Effect on the fura-2 transient of rapidly blocking the Ca^{2+} channel in electrically stimulated rabbit heart cells

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1. We used a rapid solution switcher technique to investigate mechanisms that might trigger intracellular Ca^{2+} release in rabbit ventricular myocytes. The study was carried out at 36 °C, intracellular Ca^{2+} (Ca_i^{2+}) was monitored with fura-2, and myocytes were electrically stimulated.

2. In patch-clamped cells, using the switcher to apply 20 μM nifedipine (an L-type Ca^{2+} current ($I_{Ca,L}$) blocker) 4 s before a depolarization to +10 mV reduced the amplitude of $I_{Ca,L}$ to 10·25 ± 2·25% of control (mean ± S.E.M., $n = 7$ cells).

3. In externally stimulated cells, a rapid switch to 20 μM nifedipine 4 s before a stimulus reduced the amplitude of the fura-2 transient to 64·01 ± 2·09% of control (mean ± S.E.M., $n = 19$ cells). Using an *in vivo* calibration curve for fura-2, this was equivalent to a reduction in the Ca^{2+} transient to 50% during nifedipine application. Since an identical nifedipine switch reduced $I_{Ca,L}$ to 10·25%, it would seem that blocking a large fraction of $I_{Ca,L}$ inhibited only half the Ca^{2+} transient.

4. The Na^+–Ca^{2+} exchanger is inhibited by 5 mM nickel. Switching to 20 μM nifedipine + 5 mM nickel 4 s before a stimulus abolished the fura-2 transient completely, consistent with the hypothesis that Ca^{2+} entry via reverse Na^+–Ca^{2+} exchange might trigger a fraction of the fura-2 transient that remained during nifedipine.

5. After the Na^+–K^+ pump was inhibited by strophanthidin to increase intracellular Na^+ (Na_i^+), a switch to 20 μM nifedipine became progressively less effective in reducing the fura-2 transient. This suggests that as Na_i^+ rose, other mechanisms (perhaps reverse Na^+–Ca^{2+} exchange) appeared able to substitute for $I_{Ca,L}$ in triggering the Ca^{2+} transient.

6. In cells depleted of Na_i^+ to inhibit the triggering of sarcoplasmic reticulum (SR) Ca^{2+} release by reverse Na^+–Ca^{2+} exchange, a nifedipine switch reduced the fura-2 transient to 10·9 ± 4·19% (mean ± S.E.M., $n = 7$; equivalent to 6·5% of the Ca^{2+} transient).

7. A switch to Na^+-free (Li^+) solution 100 ms before an electrical stimulus caused an increase in the fura-2 transient of 12·2 ± 1·5% (mean ± S.E.M., $n = 7$; equivalent to a 22% increase in the Ca^{2+} transient).

8. The results confirm that $I_{Ca,L}$ is an important trigger for SR Ca^{2+} release and the resulting Ca^{2+} transient. However, since 50% of the Ca^{2+} transient remained when $I_{Ca,L}$ was largely inhibited, it would seem likely that other SR trigger mechanisms might exist in addition. These data are consistent with the idea that Ca^{2+} entry via reverse Na^+–Ca^{2+} exchange during the upstroke of the normal cardiac action potential might trigger a fraction of SR Ca^{2+} release and the resulting Ca^{2+} transient.

There are many areas of general agreement about the mechanisms involved in excitation–contraction coupling in the heart, and there are also a few areas which provoke lively debate. Depolarization of the membrane with an action potential (AP) leads to a small Ca^{2+} entry into the cell. This Ca^{2+} entry then 'triggers' the rapid release of a much larger amount of Ca^{2+} from the main intracellular Ca^{2+} store, the sarcoplasmic reticulum (SR). The resulting transient rise of cytoplasmic Ca^{2+} concentration $[Ca^{2+}]_i$ (the 'Ca^{2+} transient') activates the myofilaments and produces contraction. The mechanism by which a small Ca^{2+} entry triggers a much larger SR Ca^{2+} release is referred to generally as 'Ca^{2+}-induced Ca^{2+} release' (CICR).

Previous studies have shown that CICR is likely to be the major mechanism for SR Ca^{2+} release in heart muscle (Fabiato, 1983, 1985) and the existence of CICR in intact rat cells was shown directly by Valdeolmillos, O'Neill, Smith & Eisner (1989). Ca^{2+} ions entering through the cell membrane thus appear to activate SR Ca^{2+} release, but a fundamental question is: by which pathways might Ca^{2+} enter to activate SR Ca^{2+} release? There are two main candidates, the L-type Ca^{2+} channel and the Na^+–Ca^{2+} exchanger. There is compelling evidence that the L-type Ca^{2+} current ($I_{Ca,L}$) is an important trigger for SR Ca^{2+} release (e.g. Wier, 1992). Early studies using multicellular preparations indicated an important role for $I_{Ca,L}$ in triggering CICR (e.g. Beeler & Reuter, 1970; Gibbons & Fozzard, 1975), as have a number of later studies using isolated myocytes (e.g. London & Krueger, 1986; Cannell, Berlin & Lederer, 1987; Beuckelmann & Wier, 1988).

Although there is a prevailing view that $I_{Ca,L}$ might be the only significant trigger mechanism for CICR, recent findings have begun to suggest that 'reverse' Na^+–Ca^{2+} exchange (i.e. Na^+ extrusion coupled to Ca^{2+} entry) might also be able to trigger SR Ca^{2+} release (e.g. Leblanc & Hume, 1990; Nuss & Houser, 1992; Levi, Brooksby & Hancox, 1993; Lipp & Niggli, 1994). A combined involvement of both $I_{Ca,L}$ and reverse Na^+–Ca^{2+} exchange might have important relevance in relation to the cardiac AP. Under normal physiological conditions, it is the fast AP upstroke that triggers SR Ca^{2+} release and contraction. The rapid upstroke depolarizes the membrane to +50 mV over the first 1–2 ms of the AP. However, the magnitude of Ca^{2+} entry via $I_{Ca,L}$ is well known to be maximal between 0 and +10 mV and falls off steeply at more positive potentials (e.g. London & Krueger, 1986), so that at +50 mV there will be a relatively small trigger Ca^{2+} entry via $I_{Ca,L}$ (compared with +10 mV; e.g. Stern, 1992). On the other hand, since Na^+–Ca^{2+} exchange is voltage dependent (e.g. Kimura, Miyamae & Noma, 1987), any trigger Ca^{2+} entry via reverse exchange will increase with more positive potentials, and there will be a larger exchange-mediated trigger Ca^{2+} entry at +50 mV compared with +10 mV. Thus, it is conceivable that reverse Na^+–Ca^{2+} exchange might have a greater influence as a trigger of SR Ca^{2+} release under AP conditions than it might appear to have with experiments using voltage-clamp pulses applied to +10 mV.

The purpose of the present experiments was to test, in isolated myocytes, the hypothesis that both $I_{Ca,L}$ and reverse Na^+–Ca^{2+} exchange might be involved in triggering SR Ca^{2+} release. In order that the results might not be interfered with by internal cell dialysis, resulting in an altered concentration of intracellular ions and mediators that may modulate normal excitation–contraction coupling, we used externally stimulated myocytes as an alternative to patch-clamped cells. We also performed experiments close to the normal physiological temperature of 37 °C, and monitored the Ca^{2+} transient directly with the fluorescent Ca^{2+} indicator, fura-2. An aspect of crucial importance is that we used a rapid solution switcher technique (Spitzer, 1994) to apply the L-type Ca^{2+} channel blocker nifedipine between two individual beats. In contrast to this very rapid application, a slower and more prolonged exposure to nifedipine causes reduction of Ca^{2+} entry and leads to a progressive beat-to-beat depletion of SR Ca^{2+} content. A large depletion of SR content can occur very rapidly over just two to three beats (e.g. Levi *et al.* 1993; Rakovic & Terrar, 1995), and causes difficulty in interpretation of data since when SR content has become reduced, there will be a reduced SR Ca^{2+} release directly due to this, in addition to block of trigger $I_{Ca,L}$. The very rapid application of nifedipine between two beats that we used in this study prevented any difference in SR Ca^{2+} content between control and test beats, and allowed us to test specifically the effect of blocking $I_{Ca,L}$ on the magnitude of SR Ca^{2+} release and the Ca^{2+} transient. The results suggest that $I_{Ca,L}$ might not be the only trigger of SR Ca^{2+} release in rabbit ventricular myocytes. In externally stimulated myocytes under these conditions, the results indicate that (in the absence of $I_{Ca,L}$) a fraction of SR Ca^{2+} release might be triggered by Ca^{2+} entry via reverse Na^+–Ca^{2+} exchange at the start of the AP.

METHODS

Cell isolation

Myocytes were isolated from both ventricles of the heart of male New Zealand White rabbits (1·8–2·5 kg) using a method modified from Hancox, Levi, Lee & Heap (1993). Animals were killed by cervical dislocation (a method which was sanctioned by the Home Office), the heart was removed quickly and the aorta mounted on a cannula for retrograde perfusion at a rate of 6 ml min^{-1} (g heart tissue)$^{-1}$. The basic perfusate (solution A) contained (mmol l^{-1}): NaCl, 130; Hepes, 23; glucose, 21; taurine, 20; creatine, 5; KCl, 4·5; $MgCl_2$, 5; NaH_2PO_4, 1; sodium pyruvate, 5. The heart was perfused with solution A + 750 μM Ca^{2+} for 2 min, then with solution A + 8 μM EGTA for 5 min. After this, the heart was perfused for between 12 and 16 min (depending on heart size/animal weight; the time was increased progressively to near 16 min as heart size increased) with an enzyme-containing solution consisting of solution A + 150 μM Ca^{2+} with 1 mg ml^{-1} collagenase (Type 1, Worthington Biochemical Co.) and 0·1 mg ml^{-1} protease (Type XIV, Sigma). The enzyme was then washed out by perfusing with solution A + 150 μM Ca^{2+} for a further 5 min. The heart was removed from the cannula; both ventricles were dissected and chopped into small pieces, which were then shaken for 6 min in a glass conical flask containing 50 ml of solution A + 150 μM Ca^{2+}. The cell suspension was filtered through nylon gauze (200 μm mesh), sedimented in a 50 ml glass beaker for 4 min, and then the supernatant was replaced with a higher Ca^{2+}-containing solution; [Ca^{2+}] was increased to 500 μM and finally to 1 mM. Cells were kept at room temperature in solution A + 1 mM Ca^{2+} until use, and usually survived well for up to 8 h.

Electrical recording

For recording, cells were placed in a Perspex chamber mounted on the stage of an inverted microscope (Nikon Diaphot) and superfused at 36 °C with Tyrode solution containing (mmol l^{-1}): NaCl, 140; $CaCl_2$, 2·5; KCl, 4; $MgCl_2$, 1; Hepes, 5; glucose, 10; titrated to a pH of 7·4 with 4 mmol l^{-1} NaOH. In some experiments we replaced external Na^+ by Li^+ (e.g. Figs 4 and 6) and in this solution 140 mM LiCl replaced the NaCl and the solution was titrated to pH 7·4 with LiOH. Patch pipettes (Corning 7052 glass, AM Systems Inc.) were pulled to resistances of 2·5–3 MΩ (Narashige PP 83 pipette puller) and fire-polished to between 3 and 5 MΩ (Narashige MF 83 microforge). The pipette filling solution contained (mmol l^{-1}): KCl, 110; K_2ATP, 5; Hepes, 10; $MgCl_2$, 0·4; glucose, 5; titrated to a pH of 7·1 by adding 10 mmol l^{-1} KOH. The 'pipette-to-bath' liquid junction potential for this solution was measured as −2·7 mV (using an Axoclamp-2A amplifier, Axon Instruments; with a voltage-follower head stage and recording differentially against an agar reference electrode). Since this value was small, it was not necessary to correct the membrane potential. Patch-clamp recordings were made using an Axopatch 200A amplifier and a CV202A head stage. The voltage-clamp command waveform was generated by an A/D board (CED 1401; Cambridge Electronic Design) which was controlled by a PC-based program called VGEN (Voltage Generator, written by John Dempster at the University of Strathclyde, UK).

Intracellular Ca^{2+} measurement

Since the experiments monitoring Ca_i^{2+} were carried out in externally stimulated cells, it was not possible to use the free acid form of fura-2. Therefore, myocytes were loaded with fura-2 by incubating with the acetoxymethyl (AM) ester (5 μM fura-2 AM; Molecular Probes) for 15 min. Using a digitonin–Triton X-100 permeabilization procedure, we showed that this resulted in 70% of fura located in the cytoplasmic compartment of the cell (Brooksby, Levi & Jones, 1993). The apparatus used for fluorescence measurements (Cairn Research Ltd, Newnham, Kent, UK) has been described previously (Lee & Levi, 1991; Brooksby *et al.* 1993). The filter wheel carrying the 340 and 380 nm filters was rotated at 120 Hz to improve time resolution of the fura-2 ratio measurements (this gave one 340:380 ratio measurement every 8·3 ms). For this study, the fura-2 measurements have been expressed as the 340:380 nm fura-2 ratio, since it is accepted generally that there are a number of uncertainties in accurately calibrating cells for Ca_i^{2+} when loaded with the AM ester of fura-2 (e.g. Roe, Lemasters & Herman, 1990; Williams & Fay, 1990). The design of the study was such that each experiment had its own internal control, because the amplitude and time course of the fura-2 transient directly after a rapid solution switch was compared with the fura-2 transient immediately before the switch. However, cells under these experimental conditions were also calibrated for Ca^{2+} using a modification of an ionophore procedure that we have described previously (Brooksby, Levi & Jones, 1993; see Fig. 8). In cells stimulated at 0·3 Hz, we measured a mean resting cytoplasmic Ca^{2+} level of 67 ± 26 nM and a peak systolic Ca^{2+} level of 560 ± 58 nM ($n = 10$ cells). Background fluorescence and autofluorescence was less than 1% of individual 340 nm and 380 nm signals.

To detect whether incorporation of fura-2 might alter Ca_i^{2+} buffering, we measured the shortening amplitude (normalized to resting cell length) and shortening time course of fifteen myocytes stimulated externally at 0·5 Hz before fura-2 loading, and then the shortening characteristics of a second batch of fifteen cells (from the same heart) after fura-2 loading. Under control conditions, cells shortened by 12·6 ± 1·4% of cell length, whereas cells loaded with fura-2 shortened by 11·2 ± 1·1% of cell length; there was no significant difference between these ($P = 0·45$). The time to peak contraction in unloaded cells was 133·2 ± 5·8 ms, and in fura-loaded cells it was 148 ± 14·8 ms; the difference was not significant ($P = 0·36$). Time to half-relaxation in control cells was 185 ± 6·6 ms and in fura-loaded cells it was 223 ± 31·7 ms; the difference was again not significant ($P = 0·26$). These data suggest that incorporation of fura-2 using this AM loading procedure caused only a minimal change in Ca_i^{2+} buffering.

Rapid solution switches and external stimulation

For experiments using the rapid solution switcher technique (Spitzer, 1994), myocytes were attached to the bottom of the experimental chamber with laminin. Forty micrograms of a 1 mg ml^{-1} laminin solution (Collaborative Research, Bedford, MA, USA) was added to 300 μl of normal Tyrode solution and left in the chamber for 15 min to coat the glass bottom. This solution was then removed and a suspension of cells was placed in the chamber. The cells were left for 5 min to attach to the bottom, then solution flow through the bath was started slowly and progressively increased.

For the rapid solution switcher device, two adjacent microstreams flowed simultaneously from neighbouring square glass tubes (each 200 μm wide) separated by a 70 μm glass septum. The solutions fed to the switcher outlets were preheated in a large glass jar which had separate reservoirs for each barrel, and the switcher was gravity fed from a height of 3·5 m. It was crucial to remove air bubbles from the switcher solution lines, and so solutions were passed through a heated dripper chamber whose height could be controlled remotely by venting with a solenoid valve (a device designed by Dr Ken Spitzer, University of Utah, UT, USA). The switcher was placed in position so that the control microstream flowed over a contracting myocyte, and activation of the switcher solenoid moved the streams laterally and suddenly exposed the cell to the test solution stream. Activation of the solenoid was synchronized to the voltage-clamp command or external stimulation using a pulse generator linked to the A/D board. Judging from junction potential changes recorded at the pipette tip, the bulk solution around a cell could be exchanged within 3 ms. However, when the switcher was used to rapidly change $[K^+]$ between 4 and 10 mM around a patch-clamped myocyte, inward current at −80 mV changed with a half-time of 15 ms and the solution change was complete within 100 ms. This is slower than the bulk solution change, probably due to unstirred layers around the cell which slow down diffusional exchange at the surface membrane.

Cells were externally stimulated using a miniature stimulating probe mounted on a micromanipulator. Thin (50 μm diameter) platinum wires, set 200 μm apart at their tips, were glued into each barrel of a 1·5 mm o.d. theta glass (Clark Electromedical) and connected to an isolated stimulator; 2 ms duration pulses of voltages up to 100 V could be applied between these wires to stimulate the myocytes, and the stimulation voltage for each cell was set 40% above threshold. The stimulating probe only activated a discrete area of cells and it was necessary to position it close to the bottom of the bath in order for it to stimulate cells

effectively. The precise position of the stimulating probe was also important with respect to the rapid solution switcher – it was positioned on the opposite side of the cell from the switcher outlet, so that it did not interfere with the solution stream trajectory.

Contraction

The contractile activity (unloaded cell shortening) of a myocyte was measured optically using a video-edge detector device (Crescent Electronics, Sandy, UT, USA) coupled to a TV camera mounted on the side-port of the microscope. The system ran at normal UK frame rate (50 Hz; 1 sample every 20 ms) and the time resolution of the system was adequate for measuring the contraction of rabbit cells. The TV camera image was monitored on-line and was also recorded on a video recorder so that it could be played back later to obtain the record of contraction.

Chemicals, data analysis and statistics

Nifedipine (Sigma) was made as a 20 mM stock solution in ethanol and kept in the freezer. It was added to Tyrode solution to give a final concentration of 20 μM, and was protected from light. Strophanthidin (Sigma) was made as a 50 mM stock solution in ethanol, kept at room temperature, and added to Tyrode solution to give a final concentration of 50 μM. Ryanodine was purchased from Research Biochemicals and thapsigargin from Sigma; TTX was obtained from Calbiochem. Data were recorded using a digital data recorder (Instrutech VR 100-8) and processed off-line using the CED 1401 A/D interface and the program WCP (Whole Cell Physiology; also written by John Dempster). Data are expressed as the means ± S.E.M. To compare observations within the same group, a two-sample Student's paired t test was used. To compare observations from different groups with unequal sample size, a two-sample Student's t test was used in which sample variance was not assumed equal (Microsoft Excel version 5, statistical functions). A P value of less than 0·05 was considered significant.

RESULTS

Effect on $I_{Ca,L}$ of a rapid switch to nifedipine in voltage-clamped myocytes

We assessed first whether a rapid switch to nifedipine before a single depolarization was able to block $I_{Ca,L}$ in rabbit myocytes. Patch-clamped cells were held at a membrane potential of −40 mV, to inactivate the fast Na^+ current (I_{Na}) and T-type Ca^{2+} current, and positioned in the control solution stream (Fig. 1*A*). Before each test depolarization, a train of 400 ms conditioning pulses from −40 to +10 mV was applied at a rate of 0·3 Hz to ensure $I_{Ca,L}$ was in a steady state. A switch to the nifedipine test stream was made 4 s before the 800 ms duration test depolarization. Even though we did not simultaneously measure the fura-2 signal in these experiments, cells were nevertheless continuously illuminated with UV excitation light just as they would be for a fura-2 fluorescence experiment, since we wished to determine whether UV excitation might affect the efficacy of nifedipine block.

Figure 1*A* illustrates the typical effect of a nifedipine switch on $I_{Ca,L}$. Each +10 mV conditioning pulse elicited an inward spike of $I_{Ca,L}$. A switch to 20 μM nifedipine was made shortly after the last conditioning pulse in the train, and 4 s before the following 800 ms test pulse. $I_{Ca,L}$ elicited by the test pulse was greatly reduced by the switch to 20 μM nifedipine. Very similar results were obtained in seven myocytes. The amplitude of $I_{Ca,L}$ was measured as the difference between the peak inward current at the start of the test pulse and the steady current at the end of the pulse (i.e. London & Krueger, 1986). A rapid switch to nifedipine reduced the amplitude of $I_{Ca,L}$ to 10·25 ± 2·25% of the control value ($n = 7$). The switch to nifedipine lasted 6 s, and after switching back to control solution, the amplitude of $I_{Ca,L}$ recovered over the next twelve pulses. (the first part of the recovery process can be seen in the recording illustrated in Fig. 1*A*). Three further experiments were performed in myocytes held at −80 mV and pulsed to +10 mV in the presence of TTX in order to measure $I_{Ca,L}$. Under these conditions also, a switch to 20 μM nifedipine 4 s before a single depolarization reduced the amplitude of $I_{Ca,L}$ to 10% of control. These experiments showed that (in cells also illuminated with UV light) a switch to 20 μM nifedipine 4 s before depolarization inhibited 90% of $I_{Ca,L}$.

Proportion of the fura-2 transient due to SR Ca^{2+} release

The Ca^{2+} transient in mammalian cardiac cells can be due either to SR Ca^{2+} release into the cytoplasm or direct Ca^{2+} entry from the extracellular space (Bers, 1985, 1991). In Fig. 1*B* we assessed the proportion of the fura-2 transient due to either of these sources in an externally stimulated rabbit cell under these experimental conditions. The myocyte was stimulated at 0·5 Hz until the fura-2 transient and phasic contraction had reached a steady state. We then applied 10 μM ryanodine and 1 μM thapsigargin to the bath superfusate. Ryanodine at this concentration is well known to inhibit SR Ca^{2+} release by locking the SR Ca^{2+} release channel in a sub-conductance state (e.g. Meissner & Henderson, 1987), and thapsigargin inhibits the SR Ca-ATPase (Wrzosek, Schneider, Grueninger & Chiesi, 1992). Thus, application of these agents together disables the ability of the SR to accumulate or release Ca^{2+}. After 10 min of exposure, it can be seen that the fura-2 transient had declined to 17% of the control value whilst the amplitude of phasic contraction declined to 10·5%. Very similar results were obtained in four different myocytes. The simplest interpretation of these results is that, in rabbit myocytes under these conditions, a large proportion of the fura-2 transient (80–90%) might be due to Ca^{2+} release from the SR. However, it should be noted that thapsigargin at this concentration has been reported to cause a small inhibition of the L-type Ca^{2+} channel (Buryi, Morel, Salomone, Kerger & Godfraind, 1995), and this might be responsible for a fraction of the response.

Effect on the fura-2 transient of a rapid nifedipine switch in externally stimulated myocytes

To assess the role of $I_{Ca,L}$ in triggering SR Ca^{2+} release, it is necessary to block this current between one beat and the

next, so that the only difference between the preceding control beat and the test beat is that $I_{Ca,L}$ is inhibited for the test beat. A typical result of a nifedipine switch in an externally stimulated myocyte is shown in Fig. 2*A*. The cell was stimulated at 0·3 Hz until both the fura-2 transient and contraction had reached steady state. Just after the last conditioning stimulus and 4 s before the test stimulus, a switch to 20 μM nifedipine was made that lasted 6 s. It can be seen that the fura-2 transient was reduced by the nifedipine switch to 68% of control. We found very similar results in a total of nineteen myocytes: a nifedipine switch reduced the amplitude of the fura-2 transient to 64·01 ± 2·09% of control. Thus, when precautions were taken to prevent alteration of SR Ca^{2+} content between the control and test beat, and when $I_{Ca,L}$ was largely blocked by a rapid nifedipine switch, more than half the normal fura-2 transient still remained.

Figure 2*A* also shows, however, that although more than half the fura-2 transient remained, at the same time less than half the phasic contraction was elicited. In Fig. 2*A*, only 40% of the control phasic contraction was observed during the nifedipine switch. Mean results are shown in histogram form in Fig. 2*C*. For the same nineteen cells in which the fura-2 transient was recorded, we found that the amplitude of phasic contraction declined to 24·03 ± 2·56% of control with nifedipine. The difference between the extent of decline of the fura-2 transient and that of contraction with

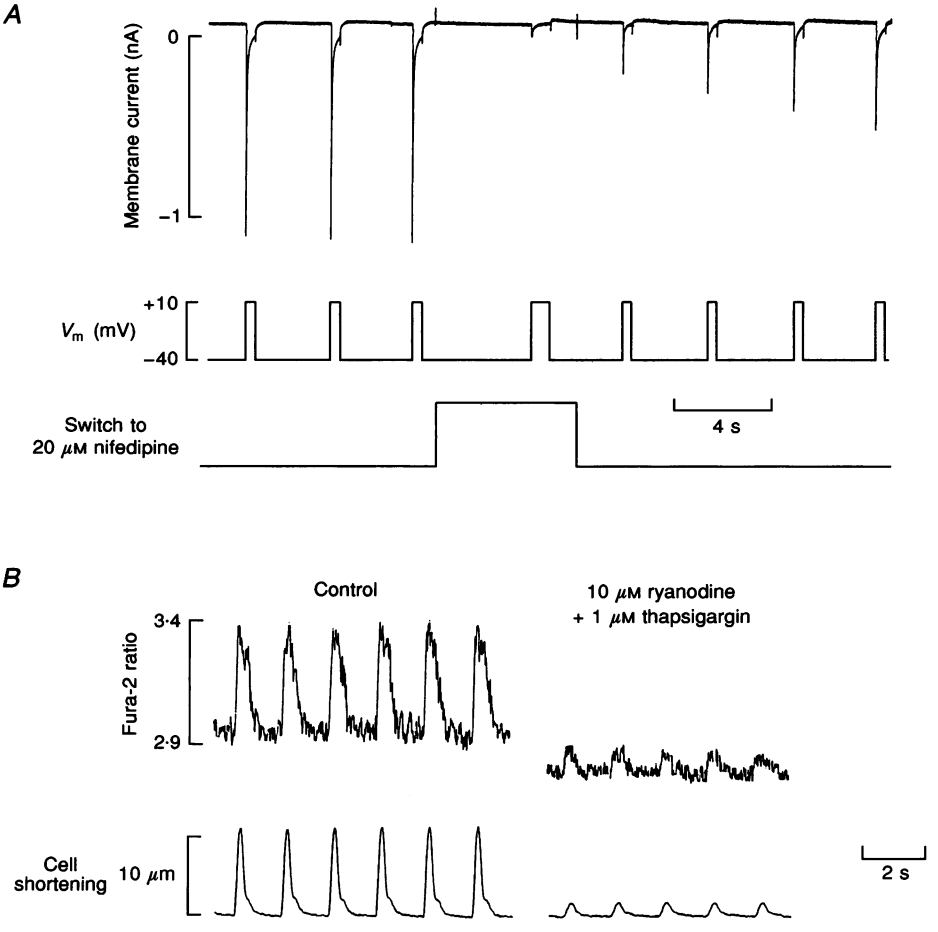

Figure 1

A, the effect of a single-beat nifedipine switch on the L-type Ca^{2+} current ($I_{Ca,L}$). The cell was held at a membrane potential (V_m) of −40 mV and a train of 400 ms conditioning pulses to +10 mV was applied to establish a steady-state amplitude of $I_{Ca,L}$. Just after the last conditioning pulse and 4 s before the 800 ms test pulse to +10 mV, a rapid switch was made to 20 μM nifedipine, which was applied for 6 s. $I_{Ca,L}$ was very largely abolished by the nifedipine switch. Here and in the following figures, the timing of the nifedipine switch is indicated by the bottom trace. *B*, the proportion of the Ca^{2+} transient due to SR Ca^{2+} release in rabbit myocytes at 36 °C . The left panel shows the 340 : 380 nm fura-2 fluorescence ratio (equivalent to the Ca^{2+} transient) and cell shortening (contraction) under control conditions at a stimulation rate of 0·5 Hz. The right panel shows the transient and contraction after 10 min exposure to 10 μM ryanodine and 1 μM thapsigargin.

nifedipine was highly significant ($P < 0{\cdot}005$). Thus, whereas the majority of the fura-2 transient remained after a nifedipine switch, only a small fraction of phasic contraction remained.

Figure 2*B* shows faster time base records of fura-2 transients and contractions recorded under control conditions and during a nifedipine switch. It is notable that although the amplitude of the fura-2 transient was

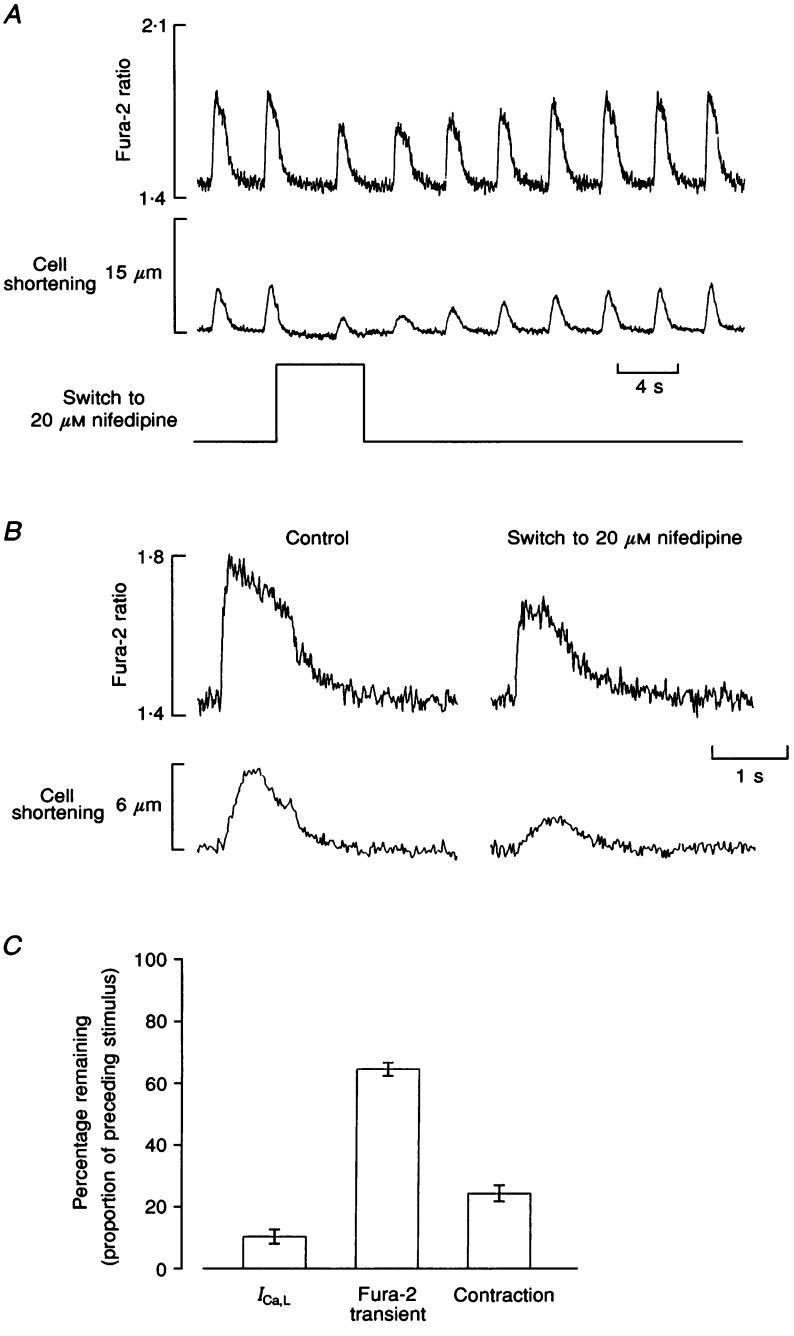

Figure 2. Effect of a single-beat nifedipine switch on the fura-2 transient and contraction of an externally stimulated myocyte

A, the cell was stimulated at 0·3 Hz until the fura-2 transient reached steady state. Shortly after the last conditioning stimulus and 4 s before the test stimulus, a switch was made to 20 μM nifedipine for 6 s duration. *B*, the faster time base records of a control fura-2 transient and contraction (left panel) and after a nifedipine switch (right panel); same cell as *A*. Note that time-to-peak of the fura-2 transient and contraction is little altered by the nifedipine switch, but both the fura-2 transient and contraction relax faster in nifedipine. *C*, mean data comparing the percentage block of $I_{Ca,L}$ by nifedipine in patch-clamped cells with the percentage inhibition of the fura-2 transient and phasic contraction in externally stimulated cells.

diminished by the nifedipine switch, the upstroke and time to peak of the transient was not affected. Under control conditions, time-to-peak of the fura-2 transient was 58·2 ± 3·8 ms; during a nifedipine switch, time-to-peak of the transient was 59·0 ± 5·6 ms ($n = 8$ cells), and the difference was not significant. However, the time course of decline of the fura-2 transient was altered by the switch. This was quantified by measuring the duration of the fura-2 transient from the start of the upstroke to the time at which it had declined to 50% of the peak value. Under control conditions, the fura-2 transient had a duration of 708 ± 84·3 ms; during a nifedipine switch, the duration of the transient declined to 503·1 ± 80·6 ms, and the difference was significant ($P < 0.05$). It is likely that the shortening of the fura-2 transient during the nifedipine switch may be related to the shortening of the AP which is known to take place (e.g. Levi, Spitzer, Kohmoto & Bridge, 1994).

Figure 2*C* illustrates in histogram form the extent to which $I_{Ca,L}$ was blocked by nifedipine in a patch-clamped cell, and the degree to which the fura-2 transient was inhibited in an externally stimulated cell. Despite the fact that a switch to nifedipine for 4 s before a depolarization inhibited almost 90% of $I_{Ca,L}$, only 36% of the fura-2 transient was abolished, thus 64% of the transient could still be elicited during the nifedipine switch. Since the fura-2 transient under these conditions appears to indicate primarily the magnitude of SR Ca^{2+} release (Fig. 1*B*), there are two explanations which might account for this finding: (a) the 10% of $I_{Ca,L}$ which remained with nifedipine might be able to trigger this large remaining fraction of SR Ca^{2+} release; or (b) other trigger mechanisms besides $I_{Ca,L}$ might exist

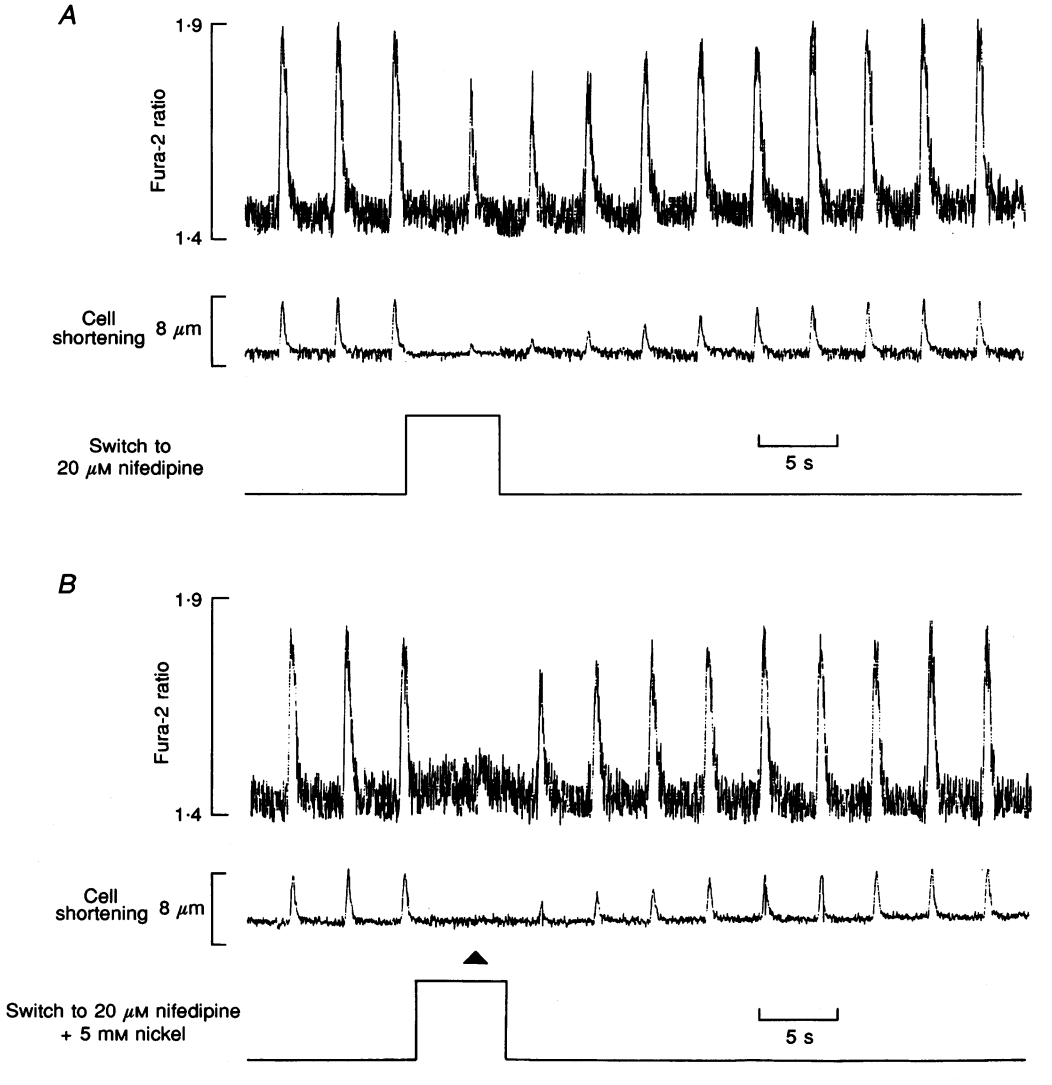

Figure 3. Blocking the Na^+–Ca^{2+} exchanger with 5 mM Ni^{2+}

A, a single-beat switch to 20 μM nifedipine; as usual, 68% of the fura-2 transient remained during the switch. *B*, 5 mM Ni^{2+} was added to the nifedipine switch solution. The cell was stimulated at the time indicated by the arrowhead, but the fura-2 transient was abolished almost completely by the addition of 5 mM Ni^{2+}.

under these conditions, and they might be responsible for triggering the significant proportion of the SR Ca^{2+} release which persists during nifedipine.

Are T-type Ca^{2+} channels involved in triggering SR Ca^{2+} release?

In externally stimulated cells that have a resting potential near −80 mV, one possibility for another SR Ca^{2+} release trigger mechanism besides $I_{Ca,L}$ is Ca^{2+} entry through the T-type Ca^{2+} channel. The functional role of the T-type channel in cardiac muscle is not certain (e.g. Vassort & Alvarez, 1994), but its voltage dependence shows that it is likely to be activated during the AP upstroke and it is possible that it might play a role in excitation–contraction coupling. Since the T-type Ca^{2+} channel is blocked by 100 μM Ni^{2+}, we used this to test for involvement of this channel in the triggering of SR Ca^{2+} release.

We could not examine the trigger role of the T-type Ca^{2+} channel by simply switching to 100 μM Ni^{2+} because of the possibility that this concentration of Ni^{2+} might also partially inhibit $I_{Ca,L}$. Therefore, we performed the experiment by comparing the effect of switching to 20 μM nifedipine alone with that of switching to 20 μM nifedipine + 100 μM Ni^{2+}. Results were obtained in six myocytes (not shown). During a switch to 20 μM nifedipine alone, the fura-2 transient declined to 71·2 ± 2·95% of the control value; in the same cells, during a switch to 20 μM nifedipine + 100 μM Ni^{2+}, the fura-2 transient declined to 70·5 ± 2·66% and the difference was not significant. Since adding 100 μM Ni^{2+} to nifedipine had no extra blocking effect on the fura-2 transient elicited during a switch, these data suggest that T-type Ca^{2+} channels are not involved in triggering SR Ca^{2+} release in externally stimulated rabbit myocytes under these conditions.

The effect of adding 5 mM Ni^{2+} to block the Na^+–Ca^{2+} exchanger

A second possible trigger for the nifedipine-resistant fura-2 transient is Ca^{2+} entry via reverse Na^+–Ca^{2+} exchange at the start of the AP (e.g. Levi, Brooksby & Hancox, 1993; Lipp & Niggli, 1994). Na^+–Ca^{2+} exchange is known to be blocked by 5 mM Ni^{2+} (Kimura, Miyamae & Noma, 1987) and Fig. 3 illustrates the effect of adding 5 mM Ni^{2+} to the nifedipine switch solution. Figure 3*A* shows a control switch to 20 μM nifedipine alone; as before, more than half (68%) of the fura-2 transient was elicited during nifedipine application. After the fura-2 transient had recovered, we made a switch to nifedipine + 5 mM Ni^{2+} (Fig. 3*B*). The cell was stimulated at the time indicated by the arrowhead, but the fura-2 transient was abolished almost completely by the added presence of 5 mM Ni^{2+}. Identical results were obtained in seven cells; the fura-2 transient was reduced to a mean of 5·5 ± 2·8%, and the contraction was abolished in every cell. This result is consistent with the hypothesis that reverse Na^+–Ca^{2+} exchange might be involved in triggering SR Ca^{2+} release in externally stimulated myocytes.

Intracellular Na^+ depletion and the effect of nifedipine switches

To investigate further whether reverse Na^+–Ca^{2+} exchange is involved in triggering SR Ca^{2+} release, we tested the effect of depleting intracellular Na^+ concentration ($[Na^+]_i$). Reduction of $[Na^+]_i$ should inhibit reverse Na^+–Ca^{2+} exchange activity (e.g. Miura & Kimura, 1989) and attenuate or abolish the trigger influence of this mechanism. $[Na^+]_i$ was depleted by a prolonged exposure to zero external Na^+. Cells were first incubated with a Ca^{2+}-free Tyrode solution (which contained 100 μM EGTA to buffer residual Ca^{2+}) for 4 min, to prevent a large Ca_i^{2+} increase on subsequent exposure to Na^+-free solution. They were then incubated for 30 min with the Na^+-free (140 mM Li^+) solution (see Methods), which during this phase was also Ca^{2+}-free and contained 100 μM EGTA. Finally, this solution was changed to a Na^+-free (140 mM Li^+) solution containing 2·5 mM Ca^{2+} (and no EGTA). Since cells by this stage were expected to be largely depleted of Na_i^+, nifedipine switches were carried out in this solution (Fig. 4). Results from two different myocytes are shown to illustrate the spectrum of responses observed. In Fig. 4*A*, a switch to 20 μM nifedipine 4 s before a stimulus caused a reduction of the fura-2 transient to 27% of the control in this Na^+-depleted myocyte. Out of seven Na^+-depleted cells, this cell had the largest remaining percentage of the fura-2 transient during nifedipine, and it is notable that a residual transient of 27% is much smaller than observed for any myocyte under normal (Na^+-containing) conditions. In Fig. 4*B*, the fura-2 transient of a different Na^+-depleted cell was abolished completely by the nifedipine switch. For the seven Na^+-depleted cells tested, we found that a mean of 10·9 ± 4·19% of the control fura-2 transient remained during nifedipine. For four Na^+-depleted cells in which contraction could be recorded without a switch artefact, we found that a mean of 6 ± 1% of phasic contraction remained during a nifedipine switch. The difference between the mean percentage fura-2 transient remaining in Na^+-depleted cells (10·9%), and that remaining under normal conditions (64·01 ± 2·09%) was highly significant ($P < 0·005$). Thus in Na^+-depleted cells, a switch to nifedipine caused a much greater reduction of the fura-2 transient than under normal conditions.

This is an important result for two reasons. (1) Under normal conditions, 64% of the fura-2 transient remained during a nifedipine switch. One possibility was that the cytoplasmic Ca^{2+} transient was actually reduced to a larger degree than this, but might not have been accurately reported by the fura-2 signal. If this was the case, then it could be predicted that in Na^+-depleted cells, where the Ca^{2+} transient might be expected to be greatly reduced by a nifedipine switch, they would also show a large remaining fura-2 transient. The fact that this did not occur argues against the possibility that fura-2 might not be accurately reporting the Ca^{2+} transient. (2) A second possibility for 64% of the fura-2 transient remaining with nifedipine

under control conditions was that a switch to nifedipine might leave more than 10% of trigger $I_{Ca,L}$ remaining in stimulated cells. Once again, if this was the case, then a similar remaining fraction of $I_{Ca,L}$ would be left in Na^+-depleted cells and it could be anticipated that a similar large fraction of the fura-2 transient would remain during a nifedipine switch. However, the fact that only a small proportion of the fura-2 transient remained during a nifedipine switch in Na^+-depleted cells is consistent with the patch-clamp results, in which only a small fraction of $I_{Ca,L}$ remained during a nifedipine switch.

The small percentage of fura-2 transient remaining during a nifedipine switch in Na^+-depleted cells is consistent with the hypothesis that reverse Na^+–Ca^{2+} exchange might trigger a proportion of SR Ca^{2+} release under normal conditions. After internal Na^+ depletion (which will attenuate reverse exchange activity), it is anticipated that $I_{Ca,L}$ becomes the primary remaining trigger for SR Ca^{2+} release, so a switch to nifedipine to inhibit $I_{Ca,L}$ might be expected to greatly attenuate the fura-2 transient. The similarity between the fractional fura-2 transient remaining in Na^+-depleted cells, and the fractional $I_{Ca,L}$ remaining with nifedipine in patch-clamped cells was striking. A mean of 10·9% of the fura-2 transient remained during a nifedipine switch in Na^+-depleted cells, compared with a mean of 10·25% of $I_{Ca,L}$. Thus in Na^+-depleted cells, a nifedipine switch reduced the fura-2 transient by approximately the same extent to which it reduced $I_{Ca,L}$ in patch-clamped cells.

Nifedipine switches after Na^+–K^+ pump inhibition

A rise in intracellular Na^+ (more specifically sub-sarcolemmal Na^+) might be expected to induce a greater degree of reverse Na^+–Ca^{2+} exchange during the AP upstroke, so it is conceivable that reverse exchange might become a more influential trigger of SR Ca^{2+} release as Na_i^+ rises. Inhibition of the Na^+–K^+ pump with strophanthidin is known to increase Na_i^+ (e.g. Lee, 1985). Figure 5*A* shows the effect of a nifedipine switch under control conditions; 70% of the fura-2 transient remained (but note reduction of phasic contraction to 40%). Then 50 μM strophanthidin

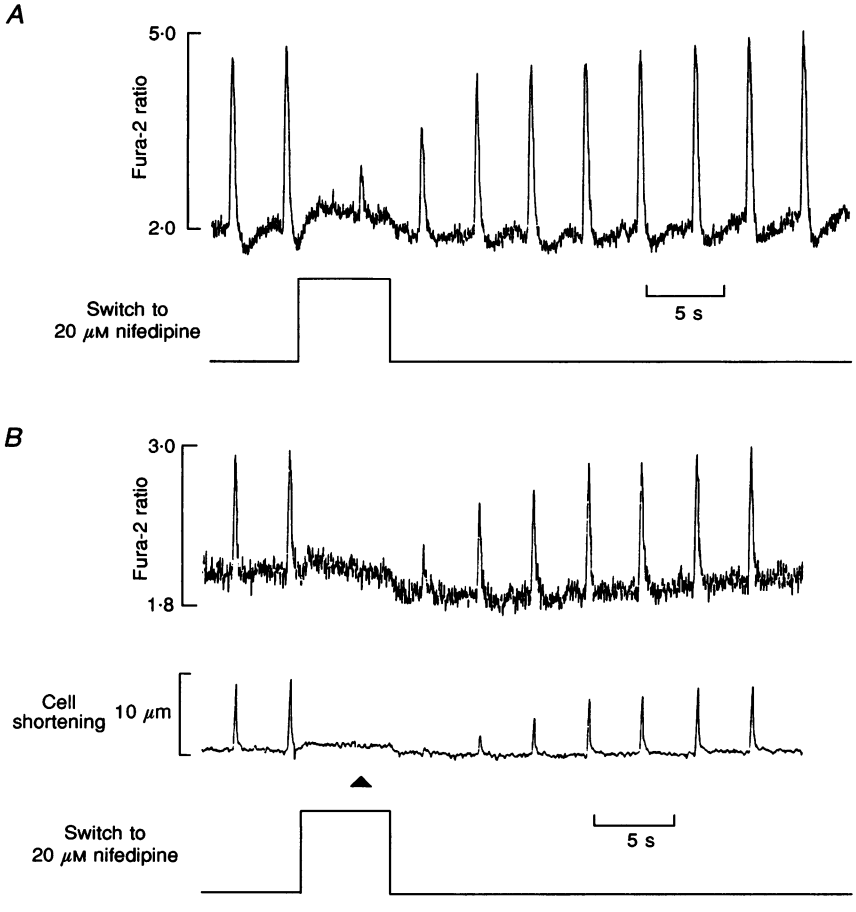

Figure 4. Recordings of single-beat nifedipine switches in two myocytes depleted of Na_i^+

A, in this myocyte the switch to nifedipine very greatly reduced the fura-2 transient and only 27% of the transient remained during nifedipine application. *B*, in another Na^+-depleted myocyte, a switch to nifedipine completely abolished the fura-2 transient and phasic contraction. The arrowhead indicates the time at which the cell was stimulated.

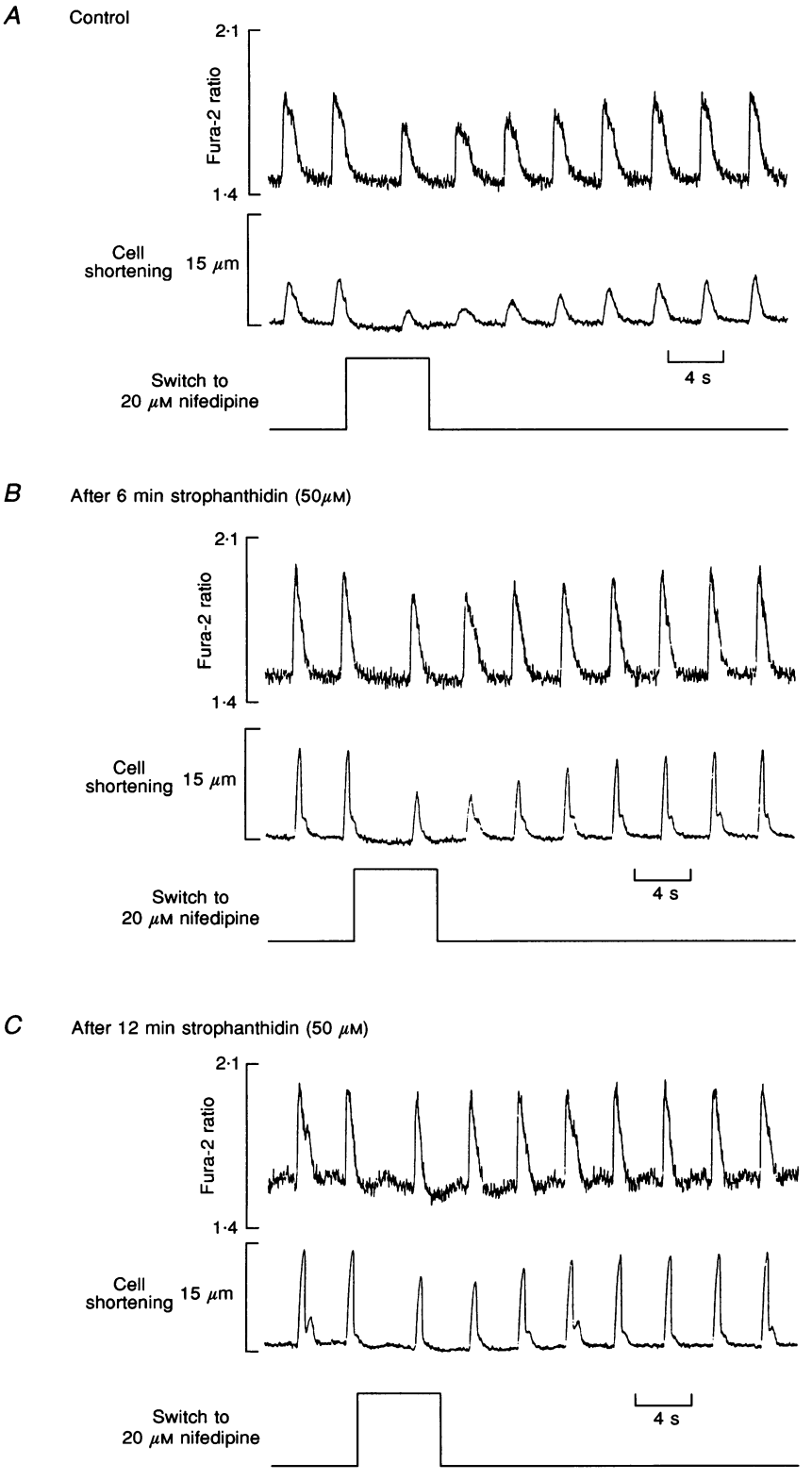

Figure 5. Effect of blocking the Na^+–K^+ pump with strophanthidin on the response to a single-beat nifedipine switch

A, the situation under normal (control) conditions; as usual, 70% of the fura-2 transient remained during an exposure to 20 μM nifedipine 4 s before the test stimulus and lasting for 6 s. *B*, the response after 6 min of 50 μM strophanthidin; note that the baseline fura-2 transient and contraction amplitude have both increased. Now a single-beat nifedipine switch reduced the fura-2 transient to 83%. *C*, the response after 12 min of 50 μM strophanthidin application; there was no detectable reduction in the amplitude of the fura-2 transient with a single-beat switch to nifedipine.

was added to the control barrel of the switcher to inhibit the Na^+–K^+ pump. After 6 min there had been a clear inotropic response with an increase of both the fura-2 transient and contraction (Fig. 5*B*). A nifedipine switch applied at this stage reduced the fura-2 transient and contraction to a lesser extent than normal; the fura-2 transient was reduced to 83% whilst contraction declined to 60%. After a further 6 min exposure to strophanthidin, the cell was just starting to exhibit symptoms of 'Ca_i^{2+} overload' – an unstable baseline Ca_i^{2+} and occasional after-contractions (Fig. 5*C*). A nifedipine switch at this stage had no detectable effect on the fura-2 transient, but contraction in this cell was reduced to 80%. Similar results were obtained in six cells – under control conditions, a nifedipine switch reduced the fura-2 transient to 61·6 ± 2·3%, whereas after 12 min exposure to strophanthidin an identical nifedipine switch only reduced the fura-2 transient to 96·8 ± 1·7%. For four cells in which contraction could be recorded without a switch artefact, after 12 min strophanthidin, phasic contraction was reduced to 70 ± 7·9% during the nifedipine switch.

The difference between the reduction of the fura-2 transient with nifedipine under normal conditions (61·6% remaining) and reduction of the transient after 12 min strophanthidin (96·8% remaining) was highly significant ($P < 0·005$). However, the reduction of the fura-2 transient during nifedipine to only 96·8 ± 1·7% after 12 min strophanthidin was not significantly different from no reduction at all. Thus, a nifedipine switch had progressively less effect in inhibiting the fura-2 transient as the inotropic response to strophanthidin developed. Two simple conclusions might be drawn from this result. First, a block of $I_{Ca,L}$ by nifedipine appears to have less effect in inhibiting the transient as strophanthidin inotropy progresses. Second, since the fura-2 transient was little affected by a nifedipine switch after 12 min strophanthidin, other SR Ca^{2+} release trigger mechanisms

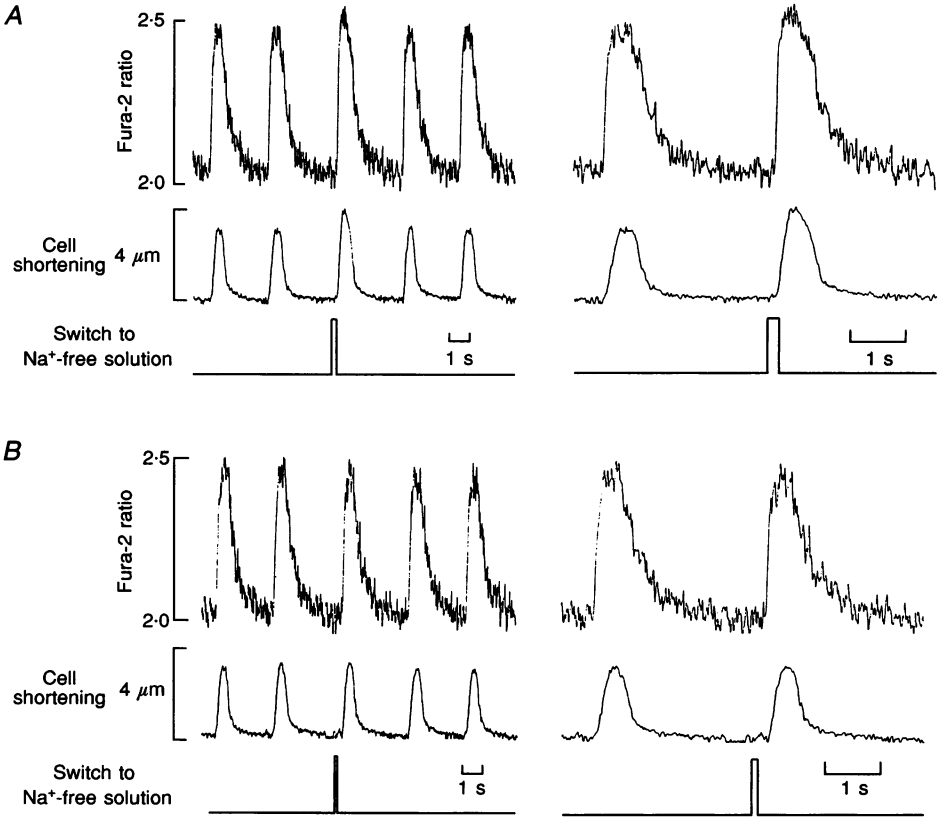

Figure 6. Effects of a rapid switch to Na^+-free solution on fura-2 transient and contraction

A, the switch to Na^+-free (140 mM Li^+, 2·5 mM Ca^{2+}) solution was made 100 ms before stimulation, and the switch lasted for 200 ms, i.e. from 100 ms before stimulation to 100 ms after. There was a clear increase in both fura-2 transient and phasic contraction. The right panel shows a faster time base record to show the precise timing of the solution switch relative to stimulation and the fura-2 transient. *B*, testing the effect on SR Ca^{2+} content of a 100 ms switch to Na^+-free solution (140 mM Li, 2·5 mM Ca^{2+}) in the same cell. The switch to Na^+-free solution was made 200 ms before stimulation and lasted for 100 ms. Normal solution (140 mM Na^+, 2·5 mM Ca^{2+}) was then applied for the remaining 100 ms before stimulation. This switch protocol had no effect on the fura-2 transient or contraction. The right panel shows faster time base records.

besides $I_{Ca,L}$ appear to be able to substitute for $I_{Ca,L}$, at least under conditions where $I_{Ca,L}$ has been largely blocked by nifedipine. These data are consistent with reverse Na^+–Ca^{2+} exchange becoming more involved as an SR Ca^{2+} release trigger as Na_i^+ becomes elevated during Na^+–K^+ pump inhibition.

Switches to Na^+-free solution just before electrical stimulation

A sudden removal of extracellular Na^+ might also be anticipated to induce a greater trigger Ca^{2+} entry via reverse Na^+–Ca^{2+} exchange, and Fig. 6 shows an experiment to test this. We used the rapid solution switcher to change from normal Tyrode solution (140 mM Na^+, 2·5 mM Ca^{2+}) to Na^+-free solution (140 mM Li^+, 2·5 mM Ca^{2+}) just before an electrical stimulus. It was crucial to make a very rapid Na^+-free application for this experiment, to prevent any rise in diastolic Ca_i^{2+} or SR Ca^{2+} content that might be expected to occur with a more prolonged application of Na^+-free solution. In Fig. 6*A*, we switched to Na^+-free solution (containing Li^+) 100 ms before the stimulus and the Na^+-free switch lasted for 200 ms (i.e. 100 ms before and 100 ms after the stimulus). It is clear that there was an increase in both the fura-2 transient and contraction with the Na^+-free solution switch. Very similar results of switching to Na^+-free solution 100 ms before stimulation were obtained in seven cells; there was a mean increase of the fura-2 transient of 12·2 ± 1·5%. For four cells in which contraction could be recorded without an artefact from the solution switch, the amplitude of phasic contraction increased by 43·8 ± 8·37% with the Na^+-free switch. An increase in the fura-2 transient (and phasic contraction) after a rapid Na^+-free solution switch supports the hypothesis that reverse Na^+–Ca^{2+} exchange might trigger SR Ca^{2+} release.

There are, however, two other possible mechanisms which could account for the observed effect of the Na^+-free solution switch on the fura-2 transient. (1) There might have been a small rise of baseline Ca_i^{2+} due to steady-state reverse exchange Ca^{2+} entry during the 100 ms of zero Na^+ exposure before stimulation. This could have led to a greater SR Ca^{2+} release by allowing Ca^{2+} entry during the AP upstroke to produce a larger peak rise in trigger $[Ca^{2+}]$ at the SR surface. However, a rise in whole-cell baseline Ca_i^{2+} can at least be excluded by examination of the recording in Fig. 6*A*; there was no rise of baseline Ca_i^{2+} during the 100 ms of Na^+-free solution before stimulation, and this was typical for all seven cells. (However, if Na^+-free solution was applied for 200 ms or longer before stimulation, then a rise in baseline Ca_i^{2+} could be observed.) (2) Ca^{2+} entry via reverse exchange might have occurred during the initial 100 ms of Na^+-free application so that SR Ca^{2+} content might have increased by the time the stimulus was applied. To test whether such a brief Na^+-free solution exposure was able to increase SR Ca^{2+} content, we performed the experiment shown in Fig. 6*B*. Two hundred milliseconds before a stimulus, we switched to Na^+-free (140 mM Li^+, 2·5 mM Ca^{2+}) solution for 100 ms and then back to normal (140 mM Na^+, 2·5 mM Ca^{2+}) solution 100 ms before the stimulus. If the 100 ms Na^+-free exposure was able to cause a Ca^{2+} entry that increased SR Ca^{2+} content, this extra Ca^{2+} would have been retained in the SR and would have been available for release on subsequent stimulation. However, this protocol applied in Fig. 6*B* did not cause an increase of either the fura-2 transient or contraction. It can therefore be concluded that 100 ms of Na^+-free exposure was not long enough to cause an increased SR Ca^{2+} content. Thus, the larger fura-2 transient caused by switching to Na^+-free solution 100 ms before stimulation was not due to increased SR Ca^{2+} content at the time of the stimulus. However, a plausible possibility is that the increased transient might result from a greater trigger stimulus for SR Ca^{2+} release.

Switches to nifedipine for longer than a single beat

A key advantage of the rapid solution switcher technique is that we were able to apply nifedipine between two individual beats, so that a large proportion of L-type Ca^{2+} channels were blocked for the subsequent stimulus without any change of SR Ca^{2+} content between the two beats. The typical effect on the fura-2 transient and cell contraction of 20 μM nifedipine application for a single-beat switch is illustrated in Fig. 7*A*. The fura-2 transient was reduced to 70% whilst contraction declined to 22% during the switch. The effect of a longer-duration nifedipine switch applied to the same cell is shown in Fig. 7*B*. The rapid solution switcher was activated between two beats, and the effect on the fura-2 transient and contraction at the first stimulus after the switch was similar to that illustrated in Fig. 7*A*. However, as the nifedipine was applied continuously for a number of beats, there was a slow, progressive decline in the fura-2 transient to a steady-state level of 27% after eight beats. Phasic contraction became undetectable after three beats. On switching back to normal solution without nifedipine, there was a rapid recovery in fura-2 transient and contraction within eight beats. Similar results were obtained in a total of seven myocytes. Since patch-clamp experiments showed that a nifedipine switch largely inhibited $I_{Ca,L}$ on the first beat, the decline in fura-2 transient over the following seven beats seemed unlikely to be due to a greatly increased degree of $I_{Ca,L}$ block. It would seem reasonable to predict that with this high nifedipine concentration, $I_{Ca,L}$ will have been abolished completely by the second or (at most) third beat. Instead, a possible explanation for the slow progressive decline in the amplitude of the fura-2 transient might be a beat-to-beat depletion of SR Ca^{2+} content. Inhibition of $I_{Ca,L}$ during the first few seconds of nifedipine application will reduce Ca^{2+} entry into the cell substantially; the SR continues to release Ca^{2+}, but with each stimulus cannot be replenished fully by the reduced Ca^{2+} entry. Thus SR Ca^{2+} content declines on a beat-to-beat basis, the lowered SR content leads to reduced

Ca^{2+} release and a smaller fura-2 transient is observed. The marked difference in fura-2 transient and contraction remaining after a nifedipine application lasting a number of beats, compared with that remaining during a single-beat nifedipine switch, shows the critical importance of using rapid solution switches applied between two individual stimuli in order to obtain results that are not contaminated by a change in SR Ca^{2+} content.

DISCUSSION

A key result of this study is that during a nifedipine switch applied for a single stimulus, approximately two-thirds of the fura-2 transient can still be elicited in externally stimulated rabbit cells. This occurs despite the fact that patch-clamp experiments show that an identical nifedipine switch blocks almost 90% of $I_{Ca,L}$. This indicates that, under these conditions, a large fractional block of $I_{Ca,L}$

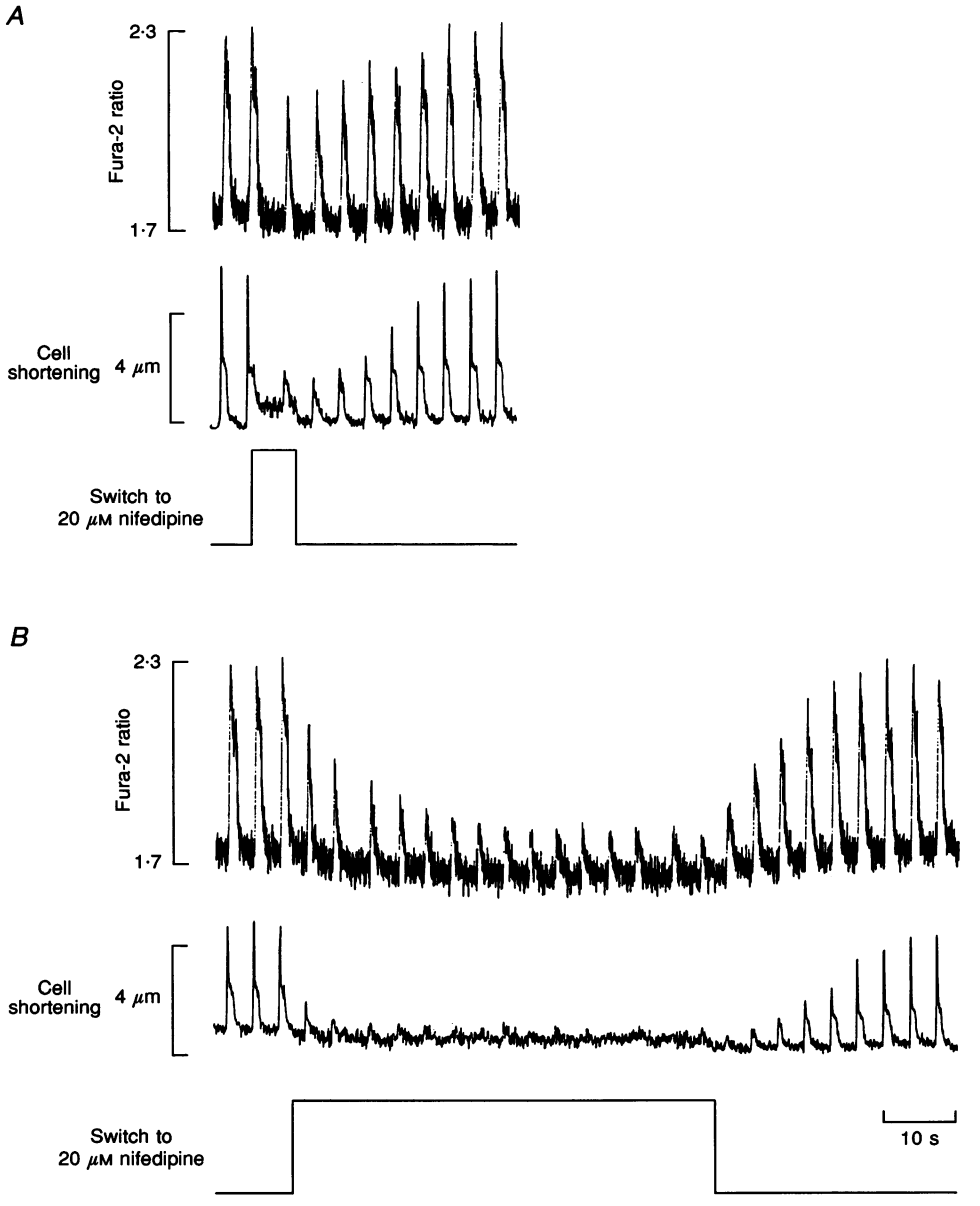

Figure 7. The effect of a more prolonged switch to nifedipine

A shows a typical single-beat nifedipine switch (same protocol as in Fig. 1); as usual, 70% of the fura-2 transient remained during nifedipine. *B*, in the same cell, the effect of a more prolonged switch to nifedipine is shown. The effect on the first beat after the nifedipine switch was similar to that shown in *A*, but as nifedipine application continued, both the fura-2 transient and contraction declined further. The fura-2 transient declined to a steady-state level of 27% after 8 beats, whereas contraction was abolished completely after 3 beats. There was a slight artefact on the contraction trace resulting from the solution switch; this was due to the cell changing position as the switcher barrels moved laterally. The scale bar applies to both *A* and *B*.

inhibits only about one-third of the fura-2 transient. The fact that two-thirds of the transient remained during nifedipine suggests that, in the absence of a large fraction of $I_{Ca,L}$, another mechanism (such as reverse Na^+–Ca^{2+} exchange) might be capable of triggering a significant proportion of the fura-2 transient.

The experiments using a prolonged nifedipine switch also suggest that $I_{Ca,L}$ might not be the only trigger mechanism for SR Ca^{2+} release. Even after nifedipine application for fifteen beats, and despite the fact that SR Ca^{2+} depletion will have occurred during the long application, there was still a residual fura-2 transient elicited by stimulation. It seems reasonable to expect that $I_{Ca,L}$ would have been abolished completely after more than two or three beats in 20 μM nifedipine (due to the known use-dependent block of $I_{Ca,L}$ by nifedipine). Therefore this might be interpreted as evidence for the existence of other mechanisms besides $I_{Ca,L}$ that are capable of triggering SR Ca^{2+} release, even from a Ca^{2+}-depleted SR. Another possibility is that the residual fura-2 transient might be caused by direct Ca^{2+} entry into the cell, perhaps via reverse Na^+–Ca^{2+} exchange during the AP. However, it has been shown clearly that the AP becomes greatly shortened during nifedipine application, to a duration of approximately 100 ms (e.g. Levi *et al.* 1994), as might be expected from the block of $I_{Ca,L}$. Other workers have measured outward Na^+–Ca^{2+} exchange currents of between 50 and 200 pA at potentials equivalent to the AP plateau (e.g. Kimura *et al.* 1987; the magnitude of outward exchange current will depend on intracellular Na^+). Taking the largest value of 200 pA, it can be calculated that $2{\cdot}7 \times 10^{-16}$ moles of Ca^{2+} will enter the cell via an exchange with 3 Na^+ : 1 Ca^{2+} stoichiometry during a 100 ms depolarization at the AP plateau. Taking a cell water volume of 50×10^{-12} l, this will result in total intracellular [Ca^{2+}] increasing by 4·1 μM during 100 ms. However, most of this Ca^{2+} will be bound and not free, and using an intracellular buffering capacity for Ca^{2+} ions of 400 bound : 1 free (e.g. Wendt-Gallitelli & Isenberg, 1991), we calculate that free cytoplasmic [Ca^{2+}] will rise by only about 10 nM over 100 ms. Since this might be an overestimate because the outward exchange current will be less than 200 pA with a normal level (about 10 mM) of Na_i^+, it would seem unlikely from this argument that direct Ca^{2+} entry via reverse Na^+–Ca^{2+} exchange might be responsible for the residual fura-2 transient during prolonged nifedipine application. An alternative possibility is that the small Ca^{2+} entry via the exchange may be able to trigger Ca^{2+} release from the depleted SR, and it is this that results in the small residual fura-2 transient during prolonged nifedipine application.

This study was designed to test the role of $I_{Ca,L}$ in triggering SR Ca^{2+} release under near normal physiological conditions. This was the prime reason for using external stimulation of the myocytes; their intracellular environment would thereby remain as normal as possible. Dialysis with a patch pipette provides a powerful means of controlling the intracellular environment, but has the accompanying consequence that many internal ions and other constituents become altered, and we do not yet know fully what the effects of internal dialysis might be for excitation–contraction coupling. The fact that myocytes were not dialysed in this study (except for determining the effect of nifedipine on $I_{Ca,L}$) excluded uncertainties due to this. In order to reproduce *in vivo* conditions more closely, we performed experiments at a temperature near to 37 °C. The Na^+–Ca^{2+} exchanger is known to have a Q_{10} (the temperature coefficient over a 10 °C temperature range) of 4 (e.g. Kimura *et al.* 1987) and it is conceivable that the exchange may play a different role in triggering SR Ca^{2+} release at 37 °C compared with room temperature (e.g. Vornanen, Shepherd & Isenberg, 1994).

Two other aspects of this study are notable. First, we used fura-2 to monitor the Ca^{2+} transient in order to have a relatively direct estimate of the quantity of SR Ca^{2+} release. Some previous investigations using the rapid solution switcher (Kohmoto *et al.* 1994; Levi *et al.* 1994) have monitored phasic contraction as an index of SR Ca^{2+} release, but this is indirect compared with the fura-2 transient. Indeed, one of the striking features of this study is the very different behaviour of phasic contraction compared with the (phasic) fura-2 transient (see later). Second, it was critical that we used a method such as the rapid solution switcher to apply nifedipine rapidly between two consecutive beats. The quantity of Ca^{2+} stored within the SR determines the size of the Ca^{2+} transient (and thus the fura-2 transient). At 37 °C, the SR Ca^{2+} content can become depleted very rapidly indeed (e.g. Fig. 7; also Rakovic & Terrar, 1995), so that if nifedipine is applied for more than just one beat, there is already a decline in the SR Ca^{2+} content before the second beat, and this by itself will cause a reduction in the fura-2 transient. The fact that we used the rapid solution switcher to apply nifedipine for just a single beat allowed us to obtain data that should not be contaminated by a change of SR Ca^{2+} content between the control and test beats.

$I_{Ca,L}$ remaining after a single-beat nifedipine switch

During a single-beat switch to nifedipine, 10·25% of the spike of inward current remained. Although the inward current activated by a +10 mV depolarization is likely to be largely $I_{Ca,L}$ (it is blocked largely by nifedipine), it is possible that there may also be a small contamination by other membrane currents. For instance, a delayed rectifier K^+ current (I_K) might also be activated by the depolarization (e.g. Carmeliet, 1992, 1993). The *I–V* relation of ventricular cells is well known to be 'N-shaped', and a combination of this with I_K might result in a depolarization from −40 to +10 mV, producing (in the complete absence of $I_{Ca,L}$) an initial step towards a more inward current level followed by a time-dependent increase of outward I_K (e.g. Carmeliet, 1992, 1993). This is not

unlike the current profile that remained after a single-beat nifedipine switch. Thus, it may be that the nifedipine switch produced a larger block of $I_{Ca,L}$ than it appeared to from the current trace, and that the appearance of a small remaining $I_{Ca,L}$ might have been an artefact caused by other overlapping currents. We prefer not to speculate further on this matter, however, and we have analysed the data assuming that 10·25% of $I_{Ca,L}$ did remain during nifedipine.

Whatever the precise proportion of $I_{Ca,L}$ which remained during a nifedipine switch, a very important question is: what proportion of the remaining fura-2 transient might this residual $I_{Ca,L}$ be capable of triggering? We did consider the possibility that a significant fraction of the L-type Ca^{2+} channels might be located in the T-tubules and, due to diffusional limitations, that nifedipine may preferentially block channels on the surface membrane rather than those in the T-tubules. This would leave L-type Ca^{2+} channels in the T-tubules available for triggering Ca^{2+} release from the SR and might partly account for the large remaining fura-2 transient under control conditions. However, in this case we would predict that in cells depleted of Na_i^+, where $I_{Ca,L}$ is expected to be the primary trigger, there would also be a large remaining fura-2 transient. However, during a nifedipine switch only 10% of the fura-2 transient remained in Na^+-depleted cells (Fig. 4), which appears to argue against this possibility.

Two other pieces of evidence suggest that 10% of $I_{Ca,L}$ might only trigger about 10% of the fura-2 transient. First, a number of previous studies have reported that, under conditions where $I_{Ca,L}$ is the primary trigger for SR Ca^{2+} release, the magnitude of $I_{Ca,L}$ grades (in a proportional manner) the amplitude of the Ca^{2+} transient (e.g. Cannell *et al.* 1987; Beuckelmann & Wier, 1988). In these studies, the voltage dependence of the Ca^{2+} transient was similar to the voltage dependence of $I_{Ca,L}$, so that presumably a small amplitude of $I_{Ca,L}$ triggers a similar small quantity of SR Ca^{2+} release. Second, in cells depleted of Na_i^+, reverse Na^+–Ca^{2+} exchange is expected to be unavailable, so the primary trigger mechanism should be $I_{Ca,L}$. Under these conditions a single-beat switch to nifedipine (which reduces $I_{Ca,L}$ to 10%) reduced the fura-2 transient to a mean of 10% (Fig. 4). The clear implication of these results would seem to be that 10% of $I_{Ca,L}$ might only trigger 10% of the fura-2 transient, at least under conditions where $I_{Ca,L}$ is the primary trigger mechanism.

Since 10% of $I_{Ca,L}$ remained during a single-beat nifedipine switch, it is necessary to take account of the role this might play in triggering the remaining fura-2 transient. Under control conditions, a single-beat nifedipine switch reduced the fura-2 transient by 36%, showing that this is the proportion of the fura-2 transient that can be inhibited by blocking 90% of $I_{Ca,L}$. However, if the remaining 10% of $I_{Ca,L}$ might trigger a further 10% of the fura-2 transient, this would indicate that a complete block of $I_{Ca,L}$ (if we could have achieved it) would have reduced the fura-2 transient by a total of 46%. Thus, as an approximate working figure, it would seem that a sudden single-beat block of $I_{Ca,L}$ might inhibit between 40 and 50% of the normal fura-2 transient in externally stimulated rabbit myocytes at 35 °C. We recognize, though, that there does appear to be some non-linearity in this scheme, since blocking 90% of $I_{Ca,L}$ appears to inhibit only 36% of the transient, compared with 10% of $I_{Ca,L}$ triggering being able to trigger 10% of the transient. It is not clear at present how this apparent non-linearity might be resolved, although it could conceivably be related to the sensitivity of the SR Ca^{2+} release channel (the ryanodine receptor) to changes of local $[Ca^{2+}]$.

Mechanisms responsible for the nifedipine-resistant fura-2 transient

A central remaining issue is the mechanism(s) which might be responsible for triggering the fura-2 transient that remains during the nifedipine switch. Ca^{2+} entry via the T-type Ca^{2+} channel is a possible candidate, but blocking this channel with 100 μM Ni^{2+} indicates that it is not involved. This conclusion is consistent with other studies, which found that the Ca^{2+} transient is not activated over the same voltage range as the T-type Ca^{2+} current (e.g. Cannell *et al.* 1987; Bouchard, Clark & Giles, 1993).

However, the results are consistent with the hypothesis that reverse Na^+–Ca^{2+} exchange might be involved in triggering the nifedipine-resistant fura-2 transient. Adding 5 mM Ni^{2+} to nifedipine completely abolished the fura-2 transient, and 5 mM Ni^{2+} is known to inhibit Na^+–Ca^{2+} exchange. The percentage of the fura-2 transient remaining during a nifedipine switch was very much smaller in myocytes depleted of Na_i^+, which is expected to abolish reverse Na^+–Ca^{2+} exchange. After applying strophanthidin to inhibit the Na^+–K^+ pump and raise Na_i^+, the fura-2 transient became progressively insensitive to a nifedipine switch and this is also consistent with the involvement of reverse exchange, since a rise of Na_i^+ is anticipated to increase trigger Ca^{2+} entry via reverse exchange (the parallel increase of baseline Ca_i^{2+} with strophanthidin might also inactivate $I_{Ca,L}$, making this a less effective trigger; e.g. Levi, 1991). Finally, a switch to Na^+-free solution just 100 ms before stimulation caused an increase in the fura-2 transient, and this is also compatible with SR triggering by reverse Na^+–Ca^{2+} exchange.

In externally stimulated myocytes, just as *in vivo*, the rapid AP upstroke depolarizes the membrane rapidly to +50 mV over the first few milliseconds (e.g. Levi *et al.* 1994; Le Guennec & Noble, 1994). The rise in whole cell cytoplasmic Ca_i^{2+} due to SR Ca^{2+} release becomes apparent 5–10 ms after the AP upstroke, so that at least over the first few milliseconds of the AP when Ca_i^{2+} remains low, Na^+–Ca^{2+} exchange is predicted to function in reverse mode and generate Ca^{2+} entry. It is this Ca^{2+} entering early during the AP that we propose might be a trigger of SR

Ca^{2+} release, in addition to a simultaneous Ca^{2+} entry via $I_{Ca,L}$. It has also been proposed that Na^+ entering via I_{Na} might accumulate under the membrane and thereby activate further Ca^{2+} entry via reverse Na^+–Ca^{2+} exchange. Any Ca^{2+} entering on reverse Na^+–Ca^{2+} exchange may thus also be amplified by involvement of I_{Na} in triggering the Ca^{2+} transient (e.g. Leblanc & Hume, 1990; Lipp & Niggli, 1994).

Relation between the fura-2 signal and changes of the cytoplasmic Ca^{2+} transient

It is an important issue for this study to consider the changes in the Ca^{2+} transient which underlie the change in the fura-2 signal we have reported, since it is the changes in the Ca^{2+} transient which will be most closely related to the magnitude of SR Ca^{2+} release in each situation. We have therefore performed further experiments to characterize the parameters of the fura-2 calibration curve for Ca^{2+} in rabbit myocytes under these conditions. For ten different cells, we used a modified ionophore calibration procedure (using both 10 μM ionomycin and 10 μM 8-Br-A23187 to permeabilize the membrane) to obtain values for the minimum fura-2 ratio with low Ca_i^{2+} (R_{min}), the maximum fura-2 ratio with saturating Ca_i^{2+} (R_{max}), and the value for β (the ratio of the 380 nm fluorescence signal in zero Ca^{2+} to that in saturating Ca^{2+}; Grynkiewicz, Poenie & Tsien, 1985). We found mean values for R_{min} of 0·85 ± 0·03, for R_{max} of 4·59 ± 0·34, and for β of 3·68 ± 0·65 ($n = 10$ cells). Using a K_D of fura-2 for Ca^{2+} of 250 nM (Grynkiewicz *et al.* 1985), the resulting calibration curve (Fig. 8) shows that the fura-2 signal will continue to respond to increases in cytoplasmic Ca^{2+} up to at least 3 μM, and this is consistent with the experimental results where procedures designed to increase or decrease Ca_i^{2+} caused a corresponding change of the measured fura-2 ratio (see Figs 2–6). From the same batch of cells, we measured a resting Ca_i^{2+} of 67 nM and a peak systolic Ca_i^{2+} (in cells stimulated at 0·3 Hz) of 560 nM. These values and the calibration curve have therefore allowed us to calculate the reduction in the Ca^{2+} transient that is reported by the change in the fura-2 signal during a nifedipine switch. For myocytes under normal conditions, we found that a single-beat nifedipine switch reduced the fura-2 transient to a mean of 64%. Using the calibration

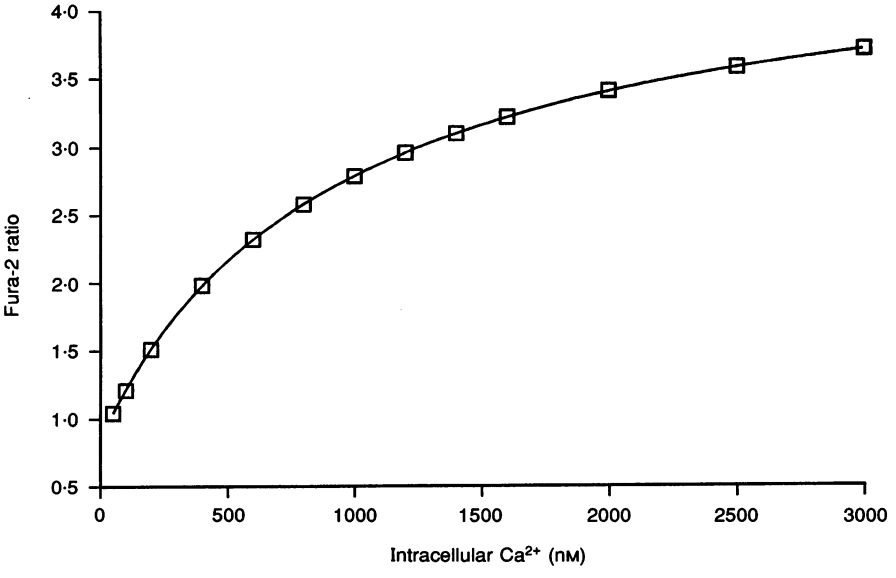

Figure 8. Mean *in vivo* calibration curve of fura-2 for intracellular Ca^{2+} in rabbit myocytes at 36 °C

The curve was plotted using the conventional relationship for fluorescence indicator ratio and Ca_i^{2+}:

$$Ca_i^{2+} = K_D \beta \frac{(R - R_{min})}{(R_{max} - R)},$$

where K_D is the dissociation constant of fura-2 for Ca_i^{2+} (*in vitro* value of 250 nM), β is the ratio of the 380 nm fluorescence intensity in zero Ca^{2+} to that in saturating Ca^{2+}, R is the fura-2 ratio at a given Ca_i^{2+}, R_{max} is the fura-2 ratio in saturating Ca^{2+}, R_{min} is the fura-2 ratio in zero Ca^{2+}. From determinations in 10 rabbit myocytes, we found a mean R_{min} of 0·85, R_{max} of 4·59, and β of 3·68. This relationship was transformed into:

$$R = \frac{Ca_i^{2+} R_{max} + K_D \beta R_{min}}{(K_D \beta + Ca_i^{2+})}$$

for plotting the calibration curve. It could then be used to estimate the fractional reduction in the actual Ca^{2+} transient indicated by the change in the fura-2 transient during a nifedipine switch.

curve, this is equivalent to a reduction in the Ca^{2+} transient to 50%, thus suggesting that 50% of SR Ca^{2+} release might persist during nifedipine. For cells depleted of Na_i^+, a nifedipine switch under these conditions reduced the fura-2 transient to a mean of 10%, which is equivalent to 6·5% of the Ca^{2+} transient remaining. When a Na^+-free switch was applied 100 ms before a stimulus, the amplitude of the fura-2 transient increased by 12·2%, which is equivalent to a 22% rise of the Ca^{2+} transient. It can be seen that, although the percentage change in the Ca^{2+} transient is always greater than the fura-2 transient, expressing the fura-2 signal in terms of Ca_i^{2+} does not alter qualitatively the central conclusion of this study, that a substantial Ca^{2+} transient and SR Ca^{2+} release still remains when a large proportion of the L-type Ca^{2+} channels are blocked between one beat and the next.

Relation between the fura-2 transient and changes in phasic contraction

One notable feature of the results is the very different behaviour of the fura-2 transient and phasic contraction. With a single-beat nifedipine switch, the fura-2 transient declined to 64%, whereas contraction amplitude was reduced to a mean of 24%. During a more prolonged switch to nifedipine, the fura-2 transient continued throughout at a reduced level, whereas contraction was abolished after three beats. With a switch to Na^+-free solution, the increase in contraction was greater than the rise in peak fura-2 transient. And during the inotropic response to strophanthidin, the percentage increase in contraction was much greater than the rise in the peak fura-2 transient. It seems most likely that this reflects the highly non-linear relation between $[Ca^{2+}]$ and contraction (e.g. Allen & Kentish, 1985; Sweitzer & Moss, 1993). There appears to be a $[Ca^{2+}]_i$ threshold below which no contraction can be activated. The $[Ca^{2+}]_i$–contraction relation also appears to be very steep (Bassani, Bassani & Bers, 1995), so that a small increase in peak Ca^{2+} transient is associated with a much larger increase in peak contraction. In terms of elucidating the mechanisms which trigger SR Ca^{2+} release, the Ca^{2+} transient would seem to indicate the quantity of SR Ca^{2+} release more directly than measurements of contraction. Perhaps from a functional viewpoint, though, the contraction measurements are of relevance, since the main purpose of the heart is to contract and pump blood. One important point that the results illustrate, however, is the potential danger of reaching conclusions about excitation–contraction coupling from measurements of contraction amplitude alone. For instance, a single-beat switch to nifedipine reduced phasic contraction to 24% of control, and from this it might have been concluded (perhaps erroneously) that a block of $I_{Ca,L}$ reduced the Ca^{2+} transient and magnitude of SR Ca^{2+} release by a very large extent. However, direct measurement of the fura-2 transient showed that this was reduced to only 64% by a nifedipine switch (equivalent to a reduction in the Ca^{2+} transient to 50%). Thus, direct measurement of changes in the fura-2 transient showed that a block of $I_{Ca,L}$ inhibits a smaller proportion of SR Ca^{2+} release than would have been concluded from measurements of phasic contraction alone.

Implications for normal physiology

Under normal *in vivo* conditions, ventricular myocytes are depolarized by an AP at a rate of 1–2 Hz in man and 2–4 Hz in rabbit. The rapid upstroke of the AP depolarizes the membrane to +50 mV over the first 1–2 ms, and it is during this very brief initial period that triggering of SR Ca^{2+} release largely takes place. The results of this study appear to suggest that in rabbit myocytes under AP conditions at 36 °C, a block of $I_{Ca,L}$ leads to an inhibition of the Ca^{2+} transient by about 50%, suggesting that SR Ca^{2+} release is also reduced by about 50%. A very important question is, how might this reflect the trigger function of $I_{Ca,L}$ under normal conditions?

It is important to note that, because a sudden block of $I_{Ca,L}$ leads to inhibition of SR Ca^{2+} release by 50%, it does not automatically follow that $I_{Ca,L}$ is normally responsible for triggering 50% of SR release. This is because in the normal situation, there may be a simultaneous trigger Ca^{2+} entry via both $I_{Ca,L}$ and reverse Na^+–Ca^{2+} exchange acting together. When $I_{Ca,L}$ is suddenly blocked by a rapid nifedipine switch, it is possible that Ca^{2+} entry via the Na^+–Ca^{2+} exchanger may then take over some of the normal trigger function of $I_{Ca,L}$, so that a significant fraction of the Ca^{2+} transient and SR Ca^{2+} release still remains. The magnitude of SR release remaining in this situation, in the absence of $I_{Ca,L}$, may be a larger fraction of the SR Ca^{2+} release than that which is normally triggered by reverse Na^+–Ca^{2+} exchange, when both $I_{Ca,L}$ and reverse exchange are functioning simultaneously as triggers. The basic conclusion from this argument is that, although the experiments reported in this study suggest a second mechanism for triggering SR Ca^{2+} release besides Ca^{2+} entry via $I_{Ca,L}$, they may not provide a precise quantitative estimate for the fraction of SR Ca^{2+} release that can be triggered separately by $I_{Ca,L}$ and reverse Na^+–Ca^{2+} exchange, under normal conditions.

Nevertheless, since the normal AP upstroke reaches +50 mV, and triggering of SR Ca^{2+} release takes place largely during the AP upstroke, it is conceivable that there may be a role for reverse Na^+–Ca^{2+} exchange in triggering SR Ca^{2+} release under normal conditions. This may be related to the fact that at a potential of +50 mV, Ca^{2+} entry via $I_{Ca,L}$ is a relatively small fraction of maximum (e.g. London & Krueger, 1986; Cannell *et al.* 1987; Beuckelmann & Wier, 1988; Stern, 1992). The fact that the AP upstroke reaches such a positive potential will tend to increase the degree to which Na^+–Ca^{2+} exchange reverses and generates trigger Ca^{2+} entry. It is possible that reverse exchange might appear to be a less influential trigger mechanism when voltage-clamp pulses to +10 mV are used (where $I_{Ca,L}$ is maximal), compared with when the normal

action potential depolarizes the membrane, as in externally stimulated cells. It must, however, be borne in mind that not all *in vivo* features could be duplicated in this study. One experimental limitation was that the stimulation rate was less than the normal rabbit heart rate, because we required at least a 4 s gap between a control and test stimulus for switching to nifedipine. Thus, although Ca^{2+} loading of the cells may have approached normal conditions, it was presumably at a lower level than *in vivo* due to the lower stimulation rate.

A proportion of triggering due directly to reverse Na^+–Ca^{2+} exchange could be due to simple depolarization-induced reverse exchange, and the remainder might be due to involvement of I_{Na}-induced reverse exchange. The relative proportions due to each of these mechanisms is not known at present. However, it does appear that when intracellular Na^+ becomes raised, reverse exchange becomes a relatively more influential trigger of SR Ca^{2+} release. This may also have relevance for *in vivo* conditions, since a higher level of intracellular Na^+ accompanies normal heart rates of 1–4 Hz (e.g. Cohen, Fozzard & Sheu, 1982) and this will tend to increase further the trigger influence of reverse exchange.

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Acknowledgements

We are grateful to The Wellcome Trust, British Heart Foundation, Royal Society and United Bristol Healthcare Trust for financial support. The study was also supported by International Travel Awards from the British Heart Foundation (No. FS 94029 and FS 94030). We are immensely indebted to Dr Ken Spitzer for the enormous help he has given us with the rapid solution switcher technique, and for his wisdom and common sense. We acknowledge the fruitful discussions A.J.L. had with John Bridge on this subject. We thank John Mitcheson and Jules Hancox for helpful comments on the manuscript. A.J.L. also wishes to thank Jackie Addison, Diane Tanner, Jenny Pasterfield and Lesley Arberry for their help during this study.

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Received 24 March 1995; accepted 4 January 1996.