Subcellular Ca^{2+} Distribution with Varying Ca^{2+} Load in Neonatal Cardiac Cell Culture

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ABSTRACT Recent work in our laboratory has investigated and modeled subcellular calcium compartmentation and Ca^{2+} movement under steady-state control conditions. This experimental study is directed to the further description and quantitation of cellular calcium compartmentation patterns and movements as correlated with contraction in neonatal rat cardiac myocytes in culture under a variety of calcium loading conditions. Compartmental contents were assessed after incubations in various $[Ca^{2+}]_o$, 0 Na^+/1 mM Ca^{2+}, and 10 μM ouabain/1.0 mM Ca^{2+} test solutions. The cellular components investigated include sarcolemmal bound, sarcoplasmic reticulum (SR), and mitochondrial calcium. The results indicate that 1) sarcolemmal calcium binding is insensitive to changes in $[Ca^{2+}]_o$ in the range tested (0.25–6.0 mM) while highly sensitive to changes in $[Na^+]_i$; 2) SR is sensitive to both changes in $[Ca^{2+}]_o$ and $[Na^+]_i$ and exhibits a maximum loading capacity of ~750 μmol Ca^{2+}/kg dw; 3) in the $[Ca^{2+}]_o$ range between 0.25 and 2.0 mM, contractile amplitude is proportional to SR content; 4) the mitochondria comprise a high-capacity calcium-containing compartment that is sensitive to changes in $[Ca^{2+}]_o$ but does not reach saturation under the conditions tested (0.25–8.0 mM $[Ca^{2+}]_o$); 5) SR calcium is divided into at least two functionally discrete pools, one of which is available for release to the myofilaments during a normal I_{Ca}-triggered contraction and other of which is caffeine releasable but unavailable for release to the myofilaments during a normal triggered release; and 6) mitochondrial calcium serves as a reservoir of calcium capable of replenishing and/or augmenting SR stores with anywhere from 10% to 50% of mitochondrial calcium cycling through SR calcium compartments.

INTRODUCTION

This paper focuses on the definition of subcellular Ca^{2+} compartmentation and movement, as correlated with contractile function in cultured neonatal rat myocardium. The work, in our laboratory, commenced with whole tissue in 1963 (Langer and Brady, 1963) and proceeded, over 35 years, to the presentation of a comprehensive cellular model in 1998 (Peskoff and Langer, 1998). Over this period, a number of techniques were developed that permitted characterization and quantitation of calcium in various organelles as they present in the intact, functional cell. The techniques include on-line monitoring of ^{45}Ca exchange, using the scintillator-disk flow cell technique (Langer et al., 1979), the instantaneous isolation of highly purified sarcolemma by high-velocity gas dissection (Langer et al., 1978), and non-perfusion-limited ^{45}Ca efflux analysis (Langer and Rich, 1992). Using these techniques, in association with various organelle-specific pharmacologic probes, we have measured Ca^{2+} content and flux of mitochondria (Marengo et al., 1997) and the sarcoplasmic reticulum (SR) (Langer et al., 1995), flux specifically via Na/Ca exchange (Langer and Rich, 1992), and Ca binding to the sarcolemma (Post and Langer, 1992) as they exist in the intact cell. This work and that of others are summarized in a recently published text (Langer, 1997).

With few exceptions, these organelle contents and fluxes were measured under conditions of normal, physiological loading conditions. It remains to define compartmentation among the organelles as they respond to a variety of Ca^{2+} loading conditions. Is Ca^{2+} distributed proportionally over a range of loading, or do different organelles (sarcolemma, SR, and mitochondria) respond differently? How does contractile state correlate with the various loading levels? Furthermore, the study examines a functional subcompartmentation of the SR as it pertains to I_{Ca}-triggered Ca release and provides further evidence for an SR-to-mitochondrial exchange route. Because the study examines interaction among subcellular organelles, it is necessary that intact, contracting whole-cell preparations be used for all investigations. The methodologies listed above were employed to separate and define organellar Ca in these cells.

MATERIALS AND METHODS

Cell culture

Hearts from 1–3-day-old Sprague-Dawley rats were harvested and culturing of myocardial cells was accomplished according to a modification of the method of Harary and Farley (1963). The culture method includes the addition of a preplating step. This methodology produces a nearly 100% pure myocyte preparation. Cells were plated on polystyrene, scintillant-containing plastic discs (Bicron, Medford, OH) for on-line $^{45}Ca^{2+}$ studies, nonscintillant 60-mm culture dishes for functional studies, or nonscintillant 70 mm × 30 mm plastic slides cut from standard culture dishes for $^{45}Ca^{2+}$ effluent studies. All three substrates had been Primaria treated (Falcon, Sumpter, SC) to increase cell adhesiveness. Within 3 days a confluent monolayer of spontaneously contractile myocytes was formed. All cell cultures were utilized between 3 and 5 days of age.

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Isotopic studies

$^{45}Ca^{2+}$ exchange was monitored in one of two ways, depending upon the components of cellular calcium exchange being measured. Measurements of $45Ca^{2+}$ effluent activity during nonisotopic washout were used for the investigation of the more rapidly exchanging cellular pools (sarcolemmal bound and SR calcium). On-line measurement of cell-associated $^{45}Ca^{2+}$ activity during nonisotopic washout was used to investigate the more slowly exchanging mitochondrial compartment.

The isotopic studies are used to define compartmental (sarcolemmal, SR, Na/Ca exchange-dependent, mitochondrial) content and flux rates as they respond to various loading conditions. Measurement of contractile function under the same conditions then allows instructive comparisons to be made; e.g., between 0.25 and 2.0 mM $[Ca^{2+}]_o$ contractile amplitude is proportional to SR content.

Effluent analysis

The technique has been described in detail previously (Langer and Rich, 1992). Briefly, slides (30 mm × 70 mm) cut from standard Primaria-treated culture dishes served as the final plating substrate for myocyte cultures used in effluent analysis experiments. The cells were $^{45}Ca^{2+}$ labeled to asymptotic activity by incubation in a buffer of known specific activity. Final specific activity of exchangeable calcium was obtained by sampling of the label solution at the end of the incubation period. The label was removed and the slide placed in a customized perfusion chamber. The chamber was constructed of Lexan and consisted of two plates. The slide, with its $^{45}Ca^{2+}$-labeled monolayer, is fit into an identically sized cutout in the bottom plate. After the slide was seated in place, a second Lexan plate was placed over the top and secured with plastic screws. Inert silicon grease was used to seal the seams and prevent leakage of the perfusate. With the cellular monolayer in place, the washout chamber has a dead space of ~2.2 ml. At a perfusion rate of 3.0 ml/s, the flow cell dead space washed out with a $t_{1/2} = 400$ ms. The inlet port was connected to the output of a series of step-motor-driven syringes via plastic tubing. The step motors are controlled by a microcomputer through which the timing, duration, and volume of the perfusate pulse could be specified. Washout was commenced by activation of the individual step motors, as specified by the experimenter. A rapid switch in perfusate composition was accomplished in this system by programming the microcomputer to activate a second step motor syringe at a specified time during washout. For these studies, a flow rate of one pulse per second delivering 3.0 ml of perfusate per pulse was used. Previous work in our laboratory had determined that this rate of flow is near non-perfusion-limited for the current investigations, thus eliminating isotopic reflux. Each pulse was then collected in a separate vial for later scintillation counting.

After a single washout or series of washouts, the cells were scraped free of the slide and placed on a preweighed filter and dried overnight in a 100°C oven for determination of cellular dry weight. The isotopic activity of each vial of collected effluent was determined by scintillation counting. The counts per minute obtained for each sample were corrected for background, efficiency of counting, and radioactive decay. Counts were normalized to a standard specific activity and cellular dry weight to allow for comparison of different experimental runs. Isotopic activity was then reported as disintegrations per minute per milligram dry weight (DPM/mg dw).

Measurement of Na^+/Ca^{2+} exchange-mediated Ca efflux

Na^+/Ca^{2+} exchange-mediated Ca^{2+} flux was measured using $^{45}Ca^{2+}$ effluent analysis and has previously been described (Langer and Rich, 1992, 1993). Briefly, cell monolayers were incubated in their respective test solutions and isotopically labeled at a specific activity of 30 μCi/μmol Ca^{2+} unless otherwise stated. An incubation period of 20 min was used. This time period was sufficient for complete labeling of all cellular compartments, with the exception of the slowest phase of exchange, which represented mitochondrial calcium (see below). The exchange rate of the mitochondrial compartment ($t_{1/2} \approx 45$ min) was sufficiently slow compared to the other cellular compartments as to render it undetectable in the time frame of the effluent system washout experiments (60–80 s). Therefore, incomplete labeling of the compartment would not be expected to affect the quantitation of the other cellular compartments.

After the label period, washout with nonisotopic buffer was initiated. During the initial phase of washout, both Na^+ and Ca^{2+} were absent from the perfusate, effectively shutting down Na^+/Ca^{2+} exchange activity. Calcium, which would normally exchange with the perfusate via this transport pathway, was thus trapped within the cell during this period (Langer et al., 1995; Langer and Rich, 1993). At the 30th second of washout, the perfusate was switched to one containing 1.0 mM Ca^{2+} and 138 mM Na^+ (control condition). As the exchanger became operative, the cellular calcium that was retained was subsequently exchanged and appeared as a transient increase in effluent isotopic activity (see Fig. 10).

The integrated area under the curve of the transient increase in effluent isotopic activity represented the amount of exchangable Ca trapped by shutting down Na^+/Ca^{2+} exchange. Upon reactivation of exchange, the integrated transient represented exchange of $^{45}Ca^{2+}$-labeled Ca with ^{40}Ca in the washout solution. Previous studies have demonstrated 100% retention of $^{45}Ca^{2+}$-specific activity in this compartment for at least 3 min in the absence of Na^+ and Ca^{2+} during nonisotopic washout. Bypass of blocked Na/Ca exchange via the sarcolemmal ATPase system was minimal, as checked by eosin inhibition during the washout experiments.

Na^+/Ca^{2+} exchange-mediated Ca^{2+} flux is derived from two cellular sources (Langer et al., 1995; Langer and Rich, 1993), the SR and binding sites on the inner sarcolemmal leaflet. The relative contribution of sarcolemma bound calcium to the Na^+/Ca^{2+} exchange-mediated flux under a variety of calcium loading conditions has been previously measured using rapid sarcolemmal isolation techniques and direct measurement of $^{45}Ca^{2+}$ binding (Langer et al., 1995).

Isolation of sarcolemma and measurement of Ca binding

The details of the technique have been described previously (Langer et al., 1978, 1995). The instantaneous isolation of sarcolemma dissected by a high-velocity stream of nitrogen directed parallel to the monolayer culture gives the highest combination of membrane yield and purity yet reported. Recovery is >40%, with a 50-fold purification of Na-K-ATPase, relative to tissue homogenate, an increase of the cholesterol/PL ratio from 0.35 to 0.49 (mol/mol), an increase of the PL/protein ratio from 0.24 to 1.4 (μmol P_i/mg protein), no detectable SR Ca-ATPase activity, and little mitochondrial contamination.

To determine Ca binding, whole cells were incubated in the selected solution (e.g., varying $[Ca]_o$) for 10 min in ^{45}Ca at 30 μCi/ml. After incubation, the solution was drained from the disk containing the monolayer, and each disk was washed in a series of five beakers, which removed all extracellular ^{45}Ca. In each experimental sequence (e.g., a particular $[Ca]_o$ level), the cell-containing disks were divided into two groups. Each group was labeled with ^{45}Ca in identical solution, but one group was washed in standard (Na and Ca present) solution, whereas the other was washed in ONa-OCa solution. Therefore, one wash was done with Na/Ca exchange "turned on" and the other with the exchange "turned off." The group washed in standard solution will have exchanged and washed out all of its Na/Ca exchange-dependent activity (including the fraction bound to the sarcolemma); the ONa/OCa group will have retained all of its Na/Ca exchange-dependent ^{45}Ca (including sarcolemmal bound) (Langer et al., 1995). With these conditions present at the end of washout, each group's sarcolemma was isolated by gas dissection, and the bound ^{45}Ca activity was counted. Lipid phosphorus content was used for normalization of ^{45}Ca activity among disks and to convert membrane-bound ^{45}Ca activity to μmol Ca/kg dry wt cells (see Langer et al., 1995).

Quantitation of sarcoplasmic reticular and sarcolemmal bound calcium pools

The absolute quantity of sarcolemmal bound calcium that contributed to the Na^+/Ca^{2+} exchange-dependent calcium pool was directly calculated from the total Na^+/Ca^{2+} exchange-dependent flux by using the previously determined ratio of SL-to-total flux for each treatment condition. The remaining nonsarcolemmal bound Na^+/Ca^{2+} exchange-dependent calcium flux has previously been identified as being of SR origin (Langer et al., 1995; Post and Langer, 1992; Langer and Rich, 1993). However, it is uncertain whether this nonsarcolemmal component represents the totality of SR calcium. Thus to obtain values that closely approximate total sarcoplasmic reticulum content, the following experimental protocol was used.

Paired experiments were conducted on each cellular monolayer within a given treatment group. In the initial experiment, myocytes were isotopically labeled and nonisotopically washed out in a manner identical to that described for the quantitation of Na^+/Ca^{2+} exchange-dependent flux. After the initial washout, the myocytes were placed in nonisotopic control buffer to allow the cells to recover control compartmentation patterns and to remove any residual isotopic activity. The same cells were then $^{45}Ca^{2+}$-labeled a second time under identical conditions. However, during the subsequent washout, 10 mM caffeine was added to the Na^+- and Ca^{2+}-containing perfusate. Caffeine is known to act specifically at the SR and promote the rapid release of calcium stores (Rousseau and Meissner, 1989). The difference in the integrated quantity of exchanged calcium under these two washout conditions is attributable to caffeine-releasable calcium, which is unequivocally of SR origin. The quantitation of sarcoplasmic reticulum calcium stores is then derived as shown below:

$$([\text{Exc}]_{\text{std}} \times [1 - \text{SL/total Exc flux}]) + ([\text{Exc}]_{\text{caff}} - [\text{Exc}]_{\text{std}}) = \text{SR } Ca^{2+}$$

where $[\text{Exc}]_{\text{std}}$ = integrated content, in μmol/kg dw, of the Na^+/Ca^{2+} exchange transient during a standard washout in the absence of caffeine, and $[\text{Exc}]_{\text{caff}}$ = integrated content, in μmol/kg dw of the Na^+/Ca^{2+} exchange-dependent transient in the presence of 10 mM caffeine, and SL/total Exc flux is the fraction of total exchange-dependent calcium flux that may be attributed to sarcolemmal binding.

Measurement of mitochondrial Ca^{2+} flux

Measurement of mitochondrial calcium flux was accomplished through the use of an on-line system that monitors cellular isotopic activity, rather than effluent isotopic activity during nonisotopic washout, as described previously (Langer and Nudd, 1984). Sixty-millimeter-diameter Primaria-treated polystyrene scintillant-containing discs (Bicron) serve as the final plating substrate for the myocyte culture. These discs, when mounted in place, form the walls of a perfusion chamber. The side of the disc with the attached cellular monolayer faces the interior of the chamber. The volume of the assembled flow cell is 5.6 ml and is turned over completely within 3–5 min at flow rates of 26 ml/min. This chamber was placed in the well of a specially constructed scintillation spectrophotometer, which consists of two photomultiplier tubes placed at the opposite faces of the chamber, in close proximity to the scintillant-containing discs.

After the discs with attached cells were secured in place, the chamber was filled with isotopic label solution, and cells were labeled to asymptote. At the end of the label period, isotopic buffer was removed and washout commenced. Nonisotopic perfusate was delivered to the flow cell via a Gilson Minipuls 2 perfusion pump. Data from the photomultiplier tubes are recorded by computer each minute for the duration of the washout. The cell-associated $^{45}Ca^{2+}$ was counted with an efficiency of 29%. Because this system was used only for the investigation of the slowly exchanging compartment, which is identified between 15 and 60 min of washout, the typical flow rate of 26 ml/min was non-perfusion-limited, indicating that the isotopic activity of the perfusate was maintained near zero. Thus the recorded isotopic activity was a true representation of cellular activity.

Once again, at the completion of the washout, cells were scraped free of the polystyrene discs, placed on a preweighed filter, and dried for determination of cellular dry weight. Counts per minute were converted to disintegrations per minute (DPM) per mg dry weight, as previously described.

The content of the slow component of exchange corresponds to the *y*-intercept of the terminal portion of the computer-fitted linear regression curve. The content is given after correction for incompleteness of labeling calculated from the equation $y = y_o\ (1 - e^{-\lambda t})$. It has previously been determined that this slow component of cellular calcium exchange is representative of mitochondrial calcium turnover on the basis of its response to proton donors and its sensitivity to specific inhibitors of mitochondrial respiration (warfarin, antimycin) (Langer and Nudd, 1984; Langer et al., 1990).

Measurement of contraction

A computerized video technique was used to quantify cell shortening and has been described in detail previously (Rich et al., 1988). For all contraction studies, sparsely plated cultures were used. The cells selected for edge detection were not in contact with other cells. This eliminated the possibility of distorting cell-to-cell interaction, which might vary under different experimental conditions.

Because these cell preparations were spontaneously contractile, the frequency of electrical pacing varied according to the inherent rate of a given monolayer preparation. In other words, the stimulation rate used for any given preparation was just sufficient to capture contractile control. The frequency of spontaneous contractile activity varies with ambient temperature; however, the coefficient of variation of the contractile frequency is ~10% at a given ambient temperature. All studies presented here were conducted at 24°C. Thus it is expected that twitch-dependent SR calcium loading is similarly maintained within a narrow range in these studies (Marengo et al., 1997). Contractile amplitude is relative and is recorded in terms of pen excursion.

Statistics

The results are expressed as mean ± SE unless otherwise indicated.

RESULTS

Cellular calcium compartmentation patterns Na^+/Ca^{2+} exchange-mediated Ca^{2+} flux and varying $[Ca^{2+}]_o$

Varying $[Ca^{2+}]_o$ from 0.25 to 4.0 mM Ca^{2+} results in a graded increase in Na^+/Ca^{2+} exchange-mediated calcium flux, which attains a maximum value of ~875 umol/kg dw at an extracellular calcium concentration of 4.0 mM. Raising $[Ca^{2+}]_o$ to 6.0 mM Ca^{2+} does not increase exchange flux further. Although the difference in the calcium content between the different treatment groups does not reach significance in all pairwise comparisons, analysis of variance (ANOVA) indicates a linear trend between treatment means and $[Ca^{2+}]_o$ with $p < 0.0001$. The values obtained in these Na/Ca exchange studies closely match those obtained previously in our laboratory (Langer et al., 1995) and provide evidence of the reproducibility of the data obtained with these techniques. It was deemed important to demonstrate reproducibility of the preparation and techniques because

current results under varying Ca load will be compared with previous studies done under physiological loading conditions.

Sarcolemmal bound, SR, and mitochondrial compartment Ca^{2+} contents with varying $[Ca^{2+}]_o$

Fig. 1 summarizes the change in the content of sarcolemmal bound, SR, and mitochondrial calcium, each derived as described in Materials and Methods, as the cellular calcium load is changed by varying the extracellular calcium concentration over the range of 0.25 to 8.0 mM. This figure clearly indicates that sarcolemmal calcium binding remains relatively constant under these conditions. In fact, the data show that there is no significant difference between the level of sarcolemmal calcium binding measured after incubations in 0.25 mM $[Ca^{2+}]_o$ and after incubations in 6.0 mM $[Ca^{2+}]_o$. Thus it appears that the sarcolemmal calcium binding is maintained at its maximum capacity (350–400 μmol Ca^{2+}/kg dw), even under low loading conditions when cellular calcium load is altered by changing external calcium concentration. In contrast, SR calcium content shows a graded increase with increasing extracellular calcium concentration in the range of 0.25–4.0 mM $[Ca^{2+}]_o$, at which point it reaches a maximum value of ~750 μmol Ca^{2+}/kg dw. Increasing extracellular calcium concentration further to 6.0 mm Ca^{2+} does not result in any further increase in SR calcium content. The maximum value of SR calcium load obtained (750 μmol/kg dw) represents an 82% increase over the control value (411 μmol Ca^{2+}/kg dw) obtained in 1.0 mM $[Ca^{2+}]_o$. Mitochondrial calcium compartment response to varying $[Ca^{2+}]_o$ differs from the other cellular calcium pools in two ways. First, the absolute calcium content of the mitochondria/kg dw cells is at least twofold and sometimes severalfold greater than any of the other cellular compartments investigated at all $[Ca^{2+}]_o$ tested. Values near 2500 μmol mitochondrial Ca^{2+}/kg dw are attained at an external calcium concentration of 8.0 mM. Thus the mitochondria may be considered a high-capacity calcium-accumulating compartment. Second, whereas mitochondrial calcium content increases with rising extracellular calcium concentration as in the SR, unlike in the SR there is no evidence of a saturation of this compartment within the range of extracellular calcium concentrations tested (0.25–8.0 mM $[Ca^{2+}]_o$). As is true for the other cellular calcium compartments, which increase in content with increasing extracellular calcium concentration, ANOVA analysis indicates a significant linear trend between treatment means and $[Ca^{2+}]_o$, $p < 0.0001$.

Response of contraction amplitude to varying $[Ca^{2+}]_o$

The functional response of the neonatal rat cardiac myocyte to increasing extracellular calcium concentration is manifested as a progressive increase in contraction amplitude relative to the control condition in the range of 0.25–2.0 mM $[Ca^{2+}]_o$ (Fig. 2). The paced contraction amplitude reaches its maximum (118% of control amplitude) at 2.0 mM $[Ca^{2+}]_o$, and further increases in $[Ca^{2+}]_o$ do not increase the degree of cell shortening beyond this value. In fact, as the extracellular calcium concentration exceeds 4.0 mM, contraction amplitude begins to decline as the cells exhibit evidence of an increase in diastolic tension. For this reason data collected at higher calcium concentrations are not included in Fig. 2 (it is interesting to note that this rise in diastolic tension is first evidenced at the same $[Ca^{2+}]_o$ at which the SR reaches its maximally loaded condition (Fig. 1)). Thus there is a clear dissociation between the pattern of SR calcium accumulation and paced contraction amplitude in the range of 2.0–4.0 mM $[Ca^{2+}]_o$.

Saturation of myofilament calcium binding sites at high SR calcium loads and release is clearly not responsible for

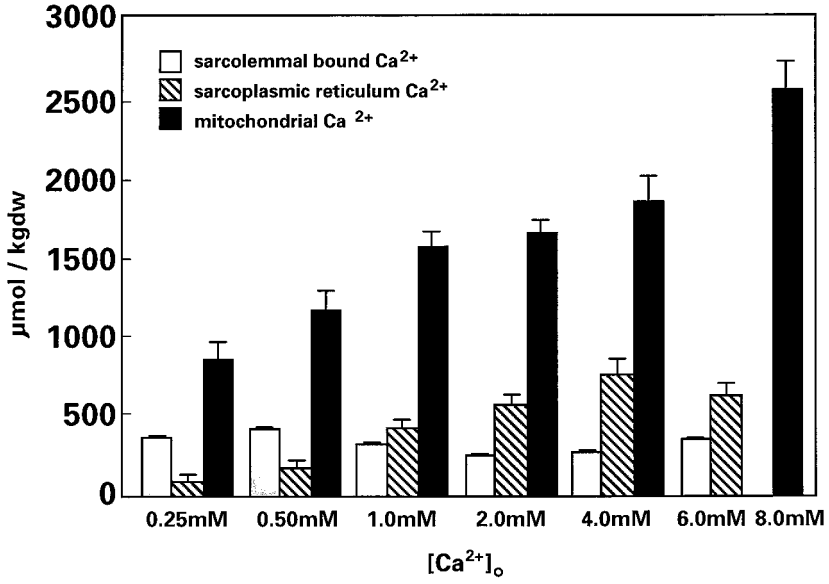

FIGURE 1 Comparison of sarcolemmal bound, sarcoplasmic reticulum, and mitochondrial compartment Ca^{2+} contents in various levels of $[Ca^{2+}]_o$.

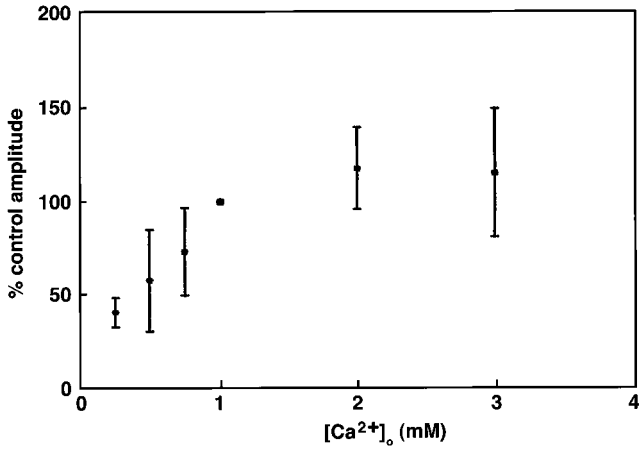

FIGURE 2 Effect of graded increase in $[Ca^{2+}]_o$ on contractile shortening response. Each point except those at 0.25 mM $[Ca^{2+}]_o$ represent mean values from at least eight cells. Cells were electrically paced at a rate just sufficient to capture contractile control. Response at 1.0 mM $[Ca^{2+}]_o$ is set at 100%.

the plateau of contraction amplitude noted at 2.0 mM $[Ca^{2+}]_o$. Introduction of 10 mM caffeine into the bathing medium results in a contracture that is significantly larger than the steady-state contraction amplitude of the same cell paced in either 1.0 mM or 2.0 mM Ca^{2+} (data not shown).

Studies exploring the relationship between extracellular calcium concentration and the absolute amount of calcium entering through the L-type channels (as determined by integration of I_{Ca}) indicate that transsarcolemmal calcium influx via the L-type channel saturates at an extracellular calcium concentration of 2.0 mM (Wang et al., 1993), the same value at which twitch amplitude plateaus in these cells. Others have reported much higher $[Ca^{2+}]_o$ levels at which I_{Ca} saturates. However, these studies were done with either EGTA in the patch pipette (Hess and Tsien, 1984) or Bay K 8644 in the bathing solution (Hess et al., 1986). Both produce significant augmentation of I_{Ca}. Neither of these additives was present in the study by Wang et al. (1993).

In an effort to clarify the extent to which transsarcolemmal derived calcium contributes to myofilament activation in the neonatal preparation, SR calcium stores were partially depleted by the addition of 1 μM thapsigargin to the perfusate. Within 30 s of the introduction of thapsigargin into the bathing medium, paced contraction amplitude declines to near zero before a slower transient oscillatory contraction begins, which does not match the external pacing rate and whose magnitude is less than 40% of the steady-state amplitude of the paced contraction under control conditions. Thus in the neonatal preparation, the introduction of thapsigargin results in almost complete inhibition of paced contractile function, indicating the predominant role of SR-derived Ca^{2+} in myofilament activation.

Ca^{2+} compartment contents with varying $[Na^+]_o/[Na^+]_i$

Fig. 3 illustrates the effect on the various cellular calcium compartments of altering $[Na^+]_o/[Na^+]i$. Maximum Na^+/Ca^{2+} exchange-dependent flux of ~875 μmol Ca^{2+}/kg dw appears to represent an absolute rather than condition-dependent maximum for this pool at room temperature (23–24°C). In contrast, maximum sarcolemmal calcium binding capacity increases by 59% (506 μmol Ca^{2+}/kg dw) relative to the control value (319 μmol Ca^{2+}/kg dw) after incubations in 0 Na^+ test solution and decreases by 45% (187 μmol Ca^{2+}/kg dw) relative to the control value after incubations in 10 μM ouabain-supplemented test solution. Therefore, sarcolemmal binding capacity, which is minimally affected by large changes in cellular calcium load induced by changing extracellular calcium concentration, is quite sensitive to changes in cellular sodium dynamics. Similar to sarcolemmal binding, the SR calcium content is sensitive to changes in cellular sodium dynamics. While

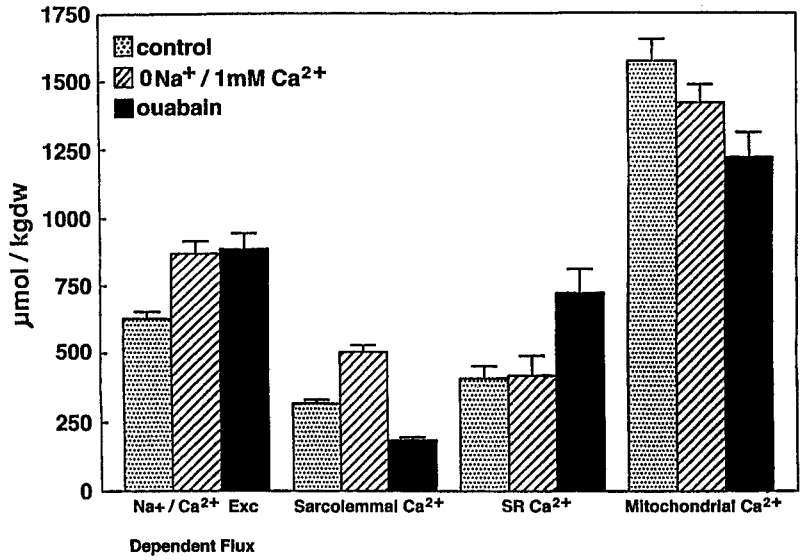

FIGURE 3 Comparison of Na^+/Ca^{2+} exchange-dependent Ca^{2+} flux, sarcolemmal bound Ca^{2+}, sarcoplasmic reticulum Ca^{2+}, and mitochondrial Ca^{2+} contents (in μmol/kg dw) in response to varying $[Na^+]_o/[Na^+]_i$. Bars = SE.

incubation in 0 Na^+ solution does not significantly alter SR calcium content relative to the control condition (420 versus 411 μmol Ca^{2+}/kg dw, respectively), incubations in 10 μM ouabain-supplemented solution results in a 76% increase in compartment content to a value of 724 μmol Ca^{2+}/kg dw. This value approximates the maximum loading value obtained by incubations in high $[Ca^{2+}]_o$. Thus, like the Na^+/Ca^{2+} exchange-dependent flux, the maximum value of SR calcium load appears to be an absolute upper limit that is independent of the method of cellular calcium loading. The mitochondrial compartment, unlike the other cellular calcium pools investigated, appears to be insensitive to changes in cellular calcium load induced by altering $[Na^+]_o/[Na^+]_i$. The mitochondrial values of Fig. 3 are not statistically different.

SR calcium pools

Cells were subject to regular electrical stimulation in control solution (1.0 mM Ca^{2+}) for a period of time sufficient to establish steady-state contraction amplitude (Fig. 4). While electrical stimulation continued, the perfusate was switched to a 0 Ca^{2+}-containing solution. The bath turnover is complete within 300 ms. After contractile activity ceased, 10 mM caffeine was applied and remained in the perfusing solution until the subsequent contracture fully relaxed. After the return to baseline diastolic tension, the caffeine was washed out, and perfusion with 0 Ca^{2+}-containing solution continued. This procedure was repeated until a subsequent caffeine pulse failed to elicit a mechanical response, indicating a depletion of SR calcium stores. The cell was then perfused for 1.2 s with a 1.0 mM Ca^{2+} solution and immediately returned to 0 Ca^{2+} perfusate. It is noteworthy that during this brief exposure to calcium-containing perfusate, diastolic tension remains unchanged and no contractile response is evident. However, subsequent exposure to 10 mM caffeine elicits a large contracture whose amplitude is ~240% larger than steady-state contraction amplitude and ~85% as large as the initial caffeine contracture obtained immediately after the switch to 0 Ca^{2+} perfusate (Fig. 4 *A*). These results are indicative of a very rapid sequestration of available cytosolic calcium by the SR. Within 1.2 s of exposure to 1.0 mM external calcium, the SR is able to sequester more than enough calcium to fully support steady-state contraction amplitude. The same cell was again perfused with 0 Ca^{2+} solution and subjected to a second series of caffeine pulses to once again deplete the SR of calcium (Fig. 4 *B*). When the cell was subsequently perfused with 1.0 mM Ca^{2+}-containing solution, recovery of steady-state contraction amplitude required 20–30 s to completely develop. The recovery of contractile parameters is characterized by a positive staircase response after a 5–6-s delay (Fig. 4 *B*). This experiment shows that not all caffeine-releasable calcium is available for release during a normal excitation-contraction cycle, supporting the theory that SR calcium is divided into a least two functionally distinct compartmental pools.

Isotopic flux studies

Additional support for the compartmentalization of SR Ca^{2+} stores is provided by the biexponential nature of the exchange kinetics of caffeine-releasable stores. Cells were labeled in standard control buffer for 20 min at a specific activity of 100 μCi $^{45}Ca^{2+}/\mu$mol Ca^{2+}. Washout with nonisotopic control buffer commenced at the end of the label period. At variable times during the washout, 10 mM caffeine was introduced into the perfusate to release SR Ca^{2+} stores. This calcium release is reflected in a transient rise in isotopic activity in the effluent. Quantitation of the released calcium is accomplished by integration of the area under the transient. Fig. 5 shows a semilog plot of caffeine-releasable $^{45}Ca^{2+}$ quantitated at different time points during washout and its best-fit nonlinear regression curve. Nonlin-

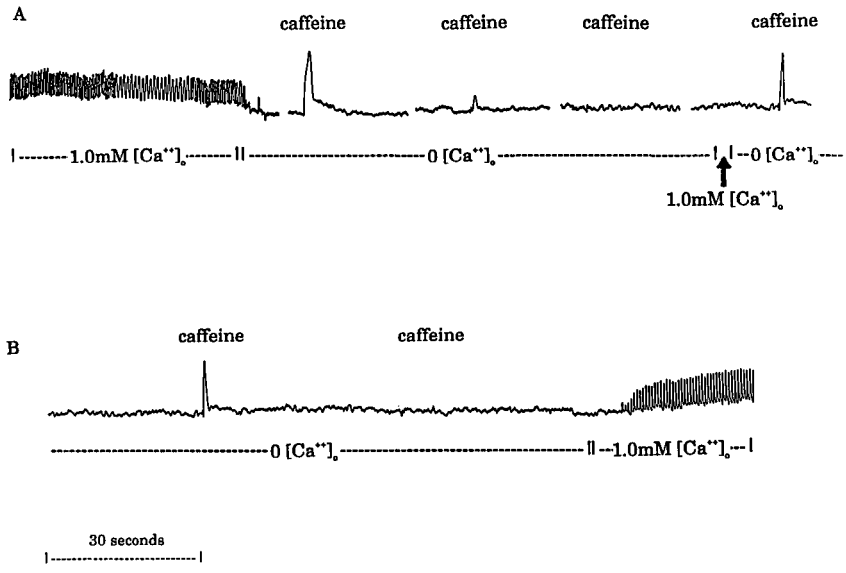

FIGURE 4 Representative chart recording demonstrating the decline of caffeine releasable SR Ca^{2+} after 0 $[Ca^{2+}]_o$ perfusion, its reaccumulation during a short Ca^{2+} pulse, and its availability for release to the myofilaments during a normal paced contraction cycle. See text for detailed explanation.

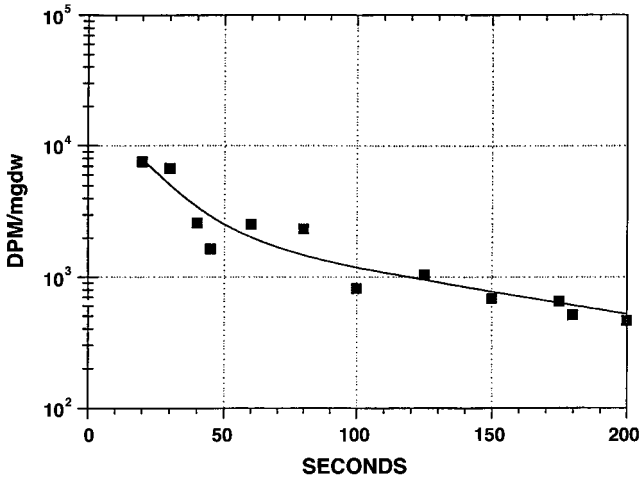

FIGURE 5 Semilog plot of total caffeine releasable $^{45}Ca^{2+}$ present at different times during washout. Each data point represents the mean of at least four caffeine additions. Nonlinear regression analysis shows the biexponential curve fit to these data points. Halftimes of turnover of the two kinetic components are 7 and 95 s, representing a total caffeine releasable pool of 666 μmol Ca^{2+}/kg dw.

ear regression analysis describes biexponential exchange kinetics for this particular cellular calcium pool with half-times of turnover of 7 and 95 s. Using these half-times, extrapolation to total releasable ^{45}Ca at time 0 indicates a caffeine-releasable pool of 666 μmol Ca^{2+}/kg dry wt.

Response of SR Ca^{2+} pools to 0 $[Ca^{2+}]_o$ functional studies

Fig. 6 is a representative example of the effect of variable times of exposure to 0 $[Ca^{2+}]_o$ on caffeine contractures. Panels *A*, *B*, and *C* were extracted from a single continuous chart recording of events taking place in a single cell. Therefore, the contractile activity noted in the left-hand side of panels *B* and *C* demonstrates that the contracture noted on return to calcium-containing solution on the right-hand side of panels *A* and *B* does not indicate irreversible changes. After 2 min (Fig. 6 *A*) and 5 min (Fig. 6 *B*) of perfusion with 0 Ca^{2+} buffer, subsequent caffeine contractures are significantly larger than the steady-state control contracture (169% and 160%, respectively). Even after 10 min (Fig. 6 *C*) in 0 $[Ca^{2+}]_o$, the caffeine contracture does not decline relative to the steady-state condition (122%). Not until a period of up to 20 minutes in 0 $[Ca^{2+}]_o$ does the magnitude of the caffeine contracture decline relative to the steady-state control contracture (data not shown). Previous studies conducted in neonatal myocytes in culture have demonstrated a continuous calcium leak from the SR under 0 $[Ca^{2+}]_o$ conditions (Post et al., 1993). Thus a discrepancy exists between the previous studies and the data obtained from the studies presented in Fig. 6, which show no evidence of a decline in SR calcium stores over a period of 10 min in 0 $[Ca^{2+}]_o$, as assessed by caffeine contracture. These seemingly disparate results can be reconciled if the SR is, in fact, accessing another internal store of calcium.

Paired caffeine contractures

Fig. 7 *A* illustrates the experimental protocol used to more definitively determine that the SR is accessing another intracellular source of calcium. After the cells had attained steady-state contraction amplitude in control conditions (1.0 mM Ca^{2+}, 138 mM Na^+), the perfusate was again switched to a 0 Ca^{2+} solution. When contraction ceased, an initial caffeine pulse was applied and maintained until the attendant contracture (T1) was complete and baseline diastolic tension had been reestablished. The caffeine was washed away, and the cells were again maintained in 0 $[Ca^{2+}]_o$ for variable periods of time before a second caffeine pulse was applied. The amplitude of the second contracture (T2) was recorded and reported as a percentage of the first contracture (T1). Fig. 8 shows the response of a representative preparation. It is evident that there is a clear time dependency to the recovery of T2 in the absence of an external source of calcium, indicating an intracellular source of calcium redistributing to caffeine-releasable SR sites.

In 10 of 10 experiments conducted in different monolayer preparations, a clear time-dependent increase in T2 is demonstrated. The time course of the recovery of T2 is quite variable between preparations; therefore a generally applicable time constant of recovery could not be obtained. Nonetheless, the qualitative results are invariant, displaying a time-dependent increase in caffeine-releasable calcium stores after depletion in the absence of an extracellular source.

Inhibitors of mitochondrial respiration

Because the largest intracellular store of Ca^{2+} is found in the mitochondria, the effect of FCCP (an inhibitor of mitochondrial respiration) (Cheung et al., 1986) on caffeine contractures obtained after variable periods in 0 Ca^{2+} was investigated. The experimental protocol and a typical response are illustrated in Fig. 9. Fig. 9 *A* demonstrates the effect of 2 min of 0 Ca^{2+} perfusion on the amplitude of a caffeine-induced contracture. In this instance, the caffeine contracture is 156% of the amplitude of the control contracture. This result is similar to the results obtained in the functional studies depicted in Fig. 6. In Fig. 9 *B*, however, the caffeine-induced contracture obtained after 2 min in 0 Ca^{2+} in the presence of 50 μM FCCP has an amplitude that is only 81% of the value of the control contracture of the same cell.

This experimental protocol was repeated in seven different cellular preparations. In each case a similar response was observed. The mean value of the amplitude of the caffeine contracture relative to control after 2 min in 0 $[Ca^{2+}]_o$ is 141% in the absence of FCCP and 64.5% in the presence of FCCP ($p = 0.01$).

Mitochondrial isotopic studies

To further investigate the role of the mitochondria in replenishing SR calcium stores, compartmentation studies

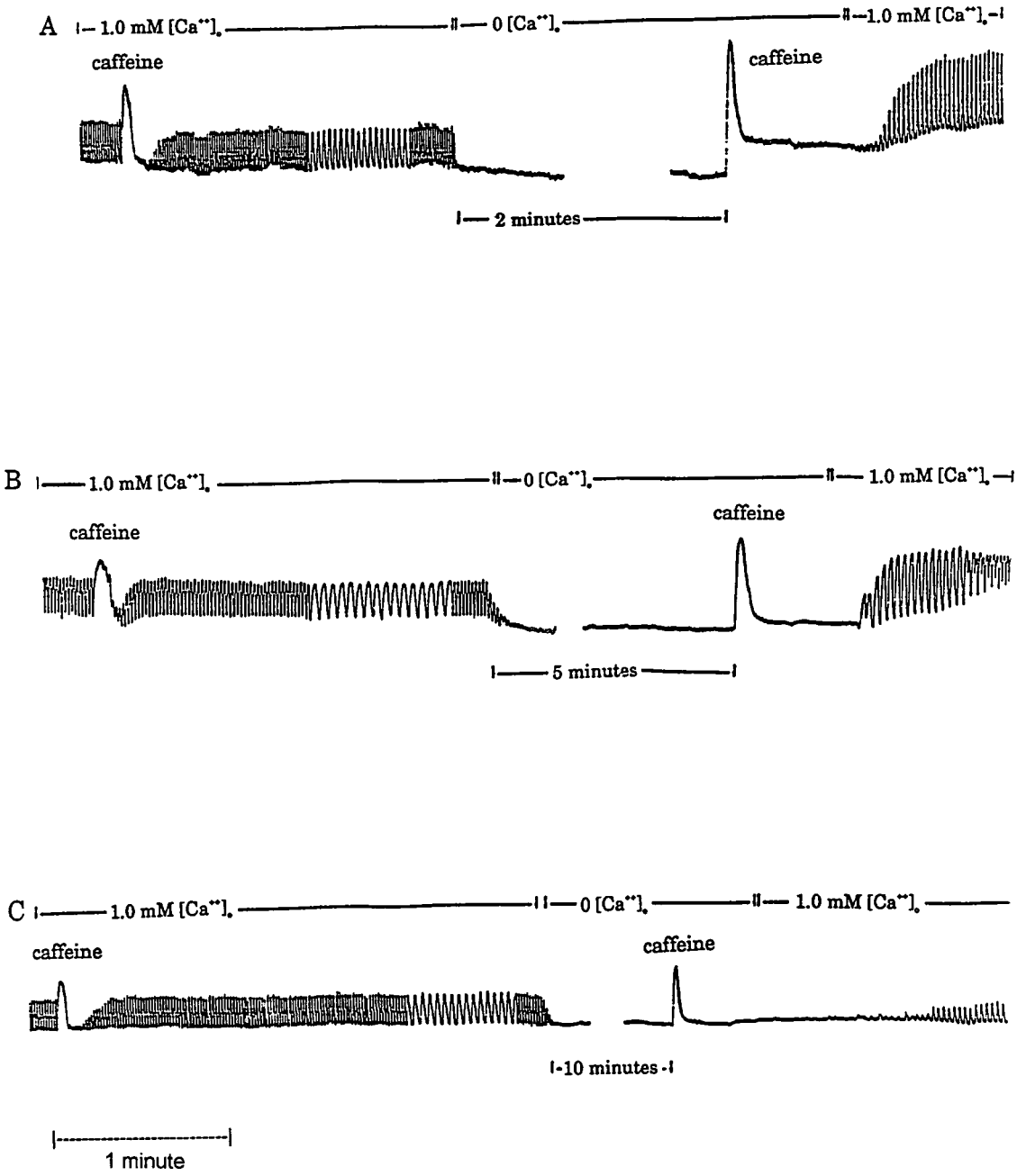

FIGURE 6 Representative example of the effect on caffeine contractures of various exposure times to 0 $[Ca^{2+}]_o$. The chart recordings shown in *A–C* were obtained from a single cell. *A*, *B*, and *C* represent 0 $[Ca^{2+}]_o$ exposure times of 2, 5, and 10 min, respectively (indicated by a break in the chart recordings).

were undertaken in which mitochondrial-specific calcium stores were manipulated and the effect on SR content was investigated.

Isotopic flux studies were conducted which utilized NaH_2PO_4 to target cellular calcium uptake specifically to the mitochondria. Addition of 10 mM NaH_2PO_4 to the extracellular medium increases the availability of protons to the electron transport chain. H_2PO_4 exchanges with mitochondrial hydroxide ions via a phosphate/hydroxide antiporter. This exchange is equivalent to the entry of H_3PO_4, followed by the loss of a proton to the mitochondrial matrix. The excess anion then allows for the accumulation of calcium as a phosphate salt (Lehninger, 1974).

Previous studies have demonstrated that inclusion of 10 mM NaH_2PO_4 in the extracellular medium promotes a net calcium uptake in cultured neonatal heart cells that is suppressed by warfarin, a specific inhibitor of mitochondrial respiration (Langer and Nudd, 1980). Furthermore, the phosphate-dependent calcium uptake has been specifically localized to the slowly exchangeable mitochondrial calcium compartment in both adult and neonatal rat myocyte preparations (Langer and Nudd, 1980; Langer et al., 1990).

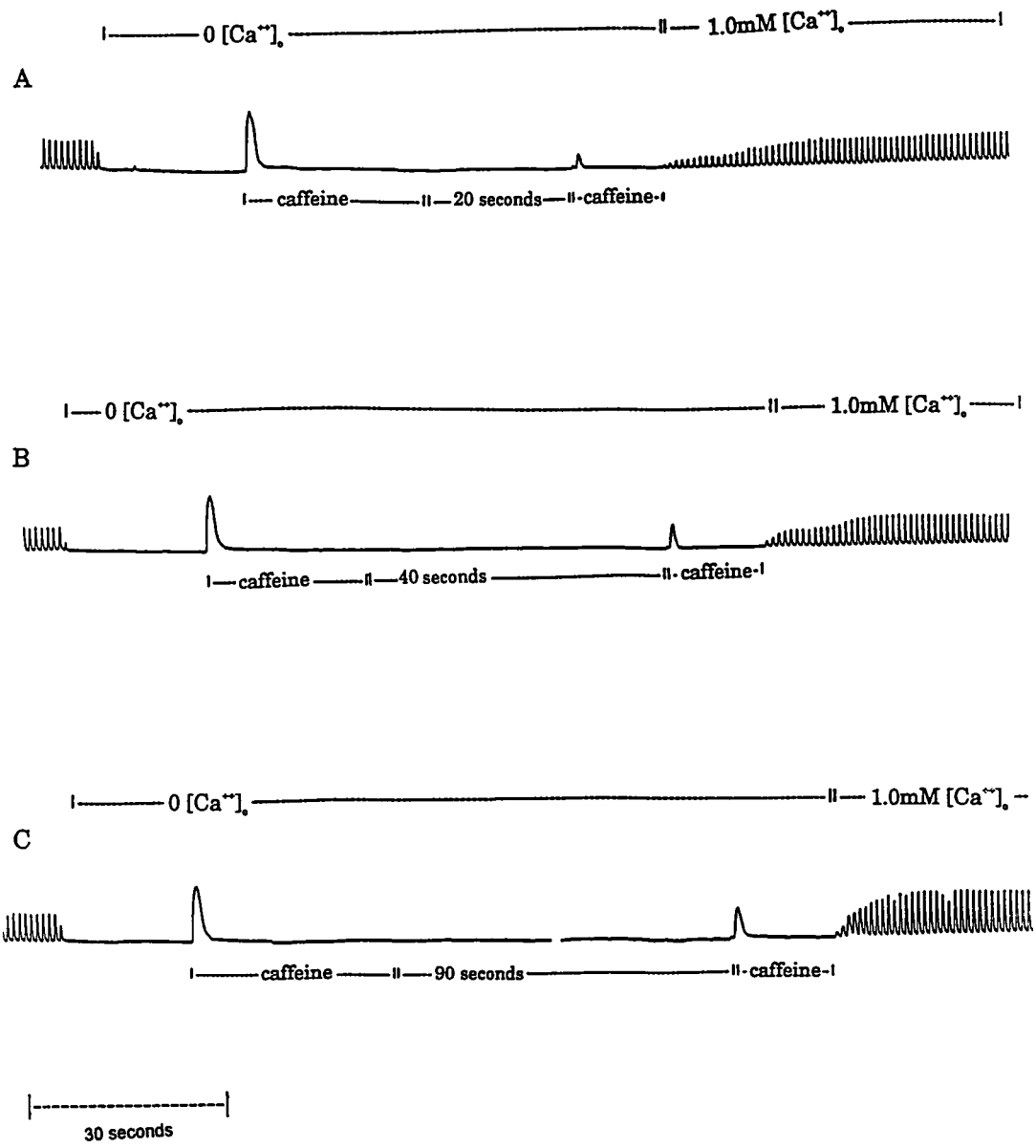

FIGURE 7 Paired caffeine contractures recorded from a single cell during 0 $[Ca^{2+}]_o$ perfusions. The time intervals between contractures are 20, 40, and 90 s in *A*, *B*, and *C*, respectively. (The time scale at bottom refers only to periods in 1 mM $[Ca^{2+}]_o$.)

Paired experiments, as in Fig. 10, were conducted in which cells were $^{45}Ca^{2+}$-labeled in either the presence or absence of 10 mM NaH_2PO_4, and the quantity of Na^+/Ca^{2+} exchange-mediated flux was measured by isotopic effluent analysis, as described in Materials and Methods. In control studies, the cells are $^{45}Ca^{2+}$-labeled in the absence of NaH_2PO_4, and a standard Na^+/Ca^{2+} exchange washout is conducted. After a period in nonisotopic control solution and washout of residual isotopic activity, the same monolayer is again $^{45}Ca^{2+}$-labeled. However, in this instance 10 mM NaH_2PO_4 is included in the label solution. Subsequent washout is conducted in the same fashion as described for the control washout, with the exception of the inclusion of 10 mM NaH_2PO_4 in the 0 Na^+/0 Ca^{2+} washout solution. Fig. 10 shows the effect of 10 mM NaH_2PO_4 on the quantity of Na^+/Ca^{2+} exchange-dependent calcium flux. The control content (no phosphate-dependent calcium loading) in the experiment shown is 687 μmol Ca^{2+}/kg dw, consistent with previously obtained values (Langer et al., 1995). In the same monolayer, the content increases to 1176 μmol Ca^{2+}/kg dw when 10 mM NaH_2PO_4 had been included in the label solution (difference = 489 μmol/kg dw). The mean values of three paired studies are 699 μmol/kg dw and 1033 μmol/kg dw, respectively, for the control and phosphate-added label conditions. The mean difference in Na^+/Ca^{2+} exchange-mediated flux between the two conditions is 334 μmol/kg dw.

Because the phosphate loading condition is not expected to alter sarcolemmal calcium binding capacity, 350–400 μmol Ca^{2+}/kg dw of the exchange-mediated flux can be

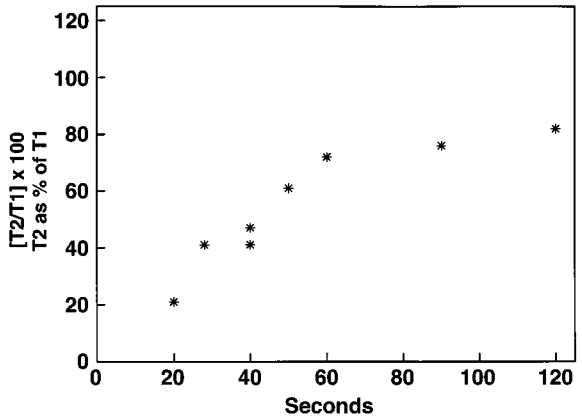

FIGURE 8 Graphical representation of the recovery of caffeine contracture (T2) amplitude as a function of time in 0 $[Ca^{2+}]_o$ obtained from a single cell. T2 is presented as a percentage of the initial caffeine contracture amplitude before exposure to 0 $[Ca^{2+}]_o$ (T1). Duplicate data points were obtained 40 s before the cell lost viability, as judged by failure to return to baseline contraction parameters in control perfusate.

considered to be of sarcolemmal origin, whereas the remaining 776–826 μmol Ca^{2+}/kg dw is of immediate SR origin, but must be derived from other sources, putatively mitochondrial. These values are remarkably close to the maximum calcium-accumulating capacity of the SR, as determined in the compartmentation studies.

Additional support for the augmented Na^+/Ca^{2+} exchange-dependent Ca^{2+} flux being assigned an immediate SR origin is provided by the character of this quantity's exchange kinetics. When the downward slope of the effluent activity transient is subject to nonlinear regression analysis, the exchange kinetics display biexponential characteristics with half-times of exchange of 4 and 46 s and 5 and 58 s for the control and phosphate-added loading conditions, respectively. These values are similar to those obtained for caffeine-releasable calcium shown in Fig. 5 (7, 95 s), which is unequivocally of SR origin.

On-line studies

Similar experiments utilizing NaH_2PO_4 were conducted in the on-line system described in Materilas and Methods, in which total cellular calcium is continuously monitored. A typical experimental protocol and the results obtained are illustrated in Fig. 11. Cells are isotopically labeled in the presence of 10 mM NaH_2PO_4 for 90 min. Washout with nonisotopic perfusate supplemented with 10 mM NaH_2PO_4 is commenced at the end of the labeling period and is continued for 40 min to ensure that all rapidly exchanging calcium compartments have been cleared of isotopic activity. The phosphate is then removed from the perfusate, and the washout is continued for an additional 40 min or until the monoexponential slow phase of exchange is reestablished. It is evident that there is a clear decrease in cell-associated $^{45}Ca^{2+}$ activity upon removal of phosphate from the perfusate. The difference in total cellular calcium content represented by this drop in isotopic activity is obtained by extrapolation of the two monoexponential phases of exchange (one established in the presence of phosphate and the other after its removal) to time 0. The difference in counts obtained at time 0 between the two extrapolated lines is then determined and converted to μmol Ca^{2+}/kg dw after correction for incompleteness of labeling. The mean value of the phosphate-dependent cellular calcium load is 942 + 113 μmol Ca^{2+}/kg dw ($n = 4$).

The total phosphate-dependent cellular calcium load is, by necessity, of mitochondrial origin. This value is greater than twice the value of phosphate-dependent calcium load obtained using the effluent analysis technique (334 μmol Ca^{2+}/kg dw). The reason for the difference in the amount of calcium released is clear. On-line monitoring of cellular calcium is capable of detecting calcium released from both the rapidly and the more slowly exchanging calcium compartments, whereas the effluent collection system only detects calcium released from the more rapidly exchanging compartment. Calcium that is specifically loaded into the mitochondria subsequently exchanges with a mixture of rapid and slow kinetic components. The more rapid component turns over with half-times typical of SR calcium exchange kinetics, whereas the more slowly exchanging component turns over with a half-time of 37 min, which is very close to the mitochondrial calcium exchange kinetics (45 min) obtained from compartmentation studies. Although this slowly exchanged mitochondrial fraction is expected to exchange via the SR, its rate is limited at the mitochondrial membrane.

DISCUSSION

Sarcolemmal bound calcium

As noted in the Results, the sarcolemmal bound calcium remains constant over a wide range of extracellular calcium concentrations, suggestive of a saturation of available binding sites. The study that first characterized sarcolemmal calcium binding sites identified both high-affinity ($K_d = 13$ μM) and low-affinity ($K_d = 1.1$ mM) sites with maximum binding capacities of 7 and 84 nmol Ca^{2+}/mg sarcolemmal protein, respectively (Post and Langer, 1992). The vast majority of sarcolemmal calcium binding sites are located on the phospholipids phosphatidylserine (PS), phosphatidylinositol (PI), and phosphatidylethanolamine (PE). Virtually 100% of PS and PI is confined to the inner sarcolemmal leaflet, whereas ~75% of the PE is found on the inner leaflet (Post et al., 1988). Given the binding capacity of these sites (84 nmol Ca^{2+}/mg sarcolemmal protein), the absolute amount of sarcolemma obtained per gram of cells (19.1 mg sarcolemmal protein/g cells) (Langer et al., 1979), and the sarcolemmal yield (43%) (Post et al., 1988), the maximum sarcolemmal binding capacity is estimated to be >3.7 mmol/kg dw. In the present studies, sarcolemmal bound calcium remains between 350 and 400 μmol Ca^{2+}/kg dw cells over a wide range of $[Ca^{2+}]_o$. However, it is clear

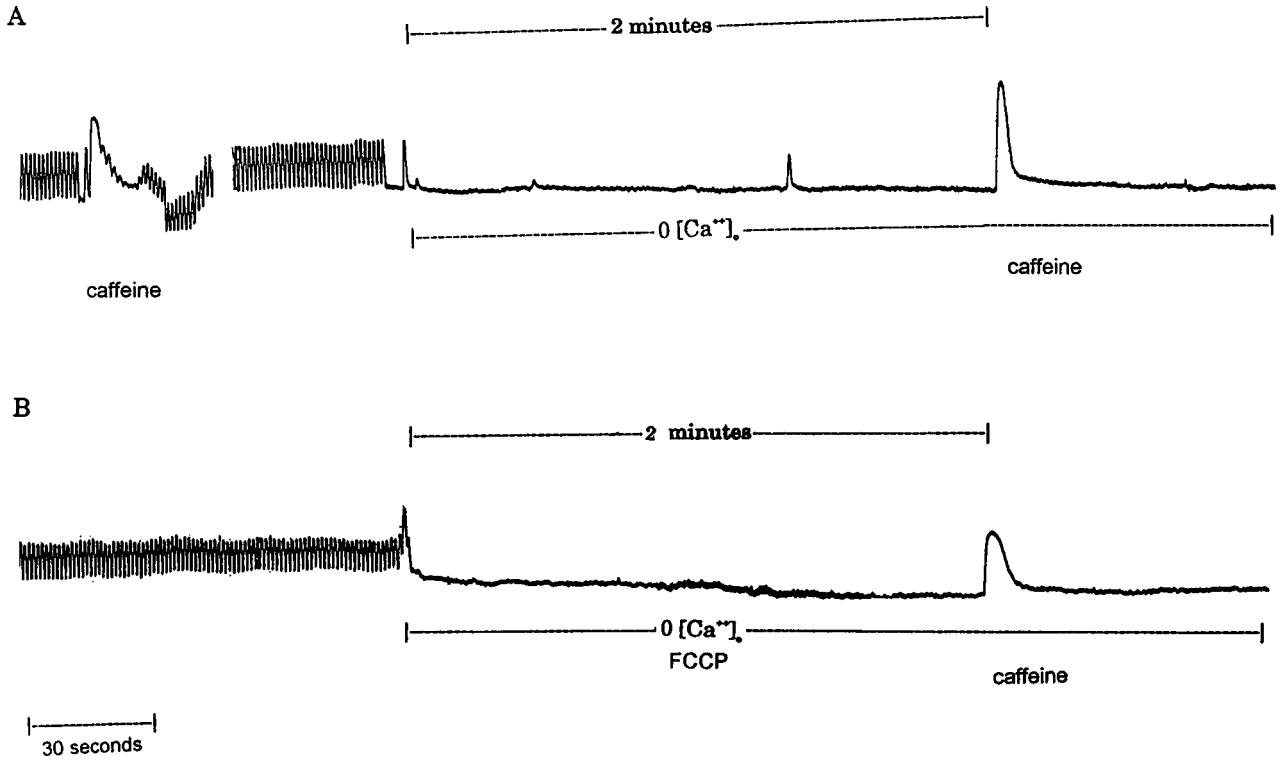

FIGURE 9 Representative chart recording illustrating the effect of 50 μM FCCP on caffeine-induced contracture amplitude after 2 min in 0 $[Ca^{2+}]_o$. (*A*) A control caffeine contracture followed by a second caffeine contracture elicited after 2 min in 0 $[Ca^{2+}]_o$. (*B*) A subsequent caffeine contracture in the cell depicted in *A* after a 2-min incubation in 0 $[Ca^{2+}]_o$ solution supplemented with 50 mM FCCP. (The time scale at bottom refers only to periods in 1 mM $[Ca^{2+}]_o$.)

that this maximum value under high extracellular calcium loading conditions represents an occupancy of only ~10% of all sarcolemmal binding sites. A reasonable conclusion is that only those sites on the inner sarcolemmal leaflet in close proximity to Na^+/Ca^{2+} exchangers participate in exchange via this transport system. The likelihood of such an occurrence is supported by the high K_d value of the low-affinity sites (Post and Langer, 1992). For any significant

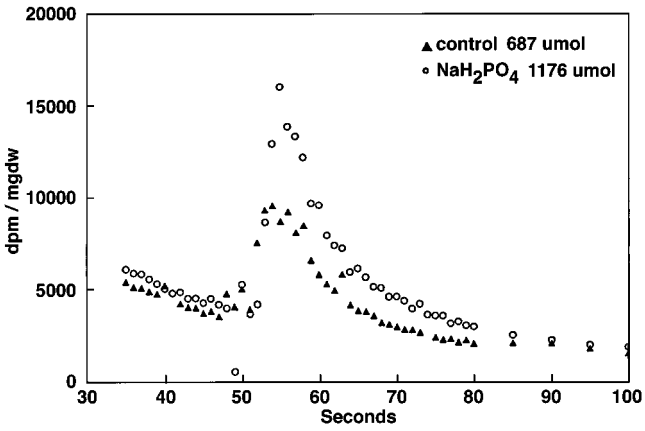

FIGURE 10 Paired effluent analysis study demonstrating the effect of 10 mM NaH_2PO_4 on the magnitude of the Na^+/Ca^{2+} exchange-dependent flux. ▲, Typical Na^+/Ca^{2+} exchange-dependent Ca^{2+} flux washout study. ○, Data from the same cellular monolayer labeled for 30 min in the presence of 10 mM NaH_2PO_4. NaH_2PO_4 was also present in the nonisotopic washout solution during the first 49 s of washout. The quantity of Na^+/Ca^{2+} exchange-dependent Ca^{2+} flux represented for each label condition is noted in the upper right-hand corner of the figure.

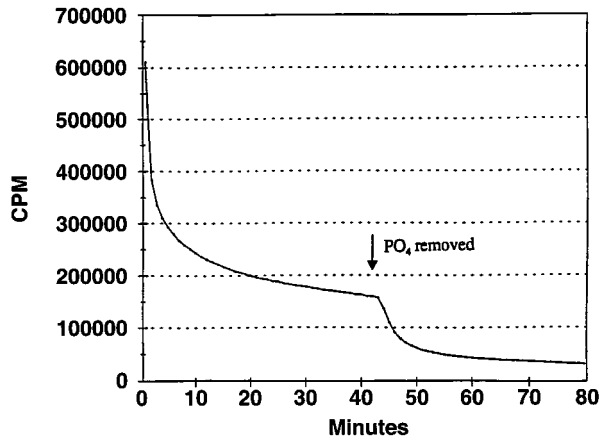

FIGURE 11 On-line $^{45}Ca^{2+}$ washout from a monolayer previously $^{45}Ca^{2+}$-labeled for 90 min in the presence of 10 mM NaH_2PO_4. The standard control buffer contained 10 mM NaH_2PO_4 during the initial 40 min of nonisotopic washout. Phosphate was removed from the buffer at the 41st minute, and washout continued for an additional 40 min. The drop in $^{45}Ca^{2+}$ cell-associated activity upon the removal of phosphate from the buffer is evident.

calcium binding to take place, a calcium concentration that is three to four orders of magnitude higher than general cytosolic levels during rest (100 nM) is required. The recently modeled diadic cleft region has the potential to provide such an environment (Peskoff and Langer, 1998; Langer and Peskoff, 1996). Even under conditions in which the cellular calcium load is decreased, this region could be expected to maintain a high local calcium concentration and maximum occupancy of available anionic binding sites in the immediate vicinity, consistent with the data obtained in the present studies.

Data obtained under conditions in which $[Na^+]_o/[Na^+]_i$ is decreased also support the idea that the calcium contribution from sarcolemmal sites is confined to those located within discrete, diffusion-restricted areas in close proximity to the Na^+/Ca^{2+} exchanger. These studies demonstrate that incubation in 0 Na^+/1.0 mM Ca^{2+} solution raises the maximum binding capacity of the sarcolemmal sites by 59% in the absence of high cellular calcium loading (no increase in sarcoplasmic reticulum or mitochondrial calcium content is noted under these conditions). Thus it is likely that the cellular calcium load has not increased sufficiently to raise general cytosolic calcium concentration to a level sufficient to recruit new sarcolemmal binding sites at more distant locations, particularly in light of the fact that incubation in 6.0 mM Ca^{2+}, which does load the other compartments, does not increase sarcolemmal calcium binding. The most likely explanation is that $[Na^+]_i$ affects sarcolemmal calcium binding by acting as a competitor for the available sites within the diffusion-restricted space.

Prolonged incubations (20 min) in 0 Na^+ solution can be expected to result in a decline in $[Na^+]_i$, thus increasing the availability of anionic binding sites to calcium and raising the maximum binding capacity in the vicinity of the exchanger. The affinity of these sites for Na^+ is, however, quite low. A study of mono- and divalent ion binding to phosphatidylserine in monolayers indicates a K_d for Na^+ binding of ~55 mM, an affinity ~2% of that for Ca^{2+} (Ohki and Kurland, 1981). However, a recent electron probe microanalysis study in guinea pig ventricular myocytes has demonstrated local sodium accumulations restricted to a narrow subsarcolemmal region (Wendt-Gallitelli et al., 1993).

The results obtained when myocytes are labeled in the presence of 10 μM ouabain are also consistent with a competitive effect of $[Na^+]_i$. Ouabain is a digitalis glycoside that selectively inhibits the Na^+/K^+ ATPase. The corresponding sodium pump inhibition increases intracellular sodium activity, which then increases intracellular Ca^{2+} via "reverse" Na^+/Ca^{2+} exchange. Thus it is the high intracellular sodium concentration that drives reverse exchange in this situation, rather than a low extracellular Na^+ concentration, as in the 0 Na^+ loading condition. The decrease in sarcolemmal calcium binding capacity attendant to Na^+ pump inhibition is consistent with increased competition for limited anionic binding sites as local intracellular sodium concentration rises.

Sarcoplasmic reticulum

Before discussion of SR Ca^{2+} distribution, it is important to note the morphology of the SR in the cultured cells. In a previous ultrastructural study (Langer et al., 1979), it was shown that all forms of SR demonstrated in adult myocardium were clearly evident in the cultured cells. There is an extensive network of SR tubules, the so-called network or free SR, that surrounds the myofibril. In addition, the cells exhibit numerous couplings formed by specialized regions of the SR as it makes close contact with the cytoplasmic side of the sarcolemma. These couplings are structurally similar to the junctional SR of adult muscle. Therefore, it seems that present findings would apply to adult myocardium.

The SR calcium content increases with increasing extracellular calcium concentration until a maximum loading condition is achieved. Qualitatively, the results obtained are as expected based on our current understanding of the conditions required to load the SR. Quantitatively, the results obtained in the present studies are consistent with those found in the literature. Studies conducted on saponin-treated ferret ventricular muscle have estimated a maximum SR calcium content of 370 μmol/liter cytoplasm (Kawai and Konishi, 1994). Using the author's own value of 0.585 as the fraction of cell volume that is cytoplasm and a tissue density of 1.07 g/cm^3, this content is the calculated equivalent of 800 μmol/kg dw. This value is remarkably close to the maximum SR calcium content measured in these studies (~750 μmol/kg dw). However, it is also true that SR calcium contents reported in the literature range from 200 to 6000 μmol Ca^{2+}/kg dw (Langer, 1997).

An unexpected finding of these compartmentation studies is the limited increase in cellular calcium load that was confined to sarcolemmal calcium binding sites in response to 0 Na^+/1.0 mM Ca^{2+} label conditions. The expectation of increased calcium influx via reverse exchange leading to an increase in SR calcium load, while reasonable, proved to be incorrect. A likely explanation for this result, which is consistent with the effect of this label condition on the sarcolemmal calcium binding capacity, is that a decrease in $[Na^+]_i$ in the vicinity of the exchanger over time results in a reestablishment of a baseline $[Na^+]_o/[Na^+]_i$. Thus the driving force for continued inward calcium movement is dissipated. Therefore, only a limited amount of calcium is transported into the cell via reverse exchange before it effectively stops. The relatively small amount of calcium that is transported into the cell appears to be directed to the newly vacated anionic sarcolemmal binding sites.

However, the fact remains that decreasing $[Na^+]_o$ is positively inotropic despite a SR content that remains constant. To explain these responses it is necessary to take a close look at the time frame of the response. The calcium content of the SR was measured after 20-min incubations in 0 Na^+ and, therefore, presumably at a time when the compartmental contents are close to steady state. In contrast, the positive inotropy noted in the functional studies was obtained after just 2–3 min in 0 Na^+ solution. Other studies of

frog atrium (Chapman and Tunstall, 1969) and rabbit interventricular septal preparations (Tillisch and Langer, 1974) have also demonstrated a relatively rapid positive inotropic response to decreasing extracellular sodium. Using interventricular septal preparations, Tillisch and Langer (1974) showed that the positive inotropic response to decreasing extracellular sodium is a transient response that returns to baseline condition after ~8 min. A similar transient inotropy in response to decreasing extracellular sodium has also been observed in our neonatal preparation in six of six experimental preparations (unpublished observations). This indicates that the positive inotropy is a non-steady-state response with return to control values well within the 20-min period at which SR content was measured.

Finally, the results obtained when cells are labeled in the presence of 10 μM ouabain are consistent with its established mechanism of action. The results support the contention that its positive inotropic effect is mediated through an increase in SR calcium load. Furthermore, the fact that the SR is maximally loaded in the presence of 10 μM ouabain attests to the importance of normal sodium homeostasis in regulating intracellular calcium loads and distribution. It also demonstrates how effectively inhibition or reversal of Na^+/Ca^{2+} exchange can alter cellular calcium dynamics when a high $[Na^+]i/[Na^+]_o$ is maintained.

Mitochondria

Mitochondrial calcium load, as in the SR, rises with increasing $[Ca^{2+}]_o$. This was not unexpected. Previous studies have indicated that mitochondrial calcium uptake is increased by time-averaged increases of cytosolic calcium (Leisey et al., 1993). However, the two features of the mitochondrial response to increasing $[Ca^{2+}]_o$ that are notable include the large calcium-accumulating capacity of this compartment relative to the other cellular compartments and the absence of any degree of impending saturation within the range of $[Ca^{2+}]_o$ tested.

As in the SR, the calcium content of the mitochondrial compartment after incubation in 0 Na^+ is not significantly different from control. This result is consistent with the concept of local intracellular sodium depletion leading to a reestablishment of control $[Na^+]_i/[Na^+]_o$, dissipating the driving force for continued calcium influx before significant calcium accumulation has occurred.

Surprisingly, incubations in control solution supplemented with 10 μM ouabain did not increase mitochondrial calcium load either. Under these conditions, the SR calcium load is at its maximum value, well above the level of calcium load at which mitochondria begin accumulating calcium when the sodium profile remains unaltered. It is established that cytosolic calcium concentration rises in response to digitalis glycosides. Thus an increase in mitochondria calcium uniporter activity is expected to accompany this rise in cytosolic calcium (Gunter et al., 1994), yet the net calcium content remains unchanged. Accepting that increased influx via the uniporter must be occurring, the only way to prevent an increase in total compartment content is to also increase movement of calcium out of the mitochondria. The primary mitochondrial calcium efflux pathway is via an electroneutral Na^+/Ca^{2+} exchange (Gunter et al., 1994). Thus, if the rise in cytosolic calcium is accompanied by a comparable rise in intracellular sodium as the Na^+/K^+ ATPase is inhibited, it is likely that this rise in sodium is prompting increased mitochondrial calcium efflux. The net effect would then be to increase calcium cycling through the mitochondria while the overall content remains relatively unchanged. As the capacity of the SR is exceeded in the absence of any net mitochondrial calcium accumulation, the expected functional response to the continued calcium influx might be expected to be contracture and negative inotropy. This is the result (not shown) frequently seen.

The results are consistent with the view that the mitochondria serve as a large-capacity slow buffer for calcium. There is no evidence that they function in the beat-to-beat release of calcium.

SR calcium availability

The morphological features of the cardiac SR and the differential localization of specific calcium uptake and release of proteins to distinct regions of the SR membrane complement the functional and kinetic data obtained in these studies. It is proposed that the Ca^{2+} that resides in the corbular and junctional SR is readily available for release to the myofilaments during an I_{Ca}-triggered opening of the SR calcium release channels. Although the longitudinal SR contains a significantly larger store of calcium, it is unavailable to the myofilaments during a normal triggered release, because it is confined, presumably by diffusional restrictions, to an area of the SR where there are few, if any, calcium release channels. Caffeine, however, is capable of triggering release of calcium from both regions. Studies conducted on canine SR vesicles incorporated into planar lipid bilayers have demonstrated that caffeine activates the calcium release channel by increasing the frequency and duration of channel opening without changing unitary conductance (Rousseau and Meissner, 1989). This would explain the caffeine-dependent release of calcium from the junctional and corbular SR, but leaves some question as to the mechanism of calcium release from the longitudinal SR. However, it is possible that the longitudinal SR is not devoid of all ryanodine-sensitive channels. It is quite possible that a relatively small number of release channels in this region are, in fact, responsible for the caffeine-mediated release. Because these channels would reside outside the diffusion-restricted area of the diadic cleft (Peskoff and Langer, 1998; Langer and Peskoff, 1996), it is possible that the local calcium rise in the vicinity of these release channels is not sufficient to trigger a regenerative release of calcium from these sites during a normal triggered contrac-

tion cycle. Caffeine, on the other hand, is indiscriminant. Moreover, it is by no means accepted that this is the only mechanism of caffeine-induced SR calcium release. Rapid cooling contractures, which also release SR calcium, behave qualitatively identically to caffeine contractures in many experimental protocols. After a sustained exposure to 10 mM caffeine, subsequent rapid cooling elicits no response, indicating that these two interventions act on the same calcium store (Bassani et al., 1993; Bers, 1989). Yet studies have shown that the mechanism of release during a rapid cooling contracture is not through a ryanodine-sensitive pathway (Feher and Rebeyka, 1994). Therefore it is quite possible that caffeine exerts a portion of its effects through an as yet unrecognized mechanism.

SR response to 0$[Ca^{2+}]_o$

The compartmentation studies have shown that when extracellular calcium is decreased from 1.0 mM to 0.25 mM, there is a net loss of 724 μmol/kg dw mitochondrial calcium during the labeling period. Because it has been demonstrated that the SR is able to rapidly sequester large amounts of available calcium, it is reasonable to theorize that during extracellular calcium depletion, the SR sequesters calcium released from the mitochondria. Initially, the loss of mitochondrial calcium is rapid and SR calcium uptake exceeds the rate of SR calcium leak. As the rate of mitochondrial calcium release slows with declining stores, the rate of SR calcium uptake also slows. Eventually, the rate of SR calcium leak exceeds that of calcium uptake, and a gradual decline in SR calcium content is noted. This would explain the initial increase in the magnitude of caffeine contracture after short periods of time in 0 $[Ca^{2+}]_o$ followed by a more gradual decline at later times. Given the large calcium content of the mitochondria ($>$1500 μmol/kg dry wt; Fig. 1), their flux rate of $>$7 μmol/kg dry/s (Langer, 1997), and the serial exchange of mitochondria and SR (Bassani et al., 1993), this sequence seems a resonable explanation.

The functional studies represented in Fig. 9 in which FCCP was used as an inhibitor of mitochondrial respiration provide evidence for a close association between SR and mitochondrial calcium pools. These studies clearly show that when normal mitochondrial calcium cycling is interrupted by inhibition of respiration, the caffeine-releasable pool of SR calcium is significantly reduced when compared to the control condition when FCCP is not present.

The current studies are consistent with a series model of calcium movement wherein calcium leaving the mitochondria must first traverse the SR before exiting the cell. Although a direct link between these organelles and the existence of specific calcium transport pathways such as channels or exchange proteins cannot be ruled out, no evidence to support the existence of such a structure exists. More likely, calcium transported out of the mitochondria is released into the general cytosolic space. Some or all of this calcium is sequestered by the SR, which then either exits the cell via Na^+/Ca^{2+} exchange or recycles to the cytoplasm.

Thus under low Ca^{2+} loading conditions, the mitochondria may serve as a source of calcium for the SR. From a teleological standpoint, maintaining short-term SR stores at the expense of the mitochondria is a reasonable mechanism for maintaining contractile strength during periods of limited calcium availability.

The results obtained from the isotopic flux studies are also consistent with those obtained from functional studies. In the Na^+/Ca^{2+} exchange-dependent flux studies, it has been shown that 10–50% of mitochondrial-specific calcium uptake subsequently exits the cell with SR exchange kinetics, whereas the functional studies have demonstrated unequivocally that the SR has access to calcium stores from at least one other cellular source. These findings complement one another and suggest that intracellular calcium movements between the SR and the mitochondria do occur.

The data obtained in these studies demonstrate that, in addition to the anatomical evidence for multiple SR compartments, there is a clear functional compartmentation of SR calcium pools that can be identified in spontaneously contractile, whole-cell preparations. The biphasic kinetics induced by caffeine and the functional responses with zero $[Ca]_o$ indicate compartmentation of SR calcium in these cells. This is consistent with the pumping and sequestration function of the longitudinal SR and the releasing function of the lateral cisterns. Thus the compartments of the SR in cardiac muscle are as in skeletal muscle, as originally shown by autoradiographic studies of Winegrad (1965). Furthermore, it is evident that the SR is able to access mitochondrial calcium stores under conditions of either SR calcium depletion or mitochondrial calcium overload. The data also suggest that a large component of mitochondrial calcium traverses the SR before it exits the cell via the sarcolemmal Na^+/Ca^{2+} exchanger.

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