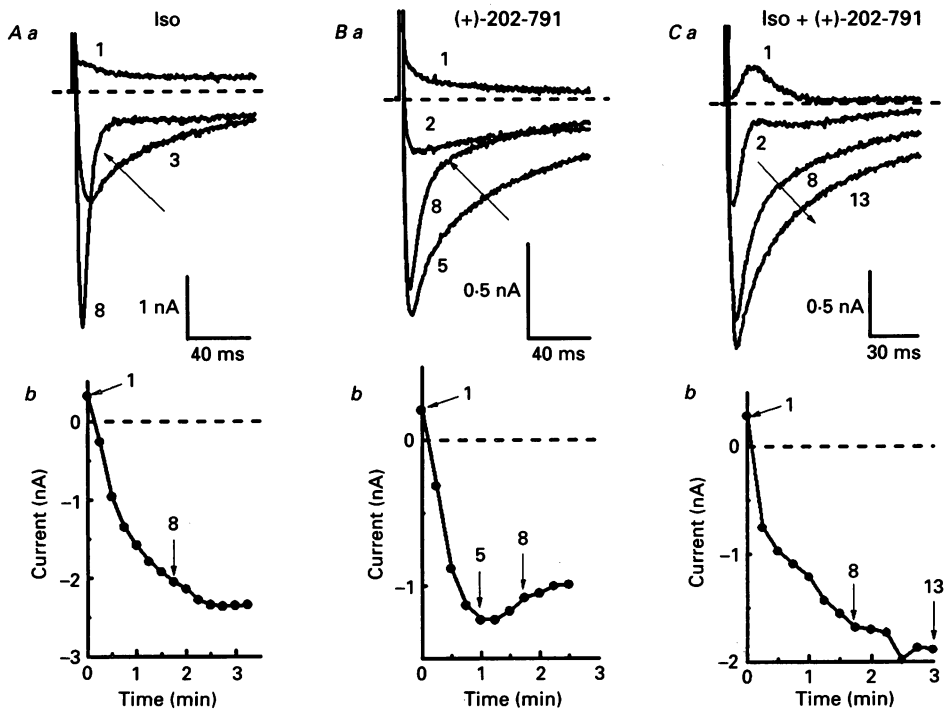

Fig. 11. Run-up of Ca^{2+} channel currents in the presence of agonists. *A*, build-up of I_{Ca} in the presence of extracellular isoprenaline (1 μM) in the bath. I_{Ca} was recorded in the same conditions as in Fig. 1*A*. *Aa*, original records. Currents shown were evoked at time $\approx$ 0, 30 and 105 s (traces 1, 3 and 8, respectively). Note the slow monoexponential decay of I_{Ca} at early stimulations (trace 3) and the prominent fast component of inactivation of the final current waveform (trace 8). *Ab*, complete kinetic of the build-up of I_{Ca} peak amplitude plotted *versus* time of recording. *B*, build-up of I_{Ca} in the presence of the dihydropyridine agonist (+)-202-791 (1 μM). *Ba*, original records of I_{Ca} evoked at time 0, 15 and 60 and 105 s (traces 1, 2, 5 and 8, respectively) in the same recording conditions as in *A*. Note the sequential development of the three distinct phases, P1 (trace 1), P2 (traces 2 and 5) and P3 (trace 8). *Bb*, complete kinetic plot of the build up of I_{Ca} peak amplitude *versus* time of recording. *C*, build-up of I_{Ca} in the concomitant presence of isoprenaline and Bay K 8644. *Ca*, original records of I_{Ca} evoked at time $\approx$ 0, 15, 105 and 180 s (traces 1, 2, 8 and 13, respectively) after establishing the WCR configuration in the standard recording conditions. Note the early appearance of a fast component of I_{Ca} (traces 1 and 2) which precedes a dramatic slowing of current decay with the time of recording (traces 8 and 13). *Cb*, complete kinetic plot of the build-up of I_{Ca} peak amplitude *versus* recording time.

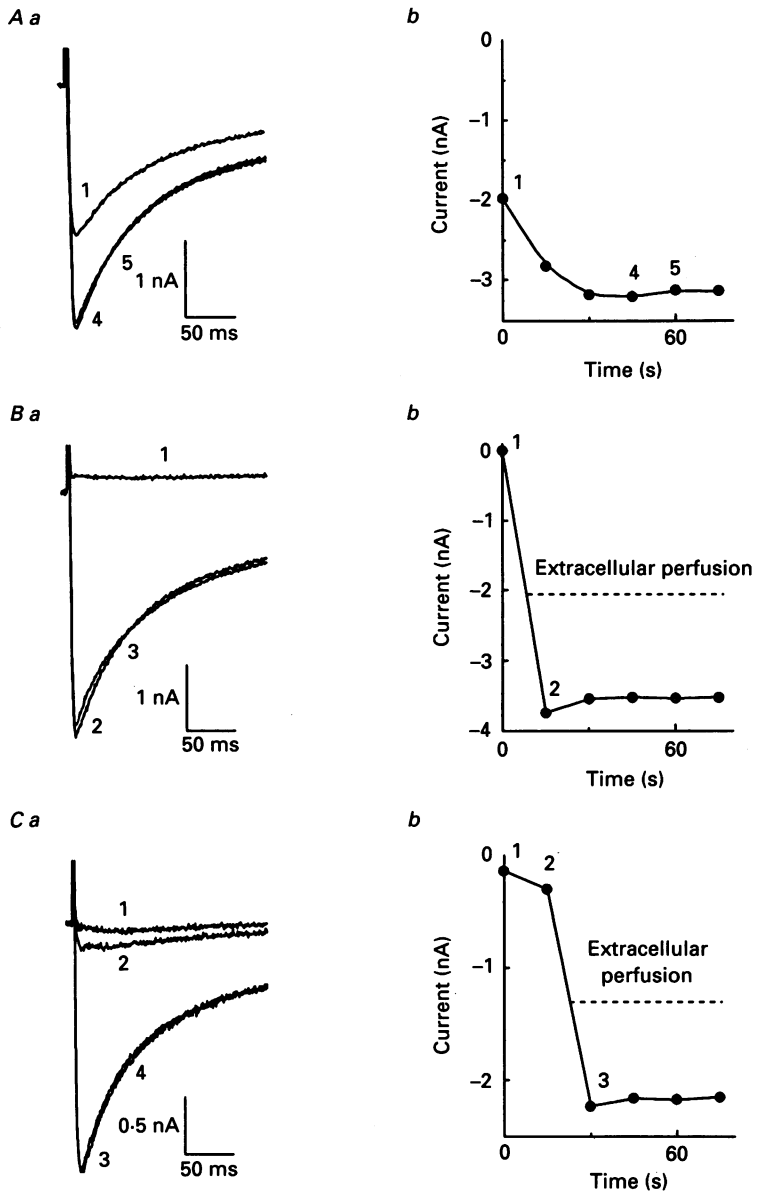

Fig. 12. Modulation of the run-up of I_{Ba} by extracellular superfusion of the myocytes. Cells were perfused at a flow rate of 0·2 ml/min prior to or after obtaining the WCR configuration. *A*, I_{Ba} was recorded in similar conditions as described in Fig. 3*A* except that it was under continuous extracellular perfusion. First stimulation (time ≈ 0) evoked a large inward current which rapidly reached its maximal peak amplitude (traces 4 and 5, recorded 45 and 60 s later, respectively). *A a*, original records. *A b*, kinetic of the variation of I_{Ba} peak as a function of time. *B*, perfusion of the cell was started between the first and the second stimulations after seal rupture. The first stimulation evoked no inward current but I_{Ba} reached its maximum amplitude at the second stimulation. I_{Ba} was recorded in similar conditions as in *A*. *C*, perfusion was started between the second and third stimulation. I_{Ba} rapidly reached its maximal amplitude. I_{Ba} was recorded in similar conditions to those in *B*.

Run-up is accelerated by extracellular superfusion

The results described above were obtained from cells bathed in a static extracellular bath solution. They demonstrate that the increase in Ca^{2+} channel currents occurring during early whole-cell clamp recordings is not markedly influenced by a variety of agents, including potent agonists. However, we found one situation where the time course of increase was dramatically altered. When, prior to obtaining the WCR, cells were submitted to extracellular perfusion of the same composition as the bath (see Methods), Ca^{2+} channel currents were rapidly augmented (Fig. 12*A*) i.e. after two or three stimulations (< 30 s). Extracellular superfusion of the cells was performed as described in the Methods. The cell was surrounded by the solution flowing out of the capillary (approximately 200 μM away from the cell) as soon as the perfusion was started (< 1 s). The rapid influence of extracellular perfusion is better illustrated in Fig. 12*B* and *C*. When cell superfusion was started between first and second stimulations, current increased from nearly zero (outward-directed) to its maximal amplitude (Fig. 12*B*). The augmentation of current was rapid and independent of the time at which the perfusion was started. This is illustrated in Fig. 12*C* where superfusion was started between the second and third stimulations. Similar results were observed in ten cells. There is no doubt that extracellular superfusion has a profound effect on the run-up of I_{Ca} during early WCR.

The effect of the external perfusion could be due to the wash-out of an inhibitory product originating in the cell itself and accumulating in the microenvironment on the extracellular side of the membrane. Since they are not well oxygenated, isolated cells may be in anaerobia which could result in (among other things) accumulation of metabolic products such as lactate, ammonia and H^+. Cardiomyocytes are permeable to these agents (Boron & De Weer, 1976; Dennis, Kohn, Anderson & Garfinkel, 1985; Trosper & Philipson, 1987). We have examined the effects of H^+, NH_4^+ and lactate at high concentrations. Neither superfusion of the cells with an extracellular solution at pH 5·5 or with 20 mM NH_4^+ suppressed I_{Ca} by more than 50% ($n = 3$ in each case). Although the superfusion of lactate (30 mM) almost suppressed I_{Ca} in three cells, no or only little effect (< 30%) was often observed (five out of eight cells). Thus none of these agents suppressed I_{Ca} consistently. Moreover, in all cases, the wash-out of the inhibitory effects upon superfusion of the control solution was slow (taking many minutes) compared to the effect described in Fig. 12. In addition, when establishing the whole-cell recording configuration in the presence of each agent (H^+, NH_4^+ and lactate), the run-up of I_{Ca} occurred with a similar time course to the control condition ($n = 3$ in each case).

DISCUSSION

Repriming of Ca^{2+} channels in static cells

This paper presents the first detailed description of the repriming of voltage-activated cardiac Ca^{2+} channels as revealed early during whole-cell clamp recordings. Indeed I_{Ca} reached what can be considered as its normal waveform only several minutes after the WCR has been established. There is little doubt that the apparent

increase of I_{Ca} reflects a genuine change in Ca^{2+} channel activity. Although a Cs^+ block of the K^+ current could account for the apparent increase of I_{Ca}, this seems unlikely (see Results). In particular, the increase of I_{Ca} (i) was observed using KCl in the patch pipette (Cs^+-free) and (ii) occurred in the presence of intracellular Cs^+ (instead of K^+), extracellular TEACl (140 mM), and 4-aminopyridine (10 mM) i.e. under conditions that ensure satisfactory suppression of K^+ currents in these cells (Richard *et al.* 1990; Apkon & Nerbonne, 1991). Involvement of other currents (Cl^-, Na^+–Ca^{2+} exchange) or artifactual contamination of Ca^{2+} channel current measurements by voltage loss or series resistance variation were also excluded.

It is clear that, once the WCR was established, not only the activity of the L-type Ca^{2+} channels was enhanced but there was also an alteration of channel functioning. After the early appearance of a slowly inactivating Ca^{2+} current (P2), a marked acceleration of current decay occurred (P3). The dramatic change in the distribution of the fast and slow inactivating components of I_{Ca} shown in Fig. 2 suggests an interconversion between two interrelated components which operate sequentially over the first minutes after the establishment of the WCR. Recently, we suggested that, based on on its biexponential decay, I_{Ca} could be divided into two alternative components of L-type current ($I_{Ca,fc}$ for the fast component, $I_{Ca,sc}$ for the slow component) which can be differentially modulated by physiological stimuli in rat ventricular cells (Richard *et al.* 1990). The present results further support the hypothesis for the existence of two interrelated gating pathways of Ca^{2+} channels.

In an attempt to identify a factor responsible for Ca^{2+} channel inhibition prior to establishment of the WCR, we eliminated a number of possibilities including voltage and intracellular free Ca^{2+}. Run-up or I_{Ca} does not reflect voltage dependent recovery of inactivated channels (as a result of membrane hyperpolarization after setting the WCR). Neither is the increase of I_{Ca} facilitated by voltage-dependent operation of channel gating (opening), and ion permeation in the channel pore is not crucial. Alternatively, it is not linked to the Ca^{2+}-buffering by EGTA and repriming is not operated through Ca^{2+}-dependent inactivation of Ca^{2+} channels. Finally, we excluded the suggestion that the increase of I_{Ca} depends upon exogenous ATP (and GTP) brought into the cell during dialysis, at least during the increase of the amplitude. Therefore, build up of Ca^{2+} channel activity is not primarily due to an increase in basal phosphorylation.

The run-up of I_{Ca} could be related to the accumulation of an endogenous factor which renders the Ca^{2+} channels inoperable prior to cell dialysis. Such a factor could be lost (washed out) during cell–pipette exchanges (Clapp & Gurney, 1991). In hippocampal neurones, this role has been devoted to a phosphatase (Mironov & Lux, 1991). However, large I_{Ca} were recorded using the perforated patch technique (see also Kurachi *et al.* 1989). In this configuration, only monovalent ions (K^+, Cs^+, Cl^-) are allowed to exchange between the cell and the pipette (for review, see Rae & Fernandez, 1991) and the wash-out of a diffusible cytoplasmic macromolecule is prevented. This is consistent with the presence of large I_{Ca} recorded in single ventricular cells by means of the single-electrode voltage-clamp technique (Josephson, Sanchez-Chapula & Brown, 1984) which also allows intracellular macromolecules to be retained.

An increase in intracellular cAMP, promoted by the exposure of cells to

isoprenaline prior to establishing the WCR, did not prevent or relieve repriming of Ca^{2+} channels. Modulation of the Ca^{2+} channel activity by phosphorylation was prevented until channels were rendered activatable during the run-up step. For example, the slowing of I_{Ca} observed in the simultaneous presence of Bay K 8644 and Iso was not instantaneous, but rather developed with a slow time course, consistent with the kinetics of the cAMP-dependent modulation of Ca^{2+} channels (Tiaho *et al.* 1990). In addition, both acceleration of I_{Ca} decay during P3 and slowing of I_{Ba} inactivation (during the corresponding phase), which are reminiscent of cAMP-dependent phosphorylation of Ca^{2+} channels (Richard *et al.* 1990; Tiaho *et al.* 1990), also occurred with a delay. It seems, therefore, that the recruitment of activatable Ca^{2+} channels is required before phosphorylation (and also binding of potent dihydropyridine agonists) can increase channel activity.

Restoration of Ca^{2+} channel activity by extracellular perfusion

The mechanism involved in the rapid relief of Ca^{2+} channel repriming by extracellular superfusion during the run-up step of I_{Ca} is puzzling. It seems unlikely that a wash-out of metabolic products, such as H^+, NH_4^+ or lactate accumulated in the microenvironment of the extracellular side of the membrane, can explain the observed effect. None of these agents suppressed I_{Ca} consistently. Although we cannot completely exclude the involvement of an unidentified agent, capable of exerting a direct blocking effect on Ca^{2+} channels, it is remarkable that even in absence of superfusion I_{Ca} runs up. Alternatively, it seems reasonable to assume that extracellular perfusion will result in mechanical disturbance of the cell membrane owing to the positive pressure. One consequence may well be alteration of Ca^{2+} channel activity. There is no doubt that the cell is subject to compression. This effect may be reminiscent of the reversible increase of I_{Ca} that is often observed by many workers in normal conditions when extracellular superfusion of the cell is started (which makes superfusion of control solutions mandatory prior to testing drugs). This hypothesis may offer a framework to explain the 'slow' increase of Ca^{2+} channel activity after the WCR is established in static myocytes. Both getting a seal and establishing the WCR (a negative pressure is applied which may tend to inflate the myocyte) are means of mechanostimulation of the cell membrane. Indeed, the whole-cell mode can be used for studies of mechanosensitive ion channels (for review, see Morris, 1990).

Conclusive remarks

When taken together, all of our results are consistent with a repriming of L-type cardiac Ca^{2+} channels in static cells prior to establishing the WCR. This repriming has an artifactual origin, possibly owing to the methodology employed, such as enzymatic dispersion of cells, abnormally long resting period of static myocytes, or even stress introduced by the patch pipette. The down-regulation of the Ca^{2+} channels is not altered by known modulators (voltage, Ca^{2+}, cAMP-dependent phosphorylation) and unrepriming is necessary before these modulations can be effective. Interestingly, such an inhibition was not observed for Na^+ and K^+ currents and it seems to be specific to Ca^{2+} channels. However, probably not all types of Ca^{2+} current would be subject to this down-regulation because at least T-type currents

reach their final waveform much more rapidly (< 30 s) than the L-type, as observed both in aortic myocytes and sensory neurones (S. Richard & J. Valmier, unpublished results) once the WCR is established. Similar run-up of L-type Ca^{2+} current was also evidenced in pulmonary arteries (Clapp & Gurney, 1991) and in hippocampal neurones (Mironov & Lux, 1991) and this behaviour may be common among various cell types. Although the mechanism involved is a matter for speculation, the effect of extracellular perfusion reported here may open the new perspective that mechano-stimulation is a way of regulating Ca^{2+} channel activity. Cardiac cells are seldom static under physiological conditions. Rather they repetitively contract, change in shape and volume and are subject to compression during blood loading of heart cavities. If this hypothesis is correct, it will be interesting to determine whether Ca^{2+} channels are sensitive to the pressure gradient or alternatively to the membrane tension induced by the pressure. These questions are currently a subject of our research.

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