Effects of Alkaline pH on Sarcoplasmic Reticulum Ca\(^{2+}\) Release and Ca\(^{2+}\) Uptake*

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Alkalization-induced Ca\(^{2+}\) release from isolated frog or rabbit sarcoplasmic reticulum vesicles appears to consist of two distinct components: 1) a direct activation of ruthenium red-sensitive Ca\(^{2+}\) release channels in terminal cisternae and 2) an increased ruthenium red-insensitive Ca\(^{2+}\) efflux through some other efflux pathway distributed throughout the sarcoplasmic reticulum. The first of these releases exhibits an alkalization-induced inactivation process and does not depend on the ruthenium red-insensitive form of Ca\(^{2+}\) release as a triggering agent for secondary Ca\(^{2+}\)-induced Ca\(^{2+}\) release. Both releases are inhibited when the extravesicular (i.e. cytoplasmic) free [Ca\(^{2+}\)] is reduced. This may reflect an increased sensitivity of the Ca\(^{2+}\) release channels to Ca\(^{2+}\) at alkaline pH. The pH sensitivity of the ruthenium red-sensitive Ca\(^{2+}\) release channels could be of significance during excitation-contraction coupling. The ruthenium red-insensitive form of Ca\(^{2+}\) release is less likely to be physiologically relevant, but it probably has contributed greatly to reports of alkalization-induced decreases in net sarcoplasmic reticulum Ca\(^{2+}\) uptake, particularly under conditions where oxalate supported Ca\(^{2+}\) uptake is much less affected, as here.

Alterations in pH have been reported to affect the ability of isolated sarcoplasmic reticulum (SR) vesicles to take up (1-11) or release Ca\(^{2+}\) (12-22). The first reports of a direct effect of an increase in Ca\(^{2+}\) permeability of isolated SR by alkalization of the medium date from 1970 (12, 13), and such alkalization-induced Ca\(^{2+}\) release has since been described in greater detail (15, 18). Recently, the pH sensitivity of SR Ca\(^{2+}\) release channels incorporated into planar lipid bilayers has been reported (23, 24), with alkalization increasing the probability that these channels will open.

Suggestions that such alkalization-induced Ca\(^{2+}\) release could have physiological significance for muscle excitation-contraction coupling (25) have been disputed by several groups reporting that myoplasmic pH transients which accompanied fiber stimulation were extremely small and quite slow in comparison to SR Ca\(^{2+}\) release (25-27), but none of these studies addressed the possibility of larger local pH transients in the triadic gap. Suggestions have been made that protons might serve as counter-ions during Ca\(^{2+}\) release, entering the SR through the same channels as Ca\(^{2+}\) exits (27, 28). If so, local alkalization might be expected even after Ca\(^{2+}\) release has started, with possible modulatory consequences.

This report was undertaken to characterize features of alkalization-induced SR Ca\(^{2+}\) release and to begin to assess its physiological significance. We report here the involvement of two different pathways for alkalization-induced Ca\(^{2+}\) release. Like heavy metal-induced Ca\(^{2+}\) release (19, 29), one of these pathways involves the Ca\(^{2+}\)-induced Ca\(^{2+}\) release channel. The other pathway is of significance to the decreased net Ca\(^{2+}\) uptake observed in isolated SR studies, and it probably helps explain much of the discrepant pH sensitivities reported for net Ca\(^{2+}\) uptake in the presence and absence of oxalate, and Ca\(^{2+}\)-stimulated ATPase activity.

**EXPERIMENTAL PROCEDURES**

Terminal cisternae and light SR subfractions were prepared from rabbit skeletal muscle according to Saito et al. (30). They were stored in liquid nitrogen until needed for up to several months. Frog SR was prepared according to similar procedures as modified by Volpe et al. (31) and Brunker et al. (29). All experiments presented here were performed with isolated frog SR subfractions, but most have also been performed with rabbit SR subfractions with very similar results. Virtually all spectrophotometric experiments have been performed at least in duplicate, with representative traces shown.

Alkalization-induced Ca\(^{2+}\) release was studied spectrophotometrically by a minor modification of the procedures of Nakamura and Schwartz (18). Frog SR (500 µg of protein) was preloaded with endogenous Ca\(^{2+}\) in the sample, usually supplemented with added CaCl\(_2\) (6.25-25 nmol), at 20-21 °C in a medium containing 150 mM KCl, 20 mM Tris-maleate, 0.25 mM antipyrilazo III, 1 mM MgATP, 5 mM Na\(_2\)phosphocreatine, and 20 µg/ml creatine phosphokinase, pH 6.5. Ca\(^{2+}\) uptake and release were monitored using A\(_{550-nm}\) on a Hewlett Packard 8451 A diode array spectrophotometer. Following completion of uptake, 0.2 M KOH (generally 35 µl) was added to the 1.0 ml in the cuvette, raising the pH from 6.5 to 7.5. Similar results were obtained if Tris base additions were substituted for the KOH. The alkalization results in an instantaneous decrease in dye absorbance at 710 nm that precedes any Ca\(^{2+}\) release that subsequently takes place, but this decrease does not interfere with measurement of the release. In every experiment a final addition of Ca\(^{2+}\) is made to recalibrate the system under precisely the conditions under which Ca\(^{2+}\) release was elicited in that experiment. This was critical since pH did alter the sensitivity of the antipyrilazo III-Ca\(^{2+}\) reaction as monitored by absorbance changes. Since maleate has also been shown to promote a spontaneous release of Ca\(^{2+}\) (32, 33) we tested PIPES, which does not support such release (32). Alkalization-induced Ca\(^{2+}\) release was also seen in PIPES solutions in the absence of maleate (not shown). Rates of Ca\(^{2+}\) release were calculated from lines hand drawn through the maximal increases of absorbance during release.

Ca\(^{2+}\) release induced by cation substitution designed to change the membrane potential was accomplished by preloading the SR with CaCl\(_2\) and then diluting it with a 9-fold excess of isoelectric Tris-substituted assay medium (complete with ATP regenerating system, antipyrilazo III, pH 6.5). Ca\(^{2+}\) uptake was monitored at 20-21 °C in the same medium in the presence of 6 mM oxalate, as described (34),

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‡The abbreviations used are: SR, sarcoplasmic reticulum; PIPES, 1,4-piperazinediethanesulfonic acid; EGTA, [ethylenebis(oxyethyl- eneinitril)]tetraacetic acid; HEDTA, N-hydroxyethylene diaminetriacetic acid; TMA, tetramethylammonium.
Effects of Alkaline pH on SR Ca\(^{2+}\) Release and Uptake

and Ca\(^{2+}\) ATPase was monitored in the same solution with light SR rendered leaky using the Ca\(^{2+}\) ionophore A23187 (2 \(\mu\)M) so as to negate indirect effects of increased Ca\(^{2+}\) efflux on the ATPase determinations (34). Reactions were terminated using 10% trichloroacetic acid, and phosphate determined according to Ottolenghi (35). Total ATPase activity was determined in the presence of 50 nM of CaCl\(_2\). Basal ATPase levels (in the presence of 1 mM EGTA) were subtracted where appropriate.

Unidirectional Ca\(^{2+}\) influx determinations were carried out by first preloading 300 \(\mu\)g of light SR samples with endogenous Ca\(^{2+}\) plus 12.5 nMol CaCl\(_2\) added exogenously at pH 6.5 or 7.5 in a final volume of 1 mL in the spectrophotometer. After Ca\(^{2+}\) uptake had reached a steady state (uptake was essentially complete at pH 6.5), 20 \(\mu\)L of a pH 6.5 Ca\(^{2+}\)/EGTA solution 100 mM in EGTA was added such that the absorbance on the spectrophotometer did not change (0.20 mM Ca\(^{2+}\)/2.0 mM EGTA final concentration, estimated free [Ca\(^{2+}\)] = 455 nM). This solution contained \(60^n{\text{Ca}}\) for the influx determinations. For unidirectional efflux determinations, \(60^n{\text{Ca}}\) was applied during the loading procedure instead and only non-radioactive Ca\(^{2+}\) added together with the EGTA buffer. At various times 100- \(\mu\)L aliquots were then removed and filtered through 0.45-\(\mu\)m cellulose nitrate filters (Millipore HAWP) and washed with 2 \(\times\) 2 mL rinses of 150 mM KCl, 20 mM Tris-maleate solution containing the same Ca\(^{2+}\)/EGTA buffer described above.

The influx and efflux measurements at pH 7.5 were performed in an analogous fashion except that more Ca\(^{2+}\) was left outside the vesicles by the time a steady-state level of loading had been reached at 3 min. This precluded the use of an EGTA-buffered solution, so 10 \(\mu\)L of a pH 7.5 Ca\(^{2+}\)/HEDTA buffer system was employed instead. In this case the buffer added corresponded to 0.57 mM Ca\(^{2+}\)/1.0 mM HEDTA; estimated free [Ca\(^{2+}\)] = 8.7 \(\mu\)M. Otherwise, determinations were performed as described for pH 6.5.

A coupled enzyme assay utilizing NADH oxidation in the presence of lactate dehydrogenase was performed as described in Palade et al. (36), with appropriate modifications for assay conditions.

The computer program used to calculate the free [Ca\(^{2+}\)] in Fig. 6 was entitled IONS, variations of which have been used previously in this laboratory and others (34, 36).

RESULTS

Alkalization-induced Ca\(^{2+}\) release can be conveniently monitored spectrophotometrically (Fig. 1), as seen by the upward movement of the trace following the instantaneous downward deflection accompanying KOH addition. Ruthenium red slows down but does not completely block this release. Using this assay system, described in detail under "Experimental Procedures," we demonstrate in Table I that all three frog SR fractions (light (R2), intermediate (R3), and heavy (R4)), when preloaded with equivalent amounts of Ca\(^{2+}\), respond to the pH change by releasing Ca\(^{2+}\). While the releases reported in Table I were greatest using terminal cisternae (R4), the presence of 1 \(\mu\)M ruthenium red in all solutions to block the Ca\(^{2+}\)-induced Ca\(^{2+}\) release channel (37, 38) reveals a near equivalence of one form of alkalization-induced Ca\(^{2+}\) releases in all fractions. Similar results were obtained using isolated rabbit SR subfractions (not shown). These results suggested that two distinct forms of alkalization-induced Ca\(^{2+}\) release existed: 1) a faster, ruthenium red-sensitive release probably involving the Ca\(^{2+}\)-induced Ca\(^{2+}\) release channels in the terminal cisternae, and 2) a slower, ruthenium red-insensitive release distributed throughout the SR, most easily studied in isolation using light SR.

In order to determine whether alkalization released Ca\(^{2+}\) which was sequestered inside the SR as opposed to that which was externally bound, we utilized the ionophore A23187. Little KOH-releasable Ca\(^{2+}\) remained associated with the SR sample after depletion of Ca\(^{2+}\) from the inside of the SR by the A23187 (not shown). Rapid release of internalized Ca\(^{2+}\) would be likely to be limited by the rate at which counter-ions move. These counter-ions would prevent the build-up of a large membrane potential that could otherwise depress the rate of Ca\(^{2+}\) release. To assess the role of counter-ion movements in these releases, we performed experiments in which choline\(^{+}\) or TMA\(^{+}\) replaced K\(^{+}\) in the medium. As seen in Table II,

![Fig. 1. Alkalization-induced Ca\(^{2+}\) release from frog SR vesicles. Solid trace: isolated SR (300 \(\mu\)g of protein, fraction R3, added at the empty arrowhead) was preloaded with 6.55 nMol of CaCl\(_2\) (three rapid upward deflections to the left of each trace) at pH 6.5 as described under "Experimental Procedures." Ca\(^{2+}\) movements were followed spectrophotometrically with antipyrylazo III. Then isosmotic KOH was added when indicated to adjust the pH to 7.5. This produced an instantaneous downward deflection in the optical trace (antipyrylazo III responds to pH as well as Ca\(^{2+}\)) followed by a slower upward movement indicative of Ca\(^{2+}\) release. Finally, the system was recalibrated at the new pH with a fourth 6.25 nMol of CaCl\(_2\) addition (arrowhead). Dashed trace, following the same preloading procedure as for the solid trace, the experiment was repeated with 1 \(\mu\)M ruthenium red added + 30 s before the KOH. Dotted trace, the same amount of KOH was added to a cuvette containing reaction mixture without SR or added CaCl\(_2\). The downward deflection demonstrates the effect of alkalization on antipyrylazo III absorbance. Thus, only the rapid downward deflection in the other traces was an artifact of KOH addition; the slower upward movements of the traces represented Ca\(^{2+}\) release observed only with a Ca\(^{2+}\) preloaded sample present.](image-url)
both K\(^+\) replacements substantially diminished the rate of Ca\(^{2+}\) release from terminal cisternae. Releases from terminal cisternae were also decreased when Cl\(^-\) was replaced with gluconate. These results suggest that H\(^+\) or OH\(^-\) permeabilities are not so high (e.g., 39) that they could substitute for alkali metal cations or for Cl\(^-\) in providing rapid counter-ion movement during Ca\(^{2+}\) release. Neither cation nor anion replacement reduced the much slower rate of OH\(^-\)-induced Ca\(^{2+}\) release from light SR, indicating that it was not rate limited by movement of counter-ions.

To assess whether the extravesicular pH alone is the sole determinant of alkalinization-induced Ca\(^{2+}\) release, a sample was preloaded with identical amounts of CaCl\(_2\) at different pH values and then alkalinized to the same pH, 7.7. The rate of Ca\(^{2+}\) release decreased strongly as the pH of the preloading medium was increased (Fig. 3). The release rate was half-maximal with preloading at pH 6.8 and was barely detectable when preloading was carried out at pH 7.4. This suggested that other factors related to the preloading pH, perhaps intravesicular pH, pH gradients (20), transmembrane potentials, redistribution of Ca\(^{2+}\) into OH\(^-\)-insensitive vesicles, or alkalinization-induced inactivation of Ca\(^{2+}\) release, play an important role in modulating alkalinization-induced Ca\(^{2+}\) release.

Neither the ruthenium red-sensitive nor the ruthenium red-insensitive alkalinization-induced Ca\(^{2+}\) release is inhibited by weak acids (citrate, acetate) or bases (chloroquine, amantadine, Ref. 40) which should influence the intravesicular pH (not shown). To assess the possible role of pH gradients (20), we increased the proton permeability of the membrane by addition of gramicidin, nigericin, or carbonyl cyanide p-chlorophenylhydrazone. These ionophores should have increased the rate of dissipation of any proton gradients formed upon alkalinization as well as the rate of intravesicular alkalinization. None of these ionophores elicited a Ca\(^{2+}\) release from terminal cisternae, nor did they stimulate the rate of a subsequent OH\(^-\)-induced Ca\(^{2+}\) release (not shown). These results suggest that dissipation of pH gradients does not cause the ruthenium red-sensitive form of alkalinization-induced Ca\(^{2+}\) release. While these ionophores elicited no Ca\(^{2+}\) release from light SR, they did increase the rate of a subsequent OH\(^-\)-induced Ca\(^{2+}\) release (not shown).

Similarly, mediation of these releases by membrane potential changes (16, 17, 41, 42) seems unlikely. Since SR proton permeability is reported to be significant (39, 43), a sudden increase in OH\(^-\) outside the SR could cause the vesicles to generate a more inside negative membrane potential. Such an effect would only be expected to speed up an electrogenic cation pump and diminish the electrochemical gradient for any passive Ca\(^{2+}\) efflux. A dominant H\(^+\) permeability of the magnitude estimated by Nunogaki and Kasai (39) is unlikely here, since removal of K\(^+\) or Cl\(^-\) from the medium attenuated rates of release (Table II), whereas H\(^+\) or OH\(^-\) should have been able to serve as counter-ions if they were so permeant. Thus, an equivalent (or greater) change in membrane potential should be generated by increasing the extravesicular [Cl\(^-\)] or by reducing the extravesicular [K\(^+\)]. Of these maneuvers, it is known that [Cl\(^-\)] additions do cause Ca\(^{2+}\) release from isolated SR (16, 17), but such increases in [Cl\(^-\)] have been shown to produce osmotic rupture of the membranes (42). Consequently, we assessed effects of reduction of [K\(^+\)] instead. A 10-fold dilution of [K\(^+\)] outside the SR by addition of nine volumes of iso-osmotic Tris/Cl medium produced only extremely slow release of Ca\(^{2+}\) (not shown). Therefore, it is unlikely that alkalinization triggers Ca\(^{2+}\) release by altering the membrane potential. The effect of alkalinization during preloading (Fig. 3) is then also less likely to be membrane potential-related, although it could involve a redistribution of Ca\(^{2+}\) into OH\(^-\)-insensitive vesicles during preloading. Signs of a possible inactivation of OH\(^-\)-induced Ca\(^{2+}\) release can be noted in the decline of release rates from terminal cisternae.

**Fig. 2.** Dependence of alkalinization-induced Ca\(^{2+}\) release from purified SR subfractions on the extent of alkalinization. Experiments with purified terminal cisternae (300 μg, fraction R4, two separate preparations identified as ○, ●), or light SR (300 μg, fraction R2, two separate preparations identified as ○, ●) were performed as described in Fig. 1, except that the amount of 0.2 M KOH added was varied. The pH was measured at the conclusion of each experimental determination.

**TABLE II**

Effects of counter-ion replacement on alkalinization-induced Ca\(^{2+}\) release

<table>
<thead>
<tr>
<th>Terminal cisternae</th>
<th>Light SR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rate of Ca(^{2+}) release</td>
<td>Extent of Ca(^{2+}) release</td>
</tr>
<tr>
<td>(μmol/mg·min)</td>
<td>(nmol/mg)</td>
</tr>
<tr>
<td>KCl medium</td>
<td>4.34 ± 0.51</td>
</tr>
<tr>
<td>Choline-Cl medium</td>
<td>0.41 ± 0.04</td>
</tr>
<tr>
<td>TMA-Cl medium</td>
<td>0.29 ± 0.04</td>
</tr>
<tr>
<td>Potassium gluconate medium</td>
<td>1.39 ± 0.51</td>
</tr>
</tbody>
</table>

Numbers in parentheses represent the % of preloaded Ca\(^{2+}\) that was released. Releases were elicited by alkalinization to pH 7.5 with 60 μl of 0.2 M Tris base. Values given represent the mean ± S.D. of duplicate determinations. In two light SR cases only individual determinations were performed.
Effects of Alkaline pH on SR Ca\textsuperscript{2+} Release and Uptake

Fig. 3. Alkalinization-induced Ca\textsuperscript{2+} release also depends on the preloading pH. SR (300 \mu g, fraction R3) was preloaded at several pH values with 12.5 nmol of CaCl\textsubscript{2} delivered as a bolus. For these experiments, part of the 60 \mu l of 0.2 M KOH used to attain a final pH of 7.7 was added before preloading. After a steady state in Ca\textsuperscript{2+} uptake had been reached with identical readings obtained with a Ca\textsuperscript{2+}-specific electrode (World Precision Instruments, New Haven), the pH of all samples was raised to 7.7 with addition of the remainder of the 60 \mu l of 0.2 M KOH. Error bars represent standard errors of two to four determinations/point. The curve was drawn by eye.

Fig. 4. Alkalinization-induced inactivation of other forms of Ca\textsuperscript{2+} release mediated by the Ca\textsuperscript{2+}-induced Ca\textsuperscript{2+} release channel. A, terminal cisternae (300 \mu g) were preloaded with endogenous Ca\textsuperscript{2+} plus 6.25 nmol of CaCl\textsubscript{2} at pH 6.5 (not shown) and then challenged with 12.5 nmol of CaCl\textsubscript{2} (Ca) to cause Ca\textsuperscript{2+}-induced Ca\textsuperscript{2+} release (control trace) or alkalinized to pH 8.0 prior to the same CaCl\textsubscript{2} challenge (pH 8.0 trace). Note that severe alkalinization to pH 8.0 elicited no Ca\textsuperscript{2+} release by itself and prevented Ca\textsuperscript{2+} from inducing the two forms of alkalinization-induced Ca\textsuperscript{2+} release. Arrowheads near the end of each trace represent 6.25 nmol of CaCl\textsubscript{2} additions for recalibration purposes.

at pH values approaching 8.0 (Fig. 2). Severe alkalinization to pH 8.0 not only inactivated OH\textsuperscript{-}-induced Ca\textsuperscript{2+} release but also was able to inactivate other forms of Ca\textsuperscript{2+} release through the same channel. As shown in Fig. 4, alkalization to pH 8.0 clearly was able to inactivate caffeine- and Ca\textsuperscript{2+}-induced Ca\textsuperscript{2+} releases from terminal cisternae.

We have tested many substances as potential inhibitors of the two forms of OH\textsuperscript{-}-induced Ca\textsuperscript{2+} release. Alkalinization-induced Ca\textsuperscript{2+} release from light SR is inhibited by oxalate (Fig. 5) and by phosphate or pyrophosphate, other Ca\textsuperscript{2+}-precipitating anions (not shown). But every other agent tested (more than 200) failed to appreciably inhibit the release from light SR (not shown). The relative lack of effects on light SR of several agents known to block Ca\textsuperscript{2+}-induced Ca\textsuperscript{2+} release (ruthenium red and others, 38), spontaneous Ca\textsuperscript{2+} release (tetracaine, 44), and two forms of Ag\textsuperscript{+}-induced Ca\textsuperscript{2+} release.

<table>
<thead>
<tr>
<th>Terminals cisternae relative rate of</th>
<th>Light SR relative rate of</th>
</tr>
</thead>
<tbody>
<tr>
<td>OH\textsuperscript{-}-induced</td>
<td>OFF-induced</td>
</tr>
<tr>
<td>Ca\textsuperscript{2+} release</td>
<td>Ca\textsuperscript{2+} release</td>
</tr>
<tr>
<td>Control</td>
<td>1.00</td>
</tr>
<tr>
<td>Inhibitors of Ca\textsuperscript{2+}-induced Ca\textsuperscript{2+} release</td>
<td></td>
</tr>
<tr>
<td>Ruthenium red (1 \mu M)</td>
<td>0.22</td>
</tr>
<tr>
<td>Tetracaine (300 \mu M)</td>
<td>0.36</td>
</tr>
<tr>
<td>Ba\textsuperscript{2+} (500 \mu M)</td>
<td>0.59</td>
</tr>
<tr>
<td>9-Aminoacridine (300 \mu M)</td>
<td>0.19</td>
</tr>
<tr>
<td>Inhibitors of spontaneous Ca\textsuperscript{2+} release</td>
<td></td>
</tr>
<tr>
<td>Tetracaine (5 min incubation, 300 \mu M)</td>
<td>0.27</td>
</tr>
<tr>
<td>Inhibitors of heavy metal-induced Ca\textsuperscript{2+} release</td>
<td></td>
</tr>
<tr>
<td>Dithiothreitol (1 \mu M)</td>
<td>0.86</td>
</tr>
<tr>
<td>Glutathione (1 mM)</td>
<td>0.78</td>
</tr>
</tbody>
</table>

TABLE II Effects of inhibitors of other forms of SR Ca\textsuperscript{2+} release
Effects of Alkaline pH on SR Ca\(^{2+}\) Release and Uptake

(dithiothreitol, glutathione, 29) are shown in Table III. Thus alkalinization-induced Ca\(^{2+}\) release from light SR likely utilizes a distinct Ca\(^{2+}\) influx pathway from these other releases. In contrast, several agents (other than ruthenium red) shown to inhibit the Ca\(^{2+}\)-induced Ca\(^{2+}\) release channel (38) greatly reduced the alkalinization-induced Ca\(^{2+}\) release from terminal cisternae (Table III). Thus, most of the alkalinization-induced Ca\(^{2+}\) release from terminal cisternae appears to be mediated by the Ca\(^{2+}\)-induced Ca\(^{2+}\) release channel localized there.

To determine whether alkalinization-induced Ca\(^{2+}\) release from terminal cisternae was due to a direct effect on the Ca\(^{2+}\) release channel or to a massive secondary Ca\(^{2+}\)-induced Ca\(^{2+}\) release brought about by a smaller Ca\(^{2+}\) release mediated by the ruthenium red-insensitive pathway, we tested whether 1 mM oxalate could completely inhibit alkalinization-induced Ca\(^{2+}\) release from terminal cisternae. The oxalate completely inhibited the ruthenium red-insensitive form of Ca\(^{2+}\) release from light SR (Fig. 5), as demonstrated also by the essential lack of release from terminal cisternae in the presence of ruthenium red. Yet in the absence of ruthenium red, an attenuated but relatively large and rapid Ca\(^{2+}\) release was still observed, indicating that alkalinization directly opened the terminal cisternae Ca\(^{2+}\) release channels without the need for a preceding smaller Ca\(^{2+}\) release to serve as a trigger. These results suggest that alkalinization per se is capable of opening the terminal cisternae Ca\(^{2+}\) release channel.

To determine the influence of extravascular Ca\(^{2+}\) on alkalinization-induced Ca\(^{2+}\) release, we preloaded the SR and then added variable quantities of either CaCl\(_2\) or EGTA in order to vary the free Ca\(^{2+}\) concentration just prior to KOH addition. The EGTA additions were too small to prevent exogenous Ca\(^{2+}\) additions from being detected by the antipyrilazo III present or to depress the pH achieved upon alkalinization. Such protocols revealed a large reduction in the rate of OH\(^-\)-induced Ca\(^{2+}\) release in the presence of even small amounts of EGTA, whether terminal cisternae or light SR was utilized (Fig. 6). The rates of OH\(^-\)-induced Ca\(^{2+}\) release in Fig. 6 are plotted as a function of the ambient free Ca\(^{2+}\) concentration at the time just after KOH addition. With both light SR and terminal cisternae, a Ca\(^{2+}\) dependence of the release is observed. In both cases, higher free [Ca\(^{2+}\)] stimulated the rate of OH\(^-\)-induced Ca\(^{2+}\) release, and lowered free [Ca\(^{2+}\)] depressed such release. Terminal cisternae appeared to require higher free [Ca\(^{2+}\)] for such stimulation than did light SR. The Ca\(^{2+}\) dependence of alkalinization-induced Ca\(^{2+}\) release indicates interaction between these two ligands and might imply that alkalinization enhances the sensitivity of the release channel(s) to Ca\(^{2+}\). We did not test for Ca\(^{2+}\)-induced inactivation or inhibition of OH\(^-\)-induced Ca\(^{2+}\) release (20, 45) from terminal cisternae, since Ca\(^{2+}\)-induced Ca\(^{2+}\) release started to take place before KOH could be added. Light SR displayed no clear signs of Ca\(^{2+}\)-induced inactivation of Ca\(^{2+}\) release at concentrations up to 10 \(\mu\)M free Ca\(^{2+}\).

We also wished to assess the effects of alkalinization-induced Ca\(^{2+}\) release on the rate and extent of Ca\(^{2+}\) uptake by isolated sarcoplasmic reticulum, which depend heavily on the pH of the medium (1–11). As seen in Fig. 7A, sarcoplasmic reticulum at pH 6.5 takes up Ca\(^{2+}\) quite quickly and completely in the absence of anions that precipitate Ca\(^{2+}\) inside the SR. At pH 7.0, Ca\(^{2+}\) uptake is of similar or faster (46) initial rate but (on the same time scale) less complete. At pH 7.5, only a small proportion of the Ca\(^{2+}\) present is taken up within this time frame, but with similar initial rate.

Identical Ca\(^{2+}\) uptake determinations in the presence of 3 \(\mu\)M ruthenium red (Fig. 7B) reveal that the inhibitory effect of alkalinization on SR Ca\(^{2+}\) uptake is reduced but not eliminated. An alkalinization-induced reduction in Ca\(^{2+}\) uptake is also present in purified terminal cisternae and light SR.
Effects of Alkaline pH on SR Ca²⁺ Release and Uptake

subfractions, whether ruthenium red is present or not (not shown). These results suggest that alkalization not only impairs Ca²⁺ uptake by opening SR Ca²⁺ release channels (23) but also by some other mechanism that is not restricted to terminal cisternae.

Recently, Dixon and Haynes (47) provided evidence that the Ca²⁺ pump of cardiac sarcolemma is an electroneutral Ca²⁺/2H⁺ exchanger. They found that such cardiac sarcolemma vesicles would lose the ability to transport Ca²⁺ if they ran out of counter-transportable H⁺ and that this effect could be reversed by introduction of the protonophores nigericin or carbonyl cyanide p-chlorophenylhydrazone. Since they also summarized evidence in favor of an electroneutral skeletal SR Ca²⁺ pump, we tested whether the alkalization-induced inhibition of SR Ca²⁺ uptake could be due to intravesicular H⁺ depletion. We found no stimulatory effect of nigericin or carbonyl cyanide p-chlorophenylhydrazone on SR Ca²⁺ transport at alkaline pH (not shown).

The pH-sensitive site in light SR could be another Ca²⁺ efflux pathway, possibly a channel, or the SR Ca²⁺ pump. Since oxalate eliminates this net Ca²⁺ release, measurements of Ca²⁺ uptake in the presence of oxalate should minimize pH effects on unidirectional efflux and allow more accurate assessment of direct pH effects on Ca²⁺ pumping. As seen in Table IV, the rate of Ca²⁺ uptake at pH 7.3–7.6 is only slightly inhibited relative to pH 6.5 or 6.6 when in the presence of oxalate. In contrast to the situation in Fig. 7 in the absence of oxalate, Ca²⁺ uptake is essentially complete at alkaline pH with oxalate present (not shown). This suggests that the SR Ca²⁺ pump is only slightly affected by alkaline pH if the intraluminal [Ca++] is held low by oxalate.

Ca²⁺-stimulated ATPase is nearly insensitive to pH when the determinations are performed in the presence of A23187 to make all vesicles leaky (Table IV). Thus again, when conditions do not permit a buildup of high free intravesicular [Ca++], the effect of moderate alkalization on the pump is relatively small. The experiments shown in Table IV demonstrate that the pump continues to hydrolyze ATP even at pH 6.0. It also does so at pH 7.5 even when oxalate and A23187 are absent and when net Ca²⁺ uptake would have halted (not shown). This observation was confirmed by measuring the ATPase with a coupled enzyme assay employing lactate dehydrogenase. The oxidation of reduced NADH was monitored continuously at 340 nm. ATP hydrolysis continued unabated even while Ca²⁺ uptake (independently monitored using antipyrilazo III) was severely curtailed (not shown).

One possible explanation for cessation of uptake after a brief period of activity was that alkaline pH might render the pump more susceptible to being inhibited by Ca²⁺ accumulated intravesicularly (3). Thus, we next assessed whether alkaline inhibition of net Ca²⁺ uptake by light SR involved a decrease in pump-mediated influx alone or, alternatively, the SR Ca²⁺ pump, we measured the rate of unidirectional ⁴⁰Ca efflux under steady-state conditions of no net Ca²⁺ movements.

Unidirectional ⁴⁰Ca efflux was determined by loading SR with ⁴⁰Ca. Then a Ca/EGTA-buffered solution (of the same free [Ca++] as that achieved by the SR Ca²⁺ uptake process was added. Samples were filtered, washed twice, and counted. As seen in Fig. 8, unidirectional ⁴⁰Ca influx and efflux rates at pH 6.5 are relatively low (<15 nmol/mg.min) in the steady state.

Table IV

**Effects of alkalization on SR Ca²⁺ pump properties**

Ca²⁺ uptake was monitored spectrophotometrically as described under "Experimental Procedures" and ATPase determinations were performed on leaky light SR in the presence of oxalate (see "Experimental Procedures"). All determinations were performed in duplicate. At the more alkaline pH values, the initial rate of uptake was very slow but then accelerated with time, as if there was delayed precipitation of calcium oxalate inside the vesicles. ND, not determined.

<table>
<thead>
<tr>
<th>pH</th>
<th>⁴⁰Ca uptake</th>
<th>ATPase</th>
<th>⁴⁰Ca uptake</th>
<th>ATPase</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>μmol/mg min</td>
<td>Total</td>
<td>Basal</td>
<td>μmol/mg min</td>
</tr>
<tr>
<td>pH 6.6</td>
<td>1.15</td>
<td>2.60</td>
<td>0.17</td>
<td>1.05</td>
</tr>
<tr>
<td>pH 7.3</td>
<td>1.54</td>
<td>2.03</td>
<td>0.33</td>
<td>1.10</td>
</tr>
<tr>
<td>pH 7.6</td>
<td>0.94</td>
<td>1.60</td>
<td>0.31</td>
<td>1.25</td>
</tr>
<tr>
<td>pH 7.8</td>
<td>0.56</td>
<td>1.48</td>
<td>0.29</td>
<td>1.19</td>
</tr>
<tr>
<td>pH 8.0</td>
<td>0.048</td>
<td>1.54</td>
<td>0.27</td>
<td>1.27</td>
</tr>
<tr>
<td>pH 6.5–7.5</td>
<td>1.71</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

³ Unidirectional ⁴⁰Ca influx and efflux should be equal under these conditions; if the data suggest otherwise, then one of the unidirectional flux determinations must be in error. A rapid enough backflux could lower a measured "unidirectional" flux sufficiently to cause errors which can be corrected for if not too severe (3, 11, 48). If the backflux is very large, it becomes nearly impossible to correct the flux data with accuracy. Under our experimental conditions of "unidirectional" influx measurement, a simultaneous efflux occurs from a small pool of poorly buffered intravesicular Ca²⁺, which can lead to underestimates of the true rate of unidirectional influx. When unidirectional efflux is measured, the corresponding influx occurs from a larger extravesicular Ca²⁺ pool which is buffered and contains a low specific activity of ⁴⁰Ca throughout. Thus unidirectional efflux is measured with greater accuracy than "unidirectional" influx.

The isotope determinations suggested that efflux occurred more rapidly than influx, despite no net Ca²⁺ flux under these conditions. Taken together, these results indicate that the "unidirectional" ⁴⁰Ca influx measurements were seriously underestimated due to the high rate of efflux. Indeed, an efflux of 50% of the accumulated Ca²⁺ in ~1.5 min at pH 6.5 causes an underestimation of the true rate of unidirectional influx. This underestimation is small at the earliest time points, but the 2 min influx time point represents less than half of the true unidirectional Ca²⁺ influx. Consequently, the rate of measured influx decreases over time, even though the true influx should remain constant under steady-state conditions.

FIG. 8. Unidirectional ⁴⁰Ca influx and efflux at pH 6.5 and 7.5 in the steady state. Determinations were performed as indicated under "Experimental Procedures." Influx determinations are shown in open circles, and efflux determinations are shown in the filled circles. Note that the rates of efflux at both pH values are significantly greater than the influx rates and that both rates are enhanced at pH 7.5.
For the pH 7.5 experiments, the SR was presented with the same load of Ca\(^{2+}\), but not all the Ca\(^{2+}\) was taken up. Consequently, the initial efflux curve (filled circles) started at a lower Ca\(^{2+}\) content than at pH 6.5. The experiments were carried out under the same conditions as at pH 6.5 except that the Ca/HEPES solution used had an appropriately higher free [Ca\(^{2+}\)]. As shown in Fig. 8, plotted on the same scale as the pH 6.5 experiments, unidirectional \(^{45}\)Ca efflux at pH 7.5 was dramatically more rapid than at pH 6.5. That the determination is credible is reflected in the fact that the rates of release from light SR in Table I would predict a similar, if not greater rate of unidirectional efflux, as would the Ca-Ca, exchange determinations of Fassold and Hasselbach at pH 7.8 (8). This suggests that the cessation of net uptake and high extravesicular free Ca\(^{2+}\) levels seen at alkaline pH are not due primarily to diminished Ca\(^{2+}\) influx (e.g., 3) but rather to enhanced Ca\(^{2+}\) efflux.

We questioned whether the small net Ca\(^{2+}\) uptake at pH 7.5 might not have reflected Ca\(^{2+}\) binding to the SR Ca\(^{2+}\) pump protein rather than sequestration inside the SR vesicles. The amount of relatively rapid Ca\(^{2+}\) uptake seen at pH 7.5 in Fig. 7 corresponds to ~5 nmol of Ca\(^{2+}\)/300 µg of protein. Assuming that light SR consists of 90% Ca\(^{2+}\) pump protein of 110,000 M\(_r\), 900 µg would equal ~3 nmol. Consequently, only about two Ca\(^{2+}\) are "taken up" per pump molecule, and this could represent occluded Ca\(^{2+}\) (49). The ionophore A23187 should liberate Ca\(^{2+}\) from the SR but would not be expected to displace Ca\(^{2+}\) bound directly to the pump. The results demonstrated that the Ca\(^{2+}\) taken up at pH 7.5 is actually internalized (released by A23187, not shown) rather than merely being bound tightly to the Ca\(^{2+}\) pump in a possibly occluded form. Most likely then, Ca\(^{2+}\) uptake proceeds until the SR fills up enough to generate a high rate of efflux.

We wished to determine whether the incomplete SR Ca\(^{2+}\) uptake at alkaline pH was due to a diminished SR capacity or to the establishment of a new [Ca\(^{2+}\)], "set-point," below which no further Ca\(^{2+}\) uptake takes place. To make this determination, we administered different amounts of Ca\(^{2+}\) to ascertain if net uptake ceased after the same amount of Ca\(^{2+}\) uptake or after the same free [Ca\(^{2+}\)], had been achieved. The [Ca\(^{2+}\)], achieved differed, but the amount of Ca\(^{2+}\) taken up was similarly small in all cases (Fig. 9). Once uptake ceased, further Ca\(^{2+}\) additions failed to promote additional Ca\(^{2+}\) uptake (not shown). Thus alkaline pH causes a diminished SR capacity.

When samples were followed at pH 7.5 for still longer periods of time, Ca\(^{2+}\) uptake actually resumed, although at a rate slower than that of the initial uptake. Eventually all the Ca\(^{2+}\) was taken up, and still more Ca\(^{2+}\) could be administered and taken up (Fig. 10, upper traces).

Calculation of ATP hydrolysis rates under similar conditions (Table IV) suggested that within 5 min, as much as 1 mM ATP could be hydrolyzed. Because of the regenerating system present, ADP levels would be buffered, but both protons and inorganic phosphate would be produced. As seen in Fig. 10, even 1 mM HCl or P\(_i\) added to a sample at the plateau phase of uptake was able to cause an immediate resumption of Ca\(^{2+}\) uptake at rates similar to samples resuming uptake at the end of the plateau phase. The HCl presumably reduces the alkalinization-induced Ca\(^{2+}\) efflux.\(^6\) By forming calcium phosphate complexes inside the SR (3, 50), phosphate would both reduce the rate of efflux and prevent any possible shutting down of the pump due to rises in intravesicular free [Ca\(^{2+}\)].

**DISCUSSION**

One part of the Ca\(^{2+}\) release induced by alkalinization appears to be mediated by the Ca\(^{2+}\)-induced Ca\(^{2+}\) release channel, since the release is much more rapid from terminal cisternae and is inhibited by ruthenium red and other blockers of Ca\(^{2+}\)-induced Ca\(^{2+}\) release. The other part of the OH\(^-\)-induced Ca\(^{2+}\) release is quite distinct from Ca\(^{2+}\)-induced Ca\(^{2+}\) release in that it is neither inhibited by ruthenium red nor preferentially expressed by terminal cisternae SR subfrac-
tions (37, 38), but instead seems to be present throughout the SR. The efflux pathway mediating this form of Ca\(^{2+}\) release also appears distinct from those mediating spontaneous Ca\(^{2+}\) release (40) or Ag\(^{+}\)-induced Ca\(^{2+}\) release from light SR (22). Two different Ca\(^{2+}\)-conducting channels have previously been reported in experiments in which SR vesicles were fused into planar lipid bilayers (51); the ruthenium red-sensitive one has previously been shown to be activated by alkalinization (23, 24). Ma et al. (23) have shown that alkaline pH (7.4) is able to increase the ryanodine receptor's probability of being open when applied to either side of the channel, but more recently Rousseau and Pinkos (24) have described pH-sensitive gating on only the cis (cytoplasmic) side of the SR Ca\(^{2+}\) release channel. This would be consistent with our protonophore results suggesting little role of intravesicular pH in alkalinization-induced Ca\(^{2+}\) release.

The involvement of a pH gradient in alkalinization-induced Ca\(^{2+}\) release has previously been proposed (20), and proton extrusion during Ca\(^{2+}\) pumping has been measured (52). However, the releases seen here cannot be generated by addition of protonophores such as gramicidin (20, 52), nor is alkalinization-induced Ca\(^{2+}\) release from terminal cisternae stimulated by such protonophores. Aspects of alkalinization-induced inactivation of OH\(^{-}\)-induced Ca\(^{2+}\) release were demonstrated in Figs. 2, 3, and 4. Alkalinization was also shown to inactivate other forms of Ca\(^{2+}\) release through the same channel, as seen in Fig. 4. This finding is completely novel. Its possible physiological significance may be reflected in the observation that channels might be partially inactivated by long term maintenance at normal myoplasmic pH levels around pH 7.0 (Fig. 3). If this occurs in vivo, moderate acidification during exercise might be expected to alleviate OH\(^{-}\)-induced inactivation of SR Ca\(^{2+}\) release.

The ruthenium red-sensitive alkalinization-induced Ca\(^{2+}\) release appears to involve a direct effect of pH on the terminal cisternae calcium release channel, as opposed to representing a secondary Ca\(^{2+}\)-induced Ca\(^{2+}\) release (Fig. 5). Nevertheless, the abolition of rapid Ca\(^{2+}\) release by EGTA suggests that OH\(^{-}\)-induced Ca\(^{2+}\) release involves a sensitization of the SR Ca\(^{2+}\) release channel to lower levels of free cytoplasmic Ca\(^{2+}\), as postulated for caffeine's effects on skinned fibers (53). This finding also suggests that alkalinization-induced Ca\(^{2+}\) release is not particularly likely to be involved in E-C coupling, since Ca\(^{2+}\) transients minimally perturbed by high concentrations of Ca\(^{2+}\)-buffering agents have been reported (25, 54). However, in these experiments no accurate determinations of resting free Ca\(^{2+}\) levels exist, particularly for the triadic gap.

The activation of alkalinization-induced Ca\(^{2+}\) release by extravascular Ca\(^{2+}\) has not previously been reported in vesicle experiments although it could have been inferred from the Ca\(^{2+}\) dependence of single channel activity performed at pH 7.4 (23) and even from skinned fiber experiments performed by Fabiato (53). In the present experiments, inactivation of alkalinization-induced Ca\(^{2+}\) release from light SR by Ca\(^{2+}\) did not occur; instead a Ca\(^{2+}\)-dependent activation of OH\(^{-}\)-induced Ca\(^{2+}\) release was observed.

Because alkalinization might have been inhibiting the SR Ca\(^{2+}\) pump, we wished to determine whether turnover of the pump might mediate the slower, ruthenium red-insensitive form of Ca\(^{2+}\) release. We consider this unlikely for the following reasons. First, 300 \(\mu\)g of SR protein composed of \(\sim 90\%\) pure 110,000 M, Ca\(^{2+}\) pump protein, would contain \(\sim 3\) nmol Ca\(^{2+}\) pump protein. The releases mediated by alkalinization are at least five times larger even in light SR. Since the releases are also time dependent in the range of several seconds in light SR, these results indicate that several turn-over cycles of Ca\(^{2+}\) pump protein would have to be involved. The process of alkalinization-induced Ca\(^{2+}\) release from light SR is not prevented by prior application of 100 \(\mu\)m quercetin (not shown), a Ca\(^{2+}\) pump inhibitor (55, 56). Additionally, we note that oxalate-supported Ca\(^{2+}\) uptake proceeds quite well at pH 7.5, and that Meissner (57) found Ca\(^{2+}\) uptake to proceed faster when a pH 6.2–7.6 change was imposed upon SR vesicles. Unless there is a strict additional intravesicular free Ca\(^{2+}\) requirement for pump uncoupling, alkalinization is unlikely to produce Ca\(^{2+}\) release via uncoupling of the SR Ca\(^{2+}\) pump, since alkalinization up to pH 7.6 does not uncouple the pump in the presence of oxalate. Finally, we note that the Ca\(^{2+}\) dependence of this release is opposite to that attributed by Inesi and de Meis (50) to slippage of the SR Ca\(^{2+}\) pump at neutral pH, where EGTA enhanced release. We conclude that the alkalinization-induced Ca\(^{2+}\) release we have been studying in light SR is not likely to principally involve any known mode of operation of the SR Ca\(^{2+}\) pump, even though the elevated pH might possibly somewhat reduce the rate of Ca\(^{2+}\) pumping during the release (10).

We also wished to provide an explanation for the near cessation of Ca\(^{2+}\) uptake at pH 7.5 after a short period of moderate activity. Oxalate, an anion which precipitates Ca\(^{2+}\) inside the SR lumen, thus prolonging Ca\(^{2+}\) pumping and inhibiting the less rapid OH\(^{-}\)-induced Ca\(^{2+}\) release, restores this incomplete Ca\(^{2+}\) uptake. This result by itself could be interpreted as an indication of either a prolonged involvement of the slow, ruthenium red-insensitive OH\(^{-}\)-induced Ca\(^{2+}\) release on the alkaline inhibition of net Ca\(^{2+}\) uptake or, alternatively, a diminished rate of Ca\(^{2+}\) influx due to the rise in internalized [Ca\(^{2+}\)]. ATPase measurements in the absence of A23187 revealed that ATP hydrolysis is not reduced concomitant with the reduction in net Ca\(^{2+}\) uptake activity. This suggested that the diminished uptake might not be due to diminished Ca\(^{2+}\) pump function. Direct measurement indicated that unidirectional 45Ca efflux is much more rapid at pH 7.5 than at pH 6.5. This indicated strongly that the primary effect of alkalinization is a large increase in unidirectional Ca\(^{2+}\) efflux. We conclude that the ruthenium red-insensitive form of OH\(^{-}\)-induced Ca\(^{2+}\) release can significantly curtail the extent of Ca\(^{2+}\) uptake by SR.

From the physiological point of view, it seems surprising that the SR is able to take up less Ca\(^{2+}\) at pH 7.0 than at pH 6.5. The isolated SR does not appear to behave as if the [Ca\(^{2+}\)], set point is altered as a function of pH, since Ca\(^{2+}\) remains approximately the same when Ca\(^{2+}\) is varied. Rather, the capacity of the SR for internalized Ca\(^{2+}\) appears to be reduced (Figs. 7 and 9). Since myoplasmic pH is much more prone to acidic activity than to alkalinization, an increase in SR capacity could have the net effect of increasing the SR's sequestration ability during fatigue-induced acidosis (58, 59) and might partly compensate for any reduced ability to release Ca\(^{2+}\) at the more acidic pH values.

However, the possible physiological consequences of such an effect appear to be nullified by the finding that Ca\(^{2+}\) uptake at alkaline pH resumes when even low concentrations of phosphate are present. Since the myoplasmic phosphate con-
centration is estimated to be several millimolar (60), it appears unlikely that the SR inside muscle fibers would take up Ca\textsuperscript{2+} only incompletely at neutral or moderately alkaline pH values. Thus, fatigue-associated acidosis would be unlikely to increase only incompletely at neutral or moderately alkaline pH values. This restorative effect of phosphate suggests that the frequent preference of biochemists for avoiding Ca\textsuperscript{2+}-precipitating anions may entail perils as serious as the overuse of such anions. Some phosphate should be included in isolated SR experiments presenting phenomena of purported physiological significance.

The physiological significance of alkalization effects on Ca\textsuperscript{2+} release may be greater than that on Ca\textsuperscript{2+} sequestration by SR. Previously, only small twitch-associated changes in bulk myoplasmic pH have been observed (25–27), a fact which has tended to negate a role for OH\textsuperscript{−}-induced Ca\textsuperscript{2+} release in skeletal muscle excitation-contraction (E-C) coupling. Involvement of OH\textsuperscript{−}-induced Ca\textsuperscript{2+} release in cardiac E-C coupling has similarly been rejected (53). In that study, pH changes smaller than those employed here did not cause SR Ca\textsuperscript{2+} release from skinned cardiac cells but did enhance subsequent Ca\textsuperscript{2+}-induced Ca\textsuperscript{2+} release (53). Nevertheless, the clear sensitivity of the skeletal SR Ca\textsuperscript{2+} release channel to alkalization observed here suggests that local pH changes in the triadic gap could assume large importance either for activation or possible inactivation of Ca\textsuperscript{2+} release. For example, moderate local alkalization could accelerate SR Ca\textsuperscript{2+} release even if it is not a primary stimulus. Alternatively, if a postulated H\textsuperscript{+} entry into the SR during Ca\textsuperscript{2+} release (27, 28) were to take place through the release channels, a local alkalization in the triadic gap might result. If large enough, this could then inactivate the release channels.

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