Luminal pH Regulates Calcium Release Kinetics in Sarcoplasmic Reticulum Vesicles†

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ABSTRACT: Calcium binding to triads isolated from rabbit skeletal muscle followed a single hyperbolic function in the pH range 5.5−8.0. Maximal binding was obtained at pH 8.0; decreasing the pH decreased the binding capacity and, at pH ≤6.0, increased K_d 2-fold. These results indicate that lowering the pH diminished calcium binding to calsequestrin, since this protein is the primary source of calcium binding sites in triads. Luminal pH had a marked effect on calcium release induced by 2 mM ATP, at pCa 5.0, pH 6.8. At a constant luminal [Ca^{2+}] of 0.1 mM, release rate constants (k) and initial rates of release increased steadily as a function of decreasing luminal pH; at luminal pH 7.5, values of $k < 0.4$ s^{-1} were found, whereas at pH 5.5 values of $k \approx 10$ s^{-1} were obtained. Increasing luminal [Ca^{2+}] from 0.05 mM to 0.7 mM had no effect on the k values measured at luminal pH 5.5. In contrast, at pH 6.8, increasing luminal [Ca^{2+}] produced a marked increase in k values, that reached maximal values of $k \approx 10$ s^{-1} at 0.7 mM luminal [Ca^{2+}]. Control experiments using fluorescent pH indicators showed that luminal pH did not change significantly during calcium release. It is proposed that luminal protons or calcium induces conformational changes in calsequestrin that in turn promote activation of the calcium release channels.

Calcium release in skeletal muscle proceeds through the ryanodine receptor-calcium release channels located in the sarcoplasmic reticulum (SR)[1] terminal cisternae regions (Melzer et al., 1995). The cytoplasmic regulation of the calcium release channels has been extensively studied. It is known that several agonists, such as micromolar calcium and millimolar ATP, or antagonists, millimolar magnesium and micromolar Ruthenium Red, act *in vitro* on the cytoplasmic side of the channel (Meissner, 1994; Coronado et al., 1994).

Cytoplasmic pH has a marked effect on single channel activity (Ma et al., 1988; Rousseau & Pinkos, 1990) and on calcium release kinetics in vesicles isolated either from rabbit or from frog skeletal muscle. Alkaline pH increases release rate constants (Meissner, 1990; Donoso & Hidalgo, 1993), and the rate of alkalinization-induced calcium release depends on the preloading pH: vesicles actively preloaded with calcium at acid pH display higher release rates than vesicles preloaded at alkaline pH (Dettbarn & Palade, 1991).

Luminal regulation of the calcium release channels has received less attention [for a review, see Hidalgo and Donoso (1995)]. Only a few studies have described activation of calcium release in vesicles by increasing the luminal Ca^{2+} concentration (Ikemoto et al., 1989; Nelson & Nelson, 1990; Gilchrist et al., 1992; Donoso et al., 1995). After fusion of SR vesicles with bilayers, the calcium channel opening probability (P_o) is also activated by increasing the *trans* (luminal) Ca^{2+} concentration (Sitsapesan & Williams, 1994, 1995). Decreasing the *trans* pH inhibits P_o after fusion of the purified ryanodine receptors with the bilayer (Ma et al., 1988) or induces a decrease in channel conductance after fusion of SR vesicles (Rousseau & Pinkos, 1990).

In the present work, we studied the effect of luminal pH on calcium release kinetics in triads isolated from rabbit skeletal muscle. We determined as a first step the effect of pH on calcium binding to triads. We found that calcium binding followed a single hyperbolic binding function in the pH range 5.5−8.0; lowering the pH decreased B_{max} but did not change K_d in the range pH 8.0−6.5, and increased the K_d 2-fold at pH 6.0 and 5.5. We investigated next the effect of luminal pH on calcium release from vesicles passively equilibrated with varying luminal [Ca^{2+}]. We found that decreasing the luminal pH from 6.8 to pH 5.5 overruled the effect of luminal calcium on release kinetics, so that after triggering release with ATP, high release rate constants were observed even at very low luminal calcium concentrations. We discuss a possible role of calsequestrin, through protein conformational changes caused either by luminal Ca^{2+} or by luminal protons, in controlling the luminal gating of the calcium release channels activated by cytoplasmic agonists.

EXPERIMENTAL PROCEDURES

Preparation of Triads. Triads were isolated from rabbit skeletal muscle in the presence of a combination of protease

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[1] Abbreviations: BCECF, 2′,7′-bis(2-carboxyethyl)-5(and 6)-carboxyfluorescein; CF, 5(and 6)-carboxyfluorescein; HEDTA, *N*-(hydroxyethyl)ethylenediaminetriacetic acid; HEPES, *N*-(2-hydroxyethyl)-piperazine-*N*′-2-ethanesulfonic acid; MES, 2-(*N*-morpholino)ethanesulfonic acid; MOPS, 3-(*N*-morpholino)propanesulfonic acid; SR, sarcoplasmic reticulum.

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inhibitors, following a procedure described in detail in previous work (Hidalgo et al., 1993). Triads were stored at −80 °C for up to 1 month.

Calcium Loading at Equilibrium. Triads (0.6 mg of protein/mL) were incubated in loading solutions containing 100 mM KCl and different concentrations of $CaCl_2$ plus $^{45}CaCl_2$ (10−20 mCi/mmol). The pH of the equilibration solution was controlled with 40 mM buffer, using HEPES titrated with Trizma base (HEPES/Tris) for pH 8.0; MOPS/Tris for pH 7.5, pH 6.8, and pH 6.5, and MES/Tris for pH 6.0 and pH 5.5. Following incubation of triads for 2 h at room temperature, luminal $[Ca^{2+}]$ and luminal pH were at equilibrium with the concentrations present in the incubation solution. After equilibration, 0.05 mL of the vesicle-containing solution was diluted in 1 mL of the same loading solution without radioactive calcium, and was filtered through Millipore filters (AA, 0.8 μm). The filters were washed with 5 mL of nonradioactive loading solution, and their radioactivity was determined in a liquid scintillation counter.

Calcium Release Studies. To measure calcium release kinetics, vesicles (0.6 mg of protein/mL) were equilibrated for 2 h at room temperature with solutions containing 100 mM KCl and variable concentrations of $^{45}CaCl_2$ (0.05−3 mM), at a specific activity of 10−20 mCi/mmol. The pH of the equilibration solution was controlled with 100 mM buffer, using MOPS/Tris for pH 7.5 and pH 6.8, and MES/Tris for pH 6.0 and pH 5.5; vesicles passively loaded with ^{45}Ca in 100 mM buffer accumulated on average 20% less ^{45}Ca than vesicles equilibrated in 40 mM buffer. In all cases, release was induced at room temperature by mixing the ^{45}Ca-loaded triads with a solution containing 2 mM ATP, pCa 5.0 (2 mM HEDTA plus 1.33 mM $CaCl_2$), 100 mM KCl, and 100 mM MOPS/Tris, pH 6.8. Release was determined in a fast filtration system (Biologic) following the procedures reported in detail elsewhere (Moutin & Dupont, 1988; Donoso & Hidalgo, 1993), except that the washing solution contained 1 mM $CaCl_2$. For release experiments done in the presence of valinomycin, 2 μM valinomycin was added both to the vesicles prior to equlibration and to the releasing solution.

Intravesicular pH Changes as a Function of Time. The fluorescent pH indicators 5(and 6)-carboxyfluorescein (CF) or 2′,7′-bis(2-carboxyethyl)-5(and 6)-carboxyfluorescein (BCECF) were used to measure the rate of change of intravesicular pH. The pH indicators were loaded into the vesicles either by three cycles of freezing and thawing or by incubation during a period of 4 h at room temperature; in both cases, solutions containing CF (1 mg/mL) or BCECF (0.5 mg/mL), 100 mM KCl and 100 mM MOPS/Tris, pH 7.0, or 100 mM MES/Tris, pH 6.0, were used. The extravesicular indicator was rapidly removed by filtration in a small Biogel A column, preequilibrated in the same buffer. A pH gradient was imposed by diluting the vesicles to 0.015 mg/mL in a solution of 100 mM KCl, 100 mM buffer at the desired pH, and the resulting fluorescence was measured as a function of time in a Shimadzu RF540 spectrofluorometer. Excitation and emission wavelengths for CF were 488 and 518 nm, respectively, and for BCECF, 495 and 525 nm. The slits for excitation and emission were 5 nm. To quench the external fluorescence due to indicator leakage from the vesicles, in all experiments 10 μL/mL of a commercial solution of anti-fluorescein rabbit antibodies was added to the dilution solution. This amount of antibody quenched efficiently the external CF or BCECF fluorescence, so that all measured fluorescence originated from indicator contained in the vesicular interior.

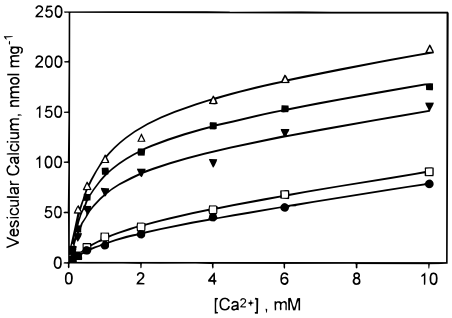

FIGURE 1: Effect of pH on the amount of calcium taken up at equilibrium by triads isolated from rabbit skeletal muscle. Triads were incubated in solutions of varying $[Ca^{2+}]$ and pH, and the total amount of calcium taken up at equilibrium by the vesicles was determined as described in the text. Standard errors for the mean of determinations done in triplicate were smaller than the size of the symbols. The best fit to the experimental points was given by the function: vesicular calcium (nmol/mg) = $B_{max}[Ca^{2+}]/(K_d + [Ca^{2+}]) + 5.44[Ca^{2+}]$. Key: open triangles, pH 8.0; solid squares, pH 6.8; solid triangles, pH 6.5; open squares, pH 6.0; solid circles, pH 5.5.

Other Procedures. Protein was determined according to Hartree (1972) using bovine serum albumin as standard. Free $[Ca^{2+}]$ was calculated with a computer program (Goldstein, 1979) using the binding constants for HEDTA and ATP reported elsewhere (Martell & Smith, 1974). To ensure accuracy, free $[Ca^{2+}]$ was always measured with a calcium electrode.

Materials. All reagents used were of analytical grade. Anti-fluorescein rabbit antibody, CF, and BCECF were obtained from Molecular Probes. Protease inhibitors (leupeptin, pepstatin A, benzamidine, and phenylmethanesulfonyl fluoride) were obtained from Sigma; Biogel A was purchased from Bio-Rad and $^{45}CaCl_2$ from DuPont−New England Nuclear Corp.

RESULTS

(1) Calcium Equilibration in the Triads as a Function of pH. A pH-dependent saturable component of calcium accumulation as a function of $[Ca^{2+}]$, plus a pH-independent linear component, was observed in the pH range 5.5−8.0 (Figure 1). Triads exhibited the highest calcium binding capacity at pH 8.0 (B_{max} = 167 nmol of calcium/mg of protein). Values of B_{max} decreased with decreasing pH but were still significant at pH 6.0 and 5.5, with 40 and 30 nmol of calcium/mg of protein, respectively (Table 1). In the range of pH 8.0−pH 6.5, the binding affinity of the saturable component remained constant, with $K_d \approx 0.65$ mM. Further decreasing the pH to 6.0 and 5.5 increased the K_d to 1.2 mM, indicating a 2-fold reduction in calcium affinity in this pH range.

We have shown in previous work that the saturable component of calcium binding to triads is due to calcium binding to calsequestrin (Donoso et al., 1995). The triads used in this work contained 20% calsequestrin, so that after correction for calsequestrin content, the number of calsequestrin binding sites as a function of pH can be calculated from the B_{max} values. The highest number of binding sites, 38 sites per calsequestrin molecule, were obtained at pH 8.0. This number decreased at lower pH values (Table 1); the

Table 1: Calcium Binding to Triads as a Function of pH[a]

pH	K_d (mM)	B_{max} (nmol/mg)	N
8.0	0.66 ± 0.05	167.0 ± 3.5	38
7.5	0.79 ± 0.06	134.8 ± 2.6	30
6.8	0.61 ± 0.04	130.4 ± 2.3	29
6.5	0.72 ± 0.10	107.8 ± 4.1	24
6.0	1.18 ± 0.17	41.5 ± 1.8	9
5.5	1.18 ± 0.21	29.7 ± 1.6	7

[a] Data are expressed as mean ± the error of the nonlinear fit to the experimental points described in the legend to Figure 1. *N* stands for the number of calcium binding sites per calsequestrin molecule, calculated according to a calsequestrin content of 20% relative to total triad proteins and a calsequestrin molecular mass of 45 kDa.

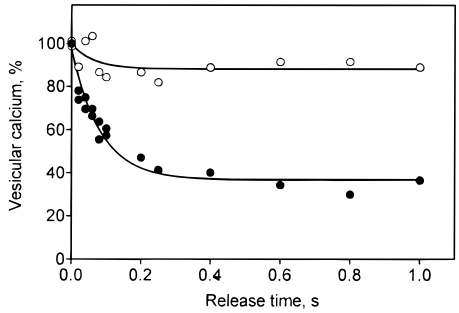

FIGURE 2: ATP-induced calcium release in triads with luminal 0.1 mM [Ca^{2+}], luminal pH 5.5. Vesicles were equilibrated with 0.1 mM [Ca^{2+}], pH 5.5, and release was induced with 2 mM ATP as stated under Experimental Procedures. Solid symbols, ATP-induced release; open symbols, release in the presence of 5 mM Mg^{2+} plus 10 μM Ruthenium Red. After ATP addition, calcium remaining in the vesicles (*N*, as % of total vesicular calcium) decayed with time as a single exponential plus an offset, such that $N = N_0 \exp(-kt)$ + offset. The best fit to the experimental data (line through solid symbols) yielded N_0 (%) = 58.3 ± 3.3; k = 10.7 ± 1.4 s^{-1}; offset (%) = 36.1 ± 2.3.

lowest number of sites was obtained at pH 5.5, with only 7 calcium binding sites per molecule.

The linear component of calcium binding to triad vesicles, that corresponds to nonspecific calcium binding plus intravesicular free calcium (Donoso et al., 1995), was not significantly affected by the change in pH.

(2) Effect of Luminal pH on Calcium Release. In all experiments, release was induced by mixing triads with well-defined luminal [Ca^{2+}] and luminal pH, with a solution of 2 mM ATP, pCa 5.0, pH 6.8. These conditions generated a positive or negative pH gradient, as well as a calcium concentration gradient that in the range of luminal [Ca^{2+}] studied, 0.05−3.0 mM, should always promote calcium efflux from the vesicles. The imposed pH gradient did not change significantly during the time course of calcium release, as described below.

Triads with luminal pH 5.5, 0.1 mM luminal [Ca^{2+}], released 60−88% of their accumulated calcium following a single exponential decay function, that in the experiment shown in Figure 2 (solid symbols) had a rate constant of 10.6 s^{-1}. Addition of 10 mM Mg^{2+} plus 10 μM Ruthenium Red thoroughly inhibited the specific release component (Figure 2, open symbols), leaving only the small release component previously shown to correspond to nonspecific calcium release (Donoso & Hidalgo, 1993). These results demonstrate that equilibration at pH 5.5 did not alter vesicular integrity, since release was still inhibited by calcium channel blockers.

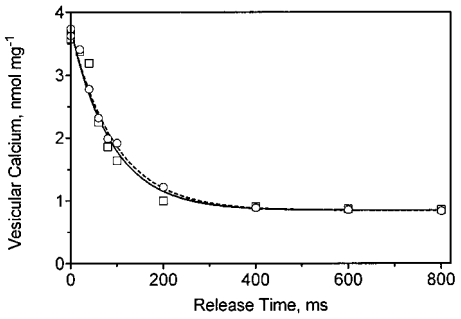

FIGURE 3: Effect of valinomycin on ATP-induced calcium release in triads with 0.1 mM luminal free [Ca^{2+}], luminal pH 5.5. Vesicles were equilibrated with 0.1 mM [Ca^{2+}], pH 5.5, and release was induced with 2 mM ATP as stated under Experimental Procedures. After ATP addition, calcium remaining in the vesicles decayed with time as a single exponential plus an offset (see above equation). The best fit to the data yielded N_0 (nmol mg^{-1}) = 2.9 ± 0.1, k (s^{-1}) = 10.4 ± 0.7, and offset (nmol mg^{-1}) = 0.8 ± 0.1 for the control; and N_0 (nmol mg^{-1}) = 2.9 ± 0.2, k (s^{-1}) = 11.1 ± 1.7, and offset (nmol mg^{-1}) = 0.8 ± 0.1 for vesicles preincubated with valinomycin. Key: open circles, control conditions; open squares, vesicles preincubated with valinomycin.

Dilution of vesicles with luminal pH 5.5 in a solution of pH 6.8 generated a significant proton diffusion potential ($[H^+]_i$ = 3.16 μM, $[H^+]_e$ = 0.16 μM, ΔV = −76.4 mV, inside − outside). To test if calcium release was inhibited by this negative proton diffusion potential, we measured release from vesicles with luminal pH 5.5, luminal 0.1 mM [Ca^{2+}], in the presence of 2 μM valinomycin as a highly effective potassium ionophore that should dissipate proton diffusion potentials by counterflow of potassium ions. The same release rates were found with and without valinomycin (Figure 3). These results indicate that even without valinomycin the permeability of the SR membrane to potassium ions was high enough to effectively compensate for the proton diffusion potential generated.

We compared next calcium release kinetics in vesicles at a constant luminal [Ca^{2+}] of 0.1 mM and at four different luminal pHs: 7.5, 6.8, 6.0, and 5.5. Vesicles within this luminal pH range had the same concentration of free luminal [Ca^{2+}], 0.1 mM, but at pH 6.0 and pH 5.5 they contained less total calcium than vesicles with luminal pH 6.8 or pH 7.5 (Figure 1). Yet decreasing the luminal pH had a marked stimulatory effect on calcium release kinetics. Vesicles with luminal pH 5.5 released calcium faster than vesicles with luminal pH 6.0, 6.8, and 7.5 (Figure 4), and had the highest rate constants, with k values in the range of 10 s^{-1} (Figure 5A). These values are about 2.5-fold higher than those measured in vesicles with luminal pH 6.0, and about 10-fold higher than the k values determined in vesicles with luminal pH 6.8; vesicles with luminal pH 7.5 had k values $<$0.4 s^{-1} (Figure 5A). From the values of release rate constants and from the total amount of calcium released, initial release rates were calculated (Figure 5B). In spite of their lower content of total releasable calcium, vesicles with luminal pH 5.5 and luminal pH 6.0 exhibited considerably higher release rates than vesicles with luminal pH 6.8 and 7.5.

The same k values were obtained in vesicles with luminal pH 5.5 when luminal [Ca^{2+}] was increased from 0.05 to 3.0 mM, showing that at this luminal pH k values were independent of luminal [Ca^{2+}] (Figure 6, filled bars). These results contrast sharply with the marked changes in rate

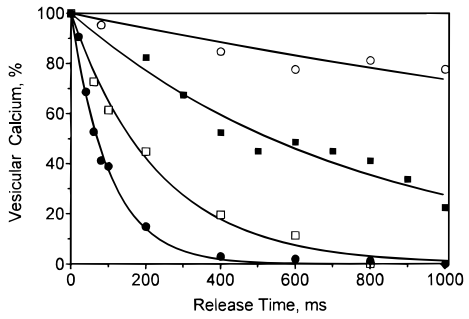

FIGURE 4: Effect of luminal pH on ATP-induced calcium release in triads with 0.1 mM luminal free [Ca^{2+}]. Vesicles were equilibrated with 0.1 mM [Ca^{2+}] at pH 5.5 (solid circles), pH 6.0 (open squares), pH 6.8 (solid squares), or pH 7.5 (open circles). Release was induced with 2 mM ATP as stated under Experimental Procedures. After ATP addition, calcium remaining in the vesicles decayed with time as a single exponential plus an offset. Only the fraction of calcium released through the channels, scaled to 100%, is given in the figure. The k values obtained from the best fit to the experimental points (lines through symbols) were (s^{-1}): $k = 9.8 \pm 0.4$ at pH 5.5; $k = 4.3 \pm 0.2$ at pH 6.0; $k = 1.3 \pm 0.1$ at pH 6.8; $k = 0.3 \pm 0.1$ at pH 7.5.

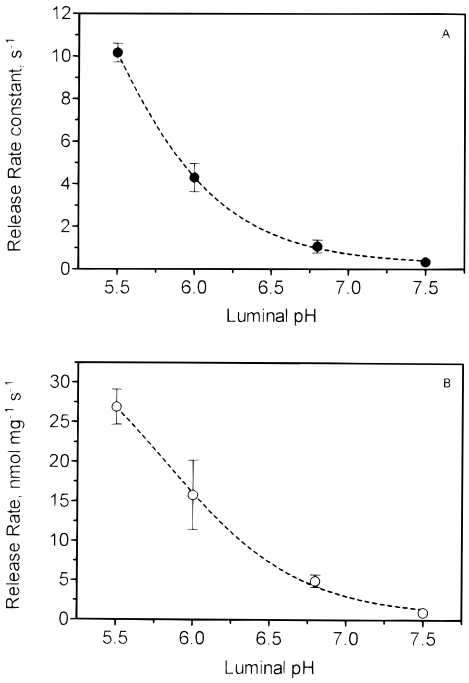

FIGURE 5: (A) Effect of luminal pH on the rate constants of ATP-induced calcium release at 0.1 mM free luminal [Ca^{2+}]. The values of k were obtained as shown in the legend to Figure 4. Mean values ± standard error of 3−5 determinations are shown. (B) Effect of luminal pH on the initial release rates of ATP-induced calcium release at 0.1 mM free luminal [Ca^{2+}]. Initial rates of calcium release were calculated by multiplying the release rate constant obtained at each pH by the corresponding total amount of calcium released. Mean values ± standard error of 3−5 determinations are shown.

constants caused by increasing luminal [Ca^{2+}] from 0.05 to 3.0 mM in vesicles with luminal pH 6.8 (Figure 6, empty bars).

(3) Luminal pH Dissipation. The time course of dissipation of pH gradients, that is a function of the vesicular proton permeability and of the buffering capacity of the vesicles, was measured in triads loaded with the fluorescent pH indicators CF or BCECF, and containing a luminal buffer concentration of 100 mM. Both indicators undergo a strong fluorescence enhancement at alkaline pH, with a reported

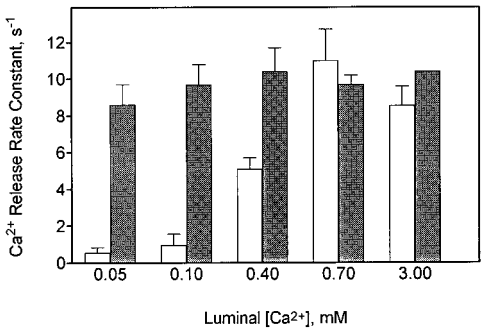

FIGURE 6: Effect of intraluminal pH on the rate constants of ATP-triggered calcium release as a function of the concentration of free luminal Ca^{2+}. Values of k were obtained as shown in the legend to Figure 4. Empty bars, pH 6.8; filled bars, pH 5.5. Mean values ± SEM of 2−5 determinations are shown.

pK_a = 6.5 for CF (Thomas et al., 1979), and a pK_a = 6.96 for BCECF (Rink et al., 1982). The same pK_a values were found in our experimental conditions for both indicators in solution, as well as for CF loaded inside the vesicles (results not shown).

To determine the time course of pH gradient dissipation, we first measured the time course of leakage of both CF and BCECF from the vesicles, after quenching the external fluorescence as described under Experimental Procedures. Both pH indicators had a slow rate of leakage, with half-times of 69 min for BCECF and of 23 min for CF at pH 7.0, indicating that at pH 7.0 BCECF is less permeable than CF, as expected from its higher negative charge density. At pH 6.0, the half-time of decay for CF decreased to 13.9 min; for BCECF, the half-time remained at 69 min at pH 6.0.

The dissipation of pH gradients was >50-fold faster than indicator leakage. Vesicles loaded with CF in 100 mM MOPS/Tris, pH 7.0, showed a decrease in fluorescence with time after dilution at pH 6.0, that followed a double exponential decay function (Figure 7A). The fast component had a half decay time of 1.15 min (rate constant = 0.6 min^{-1}). The slow component exhibited a rate constant of 0.05 min^{-1}, giving the same half-time, 13.9 min, as that of CF leakage from the vesicles at pH 6.0.

Similar results were obtained with BCECF-loaded vesicles equilibrated at pH 7.0 and diluted at pH 5.5. In the experiment shown in Figure 7B, the fast component had a half decay time of 0.6 min (rate constant = 1.15 min^{-1}), and the slow component had a half-time of 69 min, consistent with indicator leakage.

To corroborate that the fast fluorescence decay was due to internal pH changes, CF-containing vesicles equilibrated at pH 6.0 were diluted at pH 7.0 (Figure 7C). As expected for an increase in internal pH, we observed an initial increase in fluorescence with a half-time of 0.63 min ($k = 1.1$ min^{-1}); this increase was completed in 2 min, and it was followed by a slow decrease in fluorescence with a half-time of 23 min (k = 0.03 min^{-1}), the same half-time of CF leakage from the vesicles measured at pH 7.0.

A summary of the rate constant values of pH gradient dissipation (pH 6.0/pH 7.0) is shown in Table 2. In all conditions measured, the average values for vesicles containing 100 mM luminal buffer concentration were ≈0.8 min^{-1} (half-time = 0.8 min).

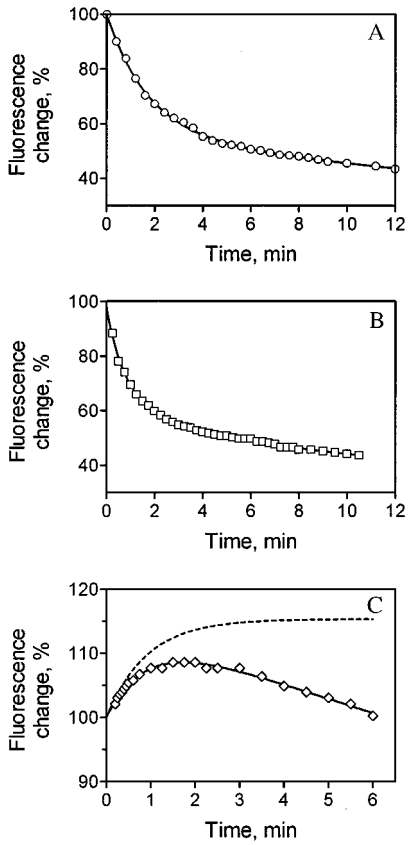

FIGURE 7: Time course of pH gradient dissipation. (A) Vesicles loaded with CF at pH 7.0 were diluted at pH 6.0 at time zero. Vesicular fluorescence followed a two-exponential decay function; the best fit to these data yielded decay rate constants of 0.60 and 0.05 min^{-1} for each exponential function. (B) Vesicles loaded with BCECF at pH 7.0 and diluted at pH 5.5. A two-exponential decay function was fitted to the data; rate constants of 1.15 and 0.01 min^{-1} were obtained from the best fit to these points. (C) Vesicles loaded with CF at pH 6.0 were diluted at pH 7.0 at time zero. These experimental points were fitted to the sum of the one-exponential increase function (dashed line) plus one-exponential decay function, plus an offset: $F(t) = F_1[1 - \exp(-k_1 t)] + F_2[\exp(-k_2 t)] + \text{offset}$. The best fit to this equation yielded a rate constant $k_1 = 1.10$ min^{-1} for the fluorescence increase, and a rate constant $k_2 = 0.03$ min^{-1} for the fluorescence decay.

Table 2: Rate Constants of Intravesicular Fluorescence Changes[a]

pH indicator	luminal pH	external pH	rate constant (min^{-1})
CF	7.0	6.0	0.73 ± 0.12 (9)
CF	6.0	7.0	0.85 ± 0.08 (4)
BCECF	7.0	6.0	0.80 ± 0.11 (3)

[a] The rate constant values for the fast component of the two exponential functions (see Figure 7) are given. Values represent mean ± SEM. The number of determinations is in parentheses.

DISCUSSION

(1) Calcium Binding to Triads. The results obtained in this work are in agreement with our previous findings that the saturable component of calcium binding to triads stems from calcium binding to calsequestrin (Donoso et al., 1995). After correction for calsequestrin content, we calculated that there are 30 Ca^{2+} binding sites per molecule at pH 7.5, with a K_d of 0.8 mM. These values are in good agreement with the values of 31 sites, $K_d = 1-2$ mM, reported for Ca^{2+} binding to calsequestrin in solution at pH 7.5 (Krause et al., 1991).

It has been questioned (Williams & Beeler, 1986) whether the changes determined in dilute calsequestrin solutions reflect the changes of the protein in the native triad structure, where it is compacted in a quasi-crystalline array (Saito et al., 1984). The present results suggest that, as far as calcium binding is concerned, calsequestrin behaves similarly in triads as it does in solution (Krause et al., 1991): in both conditions, calcium binding is noncooperative, and when measured at the same pH, similar B_{max} and K_d values are obtained.

(2) Effect of pH on Calcium Binding to Triads. Calsequestrin is a highly acidic protein with a large calcium binding capacity at neutral pH; although the structure of calcium binding sites in calsequestrin is not known, pairs of acidic residues have been implicated (Yano & Zarain-Herzberg, 1994). If so, calcium binding to calsequestrin, or to any other protein that binds calcium to negatively charged amino acids, would be expected to decrease at acidic pH by competition of protons with Ca^{2+}.

The present results showing that protons diminished Ca^{2+} binding to triads suggest that protons and Ca^{2+} compete for calsequestrin binding sites, and agree with other studies demonstrating competition of protons and calcium in inducing conformational changes of the calsequestrin molecule (Ostwald et al., 1974; Hidalgo et al., 1996). Lowering the pH from 8.0 to 6.5 decreased B_{max} with no change in K_d, indicating that in this range protons reduced the number of calcium binding sites in calsequestrin without changing the affinity of the remaining sites for Ca^{2+}. Further lowering the pH to 6.0 and 5.5 decreased B_{max} and increased K_d about 2-fold, implying reduction both in affinity and in number of calsequestrin binding sites in this pH range.

(3) Dissipation of pH Gradients and Proton Diffusion Potentials in Triads. All release experiments described in this work were done by diluting vesicles with varying luminal pH in an external solution at pH 6.8. For this reason, it was crucial to verify whether luminal pH remained constant during release, since isolated SR vesicles are very permeable to protons (Meissner & Young, 1980). Calcium release induced by ATP was completed in less than 1 s at luminal pH $\leq$6.0, and the average half-time of pH gradient dissipation in triads containing an internal buffer concentration of 100 mM was $\approx$0.8 min. These results demonstrate that in these conditions, and in spite of the significant permeability of the SR vesicles to protons (see below), the luminal pH remained essentially unchanged during the time course of calcium release.

Furthermore, in all conditions where a proton gradient was generated, a proton diffusion potential might arise, depending on the relative permeabilities of the SR membrane to protons and other ions. Thus, it became necessary to ascertain whether development of significant proton diffusion potentials affected calcium release kinetics. We found the same calcium release rates with and without valinomycin at pH 5.5, indicating that the permeability of the SR membrane to potassium ions was high enough in the absence of valinomycin to compensate for the eventual proton diffusion potential. In addition, these results indicate that the permeability of the SR membrane to potassium ions is high enough to compensate for the calcium diffusion potential generated during release (Garcia & Miller, 1984).

Thus, we established that calcium release was much faster than proton gradient dissipation, and was not affected by

diffusion potentials generated by proton or calcium gradients.

(4) Calculation of Proton Permeability. From the rates of pH dissipation, it is possible to calculate proton fluxes through the vesicular surface. The initial fluorescence changes with time, obtained when imposing a 1 unit pH gradient (pH 6.0−7.0), yielded an initial rate of H^+ movement into or out of the vesicles, V_i, equal to 1.2×10^{-11} mol s^{-1} mL^{-1}. Assuming spherical vesicles with an average radius of 100 nm (Mitchell et al., 1984), a vesicular volume of 4×10^{-15} cm^3 is obtained. One milligram of vesicles, that occupies a volume of 4 μL (Duggan & Martonosi, 1970; Kasai, 1980), would have 10^{12} vesicles. Knowing the vesicular area, the protein concentration (0.015 mg/mL), and the number of vesicles per milligram of protein, the initial rate of proton movement V_i given above can be converted into a flux of protons $J = 6.7 \times 10^{-13}$ mol s^{-1} cm^{-2}, giving a permeability coefficient $P = 0.74 \times 10^{-3}$ cm s^{-1}. This value is in reasonable agreement with the estimation of $P \approx 10^{-3}$ cm s^{-1} reported by Meissner and Young (1980).

(5) Effect of Luminal pH on Calcium Release Kinetics. The present work indicates that luminal protons, in the pH range 6.0−5.5, can effectively activate calcium release rates at 0.1 mM luminal [Ca^{2+}], a concentration that by itself does not cause channel activation at pH ≥6.8. In fact, at pH 6.8 only by increasing luminal [Ca^{2+}] to 0.7 mM are the same release rates obtained (Donoso et al., 1995). Thus, activation of calcium release rates can be effected either by luminal Ca^{2+} or by luminal protons. At a constant luminal [Ca^{2+}], release rate constants are a direct function of release channel conductance and open probability (Garcia & Miller, 1984). We propose that the increase in release rate constants caused by luminal protons in 0.1 mM luminal [Ca^{2+}] most likely reflects activation of the open probability of the release channels, since the channel conductance of SR vesicles fused in bilayers is inhibited by decreasing *trans* pH (Rousseau & Pinkos, 1990).

Proton binding to luminal regulatory sites may produce conformational changes of the channel protein itself, or of a closely related protein, causing the observed channel activation. Direct activation of the channel protein itself by luminal protons is unlikely, since inhibition instead of activation of channel activity by *trans* acid pH was found with the purified channel protein (Ma et al., 1988; Shomer et al., 1994). Hence, modifications of other luminal proteins may be responsible for channel activation by luminal protons.

(6) A Possible Role of Calsequestrin in Mediating the Effect of Luminal pH on Calcium Release Kinetics. It has been proposed that calsequestrin modulates the activity of the calcium release channels (Ikemoto et al., 1989). Increasing luminal [Ca^{2+}], concomitant with inducing calcium binding to calsequestrin, produces a marked stimulation of caffeine-induced (Ikemoto et al., 1989) or of ATP-induced calcium release rates in triads (Donoso et al., 1995) and increases the activating effect of ryanodine on calcium release (Gilchrist et al., 1992). Conformational changes of the release channel protein occur following calcium binding to calsequestrin, and are reversibly abolished by dissociation of the calsequestrin−calcium channel complex (Ikemoto et al., 1989). Transient increases in intravesicular [Ca^{2+}] precede calcium release (Ikemoto et al., 1991), suggesting that calsequestrin and the channel protein are mutually coupled so that conformational changes of one protein are transmitted to the other (Ikemoto et al., 1991; Gilchrist et al., 1992). Furthermore, SR calcium channel P_o increases after *trans* addition of calsequestrin in *trans* millimolar [Ca^{2+}] (Kawasaki & Kasai, 1994).

According to the original proposal (Ikemoto et al., 1989), increasing luminal [Ca^{2+}] produces conformational changes in calsequestrin that lead to channel activation. Protons effectively replace calcium ions in producing the conformational changes of calsequestrin that underlie changes in ellipticity (Ostwald et al., 1974) and in intrinsic fluorescence (Hidalgo et al., 1996). Based on these results and on the present findings, we propose that protons, like Ca^{2+}, produce calsequestrin conformational changes that induce channel activation even at luminal [Ca^{2+}] below the concentrations required for calcium binding to calsequestrin.

(7) Physiological Significance. In resting muscle, the calcium concentration inside the SR is in the millimolar range and far from chemical equilibrium (Winegrad, 1968), whereas the pH of the SR lumen is presumably at equilibrium with cytoplasmic pH and close to neutrality. As a consequence, calsequestrin is saturated with calcium and compacted in a quasi-crystalline array (Saito et al., 1984). In these conditions, if the above proposal is correct, luminal activation of the release channels by calsequestrin should be at its optimum.

Physiological activation of the calcium release channels by transverse tubule depolarization produces substantial calcium release from the SR, and should generate unbinding of calcium from calsequestrin as well as a large calcium diffusion potential. It has been proposed that the generation of a diffusion potential is prevented by a significant counterflow of protons (Somlyo et al., 1981) or of potassium ions (Garcia & Miller, 1984). If protons make a significant contribution as counterions, the pH within the SR lumen is likely to decrease substantially during calcium release. In these conditions, proton binding to calsequestrin would compensate for the loss of bound calcium, preserving, at least in part, luminal channel activation.

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