IONOPHORE MEDIATED EQUILIBRATION OF CALCIUM ION GRADIENTS IN FRAGMENTED SARCOPLASMIC RETICULUM

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1. Introduction

Vesicular fragments of sarcoplasmic reticulum (SR), isolated from skeletal muscle, rapidly accumulate Ca^{2+} [1–3] in the presence of adenosine triphosphate (ATP). Owing to its high specific activity, SR constitutes an outstanding system for studies on ion interactions with biological membranes [4, 5]. The kinetic behavior of Ca^{2+} uptake and the simultaneous occurrence of ATP hydrolysis suggest that Ca^{2+} is transported into the inner space of the membrane vesicles [4]. The resulting concentration gradient is made energetically possible through hydrolysis of ATP [6]. It was also shown that SR has a significant cation binding capacity, in the absence of ATP [7, 8]. Although these binding sites have an affinity for Ca^{2+} lower than that displayed by SR [9, 10] in the presence of ATP, it was proposed that part or all of the ATP-dependent Ca^{2+} accumulation may be due to simple binding of Ca^{2+} rather than resulting from ionic gradients [7, 8].

This problem may be clarified by the use of ionophores. We have now found that low concentrations of X-537 A, a Ca^{2+} ionophorous antibiotic, when added to SR suspensions after the occurrence of Ca^{2+} accumulation, produce a total release of the previously accumulated Ca^{2+}. Our results are consistent with the equilibration of the Ca ionic gradients, mediated by the ionophore X-537 A.

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2. Materials and methods

SR was prepared by differential centrifugation of homogenized skeletal muscle, obtained from rabbit hind legs [11]. Ca^{2+} movements were followed by measuring the light absorption changes undergone by murexide, a metallochromic indicator of calcium ion concentration [12]. The optical changes of the indicator were monitored by a dual wavelength (540–507 nm) spectrophotometer [13] as described previously [14].

Reaction mixtures contained 20 mM Tris-maleate pH 6.8, 50 mM KCl, 10 mM $MgCl_2$, 120 μM total Ca^{2+}, 100 μM murexide and indicated amounts of sarcoplasmic reticulum. The temperature of the reaction medium was 24°. ATPase activity was measured as described before [15] and protein was estimated by the Folin method.

Crystalline Na-ATP (lot 100C-7620) containing less than 8 ppm Ca contaminant was purchased from Sigma, St. Louis, Mo.; murexide (ammonium purpurate) from K and K Chemicals, Plainview, N.Y.; valinomycin from Calbiochem. San Diego, Calif. X-537 A was a gift from Drs. J. Berger and H.H. Leher of Hoffman-LaRoche, and carbonyl cyanide-*p*-trifluoromethoxy phenylhydrazone (FCCP) from Dr. P. Heitler of du Pont.

3. Results and discussion

X-537 A is an antibiotic obtained from an unidentified streptomyces [16], which is effective against coccidial infection in chickens [17]. The

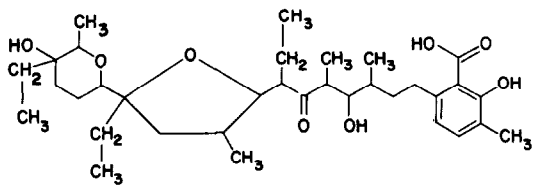

Fig. 1.

molecule is a substituted salicylic acid, with a side chain containing a high proportion of oxygen [18] (fig. 1).

X-537 A is known to form lipophilic complexes with monovalent cations thereby increasing the permeability of natural and artificial membranes to Na^+, K^+, Cs^+ and H^+ in analogy to such ionophores as nigericin and monensin [19, 20]. In addition, X-537 A is able to complex divalent cations. In fact, X-ray studies of the barium salt of X-537 A have shown that two antibiotic molecules encircle the barium ion and the water of crystallization, the antibiotic being stabilized through coordination to the metal ion and perhaps hydrogen bonding to water [21]. The resulting complex has a spherical shape, with the oxygen atoms buried inside to form a hydrophilic pocket for the cation and its water. The exterior is hydrophobic and results in a low solubility of the salt

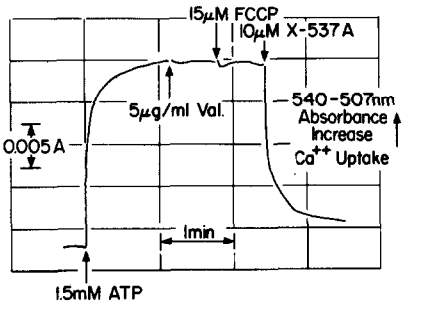

Fig. 3. ATP-dependent Ca^{2+} accumulation by SR. Ca^{2+} release is caused by X-537 A, but not by valinomycin or FCCP. SR was present at a concentration of 0.42 mg/ml.

in water and a high solubility in organic solvents. These properties would render X-537 A a carrier of Ca^{2+} through biological membranes containing hydrophobic regions. We have recently observed that X-537 A increases Ca^{2+} fluxes through isolated mitochondria, red cells and plasma membranes of isolated myoblasts [22]. For this reason, we have performed experiments to test the effect of X-537 A on the permeability of SR to Ca^{2+}.

Fig. 2A shows a calibration of the system in the absence of SR. The addition of Ca^{2+} produces a decrease in light absorbance due to the formation of a

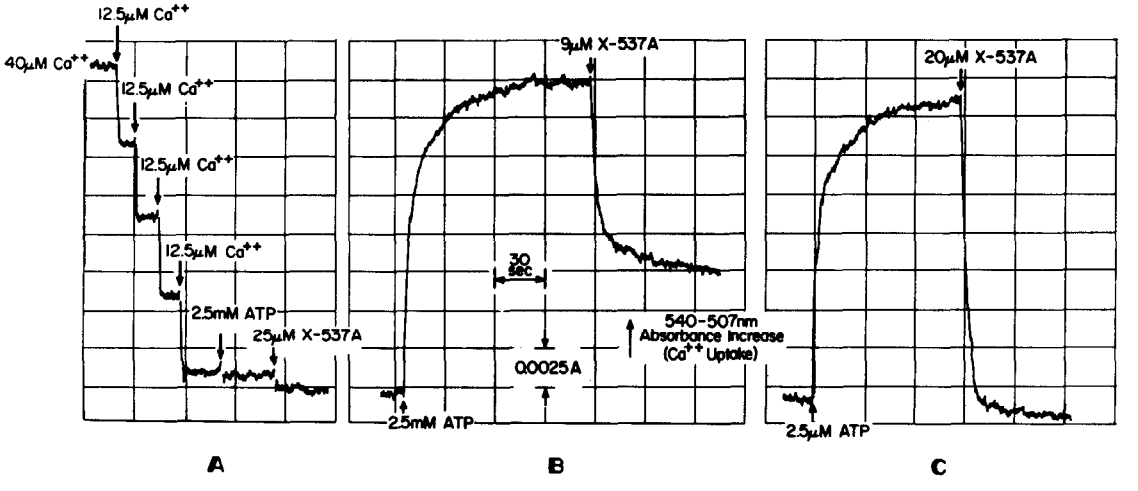

Fig. 2. A, B and C. A) Light absorbancy changes undergone by murexide on addition of $CaCl_2$, ATP and X-537 A in the experimental medium devoid of SR. B and C) ATP-dependent Ca^{2+} accumulation by SR (0.36 mg/ml) and subsequent release induced by X-537 A.

Ca–murexide complex which exhibits lower absorbance at 540 nm. This effect was quantitatively reproduced by successive additions of Ca^{2+}, indicating that the observed absorbancy changes were a linear function of the Ca^{2+} concentration. The additions of ATP and X-537 A, in the absence of SR, do not produce any significant changes and, in this mixture, their competition with murexide for calcium ion does not result in any absorbance changes.

The addition of ATP to a reaction mixture containing SR (figs. 2B and 2C) is followed by a burst of Ca^{2+} accumulation by the vesicles, reaching a steady state level of 180 μmoles Ca^{2+}/mg membrane protein. Figs. 2B and 2C also show that a rapid Ca^{2+} efflux is produced by the subsequent addition of X-537 A to the Ca^{2+} loaded SR vesicles. The resulting reduction of steady state levels is proportional to the concentration of the ionophorous antibiotic. A 20 μM concentration of X-537 A produces a release of all the accumulated Ca^{2+}.

Fig. 3 shows that other ionophores such as valinomycin and FCCP, which are known to increase the membrane permeability to K^+ and H^+, respectively [19, 20], do not alter Ca^{2+} accumulation by SR, even if used in very high concentrations. Similarly, the addition of either nigericin (25 μM) or gramicidin (25 μM) was without effect (result not shown). The lack of effect on Ca^{2+} accumulation by such monovalent cation carriers indicates that the action of X-537 A on SR is related to its specific ability to form lipophilic complexes with divalent cations and therefore to increase the membrane permeability to Ca^{2+}.

It may be ruled out that X-537 A may affect Ca^{2+} movements in SR through different mechanisms. The possibility that X-537 A interacts with nonspecific binding sites for Ca^{2+} can be excluded on the basis of $^{45}Ca^{2+}$ measurements showing that, in the absence of ATP, the amounts of Ca^{2+} bound to SR was not affected by the presence of 20 μM X-537 A. Moreover, X-537 A could interact with some specific component of the pump itself. Ca^{2+} uptake by SR is a rapid phenomena which is accompanied by a rapid burst of the ATPase activity. When the Ca^{2+} has been accumulated inside the vesicles, ATPase activity returns to the same level present before the addition of Ca^{2+} [4, 5]. We found that Ca^{2+} activated ATPase was not inhibited by X-537 A but instead stimulated and the initial burst was continued for long periods of time. No ATPase stimulation was observed in the presence of ethylene glycol (β-aminoethyl ether)-*N*, *N'*-tetracetic acid (EGTA). On the other hand, the effect of X-537 A on membrane permeability to cations is also observed in a variety of natural and artificial membranes [19, 20], not all of which possess similar Ca^{2+} binding sites or Ca^{2+} pump.

To conclude, the effect of X-537 A on SR is best explained by an increase in membrane permeability induced by the antibiotic, and a consequent equilibration of the Ca^{2+} gradient created by the pump. The reduced steady states obtained in the presence of the ionophore reflect levels at which the rates of passive efflux equal the rates of active transport.

X-537 A, in addition to offering insights into the mechanism of Ca^{2+} accumulation by SR, promises to be a very useful tool in the study of several biological control mechanisms where Ca^{2+} concentration gradients in cells or subcellular organelles are a limiting factor.

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