British Journal of Pharmacology (2002) 135, 721–734

www.nature.com/bjp

Caffeine-induced immobilization of gating charges in isolated guinea-pig ventricular heart cells

[1]Jérôme Leroy, [1]Jacques M. Lignon, [1]François Gannier, [1]Jorge A. Argibay & *,[1]Claire O. Malécot

[1]CNRS UMR 6542, Physiologie des Cellules Cardiaques et Vasculaires, Faculté des Sciences, Parc de Grandmont, 37200 Tours, France

1 The effects of 10 mM caffeine (CAF) on intramembrane charge movements (ICM) were studied in isolated guinea-pig ventricular heart cells with the whole-cell patch-clamp technique.

2 In the presence of CAF, the properties (voltage dependence, maximum Q_{ON} [Q_{max}], availability with voltage) of Q_{ON} charge activated from −110 mV were barely affected. Following a 100 ms prepulse to −50 mV to decrease the participation of charges originating from Na channels, the voltage dependence of Q_{ON} was shifted by 5 mV (negative component) and by 10 mV (positive component) towards negative potentials, and Q_{max} was depressed by 16.5%.

3 CAF drastically reduced in a time- and voltage-dependent manner Q_{OFF} on repolarization to −50 mV, the effects being greater at positive potentials.

4 CAF-induced Q_{OFF} immobilization could be almost entirely removed by repolarization to voltages as negative as −170 mV. In these conditions, the voltage-dependence of Q_{OFF} (repolarization to +30 to −170 mV) was shifted by 17 mV (negative component) and 30 mV (positive component) towards negative potentials, suggesting an interconversion into charge 2.

5 Most of CAF effects were suppressed when the sarcoplasmic reticulum (SR) was not functional or when the cells were loaded with BAPTA-AM.

6 We conclude that CAF effects on ICM are likely due to Ca^{2+} ions released from the SR, and which accumulate in the subsarcolemmal fuzzy spaces in the vicinity of the Ca channels. Because CAF effects were more pronounced on Q_{OFF} than on Q_{ON} the channels have likely to open before Ca^{2+} ions could affect their gating properties. It is speculated that such an effect on gating charges might contribute to the Ca-induced inactivation of the Ca current.

British Journal of Pharmacology (2002) **135,** 721–734

Keywords: Intramembrane charge movements; Ca channels; native cardiac cells; charge interconversion; subsarcolemmal calcium; caffeine

Abbreviations: CAF, Caffeine; ICM, Intramembrane charge movements; I_g, gating current; $I_{g,ON}$, I_g elicited on depolarization; $I_{g,OFF}$, I_g elicited on repolarization; IPP50, Inactivating prepulse to −50 mV; Q_{ON}, mobile charge during depolarization; Q_{OFF}, mobile charge during repolarization; SL, Sarcomere length; SR, Sarcoplasmic reticulum

Introduction

The methylxanthine caffeine has been widely used in the studies of excitation–contraction coupling mostly because of its sarcoplasmic reticulum (SR) Ca^{2+} releasing properties (e.g., Weber, 1968; Weber & Herz, 1968; Blayney *et al*., 1978; Meissner & Henderson, 1987). It has also other properties such as phosphodiesterase inhibition (Beavo *et al*., 1970), increase of the Ca-sensitivity of the myofilaments (Wendt & Stephenson, 1983), and non-selective adenosine antagonist (e.g., Evoniuk *et al*., 1987). Depending on the tissue studied, caffeine has been shown to either increase (e.g., Yatani *et al*., 1984; Qu & Campbell, 1998) or decrease (e.g., Hess & Wier, 1984) the L-type Ca^{2+} current and to modulate its kinetics of inactivation (e.g., Qu & Campbell, 1998).

By its Ca^{2+} releasing properties, caffeine might affect ionic channels activities. Indeed, it is well recognized that intracellular calcium modulates several cardiac ionic currents, the best known effect being the inactivation of the calcium current (I_{Ca}; e.g., Mentrard *et al*., 1984; for review, see Eckert & Chad, 1984 and Pelzer *et al*., 1990; Yue *et al*., 1990; Haack & Rosenberg, 1994), but, apart from some studies in skeletal muscle (e.g. Kovács & Szúcs, 1983; Shirokova & Ríos, 1996; Huang, 1998; Csernoch *et al*., 1999), little is known about its potential effects on intramembrane charge movements (ICM) associated with activation of voltage-dependent channels in the heart. ICM, first recorded in squid giant axon (Armstrong & Bezanilla, 1973; Keynes & Rojas, 1974) and in skeletal muscle (Schneider & Chandler, 1973; for review, see Ríos & Pizarro, 1991) have since been identified as the displacement of charged particles, corresponding to the S4 segments of voltage-gated channels, across the cell membrane during voltage steps (Catterall, 1988; Mannuzzu *et al*., 1996; Larsson *et al*., 1996; for review, see Papazian & Bezanilla, 1997). Several laboratories have found that intracellular calcium influence ICM in skeletal muscle (e.g., Pizarro *et al*., 1991; Jong *et al*., 1995a, b; Pape *et al*., 1996; Shirokova & Ríos, 1996; Stroffekova & Heiny, 1997a, b) by modulating a fraction of the total charge called Q_γ or by promoting charge

*Author for correspondence: E-mail: malecot@univ-tours.fr

1–charge 2 interconversion. Charge 1 has been described as the movement of intramembranous charges between −100 and +40 mV in normally polarized muscle fibres, whereas charge 2 has been observed in depolarized fibres, with a different voltage sensitivity, moving at hyperpolarized potentials (at least 50 to 60 mV more negative than charge 1 (Adrian & Almers, 1976; Brum & Ríos, 1987; Caputo & Bolaños, 1989; for review, see Huang, 1988; Ríos & Pizzaro, 1991; Dulhunty, 1992). It has been proposed that during prolonged depolarization charge 1 is transformed into charge 2 (charge interconversion; Brum & Ríos, 1987). Such a mechanism has also been described in heart muscle (Shirokov *et al*., 1992; 1993).

We have tested in our study whether caffeine could also affect ICM in enzymatically isolated guinea-pig ventricular heart cells, using the whole-cell patch-clamp technique. We show here for the first time, to our knowledge, that caffeine modulates ICM in ventricular heart cells by promoting charge 1–charge 2 interconversion. This effect of caffeine, strongly reduced in conditions of increased intracellular Ca^{2+} buffering or SR-Ca^{2+} depletion, might be due to a caffeine-induced SR calcium release near the membrane. The fact that caffeine mostly affects the OFF charges (i.e., charges activated on repolarization) with very little effects on the ON charges (i.e., charges activated upon depolarization) strongly suggests that the Ca^{2+} released might bind to an intracytoplasmic site of the Ca channel which is only accessible once the channel has opened. A preliminary note on these experiments has been published (Leroy *et al*., 1998).

Methods

Cell isolation

Guinea-pig heart cells were enzymatically isolated from the left ventricle as described elsewhere (Le Guennec *et al*., 1993). The experimental procedure was performed in accordance with French guidelines (authorization no 7741 to C.O.M.; animals killed by cervical dislocation). Dissociated cells were placed in a small chamber, on the stage of an inverted microscope (electrophysiological recordings: Olympus IX70, Tokyo, Japan; sarcomere length and $[Ca^{2+}]_i$ measurements: Nikon Diaphot 300, Tokyo, Japan), continuously superfused by gravity with normal Tyrode solution containing (in mM): NaCl 140, KCl 5.4, $MgCl_2$ 1, $CaCl_2$ 1.8, NaH_2PO_4 0.33, HEPES buffer 10, glucose 11, pH adjusted to 7.3 with NaOH.

Solutions

The cells were locally superfused by gravity (small capillaries positioned within 50 μm of the cell and allowing fast solution changes). To record the gating currents, all ionic currents were blocked with a solution ('gating solution') containing (in mM): TEACl 140, $MgCl_2$ 1, HEPES buffer 10, $CaCl_2$ 1, $CdCl_2$ 2, $GdCl_3$ 0.1, Tetrodotoxin (TTX) 0.001 and Glucose 11 (pH 7.3 with TEAOH). The pipette solution contained (in mM): CsCl 110, TEACl 30, HEPES buffer 10, EGTA 10, Mg^{2+}-ATP 5 (pH 7.3 with CsOH). In some of the experiments (BAPTA-AM loaded cells), the pipette solution contained 10 mM BAPTA instead of EGTA. Caffeine (10 mM) was directly added to the gating solution. Ryanodine (10^{-2} M stock solution in distilled water) was used at a final concentration of 100 μM. To deplete the sarcoplasmic reticulum Ca^{2+} content, cells were preincubated for 15 min in the presence of 1 μM thapsigargin (10^{-3} M stock solution in bidistilled water plus DMSO at the final concentration of 26%) in normal Tyrode solution (thus the final DMSO concentration during these experiments reached 0.026% – a similar final concentration of DMSO was present in all solutions used for this set of experiments). All drugs used were from Sigma Chemicals (St. Quentin Fallavier, France), except caffeine (Merck-Clévenot, Nogent sur Marne, France).

Sarcomere length and $[Ca]_i$ measurements

Contraction was monitored by following the sarcomere length (SL) as described by Gannier *et al*. (1993). Briefly, the periodicity of the cell striation pattern (A and I bands) was analysed with a Fast Fourier Transform (FFT) of a video image of the cell during the course of the experiment. A longitudinal fraction of the cell image was recorded with a commercial CCD camera (Micam VS 500, Digital Vision Technology, or Hamamatsu C3077, Hamamatsu Photonics, Japan), digitized at 50 Hz (temporal resolution of 20 ms), then transiently stored on a computer (Pentium 75 or 486 DX2 66) and used to calculate the spectrum corresponding to the distribution of the sarcomere lengths. The spectrum fundamental, continuously detected by a routine, enables on line visualization of SL during cell contraction. To have a better time resolution (2 ms) of fast events in some of the experiments, SL was also calculated by a FFT performed at 500 Hz of a video image of the cell recorded with a non-standard 218×145 pixel array two-stage CCD camera, as described by Gannier *et al*. (1998), set in parallel with the first camera. Cells were selected on the basis of the diastolic SL (between 1.8 and 2.0 μm) during field stimulation at 0.5 Hz.

For $[Ca^{2+}]_i$ measurements during caffeine contracture, myocytes were first loaded with the cell permeant fluorescent $Ca^{2+}{}_i$ indicator Indo-1 AM (Molecular Probes, Leiden, The Netherlands – 10 μM final concentration in normal Tyrode solution) for 20 min, and then superfused for at least 30 min with Indo-1 free Tyrode solution before the start of the experiment to allow de-esterification of the acetoxy-methyl-ester probe. An excitation wavelength of 360 ± 5 nm was delivered to the cells with the use of a 75 W Xenon lamp and Indo-1 emitted fluorescence was recorded by two photomultipliers (Hamamatsu R1104, Hamamatsu Photonics, Japan) at 405 ± 40 nm (Ca^{2+}-bound form) and 485 ± 40 nm (Ca^{2+}-free form). A shutter (Uniblitz D122 shutter driver, U.S.A.) was placed between the Xenon lamp and the objective of the microscope to control exposure of the cells to the excitation lamp, and thus to reduce photobleaching of Indo-1 during long recordings. This was also achieved by the use of a neutral divider (ND 64). In the present experiments, the shutter was opened for 2 s every 4 s. Fluorescence signals were filtered at 400 Hz and acquired at 1 kHz with pClamp 6.0.4 software (Axon Instruments Inc, Foster City, CA, U.S.A.) through a Labmaster TL-1 interface. The fluorescence ratio F_{405}/F_{485} was calculated on-line during the acquisition. Due to the

uncertainty of the exact dissociation constant of Indo-1 for Ca^{2+} (Hove-Madsen & Bers, 1992), no attempt was made to convert the fluorescence ratio in Ca^{2+} concentration. The average of 300 acquired data points is given as the F_{405}/F_{485} ratio in the figure.

Electrophysiological recordings and analysis

The whole-cell patch-clamp technique was used to study the gating currents at room temperature (22–25°C). Patch pipettes (1 to 2.5 MΩ) were pulled from thick wall borosilicate glass capillaries (Clark Electromedical Instruments, Reading, U.K.) with a Narishighe PB7 puller (Narishige, Tokyo, Japan) and were coated with dental wax to decrease their capacitances. An Axopatch 200A amplifier (Axon Instruments Inc.), connected to a Pentium 75 computer equipped with pClamp 6.0.4. software (Axon Instruments), through a Digidata 1200A interface (Axon Instruments), was used to control voltage and record currents. Data were analysed with Clampfit. The pipette and cell capacitances were compensated and the liquid junction potential between the pipette and bath solutions was corrected. In order to have an adequate control of the membrane voltage and to improve the clamp speed, care was taken to select relatively small cells (membrane capacitance $C_m = 75.0 \pm 4.1$ pF, mean ± s.e.mean, $n = 39$) to carry out the experiments. Cells for which the membrane capacitance varied by more than 2% during the time course of the experiments were discarded and not included in the analysis. The series resistance was also compensated by 80%: this value was chosen to avoid saturation of the electronical circuit of the Axopatch 200A with the large voltage commands used. The residual series resistance after compensation amounted to 1.03 ± 0.08 MΩ ($n = 39$). The mean time constant of the clamp speed, determined from the residual series resistance after compensation and the mean cell capacitance was estimated to 77.2 μs. Thus, in most of the cells, more than 98% of the command voltage was seen by the membrane in less than 310 μs. The data were acquired at 25 kHz and filtered with the 4-pole lowpass Bessel filter of the Axopatch at 1 kHz: these recording conditions have been successfully used in heart cells by others (e.g., Shirokov *et al*., 1992) and us (Malécot & Argibay, 1999) to record gating currents. Because of the linearity of the residual leak current between −140 mV and +20 mV in the presence of the blockers of ionic currents used to record the gating currents (not shown), a P/5 subtraction protocol (Bezanilla & Armstrong, 1977), used to remove leak and linear capacitive currents, was applied before the test pulse or before the conditioning pulse from a sub-holding potential of −120 mV (regular holding potential of −110 mV). Control pulses were of the same polarity as the test pulses. Considering the mean time constant of recovery from inactivation of the charges at −120 mV, following a 500 ms inactivating pulse, a 100 ms settling time (more than 99.9% of recovery) was used. The stimulation frequency was 0.1 Hz. These recording conditions have been shown by Shirokov and co-workers (Shirokov *et al*., 1992) to avoid contamination of the control recordings by non linear charge movements of the charge 2 type.

Mobile charges were measured by integrating the first 20 ms of gating currents during depolarization (Q_{ON}) or repolarization (Q_{OFF}), after defining the steady-state current level for at least 5 ms as baseline. The voltage dependence of charge movements was quantified according to a two-state system, as discussed in details by Ríos & Pizarro (1991) and used by several authors to describe charge movements in heart cells (e.g., Bean & Ríos, 1989; Hadley & Lederer, 1989; 1991a, b; Hanck *et al*., 1990; Shirokov *et al*., 1992; 1993). Activation curves were obtained by fitting the data with the following double Boltzman equation:

$$Q_{ON} = Q_{max,1}/[1 + \exp\{(V - V_{1/2,1})/K_1\}] + Q_{max,2}/[1 + \exp\{(V - V_{1/2,2})/K_2\}] \quad (1)$$

where $Q_{max,1}$ and $Q_{max,2}$ are the maximal charge of the two components of charges (with no assumption regarding their origin) displaced by strong depolarization and are proportional to the number (n) of mobile electron charges (e^-) and to the apparent valence of the charge moved (z'): $Q_{max} = nz'e^-$. Subscripts 1 and 2 refer to voltage-dependent components activating firstly (1 or 'negative' or 'low voltage') and secondly (2 or 'positive' or 'high voltage') with increasing the voltage. z' is related to the real charge z by the relation $z' = z\delta$, with δ ($0 \leqslant \delta \leqslant 1$) representing the distance over which the charge moves within the membrane. The slope factors K_1 and K_2 are inversely proportional to z' ($K = kT/z'e^-$ where k is the Boltzman constant and T the absolute temperature). $V_{1/2,1}$ and $V_{1/2,2}$ are the voltages for the half maximal activation of the negative and positive (i.e., less negative) components, respectively. A similar equation was used to describe the inactivation of the charge with voltage. Q_{ON} and Q_{OFF} were normalized to the cell capacitance C_m and are expressed in nC μF^{-1} when quantified. More details are given in the text when appropriate.

Statistics

Data are presented as mean values ± standard errors, with the number of experiments indicated as n. Statistical significance was assessed with the paired Student's t-test or one-way analysis of variance (ANOVA). A P value less than 0.05 was considered as statistically significant.

Results

General aspects of gating currents in heart cells

Figure 1A inset shows a typical recording of gating currents in a guinea-pig ventricular heart cell depolarized from −110 to +20 mV during 20 ms. The current in response to depolarization consisted of a transient outward current $I_{g,ON}$ (the amount of charges moved has been previously described by numerous authors as the Q_{ON}, see Introduction for references) corresponding to the displacement of intramembrane charges across the transmembrane electric field. On repolarization to −110 mV, all the charges that have moved during the depolarization returned to their initial position, giving rise to a transient inward current $I_{g,OFF}$ (amount of charges displaced: Q_{OFF}). Both $I_{g,ON}$ and $I_{g,OFF}$ increased with increasing the test potential, as shown in Figure 2A left, but Q_{ON} and Q_{OFF} reached saturation at positive potentials (see Figures 1A and 2B), as expected from charges displacements, as opposed to charging of the membrane capacitance (which does not saturate with potential).

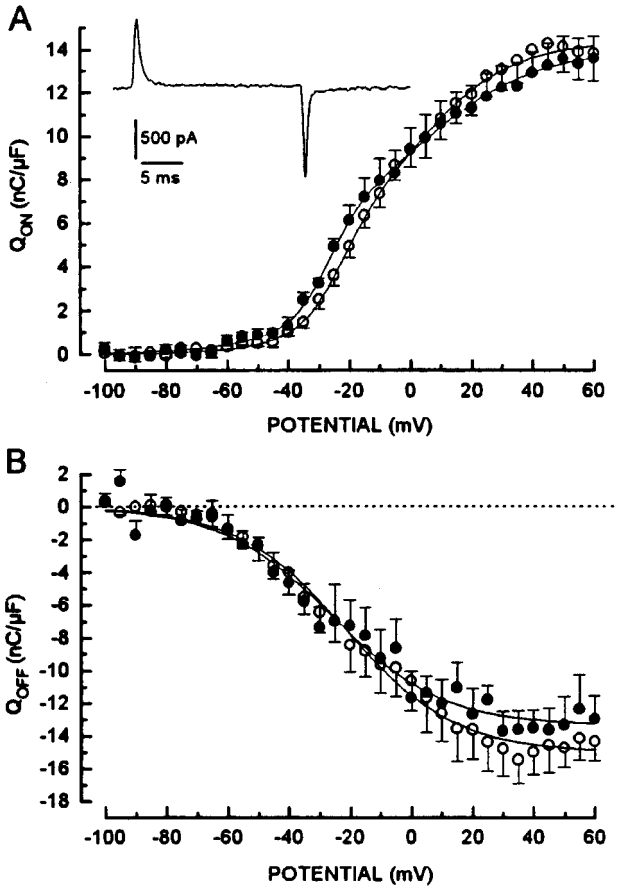

Figure 1 Caffeine effects on ICM activation from −110 mV. ICM were recorded during application of a 20 ms depolarizing pulse applied from −110 mV to voltage −100 to +60 mV in 5 mV steps. An example of gating currents elicited by a 20 ms depolarizing pulse applied from −110 mV to +20 mV is shown in inset. Upwards and downwards deflections correspond to the Q_{ON} and to the Q_{OFF} components of gating currents. The graphs show the mean charge-voltage relationships of Q_{ON} (A) and of Q_{OFF} (B) activation in control conditions (open circles) and in the presence of 10 mM caffeine (filled circles). Data points are mean values obtained in seven cells and vertical bars represent s.e.mean. The smooth lines are best fits to the mean data points with equation (1) in A and with a single Boltzman relationship in B. (A) Control: $Q_{max,1}=4.6$ nC μF^{-1}, $V_{1/2,1}=-23$ mV, $K_1=6.2$ mV; $Q_{max,2}=9.8$ nC μF^{-1}, $V_{1/2,2}=0.97$ mV, $K_2=16$ mV; caffeine: $Q_{max,1}=4.7$ nC μF^{-1}, $V_{1/2,1}=-27$ mV, $K_1=5.5$ mV; $Q_{max,2}=9.4$ nC μF^{-1}, $V_{1/2,2}=1.1$ mV, $K_2=20$ mV. (B) Control: $Q_{max}=-15$ nC μF^{-1}, $V_{1/2}=-21.5$ mV, $K=17.9$ mV; caffeine: $Q_{max}=-13$ nC μF^{-1}, $V_{1/2}=-21.5$ mV, $K=15.5$ mV.

Effects of caffeine on ICM activation

Two different protocols were used to test the effects of caffeine on ICM activation. First, 20 ms depolarizing pulses (range −100 mV to +60 mV) were applied from −110 mV to activate all channels: Q_{ON} during depolarization and Q_{OFF} on repolarization to −110 mV were calculated at each potential by integrating during 20 ms $I_{g,ON}$ and $I_{g,OFF}$. Second, a 100 ms inactivating prepulse to −50 mV (IPP50) applied from −110 mV was used before applying 30 ms depolarizing pulses (range −50 to +60 mV). In this case, Q_{OFF} was calculated by integration of $I_{g,OFF}$ at −50 mV during 30 ms. This second protocol allows to decrease the participation of Na channels to the total ICM (i.e., Malécot & Argibay, 1999), and thus to better evaluate the potential effect of caffeine on ICM mostly originating from Ca channels.

Figure 1 shows the average charge-voltage (Q-V) relationships in control conditions and in the presence of 10 mM caffeine, when gating currents were elicited by depolarizing the cells from a holding potential of −110 mV. Caffeine did not affect the total amount of ON charges displaced with strong depolarizations (Figure 1A). The only effect was a small but not statistically significant negative shift (−4 mV) of the mid activation potential of the first negative component of charge movement. OFF charges seen on repolarization to −110 mV (Figure 1B) were also barely affected except a statistically not significant decrease at activating potentials more positive than +20 mV. However, when the participation of ICM arising from Na channels was decreased by a 100 ms inactivating prepulse to −50 mV (IPP50), caffeine significantly affected the gating currents, as shown in Figure 2. Figure 2A shows typical recordings of a family of gating currents recorded at different potentials applied after a 100 ms IPP50, using the protocol shown in Figure 2Ba inset. Both $I_{g,ON}$ and $I_{g,OFF}$ increased with increasing the test potential, as shown in Figure 2A left, and Q_{ON} and Q_{OFF} reached saturation at positive potentials (see Figure 2B). In the presence of 10 mM caffeine (Figure 2A right), only the $I_{g,OFF}$ amplitudes appeared to be decreased, especially for the more positive test potentials. Thus, caffeine apparently decreased in these conditions the gating currents with voltage. Average Q-V relationships for the ON and the OFF components of ICM are respectively given in Figure 2Ba,Bb, in control conditions (open circles), and in the presence of 10 mM caffeine (filled circles). In the presence of caffeine, the maximum ON charges displaced by strong depolarizations was decreased by 1.6 nC μF^{-1}. Moreover, the application of caffeine induced a consistent negative shift (5 to 10 mV) of the voltage dependence of Q_{ON} recorded from −50 mV: both components of charge activation with voltage were affected as their half-activation potentials were shifted from -25 ± 2 mV to -30 ± 10 mV for the first negative one, and from 14 ± 3 mV to 4.4 ± 10.0 mV for the second one. Although more pronounced than at −110 mV, caffeine effects on Q_{ON} following a 100 ms IPP50 were not statistically significant. The most important effect of caffeine was on the OFF component recorded on repolarization to −50 mV. Because of the relatively 'noisy' Q-V relationship of the OFF component, caffeine effect on Q_{OFF} was arbitrarily quantified by two parameters: the percentage of block induced at +40 mV, and V_B, the apparent voltage at which Q_{OFF} decreased in the presence of caffeine. When available Q_{OFF} charges moved at +40 mV were compared, caffeine significantly decreased Q_{OFF} by $46.5\pm20.3\%$ on average ($n=7$; from -5.49 ± 1.20 nC μF^{-1} in control conditions to -2.65 ± 0.85 nC μF^{-1} in the presence of caffeine), and apparent blockade appeared at $V_B=-14\pm5$ mV ($n=7$).

Effects of caffeine on ICM immobilization

The voltage dependence of Q_{ON} availability, or immobilization, was assessed as described in the legend of Figure 3, i.e., using a 1 ms return to the holding potential between the 500 ms conditioning pulse and the test pulse to +20 mV (see

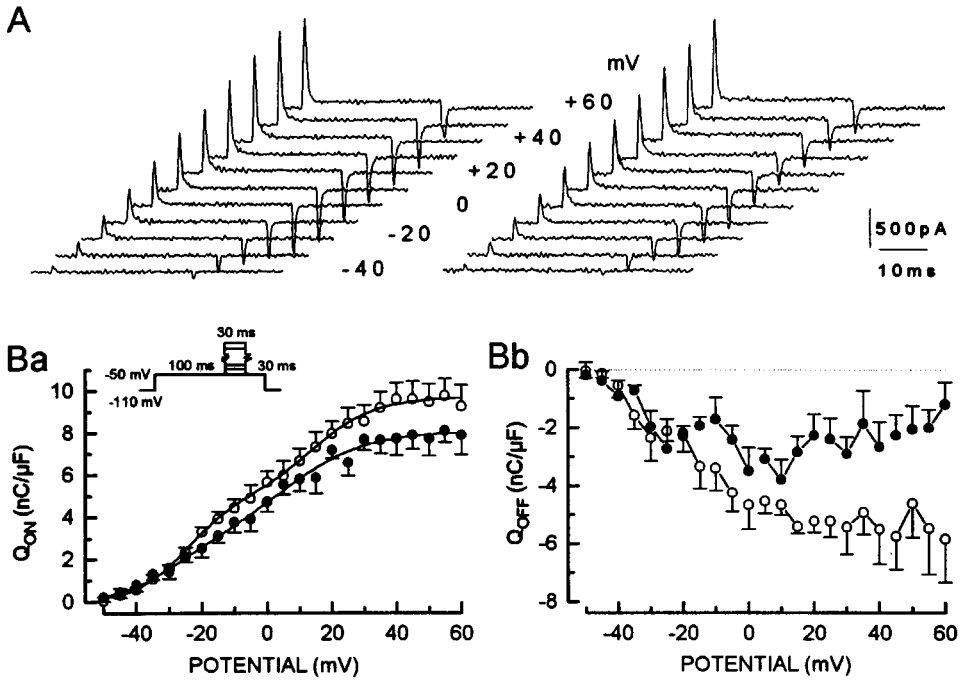

Figure 2 Caffeine effects on ICM activation following a 100 ms inactivating prepulse to −50 mV applied from −110 mV. (A) Typical recording of gating currents under control conditions (left) and after application of 10 mM caffeine (right). The currents were elicited by depolarizing the membrane for 30 ms to the potentials −40 to +60 mV (protocol shown in Ba, inset). (B) Mean charge-voltage relationships of Q_{ON} (panel Ba) and of Q_{OFF} (panel Bb) activation in control conditions (open circles) and in the presence of 10 mM caffeine (filled circles). Data points are mean values obtained in seven cells and vertical bars represent s.e.mean. The smooth lines in Ba are best fits to the mean data points with equation (1) Control: $Q_{max,1}=4.8$ nC μF^{-1}, $V_{1/2,1}=-25$ mV, $K_1=7.8$ mV; $Q_{max,2}=4.9$ nC μF^{-1}, $V_{1/2,2}=14$ mV, $K_2=9.7$ mV; caffeine: $Q_{max,1}=2.4$ nC μF^{-1}, $V_{1/2,1}=-30$ mV, $K_1=8.3$ mV; $Q_{max,2}=5.7$ nC μF^{-1}, $V_{1/2,2}=4.4$ mV, $K_2=13$ mV.

also Figure 3A inset). Complete immobilization of Q_{ON} occurred in these conditions (Figure 3A), was biphasic and well described by a double Boltzman function as previously reported (Malécot & Argibay, 1999). In control conditions, the mean $V_{1/2}$ and slope factors K calculated for seven cells were -69 ± 1 mV and -6.3 ± 0.8 mV for the first (negative) component of immobilization, and -19 ± 1 mV and -8.0 ± 0.8 mV for the second (positive) one, respectively. In the presence of 10 mM caffeine, only the amplitude of the second (positive) component was decreased from 6.7 ± 0.3 nC μF^{-1} to 5.4 ± 0.4 nC μF^{-1}. This decrease corresponds to the one occurring during Q_{ON} activation with voltage in the presence of caffeine (see Figure 1A). Neither the $V_{1/2}$ nor slope factors K were affected in the presence of caffeine.

Figure 3B shows the effect of a progressive increase of the pulse length on OFF gating currents in control conditions and in the presence of 10 mM caffeine for two test potentials. In control conditions, when the cell was depolarized by applying a voltage pulse from −110 mV to −5 or to +10 mV and the pulse duration increased from 2 to 38 ms, there was little variation in the amplitudes of $I_{g,ON}$ and of $I_{g,OFF}$ seen on repolarization to −110 mV (Figure 3Ba,Bc). However, in the presence of caffeine, although $I_{g,ON}$ amplitude remained the same from pulse to pulse (like in control conditions), $I_{g,OFF}$ amplitude markedly decreased with increasing the pulse length (Figure 3Bb,Bd). This effect was significant for potentials more positive than ≈ -10 mV, i.e. in the voltage range of calcium channels activation, and was more pronounced when the pulse amplitude was larger (compare Figure 3Bb,Bd). Thus, caffeine effect on $I_{g,OFF}$ depends on the pulse duration (i.e., on the channel opening) and voltage. It should be noted that on repolarization to −110 mV, the total Q_{OFF} charge was only slightly affected when the pulse length was increased, as could be expected from the results presented Figure 1B. Analysis of the decay of $I_{g,OFF}$ in every cell with a double exponential revealed that both components were affected in their amplitude and kinetics. For example, in the case of Figure 3B for the 19th pulse (38 ms duration), the amplitude of the fast component was decreased from −805 to −583 pA at +10 mV (only from −699 to −688 pA at −5 mV) and that of the slow one increased from −46 to −145 pA (−4 to −47 pA at −5 mV). At +10 mV, the fast time constant was slightly increased (from 218 to 271 μs) whereas that of the slow one was markedly increased from 3.8 to 52.3 ms (at −5 mV: the values of the fast and slow time constants were slightly increased from 241 to 273 μs and from 3.2 to 5.8 ms, respectively). Thus, caffeine slows the return of the gating charges to their initial position on repolarization to −110 mV, and this effect is more pronounced at positive activating potentials.

The experiments described in the remaining part of the paper were carried out to test whether the decreases of Q_{OFF} on repolarization to −50 mV and of $I_{g,OFF}$ amplitude together with the slowing of its kinetics of decay with increasing the pulse length were due to a direct effect of caffeine on the channels proteins or to a caffeine-induced increase of Ca^{2+} content in the vicinity of the inner mouth of

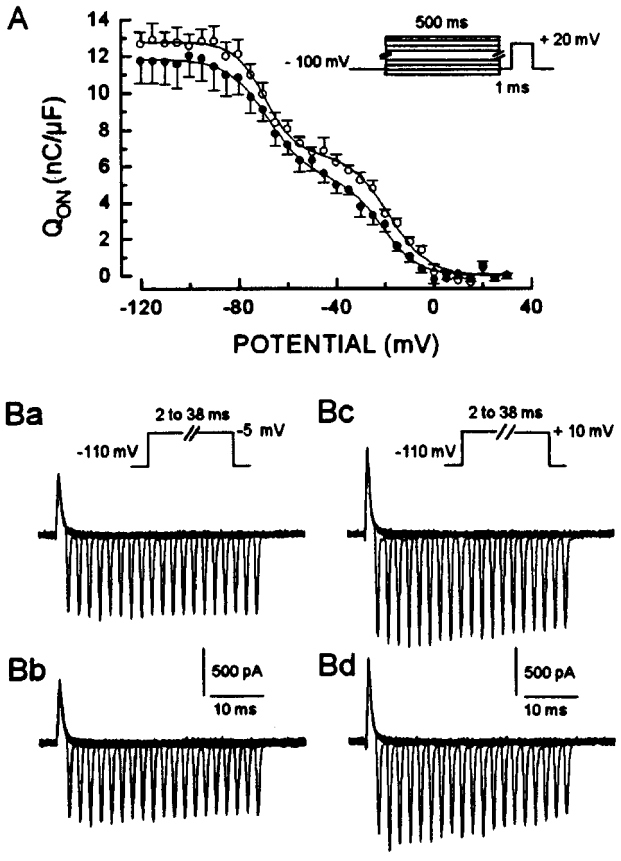

Figure 3 Effects of caffeine on ICM inactivation. (A) Mean voltage dependencies of inactivation of the Q_{ON} component of ICM in control conditions (open circles) and in the presence of 10 mM caffeine (filled circles). The cells were polarized from −100 mV to between −120 and +30 mV for 500 ms (5 mV steps) to inactivate the charges, and briefly repolarized to −100 mV for 1 ms before applying a test pulse to +20 mV (see inset). Data points are mean values obtained in seven cells and vertical bars represent s.e.mean. The smooth lines are best fits to the mean data points with equation (1). Control: $Q_{max,1}=6.1$ nC μF^{-1}, $V_{1/2,1}=-69$ mV, $K_1=-6.3$ mV; $Q_{max,2}=6.7$ nC μF^{-1}, $V_{1/2,2}=-19$ mV, $K_2=-8$ mV; caffeine: $Q_{max,1}=6.4$ nC μF^{-1}, $V_{1/2,1}=-68$ mV, $K_1=8.1$ mV; $Q_{max,2}=5.4$ nC μF^{-1}, $V_{1/2,2}=-22$ mV, $K_2=-7.6$ mV. (B) Superimposed gating currents elicited by depolarizing the cell from −110 to +20 mV and increasing the pulse duration from 2 to 38 ms in 2 ms steps in control conditions (Ba) and in the presence of 10 mM caffeine (Bb). Upwards and downwards deflections correspond to the Q_{ON} and to the Q_{OFF} components of gating currents seen on depolarization and on repolarization, respectively. Note the progressive decrease in the OFF current amplitude as the pulse length is increased in the presence of caffeine.

the ionic channels. It should be noted that all the caffeine effects described so far were mostly reversed after 5 min washout of caffeine with the 'gating' solution.

Assessment of Sr Ca^{2+} content in rested myocytes

In our gating currents recordings conditions, all ionic conductances are blocked, including the Na/Ca exchange both by the absence of external sodium and the use of cations known to affect the exchanger (Trosper & Philipson, 1983; Zhang & Hancox, 2000). The cardiomyocytes used for the experiments are in a rested state and SR Ca^{2+} content should, in theory, be low. Thus, the first point that needed to be verified was whether the SR still contained some Ca^{2+} that could be released by caffeine application. This was assessed by the study of caffeine-induced contracture (evaluated by the measurement of sarcomere length, SL) and associated rise in $[Ca^{2+}]_i$ (measured with Indo-1 fluorescence) in intact rested cells superfused with the same extracellular solutions as those used for ICM recordings. In rested cells bathed in normal Tyrode solution, the simultaneous measurements of SL and of the Indo-1 fluorescence ratio F_{405}/F_{485} (R) yielded respective mean values of $SL=1.88\pm0.01$ μm and $R=0.74\pm0.01$ ($n=32$ cells). During a twitch elicited at 0.25 Hz, the cells shortened to a mean SL of 1.82 ± 0.01 μm and the fluorescence ratio R increased to 0.94 ± 0.02 ($n=30$ cells).

Figure 4B shows a typical recording of the effects of 10 mM caffeine in a rested cell bathed in the 'gating' solution. In the example shown, the cell has been kept at rest for 12 min in normal Tyrode solution before superfusion of the 'gating' solution. This produced either no changes of the sarcomere length, or a very small not statistically significant increase of SL (<0.01 μm, $n=19$) corresponding to a slight decrease of resting tension (Figure 4B, bottom panel). In the mean time, the fluorescence ratio slowly increased from 0.75 (Figure 4B, top panel; on average: 0.74 ± 0.01, $n=19$) to a new steady-state of 1.09 (on average: 1.29 ± 0.08, $n=19$). This was due to a slow decrease of the fluorescence signal at 485 nm (not shown) induced by the presence of Cd^{2+} in the gating solution. Indeed, control experiments carried out with a Cd^{2+}-free 'gating' solution showed no increase of the fluorescence ratio, as shown in Figure 4A.

Upon application of 10 mM caffeine, a relatively large instantaneous fast initial contracture developed and partially relaxed after a few oscillations. It was immediately followed by a secondary important maintained contracture, which slowly increased throughout caffeine exposure, as shown in Figure 4B, bottom panel. In the mean time, caffeine induced a marked transient increase of the fluorescence ratio which, however, remained high (and even slightly increased) during the whole caffeine application. It should be noted that these transient responses to caffeine likely result from Ca^{2+} uptake by intracellular Ca buffers (mostly mitochondria in our experimental conditions). Upon caffeine removal, the cell partially relaxed and the fluorescence ratio decreased. However, SL and fluorescence ratio usually never completely returned to their pre-caffeine values and most of the time waving of the cell, associated to a secondary increase of the fluorescence ratio, occurred, leading to cell death on washout of the 'gating' solution with normal Tyrode solution. It should be noted that longer exposure to caffeine (up to 12 min) gave qualitatively the same results, i.e., a maintained contracture associated with a maintained increase of Indo-1 fluorescence (not shown). The decrease of SL and concomitant maintained increase of the fluorescence ratio during the whole caffeine application testify of a caffeine-induced increase of $[Ca^{2+}]_i$, since 10 mM caffeine has been shown not to affect the Indo-1 fluorescence ratio (O'Neill *et al*., 1990; McKemy *et al*., 2000). Moreover, the lack of development of a contracture associated with the increase of the fluorescence ratio during application of the 'gating' solution alone argue against an increase of $[Ca^{2+}]_i$ induced by this solution: the best explanation for the observed increase in fluorescence is likely a modification of

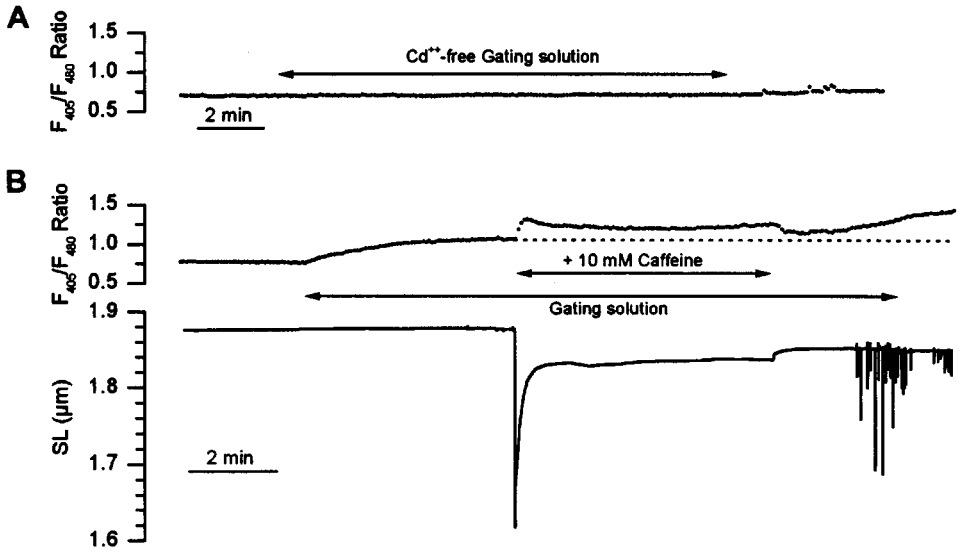

Figure 4 Caffeine-induced contracture and rise in Indo-1 fluorescence ratio in a representative intact rested cell superfused with the 'gating solution'. (A) Typical control experiment showing the absence of modification of the Indo-1 fluorescence ratio F_{405}/F_{485} in a rested cell superfused with a Cd^{2+}-free gating solution. (B) The top panel shows the evolution of Indo-1 fluorescence ratio F_{405}/F_{485} during application of the Cd^{2+}-containing gating solution and further application of 10 mM caffeine. The bottom panel shows in the same cell the concomitant evolution of the sarcomere length (SL). The cell has been rested for 12 min in normal Tyrode, and for an additional 4 min in the 'gating solution' before addition of 10 mM caffeine. Cell contracture is visualized as the decrease of the SL shown in ordinate. An initial fast contracture is followed by a secondary large maintained contracture, slightly increasing throughout caffeine application. Note the concomitant maintained increase in fluorescence during caffeine application. Caffeine effects are partially reversed upon washout with the gating solution.

the Indo-1 fluorescence emission spectrum by the high concentration of Cd^{2+} used (Owen & Dever, 1995).

It should be mentioned that gating currents were usually recorded 2–5 min after caffeine application, i.e., during the maintained contracture associated with an elevation of $[Ca^{2+}]_i$. Thus, at least in intact rested cells, the SR still contains some calcium that could be released by the caffeine solution used in our experiments. Because of the very close contact between the sarcolemmal membrane and SR in cardiac cells, we can thus expect that even in the presence of EGTA in the pipette solution which will chelate the bulk Ca^{2+} concentration (with the existence of a decreasing efficacy from the tip of the pipette towards the extremities of the cell, due to the gradient of diffusion of EGTA from the pipette into the cell), caffeine could nevertheless increase the Ca^{2+} concentration in the fuzzy-spaces (Lederer *et al.*, 1990), i.e., in the very limited restricted space lying just beneath the sarcolemmal membrane and the SR (see Discussion).

Caffeine effects on ICM in BAPTA-AM loaded cells

To test whether the caffeine effect we observed so far on Q_{OFF} could likely be due to an increase of the subsarcolemmal calcium concentration, we have recorded gatings currents in BAPTA-AM loaded cells (15 min of incubation in normal Tyrode solution containing 10 μM BAPTA-AM) and used 10 mM BAPTA instead of 10 mM EGTA in the pipette solution. In these conditions, subsarcolemmal Ca^{2+} in the fuzzy-spaces should be better chelated, as BAPTA-AM was allowed to diffuse from the outside to the inside of the cell. Figure 5A shows the typical effect of 10 mM caffeine in these cells, when the pulse length was increased at +20 mV from 2 to 38 ms. When compared to the control conditions (Figure 5Aa), caffeine had no further effect: $I_{g,OFF}$ amplitude remained constant as the pulse length was increased (Figure 5Ab), like in control conditions (compare with Figure 3B).

Average Q-V relationships in BAPTA-AM loaded cells ($n=5$) have been established using a 100 ms IPP50 to decrease the participation of ICM originating from Na channels, especially because we noticed that caffeine had much less effect when the depolarizing pulses were directly applied from the holding potential of −110 mV. When compared to the control cells studied in the presence of EGTA, the voltage dependence of Q_{ON} activation in BAPTA-AM loaded control cells (not shown) presented the same characteristics, as to the mid-activation potentials of both components and slope factors K of the Boltzman relationships. On the contrary to control (EGTA) cells, caffeine application in BAPTA-AM loaded cells did not induce any significant shift of the mid-activation potentials: from -28.0 ± 0.8 mV to -34.0 ± 3.0 mV for the first negative component, and from $+7.6\pm2.0$ mV to $+5.8\pm3.0$ mV for the second positive one (not shown). Caffeine decreased also the total amount of charges displaced by strong depolarizations by 2.3 ± 0.6 nC μF^{-1}, like in control EGTA cells. However, OFF charges measured on repolarization to −50 mV were much less depressed in BAPTA-AM loaded cells (Figure 5B): although the blockade of OFF charges at +40 mV ($16.6\pm5.5\%$, from -7.06 ± 0.22 nC μF^{-1} in control to -5.84 ± 0.25 nC μF^{-1} in the presence of 10 mM caffeine; $n=7$) was not statistically significant, an apparent blockade appeared at $V_B=15\pm5$ mV, a potential significantly more positive than in control EGTA cells ($V_B=-14\pm5$ mV).

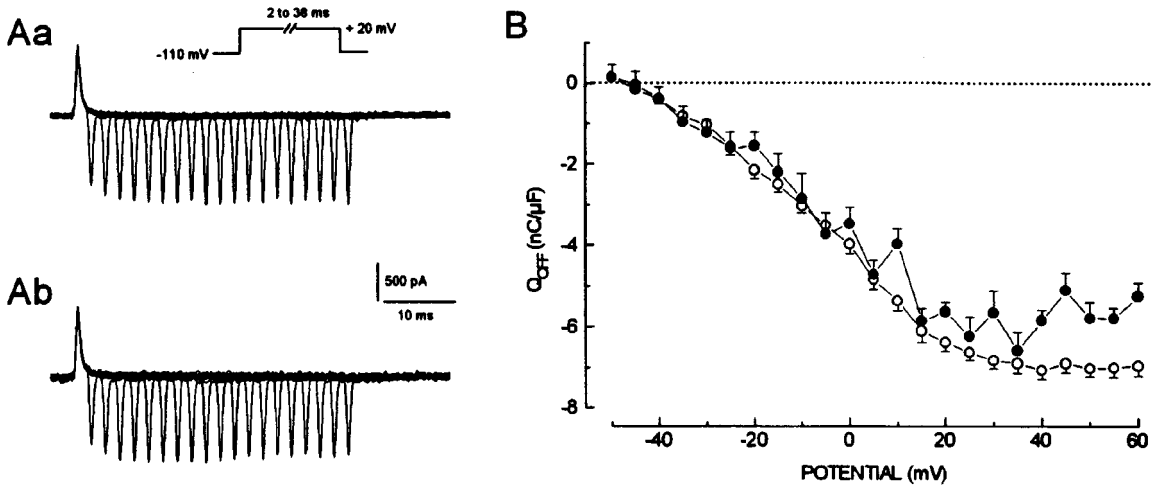

Figure 5 Effects of caffeine on ICM in cells incubated in the presence of BAPTA-AM. (A) Superimposed gating currents elicited by depolarizing the cell from −110 to +20 mV and increasing the pulse duration from 2 to 38 ms in 2 ms steps in control conditions (Aa) and in the presence of 10 mM caffeine (Ab). Upwards and downwards deflections correspond to the Q_{ON} and to the Q_{OFF} components of gating currents seen on depolarization and on repolarization, respectively. Note that in these conditions, the OFF current amplitude does not decrease as the pulse length is increased in the presence of caffeine (compare with Figure 3B). The pipette solution contained BAPTA instead of EGTA. (B) Caffeine effects on charge-voltage relationship. ICM were recorded after a 100 ms IPP50 with the protocol described in Figure 2. The graphs show the mean charge-voltage relationship Q_{OFF} (recorded during repolarization to −50 mV) in control conditions (open circles) and in the presence of 10 mM caffeine. Data points are mean values obtained in five cells and vertical bars represent s.e.mean.

Contribution of SR Ca^{2+} to the caffeine effects

To assess the participation of SR Ca^{2+} to the observed effects of caffeine when the IPP50 protocol was used, experiments were carried out either in the presence of a very high concentration of ryanodine (100 μM) to block SR Ca channels (and thus caffeine-induced Ca^{2+} release), or in SR Ca^{2+} depleted cells with the use of thapsigargin (1 μM).

When caffeine was applied in the presence of 100 μM ryanodine, Q_{ON} elicited with the 100 ms IPP50 protocol was only slightly decreased by about 0.9 ± 0.6 nC μF^{-1} ($n=5$). Half-activation potentials were shifted to more negative potentials, from -23 ± 2 mV to -34 ± 2 mV for the first negative component and from $+14\pm3$ mV to $+7.8\pm3.0$ mV for the second positive one (not shown). These differences are statistically significant only for the second component. Figure 6A shows that there was always a block of Q_{OFF} when caffeine was applied, in spite of the presence of ryanodine in the solution. The available charges moved at +40 mV in the presence of 10 mM caffeine were significantly decreased by $37.9\pm5.9\%$ (control ryanodine: -5.4 ± 0.4 nC μF^{-1}; caffeine + ryanodine: -3.4 ± 0.4 nC μF^{-1}; $n=6$). The apparent voltage V_B at which blockade of OFF charges appeared ($+5\pm5$ mV) was also significantly more positive than when caffeine alone is applied (-14 ± 5 mV).

To deplete SR Ca^{2+} content, we have pre-incubated the myocytes in normal Tyrode solution containing 1 μM thapsigargin for 15 min. Depletion was achieved in these resting non voltage-clamped cells by applying a short pulse of 1 mM caffeine in the same solution, to allow the Ca^{2+} released by the SR to be extruded from the cell by the Na/Ca exchange and the sarcolemmal Ca-ATPase. No electrical stimulation was used to avoid Ca^{2+} entry into the cells. Thereafter, these treated cells were superfused with the 'gating' solution in the continuous presence of 1 μM thapsigargin and gating currents were recorded after establishment of the whole-cell recording. In thapsigargin treated cells, the voltage dependence of Q_{ON} (100 ms IPP50 protocol) was shifted to more positive potentials, as compared to control cells. In the presence of thapsigargin, half activation potentials were shifted from -25 ± 2 mV to -9.5 ± 6.0 mV for the first negative component and from $+14\pm3$ mV to $+45\pm9$ mV for the second positive component ($n=5$). In these SR depleted cells, application of 10 mM caffeine did not significantly affect the voltage dependence of Q_{ON}. Half-activation potentials were shifted from -9.5 ± 6 mV to $+14\pm10$ mV for the first negative component and from $+45\pm9$ mV to $+41\pm5$ mV for the second positive one (not shown). The Q_{OFF}-voltage relationship of these cells is shown in Figure 6B. Apparent block of Q_{OFF} in the presence of caffeine appeared at $V_B=+28\pm9$ mV, a potential significantly more positive than that in control conditions (-14 ± 5 mV; see Figure 2Bb). However, the caffeine-induced decrease of Q_{OFF} at +40 mV (from -5.0 ± 0.7 nC μF^{-1} to -4.2 ± 0.9 nC μF^{-1}) was no longer significant (mean decrease of $15.4\pm20.2\%$, $n=5$) when the SR Ca^{2+} content has been previously depleted by thapsigargin pretreatment. It should be noted that like in BAPTA-AM loaded cells (Figure 5A), when the SR Ca channels were blocked by ryanodine or when the SR was depleted with the use of thapsigargin, caffeine did not decrease $I_{g,OFF}$ amplitude as the pulse length was increased from 2 to 38 ms from a holding potential of −110 mV (not shown).

Mechanism of action of caffeine

The results presented so far show that when caffeine is applied to myocytes, some of the charges moved by depolarizing the cell from −50 mV to positive potentials

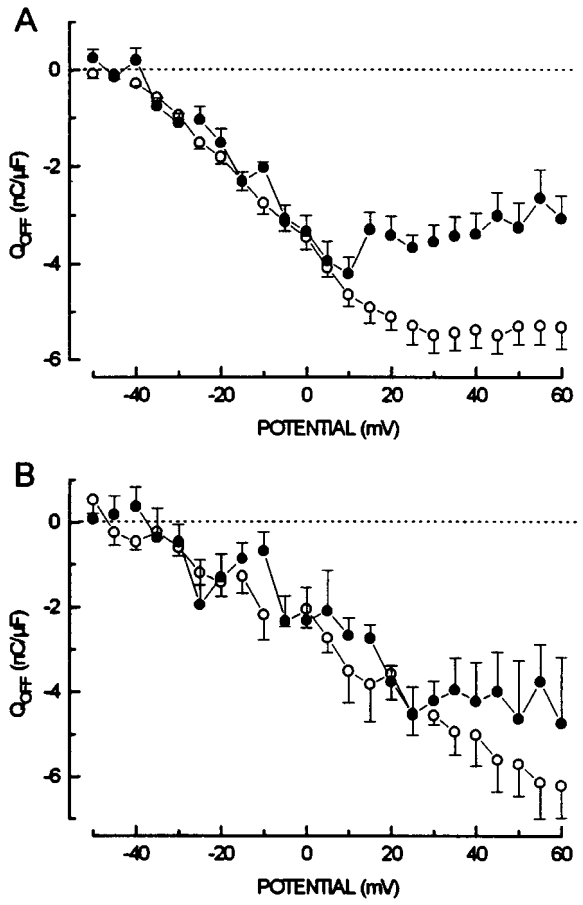

Figure 6 Effects of caffeine on charge-voltage relationships in the presence of agents affecting the sarcoplasmic reticulum (SR). ICM were recorded after a 100 ms IPP50 with the protocol described in Figure 2. The graphs show the mean charge-voltage relationships of Q_{OFF} (recorded during repolarization to −50 mV) in control conditions (open circles) and in the presence of 10 mM caffeine. (A) Cells preincubated with 100 μM ryanodine and continuously superfused with ryanodine for the control conditions. (B) Cells in which the SR has been depleted by preincubation with 1 μM thapsigargin and continuously superfused with thapsigargin for the control conditions. Data points are mean values obtained in five (A,B) cells and vertical bars represent s.e.mean.

(100 ms IPP50 protocol) do not come back to their initial state when cells are repolarized to −50 mV: Q_{OFF} is depressed, especially at positive potentials. This apparent immobilization during depolarization could be due either to a caffeine-induced voltage-dependent block likely mediated by Ca^{2+} ions, or to a shift to more negative potentials of the voltage dependence of the return of the charges to their initial positions (known as the interconversion of charge 1 into charge 2, see Introduction for references). To test this hypothesis, we have used a protocol in which the cells were repolarized to potentials +30 to −170 mV, after a 30 ms depolarization to +40 mV was applied from −50 mV (see protocol in Figure 7A right inset) to induce charges inactivation. Figure 7A shows the mean ($n=9$) voltage dependence of Q_{OFF} recorded with this protocol in control conditions and in the presence of 10 mM caffeine. As can be seen, caffeine markedly affected the return of the OFF charges, by inducing a negative shift of their voltage dependence: mid activation potentials of Q_{OFF} were shifted from -93 ± 4 mV to -110 ± 10 mV for the first negative component, and from -28 ± 7 mV to -58 ± 10 mV for the second positive one. However, in both control conditions and in the presence of caffeine, the maximum Q_{OFF} charges remained the same at −150 mV (-13.3 ± 0.7 nC μF^{-1} and -12.7 ± 1.1 nC μF^{-1}, respectively). It should be noted that caffeine completely blocked the return of the OFF charges for potentials between +30 mV and −40 mV. The left inset in Figure 7A shows that caffeine significantly reduced the OFF charges evoked on repolarization from +40 mV to −80 mV: on average, OFF charges at −80 mV amounted to -7.65 ± 0.52 nC μF^{-1} in control conditions and to -5.05 ± 0.54 nC μF^{-1} in the presence of 10 mM caffeine. Thus, caffeine did not really block the return of the OFF charges on repolarization, but induced a shift towards negative potentials of the voltage dependence of their return: this shift is the manifestation of a caffeine-induced interconversion between charge 1 and charge 2.

We have tested the potential role of the Ca^{2+} released by the SR in the decrease of OFF charges (and thus in the charge interconversion) using the same protocol in three experimental conditions: cells loaded with BAPTA-AM to improve subsarcolemmal Ca^{2+} chelation, cells with SR Ca channels blocked by ryanodine and cells whose SR has been previously depleted with thapsigargin. Q-V relationships of Q_{OFF} realized in these conditions show that when intracellular calcium chelation was more efficient with BAPTA, caffeine had much less effect on the voltage dependence of Q_{OFF} (Figure 7B), especially for positive potentials. However, half-activation potentials of Q_{OFF} were still significantly shifted from -87.0 ± 3.8 mV to -102.9 ± 5.8 mV for the first negative component and from -7.7 ± 4.2 mV to -18.5 ± 2.8 mV for the second positive one ($n=7$). Apparent block of the charges recorded on repolarization was only obtained between +40 mV and +20 mV. An example of OFF charges at −80 mV is shown in Figure 7B inset: in the presence of BAPTA, caffeine has little effect on the OFF charges recorded at −80 mV.

When the SR Ca channels were blocked with 100 μM ryanodine, caffeine did not induce a negative shift of the voltage dependence of OFF charges (Figure 7C): in five cells, half-activation potentials were not significantly shifted from -98 ± 6 mV to -90 ± 3 mV for the first negative component and from -24 ± 10 mV to -19 ± 6 mV for the second positive component. Apparent block of the charges recorded on repolarization was only obtained between +40 mV and 0 mV. An example of OFF charges at −80 mV is shown in Figure 7C inset. The total charge measured at −170 mV was slightly depressed by 1.1 nC μF^{-1} in the presence of caffeine. Similar results were obtained in cells whose SR has been previously depleted with thapsigargin: caffeine did not induce any statistically significant modifications of the Q_{OFF} voltage dependence, as illustrated in Figure 7D. Mid activation potentials of Q_{OFF} were shifted from -97 ± 10 mV to -83 ± 10 mV for the first negative component, and from -30 ± 20 mV to -15 ± 20 mV for the second positive one ($n=7$). Like in control cells (i.e., non pretreated with thapsigargin), in both control conditions and in the presence of caffeine, the maximum Q_{OFF} charges remained the same at −150 mV (-15.01 ± 0.97 nC μF^{-1} and -13.7 ± 1.3 nC μF^{-1}, respectively). Apparent block of the charges recorded on repolarization was only obtained between +40 mV and

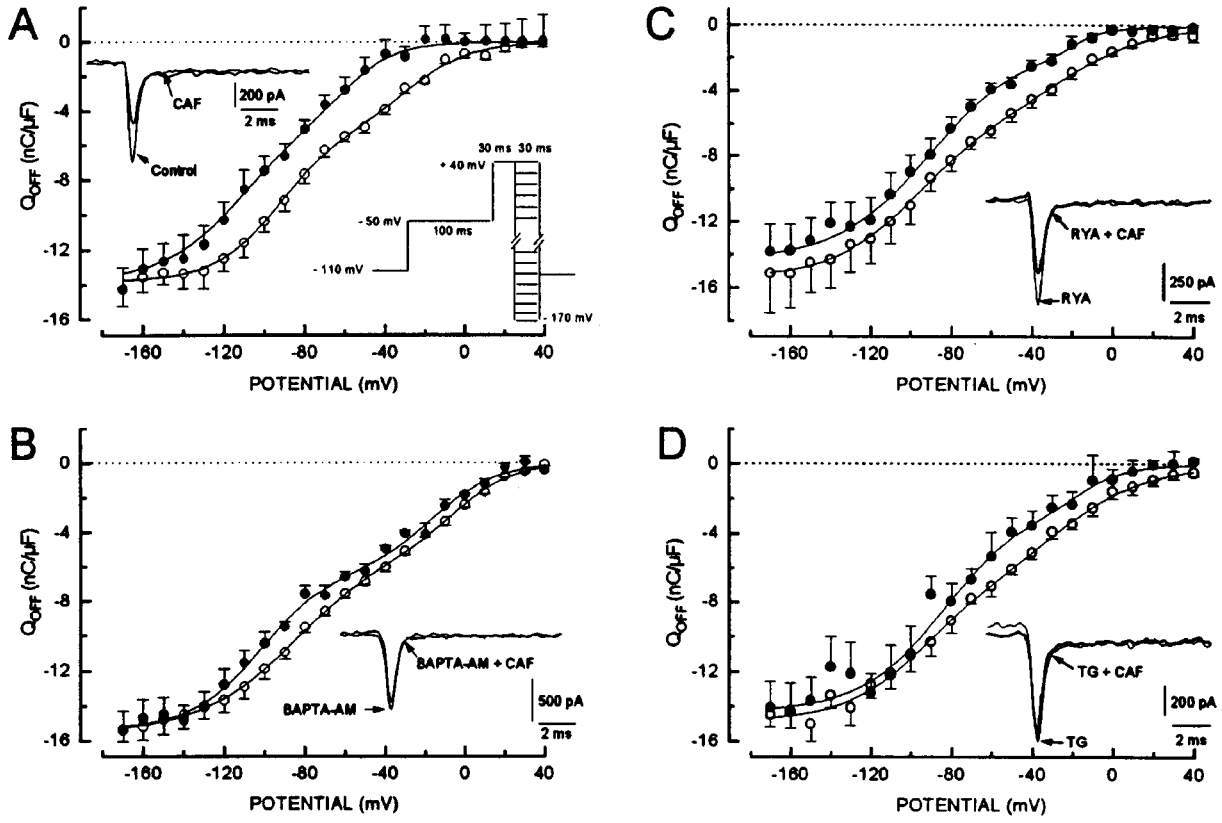

Figure 7 Effects of caffeine on the voltage-dependence of OFF charges activation following a depolarization to +40 mV according to the protocol shown in bottom right inset in A (see text for details). In each panel, data points are mean values obtained in nine (A), six (B), five (C) and seven (D) cells and vertical bars represent s.e.mean. The smooth lines are best fits to the data points with equation (1) The top left inset in A or bottom right insets in B, C and D show superimposed typical recordings of OFF gating currents at −80 mV in corresponding control conditions (thin line) and in the presence of 10 mM caffeine (thick line). (A) Mean charge-voltage relationships of Q_{OFF} in control conditions (open circles) and in the presence of 10 mM caffeine. The pipette solution contained EGTA. Control EGTA: $Q_{max,1}=-8.6$ nC μF^{-1}, $V_{1/2,1}=-93$ mV, $K_1=-16$ mV; $Q_{max,2}=-5.2$ nC μF^{-1}, $V_{1/2,2}=-28$ mV, $K_2=-16$ mV; caffeine: $Q_{max,1}=-10$ nC μF^{-1}, $V_{1/2,1}=-110$ mV, $K_1=-20$ mV; $Q_{max,2}=-3.8$ nC μF^{-1}, $V_{1/2,2}=-58$ mV, $K_2=-12$ mV. (B) Same representation as in A, for cells preincubated in the presence of 5 μM BAPTA-AM and patched with BAPTA-containing pipette solution. Control BAPTA: $Q_{max,1}=-13$ nC μF^{-1}, $V_{1/2,1}=-70$ mV, $K_1=-19$ mV; $Q_{max,2}=1.7$ nC μF^{-1}, $V_{1/2,2}=-8.2$ mV, $K_2=-5.2$ mV; caffeine: $Q_{max,1}=-13$ nC μF^{-1}, $V_{1/2,1}=-76$ mV, $K_1=-15$ mV; $Q_{max,2}=-1.5$ nC μF^{-1}, $V_{1/2,2}=-25$ mV, $K_2=-5.4$ mV. (C,D) Effects of caffeine in the presence of agents affecting the SR on the voltage-dependence of OFF charges activation following a depolarization to +40 mV. Same representation as in A. (C) In the presence of 100 μM ryanodine (RYA). Control RYA: $Q_{max,1}=-9.4$ nC μF^{-1}, $V_{1/2,1}=-98$ mV, $K_1=-21$ mV; $Q_{max,2}=-6$ nC μF^{-1}, $V_{1/2,2}=-24$ mV, $K_2=-23$ mV; caffeine: $Q_{max,1}=-13$ nC μF^{-1}, $V_{1/2,1}=-90$ mV, $K_1=-23$ mV; $Q_{max,2}=-1.3$ nC μF^{-1}, $V_{1/2,2}=-19$ mV, $K_2=-6.6$ mV. (D) In the presence of 1 μM thapsigargin (TG) in SR Ca-depleted cells. Control TG: $Q_{max,1}=-6.9$ nC μF^{-1}, $V_{1/2,1}=-97$ mV, $K_1=-19$ mV; $Q_{max,2}=-7.9$ nC μF^{-1}, $V_{1/2,2}=-30$ mV, $K_2=-24$ mV; caffeine: $Q_{max,1}=-12$ nC μF^{-1}, $V_{1/2,1}=-83$ mV, $K_1=-21$ mV; $Q_{max,2}=-2.3$ nC μF^{-1}, $V_{1/2,2}=-15$ mV, $K_2=-11$ mV.

+5 mV (Figure 7D). Figure 7D inset shows a typical example of OFF charges recorded at −80 mV in thapsigargin-treated cells: caffeine barely affected Q_{OFF} at −80 mV (-9.1 ± 0.7 nC μF^{-1} in control thapsigargin and -7.9 ± 1.1 nC μF^{-1} in the presence of both thapsigargin and caffeine, $n=7$).

Discussion

The main result of our study is that, in freshly isolated guinea-pig ventricular heart cells, under our experimental conditions, caffeine markedly depressed OFF charges on repolarization to −50 mV with little effect on the ON charges on depolarization from −50 mV. This inhibitory effect can be explained by charge 1−charge 2 interconversion likely mediated *via* the Ca^{2+} released from the SR which accumulate in the fuzzy spaces to produce an increase of subsarcolemmal calcium concentration.

The effects of caffeine we observed on OFF charges could be due either to a direct effect of the molecule on the channels proteins, or to an indirect effect *via* its two well-known cellular actions. First, like other methylxanthines, caffeine is a phosphodiesterase inhibitor (Beavo *et al*., 1970) and thus increases the cyclic AMP-dependent phosphorylation processes. Second, caffeine induces the release of Ca^{2+} from the SR (e.g., Weber, 1968; Wier, 1990). The suppression of most caffeine effects in BAPTA-AM loaded cells and in cells whose SR has been depleted with thapsigargin argue against a direct effect of caffeine on the channels proteins, as well as against an effect mediated *via* an increased cyclic AMP level. On the contrary, they are more likely in favour of a potential effect of the caffeine-induced Ca^{2+}-release by the

SR, and thus of an effect mediated by the elevation of intracellular Ca^{2+} concentration. Indeed, preliminary experiments carried out in guinea-pig ventricular heart cells have revealed that OFF charges were not decreased by phosphorylation induced by 1 μM isoprenaline or 10 μM forskolin (authors' unpublished results). A screening of surface charges by the calcium released from the SR is also unlikely to explain our results since ON and OFF charges are differently affected.

SR Ca^{2+} content in rested cells bathed in gating solution

An important question raised is whether there is still some Ca^{2+} in the SR that could be released by caffeine in our experimental conditions, as SR Ca^{2+} is well known to decrease in rested cells. We show in our study (Figure 4) that in intact ventricular cells rested for 12 min in normal Tyrode before application of our 'gating solution' for an additional 3–4 min, the subsequent application of 10 mM caffeine produces a marked contracture (assessed by the sarcomere length shortening) associated with a maintained rise in Indo-1 fluorescence ratio (F_{405}/F_{485}). This observation demonstrates that although the myocytes are quiescent, there remains a substantial fraction of Ca^{2+} in the SR that can be released by application of caffeine in our experimental conditions. This result is in agreement with the findings of Terracciano *et al.* (1995) who have reported that the SR-Ca^{2+} content of guinea-pig ventricular myocytes exponentially decreases when a cell is kept at rest (rate constant of 0.029 s^{-1}) and that, thereafter, there remained a portion (56%) of caffeine-releasable Ca^{2+} that leaves the SR more slowly.

Does caffeine efficiently increase the subsarcolemmal Ca^{2+} concentration?

In our experimental conditions, several factors will interact to control the intracellular Ca^{2+} concentration. First, the blockade of the Na/Ca exchange by the absence of Na^+ in the gating solution and the presence of cations known to affect the exchange current (Gd^{3+}, Cd^{2+}; Trosper & Philipson, 1983; Zhang & Hancox, 2000) should contribute to keep the intracellular Ca^{2+} concentration high during caffeine application (as indeed observed, see Figure 4B). Second, the sarcolemmal Ca^{2+}-ATPase, although more efficient when the Na/Ca exchange is blocked, has likely a too small Ca^{2+} transport capacity to properly extrude the Ca^{2+} (e.g., Bassani *et al.*, 1995). Moreover, it has been shown that Cd^{2+} is an effective blocker of the sarcolemmal Ca^{2+}-ATPase at a concentration of 20 nM (Verbost *et al.*, 1988), i.e., at a concentration much lower than that used in the present study (2 mM). On the other hand, cell Ca^{2+} content should be noticeably decreased as part of the Ca^{2+} released from the SR in the presence of caffeine will be chelated by the EGTA (or BAPTA) present in the pipette solution and diffusing into the cell: bulk Ca^{2+} concentration will therefore be kept to a very low level, not sufficient to induce contractures like that presented in Figure 4B. However, although found in the rat, it has been reported that even in ventricular cells in which the myoplasmic Ca^{2+} concentration was buffered with 10 mM EGTA (as in our experiments), Ca channels were inactivated by the Ca^{2+} released from the SR (e.g., Sham *et al.*, 1995; Adachi-Akahane *et al.*, 1996), because of the existence of a functional coupling of Ca channels and ryanodine receptors in these cardiac myocytes. Such a coupling might also exist in the guinea-pig heart as suggested by the results of an ultrastructural study combining confocal microscopy and label-fracture electron microscopy (Takagishi *et al.*, 1997). Ca channels of the sarcolemmal membrane and of the T-tubules appeared to be organized in cluster, each cluster lying adjacent to junctional SR, thus permitting the close coupling of influx of calcium through plasma membrane Ca channels to trigger SR Ca^{2+} release. Other studies have also suggested the existence of micro-domains ('fuzzy-spaces': e.g., Leblanc & Hume, 1990; Lederer *et al.*, 1990; Bielen *et al.*, 1991; see also for review Ríos & Stern, 1997) lying just beneath the inner leaflet of the sarcolemmal membrane and the SR membrane and in which diffusion of ions and molecules is limited (e.g., Stern, 1992; Adachi-Akahane *et al.*, 1996; Naraghi & Neher, 1997; Neher, 1998). In particular, large molecules such as EGTA and BAPTA will not have free access to the micro-domains to rapidly chelate Ca^{2+} ions, and will therefore control only the bulk cytoplasmic Ca^{2+} concentration, and not the subsarcolemmal one. However, it appears that Ca^{2+} leaving the SR can accumulate in these subsarcolemmal micro-domains from which it will be extruded, in normal conditions, mostly by the Na-Ca exchange (see Callewaert, 1992). During caffeine application in our experiments, part of the Ca^{2+} released from the SR will therefore mostly remain trapped in these subsarcolemmal micro-domains, because of the blockades of the Na/Ca exchange (Gd^{3+}, Cd^{2+}, lack of external Na^+; Trosper & Philipson, 1983; Zhang & Hancox, 2000) and of the sarcolemmal Ca^{2+}-ATPase by Cd^{2+} (Verbost *et al.*, 1988). Unfortunately, to our knowledge, there are no convenient techniques available for the quantification of subsarcolemmal Ca^{2+} concentration in these subsarcolemmal micro-domains. Moreover, in the presence of caffeine and internal EGTA or BAPTA, fluorescence measurement of bulk intracellular Ca^{2+} or measurement of sarcomere length will not give any information on the Ca^{2+} concentration in these restricted domains.

Mode of action of subsarcolemmal Ca^{2+}

As shown in Figure 7A, prolonged depolarization in the presence of caffeine shifted the OFF charge distribution to negative voltages, without significantly affecting the total amount of charges displaced. These observations are consistent with the known property of charge interconversion first described in detail in skeletal muscle (see Introduction for references), and later on in heart cells by Shirokov *et al.* (1992; 1993). Charge 1 has been described as the movement of intramembranous charges between -100 and $+40$ mV in normally polarized cells, whereas charge 2 has been observed in depolarized fibres, with a different voltage sensitivity, moving at hyperpolarized potentials. It has been proposed that during prolonged depolarization charge 1 is transformed into charge 2 (charge interconversion; Brum & Ríos, 1987). Thus, the effect of Ca^{2+} is to promote this charge interconversion, i.e., to increase the proportion of charges in the immobilized state (or charge 2). This is also seen when the duration of the depolarizing pulse is increased (as in Figure 3B): only OFF gating current amplitude is decreased. This observation, together with the fact that only OFF

charges appear to be affected with voltage, strongly suggest that ionic channels likely have to open before Ca^{2+} ions could affect their gating properties.

In contrast to our present observations, no effect of increased intracellular Ca^{2+} concentration has been previously found in native cardiac cells (Hadley & Lederer, 1991b; Shirokov *et al*., 1993). Hadley & Lederer (1991b) have shown using flash photolysis of DM-nitrophen that an increase of intracellular Ca^{2+} concentration induced an inactivation of L-type Ca^{2+} current, but without any effect on ON charges (they did not study the OFF charges). Our findings do not contradict their results, at least for one point, as we also found almost no effect on ON charges. In their study, Shirokov and co-workers (1993) have measured in the same cell calcium influx through Ca channels and ICM at -150 mV (a potential at which charge 2 is already maximum) to determine if Ca^{2+} influx was promoting charge 1–charge 2 interconversion, i.e., charge immobilization. As the amount of charge measured at -150 mV was not increased when Ca^{2+} was entering the cell, these authors concluded that the Ca^{2+}-dependent inactivation of L-type Ca channels does not affect their voltage sensor. However, we have also found that the total charge displaced at -150 mV was not increased in the presence of caffeine: this is because at this potential, all charges that have moved during depolarization have already returned to their initial positions. Thus, charge interconversion can be seen only at more positive potentials, where they are in 'intermediate' positions. This is shown by the negative shift of the Q_{OFF}-voltage relationship in the presence of caffeine (Figure 7A).

Our results in native cells are in agreement with those of Ferreira *et al*. (1998) on CHO cells co-expressing a mostly cardiac α_1(CSk3), β_{1a}, $\alpha_2\delta$ and γ Ca channel subunit. These authors found that 10 μM intracellular Ca^{2+} immobilized gating currents, but only after opening transitions had taken place. However, two recent studies on expressed N-type Ca channels (Jones *et al*., 1999; Shirokov, 1999) report contradictory results on the effect of Ca^{2+} on inactivation of the Ca channels at the level of the gating currents. For Jones *et al*. (1999), inactivation of N-type Ca channels is purely voltage-dependent (as the degree of charge immobilization was unchanged by the block of ionic current and was closely matched by the degree of current inactivation) and occurs from intermediate closed conformations along the activation pathway (no need for the channel to open before it inactivates). On the contrary, Shirokov (1999) found no immobilization of the gating current with voltage (but evidence for charge 1-charge 2 interconversion) and a current-dependent inactivation of the N-type Ca channels (Ca^{2+} would act in the permeation pathway).

In conclusion, since (i) ON and OFF charges are differently affected and (ii) caffeine effect on OFF charges is dependent upon the duration and voltage (Figure 3B) of the depolarizing pulse (in the voltage range of activation of Ca channels, thus precluding that the observed effects reflected the kinetics of binding of Ca^{2+} on an intracellular site), our results strongly support that Ca channels have to open before Ca^{2+} can affect their gating. We postulate that such a mechanism might be involved in the Ca^{2+}-dependent inactivation of the Ca^{2+} current under normal physiological conditions, as high Ca^{2+} concentration can be reached in the subsarcolemmal spaces. Our results are the first evidence in native cardiac cells for a role played by the Ca^{2+} released from the SR in modulating Ca channels inactivation at the level of their gating currents. Whether Ca^{2+} acts by itself or in association with a modulatory protein such as calmodulin, as recently suggested for several Ca channels types (Ehlers & Augustine, 1999; Lee *et al*., 1999; Peterson *et al*., 1999; Zühlke *et al*., 1999) awaits further study. Our findings might nevertheless lead to a better understanding of the modulatory role played by Ca^{2+} in cardiac excitation–contraction coupling.

Part of this work was supported by special grants from the 'Conseil Général d'Indre et Loire', the 'Conseil Régional du Centre', and the 'Fondation pour la Recherche Médicale'. J. Leroy is the recipient of a Doctoral 'Allocation de Recherche' from the Ministère de la Recherche. J.M. Lignon and F. Gannier have equally contributed to this work and can therefore be inverted in the authors listing, as needed.

References

ADACHI-AKAHANE, S., CLEEMAN, L. & MORAD, M. (1996). Cross-signaling between L-type Ca2+ channels and ryanodine receptors in rat ventricular myocytes. *J. Gen. Physiol.,* **108,** 435–454.

ADRIAN, R.H. & ALMERS, W. (1976). Charge movement in the membrane of skeletal muscle. *J. Physiol. (Lond.),* **254,** 339–360.

ARMSTRONG, C.M. & BEZANILLA, F. (1973). Currents related to movement of the gating particles of the sodium channels. *Nature,* **242,** 459–461.

BASSANI, R.A., BASSANI, J.W.M. & BERS, D.M. (1995). Relaxation in ferret ventricular myocytes: role of the sarcolemmal Ca ATPase. *Pflügers Arch.,* **430,** 573–578.

BEAN, B.P. & RÍOS, E. (1989). Nonlinear charge movement in mammalian cardiac ventricular cells. Components from Na and Ca channel gating. *J. Gen. Physiol.,* **94,** 65–93.

BEAVO, J.A., ROGERS, N.L., CROFFORD, J.G., HARDMAN, E.W., SUTHERLAND, E.W. & NEWMAN, E.V. (1970). Effects of xanthine derivatives on lipolysis and on adenosine 3′,5′-monophosphate phosphodiesterase activity. *Mol. Pharmacol.,* **6,** 597–603.

BEZANILLA, F. & ARMSTRONG, C.M. (1977). Inactivation of the sodium channel. I. Sodium currents experiments. *J. Gen. Physiol.,* **70,** 549–566.

BIELEN, F.V., GLITSCH, H.G. & VERDONCK, F. (1991). Changes of the subsarcolemmal Na^+ concentration in internally perfused cardiac cells. *Biochim. Biophys. Acta,* **1065,** 269–271.

BLAYNEY, L., THOMAS, H., MUIR, J. & HENDERSON, A. (1978). Action of caffeine on calcium transport by isolated fractions of myofibrils, mitochondria, and sarcoplasmic reticulum from rabbit heart. *Circul. Res.,* **43,** 520–526.

BRUM, G. & RÍOS, E. (1987). Intramembrane charge movement in frog skeletal muscle fibres: properties of charge 2. *J. Physiol. (Lond.),* **387,** 489–517.

CALLEWAERT, G. (1992). Excitation-contraction coupling in mammalian cardiac cells. *Cardiovasc. Res.,* **26,** 923–932.

CAPUTO, C. & BOLAÑOS, P. (1989). Effects of D600 on intramembrane charge movement of polarized and depolarized frog muscle fibers. *J. Gen. Physiol.,* **94,** 43–64.

CATTERALL, W.A. (1988). Structure and function of voltage-sensitive ion channels. *Science,* **242,** 50–61.

CSERNOCH, L., SZENTESI, P. & KOVÁCS, L. (1999). Differential effects of caffeine and perchlorate on excitation-contraction coupling in mammalian skeletal muscle. *J. Physiol. (Lond.),* **520,** 217–230.

DULHUNTY, A.F. (1992). The voltage-activation of contraction in skeletal muscle. *Prog. Biophys. Mol. Biol.,* **57,** 181–223.

ECKERT, R. & CHAD, J.E. (1984). Inactivation of Ca channels. *Prog. Biophys. Mol. Biol.,* **44,** 215–267.

EHLERS, M.D. & AUGUSTINE, G.J. (1999). Calmodulin at the channel gate. *Nature,* **399,** 105–108.

EVONIUK, G., JACOBSON, K.A., SHAMIM, M.T., DALY, J.W. & WURTMAN, R.J. (1987). A1- and A2-selective adenosine antagonists: in vivo characterization of cardiovascular effects. *J. Pharmacol. Exp. Ther.,* **242,** 882–887.

FERREIRA, G., TAKESHIMA, H., RÍOS, E. & GONZÁLEZ, A. (1998). High intracellular calcium affects L-type calcium channel gating. *Biophys. J.,* **74,** A101.

GANNIER, F., BERNENGO, J.-C., JACQUEMOND, V. & GARNIER, D. (1993). Measurement of sarcomere dynamics simultaneously with auxotonic force in isolated cardiac cells. *I.E.E.E. Trans. Biomed. Engin.,* **40,** 1226–1232.

GANNIER, F., LIGNON, J., PAYANDEH, B. & BERNENGO, J.-C. (1998). High-speed sarcomere measurements in isolated cardiomyocytes during contraction. *Biophys. J.,* **74,** A153.

HAACK, J.A. & ROSENBERG, R.L. (1994). Calcium-dependent inactivation of L-type calcium channels in planar lipid bilayers. *Biophys. J.,* **66,** 1051–1060.

HADLEY, R.W. & LEDERER, W.J. (1989). Intramembrane charge movement in guinea pig and rat ventricular myocytes. *J. Physiol.,* **415,** 601–624.

HADLEY, R.W. & LEDERER, W.J. (1991a). Properties of L-type calcium channel gating current in isolated guinea-pig ventricular myocytes. *J. Gen. Physiol.,* **98,** 265–285.

HADLEY, R.W. & LEDERER, W.J. (1991b). Ca^{2+} and voltage inactivate Ca^{2+} channels in guinea-pig ventricular myocytes through independent mechanisms. *J. Physiol.,* **444,** 257–268.

HANCK, D.A., SHEETS, M.F. & FOZZARD, H.A. (1990). Gating currents associated with Na channels in cardiac Purkinje cells. *J. Gen. Physiol.,* **95,** 439–457.

HESS, P. & WIER, W.G. (1984). Excitation-contraction coupling in cardiac Purkinje fibers. Effects of caffeine on the intracellular $[Ca^{2+}]$ transient, membrane currents, and contraction. *J. Gen. Physiol.,* **83,** 417–433.

HOVE-MADSEN, J. & BERS, D.M. (1992). Indo-1 binding to protein in permeabilized ventricular myocytes alters its spectral and Ca binding properties. *Biophys. J.,* **63,** 89–97.

HUANG, C.L.H. (1988). Intramembrane charge movements in skeletal muscle. *Physiol. Rev.,* **68,** 1197–1246.

HUANG, C.L.H. (1998). The influence of caffeine on intramembrane charge movements in intact frog striated muscle. *J. Physiol. (Lond.),* **512,** 707–721.

JONES, L.P., DEMARIA, C.D. & YUE, D.T. (1999). N-type calcium channel inactivation probed by gating current analysis. *Biophys. J.,* **76,** 2530–2552.

JONG, D.S., PAPE, P.C., BAYLOR, S.M. & CHANDLER, W.K. (1995a). Calcium inactivation of calcium release in frog cut muscle fibers that contain millimolar EGTA or fura-2. *J. Gen. Physiol.,* **106,** 337–388.

JONG, D.S., PAPE, P.C. & CHANDLER, W.K. (1995b). Effects of sarcoplasmic reticulum calcium depletion on intramembranous charge movement in frog cut muscle fibers. *J. Gen. Physiol.,* **106,** 659–704.

KEYNES, R.D. & ROJAS, E. (1974). Kinetics and steady-state properties of the charged system controlling sodium conductance in the squid giant axon. *J. Physiol. (Lond.),* **239,** 393–434.

KOVÁCS, L. & SZÚCS, G. (1983). Effect of caffeine on intramembrane charge movement and calcium transients in cut skeletal muscle fibres of the frog. *J. Physiol. (Lond.),* **341,** 559–578.

LARSSON, H.P., BAKER, O.S., DHILLON, D.S. & ISACOFF, E.Y. (1996). Transmembrane movement of the *Shaker* K^+ channel. *Neuron,* **16,** 387–397.

LEBLANC, N. & HUME, J.R. (1990). Sodium current-induced release of calcium from cardiac sarcoplasmic reticulum. *Science,* **248,** 372–376.

LEDERER, W.J., NIGGLI, E., HADLEY, E. & HADLEY, R.W. (1990). Sodium-calcium exchange in excitable cells: fuzzy space. *Science,* **248,** 283.

LEE, A., WONG, S.T., GALLAGHER, D., LI, B., STORM, D.R., SCHEUER, T. & CATTERALL, W.A. (1999). Ca^{2+}/calmodulin binds to and modulates P/Q-type calcium channels. *Nature,* **399,** 155–159.

LE GUENNEC, J.-Y., PEINEAU, N., ESNARD, F., LACAMPAGNE, A., GANNIER, F., ARGIBAY, J., GAUTHIER, F. & GARNIER, D. (1993). A simple method for calibrating collagenase/pronase E ratio to optimize heart cell isolation. *Biol. Cell,* **79,** 161–165.

LEROY, J., ARGIBAY, J.A. & MALÉCOT, C.O. (1998). Local control of ion channels gating currents by subsarcolemmal calcium in isolated guinea-pig heart cells. *J. Gen. Physiol.,* **112,** 17a.

MALÉCOT, C.O. & ARGIBAY, J.A. (1999). Block of gating currents related to K^+ channels as a mechanism of action of clofilium and d-sotalol in isolated guinea-pig ventricular heart cells. *Br. J. Pharmacol.,* **128,** 301–312.

MANNUZZU, L.M., MORONNE, M.M. & ISACOFF, E.Y. (1996). Direct physical measure of conformational rearrangement underlying potassium channel gating. *Science,* **271,** 213–216.

MCKEMY, D.D., WELCH, W., AIREY, J.A. & SUTKO, J.L. (2000). Concentrations of caffeine greater than 20 mM increase the indo-1 fluorescence ratio in a Ca^{2+}-independent manner. *Cell Calcium,* **27,** 117–124.

MEISSNER, G. & HENDERSON, J.S. (1987). Rapid calcium release from cardiac sarcoplasmic reticulum vesicles is dependent on Ca^{2+} and is modulated by Mg^{2+}, adenine nucleotide, and calmodulin. *J. Biol. Chem.,* **262,** 3065–3073.

MENTRARD, D., VASSORT, G. & FISCHMEISTER, R. (1984). Calcium-mediated inactivation of the calcium conductance in cesium-loaded frog heart cells. *J. Gen. Physiol.,* **83,** 105–131.

NARAGHI, M. & NEHER, E. (1997). Linearized buffered Ca^{2+} diffusion in microdomains and its implications for circulation of $[Ca^{2+}]$ at the mouth of a calcium channel. *J. Neurosci.,* **17,** 6961–6973.

NEHER, E. (1998). Usefulness and limitations of linear approximations to the understanding of Ca^{++} signals. *Cell Calcium,* **24,** 345–357.

O'NEILL, S.C., DONOSO, P. & EISNER, D.A. (1990). The role of $[Ca^{2+}]_i$ and $[Ca^{2+}]$ sensitization in the caffeine contracture of rat myocytes: measurement of $[Ca^{2+}]_i$ and $[Caffeine]_i$. *J. Physiol. Lond.,* **425,** 55–70.

OWEN, C.S. & DEVER, S. (1995). Indo-1 can simultaneously detect Ba^{2+} entry and Ca^{2+} blockade at a plasma membrane calcium channel. *Mol. Cell. Biochem.,* **151,** 91–98.

PAPAZIAN, M. & BEZANILLA, F. (1997). How does an ion channel sense voltage? *News Physiol. Sci.,* **12,** 203–210.

PAPE, P.C., JONG, D.S. & CHANDLER, W.K. (1996). A slow component of intramembranous charge movement during sarcoplasmic reticulum calcium release in frog cut muscle fibers. *J. Gen. Physiol.,* **107,** 79–101.

PELZER, D., PELZER, S. & MCDONALD, T.F. (1990). Properties and regulation of calcium channels in muscle cells. *Rev. Physiol. Biochem. Pharmacol.,* **114,** 107–207.

PETERSON, B.Z., DEMARIA, C.D., ADELMAN, J.P. & YUE, D.T. (1999). Calmodulin is the Ca2+ sensor for Ca2+-dependent inactivation of L-type calcium channels. *Neuron,* **22,** 549–558.

PIZARRO, G., CSERNOCH, L., URIBE, I., RODRÍGUEZ, M. & RÍOS, E. (1991). The relationship between Q_γ and Ca release from the sarcoplasmic reticulum in skeletal muscle. *J. Gen. Physiol.,* **97,** 913–947.

QU, Y. & CAMPBELL, D.L. (1998). Modulation of L-type calcium current kinetics by sarcoplasmic reticulum calcium release in ferret isolated right ventricular myocytes. *Can. J. Cardiol.,* **14,** 263–272.

RÍOS, E. & PIZARRO, G. (1991). Voltage sensor of excitation-contraction coupling in skeletal muscle. *Physiol. Rev.,* **71,** 849–908.

RÍOS, E. & STERN, M.D. (1997). Calcium in close quarters: microdomain feedback in excitation-contraction coupling and other cell biological phenomena. *Ann. Rev. Biophys. Biomol. Struct.,* **26,** 47–82.

SCHNEIDER, M.F. & CHANDLER, W.K. (1973). Voltage-dependent charge movement in skeletal muscle: a possible step in excitation-contraction coupling. *Nature,* **405,** 727–745.

SHAM, J.S.K., CLEEMANN, L. & MORAD, M. (1995). Functional coupling of Ca^{2+} channels and ryanodine receptors in cardiac myocytes. *Proc. Natl. Acad. Sci. U.S.A.,* **92,** 121–125.

SHIROKOV, R. (1999). Interaction between permeant ions and voltage sensor during inactivation of N-type Ca^{2+} channels. *J. Physiol. (Lond.),* **518,** 697–703.

SHIROKOV, R., LEVIS, R., SHIROKOVA, N. & RÍOS, E. (1992). Two classes of gating current from L-type Ca channels in guinea-pig ventricular myocytes. *J. Gen. Physiol.,* **99,** 863–895.

SHIROKOV, R., LEVIS, R., SHIROKOVA, N. & RÍOS, E. (1993). Ca^{2+}-dependent inactivation of cardiac L-type Ca^{2+} channels does not affect their voltage sensor. *J. Gen. Physiol.,* **102,** 1005–1030.

SHIROKOVA, N. & RÍOS, E. (1996). Caffeine enhances intramembranous charge movement in frog skeletal muscle by increasing cytoplasmic Ca^{2+} concentration. *J. Physiol. (Lond.),* **493,** 317–339.

STERN, M.D. (1992). Buffering of calcium in the vicinity of a channel pore. *Cell Calcium,* **13,** 183–192.

STROFFEKOVA, K. & HEINY, J.A. (1997a). Triadic Ca2+ modulates charge movement in skeletal muscle. *Gen. Physiol. Biophys.,* **16,** 59–77.

STROFFEKOVA, K. & HEINY, J.A. (1997b). Stimulation-dependent redistribution of charge movement between unavailable and available states. *Gen. Physiol. Biophys.,* **16,** 79–89.

TAKAGISHI, Y., ROTHERY, S., ISSBERNER, J., LEVI, A. & SEVERS, N.J. (1997). Spatial distribution of dihydro-pyridine receptors in the plasma membrane of guinea pig cardiac myocytes investigated by correlative confocal microscopy and label-fracture electron microscopy. *J. Electron Microscopy,* **46,** 165–170.

TERRACIANO, C.M., NAQVI, R.U. & MACLEOD, K.T. (1995). Effects of rest interval on the release of calcium from the sarcoplasmic reticulum in isolated guinea pig ventricular myocytes. *Circ. Res.,* **77,** 354–360.

TROSPER, T.L. & PHILIPSON, K.D. (1983). Effect of divalent and trivalent cations on Na^{+}-Ca^{2+} exchange in cardiac sarcolemmal vesicles. *Biochim. Biophys. Acta,* **731,** 63–68.

VERBOST, P.M., FLIK, G., LOCK, R.A. & WENDELAAR-BONGA, S.E. (1988). Cadmium inhibits plasma membrane calcium transport. *J. Membrane Biol.,* **102,** 97–104.

WEBER, A. (1968). The mechanism of action of caffeine on sarcoplasmic reticulum. *J. Gen. Physiol.,* **52,** 760–772.

WEBER, A. & HERZ, R. (1968). The relationship between caffeine contracture in intact muscle and the effect of caffeine on reticulum. *J. Gen. Physiol.,* **52,** 750–759.

WENDT, I.R. & STEPHENSON, D.G. (1983). Effects of caffeine on Ca-activated force production in skinned cardiac and skeletal muscle fibre of the rat. *Pflügers Arch.,* **398,** 210–216.

WIER, W.G. (1990). Cytoplasmic [Ca^{2+}] in mammalian ventricle: dynamic control by cellular processes. *Ann. Rev. Physiol.,* **52,** 467–485.

YATANI, A., IMOTO, Y. & GOTO, M. (1984). The effects of caffeine on the electrical properties of isolated, single rat ventricular cells. *Jpn. J. Physiol.,* **34,** 337–349.

YUE, D.T., BACKX, P.H. & IMREDY, J.P. (1990). Calcium-sensitive inactivation in the gating of single calcium channels. *Science,* **250,** 1735–1738.

ZHANG, Y.H. & HANCOX, J.C. (2000). Gadolinium inhibits Na(+)-Ca(2+) exchanger current in guinea-pig isolated ventricular myocytes. *Br. J. Pharmacol.,* **130,** 485–488.

ZÜHLKE, R.D., PITT, G.S., DEISSEROTH, K., TSIEN, R.W. & REUTER, H. (1999). Calmodulin supports both inactivation and facilitation of L-type calcium channels. *Nature,* **399,** 159–162.

(Received August 6, 2001
Revised November 15, 2001
Accepted November 22, 2001)