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Fluorescence Probe Study of the Lumenal Ca^{2+} of the Sarcoplasmic Reticulum Vesicles during Ca^{2+} Uptake and Ca^{2+} Release

Yukio Saiki* and Noriaki Ikemoto*,†
**Boston Biomedical Research Institute and †Department of Neurology, Harvard Medical School, Boston, Massachusetts 02114*

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A limited amount of information is available about the lumenal Ca^{2+} kinetics of the sarcoplasmic reticulum (SR). Incubation of mag-fura-2AM permitted to incorporate a sufficient amount of the probe into the SR vesicles, as determined by Mn^{2+} quenching. Rapid changes in the lumenal $[Ca^{2+}]$ ($[Ca^{2+}]_{lum}$) during Ca^{2+} uptake and release could be monitored by following the signal derived from the lumenal probe while clamping the extra-vesicular Ca^{2+} ($[Ca^{2+}]_{ex}$) at various desired levels with a BAPTA/Ca buffer. Changes in the $[Ca^{2+}]_{lum}$ during uptake and release show the characteristics intrinsic to the SR Ca^{2+} pump (the $[Ca^{2+}]_{ex}$-dependence of the activation and inhibition by thapsigargin) and the Ca^{2+} release channel (blocking by ruthenium red), respectively. A new feature revealed by the $[Ca^{2+}]_{lum}$ measurement is that during the uptake reaction the free $[Ca^{2+}]_{lum}$ showed a significant oscillation. Several pieces of evidence suggest that this is due to some interactions between the Ca^{2+} pump and lumenal proteins. © 1997 Academic Press

There are a number of excellent chemical probes highly suited for the assay of the Ca^{2+} concentration within the cytoplasm and in the reaction solution. The application of some of the fluorescent Ca^{2+} probes to muscle research has permitted a remarkable progress in resolving, for example, the intra-cellular Ca^{2+} transients with very high temporal and spatial resolutions (1- 3). A considerable amount of information has also been obtained about the kinetics of Ca^{2+} transport into and Ca^{2+} release from isolated SR vesicle using these cytoplasmic probes (4, 5). However, a very limited amount of information (6-9) is available about the kinetics of lumenal Ca^{2+} during Ca^{2+} uptake and release.

Low-affinity Mg^{2+} fluorescent probes mag-fura-2 tetra-potassium (a membrane-inpermeable form) and mag-fura-2AM (a membrane-permeable form) have a relatively high affinity for Ca^{2+} (Kd = 44-53 μM)(10-12), and have been widely used for the assays of the cytoplasmic Ca^{2+} of intact muscle cells (13, 14). The usefulness of mag-fura-2AM as a lumenal Ca^{2+} probe was recognized by earlier reports that the probe could be incorporated in the endoplasmic Ca^{2+} stores (15-17). In the recent study by Shannon and Bers (18), mag-fura-2 tetra-potassium was trapped in the SR lumen during the process of homogenization of the cardiac muscle tissue. They demonstrated that a large Ca^{2+} concentration gradient (~7,000), which was in the vicinity of the theoretically expected level, was established across the SR membrane during cardiac SR Ca^{2+} uptake. The aims of the present study are (a) to devise appropriate conditions to incorporate mag-fura 2AM into the SR vesicles after their isolation from the skeletal muscle tissue, (b) to characterize and calibrate the fluorescence signal of the lumenal Ca^{2+}, and (c) to employ the methods for the Ca^{2+} uptake and release studies.

Abbreviations: BAPTA, 1,2-bis(o-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid; mag-fura-2AM, 5-oxazolecarboxylic acid, 2 - [5 - [2 - [acetyloxymethoxy] - 2 - oxoethoxy] - 6 - [bis[2 - [acetyloxymethoxy]-2-oxoethyl]amino]-2-benzofuranyl]-acetyloxymethyl ester; MES, 2-(*N*-morpholino)ethanesulfonic acid; SR, sarcoplasmic reticulum.

EXPERIMENTAL PROCEDURES

Preparation and lumenal probe incorporation. The triad-enriched microsomal fraction (triad) was prepared from rabbit leg and back muscle by differential centrifugation as described previously (19). After the final centrifugation, the sedimented fraction was homogenized in a solution containing 0.3 M sucrose, 0.15 M K gluconate, proteolytic enzyme inhibitors (0.1 mM phenylmethanesulfonyl fluoride, 10 μg/ml aprotinin, 0.8 μg/ml antipain, 2 μg/ml trypsin inhibitor) and 20 mM MES, pH 6.8, to a final protein concentration of 20-30 mg/ml. The preparation was quickly frozen in liquid nitrogen and stored at −70°. The triads (4 mg/ml) were incubated with 2 μM mag-fura-2AM (a membrane-permeable form), or mag-fura-2 tetra-

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potassium (a membrane-impermeable form) as a control, in a solution of 0.15 M K gluconate, 40 mM imidazole (pH 6.8) for 30 min at 22°. The incubated vesicles were centrifuged at 195,000 × g for 15 min at 4°. The sedimented vesicles were homogenized in a solution containing 0.15 M K gluconate and 40 mM imidazole, pH 6.8 and centrifuged again. The supernatant was discarded and the centrifuge tubes containing the sedimented vesicles were rinsed several times, and the sedimented vesicles were homogenized in the same solution (the final concentration of 16 mg/ml). The preparation labeled with mag-fura-2AM is designated as labeled vesicles hereafter.

Calibration of the lumenal $[Ca^{2+}]$ ($[Ca^{2+}]_{lum}$). To determine the fluorescence intensity of the labeled vesicles as a function of the $[Ca^{2+}]_{lum}$, the vesicles (8.0 mg/ml) were incubated in a solution containing 0.15 M K gluconate, 40 mM imidazole (pH 6.8) and various concentrations of Ca^{2+} (0-5 mM) for 12 hrs at 4°. After equilibration at various levels of Ca^{2+}, the vesicles were mixed with a solution containing 20 mM BAPTA , 2.5 mM Mg ATP (to mimic the conditions for Ca^{2+} uptake/release experiments, but there was no Ca^{2+} uptake under such conditions, since $[Ca^{2+}]_{ex}$ was 0) and 1 μM ruthenium red (to prevent Ca^{2+} release). Then, the fluorescence intensity at $\lambda_{emission}$= 510 nm was determined at $\lambda_{excitation}$ = 330 nm and 370 nm at each Ca^{2+} concentration with a spectrophotometer (SPEX Fluorolog).

Assays of the $[Ca^{2+}]_{lum}$ *during* Ca^{2+} *uptake and release.* For Ca^{2+} uptake assays, one volume of solution containing the labeled vesicles (0.4 mg/ml), 0.15 M K gluconate, an ATP-regenerating system (5.0 mM phosphoenyolpyruvate, 10 units/ml pyruvate kinase), 40 mM imidazole (pH 6.8) was mixed with an equal volume of solution containing 0.15 M K gluconate, 5.0 mM Mg ATP, 2.5 mM BAPTA, various concentrations of $CaCl_2$, 40 mM imidazole (pH 6.8) in a stopped-flow spectrophotometer system (BioLogic, model MPS-4). For Ca^{2+} release assays, the labeled vesicles (0.4 mg/ml) were incubated in a Ca^{2+} loading solution containing 0.15 M K gluconate, 5.0 mM Mg ATP, an ATP-regenerating system, 50 μM $CaCl_2$, 40 mM imidazole (pH 6.8) at 22° for 5 mins. Then, one volume of the incubated solution was mixed with an equal volume of 0.15 M K gluconate solution (buffered at pH 6.8 with 40 mM imidazole; $[Ca^{2+}]_{ex}$=0.2 μM adjusted by a mixture of 2.5 mM BAPTA and 1.437 mM $CaCl_2$) containing various concentrations of polylysine (Mr=27,000). Changes in the $[Ca^{2+}]_{lum}$ during Ca^{2+} uptake and release reactions were monitored by following the fluorescence intensity of the trapped mag-fura-2AM (emission at 510 nm, excitation at 370 nm, with a 430 nm cut-off filter).

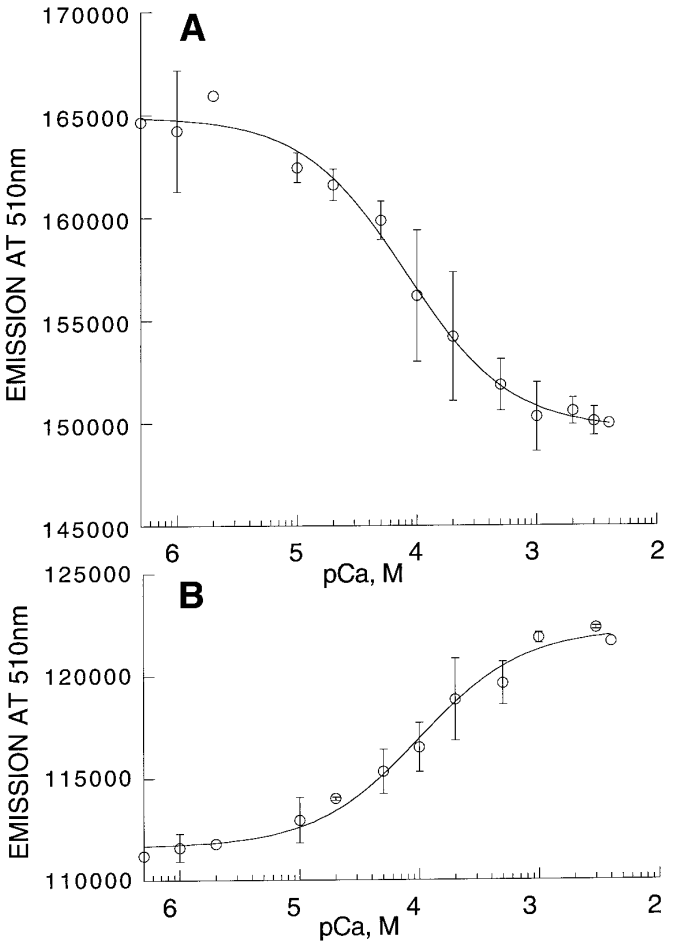


FIG. 1. $[Ca^{2+}]$-dependent changes in the emission intensities at two major excitation wavelengths (A at 370 nm; B at 330 nm) of the mag-fura-2 AM trapped in the lumen of the labeled vesicles. The labeled vesicles were equilibrated with various Ca^{2+} concentrations, and the $[Ca^{2+}]_{ex}$ was clamped to approximately zero by adding 20 mM BAPTA. Immediately after the addition of BAPTA, the fluorescence intensities ($\lambda_{emission}$ = 510 nm) excited at $\lambda_{excitation}$ =330 nm (E_{330}) and that at $\lambda_{excitation}$ =370 nm (E_{370}) were recorded as a function of the $[Ca^{2+}]$ to which the labeled vesicles had been equilibrated.

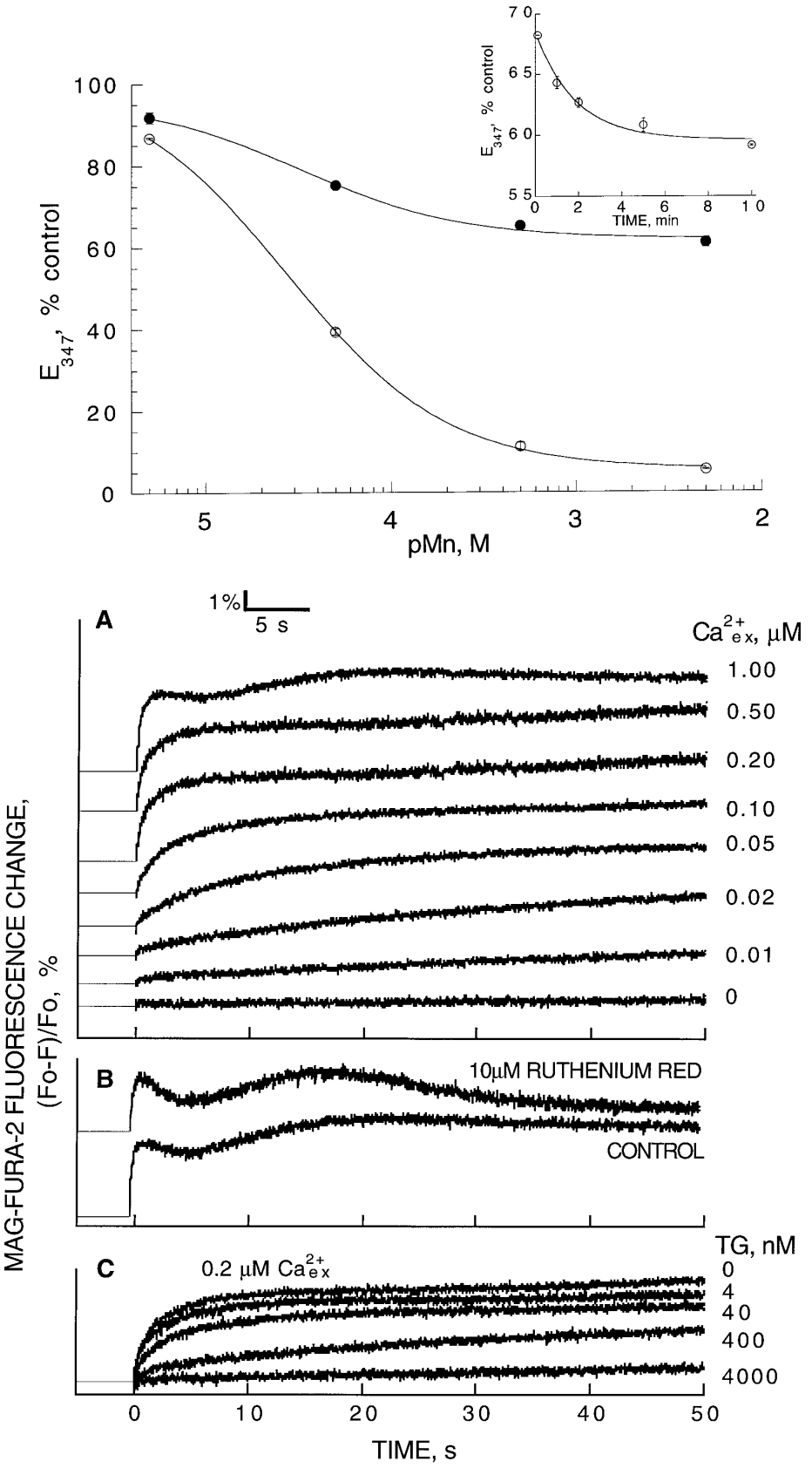
RESULTS AND DISCUSSION

Characterization of the Lumenally Trapped Probe

In the $[Ca^{2+}]$ calibration experiment shown in Fig. 1, the labeled vesicles were incubated with various concentrations of Ca^{2+} for 12 hrs to generate various levels of $[Ca^{2+}]_{lum}$ by permeation. Then, the $[Ca^{2+}]_{ex}$ was adjusted to ~ 0 μM by adding 20 mM BAPTA and 2.5 mM Mg ATP ; MgATP was added to mimic the conditions for Ca^{2+} uptake/release experiments, although there was no Ca^{2+} uptake under these conditions, since $[Ca^{2+}]_{ex}$ was 0. Immediately after the addition of BAPTA, the

FIG. 2. The $[Mn^{2+}]$-dependence of fluorescence quenching of the vesicles labeled with mag-fura-2 AM (solid symbols). The labeled vesicles were equilibrated with 100 μM Ca^{2+}, and various concentrations of Mn^{2+} were added. About 3 s after the addition of Mn^{2+}, the fluorescence intensity ($\lambda_{emission}$ = 510 nm, $\lambda_{excitation}$ = 347 nm: E_{347}) was determined at each concentration of Mn^{2+}. To determine the efficiency of Mn^{2+} quenching, various concentrations Mn^{2+} were added to a solution of mag-fura-2 tetra-potassium in the absence of SR vesicles (open symbols). The inset shows the time course of the signal change after the addition of 500 μM Mn^{2+} to the labeled vesicles.

FIG. 3. A: Changes in the lumenal $[Ca^{2+}]$ during Ca^{2+} uptake at different levels of extra-vesicular $[Ca^{2+}]$ ($[Ca^{2+}]_{ex}$). The vesicles labeled with mag-fura-2AM were mixed with 2.5 mM Mg ATP, 1.25 mM BAPTA and various concentrations of $CaCl_2$ in a stopped-flow apparatus, and the change in E_{370} was monitored. B: Lack of effect of ruthenium red on the oscillation in the $[Ca^{2+}]_{lum}$ during Ca^{2+} uptake. The Ca^{2+} uptake reaction was carried out at $[Ca^{2+}]_{ex}$ =2.0 μM in the presence or in the absence of 10 μM ruthenium red. C. Inhibition of the MgATP-induced change in the lumenal $[Ca^{2+}]$ by thapsigargin. The Ca^{2+} uptake reaction was carried out at $[Ca^{2+}]_{ex}$ = 0.2 μM in the presence of various concentrations of thapsigargin (TG) as indicated.


E347, % control
pMn, M
TIME, min
1%
5 s
A
Ca2+ex, µM
1.00
0.50
0.20
0.10
0.05
0.02
0.01
0
B
10µM RUTHENIUM RED
CONTROL
C
0.2 µM Ca2+ex
TG, nM
0
4
40
400
4000
MAG-FURA-2 FLUORESCENCE CHANGE, (Fo-F)/Fo, %
TIME, s

emission intensity at 510 nm excited at 370 nm (E_{370}) and that excited at 330 nm (E_{330}) were recorded at each Ca^{2+} concentration, and were plotted as a function of the $[Ca^{2+}]_{lum}$. The signals obtained under such conditions should represent primarily the $[Ca^{2+}]_{lum}$, since the $[Ca^{2+}]_{ex}$ was clamped at ~ 0 μM by BAPTA. This $[Ca^{2+}]_{ex}$-clamping method seems to be the most feasible approach for the specific monitoring of the $[Ca^{2+}]_{lum}$, since a significant amount of probe remained bound to the external surface of the SR membrane even after washing (see below). The Kd values for Ca^{2+} determined by fitting to the E_{330} and E_{370} curves were 81 μM and 94 μM, respectively. As a control, we carried out the same experiment with the sample that had been incubated with mag-fura-2 tetra-potassium instead of mag-fura-2AM. Both E_{330} and E_{370} curves showed no appreciable $[Ca^{2+}]_{lum}$ -dependence (data not shown), indicating that a negligible amount of mag-fura-2 tetra-potassium entered the SR lumen.

The efficiency of incorporation of mag-fura-2AM into the SR lumen was determined by the fluorescence quenching of the extra-vesicular probe with a commonly used quencher of mag-fura-2, Mn^{2+} (17,18). Fig. 2 depicts the results of the $[Mn^{2+}]$-dependent quenching pattern of the vesicles incubated with mag-fura-2AM (solid symbol). Each datum point represents the fluorescence intensity, E_{347} (the 510 nm emission at $\lambda_{excitation}$ =347 nm, which is the isobesic wavelength for mag-fura-2 spectra at various Ca^{2+} concentrations (11)). The E_{347} values were determined immediately after the addition of the quencher (3 s), since the fluorescence gradually decreased probably due to a gradual entry of Mn^{2+} into the SR lumen (cf. Inset to Fig. 2). The relative intensity at a maximally quenching concentration of Mn^{2+} (5 mM), $(E_{347})_{Mn\,=\,5\,mM}/(E_{347})_{Mn\,=\,0}$, was 0.611±0.007 (n=3). Fig. 2 also shows that Mn^{2+} quenching was not complete even at 5 mM, as indicated by the control in which increasing concentrations of Mn^{2+} were added to a solution of mag-fura-2 tetra-potassium in the absence of SR vesicles (open symbols). The difference in the $(E_{347})_{Mn=5\,mM}/(E_{347})_{Mn\,=\,0}$, values between the labeled sample and the control was 0.557±0.037 (n=3), indicating that 55.7% of the total mag-fura-2AM was trapped in the SR lumen.

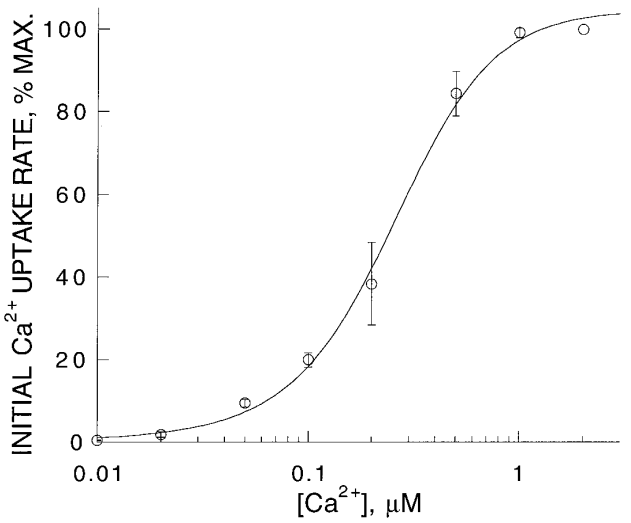


FIG. 4. The $[Ca^{2+}]_{ex}$ -dependence of the initial rate of the increase of the lumenal $[Ca^{2+}]$. The initial Ca^{2+} uptake rates were determined from $\{d(uptake)/dt\}_{t\to 0}$.

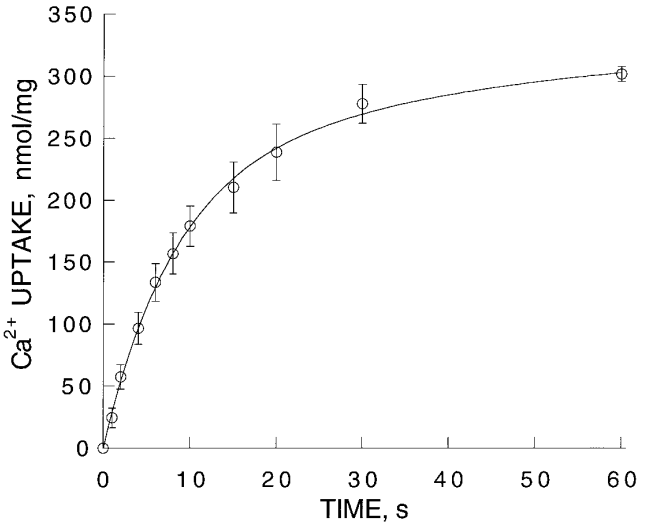


FIG. 5. The time course of Ca^{2+} uptake determined by the chemical-quench technique using $^{45}Ca^{2+}$. Ca^{2+} uptake reactions were carried at $[Ca^{2+}]_{ex}$ =1.0 μM in the same way as done in Fig. 3A, except that $^{45}Ca^{2+}$ was included in the reaction solution. The reaction was quenched by mixing with the quench solution containing 0.15 M K gluconate, 30 mM EGTA, 10 μM ruthenium red, 5mM $MgSO_4$ and 40 mM imidazole (pH 6.8) at various times (0 to 60 s). The quenched reaction mixtures were filtered through Millipore filters (ϕ 0.45μM), and the radioactivity of ^{45}Ca retained on the filter was counted with a scintillation counter.

Changes in the Lumenal Ca^{2+} during Ca^{2+} Uptake and Ca^{2+} Release

Using the vesicles labeled with mag-fura-2AM, we carried out Ca^{2+} uptake reaction (Fig. 3), and followed the changes in the $[Ca^{2+}]_{lum}$ by monitoring E_{370} in a stopped-flow fluorometer. In this experiment the uptake reaction was started with 2.5 mM MgATP in the presence of the BAPTA/Ca mixtures to clamp the $[Ca^{2+}]_{ex}$ at various concentrations as indicated. At $[Ca^{2+}]_{ex}$ = 0 there was no appreciable change in the $[Ca^{2+}]_{lum}$. Upon increasing the $[Ca^{2+}]_{ex}$ the initial rate of uptake determined by the $[Ca^{2+}]_{lum}$ signal became faster and leveled off at $[Ca^{2+}]_{ex}$ = 0.5-1.0 μM (Fig. 4), showing the pattern essentially identical to the previously reported $[Ca^{2+}]$-dependence of activation of Ca^{2+} ATPase and transport (20, 21). The $[Ca^{2+}]_{lum}$ change was blocked by thapsigargin with a IC_{50} = 0.45 μM (Fig. 3 C) (22). These results indicate that

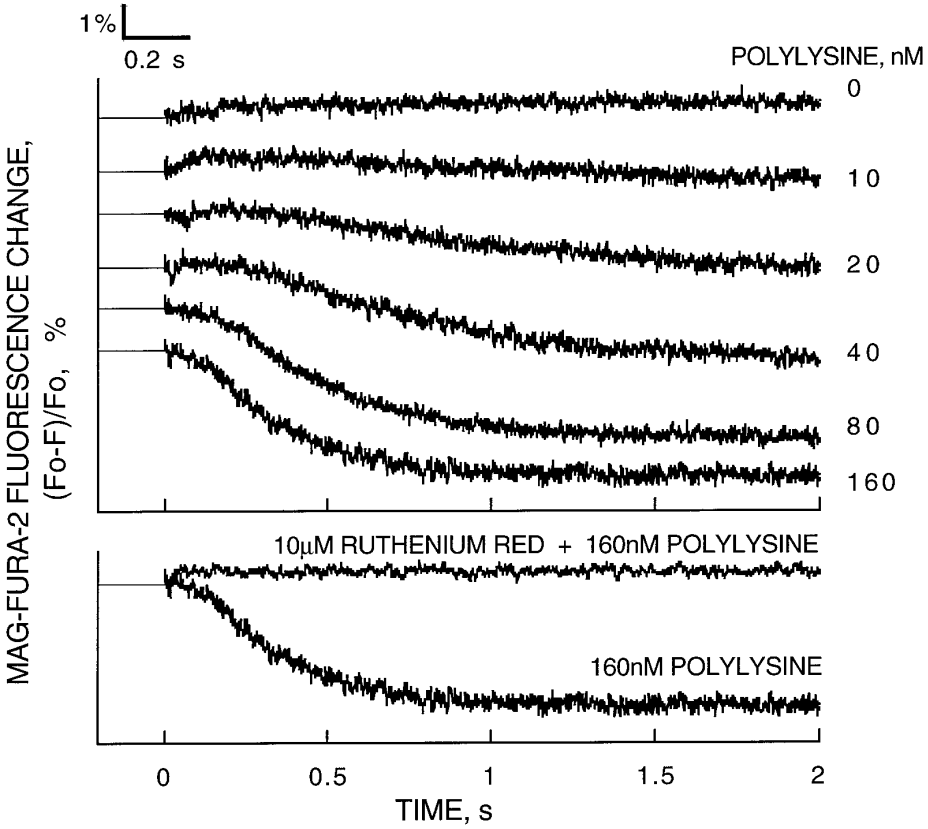


FIG. 6. Top panel: Changes in the lumenal $[Ca^{2+}]$ during Ca^{2+} release induced by various concentrations of the RyR-specific release trigger polylysine. The labeled vesicles, which had been loaded with Ca^{2+} by mediation of ATP-dependent Ca^{2+} uptake, were mixed with various concentrations of the RyR-specific Ca^{2+} release trigger polylysine at $[Ca^{2+}]_{ex}$ = 0.2 μM. Bottom panel: Inhibition of polylysine-induced decrease in the the lumenal $[Ca^{2+}]$ by ruthenium red. Ca^{2+} release was induced with 0.16 μM polylysine in the presence of 10 μM ruthenium red. The reference curve represents the time course of Ca^{2+} release induced by 0.16 μM polylysine.

the change in the $[Ca^{2+}]_{lum}$ is primarily due to Ca^{2+} transport *via* the SR Ca^{2+} ATPase. However, we consistently observed an oscillation in the $[Ca^{2+}]_{lum}$ when the uptake reaction was carried out at higher $[Ca^{2+}]_{ex}$ ($\geq$ 0.5 μM, cf. Fig. 3 A). Interestingly, the calcium uptake showed a monotonic increase when the total amount of calcium taken up was monitored by the chemical-quench technique using ^{45}Ca (Fig. 5). Furthermore, the oscillation could not be stopped by 10 μM ruthenium red (Fig. 3 B), although the amplitude of the signal slightly decreased due to the absorption by ruthenium red. This clearly distinguishes the lumenal Ca^{2+} oscillation described here from the previously reported oscillation in the $[Ca^{2+}]_{ex}$ after induction of Ca^{2+} release by caffeine (23). These results indicate that the lumenal Ca^{2+} oscillation is not due to an alteration of uptake and release, but due to e.g. some counteractions between the pump flux and lumenal Ca^{2+} binding proteins. Partially inhibiting concentrations of thapsigargin reduced the initial rate of Ca^{2+} uptake and concomitantly reduced the degree of the lumenal Ca^{2+} oscillation (data not shown). This suggests that the rate of increase in the $[Ca^{2+}]_{lum}$ may be one of the factors regulating the lumenal Ca^{2+} oscillation.

In order to assess the actual changes in the $[Ca^{2+}]_{lum}$ in the above experiments, we carried out Ca^{2+} uptake reaction in a Spex fluorometer under the same conditions as the stopped-flow experiment at $[Ca^{2+}]_{ex}$ = 0.2 μM to avoid lumenal Ca^{2+} oscillation, which would disturb a stable calibration. The lumenal concentrations of the free Ca^{2+} at the beginning and at the steady state of uptake (50 s), were 0.463 mM and 0.677 mM (average of two experiments), respectively, as calibrated by the same type of experiments as Fig. 1.

The results of the Ca^{2+} release experiment are shown in Fig. 6. In this experiment, the labeled vesicles that had been loaded with Ca^{2+} were mixed with solutions containing various concentrations of the RyR-specific release trigger polylysine (24, 25) and the BAPTA/calcium buffer to clamp the $[Ca^{2+}]_{ex}$ at 0.2 μM (Fig. 6 top panel). Polylysine decreased the $[Ca^{2+}]_{lum}$ in a dose-dependent manner after a small transient increase in the $[Ca^{2+}]_{lum}$ at lower polylysine

concentrations or an appreciable lag phase at higher concentrations (cf. ref. (6)). The change in the $[Ca^{2+}]_{lum}$ was blocked by ruthenium red (Fig. 6 bottom panel), suggesting that the polylysine-induced fluorescence change is primarily due to Ca^{2+} release through the SR Ca^{2+} release channel.

In conclusion, incubation of mag-fura-2AM with the isolated SR vesicles resulted in the incorporation of a sufficient amount of the probe into the SR lumen for the assay of the lumenal $[Ca^{2+}]$. The conditions devised in this study (e.g. clamping of the extra-vesicular $[Ca^{2+}]$ and the use of gluconate with a Ca^{2+}-chelating capacity) permitted a specific monitoring of the changes in the lumenal $[Ca^{2+}]$ during the Ca^{2+} uptake and release. An interesting phenomenon revealed in this study is an oscillatory change in the lumenal $[Ca^{2+}]$ occurring during Ca^{2+} transport. The detailed mechanism for the lumenal Ca^{2+} oscillation remains to be investigated.

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