The Effect of Calcium Ionophores on Fragmented Sarcoplasmic Reticulum

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ABSTRACT X-537 A and A 23187, two antibiotics which form lipophilic complexes with divalent cations, function as ionophores in vesicular fragments of sarcoplasmic reticulum (SR). Addition of either ionophore to SR preloaded with calcium in the presence of adenosine triphosphate (ATP), causes rapid release of calcium. Furthermore, net calcium accumulation by SR is prevented, when the ionophores are added to the reaction mixture before ATP. On the contrary, ATP-independent calcium binding to SR is not inhibited. This effect is specific for the two antibiotics and could not be reproduced, either by inactive derivatives, or by other known ionophores. Neither ionophore produces alterations of the electron microscopic appearance of SR membranes or inhibition of the calcium-dependent ATPase. In fact, the burst of ATP hydrolysis obtained on addition of calcium, is prolonged in the presence of the ionophores. Lanthanum inhibits ATP-independent calcium binding to SR, ATP-dependent calcium accumulation and calcium-dependent ATPase. However, addition of lanthanum to SR preloaded in the presence of ATP, does not cause calcium release. The reported experiments indicated that: (a) ATP-dependent calcium accumulation by SR results in primary formation of calcium ion gradients across the membrane. (b) Most of the accumulated calcium is not available for displacement by lanthanum on the outer surface of the membrane. (c) Calcium ionophores induce rapid equilibration of the gradients, by facilitating cation diffusion across the membrane.

INTRODUCTION

Vesicular fragments of sarcoplasmic reticulum membranes (SR) display a specific ability to accumulate Ca++ in the presence of ATP (1–3). Ca++ accumulation by SR proceeds very rapidly (4), and steady-state levels of 140–160 nmoles Ca++/mg of SR protein are reached within 60 sec at 25°C. Presently, it is not clear to what extent calcium is accumulated as a free ion against a concentration gradient, and to what extent it is simply bound to the membrane (5, 6). We have attempted to clarify this point with the aid of ionophores that produce equilibration of Ca++ gradient across the membrane,
and by the use of La\textsuperscript{3+} which competitively inhibits Ca\textsuperscript{2+} binding to the membrane.

During the last 10 years a number of antibiotics have been found to increase the permeability of natural and artificial membranes to monovalent cations, due to selective formation of cation antibiotic complexes (7–9). Such complexes display a high lipid solubility and function as carriers of ions ("ionophores") (10) across the lipid barrier of the membrane, facilitating diffusion and rapid equilibration of ionic gradients.

Based on the charge of the antibiotic and on the nature of the complex with monovalent cations, the known ionophores have been classified in at least two categories (8, 11):

(a) electrically neutral molecules of cyclic configuration, able to form charged 1:1 complexes with monovalent cations through dipolar interaction with oxygen atoms. The group includes valinomycin, which facilitates diffusion of K\textsuperscript{+}, Rb\textsuperscript{+}, and Cs\textsuperscript{+} across lipid barriers (7, 8, 10, 11).

(b) molecules of partially linear configuration, containing negatively charged groups involved in complex formation with cations. An example of this group is nigericin, a carboxylic antibiotic which catalyzes an electroneutral exchange of cations across bulk lipid phases of artificial and natural membranes (7–10).

A particularly interesting member of the second group is the antibiotic X-537 A, which is able to complex not only monovalent, but also divalent cations (12–14). The structure of the X-537 A complex with barium has been studied by X-ray diffraction and found to consist of two molecules of antibiotics, bent over one molecule of cation and one of water (14).

The ability of X-537 A to form a lipophilic complex with Ca\textsuperscript{2+} renders this antibiotic an ideal carrier for Ca\textsuperscript{2+} through lipid phases. In preliminary experiments we found that addition of X-537 A to suspension of SR is followed by immediate release of the Ca\textsuperscript{2+} accumulated by the vesicles in the presence of ATP (15). With the present work we have extended our studies to clarify the mechanism of the effects of X-537 A and other Ca\textsuperscript{2+} ionophores on SR vesicles.

In some of the experiments reported here, we have also studied the effect of La\textsuperscript{3+} on the activity of SR, attempting to interfere with binding of Ca\textsuperscript{2+} to the membrane. In fact La\textsuperscript{3+} competitively inhibits Ca\textsuperscript{2+} binding in various systems (16–18), including SR (19) Furthermore La\textsuperscript{3+} inhibits Ca\textsuperscript{2+} transport in mitochondria, probably competing for binding to the carrier (20, 21).

**MATERIALS AND METHODS**

**Methods**

SR was obtained by homogenization and differential centrifugation of rabbit white muscle, as previously described (22). Protein concentration was estimated by the biuret reaction, standardized with Kjeldahl nitrogen determination.
Ca++ uptake by SR was measured either by isotopic or spectrophotometric methods. In the former case, SR vesicles were incubated with 44Ca-CaCl₂ in appropriate reaction mixtures. At different time intervals after the addition of ATP, SR was removed by rapid filtration (23); the residual isotope in the filtrate was then determined by scintillation counting.

Alternatively, Ca++ uptake by SR was followed by monitoring the changes in absorbance undergone by murexide, a metallachromic indicator sensitive to the Ca++ concentration in the medium (24, 25). The measurements were performed with a filter dual wavelength (540-507 nm) double beam spectrophotometer (26) in a 1 mm light-path cuvette and continuously recorded. The reaction mixtures contained 20 mM tris(hydroxymethyl)aminomethane (Tris)-maleate (pH 6.8), 50 mM KCl, 10 mM MgCl₂, 100 mM total CaCl₂, 100 μM murexide, and 200-350 μg SR protein/ml.

ATPase activity was assayed by following the liberation of inorganic phosphate (27). The reaction was carried out in a constantly stirred reaction mixture thermostated at 25°C. 0.3 ml of 0.1 mM ATP was added at 0 time to 9.7 ml of the reaction mixture (20 mM Tris maleate [pH 6.8], 50 mM KCl, 10 mM MgCl₂, 100 μM ethylene glycol bis(β-aminoethyl ether) N,N',N''-tetraacetic acid (EGTA), 270 μg SR protein/ml). At suitable times after the addition of ATP a 0.5 ml sample was withdrawn and the reaction was interrupted by equal volumes of 10% trichloroacetic acid.

Structural observations were made with a Philips 300 electron microscope (Philips Electronic Instruments, Mount Vernon, N.Y.), after negatively staining the SR vesicles with 1% phosphotungstic acid or 1% uranyl acetate as described previously (28).

Materials

Crystalline Na-ATP containing less than 8 ppm calcium contaminant was purchased from Sigma Chemical Co., St. Louis, Mo. Murexide (ammonium purpurate) was obtained from K and K Labs, Inc., Plainview, N. Y., valinomycin from Calbiochem, Los Angeles, Calif., LaCl₃ from Alfa Inorganics, Inc., Beverly, Mass.

X-537 A and derivatives were generous gifts of Dr. J. Berger of Hoffman-La Roche, Nutley, N. J. A 23187 was kindly supplied by Dr. R. Hamill of Eli Lilly and Company, Indianapolis, Ind. These ionophores (free acids) were dissolved in ethanol and the volume per each experiment did not exceed 3 μl/ml reaction mixture.

RESULTS

Effect of X-537 A on Ca++ Release by Sarcoplasmic Reticulum

A calibration of the murexide method for Ca++ determination is shown in Fig. 1 A. In the absence of SR, the addition of 12.5 μM CaCl₂ to the medium used in studies of Ca++ uptake produces a decrease in light absorbance at 540 nm, due to the formation of a Ca-murexide complex which absorbs less light than murexide alone. Subsequent additions of CaCl₂ in equal amounts produce identical effects, demonstrating a linear relationship between absorbance changes and Ca++ concentration in the medium.

ATP, in the absence of SR, does not cause any absorbance changes, due
FIGURE 1. (A) Light absorbancy changes undergone by murexide on addition of CaCl₂, ATP, and X-537 A in a medium devoid of SR. (B). ATP-dependent Ca²⁺ accumulation by SR and subsequent release induced by X-537 A. The reaction mixtures (2 ml) contained 20 mm Tris-maleate (pH 6.8), 50 mM KCl, 10 mM MgCl₂, and 100 μM murexide. In (B) 280 μg SR protein/ml were also present. The amount of CaCl₂ present and subsequent additions are reported in the figure. Temperature 24°C.

to the presence of MgCl₂ (10 mM) which prevents formation of Ca-ATP complex. Similarly, in the absence of SR, hardly any change is observed on addition of the ionophore X-537 A, indicating that the concentrations of Ca-ionophore complex formed in the control reaction medium are at the limits of the murexide method sensitivity.

In the presence of SR, addition of ATP is followed by an abrupt increase in light absorbance (Fig. 1 B), corresponding to Ca²⁺ uptake by SR. Steady-state levels of 150 nmoles Ca²⁺ accumulation/mg SR protein are reached within 2–3 min. At this point, addition of 15 μM X-537 A prompts rapid release of the entire amount of calcium accumulated by SR.

Fig. 2 A shows the effect of various concentrations of X-537 A on release of calcium accumulated by SR. By varying the amounts of ionophore added, different steady-state levels of Ca²⁺ accumulation were obtained and, upon addition of 20 μM X-537 A, all the calcium was released within few seconds. Moreover if X-537 A is added before ATP, net accumulation of calcium is reduced or totally prevented (Fig. 2 B).

The effect of X-537 A on Ca²⁺ accumulation by SR was also checked by measuring the distribution of radioactive calcium (Fig. 3). The results are similar to those obtained with the murexide method, indicating that lower
steady-state levels are obtained with increasing concentrations of the ionophore. Furthermore, isotopic measurements allow detection of a small amount of ATP-independent calcium binding to SR (12 nmoles/mg protein in the presence of 10 mM MgCl₂). Contrary to the ATP-dependent calcium accumulation, ATP-independent calcium binding to the membrane is not affected by X-537 A (Fig. 3).

In suitable experimental conditions, it can be shown that the calcium-releasing effect of X-537 A is temperature dependent. In the experiment reported in Fig. 4, ATP-dependent calcium accumulation by SR vesicles was obtained at 23°C, in the presence of a standard medium. After reaching steady-state levels of Ca++ uptake, at constant time the reaction mixtures were brought to the experimental temperatures with the aid of a perfused cell cavity. X-537 A (9 μM) was then added and the subsequent Ca++ released monitored by double beam spectrophotometry and recorded on a storage oscilloscope. When the logarithm of the initial rate of Ca++ release from SR was plotted against the reciprocal of the absolute temperature (29), a slope formed by two straight lines meeting at an angle was obtained. The initial rates of calcium release were increased by temperature increments between 8° and 25°C, but not above 25°C. It should be pointed out that such rates are obtained from measurements of net release, and the derived Arrhenius
FIGURE 3. Ca\(^{++}\) uptake by SR determined by isotope distribution and the effect of X-537 A. The reaction mixture (2 ml) contained 20 mM Tris-maleate (pH 6.8), 50 mM KCl, 10 mM MgCl\(_2\), 100 \(\mu\)M CaCl\(_2\), and 280 \(\mu\)g/ml SR protein. The reaction was initiated by the addition of 1 mM ATP. The control experiments without ATP either in the presence \(\square\) or in the absence \(\bigcirc\) of 10 \(\mu\)M X-537 A are also reported. Temperature 24°C.

FIGURE 4. Arrhenius plot of the Ca\(^{++}\) release by SR vesicles induced by X-537 A. Ca\(^{++}\) uptake by SR vesicles was induced by the addition of 2 mM ATP in a reaction mixture identical to that of Fig. 2 A. 2 min after the addition of ATP the reaction mixture was quickly (less than 30 sec) brought to the temperature indicated in the figure and then 9 \(\mu\)M X-537 A was added. The subsequent release was monitored by murexide technique in a storage oscilloscope and the logarithm of the initial rate of Ca\(^{++}\) release from SR was plotted versus the reciprocal values of the absolute temperature.

plot merely reflects the effect of energy of activation, rather than a real value. Nevertheless, an interesting feature of the plot in Fig. 4, is a sharp break at 25°C.

**Effect of X-537 A on the Structure and ATPase Activity of SR**

The ionophore X-537 A, in concentrations causing calcium release from loaded vesicles or interfering with ATP-dependent calcium accumulation did not produce obvious changes in the structure of SR vesicles and did not inhibit the membrane ATPase.

Several electron microscopic examinations of SR vesicles treated with X-537 A before negative staining, failed to show any evidence of damage or interruption of the membrane structure (Fig. 5 A). The fine granular detail of the outer membrane layer, previously described (28, 30) is prevented in treated vesicles (Fig. 5 B) just as well as in control preparations.

Fig. 6 shows the ATPase activity obtained on addition of CaCl\(_2\) to suspen-
FIGURE 5. Electron micrographs of SR vesicles, treated with 20 μm X-537 A before negative staining. (A), 1% phosphotungstic acid, ×220,000 (B), 1% uranyl acetate, ×336,000.
**FIGURE 6.** ATPase activity of SR vesicles in the presence of X-537 A. ATPase activity was assayed by following the liberation of inorganic phosphate as described in Methods. 0.3 ml of 0.1 M ATP were added to 9.7 ml of reaction mixture (see Methods) at 0 time. 0.1 ml of 0.01 M CaCl₂ were added at the time indicated by the arrow to reach the final concentration of 100 μM. At the times indicated 0.5 ml of reaction mixture was quickly withdrawn and the reaction was stopped by addition of equal volume of 10% trichloroacetic acid. Temperature 25°C.

In control experiments, a burst of ATP hydrolysis is obtained simultaneous to calcium uptake; the activity subsides when steady-state levels of accumulated calcium are reached. In the presence of the ionophore X-537 A, ATPase activity is not inhibited. On the contrary, the burst of calcium-dependent ATP hydrolysis is prolonged, and it is maintained at high constant rates in the presence of X-537 A concentration (20 μM) that totally prevent retention of accumulated calcium.

**Effect of Other Ionophores on Ca²⁺ Movement**

In order to test the specificity of X-537 A in inducing Ca²⁺ released from SR vesicles, the effect of X-537 A was compared with that of other ionophores with well defined properties. Fig. 7 shows that other ionophores, which are known to increase the permeability of natural and artificial membranes to K⁺, Na⁺, and H⁺, have no effect on Ca²⁺ accumulation by the SR vesicles. Nigericin, valinomycin, FCCP (carbonyl cyanide-p-trifluoromethoxy phenylhydrazone), and gramicidin are ineffective compared to the calcium release induced by X-537 A. Of particular interest is the lack of effect of nigericin, which has a structure very close to that of X-537 A.

This specificity is stressed by the experiment reported in Fig. 8, where the effect of X-537 A on Ca²⁺ release by SR vesicles (A) is compared with that of X-537 A-3Ac. This derivative does not form complexes with divalent
FIGURE 7. Comparison of the effect of nigericin and valinomycin (A), valinomycin and FCCP (B), gramicidin (C), and X-537 A (D) on Ca++ release by SR vesicles. The reaction mixture was identical to that of Fig. 2 A, except for SR which was 370 µg protein/ml. The reaction was started by the addition of 2.5 mM ATP and the concentrations of ionophore added are reported in the figure.

FIGURE 8. The effect of X-537 A, X-537 A-3Ac, and A 23187 on Ca++ release by SR vesicles. Experimental conditions were that of Fig. 2A except for the SR, which was 420 µg protein/ml. The titration of the reaction mixture in the absence of SR is reported in (D). The reaction started by the addition of 400 µM ATP and successive additions of ionophores are reported on the figure.

cations\(^1\) and has little effect on Ca++ release by SR even if added at a concentration 4 times higher than that of the nonacetated form, (Fig. 8 B). By contrast A 23187, an ionophore of different structure which has been recently used to permeabilize mitochondrial membranes to Ca++ and Mg++ (31), is able to produce Ca++ release from SR at very low concentrations (Fig. 8 C).

\(^1\) Berger, J. 1971. Personal communication.
Effect of La\(^{3+}\)

A series of experiments was carried out to study if La\(^{3+}\) inhibition of calcium binding would interfere with Ca\(^{2+}\) accumulation and ATPase activity of SR. These experiments were carried out with techniques involving isotopic measurements, since La\(^{3+}\) interferes with the murexide method for Ca\(^{2+}\) determination. Some results are reported here, which contribute to understanding the mechanism of accumulation by SR, the partition of the accumulated calcium, and the effect of ionophores.

When SR is incubated in the presence of LaCl\(_3\), ATP-independent Ca\(^{2+}\) binding to the membrane is reduced, as reported by Chevallier and Butow (19). On addition of ATP, we found that the ATP-dependent Ca\(^{2+}\) uptake by SR is also inhibited by LaCl\(_3\), at concentrations varying between 0.1 and 1 mM, (Fig. 9). The inhibition is prevalently on the initial rates of calcium uptake, rather than on the steady-state levels of calcium accumulation. It should be pointed out that throughout all the experiments the ATP was added in excess, in order to minimize inactivation of ATP due to the formation of a lanthanum-ATP complex.

Contrary to what is observed with the ionophore X-537 A, LaCl\(_3\) produces inhibition of calcium-dependent ATPase (Fig. 10) in addition to its effect on calcium uptake. Furthermore, if LaCl\(_3\) is added to SR vesicles after the occurrence of ATP-dependent calcium accumulation, no sizeable release of calcium is obtainable (Fig. 11), but simply inhibition of further uptake.

**Figure 9.** ATP dependent Ca\(^{2+}\) accumulation by SR vesicles and the effect of lanthanum. The reaction mixture was similar to that of Fig. 2 A except for the pH which was 6.6 and the SR which was 310 \(\mu\)g protein/ml. To obtain time resolution of the initial phases of \(4^{\text{Ca}}\)\(^{2+}\) uptake, the temperature of the reaction mixture was 15\(^\circ\)C. The reaction was initiated by 2 mM ATP and Ca\(^{2+}\) uptake by SR vesicles was measured radiochemically as described in Methods.
FIGURE 10. ATPase activity of SR vesicles and the effect of lanthanum. The reaction mixture (2 ml) contained 50 mM KCl, 20 mM Tris-malate pH 6.5, 10 mM MgCl₂, 100 µM CaCl₂, 310 µg SR protein/ml. The reaction was initiated with 2 mM ATP and the temperature was 15°C. The dashed curve is that of a reaction mixture containing 100 µM EGTA instead of CaCl₂.

FIGURE 11. The effect of lanthanum on the Ca²⁺ accumulated by SR vesicles. Experimental conditions as in Fig. 9. The reaction was initiated with 2 mM ATP and 0.5 mM LaCl₃ was added at the point indicated on the figure.

DISCUSSION

X-537 A is an antibiotic with well defined structure obtained in crystalline form from an identified Streptomyces (12). The molecular structure is that of a monocarboxylic acid containing a number of cyclic ether moieties and an aromatic chromophore group. X-537 A is known to form lipophilic complexes with monovalent cations, thereby rendering artificial and natural membranes permeable to Na⁺, K⁺, Rb⁺, Cs⁺, and H⁺, by a mechanism similar to that of nigericin and monensin (8, 11). Recent observations by Johnson et al. (14) showed that X-537 A forms lipophilic complexes also with divalent cations. This property renders X-537 A an ionophore capable of facilitating diffusion of divalent cations across lipid barriers, as in mitochondria (32), plasma,² and SR (15) membranes.

A 23187 is another antibiotic obtained from Streptomyces chartrensensis which also forms lipophilic complexes with Ca²⁺ and Mg²⁺, facilitating their diffusion through lipid phases of model systems and mitochondrial membranes (31).

The release of accumulated calcium from SR vesicles, induced by X-537 A

² Scarpa, A. 1972. Ionophore mediated Ca²⁺ equilibration in mitochondria and intact cells. Manuscript in preparation.
and A 23187 in our experiments, may be explained with facilitation of calcium diffusion from the inner vesicular space to the outer medium and equilibration of a transmembrane gradient created by the calcium pump. With increasing concentrations of ionophore, lower steady states of accumulated calcium are obtained, corresponding to levels where the rates of passive diffusion are equal to the rates of active transport. Maximal ionophore effects produce complete equilibration of the calcium gradients.

The inhibition of net calcium accumulation by SR, observed when the ionophores are added to the reaction mixture before ATP, may also be explained by an ionophore-induced calcium "leak," which prevents retention of the actively transported calcium. In fact, the process of active transport, as indicated by calcium-activated ATPase, is not inhibited by the ionophores. On the contrary, the burst of ATP hydrolysis is prolonged, as it occurs in experimental conditions where calcium filling of the vesicles is prevented by induction of calcium "leak" (28, 33). Furthermore the continuous draining off of Ca++ from SR vesicles induced by the ionophore prevents the inhibitory effect of accumulated Ca++ on ATPase activity (34).

It can be ruled out that in the experiments with X-537 A and A 23187, the induction of calcium "leak" in SR is due to gross structural effects, such as interruption of membrane continuity. In fact, the ionophore-treated vesicles were always found to be of normal appearance on electron microscopic examination.

It can also be ruled out that significant calcium binding by the ionophores may occur in the outside medium so as to interfere with ATPase activation and calcium uptake by SR. Based on the reported affinity constant for the Ca-X-537 A and Mg-X-537 A complexes (32), it can be calculated that in the presence of 10 mM Mg++, 0.1 mM Ca++ and 20 μM X-537 A, the concentration of Ca-X-537 A complex should be lower than 5 μM. This estimate was in fact confirmed by measurements made by the murexide method (Fig. 1 A). The remaining free calcium ion (more than 95 μM) is certainly sufficient to saturate the active transport system of SR, whose half maximal activation occurs at Ca++ concentrations of the order of 10\(^{-7}\) M (35).

On the other hand, the amounts and the rates of Ca-ionophore complex formation are evidently sufficient to facilitate diffusion of calcium across the membrane. When the intravesicular concentration of calcium ion is increased as a consequence of active transport, passive diffusion of the Ca-ionophore complex occurs at rapid rates, and results in equilibration of the calcium gradient.

The observed temperature dependence of the rates of ionophore-assisted calcium release from the SR vesicles may be due to activation energy necessary for motion of the Ca-ionophore complex within the lipid layer of the membrane. In fact, the abrupt change in slope obtained in the Arrhenius
plot (Fig. 4), is also observed, at the same temperature, in plots expressing the temperature dependence of motion parameters of lipophilic spin labels in SR (36) and reflecting order-disorder transitions in the lipid component of the membrane.

Our experiments with ionophores indicate that calcium accumulation in SR results primarily in the establishment of calcium ion gradients across the membrane. In fact, ionophore-induced facilitation of passive diffusion produces nearly total release of the calcium accumulated by SR in the presence of ATP. It should be pointed out that, even in the presence of the ionophore, a small amount of calcium must be still bound to specific sites of the transport system, as indicated by ATPase activation.

This conclusion is also consistent with the experiments on the effect of lanthanum. It was found that concentrations of lanthanum, known to competitively inhibit high affinity calcium binding to SR (19), reduce the rates of ATP-dependent calcium accumulation (Fig. 9). It is apparent that the lanthanum effect is an inhibition of the calcium transport system proper, since calcium-dependent ATPase is also inhibited (Fig. 10).

Contrary to the effect obtained with the ionophore, addition of lanthanum to SR vesicles after the occurrence of ATP-dependent calcium accumulation does not result in calcium release (Fig. 11). This indicates that the accumulated calcium is not available for displacement by La³⁺, and therefore is not bound on the outer surface of the SR vesicles. As previously suggested (4), it cannot be excluded that some calcium binding may occur on the inner surface of the vesicles, secondary to the establishment of high intravesicular concentration of calcium ion.

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