Assessment of Intra-SR Free [Ca] and Buffering in Rat Heart

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ABSTRACT To measure the free intrasarcoplasmic reticulum [Ca] ($[Ca]_{SR}$) in isolated rat cardiac microsomes, ventricular tissue was homogenized in the presence of the low-affinity Ca indicator furaptra. Stepwise increases in cuvette [Ca] ($[Ca]_c$) in the presence of ATP caused progressive increases in steady-state intravesicular fluorescence ratio to a maximum (R_{max}). Steady-state $[Ca]_{SR}/[Ca]_c$ was ~7000. Therefore the resting $[Ca]_{SR}$ may approach 700 μM in the rat cardiac myocyte at $[Ca]_c$ = 100 nM. The sarcoplasmic reticulum (SR) Ca pump requires a free energy of $\Delta G \approx 44$ kJ · mol^{-1} to generate this [Ca] gradient (e.g., ~74% of ΔG_{ATP}). Total SR ^{45}Ca uptake was also measured in digitonin-permeabilized myocytes as a function of $[Ca]_c$ in the absence of precipitating ions. The steady-state SR Ca content at 100 nM $[Ca]_c$ was ~400 μmol/liter cytosolic volume. Used together, these data allowed evaluation of the in situ SR Ca-buffering properties. The SR Ca-binding site concentration was ~14 mM, and $K_{d(Ca)} \approx 0.638$ mM $[Ca]_{SR}$.

INTRODUCTION

Cardiac myocytes contain a number of Ca transport mechanisms. Many of these play central roles in Ca homeostasis, and thus cardiac contractility, by pumping ions out of the cytosol. One of the most important of these mechanisms is the sarcoplasmic reticular Ca pumping ATPase (SR Ca pump), a membrane transport process that results in SR Ca sequestration at the expense of ATP.

The first aim of the present work was to measure the free [Ca] gradient that can be generated across the SR membrane by the SR Ca pump. This required measurement of the free intra-SR [Ca] ($[Ca]_{SR}$) as a function of cytosolic [Ca] ($[Ca]_c$), a relationship that has been difficult to measure.

The expected $[Ca]_{SR}$ may be estimated if the main determinant of steady-state SR Ca uptake is the thermodynamic limit of the pump. In this case the $[Ca]_{SR}$ would be dependent primarily upon a balance between the forward and reverse fluxes of the SR Ca pump. Assuming a stoichiometry of 2 Ca/ATP and no voltage gradient across the SR membrane (Beeler, 1980; Meissner, 1981; Somlyo et al., 1977), the limiting $[Ca]_{SR}$ may then be calculated for a given $[Ca]_c$:

$$\Delta G = 2RT \cdot \ln\left(\frac{[Ca]_{SR}}{[Ca]_c}\right) \quad (1)$$

For instance, if the ΔG is 78% of the ΔG of ATP (ΔG_{ATP}) (Allen et al., 1985) or 46 kJ · mol^{-1}, the lumenal $[Ca]_{SR}$ could approach a calculated thermodynamic limit of ~1 mM in the resting cardiomyocyte or a ~10,000:1 $[Ca]_{SR}$: $[Ca]_c$ gradient.

The second aim of this study was to measure SR Ca uptake in a relatively intact state under ionic conditions comparable to those of the $[Ca]_{SR}$ measurements. Thus once the free and total SR [Ca] are known, in situ Ca buffering can be estimated. The buffering characteristics of cardiac SR lumen in situ have not been measured directly.

There have been a number of studies in which a specific effort has been made to estimate the functional capacity of the SR. Estimates have ranged from 87 to 900 μmol · liter cytosol^{-1} under varying conditions (Solaro and Briggs, 1974; Dani et al., 1979; Hunter et al., 1981; Levitsky et al., 1981; Fabiato, 1983; Bridge, 1986; Hove-Madsen and Bers, 1993; Bassani et al., 1995b; Delbridge et al., 1996; Bassani and Bers, 1995). Most of this Ca is probably bound to low-affinity Ca buffers such as calsequestrin, although the actual concentrations of these buffers are unknown (Bers, 1991).

We would expect the overall SR Ca-binding properties to be similar to those of calsequestrin, given its presence as a major buffering protein. Cardiac calsequestrin has been purified (Campbell et al., 1983), and the primary structure has been deduced by cDNA cloning (Scott et al., 1988). Its apparent $K_{d(Ca)}$ is ~0.5 mM, with ~40 Ca-binding sites per mole (Mitchell et al., 1988; Slupsky et al., 1987).

Measurements such as these will be useful in considering the state of the cardiac SR under a variety of physiological and pathophysiological conditions. For instance, such results may be relevant for estimation of the functional capacity of the SR in pathological states where the ΔG_{ATP} or the number of pumps may be reduced (Allen et al., 1985; McCall and Bers, 1996; Levitsky et al., 1991; de la Bastie et al., 1990).

MATERIALS AND METHODS

All chemicals were from Sigma Chemical Co. (St. Louis, MO), except as indicated.

Preparation of cardiac microsomes

Sprague-Dawley rats were anesthetized with sodium pentobarbitol and their hearts were excised. Rat hearts were rapidly perfused with 20 ml cold

Received for publication 23 December 1996 and in final form 6 June 1997.

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0006-3495/97/09/1524/08 $2.00

140 mM KCl, and 40 mM HEPES buffer (pH 7.2) to rinse out residual blood. The atria were trimmed away, and the ventricular tissue was homogenized with a Polytron homogenizer in 50 μM furaptra tetrapotassium salt (Molecular Probes, Eugene, OR), 250 mM sucrose, 20 mM 3-(*N*-morpholino)propanesulfonic acid (MOPS) brought to pH 7.4 with Tris. Homogenates were centrifuged at 6500 $\times$ $g_{(avg)}$ for 20 min at 4°C to sediment unbroken membranes and mitochondria. The supernatant was again centrifuged, at 165,000 $\times$ $g_{(avg)}$, and the pellet was resuspended in 100 μM 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*,*N*-tetraacetic acid (BAPTA) (free [Ca] = 10 nM), 140 mM KCl, 40 mM HEPES (pH 7.2) and quick frozen with liquid N_2.

Intravesicular fluorescence measurement

All fluorescent measurements were performed with a 8100 Series 2 fluorimeter (SLM-Aminco). Microsomes were thawed, washed, and resuspended with the Ca-BAPTA buffer above containing 10 nM free [Ca] and added to a cuvette with constant stirring. A Lowry protein assay was performed to obtain an average protein concentration of ~1 mg/ml (Lowry et al., 1951). Furaptra concentration outside the microsomes in the cuvette was negligible because added Mg failed to increase fluorescence in control experiments (Fig. 1 *B*) and 200 μM Mn failed to quench (not shown). ATP- and Ca-dependent increases in fluorescence were sensitive to A23187 (Fig. 1 *B*). ATP (3 mM with 3.3 mM Mg) was added to start Ca uptake. Experiments were performed in BAPTA buffer to minimize uptake-mediated change in free cuvette [Ca] or $[Ca]_c$ (because this concentration is analogous to the free cytosolic [Ca] in an intact cell). Ca uptake into SR microsomes was measured as an increase in the fluorescence ratio (R, 340/380 nm excitation, 497 emission) until steady state was reached (Fig. 1 *A*). $CaCl_2$ was added to bring $[Ca]_c$ to a new level. The new free Ca was calculated using the computer program MaxChelator (Bers et al., 1994; available at http://www-leland.stanford.edu/~cpatton/). All of the constants used were taken from Martell and Smith (1974) except for those related to EGTA (von Schwarzenbach et al., 1957; Fabiato and Fabiato, 1979). Fluorescence ratio was again measured and this procedure repeated until the dye became saturated (R_{max}). All uptake experiments were performed in the presence of 4 μM oligomycin and 20 μM ruthenium red to inhibit any residual mitochondrial uptake and to block SR Ca release. Contamination of the signal by sarcolemmal and mitochondrial Ca transport was minimal because 20 μM thapsigargin inhibited all Ca-dependent increases in fluorescence in the presence of ATP (not shown).

The minimum R (R_{min}), R_{max}, and the $K_{d(Ca)}$ of the dye were determined as described in the Results.

Ion-selective Ca mini-electrodes

Ca minielectrodes were prepared as described (Baudet et al., 1994). Briefly, polyethylene tubes were dipped in a membrane solution containing 25 mg ETH129 (Fluke Chemika-BioChemika, Buchs, Switzerland), 470 mg nitrophenyl octyl ether, 12.9 mg potassium tetrakischlorophenyl borate, and 250 mg polyvinyl chloride, all dissolved in ~5 ml tetrahydrofuran. The membranes were air dried and filled with a solution containing 140 mM KCl, 10 mM NaCl, 10 mM HEPES (pH 7.2), and 5 mM EGTA (pCa 6).

Myocyte isolation

Rat ventricular myocytes were isolated as described previously (Hryshko et al., 1989). Briefly, Sprague-Dawley rats were anesthetized with sodium pentobarbitol and their hearts were excised. Hearts were perfused with a nominally Ca-free Tyrode solution for 5 min followed by 20 min of perfusion with 1 mg · ml^{-1} collagenase B (Boehringer Mannheim Corp., Indianapolis, IN) and 0.16 mg · ml^{-1} protease (type XIV; Sigma). After enzymatic digestion, the myocytes were filtered and washed twice in a nominally Ca-free Tyrode solution.

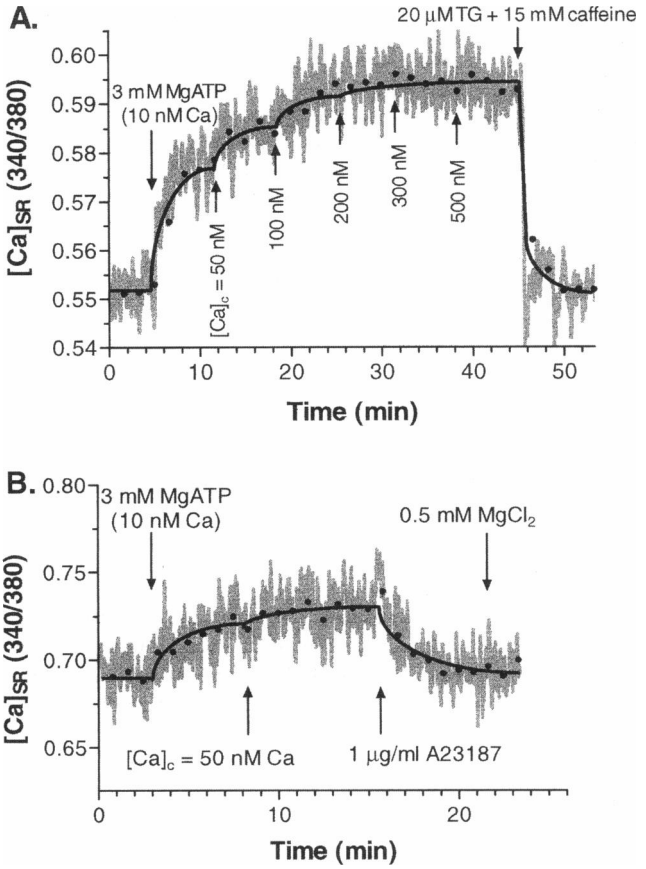

FIGURE 1 Protocol to measure $[Ca]_{SR}$. (*A*) Data are from a typical experiment. The low-affinity Ca indicator furaptra was entrapped within SR vesicles to measure Ca transported by the SR Ca pump. ATP was added to start Ca uptake at $[Ca]_c$ = 10 nM. After steady state was achieved, Ca was added to the cuvette to bring $[Ca]_c$ to the next indicated value. Ruthenium red was present to prevent release at high $[Ca]_c$. The furaptra fluorescence from the intra-SR indicator appeared to saturate when $[Ca]_c$ was at 200–300 nM. Thapsigargin stopped further uptake and 15 mM caffeine caused subsequent Ca release. The shaded area indicates noise in a data trace after minimal (five-point average) smoothing. The closed circles are the average of 50 points, and the solid curve is drawn by eye. (*B*) Control experiment in which all $[Ca]_{SR}$ is released by the addition of 4-bromo A23187, returning R to R_{min} before ATP addition. The addition of 0.5 mM $MgCl_2$ does not cause an increase in fluorescence, indicating that all of the dye is inside the vesicles. The fluorescence drops to the level before ATP addition, indicating that the experiment begins at R_{min}. The ratio data are offset in *B* because of a change in the instrument settings.

ATP-dependent ^{45}Ca uptake

^{45}Ca uptake was measured in digitonin-permeabilized, freshly isolated cardiac myocytes (Hove-Madsen and Bers, 1993). Briefly, isolated myocytes were washed once in a solution containing 100 μM BAPTA (0 Ca), 140 mM KCl, 40 mM HEPES (pH 7.2, BAPTA buffer). They were next washed again in the same solution containing 20 μM digitonin and 10 mM caffeine to release the residual Ca stored in the SR. The Ca-depleted myocytes were washed three times in 20 μM digitonin, BAPTA buffer. These myocytes were diluted into a solution containing 4 μM oligomycin, 20 μM ruthenium red, and BAPTA buffer with indicated $[Ca]_c$. Uptake was initiated after a 5.5-min equilibration period by the addition of 3 mM ATP and 3.3 mM $MgCl_2$. The final protein concentration was ~2 mg · ml^{-1}, as determined by the Bio-Rad Protein Assay (Bio-Rad Laboratories, Richmond, CA). ^{45}Ca uptake into the SR was therefore measured under

conditions nearly identical to those used for the intravesicular fluorescence experiments. Uptake was stopped in 25-μl aliquots of the incubate every 5 min by addition to 3 ml ice-cold 1 mM EGTA, 200 mM KCl, MOPS, Tris (pH 7.4). The myocytes were immediately collected by vacuum filtration. Residual radioactivity on the filters was measured by β-scintillation spectroscopy. Parallel uptakes were measured in the same way on myocytes pretreated with ~10 nmol · mg^{-1} thapsigargin. Reported values are therefore given as thapsigargin-sensitive Ca uptake. Background ^{45}Ca binding in the presence of thapsigargin did not vary with time, but did vary with $[Ca]_c$ (~33% at low [Ca] and 10% at high [Ca]).

Conversion factors

The Ca uptake (measured in nmol · mg myocyte protein^{-1}) was converted to μmol Ca · kg wet weight^{-1} by using 0.57 mg myocyte protein · mg homogenate protein^{-1} and 120 mg homogenate protein · g wet weight^{-1} as conversion factors. The former was estimated based on purification of dihydropyridine receptors and ryanodine receptors in isolated myocytes over ventricular homogenates (Bers and Stiffel, 1993; Lew et al., 1991). Measurements in μmol · kg wet weight^{-1} were changed to μmol · liter cytosol^{-1} by using factors of 0.77 g total water/g wet weight, 0.75 g intracellular water/g total water, 0.36 liter mitochondria/liter cell volume, and 0.65 kg mitochondrial water/liter mitochondria (Fabiato, 1983). Therefore the final conversion factor is $0.77 \times [0.75 - (0.36 \times 0.65)] = 0.4$ liter nonmitochondrial volume · kg wet weight^{-1} (Fabiato, 1983; Bers, 1991). The SR volume was assumed to be 3.5% of the total cell volume in the rat cardiomyocyte (Page et al., 1971; Page, 1978).

RESULTS

Intra-SR free [Ca]

Fig. 1 *A* shows the protocol used to measure intra-SR fluorescence with the entrapped low-affinity Ca indicator furaptra ($K_{d(Ca)} \approx 50$ μM) in a typical experiment. ATP was added as indicated to initiate Ca uptake ($[Ca]_c$ = 10 nM). As Ca was taken up into the microsomes, $[Ca]_{SR}$ rose as indicated by an increase in *R*. Uptake was allowed to reach steady state. Ca was subsequently added to bring $[Ca]_c$ to the indicated concentrations (50, 100, 200, 300, and 500 nM). Additional Ca uptake took place, and the new steady-state *R* was measured after each addition. The intravesicular fluorescence signal appeared to saturate at an extravesicular $[Ca]_c$ of 200–300 nM (Fig. 2*A*). This maximum *R* probably reflects saturation of the indicator with millimolar $[Ca]_{SR}$ rather than $[Ca]_{SR}$ reaching a true limit.

The addition of A23187 to the vesicles after Ca loading at low $[Ca]_c$ (50 nM) decreased the fluorescence back to the level before ATP addition (Fig. 1 *B*). This confirms that the *R* at $[Ca]_c$ = 10 nM before ATP addition can be defined as the minimum fluorescence ratio (R_{min}). Note that the vesicles are washed in this Ca-BAPTA buffer before the beginning of the experiment to deplete the vesicular lumen of Ca.

Fig. 1 *B* also shows that the addition of 0.5 mM Mg did not produce any detectable change in *R*. If any furaptra was not trapped in microsomes, this would have produced a large increase. Similarly, the addition of 200 μM Mn (which quenches furaptra fluorescence) has no effect when added to the cuvette (not shown). If microsomes were exposed to 10 nmol · mg^{-1} thapsigargin, there was no increase in *R* upon Ca addition (not shown). This confirms

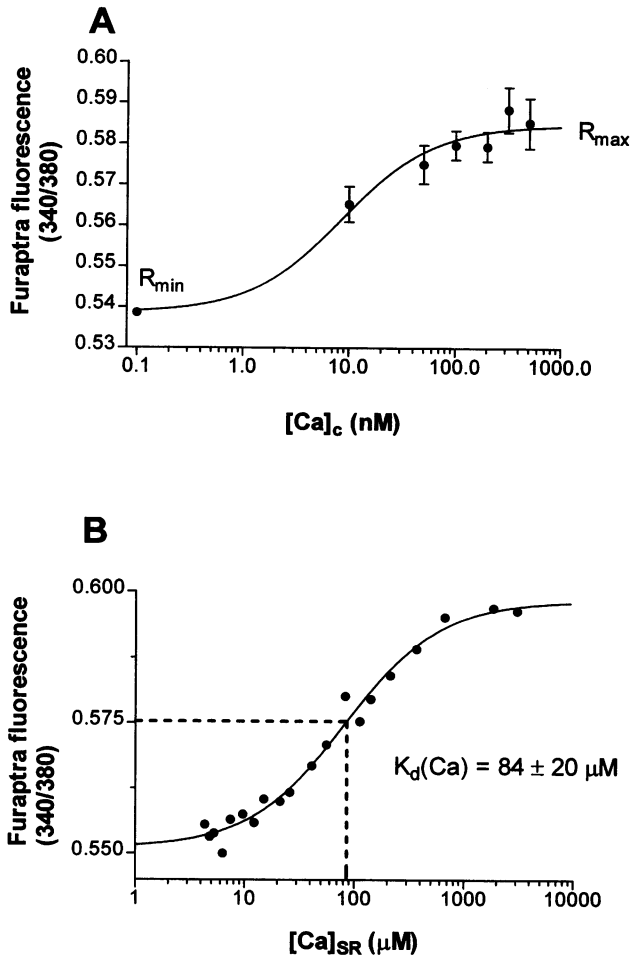

FIGURE 2 Fluorescence calibration. (*A*) Pooled fluorescence results. Data are from four experiments, as in Fig. 1 *A* (averages ± SE, n = 3), normalized to the average R_{min} of 0.5386. R_{max} was determined by sigmoid fit (0.5848). (*B*) Intra-SR furaptra calibration. A typical experiment is shown, where K_d for the intra-SR furaptra was determined after the addition of 15 mM caffeine by equilibration of $[Ca]_c$ with the vesicular lumen through the open Ca release channel. The indicated K_d is the average ± SE of four experiments.

that fluorescence changes are entirely dependent on the SR Ca ATPase.

The dynamic range of the fluorescence signal was small. This is likely due to a large fraction of furaptra trapped in "microsomes" without Ca-ATPase molecules (e.g., mitochondria). The fluorescence from these microsomes that do not have SR Ca pumps is high, but the Ca-dependent fluorescence within them stays at the minimum value. This adds a background at both wavelengths which, although unchanging during the experiment, limits the dynamic range of the ratio signal. Put another way, only a small fraction of the trapped furaptra is in the compartment that produces Ca- and ATP-dependent Ca uptake.

After all values were normalized to the mean R_{min}, R_{max} was determined by fitting the *R* versus $[Ca]_c$ data to a sigmoid curve (Fig. 2 *A*). Fluorescence calibration was subsequently performed in situ (Fig. 2 *B*) to determine the

$K_{d(Ca)}$ of the dye within the SR. After R reached a maximum in Fig. 1 *A*, 15 mM caffeine was added to overcome the ruthenium red block of the SR release channels. This caused Ca efflux at 500 nM $[Ca]_c$ and brought R back down toward R_{min} (see Fig. 1 *A*). Ca was added to the cuvette as in Fig. 1 *A*, but the Ca now equilibrated with the intravesicular compartment through the open Ca release channels. R was measured at each step increase in $[Ca]_c$. $[Ca]_c$ was measured at these high Ca concentrations with a Ca minielectrode. The measured $K_{d(Ca)}$ of the dye in situ was 84 ± 20 μM (n = 4), which agrees reasonably well with the published $K_{d(Ca)}$ of 50 μM (Fig. 2 *B*) (Kurebayashi, 1992).

By using this $K_{d(Ca)}$ and the R_{min} and R_{max} from Fig. 2 *A*, the mean R values can be converted to $[Ca]_{SR}$ as a function of $[Ca]_c$. These data (shown in Fig. 3) form a line with a slope of 7000 (data too near the R_{max} of the dye in Fig. 2 *A* could not be used reliably in this calculation). This relationship suggests a value of 700–1050 μM $[Ca]_{SR}$ for cellular values of resting $[Ca]_c$ (100–150 nM). The slope of the line in Fig. 3 (~7000) is the steady-state [Ca] gradient across the SR membrane ($[Ca]_{SR}/[Ca]_c$) generated by the SR Ca pump through the breakdown of ATP. For a Ca pump stoichiometry of 2 Ca/ATP, generation of this gradient would require 44 kJ · mol^{-1}, as defined by Eq. 1 (Fig. 4). This is 74% of the energy available from ATP if ΔG_{ATP} is assumed to be 59 kJ · mol^{-1} (Allen et al., 1985), although no measurements of pH, ADP, or P_i were made in the present study. In the limit of thermodynamic reversibility the SR Ca pump may therefore function very efficiently under these experimental conditions.

Total SR [Ca]

We used ^{45}Ca to determine total steady-state SR Ca content ($[Ca]_{SR-tot}$) in digitonin-permeabilized myocytes under conditions similar to the above measurements of $[Ca]_{SR}$ in microsomes. Washed, digitonin-permeabilized cells were added to a solution containing 20 μM digitonin and BAPTA buffer. Enough ^{45}Ca was present in the buffer to bring the final $[Ca]_c$ to the indicated concentration. Ruthenium red (20 μM) and 4 μM oligomycin were also present to block the SR Ca release channels and inhibit mitochondrial uptake. ^{45}Ca uptake took place for a period of 10 min, to allow steady state to be achieved (verified by time-course measurements). Parallel experiments were done on thapsigargin-pretreated cells.

Fig. 5 summarizes the mean thapsigargin-sensitive steady-state total Ca uptake data. The half-maximum uptake ($K_{1/2}$) was obtained at 137 nM $[Ca]_c$, and the maximum steady-state SR Ca uptake level was 434 μmol · kg wet $weight^{-1}$ (1000 μmol · liter $cytosol^{-1}$). The maximum SR capacity may therefore be calculated to be 19 mM, assuming 3.5% of the total cell volume is SR and 35% is mitochondria (Page et al., 1971; Page, 1978).

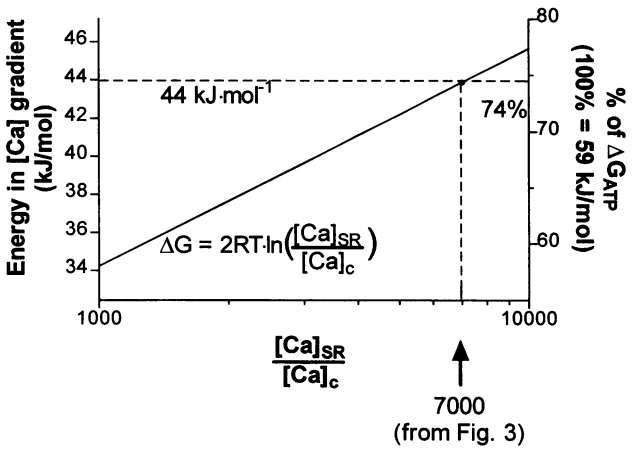

FIGURE 4 Free energy of SR [Ca] gradient production. The free energy used to generate the SR [Ca] gradient was calculated by Eq. 1 (*solid line*). The apparent efficiency is a percentage where ΔG_{ATP} (i.e., 100%) is assumed to be 59 kJ mol^{-1} (Allen et al., 1985). The arrow and the dotted line are based on the $[Ca]_{SR}/[Ca]_c$ slope measured in Fig. 3.

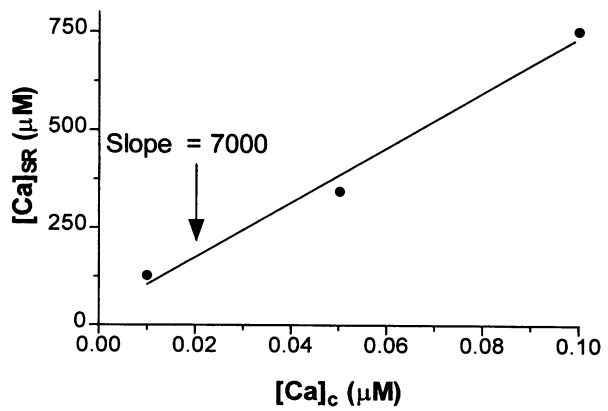

FIGURE 3 SR free [Ca] gradient. Mean $[Ca]_{SR}$ values are plotted as a function of $[Ca]_c$. The slope is the [Ca] gradient across the SR membrane generated by the SR Ca pump. The line is the best-fit linear regression. Data at higher $[Ca]_c$ (Fig. 2) were consistent with these lower values, but were more variable as R approached R_{max}.

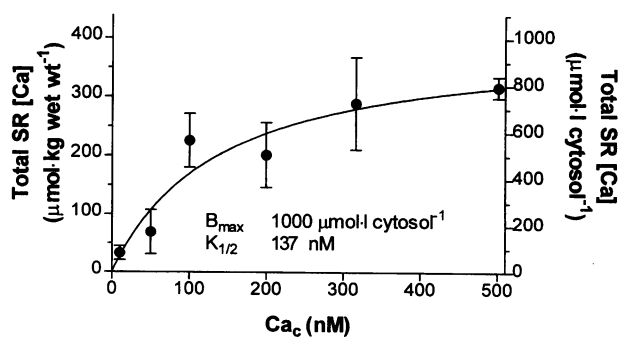

FIGURE 5 Steady-state $[Ca]_{SR-tot}$ in permeabilized myocytes. ^{45}Ca uptake was allowed to come to steady state (10 min) at the $[Ca]_c$ shown. Each experimental data point is the average of the 10- and 15-min values for one experiment. Values are mean ± SE, n = 3 experiments at 500 nM, n = 4 experiments at all other $[Ca]_c$. The curve is a hyperbolic fit to the data ($[Ca]_{SR-tot} = B_{max}/(1 + K_{1/2}/[Ca]_c)$) for the values indicated.

SR Ca buffering

We have estimated the SR Ca-buffering capability by using the present data generated for free and total SR [Ca] as evaluated above (Fig. 6 *A*). Because SR-bound [Ca] ($[Ca]_{SR-b}$) is $[Ca]_{SR-tot} - [Ca]_{SR}$, and both $[Ca]_{SR}$ and $[Ca]_{SR-tot}$ have been measured as a function of $[Ca]_c$, these curves may be manipulated to determine $[Ca]_{SR-b}$ as a function of $[Ca]_c$ (difference between curves in Fig. 6 *A*). This function yields a B_{max} of 740 μmol · liter cytosol^{-1}, and for a resting $[Ca]_c$ of 100 nM the amount bound would be ~375 μmol · liter cytosol^{-1}. The $K_{1/2}$ (90 nM $[Ca]_c$) suggests that the $[Ca]_{SR-tot}$ is at about one-half the SR buffering capacity at rest, allowing room for protection against both Ca overload and depletion.

$[Ca]_{SR-b}$ may also be determined as a function of $[Ca]_{SR}$, thus forming a binding curve for intra-SR buffering proteins (Fig. 6 *B*). Such a function yields a $K_{d(Ca)}$ of 638 μM and a B_{max} of 14 mM (using SR = 3.5% total cell volume).

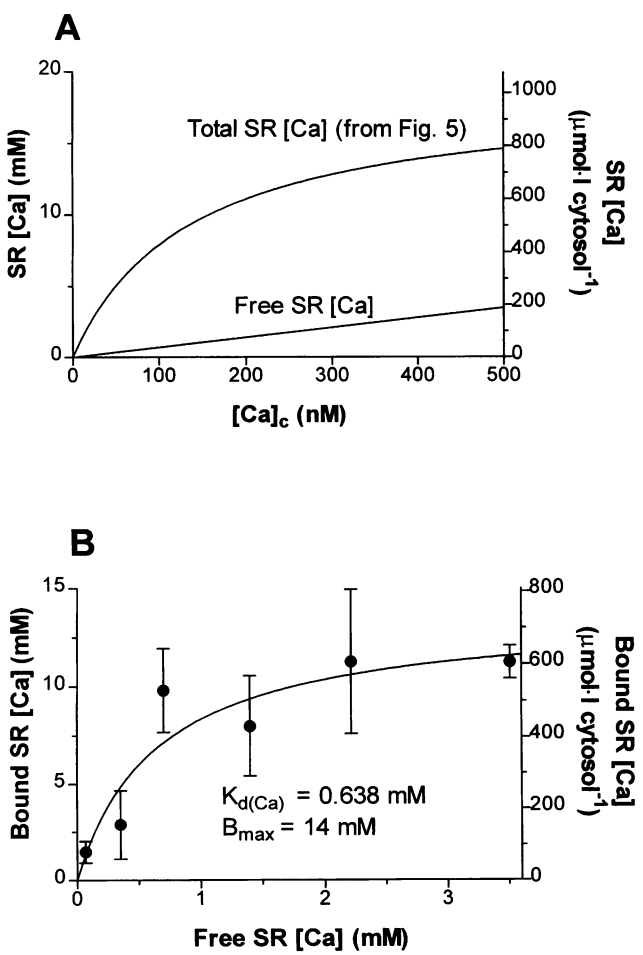

FIGURE 6 SR Ca buffering curve. (*A*) $[Ca]_{SR}$ and $[Ca]_{SR-tot}$ in relation to $[Ca]_c$. Values are converted from μmol · liter cytosol^{-1}, assuming 35% and 3.5%, respectively, of the cell volume are mitochondria and SR (Page et al., 1971; Page, 1978). The $[Ca]_{SR}$ is extrapolated from Fig. 3. (*B*) SR [Ca] bound (based on the difference between the curves in *A*) as a function of $[Ca]_{SR}$. These data are described by $[Ca]_{SR-tot} = B_{max}/(1 + K_{d(Ca)}/[Ca]_{SR})$ for the parameters indicated.

DISCUSSION

In the present study, we have partially characterized the steady-state Ca uptake characteristics of rat cardiac SR. Specifically, we have determined the free and total SR [Ca] as a function of $[Ca]_c$ under similar conditions. We have also used this information to construct a Ca binding curve to describe the buffering characteristics of the SR in situ. This information has previously been unavailable.

Technical considerations

$[Ca]_{SR}$ was determined as a function of $[Ca]_c$ in furaptra-loaded SR vesicles rather than in a more intact preparation. There are two advantages to this method: 1) Homogenization allows trapping of furaptra in SR vesicles, eliminating the need for AM-loading. 2) The partial purification of SR is helpful in limiting fluorescence from indicator entrapped within membranes that do not contain the SR Ca pump (for instance, from mitochondria, which make up ~35% of the cardiac cell volume). This problem is smaller in cytosolic Ca store measurements with most other cell types (Blatter, 1995; Steenbergen and Fay, 1996; Tse et al., 1994).

Furaptra was the dye of choice because its $K_{d(Ca)}$ is among the highest of the Ca-dependent fluorescent dyes (Fig. 2 *B*). This makes it adequate for measuring Ca concentrations up to ~1 mM. We made our measurements at intra-SR Ca concentrations less than this maximum by starting $[Ca]_{SR}$ measurements at very low $[Ca]_c$ (10 nM). This allowed three values of $[Ca]_{SR}$ versus $[Ca]_c$ to be assessed before saturation of the intra-SR indicator.

$[Ca]_{SR-tot}$ was also measured as a function of $[Ca]_c$ under conditions similar to those of the $[Ca]_{SR}$ measurements. In this case digitonin-permeabilized isolated ventricular myocytes were used. This technique eliminates concerns about loss or gain of lumenal SR protein during homogenization and purification. In addition, the use of ^{45}Ca avoids the addition of exogenous buffers (such as furaptra). Changing intra-SR Ca buffering was not a consideration when measuring the $[Ca]_{SR}$ in membrane vesicles, because this uptake was limited by the free [Ca] gradient across the SR membrane (not the amount bound). It is an important consideration, however, when measuring $[Ca]_{SR-tot}$, because much of this Ca is bound to intra-SR proteins.

Ruthenium red (20 μM) was added to all incubates to inhibit any residual mitochondrial Ca uptake and to block SR Ca release in the presence of high $[Ca]_c$. SR Ca uptake cannot take place at high $[Ca]_c$ if the SR Ca release channel is open. We found that blockage of SR Ca release by ruthenium red was easier to overcome with caffeine than when blockage was by high ryanodine concentration. Ruthenium red can also partially inhibit oxalate-supported Ca uptake in rat cardiac homogenate, but this effect is only seen at concentrations greater than the 20 μM used here (Davis et al., 1992). It is not known whether ruthenium red has any effect on steady-state SR Ca content in cardiac tissue (i.e.,

when forward and reverse pump fluxes both take place and net uptake is limited by build-up of $[Ca]_{SR}$).

Phospholamban is an endogenous inhibitor of SR Ca pump rate (Tada et al., 1975; James et al., 1989; Suzuki and Wang, 1986). When phosphorylated, it dissociates from the pump, increasing Ca uptake rate. The phosphorylation state of phospholamban within our preparations has not been determined; however, it is not clear whether this would change the steady-state Ca content (rather than just the time required to achieve that steady state). The actual rate of Ca transport may not be important in the steady-state $[Ca]_{SR}/[Ca]_c$ gradient or net ^{45}Ca uptake as reported here (see Implications, below).

Intra-SR free Ca

Given the $[Ca]_{SR}$:$[Ca]_c$ reported here, a resting $[Ca]_{SR}$ of 0.7–1.05 mM at a resting cytoplasmic [Ca] of 100–150 nM might be expected in intact cells. This resting $[Ca]_{SR}$ value agrees reasonably well with a recent report of diastolic $[Ca]_{SR}$ in the perfused working heart obtained by ^{19}F-NMR (1.5 mM) (Chen et al., 1996).

It should be pointed out that to apply our estimate to the heart under physiological conditions, the following assumptions must be true: 1) that SR Ca uptake is fast enough to reach steady state during the diastolic period in the beating heart; 2) that the $[Ca]_{SR-tot}$ must be primarily dependent upon the thermodynamic limit of the pump rather than a balance between a large Ca pumping rate and a large steady-state leak (mediated, for example, through the Ca release channel). If the steady-state $[Ca]_{SR}$ were primarily dependent upon large but balanced Ca pump and leak rates, then the leak from the SR should be close to the expected unloaded SR Ca pump transport velocity at rest (dictated by $[Ca]_c$). The forward transport velocity at $[Ca]_c$ = 100 nM can be calculated assuming the usual Hill equation and using data from intact cells (Bassani et al., 1994) (208 μmol l^{-1} cytosol $s^{-1}/(1 + ((184\ nM)/(100\ nM))^{3.9}) = 18$ μmol · liter^{-1} cytosol · s^{-1}). This value is 60 times larger than the rate of unidirectional Ca leak from SR in intact rat and rabbit cardiomyocytes (0.32 and 0.27 μmol · liter cytosol^{-1} · s^{-1}, respectively) (Bassani and Bers, 1995). Thus the leak at rest, and therefore the actual SR Ca pumping rate to compensate for it, are very low with respect to the expected transport rate (<2% of the value calculated above). Furthermore, the observation that this situation occurs in intact myocytes and heart (Bassani et al., 1994; Chen et al., 1996) implies that the first assumption above may also be valid. We infer that the steady-state $[Ca]_{SR}$ is most likely limited thermodynamically by the free SR [Ca] gradient.

Total SR [Ca]

Direct measurements of SR [Ca] have been difficult to obtain under relatively physiological conditions. Various values for $[Ca]_{SR-tot}$ in mammalian cardiac muscle have been reported by numerous investigators (87–884 μmol · liter cytosol^{-1}) (Solaro and Briggs, 1974; Levitsky et al., 1981; Hove-Madsen and Bers, 1993; Dani et al., 1979; Hunter et al., 1981; Fabiato, 1983; Bridge, 1986; Bassani et al., 1995b; Delbridge et al., 1996; Bassani and Bers, 1995). In many cases values have been obtained at near-physiological $[Ca]_c$. Our value at 100 nM $[Ca]_c$ of ~415 μmol · liter cytosol^{-1} is in this range.

SR Ca buffering

We have combined our measurements of $[Ca]_{SR}$ and $[Ca]_{SR-tot}$ in a novel way to construct a Ca binding curve describing the in situ buffering characteristics of the SR.

We expect the characteristics of overall SR Ca binding to be similar to those of calsequestrin. Calsequestrin is a major SR Ca-binding protein and may be responsible for a large portion of its buffering capacity. Cardiac calsequestrin is a highly acidic molecule that binds ~35–40 Ca per molecule (900 nmol · mg protein^{-1}) with an apparent $K_{d(Ca)}$ in vitro of 400–600 μM (Mitchell et al., 1988; Slupsky et al., 1987) under experimental conditions similar to ours (100–150 mM KCl, pH 7.0–7.5). Although the calsequestrin-binding properties vary considerably depending upon the environment (e.g., the affinity is known to increase sharply with decreasing KCl; Mitchell et al., 1988), our results are consistent with the $K_{d(Ca)}$ for calsequestrin above and provide a measurement of its in situ binding characteristics. For example, if we assume that there are 2.1 g SR protein · kg wet weight^{-1} (Levitsky et al., 1981), 0.2 g calsequestrin · g SR protein^{-1} (as in skeletal muscle; Campbell, 1986), and a molecular weight of 45,269 g · mol calsequestrin^{-1} (from cDNA cloning) (Scott et al., 1988), a value of 750 μmol Ca binding sites · liter cytosol^{-1} is obtained. Further assuming a tissue density of 1.06 g · ml^{-1} and a fractional volume occupied by the SR of 3.5% (Page et al., 1971; Page, 1978), we may estimate the concentration of Ca-binding calsequestrin sites within the SR to be ~15 mM. This is remarkably close to our maximum $[Ca]_{SR-b}$ value of 14 mM and is consistent with the major role of calsequestrin in intra-SR Ca buffering.

Implications

Among the additional conclusions that can be drawn from the data is that the final $[Ca]_{SR-tot}$ (and the data in Fig. 5) will be independent of the rate of uptake (if the SR Ca uptake reaches a steady state during diastole). It will instead depend only upon: 1) the intra-SR Ca-buffering characteristics and 2) the thermodynamic free [Ca] gradient that can be generated across the SR membrane. In this context, the Ca pump rate does not affect the load at steady state, but only how quickly it is achieved. Furthermore, thermodynamic changes could have major consequences. If the ΔG_{ATP} within the cardiomyocyte drops from 59 kJ · mol^{-1}

to 47 kJ · mol^{-1} during hypoxia (Allen et al., 1985), the SR Ca pump at the apparent efficiency calculated from the present data (74%) will generate a $[Ca]_{SR}$:$[Ca]_c$ gradient of only 1100:1. This sevenfold decline in the gradient (from 7000:1) would lead to a drop in $[Ca]_{SR}$ from 700 to 110 μM and in $[Ca]_{SR-tot}$ from 440 to 90 μmol · liter cytosol^{-1} at 100 nM $[Ca]_c$, using the present values for SR buffering. In this scenario, factors that increase uptake velocity might still be important for sustaining a transport rate sufficient to bring the SR Ca content to steady state within the diastolic period. However, these factors could do nothing to increase the SR Ca load at steady state (which is limited thermodynamically).

It may be noted that several recent estimates of SR Ca content in intact ventricular myocytes have clustered around ~100 μmol · liter cytosol^{-1} (Bassani et al., 1995a,b; Delbridge et al., 1996; Callewaert et al., 1989; Terracciano et al., 1995; Varro et al., 1993). It is not clear why these values are only about one-half to one-quarter of those measured recently in digitonin-permeabilized preparations (Hove-Madsen and Bers, 1993; Kawai and Konishi, 1994; and present study). It is possible that even the small SR Ca leak flux in intact cells (without release channel blockers; Bassani and Bers, 1995) is sufficient to prevent the SR from reaching the limit reported here. This may also be complicated by the spatially heterogeneous nature of the SR Ca leak as Ca sparks (Cheng et al., 1993).

In summary, we have determined the cardiac intra-SR free [Ca] as a function of [Ca] outside the SR and the free energy of the generated [Ca] gradient across the SR membrane and estimated the apparent efficiency of the SR Ca pump. We have used these data in conjunction with measurements of total SR [Ca] under identical conditions to determine the buffering characteristics of the intra-SR Ca binding molecules in situ. These near-physiological measurements are consistent with calculated estimates and have significant physiological and pathophysiological implications.

The authors thank Mrs. Christina Hovance for her careful technical work in isolating the myocytes for some of the experiments performed.

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