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Rapid Kinetic Analysis of the Calcium-Release Channels of Skeletal Muscle Sarcoplasmic Reticulum: The Effect of Inhibitors

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ABSTRACT: During excitation of skeletal muscle fibers, Ca ions stored in the cisternal compartments of the sarcoplasmic reticulum (SR) are released to the cytosol within milliseconds. In this study, the kinetics of the fast release of Ca were analyzed by means of a newly developed rapid filtration apparatus. Isolated SR vesicles of cisternal origin were preloaded with 1 mM $^{45}CaCl_2$, and Ca efflux was studied (between 20 and 1000 ms) after dilution into media of various composition. The effect of extravesicular Ca on the gating of the Ca-release channels and its susceptibility to the influence of drugs were thoroughly investigated. In the presence of 1 mM $MgCl_2$ and 3 mM ATP, highest rates of Ca release were observed at a free Ca concentration between 1 and 50 μM. In the lower micromolar Ca range, compounds such as neomycin and FLA 365 inhibited the release monophasically and with an IC_{50} of 0.37 and 3.4 μM, respectively. At Ca concentrations between 10 and 50 μM, the inhibitors could not block Ca release effectively. Close analysis of the dose–response curves revealed a biphasic pattern, indicative of the presence of two substates of the Ca-release channel, displaying high- and low-affinity binding sites for the inhibitors. Interestingly, neomycin (or ruthenium red) and FLA 365 at low concentrations acted synergistically and blocked release completely. The results indicate the existence of various open substates of the Ca channels that can be distinguished pharmacologically. Effective blockade of rapid Ca release requires inhibition of all substates coexisting under a given condition.

Rapid Ca uptake and release from the sarcoplasmic reticulum (SR) play a key role in the regulation of the kinetics of relaxation and contraction of the myofilaments in striated muscle (Endo, 1977). The basic mechanism of Ca translocation into the SR compartment, which is effected by a Ca-pumping ATPase, is well characterized [for a review see, e.g., Inesi (1985) or Ikemoto (1982)]. On the other hand, the coupling of the excitation wave spreading along the plasma membrane and into the transverse tubular system with massive release of Ca ions from the adjacent terminal cisternae of the SR is not yet fully understood [for a review see Martonosi (1984)]. In-depth characterization of the phenomenon has long been impeded by its morphological and organizational complexity as well as the high rates of Ca release, for the investigation of which special rapid kinetic methods are required. Only very recently have consistent advances in our knowledge of the molecular organization of the Ca-release process been achieved, thanks to the availability in radiolabeled form of ryanodine, a plant alkaloid capable of binding with nanomolar affinity to the cisternal compartments of the SR (Fleischer et al., 1985; Pessah et al., 1987) and of interfering with the operation of the Ca channels (Jones et al., 1979; Alderson & Feher, 1987). Solubilized ryanodine receptors have been purified by immunoaffinity chromatography (Imagawa et al., 1987), by centrifugation through a linear sucrose gradient (Lai et al., 1988), or by differential affinity chromatography (Inui et al., 1987) procedures. The identity of receptor and Ca-release channels has been proved by conductance-recording studies of the purified ryanodine receptor reconstituted in planar lipid bilayers (Imagawa et al., 1987; Lai et al., 1988). These investigations shed light on the electrical properties of the single Ca channel and provided useful information on the Ca-, Mg-, and nucleotide-dependent regulation of its activity. The influence of the latter compounds and of other agents, such as calmodulin and caffeine, on the activity of the Ca channels was also successfully investigated by using native SR vesicles with the help of rapid kinetic procedures (Nagasaki & Kasai, 1983; Meissner et al., 1984; Kim et al., 1983; Moutin & Dupont, 1988). In the present study, we used a recently developed rapid-filtration apparatus (Dupont & Moutin, 1987) to study the effect of some inhibitors of the Ca-release process in skeletal muscle SR. The method can resolve Ca-release kinetics from passively loaded SR cisternae in the millisecond time range. Among the compounds investigated, ruthenium red (RR) and the antibiotic neomycin have previously been described as inhibitors of Ca release from skeletal SR (Palade, 1987). Another inhibitor tested in this study was the recently discovered compound FLA

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365 ([2,6-dichloro-4-(dimethylamino)phenyl]isopropylamine), the properties of which have been characterized by using cardiac SR preparations (Chiesi et al., 1988). The results obtained show the existence of two classes of compounds (i.e., RR and neomycin on the one hand and FLA 365 on the other) displaying complementary inhibitory properties. A strong synergistic effect can be obtained by combining compounds belonging to these different classes. These observations serve to demonstrate a heterogeneity in the properties of the open Ca-release channels of skeletal SR cisternae.

Materials and Methods

Materials. Ruthenium red (RR) and neomycin were purchased from Fluka Chemie AG (Buchs, Switzerland); other drugs were synthesized in the Chemistry Department of Ciba-Geigy (Basel). All other reagents were of the best quality commercially available.

Isolation of Heavy SR Membranes. White muscles from the hind legs of rabbits were used for the preparation of skeletal SR membranes enriched in cisternal compartments. A heavy subfraction was obtained by differential centrifugation at 10000*g* for 30 min according to the method described by Meissner and Henderson (1987). The final pellet was resuspended at a protein concentration of about 20–30 mg/mL in a medium containing 100 mM KCl, 5 mM Tris–maleate, pH 6.8, and 300 mM sucrose. After rapid freezing in liquid nitrogen, the preparation was stored at −80 °C until use. About 70–80% of the vesicles thus obtained derived from the cisternal compartments and contained Ca-release channels. The protein concentration was determined by the procedure described by Lowry et al. (1951) using bovine serum albumin as a standard.

Ca-Uptake Measurements. Energy-dependent Ca uptake into heavy SR membranes was determined by the Millipore filtration technique (Martonosi & Feretos, 1964). The uptake medium contained 100 mM KCl, 1 mM $MgCl_2$, 20 mM HEPES, pH 7, 5 mM NaN_3, 60 mM sucrose, 3 mM potassium oxalate, 0.5 mM EGTA, and trace amounts of $^{45}CaCl_2$. The ATP concentration in the medium was either 0.5 or 3 mM. The amount of total cold $CaCl_2$ needed to yield the desired free Ca concentration was calculated by a computer program using the dissociation constants published by Fabiato and Fabiato (1979). When required, drugs were added to the uptake medium. Ca-uptake reactions were started by the addition of SR membranes (20–40 μg of protein/mL) at room temperature and terminated at various time intervals (every 30 s up to 5 min) by filtration (0.22-μm filters). Filters were immediately washed with ice-cold 100 mM KCl, 1 mM $MgCl_2$, 5 mM NaN_3, 20 mM HEPES, pH 7, and 0.5 mM EGTA (stop solution). Radioactivity associated with the filters was measured in a liquid scintillation counter. The initial rates of the uptake reactions were determined.

Ca-Release Measurements by a Rapid-Filtration Technique. Heavy SR vesicles were washed in a medium composed of 100 mM KCl, 20 mM HEPES, pH 7, and 60 mM sucrose (medium A). The membranes were resuspended at a protein concentration of about 3 mg/mL in the same medium supplemented with 1 mM $^{45}CaCl_2$. Passive ^{45}Ca loading was carried out at room temperature for 1 h. The preparation was then kept on ice and utilized in the release reaction within 2 h. For the measurement of rapid Ca release from the loaded vesicles, a recently developed fast-filtration system (Dupont & Moutin, 1987; distributed by Bio-Logic, Meylan, France) was used. The instrument can resolve filtration times down to about 10–20 ms, making it possible to follow satisfactorily the rapid efflux kinetics of Ca from SR as shown recently by Moutin and Dupont (1988). Loaded SR vesicles were first distributed evenly on a Millipore filter (0.8 μm) under conditions allowing removal of ^{45}Ca from the outside medium while preventing any loss of ^{45}Ca from the vesicles. For this purpose, 10 μL of loaded membranes was rapidly diluted in "nonrelease" medium (medium A supplemented with 5 mM $MgCl_2$ and 1 mM EGTA) and then placed on a prewetted filter under vacuum. The filter was then washed twice with 1 mL of nonrelease medium. Immediately thereafter (within 10–20 s), release solutions were forced through the filters for the desired periods of time (ranging from 20 to 5000 ms). The release solutions contained medium A supplemented with various effectors such as Ca and ATP, as mentioned under Results. The radioactivity remaining on the filter was then directly counted by using a scintillation cocktail (Optifluor, Packard Instruments). Inhibitors were added at the desired concentration to all solutions used, i.e., to the concentrated membrane suspension containing 1 mM $^{45}CaCl_2$, to the nonrelease medium, and to the release medium. The use of 0.8-μm filters was found to be optimal since it allowed sufficient loading of biological material while permitting high filtration rates. The loss of vesicles through the filters was constant and limited (12–17%) and was comparable to that obtained with 0.22- or 0.44 μm filters.

Data Analysis. Initial Ca-release rates were determined from semilogarithmic plots of the data. The effect of inhibitors was quantified by determining the percent inhibition of the initial release rate measured with a release medium of identical composition but without inhibitors present.

Fitting of data points was obtained by using the curve-fitting routine of RS/1 (BBN Software Products Corp.), which adjusts the parameters to minimize the sum of the relative squared errors. A collection of models used for curve-fitting routines (considering from one to three different populations of binding sites) was utilized. The model giving fittings with the highest significance level was chosen to analyze the data.

Results

Figure 1 shows the stimulation of the rate of Ca uptake into SR vesicles enriched in cisternal elements by three blockers of Ca release under various experimental conditions. When Ca uptake was measured in the presence of 0.5 mM ATP and 1 mM $MgCl_2$, the stimulatory effect was reduced to a minimum (panel A). Only when the free Ca concentration in the uptake medium was above 10^{-5} M could some stimulation by the Ca-release blockers be detected. On the other hand, the presence of 3 mM ATP in the medium permitted a clear stimulation of the Ca-uptake reaction by all three blockers tested (see Figure 1B). The stimulation was Ca dependent and gave a typical bell-shaped curve when any one of the inhibitors was present. The stimulation curve reached a maximum at about 2–3 μM Ca and then rapidly decreased to very low values at Ca concentrations above 10 μM.

A series of Ca-release experiments was performed to investigate directly the effect of the medium composition (and, in particular, of extravesicular Ca concentration) on the opening of the Ca-release channels and the susceptibility of that effect to the action of inhibitors. The experimental conditions and the solutions used were identical with those employed for the uptake reactions presented in Figure 1. The measurements were carried out by using a newly developed fast-filtration system (Dupont & Moutin, 1987). The procedure, which is described under Materials and Methods, allows a clear and reproducible resolution of the rapid Ca-release kinetics (millisecond time scale). In the absence of free Ca ions and nucleotides and in the presence of 1 mM

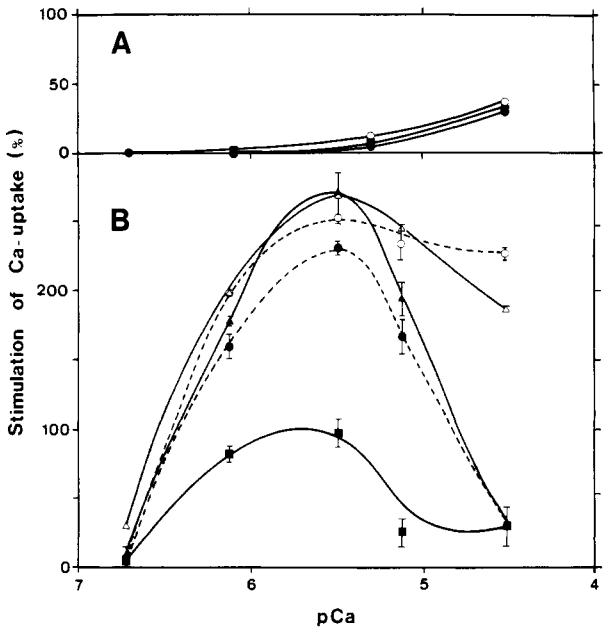

FIGURE 1: Ca concentration dependence of the stimulating effect of Ca-release blockers on Ca uptake by heavy SR from skeletal muscle. ^{45}Ca uptake was measured by the Millipore filtration technique as described under Materials and Methods. The incubation medium contained 100 mM KCl, 1 mM $MgCl_2$, 20 mM HEPES, pH 7, 5 mM NaN_3, 60 mM sucrose, 3 mM potassium oxalate, 0.5 mM EGTA, different concentrations of $CaCl_2$ to give the desired free Ca concentration, and either 0.5 mM ATP (A) or 3 mM ATP (B). The following concentrations of inhibitors were added: (■) 50 μM FLA 365; (●) 50 μM neomycin; (▲) 0.5 μM RR; (○) 25 μM neomycin and 25 μM FLA 365; (△) 0.25 μM RR and 25 μM FLA 365. In a typical experiment, the uptake rates measured under control conditions (3 mM ATP, no inhibitor) were 230, 450, 710, 1050, 720, and 160 nmol/(mg·min) at 0.2, 0.75, 3.2, 7.5, 30, and 100 μM free Ca concentration, respectively.

$MgCl_2$, Ca efflux from the passively loaded vesicles was extremely slow (see Figure 2A). Addition of 3 mM ATP to the medium induced a stimulation of Ca release. Both a direct effect of the nucleotide on the channels and the complexation of the inhibitory Mg ions in the release medium might have contributed to the stimulation observed. The rate of rapid Ca release was also dependent on the concentration of free Ca ions in the dilution medium (Figure 2A). Figure 3 shows the distinctly bell-shaped course of the Ca-dependency curve, as measured in the presence of 3 mM ATP and 1 mM Mg. At micromolar Ca concentrations the release channels are optimally open and the Ca efflux rate is maximal. On the other hand, at millimolar Ca concentrations the channels have a tendency to close again. These observations are basically consistent with previous reports on the regulation of the channels by ions (Kim et al., 1983; Meissner et al., 1986). In the light of the results obtained, the variability of the stimulatory effect of Ca-release blockers on Ca-uptake activity is easier to interpret. Low ATP and high free Mg concentrations in the uptake buffer (Figure 1A) favor the closed configuration of the Ca-release channels. Therefore, little or no further stimulation by the release blockers can be observed. Micromolar Ca combined with high adenosine nucleotide and low free Mg concentrations optimally activates the Ca-release channels, thus allowing the release blockers to evoke a strong stimulatory effect on Ca uptake. Indeed, the stimulation of Ca uptake shown in Figure 1B follows very closely the Ca-induced opening of the channels (Figure 3).

The capacity of neomycin and FLA 365 to interfere directly with the channels when they are optimally open (i.e., in the presence of 1 mM Mg, 3 mM ATP, and 2 μM Ca) was further investigated. A typical experiment, performed in the presence of increasing concentrations of FLA 365 in the release medium, is shown in Figure 2B. The dose dependency of inhibition of Ca release by the two compounds is summarized in Figure 4A. Both neomycin and FLA induced nearly full inhibition of Ca release at concentrations of about 2 and 50 μM, respectively. The data points presented in Figure 4A could be best fitted by monophasic curves, of which parameters are shown in Table I. The calculated IC_{50} values were 0.37 μM for neomycin and 3.4 μM for FLA 365.

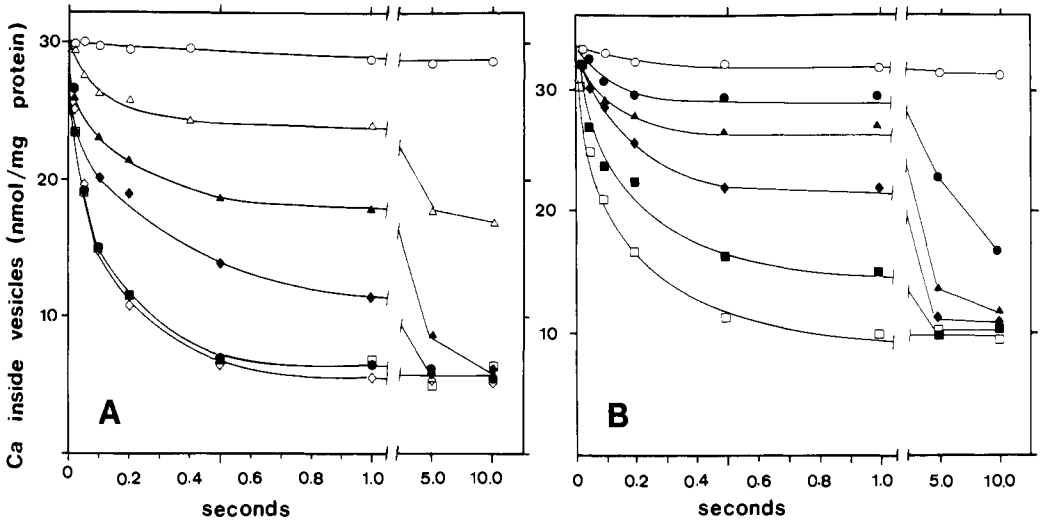

FIGURE 2: (A) Rapid Ca release induced by different concentrations of Ca. Passive ^{45}Ca loading was carried out by incubation of SR vesicles for 1 h at room temperature in a medium containing 100 mM KCl, 20 mM HEPES, pH 7, 60 mM sucrose, and 1 mM $^{45}CaCl_2$. Ca-release media contained 100 mM KCl, 20 mM HEPES, pH 7, 60 mM sucrose, 1 mM $MgCl_2$, 3 mM Na_2ATP, 0.5 mM EGTA, and different $CaCl_2$ concentrations to obtain the following free Ca: (△) 10^{-9} M; (▲) 2×10^{-7} M; (♦) 5×10^{-7} M; (●) 3×10^{-6} M; (◇) 10^{-5} M; (□) 5×10^{-5} M. The control trace (○) was obtained by using a release medium lacking ATP and $CaCl_2$. Rapid ^{45}Ca release was measured by the fast-filtration technique as described under Materials and Methods. (B) Inhibition of rapid Ca release by FLA 365. ^{45}Ca-release experiments were carried out basically as described in (A). Ca-release media contained 100 mM KCl, 20 mM HEPES, pH 7, 60 mM sucrose, 1 mM $MgCl_2$, 3 mM ATP, 0.5 mM EGTA, 0.430 $CaCl_2$ (to obtain a free Ca concentration of 2 μM), and the following concentrations of FLA 365: (□) none; (■) 5 μM; (♦) 10 μM; (▲) 30 μM; (●) 100 μM. The control trace (○) was obtained by using a release medium lacking $CaCl_2$ and ATP. The slight difference in the total amount of rapidly released Ca obtained in (A) and (B) is due to the fact that different SR preparations were used.

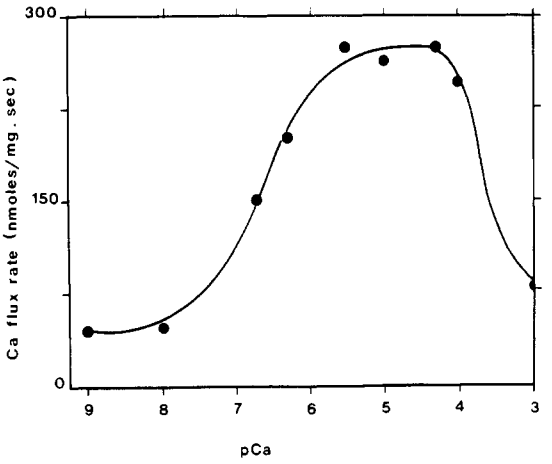

FIGURE 3: Dependence of Ca-release rate on the external free Ca concentration. ^{45}Ca loading of heavy SR vesicles and ^{45}Ca release in the presence of various free Ca concentrations in the dilution buffer were performed as described in the legend to Figure 2A. The initial rate of rapid Ca efflux was used for the plot.

Table I: Parameters of the Curves Presented in Figure 4[a]

	final value	sig level
Ca_{free} = 2 μM		
curve 1[b]		
K_i (μM)	0.37 ± 0.024	0.0001
n_{Hill}	2.33 ± 0.34	0.0010
curve 2[b]		
K_i (μM)	3.39 ± 0.24	0.0010
n_{Hill}	1.43 ± 0.1	0.0001
Ca_{free} = 30 μM		
curve 1 (n_{Hill} = 4)[c]		
B_{max1} (%)	45.8 ± 4.5	0.001
K_{i1} (μM)	0.24 ± 0.002	0.001
K_{i2} (μM)	4.49 ± 1.5	0.008
curve 2 (n_{Hill} = 4)[c]		
B_{max1} (%)	34.39 ± 6.0	0.001
K_{i1} (μM)	4.5 ± 1.0	0.001
K_{i2} (μM)	28.0 ± 2.4	0.0001
curve 3[b]		
K_i (μM)	0.20 ± 0.01	0.001
n_{Hill}	3.3 ± 0.59	0.05

[a] The inhibitory constants (K_i) and Hill coefficients (n_{Hill}) were obtained by best-fitting procedures as described under Materials and Methods. [b] The equation used for fitting was $f(X) = 100/(1 + (K_i/X)^n)$. [c] The curves were fitted by using a model that takes into account two populations of binding sites. Good fittings were obtained giving a Hill coefficient of 4. The equation used was $f(X) = [B_{max1}/(1 + (K_{i1}/X)^4)] + [(100 - B_{max1})/(1 + (K_{i2}/X)^4)]$.

Concentrations (50 μM) of FLA 365 and neomycin, which have been shown to inhibit Ca release completely at 2 μM Ca, did not stimulate Ca uptake when tested at slightly higher free Ca concentrations (e.g., 30 μM Ca in Figure 1B). The absence of any stimulatory effect was also observed with 0.5 μM RR (see Figure 1B). This observation was quite unexpected, since the Ca-release rate at 30 μM Ca was still maximal and comparable to that measured at 2 μM Ca (see Figure 3). Therefore, the lack of stimulation was not due to a reduced Ca efflux rate at 30 μM free Ca (a phenomenon that occurs at much higher Ca concentrations, as shown in Figure 3) but rather to the incapacity of the drugs to effectively close the channels. This was demonstrated by the experiments summarized in Figure 5, in which the effects of the compounds on the rapid Ca release at 2 and 30 μM Ca are compared; 50 μM neomycin or FLA 365 induced a nearly complete inhibition of the rapid Ca release when measured in the presence of 2 μM Ca_{free}, while at higher Ca concentrations (i.e., 30 μM) the inhibition was reduced to less than 80%. Ca-release

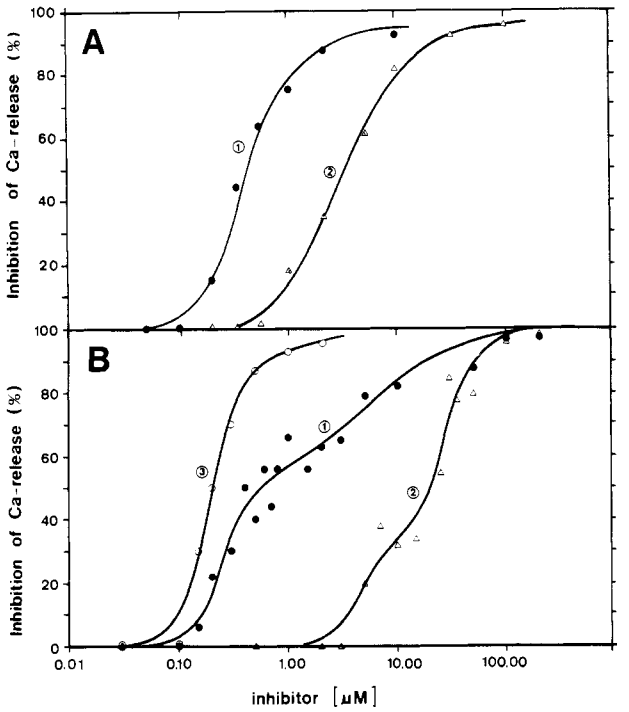

FIGURE 4: (A) Dose dependence of the inhibition of rapid Ca release by neomycin and FLA 365 at a free Ca concentration of 2 μM. The ^{45}Ca-release medium contained 100 mM KCl, 20 mM HEPES, pH 7, 60 mM sucrose, 1 mM $MgCl_2$, 3 mM Na_2-ATP, 0.5 mM EGTA, and 0.430 mM $CaCl_2$ to give a 2 μM free Ca concentration. ^{45}Ca release was measured as described in the legend to Figure 2B in the presence in the release medium of various concentrations of either FLA 365 (curve 2) or neomycin (curve 1). The rate of Ca release was calculated as described under Materials and Methods. The parameters of the curves fitting the experimental data are given in Table I. (B) Dose dependence of the inhibition of rapid Ca release by neomycin and FLA 365 at a free Ca concentration of 30 μM. ^{45}Ca-release media were composed of 100 mM KCl, 20 mM HEPES, pH 7, 60 mM sucrose, 1 mM $MgCl_2$, 3 mM Na_2-ATP, 0.5 mM EGTA, 0.719 mM $CaCl_2$ (to obtain 30 μM free Ca), and increasing concentrations of FLA 365 (curve 2) or neomycin (curve 1). Ca efflux rates were determined as described under Materials and Methods. Curve 3 was obtained in the presence of 10 μM FLA 365 and increasing concentrations of neomycin. The Ca-release rate in the presence of 10 μM FLA 365 was reduced to about 70% of the control value. This reduced release rate was considered as 100% for the calculation of the data points presented in curve 3. The parameters of the curves fitting the data points are shown in Table I.

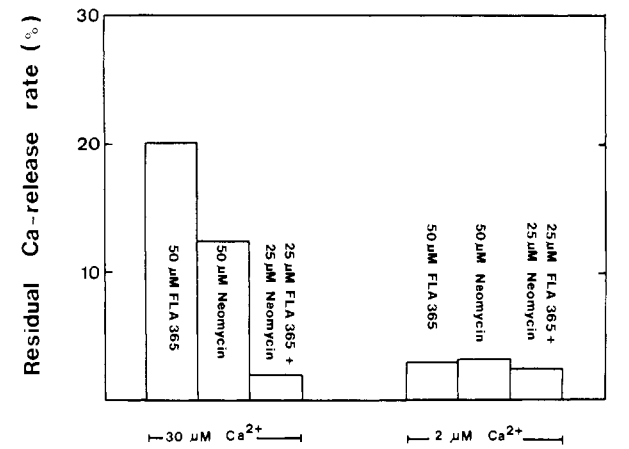

FIGURE 5: Additive action of neomycin and FLA 365 on the rapid Ca release. Release of Ca ions from passively loaded heavy SR vesicles was measured in the presence of either 0.430 or 0.719 mM $CaCl_2$ to obtain either 2 or 30 μM free Ca in the release medium, respectively. When required, inhibitors were added at the concentrations indicated. Rates of Ca release were obtained from efflux curves such as those presented in Figure 2.

channels must be completely blocked to induce a measurable stimulation of the uptake reaction (Alderson & Feher, 1987). The release rate is orders of magnitude higher than the uptake rate, so that a single open channel/vesicle may be sufficient to completely inhibit Ca uptake. Therefore, a residual release rate of 13–20%, as was observed in the presence of 50 μM neomycin or FLA 365 at a free Ca concentration of 30 μM, was sufficient to prevent the manifestation of any stimulatory effect on the Ca uptake. The rate of the Ca-pumping ATPase becomes relevant only when the inhibition of Ca release is near 100%. At 30 μM free Ca this condition was achieved by a combination of 25 μM neomycin and FLA 365, as shown in Figure 5, thus explaining the strong synergistic stimulatory effect of the two compounds on Ca uptake (Figure 1B). The same synergistic action was noted when 0.25 μM RR and 25 μM FLA 365 were combined (Figure 1B).

The dose dependency of the inhibitory effects of neomycin and FLA 365 on Ca-release rate was also thoroughly investigated in the presence of 30 μM free Ca in the release medium (Figure 4B). In contrast to the response observed at 2 μM free Ca (see Figure 4A), the data points obtained with both compounds could not be satisfactorily fitted by monophasic functions. On the other hand, optimal curve fittings were obtained by using biphasic functions with relatively high slope values ($n_{Hill} = 4$), as shown in Table I. The data are indicative of the existence of two different populations of Ca channels displaying low- and high-affinity binding sites for the drugs. The calculated K_i values corresponding to the high-affinity effects of neomycin (0.24 μM) and FLA 365 (4.5 μM) were very similar to those obtained with a free Ca concentration of 2 μM in the release medium (see Table I). When the release experiments were performed in the presence of a low concentration of FLA 365 (i.e., 10 μM) saturating only its high-affinity sites, the remaining rapid release of Ca could be blocked completely and monophasically by low concentrations of neomycin ($K_i = 0.2$ μM) (see Figure 4B, curve 3, and Table I).

Discussion

The Ca-release channels are located exclusively on that portion of the cisternal compartments of the SR that is adjacent to the transverse tubular membrane. This specialized region is termed junctional SR and is characterized by the absence of Ca-pumping ATPase molecules, which otherwise are very abundant on the SR membrane, including the cisternal compartments (Zubrzycka-Gaarn et al., 1984). During homogenization of muscle cells, a population of membrane vesicles is formed that contains both Ca-ATPase and Ca-release channel units. The capacity of that portion of vesicles to accumulate Ca ions by the action of the Ca-pumping ATPase evidently depends on the conductivity state of the release channels. When the channels are open, Ca uptake is arrested, whereas under conditions inducing the closure of the release channels it is optimal. In the past, Ca-release blockers have been characterized mainly by their capacity to stimulate Ca uptake, since direct measurement of their effect on the rapid Ca release is difficult and requires special equipment.

In the present investigation, three different compounds known to inhibit Ca release were analyzed: ruthenium red, neomycin, and FLA 365, a newly discovered blocker of Ca release from cardiac SR cisternae (Chiesi et al., 1988). The former two molecules share some common properties, such as a large number of positive charges at neutral pH, concentrated over a relatively small area. On the basis of this similarity, their mode of action on the Ca-release channels is supposed to be the same (Palade, 1987). Throughout our study we also found that the characteristics of RR and neomycin are basically identical. The experiments shown in Figure 1 were designed to demonstrate the high variability of the effects of Ca-release blockers on the stimulation of Ca uptake, depending on the experimental conditions employed. Ca release is strictly under the control of ions commonly present in the uptake medium. Millimolar Mg and Ca ions block Ca release, while ATP and micromolar Ca activate it (Kim et al., 1983; Nagasaki & Kasai, 1983; Meissner et al., 1986). The effect of these ions on rapid Ca release was found to be basically consistent with previous papers also when analyzed under the particular conditions used in this study (see Figures 2A and 3). The results clearly show that the extent of stimulation of Ca uptake by the release blockers correlates closely with the rate of Ca release. An interesting exception to this rule, however, was noticed at free Ca concentrations between 10^{-5} and 10^{-4} M. Under such conditions, Ca channels were found to be maximally open, but the blockers were not effective in stimulating the uptake reaction (Figure 1B). A closer inspection of the phenomenon revealed that the capacity of the blockers to inhibit Ca release is Ca dependent. At 2 μM free Ca, neomycin completely blocked Ca release at low concentrations by acting on a single set of binding sites (Figure 4A and Table I). Similarly, Ca release was blocked monophasically by FLA 365 with high affinity. At slightly higher Ca concentrations (e.g., 30 μM), the dose dependency of Ca-release inhibition by both FLA 365 and neomycin showed a more complex pattern, which could be satisfactorily fitted by assuming the existence of two binding sites for each ligand (Figure 4B and Table I). Under such conditions two populations of channels could apparently be distinguished. FLA 365 or neomycin blocked only a portion of the Ca-release channels with high affinity. A low-affinity site must also be saturated to obtain complete inhibition of Ca release (see curves 1 and 2 in Figure 4B).

A further interesting corollary to this study concerns the particular complementarity of action of the two types of drug represented by FLA 365, on the one hand, and by neomycin or RR on the other. Figure 1B shows that they can act synergisticaly in stimulating Ca uptake when tested, for instance, at a free Ca concentration of 30 μM. Synergism could be accounted for if the population of Ca channels possessing low-affinity binding sites for one type of drug interacted with the other compound with high affinity and vice versa. The experiments summarized in curve 3 of Figure 4B were designed to prove this possibility. A portion of the release channels were blocked by low concentrations of FLA 365 (10 μM). The remaining Ca-release activity could be completely blocked monophasically by low concentrations of neomycin. Interestingly, the high Hill coefficients (3–4) obtained for most of the curve fittings (see Table I) are consistent with the tetrameric structure of functional Ca channels recently proposed by Lai et al. (1988).

In conclusion, Ca ions induce the opening of the Ca-release channels of skeletal SR cisternae in a complex way. The schematic representation in Figure 6 depicts the minimal number of Ca-induced conformational states that the Ca-release channels must assume to account for the observations made so far. The effect of Mg and nucleotides is not considered for simplicity. According to the model, an increase of the free Ca concentration to the micromolar range induces opening of the channel. In this open conformation the channel presents high-affinity (H) binding sites for the two categories of drugs represented by neomycin and FLA 365. When the free Ca concentration is increased above 10^{-5} M, the channel

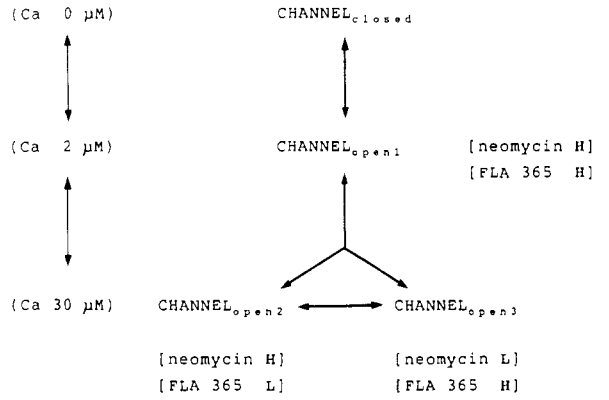

FIGURE 6: Model on the Ca-dependent interconversion of the Ca-release channel into various open substates. H and L stand for high-affinity and low-affinity binding of the drugs to the specific channel conformational states.

can assume one of two possible open conformations, displaying different affinities for the two types of drug. A complete block of Ca-release activity can be achieved by each of the two drugs if administered at sufficiently high concentration. Alternatively, low concentrations of each drug can act synergistically to close both conformational states of the channel. In a preliminary study, we observed that drugs such as RR prevented Ca release from cardiac cisternae only in the presence of FLA 365 (Chiesi et al., 1988). The experiments were carried out in the presence of 0.05 mM EGTA and 0.05 mM $CaCl_2$ in the efflux medium (Ca_{free} = 4.3 μM). Under the same conditions, RR alone blocked completely Ca release from skeletal muscle preparations. The possibility was postulated, therefore, that cardiac SR might contain a hitherto unknown channel (FLA 365 sensitive) in addition to the RR-sensitive channel also present in skeletal muscle. By means of fast kinetic analysis of Ca release under a variety of experimental conditions, we have now obtained evidence for a similar synergistic action of the two drugs in skeletal muscle preparations also. The present analysis is more consistent with the existence of various open substates of the same channel modulated by the ionic composition of the medium rather than with the hypothesis of different channel entities. Various types of channel are known to assume several different conformational states and sometimes show substates of the open conformation. For instance, open substates were identified for the chloride channel (Miller, 1982) by the detection of various levels of single-channel conductance. Activity recordings of the purified Ca-release channel from skeletal muscle, after reconstitution into planar lipid bilayers, have also shown the existence of various subconductance states (Lai et al., 1988). A different Ca sensitivity of the substates could account for the different behavior of the channels present in cardiac and skeletal muscle cisternal compartments observed previously (Chiesi et al., 1988).

Registry No. FLA 365, 67759-58-4; RR, 25125-46-6; Ca, 7440-70-2; neomycin, 1404-04-2.

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