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Ionophorous Properties of the 20,000-Dalton Fragment of $(Ca^{2+} + Mg^{2+})$-ATPase in Phosphatidylcholine: Cholesterol Membranes

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Summary. The purified 20,000-dalton fragment of sarcoplasmic reticulum $(Ca^{2+} + Mg^{2+})$-ATPase has been shown by us (A.E. Shamoo, T.E. Ryan, P.S. Stewart, D.H. MacLennan, 1976. J. Biol. Chem. **251**:4147) to have Ca^{2+}-selective ionophoric activity. The Ca^{2+}-ionophoric fragment has been purified by either SDS-column chromatography or SDS-preparative gel electrophoresis. The Ca^{2+}-ionophoric fragment has been subjected to prolonged dialysis to insure the removal of bound SDS from the fragment. The selectivity sequence of this fragment in black lipid membranes (BLM) formed from either oxidized cholesterol or phosphatidylcholine/cholesterol is the same, $P_{Ba} > P_{Ca} > P_{Sr} > P_{Mg} > P_{Mn}$. This selectivity sequence is the same as that for the intact $(Ca^{2+} + Mg^{2+})$-ATPase. Treatment of the fragment with cholate to absolutely insure the removal of bound SDS resulted in the fragment having a selectivity sequence as above except that $P_{Mn} > P_{Mg}$. This and other data indicate that the 20,000-dalton fragment is the site containing the Ca^{2+}-ionophoric activity of the $(Ca^{2+} + Mg^{2+})$-ATPase.

$(Ca^{2+} + Mg^{2+})$-ATPase[1] is known to be the primary "pump" protein for translocation of Ca^{2+} into rabbit white skeletal muscle sarcoplasmic reticulum (Racker, 1972; 1973). The enzyme has been reconstituted into artificial vesicles following its purification. These vesicles have been shown to catalyze ATP-dependent Ca^{2+} transport. The reconstitution data and other mounting evidence indicating that membrane-bound ATPases contain the entire mechanism necessary for ion transportation prompted our laboratory to look for and find an ion-bearing and ion-translocating site more commonly known as an ionophoric site (Shamoo & Ryan, 1975). In the case of the $(Ca^{2+} + Mg^{2+})$-ATPase molecule, we were able to show that the Ca^{2+} ionophorous property resides as part of the intact

1 *Abbreviations:* $(Ca^{2+} + Mg^{2+})$-ATPase = $(Ca^{2+} + Mg^{2+})$-dependent adenosine-triphosphase.

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polypeptide ($Ca^{2+} + Mg^{2+}$)-ATPase (Shamoo & MacLennan, 1974). Furthermore, it was shown that tryptic digestion of the enzyme results first in 55,000 (*A*) and 45,000 (*B*) dalton fragments, followed by further cleavage of the 55 K-dalton fragment into 30,000 (A_1) and 20,000 (A_2) dalton fragments (Stewart, MacLennan, & Shamoo, 1976). It was also shown by our laboratory and others that the hydrolytic site resides in fragments *A* and A_1, whereas the ionophoric site resides in fragments *A* and A_2 (Shamoo *et al.*, 1976). The fragments are held together strongly through hydrophobic interactions. Only strong detergents, e.g., sodium dodecylsulfate (SDS), have been successful in separating the fragments (Stewart *et al.*, 1976). Our laboratory has separated the fragments with either SDS-column chromatography or SDS-preparative gel electrophoresis (Ryan *et al.*, 1976). It was found that the fragments isolated by SDS-preparative gel electrophoresis were the "cleanest" fragments when compared with those isolated by other methods on analytical SDS polyacrylamide gels. SDS was removed from the fragments by prolonged dialysis in 8 M urea followed by water (Shamoo *et al.*, 1976).

The purpose of this paper is to show that cholate added during the final stages of dialysis to ensure complete SDS removal has no effect on the ionophorous properties of the A_2 fragment (the cholate is dialyzed away before ionophorous activity is assayed), to study the ionophorous activity of the A_2 fragment in black lipid membranes (BLM) formed from phosphatidylcholine/cholesterol (PC:chol) and compare the results with those obtained using oxidized cholesterol membranes (Shamoo *et al.*, 1976), and to further characterize the selectivity of A_2 in bilayers.

Materials and Methods

Preparation

Sarcoplasmic reticulum (SR) was prepared from rabbit white skeletal muscle by the method of MacLennan (1970). The tryptic fragments of the ATPase derived from SR were prepared and purified by the method of Stewart *et al.* (1976) where a Bio-Gel A 1.5-M column equilibrated with 0.5% SDS was used. Fractions rich in the A_2 fragment were concentrated by Amicon ultrafiltration with a PM 10 membrane and passed through a Bio-Gel P-100 column (180 × 2.5 cm) equilibrated with 0.5% SDS, 50 mM Tris-HCl, pH 7.0, 1 mM dithiothrietol, 0.02% NaN_3. The column was eluted with the same column buffer, and 2-ml fractions were collected every 20 min.

Conductance Measurements

The lipid bilayer was formed from either egg phosphatidylcholine/cholesterol (5:1 mg/mg in *n*-decane) or from oxidized cholesterol prepared according to Tien *et al.*

(1966). Conductance, capacitance, and ionic selectivities were all measured according to our published methods (Shamoo *et al.*, 1976). The various divalent ionic selectivities were calculated from the equation:

$$V=\frac{RT}{F}\ln\left\{\frac{\sqrt{\beta^2+4\alpha\gamma}-\beta}{2\alpha}\right\}$$

with α, β, and γ defined as:

$$\alpha\equiv 4\sum_{i(-2)} P_i C_{i1}+\sum_{i(-1)} P_i C_{i1}+\sum_{i(+1)} P_i C_{i2}+4\cdot\sum_{i(+2)} P_i C_{i2}$$

$$\beta\equiv \sum_{i(-1)} P_i C_{i1}-\sum_{i(-1)} P_i C_{i2}+\sum_{i(+1)} P_i C_{i2}-\sum_{i(+1)} P_i C_{i1}$$

$$\gamma\equiv 4\sum_{i(-2)} P_i C_{i2}+\sum_{i(-1)} P_i C_{i2}+\sum_{i(+1)} P_i C_{i1}+4\sum_{i(+2)} P_i C_{i1}$$

where

$P_i\equiv$ the permeability of the ith substance in $cm\cdot s^{-1}$.

$C_{ij}\equiv$ the concentration of the ith substance on the jth side of the membrane in $mole\cdot cm^{-3}$.

V is the membrane potential in volts; F is the Faraday; R is the gas constant; and T is the absolute temperature.

Details of calculating various selectivities under various experimental conditions are given in our review paper (Shamoo & Goldstein, 1977).

Cholate Treatment and Estimation of SDS and Cholate Bound

The 20,000-dalton fragment isolated in 0.5% SDS was subjected to urea, followed by water dialysis as previously described (Shamoo, *et al.*, 1976). To remove remaining traces of SDS, the 20,000-dalton fragment was sonicated in 2% potassium cholate and dialyzed against 1% potassium cholate, pH 8.0, for four days, followed by dialysis against distilled H_2O for up to five days. The removal of detergents by dialysis was followed isotopically with ^{3}H-cholate and ^{35}S-SDS. The amounts of cholate and SDS originally bound were determined by equilibrium dialysis against 0.5% SDS, as previously described (Shamoo *et al.*, 1976), or 1% potassium cholate, pH 8.0. As controls, both bovine serum albumin (Sigma) and Soybean trypsin inhibitor were subjected to identical treatment.

Analytical Methods

Protein was determined by the method of Lowry *et al.*, (1951). SDS polyacrylamide gel electrophoresis was carried out according to the methods of Laemmli (1970) and Swank and Munkres (1971).

Results

Cholate Treatment and Estimation of Bound SDS and Cholate

Table 1 shows the amount of SDS and cholate bound to the A_2 fragment and the control proteins, bovine serum albumin and soybean

Table 1. Equilibrium dialysis with ^{35}S-SDS and ^{3}H cholate

	A_2	Bovine serum albumin	Soybean trypsin inhibitor
SDS bound			
mg SDS/mg protein	20.7	1.94	1.44
Mole SDS/mole Protein	14.3×10^2	4.42×10^2	1.01×10^2
Cholate bound			
mg cholate/mg protein	10.49	1.07	1.74
Mole cholate/mole protein	4.7×10^2	1.6×10^2	0.8×10^2

SDS and cholate bound to the A_2 fragment and controls under conditions used for isolation and purification.

trypsin inhibitor, under conditions similar to those used for A_2 purification and SDS removal. The SDS binding is identical to that reported previously (Shamoo *et al.*, 1976), with the water soluble controls binding the expected amount of SDS (Weber & Osborn, 1975) and the membrane-bound A_2 fragment binding 10-fold more. The amount of bound cholate is also consistent with this pattern.

The time course (not shown) of SDS and cholate removal from the A_2 fragment was identical to the time course of SDS and cholate removal from BSA and soybean trypsin-inhibitor. The proteins were tested for ionophoric activity following urea $\rightarrow H_2O \rightarrow$ cholate $\rightarrow H_2O$ dialysis. At a later time point, cold SDS was added back in an attempt to exchange for the remaining radioactivity. It was impossible to remove the last traces of radioactivity by this method. This indicates the presence of nondialyzable radioactive contamination. Attempts to remove the remaining radioactivity in the cholate removal experiment by exchanging with cold cholate also failed.

Table 2 shows data taken from the detergent removal experiments, giving the residual SDS and cholate-bound normalized both per milligram and per mole of protein. Residual SDS and cholate are consistent with expected differences between hydrophobic and water soluble proteins. It should be noted that identical amounts of radioactive tracer were added to every sample at the start of an experiment and identical amounts of radioactivity were recovered regardless of the amount of protein present. This is consistent with the data mentioned above indicating the presence of nondialyzable radioactive contamination.

Table 2. Residual SDS and cholate bound following dialysis

SDS or cholate bound	A_2	Bovine serum albumin	Soybean trypsin inhibitor
mg SDS/mg protein	15.94×20^{-3}	2.00×10^{-3}	1.51×10^{-3}
Mole SDS/mole protein	1.11	0.46	0.10
mg cholate/mg protein	30.4×10^{-2}	3.6×10^{-2}	3.9×10^{-2}
Mole cholate/mole protein	13.5	5.4	1.75

SDS and cholate bound to the A_2 fragment and controls following dialysis, as described in *Materials and Methods.*

Effect of Cholate on Bilayer Conductance

The cholate treatment of the A_2 fragment necessitated the testing of the effect of cholate on the BLM conductance. The threshold effect of cholate was at 5×10^{-4} molar; much greater than the maximum estimation of residual cholate bound to the A_2 fragment following dialysis. The $P_{Ca^{2+}}$: P_{Cl^-} was 1:2, the free mobility ratio in bulk solution. There was absolutely no selectivity between any of the divalent cations reported here. Similar results at much lower concentrations have been reported for SDS (Shamoo *et al.*, 1976).

Ionophorous Properties of A_2 in Phosphatidylcholine/Cholesterol Membranes

The A_2 preparation was tested for ionophorous activity following the described dialysis procedure. Bi-ionic potentials were used to measure selectivity. The bi-ionic potential was measured as the voltage intercept in the current-voltage (I–V) curve shown in Fig. 1. Once the conductance increased more than several times, the voltage intercept for a given experiment was constant regardless of the level of conductance or the fluctuation in conductance during the experiment. Thus the selectivity parameter calculated are independent of rate of incorporation of the protein. Since "true" steady-state conductance levels in the presence of ionophore is rarely achieved, the voltage intercept therefore becomes the most reliable parameter for measuring selectivity. Figure 1 is a composite of several bi-ionic experiments. Table 3 gives the average $\pm$SE bi-ionic

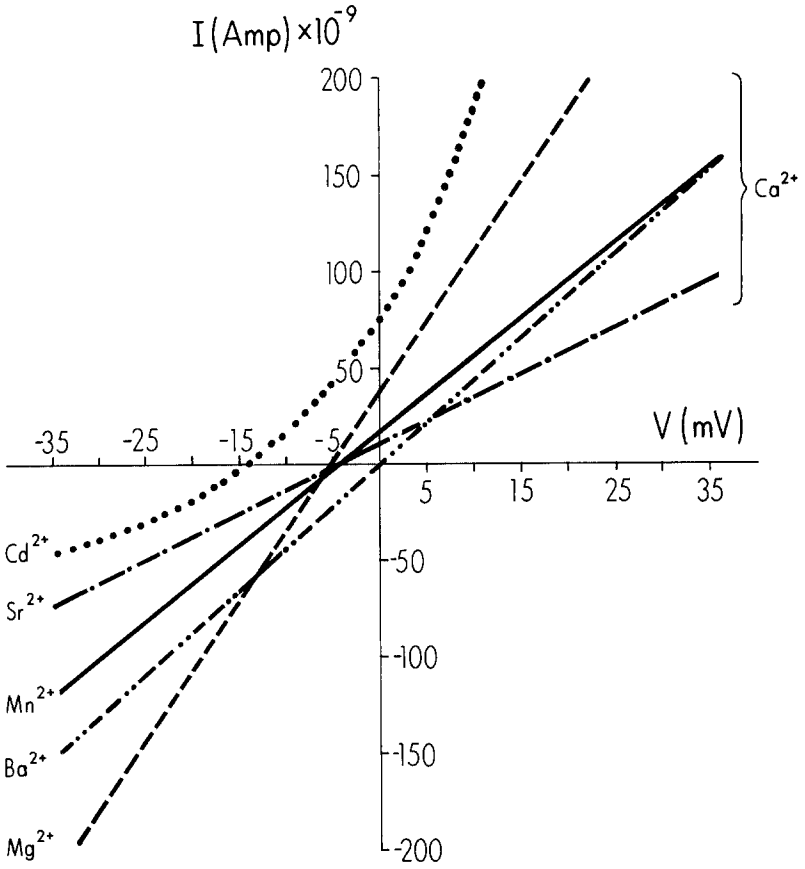

Fig. 1. A composite of representative data of several current voltage characteristics of BLM's formed from PC-chol and in the presence of cholate-treated 20,000-dalton fragment. The BLM was bathed on both sides with 5 mM histidine, pH 7, and on one side with 5 mM $CaCl_2$ and on the other side with 5 mM of the indicated divalent cation. The statistical data of the intercept (bi-ionic potential) experiments of this kind are shown in Table 5

potential from these experiments. The selectivity sequence is the same as that reported previously for the intact enzyme and the A_2 fragment in oxidized cholesterol membranes (Shamoo & MacLennan, 1974; Shamoo *et al.*, 1976). In the case of Ca/Mn, we report the data from two different bi-ionic potentials for the two buffers used (i.e., histidine and HEPES). With all the other ions reported here, we found no difference in the bi-ionic potentials when the two different buffers were used. This result was somewhat surprising, especially for Cd^{2+} since histidine is known to strongly bind divalent cations such as Cd^{2+} and Mn^{2+}.

Table 3. Selectivity of the A_2 fragment in phosphatidylcholine-cholesterol membranes

Ionic condition	Final dosage (mg/ml)	No. of membranes per bath	Total No. of readings	Average PD $\pm$ SE (mV)	Calculated selectivity
Ca^{2+} *vs.* Ba^{2+}	9.8×10^{-4}	2 2 4 5 3 3	11	0.0	1.0
Ca^{2+} *vs.* Ca^{2+}	3.0×10^{-4}	2 2 4 5 3 3	14	0.0	1.0
Ca^{2+} *vs.* Sr^{2+}	5.5×10^{-4}	5 2 1 3 2 2	15	3.4 ± 0.43	1.40
Ca^{2+} *vs.* Mn^{2+}	2.8×10^{-4}	7 2 1	10	7.4 ± 0.83	2.16
Ca^{2+} *vs.* Mg^{2+}	5.5×10^{-4}	2 2 3 0 1 0 3	11	4.1 ± 0.52	1.50
Ca^{2+} *vs.* K^{+}	1.4×10^{-4}	6 4 4 6 4	24	18.3 ± 0.82	1.5
Ca^{2+} *vs.* Na^{+}	1.4×10^{-4}	8 3 3 4	18	20.38 ± 1.3	1.7

Bi-ionic potentials (in mV) are shown under various ionic conditions. The BLM was formed from phosphatidylcholine/cholesterol (5:1 mg/ml in *n*-decane) in the presence of 5 mM histidine, pH 7.2, on both sides and on one side with 5 mM $CaCl_2$, and on the other side 5 mM of the indicated cation. The number of membranes per bath indicates that the bilayer was formed over again rapidly (sometimes before it breaks or immediately after) with little mixing of the two baths. The selectivity was calculated according to equation given in the text and using $P_{Ca^{2+}}/P_{Cl^-}=2.3$.

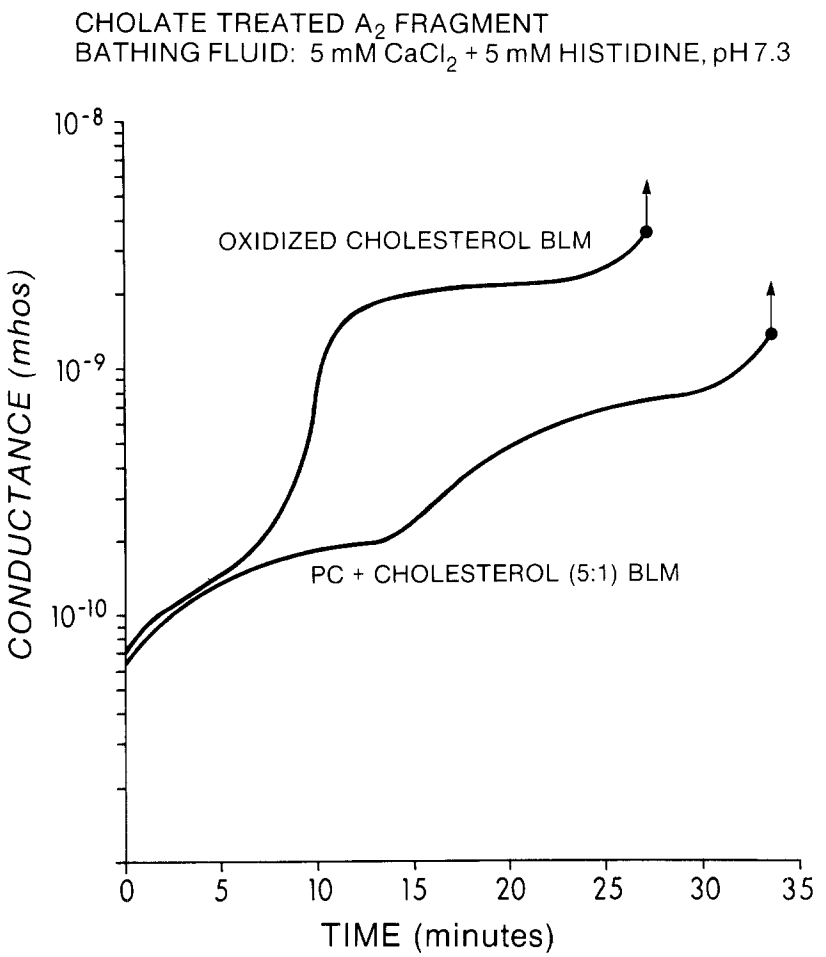

Fig. 2. BLM conductance in mho's *vs.* time in minutes in the presence of cholate-treated 20,000-dalton fragment and 5 mM $CaCl_2$ + 5 mM histidine, pH 7.3. The data in the upper and lower curves were derived from membranes formed from oxidized cholesterol and PC-cholesterol (5:1), respectively

Figure 2 shows two representative experiments of BLM conductance *vs.* time. In one experiment, the BLM was formed from oxidized cholesterol, while the other BLM was formed from phosphatidylcholine–cholesterol (PC–chol) as described. In both cases the BLM's conductance increased. The oxidized cholesterol's final conductance was greater than that of the PC–chol. This may be due to the fact that protein may incorporate more into oxidized cholesterol membrane than PC–chol membranes.

Table 4 gives the data on the selectivity of cholate-treated A_2 under various ionic conditions in oxidized cholesterol membranes. The selec-

Table 4. Selectivity of cholate-treated A_2 fragment in oxidized cholesterol membranes

Ionic condition		Final dosage (mg/ml)	No. of membranes per bath	Total No. of readings	Average PD ± SE (mV)	Calculated selectivity
Ca^{2+} *vs.* Ba^{2+}		8.3×10^{-4}	4	4	-4.9 ± 0.24	0.64
Ca^{2+} *vs.* Ca^{2+}	1:1	8.3×10^{-4}	2 1	3	0.0	1.0
	2:1	1.1×10^{-4}	2 1	3	4.0 ± 0.38	2.30
	3:1	1.1×10^{-4}	2 3 2	7	6.1 ± 0.39	2.30
Ca^{2+} *vs.* Sr^{2+}		8.3×10^{-4}	2 3	4	0.9 ± 0.36	1.09
Ca^{2+} *vs.* Mn^{2+}		7.5×10^{-4}	3 2 1	6	3.3 ± 0.20	1.38
Ca^{2+} *vs.* Mg^{2+}		8.3×10^{-4}	2	2	3.0 ± 0.35	1.34

Bi-ionic potentials (in mV) under various ionic conditions. The BLM was formed from oxidized cholesterol in the presence of 5 mM histidine, pH 7.3, on both sides and on one side 5 mM $CaCl_2$ and on the other side 5 mM of the indicated cation. The rest of the conditions were similar to those of Table 3.

tivity sequence and magnitudes are consistent with what we have reported previously for the entire $(Ca^{2+}+Mg^{2+})$-ATPase molecule and the A_2 fragment (Shamoo & MacLennan, 1974; Shamoo *et al.*, 1976).

Figure 3 gives the potential difference in millivolts *vs.* $\log\frac{[Ca]_1}{[Ca]_2}$ in PC–chol membranes in the presence of cholate-treated A_2. The $P_{Ca^{2+}}/P_{Cl^-}$ is not constant but varies from about 2.2 to 3.7.

Table 5 gives the data on the selectivity of cholate-treated A_2 under various ionic conditions in PC-chol membranes. The selectivity sequence and magnitudes are consistent with what we have reported previously.

Properties of Ca^{2+} *Conductance Inhibitors*

We have previously shown that several divalent cations such as Zn^{2+}, Cd^{2+}, Hg^{2+}, Mn^{2+}, etc., inhibit the Ca^{2+} conductance of the intact enzyme or the A_2 fragment (Shamoo *et al.*, 1976; Shamoo, MacLennan,

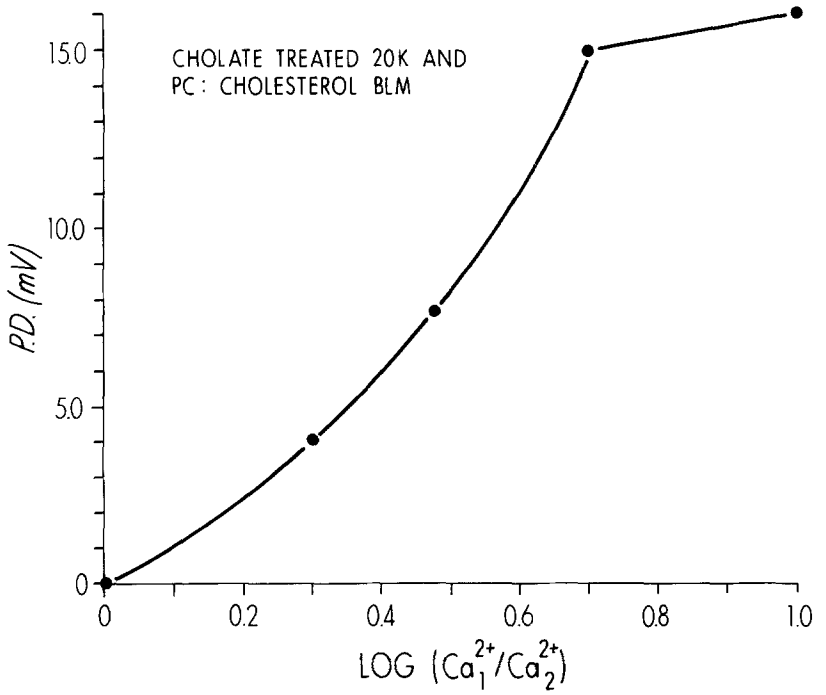

Fig. 3. Diffusion potentials in mV *vs.* log of calcium concentration ratios across BLM formed from PC-chol and with cholate-treated 20,000-dalton fragments. The figure is obtained from the data presented in Table 5

& Eldefrawi, 1976). The stoichiometry of inhibition of Ca^{2+} conductance was shown to be one to one. All of the divalent cations previously reported to inhibit the ionophorous activity of the intact enzyme also inhibit the activity of the A_2 fragment in the BLM. Two examples, (Zn^{2+}, Cd^{2+}) are shown here in detail.

Figure 4 gives data of a representative experiment of conductance *vs.* time in the presence of 5 mM Ca^{2+} and varying concentration of Cd^{2+} or Zn^{2+}. The BLM was formed from PC-cholesterol. It can be seen from the figure that increasing Cd^{2+} or Zn^{2+} concentrations decreases the rate of the conductance increase and the final steady or quasi-steady state conductance level.

The number of conducting units in the membrane may be written as

$$A = A_0 - B \tag{1}$$

where A_0 is the original number of conducting units and B is the number of conducting units closed by the inhibitor.

Table 5. Selectivity of cholate-treated A_2 fragment in phosphatidylcholine cholesterol membranes

Ionic condition	Final dosage (mg/ml)	No. of membranes per bath	Total No. of readings	Average PD ± SE (mV)	Calculated selectivity
Ca^{2+} *vs.* Ca^{2+} 1:1	4.5×10^{-4}	1 3	4	0.0	
2:1		3 3	6	3.8 ± 0.54	2.19
3:1		2 1 3 1 1	8	7.1 ± 0.50	2.74
5:1	3.0×10^{-4}	3 4	7	11.9 ± 1.09	3.68
10:1	3.0×10^{-4}	1 2	3	14.7 ± 2.48	3.42
Ca^{2+} *vs.* Ba^{2+}	4.5×10^{-4}	4 3	7	−2.8 ± 0.43	0.77
Ca^{2+} *vs.* Sr^{2+}	6.0×10^{-4}	3 1 4	8	0.9 ± 0.19	1.09
Ca^{2+} *vs.* Mg^{2+}	3.8×10^{-4}	3 5 3	11	7.0 ± 0.6	2.08
Ca^{2+} *vs.* Mn^{2+} (Histidine buffer)	4.5×10^{-4}	3 4	7	5.2 ± 0.40	1.70
(Hepes buffer)	3.0×10^{-4}	3 3 1	7	4.8 ± 0.48	1.63
Ca^{2+} *vs.* Cd^{2+}	6.8×10^{-4}	3 3 3 1 3	13	6.2 ± 0.36	1.90

Diffusion and bi-ionic potential (in mV) under various ionic conditions. The BLM was formed from phosphatidylcholine/cholesterol membranes, in the presence of 5 mM histidine, pH 7.3, on both sides. The first row gives the data of diffusion potential under the conditions of 5 mM $CaCl_2$ on both sides and increasing the ratio as indicated by increasing the calcium concentration on one side only. On one side 5 mM $CaCl_2$ and on the other side 5 mM of the indicated cation.

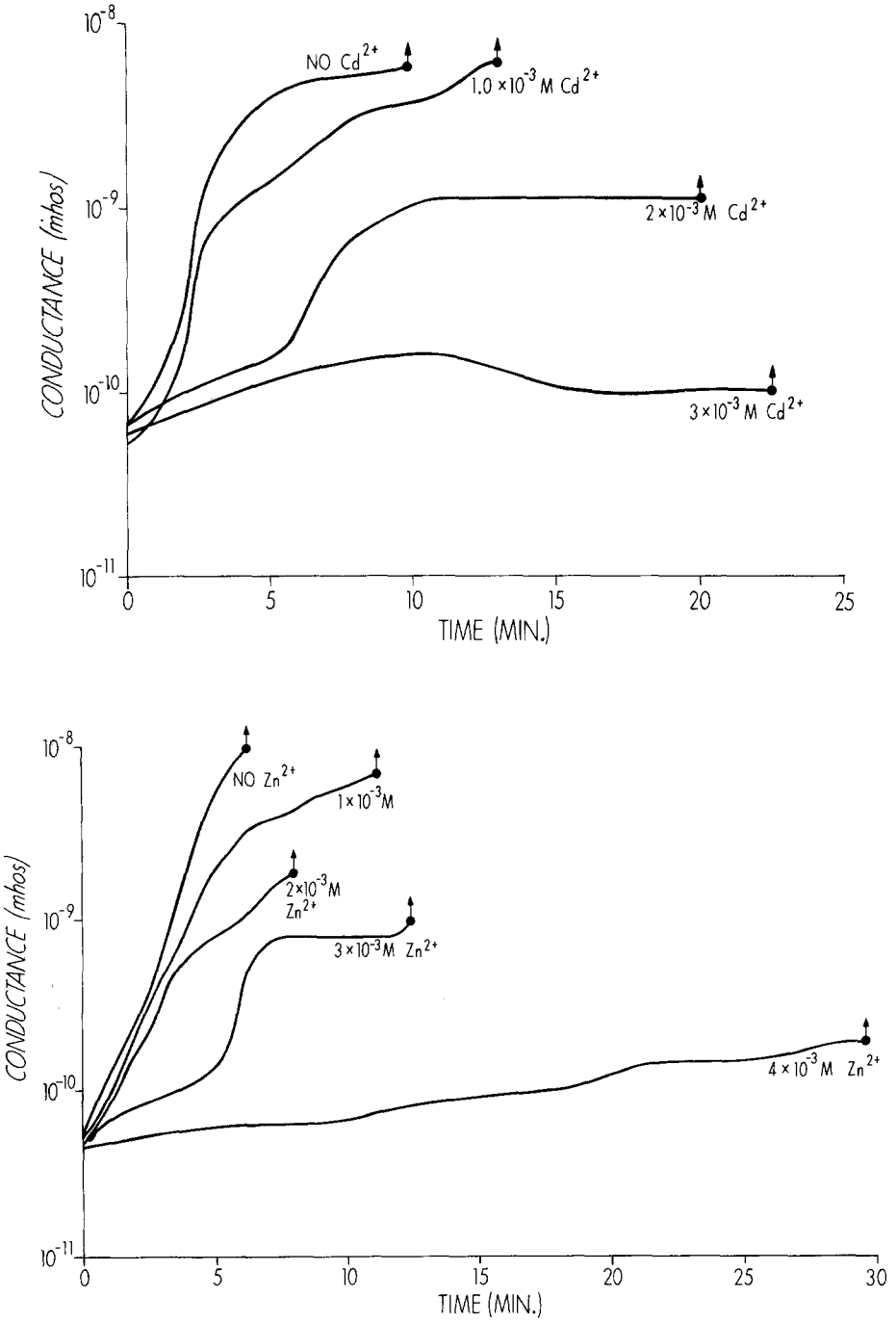

Fig. 4. Representative experiments of conductance *vs.* time of PC-chol. BLM's formed in the presence of cholate-treated 20,000-dalton fragment. Conductance was allowed to increase in the absence and the presence of various concentrations of either Ca^{2+} or Zn^{2+}

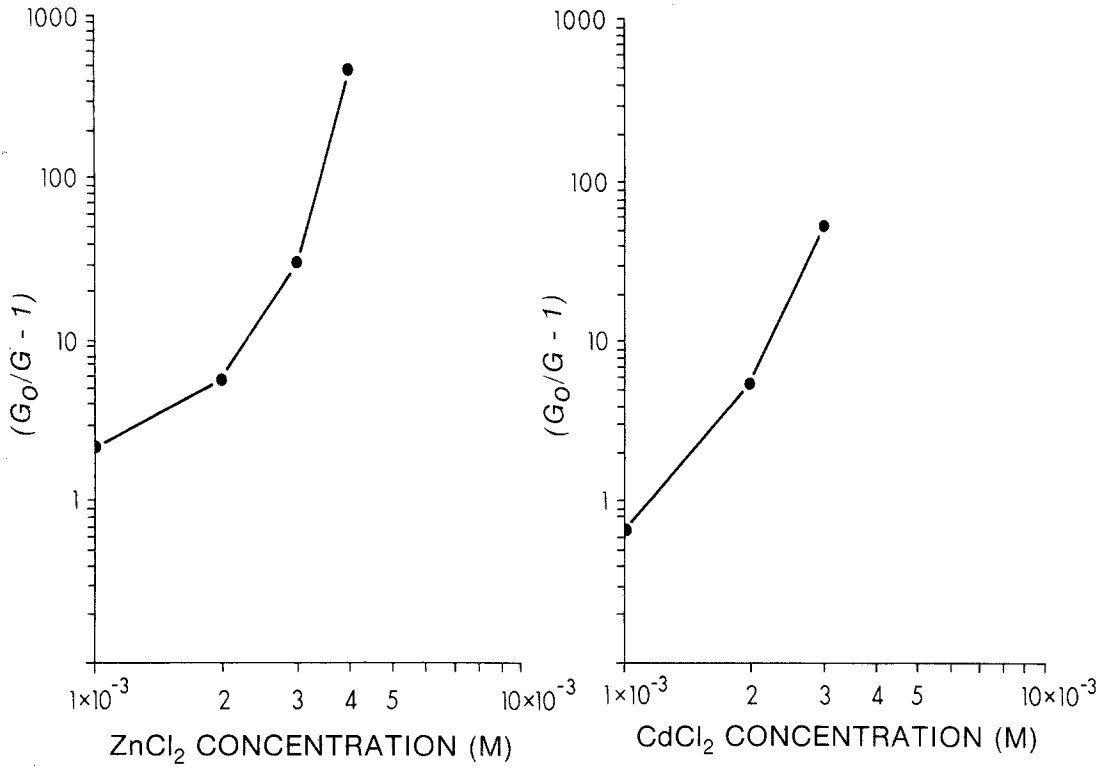

Fig. 5. Plot of the log of the ratio of the membrane conductance with no inhibitor present over the membrane conductance with inhibitor *vs.* the log of the inhibitor concentration. These graphs yield rough approximations for n greater than 1 (*see Results*)

The reaction between a conducting unit and the inhibitor may be written as

$$A+nI \rightleftharpoons B \tag{2}$$

where I is the inhibitor and n is the number of moles of inhibitor. The equilibrium expression for this equation may be written as

$$K=\frac{B}{AI^n}=\frac{A_0-A}{AI^n}. \tag{3}$$

If we assume the electrical conductance of the membrane (G) is proportional to the number of conducting units (A) we can rearrange Eq. (3) to yield:

$$\frac{G_0}{G}-1=K\,I^n \tag{4}$$

where G_0 is the membrane conductance with no inhibitor present. n may be calculated from the slope of a $\left(\log\left(\frac{G_0}{G}-1\right)\right)$ *vs.* $\log [I]$ plot. Figure 5 shows the conductance data plotted against the inhibitors Zn^{2+} and Cd^{2+} in this manner. These graphs are not simple straight lines; however, rough approximations yield values for n greater than 1.

Discussion

Phosphatidylcholine is a major lipid constituent of sarcoplasmic reticulum (Meissner & Fleischer, 1977). This fact prompted us to use a BLM composition more closely related to sarcoplasmic reticulum than oxidized cholesterol. Therefore, the ionophorous activity of A_2 in BLM's formed from PC-chol (5:1 mg/ml) were compared to those BLM's formed from oxidized cholesterol. The ionophorous activity, ionic dependency, and selectivity of the A_2 fragment were practically the same. The A_2 fragment was treated with cholate to insure the complete removal of SDS, and the ionophorous properties of A_2 were again practically the same. For example, the divalent cation selectivity of the intact enzyme (100,000); A (55,000) fragment; A_2 (20,000) fragment, and cholate-treated A_2 fragment in oxidized cholesterol membranes were all as follows: $Ba^{2+} > Ca^{2+} > Sr^{2+} > Mg^{2+} > Mn^{2+}$ (Shamoo & MacLennan, 1974; Shamoo *et al.*, 1976). Also the selectivity sequence of the A_2 fragment in PC-chol was the same as above (*see* Table 5).

The only deviation from this selectivity sequence occurred for the cholate-treated A_2 fragment in PC-chol membranes where we had $P_{Mn^{2+}} > P_{Mg^{2+}}$ rather than $P_{Mg^{2+}} > P_{Mn^{2+}}$. The following account indicates that this reversal of permeability sequence cannot be attributed to contaminant SDS: the intact enzyme has never been exposed to SDS, the cholate-treated A_2 resulted in near nil SDS bound, as we have shown isotopically, and finally those fragments having some bound SDS all have the same selectivity sequence. It is unlikely that this reversal is due to the lipid-forming solution of the BLM since the A_2 fragment selectivity sequence is the same in oxidized cholesterol and PC-chol membranes. The difference in the $P_{Mg^{2+}}$ and $P_{Mn^{2+}}$ selectivity of the cholate-treated A_2 fragment in oxidized cholesterol membranes is not statistically significant. The cholate-treated A_2 fragment selectivity in PC-chol membranes is $P_{Mn^{2+}} > P_{Mg^{2+}}$. We cannot discount the possibility that cholate treatment might have introduced this change in selectivity. Cholate alone

has no selectivity among divalent cations that could account for the difference (*unpublished data*).

We have previously shown that Ca^{2+}, Zn^{2+}, Mn^{2+}, and La^{3+} all inhibited the ionophorous activity of the intact enzyme by 1:1 competition ($n=1.0$) with calcium. Data shown here for the Cd^{2+} and Zn^{2+} inhibitory effect on the A_2 fragment indicate that such competition is greater than 1. It appears that at low concentrations of the inhibitor the competition is close to 1:1. The inhibitory effect of these ions still resides in the A_2 fragment; however, the stoichiometry has increased. This increase in stoichiometry may be due to the introduction of nonspecific negative site groups due to tryptic digestion. These may bind Cd^{2+} and Zn^{2+} at higher concentrations.

In conclusion, we have shown that the A_2 (20,000) fragment is the Ca^{2+}-ionophoric site in the overall Ca^{2+} transport system with the ionophorous properties consistent with the ionophorous properties of the intact enzyme and consistent with the overall Ca^{2+} transport of sarcoplasmic reticulum.

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