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ROLE OF Ca^{2+} CHANNEL IN CARDIAC EXCITATION–CONTRACTION COUPLING IN THE RAT: EVIDENCE FROM Ca^{2+} TRANSIENTS AND CONTRACTION

BY LARS CLEEMANN AND MARTIN MORAD*

From the Department of Physiology, University of Pennsylvania, Philadelphia, PA 19104-6085, USA and Mount Desert Island Biological Laboratory, Salsbury Cove, ME 04672, USA

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SUMMARY

1. Optical methods were used to measure simultaneously unloaded cell shortening and intracellular Ca^{2+} transients in whole-cell voltage clamped rat ventricular myocytes. Red light (> 670 nm) was used to measure cell shortening with a linear photodiode array. The dyes Fura-2 (K_d = 140 nM) and Mag-Fura-2 (K_d = 44 μM) were used as Ca^{2+} indicators with fluorescence excitation at 340 and 410 nm and emission at 510 nm.

2. Repeated measurements at 6 s intervals as 0·4 mM-Fura-2 diffused into the cell from the tip of the voltage clamp pipette showed no decrease in the rate of rise and peak value of the intracellular Ca^{2+} transient and only a small suppression of cell shortening, suggesting that the molecular mechanisms regulating the Ca^{2+} release were not significantly altered by the buffering capacity of the Fura-2.

3. Experiments in which the sarcoplasmic reticulum (SR) was depleted of Ca^{2+} either by exposure to caffeine or by repeated brief (20 ms) voltage clamp depolarizations confirm that the SR is the major source of activator Ca^{2+}.

4. Mag-Fura-2 (1 or 5 mM) was used to register the initial rapid development of the $[Ca^{2+}]_i$ transient but the later time course of the Ca^{2+} transients measured with this dye was obscured by motion artifacts resulting from cell shortening.

5. Both Fura-2 and Mag-Fura-2 showed that depolarization to 0 mV from a holding potential of −80 mV resulted in a $[Ca^{2+}]_i$ transient which developed with a delay of 3–9 ms and approached its peak value in an additional 8–19 ms. Both Ca^{2+} indicators also showed that the Ca^{2+} transient approached its peak value more slowly as the clamped membrane potential was made increasingly more positive.

6. The voltage dependencies of the Ca^{2+} signal (Fura-2) and cell shortening were both bell-shaped and were qualitatively similar to the voltage dependence of Ca^{2+} current simultaneously measured. This was observed with holding potentials of both −40 and −80 mV.

7. Comparison of the temporal relation of the Ca^{2+} current, I_{Ca}, and intracellular Ca^{2+} transient (Fura-2) and cell shortening at different membrane potentials showed

* To whom all correspondence should be addressed at the Department of Physiology, University of Pennsylvania, Philadelphia, PA 19104–6085, USA.

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that Ca^{2+} transient measured 25 ms into the depolarization correlated closely to the integral of the Ca^{2+} current measured prior to this time. Cell shortening, on the other hand, peaked about 100 ms later and correlated with measurements of the Ca^{2+} activity at the later time.

8. The slower development of the Fura-2 transient at +30 mV than at −20 mV was mirrored in the time course of the inward Ca^{2+} current which was smaller but longer lasting at +30 mV than at −20 mV. At the same time the initial delay of the Ca^{2+} transient was shorter at +30 than at −20 mV.

9. The initial delay was briefer for Ca^{2+} transients (Fura-2) evoked by repolarization from +100 to −40 mV than for Ca^{2+} transients evoked by depolarization from −40 to 0 mV.

10. The amplitude of the Fura-2 transient was reduced as the duration of a depolarizing clamp pulse (e.g. to 0 mV) was reduced to values below 10–20 ms. Similarly the development of the Ca^{2+} transient could be prematurely terminated by further depolarization to 100 mV.

11. We conclude that simultaneous measurements of both cell shortening and intracellular Ca^{2+} transients provide detailed data on excitation–contraction coupling in voltage clamped rat ventricular myocytes. Our results support the hypothesis that the release of Ca^{2+} from the SR is under direct, continuous control of the Ca^{2+} influx through the Ca^{2+} channel.

INTRODUCTION

Cardiac muscle is activated by the depolarization-dependent Ca^{2+} current and the release of Ca^{2+} from the sarcoplasmic reticulum (SR) which in turn elevate the myoplasmic calcium and allow the myofilaments to contract (Morad & Goldman, 1973; Chapman, 1979; Morad & Cleemann, 1987). When examining these processes in intact single cardiac cells the degree of activation has been estimated either from the isometric force development (Tung & Morad, 1988), from the unloaded shortening (London & Krueger, 1986) or from measurements of $[Ca^{2+}]_i$ transients (Blinks, Wier, Hess & Prendergast, 1982; Grynkiewicz, Poenie & Tsien, 1985; Cannell, Berlin & Lederer, 1987). We decided to measure both unloaded cell shortening and $[Ca^{2+}]_i$ transients in the same experiment. This direct approach eliminates uncertainties resulting from comparison of results obtained with different sets of cells, which may have been subjected to different intra- and extracellular experimental conditions. A linear array of 256 photodiodes was used to monitor the movement of the free end of the cell (London & Krueger, 1986) and Fura-2 (Grynkiewicz *et al.* 1985) or Mag-Fura-2 (Raju, Murphy, Levy, Hall & London, 1989) was used to monitor $[Ca^{2+}]_i$. These two optical techniques can be conveniently combined by colour coding the signals so that red light is used for the length measurements while ultraviolet and green light is used to measure the $[Ca^{2+}]_i$ transients. A similar approach has been used to combine measurements of cell shortening with Indo-1 measurements of $[Ca^{2+}]_i$ (Stern, Silverman, Houser, Josephson, Capogrossi, Nichols, Lederer & Lakatta, 1988; Spurgeon, Stern, Baartz, Raffaeli, Hansford, Talo, Lakatta & Capogrossi, 1990).

It has long been recognized that the phasic component of tension development in heart muscle has a bell-shaped voltage relation which closely follows that of I_{Ca}

(Vassort & Rougier, 1972; Morad & Goldman, 1973; Trautwein, McDonald & Tripathi, 1975; Chapman, 1979). The simplest explanation would be that I_{Ca} acts directly as the major source of activator Ca^{2+} (Horackova & Vassort, 1976). There is now considerable evidence, however, that I_{Ca} in addition acts by releasing Ca^{2+} from the SR (e.g. Morad & Goldman, 1973; Beuckelmann & Wier, 1988). The role of the SR in activation of mammalian myocardium is suggested by the finding that I_{Ca} carries too little Ca^{2+} to activate the contractile filaments, by rate-stair case, post-extrasystolic potentiation and other indications of a 'contractile memory' (Morad & Cleemann, 1987), and by the Ca^{2+}-induced Ca^{2+} release observed in skinned cardiac fibres (Fabiato, 1985*a*, *b*, *c*). The involvement of the SR is demonstrated also by the caffeine-induced release of Ca^{2+} (Callewaert, Cleemann & Morad, 1989) and by the suppression of $[Ca^{2+}]_i$ transients by ryanodine (Barcenas-Ruiz & Wier, 1987; Beuckelmann & Wier, 1988; Callewaert, Cleemann & Morad, 1988).

But while the release of Ca^{2+} from the SR is well documented, questions remain as to how it is controlled. Ca^{2+}-induced Ca^{2+} release is a popular hypothesis (Fabiato, 1985*a*, *b*, *c*; Beuckelmann & Wier, 1988) but it might involve a positive feedback mechanism which could bring the release process to completion once it were started. It is of interest, therefore, to examine whether the SR acts simply as an amplification station for the Ca^{2+} which enters through the Ca^{2+} channel or whether there is indication of positive or negative feedback. At the same time it may be questioned if a voltage-dependent and gating-charge-mediated release process, of the type operating in skeletal muscle (Schneider & Chandler, 1973; Bean & Rios, 1989), could play, at least a supplementary role, in cardiac muscle (Cannell *et al.* 1987).

The experiments described below show that reliable simultaneous measurements of cell shortening and $[Ca^{2+}]_i$ transients may be obtained in whole-cell clamped mammalian ventricular myocytes. The voltage dependence of both cell shortening and $[Ca^{2+}]_i$ transient were in agreement with the current–voltage relation for the Ca^{2+} current, I_{Ca}. The detailed time course of I_{Ca}, as expressed in terms of activation and inactivation, was reflected in the time course of $[Ca^{2+}]_i$ transient but generally was beyond the time resolution of the measurements of cell shortening. Mag-Fura-2, which equilibrates rapidly with Ca^{2+} (Baylor, Hollingworth & Konishi, 1989; Konishi, Hollingworth, Harkins & Baylor, 1991), did not reveal events faster than those detected with Fura-2. Our results indicate that the release of Ca^{2+} from the SR is under continuous and direct control of I_{Ca}.

METHODS

Preparation and measurement of membrane current

The heart was removed from rats deeply anaesthetized with sodium pentabarbitone. Ventricular myocytes, which were isolated by enzymatic dissociation (Mitra & Morad, 1985), were placed in a perfusion chamber on the stage of an inverted microscope (Nikon Diaphot, box in a dashed line in Fig. 1). Quiescent cells were voltage clamped using the whole-cell patch clamp technique (Hamill, Marty, Neher, Sakmann & Sigworth, 1981). The head stage of the patch clamp amplifier is shown schematically in Fig. 1. It measured the membrane current (i_m) which was required to clamp the membrane potential (V_m) to the command potential. The patch clamp pipette (E) had a resistance of 2–5 MΩ and was filled with a dialysing solution which normally contained 120 mM-CsCl, 5 mM-magnesium adenosine triphosphate (MgATP), 0·1 mM-adenosine 3,5′-monophosphate (cyclic AMP), 0·4 mM-Na_5Fura-2 (Molecular Probes, Eugene, OR, USA) and 20 mM-HEPES buffer titrated to pH 7·2 by addition of CsOH. The intracellular concentration of Na^+ may have been as low as 2 mM

since this ion was added only as its Fura-2 salt. In some experiments Fura-2 was replaced by Mag-Fura-2 (Molecular Probes; 1 or 5 mM) and MgATP by K_2ATP so that the internal solution was Mg^{2+} free. The external Tyrode solution used under control conditions contained 137 mM-NaCl, 5·4 mM-KCl, 1 mM-$MgCl_2$, 2 mM-$CaCl_2$, 10 mM-glucose, 0·01 mM-tetrodotoxin (TTX) and 10 mM-HEPES buffer at pH 7·4. In some experiments 2 mM-caffeine was added to the perfusate. A

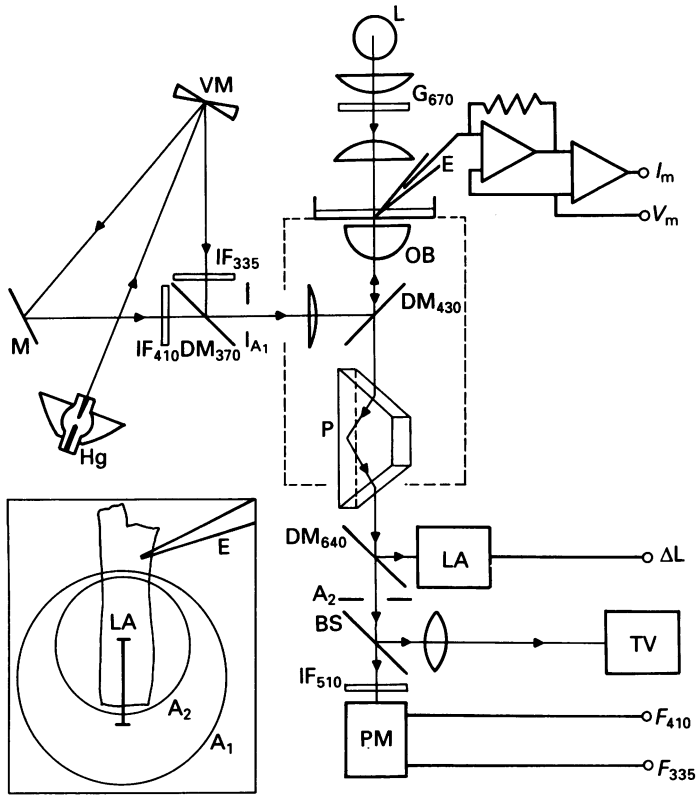

Fig. 1. Experimental setup. This simplified diagram shows the optics used for measurement of cell shortening, ΔL, and fluorescence signals, F_{410} and F_{335}, and the electronics used for measurement of membrane current, i_m, and membrane potential, V_m. For details see the text. Some optical components such as a shutter, the beam splitter used to direct light to the oculars of the microscope and some lenses and mirrors are not shown in the diagram.

multibarrelled pipette with a common outlet was used to rapidly exchange the solution around the cell (Tang, Dichter & Morad, 1989). All experiments were performed at room temperature (20–25 °C). Clamp pulses were applied every 6 s.

The inward Ca^{2+} current, I_{Ca}, was measured as the difference between the peak inward current and the current at the end of the clamp pulse. This value approximates the peak Ca^{2+} current since the inward Na^+ current was blocked by 10 μM-TTX and the channel generally was inactivated by holding the membrane potential at −40 mV. Contamination from outward K^+ currents (the delayed rectifier and the transient outward current) was also minimized by using Cs^+ instead of K^+ in the dialysing solution.

Each finding reported in this paper is typical of three or more experiments in which intracellular Ca^{2+} transients and cell shortening were recorded simultaneously and, in general, is supported also by a larger pool of experiments in which either Ca^{2+} transients or cell shortening were recorded.

Optical measurements of cell shortening and intracellular Ca^{2+} activity

Red light was used to measure the cell shortening while ultraviolet and green light was used in the measurements of intracellular Ca^{2+} activity with Fura-2 or Mag-Fura-2.

The red light used for measurement of cell length came from an incandescent lamp (L, Fig. 1) and passed through a collimating lens, a red glass filter (G_{670}, 670 nm long pass) and a focusing lens before it hit the cells in the perfusion chamber. From there it passed through the objective (OB) and was reflected by a dichroic mirror (DM_{640}, 640 nm short pass) before it was focused onto a linear array of 256 photodiodes (LA). A dove prism (P) which could be rotated along its long axis was used to align the image of the examined cell with the linear array. The linear array was read every 4 ms by an electronic edge detector which gave an analog signal (ΔL) indicating the position of the end of the cell (Naebauer, Callewaert, Cleemann & Morad, 1989).

The ultraviolet light used for excitation of the calcium-sensitive dyes Fura-2 and Mag-Fura-2 came from 100 W mercury arc lamp with an ellipsoidal reflector (Hg). The beam was split in two by a vibrating mirror (VM, 1150 Hz) which acted as a multiplexer. One beam was reflected by an adjustable mirror (M) and passed through an interference filter (IF_{410}, 410 nm, 20 nm band width), a dichroic mirror (DM_{370}, 370 nm long pass) and a field aperture (A_1) before it entered the microscope through the port used for epi-illumination. The other beam had a wavelength defined by an interference filter (IF_{335}, 335 nm, 20 nm band width) and it joined the first beam after being reflected in the dichroic mirror DM_{370}. Within the microscope the dichroic mirror DM_{430} reflected the ultraviolet light toward the objective but transmitted the returning fluorescent green light toward the detection port. After passing through the dove prism (P) and the dichroic mirror (DM_{640}) the light was focused onto an aperture (A_2, moveable and with adjustable diameter) which defined the detection area used in the fluorescence measurements. Most of the fluorescent light was transmitted through a beam splitter (BS) and an interference filter (IF_{510}, 510 nm, 70 nm band width) and hit a photomultiplier (PM). The signal from the photomultiplier was demultiplexed (Cleemann & Morad, 1991) and yielded two signals corresponding to the two wavelengths of excitation (F_{410} and F_{335}).

A small part of the light was reflected by the beam splitter (BS) and was focused onto a TV camera which was connected to a monitor used in the alignment of the system. The inset shows the confocal planes of the system as seen on the TV monitor. One end of the roughly rectangular cell was first aligned with the linear array (LA) used for measurements of cell length by rotating the dove prism (P) and moving the stage of the microscope. The whole-cell patch clamp electrode was then placed over the other end of the cell and a giga-seal was formed. The moveable, adjustable aperture (A_2) was now placed over the cell extending from the free end almost all the way to the point of attachment of the electrode. In general this area was completely within the area of fluorescence illumination (A_1). The closing of the second aperture (A_2) was the last step in the adjustment process since only the area within this aperture was visible on the TV monitor. The background fluorescence was measured after all optical adjustments were made but before breaking into the cell.

Calculation of the intracellular Ca^{2+} activity

The intracellular Ca^{2+} activity, $[Ca^{2+}]_i$, measured with Fura-2 was calculated using equations equivalent to those of Grynkiewicz *et al.* (1985):

$$[Ca^{2+}]_i = K_d B(R_{EGTA} - R)/(R - R_{Ca}), \quad (1)$$

$$R = (F_{410} - F_{410,bg})/(F_{335} - F_{335,bg}), \quad (2)$$

$$B = F_{335,EGTA}/F_{335,Ca}. \quad (3)$$

The fluorescence ratio (R) was calculated from the F_{410} and F_{335} after subtraction of the respective background fluorescences ($F_{410,bg}$ and $F_{335,bg}$). The limiting fluorescence ratios (R_{EGTA} and R_{Ca}) were measured with samples of solution with free Fura-2 (0·4 mM-Fura-2 and 10 mM-EGTA) or Ca^{2+}-saturated Fura-2 (0·4 mM-Fura-2 and 2 mM-Ca^{2+}) placed on the stage of the microscope. The fluorescence measurements with these fluid samples did not require background subtraction and were used also to determine the factor B. As dissociation constant for Fura-2 we used a K_d value of 140 nM (Grynkiewicz *et al.* 1985).

The change in the intracellular Ca^{2+} activity measured with Mag-Fura-2 was calculated from the fluorescence signal obtained with excitation at 410 nm where the fluorescence is quenched almost completely if the dye is saturated with Ca^{2+}. Considering the pertinent range of $[Ca^{2+}]_i$ (1–2 μM) and the relatively high dissociation constant for binding of Ca^{2+} to Mag-Fura-2 (K_d = 44 μM at 16 °C,

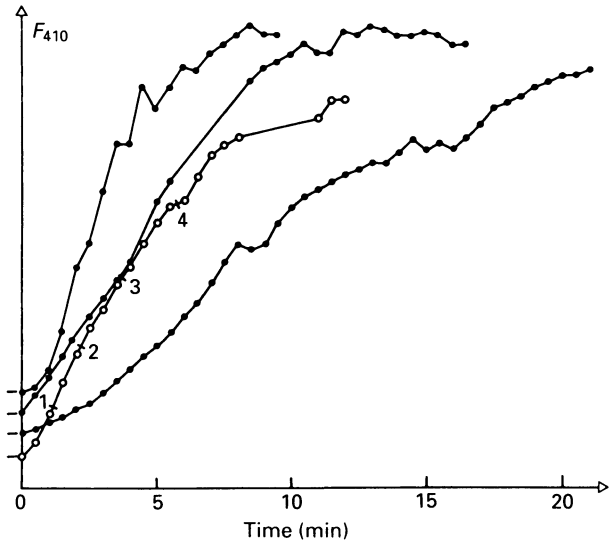

Fig. 2. The fluorescence intensity measured within four cells during equilibration with Fura-2. Horizontal bars next to the vertical axis indicate the background fluorescence measured with excitation at 410 nm (F_{410}) just prior to breaking into the cell at time zero. The curve based on open circles corresponds to the experiment illustrated in Fig. 3 and is labelled with numbers corresponding to the traces in this figure.

Baylor *et al.* 1989), it may be assumed that only a small fraction of the dye molecules bind Ca^{2+} and that this fraction is linearly related to $[Ca^{2+}]_i$. Under these conditions it is estimated that a 1 μM increase in $[Ca^{2+}]_i$ will reduce the fluorescence intensity by 1 $\mu M/K_d$ = 1/44 = 2·27 %. While Mag-Fura-2 may be used to measure changes in $[Ca^{2+}]_i$ it should be noticed, that this dye is not a suitable indicator for accurate measurements of the resting $[Ca^{2+}]_i$.

Data acquisition

The membrane current, i_m, the membrane potential, V_m, the cell shortening, ΔL, and the two fluorescence signals, F_{410} and F_{335} were sampled, analysed and displayed using a data acquisition system based on an IBM compatible Personal Computer. The data acquisition process was synchronized to the vibrating mirror so that each channel was sampled every 0·87 ms. All figures show unfiltered signals recorded without signal averaging during a single sweep. Integrators and sample and hold circuits (Cleemann & Morad, 1991) delayed the fluorescence signals by 1·2 ms and the cell shortening signal by 4 ms relative to measured membrane current and membrane potential.

RESULTS

Diffusion of Fura-2 into the cell

The procedure illustrated in Figs 2 and 3 was designed to test if the addition of 0·4 mM-Fura-2 to the intracellular compartment significantly altered the excitation–contraction process. A gigaseal was formed between the patch electrode and the cell and the detection aperture (A_2 in the inset of Fig. 1) was adjusted. The

purpose of the latter step was to reduce the background fluorescences (B in panels *C* and *D* of Fig. 3). Upon breaking the membrane under the electrode, Fura-2 started to diffuse from the electrode into the cell, resulting in gradual increase of the cellular fluorescence intensity (Fig. 2). The half-time for equilibration was 2–10 min in rough

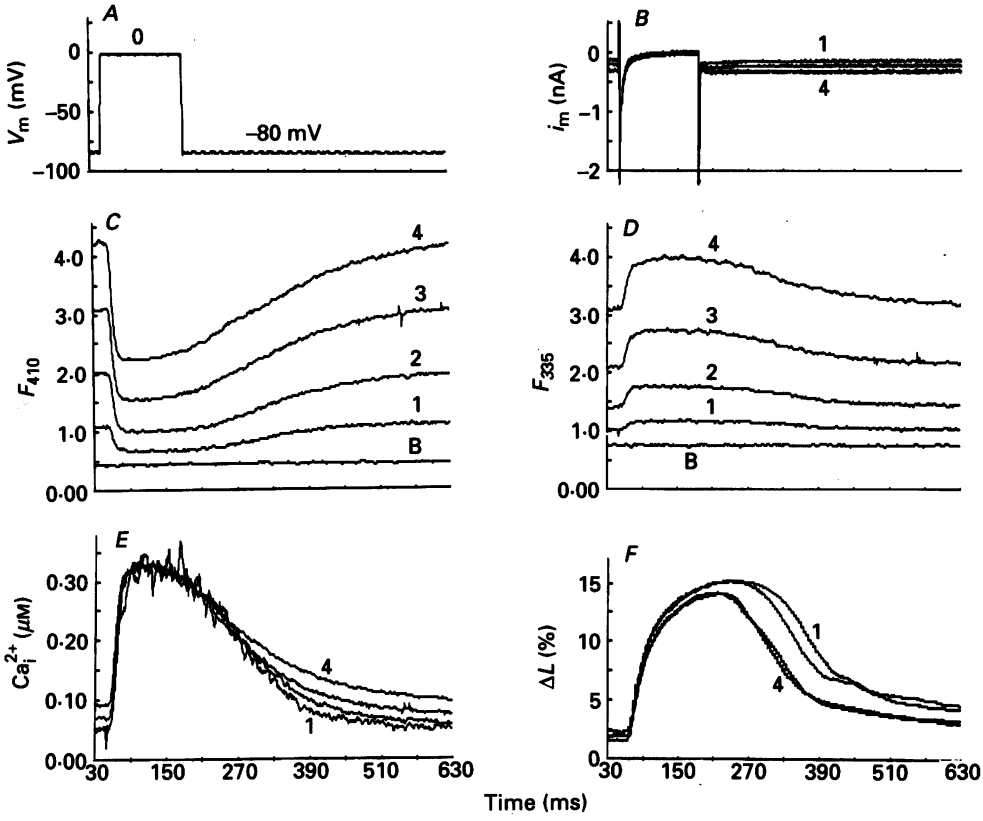

Fig. 3. Measurements during diffusion of Fura-2 into the cell. *A*, the membrane potential (V_m). *B*, membrane current (i_m). *C*, fluorescence measured with excitation at 410 nm (F_{410}). *D*, fluorescence measured with excitation at 335 nm (F_{335}). *E*, intracellular Ca^{2+} activity (Ca_i^{2+}) measured from these two fluorescences. *F*, cell shortening (ΔL). The background fluorescence signals (B) were measured with the patch clamp pipettes attached to the cell but before breaking into the cell. The traces labelled 1–4 were measured 1, 2, 4 and 6 min after breaking into the cell. The holding potential was −80 mV and the cell was depolarized to 0 mV by a 150 ms clamp pulse every 6 s.

agreement with estimates based on the electrode resistance and the molecular weight of Fura-2 (Klein, Simon, Szucs & Schneider, 1988; Pusch & Neher, 1988). Typically an experiment lasted 10–20 min from the time of penetration until the onset of spontaneous contractions and subsequent irreversible contracture. The first 5 min of this period was used to study the loading process and was followed by measurement of voltage relations and other interventions.

While Fura-2 equilibrated within the cell, and presumably approached the 0·4 mM found in the dialysing solution, we observed no reduction in the peak value of the

$[Ca^{2+}]_i$ transient or its rate of rise (Fig. 3*E*). Within the accuracy of the measurements the peak value remained constant at 0·32 μM from the first recording at the 1 min mark (trace 1) to later measurements at time 2 (trace 2), 4 (trace 3) and 6 min (trace 4) and until the end of the experiment at 13 min (○ in Fig. 2). The accuracy of the

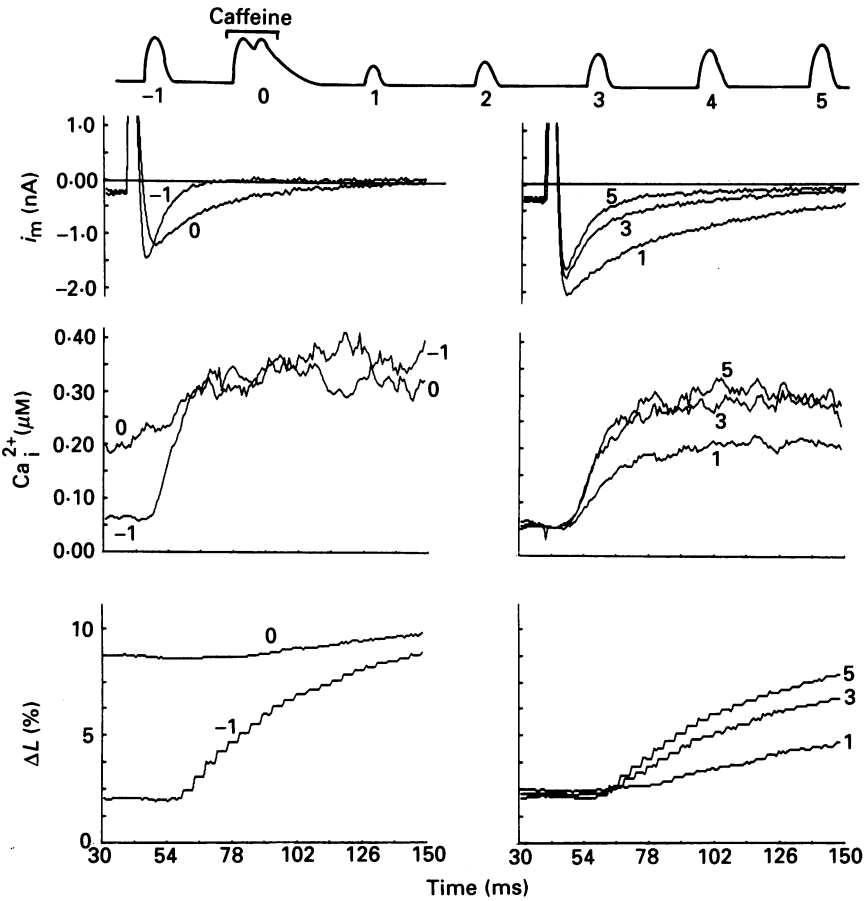

Fig. 4. The effect of rapid exposure to 2 mM-caffeine on membrane current (i_m), intracellular Ca^{2+} transient (Ca_i^{2+}), and cell shortening (ΔL). The protocol is shown at the top. The left panels show responses recorded during voltage clamp pulses before (−1) and during (0) a caffeine contracture. The right panels show the first (1), the third (3) and the fifth (5) beat during recovery. The holding potential was −70 mV and the voltage clamp pulses depolarized the membrane to 0 mV for 200 ms.

Fura-2 measurements improved as the intracellular dye concentration increased more than eightfold from the first recording where the dye concentration was estimated to be about 50 μM. At the same time the amplitude of the cell shortening was only slightly reduced from 13% at 1 min (trace 1) to 12% at 6 min (trace 4) and 9% at the end the experiment. These findings suggest that the degree of Ca^{2+} release was not significantly altered by the build-up of the dye within the cell.

The $[Ca^{2+}]_i$ transients reached their peak value about 50 ms after depolarization, while the peak value of the cell shortening occurred about 100 ms later. It is also of interest to note that the cell started to relax rapidly at a time when the $[Ca^{2+}]_i$ transient fell below a value of approximately 0·2 μM.

It was generally noticed that the background value of $[Ca^{2+}]_i$ increased slowly throughout the experiment. This may be due, in part to a slow leak of Ca^{2+} into the cell as suggested by the holding current (Fig. 3*B*).

Ca^{2+} depletion of the SR by exposure to caffeine or by abbreviation of the clamp pulse

The experiments presented here will be described in terms which presupposed that most of the activator Ca^{2+} is released from the SR (Morad & Goldman, 1973; Fabiato, 1985*a–c*; Morad & Cleemann, 1987; Beuckelmann & Wier, 1988). To examine this point we attempted to deplete the SR of Ca^{2+} by exposure to caffeine (Callewaert *et al.* 1989; Naebauer *et al.* 1989) or by repeated brief voltage clamp depolarizations (Morad & Goldman, 1973). Figure 4 illustrates an experiment where the cell was voltage clamped at 6 s intervals as indicated in the inset of the figure. After a control beat (−1), the myocyte was briefly exposed to 2 mM-caffeine (0) and then allowed to re-equilibrate (Beats 1–5). The $[Ca^{2+}]_i$ transient and cell shortening during the first beat (1) following the caffeine contracture were greatly suppressed, even though the Ca^{2+} current inactivated much more slowly, thus increasing the estimated influx of Ca^{2+} through the Ca^{2+} channel severalfold. This experiment suggests that caffeine depletes the SR of a major part of its Ca^{2+}, such that subsequent depolarizations, even with an enhanced Ca^{2+} current, are insufficient to activate the myocyte normally.

The prolonged inward currents following the caffeine contracture (Fig. 4) may be attributed to the absence of Ca^{2+}-induced inactivation of the Ca^{2+} current (Kohlhardt, Herdey & Kubler, 1973; Kass & Sanguinetti, 1984; Lee, Marban & Tsien, 1985). In this context, it is of interest to note that the inward current recorded during the caffeine contracture (0), though of slightly smaller peak value, inactivates significantly slower than the current during the control beat (−1) even though $[Ca^{2+}]_i$ is elevated prior to the depolarization and reaches a peak value comparable to that of the control beat.

Figure 5 illustrates an experiment where we attempted to deplete the SR by shortening the duration of the voltage clamp depolarization from 220 to 20 ms. This is based on the idea that the Ca^{2+} current during the first 20 ms is sufficient to release the internal stores but that a longer lasting depolarization is required to replenish these stores (Morad & Goldman, 1973; Fabiato, 1985*c*). The left panels show that the shortening of the depolarization resulted in a gradual decline in both the $[Ca^{2+}]_i$ transient (Ca_i^{2+}) and the cell shortening (ΔL). A new steady state was reached only after five to ten abbreviated depolarizations. The right panels show that recovery was equally slow when the full duration of the clamp pulse was re-established. These findings are consistent with previously published measurements of isometric force in intact voltage clamped ventricular strips (Morad & Goldman, 1973).

The experiments illustrated in Figs 4 and 5 show the type of slow beat-dependent kinetics which has been associated with a functional SR which contributes significantly to the activation of the cell.

Kinetics of SR Ca^{2+} release: comparison of measurements with Fura-2 and Mag-Fura-2

It may be questioned whether Fura-2 responds sufficiently rapidly to resolve the characteristics of the rising phase of the $[Ca^{2+}]_i$ transient. Fura-2 has an off-rate of

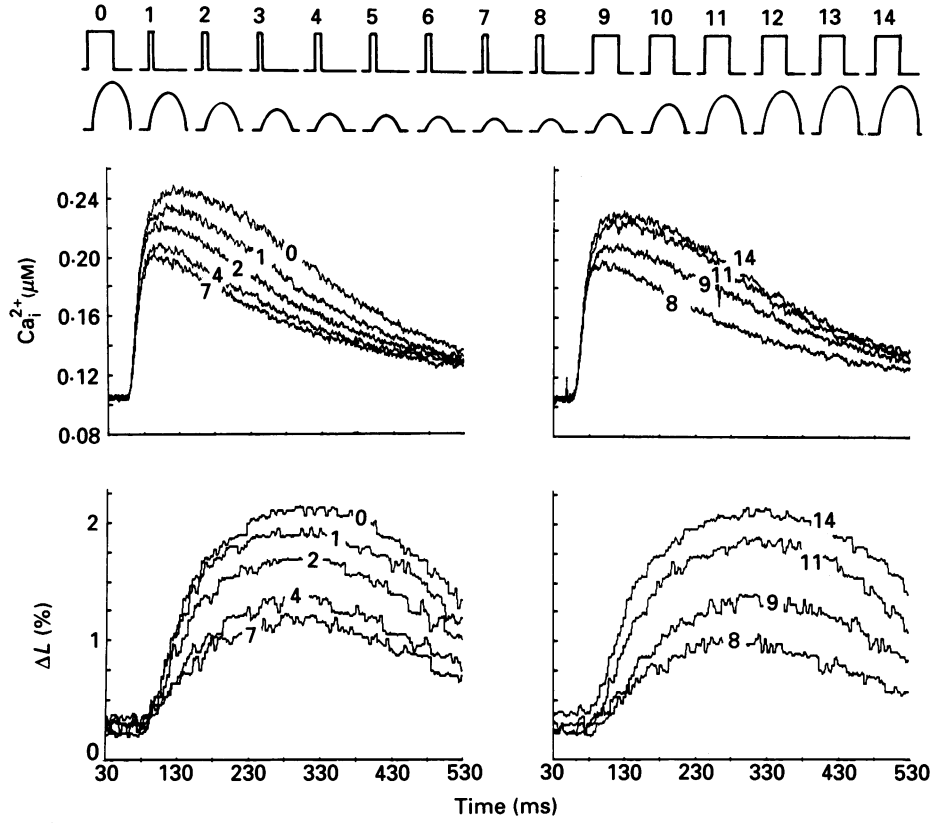

Fig. 5. The effect of transient abbreviation of the clamp pulse on intracellular Ca^{2+} activity (Ca_i^{2+}) and cell shortening (ΔL). The schematic at the top illustrates that the voltage clamp pulse after a control beat (0) was shortened from 220 to 20 ms during the following eight beats (1–8) and then returned to its initial value during the following beats (9–14). The left panels show the successive decline of the Ca^{2+} transient and cell shortening during the shortened clamp pulses while the right panels show the recovery as the original clamp duration was re-established. The traces are labelled with the number of the beat. The membrane was depolarized from −80 to 0 mV every 6 s.

about 80 s^{-1} *in vitro* (Jackson, Timmerman, Bagshaw & Ashley, 1987) and, in frog skeletal muscle, this indicator dye completely misses the initial rapid $[Ca^{2+}]_i$ transient which had been measured with Antipyrylazo III (Baylor & Hollingworth, 1988; off-rate = 700 s^{-1}, Baylor, Hollingworth, Hui & Quinta-Ferreira, 1985). Thus in some experiments we used Mag-Fura-2 to check the time course of the rising phase of the Ca^{2+} transients. Mag-Fura-2 binds Mg^{2+} 1:1 with a K_d of 1·5 mM (Raju *et al.* 1989) but it can also, in the absence of Mg^{2+}, be used as a 1:1 Ca^{2+} probe (K_d = 53 μM

at 37 °C, Raju *et al.* 1989; $K_d = 44$ μM at 16 °C, Baylor *et al.* 1989) with absorption and emission spectra similar to those of Fura-2. In this capacity Mag-Fura-2 tracks the intracellular Ca^{2+} transient in skeletal muscle as fast as Antipyrylazo III (Baylor *et al.* 1989; Konishi *et al.* 1991).

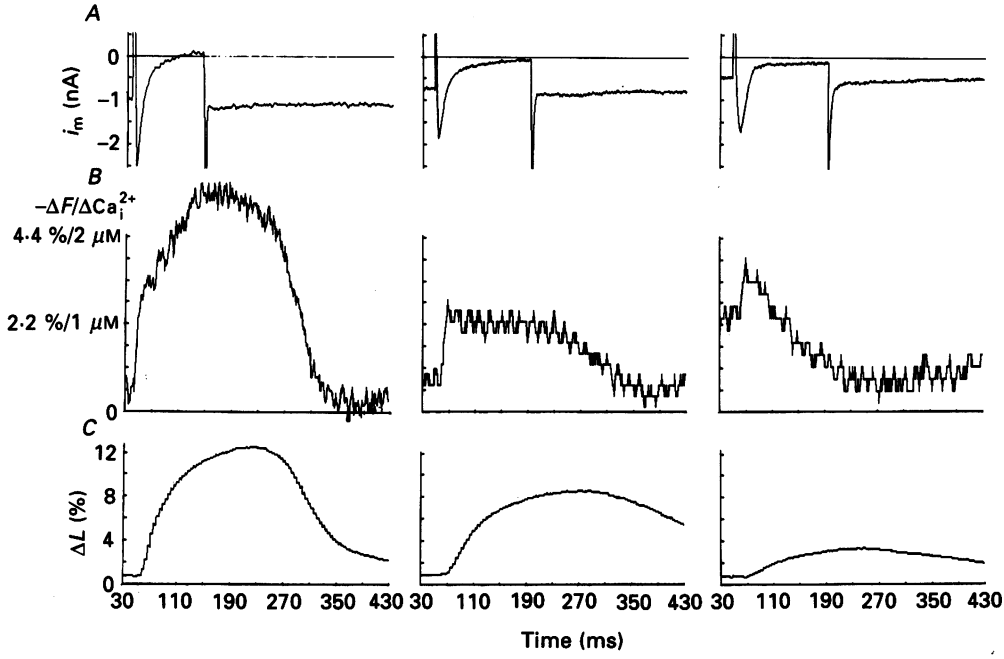

Fig. 6. Measurement of intracellular Ca^{2+} transients with Mag-Fura-2. *A*, membrane current (i_m). *B*, the fluorescence signal ($-\Delta F/\Delta Ca_i^{2+}$). *C*, cell shortening (ΔL). The fluorescence signal was measured with excitation at 410 nm and emission at 510 nm.The Ca^{2+} transient is seen as a rapid upward deflection (decrease in fluorescence) which follows shortly after depolarization from −80 to 0 mV. Superimposed on the Ca^{2+} signal is a motion artifact which changes from cell to cell and depends critically on the adjustment of the visual field. The left panels were recorded with 5 mM-Mag-Fura-2 in the dialysing solution. The middle and right panels were recorded with different adjustment of the visual field from the same cell with 1 mM intracellular Mag-Fura-2.

In five out of fifteen cells where 1 or 5 mM-Mag-Fura-2 was loaded through the pipette we found indication of a Ca^{2+} transient which was seen as a rapid decrease in the fluorescence signal produced by excitation at 410 nm (Fig. 6). Excitation at 340 nm never produced a clear-cut Ca^{2+} signal. Our failure to observe consistently a signal at 410 nm, and to detect it at all at 340 nm, may be, in part, due to limited stability of the mercury arc lamp which served as primary light source. Following the rapid Ca^{2+} transient a motion artifact with an amplitude and a polarity which depended critically on the adjustment of the detection aperture (Fig. 1, A_2) was often observed. Thus only that part of the Mag-Fura-2 signal which precedes the contraction can be used as a reliable indicator of the intracellular Ca^{2+} transient. The initial amplitude of the Ca^{2+} transient detected with Mag-Fura-2 was $1{\cdot}07 \pm 0{\cdot}37$ μM

mean ± S.D., $n = 5$) but this number is likely to be uncharacteristically high considering that in ten out of fifteen cells we failed to observe a signal.

Figure 7 compares the time course of the Ca^{2+} signals measured with Mag-Fura-2 (left panels) and Fura-2 (right panels). Both cells were depolarized to 0 mV from a

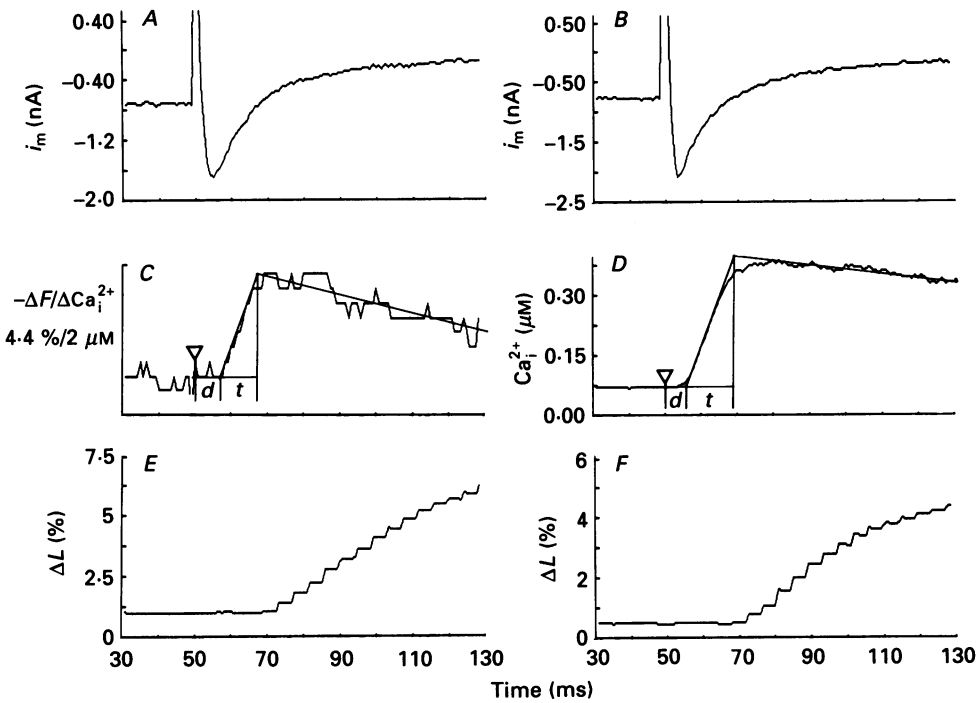

Fig. 7. Comparison of the rising phase of the Ca^{2+} transients measured either with 1 mM-Mag-Fura-2 (left panels) or with 0·4 mM-Fura-2 (right panels). *A* and *B*, membrane current (i_m). *C* and *D*, the Ca^{2+} signal. *E* and *F* cell shortening (ΔL). The cells were depolarized to 0 mV from a holding potential of −80 mV at time 50 ms. The Ca^{2+} signal measured with Mag-Fura-2 (*C*, $-\Delta F/\Delta Ca_i^{2+}$) is the fluorescence intensity measured with excitation at 410 nm while the Ca^{2+} signal measured with Fura-2 (*D*, Ca_i^{2+}) is derived from the optical signals corresponding to excitation at both 340 and 410 nm. The arrow-heads in *C* and *D* indicate the time of depolarization. They are delayed 1·2 ms relative to the capacitive transients in *A* and *B* (i_m) in order to compensate for the delay of the integrators and sample and hold circuits used in the fluorescence measurements (Cleemann & Morad, 1991). The Ca^{2+} signals were approximated with three straight lines of which the first defines the resting Ca^{2+} activity, the second the maximal rate of rise and the third the relatively steady plateau. The delay (*d*) was defined as the time from depolarization (arrow-head) to the first intersection of the lines and the rise time (*t*) as the time from this intersection to the second intersection. The delay and the rise time of the Ca^{2+} transients with the two dyes were not significantly different.

holding potential of −80 mV and both Ca^{2+} signals show that, following depolarization, there is first a delay (*d*) of about 6–7 ms (Mag-Fura-2, 7·7 ± 1·6 ms (mean ± S.D.) $n = 5$; Fura-2, 5·5 ± 2·8 ms, $n = 8$) and then the Ca^{2+} signal rises rapidly approaching a plateau with a rise time (*t*) of about 10–13 ms (Mag-Fura-2, 14·3 ± 4·8 ms (mean ± S.D.) $n = 5$; Fura-2, 13·3 ± 4·9 ms, $n = 8$). The delay and the

rise time were measured as indicated in Fig. 7 and explained in its legend. These events are largely over before the onset of contraction. They do, on the other hand, coincide with, or follow slightly after, the interval where the inward current is maximum. The only noticeable differences between the two Ca^{2+} signals is that the amplitude registered with Mag-Fura-2 is $1{\cdot}07 \pm 0{\cdot}37$ μM compared to the $0{\cdot}32 \pm 0{\cdot}05$ μM(mean $\pm$ S.D., $n = 9$, $V_H = -80$ mV) registered with Fura-2. The

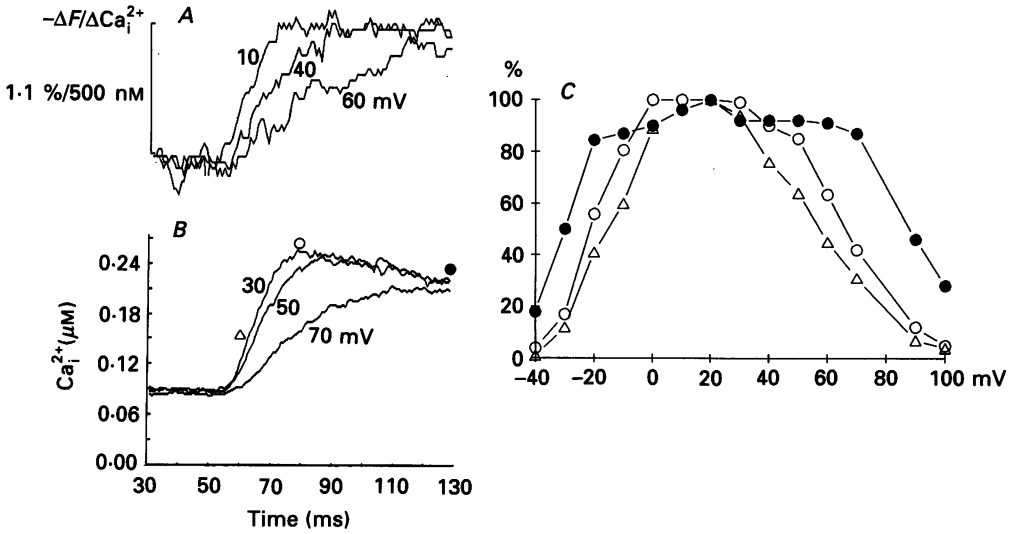

Fig. 8. Both Mag-Fura-2 (A, $-\Delta F/\Delta Ca_i^{2+}$) and Fura-2 ($B$, Ca_i^{2+}) show that the intracellular Ca^{2+} transients develop more slowly as the clamped membrane potential is made increasingly more positive. The Ca^{2+} signals are labelled with the clamped membrane potential. The holding potential in both experiments was -80 mV. C, shows the voltage dependence of the intracellular Ca^{2+} transients measured with Fura-2. The normalized curves correspond to the maximal rate of rise (△) and to the change in Ca^{2+} activity measured 25 ms (○) and 80 ms (●) after depolarization.

agreement between the time course of the Ca^{2+} signals measured with Fura-2 and the faster Mag-Fura-2 suggest that the $[Ca^{2+}]_i$ transients in cardiac muscle do not develop at rates significantly faster than the responses time of Fura-2.

As the membrane was depolarized to more positive potentials we found that Ca^{2+} transient developed more and more slowly. In time, however, they often approached a value which was fairly independent of the clamped membrane potential. This was seen both with Mag-Fura-2 (Fig. 8A) and with Fura-2 (Fig. 8B). The signals recorded with Mag-Fura-2 were more noisy than the signals measured with Fura-2 and did not lend themselves to numerical analysis. The Ca^{2+} transients measured with Fura-2 were analysed in terms of maximal slope (△) and values measured 25 ms (○) and 80 ms (●) following depolarization. The normalized curves (Fig. 8C) show bell-shaped relations with broadening plateaux as the measurements were made with larger delays after depolarization.

The voltage dependence of cell shortening and intracellular Ca^{2+} transients

With a holding potential of −40 mV, the cell was depolarized every 6 s by a sequence of 150 ms voltage clamp pulses which were increased in steps of 10 mV from one test pulse to the next. Standard depolarizations to 0 mV immediately before and

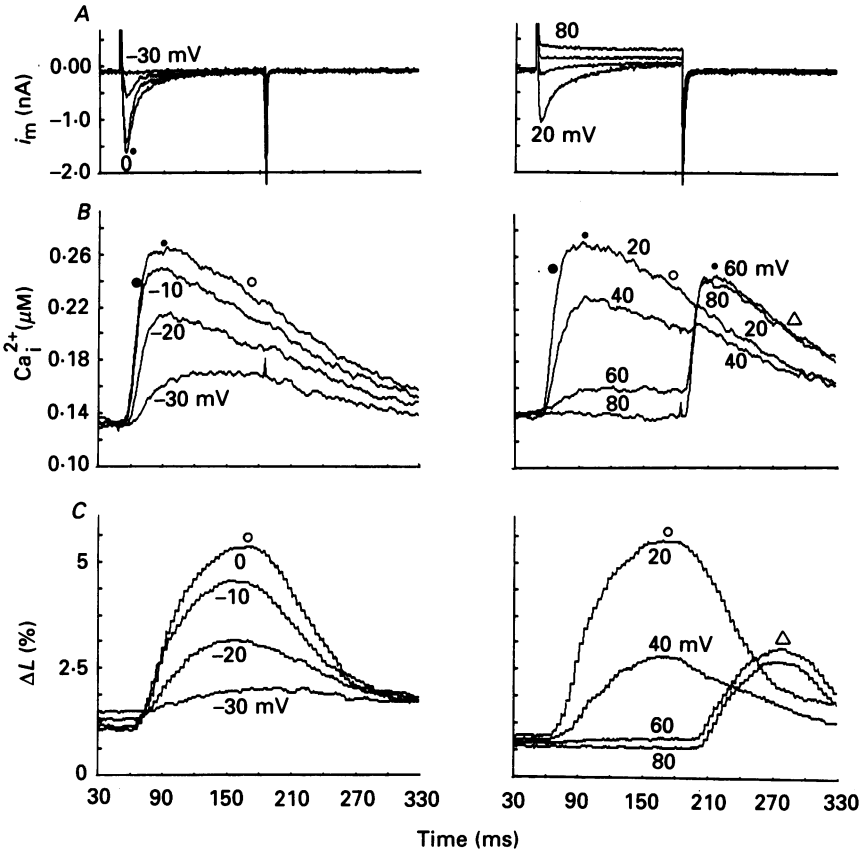

Fig. 9. *A*, voltage dependence of the membrane current (i_m). *B*, the intracellular Ca^{2+} activity (Ca_i^{2+}). *C*, cell shortening (ΔL). The left panels show records obtained at potentials from −30 to 0 mV and the right panels show records obtained at positive potentials. The holding potential was −40 mV and the duration of the clamp pulses was 140 ms. The symbols indicate the time of the different measurements and correspond to the curves shown in Figs 10 and 11 which are based on the same experiment.

after this sequence produced currents, Ca^{2+} transients and contractions which were similar in magnitude to those obtained when the membrane was depolarized to 0 mV during this sequence. For this reason we judged it permissible to use this abbreviated protocol instead of the more time consuming sequence where depolarizations to different test potentials were separated by a train of four or more standard depolarizations (Beuckelmann & Wier, 1988). The left panels in Fig. 9 show that successive depolarizations to potentials from −30 to 0 mV increased I_{Ca} (Fig. 9*A*), the $[Ca^{2+}]_i$ transient (Fig. 9*B*), and the cell shortening (Fig. 9*C*). Clamp pulses to

positive potentials reversed this trend such that I_{Ca}, the $[Ca^{2+}]_i$ transient and the cell shortening all decreased (Fig. 9, right panels) as the amplitude of the clamp pulse increased and approached the reversal potential for Ca^{2+} ($E_{Ca} \simeq RT/2F \log_e(2\ mM/0{\cdot}15\ \mu M) = 120$ mV). Both the Fura-2 measurements and the measure-

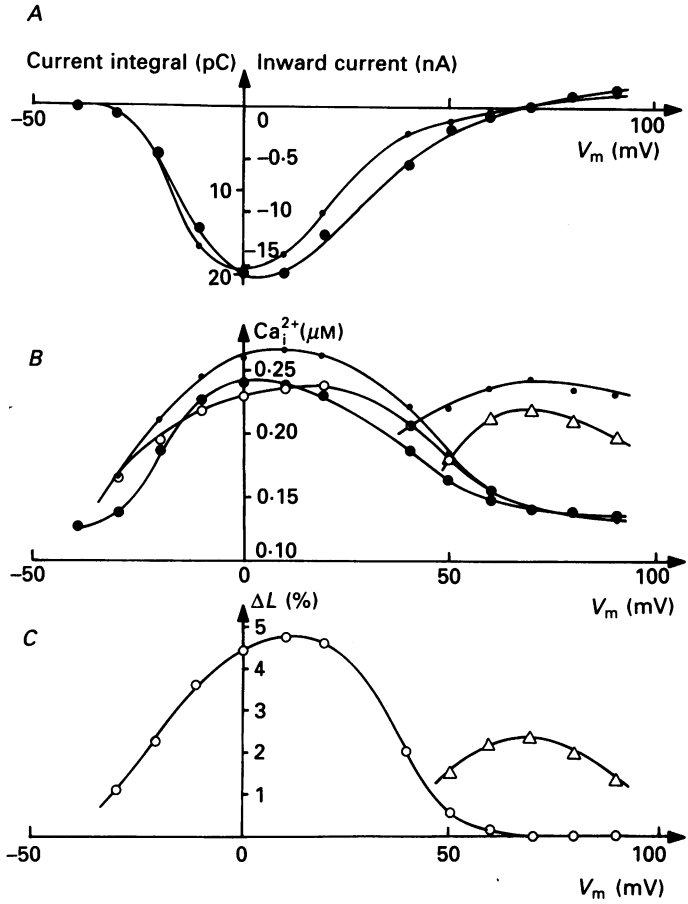

Fig. 10. Voltage dependence of the membrane current, the intracellular Ca^{2+} transient and the cell shortening. *A*, peak inward current (●) and the current integral at 25 ms (●). *B*, intracellular Ca^{2+} activity measured 25 ms after depolarization (●), at its peak (●), and at the time when the cell shortening is at its peak (○). *C*, peak cell shortening (○, △). ○, responses elicited by depolarization; △, responses elicited by repolarization.

ments of cell shortening indicate that repolarizing clamp pulses from potentials positive to +40 mV reactivated the myocyte. It has been suggested that the reactivation of Ca_i^{2+} transient is caused by influx of Ca^{2+} through the incompletely inactivated Ca^{2+} channel in the first few milliseconds following repolarization (Barcenas-Ruiz & Wier, 1987; Beuckelmann & Wier, 1988). These results therefore indicate that there is at least a rough agreement between the Ca^{2+} current and the two indicators of activation, cell shortening, and intracellular Ca^{2+} transients.

Figure 10 compares the voltage dependence of I_{Ca} (*A*, ●) and the integral of I_{Ca} (*A*,

●) to the voltage dependence of the various indicators of activation; that is the Ca^{2+} transient at 25 ms after depolarization (*B*, ●), the Ca^{2+} transients at its peak (•), and the intracellular Ca^{2+} activity (*B*) at the time of peak cell shortening (*C*) activated either by depolarizing (○) or repolarizing (△) pulses. It appears that the

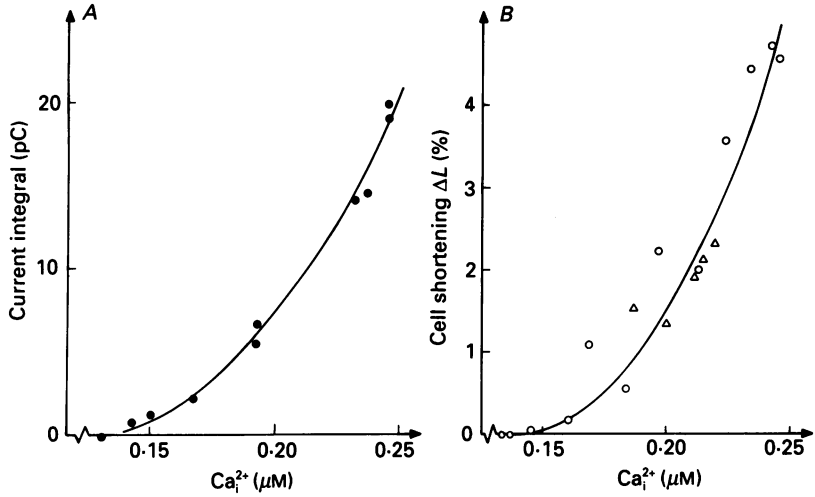

Fig. 11. Correlation between the intracellular Ca^{2+} transient and the current integral (*A*) and between the intracellular Ca^{2+} transient and cell shortening (*B*). The values in *A* were measured 25 ms after depolarization while the values in *B* were measured at the peak of the cell shortening produced either by depolarization (○) or repolarization (△).

bell-shaped curves recorded with depolarizing pulses fall off at different rates as the potential is raised to increasingly positive values. In general, the later the measurement, the more is the shift of the optimum of the curve toward more positive potentials. Thus the optimum of the curve representing the current integral (*A*, ●) is to the right of optimum of the peak current curve (*A*, •). Similarly, the maximum of the curve representing the $[Ca^{2+}]_i$ signal at peak cell shortening (*B*, ○) is shifted to the right of the maximum of the curve representing the intracellular Ca^{2+} transients 25 ms after depolarization (*B*, ●). The shift of the optimum of the curves is also accompanied by a broadening of the bell shaped relations. Thus the integral of I_{Ca} has bell-shaped relation (*A*, ●) which is wider than that of I_{Ca} (*A*, •) and the Ca^{2+} activity at the peak of the contraction (*B*, ○) has a bell-shaped relation which falls outside that of the early Ca^{2+} transient at both positive and negative potentials.

Figure 11 correlates the three types of signals, I_{Ca}, intracellular Ca^{2+} transients, and cell shortening plotted in Fig. 10. The left panel shows that the $[Ca^{2+}]_i$ activity measured 25 ms after depolarization is well correlated to the integral of I_{Ca} measured prior to this time. The right panels shows that the cell shortening is also correlated fairly well to $[Ca^{2+}]_i$ when measured at its peak. In other words, there is a satisfactory correlation between the signals if the comparison is based on estimates obtained at the same time and from the same cell.

Both relations of Fig. 11 are non-linear suggesting that the smaller I_{Ca} is relatively more effective than the larger I_{Ca} in releasing intracellular Ca^{2+}. This finding is

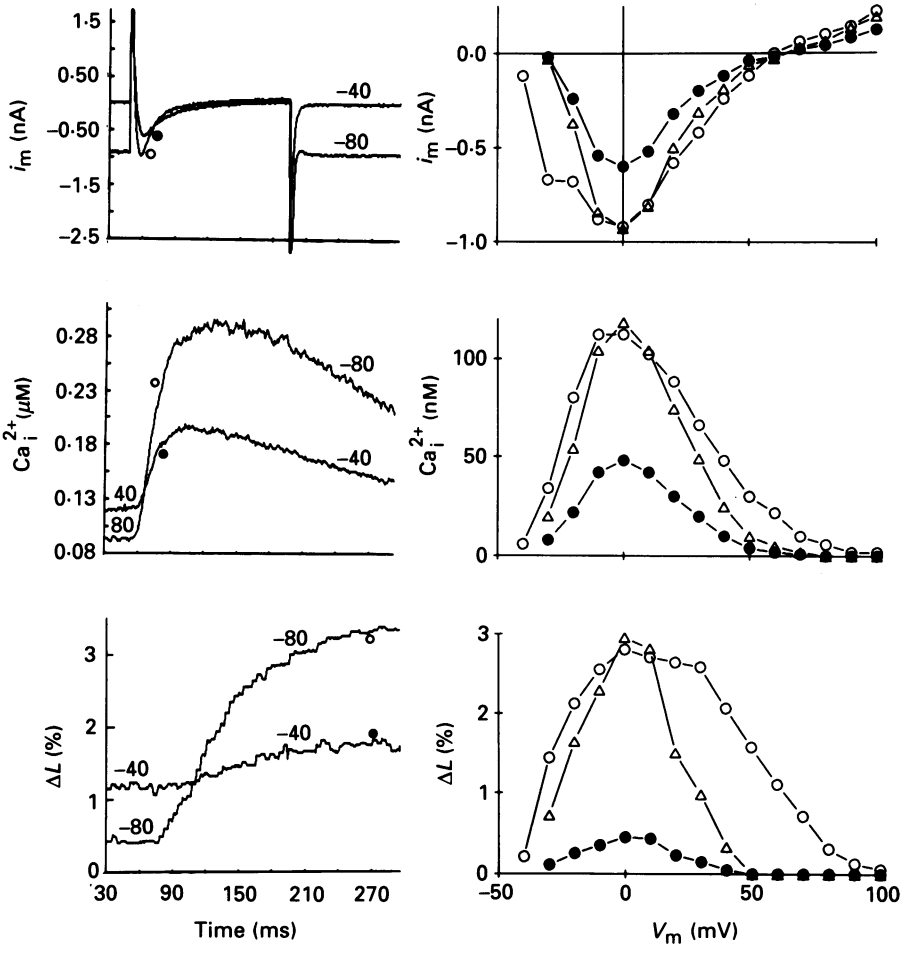

Fig. 12. Effect of the holding potential on the membrane current (i_m), the intracellular Ca^{2+} transient (Ca_i^{2+}) and the cell shortening (ΔL). The left panels show sample records obtained during depolarization to 0 mV from a holding potential of either −80 mV (−80) or −40 mV (−40). The graphs in the right panels show the voltage dependence of the three parameters. ●, transients measured following depolarization from −40 mV; ○, values measured following depolarization from −80 mV; △ values obtained with a holding potential of −40 mV but are scaled to give the same maximum as obtained with a holding potential of −80 mV. The 'maximum' was estimated as the average value of the three measurements at −10, 0 and +10 mV.

consistent with the idea that the higher Ca^{2+} influxes may also inactivate the Ca^{2+} release mechanism (Fabiato, 1985*b*). Figure 11*B* shows that $[Ca^{2+}]_i$ has to reach a threshold before contraction develops, but thereafter contraction rises steeply as $[Ca^{2+}]_i$ is increased further. This finding is consistent with the well-known co-operativity of myofibrillar responses to $[Ca^{2+}]_i$ (Chapman, 1979; Yue, Marban & Wier, 1986).

The effect of holding potential on activation

We tested the effect of the holding potential, V_H, on the voltage dependence of the $[Ca^{2+}]_i$ signal and cell shortening. This was deemed essential since some of the discrepancies in the literature may have arisen from the use of different holding potentials (Cannell *et al.* 1987; Callewaert *et al.* 1988).

Figure 12 shows results from an experiment where holding potentials of −80 mV and −40 mV were used in the same cell. The left panels show sample records of membrane current (i_m), Fura-2 transient (Ca_i) and cell shortening (ΔL) recorded during depolarization to 0 mV. Changing V_H from −80 to −40 mV reduced the holding current and the peak inward current and slowed its inactivation. This was accompanied by the elevation of baseline $[Ca^{2+}]_i$ and tonic shortening of the cell. The Ca^{2+} transient and cell shortening measured from a V_H of −40 mV were also significantly reduced.

The voltage dependence of the peak inward current, the $[Ca^{2+}]_i$ transient measured 25 ms after depolarization, and the peak value of the cell shortening at the two different holding potentials is shown in the graphs of Fig. 12. In all cases we obtained similar bell-shaped curves with a maximum around 0 mV. The values obtained with a V_H of −40 mV (●) were less than the values obtained with a V_H of −80 mV (○). To compare the voltage dependencies of the measured parameters at the different holding potentials the values obtained with a V_H of −40 mV were scaled up (△) to match the maxima of the curves obtained with a V_H of −80 mV. Such a comparison showed that the bell-shaped relations had a broader maximum when measured with a V_H of −80 than with −40 mV. With a V_H of −40 mV, the width of the bell-shaped relations (at the 50% level) was nearly the same for I_{Ca} (−18 to +23 mV), the Ca^{2+} transient (−20 to +27 mV) and cell shortening (−22 to 22 mV). The width of the voltage dependence of the same three parameters, at a V_H of −80 mV, no longer had the same approximate range (I_{Ca}, −34 to +28 mV; Ca_i^{2+}, −25 to +35 mV; ΔL, −30 mV to +54 mV). In particular the cell shortening (measured 200 ms after depolarization) had a broader plateau than the Fura-2 signal (measured at 25 ms). Such broadening reflects the time dependence of activation seen in Fig. 8 and was generally more prominent at the more negative holding potential (cf. Figs 8 and 10).

At a V_H of −80 mV the current–voltage relation often displayed a shoulder at potentials around −30 mV suggesting the presence of either the low-threshold Ca^{2+} channel or residual Na^+ current not blocked by TTX. At any rate the activation of this current component did not result in a rapid rise in $[Ca^{2+}]_i$.

The time course of the development of $[Ca^{2+}]_i$ transients

If I_{Ca} were to control the release of Ca^{2+} from the SR, then it might be expected that changes in the time course and voltage dependence of I_{Ca} should also be reflected in the time course and the voltage dependence of the development of the $[Ca^{2+}]_i$ transient.

Figure 13 compares the time course of $[Ca^{2+}]_i$ and I_{Ca} obtained with depolarizing pulses to −20 and +30 mV. These potentials were chosen because the final rise in $[Ca^{2+}]_i$ was the same. The Ca^{2+} transient activated by a clamp pulse to −20 mV developed more rapidly than that elicited by depolarization to +30 mV. This is

consistent with the observation that I_{Ca} was larger and inactivated more rapidly at -20 mV than at $+30$ mV. Furthermore the delay in the development of the $[Ca^{2+}]_i$ transient was 1–2 ms shorter at $+30$ mV than at -20 mV ($n = 3$) consistent with the idea that the Ca^{2+} current activates faster at the more depolarized potential (Cavalié,

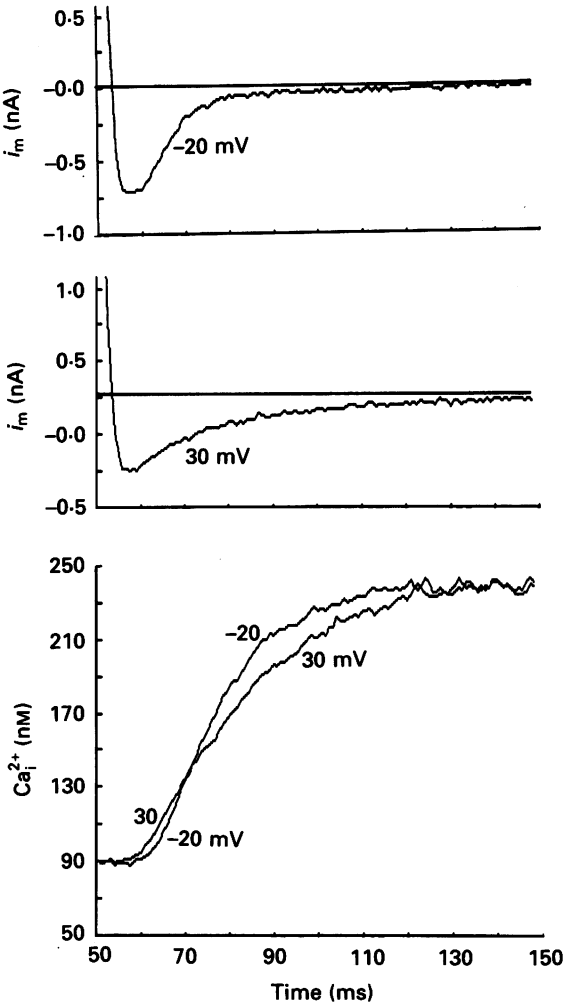

Fig. 13. Comparison of the time course of the membrane current (i_m) and the Ca^{2+} transient (Ca_i^{2+}) measured with Fura-2. At the beginning of the traces the membrane was depolarized from a holding potential of -80 mV either to -20 mV or to $+30$ mV. The inward current at -20 mV has a larger peak value but is briefer than the inward current at $+30$ mV. Correspondingly the Ca^{2+} transient measured at -20 mV approaches its final level faster than the Ca^{2+} transient measured at $+30$ mV.

Ochi, Pelzer & Trautwein, 1983; Lee & Tsien, 1984; Kay & Wong, 1987). Differences in the rate of activation of I_{Ca} could not be verified in the current records of Fig. 13 due to the presence of capacitive currents lasting about 3 ms. The cell shortenings recorded at the two potentials were not noticeably different.

Figure 14 compares the intracellular Ca^{2+} transient activated by depolarization from −40 to 0 mV (D) to that obtained with repolarization from +90 to −40 mV (R). The $[Ca^{2+}]_i$ transient produced by repolarization occurred 3 ms earlier than the transient produced by depolarization. Thus the 'tail transient' occurred with less

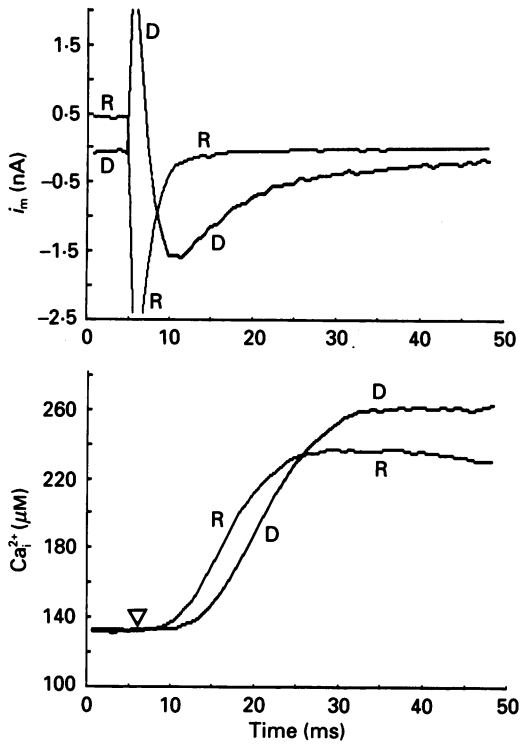

Fig. 14. Comparison of membrane currents (i_m) and Ca^{2+} transients (Ca_i^{2+}) produced either by depolarization (D) or repolarization (R). The holding potential was −40 mV. The membrane potential during the depolarizing clamp pulse was 0 mV. The repolarizing clamp pulse represent the return to the holding potential at the end of a 140 ms clamp pulse to +90 mV.

initial delay than the transient activated at 0 mV where some time is thought to be required for the opening of the Ca^{2+} channel. This is consistent with the idea that the Ca^{2+} channel was already open at the time when the membrane was repolarized from +90 to −40 mV and it suggests that the initial delay in the development of depolarization-induced Ca^{2+} transients is due, in part, to delay in the opening of the Ca^{2+} channel. No significant difference in the delay of the development of the contraction was detected.

If the release of Ca^{2+} from the SR is initiated by the influx of Ca^{2+} through the Ca^{2+} channel, then it is likely that termination of the Ca^{2+} current will also stop the Ca^{2+} current. This possibility was tested in the experiment illustrated in Fig. 15. In the left panels a long voltage clamp depolarization (L) was shortened to 8, 5 and 2 ms by repolarization to the holding potential. This procedure resulted in deactivation of I_{Ca} after a brief tail current and in abbreviation of the rising phase of the $[Ca^{2+}]_i$

transient. This finding (cf. Cannell *et al.* 1987; Beuckelmann & Wier, 1988) though compatible with the idea that the release of Ca^{2+} depends on the magnitude and duration of I_{Ca} has been interpreted to implicate a direct role for the membrane potential in regulation of Ca^{2+} release (Cannell *et al.* 1987, 1990). To differentiate

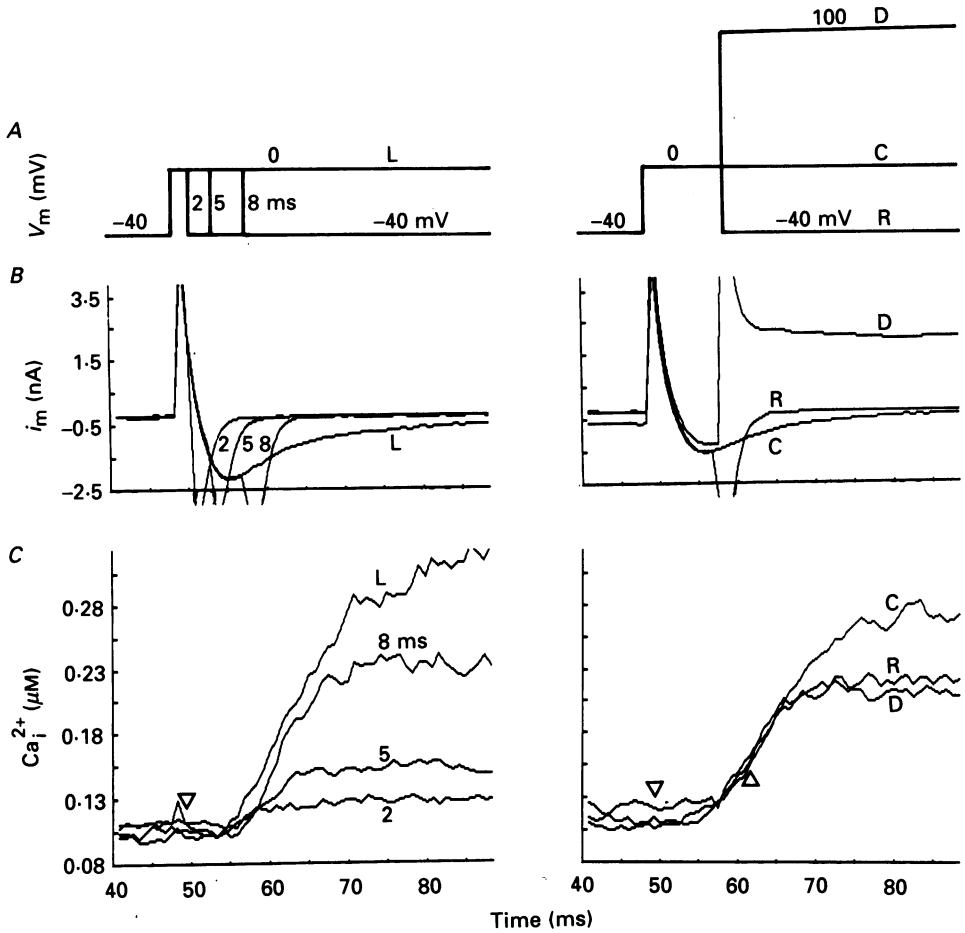

Fig. 15. Interruption of the development of the Ca^{2+} transient. *A*, schematic of the voltage clamp protocol (V_m). *B*, membrane current (i_m). *C*, intracellular Ca^{2+} transient (Ca_i^{2+}). The left panels show that the amplitude of the Ca^{2+} transient is reduced as the duration of a depolarizing clamp pulse to 0 mV is reduced from a large value (L) to 8, 5 and 2 ms (8, 5 and 2). The right panels show that the development of Ca^{2+} transient at 0 mV (C) can be terminated after 10 ms equally well by repolarization to the holding potential (R) or by further depolarization to +100 mV (D). The holding potential was −40 mV.

between the effects of Ca^{2+} influx and voltage we carried out a series of experiments where Ca^{2+} influx was terminated either by deactivation or by reducing the driving force for influx of Ca^{2+}. Figure 15 (right panels) compares the effects of these two interventions applied 10 ms after the initial depolarization from −40 to 0 mV. When compared to an uninterrupted clamp pulse to 0 mV (trace C), it is seen that

repolarization (trace R) and depolarization to +100 mV (trace D) were equally effective in terminating the Ca^{2+} release. The similarity in the regulation of the Ca^{2+} release produced either by deactivation of I_{Ca} or by clamping to a potential near E_{Ca} suggests that Ca^{2+} release is modulated primarily by the influx of Ca^{2+} through the Ca^{2+} channel rather than by charge movement associated with voltage change across the membrane.

DISCUSSION

Sensitivity, possible saturation, buffering capacity and response time of Fura-2

Sensitivity of Fura-2

The accuracy of the measured $[Ca^{2+}]_i$ depends mainly on the fluorescence intensity, the stability of the background fluorescence and the effective K_d of Fura-2. We used a relatively high concentration of Fura-2 (0·4 mM) in order to measure rapid Ca^{2+} transients with minimal noise and introduced an adjustable detection aperture (A_2 in Fig. 1) which proved quite effective in reducing the background fluorescence (Fig. 3).

The calculated values of $[Ca^{2+}]_i$ are directly proportional to the assumed value of the K_d (eqn (1)). Thus we used a K_d of 140 nM (Grynkiewicz *et al.* 1985) and found, on average, a maximal $[Ca^{2+}]_i$ of 0·32 μM while Beuckelmann & Wier (1988) used a K_d of 200 nM (Williams, Fogarty, Tsien & Fay, 1985) and found a peak $[Ca^{2+}]_i$ of 0·5 μM. Such values are in mutual agreement indicating that peak $[Ca^{2+}]_i$ was 2·3 or 2·5 times K_d but they are lower than some earlier results (Cannell *et al.* 1987; Callewaert *et al.* 1988). The relevant, *in vivo* value of K_d is difficult to determine since Fura-2, like other Ca^{2+}-sensitive dyes, is likely to bind to intracellular constituents (Maylie, Irving, Sizto & Chandler, 1987). As a first correction one may account for such binding by adjusting K_d upwards, possibly to a value as high as 400 or 700 nM (Konishi, Olsen, Hollingworth & Baylor, 1988). This would make our peak $[Ca^{2+}]_i$ 0·9 or 1·6 μM and could account for the discrepancy with the value of 1·03 μM measured with Mag-Fura-2. While such an explanation is plausible it should be remembered that the physiological K_d of Mag-Fura-2 could also be altered by binding and that only one-third of the experiments with Mag-Fura-2 yielded detectable signals.

Possible saturation of Fura-2

More serious than the uncertainty of K_d is the possibility that Fura-2 might bind tightly to intracellular proteins or be taken up by cellular organelles (Spurgeon *et al.* 1990) thereby introducing a pool of Fura-2 which fluoresces but is insensitive to Ca^{2+}. The remaining, Ca^{2+}-sensitive pool of Fura-2 could then be completely saturated with Ca^{2+} even though the measured fluorescence ratio were far from its theoretical limit. Such failure to detect saturation of Fura-2 implies that significant changes in $[Ca^{2+}]_i$ might remain undetected. It could be argued, for instance, that the more or less pronounced plateau of the voltage dependence of the Ca_i^{2+} transient (Figs 8 and 12) represents dye saturation rather than an upper limit for the Ca^{2+} release. This possibility can be ruled out since a plateau could also be detected with Mag-Fura-2 (Fig. 8) and measurement of contraction (Fig. 12). Furthermore, whenever the Fura-2-signal was steady, so was the twitch (Fig. 3), and whenever one changed, so did the other (Figs 4, 5, 9 and 12). We conclude, therefore, that Fura-2 was not used outside the region where it is sensitive to changes in $[Ca^{2+}]_i$.

Buffering capacity of Fura-2

Another possible problem with Fura-2 is that its concentration (0·4 mM) might be sufficiently high to buffer $[Ca^{2+}]_i$ (Cannell *et al.* 1987) and alter the qualitative nature of excitation–excitation coupling. The amount of activator Ca^{2+} in cardiac muscle is though to be in the range of roughly 30 μmol (contractile filaments only, Solaro, Wise & Briggs, 1974) or 60 μmol (Fabiato, 1983) to 200 μmol (Jorgensen, Broderick, Somlyo & Somlyo, 1989) per litre tissue. In comparison the amount of Ca^{2+} which binds to Fura-2 is estimated to be 64 μmol per litre tissue based on a concentration of 0·4 mM in the pipette, an accessible intracellular space corresponding to about 40% of the tissue volume (Fabiato, 1983) and a degree of saturation which typically increases from 30% at rest to 70% following activation (0·4 mM $0{\cdot}4(0{\cdot}7-0{\cdot}3) = 64$ μM). Although little can be concluded from such numbers they do provide limits for the maximal buffering attenuation by Fura-2 *vs.* the endogenous buffers. More to the point, perhaps, are the experimental data which show that the rate of rise and the peak value of the Fura-2 transient as well as the twitch were stable as the concentration of the dye build up inside the cell (Fig. 3). This may mean that the buffering capacity of Fura-2 is small relative to that of the endogenous Ca^{2+} buffers of cardiac cells (Fabiato, 1983) even if these are less abundant than in skeletal muscle fibres (Baylor, Chandler & Marshall, 1983). Alternatively it is possible that the release of Ca^{2+} is regulated in such a way as to overcome the buffering capacity of Fura-2 and keep the $[Ca^{2+}]_i$ transient within narrow limits.

Response time of Fura-2

It was thought that Mag-Fura-2 might follow the time course of the $[Ca^{2+}]_i$ transient more faithfully than Fura-2. This has been observed in frog skeletal fibres (Baylor *et al.* 1989; Konishi *et al.* 1991). We, on the other hand, found no difference in the delay and rise time of the intracellular Ca^{2+} transients using Fura-2 or Mag-Fura-2 in rat ventricular myocytes (Figs 7 and 8). This is consistent with a release process which, in heart muscle, is a relatively slow process lasting 10–20 ms (Figs 13 and 14) or as long as the influx of Ca^{2+} through the Ca^{2+} channel (Fig. 15). Fura-2 apparently responds fast enough to resolve such slow activation. Fura-2, rather than Mag-Fura-2, therefore was used in the majority of the experiments since it is more sensitive and less prone to yield motion-induced artifacts (Fig. 6). The motion artifacts measured with Mag-Fura-2 typically were 1–3% or $\frac{1}{2}$ to $\frac{1}{4}$ of the contraction measured with the length detector. Assuming that the Fura-2 measurements had similar motion artifacts superimposed on the fluorescence intensities, and that these were further suppressed by the calculation of fluorescence ratios, we conclude that the Fura-2 measurements of $[Ca^{2+}]_i$ were virtually free of motion-induced artifacts.

Relationship between $[Ca^{2+}]_i$ and cell shortening

Time course of activation

The delay in the development of the $[Ca^{2+}]_i$ transient was typically 3–9 ms (Figs 7, 13, 14 and 15) but could be shortened by clamping the myocytes to more positive potentials (Fig. 13) or by first holding the potential at +100 mV and then returning to the test potential (Fig. 14). In this way the $[Ca^{2+}]_i$ transient reflects the activation

kinetics of the Ca^{2+} channel which activates faster at more positive potentials (Cavalié *et al.* 1983; Lee & Tsien, 1984; Kay & Wong, 1987) and is assumed to be open at +100 mV even though there it carries no significant Ca^{2+} influx (fig. 14).

While the $[Ca^{2+}]_i$ transient clearly reflected the detailed time course of the Ca^{2+} current, we found that the cell shortening was delayed significantly and developed too slowly to yield mechanistic information.

Voltage dependence of activation

Our results show that both the $[Ca^{2+}]_i$ transient and cell shortening have bell-shaped voltage dependencies similar to that of the inward Ca^{2+} current (Figs 8–12). Cell shortening resulting from a rise in $[Ca^{2+}]_i$ may also be activated following repolarization from positive potentials. The results confirm previous reports where either the $[Ca^{2+}]_i$ transient (Beuckelmann & Wier, 1988; Callewaert *et al.* 1988) or mechanical activity (London & Krueger, 1986) were measured separately. The strength of the present approach is therefore not to be found in the qualitative nature of the two types of measurements but in a quantitative comparison. When performing such comparisons it was noticed that the various bell-shaped curves had slightly different width and location of their maxima (Figs 8, 10 and 12) depending on how the parameter was measured. In general the maximal moved toward more positive potentials and broadened into a plateau as the measurement was taken later and later into the depolarization pulse. We also noticed that the cell shortening which follows repolarization is noticeably smaller than that caused by depolarization to 0 mV (Figs 9 and 10) even though the peak values of the $[Ca^{2+}]_i$ transients are only slightly different. To resolve this type of discrepancy, one must take into account that the intracellular Ca^{2+} transient precedes the contractile response by about 100 ms and that the time course of the Ca^{2+} currents and the $[Ca^{2+}]_i$ transients depend on membrane potential. Thus we compared the $[Ca^{2+}]_i$ transients measured after 25 ms to the integral of I_{Ca} and the peak shortening to simultaneous estimates of $[Ca^{2+}]_i$. When done in this manner (Fig. 11), there was little discrepancy between the two indicators of activation of myocytes and the results were fully compatible with the hypothesis that release of Ca^{2+} from the SR is regulated by influx of Ca^{2+} through the Ca^{2+} channel. We suggest therefore that the reported discrepancies between the voltage dependence of I_{Ca} and $[Ca^{2+}]_i$ transients (Cannell *et al.* 1987) may result from comparing the peak value of I_{Ca} to the maximal value of the $[Ca^{2+}]_i$ transient occurring at a later time.

Changing the holding potential from −40 to −80 mV somewhat broadened the bell-shaped curves representing the voltage dependence of the inward Ca^{2+} current, the $[Ca^{2+}]_i$ transient and the cell shortening (Fig. 12). It did not, however, introduce deviations between the three parameters beyond what could be explained either by their different time course or by activation of other inward current components (residual Na^+ current or T-type Ca^{2+} current).

With 200 ms clamp pulses in rat ventricular myocytes (and 2 mM-Na^+ in the dialysing solution) we did not observe slowly developing $[Ca^{2+}]_i$ transients at positive potentials of the type linked to Na^+–Ca^{2+} exchange in guinea-pig ventricular myocytes (Barcenas-Ruiz, Beuckelmann & Wier, 1987).

Non-linear relationship between $[Ca^{2+}]_i$ *and cell shortening*

The curves in Fig. 11 may be interpreted in terms of stoichiometry of the activation process. The relation between $[Ca^{2+}]_i$ and cell shortening may correspond to the lower part of the sigmoid relationship between $[Ca^{2+}]_i$ and force development in skinned and intact cardiac fibres (Chapman, 1979; Fabiato, 1985*a*; Yue *et al.* 1986). The curve on the left shows that larger changes in $[Ca^{2+}]_i$ require unproportionally larger influxes of Ca^{2+} through the Ca^{2+} channel. This may indicate that Ca^{2+}-induced inactivation of the Ca^{2+} release channel early in the release process (Fabiato, 1985*b*). However, it should be noted that the $[Ca^{2+}]_i$ transients were measured before they reached their peak value. It is also possible that the Ca^{2+} release from the SR is linearly related to the integral of the Ca^{2+} current and that the non-linear curve reflects the buffering capacity of the cell. Here it should be considered that the curvature of the relationship cannot be explained merely by the buffering of $[Ca^{2+}]_i$ by Fura-2 since a simple one-to-one stoichiometry would make the curve less steep as the saturation was approached. A sigmoid binding curve, such as those displayed for the myofilaments or other endogenous Ca^{2+} buffers (Baylor *et al.* 1983), might well be the result of higher order stoichiometry and could result in the observed curvature. It is possible, therefore, that both panels of Fig. 11 reflect the sigmoid binding curve for the contractile filaments.

In agreement with the non-linear relationship between $[Ca^{2+}]_i$ and cell shortening (Fig. 11) it was observed that the beat-dependent modulation of contraction was more pronounced than that of $[Ca^{2+}]_i$ (Figs 4 and 5).

Evidence for release of Ca^{2+} *from the SR*

The measurements of cell shortening, and especially of $[Ca^{2+}]_i$ show that the activation process closely mirrors both the time course and the voltage dependence of the Ca^{2+} current. It is important therefore to keep in mind that the Ca^{2+} current in mammalian cardiac cells does not act primarily as a direct activator of the contractile filaments but that it acts indirectly by releasing Ca^{2+} from the SR. This concept is supported by the finding that ryanodine greatly suppresses the $[Ca^{2+}]_i$ transients without suppressing I_{Ca} (Barcenas-Ruiz & Wier, 1987; Beuckelmann & Wier, 1988; Callewaert *et al.* 1988). In the present report this idea is supported by the finding that cell shortening and $[Ca^{2+}]_i$ transients are suppressed when attempts are made to deplete the SR of Ca^{2+}, either by caffeine (Fig. 4) or by repeated abbreviations of the depolarizing pulses (Fig. 5). These findings supplement previous measurements where cell shortening was suppressed following exposure to caffeine (Naebauer *et al.* 1989). They are also consistent with the gradual decline in isometric force seen in syncytial cardiac preparations following abbreviations of the voltage clamp depolarization (Morad & Goldman, 1973), with the gradual build up of isometric force when I_{Ca} has been increased stepwise by photolysis of nifedipine (Morad & Cleemann, 1987), and finally with the finding that a small elevation in $[Ca^{2+}]$ can release Ca^{2+} in skinned myocytes while longer exposure to Ca^{2+} is necessary to load the SR (Fabiato, 1985*c*).

Ca^{2+}-induced inactivation of the Ca^{2+} current

It is thought that inactivation of the Ca^{2+} current is partly a potential dependent process and is partly accelerated by elevation of $[Ca^{2+}]_i$ (Kohlhardt *et al.* 1973). In support of this hypothesis it has been observed that I_{Ca} inactivates more slowly when the $[Ca^{2+}]_i$ transients is nearly abolished by ryanodine (Callewaert *et al.* 1988) or caffeine (Naebauer *et al.* 1989). The present study also clearly demonstrates the prolongation of I_{Ca} when the $[Ca^{2+}]_i$ transient is suppressed by exposure to caffeine (Fig. 4) and by changing the holding potential from -80 to -40 mV (Fig. 12).

Inspection of Fig. 4 shows that current following a brief exposure to caffeine (trace 0) inactivates slowly even though $[Ca^{2+}]_i$ is still elevated, and the cell still contracted. This might suggest that Ca^{2+} released from the SR has a more immediate effect at the activation site than the Ca^{2+} scattered throughout the myoplasm. Such a possibility may be compatible with the idea that Ca^{2+} which enters through the sarcolemmal Ca^{2+} channel communicates directly with the release channel of the SR. The increased magnitude and even slower inactivation of the inward current during the subsequent beat (trace 1) might then be taken as evidence that SR does not release Ca^{2+}. This absence of SR release may either indicate that the SR is nearly empty, or that the SR, though partially filled, is in a refractory state.

Is Ca^{2+} release from the SR under direct and continuous control of the Ca^{2+} channel?

The Ca^{2+}-induced Ca^{2+}-release (CIRC) hypothesis (Fabiato, 1983, 1985*a–c*) states that the release of Ca^{2+} from the SR is not only promoted by a rapid elevation of the Ca^{2+} activity ($d[Ca^{2+}]_i/dt$) but is also inactivated by a moderate or prolonged elevation of $[Ca^{2+}]_i$. The first component of this scheme has been widely used to explain how the Ca^{2+} current, by locally increasing $[Ca^{2+}]_i$, may induce release from the SR (Beuckelmann & Wier, 1988; Callewaert *et al.* 1988). The present experiments show that the SR release not only reflects the voltage dependence of I_{Ca} (Figs 9–12) but also reflects the detailed activation and inactivation kinetics of this current component (Figs 13–15).

It has been suggested that membrane potential in intact cardiac myocytes modulates Ca^{2+} release from SR (Cannell *et al.* 1987) in a manner similar to that observed in the skeletal muscle (Hodgkin & Horowicz, 1960; Schneider & Chandler, 1973; Baylor *et al.* 1983). In particular it was proposed that suppression of the Ca^{2+} transient by premature repolarization (Fig. 15, left panels) includes a potential-dependent closing of the SR release channel (Cannell *et al.* 1990). This finding is contradicted by the observations (Fig. 15, right panels) that an on-going release can also be interrupted by further depolarization to a potential near E_{Ca}, suggesting that Ca^{2+} influx rather than potential controls the release of process. These results are consistent with the recent findings of Naebauer *et al.* (1989) who showed that the influx of Ca^{2+}, but not Na^+ or Ba^{2+}, through the Ca^{2+} channel was a necessary condition for the release of Ca^{2+} from the SR.

If Ca^{2+} directly regulates the release process, it may then be asked whether the sarcolemmal Ca^{2+} channel is in a unique position to supply the Ca^{2+} which promotes SR release or whether any intracellular source of Ca^{2+} could cause release. The latter seems to be the case since SR release can also be caused by caged Ca^{2+} liberated by

photolysis into the intracellular compartment (Naebauer & Morad, 1990) and, apparently, by Ca^{2+} which enters the cell through the Na^+–Ca^{2+} exchanger (Leblanc & Hume, 1990).

Such a general role of intracellular Ca^{2+} may suggest, however, that Ca^{2+} released from the SR can also cause SR release, thereby establishing a positive feedback loop which, contrary to all observations, sustains itself until the release process has run to completion. This problem may be resolved by the Ca^{2+}-dependent inactivation of release process which is the second component of the CIRC hypothesis (Fabiato, 1983, 1985*b*). Thus one might propose that the SR release channel first goes through a $[Ca^{2+}]_i$-sensitive phase where the magnitude of the subsequent Ca^{2+} release is determined and then proceeds into a $[Ca^{2+}]_i$-insensitive phase during which the predetermined Ca^{2+} release is meted out. Such a temporal separation of Ca^{2+} sensing and Ca^{2+} release might appear at first to be in qualitative agreement with the present experiments since (1) the initial delay of the Ca^{2+} signal could correspond to the $[Ca^{2+}]_i$ sensing phase, and (2) elevation of $[Ca^{2+}]_i$ caused by a change of holding potential from -80 to -40 mV could suppress the Ca^{2+} transients (Fig. 12) by partial inactivation of the Ca^{2+} release process. Quantitative analysis, however, does not support this view. An upper time limit for a hypothetical $[Ca^{2+}]_i$-sensing phase can be determined from Fig. 14 which shows that the Ca^{2+} release is well under way 3–5 ms after repolarization. Within this time period it would seem that the release process must become regenerative unless the release process is now largely insensitive to $[Ca^{2+}]_i$. Figure 15 on the other hand, shows that the release can be still stopped 10 ms after depolarization by termination of I_{Ca}. Furthermore, changes in the time course of I_{Ca} are well reflected in the time course of a submaximal Ca^{2+} release 30 ms after depolarization (Fig. 13). From such findings we conclude that the Ca^{2+} release is not just determined by the initial (< 5 ms) value of $d[Ca^{2+}]_i/dt$ or I_{Ca} but continues to be controlled by I_{Ca} for tenths of milliseconds. It is natural then to relate the measured delay between I_{Ca} and the Fura-2 signal, not to a $[Ca^{2+}]_i$-sensing interval, but rather, to the time required for opening of the release channel, diffusion of Ca^{2+} throughout the myoplasm and binding of Ca^{2+} to Fura-2. This interpretation is consistent with the finding that a similar delay of a few milliseconds is also observed when the development of the Ca^{2+} signal is terminated by interruption of I_{Ca} (Fig 15).

The duration of the SR release (10–30 ms, Figs 8 and 13) tends to rule out a rapid suppression of the release function by elevated $[Ca^{2+}]_i$. Such a mechanism also seems unlikely since the amplitude of the Ca^{2+} 'tail' transients is fairly independent of a longer lasting elevation of $[Ca^{2+}]_i$ during a previous depolarization (cf. traces labelled 60 and 80 mV in Fig. 9). This is consistent with the observations that the elevation of $[Ca^{2+}]_i$ during a 500 ms pre-pulse does not suppress a subsequent release (Naebauer & Morad, 1990). These findings suggest that Ca^{2+}-induced inactivation of the release process does not play a significant role in regulation of the Ca^{2+} release of intact cardiac myocytes.

The weight of the experimental evidence supports the view that Ca^{2+} ions entering through the Ca^{2+} channel play a pivotal role in promoting the graded release of Ca^{2+} from the SR and that this control mechanism is not limited to the initiation of the release process but is still in effect as the intracellular Ca^{2+} transient approaches its final level. What terminates the Ca^{2+} release or prevents the Ca^{2+}-induced release

from driving the system into a positive feedback loop remains a mystery. Although the Ca^{2+}-mediated inactivation process remains a very attractive hypothesis, we find no direct evidence to support it in our isolated, whole-cell clamped ventricular myocytes.

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