Changes in Luminal pH Caused by Calcium Release in Sarcoplasmic Reticulum Vesicles

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ABSTRACT Fast (milliseconds) Ca^{2+} release from sarcoplasmic reticulum is an essential step in muscle contraction. To electrically compensate the charge deficit generated by calcium release, concomitant fluxes of other ions are required. In this study we investigated the possible participation of protons as counterions during calcium release. Triad-enriched sarcoplasmic reticulum vesicles, isolated from rabbit fast skeletal muscle, were passively loaded with 1 mM $CaCl_2$ and release was induced at pCa = 5.0 and pH = 7.0 in a stopped-flow fluorimeter. Accompanying changes in vesicular lumen pH were measured with a trapped fluorescent pH indicator (pyranin). Significant acidification (~0.2 pH units) of the lumen occurred within the same time scale ($t_{1/2}$ = 0.75 s) as calcium release. Enhancing calcium release with ATP or the ATP analog 5′-adenylylimidodiphosphate (AMPPNP) produced >20-fold faster acidification rates. In contrast, when calcium release induced with calcium with or without AMPPNP was blocked by Mg^{2+}, no acidification of the lumen was observed. In all cases, rate constants of luminal acidification corresponded with reported values of calcium release rate constants. We conclude that proton fluxes account for part (5–10%) of the necessary charge compensation during calcium release. The possible relevance of these findings to the physiology of muscle cells is discussed.

INTRODUCTION

In skeletal muscle, calcium is stored in the lumen of the sarcoplasmic reticulum (SR), partly bound to calsequestrin and partly free, presumably reaching up to millimolar concentrations. The junctional SR membrane contains the ryanodine receptor Ca^{2+} release channels (Melzer et al., 1995), which allow fast (milliseconds) release of Ca^{2+} ions from the lumen into the myoplasm to trigger muscle contraction. Upon relaxation, the Ca^{2+} gradient is restored by the powerful SR Ca-ATPase (see Mintz and Guillain, 1997, for a recent review). The SR membrane also contains Cl^- and K^+ channels (Miller, 1978; García and Miller, 1984; Tanifuji et al., 1987; Dulhunty et al., 1996; Kourie et al., 1996) and has a limited permeability for H^+ (Nunogaki and Kasai, 1986; Donoso et al., 1996; Meissner and Young, 1980) as well as for nucleotides such as ATP (Shoshan-Barmatz et al., 1996).

Rapid release of Ca^{2+} ions from the SR lumen requires concomitant flux of other ions (e.g., Mg^{2+}, Na^+, K^+, or H^+ and/or Cl^-, OH^-, HCO_3^-, $H_2PO_4^-$, or HPO_4^{2-}) to prevent significant charge build-up across the membrane (Dulhunty et al., 1996). Little is known about the actual movement of ions accompanying the calcium flux during physiological release. Because of the high K^+ conductance of the SR membrane, García and Miller (1984) concluded that rapid influx of K^+ probably maintains the membrane potential near zero at all times, even during the peak of Ca^{2+} release. On the other hand, by measuring changes in myoplasmic pH that coincide with changes in myoplasmic free Ca^{2+} after action potential stimulation of muscle fibers, it was proposed that protons might contribute 10–15% of the necessary charge compensation during calcium release (Pape et al., 1990). Furthermore, by measuring in intact fibers the uptake of Mg^{2+} and K^+ into the terminal cisternae during calcium release, it was concluded that the amount of counterion movement was insufficient to account for the necessary total charge compensation, suggesting that proton fluxes might make up for the deficit (Somlyo et al., 1981; Fink and Veigel, 1996). However, no direct evidence for luminal acidification of the SR, indicating the existence of proton influx during calcium release, has been provided (Dulhunty et al., 1996).

In this study we followed changes in the pH of the lumen of SR vesicles during calcium release by trapping a fluorescent pH indicator, pyranin, inside the vesicles. Calcium release was induced in a stopped-flow fluorimeter under various conditions. Significant acidification (~0.2 pH units) of the lumen occurred within the same time scale as calcium release in all conditions tested. Hence, it is proposed that protons partially compensate for the charge deficit generated by calcium release.

MATERIALS AND METHODS

Isolation of vesicles

Heavy SR vesicles, enriched in triads, were isolated from rabbit fast skeletal muscle as described (Hidalgo et al., 1993).

Calcium release experiments

The kinetics and the amount of calcium released per mg protein was determined by rapid filtration as before (Donoso et al., 1995).

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Buffering capacity of the lumen of SR vesicles

SR vesicles were incubated (2 mg of protein/ml) in 2 ml of 1 mM $CaCl_2$, 100 mM KCl, and 10 mM MOPS-KOH, pH 7.00, at 4°C overnight. Vesicles were washed twice by sedimentation for 2 min at 14,000 × *g* in an Eppendorf centrifuge (model 5415C), followed by resuspension in 1 ml of 110 mM KCl, 50 μM MOPS-KOH, pH 7.2. This low external buffer concentration was chosen to optimize the contribution of SR luminal buffering capacity. The luminal buffering capacity was determined according to Mitchell and Moyle (1967). Changes in external pH were monitored by adding pyranin (10 μM final) to the vesicle suspension and measuring the fluorescence with a FluoroMax fluorimeter (SPEX Industries, Edison, NJ), using excitation at 455 nm and measuring fluorescence at 509 nm. The relation between pyranin fluorescence and external pH was calibrated by measuring the minimal and maximal fluorescence of pyranin in solution at very acidic and very alkaline pH and using the known slope of fluorescence between pH 6.5 and pH 8.0.

Pyranin loading

SR vesicles (5 mg of protein/ml) were passively loaded with pyranin and calcium by incubating overnight at 4°C in column buffer (100 mM KCl, 1 mM $CaCl_2$, 10 mM MOPS-KOH, pH 7.00 ± 0.01), containing 1 mM pyranin. External pyranin was routinely removed by loading 0.25 ml of the incubation mixture on a 3-ml Biogel A-1.5m (200–400 mesh) column; elution was done with column buffer. All experiments were carried out immediately with the first fraction (0.25 ml) coming off the column that displayed turbidity. The protein concentration of this fraction was approximately 2 mg/ml.

Escape of trapped pyranin

Pyranin-loaded SR vesicles (0.25 ml) were immediately diluted in 25 ml of column buffer at room temperature. Upon dilution, 3-ml samples were extracted at 5-min intervals and were filtered through 0.2-μm Millipore filters to separate the SR vesicles from the external buffer. Subsequent determinations of the pyranin fluorescence of the filtrate indicated that the fluorescence of the external buffer increased threefold with respect to its initial value (i.e., from the sample taken at $t = 0$) over a time course of 60 min with a rate constant of 0.043 min^{-1} (half-time of ~16 min, not shown). These observations show that, initially, ~60% of the pyranin was inside the vesicles and that it escaped with a half-time of 16 min. Thus, the stopped-flow experiments had to be done rapidly (within 10 min) to get reliable measurements of luminal pH and to avoid corrections for pyranin depletion.

Calibration of luminal pyranin fluorescence versus pH

Pyranin-loaded vesicles were prepared by incubating overnight SR vesicles in aliquots of 0.3 ml of buffer (100 mM KCl, 1 mM $CaCl_2$, 10 mM MOPS-KOH, 1 mM pyranin, and SR vesicles at 1 mg/ml protein) at various pH values in the range of pH 6.0 to pH 8.5. For the incubations at pH 6.0 and 6.25, MOPS was replaced by MES, and for the incubations at pH 7.75, 8.0, and 8.5, HEPES was used instead of MOPS. External pyranin was removed from every aliquot by washing the vesicles three times by sedimentation for 2 min at 14,000 × *g* in an Eppendorf centrifuge, followed by resuspension in 1 ml of the incubation buffer without pyranin. Upon washing, the vesicles were diluted in a final volume of 3 ml, and the protein concentration (±0.1 mg/ml) was measured. An aliquot of 1 ml was taken and the fluorescence of pyranin measured in a SPEX fluorimeter using excitation at 455 nm and measuring fluorescence at 509 nm. This reading corresponded to the fluorescence of trapped pyranin plus external pyranin. To measure only external pyranin fluorescence, the remaining 2 ml was filtered immediately through a 0.20-μm Sartorius filter to separate the SR vesicles from the external buffer, and the pyranin fluorescence of the filtrate was measured. The fluorescence of the trapped pyranin was calculated from the difference of the two measurements and was normalized for the protein concentration. In a control experiment, SR vesicles (0.1 mg of protein/ml) were incubated at pH 6.0, pH 7.0, and pH 8.0 in incubation buffer without pyranin, and the emission at 509 nm (excitation, 455 nm) due to light scattering was measured. Light scattering increased with increasing pH, but the levels were negligible relative to the increase in fluorescence at increasing pH when pyranin was trapped in the SR vesicles.

Stopped-flow experiments

Pyranin-loaded SR vesicles were diluted 10 times in column buffer and were mixed in a sequential stopped-flow fluorimeter (type DX .17 MV, Applied Photophysics, Leatherhead, UK) with different solutions, as detailed in the figure legends. After a dead time of ~1–5 ms, the changes in pyranin fluorescence were measured using an excitation wavelength of 455 nm and a 495-nm cutoff long-pass filter (Oriel, Stratford, CT) in front of the photomultiplier detecting emission light. Although a significant fraction (30–50%) of the total pyranin was present in the external solution, all changes in fluorescence represented only changes in luminal pH as the pH of the external solution was strongly buffered relative to the small volume of the vesicular lumen. All stopped-flow experiments were carried out at room temperature.

RESULTS

Buffering capacity of the lumen of SR vesicles

The buffering capacity of the lumen of the SR vesicles was determined according to Mitchell and Moyle (1967). As illustrated in Fig. 1, 100 nmol of H^+ (i.e., 5 μl of 10 mM oxalic acid stock solution) was added to the external medium of SR vesicles (3 mg/ml) that were suspended in 1 ml of 110 mM KCl, 50 μM MOPS-KOH, pH 7.2, and 10 μM pyranin (see Materials and Methods). Upon addition of 100 nmol H^+, the external pH dropped instantly 0.208 units ($\Delta pH_{initial}$) due to the buffering of the external medium only. Thus, the external buffering capacity amounted to 100 nmol of $H^+/\Delta pH_{initial}$ = 480 nmol/pH unit. At a time interval long enough to allow pH equilibration of the lumen (6 min), a slight

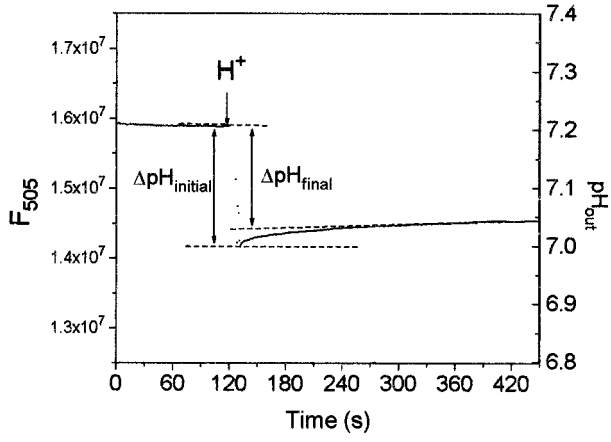

FIGURE 1 Determination of buffering capacity of the lumen of SR. SR vesicles were resuspended in 1 ml of 110 mM KCl, 50 μM MOPS-KOH, pH 7.2. Changes in external pH were followed upon addition of a small amount of protons.

recovery of the drop in external pH ($\Delta pH_{final} = 0.185$) was observed. The sum of luminal and external buffering capacity amounted to 100 nmol of $H^+/\Delta pH_{final} = 540$ nmol/pH unit. Thus, the buffering capacity of the lumen of SR vesicles, evaluated from the difference, was 60 nmol/pH unit, yielding per milligram of protein a luminal buffering capacity of 20 nmol of H^+/pH unit. On average ($N = 3$), we obtained per milligram of protein a luminal buffering capacity of 20 ± 5 nmol of H^+/pH unit).

Calibration of pyranin fluorescence

Fig. 2 shows the effect of pH in the range 6.0–8.5 on the fluorescence of a solution of 0.5 μM pyranin dissolved in buffer (1 mM $CaCl_2$, 100 mM KCl, and 10 mM MOPS-KOH) as well as the fluorescence of trapped pyranin (1 mM) in the lumen of SR vesicles, determined as described in Materials and Methods. Although the pK_a of trapped pyranin ($pK_a = 7.0$) was more acidic than the pK_a of pyranin in solution ($pK_a = 7.3$), a linear dependence was found between pyranin fluorescence and luminal pH between pH 6.5 and 7.5, which was the pH range of our experiments. The slight shift in pK_a of trapped pyranin may reflect changes in the activity coefficient of H^+ inside the vesicles due to the high protein content of the SR lumen. Alternatively, quenching of fluorescence in the alkaline pH range may take place due to the high concentration (1 mM) of pyranin trapped in the lumen.

Proton leak

Before measuring changes in SR luminal pH (pH_{in}) that might occur during calcium release, it was necessary to establish the leakiness of the SR vesicles for protons in the current experimental conditions. Fig. 3 illustrates stopped-flow experiments with different time frames in which pH gradients were imposed on SR vesicles with pyranin trapped inside. The pH of the outer solution was increased to pH 7.15 by mixing SR vesicles in column buffer pH 7.00 with column buffer pH 7.30 in equal volumes. A very fast immediate increase in luminal pH, which followed a single exponential function with a rate constant of 108.3 s^{-1} ($t_{1/2} = 6.4$ ms) was observed (Fig. 3 *A*). This initial increase was followed by a second and slower exponential increase with a rate constant of 0.0044 s^{-1} ($t_{1/2} = 2.6$ min; Fig. 3 *B*). The first and second components represented 54% and 46%, respectively, of the total fluorescent change. When SR

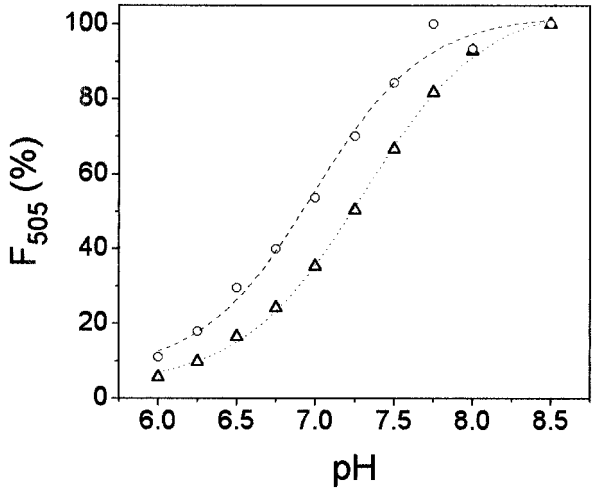

FIGURE 2 Calibration of pyranin fluorescence. △, fluorescence of 0.5 μM pyranin in buffer solution (100 mM KCl, 1 mM $CaCl_2$, 10 mM MOPS-KOH) at different pH values; ○, fluorescence of 1 mM pyranin trapped in the lumen of SR vesicles. The curves drawn represent the best fitting to a pK_a function.

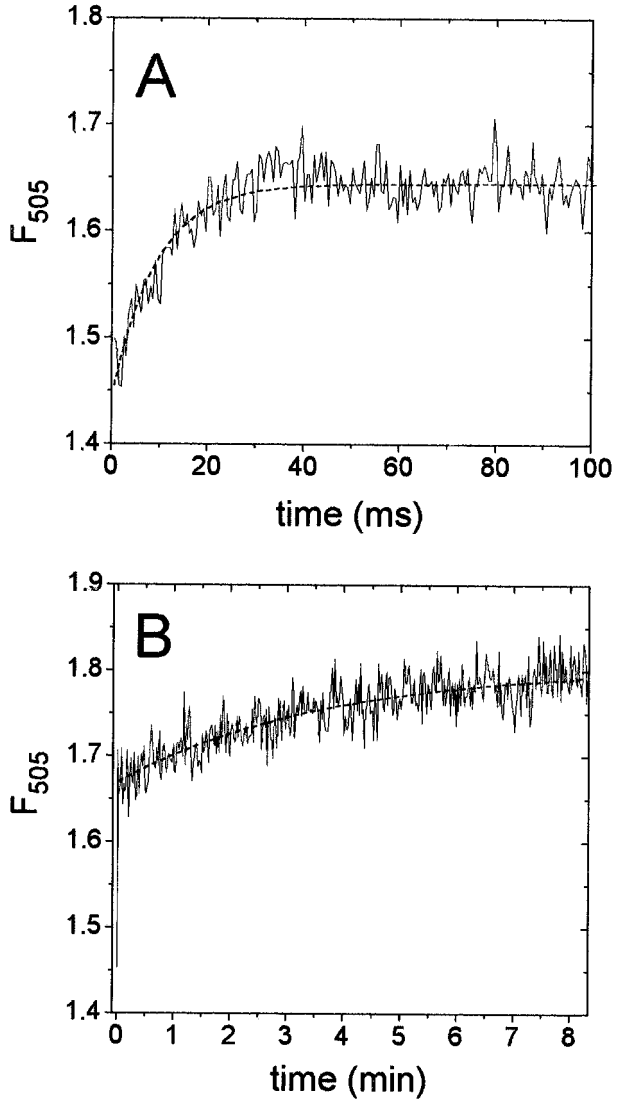

FIGURE 3 Measurement of proton leak. Two identical stopped-flow experiments with different time frames in which pH gradients were imposed on SR vesicles with 1 mM pyranin trapped inside are illustrated. The pH of the external solution increased to 7.15 by 1:1 mixing of SR vesicles in column buffer pH 7.00 with column buffer pH 7.30. (*A*) The increase in pyranin fluorescence revealed a fast immediate increase in luminal pH ($t_{½} = 6.4$ ms). (*B*) The initial increase in fluorescence was followed by a slow increase to a final value ($t_{1/2} = 2.6$ min).

vesicles were mixed with column buffer with lower pH (final pH_{out} of 6.70), a very fast initial decrease in pH_{in} followed by a slow decrease were found, with similar kinetics to those illustrated in Fig. 3 (not shown). The fast component of change in pyranin fluorescence was not due to pyranin present in the external medium as a control experiment, in which a buffered aqueous solution of pyranin (10 μM final) was mixed with buffer at different pH values showed that the response time of pyranin fluorescence to changing pH was faster than the mixing time of the stopped-flow instrument (not shown). We think that these two components represent noncyclic and cyclic proton gradient dissipation, respectively, as argued in detail in the Discussion.

Calcium-induced calcium release

Vesicles passively equilibrated in 1 mM $CaCl_2$ released as a rule 40 $\pm$ 5 nmol of Ca^{2+}/mg of protein after dilution in 10 μM Ca^{2+} at pH 7.0, with rate constants in the range of 0.5 to 1 s^{-1}, as determined by the rapid filtration technique. By measuring the changes in luminal pH that accompany calcium release, we investigated the contribution of influx of protons to the necessary charge compensation. Fig. 4 shows the results of stopped-flow experiments in which calcium release was induced by mixing 1:1 SR vesicles (with 1 mM pyranin inside) in column buffer (pH 7.02 $\pm$ 0.01) with release buffer (i.e., 50 mM MOPS-KOH, pH 7.00, 100 mM KCl, 2.5 mM HEDTA, and 1 mM $CaCl_2$). After mixing, the composition of the external solution changed to 30 mM MOPS, 100 mM KCl, 1.0 mM $CaCl_2$, and 1.25 mM HEDTA, decreasing the free Ca^{2+} concentration to 10^{-5} M; in this particular experiment, the external pH upon mixing was 7.00 $\pm$ 0.01. As illustrated in Fig. 4 (lower trace), pH_{in} dropped with single exponential kinetics, with $t_{1/2}$ = 0.75 s (k = 0.92 s^{-1}), a similar value to that determined for calcium-induced calcium release with the rapid filtration technique under comparable conditions (Moutin and Dupont, 1988; Donoso and Hidalgo, 1993). The pH gradient that was generated during Ca^{2+} release dissipated within minutes (not shown), with a rate constant similar to that of the slow component of proton leak illustrated in Fig. 3 *B*. When calcium release was inhibited by blocking the Ca^{2+} channels with 1 mM Mg^{2+}, no acidification upon imposing a Ca^{2+} gradient was observed (upper trace in Fig. 4).

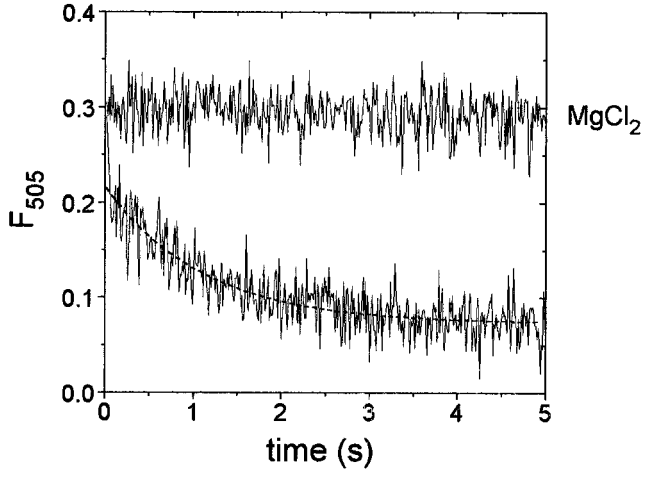

FIGURE 4 Luminal pH changes during calcium-induced calcium release: stopped-flow experiments in which calcium release was induced by mixing SR vesicles in column buffer (containing 1 mM $CaCl_2$ and 1 mM pyranin inside) with release buffer. Upon mixing, the external solution bathing the SR vesicles changed to 30 mM MOPS, 100 mM KCl, 1.0 mM $CaCl_2$, and 1.25 mM HEDTA, decreasing the free calcium concentration to 10^{-5} M. The lower trace shows changes in luminal pH accompanying calcium release. The dashed line represents a single exponential fit to the data points. The upper trace shows that the presence of 1 mM Mg^{2+} inhibited calcium release and also the acidification of pH_{in}.

Effect of ATP

The presence of ATP enhances calcium release rates by stimulating the opening of the Ca^{2+} channels (Meissner, 1994). Thus, if the acidification observed in Fig. 4 is caused by influx of protons as part of the necessary charge compensation during calcium release, accelerating calcium release should accelerate the influx of protons. To test this prediction, calcium release was triggered by mixing (1:1) pyranin-loaded SR vesicles as described for the experiment shown in Fig. 4 but in the presence of ATP (sodium salt, 2 mM final concentration). Upon mixing, the external pH was 6.92. As illustrated in Fig. 5, the changes in luminal pH were followed in three time frames: 200 ms (Fig. 5 *A*), 20 s (Fig. 5 *B*), and 8 min (Fig. 5 *C*). The experimental record illustrated in Fig. 5 *A* shows that pH_{in} dropped very fast, exponentially, with $t_{1/2}$ = 28 ms (k = 25 s^{-1}). After 200 ms, pH_{in} reached a new level. The fast initial luminal pH decrease was followed by a further and slower decrease that was completed within 20 s (Fig. 5 *B*). This second decrease, which was not seen in all experiments, amounted to less than 20% of the initial fluorescence decrease. As shown in Fig. 5 *C*, the pH gradient generated by Ca^{2+} release dissipated with a rate constant of 0.0054 s^{-1} ($t_{1/2}$ = 2.13 min), a value similar to that obtained for the slow component of proton leak illustrated in Fig. 3 *B*.

In the particular experiment depicted in Fig. 5, *A–C*, at t = 0 the pH_{out} dropped to 6.92. Thus, a fast initial drop in pH_{in}, as shown in Fig. 3 *A*, should be expected. However, if the initial drop in pH_{in} were only due to the change in external pH, it should have been completed within ~20 ms and should have been followed by slow further acidification, as in the experiment of Fig. 3. However, Fig. 5 *C* shows that, at a longer time interval, the initial decrease in pH_{in} did not continue but dissipated. Thus, these results show that significant extra proton influx occurred during the first 200 ms. Moreover, when the experiment was repeated under conditions where the pH_{out} became slightly alkaline (~7.05) upon mixing, an initial alkalization of the lumen of the SR vesicles due to proton leak that was completed within 20 ms was followed by acidification (Fig. 5 *D*). The

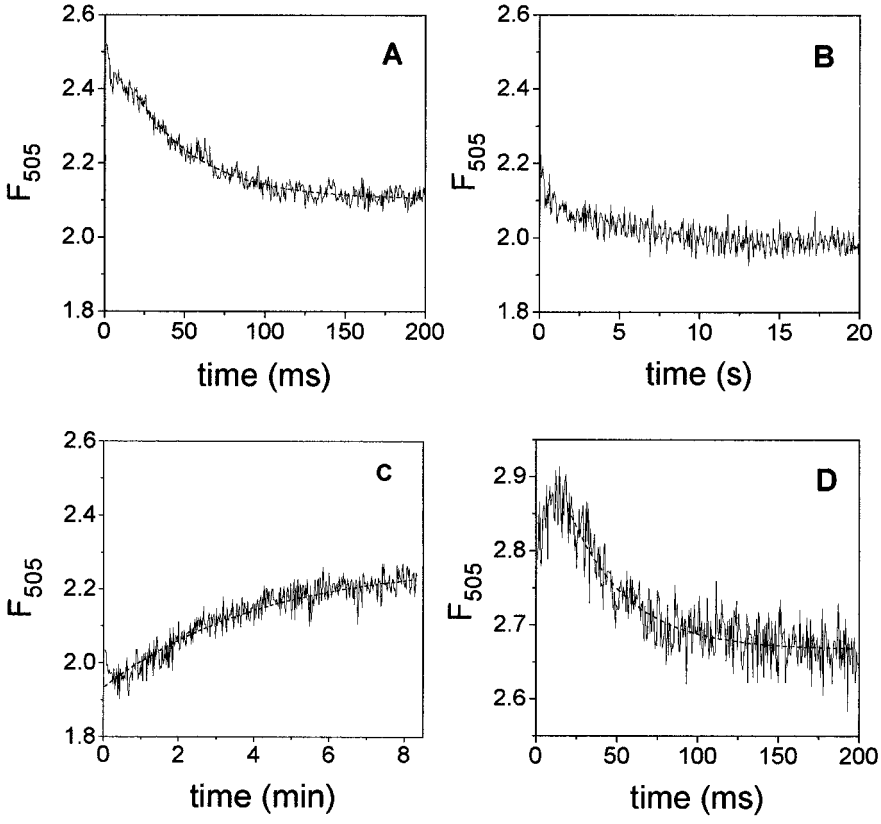

FIGURE 5 Luminal pH changes during ATP-induced calcium release. Changes in pH_{in} were followed in three sequential experiments done with different time frames (*A*, *B*, and *C*). Calcium release was induced as in Fig. 4, except that ATP (sodium salt, 2 mM final concentration) was added to the release buffer to stimulate the opening of the Ca^{2+} channels. (*D*) Similar experimental conditions as in *A*, except that, upon mixing, the external pH was slightly alkaline. The dashed lines in *A* and *D* represent single exponential fittings to the data.

rate constant of the latter acidification was 17 s^{-1}, similar to that obtained from the trace shown in Fig. 5 *A*. We chose to present the data illustrated in Fig. 5, *A–C*, as they allowed us to estimate in the same experiment the amount of acidification that occurred in the first 200 ms. Assuming, from the data shown in Fig. 5 *C*, that $pH_{in} = 7.00$ at $t = 0$ and $pH_{in} = pH_{out} = 6.92$ at $t = \infty$, and as pyranin fluorescence was a linear function of pH in this range (Fig. 2), the extent of the drop in luminal pH during the first 200 ms, extrapolated from the fluorescence readings, amounted to ~0.2 pH units.

Effects of the ATP analog AMPPNP and Mg^{2+}

If the fast luminal acidification observed in the presence of ATP is a consequence of calcium release, it should be inhibited when blocking the Ca^{2+} channels with Mg^{2+}. However, Mg^{2+} plus ATP activate the SR Ca^{2+}-ATPase, which also pumps protons, complicating the interpretation of the experimental results. Therefore we activated the Ca^{2+} channels with the nonhydrolyzable ATP analog 5′-adenylylimidodiphosphate (AMPPNP) and evaluated the effect of Mg^{2+} under these circumstances. As shown in Fig. 6, the initial luminal acidification ($t_{1/2}$ = 26 ms) of the vesicles was much faster in the presence of 2 mM AMPPNP than without AMPPNP ($t_{1/2}$ = 750 ms; Fig. 4) and had a very similar time course as that observed with ATP ($t_{1/2} = 25 \pm 5$ ms). In the combined presence of AMPPNP and Mg^{2+} (2 mM), no acidification of the lumen was observed (top trace in Fig. 6).

DISCUSSION

Passive proton permeability of SR vesicles

Our findings of a fast ($t_{1/2}$ = 6.4 ms, complete within 20 ms) and a slow ($t_{1/2}$ = 2.6 min) component of proton leak across

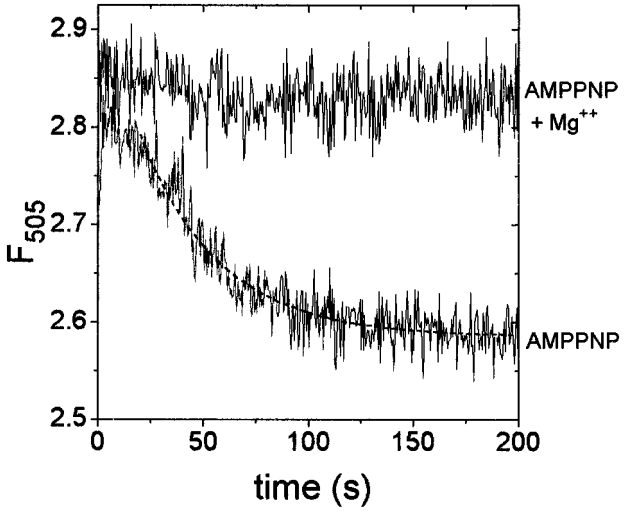

FIGURE 6 Luminal pH changes during AMPPNP-induced calcium release. Experimental conditions were as in Fig. 4 except that AMPPNP (2 mM final concentration) was added to the release buffer. The dashed line represents a single exponential fit to the data points. The upper trace reveals that the acidification of the lumen of the SR vesicles caused by the release of calcium was prevented by 2 mM Mg^{2+}.

the membrane of SR vesicles when a pH gradient was imposed are at variance with the stopped-flow results reported by Nunogaki and Kasai (1986). These authors reported only very fast changes in pH_{in} (such as those shown in Fig. 3 *A*) when altering pH_{out} and concluded that these changes are due to a high proton permeability of the membrane of SR vesicles and represent complete equilibration of the pH gradient. Yet Meissner and Young (1980) had previously reported a lower proton permeability of the SR membrane, which we confirmed (Donoso et al., 1996); furthermore, pH equilibration across the SR membrane has similar kinetics to the slow component found in the stopped-flow experiment shown in Fig. 3. We conclude that the very fast initial component of pH dissipation reflects the capacity of the SR membrane to transfer very rapidly a limited amount of protons in a noncyclic fashion, as this transfer did not dissipate the pH gradient completely. We tentatively propose that this noncyclic proton transfer arises either from redistribution of lipophilic acids in the membrane (Kamp and Hamilton, 1992; Kamp et al., 1995) or from redistribution of COOH or NH_3 groups on proteins that flip-flop rapidly across the membrane. As reported earlier (Meissner and Young, 1980; Donoso et al., 1996), cyclic proton transfer, resulting in a complete dissipation of the imposed pH gradient, was much slower (Fig. 3 *B*) and took minutes to complete.

Luminal pH changes during calcium release

The observed rates of acidification of the lumen of SR vesicles that were obtained under various calcium release conditions are listed in Table 1. In general, it was difficult to achieve experimental conditions during which the luminal and external pH were exactly the same at $t = 0$. Therefore, to avoid potential interference of the fast noncyclic component of proton leak, rate constants of luminal acidification during Ca^{2+} release were obtained from single exponential fittings of the data points collected 15 ms after mixing. The rates of calcium release from SR vesicles measured under similar conditions (i.e., control calcium-induced calcium release or plus ATP or AMPPNP) with the rapid filtration technique (Moutin and Dupont, 1988; Donoso and Hidalgo, 1993) or with other methods (Meissner, 1994) are in close correspondence to the rates of acidification of the lumen reported in this work. Moreover, when calcium release was inhibited by blocking the Ca^{2+} release channels with Mg^{2+}, no drop in pH_{in} was found (Table 1). We infer from these results that an influx of H^+ ions accompanies the efflux of Ca^{2+} ions, contributing to the necessary charge compensation during calcium release. The decrease in luminal pH during calcium release was approximately 0.2 pH units (Fig. 5). As the buffering capacity of the lumen per milligram of protein was 20 ± 5 nmol H^+/pH unit, the influx of protons amounted to 4 nmol of H^+/mg of protein. The amount of calcium released from the vesicles under the same circumstances is 40 ± 5 nmol of Ca^{2+}/mg of protein (Donoso et al., 1995). Hence, we conclude that the influx of protons that accompanies the efflux of Ca^{2+} accounts for only a fraction (5–10%) of the necessary charge compensation. Most of the charge compensation is probably accounted for by K^+ ions (García and Miller, 1984).

TABLE 1 Rates of acidification during calcium release from the lumen of SR vesicles

	Luminal acidification		
	$t_{1/2}$ (s)	Rate constant (s^{-1})	Calcium release* Rate constant (s^{-1})
Control	0.75	0.91 ± 0.2	0.5–1.0
Control + Mg^{2+}		<0.01	<0.01
ATP	0.028	25 ± 5	10–100
AMPPNP	0.026	26 ± 5	10–100
AMPPNP + Mg^{2+}		<0.01	<0.01

All experiments were done at least three times.
*Data taken from Moutin and Dupont (1988) and Donoso and Hidalgo (1993) and from the review by Meissner (1994).

The buffering capacity of the SR lumen, measured in vesicles that did not contain pyranin, was approximately two times larger than the buffering capacity one would expect on the basis of the internal volume of the vesicles (1.6 μl/mg protein; Donoso et al., 1995) and the concentration of trapped buffer (10 mM MOPS). This additional luminal buffering capacity is probably due to the presence of calsequestrin inside the vesicles, as the presence of proton-binding sites in calsequestrin has been inferred on the basis of proton-induced calsequestrin conformational changes (Hidalgo et al., 1995). Moreover, the buffering strength of the lumen of the vesicles containing trapped pyranin (1 mM during incubation) may be higher than 20 nmol/(pH unit, mg protein) due to accumulation of the probe, although pyranin is a very charged molecule with very low affinity for membranes (Kamp and Hamilton, 1992). Thus, it is possible that the determined fraction (5–10%) of charge compensation accounted for by protons, during calcium release, represents an underestimation of the total proton contribution.

After the initial luminal acidification due to influx of protons coinciding with the efflux of Ca^{2+}, the pH gradient dissipated very slowly (minutes; Fig. 5 *C*). In vivo, a much faster restoration of the pH gradient is required to prevent significant acidification of the lumen of the SR during repeated muscle contractions. It is possible that the pH gradient is restored by the action of the Ca^{2+}-ATPase, which also pumps protons out of the SR (Mintz and Guillain, 1997), or by other proton pathways (Geers et al., 1991; Decker et al., 1996) that were not characterized in our experimental conditions.

Physiological significance

Characterization of proton fluxes across the SR membrane is important in understanding the physiology of muscle

cells. Recently, it was shown that lowering SR luminal pH compensates the decrease in calcium release rates due to vesicular luminal Ca^{2+} depletion (Donoso et al., 1996). These results indicate that luminal protons might have a controlling effect on calcium release rates. Thus, influx of protons into the vesicular lumen may play a role in modulating physiological calcium release rates. Furthermore, dysfunctional restoration of pH gradients generated during Ca^{2+} release may cause malfunctioning of the muscle cells, such as fatigue. Traditionally, only acidification of myoplasmic pH has been related to fatigue (Westerblad et al., 1991). The present results suggest that luminal acidification of the SR has to be taken into account as well.

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