A role for the sarcoplasmic reticulum in Ca\(^{2+}\) extrusion from rabbit inferior vena cava smooth muscle

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The extrusion of Ca\(^{2+}\) maintains Ca\(^{2+}\) homeostasis in vena cava smooth muscle cells. Removal of Ca\(^{2+}\) from the cytosol is an active process that depends primarily on the plasma membrane Ca\(^{2+}\)-adenosinetriphosphatase (ATPase; PMCA) and the Na\(^{+}\)-Ca\(^{2+}\) exchanger, which directly extrude intracellular Ca\(^{2+}\), and the sarcoendoplasmic reticulum Ca\(^{2+}\)-ATPase (SERCA). PMCA is an ATP-dependent pump activated by Ca\(^{2+}\) and calmodulin and represents one of the two Ca\(^{2+}\)-exporting systems in the plasma membrane (PM). It is referred to as a high-affinity, low-capacity pump. This 130-kDa phosphoprotein consisting of PMCA 1b and 1a isoforms in vascular smooth muscle (VSM) of the rabbit mediates an active transport of Ca\(^{2+}\) at a Ca\(^{2+}\)-to-ATP ratio of one. Hardin et al. (15) found that ATP produced by membrane-associated glycolysis may be the primary fuel source for the PMCA. Na\(^{+}\)-Ca\(^{2+}\) exchange is the second Ca\(^{2+}\) translocator in the PM, but, unlike the PMCA, it is known as a high-velocity, low-affinity antiporter. It electrogically exchanges three Na\(^{+}\) for one Ca\(^{2+}\), thus it responds to Na\(^{+}\) and Ca\(^{2+}\) gradients as well as to the transmembrane electrical potential (6). One possible reason why the Na\(^{+}\)-Ca\(^{2+}\) exchanger can be effective in spite of its low affinity is because it is exposed to a localized high intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_i\); see Ref. 6) that is due to the unloading of Ca\(^{2+}\) from the sarcoplasmic reticulum (SR) into the restricted space between the SR and PM.

By convention, the PMCA and Na\(^{+}\)-Ca\(^{2+}\) exchange are thought to be entirely responsible for Ca\(^{2+}\) extrusion; however, an additional more complex pathway for the removal of [Ca\(^{2+}\)]\(_i\) in which the SR plays a key role has been proposed. It has already been established that the SR is a transient source and sink for Ca\(^{2+}\) during fluctuations in contractile tension (30) and may act as a "buffer barrier" (32) by taking up a fraction of the Ca\(^{2+}\) that enters the cells through the plasmalemma before it reaches the myofilaments. It is postulated that, in order for the SR to buffer Ca\(^{2+}\) on a steady-state basis, it is required that Ca\(^{2+}\) be released from the SR lumen and extruded into the extracellular space (ECS; see Ref. 31). This extrusion process is thought to be mediated by the transport of Ca\(^{2+}\) from the SR where ryanodine and inositol 1,4,5-trisphosphate (IP\(_3\)) receptor-operated Ca\(^{2+}\) channels release Ca\(^{2+}\) into the subsarcolemmal space between the SR and the plasmalemma (this process is referred to as "vectorial release") from where Na\(^{+}\)-Ca\(^{2+}\) exchangers complete the extrusion process (22). Thus it is implied that SERCA functions in concert with Na\(^{+}\)-Ca\(^{2+}\) exchangers closely apposed to the peripheral SR, thereby contributing significantly to Ca\(^{2+}\) extrusion. The SERCA is an ATP-dependent and azide-insensitive (i.e., nonmitochondrial) protein (105 kDa), like the PMCA, which expresses the SERCA 2a and 2b (and possibly SERCA 3) isoforms in rabbit VSM (25). Endogenous phospholamban, in its dephosphorylated state, inhibits SERCA, but, when phosphorylated by various protein kinases, its activity is blocked, resulting in the activation of SERCA. According to Eggemont et al. (11), stimulated Ca\(^{2+}\)-ATPase activity in the SR and PM is approximately the same in bovine pulmonary VSM; however, it may differ widely between different smooth muscle types and depends on several factors, including regulation by protein kinases, proteins, lipids, and cytosolic pH.

In VSM, mitochondrial Ca\(^{2+}\) uptake plays a minor role in Ca\(^{2+}\) homeostasis under physiological conditions (41), but, when [Ca\(^{2+}\)]\(_i\) rises to pathological levels, the mitochondria begin to sequester significant amounts of Ca\(^{2+}\) (21). The apparent Michaelis constant for Ca\(^{2+}\) uptake in VSM mitochondria, corresponding to a value of 17 \mu M, is ~200-fold higher than the resting [Ca\(^{2+}\)]\(_i\) in these cells. Thus it is improbable that the mitochondria act as a Ca\(^{2+}\) sink even when [Ca\(^{2+}\)]\(_i\) is elevated during stimulation.

In the present study, our objective was to determine the mechanism of Ca\(^{2+}\) extrusion using vena cava smooth muscle cells from the rabbit as a model. With the help of various pharmacological tools, we were able to block SERCA, the Na\(^{+}\)-Ca\(^{2+}\) exchanger, and the
PMCA. For inactivating the PMCA, it was necessary to prevent ATP from being generated through a membrane-associated glycolytic pathway that preferentially fuels the Ca\(^{2+}\) pump (15). Iodoacetate (IAA), a metabolic inhibitor that inhibits ATP synthesis, was used to target the glycolytic machinery associated with the PMCA by way of arresting the glycolytic enzyme glyceraldehyde phosphate dehydrogenase (5). IAA also affects other enzymes due to its capability to alkylate sulfhydryl groups; however, this reaction likely occurs after 1–2 h of incubation with IAA (5). Carbonyl cyanide p-trifluoromethoxyphenylhydrazone (FCCP) was used in combination with IAA as a mitochondrial poison or protonophoric uncoupler of energy-dependent respiration (31) via the dissipation of the mitochondrial proton gradient and membrane potential (2). We employed a protonophoric coupling dye (mitotracker) that targets the glycolytic machinery associated with the mitochondrial Ca\(^{2+}\) sequestration (31) via the dissipation of the mitochondrial proton gradient and membrane potential (2). We employed a protonophoric coupling dye (mitotracker) that targets the glycolytic machinery associated with the mitochondrial Ca\(^{2+}\) sequestration (31) via the dissipation of the mitochondrial proton gradient and membrane potential (2).

**MATERIALS AND METHODS**

Tissue preparation. New Zealand White rabbits (1.5–2.5 kg) were killed by CO2 asphyxiation then exsanguinated. The inferior vena cava was promptly excised, and connective tissue and adventitia were removed in a petri dish containing normal physiological saline solution (PSS), pH 7.4, at room temperature (22–25°C). The tissue was opened longitudinally, cut into a 25 by 7-mm rectangular sheet, and the luminal endothelial layer teased off by a piece of wet filter paper. A rectangular plastic frame with a channel of silicon elastomer near each edge was used to pin the smooth muscle strip into place.

[Ca\(^{2+}\)]\(_i\) measurement. Background tissue autofluorescence was measured before loading the tissue with the fluorescent Ca\(^{2+}\) indicator fura 2. In a spectrofluorimeter (Spex Fluorolog; Spex Industries), light from a xenon lamp was beamed into the tissue on its support frame. The luminal side of the tissue was illuminated with alternating wavelengths of 340 and 380 nm (i.e., F\(_{340}/F_{380}\)). The luminal endothelial layer was then teased off by a piece of wet filter paper, and the measured fluorescence was collected in the front face mode via a photomultiplier. The venous circulation was then transferred to a cuvette and placed in the sample chamber of the spectrofluorimeter. The bathing solutions were changed by a peristaltic pump, perfusing the tissue at a rate of 0.37 ml/s, and by vacuuming off the overflow. All experiments were carried out at room temperature.

Fluorescence due to excitation at 340 and 380 nm (i.e., F\(_{340}\) and F\(_{380}\)) was calculated by subtracting background autofluorescence, then converting the ratio F\(_{340}/F_{380}\) to [Ca\(^{2+}\)]. We used the equation from reference 14: [Ca\(^{2+}\)] = K\(_D\) (R – R\(_{\text{min}}\))/(R\(_{\max}\) – R) where K\(_D\) is the dissociation constant, R is the ratio F\(_{340}\)/F\(_{380}\), and R\(_{\text{min}}\) is the minimum value of the ratio R when all of the Ca\(^{2+}\) is in the Ca\(^{2+}\)-free form, R\(_{\text{max}}\) is the maximum value of R when fura 2 is saturated with Ca\(^{2+}\), and β = S\(_{F380}/S_{380}\); Fura 2 was assumed to have an apparent K\(_D\) for Ca\(^{2+}\) of 200 nM (20, 34). R\(_{\text{min}}\), R\(_{\text{max}}\), and β were 1.16, 4.50, and 2.25, respectively, as determined by perfusing with a solution containing ionomycin (10 µM) and 20 mM Ca\(^{2+}\) then switching to a Ca\(^{2+}\)-free [2 mM ethylene glycol-bis(β-aminoethyl ether)N,N,N’,N’-tetraacetic acid (EGTA)] milieu. The K\(_D\) estimated by Grynkiewicz et al. (14) at 20°C corresponds to fura 2 in solution rather than in smooth muscle tissue itself; therefore, this value is often, incorrectly, used with cell preparations and must be adjusted for protein interactions, and changes in pH, and temperature that occur in smooth muscle.

Curve-fitting analysis. A nonlinear, biexponential curve-fitting equation (Can and Scientific-GraphPlot) of the form, f = ae\(^{-bx}\) + ce\(^{-dx}\) where f is [Ca\(^{2+}\)], a and c are compartment sizes, x is time in seconds, and b and d are the fast and slow rate constants, respectively, was used to estimate the rate of [Ca\(^{2+}\)]\(_i\) decline (a single exponential did not produce a sufficient fit). The fast component of the curve, corresponding to the rate constant b, was used for comparison between the various experiments. The slow component, d, was consistently the same between control and experimental curves (Table 1), indicating a less important role. Two components suggest that several different processes are involved in the extrusion of Ca\(^{2+}\).

**Table 1.** Rate of [Ca\(^{2+}\)]\(_i\) decline fitted to the equation, f = ae\(^{-bx}\) + ce\(^{-dx}\) (see MATERIALS AND METHODS) with blockade of the Na\(^{+}\)-Ca\(^{2+}\) exchanger and SERCA

<table>
<thead>
<tr>
<th>Constants</th>
<th>Control (0 Na(^{+}) PSS)</th>
<th>0 Na(^{+})</th>
<th>Control (0 Na(^{+}) PSS)</th>
<th>CPA</th>
<th>Control (0 Na(^{+}) PSS)</th>
<th>0 Na(^{+}) + CPA</th>
</tr>
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<tr>
<td>b (10(^{-2}), s(^{-1}))</td>
<td>7.64 ± 1.24</td>
<td>4.09 ± 0.72</td>
<td>14.23 ± 1.94</td>
<td>10.85 ± 1.07</td>
<td>5.46 ± 1.07</td>
<td>4.76 ± 0.91</td>
</tr>
<tr>
<td>d (10(^{-3}), s(^{-1}))</td>
<td>2.22 ± 0.70*</td>
<td>1.78 ± 0.72*</td>
<td>1.16 ± 0.39</td>
<td>0.62 ± 0.02*</td>
<td>2.46 ± 0.40*</td>
<td>2.53 ± 0.47*</td>
</tr>
<tr>
<td>a (10(^{4}), nM)</td>
<td>1.83 ± 0.25</td>
<td>1.91 ± 0.27</td>
<td>1.90 ± 0.31</td>
<td>2.04 ± 0.29</td>
<td>2.01 ± 0.32</td>
<td>2.05 ± 0.39</td>
</tr>
<tr>
<td>c (10(^{4}), nM)</td>
<td>0.93 ± 0.11</td>
<td>0.94 ± 0.10</td>
<td>1.14 ± 0.13</td>
<td>1.19 ± 0.17</td>
<td>1.04 ± 0.11</td>
<td>1.13 ± 0.09</td>
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Values expressed as means ± SE; n ≥ 6 experiments. [Ca\(^{2+}\)], intracellular Ca\(^{2+}\) concentration; SERCA, sarcoendoplasmic reticulum Ca\(^{2+}\)-ATPase; PSS, physiological saline solution; CPA, cyclopiazonic acid; a and c, compartment sizes; x, time in seconds; b and d, fast and slow rate constants, respectively. * ANOVA, P < 0.20.
were dissolved in dimethyl sulfoxide. IAA, phenolamine, and phentolamine (PE; all from Sigma) were dissolved in double-distilled H2O. Caffeine was solubilized in the appropriate PSS solution.

Statistics of experiments. All traces are typical of at least four experiments. The data in bar graphs were presented as means ± SE. Differences of means were verified with analysis of variance, the two-sample t-test, and the Newman-Keuls test, where appropriate.

RESULTS

Total inhibition of Na+-Ca2+-exchanger and Ca2+ pump activity abolishes Ca2+ extrusion. To determine whether Ca2+ extrusion could be blocked, the major contributing mechanisms, which include the Na+-Ca2+-exchanger and the PMCA, as well as the mechanism thought to be partly involved in SR-mediated extrusion, the SERCA, were inhibited (Fig. 1). A control response was obtained by recording changes in [Ca2+]i while perfusing with 80 mM K+-PSS and PE (20 µM; loading stimulus) followed by the addition of zero Ca2+ PSS and phentolamine (10 µM), a competitive antagonist of PE, to abolish activation and Ca2+ entry. The initial rise in [Ca2+]i was due to the Ca2+-loading effect of K+ and PE, whereas the subsequent fall in [Ca2+]i was assumed to be due to active extrusion in the absence of Ca2+ influx. The experimental curve in Fig. 1 was obtained by following the same protocol as in the control, except for the addition of CPA (20 µM), a SERCA blocker, IAA (1 mM), an inhibitor of membrane-associated glycolysis, and FCCP (3 µM), an uncoupler of oxidative metabolism in the mitochondria, at the time of activation of Ca2+ influx to allow for preincubation and, in addition, removal of external Na+ to block the Na+-Ca2+-exchanger upon inactivation and Ca2+ removal. Within 120 s, the height of the activation curve had reached its peak of 352.6 ± 49.4 nM, n = 6 (compared with the peak control value of 201.8 ± 32.2 nM, n = 6), and then, by blocking Ca2+ influx (as in the control), it could be determined whether all of the Ca2+-extruding components would be inhibited by the combination of CPA, IAA, FCCP, and zero Na+. The resulting plateau, lasting until the end of the experiment, made it clear that the combination of blockers used to target SERCA, the PMCA, and the Na+-Ca2+-exchange were sufficient to completely block Ca2+ efflux. Without an apparent decay in [Ca2+]i, it is obvious that the passive leak of Ca2+ out of the cells is negligible. Controls for the effects of FCCP and IAA in a normal PSS and zero Ca2+ PSS medium are shown in Fig. 1, inset. There was no change in [Ca2+]i with IAA alone. However, in the FCCP control, a delayed upward deflection in [Ca2+]i led to a sustainable plateau phase that indicated inhibition of active Ca2+ removal from the cytoplasm. The FCCP-induced rise in [Ca2+]i was responsible for the higher experimental peak when compared with the control.

SERCA and Na+-Ca2+-exchanger blockade slows the rate of [Ca2+]i decline. Typical responses to inhibitors of SERCA and the Na+-Ca2+-exchanger are shown in Fig. 2, A-C. After establishment of basal [Ca2+]i (54.1 ± 9.8 nM, n = 6), each strip of smooth muscle was loaded with Ca2+ by the addition of 80 mM K+ PSS and 20 µM PE (as before) and then washed with zero Ca2+ PSS and 10 µM phenolamine to abolish the influx of Ca2+. These manipulations caused a sustained increase in [Ca2+]i followed by a decline upon removal of external Ca2+, revealing the process of Ca2+-extrusion. The data collected from these experiments are summarized in Fig. 3.

We set out to quantify the fractional contribution of the Na+-Ca2+-exchanger to Ca2+ extrusion. Tissue responsiveness was tested by means of Ca2+ loading and then by observing the decline in [Ca2+]i, as shown in Fig. 2A, left (control). The experimental curve was obtained by the same method as the control, except that external Na+ was removed at the start of the decline in [Ca2+]i. The main effect of blocking the Na+-Ca2+-exchanger was a decrease in the rate of [Ca2+]i decline (rate of decline = 0.041 ± 0.007 s−1, n = 8; rate in the control = 0.076 ± 0.012 s−1, n = 8). When Na+ was removed from a normal PSS-bathed tissue (Fig. 2A, inset), a small increase in [Ca2+]i was produced, which may indicate that reversal of the Na+ gradient is responsible for the operation of the Na+-Ca2+-exchanger in the reverse mode, enhancing Ca2+ influx, as long as sufficient intracellular Na+ and extracellular Ca2+ are available. The increase seen in Fig. 2A, inset, was not apparent in the graph below (no difference between the peak of the control, 234.0 ± 31.7 nM, n = 8, and the experimental peak, 234.00 ± 30.3 nM, n = 8) because Na+ was removed along with Ca2+, and, there-
Therefore, inward Ca\(^{2+}\) transport by the Na\(^{+}\)-Ca\(^{2+}\) exchanger (i.e., the reverse mode) was not observed.

Figure 2B illustrates a typical response to CPA. The purpose of blocking SERCA with CPA was to determine if the SR played a role in the Ca\(^{2+}\) extrusion process. To check the responsiveness of the tissue, a control curve (as in Fig. 2A, left) was obtained first. Next, the effect of CPA on the rate of [Ca\(^{2+}\)]\(_i\) decline was ascertained by incubating the tissue with CPA (20 µM) and at the same time adding the K\(^{+}\) and PE stimulus. The stimulus was then removed by washing with zero Ca\(^{2+}\) PSS and CPA, which marked the start of Ca\(^{2+}\) decay within the cells. The rate of [Ca\(^{2+}\)]\(_i\) decline decreased with CPA present (rate = 0.108 ± 0.011 s\(^{-1}\), n = 7) when compared with control conditions (rate = 0.142 ± 0.019 s\(^{-1}\), n = 7). An additional control experiment for the effect of CPA in a normal PSS and zero Ca\(^{2+}\) PSS medium was done (see Fig. 2B, inset). The resulting, delayed transient rise in [Ca\(^{2+}\)]\(_i\) showed that CPA had a slow onset of action making it necessary to preincubate the tissue. This increase in [Ca\(^{2+}\)]\(_i\) by CPA accounted for the enhancement in the peak [Ca\(^{2+}\)]\(_i\) signal of the experimental curve (peak = 290.8 ± 38.0 nM, n = 7) compared with the control value of 243.8 ± 29.4 nM, n = 7 (Fig. 2B).

To test if the Na\(^{+}\)-Ca\(^{2+}\) exchanger was working in parallel or in sequence with SERCA, we looked at whether inhibition of SERCA had any effect on the decline in [Ca\(^{2+}\)]\(_i\) if the Na\(^{+}\)-Ca\(^{2+}\) exchanger was blocked (Fig. 2C). The control, as before, consisted of a Ca\(^{2+}\)-loading stimulus, followed by perfusion with zero Ca\(^{2+}\) PSS and removal of external Na\(^{+}\). The experimental curve consisted of an application of stimulant and CPA, as in Fig. 2B, followed by removal of the stimulus and addition of zero Na\(^{+}\), zero Ca\(^{2+}\) PSS. In a comparison between curves, it was shown that there was no significant difference in the rate of [Ca\(^{2+}\)]\(_i\) decline between tissue with the Na\(^{+}\)-Ca\(^{2+}\) exchanger blocked and tissue

Fig. 2. Blockade of Na\(^{+}\)-Ca\(^{2+}\) exchange and SERCA attenuates the rate of decline in [Ca\(^{2+}\)]\(_i\). Time course (seconds) changes in [Ca\(^{2+}\)]\(_i\) (nM) were monitored during stimulation with 80 mM K\(^{+}\) PSS and PE (20 µM) and then by washing out with 0 Ca\(^{2+}\) PSS and phentolamine (10 µM) as shown on left. On the right, this experiment is repeated (for comparison, the control decay is superimposed as shown by the broken line) with external Na\(^{+}\) removed (A), which reveals Na\(^{+}\)-independent Ca\(^{2+}\) extrusion, CPA (20 µM) preincubation to selectively block SERCA (B), and a combination of CPA and 0 Na\(^{+}\) (C). The effect of Na\(^{+}\) removal and CPA on the tissue preparation appears in the insets of A and B, respectively. Exposure to normal PSS (solid bar) induces a rise in [Ca\(^{2+}\)]\(_i\) followed by a fall upon removal of external Ca\(^{2+}\) (broken bar). These results are representative of 6 or more experiments.

Fig. 3. Comparison of the effects of CPA, 0 external Na\(^{+}\), and their combination on the rate of [Ca\(^{2+}\)]\(_i\) decline. With blockade of SERCA, the rate of [Ca\(^{2+}\)]\(_i\) decline was 77.72 ± 5.08% of control, n = 7. With blockade of Na\(^{+}\)-Ca\(^{2+}\) exchange, the rate of decline in [Ca\(^{2+}\)]\(_i\) was further reduced to 53.35 ± 4.56% of control, n = 8, similar to a value of 48.49 ± 3.52%, n = 6, which was obtained by blocking both of these Ca\(^{2+}\) extrusion mechanisms. §P < 0.0009 vs. control and *P < 0.0007 vs. CPA-, 0 Na\(^{+}\)-, and 0 Na\(^{+}\) + CPA-treated tissue by analysis of variance (ANOVA) and Newman-Keuls test.
with the Na\(^+\)-Ca\(^{2+}\) exchanger and SERCA blocked (experimental rate of decline = \(0.048 \pm 0.009\) s\(^{-1}\), \(n = 6\); in the control, the rate of \([\text{Ca}^{2+}]\) decline = \(0.054 \pm 0.011\) s\(^{-1}\), \(n = 6\)). This indicates that the Na\(^+\)-Ca\(^{2+}\) exchanger- and SR-mediated Ca\(^{2+}\) extrusion are non-additive. The peak of the control curve (255.0 \(\pm 53.5\) nM, \(n = 6\)) was less than that of the experimental curve (291.3 \(\pm 66.9\) nM, \(n = 6\)) because of the presence of CPA in the latter.

Summarizing the effects of CPA, zero Na\(^+\), and CPA plus zero Na\(^+\) on their ability to block Ca\(^{2+}\) extrusion. Figure 3 illustrates the extent to which the SERCA, Na\(^+\)-Ca\(^{2+}\) exchange, and the PMCA each contributed to the \([\text{Ca}^{2+}]\) decline. SERCA-mediated Ca\(^{2+}\) extrusion accounted for \(~23\%\), whereas the remaining 77\% (\(n = 7\)) was due to extrusion by the PMCA and the Na\(^+\)-Ca\(^{2+}\) exchanger. The Na\(^+\)-Ca\(^{2+}\) exchanger, with or without SERCA activity present, contributed to approximately one-half (47\%) of the extruded Ca\(^{2+}\) as revealed by blockade of the Na\(^+\)-Ca\(^{2+}\) exchanger with zero Na\(^+\). The other one-half, 53\% (\(n = 8\)), was extruded by the PMCA. This was confirmed by inactivating SERCA and the Na\(^+\)-Ca\(^{2+}\) exchanger simultaneously (no significant differences between 53 and 49\%, \(n = 6\)), which revealed that about one-half of the Na\(^+\)-Ca\(^{2+}\) exchanger-mediated Ca\(^{2+}\) extrusion involved Ca\(^{2+}\) uptake by SERCA (23/47 = 50\%). An alternative method for determining the rate of Ca\(^{2+}\) extrusion was to calculate the slope of the \([\text{Ca}^{2+}]\) decline curve at a specific elevated \([\text{Ca}^{2+}]\) (e.g., 200 or 250 nM) using statistical tests to compare the slopes of the control decays with those of the experimental decays. With respect to statistically significant differences, the outcomes, regardless of using rate constants or slopes, were the same (data not shown).

SERCA blockade empties the Ca\(^{2+}\) store within the SR, an effect that is opposite to that seen with Na\(^+\)-Ca\(^{2+}\) exchanger blockade. To investigate the possibility that SR Ca\(^{2+}\) accumulation, without Ca\(^{2+}\) extrusion, was responsible for a significant decline in \([\text{Ca}^{2+}]\), we measured caffeine-induced \([\text{Ca}^{2+}]\) increases as an indicator of the SR Ca\(^{2+}\) content (Fig. 4). A bolus of caffeine (25 mM) was administered in Ca\(^{2+}\)-free PSS as a control (peak 1). After the first exposure to caffeine, Ca\(^{2+}\) was replenished in the absence of caffeine to readjust the baseline. The tissue was then exposed to 80 mM K\(^+\) PSS and PE (20 µM) to reproduce the conditions of the previous experiments. After perfusion with zero Ca\(^{2+}\)
PSS and phenolamine (10 µM; and CPA and/or zero Na+ if blockade is required), a second caffeine-induced Ca2+ release was elicited (peak2).

The experiment in Fig. 4A represents a control for the experiments in Fig. 4, B-D, because it does not include any blockers. With the Na+-Ca2+ exchanger, SERCA, and the PMCA actively extruding Ca2+, peak1 and peak2 were observed. The results showed that, in contrast to the control peak (peak1), peak2 was nearly abolished, indicating that 1) Ca2+ was being emptied from the SR, into the ECS, and 2) SR Ca2+ was not replenished in a Ca2+-free medium. Figure 4, inset, represents the control for the caffeine-induced Ca2+ release, illustrating how reaplication of external Ca2+ can induce a subsequent caffeine response of similar magnitude to the first if Ca2+ is present in the bathing solution.

Figure 4B illustrates how blockade of the Na+-Ca2+ exchanger can affect the Ca2+ content within the SR. Following the same protocol as in the control (see Fig. 4A), except that external Na+ was removed along with external Ca2+, we were able to observe a large second peak, suggesting that the Na+-Ca2+ exchanger controls the emptying of the caffeine-sensitive SR Ca2+ store. With the Na+-Ca2+ exchanger blocked, there was a large buildup of Ca2+ in the SR probably as a result of the reuptake of released Ca2+ without the possibility of unloading via the Na+-Ca2+ exchanger.

In Fig. 4C, determination of SR activity, in the face of SERCA blockade, was pursued. Because SERCA is thought to be a significant player in removal of cytoplasmic Ca2+, its absence should clearly alter the Ca2+ content within the SR. The experimental protocol of Fig. 4A was repeated, except that CPA (20 μM) was added with the Ca2+ loading stimulus. The first caffeine-induced Ca2+ peak was of standard height; however, the second caffeine stimulus produced no response. This is to be expected with SERCA blockade, since SERCA, under this condition, is unable to refill the SR.

When SERCA was blocked with CPA the SR Ca2+ sparing effects of zero Na+ were abolished (Fig. 4D).

Comparing the refilling capacity of the SR in the presence of absence of SERCA and Na+-Ca2+ exchanger inhibitors. The amplitude of the first and second caffeine-induced Ca2+ responses were ratioed (peak2/peak1) and are compared in Fig. 5. With the activity of all the extrusion pathways intact (control), peak2/peak1 of 13.2 ± 6.7%, n = 4, was very low. SERCA blockade appeared to further reduce SR Ca2+ by preventing reuptake (peak2/peak1 = 1.7 ± 1.7%, n = 4, though not significantly different from the control, P < 0.3). When SERCA and the Na+-Ca2+ exchanger were blocked simultaneously, the Ca2+ replenishing effect of Na+-Ca2+ exchanger blockade was nullified by the depleatory effect of CPA (peak2/peak1 = 5.1 ± 2.5%, n = 4), indicating that SERCA is an upstream effector of SR-mediated Ca2+ extrusion. In contrast to the responses seen in the control and those with CPA, the removal of external Na+ by itself markedly enhanced Ca2+ reuptake into the SR as seen by the large peak2-to-peak1 ratio of 85.5 ± 13.5%, n = 4.

**DISCUSSION**

Ca2+ extrusion is a physiological process that is essential for keeping the resting [Ca2+]i low in response to the Ca2+ entry that is due to the activity of a variety of Ca2+ channels in the presence of a steep electrochemical gradient across the PM. Our aim in designing this study was to better understand the mechanisms involved in Ca2+ extrusion. Total impairment of Ca2+ pumping activity (i.e., PMCA, SERCA, and mitochondrial) and Na+-Ca2+ exchange completely abolished Ca2+ extrusion as seen by a plateau in the elevated steady-state [Ca2+]i, after high K+ stimulation and the subsequent perfusion with a Ca2+-free bathing solution. PMCA blockade was achieved through the reduction of ATP synthesis (using FCCP and IAA; see Ref. 25), which also inhibits SERCA (29) and is reported to markedly reduce the bidirectional operation of the Na+-Ca2+ exchanger in the squid axon (9). In determining the relative contribution of each of the distinct Ca2+ translocators, we found that, of the total Ca2+ extrusion that could be blocked by complete metabolic inhibition, 47% was extruded by the Na+-Ca2- exchangers, and 53% was extruded via the PMCA. Similar values were obtained by Furukawa and colleagues (12) and Mccarren and co-workers (20), using the stomach smooth muscle of Bufo marinus, observed a Na+-dependent [Ca2+]i decline accounting for 38–52% of the total Ca2+ removal between a [Ca2+]i of 300 and 500 nM (20). Although there is excellent agreement between the results quoted above, it warrants considering the experimental conditions before extrapolation to physiological conditions in vivo. In our study, temperature was lowered from 37°C to room temperature, which may have had differential effects on the Ca2+-ATPases and...
the Na\(^+\)-Ca\(^{2+}\) exchanger (10). Another factor is the removal of external Na\(^+\), which, when used to block Na\(^+\)-Ca\(^{2+}\) exchange, can alter pH\(_i\), leading to a change in the affinity of SERCA, PMCA, and the Na\(^+\)-Ca\(^{2+}\) exchanger for Ca\(^{2+}\). However, in a study by Motley et al. (23), switching to a Na\(^+\)-free solution was associated with negligible changes in pH\(_i\). An additional potential problem is whether the Na\(^+\) substitutes, Li\(^+\) or N-methyl-d-glucamine, have different effects on the inferior vena cava, although, according to one study, no differences were observed (13). Further complexity is introduced by the use of multicellular preparations, which, unlike single cells, exhibit an averaged response but which, on the other hand, do not require isolation with digesting enzymes that may alter cell physiology. The multicellular preparation may have contributed to the requirement for two exponential components in fitting the rates of [Ca\(^{2+}\)]\(_i\) decline. However, the fact that several mechanisms contribute to Ca\(^{2+}\) extrusion and that cellular Ca\(^{2+}\) redistribution may take place would also tend to complicate the kinetics. For this reason, we adopted a second method by measuring the instantaneous rate of [Ca\(^{2+}\)]\(_i\) decline at a fixed elevated [Ca\(^{2+}\)]\(_i\) value, which led to conclusions identical to those derived from measuring changes in the fast rate constant.

Aside from the PMCA and the Na\(^+\)-Ca\(^{2+}\) exchanger, no other extrusion pathway has been clearly established. Nonetheless, previous work from this laboratory has eluded to a complementary extrusion mechanism that involves the SR and structurally linked Na\(^+\)-Ca\(^{2+}\) exchangers (30). It was postulated that the superficial SR acts as a buffer barrier to Ca\(^{2+}\) entry and that, to maintain this function, the SR must release Ca\(^{2+}\) into the subplasmalemmal space from where it is subsequently extruded (31). Chen and van Breemen (7) also suggested the presence of an extrusion pathway between the SR and the PM when they found that inhibition of Ca\(^{2+}\) accumulation by the SR increases [Ca\(^{2+}\)]\(_i\), which was not accompanied by an increase in Ca\(^{2+}\) influx. Suzuki et al. (28) have observed reduced Ca\(^{2+}\)-activated K\(^+\) channel activity (an indicator for localized Ca\(^{2+}\) increases derived from the SR) in smooth muscle treated with CPA, suggesting that Ca\(^{2+}\) uptake is immediately followed by extrusion of Ca\(^{2+}\) toward the ECS. Several other studies (24, 33) lead to the conclusion that this unique extrusion pathway does indeed exist. One study supports the idea that Ca\(^{2+}\) taken up into the SR is later extruded through an interaction between the SR and the PM and that, when SERCA is blocked, cytosolic Ca\(^{2+}\) is removed by the PM extrusion mechanisms not involved in SR-mediated Ca\(^{2+}\) extrusion (19).

The proposed participation of the SR in Ca\(^{2+}\) extrusion from the cells is consistent with the observations made in this study in which SERCA blockade reduced the rate of [Ca\(^{2+}\)]\(_i\) decline by 23%. An alternative interpretation of this result, which does not involve a multistep Ca\(^{2+}\) transport between SERCA and Na\(^+\)-Ca\(^{2+}\) exchange, would be that the SR is accumulating and retaining cytoplasmic Ca\(^{2+}\) during exposure to zero external Ca\(^{2+}\). In this case, SERCA would function additively to Na\(^+\)-Ca\(^{2+}\) exchange and the PMCA. However, our finding that the effects of CPA and zero external Na\(^+\) were not additive is not consistent with this interpretation. Other findings reported in results also diminish the likelihood of simple SR accumulation and retention as a possible explanation. We found that there was no significant difference in caffeine-sensitive SR Ca\(^{2+}\) content when comparing the [Ca\(^{2+}\)]\(_i\) decline under control and CPA conditions (Fig. 4, A and C). Removal of external Na\(^+\) greatly enhanced SR Ca\(^{2+}\) retention, but its abolition by CPA did not significantly slow the rate of [Ca\(^{2+}\)]\(_i\) decline. Results first obtained by Aaronson and van Breemen (1) then later confirmed by Blaustein and colleagues (3) established that, in VSM, the Na\(^+\)-Ca\(^{2+}\) exchanger plays a key role in controlling the amount of Ca\(^{2+}\) stored in the SR. One reason for the high SR Ca\(^{2+}\) content in zero Na\(^+\) media may be that Ca\(^{2+}\) released from the SR into the junctional space is unable to leave the cell because of the blocked Na\(^+\)-Ca\(^{2+}\) exchanger and is quickly pumped back into the SR without being detected by cytoplasmic fura 2, as Wol ska and Lewartowski (34) demonstrated. Such an Na\(^+\)-sensitive pool has also been described in guinea pig taeni coli (1). In parallel experiments by Chen and van Breemen (8; using thapsigargin as a SERCA inhibitor in place of CPA), similar data were obtained. The results presented here are best explained by the model presented in Fig. 6. This model assumes that a fraction (~50%) of the Na\(^+\)-Ca\(^{2+}\) exchangers is positioned close to the peripheral SR and functions mainly to remove Ca\(^{2+}\) from the junctional space between PM and SR membranes (denoted [Ca\(^{2+}\)]\(_j\)). [Ca\(^{2+}\)]\(_j\) is thought to be elevated above the bulk myoplasmic Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_m\); see Ref. 27) because of asymmetric release or impaired diffusion away from the release sites. The low-affinity, high-velocity Na\(^+\)-Ca\(^{2+}\) exchanger...
transports Ca\(^{2+}\) from the junctional space to the ECS. This assumption is supported by our observation that inhibition of this process allows reuptake of Ca\(^{2+}\) into the SR via SERCA. Additional evidence in support of SR-mediated Ca\(^{2+}\) extrusion is presented by Stehno-Bittel and Sturek (27), who also demonstrated the presence of peripheral Ca\(^{2+}\)-sensitive Na \(^{+}\)-coupled transport across the sarcolemma. Although experimental data was recently confirmed by Blaustein and co-workers (28), it has also been suggested that local IP \(_{3}\) gradients may promote the asymmetrical Ca\(^{2+}\) release by the peripheral SR. Although experimental data support the interaction between the peripheral SR and the Na\(^{-}\)-Ca\(^{2+}\) exchanger has also been proposed on the basis of ultrastructure. Moore et al. (22), using digital imaging fluorescence microscopy and a constrained deconvolution algorithm for processing the data, showed colocalization and suggested functional coupling of the Na\(^{-}\)-Ca\(^{2+}\) exchanger and the SR in smooth muscle. This was recently confirmed by Blaustein and co-workers (16, 17). The pathway for SR unloading relies on an asymmetrical arrangement of SERCA and SR Ca\(^{2+}\) release sites such that the Ca\(^{2+}\) uptake from the myoplasm is preferentially released by Ca\(^{2+}\) channels facing the junctional space rather than by those facing the myoplasm. Additional evidence suggests that the Na\(^{-}\)-Ca\(^{2+}\) exchanger may have selective access to SR Ca\(^{2+}\) (17). It has also been suggested that local IP \(_{3}\) gradients may promote the asymmetrical Ca\(^{2+}\) release by the peripheral SR. Although experimental data support the interaction between the Na\(^{-}\)-Ca\(^{2+}\) exchanger and SR Ca\(^{2+}\) transport, the detailed mechanism of this process is, at present, unclear.

In conclusion, our findings support a role for the VSM SR in Ca\(^{2+}\) extrusion by a sequential coupling of SERCA, Ca\(^{2+}\) release channels, and the Na\(^{-}\)-Ca\(^{2+}\) exchanger.

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