Coordination between Ca^{2+} Release and Subsequent Re-Uptake in the Sarcoplasmic Reticulum†

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ABSTRACT: We here report the results of our recent effort to produce, in the isolated sarcoplasmic reticulum (SR), a biphasic Ca^{2+} release and Ca^{2+} re-uptake transient and to resolve the kinetic relationship between Ca^{2+} release and re-uptake of the released Ca^{2+}. Ca^{2+} release from the SR was induced by polylysine (the ryanodine receptor-specific Ca^{2+} release trigger) at various levels of calcium loading, or at various doses of the trigger. The changes in the Ca^{2+} concentration in the reaction solution and in the lumenal Ca^{2+} concentration were determined by stopped-flow spectroscopy using fluo-3 and mag-fura-2AM, respectively. At higher levels of calcium loading (>150 nmol/mg), polylysine induced monophasic Ca^{2+} release curves (without an appreciable re-uptake phase) as reported in most studies in the literature. However, lowering the calcium loading level to an intermediate range (100−150 nmol/mg) produced the desired biphasic transient curves consisting of Ca^{2+} release and Ca^{2+} re-uptake phases. Under these conditions, the increase in the polylysine concentration resulted in the increase of both the rate of Ca^{2+} release and that of re-uptake of the released Ca^{2+}. The maximal rate of Ca^{2+} release and that of re-uptake showed a parallel relationship in the polylysine concentration range of 0−10 μM. This indicates that Ca^{2+} release from the SR and re-uptake of the released Ca^{2+} via the SR Ca^{2+} pump are well-coordinated processes. The changes in the lumenal Ca^{2+} concentration during the release and re-uptake reaction were monitored at an optimum level of calcium loading while clamping the extravesicular Ca^{2+} concentration at a constant value. There was again a tight correlation between Ca^{2+} release (decrease of the lumenal Ca^{2+} concentration) and re-uptake (increase of the lumenal Ca^{2+} concentration), indicating that acceleration of the re-uptake is controlled by the rate of decrease of the lumenal Ca^{2+} concentration. We propose that one of the mechanisms, by which the mode of coordination between the two components of the biphasic Ca^{2+} transient (viz. Ca^{2+} release via the ryanodine receptor and Ca^{2+} re-uptake via the SR Ca^{2+} pump) is controlled, is the change in the Ca^{2+} concentration gradient across the SR membrane.

The depolarization signal elicited in the T-tubule triggers biphasic changes in the cytoplasmic Ca^{2+} concentration, the so-called intracellular Ca^{2+} transient. The Ca^{2+} transient consists of two kinetic components: the initial rapid increase in the cytoplasmic Ca^{2+} concentration due to a rapid Ca^{2+} release from the SR[1] and its subsequent decrease due to several mechanisms described below (*1*−*4*). Compared with the extensive work done on the first Ca^{2+} release phase of the Ca^{2+} transient (*5*−*14*), only a limited amount of work has been done on the second phase. As a matter of fact, straightforward analysis of the second phase of the Ca^{2+} transient has been rather difficult, particularly in the fiber system, because of the involvement of complex factors, such as (a) binding of the released Ca^{2+} to cytoplasmic and myofibrillar proteins (*15*−*20*), (b) re-uptake of the released Ca^{2+} via the SR Ca^{2+} pump (*18*−*20*), (c) inactivation of the SR Ca^{2+} channel (*21*−*26*), and (d) oscillatory changes in the myofibrillar tension and in the cytoplasmic Ca^{2+} concentration (*27*−*32*).

The goals of this study are first to devise appropriate in vitro assay conditions that permit us to produce biphasic release−re-uptake curves and then to analyze the kinetic relationship between the release and re-uptake processes. There are definite advantages in utilizing the isolated vesicular preparation for this purpose, since it is freed from cytoplasmic and myofibrillar calcium-binding proteins, permitting the straightforward analysis of the Ca^{2+} release flux (*33*, *34*). The system is also well suited for the Ca^{2+} influx (re-uptake flux) analysis, as confirmed by the successful utilization of the vesicular SR preparation in the majority of the Ca^{2+} transport studies in the literature (*35*−*40*).

In this study, we investigated various factors affecting the shape of Ca^{2+} release−re-uptake time course using the RyR-specific ligand polylysine as a Ca^{2+} release trigger (*41*, *42*). As described here, there are at least two key parameters for the production of the aimed SR Ca^{2+} release−re-uptake time course: (a) optimum levels of SR Ca^{2+} loading and (b)

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[1] 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.

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optimum doses of the Ca^{2+} release trigger. Under the optimized conditions, the rate of Ca^{2+} release and the rate of re-uptake of the released Ca^{2+} increased in a parallel fashion when the polylysine dose was increased. These results suggest that Ca^{2+} release through the release channel and re-uptake of the released Ca^{2+} through the SR Ca^{2+} pump are operating in a coordinated manner, under such optimized experimental conditions. As indicated from several pieces of evidence shown here, the re-uptake phase appears to be accelerated by the faster Ca^{2+} release by mediation of some mechanisms, one of which would be a rapid decrease in the Ca^{2+} concentration gradient across the SR membrane. These data provide a new clue for the better understanding of the transient contraction−relaxation mechanism in muscle, although whether a similar mechanism is operating in the regulation of the Ca^{2+} transient in situ remains unresolved.

EXPERIMENTAL PROCEDURES

Preparation. The heavy microsomal fraction enriched in triadic vesicles (triads) was prepared from rabbit leg and back muscle by differential centrifugation as described previously (*43*). After the final centrifugation, the sedimented fraction was homogenized in a solution containing 0.3 M sucrose, 0.15 M potassium gluconate, proteolytic enzyme inhibitors (0.1 mM phenylmethanesulfonyl fluoride, 10 μg/mL aprotinin, 0.8 μg/mL antipain, and 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 °C.

Stopped-Flow Assay of Ca^{2+} Release and Re-Uptake. To load the SR moiety with Ca^{2+}, the triads (0.4 mg/mL) were incubated in solution A (see below) for at least 5 min, at which time the Ca^{2+} concentration of solution A was reduced to 0.04−0.05 μM. The level of SR calcium loading (the endogeneous lumenal calcium level of 35−50 nmol/mg plus that of the actively transported calcium) was determined by the EGTA back-titration method described previously (*44*). Then, 1 volume of solution A was mixed with 1 volume of solution B loaded containing various concentrations (0−50 μM) of polylysine (M_r = 4000) to induce Ca^{2+} release. The time course of Ca^{2+} release and the subsequent re-uptake of the released Ca^{2+} were monitored in a stopped-flow fluorometer (BioLogic SFM4, Figure 1) using fluo-3 as the Ca^{2+} indicator (excitation at 437 nm and emission at 530 nm with a 510-nm cutoff filter).

The base solution was 0.15 M potassium gluconate and 40 mM imidazole (pH 6.8).

Solution A was base solution with 0.4 mg/mL triad, 5.0 mM $MgSO_4$, 5.0 mM ATP, an ATP-regenerating system (5.0 mM phosphoenyolpyruvate and 10 units/mL pyruvate kinase), and various concentrations of $CaCl_2$.

Solution B was base solution with 5.0 μM fluo-3 and various concentrations of polylysine (M_r = 4000).

The free Ca^{2+} concentration in the reaction solution was determined from the curve of the fluo-3 fluorescence intensity versus the free Ca^{2+} concentration obtained by calibration of the fluorescence intensity against various concentrations of Ca^{2+} (Ca^{2+} buffers containing 1 mM BAPTA and various concentrations of added calcium). In all of these traces, upon both solutions being mixed $[Ca^{2+}]_{ex}$ abruptly increased from 0.05 μM (the Ca^{2+} level of solution A that had been reached as a result of active calcium loading) to 0.15 μM because of the endogenous calcium that was present in solution B. To obtain the polylysine-dependent component proper of the Ca^{2+} release−re-uptake time course, the control curve (i.e., the 0-polylysine curve) was subtracted from the curves obtained with various concentrations of polylysine (Figure 1A).

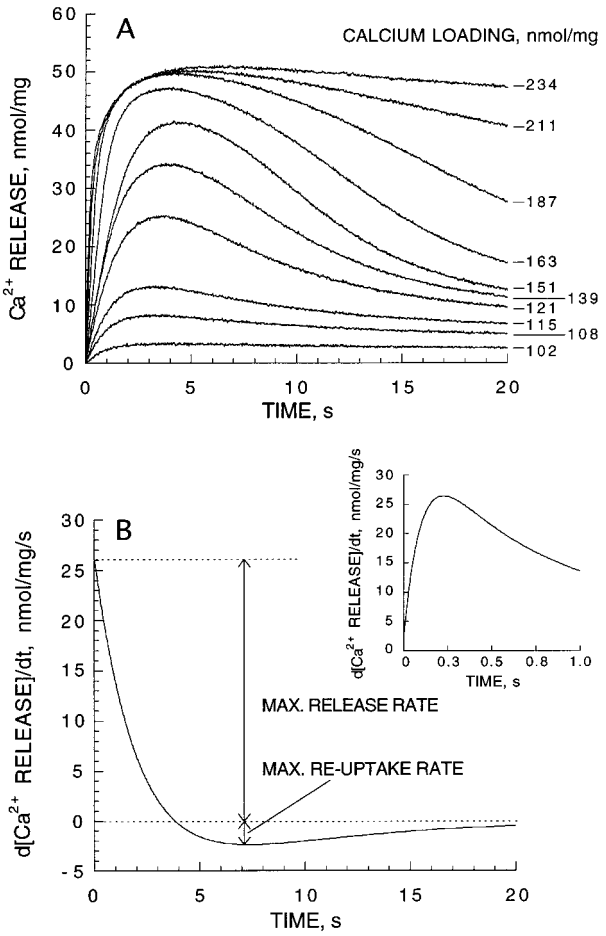

FIGURE 1: (A) Stopped-flow traces of polylysine-induced Ca^{2+} release from and re-uptake of the released Ca^{2+} into the SR vesicles, which had been subjected to various levels of Ca^{2+} loading. The SR was incubated in a solution containing various concentrations of $CaCl_2$, and the level of calcium loading (i.e., the total level of lumenal calcium including the endogeneous lumenal calcium) was determined by the EGTA back-titration method (*44*). Ca^{2+} release was induced by mixing the Ca^{2+}-loaded SR with 10 μM polylysine. The time course of Ca^{2+} release was monitored using fluo-3 as a Ca^{2+} probe. Each trace was obtained by signal averaging a total of 25−30 traces originating from three experiments. For details, see Experimental Procedures. (B) First derivative of the Ca^{2+} release−re-uptake curve (20 s reaction time; inset, 1.0 s reaction time) obtained at the calcium loading level of 139 nmol/mg.

The amount of calcium released (or the amount of re-uptake) was computed from the changes in the free Ca^{2+} concentration using the association constants of various metal−ligand complexes present in the solution (*45*, *46*) and the association constant of gluconate (a major Ca^{2+}-chelating component in the reaction solution) for Ca^{2+} (2.617×10^2 M^{-1}). The association constant of gluconate was determined from two quantities: the amount of calcium obtained from the atomic absorption spectrometry and the free Ca^{2+} concentration obtained from the fluo-3 fluorometry (see above).

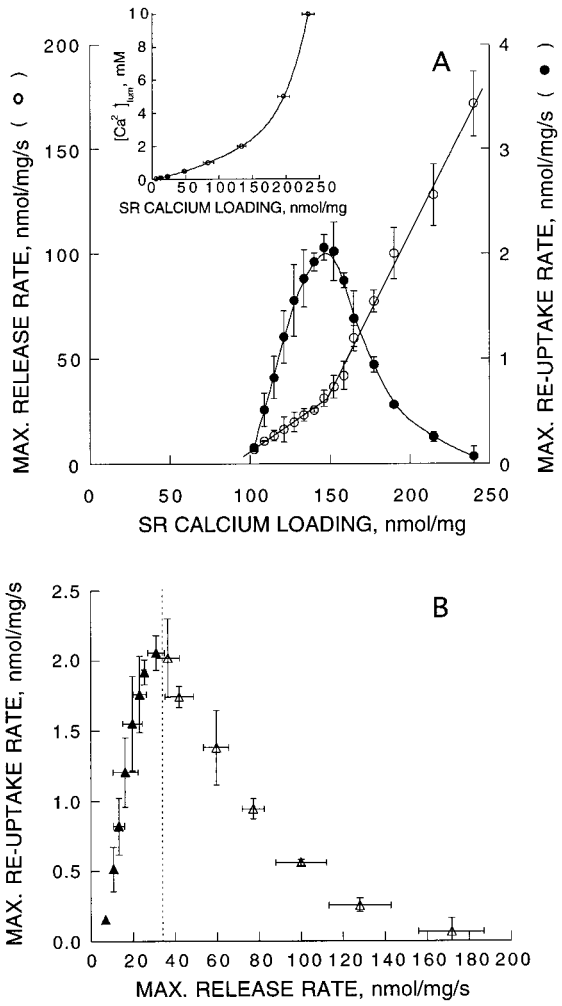

FIGURE 2: (A) Apparent maximal Ca^{2+} release rate and the apparent maximal Ca^{2+} re-uptake rate as a function of the amount of loaded calcium. The inset shows the estimated lumenal Ca^{2+} concentrations at various levels of calcium loading. The apparent maximal rate of Ca^{2+} release was obtained from the highest positive value of the curves as shown in Figure 1B, and the apparent maximal rate of Ca^{2+} re-uptake was obtained from the absolute value of the lowest negative point of the corresponding derivative curve. The lumenal Ca^{2+} concentrations at various levels of calcium loading were calculated as described in Experimental Procedures. Data represent the mean ± standard deviation of four experiments. (B) Relationship between the apparent maximal Ca^{2+} release rate and the apparent maximal Ca^{2+} re-uptake rate. The data obtained at the optimum loading range (100−150 nmol/mg) are depicted as black symbols, while those obtained in the overloading range (>150 nmol/mg) are depicted as white symbols. Data represent the mean ± standard deviation of four experiments.

Assays of Lumenal Ca^{2+} during Ca^{2+} Release and Re-Uptake. For the assay of lumenal Ca^{2+}, mag-fura-2AM (a membrane-permeable form) was loaded into the triads as described previously (*47*). Briefly, the triads (4 mg/mL) were incubated with 2 μM mag-fura-2AM in a solution of 0.15 M potassium gluconate and 40 mM imidazole (pH 6.8) for 30 min at 22 °C. The incubated vesicles were centrifuged at 195000*g* for 15 min at 4 °C. The sedimented vesicles were homogenized in a solution containing 0.15 M potassium gluconate and 40 mM imidazole (pH 6.8) and centrifuged again. The supernatant was discarded; the centrifuge tubes containing the sedimented vesicles were washed several times, and the sedimented vesicles were homogenized in the same solution (final concentration of 16 mg/mL).

For Ca^{2+} release and re-uptake assays, the labeled vesicles (0.4 mg/mL) were incubated in a calcium loading solution containing 0.15 M potassium gluconate, 5.0 mM MgATP, an ATP-regenerating system (5.0 mM phosphoenolpyruvate and 10 units/mL pyruvate kinase), and 40 mM imidazole (pH 6.8) at 22 °C for 5 min. Then, 1 volume of the incubated solution was mixed with an equal volume of 0.15 M potassium gluconate solution (buffered at a $[Ca^{2+}]_{ex}$ of 0.15 μM with a mixture of 2.5 mM BAPTA and 1.259 mM $CaCl_2$) containing various concentrations of polylysine (M_r = 4000) and 40 mM imidazole (pH 6.8). Changes in the $[Ca^{2+}]_{lum}$ during Ca^{2+} release and subsequent re-uptake reactions were monitored by following the fluorescence intensity of the trapped mag-fura-2AM in a stopped-flow spectrophotometer system (BioLogic, model MPS-4, emission at 510 nm and excitation at 370 nm with a 430 nm cutoff filter) as described in ref *47*.

Determination of the Lumenal Ca^{2+} Concentration at Various Calcium Loading Levels. For the determination of the lumenal Ca^{2+} concentration, SR vesicles (0.1 mg/mL) were incubated in 0.15 M potassium gluconate and 40 mM imidazole (pH 6.8) at various concentrations of $CaCl_2$ (1.98−111.11 mM) containing $^{45}Ca^{2+}$ (5 μCi/mL) for 120 min at 22 °C. A 0.1 mL portion of the solution was filtered through the Millipore filter (0.45 μm) and washed with 2 × 5 mL of a solution containing 0.15 M potassium gluconate, 40 mM imidazole (pH 6.8), 1 mM EGTA (to remove the externally bound $^{45}Ca^{2+}$), and 5 μM ruthenium red (to prevent a leak of the internally loaded $^{45}Ca^{2+}$). The filter was air-dried, and the amount of $^{45}Ca^{2+}$ trapped within the vesicles was determined by scintillation counting. The level of lumenal calcium loading was determined from the amount of ^{45}Ca trapped within the vesicles. The lumenal Ca^{2+} concentrations at various levels of calcium loading were determined from the calculated amounts of the lumenal calcium and the various concentrations of Ca^{2+} in the passive loading solution as described previously (*48*).

RESULTS

In most of the published in vitro studies of Ca^{2+} release, little attention has been paid to the events occurring after Ca^{2+} release. This is chiefly due to the fact that the Ca^{2+} release time course has been monophasic under the conventional release assay conditions. In many reports, as a matter of fact, release assays were carried out with a passively loaded SR preparation in the absence of added MgATP, conditions under which there was no re-uptake of the released Ca^{2+} (*49*, *50*). As described below, a relatively rapid SR Ca^{2+} release−re-uptake time course could be produced if (a) the level of SR calcium loading and (b) the dose of the Ca^{2+} release trigger were optimized.

In this study, we used polylysine rather than T-tubule depolarization as a Ca^{2+} release-triggering agent for two important reasons. First, in the case of stimulation via T-tubules, Ca^{2+} release takes place from a limited population of SR vesicles linked with T-tubules (triads), while the re-uptake of the released Ca^{2+} occurs not only in the coupled triads but also in a large population of the SR vesicles that are not linked with T-tubules. In contrast to T-tubule depolarization, polylysine will activate all RyRs that are distributed to the majority of heavy SR vesicles. This

minimizes the possibility that Ca^{2+} release and re-uptake are occurring in separate populations of SR vesicles. Second, polylysine is the RyR-specific ligand as described previously (*41*, *42*), and hence, the polylysine-dependent activation of Ca^{2+} pumping described in this study is not a result of direct stimulation of the SR Ca^{2+} ATPase, but is produced by stimulation of the RyR.

Figure 1A depicts the time courses of Ca^{2+} release and re-uptake induced by 10 μM polylysine (a submaximally activating concentration) at various levels of SR calcium loading as monitored with fluo-3 as an extravesicular (cytoplasmic) Ca^{2+} ($[Ca^{2+}]_{ex}$) probe. As seen, Ca^{2+} release began to occur only after a sufficient amount of calcium (e.g., 102 nmol/mg) had been loaded. Upon the increase of calcium loading above this level, Ca^{2+} release became faster and the magnitude larger. Interestingly, this was followed by re-uptake of the released Ca^{2+}, whose rate increased with the increase in the rate of the preceding Ca^{2+} release. Upon the loading level being increased further above certain levels (e.g., 187−211 nmol/mg), it accelerated the Ca^{2+} release phase even more, but the Ca^{2+} re-uptake phase was sharply inhibited with the increase in the loading level. To assess the maximal flux rates in the Ca^{2+} release and re-uptake phases, the first derivatives of these curves were obtained. Figure 1B illustrates an example of the derivative curve obtained at the calcium loading level of 139 nmol/mg. As seen, the $d[Ca^{2+}]/dt$ value rapidly increased to the maximal level within about 200 ms (see the inset of Figure 1B), fell sharply to a more negative value, and then increased again toward zero. The highest positive value in these curves was taken as the apparent maximal rate of Ca^{2+} release [$(k_{rel})_{max}$], and the absolute value of the most negative point was taken as the apparent maximal rate of Ca^{2+} re-uptake [$(k_{up})_{max}$]. Figure 2A illustrates how the rates of release and re-uptake change as a function of the level of calcium loading. As seen here, there are three clearly distinguishable phases in the calcium load-dependent regulation of the release and re-uptake activities. In the lowest range of loading below the threshold level (≤100 nmol of calcium/mg of protein), no Ca^{2+} release, and consequently no re-uptake, took place. In the middle range of loading (100−150 nmol/mg), an increase in the loading level increased both Ca^{2+} release and re-uptake activities (a range of "optimum loading"). In the higher range (>150 nmol/mg), an increase in the loading level produced a pronounced activation of the Ca^{2+} release activity, but it produced a severe inhibition of re-uptake (a range of "overloading").

The plots of the re-uptake rate versus the release rate obtained at various loading levels are shown in Figure 2B. In the optimum range of Ca^{2+} loading (black symbols; calcium loading = 100−150 nmol/mg), there is a good correlation between both rates. This indicates that Ca^{2+} release and pumping of the released Ca^{2+} are well-coordinated phenomena at least under these conditions. In the range of overloading (white symbols; calcium loading = 160−240 nmol/mg), however, such coordination is lost. The estimated Ca^{2+} concentrations in the SR lumen ($[Ca^{2+}]_{lum}$) at various levels of Ca^{2+} loading (for the method of assessment, see Experimental Procedures) are plotted in the inset of Figure 2A. As seen, the degree of increase in the $[Ca^{2+}]_{lum}$ per an increased level of calcium loading is rather small in the optimum range of loading. However, it becomes very large in the range of Ca^{2+} overloading. The loss of coordination between the release and re-uptake rates occurring in this range appears to be well-correlated with this sharp increase in the $[Ca^{2+}]_{lum}$ per an increased level of calcium loading. Thus, the inhibition of the re-uptake phase occurring in this range seems to be caused by the increase in the $[Ca^{2+}]_{lum}$. Since the extravesicular Ca^{2+} concentration ($[Ca^{2+}]_{ex}$) was about the same regardless of the level of calcium loading in those experiments, the inhibition of re-uptake is presumably caused by an increase in the Ca^{2+} concentration gradient across the SR membrane (see the Discussion).

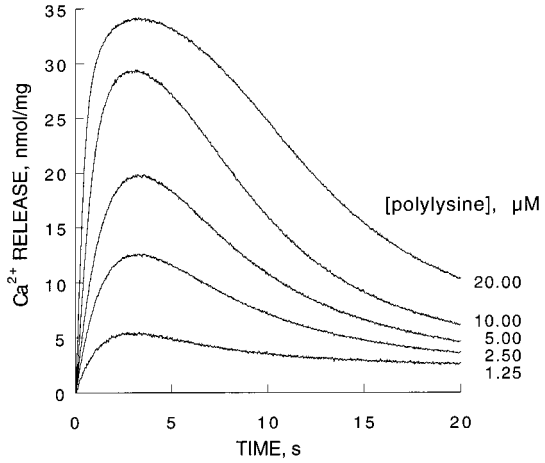

FIGURE 3: Stopped-flow traces of Ca^{2+} release from and re-uptake of the released Ca^{2+} into the SR vesicles loaded to an optimum level, when Ca^{2+} release was induced with various concentrations of polylysine. The SR moiety was loaded with Ca^{2+} by incubating SR with 5.0 mM MgATP. Ca^{2+} release was induced by mixing the Ca^{2+}-loaded SR with various concentrations of polylysine. The time course of Ca^{2+} release was monitored using fluo-3 as a Ca^{2+} probe. Each trace was obtained by signal averaging 7−12 traces. For details, see Experimental Procedures.

Figure 3 depicts the time courses of the release−re-uptake reaction induced by various concentrations of polylysine at an optimum level of Ca^{2+} loading (140 nmol/mg). Figure 4A shows the polylysine concentration dependence of the values of $(k_{rel})_{max}$ and $(k_{up})_{max}$ obtained from the data of Figure 3. Importantly, potentiation of Ca^{2+} release by increasing the polylysine dose resulted in the potentiation of the subsequent re-uptake of the released Ca^{2+} up to 10 μM in a parallel fashion. Further increases in the polylysine concentration increased the $(k_{rel})_{max}$, but it produced a sharp decrease in the $(k_{up})_{max}$ value (Figure 4A). In Figure 4B, the values of $(k_{up})_{max}$ are plotted as a function of $(k_{rel})_{max}$. The data from a number of the same type of experiments are also included in this figure. As seen, there is a linear relationship between the two rates when Ca^{2+} release was induced with moderate concentrations of polylysine (0−10 μM). Thus, in the range of $0 < (k_{rel})_{max} \leq 25$ nmol mg^{-1} s^{-1}, there appears to be a tight correlation between the maximal rates of Ca^{2+} release and re-uptake, again indicating that Ca^{2+} release and re-uptake are coordinated. Upon $(k_{rel})_{max}$ being increased further, however, the coordination between the two is lost.

Then, what is the mechanism by which the kinetic relation between Ca^{2+} release and re-uptake is regulated? The experiment whose results are shown in Figure 5 provides a clue to this question. In this experiment, we monitored the changes in the $[Ca^{2+}]_{lum}$ using the lumenally trapped mag-fura-2AM according to our recently described method (*47*).

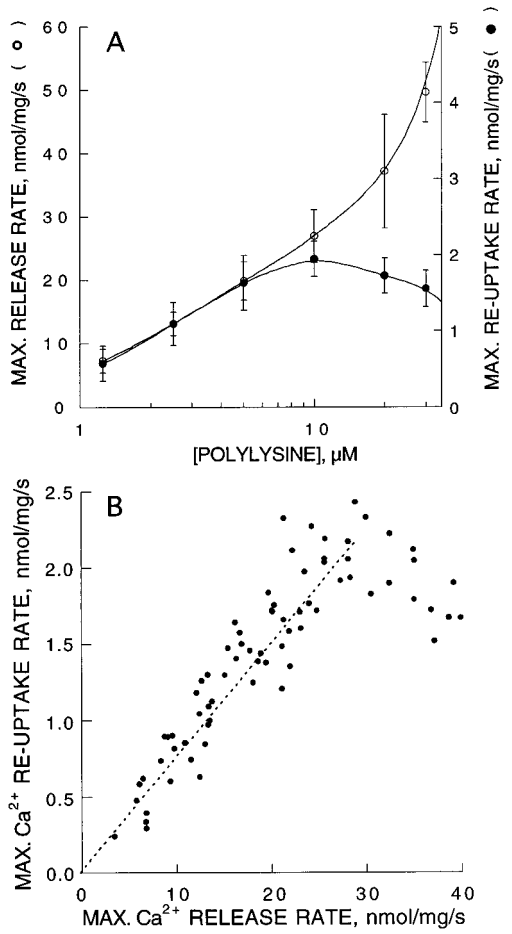

FIGURE 4: (A) Maximal rates of Ca^{2+} release and subsequent re-uptake as a function of the concentration of polylysine. Ca^{2+} release was induced by various concentrations of polylysine as described in Experimental Procedures. Data represent the mean ± standard deviation of 12 experiments. (B) Relationship between the apparent maximal Ca^{2+} release rate and the apparent maximal Ca^{2+} re-uptake rate.

In this assay, the presence of a strong BAPTA/Ca buffer in the reaction solution (see Experimental Procedures) permits specific monitoring of the changes in the $[Ca^{2+}]_{lum}$ while clamping the $[Ca^{2+}]_{ex}$ at a constant level. Furthermore, in this assay the Ca^{2+} that is released from the SR will be chelated instantly by the strong BAPTA buffer, and the released Ca^{2+} from the particular SR vesicle would have no influence on the other SR vesicles. Therefore, the time courses of Ca^{2+} release and re-uptake obtained in this assay must represent coordination phenomena occurring within the same vesicles. Figure 5 depicts the time courses of the change in the mag-fura-2 signal induced by the addition of various concentrations of polylysine at the optimum level of Ca^{2+} loading (110−130 nmol/mg). The general pattern of the dose-dependent changes in the fluorescence signal corresponding to the $[Ca^{2+}]_{lum}$ (a decrease and then an increase in the $[Ca^{2+}]_{lum}$) is quite similar to that of the fluo-3 traces of the $[Ca^{2+}]_{ex}$, except that the direction of the Ca^{2+} concentration change is opposite. As seen, the higher the extent of dose-dependent activation of the initial decrease in the $[Ca^{2+}]_{lum}$, the higher the activation of the subsequent increase in the $[Ca^{2+}]_{lum}$. To facilitate the analysis, the apparent maximal rates of Ca^{2+} release and re-uptake were calculated from the first derivatives of the lumenal Ca^{2+} concentration traces in a manner similar to that used for the fluo-3 ($[Ca^{2+}]_{ex}$) traces.

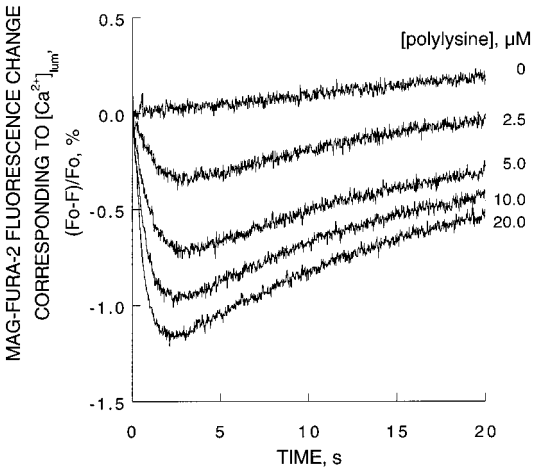

FIGURE 5: Time courses of the changes in the lumenal Ca^{2+} concentration during polylysine-induced SR Ca^{2+} release and the subsequent uptake of the released Ca^{2+}. Ca^{2+} release was induced by various concentrations of polylysine with the triads labeled with mag-fura-2AM in a solution containing a strong BAPTA/calcium buffer to clamp the $[Ca^{2+}]_{ex}$. The changes in the $[Ca^{2+}]_{lum}$ were monitored by following the intensity of fluorescence emission at 510 nm obtained by excitation at 370 nm (for details, see ref *47*). Each trace was obtained by signal averaging a total of 10−15 traces.

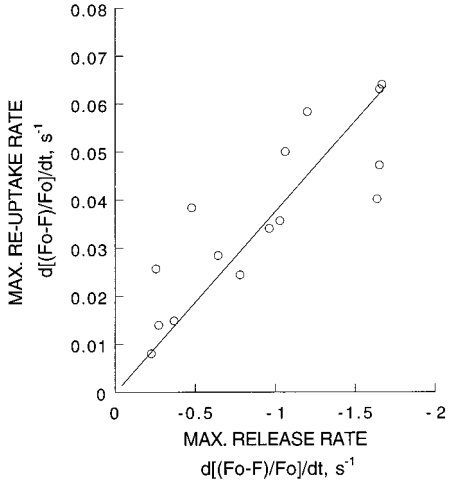

FIGURE 6: Relationship between the maximal rate of Ca^{2+} release (decrease in $[Ca^{2+}]_{lum}$) and that of Ca^{2+} re-uptake (increase in $[Ca^{2+}]_{lum}$). Both rates were calculated from the first derivative of the curves shown in Figure 5 and those of four similar experiments. Data represent the mean ± standard deviation of five experiments.

As shown in Figure 6, there is again a good correlation between the release and re-uptake rates, indicating that the release-induced activation of Ca^{2+} re-uptake is regulated as a function of the rate of decrease of $[Ca^{2+}]_{lum}$. Since in these experiments the $[Ca^{2+}]_{ex}$ was clamped, these results suggest that a rapid reduction of the Ca^{2+} concentration gradient across the SR membrane is at least one of the factors involved in the mechanism of acceleration of the subsequent re-uptake process (see the Discussion).

DISCUSSION

In an intact muscle cell, the twitch contraction is followed by a rapid tension decay because the Ca^{2+} released from the SR is rapidly removed from the cytoplasm by re-accumulation into the SR via the SR Ca^{2+} ATPase and binding to the cytoplasmic Ca^{2+} binding proteins (*1*−*4*). The important unresolved question is whether the two-way Ca^{2+} fluxes

across the SR membrane (viz. Ca^{2+} release and re-uptake of the released Ca^{2+}) are coordinated or are independently operating processes. As described in the introductory section, the isolated SR vesicle preparation which is freed from the cytoplasmic Ca^{2+} binding proteins would permit straightforward analysis of the Ca^{2+} release and re-uptake fluxes and their kinetic relationship. However, virtually no such studies have been carried out chiefly due to the difficulties in producing a relatively fast release−re-uptake transient in the isolated vesicular system. Many studies of Ca^{2+} release in vitro reported in the literature have been carried out even intentionally under the conditions where there was no Ca^{2+} re-uptake.

The aims of this study were first to establish appropriate conditions that permit one to produce an in vitro model of the intracellular Ca^{2+} transient consisting of a biphasic Ca^{2+} release−re-uptake time course, and then to investigate the kinetic relationship between Ca^{2+} release and subsequent re-uptake. One of the important criteria for achieving the first aim was to adjust the level of SR calcium loading to an optimal level. As shown in the loading dependence of release and re-uptake activities (Figure 1A), there was no Ca^{2+} release and hence no re-uptake until the level of calcium loading reached the threshold level (about 100 nmol/mg). In a relatively narrow range (100−150 nmol/mg) of calcium loading above the threshold, both Ca^{2+} release and re-uptake were activated in a coordinated manner. Upon the calcium loading level being increased further, there was a substantial further activation of Ca^{2+} release, but this produced a severe inhibition of re-uptake. In most of the Ca^{2+} release studies with isolated vesicles, there was a general tendency to load the SR with larger amounts of calcium in an attempt to produce a significant amount of Ca^{2+} release. Perhaps this was the reason for the difficulty in producing the Ca^{2+} release−re-uptake transient in the past.

The most important aspect of this paper is the finding that the dose-dependent activation of polylysine-induced Ca^{2+} release produces a consequent activation of re-uptake of the released Ca^{2+} in a well-coordinated manner, if the SR is not overloaded (see above). As shown in the fluo-3 traces of the $[Ca^{2+}]_{ex}$, polylysine induced a rapid increase of the $[Ca^{2+}]_{ex}$ in a dose-dependent fashion. This was followed by a spontaneous decrease in the $[Ca^{2+}]_{ex}$. Mirroring these changes in the $[Ca^{2+}]_{ex}$, the $[Ca^{2+}]_{lum}$ first decreased in a dose-dependent fashion, and then increased. Importantly, the maximal rate of Ca^{2+} release and that of re-uptake showed a close parallelism in both $[Ca^{2+}]_{ex}$ and $[Ca^{2+}]_{lum}$ traces, suggesting that the Ca^{2+} release and the re-uptake are in fact well-coordinated processes. However, it should be noted that the maximal rates of both Ca^{2+} release and re-uptake determined here actually represent the net values of the combined unidirectional efflux and influx rates. Therefore, the Ca^{2+} release−re-uptake coordination described here does not necessarily represent a direct coupling between the two related proteins (the RyR and the SR Ca^{2+} ATPase). Actually, the things that are changing in a coordinated manner are the prevalence of the net efflux in the release phase and that of the net influx in the re-uptake phase.

Polylysine used in this study as the Ca^{2+} release trigger is the RyR-specific ligand. That is, of proteins present in the triad, the RyR is only the protein to which polylysine binds in its activating concentration range (*41*, *42*). This has an important bearing in interpreting these results, since the release-induced activation of the SR Ca^{2+} pump revealed in this study is a result of stimulation of the RyR rather than of stimulation of the Ca^{2+} ATPase. However, one problem with polylysine is that unlike the physiological trigger (viz. transient T-tubule depolarization), polylysine presumably remains bound to the RyR after inducing Ca^{2+} release and the resultant prolonged open state of the Ca^{2+} channel will inhibit the subsequent Ca^{2+} re-uptake. This would explain at least partly why the rate of the re-uptake phase in the in vitro Ca^{2+} transient model is considerably slower than that of the relaxation phase of the intracellular Ca^{2+} transient. The lack of cytoplasmic Ca^{2+} binding proteins in the isolated vesicular system (see above) would also account for the slow Ca^{2+} removal in the in vitro model of the Ca^{2+} transient.

As described above, the transient activation of the Ca^{2+} pump observed after polylysine-induced Ca^{2+} release is controlled by a RyR-mediated mechanism. There appears to be no physical connection between the RyR and the SR Ca^{2+} ATPase. Then, what is mediating the coordination between the two? Since the Ca^{2+} ATPase activity is dependent upon the $[Ca^{2+}]_{ex}$, the increase in the $[Ca^{2+}]_{ex}$ due to Ca^{2+} release is certainly one of the important mechanisms for the acceleration of the re-uptake flux. However, this is not the only mechanism, since the acceleration of Ca^{2+} re-uptake also took place when the $[Ca^{2+}]_{ex}$ was clamped at a constant value, as shown in the lumenal calcium probe experiment. This suggests that a rapid reduction of the Ca^{2+} concentration gradient across the SR membrane, rather than the released Ca^{2+} itself, is a major factor controlling the activation of re-uptake at least in this lumenal probe experiment. In further support of this idea, higher levels of $[Ca^{2+}]_{lum}$ (viz. high Ca^{2+} concentration gradients) produced a severe inhibition of the re-uptake phase (cf. the $[Ca^{2+}]_{lum}$ vs calcium loading plot in Figure 1A). This is because a large Ca^{2+} concentration gradient across the SR membrane suppresses (*35*, *37*) or even reverses (*38*) Ca^{2+} influx. With respect to the mechanism by which the reduction in the Ca^{2+} gradient accelerates the net Ca^{2+} re-uptake flux, several possibilities can be considered. One of these possibilities is that a rapid reduction in the $[Ca^{2+}]_{lum}/[Ca^{2+}]_{ex}$ gradient relieves the Ca^{2+} ATPase from an inhibited state. However, the gradient-dependent acceleration of the uptake flux will also occur without causing any change in the Ca^{2+} pump per se. For instance, the reduction in the Ca^{2+} concentration gradient reduces the passive Ca^{2+} leak flux which is also dependent upon the Ca^{2+} concentration gradient, and in turn increases the rate of net Ca^{2+} re-uptake. Similarly, the decrease in the gradient will also reduce the release flux because of the reduction of the calcium loading level, resulting in the accelerated re-uptake flux. The Ca^{2+} re-uptake rate determined in this study is the net rate representing the combination of the Ca^{2+} uptake flux per se and the Ca^{2+} efflux components of at least two types mentioned above. Therefore, the changes in any of these flux components and their combinations would produce the observed effect. However, whichever of these flux components is making a major impact, the acceleration of the net uptake flux would not occur under calcium overloaded conditions as shown in this study, because both (a) inhibition of Ca^{2+} pumping and (b) an increase in the Ca^{2+} efflux components will take place.

The finding in this study that the release−re-uptake kinetics considerably varies depending on the levels of calcium loading of the SR (or Ca^{2+} concentration gradients across the SR membrane) may have an important physiological significance. As shown here, no Ca^{2+} release takes places until the SR is filled to a threshold level, indicating that in underloaded conditions Ca^{2+} release is not functional. Conversely, in the overloaded conditions Ca^{2+} release becomes highly active but Ca^{2+} pumping is severely inhibited. It is only the intermediate level of calcium loading that permits a tight coordination between Ca^{2+} release and re-uptake. Several suggestions can be made about physiological implications of such loading (or gradient)-dependent regulation, although the details remain to be resolved. First, it is tempting to speculate that the level of calcium loading of the SR in situ would not exceed the intermediate level, thus permitting an efficient re-uptake of the released Ca^{2+} in the muscle cell. Alternatively, the cytoplasmic Ca^{2+} binding proteins, which are present in the muscle fiber but are absent in the isolated vesicles (see above), might come to play a more important role in removing the released Ca^{2+} than SR Ca^{2+} re-uptake in the overloaded conditions. Another important point related to the loading dependence is that there was no Ca^{2+} release below the threshold level of calcium loading. The threshold was as high as 100 nmol/mg under the conditions of this study (namely, stimulation with a partially activating concentration of polylysine in the presence of 0.15 M potassium gluconate that has a relatively high Ca^{2+} buffering capacity), although it became significantly smaller when release was triggered at a maximally activating concentration of polylysine (data not shown). The presence of such a latent phase of calcium loading for Ca^{2+} release, which is already known to exist in caffeine-induced Ca^{2+} release (*51*, *52*), can account for the inactivation of the Ca^{2+} release flux upon partial depletion of the SR calcium, a well-known phenomenon in the muscle fiber (*24*, *53*).

In conclusion, we succeeded in producing, in the isolated SR vesicles, a reasonably fast Ca^{2+} transient consisting of release and re-uptake phases, provided that the SR calcium loading level is set to an optimum range. As suggested from the analysis of the efflux and influx components of the Ca^{2+} transient, re-uptake of the released Ca^{2+} is accelerated by a rapid reduction of the $[Ca^{2+}]_{lum}/[Ca^{2+}]_{ex}$ gradient due to Ca^{2+} release, resulting in a tight coordination between the release and re-uptake kinetics. To further resolve the unidirectional release and re-uptake fluxes during the Ca^{2+} transient is the next important task. Whether a similar mechanism is operating in the regulation of the Ca^{2+} transient in situ remains unresolved, but the data presented here provide a new clue for the understanding of the contraction−relaxation mechanism in muscle.

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