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INHIBITION OF RAPID Ca-RELEASE FROM ISOLATED SKELETAL AND CARDIAC SARCOPLASMIC RETICULUM (SR) MEMBRANES

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Rapid Ca-release from the cisternal compartments isolated from skeletal and cardiac muscle SR was characterized by the use of inhibitors. Ruthenium red (RR) completely blocked (IC_{50} = 90 nM) the Ca-channels of skeletal SR. Its effect on the rapid Ca-release from cardiac SR was marginal but became optimal (IC_{50} = 200 nM) in the presence of FLA 365 ([2,6-dichloro-4-dimethyl-aminophenyl] isopropylamine) which by itself had no measurable effect. The antibiotic neomycin mimicked the properties of RR. The strong synergistic effect of RR or neomycin and FLA 365 indicates that either cardiac cisternae contain two distinct isoforms of Ca-release channel, or that different drugs are needed to effectively block the same channel.

The opening of Ca-release channels localized on the SR membrane is a basic event in the development of excitation-contraction coupling in striated muscles. The process is completely confined to the cisternal compartments of the SR network (1). Although it is not yet possible to define its physiological mechanism at the molecular level, a wealth of important information on the topic has recently become available. The use of rapid spectrophotometric methods (2,3) and single-channel recordings obtained after incorporation of SR membranes into planar lipid bilayers (4,5) have shown that Ca-release is a Ca-dependent process, stimulated by millimolar adenine nucleotides and inhibited by Mg. It is carried out by high-conductance channels showing very similar, but distinct properties in skeletal and cardiac cisternae. Information pertinent to the structure of the Ca-release channel

ABBREVIATIONS
EGTA: ethyleneglycol-bis-(beta-aminoethylether)N,N,N′,N′-tetraacetic acid, HEPES: N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid, RR: ruthenium red, FLA 365: [2,6-dichloro-4-dimethylaminophenyl] isopropylamine.

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was obtained by exploiting the capacity of radioactively labeled ryanodine to bind with high affinity to receptors situated on the cisternal compartments. The plant alkaloid ryanodine was originally known for its various pharmacological effects on both skeletal and cardiac muscles (6), which were indicative of a modification of the properties of Ca-release from the SR. Very recently, the ryanodine receptors from skeletal (8,9,10) and cardiac (11) muscles have been isolated to purity and shown to contain Ca-channel activity with characteristics very similar to those recorded using native SR membranes.

In this report, the action of various drugs on the process of rapid Ca-release in cardiac and skeletal muscle has been investigated and compared. The results demonstrate the existence of two classes of compounds showing a very pronounced synergistic action on the properties of the Ca-release channels localized on the cardiac cisternal compartments.

MATERIALS AND METHODS

Materials: ^{45}Ca was purchased from New England Nuclear, ruthenium red and neomycin from Fluka (Buchs), ryanodine from Penick Corp. (Lindhurst, NJ). Other drugs were obtained from the Chemistry departement of Ciba-Geigy, Basel. All other reagents were of the best quality commercially available.
Biological material: Rabbit white skeletal muscles were obtained from the hind legs. SR subfractions were obtained basically according to Meissner (3) by means of differential centrifugation. A heavy preparation sedimenting at 10000g x 30 min was routinely obtained with satisfactory properties: 70-80% of the Ca associated with these membranes could be rapidly released after activation of the Ca-release channels (see Figure 4).
Canine cardiac SR was isolated according to (12) as modified in (13). The crude heavy fraction sedimenting at 23000g x 15 min was subfractionated on linear dextran/sucrose gradients prepared as described in (12). The material banding with the highest density contained approximately 40% of cisternal elements, as demostrated by the passive efflux experiments shown in Figure 4.
Ca-uptake: SR membranes (50 ug/ml for the cardiac and 10 ug/ml for the skeletal preparation) were incubated at 37 °C in a medium composed of 100 mM KCl, 1 mM $MgCl_2$, 50 mM HEPES, pH 7, 3 mM K-oxalate, 200 uM antipyrylazo III, and 10 µM $CaCl_2$. When required, the uptake medium was supplemented with various drugs. Active uptake was started by the addition of 0.5 mM Na_2-ATP. The measurements were performed in a dual-wavelength spectrophotometer (Shimadzu UV-3000). Wavelength settings; 720 and 790 nm. The initial rate of Ca-uptake was considered for the calculations.
Ca-release measurements: Heavy SR preparations (5-10 mg/ml) were passively loaded for 2 hours at room temperature with 1 mM $^{45}CaCl_2$ in the presence of 100 mM KCl, 20 mM HEPES, pH 7. Release of Ca ions from the store was started by diluting 10 ul of loaded vesicles into 1 ml of 100 mM KCl, 20 mM HEPES, pH 7, supplemented with 1 mM $MgCl_2$ and 1 mM EGTA (control release), or with 0.05 mM EGTA and 0.05 mM $CaCl_2$ (Ca-induced Ca-release). When required, drugs were added to the release medium. Passive release was stopped by rapid filtration (Millipore 0.22 um). Filters were rinsed with ice-cold solution containing EGTA and $MgCl_2$. Radioactivity associated with the vesicles was counted after dissolving the filters in Opti-fluor (Packard Instruments).

RESULTS

In the present study, blockers of the process of rapid Ca-release from SR membranes were investigated. For this purpose, the capacity of compounds to stimulate energy-dependent Ca-uptake into heavy subfractions of cardiac and skeletal muscle SR was determined. Such measurements can be rapidly performed spectrophotometrically using the metallochromic Ca-indicator antipyrylazo III, thus permitting many compounds with a putative Ca-release blocking action to be screened. The membranes used were enriched in the cisternal compartments of the SR, which contain both Ca-release channels and Ca-ATPase. Uptake was measured in the presence of millimolar ATP and micromolar Ca in the uptake medium, i.e. under conditions in which the Ca-release channels are mainly in the open state (3). Figure 1 shows the effect of ryanodine and ruthenium red (RR) on the Ca-uptake kinetics of skeletal and cardiac SR preparations. In skeletal SR vesicles, the rate of Ca-uptake was stimulated by high concentrations of the alkaloid ryanodine to the maximal level observed (i.e. about 300% of the control value). This effect of ryanodine was due to its well-known ability to bind specifically to the SR Ca-channels and to inhibit Ca-release at concentrations higher than 10 μM (7). RR, another compound known for its strong inhibitory effect on Ca-release (4,8), also induced a maximal stimulation of Ca-uptake into skeletal muscle SR at a concentration of about 200 nM (EC_{50} = 90 nM). In cardiac SR preparations, ryanodine produced a similar stimulation of Ca-uptake (about 260% of the control value). On the other hand, up to 2 μM RR induced only a marginal stimulation (110-130% of control, depending on the preparation) when tested under the same experimental conditions. Higher concentrations of RR could not be investigated, as they also inhibit the Ca-pumping ATPase activity. The screening of various synthetic compounds for effects on Ca-release from cardiac SR led to the discovery of [2,6-dichloro-4-dimethylaminophenyl]isopropylamine (FLA 365), a drug whose curious properties are shown in Figure 2. FLA 365, when tested alone, did not induce any stimulation of Ca-uptake. Interestingly, however, submicromolar concentrations of RR were found to induce full stimulation of Ca-uptake when acting in combination with FLA 365.

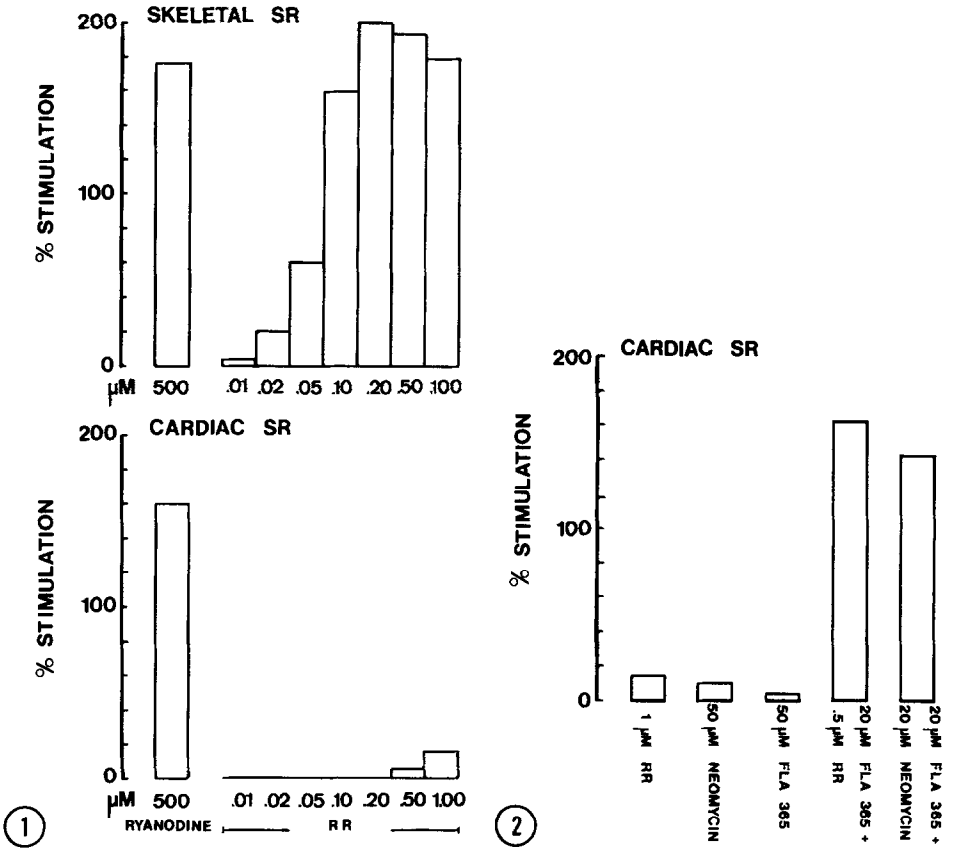


Figure 1: Stimulation of Ca-uptake by ryanodine and RR in skeletal and cardiac heavy SR preparations. Ca-uptake was measured spectrophotometrically as described in the Methods section in the presence of 100 mM KCl, 50 mM HEPES, pH 7, 1 mM $MgCl_2$, 3 mM K-oxalate, 30 μM added $CaCl_2$, 200 μM antipyrylazo III, 0.5 mM Na_2-ATP, and the indicated concentrations of ryanodine or RR.

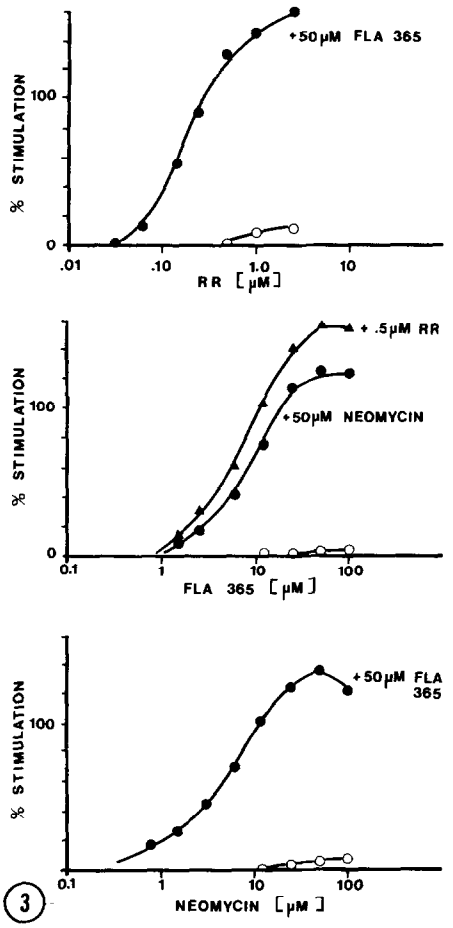
Figure 2: Concerted action of drugs on the Ca-uptake rates in heavy cardiac SR. Ca-uptake experiments were carried out as described in the legend to Figure 1. The various compounds were added, alone or in combination, to the uptake medium in the concentrations indicated in the figure.

Neomycin is another substance of interest which was found to mimic the effects of RR on the cardiac SR preparation. Submicromolar concentrations of this antibiotic have recently been shown to block Ca-release from skeletal muscle SR induced by various releasing agents (14). Like RR, neomycin failed to stimulate Ca-uptake into the cardiac SR preparation consistently, even at concentrations as high as 50 μM. On the other hand, in the presence of FLA 365 Ca-uptake was optimally stimulated by this drug. As expected, RR and neomycin also remained ineffective when tested together (data not shown).

The concentration-dependence of the stimulatory action of the three different compounds added individually or in combination is presented in

Figure 3. The upper panel of the Figure shows that RR, inactive up to a concentration of 2 μM, maximally stimulated Ca-uptake when combined with 50 uM FLA 365 with an EC_{50} of about 200 nM. Similarly, neomycin, which had no effect even at a concentration of 100 μM, induced an optimal stimulation of the uptake reaction when combined with 50 μM FLA 365 with an EC_{50} of 5-6 μM (Figure 3, lower panel). The concentration of FLA 365 needed to potentiate the effect of RR and neomycin was also determined. Figure 3, middle panel, shows that in the presence of either 0.5 μM RR or 50 μM neomycin, similar amounts of FLA 365 (EC_{50} = 8 μM) were required to stimulate Ca-uptake. Apparently the situation in cardiac cisternae is quite different from that seen in skeletal cisternae. In the former membranes the capability of RR or neomycin to stimulate Ca-uptake was dependent on the presence of FLA 365.

Ca-ATPase activity was not stimulated but rather suppressed by the compounds (not shown), indicating that they acted by inhibiting the passive permeability to Ca. This conclusion borne out by direct measurements of Ca-induced Ca-release. SR cisternal fractions were preloaded with 1 mM ^{45}Ca and release was started upon dilution into an isoosmotic medium of various composition. The time-course of Ca-release is shown in Figure 4, the shortest time-point considered being 10 sec (i.e. the limit of manual sampling and quenching). Ca-efflux from skeletal SR vesicles was found to be very slow when the dilution medium contained 1 mM Mg and no Ca (10^{-9}M). On the other hand, 80% of the Ca associated with the vesicles was rapidly lost in the presence of 10^{-5}M Ca. This is a condition known to open the Ca-release channels of the terminal cisternae (3). As expected, the rapid Ca-release component was completely inhibited by 5 μM RR. The passive permeability of the cardiac SR preparation to Ca ions was relatively high in the absence of Ca and the presence of Mg in the dilution medium also. Nevertheless, activation of the Ca-release channels by Ca clearly induced a further rapid loss of about 40% of the total Ca, indicating that about 40% of the vesicles in this preparation were actually of cisternal origin. Neither 5 μM RR nor 30 μM FLA 365 were able to block rapid Ca-release from cardiac cisternae. Their blocking action,



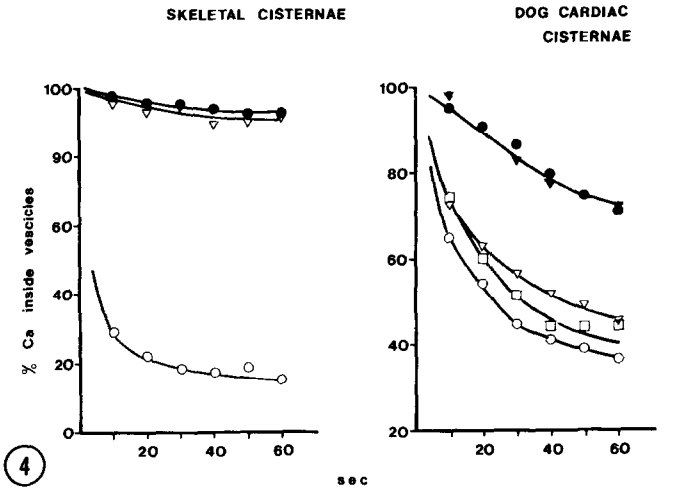


Figure 3: Concentration dependence of the stimulatory effect of drugs on the Ca-uptake of heavy cardiac SR. Ca-uptake was measured as described in the Legend to Figure 1. Upper panel: dose response curve of the stimulation of Ca-uptake by RR alone (○) or in combination with 50 μM FLA 365. Middle panel: dose response curve of FLA 365 alone (○), in the presence of 0.5 μM RR (▲) or in combination with 50 μM neomycin (●). Lower panel: dose response curve of neomycin alone (○) or in the presence of 50 μM FLA 365.

Figure 4: Inhibition of Ca-release from skeletal and cardiac SR preparations by different blocking agents. Heavy SR vesicles were passively preloaded with 1 mM $^{45}CaCl_2$ and then diluted in release medium containing: 1 mM Mg and no Ca (●); 10^{-5}M Ca (○); 10^{-5}M Ca and 10 μM RR (▽); 10^{-5}M Ca and 50 μM FLA 365 (□)); or 10^{-5}M Ca, 5 μM RR and 30 μM FLA 365 (▼). Passive efflux was investigated with the Millipore filtration technique as described in the Methods section.

however, became fully evident when the compounds were added together to the Ca-release medium.

DISCUSSION

The cisternal compartments of skeletal and cardiac muscle SR contain similar Ca-release channels. In both tissues, Ca-release is mediated by

high-conductance channels consisting of 350-400 kDa polypeptides, activated by uM Ca-ions and mM ATP and inhibited by mM Mg. The two channel types, however, are not identical, as shown by their different affinities for ryanodine (15), the small differences in Mr (11) and slope conductance when reconstituted into planar lipid bilayers (4,5), and the lower sensitivity of the cardiac Ca-channel to inhibition by RR (3,5). The inability of RR to inhibit effectively Ca-release from cardiac preparations was confirmed in this study. Interestingly, however, we found that RR becomes fully effective in the presence of the drug FLA 365. The combined observations of the large synergistic effect of the two drugs and of the remarkable similarity of the IC_{50} values for RR in cardiac and skeletal muscle SR (200nM and 90 nM, respectively), strongly suggest that cardiac SR cisternae may contain two distinct Ca-release channels. One channel is sensitive to RR-blockade and presumably corresponds to that found in skeletal muscle SR: the other is inhibited selectively by the newly discovered compound FLA 365. Both channel types would be activated by uM Ca and inhibited by high ryanodine or Mg concentrations. Neomycin and RR very likely belong to the same category of drugs: indeed, both share the property of being small molecules with an unusually large number of positive charges at neutral pH. The mechanism of action of the two drugs on the SR is likely to be the same, as demonstrated by the lack of synergism. The data are also open to other interesting interpretations which take into account only one type of Ca-release channel. RR (or neomycin) and FLA 365 could bind independently to the same channel on different sites and inhibit Ca-release only partially (i.e. the channel assumes a state of lower conductance). Only the concerted action of the two categories of drugs could then induce complete blockade. Alternatively, a particular channel conformation sensitive to the low concentrations of RR might occur only after binding of FLA 365.

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