Rate of Diastolic Ca Release from the Sarcoplasmic Reticulum of Intact Rabbit and Rat Ventricular Myocytes

Rosana A. Bassani and Donald M. Bers
Department of Physiology, Loyola University School of Medicine, Maywood, Illinois 60153 USA

ABSTRACT The sarcoplasmic reticulum (SR) of cardiac myocytes loses Ca during rest. In the present study, we estimated the rest-dependent unidirectional Ca efflux from the SR in intact rabbit and rat ventricular myocytes. We determined the time course of depletion of the SR Ca content (assessed as the amount of Ca released by caffeine) after inhibition of the SR Ca-ATPase by thapsigargin. Before rest intervals in Na-containing, Ca-free solution, a 3-min preperfusion with 0Na,0Ca solution was performed to deplete Na_i but keep the SR Ca content constant. The decrease in Na_i should stimulate Ca efflux via Na/Ca exchange when Na_o is reintroduced. Thapsigargin treatment was limited to the last 2 min of preperfusion with 0Na,0Ca solution to minimize SR Ca loss before addition of Na, while attaining complete block of the SR Ca pump. Total SR Ca content was estimated from the $[Ca]_i$ transient evoked by caffeine, taking into account passive cellular Ca buffering. The time constants for SR Ca loss after thapsigargin were 385 and 355 s, whereas the pre-rest SR Ca content was estimated to be 106 and 114 μM (μmol/l nonmitochondrial cell volume) in rabbit and rat myocytes, respectively. The unidirectional Ca efflux from the SR was similar in the two cell types (rabbit: 0.27 μM s^{-1}; rat: 0.32 μM s^{-1}). These values are also comparable with that estimated from elementary Ca release events ("Ca sparks," 0.2–0.8 μM s^{-1}). Thus, resting leak of Ca from SR may be primarily via occasional openings of SR Ca release channels. Finally, this flux is very slow compared with other Ca transporters in ventricular myocytes.

INTRODUCTION

The sarcoplasmic reticulum (SR) plays an essential role in the physiology of striated muscle. Ca sequestered into this organelle can be rapidly released through the SR Ca channels during electrical excitation, providing the major fraction of the Ca that activates contraction. In cardiac muscle, SR Ca release seems to be evoked by increase in intracellular Ca concentration ($[Ca]_i$) due to Ca influx during the action potential ("Ca-induced Ca release," Fabiato, 1983).

When isolated and reconstituted into lipid bilayers, the SR Ca channels display activity that depends on the composition of the medium. The activity of the isolated SR Ca channel, as well as Ca efflux from isolated SR vesicles, can be modified by several endogenous ligands (e.g., Ca, Mg, adenine nucleotides, calmodulin) (Meissner et al., 1986; Meissner and Henderson, 1987; Lai et al., 1988; Coronado et al., 1994), which are probably important modulators of the SR Ca release in intact cells. Although these bilayer studies have been extremely valuable in understanding SR Ca release channel gating, it is difficult to extrapolate these results to physiological conditions.

In cardiac muscle, Ca release by the SR appears to take place during rest, albeit at a much lower rate than during excitation-contraction coupling. Ca "leakage" from the SR (followed by Ca extrusion via sarcolemmal Ca transport) is considered to be the mechanism responsible for the rest-dependent decline of twitch amplitude and SR Ca content, a phenomenon characteristic of cardiac tissue from several species (e.g., Bridge, 1986; Janczewski and Lewartowski, 1986; Bers, 1989; Bers et al., 1993; Bassani and Bers, 1994). Recently, Cheng et al. (1993) provided evidence of discrete SR Ca release events ("Ca sparks") in intact, resting rat ventricular myocytes. Such Ca sparks are apparently caused by the opening of SR Ca channels (either a single channel or a cluster of channels in concert) and may represent the elementary event underlying the excitation-contraction coupling process in cardiac muscle.

Although there is evidence that spontaneous SR Ca release does occur during rest, little information is available regarding the rate at which this process occurs. Even though time-dependent changes in SR Ca content have been reported in several studies (e.g., Bridge, 1986; Bers, 1989; Banijamali et al., 1991; Bers et al., 1993; Bassani and Bers, 1994), the time course of these changes does not allow accurate estimates of the rate of the unidirectional Ca flux from the SR due to spontaneous Ca release for the following reason: they reflect *net* changes in the SR Ca content (i.e., part of the released Ca is taken back up by the SR, which tends to limit the SR Ca loss). An example of this is the fact that SR Ca content (as assessed with rapid cooling or caffeine contractures) does not decline after moderate rest (up to 5 min) in rat ventricle under control conditions (Bers, 1989; Banijamali et al., 1991; Bers et al., 1993; Bassani and Bers, 1994). However, a clear rest-decay of SR Ca can be observed in the same preparation when Ca efflux via the Na/Ca exchange during rest is stimulated by manipulation of [Na] and [Ca] gradients (Bassani and Bers, 1994). This indicates that in control conditions in rat ventricular myocytes, practically all Ca released from the SR during rest undergoes reuptake, which masks the unidirectional flux from the SR.

In the present study, we have determined the time course of rest-dependent decline in SR Ca content, as estimated

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Address reprint requests to Dr. Donald M. Bers, Department of Physiology, Loyola University School of Medicine, 2160 S. First Ave, Maywood, IL 60153. Fax: 708-216-6308; E-mail: dbers@luc.edu.

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from the $[Ca]_i$ transients evoked by a supra-maximal concentration of caffeine in ventricular myocytes. We have also devised an approach to overcome the complicating effects of SR Ca reuptake during rest, which entails complete inhibition of the SR Ca-ATPase by thapsigargin, a selective and irreversible SR Ca-ATPase inhibitor in cardiac myocytes (Kirby et al., 1992; Wrzosek et al., 1992; Bassani et al., 1993a). These experiments were performed in ventricular myocytes from two species that typically display opposite contractile responses to rest (rabbit ventricle, which shows rest-decay of twitch amplitude, and rat ventricle, which responds to rest with twitch potentiation). Our results showed that, once the species-dependent differences in SR Ca uptake and Na/Ca exchange are minimized, the rate of spontaneous SR Ca release is very low and similar in both cell types.

MATERIALS AND METHODS

Myocyte isolation

Ventricular myocytes were isolated from rabbit and rat hearts as described previously (Bassani et al., 1994a). Briefly, the heart was dissected, mounted in a Langendorff apparatus, and perfused with nominally Ca-free solution for 5 min. Collagenase (type B, Boehringer Mannheim, Mannheim, Germany, lot FCA134, 1 mg ml^{-1}) and pronase (Boehringer Mannheim, 0.12 mg ml^{-1}) were then added to the solution. After the heart was perfused with the enzymes for 12–20 min, the ventricular tissue was mechanically dispersed and filtered. The cell suspension was repeatedly rinsed to remove the enzymes, with gradual increase in extracellular [Ca] ($[Ca]_o$) up to 1 (rat) or 2 mM (rabbit). Cells were plated on laminin-treated chambers.

Fluorescence measurements

Cells were loaded with indo-1 AM (10 μM, Molecular Probes, Eugene, OR) for 15 min at room temperature (to minimize intracellular indo-1 compartmentalization), after which they were superfused with control Tyrode's solution for 40 min at room temperature (22–23°C), to allow indo-1 washout and intracellular deesterification (Bassani et al., 1992). Using these loading conditions indo-1 is primarily cytosolic, based on fluorescence loss after digitonin treatment and dialysis with patch pipettes lacking indicator (Bassani et al., 1992).

The apparatus for fluorescence measurements was the same as described elsewhere (Bassani et al., 1993b). The excitation wavelength was 365 nm, and fluorescence emission was recorded at 405 and 485 nm. An adjustable window allowed restriction of the microscopic field to the size of the cell. Background fluorescence at both emission wavelengths of an empty field of the same size was subtracted before calculation of the ratio 405/485 (R). R was then converted to $[Ca]_i$ according to the equation (Grynkiewicz et al., 1985): $[Ca]_i = K_d \cdot \beta (R - R_{min})/(R_{max} - R)$, where the dissociation constant, K_d, was measured to be 0.441 ± 0.009 μM in vitro using simultaneous [Ca] measurement with Ca minielectrodes (as described by Hove-Madsen and Bers, 1992). The minimum and maximum R values (R_{min} and R_{max}) and the scaling factor β were determined in vivo (Bassani et al., 1994a).

Experimental procedure

After the indo-1 loading and washout protocol was complete, electrical field stimulation (square waves, 1.5 ms duration, 1.2 × threshold voltage, 0.5 Hz) through a pair of platinum electrodes was started. SR Ca content was evaluated by application of 10 mM caffeine dissolved in 0Na,0Ca solution. SR Ca content was measured under four experimental conditions in each cell. In each case, stimulation was interrupted and cells were immediately perfused with 0Na,0Ca solution to wash out extracellular Na (Na_o) and prevent Na/Ca exchange. Later, SR Ca content was released with 10 mM caffeine in 0Na,0Ca solution that was rapidly switched on using a special switching device (Bassani et al., 1992). Before each caffeine application, cells were stimulated until $[Ca]_i$ transients attained a steady-state value (3–5 min). After caffeine was washed out, cells were rested in control solution for 1 min before resumption of stimulation.

The four basic protocols were as follows:

Steady-state (SS) SR Ca content

Cells were perfused with 0Na,0Ca for 15 s before caffeine application (Figs. 1 *A* and 2 *A*).

SS SR Ca content after long-preperfusion with 0Na,0Ca

In this case, perfusion with 0Na,0Ca was extended to 3 min before caffeine application (Fig. 1 *B*).

Control post-rest SR Ca content

In this protocol, perfusion with 0Na,0Ca (3 min) was followed by perfusion with Na-containing, 0Ca solution for variable periods (30 s to 7.5 min), during which the cells were rested (Fig. 1 *C*). The preperfusion with 0Na,0Ca maintains the SR Ca content constant while Na_i is depleted. Subsequent perfusion with Ca-free solution containing 140 Na then stimulates Ca efflux via Na/Ca exchange and maximally accelerates rest decay in rabbit and rat ventricular myocytes (Bassani and Bers, 1994). However, the SR Ca-pump still competes with the Na/Ca exchange so that rest decay does not represent unidirectional SR Ca efflux.

Post-rest SR Ca content after inhibition of the SR Ca-ATPase

This protocol was similar to those in the second and third steps, except that cells were treated with thapsigargin (5–10 μM) during the last 2 min of the period of preperfusion with 0Na,0Ca to block completely the SR Ca-pump

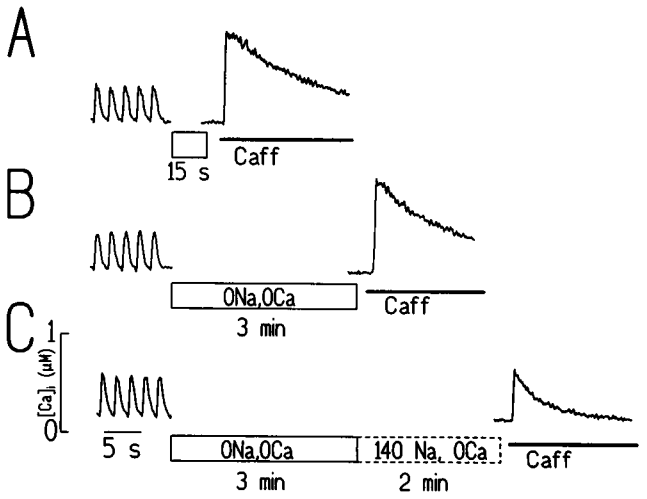

FIGURE 1 $[Ca]_i$ transients obtained in rabbit ventricular myocytes. To assess steady-state SR Ca content, 10 mM caffeine in 0Na,0Ca solution was applied (*solid bars*) after the interruption of electrical stimulation and after 15 s (*A*) or 3 min (*B*) of preperfusion with 0Na,0Ca solution (*open horizontal bars*). Note that the amplitude of the transients was virtually the same. To assess post-rest SR Ca content, caffeine was switched on after an additional rest interval in 140 mM Na, Ca-free solution (*dashed open horizontal bar*) that followed the preperfusion period in 0Na,0Ca (*C*). Experimental traces are all on the same time scale, but breaks in record indicated by bars are not to scale.

(Bassani et al., 1993a). Caffeine was then applied immediately after the period of thapsigargin treatment (Fig. 2 *B*), or after a rest interval in 0Ca solution (Fig. 2 *C*). When the protocol was over, the effectiveness of the thapsigargin treatment was tested with caffeine after 2 min of electrical stimulation (Fig. 2 *B*, last trace). Cells showing any SR Ca reaccumulation after thapsigargin treatment (i.e., that responded to caffeine with a $[Ca]_i$ transient after stimulation) were discarded.

All four protocols were run with each cell. However, because of the irreversible nature of the inhibition of the SR Ca ATPase by thapsigargin, only one rest interval in 0Ca solution was tested per cell (4–6 different cells were used for each rest interval). Thus, we were able to determine the post-rest SR Ca content before and after thapsigargin in the same set of cells (although a different set of cells was used for each rest period).

Solutions

The control Tyrode's solution contained (mM): 140 NaCl, 6 KCl, 1 $MgCl_2$, 1 (rat) or 2 (rabbit) $CaCl_2$, 10 glucose, and 5 HEPES. In the 0Na,0Ca solution, LiCl replaced NaCl, $CaCl_2$ was omitted, and 1 mM EGTA was added. The 0Ca solution was nominally Ca-free. The pH of all solutions was adjusted to 7.4 at 22°C. Thapsigargin stock solution was made in DMSO and diluted (at least 1000-fold) in 0Na,0Ca solution immediately before use (Bassani et al., 1993a). Caffeine was also dissolved in the 0Na,0Ca solution.

Data analysis

Total [Ca] in the cytosol ($[Ca]_t$) was calculated as:

$$[Ca^{2+}]_t = [Ca^{2+}]_i + \frac{B_{max1}}{1 + (K_1/[Ca^{2+}]_i)} + \frac{B_{max2}}{1 + (K_2/[Ca^{2+}]_i)} + \frac{[Indo]_i}{1 + (K_{in}/[Ca^{2+}]_i)}, \quad (1)$$

where B_{max1}, B_{max2}, K_1, and K_2 are the empirical constants for cellular Ca buffering in rabbit myocytes (from Hove-Madsen and Bers (1993a), B_{max1} and B_{max2} = 215 and 702 μmol/l of nonmitochondrial water and K_1 and K_2 = 0.42 and 79 μM, respectively) and was not detectably different in rat myocytes (Hove-Madsen and Bers, 1993b). The last term in Eq. 1 reflects Ca binding to intracellular indo-1 (K_{in} = 0.44 μM). The $[indo\text{-}1]_i$ is not known, but is assumed to be 50 μM because the fluorescence is similar to that where we have estimated $[indo\text{-}1]_i$ more directly with 50 μM indo-1 in patch pipettes (Berlin et al., 1994).

Total SR Ca content ($[Ca]_{SR}$) was considered to be the difference between $[Ca]_t$ at the peak of the $[Ca]_i$ transient evoked by caffeine and diastolic $[Ca]_t$ immediately before caffeine application. $[Ca]_t$ was expressed in μM (μmol/l nonmitochondrial cell water) (Fabiato, 1983; Hove-Madsen and Bers, 1993a). An inherent assumption is that the passive cytosolic Ca buffering is in equilibrium during the peak of these $[Ca]_i$ transients. There may be a fraction of the equilibrium passive intracellular Ca buffering that equilibrates more rapidly (Berlin et al., 1994), but because $[Ca]_i$ declines slowly during these caffeine contractures in 0Na,0Ca, this is unlikely to be a major limitation.

Although use of a different K_d for intracellular indo-1 (e.g., 844 nM) (Bassani et al., 1995) would increase proportionally both the resting and peak free $[Ca]_i$ values, it changes the calculated $[Ca]_{SR}$ by only about 10% (from 114 to 111 μM in rat and from 106 to 91 μM in rabbit). Using the higher K_d also prolongs the time constants estimated for total SR Ca decline by ~30%, but does not change the interpretation of the results.

The amplitude of the $[Ca]_i$ transients (or total SR Ca content released) evoked by caffeine after different rest periods in 0Ca solution was normalized to the steady-state $[Ca]_{SR}$ ($[Ca]_{SRo}$) determined with the second protocol in the same cell (i.e., Fig. 1B, after 3-min preperfusion with 0Na,0Ca solution before inhibition of the SR Ca uptake). The SR Ca rest-decay was fitted with a monoexponential curve with an asymptotic value of zero ($r > 0.9$ for all curves).

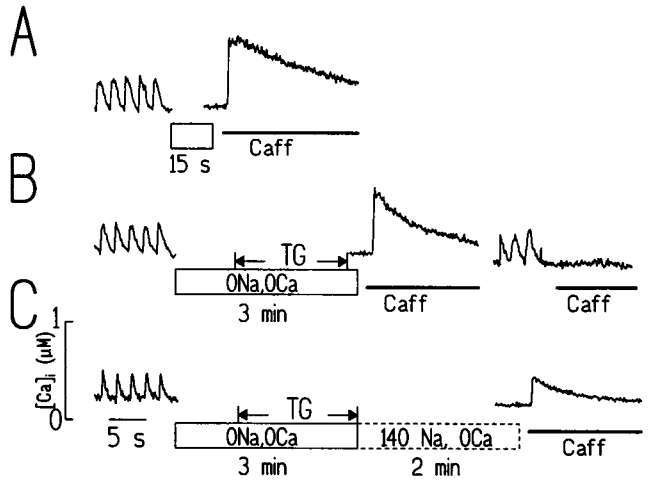

FIGURE 2 $[Ca]_i$ transients in rabbit ventricular myocytes. *A* shows release of the steady-state SR Ca content (similar to Fig. 1 *A*). In *B*, the protocol was similar to that in Fig. 1 *B*, except that the cell was exposed to thapsigargin (TG, to inhibit the SR Ca-ATPase) during the last 2 min of the preperfusion period with 0Na,0Ca solution. Note that this treatment produced only a small decrease in the transient amplitude. Later, the cell was electrically stimulated and again challenged with caffeine (*solid bars*). The lack of response to caffeine confirmed the effectiveness of the thapsigargin treatment. After thapsigargin treatment Ca_i transients during twitches were reduced to an average of 75 and 10% of control amplitude in rabbit and rat myocytes, respectively. Finally, *C* shows release of SR Ca after rest in Ca-free solution (similar to Fig. 1 *C*) in a cell that had been treated with thapsigargin. Note the considerable reduction in the $[Ca]_i$ transient amplitude. Experimental traces are all on the same time scale, but breaks in record indicated by bars are not to scale.

RESULTS

Application of 10 mM caffeine in 0Na,0Ca solution evokes a large $[Ca]_i$ transient (Fig. 1 *A*) due to massive SR Ca release (SR Ca channel activity is greatly increased by caffeine; Rousseau and Meissner, 1989). The slow decline of this transient depends on mitochondrial Ca uptake and Ca extrusion by the sarcolemmal Ca-ATPase (Bassani et al., 1992; Negretti, 1993). The amplitude of this $[Ca]_i$ transient apparently reflects the total caffeine-releasable Ca pool in the SR because: 1) these Ca transport systems do not seem to be sufficiently rapid to curtail the peak of the transient, and 2) subsequent caffeine application after a short interval is unable to evoke increase in $[Ca]_i$ unless Ca influx has been promoted between caffeine applications (Bassani et al., 1992, 1993b).

Inhibition of the Na/Ca exchange during rest prevents net SR Ca loss (Bers et al., 1989; Bassani et al., 1992; Bers et al., 1993; Bassani and Bers, 1994). Accordingly, Fig. 1 shows that the $[Ca]_i$ transient evoked by caffeine in rabbit myocytes is virtually the same whether the cell has been rested in 0Na,0Ca for 15 s (Fig. 1 *A*) or for as long as 3 min (Fig. 1 *B*). The increase of $[Ca]_i$ in response to caffeine was 929 ± 59 nM and 870 ± 53 nM after 15-s and 3- min rest in 0Na,0Ca, respectively,in rabbit cells ($p > 0.05$, $n = 34$). In rat cells, similar results were observed (998 ± 63 and 892 ± 55 nM after 15-s and 3-min rest, respectively;

$p > 0.05$, $n = 37$). Thus, we can conclude that the SR Ca content is reasonably well preserved in these conditions, which allows us to consider the response to caffeine after long (3-min) preperfusion to be representative of the SS SR Ca content. The average SS $[Ca]_{SR}$ was estimated to be 106 ± 3 and 114 ± 4 μM in rabbit and rat myocytes, respectively. Again, it is important to stress that this preperfusion period is not included in the rest interval, which was taken as the interval during which cell Ca loss actually occurs (i.e., when the cell was bathed with Na-containing, Ca-free solution).

Unlike rabbit cells, rat myocytes do not show rest-decay of SR Ca content even after 5-min quiescence in control solution. The reason for this seems to be that in this cell type the Na/Ca exchange is a poor competitor for the Ca released during rest, compared with the SR Ca-ATPase (Bassani et al., 1994a). But if Ca efflux by the exchange is thermodynamically favored by removal of Ca_o, especially after $[Na]_i$ has been depleted by preperfusion with 0Na,0Ca solution, rat myocytes can display rest-decay of twitches and SR Ca, very much like rabbit myocytes (Bassani and Bers, 1994).

Even after stimulation of Ca efflux by the Na/Ca exchange, Ca uptake by the SR Ca pump still takes place during rest (the pump can transport Ca much faster than the exchanger in both rat and rabbit myocytes (Negretti et al., 1993; Bassani et al., 1994a), and this can mask the diastolic SR Ca release. To circumvent this difficulty, we treated the cells with thapsigargin to inhibit the SR Ca-ATPase. This treatment was performed during the last 2 min of the preperfusion period with 0Na,0Ca to minimize SR Ca loss. Even so, Ca could leak from the SR during the treatment, decreasing the SR Ca content even before the rest interval in 140Na solution had started. To evaluate this loss, we applied caffeine to some cells immediately after thapsigargin was washed out. The amplitude of the $[Ca]_i$ transient was changed very little in this condition (compare traces *A* and *B* in Fig. 2). Only a small decrease in the $[Ca]_t$ released by caffeine was observed compared with thapsigargin-free solution (7.5 ± 3.3 and 2.4 ± 2.6% in rabbit and rat cells, respectively, $n = 5$). This Ca loss was taken into account for the determination of the time-constants of SR Ca loss (i.e., as % of the SS $[Ca]_{SR}$ after a rest interval of 0 s). Throughout thapsigargin treatment and the subsequent rest period, diastolic $[Ca]_i$ decreased slowly and monotonically to ~50–80 nM after 7.5-min rest. This was true even when cells remained in Na-free medium (not shown). This indicates that the rate of diastolic Ca release from the SR could probably be matched by the remaining Ca transport systems that remove Ca from the myoplasm (mitochondrial Ca uptake and sarcolemmal Ca-pump) (Bassani et al., 1992) and certainly by the Na/Ca exchanger in 140Na solution.

After inhibition of the SR Ca pump, rest in 0Ca solution produced a faster decline of SR Ca content than when the pump was not inhibited (compare Figs. 1 *C* and 2 *C*). This occurred in both rabbit and rat cells and indicates that Ca reuptake into the SR can indeed limit the SR Ca loss during rest.

The time-courses of decline in the SR Ca content before and after thapsigargin treatment in rabbit and rat myocytes are shown in Figs. 3 and 4, respectively. The time-constants (τ) of SR Ca depletion were higher in the control condition compared with those after thapsigargin treatment. That was expected, because the former probably reflects the net SR Ca loss during rest (SR Ca reuptake of part of the released Ca), whereas the latter is probably indicative of the unidirectional Ca flux from the SR to the cytosol.

Another factor influencing τ is the manner in which the SR Ca content was evaluated. When the increase in $[Ca]_i$ evoked by caffeine after different rest intervals in 0Ca solution was used as the index of the SR Ca content, τ was 316 and 443 s in rabbit and rat myocytes, respectively, before thapsigargin, whereas after SR Ca pump inhibition, it was decreased to 203 and 214 s in rabbit and rat, respectively (Figs. 3 *A* and 4 *A*). On the other hand, τ was prolonged when the SR Ca content was estimated as the increment of $[Ca]_t$ caused by caffeine after each rest interval (687 and 1056 s before thapsigargin in rabbit and rat, respectively; 385 and 355 s after thapsigargin in rabbit and rat, respectively; Figs. 3 *B* and 4 *B*). The larger τ values are due to the nonlinearity of Ca buffering by cellular proteins, which is taken into account in the estimation of $[Ca]_t$. The correction for cell Ca buffering activity thus makes a difference and should allow a more accurate estimate of the rest-dependent changes in total SR Ca content.

However, two interesting points emerge from these results, whatever approach was used to estimate the SR Ca content. First, the control τ values (net flux) were higher in rat than in rabbit cells, despite the procedures used to stimulate Ca efflux by the Na/Ca exchange. Second, after the SR Ca uptake was irreversibly inhibited, both cell types showed comparable time-courses of rest-decay of the SR Ca content.

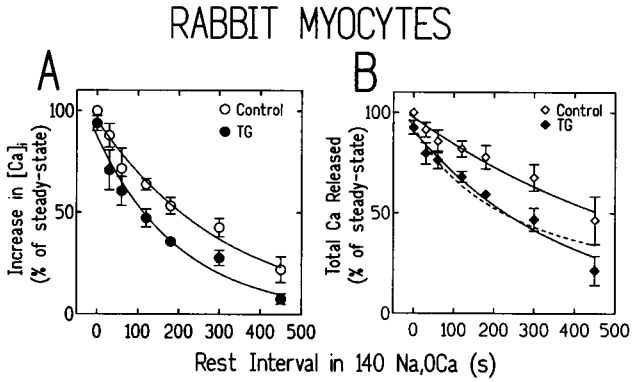

FIGURE 3 Time course of post-rest depletion of the SR Ca content (normalized to the steady-state content determined after 3-min preperfusion with 0Na,0Ca solution) in rabbit ventricular myocytes. Means ± SEM of the same set of cells (4–6 cells/time point) are shown before and after thapsigargin treatment (TG). In *A*, the SR Ca content was evaluated by the increase in myoplasmic free [Ca] in response to caffeine. In *B*, SR Ca was calculated as the total Ca released by caffeine (after correction for cell Ca buffering, see Materials and Methods). Data points were fit with a monoexponential curve, except for the alternative hyperbolic dashed curve in *B* (which is described in Discussion).

These findings indicate that the rate of spontaneous diastolic Ca release is similar in rabbit and rat myocytes. However, in the absence of thapsigargin the SR Ca is lost more slowly in rat. This may be due to the stronger SR Ca-pump in rat, which may compete better with Na/Ca exchange in rat, compared with rabbit myocytes (Hove-Madsen and Bers, 1993b; Bassani et al., 1994a).

DISCUSSION

This study demonstrates that during rest the unidirectional efflux of Ca from the SR in ventricular myocytes is slow. We also demonstrate that in rat ventricular myocytes, which do not ordinarily display rest-decay of twitches or SR Ca content, the unidirectional flux of Ca from the SR during diastole is very similar to that observed in rabbit myocytes, which typically shows rest-decay of twitches and SR Ca content.

Assumptions necessary for the interpretation of results

So that the results obtained in this study can be used for a quantitative estimation of the net and unidirectional fluxes of Ca due to spontaneous SR Ca release in resting ventricular myocytes, several assumptions are required. In addition to the relationship between free and total cytosolic [Ca] (see Materials and Methods), these include the following:

10 mM caffeine can induce release of all the SR Ca content

This caffeine concentration is well above the threshold intracellular caffeine concentration to produce regenerative SR Ca release in cardiac myocytes (1 mM) (O'Neill and Eisner, 1990), and little difference is seen between Ca_i transients evoked by 5–50 mM caffeine (Bassani et al., 1994b). Moreover, shortly after a sustained 10 mM caffeine exposure, a second caffeine application or rapid cooling fails to evoke a $[Ca]_i$ transient (Bassani et al., 1993b; Bers et al., 1989). This indicates that 10 mM caffeine is able to empty the caffeine-sensitive Ca pool in the SR. We are also considering only caffeine-sensitive stores, which we assume represent the SR. There may be other intracellular stores that are, for example, $Ins(1,4,5)P_3$-sensitive. However, thapsigargin can also block Ca uptake into $Ins(1,4,5)P_3$-sensitive pools, but it does not alter the peak or decline of the Ca_i transient evoked by caffeine (Fig. 2) (Bassani et al., 1994b). This suggests that such stores are not significant in the present context.

The SR Ca-ATPase was completely inhibited by thapsigargin

This assumption is essential for determination of the unidirectional Ca efflux from the SR. The protocol of thapsigargin treatment used here was shown previously to cause complete and irreversible inhibition of SR Ca reaccumulation in cardiac myocytes, even after prolonged electrical stimulation (Bassani et al., 1993a). Moreover, care was taken to test a posteriori the degree of inhibition of SR Ca reloading in each cell studied. Thus, we have reason to believe that this requirement has been met satisfactorily. Also, it has been shown that thapsigargin does not affect the rate of Ca release from SR vesicles (Kirby et al., 1992) or the activity of isolated SR Ca channels (Dr. H. H. Valdivia, personal communication).

Ca efflux by the Na/Ca exchange did not affect the SR Ca release rate

This could have been a serious complication if Ca removal via Na/Ca exchange was unable to keep up with the rate of SR Ca efflux. If cytosolic [Ca] increased, it could alter SR Ca release channel gating and complicate the interpretation of the results. Especially in rat cells, where the Na/Ca exchange is weaker, this might be a real concern. However, we have attempted to accelerate Ca efflux via the Na/Ca exchange by Na_i depletion (preperfusion with 0Na,0Ca solution) and removal of Ca_o during the rest period. It should also be noted that there was no increase in $[Ca]_i$ after application of thapsigargin, even in Na- and Ca-free solution. This contrasts with observations in other cell types, where thapsigargin evokes a $[Ca]_i$ transient, part of which is ascribed to Ca release from intracellular stores (Law et al., 1990; Vigne et al., 1992). The reason for this may be that the rate of SR Ca leak is intrinsically low in ventricular cells, which allows even the relatively slow Ca-removing systems (e.g., mitochondrial Ca uptake and sarcolemmal Ca pump, in the absence of Na_o) to keep up with the leak.

SR Ca efflux was not affected by time-dependent changes in endogenous modulators of the SR Ca channels

The main cause of concern here would be modulation of the SR Ca channel activity by, e.g., calmodulin or protein kinases (for a recent review, see Coronado et al., 1994), which may remain activated for several seconds even at the diastolic $[Ca]_i$ observed here (Meyer et al., 1992). However, in our protocols SR Ca efflux assessment started after 3-min preperfusion with 0Na,0Ca solution. This may have allowed complete dissipation of the Ca-dependent effects of these endogenous modulators and provided an adequate steady state for these measurements.

Ca flux from the SR in intact cells: quantitative aspects

In this study, we observed that the total amount of Ca in the SR available for release by caffeine ($[Ca]_{SR}$) declines as a monoexponential function of the rest interval, t:

$$[Ca]_{SR} = [Ca]_{SRo} \cdot e^{-t/\tau}, \quad (2)$$

where $[Ca]_{SRo}$ represents the initial, steady-state $[Ca]_{SR}$ (i.e., after 0 s rest) and τ is the time constant of SR Ca depletion. In rabbit myocytes, $[Ca]_{SRo}$ was estimated to be 106 ± 3 μM ($n = 34$). The values of τ determined in rabbit cells (Fig. 3

B) were 687 (τ_n, i.e., for net SR Ca flux) and 385 s (τ_u, i.e., for unidirectional SR Ca efflux) before and after inhibition of the SR Ca-pump by thapsigargin, respectively. In rat myocytes, $[Ca]_{SRo}$ was 114 ± 4 μM (n = 37). Values of τ_n and τ_u experimentally obtained in rat cells (Fig. 4 *B*) were 1056 and 355 s, respectively.

The $[Ca]_{SRo}$ values estimated in this study are in reasonable agreement with others reported previously using different methodological approaches. For instance, Varro et al. (1993) calculated that caffeine can release 120 μM Ca from SR Ca-loaded rat ventricular myocytes, by integration of the Na-Ca exchange inward current. The peak total SR Ca content in guinea pig myocytes may be estimated to be ~105 μM (after correction for the volume fraction of the SR, 3.5%), from the measurements obtained by Wendt-Gallitelli and Isenberg (1991), using electron-probe microanalysis. An extrapolation from data reported by Hove-Madsen and Bers (1993a, their Fig. 6) for rabbit ventricular myocytes would yield an SR Ca content of ~150 μM at ~0.2 μM diastolic $[Ca]_i$. These and other recent estimates are somewhat lower than most historical estimates (for a review, see Bers, 1991), but may reflect a current convergence toward values of 100–150 μM for SR Ca content in intact cells at normal $[Ca]_i$.

The steady-state rate of SR Ca leak might be expected to be the same as the initial rate of SR Ca efflux at $t = 0$ in our experiments. This is the time derivative of Eq. 2, at $t = 0$ (i.e, $-[Ca]_{SRo}/\tau$). Thus, both net (i.e., with part of the leaked Ca taken back up) and unidirectional Ca flux from the SR (i.e., leak only) can be estimated using τ_n and τ_u, respectively.

Because $[Ca]_{SRo}$ and τ_u (after thapsigargin) were similar in rabbit and rat myocytes, the estimated unidirectional SR Ca flux was comparable in the two cell types: 0.27 and 0.32 μM s^{-1}, respectively. This confirms our previous proposal (Bassani and Bers, 1994) that a lower SR Ca release rate is not the limiting factor that prevents rest-dependent SR Ca loss in rat ventricle.

We have also explored the possibility that the rate constant of SR Ca leak ($1/\tau$) is not constant, but a linear function of the $[Ca]_{SR}$ and (i.e., $1/\tau = a \cdot [Ca]_{SR}$). In this case, the initial rate of SR Ca efflux would be: $d[Ca]_{SR}/dt = -a \cdot ([Ca]_{SRo})^2$, and

$$[Ca]_{SR} = [Ca]_{SRo}/(1 + a \cdot t \cdot [Ca]_{SRo}) \quad (3)$$

This function is expected to be hyperbolic rather than exponential, and the fitting of the experimental points (unidirectional flux, i.e., after thapsigargin) to it can be seen as the dashed lines in Figs. 3 and 4. Reasonably good fits were obtained with both functions, although for long intervals $[Ca]_{SR}$ did not stabilize, as predicted by the hyperbolic function.

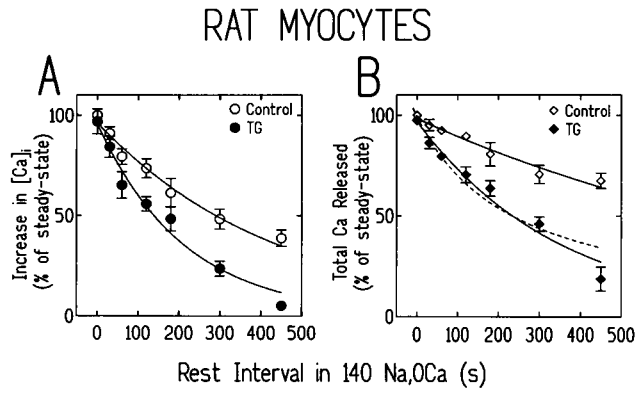

FIGURE 4 Time course of post-rest SR Ca depletion in rat ventricular myocytes (details as in Fig. 3).

Comparison with elementary release estimates

It is possible to extrapolate an estimate for the unidirectional SR Ca flux in rat myocytes from the Ca spark data reported by Cheng et al. (1993). Using the assumptions below, the resting SR Ca flux from these Ca sparks would range from 0.2 to 0.8 μM s^{-1}, which is consistent with our estimate of 0.32 μM s^{-1} in rat cells. For these calculations, we considered the following: 1) the concentration of SR Ca release channels or ryanodine receptors (RyR) was estimated as ~8×10^{16} RyR/l nonmitochondrial water, based on the number of RyR in rat ventricle (833 fmol mg^{-1} protein) (Bers and Stiffel, 1993) after correction for protein concentration (i.e., 100 mg protein/ml ventricular cell) and nonmitochondrial volume (i.e., 65% of cell volume) (Page, 1978); 2) the characteristics of the release channel were assumed to be an opening rate of 10^{-4} opening events · RyR^{-1} · s^{-1} (Cheng et al., 1993), a Ca current of 2 pA at 0 mV (Tinker et al., 1993), and a mean open time of 2.5 or 10 ms · $opening^{-1}$ (Ashley and Williams, 1990; Cheng et al., 1993).

The present study provides a more direct assessment of overall SR Ca efflux rate for the whole cell. However, the similarity to extrapolations from the Ca spark experiments may indicate that the diastolic Ca efflux from the SR in ventricular cells is mostly accounted for by occasional openings of SR Ca release channels.

Comparison with other cellular Ca fluxes

Our SR Ca flux (~0.3 μM s^{-1}) estimate is ~10,000-fold smaller than the peak flux of Ca from the SR calculated during excitation-contraction coupling in mammalian ventricular myocytes (Sipido and Wier, 1991; Wier et al., 1994). This means that if each release event is unitary (i.e., 2 pA Ca current through a single SR Ca release channel) the peak SR Ca release flux (3 mM/s) would require 0.4% of the release channels to be open simultaneously (or 5800 of the 1.6×10^6 ryanodine receptors in a cell with 20-pl nonmitochondrial volume). Furthermore, the total integrated Ca release flux expected during E-C coupling (~50 μM) would require a total of 10,000–50,000 release channel openings (based on 10- or 2-ms mean open time).

The diastolic unidirectional Ca efflux from the SR determined here is approximately 0.15 and 0.33% of the maximal rates of SR Ca uptake estimated in intact rat and rabbit ventricular myocytes, respectively (~100–200 μM s^{-1}) (Bassani

et al., 1994a; Wier et al., 1994). Also, in both cells types the unidirectional SR Ca efflux is still lower (~13-fold) than the lumped maximal rate of Ca transport by the slow systems (mitochondrial Ca uptake and sarcolemmal Ca-pump) (Bassani et al., 1994a), which explains the monotonic decline of diastolic $[Ca]_i$ in the myocytes even after thapsigargin treatment and in 0Na,0Ca solution (i.e., in absence of SR Ca reuptake and Ca extrusion via Na/Ca exchange).

Although the unidirectional Ca efflux from the SR was found to be similar in rat and rabbit ventricular myocytes, the net Ca flux (i.e., that determined before treatment with thapsigargin) was not. Although in rabbit cells the net efflux was estimated at 0.15 $\mu M\ s^{-1}$, in rat myocytes it was only 60% of this value (0.11 $\mu M\ s^{-1}$). If we assume that the difference between the net and the unidirectional SR Ca efflux rates represents a rough approximation of the SR Ca reuptake flux, we could infer that in rabbit myocytes the SR reaccumulates ~45% of the leaked Ca, whereas in rat myocytes this fraction is as great as ~65%, even after biasing the competition in favor of Ca extrusion by Na/Ca exchange. This supports previous results indicating that the SR Ca-ATPase is more powerful in rat than in rabbit ventricular myocytes (Hove-Madsen and Bers, 1993b; Bassani et al., 1994a).

In conclusion, we have estimated the diastolic rate of unidirectional SR Ca efflux in intact rabbit and rat ventricular myocytes by determination of rest-dependent rate of SR Ca depletion. Our estimates generally agree with those projected from experiments analyzing the occurrence of local $[Ca]_i$ transients attributed to elementary, spontaneous SR Ca release events (Cheng et al., 1993). From our data, we are able to infer that, under the present experimental conditions, spontaneous SR Ca release rate during rest is very low, similar in both cell types, and within the capacity of the slow Ca transport systems that remove cytosolic Ca in ventricular cells.

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