Journal of Physiology (1989), **417**, *pp.* 537–553
With 8 text-figures
Printed in Great Britain

INTRACELLULAR Ca^{2+} TRANSIENTS DURING RAPID COOLING CONTRACTURES IN GUINEA-PIG VENTRICULAR MYOCYTES

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(*Received 28 February 1989*)

SUMMARY

1. We measured intracellular Ca^{2+} transients during rapid cooling contractures (RCCs) in guinea-pig ventricular myocytes using the fluorescent Ca^{2+} indicator, Indo-1.

2. Rapid cooling of myocytes from 22 to 0–1 °C induced a rapid increase in $[Ca^{2+}]_i$ which preceded the peak of the contraction and was sometimes large enough to saturate Indo-1. This indicates that $[Ca^{2+}]_i$ may reach > 10 μM during an RCC.

3. The $[Ca^{2+}]_i$ during the RCC slowly declined from its peak value and most of this decline in $[Ca^{2+}]_i$ can be attributed to slow reaccumulation of Ca^{2+} by the sarcoplasmic reticulum (SR) in the cold. RCCs induced in the absence of Ca_o^{2+}, were not different from control, supporting previous conclusions that RCCs depend exclusively on intracellular Ca^{2+} stores.

4. RCCs are depressed by long rest periods (rest decay) or by exposure to ryanodine or caffeine, which supports conclusions that RCCs are due to Ca^{2+} release from the SR. The rest decay of RCCs can be almost completely prevented by applying Na_o^+-free solution during the rest period. This implies that the loss of SR Ca^{2+} during rest depends on the sarcolemmal Na^+–Ca^{2+} exchange (and not the sarcolemmal Ca^{2+}-ATPase pump).

5. Rapid rewarming during an RCC normally leads to an additional transient contraction (or rewarming spike), without any increase in $[Ca^{2+}]_i$. Thus, the rewarming spike might be attributable to an increase in myofilament Ca^{2+} sensitivity induced by rewarming.

6. A second RCC is used to assess the fraction of Ca^{2+} which is re-sequestered by the SR during relaxation from the first RCC. In control solution progressive RCCs decline in amplitude, but in Na^+-free, Ca^{2+}-free solution they are of constant amplitude. We conclude that the SR Ca^{2+} pump and Na^+–Ca^{2+} exchange are responsible for relaxation and that the latter may account for 20–50 % of relaxation.

7. These results support the use of RCCs as a useful means of assessing SR Ca^{2+} content in intact cardiac muscle cells.

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INTRODUCTION

Rapid cooling of mammalian cardiac muscle to 1 °C induces contractures which are attributable to Ca^{2+} release from the sarcoplasmic reticulum (SR) and subsequent activation of the myofilaments (Kurihara & Sakai, 1985; Bridge, 1986). The amplitude of these rapid cooling contractures (RCCs) may reflect the amount of Ca^{2+} in the SR which is available for release (Kurihara & Sakai, 1985; Bridge, 1986; Bers, Bridge & MacLeod, 1987; Bers, 1987). The evidence that these RCCs depend on SR Ca^{2+} content is that (1) RCCs can be abolished by caffeine or ryanodine, though the latter is condition dependent (Bridge, 1986; Bers *et al.* 1987); (2) RCCs amplitude changes in a similar manner to changes in contractions thought to be highly dependent on SR Ca^{2+}, i.e. post-rest contractions and caffeine-induced contractures (Bridge, 1986; Bers *et al.* 1987; Bers, 1987, 1989). RCC amplitude does not appear to depend on Ca^{2+} influx across the sarcolemma, since changes in the $[Ca^{2+}]$ or $[Na^{+}]$ of the cooling solution do not alter the RCC amplitude (Kurihara & Sakai, 1985; Bridge, 1986). On the other hand, changes in ionic composition prior to cooling can change the amplitude of a subsequent RCC (Kurihara & Sakai, 1985; Bridge, 1986; Bers, 1987). These latter changes in RCC amplitude can be explained if changes in ionic gradients alter cellular (and SR) Ca^{2+} content.

A crucial aspect of the dynamic regulation of Ca^{2+} in cardiac muscle cells is the quantity of SR Ca^{2+} which is available for release. Electrically evoked twitch contractions are likely to be activated by a combination of both transsarcolemmal Ca^{2+} influx and SR Ca^{2+} release (Bers, 1985). Furthermore, the fraction of SR Ca^{2+} release at each stimulated contraction may vary under different conditions (Fabiato, 1985). While it is not yet known whether the entire SR Ca^{2+} content is released upon cooling to 0 °C, it seems likely, based on quantitative considerations (Bers, 1989). Thus RCCs may provide important information about SR Ca^{2+} content in intact cardiac muscle. The SR Ca^{2+} content can also be assessed by rapid application of caffeine and measurement of the contraction so induced (Smith, Valdeolmillos, Eisner & Allen, 1988). RCCs and caffeine-induced contractures are valuable complementary approaches for assessing SR Ca^{2+} content as they have different complicating factors (e.g. myofilament Ca^{2+}-sensitivity is decreased by cooling, but increased by caffeine, Wendt & Stephenson, 1983; Harrison & Bers, 1989). RCCs may have some advantage in multicellular preparations because heat is transferred through the thickness of the muscle much faster than one could expect equilibration of a small molecule at the core (Bridge, 1986). During RCCs, the cold also depresses other transport processes, which can be advantageous for switching bath solutions during an RCC (Bers & Bridge, 1989). The only other way to get this information about SR Ca^{2+} is electron probe microanalysis (which is technically difficult and also destructive; Tormey, 1983). Thus, RCCs are an experimentally simple way to assess the SR Ca^{2+} which is available for release and can be highly reproducible.

Here we report measurements of Ca_i^{2+} transients during RCCs in mammalian cardiac muscle using the intracellular fluorescent Ca^{2+} indicator, Indo-1. A preliminary account of these results was presented to the Physiological Society (Bers, Bridge & Spitzer, 1988).

METHODS

Hearts were removed from guinea-pigs anaesthetized with sodium pentobarbitone (~ 40 mg/kg, I.P.). The heart was immediately attached to an aortic cannula providing continuous retrograde perfusion at 37 °C by gravity at a pressure of ~ 60 cmH_2O. The hearts were first perfused with a nominally Ca^{2+}-free solution (no added Ca^{2+}) for 3–5 min, followed by 15 min of perfusion with the same solution containing collagenase (1 mg/ml, Class II, Worthington Biochemical, Freehold, NJ, USA). Both solutions contained (in mM): 126 NaCl, 4·4 KCl, 5 $MgCl_2$, 18 $NaHCO_3$, 0·33 NaH_2PO_4, 11 glucose, and were gassed with 5% CO_2 and 95% O_2 (pH 7·3). The heart was detached from the cannula and the ventricles were cut into small pieces, then gently shaken for about 5 min at 37 °C in the enzyme solution to disperse the cells. The [Ca^{2+}] was then gradually changed to 2·7 mM with normal Tyrode solution (see below) while the cells settled on a glass cover-slip which formed the bottom of the Plexiglas experimental chamber (mounted on the stage of an inverted microscope, Nikon Diaphot, Tokyo). The cover-slips had been pre-treated with 1 μg/ml laminin (Collaborative Research, Bedford, MA, USA) to increase cell adhesion during experiments at high flow rates. After the cells were allowed to attach for about 30 min, they were loaded with the fluorescent Ca^{2+} indicator, Indo-1, by incubation with the acetoxymethyl ester form of the indicator, Indo-1-AM (10 μM for 30–45 min; CalBiochem, La Jolla, CA, USA). The cells were field stimulated at 0·25–0·5 Hz and the flow rate in the chamber was ~ 5 ml/min (or ~ 1 cm/s) during RCC protocols and solution depth was maintained at ~ 3 mm.

The optical system was similar to that described by Peeters, Hladky, Bridge & Barry (1987). A 200 W mercury arc lamp (Oriel, Stratford, CT, USA) provided the excitation light via a 350 nm bandpass filter, through a 400 nm dichroic mirror and 40 × Fluor objective to the cells. Optical signals from a cell were directed to two photomultiplier tubes (R268UHHA, Hamamatsu Corp., Bridgewater, NJ, USA) and to a CCD televison camera (TM-540, Pulnix America Inc., Sunnyvale, CA, USA). An adjustable rectangular window was used to restrict the optical image to the cell of interest, thereby minimizing background fluorescence from other cells and debris. The fluorescent signals to the two photomultipliers were filtered with bandpass filters at 400 and 500 nm, respectively. The cell image was displayed on a 19 in TV monitor and changes in cell length were measured with a video motion detector (Steadman, Moore, Spitzer & Bridge, 1988). The ratio of emitted fluorescence at 400 nm : 500 nm (F_r) was obtained on-line through an analog divider circuit and, along with the video image, was recorded on a modified video recorder.

Thin papillary muscles or ventricular trabeculae (0·1–0·5 mm in diameter) were dissected from the hearts of guinea-pigs. The ends of the muscle were tied with fine suture. One end of the muscle was attached to a fixed post and the other to a piezoresistive transducer (AE 875, SensoNor, Horten, Norway) in a 0·15 ml superfusion chamber. The muscle was stimulated at 0·5 Hz by platinum plates in the lateral chamber walls during equilibration (~ 1 h) and between protocols. The flow rate in the chamber was ~ 35 ml/min (or ~ 5 cm/s).

The control superfusate was a modified normal Tyrode solution (NT) containing (in mM): 140 NaCl, 6 KCl, 1 $MgCl_2$, 2·7 $CaCl_2$, 10 glucose, 5 HEPES and pH was adjusted to 7·40 at 1 or 29 °C as appropriate. All solutions were equilibrated with 100% O_2 and the bath temperature was 22 °C for myocytes and 29 °C for muscles (except during cooling contractures). In Na^+-free, Ca^{2+}-free solutions, NaCl was replaced isosmotically with LiCl and $CaCl_2$ was replaced with 500 μM-EGTA. Solenoid valves were situated at the bath inlet and the perfusion lines leading to these valves were jacketed with either water (at 22 or 30 °C) or propylene glycol : water (1 : 3 at −2·0 °C). At the flow rates used, switching to the cold solution cooled the muscle surface or myocyte to below 3 °C in < 1 s. Rewarming was similarly rapid. A stream of dry air directed underneath the chamber prevented water condensation on the cover-slip during rapid cooling.

With rapid introduction of cold solution, the glass bottom of the chamber sometimes moved up by ~ 5 μm. This movement resulted from either thermal contraction of the silicone interface between the glass and Plexiglas walls and/or contraction of small air bubbles trapped in the silicone itself. The effect of this movement on F_r was estimated in six stimulated myocytes at 22 °C in NT. To simulate a 5 μm upward movement of the chamber, the objective lens was rapidly lowered 5 μm using the fine focus adjustment. This manoeuvre caused a slight decrease in the diastolic F_r (5·4 ± 3·4%) and a slight increase in the peak transient F_r during a twitch (7·0 ± 6·9%). It is

not clear why this movement alters F_r, but the effect of these changes does not alter any of the conclusions in the present study and is considered in the results.

Calibrations of the free acid form of the indicator, Indo-1, at both 1 and 22 °C were carried out in a Perkin-Elmer MPF-66 spectrofluorometer. Excitation was set at 355 nm and emission spectra were recorded from 380 to 540 nm (2 nm bandwidth) at each $[Ca^{2+}]$. Ca^{2+} was buffered with 10 mM-EGTA and 5 μM-Indo-1 was included with 140 mM-KCl and 10 mM-HEPES at pH 7·2. The association constant of EGTA for Ca^{2+} was adjusted for temperature and ionic strength as described by Harrison & Bers (1989) for preparing the solutions. The free $[Ca^{2+}]$ was also directly measured in the calibration solutions using a Ca^{2+} electrode (Bers, 1982). The wavelength for maximum fluorescence intensity in nominally Ca^{2+}-free and high $[Ca^{2+}]$ (1 mM free $[Ca^{2+}]$) were 480 and 404 nm respectively (at both temperatures). The ratio of emitted fluorescence at these two wavelengths (F_{404}:F_{480}), was plotted in the calibration curves, while the fluorescence at either individual wavelength (or the ratio of F_{404} or F_{480} to that at the Ca^{2+}-independent wavelength, 444 nm) is used to determine the affinity of Indo-1 for Ca^{2+} (Grynkiewicz, Poenie & Tsien, 1985).

RESULTS

Temperature modifies Ca^{2+} sensitivity of Indo-1 and myofilaments

To evaluate the influence of temperature on the Ca^{2+} indicator we performed *in vitro* calibrations for Indo-1 at 1 and 22 °C (Fig. 1). Five individual calibrations were done at each temperature and were well fitted with a modified Hill equation. The fit parameters (K_d, n, F_{min} and F_{max}) for each temperature were averaged to generate the upper curves in Fig. 1. Cooling from 22 to 1 °C decreased the Ca^{2+} affinity of Indo-1 from $K_d = 392 \pm 34$ nM to $K_d = 509 \pm 27$ nM. The shift in the half-maximal value of F_r was 0·18 pCa units (or from 794 nM at 22 °C to 1·20 μM at 1 °C), but the maximum value of F_r did not change with cooling. The half-maximum F_r for Indo-1 (free acid) measured in the bath on the microscope stage was 750–800 nM at 22 °C (similar to that above). Thus, while we have not routinely performed *in vivo* calibrations in the cells which are loaded with Indo-1-AM, we know what shifts to expect from the free acid form of the indicator in calibration solutions. We have chosen to present the fluorescent results in cells in arbitrary units (F_r) due to the difficulty in obtaining reliable *in vivo* calibrations at both temperatures and with the multiple forms of the indicator which may be present in the cells and potential complications due to intracellular binding and compartmentalization of the indicator (Konishi, Olson, Hollingworth & Baylor, 1988).

The background and autofluorescence of the cell prior to indicator loading was 2–3% of the fluorescence in the loaded cell and was not corrected for. In a series of five cells where diastolic and peak F_r were consistent and calibrations were performed under identical conditions, and with the above reservations, we can estimate the typical diastolic and peak $[Ca^{2+}]_i$ (300 nM and 1·3 μM respectively). These values are slightly higher than those reported by Beuckelmann & Weir (1988) in guinea-pig myocytes, but may serve as a rough index of the $[Ca^{2+}]_i$ transient during steady-state twitches in other cells (where independent calibrations were not always done).

Cooling also decreases both the Ca^{2+} sensitivity and maximum force generated by cardiac muscle myofilaments (Harrison & Bers, 1989). For comparative purposes, the results obtained by Harrison & Bers (1989) for isometric force in Triton-skinned rabbit ventricular muscle at 1 and 22 °C are included in Fig. 1 (lower curves). Cooling from 22 to 1 °C decreased the maximum force to 29·3 ± 5·4% and shifted the pCa for

half-maximal activation from 5·34±0·05 to 4·73±0·04. Preliminary results in guinea-pig fibres indicate similar shifts in Ca^{2+} sensitivity, but a larger depression of maximal force at 1 °C (Harrison & Bers, 1988). These curves show that for a given [Ca^{2+}], tension is more sensitive to temperature changes than is F_r.

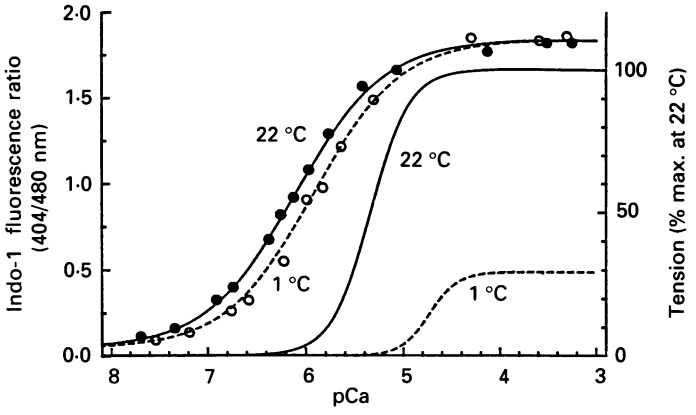

Fig. 1. *In vitro* calibration curves for Indo-1 at 22 and 1 °C (top two curves). Myofilament Ca^{2+} sensitivity (lower two curves) measured in Triton-skinned rabbit ventricular muscle by Harrison & Bers (1989) is also illustrated. Data for all four curves were fitted with a modified Hill equation $\{(F = F_{min} + (F_{max} - F_{min})/(1 + (K_d/[Ca^{2+}])^n)\}$. After averaging the parameters obtained from several calibrations, the mean values were used to plot the curves shown. The points on the Indo-1 calibrations are from a typical calibration at 22 °C (●) and at 1 °C (○).

Rapid cooling contractures in isolated myocytes

When 0·25 Hz stimulation of a myocyte is terminated and the cell is rapidly cooled, both the amplitude of cell shortening and the increase in fluorescence ratio signal (F_r) are greater than during the preceding twitch (Fig. 2*A*). The increase in F_r upon cooling probably underestimates the rise in free $[Ca^{2+}]_i$ since cooling decreases the Ca^{2+} affinity of Indo-1. The Ca_i^{2+} signal (F_r) rises rapidly to a peak and then declines more slowly while the cell is maintained at 1 °C. Shortening begins after the rise in $[Ca^{2+}]_i$ and takes several seconds to reach its maximum extent. When the cell is rested for 30 s or 2 min prior to cooling (Fig. 2*B* and *C*), the $[Ca^{2+}]_i$ transient and the extent of shortening are both smaller. This presumably reflects the process known as rest decay, where in some cardiac muscle preparations (such as guinea-pig ventricle) the amplitude of post-rest contractions (and the SR Ca^{2+} content) declines as a function of rest interval duration (Allen, Jewell & Wood, 1976; Bers, 1985, 1989; Bridge, 1986).

Following rest periods of 2–5 min, rapid cooling sometimes elicited a small contracture despite an accompanying decrease in F_r (e.g. Fig. 2*C*). The two most likely explanations for this decline in F_r are: (a) a cooling-induced upward shift in the bottom of the cell chamber (as noted in the Methods this effect alone can cause diastolic F_r to decrease by ~ 6% of the F_r transient at 22 °C, but is not sufficient to explain the entire decrease in F_r seen upon cooling in some cells and, (b) a cooling-induced decrease in Indo-1 affinity for Ca^{2+} (see Fig. 1). Thus, cooling from 22 to 1 °C

without a change in $[Ca^{2+}]$ would lead to a decrease in F_r. The magnitude of this effect can be addressed using the calibrations in Fig. 1. At a constant resting $[Ca^{2+}]_i$ of 150 nM, cooling would lead to a 26% decrease in F_r. This decrease would also correspond to 12–21% of the amplitude of the F_r transient during a twitch at 22 °C (assuming $[Ca^{2+}]_i$ increases from 150 nM to 500–1000 nM). Furthermore, $[Ca^{2+}]_i$ would have to increase from 150 nM at 22 °C to 230 nM at 1 °C for F_r to remain constant. The combination of these two effects is sufficient to explain the decreases of F_r sometimes observed after rests.

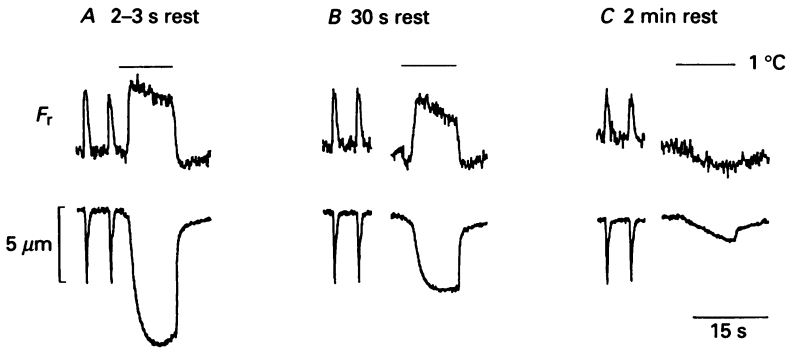

Fig. 2. Shortening (μm) and $[Ca^{2+}]_i$ (F_r) during the last two stimulated twitches (at 0·25 Hz) and during RCCs induced 2–3 s after the last twitch (*A*), after 30 s rest (*B*) and after 2 min rest (*C*). The horizontal bar indicates the time during which the superfusate was at 1 °C. During the break in the record in *B* and *C* the cell was at rest. The F_r signal was filtered at 3 Hz.

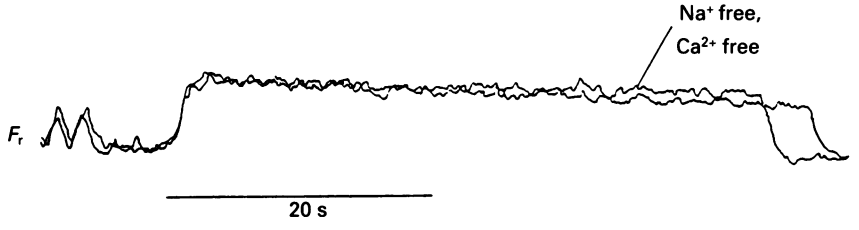

Fig. 3. Superimposed Ca_i^{2+} transients (F_r) from two successive RCCs in one myocyte. One RCC was induced with NT and the other in Na^+-free, Ca^{2+}-free (+500 μM-EGTA) solution at 1 °C. The cold solution was applied at the start of the time bar and 22 °C NT was reintroduced near the end of each trace. The two final twitches in NT (0·5 Hz) prior to cooling are shown at the start of each trace. The F_r signal was filtered at 1 Hz.

When a cell is cooled in a Na^+-free, Ca^{2+}-free solution (rather than NT), the amplitude of the F_r change is not altered. (Fig. 3). This supports earlier conclusions from contraction measurements in multicellular preparations, that RCCs do not depend on Ca^{2+} entry from the extracellular space (Kurihara & Sakai, 1985; Bridge, 1986). The slow decline in $[Ca^{2+}]_i$ during the RCC is only slightly slowed in the absence of extracellular Na^+ (Fig. 3). In this cell F_r declined by 27% during the RCC in Na^+-free, Ca^{2+}-free solution and by 32% during the same time in NT. This suggests that some other mechanism than Na^+–Ca^{2+} exchange is responsible for most of the decline in $[Ca^{2+}]_i$ during the RCC. If the cell in Na^+-free, Ca^{2+}-free solution is re-cooled right after rewarming, the peak F_r at the second RCC is nearly

the same as that at the first RCC (even when F_r has declined substantially during the first RCC). Thus, it seems likely that the SR can slowly reaccumulate Ca^{2+} at 1 °C.

In several cells, the process of rest decay of RCCs was investigated in both NT and in Na^+-free, Ca^{2+}-free solution (e.g. Fig. 4). Figure 4*A–C* is similar to Fig. 2*A–C* except that the steady-state stimulation rate prior to the rest period in Fig. 4 was 0·5 Hz. Rest decay of both F_r and shortening occurs when the muscle is rested in NT.

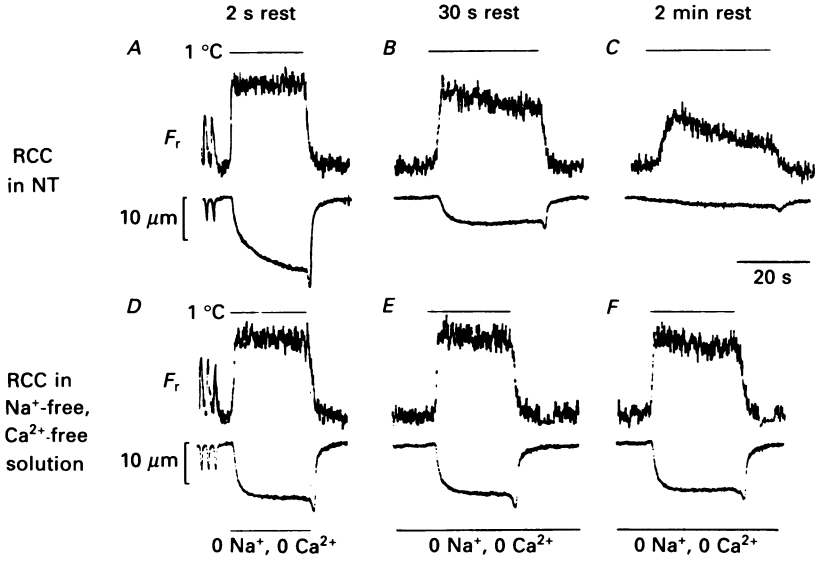

Fig. 4. Shortening (μm) and $[Ca^{2+}]_i$ (F_r) during the last stimulated twitches (at 0·5 Hz in *A* and *D*) and during RCCs induced 2 s after the last twitch (*A* and *D*), after 30 s rest (*B* and *E*) and after 2 min rest (*C* and *F*). In *A–C* the superfusate during rest and RCC was NT. In *D–F* the superfusate during rest and RCC was Na^+-free Ca^{2+}-free solution (with 500 μM-EGTA). The horizontal bar indicates the time during which the superfusate was at 1 °C. The F_r signal was filtered at 3 Hz.

When extracellular Na^+ is absent during the rest (Fig. 4*D–F*) there is very little decline in the amplitude of the F_r transient or shortening during RCCs induced after the rest (see also Fig. 7). These results indicate that SR Ca^{2+} lost during rest decay is principally dependent on Na^+–Ca^{2+} exchange as suggested by Sutko, Bers & Reeves (1986). Furthermore, other means of extruding Ca^{2+} from the cell are unable to induce substantial decline in SR Ca^{2+} even after 5 min of rest in Ca^{2+}-free solution. For example, the sarcolemmal Ca^{2+}-ATPase pump does not appear to be capable of extruding enough Ca^{2+} from the cell during rest to unload the SR, despite the fact that Ca^{2+} extrusion is probably favoured by an outwardly directed chemical gradient (in Ca^{2+}-free, 500 μM-EGTA solution).

During the RCC in Fig. 4*A*, F_r rises to a level much greater than during the twitch and does not appear to decline. It seems likely that the indicator is nearly saturated with Ca^{2+} and that $[Ca^{2+}]_i$ is declining during this RCC, for the following reasons. First, all RCCs except those reaching an apparent maximum exhibit gradual decline during the period at 1 °C (see Figs 4*A* and *D* and 5*B vs.* Figs 2 and 4*B* and *C*). Second,

the F_r reached during this RCC is almost twice as large as that reached during the steady-state twitch. This may be expected to be close to saturation of Indo-1 if one considers the $[Ca^{2+}]_i$ and F_r transient during the normal twitch. An increase in $[Ca^{2+}]_i$ from ~ 100 to ~ 800 nM during a twitch (Cannell, Berlin & Lederer, 1987; Beuckelmann & Weir, 1988) would correspond to an F_r increase from 0·24 to 0·95 in Fig. 1. Doubling the ΔF_r would increase F_r to ~ 1·66 (near the maximum) which corresponds to 90% saturation of Indo-1 and to 10 μM-Ca (at 1 °C). If $[Ca^{2+}]_i$ nearly saturates Indo-1 during RCCs and the calibrations in Fig. 1 are at least approximately valid for the indicator in the cells, then $[Ca^{2+}]_i$ may reach > 10 μM during the RCC. Results with multicellular preparations also support this conclusion (see Fig. 8 and below).

When papillary muscles or ventricular trabeculae are rewarmed after an RCC a transient additional contraction (or rewarming spike) is normally observed and has been attributed to an increase in myofilament Ca^{2+} sensitivity induced by warming (see Fig. 1 and Bridge, 1986; Bers, 1989; Harrison & Bers, 1989). These rewarming spikes are usually, but not always, observed on the shortening records during RCCs in myocytes (e.g. Fig. 4 *vs.* Fig. 2). The results in Fig. 4 indicate that the rewarming spike in the shortening record is not associated with any increase in F_r (or $[Ca^{2+}]_i$). Indeed, since warming slightly increases the Ca^{2+} sensitivity of the dye (Fig. 1), a small increase in F_r might have been expected even at constant $[Ca^{2+}]_i$. Thus the rewarming spike is not due to any transient rise of $[Ca^{2+}]_i$. In fact, $[Ca^{2+}]_i$ is declining at the time the rewarming spike is observed.

SR Ca^{2+} pump vs. Na^+–Ca^{2+} exchange during relaxation

Bers & Bridge (1989) recently demonstrated that *either* the SR Ca^{2+} pump *or* the Na^+–Ca^{2+} exchange can produce relaxation at relatively normal rates, but that one of these processes must be available otherwise relaxation is extremely slow. Thus, it is expected that the SR Ca^{2+} pump and Na^+–Ca^{2+} exchange compete for myoplasmic Ca^{2+} during relaxation. Here we use a series of RCCs to assess the relative contributions of these competing processes to relaxation from an RCC (Fig. 5).

In NT, the Ca_i^{2+} transients associated with successive RCCs in a myocyte

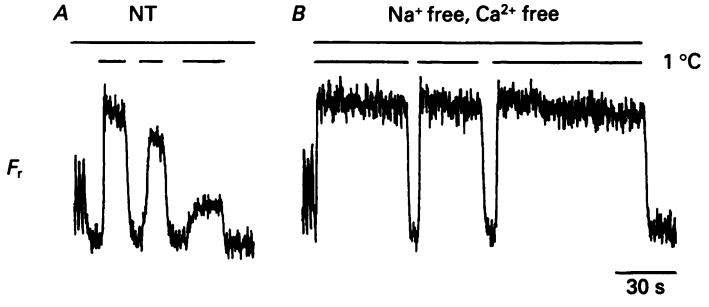

Fig. 5. $[Ca^{2+}]_i$ (F_r) during the last stimulated twitches (at 0·5 Hz) and during a series of RCCs started ~ 2 s after the last stimulated twitch. In *A* the superfusate during rest and RCC was NT. In *B* the superfusate during the RCCs and rewarming was Na^+-free, Ca^{2+}-free solution (with 500 μM-EGTA). The horizontal bar indicates the time during which the superfusate was at 1 °C. The F_r signal was filtered at 3 Hz.

progressively decline in amplitude from one to the next (Fig. 5*A*). We interpret this to reflect the competition between the SR Ca^{2+} pump and the Na^+–Ca^{2+} exchange. That is, during relaxation from the first RCC (in Fig. 5*A*) some Ca^{2+} is pumped into the SR and some is extruded from the cell. The subsequent RCC is then indicative of the amount of Ca^{2+} resequestered by the SR. The F_r change in this cell at the second and third RCC are 64 and 49% of those at the preceding RCCs respectively. This suggests that a substantial fraction of the myoplasmic Ca^{2+} during an RCC is not resequestered by the SR. When a similar series of RCCs is induced in the absence of extracellular Na^+ to prevent Ca^{2+} extrusion via Na^+–Ca^{2+} exchange (Fig. 5*B*), there is almost no decrement in the F_r transient at the second or third RCC in this same cell. We conclude that Na^+–Ca^{2+} exchange was reponsible for the decrement in successive RCCs in NT (Fig. 5*A*) and that this system can compete rather effectively with the SR Ca^{2+} pump under these conditions.

Ryanodine and rest decay of rapid cooling contractures (RCCs)

The effect of ryanodine on RCCs in myocytes was also examined (Fig. 6). After a cell was equilibrated for 20 min with 1 μM-ryanodine, RCCs and $[Ca^{2+}]_i$ transients could only be elicited immediately after a train of stimulated twitches (Fig. 6*A*) or after short periods of rest (e.g. < 5 s). This is consistent with RCC results in multicellular preparations where only force development was measured (Bers *et al.* 1987). Caffeine can also abolish RCCs in myocytes just as in multicellular preparations (Bridge, 1986; Bers *et al.* 1987; Bers & Bridge, 1988; Hryshko, Stiffel & Bers, 1989).

Pooled results from fifteen rest decay experiments (Fig. 7) indicate that rest decay of both the fluorescence transient (F_r) and the shortening (Δl) associated with post-rest RCCs is markedly slowed in Na^+-free, Ca^{2+}-free solution. Rest decay of both F_r and Δl is dramatically accelerated in the presence of ryanodine. The extent of shortening declines more rapidly as a function of rest duration than does the F_r. We expect this because the $[Ca^{2+}]$ dependence of tension (at 1 °C) is much steeper than that of F_r. That is, the Hill coefficients for the F_r curve and isometric force curves at

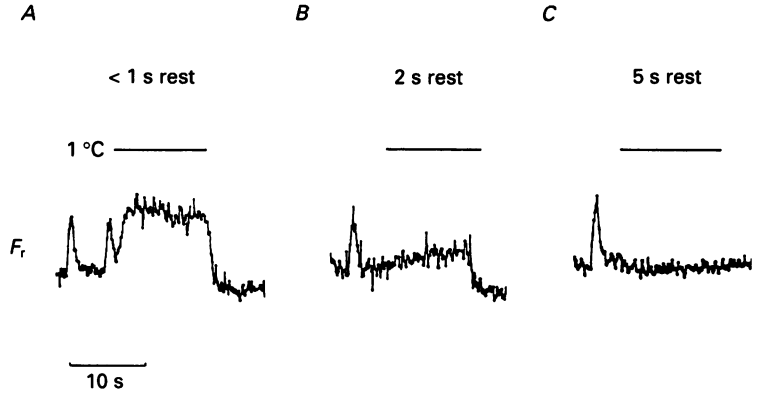

Fig. 6. This cell was equilibrated with 1 μM-ryanodine for 20 min prior to this series of RCCs. Stimulation was at 0·5 Hz and the time from the last stimulus to the start of the cold solution (horizontal bar) is indicated over the F_r traces in each panel.

1 °C in Fig. 1 are 0·98 and 2·94 respectively. Thus for a given decrease in $[Ca^{2+}]$, a larger decline in contraction than F_r is expected.

$[Ca^{2+}]_i$ changes inferred from force transients in multicellular preparations

The foregoing results with Indo-1 have indicated that $[Ca^{2+}]_i$ rises rapidly to a peak during an RCC and then gradually declines while the cell is maintained at 1 °C.

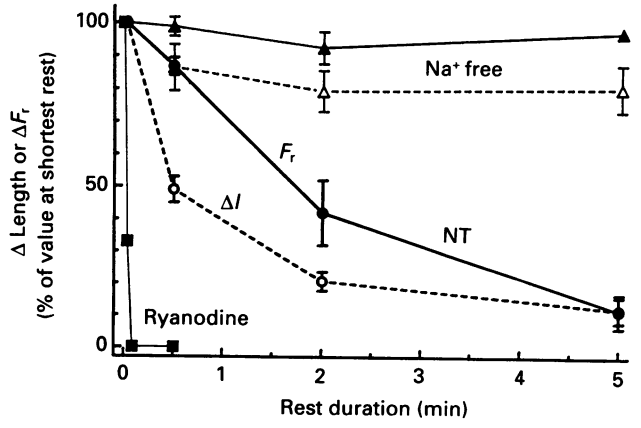

Fig. 7. Pooled results for F_r (continuous lines and filled symbols) and shortening (dashed lines and open symbols) from fifteen rest decay experiments like those shown in Figs 4 and 5. Rest and cooling was either in NT (circles), Na^+-free, Ca^{2+}-free + EGTA (triangles) or NT + ryanodine (after equilibration, squares). Data were normalized to the amount of shortening or F_r observed after the shortest rest interval (2 s).

We have used an independent (and more indirect) method to assess the time course of the $[Ca^{2+}]_i$ transient in intact, guinea-pig ventricular multicellular preparations (Fig. 8). The lower continuous trace shows the last stimulated twitch (0·5 Hz) prior to a 10 s rest and subsequent RCC and rewarming spike. During the rapid rewarming the myofilaments are expected to respond rapidly to the local $[Ca^{2+}]_i$ at the time of rewarming. Thus, by rewarming at different times after initial cooling (0·6–180 s), the amplitude of the rewarming spike should be indicative of the $[Ca^{2+}]_i$ at the time of rewarming. The upper curve in Fig. 8 is the superimposed 'envelope' of rewarming spike peaks obtained when the fibre was rewarmed at different times after cooling (indicated by the points). This indicates that $[Ca^{2+}]_i$ rises to a peak very rapidly upon cooling and then declines while the muscle is maintained at 1 °C. Although the technique is indirect the conclusion is the same as for the myocytes in Figs 2–6. Thus, the $[Ca^{2+}]_i$ changes observed during RCCs in myocytes are probably similar to those in multicellular preparations in which most RCC studies have been performed. The amplitude of the maximum rewarming spike (70 mN/mm^2) is about the maximum isometric force guinea-pig ventricular muscle can generate, even in *skinned* and fully activated muscle (S. M. Harrison & D. M. Bers, unpublished observation). This provides independent support for the earlier conclusion about the $[Ca^{2+}]_i$ reached during an RCC. That is, such saturation of guinea-pig ventricular myofilaments at 29 °C requires $\sim$ 10 μM $[Ca^{2+}]$ (Harrison & Bers, 1988).

The envelope of rewarming spikes in Fig. 8 declines during the RCC more rapidly than the F_r signals in Figs 2–6. This may again be attributable to the steeper Ca^{2+} dependence of tension (at 29 °C Hill coefficient = 2·15, Harrison & Bers, 1989) than of F_r (at 1 °C).

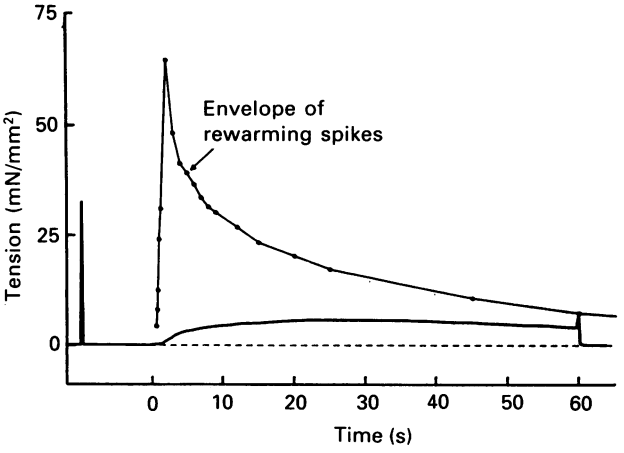

Fig. 8. Isometric force record from a multicellular preparation showing the last stimulated twitch (0·5 Hz) and an RCC (lower trace). The points on the upper curve are the peak values of force reached when the muscle was rapidly rewarmed at the times after cooling as indicated along the abscissa in nineteen consecutive RCCs. The RCCs are highly reproducible and the one shown corresponds to the last point shown on the upper curve (see text for further detail).

The decline of $[Ca^{2+}]_i$ during RCCs and the time required for RCCs to reach the maximum extent of shortening or isometric force emphasizes the discrepancy between the time course of $[Ca^{2+}]_i$ changes and force changes during an RCC. Thus, while RCCs may still be a useful means of assessing the amount of SR Ca^{2+} available for contraction, they do not reflect the time course of, or peak value of $[Ca^{2+}]_i$ reached during an RCC.

DISCUSSION

By using an intracellular Ca^{2+} indicator, we confirm numerous previous conclusions about RCCs in mammalian cardiac muscle (Kurihara & Sakai, 1985; Bridge, 1986; Bers, 1987, 1989; Bers *et al.* 1987). For example, it seems clear that RCCs depend on Ca^{2+} release from the SR and not transsarcolemmal Ca^{2+} influx. This is supported by the observation that RCCs and associated $[Ca^{2+}]_i$ transients are suppressed by ryanodine, caffeine and long rest intervals (in the presence of Na_o^+) and are not depressed in the absence of Ca_o^{2+}. The latter effect is particularly important since similar effects in multicellular preparations were complicated by diffusional limitations (Kurihara & Sakai, 1985; Bridge, 1986; Bers & Bridge, 1989).

RCCs in myocytes are similar to those in multicellular preparations, but differ in amplitude compared to twitches (Hryshko *et al.* 1989). Shortening during RCCs in

myocytes generally exceeds that at stimulated twitches, whereas isometric force during RCCs in guinea-pig are usually smaller than at twitches (see Fig. 4 *vs.* Fig. 8 and Bers, 1989; Hryshko *et al.* 1989). This may simply be due to the prolonged active state in the cold, allowing continued shortening in myocytes against a small (primarily internal) load.

Magnitude and time course of $[Ca^{2+}]_i$ *transients induced by cooling*

Two prominent features of the $[Ca^{2+}]_i$ transients induced by cooling are their large amplitude (compared to that during a twitch) and the rapid rate of rise (compared to the subsequent contraction). The rise in the F_r signals in Figs 2*A* and 4*A* were half-maximal in about 150 ms (but is slower for smaller RCCs). This is likely to be lower than the rate of SR Ca^{2+} release induced by cooling to < 5 °C for three reasons. First, the signals were filtered at 15 Hz and the kinetics are consequently bandwidth limited. Second, the temperature change is not instantaneous and occurs over several hundred milliseconds. Third, while the cell is being cooled the sensitivity of the indicator is simultaneously decreasing, which would tend to slow the upstroke of the F_r signal. Thus, within the kinetic constraints of the present methodology, it would appear that the rate of Ca^{2+} release from the SR during an RCC is remarkably fast.

It is clear that the $[Ca^{2+}]_i$ reached during an RCC can be considerably higher than that reached during a twitch under the same experimental conditions (particularly if one considers that cooling decreases the Ca^{2+} sensitivity of Indo-1). Detailed analysis of the amplitude of the $[Ca^{2+}]_i$ transient is limited by our reliance on *in vitro* calibration curves for Indo-1. However, since the $[Ca^{2+}]_i$ appears to saturate the indicator under some circumstances (e.g. Fig. 4), some quantitative considerations may be warranted. If the calibration of Indo-1 in Fig. 1 is valid for the indicator in the cell, 90–95% saturation would be reached at 13–32 μM-Ca^{2+}. To estimate the amount of SR Ca^{2+} release required for $[Ca^{2+}]_i$ to reach this level, one must consider the ability of cellular contents to buffer intracellular Ca^{2+} (Fabiato, 1983; Pierce, Philipson & Langer, 1985). Although the values measured by Pierce *et al.* (1985) and computed by Fabiato (1983) are for 22 °C, they indicate that release of 140–235 or 85–115 μmol Ca^{2+}/kg wet weight (respectively) would be required to bring free $[Ca^{2+}]_i$ to 13–32 μM. These values are similar in magnitude to various estimates of the SR Ca^{2+} content in mammalian cardiac muscle (in μmol Ca^{2+}/kg wet weight): 170 (Solaro & Briggs, 1974), 100–300 (Dani, Cittadini & Inesi, 1979), 125 (Hunter, Haworth & Berkoff, 1981), $\sim$ 160 (Levitsky, Benevolensky, Levchenko, Smirnov & Chazov (1981), > 57 (Fabiato, 1983). While it is not possible to know with certainty, it would seem likely that the entire SR Ca^{2+} content may be released during an RCC, but *not* normally during a twitch.

Comparison with $[Ca^{2+}]_i$ *during RCCs in skeletal muscle*

RCCs can also be induced in skeletal muscle fibres (Sakai, 1965; Konishi *et al.* 1985), but only after pre-treatment with caffeine (0·3–1·4 mM). This is in contrast to mammalian cardiac muscle, where caffeine only depresses RCCs (half-maximal effect at 1 mM; Bers & Bridge, 1988). Nevertheless, RCCs in both preparations appear to be due to SR Ca^{2+} release. The $[Ca^{2+}]_i$ transients in skeletal muscle fibres measured by Konishi *et al.* (1985) using aequorin, exhibited a complex time course and

oscillations. The time course of the $[Ca^{2+}]_i$ changes during RCCs in cardiac muscle appear considerably simpler and without oscillations.

Decline in $[Ca^{2+}]_i$ during RCCs

The slow decline in $[Ca^{2+}]_i$ during RCCs appears to be due in large part to slow reaccumulation of Ca^{2+} by the SR in the cold. This conclusion is based on two observations: (1) Na^+-free superfusion during the cold only slows the decline in $[Ca^{2+}]_i$ slightly (Fig. 3); (2) if a second RCC is induced immediately after the first (when Ca^{2+} extrusion during the warm period is prevented by Na^+-free solution), the maximal contraction observed is almost the same whether the first RCC was brief (without much decay, e.g. Fig. 5*B*) or prolonged (with substantial decay; Bers, 1989; Hryshko *et al.* 1989). This suggests that most of the Ca^{2+} removed from the cytoplasm during a long first RCC is resequestered by the SR and thus available for the second RCC. In the presence of Na_o^+ this conclusion is slightly complicated by the fact that Na^+–Ca^{2+} exchange may also extrude some Ca^{2+} during rewarming (Bers & Bridge, 1989). Na^+–Ca^{2+} exchange also appears to make a minor contribution to the decline in $[Ca^{2+}]_i$ during the RCC (Fig. 3). Thus, while cooling to 0–1 °C may strongly inhibit both the SR Ca^{2+} pump and Na^+–Ca^{2+} exchange, neither process is abolished.

It has also been reported that the rate of relaxation of RCCs at 0–1 °C is markedly different in different mammalian cardiac muscle preparations (e.g. rat ventricle and rabbit atrium relax much faster at 1 °C than rabbit or guinea-pig ventricle; Bers, 1989). This may indicate differences in the temperature sensitivity of the Ca^{2+} transport systems in different preparations.

The present results indicate that during an RCC in guinea-pig ventricular muscle, $[Ca^{2+}]_i$ declines from its peak value, while the contractile amplitude (shortening or isometric force) is still increasing (see Figs 2, 4 and 8). While cooling would slow the steps between the $[Ca^{2+}]_i$ rise and force generation (or shortening), we cannot exclude the possibility that the $[Ca^{2+}]_i$ peak (sensed by Indo-1) is different from the $[Ca^{2+}]_i$ which determines the amplitude of the contraction. However, we have never observed a situation where the peak $[Ca^{2+}]_i$ during an RCC is changed in an opposition direction to that observed for the contracture peak (provided that the RCC is long enough to reach its maximal value). Thus, while the time course of the contracture during an RCC does not reflect the time course of $[Ca^{2+}]_i$ change, the amplitude of the RCC is still a useful index of SR Ca^{2+} available for release. Of course if an agent which modifies myofilament Ca^{2+} sensitivity (e.g. catecholamines) is to be added, the $[Ca^{2+}]_i$ and contracture results may not change in a parallel fashion.

The contributions of Na^+–Ca^{2+} exchange and the SR Ca^{2+} pump to relaxation

We conclude from our results that the SR Ca^{2+} pump and the Na^+–Ca^{2+} exchange are the main processes responsible for reducing myoplasmic $[Ca^{2+}]$ during relaxation from RCCs (Fig. 5). Furthermore, these two processes compete with each other, with the SR Ca^{2+} pump dominanting only slightly under our conditions. We cannot be more quantitative without accurate *in vivo* calibrations, but it seems reasonable to conclude that Na^+–Ca^{2+} exchange may be responsible for removing 20–50% of the Ca^{2+} which is removed from the myoplasm during relaxation. This is a broad range, but is consistent with similar types of experiments in rabbit ventricular muscle and

myocytes (Bers & Bridge, 1989; Hryshko *et al.* 1989) where only contractions were measured. The relative contributions of the SR Ca^{2+} pump and Na^+–Ca^{2+} exchange to relaxation may also be expected to vary with conditions and in different cardiac muscle preparations, since the Na^+–Ca^{2+} exchange is voltage dependent (e.g. Bridge *et al.* 1988). In our experiments with RCCs, where the membrane is polarized throughout the period of decline in $[Ca^{2+}]$, the Na^+–Ca^{2+} exchange would be more effective than during an action potential in guinea-pig (or rabbit) ventricle where the plateau phase of the action potential (near 0 mV) would limit Ca^{2+} extrusion via the Na^+–Ca^{2+} exchange (Bers & Bridge, 1989). On the other hand, in rat ventricle where the action potential repolarizes very early, Ca^{2+} extrusion via Na^+–Ca^{2+} exchange would be more strongly favoured. Indeed, Ca^{2+} extrusion attributable to Na^+–Ca^{2+} exchange is observed during normal contractions in rat ventricle (Shattock & Bers, 1989).

The contributions of Na^+–Ca^{2+} exchange and the sarcolemmal Ca^{2+} pump to rest decay

The decline in the amplitude of post-rest contractions as a function of rest interval in mammalian cardiac muscle has been referred to as rest decay (e.g. Allen *et al.* 1976) and is thought to represent the gradual loss of Ca^{2+} from the SR at rest. Results from RCC experiments have supported this interpretation (Bridge, 1986; Bers *et al.* 1987; Bers, 1989). Cellular Ca^{2+} loss has also been demonstrated to occur during rest (Bridge, 1986; Janczewski & Lewartowski, 1986; MacLeod & Bers, 1987; Pierce, Rich & Langer, 1987). The rate of rest decay can be reduced or even reversed by decreasing the transsarcolemmal $[Na^+]$ gradient (Sutko *et al.* 1986), as can the loss of SR and cellular Ca^{2+} (Wendt & Langer, 1977; Bridge, 1986; Bers & Bridge, 1988). These results implicate the Na^+–Ca^{2+} exchange as a major determinant of SR Ca^{2+} content during rest decay.

Barry & Smith (1984) suggested that a Na_o^+-independent Ca^{2+} extrusion was the dominant means of ^{45}Ca efflux in cultured chick heart cells. Those initial studies were complicated by unknown Ca^{2+}–Ca^{2+} exchange fluxes. Barry, Rasmussen, Ishida & Bridge (1986) extended these findings and concluded that the Na_o^+-independent Ca^{2+} extrusion (due probably to the sarcolemmal Ca^{2+}-ATPase pump) was about 20% of that extruded by the Na^+–Ca^{2+} exchange. The present results indicate that rest decay in guinea-pig ventricular myocytes can be almost abolished if Na^+–Ca^{2+} exchange is prevented from extruding Ca^{2+} (i.e. in Na^+-free solution, Figs 4 and 7). Furthermore, the sarcolemmal Ca^{2+}-ATPase pump appears unable to extrude Ca^{2+} at a rate sufficient to produce rest decay, even though the gradient for Ca^{2+} extrusion is greatly reduced (in the Na^+-free, Ca^{2+}-free medium). Thus the ability of the Na^+–Ca^{2+} exchanger to extrude Ca^{2+} from mammalian cardiac myocytes at resting levels of $[Ca^{2+}]_i$ would appear to be very much greater than the sarcolemmal Ca^{2+}-ATPase pump.

Mechanism by which cooling induces SR Ca^{2+} release

The molecular mechanism by which rapid cooling induces SR Ca^{2+} release in cardiac muscle is not known. It was previously argued (Bridge, 1986) that simple inhibition of the SR Ca^{2+} pump by the cold might allow an unopposed SR Ca^{2+} *leak* to

provide enough Ca^{2+} for the RCC. This now seems unlikely since the SR may release the entire SR Ca^{2+} content in < 1 s during an RCC (see above). Were the SR Ca^{2+} pump inhibition the only factor responsible for the release of Ca^{2+}, it is implicit that this high rate of SR Ca^{2+} release must be compensated by the pump under normal resting conditions. However, this would imply that the SR Ca^{2+} pump works at a very high rate in resting heart cells (see discussion of SR Ca^{2+} capacity above). As a working hypothesis we suggest that the SR Ca^{2+} release during rapid cooling is via *the* SR Ca^{2+} release channel and that rapid cooling to < 5 °C increases the open probability of this channel (at least transiently).

Conclusion

Rapid cooling of ventricular myocytes leads to a very large and rapid increase of $[Ca^{2+}]_i$ which depends on SR Ca^{2+} and not on $[Ca^{2+}]_o$. This increase of $[Ca^{2+}]_i$ is likely to be sufficient to activate the observed RCCs. During an RCC, $[Ca^{2+}]_i$ declines from its peak value prior to the peak of contraction (primarily due to residual SR Ca^{2+} pump activity in the cold). Rest decay of RCCs and $[Ca^{2+}]_i$ transients is dependent on Ca^{2+} extrusion via Na^+–Ca^{2+} exchange. The results support the use of RCCs as a useful index of the quantity of SR Ca^{2+} available for release in intact myocytes.

This work was supported by grants from the National Institutes of Health (HL30077, HL31140 and HL34288). J. H. B. B. and K. W. S. are also supported by the Nora Eccles Treadwell Foundation and the Richard A. and Nora Eccles Harrison Fund for Cardiovascular Research. D. M. B. is the recipient of a National Institutes of Health Research Career Development Award (HL01526). We thank Bruce M. Steadman and Kent B. Moore for designing and constructing many of the electronic and mechanical devices required for fluorescence measurements, signal processing and solution switching.

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