KINETICS OF TRANSPORT OF DIVALENT CATIONS ACROSS SARCOPLASMIC RETICULUM VESICLES INDUCED BY IONOPHORES

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SUMMARY: The fluorescent chelate probe technique is employed in exploring the kinetics of Ca^{++} and Sr^{++} release from sarcoplasmic reticulum induced by two divalent cation ionophores, A23187 and X537A. Both reagents are shown to release Ca^{++} that has been previously accumulated. A23187 is 60X more effective than X537A, while X537A is 100X more effective than A23187 in sequestering Ca^{++} into an organic phase in two phase systems. An explanation for the discrepancy between the effectiveness of the antibiotics in the two phase system and the transport rates in sarcoplasmic reticulum is provided by the observation of a particularly low affinity of X537A for Ca^{++} and Sr^{++} in polar media. The fluorescence properties of A23187 in the vesicle membrane are described.

Two new antibiotics have been described recently which possess the property of transporting divalent cations across membranes. Pressman (1) has described the complexation of X537A with monovalent and divalent cations and shown permeability changes in biological membranes. Reed and Lardy (2) showed complexation by A23187 of divalent cations, but not monovalent cations and have demonstrated an action of the ionophore in releasing Ca^{++} and Mg^{++} from mitochondria. The release by X537A of Ca^{++} that has been accumulated within isolated sarcoplasmic reticulum vesicles (SR) has also been demonstrated (3,4).

In this paper we describe the application of fluorescence techniques to the monitoring of Ca^{++} movements in isolated SR under the influence of Ca^{++} ionophores. The operation of the fluorescent chelate probe has been described in detail elsewhere (5-7). The technique depends on the property of chlorotetracycline to form highly fluorescent complexes with Ca^{++} or Mg^{++} attached to the vesicle membrane. When Ca^{++} is accumulated within the vesicle it binds to the internal surface of the membrane and gives rise to a fluorescent adduct with chlorotetracycline. This active accumulation of Ca^{++} is visualized as an increase in fluorescence of chlorotetracycline. The operation of this technique in exploring the mode of action and selectivity of divalent

cation ionophores is presented here.

MATERIALS AND METHODS

SR vesicles were isolated from rabbit soleus muscle and fluorescence measurements made as described previously by Caswell and Warren (7) in a quartz cuvette containing a stainless steel vibrating stirrer. Antibiotics were applied to the chamber from Hamilton syringes as concentrated solutions in alcohol. ATP determinations were made using the firefly procedure of Strehler and Totter (8).

Complexation studies of the antibiotics were effected in a two phase system as described previously (9). Determination of affinity of cations for the antibiotics was carried out by fluorescence titrations in a single phase using the technique described by Caswell and Hutchison (5).

A23187 was a gift of Eli Lilly and X537A was a gift of Hoffman La-Roche.

RESULTS AND DISCUSSION

Figure 1 shows that the fluorescence of the chlorotetracycline increases when Ca^{++} accumulation has been initiated in the presence of 20 μM external Ca^{++} with 50 μM Mn^{++} to serve as a cofactor for the ATP induced Ca^{++} transport. Subsequent addition of either A23187 or X537A causes a decrease in fluorescence which is indicative of a release of the accumulated Ca^{++}. If low concentrations of either reagent are added (Fig. 1, A and C) then the release is only partial and a new steady state of fluorescence is attained. Further additions of ionophore cause steady states to be established at decreased fluorescence levels implying that the steady state of Ca^{++} accumulation is decreased progressively by higher concentrations of either ionophore. On the other hand, if a large concentration of A23187 is added initially then a very rapid decline in fluorescence is observed until the fluorescence has dropped to the baseline implying complete release of accumulated Ca^{++} (Fig. 1B). These data are indicative of a competition between the ATP induced accumulation of Ca^{++} and the ionophore induced release of this Ca^{++}. If this is the case, then inhibition of the energy linked Ca^{++} uptake should cause complete re-

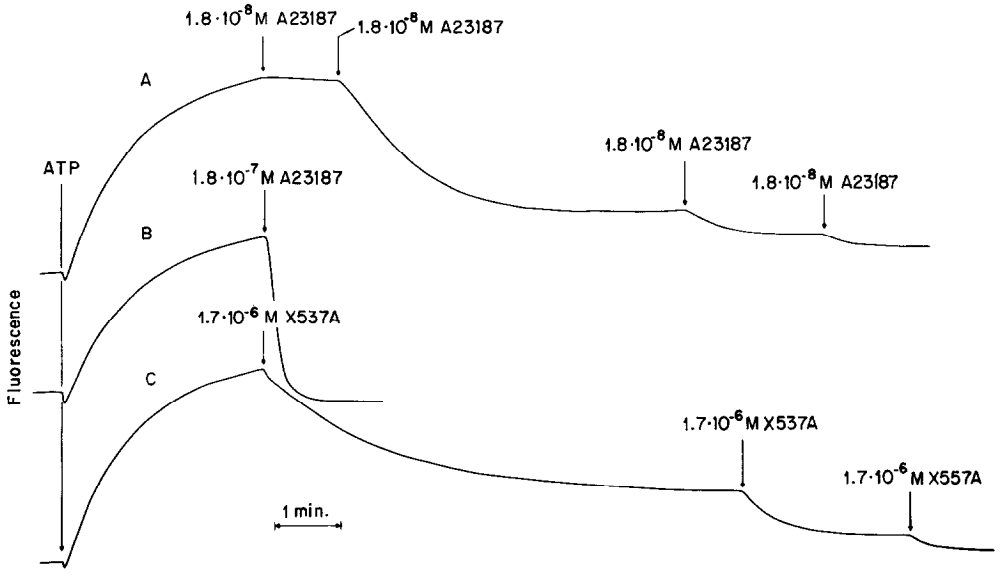

Fig. 1. Release of Ca^{++} induced by divalent cationophores. Incubation conditions are: 5mM histidine; 350mM sucrose; 50 µM $MnCl_2$; 20 µM $CaCl_2$; 10 µM chlorotetracycline; microsomes; 0.1 mg protein/ml. pH is 6.8 and temperature 22°. Ca^{++} uptake is initiated by 500 µM ATP. Fluorescence is determined at excitation wavelength of 390 nm and emission of 530 nm.

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lease of Ca^{++} at all effective doses of the antibiotic. The inhibition of further Ca^{++} accumulation has been achieved here by administration of EGTA immediately prior to the ionophore in order to eliminate free exogenous Ca^{++}. The subsequent addition of either antibiotic now causes fluorescence decrease back to baseline implying complete Ca^{++} release. The data from such experiments are shown in Fig. 2 in which log dose/log response curves are plotted for both A23187 and X537A. The following information may be gathered from these plots: 1) The rate of Ca^{++} release is first order with respect to the ionophore concentration over the range of concentrations tested. 2) The antibiotic A23187 is more potent by a factor of 60 than X537A in releasing Ca^{++} from the SR. A further prediction may be made on the mode of action of the ionophores that they should increase the Ca^{++} induced ATPase. This action has been observed by Scarpa and Inesi with X537A (3). We are able to

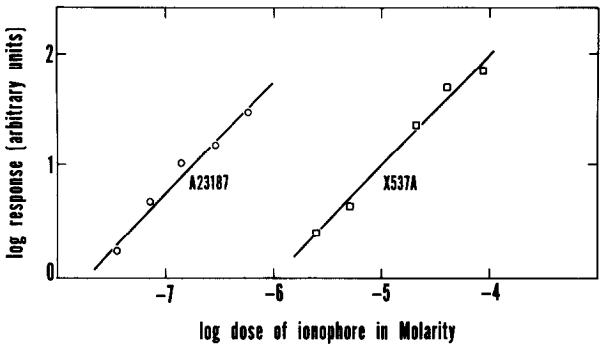

Fig. 2 Kinetics of Ca^{++} release induced by ionophores. Incubation medium contains: 5mM histidine; 350mM sucrose; 500 µM $MgCl_2$; 100 µM $CaCl_2$; 10 µM chlorotetracycline; microsomes 0.1 mg protein/ml. pH is 6.8 and temperature 22º Ca^{++} accumulation is initiated by 500 µM ATP. The release is initiated by simultaneous addition of 1mM EGTA and the ionophore.

confirm that an enhancement also occurs on administration of A23187. The enhancement by a factor of 1.6 caused by 1 µM A23187 indicates that the mode of action of the reagent is to release by passive outflow the Ca^{++} that has been accumulated through energy linked ATP hydrolysis.

Both A23187 and X537A are fluorescent, but A23187 fluoresces more intensely and at a more suitable wavelength for observation within biological membranes. The excitation and emission spectra of A23187 in SR are shown in Fig. 3. In the presence of Ca^{++} there is a diminution in fluorescence at the wavelengths corresponding to the free antibiotic, and an increase in fluorescence associated with the complexed form of the antibiotic. Thus decreases in the excitation spectra occur at 292, 383 and 393 nm while new peaks appear at 310 and 370 nm. The emission spectrum is shifted from 435 nm to 422 nm. Since the antibiotic is insoluble in water, it is most likely that these shifts are reflecting complexation of the antibiotic within membranes or at their surface. The fluorescence of A23187 reflects not only the chemical nature of the molecule, but also is responsive to environmental influences and should prove valuable in describing the physical nature of the environment of the ionophore in biological membranes.

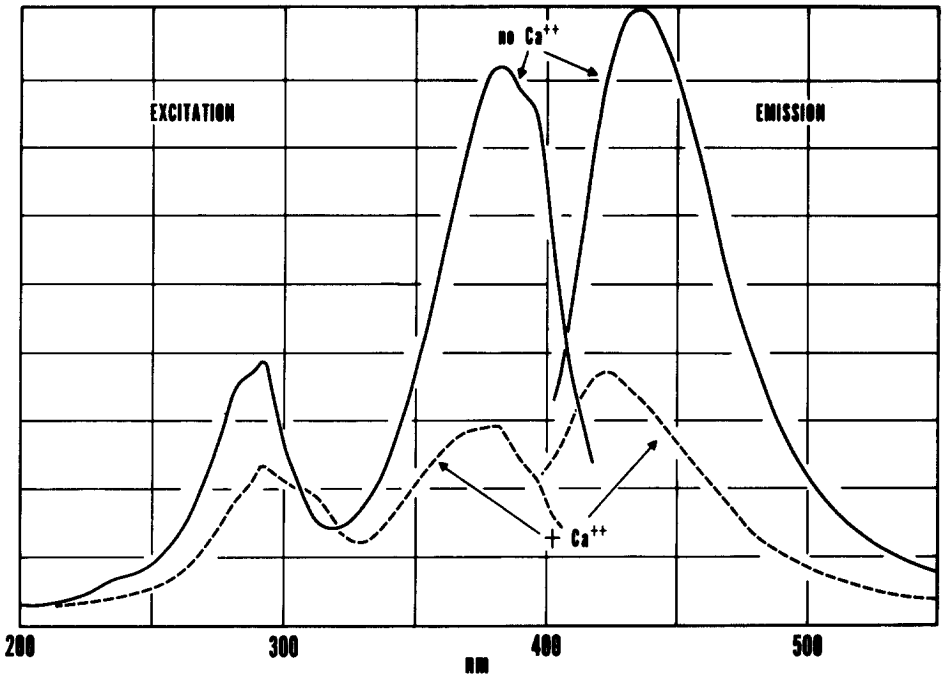

Fig. 3 Fluorescence spectra of A23187 is SR vesicles. The medium contains 5mM Tris Cl; 350mM sucrose; 0.5 μM A23187; microsomes, 0.1 mg protein/ml; 1mM EGTA for the solid line traces and 5 mM $CaCl_2$ for the dashed line trace.

One of the most intriguing aspects of the comparison of the two antibiotics is the lack of correlation between the transport rate of the antibiotic in the membrane and the ability of the reagents to sequester Ca^{++} or Sr^{++} from an aqueous into an organic phase. This is shown in Table I. It is seen that, whereas A23187 is 60 fold more effective in releasing Ca^{++} from SR it is 100 fold less effective in extracting Ca^{++} from water into butanol/toluene. Complexation selectivity between Ca^{++} and Sr^{++} does parallel the selectivity for the induction of cation release from the SR for each of the ionophores. It appeared that the kinetics of divalent cation transport might be limited for X537A by the ability of the antibiotic to complex with Ca^{++} at the membrane/water interface. Accordingly the affinities of Ca^{++} and Sr^{++} for X537A and A23187 in polar media were determined by fluorescence titrations of the

TABLE I

Selectivity of transport and of complexation induced by divalent cationophores

	A23187			X537A		
	Mg^{++}	Ca^{++}	Sr^{++}	Mg^{++}	Ca^{++}	Sr^{++}
1. Transport turnover numbers sec^{-1}	----	5.0	0.49	----	.083	0.17
2. Extraction into toluene/butanol K_A mM^{-2}	----	.022	.0095	----	2.1	11
3. K_A in 50% methanol mM^{-1}	1.2	1.2	0.42	----	----	----
4. K_A in 80% ethanol mM^{-1}	36	30	4.0	0.36	0.62	----

Turnover numbers are estimated in muscle microsomes based on the initial rate of fluorescence decrease of chlorotetracycline induced by the antibiotics and the estimated Ca^{++} accumulation based on ^{45}Ca measurements. The data are referred to a microsome concentration of 0.1 mg protein/ml. The K_A for extraction are estimated using radioisotope distribution between an aqueous phase pH 8.0 and toluene/butanol. The K_A is $\frac{[M^{++}\text{-ionophore}_2]_{org}}{[M^{++}]_{aq} \cdot [\text{ionophore}]^2_{org}}$. The K_A in polar media are determined at pH 6.8 by titrations of a fluorescence decrease induced by divalent cations on the fluorescence of A23187 at excitation of 380nm and emission of 440nm and of X537A at excitation of 315nm and emission at 420nm. The K_A is $\frac{[M^{++}\text{-ionophore}]}{[M^{++}] \cdot [\text{ionophore}]}$. Estimates for K_A of Sr^{++} with X537A were not obtained since no fluorescence decrease accompanied binding by this ion.

antibiotics. The results (Table I) show that in 50% methanol the K_A of A23187 for Ca^{++} and Mg^{++} is $1.2mM^{-1}$ while the K_A for Sr^{++} is $0.42mM^{-1}$. Thus the ion selectivity follows qualitatively that for transport across the SR. A medium of 50% methanol was chosen since this corresponds to the environment of divalent cations bound to the membrane surface (7) and complexation to the cation in this environment is a necessary preliminary for transport. In contrast the affinities of X537A for Ca^{++} or Sr^{++} in 50% methanol were too low to permit accurate estimates of K_A. Accordingly the K_A in 80% ethanol were determined and then show good correspondence between complexation affinity and turnover number for transport. There exists therefore a dramatic difference in response to solvent polarity of these two anti-

biotics. The low affinity of X537A for divalent cations in polar media provides a ready explanation for the low transport turnover number for this reagent, since it is necessary for the ionophore to complex cations in the relatively polar environment of the membrane surface before movement into the apolar interior, and hence transport,can occur. In these polar environments titrations show (Table I) that the stoichiometry of complexation of antibiotic to divalent cations is 1:1 while in apolar media it approaches 2:1.

These data enable one to comprehend the kinetics of carrier mediated divalent cation transport as being determined by a sequence of events beginning with 1:1 complexation of the ionophore with the cation that is attached to the membrane surface. This must presumably be followed by interaction with a second ionophore molecule as the complex moves in to the apolar interior of the membrane. Release of the cation on the other surface and return of the uncomplexed ionophore completes the cycle. In this elaborate sequence of transport processes any stage may prove rate limiting depending on the nature of the ion and of its carrier.

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