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The sarcoplasmic reticulum is permeable to a number of ions although the permeability can change depending on the physiological state of the membrane. In the resting state, the sarcoplasmic reticulum is relatively impermeable to Ca^{2+} but this ion is pumped across the membrane when ATP is hydrolyzed by the (Ca^{2+} + Mg^{2+})-dependent ATPase.[1] Under the appropriate physiological stimulus, Ca^{2+} flows out of the sarcoplasmic reticulum at rates greatly exceeding those at which it was pumped inward.[2] The membrane has permeability to monovalent cations. These ions appear to flow through sites so sparsely distributed that only about two thirds of any population of small vesicles are permeable to K^{+} or Na^{+}.[3] The membrane is also permeable to anions, and, when Ca^{2+} uptake is assayed in the presence of phosphate or oxalate, Ca^{2+} oxalate or Ca^{2+} phosphate precipitates can be found in the interior of vesicles.[4] The sites for anion passage may be blocked by anion transport inhibitors such as SITS.[5]

FRAGMENTATION OF THE ATPASE AND THE SITE OF CA^{2+} IONOPHORIC ACTIVITY

We have studied a number of sarcoplasmic reticulum proteins for their ability to conduct Ca^{2+} across a lipid bilayer in an electrical field. While calsequestrin, the high-affinity calcium-binding protein and the proteolipid did not display ionophoric activity, the ATPase was found to act as a Ca^{2+}-dependent and selective ionophore.[6] This assay has permitted us to trace the activity, which may represent a pathway through which Ca^{2+} moves in or out of the sarcoplasmic reticulum, to a particular part of the ATPase molecule. Investigations in our own and other laboratories[7-9] have shown that when the sarco-

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plasmic reticulum is exposed to trypsin for short periods, the 100,000-dalton ATPase is cleaved, first to 55,000- and 45,000-dalton fragments, and then the 55,000-dalton fragment is cleaved to 25,000-† and 30,000-dalton fragments. We isolated each of these four fragments by a combination of molecular sieving and hydroxylapatite chromatography in the presence of SDS.[10] The Ca^{2+}-dependent and selective ionophoric activity was located in the 55,000-dalton fragment and in the 25,000-dalton fragment derived from it.[11]

These observations led us to concentrate on the structure of the 25,000-dalton fragment.[12] We found that the 25,000- and 55,000-dalton fragments, like the 100,000-dalton ATPase,[13] had a blocked N-terminal methionine. We were, however, able to obtain N-terminal sequences of the 30,000- and 45,000-dalton fragments. These observations permitted us to align the fragments as N-25,000-30,000-45,000-COOH (FIGURE 1).

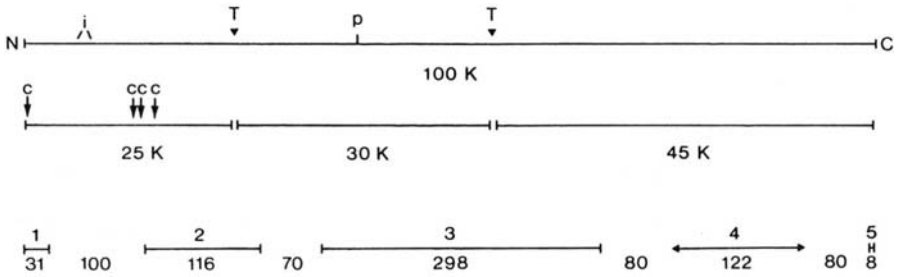

FIGURE 1. Structure of ATPase. Alignment of tryptic and CNBr fragments and of known hydrophilic sequences[14] within the (Ca^{2+} + Mg^{2+})-ATPase. The first line indicates the positions of the ionophoric site (i), the tryptic cleavage sites (T), and the phosphorylation site (P) relative to the amino (N) and carboxyl (C) terminal amino-acid residues. The second line indicates the sites of CNBr cleavage in the 25,000-dalton tryptic fragment. The third line positions Allen's hydrophilic sequences within the molecule. The position of the sequence of 122 amino acids in the 45,000-dalton fragment is uncertain. We have assumed that the molecular weight of the molecule is about 100,000-daltons, that it contains about 905 amino acids and that about 330 residues are unsequenced. Of these 100 can be assigned to the first hydrophilic gap and 70 to the second gap. We have arbitrarily assigned 80 amino acids to each of the third and fourth gaps on either side of the 122 residues in the 45,000-dalton fragment.

Drs. Geoffrey Allen and N. M. Green [14,36] have succeeded in sequencing five large segments of the ATPase molecule including the N- and C-terminal fragments. The alignment of the internal sequences in the ATPase molecule was not obvious. From our N-terminal sequences of the large fragments it was possible to align Allen's known sequences in proper order (FIGURE 1).

We have used CNBr as a reagent for further cleavage of the 25,000-dalton fragment. This fragment has 4 methionine residues of which one is N-terminal. Cleavage with CNBr released fragments of 13,000, 7,500, 3,000, and less than 1,000 daltons, in line with the expectation that there are 3 internal methionines in the fragment. The amino-terminal sequence of the 13,000-dalton fragment

† We have previously referred to this fragment as the 20,000-dalton fragment but recent data have indicated that its molecular weight is nearer 25,000.

revealed that it began at the second residue of the ATPase, confirming that it was derived from the N-terminus of the ATPase molecule by methionyl cleavages on either end. The 7,500-dalton CNBr fragment lacked homoserine and its amino-terminal sequence and composition showed that it was contained in one of Allen's sequences (sequence 3), which also contained the site of tryptic cleavage within the 55,000-dalton fragment. The two smaller CNBr fragments have not been so well defined although their alignment is clearly 13,000-1,000-3,000-7,500 (FIGURE 1).

The four CNBr-released fragments have been recently assayed for lonophoric activity in black lipid membranes (BLM). Only the 13,000-dalton, amino-terminal fragment exhibited Ca^{2+}-dependent ionophoric activity, as illustrated in FIGURE 2. The other fragments produced small conductance increases only at higher dosages than were used for the 13,000-dalton fragment. TABLE 1 shows the selectivity sequence for divalent metals obtained using the 13,000-dalton fragment incorporated into BLM. This sequence is different from that for the 100,000-, 50,000-, and 25,000-dalton fragments: these larger fragments had the selectivity sequence $Ba^{2+} > Ca^{2+} > Sr^{2+} > Mg^{2+} > Mn^{2+}$, whereas the 13,000-dalton fragment had the selectivity sequence $Mn^{2+} > Ca^{2+} > Ba^{2+} > Sr^{2+} > Mg^{2+}$. This change in selectivity may reflect an alteration of the ionophoric site by CNBr cleavage.

How do we visualize the ionophoric site within the ATPase molecule? Data relevant to the understanding of this problem are presented in FIGURE 1 where we have aligned Allen's sequences with known markers in the ATPase, two

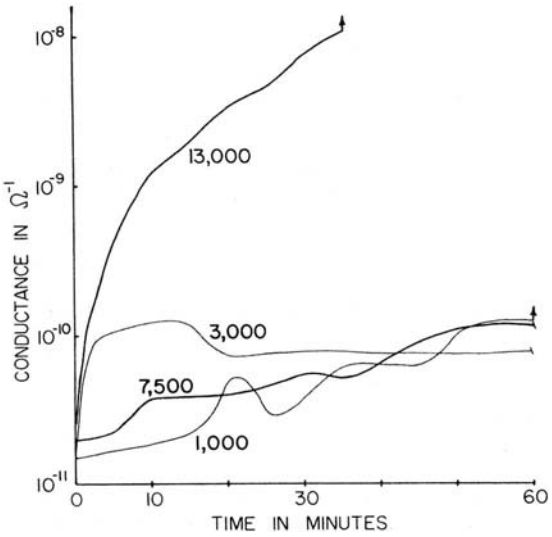

FIGURE 2. The time response of BLM conductance in the presence of CNBr fragments from the 25,000-dalton tryptic fragment of the (Ca^{2+} + Mg^{2+}-ATPase. The membranes were formed from phosphatidylcholine:cholestrol [5:1 (mg/mg), 10 mg/ml in *n*-decane]. The membranes were bathed on both sides in 5 mM $CaCl_2$, 10 mM HEPES (Tris), pH 7.2. Upward arrows indicate the point at which the membranes broke. Concentration of peptides in the bathing solution were: the 13,000-dalton protein, 1.5 μg/ml; 7500, 17 μg/ml; 3000, 20 μg/ml; and 1000, 20 μg/ml.

TABLE l

RELATIVE PERMEABILITY TO CATIONS OF BLM FORMED IN THE PRESENCE OF THE 13,000-DALTON CNBR FRAGMENT

Ionic Condition	Number of Measurements	Mean Potential ±SD	Mean P_{Ca}/P_M* ±SD
Ca^{2+} vs Mn^{2+}	15	+0.8±1.6	0.905±0.18
Ca^{2+} vs Mn^{2+}	4	-3.0±0.4	1.30±0.06
Ca^{2+} vs Mn^{2+}	8	-3.7±2.3	1.43±0.31
Ca^{2+} vs Mn^{2+}	7	-7.0±2.5	1.94±0.44

*Calculated using P_{Ca}/P_{C1} =1.87±0.5, n=6.

tryptic cleavage sites, the phosphorylation site, the ionophoric site, and the amino and carboxyl termini of the polypeptide chain. The trypsin-sensitive sites are cleaved in sealed vesicles of the sarcoplasmic reticulum and must lie on the cytoplasmic surface. The phosphorylation site labeled by cytoplasmic ATP must also lie on the cytoplasmic surface. The ionophoric site, because of its ability to interact with lipid bilayers, must lie within the membrane.

We have no information on the localization of the carboxyl-terminal portion of the ATPase. However, we have deduced that the amino terminus lies on the cytoplasmic surface of the membrane from experiments concerned with labeling amino acids in the amino-terminal sequence and from experiments on biosynthesis of the ATPase. An amino-terminal peptide containing the first 31 amino acids of the ATPase can be readily purified from a tryptic digest of the succinylated enzyme.[14] This fragment contains a single cysteine at position 12 and it is reactive with *N*-ethylmaleimide (NEM) added to sealed sarcoplasmic reticulum vesicles. If the sealed vesicles are pretreated with glutathione-maleimide, a reagent that cannot penetrate to the interior of sealed vesicles,[15] NEM labeling of Cys 12 is prevented. These observations suggest that Cys 12 of the ATPase lies on the cytoplasmic surface of the membrane.

In our studies of the biosynthesis of the ATPase,[16,17] we have asked the question whether the ATPase is synthesized with an N-terminal signal sequence that would initiate movement of the N-terminus into the bilayer. In the mature ATPase molecule,[14] the sequence of the 32 N-terminal amino acids begins with *N*-acetyl-methionine and ends with three basic amino acids. The sequence contains 18 polar and 14 nonpolar amino acids and the longest nonpolar sequence is 7. This is unlikely to comprise a signal sequence and, indeed, it is a water-soluble fragment. The ATPase is synthesized on membrane bound polyribosomes,[16] but present evidence[17] suggests that it is formed without an N-terminal signal sequence. We have found that the amino-terminal methionine residue is donated by initiator methionyl tRNA and that acetylation takes place during translation. Thus, the first interaction of the ATPase with the membrane is probably not with an N-terminal signal sequence but rather with an internal signal sequence as has been recently postulated for other proteins synthesized on membrane-bound ribosomes.[18,19] These observations would suggest that the N-terminus of the ATPase would remain cytoplasmic and that the residues after residue 32 would provide the first interaction with the membrane. An interesting possibility in this postulate is that the sequence of basic amino acids near

residue 30 might act as an anchor for the initial penetration of the chain into the membrane.

These observations suggest that the peptide chain begins in the cytoplasm and passes an even number of times through the lipid bilayer anchoring the 25,000-dalton fragment and creating a cytoplasmic, tryptic-sensitive site. It again passes an even number of times through the bilayer, anchoring the 30,000-dalton fragment and leaving the second tryptic cleavage site and the site of phosphorylation on the cytoplasmic surface. The chain must pass through the lipid bilayer again in the 45,000-dalton region to account for its attachment to the lipid bilayer. Thus, the minimal number of passages in this region would be one. However, there are two possible hydrophobic gaps

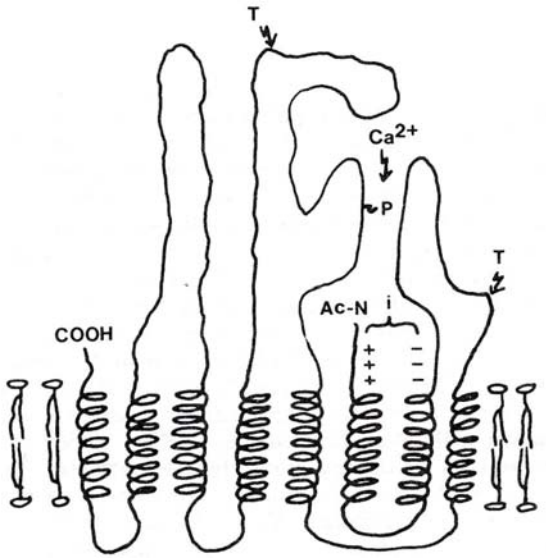

FIGURE 3. Diagrammatic representation of folding of the ATPase molecule within the phospholipid bilayer of the sarcoplasmic reticulum membrane. T, trypsin sensitive sites; P, phosphorylation site; i, ionophoric region; Ac-N, amino terminal of peptide; COOH, carboxyl terminal of peptide. Regions of basic (plus signs) or acidic (minus signs) amino acids are indicated.

amongst the hydrophilic Sequences in the 45,000-dalton region and this would suggest two sites of interaction with the membrane. Accordingly, the number of passages is more likely to be 3 or, if the C-terminal were cytoplasmic, 4. In assessing the number of transmembrane passages possible at each site, it becomes apparent that each of the four hydrophobic gaps contains between 70 and 100 residues. If each passage were between 25 and 35 amino-acid residues in length, with some residues on the luminal surface, then the chain could only pass two times through the lipid bilayer at each site. In FIGURE 3, we have indicated this diagrammatically by showing the ATPase passing 8 times through the membrane in 4 distinct loops.

In FIGURE 3, the loops are drawn in a linear sequence which does not attempt to show how a channel for Ca^{2+} passage might actually he formed. lonophoric activity measured in a BLM is relatively unchanged in going from 100,000 to 55,000 to 25,000 daltons. The contribution of the 45,000-dalton fragment to the Ca^{2+} selectivity appears to be negligible. In going from the 25,000- to 13,000-dalton fragments, a change was observed in the selectivity sequence for ions. This cleavage almost certainly did not affect the primary structure of the transmembrane segment, but it did cleave off a very hydrophilic and highly charged region just distal to it (indicated by minus signs in FIGURE 3). This charged sequence, the amino-terminal end of Allen's sequence 3,14 could be involved in calcium binding and might provide a gating function for the ionophoric activity measured in BLMs.

Pick and Packer[20] have shown that dicyclohexylcarbodiimide (DCCD) is an inhibitor of the ATPase when added in the absence of Ca^{2+}. Accordingly, they have suggested that DCCD might act at a Ca^{2+} binding site. They have shown that bound, Ca^{2+}-sensitive [^{14}C]DCCD is concentrated in the 25,000-dalton fragment following tryptic cleavage of the inhibited enzyme. These observations, which we have confirmed, point to a Ca^{2+} binding site as being very close to, or within, the hydrophobic sector, which we have found to possess Ca^{2+} ionophoric activity. These observations are incorporated into the model outlined in FIGURE 3, which shows sites of Ca^{2+} interaction in the 25,000-dalton fragment.

Although the ionophoric activity can be measured in the 25,000-dalton fragment alone, Abramson and Shamoo[21] have found evidence for an interaction among several transmembrane segments in Ca^{2+} ionophoric activity. They have found that the isolated 45,000-dalton fragment endows BLM with large, nonselective pathways for Ca^{2+} and Cl^-. This activity was abolished by dithiothreitol (DTT), which they believe disrupted a known disulfide bridge in the fragment. Treatment of the 55,000-dalton and 25,000-dalton fragments with DTT did not affect Ca^{2+} conductance, but treatment of the 100,000-dalton ATPase with DTT again blocked the Ca^{2+} conducting pathway. There is further evidence for interaction between the regions encompassed by the 25,000- and 30,000-dalton fragments in physiologically relevant Ca^{2+} transport. Tryptic cleavage of the 55,000-dalton fragments to its subfragments can be correlated with uncoupling of ATP hydrolysis from Ca^{2+} transport.[7,22] The cleavage occurs at a single Arg-Ala bond without loss of peptide material.[12] It appears that physical disruption of the peptide chain can disrupt concerted activity of these two regions of the molecule.

It is still premature to equate the ionophoric activity that we have measured with the physiologically relevant movement of Ca^{2+} to the interior of the sarcoplasmic reticulum. It is quite clear that ions to be transported must first activate ATP hydrolysis. Of the series Ba^{2+}, Ca^{2+}, Sr^{2+}, Mg^{2+}, and Mn^{2+}, only Ca^{2+} and Sr^{2+} activate ATP hydrolysis, and only Ca^{2+} and Sr^{2+} are transported. In FIGURE 3 we have indicated that Ca^{2+} must pass this initial barrier of triggering ATP hydrolysis. It is probable that ATP hydrolysis brings about a conformational change carrying Ca^{2+} close to the site of transmembrane passage. Ca^{2+} may then have to pass through a second gate, consisting of charged regions at the mouth of the Ca^{2+} channel and then through a transmembrane channel provided by the transmembrane passages of the peptide chain. The details of these sites have yet to be worked out.

ROLE OF THE PROTEOLIPID IN Ca^{2+} UPTAKE

Racker and Eytan[23] have suggested, on the basis of reconstitution experiments, that the proteolipid might act as a Ca^{2+} ionophore. Racker[24] has developed an attractive model for Ca^{2+} transport in which the proteolipid is the site through which Ca^{2+} ultimately flows through the membrane. The model would imply that there would be little or no Ca^{2+} transport in the absence of proteolipid.

We have recently developed a method for removal of the proteolipid from preparations of the ATPase, leaving the enzyme in a form that can be reconstituted for Ca^{2+} transport. In brief, sarcoplasmic reticulum is dissolved with a solution of Triton X-100 and then phospholipid is added. When phospholipid exchange is complete, the ATPase is precipitated with ammonium acetate leaving the bulk of the proteolipid in solution and butanol extracts of this preparation of ATPase contain virtually no proteolipid (FIGURE 4). The proteolipid-depleted ATPase is resuspended, treated to remove Triton, and reconstituted by freeze-thaw-sonication[26] into phospholipid vesicles for Ca^{2+} transport assay. The reconstituted, proteolipid-depleted ATPase preparation transported Ca^{2+} very well (TABLE 2), and we have obtained a Ca^{2+}/ATP ratio as high as 0.7 with the preparation. Under comparable conditions this value was the same as that obtained for the purified ATPase containing proteolipid. The efficiency undoubtedly reflected a mixture of leaky and tightly sealed vesicles.

These experiments indicate that there is no obvious, essential role for the proteolipid in providing a channel for the Ca^{2+} uptake process. Moreover, in earlier experiments[6] we were unable to measure ionophoric activity in the proteolipid using the black lipid membrane assay. It is our view, then, that it is unlikely that the proteolipid provides a channel for the Ca^{2+} uptake process.

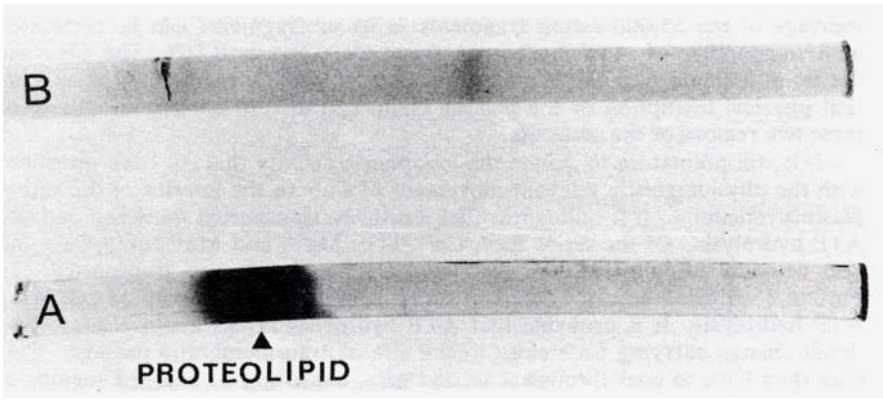

FIGURE 4. Butanol extraction of proteolipid from (A) sarcoplasmic reticulum and (B) Triton-phospholipid-treated sarcoplasmic reticulum. The proteolipid was extracted from equal amounts of the two preparations with butanol [25] and precipitated with ether and equal aliquots were separated in SDS-urea gels by electrophoresis.

TABLE 2

CALCIUM UPTAKE BY PROTEOLIPID-DEPLETED ATPASE *

Enzyme Preparation	Ca^{2+} Uptake †		ATP Hydrolysis †		Ca^{2+}:ATP	
	Exp. I	II	I	II	I	II
Sarcoplasmic reticulum	1.35	1.86	1.35	0.88	1.00	2.10
ATPase	0.65	—	2.0	—	0.33	—
ATPase, minus proteolipid	0.55	1.27	1.75	1.78	0.31	0.71

* Purified ATPase[27] or Triton X-100-lipid-treated ATPase were reconstituted into liposomes using the freeze-thaw-sonication method.[26] Ca^{2+} uptake and ATPase activities were measured at pH 6.8 under identical conditions except that $^{45}Ca^{2+}$ was used in one assay and [γ^{32}-P]ATP was used in the other.

† μmol per mg protein per min.

QUERCETIN INHIBITION OF CA^{2+} UPTAKE

We have found[28] that quercetin, which has previously been found to inhibit ATP hydrolysis in mitochondria,[30] chloroplasts,[31] and sarcopalsmic reticulum,[32,33] is an effective inhibitor of ATP-dependent Ca^{2+} uptake in sarcoplasmic reticulum. A generalized scheme of the partial reactions of ATP-dependent Ca^{2+} uptake has been developed over a period of years from work in a number of laboratories.[34] This scheme shows binding of two molecules of Ca^{2+} and one molecule of ATP to the ATPase. ATP is hydrolyzed to form $E\text{-}P_I$ with transfer of Ca^{2+} to the inner surface of the membrane. Release of Ca^{2+} is accompanied by transition of $E\text{-}P_I$ to $E\text{-}P_{II}$ and phosphate is subsequently released. $E\text{-}P_{II}$ can be formed from inorganic phosphate in the absence of Ca^{2+} and the presence of Mg^{2+}. When Ca^{2+} is added subsequently, $E\text{-}P_I$ is formed and, in the presence of ADP, phosphate transfer occurs, yielding ATP. ATP can also be synthesized by a reversal of the overall procedure. Once a Ca^{2+} gradient is established, release of Ca^{2+} will drive net ATP synthesis from ADP and P_i. This overall reaction leads to an active ATP-P_i exchange reaction.

Quercetin inhibits ATP hydrolysis, Ca^{2+} uptake, ATP-P_i exchange, ATP synthesis by a Ca^{2+} gradient, $E\text{-}P_{II}$ formation from P_i and ATP-ADP exchange but it does not inhibit $E\text{-}P_I$ formation from ATP (TABLE 3). These observations lead us to suspect that quercetin stabilizes the $E\text{-}P_I$ form of the enzyme so that the phosphoryl group cannot energize the release of Ca^{2+} to the interior of the vesicle nor be donated back to ADP and that this is the basis of its effect on energetic reactions of the ATPase molecule.[35]

QUERCETIN DOES NOT INHIBIT CA^{2+} RELEASE

The finding that it is possible to inhibit the (Ca^{2+} + Mg^{2+}-ATPase so that Ca^{2+} does not flow backwards through its channels of uptake (TABLE 3) has permitted us to ask the question whether Ca^{2+} release initiated by caffeine, Cl^-, or Ca^{2+} itself occurs through the reversal of the Ca^{2+} pump system.[28] We have used a skinned fiber system in which the sarcoplasmic reticulum retains its natural orientation with regard to the muscle fiber. Release of Ca^{2+} was

monitored by fiber contraction and uptake was monitored by fiber relaxation. We have been able to show that quercetin inhibits Ca^{2+} uptake in this system since it prevents Ca^{2+} loading by the sarcoplasmic reticulum (thereby eliminating caffeine-induced fiber contraction), and, moreover, when added together with caffeine, relaxation of the contracted fiber was greatly prolonged. In spite of these clear effects on Ca^{2+} uptake, we found that calcium release was not inhibited. In fact, the rate of release was apparently enhanced but this could be explained on the basis that quercetin inhibited the reuptake that occurred simultaneously with release. From these simple observations we have deduced that the channel for Ca^{2+} uptake is different from that for Ca^{2+} release.

TABLE 3

QUERCETIN INHIBITION OF SARCOPLASMIC RETICULUM ACTIVITIES *

Reaction	Quercetin, μM	Activity †	
		-Quercetin	+Quercetin
ATP hydrolysis	50	250	16
Ca^{2+} uptake	50	363	13
ADP-ATP exchange	50	388	22
ATP synthesis	100	73	12
Ca^{2+} release‡	100	110	20
ATP-P_i exchange	100	23	2
E-P_I formation	100	3.1	3.3
E-P_{II} formation	100	3.0	0.5
Ca^{2+} release	100	7.0§	26§

* The assays for ATP hydrolysis, Ca^{2+} uptake, ATP synthesis, Ca^{2+} release coupled to ATP synthesis, and Ca^{2+} release in skinned muscle fibers were previously described in Reference 28, for ADP-ATP exchange in Reference 27, and for ATP-P_i exchange, E-P_I and E-P_{II} formation in Reference 29.

† nmol per mg protein per min.

‡ Coupled to ATP synthesis.

§ Tension, mg/sec in skinned muscle fibers.

ANION TRANSPORT

In another section of this book,[37] we have reported on our studies with the anion transport inhibitor DIDS. This compound inhibits anion efflux from sarcoplasmic reticulum. It also inhibits Ca^{2+} transport, and it is possible, although not yet proven, that its effect on Ca^{2+} transport is linked to its ability to inhibit the cotransport of anions. In this respect, DIDS might be a useful probe for analysis of an anion channel in the sarcoplasmic reticulum. Preliminary experiments show that [^{3}H]H_2DIDS binds to the ATPase.

SUMMARY

In summary, we have begun to characterize three different ion pathways in the sarcoplasmic reticulum. Ca^{2+}-ionophoric activity has been traced to a

13,000-dalton CNBr fragment localized at the amino terminus of the ATPase molecule. The pathway involved in Ca^{2+} release can be distinguished from the pathway involved in Ca^{2+} uptake by its insensitivity to quercetin. An anion pathway is sensitive to DIDS and appears to be localized in the ATPase molecule.

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