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Trypsin-Induced Calcium Efflux from Sarcoplasmic Reticulum: Evidence for the Involvement of the (Ca^{2+} + Mg^{2+})-ATPase

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Summary. Trypsin digestion of the sarcoplasmic reticulum membrane at 35 to 43°C leads to an increased calcium permeability, the temperature dependence of which suggests tryptic exposure or creation of a channel rather than tryptic release of a mobile carrier (K.C. Toogood et al., *Membr. Biochem.* 5:49–75, 1983). Here we show that: (1) the digested vesicles both pump and leak calcium, demonstrating that the vesicles remain intact; (2) an increased rate of efflux is not observed for membranes digested and kept at 15°C, but a temperature shift to 35°C following arrested digestion leads to the development of increased calcium permeability, indicating that a digestion step at the lower temperature potentiates increased permeability which develops rapidly as a result of a trypsin-facilitated protein conformational change at the higher temperature; (3) two inhibitors of the ATPase, adenyl-5′-yl imidodiphosphate and dicyclohexyl-carbodiimide, both measurably retard the development of increased permeability at the higher temperature following arrested digestion, suggesting that these inhibitors bind to the target protein and prevent the conformational change responsible for the permeability increase, and further suggesting that the ATPase is the target for the trypsin; (4) digestion of the ATPase at 15°C follows the same initial cleavage pattern as at 35°C, but the cleavage stops or drastically slows down after the second digestion step at the lower temperature, whereas the digestion continues beyond the second step at the higher temperature, showing that an early digestion step may be responsible for potentiating increased permeability; (5) the permeability increase following digestion at 15°C and incubation at 35°C correlates ($r > 0.98$) with the second tryptic cleavage step of the calcium ATPase, providing more support for the ATPase as the trypsin-sensitive efflux site; and (6) the rate of efflux depends on the concentration of the doubly cleaved ATPase molecules to the first power; the null hypothesis that the efflux actually depends on the cleaved ATPase concentration to the second or higher power was examined using the F test and can be rejected (confidence > 0.90 to 0.98), suggesting that the efflux pathway is through a single ATPase molecule. We speculate that the pathway for increased calcium permeability is the one employed during calcium uptake and that there is a functional separation of the ATPase and calcium channel activities by trypsin digestion at 15°C followed by incubation at 35°C.

Key Words sarcoplasmic reticulum · trypsin digestion · calcium ATPase

Introduction

The sarcoplasmic reticulum (SR)[1] is well-studied owing to its ATP-dependent calcium pumping, ease of isolation, and simple protein composition (MacLennan, 1970; MacLennan & Holland, 1975; Ikemoto, 1982). The ATP-driven accumulation of calcium by this membrane has been shown to be responsible for muscle relaxation (Marsh, 1952; Hasselbach & Makinose, 1961; Ebashi & Kodama, 1965). A great deal has been learned about the kinetic steps involved in this ATP-driven calcium uptake (Hasselbach & Makinose, 1961; McFarland & Inesi, 1971; Martonosi & Halpin, 1971; Carvalho, de Sousa & de Meis, 1976; Pick & Racker, 1979; Tanford, 1984). The relationship of these kinetic steps to specific structural changes in the calcium ATPase has been the subject of intensive investigation in many laboratories (Dupont, 1976; Murphy, 1976; Yamada & Ikemoto, 1978; Guillain et al., 1980; Andersen et al., 1982, 1985, 1986; Pick & Karlish, 1982).

One fruitful approach to the investigation of the structure/function relationships in the calcium pump protein has been the use of trypsin digestion. The first of these studies demonstrated that the tryptic digestion of the ATPase proceeds initially in

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[1] *Abbreviations:* AMP-PNP, adenyl-5′-yl imidodiphosphate; CD, circular dichroism; DCCD, dicyclohexylcarbodiimide; EGTA, ethylene-bis-β-(aminoethyl ether)-N,N,N′, N′ tetra-acetic acid; SDS, sodium dodecyl sulfate; SR, sarcoplasmic reticulum; STI, soybean trypsin inhibitor; T_1, the first tryptic cleavage of the calcium ATPase; T_2, the second tryptic cleavage step.

a stepwise fashion (Thorley-Lawson & Green, 1973; Migala, Agostini & Hasselbach, 1973; Inesi & Scales, 1974; Stewart & MacLennan, 1974) with the first cleavage (T_1) quantitatively yielding two fragments A and B, followed by a second cleavage (T_2) occurring in fragment A, yielding subfragments A_1 and A_2. The approximate locations of T_1 and T_2 were identified (Klip, Reithmeier & MacLennan, 1980). The amino acid sequence of the calcium ATPase determined from the DNA sequence provided the basis to pinpoint T_1 and T_2 in the primary structure (MacLennan et al., 1985; Andersen et al., 1986).

More recent use of trypsin digestion has been to probe the function of the ATPase molecule. No change in the structure or function of the calcium ATPase has yet been associated with T_1, whereas T_2 has been shown to uncouple ATPase activity from calcium pumping (Scott & Shamoo, 1982), to alter one of the high-affinity calcium binding sites (Scott & Shamoo, 1984), and to inhibit the ability of the ATPase to form two-dimensional arrays in the presence of vanadate (Dux & Martonosi, 1983).

Our previous investigation showed that trypsin digestion creates the potential for increased calcium efflux (Toogood et al., 1983). In our initial study, no attempt was made to identify the molecule or molecules responsible for the trypsin-sensitive efflux. The aim of the present study was to determine the identity of the protein or proteins responsible for the increased calcium efflux rate following trypsin digestion.

The data reported here suggest T_2 may be the critical step to potentiate the increased calcium permeability, with the actual development of the increased permeability strongly dependent on the temperature. An ATPase inhibitor and an ATP substrate analogue both slow the development of increased permeability following the digestion step. This retardation by molecules that bind to the ATPase provides one line of evidence that this molecule is the target for the digestion-induced permeability increase. The increased permeability was also found to exhibit a first-power dependence on the amount of ATPase molecules that have undergone the second cleavage, T_2, thus providing a second line of evidence that the ATPase is the site responsible for the trypsin-stimulated efflux. A first-power dependence on concentration suggests that the calcium pathway is almost certainly through a single ATPase molecule. It is therefore proposed that the calcium ATPase contains a cryptic ion pathway that can be exposed by proteolytic digestion.

Materials and Methods

All Chemicals were reagent grade. Trypsin from bovine pancreas, soybeam trypsin inhibitor, alkaline phosphatase (AP), ATP, and calcium ionophore (A23187) were purchased from Sigma and $^{45}CaCl_2$ from New England Nuclear.

SR vesicles were isolated from the homogenized skeletal muscles of female white rabbits by the method of differential centrifugation as described by Martonosi, Donley and Halpin (1968); all operations were carried out at pH 6.8 and at 4°C or colder. The resulting membranes at 5 to 10 mg/ml protein (estimated by the Bradford, 1976, assay) were suspended in 1.0 M sucrose, 0.1 M KCl, 50 mM Tris, pH 8.0 using a Dounce homogenizer, quickly frozen in 1.0-ml aliquots using a cold methanol bath, stored at −70°C, and then thawed as needed at room temperature. If further purification was desired, the vesicles were sedimented into discontinuous sucrose gradients as described by Mrak and Fleischer (1982) and collected at the 43% sucrose step.

ATPase activity was estimated by liberation of inorganic phosphate, which was complexed as phosphomolybdate and measured spectrophotometrically as described previously (Chen, Toribara & Warner, 1956).

Calcium loading into vesicles was done at 35 or 37°C in the presence of oxalate. The usual uptake medium contained 0.16 M sucrose, 0.1 M KCl, 0.1 M NaCl, 5.0 mM ATP, 2.5 mM oxalate, 0.167 mM $^{45}Ca^{2+}$, 5.0 mM Mg^{2+}, 0.16 M histidine (or other buffer) at pH 6.8 and an SR protein concentration of 0.16 mg/ml. Generally greater than 90% of the calcium was accumulated by the vesicles within 15 sec after ATP addition as measured by the Millipore filtration technique (Martonosi & Feretos, 1964), corresponding to an uptake rate of 2 to 4 μM Ca^{2+}/mg protein-min.

In one experiment calcium uptake was determined in the absence of oxalate using the method in Scott and Shamoo (1982). The medium consisted of 0.2 mg/ml SR protein in 100 mM HEPES, pH 6.8, 0.1 M KCl, 5 mM $MgCl_2$ and 0.1 mM $^{45}CaCl_2$. The uptake was initiated by the addition of ATP (5 mM) and the calcium accumulation was estimated by filtration of 1-ml aliquots at various times after ATP addition. The filters were washed, dried and counted.

Calcium radioactivity for uptake assays with or without oxalate was estimated using a Beckman LS 9000 liquid scintillation counter.

Trypsin digestion was carried out at 35 or 15° on previously loaded vesicles in uptake medium at a trypsin/SR protein ratio of 1:20, mg/mg, for varying lengths of time as specified. Digestion was stopped by the addition of soybean trypsin inhibitor, STI/trypsin, 4:1, mg/mg. Following STI addition, samples were incubated for at least 5 min before further use.

Membrane samples for polyacrylamide gel electrophoresis were actively loaded in 0.48 mM Ca^{2+}, 9.0 mM oxalate, 5 mM Mg^{2+}, 0.9 mg/ml SR protein, 1 mM ATP, and 0.1 M HEPES at pH 7.5. The higher calcium and oxalate concentrations were used to prevent premature calcium efflux, so that calcium efflux rates could be measured after an alkaline phosphatase incubation to remove the residual ATP. The higher pH was used to improve the hydrolysis of ATP by the alkaline phosphatase. The necessity of removing the residual ATP is explained in the text. Trypsin was added to initiate digestion and at appropriate times STI was added to aliquots to stop the digestion.

The vesicles were diluted with 10 mM Tris buffer (pH 7.5) and sedimented at 90,000 rpm for 20 min in a Beckman Airfuge. The pellets were washed twice with 5% glycerol and resedi-

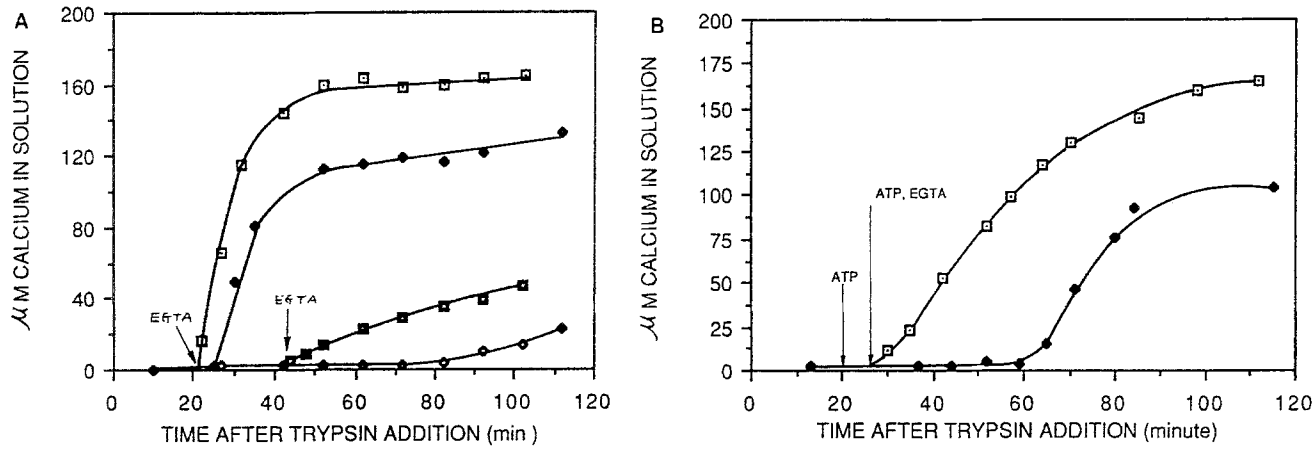

Fig. 1. Effect of EGTA on initiation of calcium efflux. Vesicles were loaded using 1 mM ATP as described in Materials and Methods. One portion of membranes was digested with trypsin for 20 min and the digestion was stopped with STI as described in Materials and Methods. To the other portion, the same amount of premixed trypsin plus STI inhibitor was added; these membranes show no evidence of digestion and serve as undigested controls. The digested and undigested membranes were further subdivided and efflux monitored with and without added EGTA using the Millipore filtration assay to determine calcium external to the membranes: (*A*): undigested membranes, no additions (◇); undigested membranes, addition of EGTA (□); digested membranes, no additions (◆); digested membranes, addition of EGTA (⊡). To enhance the distinction between plus and minus EGTA in the digested membranes, the experiment was repeated with the addition of 1 mM extra ATP to delay onset of calcium efflux (*B*): ATP added alone (◆); ATP added with EGTA (⊡)

mented for 10 min. The pellets were soaked in 1% mercaptoethanol in preparation for electrophoresis. The electrophoresis was performed according to Laemmli (1970) or according to Dunker and Rüeckert (1969) and Weber and Osborn (1969) using 10% gels containing 0.8% bisacrylamide.

Results

Trypsin Digestion Yields Vesicles that both Pump and Leak Calcium

Our previous studies revealed that trypsin digestion at 35°C or above leads to increased calcium efflux, but only after the ATP became depleted (Toogood et al., 1983). It was left unresolved whether the residual ATP promotes a calcium pumping that compensates for the increased efflux rate, thereby simply masking the increased permeability, or whether the residual ATP binds to the relevant protein in a way so as to prevent efflux.

If ATP prevents efflux by a compensating pumping of calcium, then addition of EGTA should block uptake by chelating the external calcium (Weber, Herz & Reiss, 1966) and thereby immediately unmask the calcium efflux rate. If ATP acts by binding to a protein, then EGTA should not lead to an immediate increase in permeability.

Undigested control membranes show that EGTA addition leads to the unmasking of the same slow efflux rate as is observed following ATP depletion (Fig. 1*A*). Both undigested control and digested membranes are induced to develop increased permeability immediately following EGTA addition (Fig. 1*A*). Added ATP delays the onset of efflux in digested membranes, but that delay is prevented if EGTA is added concomitantly with the extra ATP (Fig. 1*B*). These results suggest that ATP-driven calcium uptake occurs in membranes that have been made leaky by trypsin digestion and that the ATP-driven uptake masks the increased permeation rates that become observable following ATP depletion.

If indeed the trypsin-digested membranes can continue to actively transport calcium, then it should be possible to observe this directly by the addition of more ATP after leakage has developed. This experiment with variations and controls is shown in Fig. 2.

Both at 35 (Fig. 2*A*) and 43°C (Fig. 2*B*), addition of oxalate alone is found to induce accumulation of calcium into the digested vesicles. This oxalate-induced uptake is due to the precipitation of calcium onto the residual calcium oxalate crystals inside the membrane vesicles, a precipitation resulting from the lowered calcium solubility following the jump in oxalate concentration. These results reconfirm the leakiness of the digested membranes

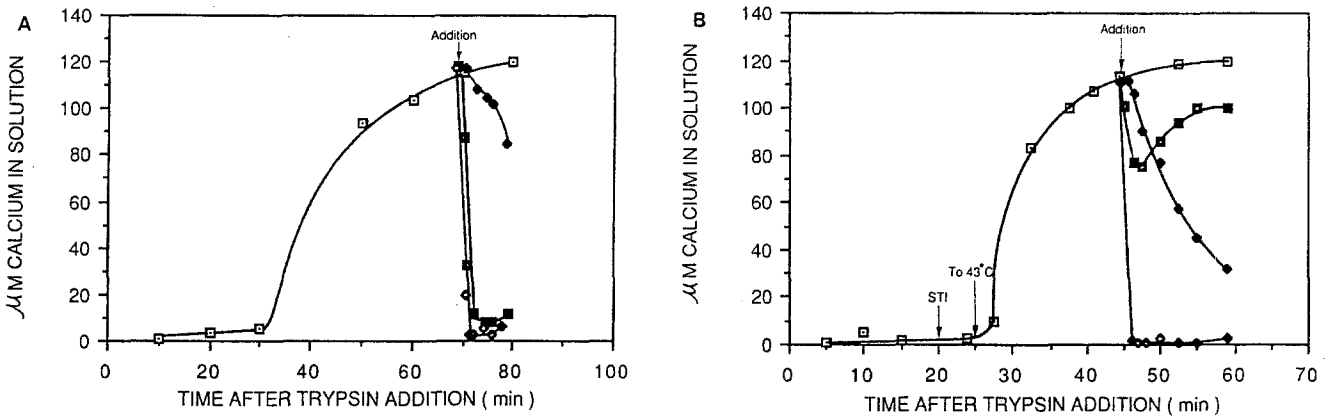

Fig. 2. Calcium uptake after trypsin-induced leakage. SR membrane vesicles were loaded, treated with trypsin for 20 min and digestion was stopped with STI as described in Fig. 1. Just before the expected onset of rapid calcium efflux at 35°C, one portion of the membranes was shifted to 43°C. Re-uptake of calcium was studied both at 35°C (*A*) and at 43°C (*B*). The effects of three different additions were determined at both temperatures: addition of oxalate alone (final concentration, 9 mM) (◆); addition of ATP alone (final added concentration, 1 mM) (□); addition of both ATP and oxalate at the indicated concentrations (◇); control with no additions (⊡)

Table. ATP hydrolysis rates in digested and undigested SR membranes[a]

Sample	Total ATPase	Ca^{2+}-ATPase	Mg^{2+}-ATPase
35°C (before digestion)	2.04	1.08	0.96
35°C (after digestion)	2.10	1.55	0.55
43°C (after digestion)	3.45	2.42	1.02

[a] All ATPase activities are expressed as μmol P/mg protein/min. Phosphate liberation was determined by yield of inorganic phosphate (Chen et al., 1956) in 0.16 M histidine, pH 6.8, 0.1 M KCl, 0.167 M sucrose, 1 mM $MgCl_2$, 0.2 mg/ml SR protein and 0.167 M $CaCl_2$ for total ATPase rate or 1.0 mM EGTA in place of the calcium for the Mg^{2+}-ATPase rate. After the ATPase rates were determined for undigested vesicles, trypsin was added (1 mg trypsin/20 mg SR protein) and the vesicles were digested for 11 min. STI (10 mg STI/1 mg trypsin) was added to stop the digestion. Half the vesicles were shifted to 43°C. The ATP hydrolysis rates were determined from the initial slopes of the curves of liberated phosphate versus time of incubation. These particular membranes were not subjected to sucrose gradient centrifugation and so contain a considerable amount of contaminants, which result in the high background Mg^{2+}-ATPase activities and the low Ca^{2+}-ATPase values, but nevertheless these data are sufficient to form the basis for the conclusions reached in the text.

and also that they are still intact and capable of harboring the calcium oxalate crystals inside.

In the presence of both ATP and oxalate, the vesicles very rapidly pump and retain the calcium at both 35 and 43°C (Fig. 2*A* and *B*). The high oxalate concentration prevents the accumulated calcium from leaking back out again, and thus the rapid calcium pumping can be observed even in the leaky, trypsin-digested vesicles.

In the presence of added ATP alone, rapid calcium uptake is observed at both 35 and 43°C, but at 43°C the net uptake is observed to be transient. Our explanation of these results is that the vesicles can accumulate calcium only so long as there is sufficient ATP. At 43°C the rapid hydrolysis of ATP causes a fast depletion of this molecule, thereby leading to a transient uptake of Ca^{2+} at the higher temperature. Further support for this interpretation is provided by comparing ATP hydrolysis rates in undigested membranes with those rates in digested membranes at 35 and at 43°C (Table). As required by the explanation based on ATP depletion, the ATP hydrolysis rates are higher at 43°C as compared to 35°C in the digested membranes. Note also that the trypsin digestion does not lower the ATP hydrolysis rate.

Removal of ATP by Alkaline Phosphatase

Since the SR membranes can continue to pump calcium even when digested, the measurement of calcium efflux requires either the removal of the ATP or else the use of an ATP-independent assay such as the measurement of calcium efflux following passive accumulation of labeled calcium (Inesi & Scales, 1974; Millman, 1980). The advantages of calcium oxalate-loaded vesicles for our purposes are: (1) the measurement of uptake and efflux can be carried out under very similar assay conditions (*see* Fig. 2); (2) calcium oxalate-loaded vesicles require smaller amounts of membrane and radioactivity compared to the passive assay; and (3) the low

levels of free calcium and high content of calcium in the calcium oxalate-loaded vesicles slow down and prolong the calcium efflux, thus facilitating the rate measurement in our very leaky trypsin-digested membranes. It should be noted, however, that the free calcium levels are so low that characteristics of the release pathway such as determination of K_m cannot be accomplished by our assay conditions and also that the measured leakage rates are exceptionally slow—but for our experiments the advantage of controlled release from very leaky vesicles probably outweigh the shortcomings due to the presence of calcium oxalate. Therefore, we elected the approach of removing the unused ATP.

Figure 3 shows that incubating previously loaded SR membranes in alkaline phosphatase leads to the loss of the ability of these membranes to accumulate freshly added calcium. After about 20 min of incubation under these conditions, the ATP levels have dropped sufficiently so that calcium uptake is drastically reduced. Because the residual accumulation is not eliminated by prolonged incubation with alkaline phosphatase, this slight calcium uptake is probably due to passive calcium influx onto the residual calcium oxalate crystals (*see* Fig. 2), in which case even this slight accumulation rate would completely disappear under efflux conditions. Thus, alkaline phosphatase seems to provide a simple, reliable method for removing residual ATP so that calcium efflux can be cleanly determined in calcium oxalate-loaded, partially digested membranes.

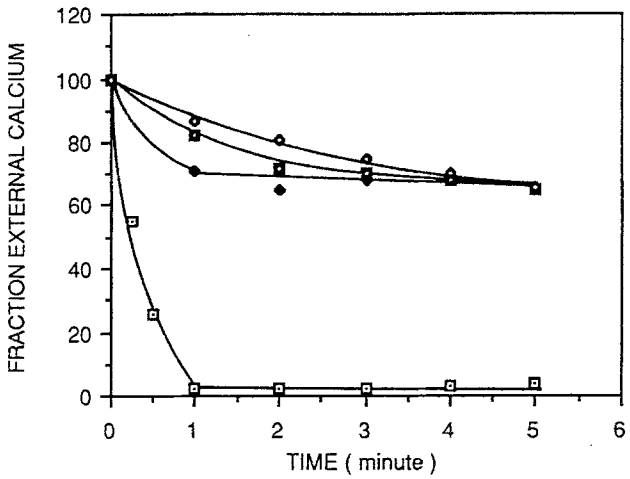

Fig. 3. Characterization of alkaline phosphatase treatment. SR vesicles were preloaded with calcium at 35°C for 3 min. Membrane solutions were then incubated with alkaline phosphatase (AP) at 35°C. At different time intervals, uptake of calcium was monitored for control and AP-treated vesicle solutions: control (⊡); AP 20 min (◆), AP 40 min (□), AP 50 min (◇)

Characterization of Calcium Efflux Following Trypsin Digestion at 15° and 35°C

Previous work by Scott and Shamoo (1982) suggested that trypsin digestion at room temperature leads to an uncoupling of calcium pumping and ATP hydrolysis concomitant with T_2. It was suggested that the uncoupling is an intramolecular event (Berman, 1982) because the trypsin digestion did not lead to an increase in membrane permeability. In contrast, our results (Figs. 1 & 2; and Toogood et al., 1983) show that trypsin digestion does lead to an increase in calcium permeability. The most obvious difference between our work and that of Scott and Shamoo (1982) is the higher temperature that we used.

Assuming temperature to be the critical variable, two possibilities are: (1) the critical digestion step to potentiate leakage simply does not occur at the lower temperature, or (2) the critical digestion step does occur at the lower temperature, but the higher temperature is necessary for a conformational change that causes the increased efflux rate to develop.

Digestion at 15°C alone was found to not affect calcium permeability and short-term incubation at 35°C alone was also found to not affect calcium permeability. But the combination of digestion at 15°C followed by short-term incubation at 35°C was found to lead to a rapid increased calcium permeation rate (Fig. 4). These data show that the digestion step critical for increased permeability does occur at low temperature, but that the incubation at an elevated temperature is necessary for the expression of the increased efflux rate. We speculate that the digestion step reduces the stability of the target protein and that the elevated temperature promotes a conformational change that results in increased permeability. These data provide a possible reconciliation of our data with the previous work of Scott and Shamoo.

Scott and Shamoo (1982, 1984) also showed that trypsin digestion at room temperature is associated with an uncoupling of calcium pumping from ATP hydrolysis that correlates with T_2, yet our data seem to show undiminished calcium uptake despite the trypsin digestion (Figs. 1 & 2). We used oxalate in our pumping assays; Scott and Shamoo (1982) did not. To test whether oxalate is responsible for the apparent difference in our results, we compared calcium uptake of trypsin-digested membranes with and without oxalate (Fig. 5). These data suggest that oxalate is indeed reponsible for the difference between our work and the previous reports of Scott and Shamoo (1982, 1984).

When the calcium uptake is carried out in the absence of oxalate, the membranes digested for 3 hr show a substantially diminished calcium uptake,

and membranes that have been digested and shifted to 35°C show no calcium pumping at all (Fig. 5*A*). Although we have not attempted to quantitate the rate of calcium uptake and the amount of tryptic digestion in this experiment, we have determined that the T_2 cleavage is not quite complete by 3 hr of digestion under our conditions (*see below*), suggesting that the residual uptake is due to the residual singly cleaved ATPase molecules. These results are in qualitative agreement with the previous work of Scott and Shamoo; qualitative agreement is sufficient for our verification since the detailed quantitative correlation has already been established in the previous work. On the other hand, if oxalate is included in the uptake assay, then control, trypsin-digested and trypsin-digested/temperature-shifted membranes all exhibit similar rates of calcium pumping (Fig. 5*B*). The slight difference in uptake rates follows the same order that is much more apparent in the absence of oxalate, namely control > digested > digested/temperature-shifted, suggesting that uncoupling also occurs in the presence of oxalate, but that the observation of the effect is greatly masked by the oxalate.

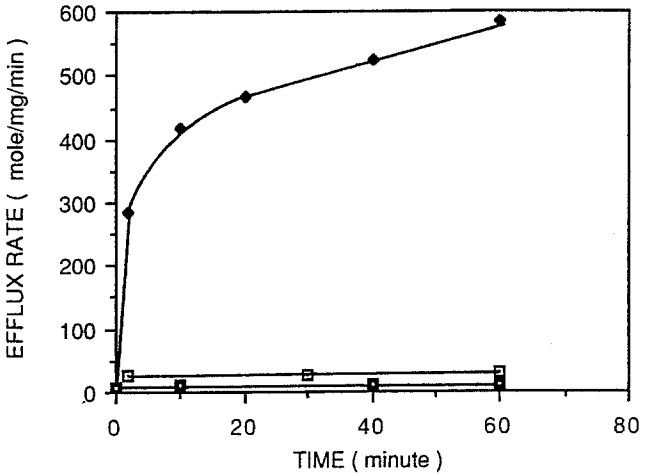

Fig. 4. Effect of trypsin digestion at 15°C followed by a temperature shift. Sarcoplasmic reticulum membranes were loaded with labeled calcium as described in Materials and Methods. Control membranes were incubated without digestion. To a second aliquot, trypsin (1 mg trypsin/20 mg SR protein) was added and allowed to incubate at 15°C. Digestion was stopped after 4 hr with the addition of soybean trypsin inhibitor. Aliquots of both the digested and undigested membranes were shifted to 35°C for various periods of time and then re-equilibrated at 15°C for measurement of efflux rates; in addition, aliquots of the digested membranes were maintained at 15°C without the temperature shift. The resulting data are plotted for: membranes subjected to trypsin digestion and shifted to 35°C (◆); control membranes with no digestion but shifted to 35°C (⊡); and control membranes subjected to digestion but no temperature shift (□)

Identification of the Trypsin-Sensitive Efflux Protein

All of the calcium in both light and heavy fractions of SR membranes has been found to be subject to trypsin-stimulated release, indicating that the trypsin-sensitive protein is found in all the vesicles (Z. He, *personal communication*). Thus, an obvious candidate for the trypsin-sensitive release site is the calcium ATPase molecule. The data of the Table suggest that the calcium stimulation of ATPase activity survives the trypsin digestion, suggesting that

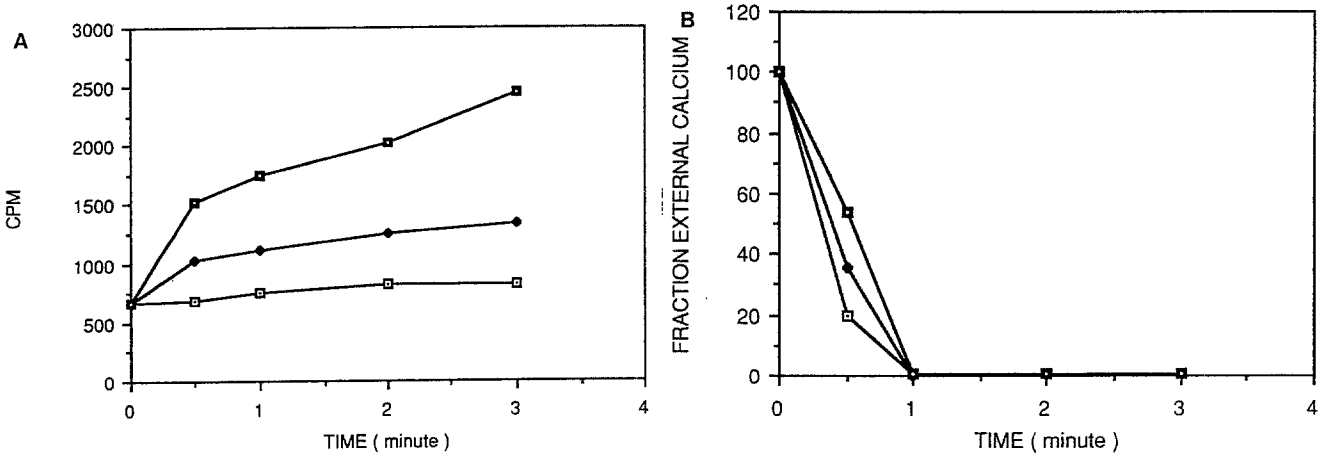

Fig. 5. Effects of oxalate on calcium uptake in digested membranes. SR membranes were digested for 4 hr under the usual conditions or the membranes were left undigested; next the membranes were variously treated (*see below*) and finally the membranes were assayed for calcium uptake in the absence of oxalate as described in Materials and Methods (*A*); the filter was counted in these experiments, so calcium uptake is represented by an upward curve. The three uptake assays were carried out at 15°C using: control membranes with no treatment at all (□); membranes digested for 4 hr at 15°C and kept at 15°C for the uptake assay (◆); and membranes shifted to 35°C for 15 min following the digestion at 15°C (⊡). The experiment was repeated with oxalate as described in Materials and Methods (*B*); the filtrate was counted in these experiments and so calcium uptake is represented as a downward curve. As before the uptake assays were carried out at 15°C using: control membranes (⊡); membranes digested for 3 hr and kept at 15°C (◆); and membranes incubated at 35°C for 15 min following the digestion at 15°C (□)

both the ATPase and calcium binding sites remain intact following the critical tryptic digestion step. If the trypsin-sensitive site for calcium release is the calcium ATPase, then substrate analogues or ATPase inhibitors would be expected to stabilize the protein, which in turn would retard or prevent the conformational change at the elevated temperature and thereby slow the development of the permeability increase following the tryptic digestion step. AMP-PNP is a well-characterized nonhydrolyzable analogue of ATP (Yount et al., 1971) and DCCD is a specific inhibitor of the ATPase that apparently works by blocking the high-affinity calcium binding sites (Pick & Racker, 1979; Murphy, 1981).

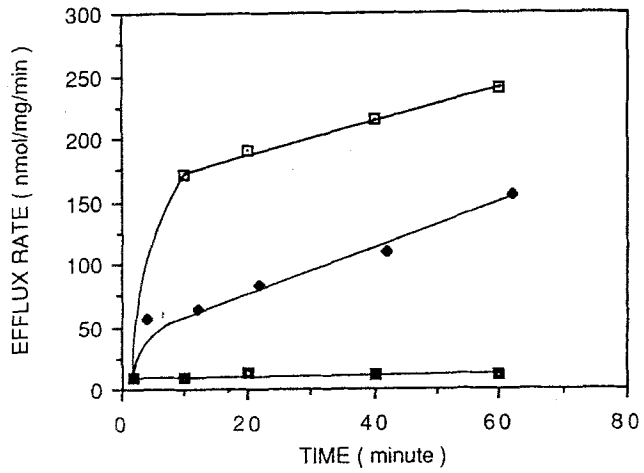

Fig. 6. Effects of AMP-PNP and DCCD on the development of increased permeability in membranes digested at 15°C and shifted to 35°C. SR membranes were trypsin digested at 15°C for 4 hr as described previously. Five min prior to the temperature shift, AMP-PNP at 10 mM or DCCD at 0.6 mM was added to respective aliquots. The membranes were then shifted to 35°C for the times indicated and cooled back to 15°C for the measurement of efflux rates. The data are plotted for: digested control membranes with no additions (⊡); digested membranes with added AMP-PNP (◆); and digested membranes with added DCCD (□)

SR membranes were digested at 15°C for 4 hr, then shifted to 35°C for various periods of time with and without the addition of AMP-PNP or DCCD just prior to the temperature shift. Following the incubation at 35°C, the membranes were returned to 15°C for the measurement of calcium efflux. AMP-PNP and DCCD were both found to markedly slow the development of increased calcium permeability, with DCCD exhibiting a much larger effect (Fig. 6). The more pronounced effect of DCCD could be due to its covalent binding to the target molecule, whereas AMP-PNP binds reversibly. These data support the supposition that the calcium ATPase is a likely site for the trypsin-sensitive permeability pathway. For trypsin-induced efflux developed under slightly different conditions (but yielding a channel almost certainly equivalent to the one described here), DCCD and AMP-PNP do not retard efflux if added after the development of the increased permeation rates (Folsom, 1984), suggesting that both of these reagents act by preventing the conformational change at 35°C, not by direct inhibition of calcium efflux through the channel.

Correlation of Trypsin Digestion Pattern with Calcium Efflux

Polyacrylamide gels of membranes digested for various times at 15°C are shown in Fig. 7(*A*) and those from samples digested at 35°C are shown in Fig. 7(*B*). Note that the first two cleavage steps of the

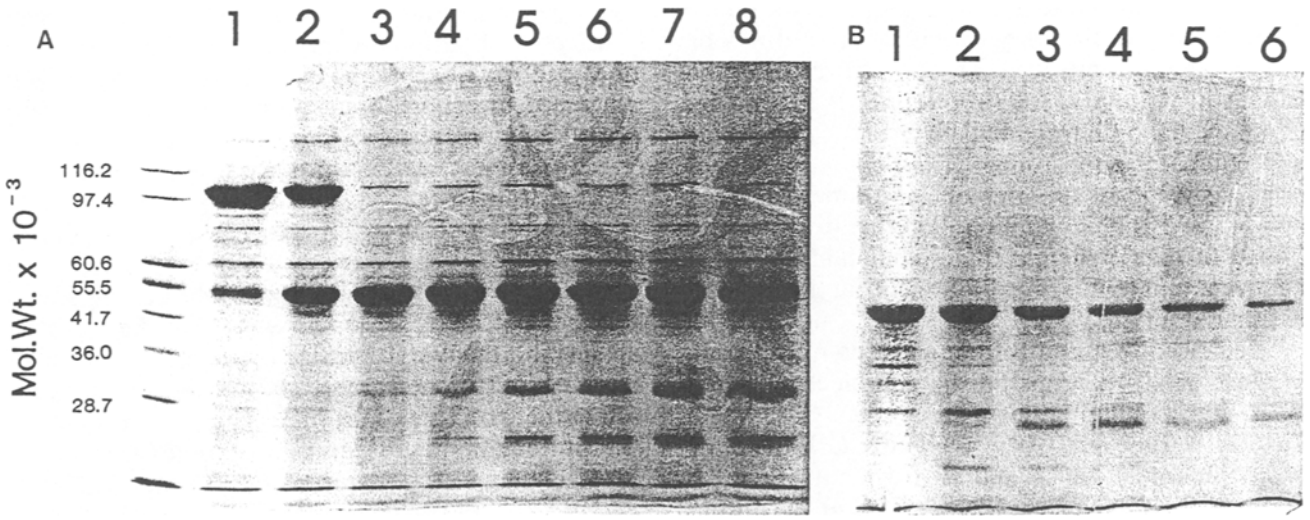

Fig. 7. Polyacrylamide gels of digested membranes at 15 and 35°C. SR vesicles were prepared as stated in Materials and Methods. For the 15°C digestion (*A*), the times of digestion were (1) 0.0; (2) 1.0; (3) 10.0; (4) 20.0; (5) 40.0; (6) 60.0; (7) 120.0; and (8) 180.0 min. For the 35°C digestion (*B*), the times of digestion were (1) 1.0; (2) 5.0; (3) 20.0; (4) 30.0; (5) 40.0; and (6) 50.0 min

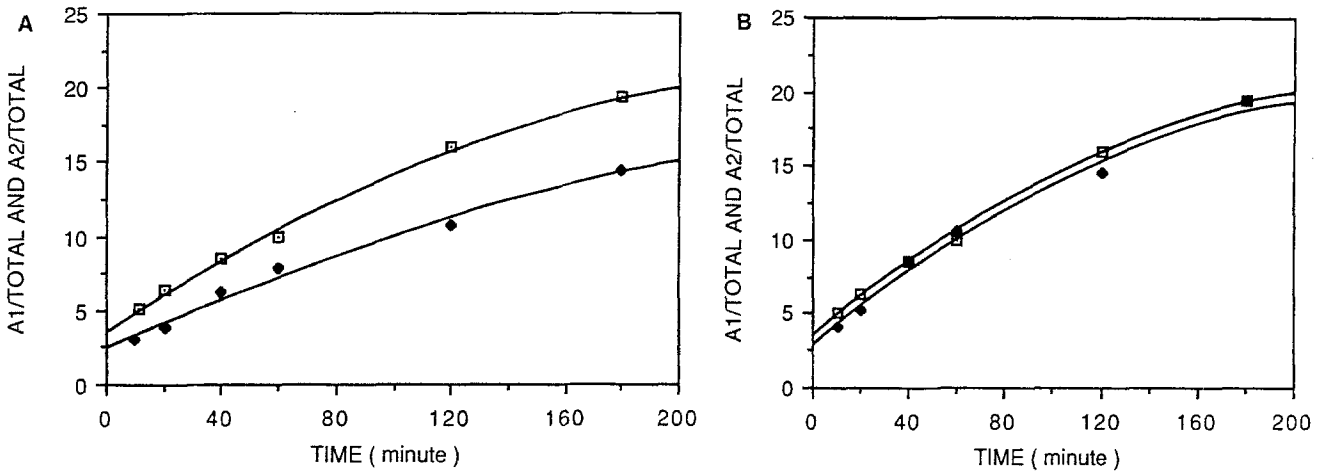

Fig. 8. Amount of digestion versus time. SR vesicle solutions were loaded as described previously. Trypsin (1 mg trypsin/20 mg SR protein) was added and allowed to incubate at 15°C and digestion was stopped at 10, 20, 40, 60, 120, and 180 min. The aliquots were treated and subjected to gel electrophoresis as stated in Materials and Methods. The resulting sample lanes were scanned using an Ultroscan XL Laser Densitometer. The A/B, A_1 and A_2 bands were cut and weighed. The total weight of the three bands was used to normalize the data from the various gels. In 8(*A*) the data are expressed as A_1/(Total Weight) (⊡) and as A_2/(Total Weight) (◆). In 8(*B*) the data are normalized assuming equivalent completeness of digestion at 3 hr for both A_1 (⊡) and A_2 (◆)

calcium ATPase are apparently the same at both temperatures.

For the gels from the samples incubated at 35°C, the initial A_1 fragment decreases in intensity and at the same time a new fragment of slightly lower molecular weight appears (Fig. 7*B*), suggesting that A_1 is cleaved to generate the new band. This extra digestion step has been noted previously (Andersen et al., 1985). The lack of any trace of cleavage of the A_1 band in the samples incubated at 15°C (Fig. 7*A*) suggests that digestion beyond T_2 does not occur or is greatly slowed at the lower temperature.

The band intensities of A_1 and A_2 were estimated as a fraction of the total stain intensity. Plotting the fractional intensities as a function of time of digestion shows that the amount of A_1 and A_2 both increase monotonically (Fig. 8*A*). Normalizing the amount of A_1 and A_2 and replotting the data (Fig. 8*B*) show that both fragments have exactly the same time course of appearance. If either A_1 or A_2 suffered a significant amount of additional cleavage, then the two curves would not coincide as they do. This is further evidence that significant additional digestion apparently does not occur at 15°C during this time interval.

Because the A and B fragments do not separate on these gels, we repeated the gel electrophoresis using the sodium phosphate tube gel system (Dunker & Rueckert, 1969; Weber & Osborn, 1969) which separates the A and B fragments (Scott & Shamoo, 1982). These gels (*not shown*) reveal that T_2 is not yet complete even after 4 hr of digestion. Thus, at the low temperature under our conditions, the ATPase evidently remains well-folded and thus shows considerable resistance to tryptic digestion.

Quantitative comparison of the amounts of A_1 or A_2 with the calcium efflux rate provides a means of testing for a correlation between these two functions (Fig. 9). The efflux rates show a straight-line dependence on the amounts of A_1 and A_2, with correlation coefficients of 0.989 and 0.970, respectively.

Discussion

In this paper we show that, at the early stages of trypsin digestion, SR vesicles can both pump and leak calcium (Figs. 1 & 2). The ability of the same vesicles to both pump and leak calcium is probably due to a mixture of ATPase molecules at various stages of digestion. The polyacrylamide gels support this interpretation (Fig. 8).

It is almost certain that the trypsin-induced increases in permeability are due to a change in the protein rather than some indirect effect leading to disruption of the bilayer. Such an interpretation is consistent with electron micrographs of extensively digested SR membranes showing that the digestion seems to remove particulate structures from the surfaces of the membrane, but otherwise does not affect the structure of the bilayer or the structure of the intramembranous protein particles (Stewart & MacLennan, 1974; Yamanaka & Deamer, 1976).

It has been shown that the approximately 20,000 dalton fragment A_2 can function as an

ionophore when isolated (Shamoo et al., 1976; Stewart, MacLennan & Shamoo, 1976), presumably by a mobile carrier mechanism. Thus, trypsin digestion could act by releasing a hydrophobic mobile carrier peptide. Alternatively, trypsin digestion could act by creating or exposing a channel.

Measuring the temperature dependence of conductance over temperatures corresponding to lipid phase transitions has been previously used to distinguish channels from mobile carriers (Krasne, Szabo & Eisenman, 1971; Boheim, Hanke & Eibl, 1980). Martonosi (1974) previously showed that the SR membranes exhibit a peak in the differential scanning calorimetry trace near 0 to 4°C, evidently due to a lipid phase transition. A lipid phase transition near 3°C has very recently been confirmed by X-ray diffraction measurements (Pascolini, Asturias & Blasie, 1987). Thus, calcium efflux by a mobile carrier would be expected to exhibit a significant drop in rate near 0°C as the lipids undergo the phase transition, whereas, if the lipid phase change does not alter the structure of the channel, then channel-mediated calcium efflux would be less sensitive to temperature changes as the temperature is dropped to 0°C.

The time course of calcium efflux as a function of temperature between 10 and 0°C for the trypsin-digested membranes has been compared with the efflux induced by A23187. A23187-mediated calcium permeation shows a substantial drop in rate as the temperature is lowered from 10 to 0°C, a result that is consistent with its known mobile carrier mechanism and the known lipid phase change. The trypsin-digested membranes on the other hand exhibit only a very small change in the efflux rate, indicating that the increased efflux rate in the digested membranes is due to a channel mechanism, not the result of the trypic release of a mobile carrier (Toogood et al., 1983).

An apparent difference between our work (Toogood et al. 1983) and that of Scott and Shamoo (1982) is that they reported trypsin digestion did not increase membrane leakiness whereas we reported that trypsin digestion leads to increased permeability. Here we show that the critical difference between our two experiments lies in the temperature difference—at lower temperatures, the early stages in digestion do not promote leakiness, whereas at the higher temperatures digestion does promote increased leakiness (Fig. 4).

Also, Scott and Shamoo (1982) reported that digestion uncouples calcium pumping whereas our data (Figs. 1 & 2) show that the digested membranes continue to pump at an apparently undiminished rate. In Fig. 5 we show that this apparent discrepancy is due to oxalate. It is interesting that

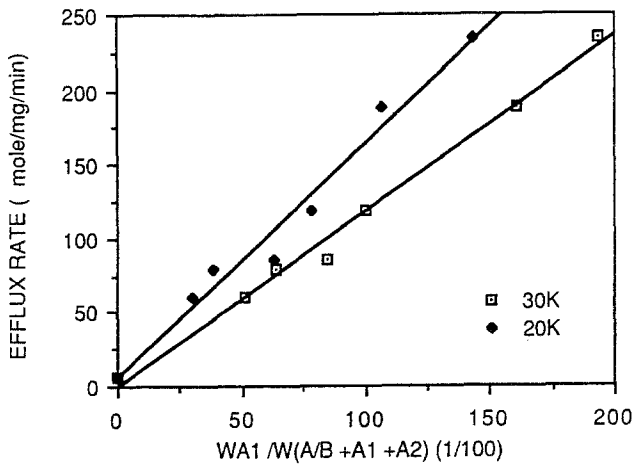

Fig. 9. Correlation between efflux rate and amount of digestion. SR membranes were loaded with $^{45}Ca^{2+}$ as described previously. Trypsin (1 mg trypsin/20 mg SR protein) was added and allowed to incubate at 15°C and digestion was stopped at different times with the addition of soybean trypsin inhibitor. Aliquots were taken and treated as described in Materials and Methods to run the SDS gel. The resulting gel was scanned by an Ultroscan XL Laser Densitometer. A, B, A_1, A_2, bands were cut and weighed. Meanwhile aliquots at the same time intervals of digestion were shifted to 35°C and incubated for 10 min before measuring the efflux rate. Fractions of A_1 (⊡) and of A_2 (◆) versus efflux rates at the corresponding times of digestion are indicated in the plot

the digested membranes become obviously uncoupled in the absence of oxalate, but retain the ability to pump in the presence of oxalate. The slight reduction in calcium pumping by the digested membranes and by the digested/temperature-shifted membranes suggests that uncoupling does occur, but is largely masked by oxalate trapping.

A major question left unresolved in the previous studies (Toogood et al., 1983) was the identity of the protein responsible for the trypsin-induced increase in permeability. Because channels can transport up to about 10^7 ions/channel/sec (Hille, 1982), even one to two channel proteins per vesicle might be enough to account for the trypsin-stimulated transport activity. Given the difficulty in achieving a high degree of purity of membrane proteins, we believed that isolated and reconstitution would have been unlikely to yield preparations that are pure enough to unambiguously identify the trypsin-sensitive channel site. Thus, we have taken an alternative approach in our attempts to identify the protein responsible.

Two lines of evidence suggest that the ATPase molecule may be the trypsin-sensitive site. The data here show that DCCD, an inhibitor of the ATPase that is believed to act at the calcium binding sites (Pick & Racker, 1979; Murphy, 1981), and AMP-PNP, an ATP analogue (Yount et al., 1971), both

slow the development of increased permeability at 35°C following trypsin digestion at 15°C (Fig. 6). The finding with DCCD is especially significant because labeling with radioactive DCCD followed by gel electrophoresis indicated that the ATPase is the only protein that becomes clearly labeled with DCCD (Pick & Racker, 1979). These data support the supposition that the trypsin-sensitive site is likely to be the calcium ATPase. The second line of evidence is that the trypsin-stimulated efflux correlates exactly with the second cleavage of the calcium ATPase molecule (Fig. 9).

It seems unlikely that there is a protein, other than the ATPase, that could account for all these data. The trypsin-sensitive site would have to be a protein that binds both AMP-PNP and DCCD and that also undergoes trypsin digestion at the same rate as the second cleavage step of the ATPase. This hypothetical other protein would also have to be abundant enough to be present in all the vesicles (because all the calcium leaks out following trypsin digestion) and yet not be clearly labeled by radioactive DCCD. Thus, we conclude that the T_2 cleavage step of the ATPase is very likely to be the critical event associated with the development of increased permeability.

Assuming that T_2 is indeed the critical digestion step, then perhaps the single most important result of this paper is that calcium permeability depends on the concentration of doubly cleaved ATPase molecules to the first power (Fig. 8). We subjected these data to the F test (for example, Bhattaharyya & Johnson, 1977). We assumed the null hypothesis that the efflux rate actually depends on the concentration of the doubly cleaved ATPase molecule to the second power, and so determined the best fit of our data to parabolic curves. Comparing the linear and parabolic fits by the F test leads to the conclusion that the null hypothesis can be rejected with a confidence > 0.95 if the data from A_1 are used alone, > 0.90 if the data from A_2 are used alone and > 0.98 if the data from A_1 and A_2 are combined. Null hypotheses assuming a third or higher power dependence are even more strongly rejected. These results support the first power correlation shown in Fig. 8.

The dependence on concentration to the first power has been used to suggest that a monomer of valinomyocin transports potassium (Tosteson et al., 1968), a second power dependence indicates that a dimer of gramicidin transports monovalent cations (Tosteson et al., 1968) and a sixth or higher power dependence indicates that an oligomer of six or more alamethecin molecules is responsible for its transport function (Mueller & Rudin, 1968). By the same logic, the first power dependency argues very strongly that the trypsin-induced efflux depends on a monomer of the (doubly cleaved) ATPase molecule—that is, efflux is almost certainly through a channel enclosed by a single ATPase molecule.

It has been shown that the calcium ATPase probably exists as an oligomer in the SR membrane (Stewart & MacLennan, 1974; Scales & Inesi, 1976; Ikemoto, Miyao & Kurobe, 1981; Gingold, Rigaud & Champeil, 1981; Hymel et al., 1984; Taylor, Dux & Martonosi, 1984; Silva & Verjovski-Almeida, 1985) and thus there are various suggestions that calcium pump requires an oligomeric form to carry out its function. In contrast, based on the behavior of detergent-solubilized material, other workers have suggested that the functional form of the ATPase for calcium pumping might be monomeric (Martin, Tanford & Reynolds, 1984; Andersen et al., 1986). Also, re-analysis of the tryptic digestion data of Scott and Shamoo (1982) suggests a monomer for the functional pump because the loss of pumping activity depends on the first power of the amount of T_2 (Blumental, Scott and Shamoo, *personal communication*). Our data suggesting a channel enclosed by a single ATPase molecule are at least consistent with the suggestion that the functional form of the ATPase is a monomer.

In several natural membranes and reconstituted systems it has been found that protein aggregation is associated with increased permeability. In the particular case of the SR membranes, chemical cross-linking of the proteins has been shown to increase membrane permeability (Chiesi, 1984). Thus, one model might be that trypsin digestion potentiates a protein aggregation step that leads to the creation of artifactual channels. A second model is that trypsin digestion facilitates the unfolding of the ATPase domain, thus exposing an ion pathway underneath. The inhibition of the protein conformational change by DCCD and AMP-PNP are both consistent with the prevention of a protein unfolding step. Also, the data showing a first-power dependence for the efflux rate supports the unfolding model over the aggregation model.

Shamoo and co-workers over the years have published a model for the calcium ATPase with separate channel, gate and hydrolytic domains (Shamoo & Ryan, 1975; Shamoo et al., 1976, 1977, 1987). Lack of information required educated guesses and so it is not surprising that early versions of this model contained errors, such as the proposal that the channel domain resided entirely within the B fragment and that the A fragment comprised a domain located entirely outside the membrane. Nevertheless, the idea of separate domains continues to be a useful hypothesis for the structure of the calcium ATPase. The data in this paper, sug-

gesting that trypsin digestion at 15°C followed by incubation at 35°C leads to the exposure of a calcium channel in the ATPase without loss of ATPase activity, indicate a possible separation of the ATPase and channel domains—a result broadly consistent with the models of Shamoo and coworkers.

If the separate domain model is indeed the correct interpretation, then our approach also provides a new method for probing the molecular details of the channel and the forces that hold the channel and ATPase domains together. For example, the inhibition of the development of calcium permeability by AMP-PNP suggests that the ATP binding site might be located between the ATPase and channel domains, and thus AMP-PNP binding slows the separation of the two domains. The inhibition of the development of increased calcium permeability by DCCD, which is believed to react with the calcium binding sites (Pick & Racker, 1979; Murphy, 1981), likewise suggests that the calcium binding sites might also be located between the channel and ATPase domains. Of course, AMP-PNP and DCCD could be exerting their effects indirectly as well. Clearly, however, further studies on the putative uncoupling of the channel and ATPase functions promise to yield additional information about structure/activity relationships in the calcium ATPase molecule.

If the channel described in this paper is actually a functional part of the calcium ATPase, a likely possibility is that this channel is the pathway for calcium uptake. Despite arguments to the contrary and the recent ryanodine data implicating the terminal cisternae "feet" structures as the possible site of the calcium release channel (Inui, Saito & Fleischer, 1987; Lattanzio et al., 1987), some observations suggest that the ATPase might be at least a contributor to physiological calcium release (Mészáros & Ikemoto, 1985; Gould et al., 1987; McWhirter et al., 1987), which raises the possibility that the channel described in this paper might also be involved in calcium release. If indeed the ATPase plays any role in calcium release, it seems possible that the same channel could be involved in both uptake and release, with the binding of regulatory factors controlling the function.

Whether the channel described in this paper may be involved in calcium uptake or in calcium release, in either case it would seem to be worthwhile to compare the characteristics of the channel described in this paper with the one induced by heavy metals (Abramson et al., 1983; Bindoli & Fleischer, 1983). It has recently been suggested that the silver-induced efflux is apparently by means of the calcium ATPase (Gould et al., 1987). If the thermally induced channel in the doubly cleaved ATPase described in this paper and the silver-induced calcium efflux (which is only assumed to be by means of a channel) are found to exhibit the same characteristics, it would further show that our activity is indeed mediated by the ATPase, it would confirm the supposition that silver acts by exposing a channel, and it would show that the same channel function can be regulated in two very distinctive ways. Such comparisons have been initiated in our laboratory.

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Note Added in Proof

After this work was completed, a new paper was published suggesting that the Ca^{2+}-ATPase is not the site of trypsin-stimulated efflux (Shoshan-Barmatz, V., Ouziel, N. and Chipman, D.M., 1987, trypsin digestion of sarcoplasmic reticulum inhibits Ca^{2+} accumulation by action on a membrane component other than the Ca^{2+}-ATPase, *J. Biol. Chem.* **262**:11559–11564). If the conclusion of Shoshan-Barmatz et al. (1987) is correct, then DCCD labeling should help to identify the putative other protein. We are attempting to carry out this experiment. Alternatively, despite the work of Shoshan-Barmatz et al. (1987) the tryptic digestion of the ATPase could be the critical step to potentiate the efflux as suggested herein. If so, there are several possible alternative explanations of the data of Shoshan-Barmatz et al. (1987) including: (i) trypsin-stimulated efflux could require a second polypeptide chain in addition to the ATPase, a polypeptide that becomes lost during isolation and reconstitution of the ATPase; (ii) the detergent treatment and isolation of the ATPase could cause a conformational change that inhibits the trypsin-stimulated efflux in the reconstituted system; (iii) it is well known that anion channel activity is lost during reconstitution—a very slight amount of calcium efflux in the absence of the anion channel could lead to voltage gradients that inhibit trypsin-stimulated efflux; and, (iv) finally, in our hands, elevated temperature is necessary to bring about trypsin-stimulated efflux, whereas Shoshan-Barmatz et al. (1987) observed trypsin-stimulated efflux at a reduced temperature, suggesting that there is a very basic difference in the stability of the efflux protein in our hands as compared with theirs (*see* ii above). Sorting out the differences between our data and that of Shoshan-Barmatz et al. (1987) will provide important insight about the functioning of the Ca^{2+}-ATPase.