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Disruption of Energy Transduction in Sarcoplasmic Reticulum by Trypsin Cleavage of $(Ca^{2+}+Mg^{2+})$-ATPase

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Summary. Proteolytic digestion of sarcoplasmic reticulum vesicles with trypsin has been used as a structural modification with which to examine the interaction between the ATP hydrolysis site and calcium transport sites of the $(Ca^{2+}+Mg^{2+})$-ATPase. The kinetics of trypsin fragmentation were examined and the time course of fragment production compared with ATP hydrolytic and calcium uptake activities of the digested vesicles. The initial cleavage (TD 1) of the native ATPase to A and B peptides has no effect on the functional integrity of the enzyme, hydrolytic and transport activities remaining at the levels of the undigested control. Concomitant with the second tryptic cleavage (TD 2) of the A peptide to A_1 and A_2 fragments, calcium transport is inhibited. Kinetic analysis demonstrates that the rate constant for inhibition of calcium uptake is correlated with the rate constant of a fragment disappearance. Both Ca^{2+}-dependent and total ATPase activities are unaffected by this second cleavage. Passive loading of vesicles with calcium and subsequent efflux measurements show that transport inhibition is not due to increased permeability of the membrane to calcium even at substantial extents of digestion. Steady-state levels of acid-stable phosphoenzyme are unaffected by either TD 1 or TD 2, indicating that uncoupling of the hydrolytic and transport functions does not increase the turnover rate of the enzyme and that TD 2 does not change the essential characteristics of the ATP hydrolysis site. Sarcoplasmic reticulum (SR) vesicles were examined for the presence of "tightly bound" nucleotides and are shown to contain 2.8–3.0 nmol ATP and 2.6–2.7 nmol ADP per mg SR protein. The ADP content of SR remains essentially unchanged with TD 1 cleavage of the ATPase enzyme to A and B peptides, but declines upon TD 2 in parallel with the digestion of the A fragment and the loss of calcium uptake activity of the vesicles. The ATP content is essentially constant throughout the course of trypsin digestion. The results are discussed in terms of current models of the SR calcium pump and the molecular mechanism of energy transduction.

Key words sarcoplasmic reticulum · $(Ca^{2+}+Mg^{2+})$-ATPase · energy coupling · Ca^{2+}-ionophore · Ca^{2+}-uptake · uncoupling

Introduction

The calcium pump system of sarcoplasmic reticulum (SR)[1] has emerged in recent years as the most extensively characterized active transport system on the molecular level. The system exhibits a tight coupling of two calcium ions transported per mole ATP hydrolyzed under ideal conditions (Hasselbach & Makinose, 1961). The ionic and substrate conditions for ATP hydrolysis have been documented and the partial steps of the reaction cycle have been deduced in general form over the last decade.

Recent evidence has provided insights into the molecular mechanism of ion translocation by the SR ATPase. This has been achieved through the synthesis of approaches involving both kinetic methods and examination of the protein structure of the ATPase enzyme. The functional activities of the enzyme have been localized within the general features of the molecular structure as deduced from proteolytic fragmentation of the ATPase peptide. Structural studies have used digestion of the enzyme with trypsin in order to characterize the morphological features of SR and to elucidate which portions of the enzyme peptide interact with the lipid bilayer (Ikemoto, Sreter & Gergely, 1971; Stewart & MacLennan, 1974; Thorley-Lawson & Green, 1975). Studies of the tryptic fragments have shown that the phosphorylation site is located in the A and A_1 fragments of the enzyme (Thorley-Lawson & Green, 1973; Allen & Green, 1976; Stewart, MacLennon & Shamoo, 1976). The A and A_2 fragments have been shown to exhibit Ca^{2+}-dependent and Ca^{2+}-selective ionophorous activity in artificial bilayer membranes (Shamoo, Ryan, Stewart & MacLennan, 1976; Shamoo & Goldstein, 1977; Shamoo, 1978). While the studies outlined above strongly suggest that the functional centers of the

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[1] The abbreviations used are: SR, sarcoplasmic reticulum vesicles; ATP, adenosine 5′-triphosphate; EGTA, ethylene glycol bis (β-aminoethyl ether) N,N′-tetraacetic acid; Tris, tris(hydroxymethyl) aminomethane; Hepes, 4-(2-hydroxyethyl)-1 piperazineethanesulfonic acid; Pi, inorganic orthophosphate; EP, acid-stable phosphorylated intermediate; SDS, sodium dodecyl sulfate.

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enzyme are localized in different portions of the peptide structure by examination of isolated tryptic fragments, there exists a gap in our understanding of the dynamic interaction between these domains in the functional enzyme.

This study attempts to correlate the extent of trypsin digestion and the disposition of the fragments with changes in the functional activities of the calcium pump of SR. This use of trypsin cleavage provides a modification tool with which to examine the events involved in energy transduction between ATP hydrolysis and active calcium transport.

Materials and Methods

Preparation of SR

SR vesicles were prepared essentially by the method of MacLennan (1970), homogenized at 20–30 mg/ml in 0.01 M Tris-Cl, 0.25 M sucrose, pH 8.0, frozen rapidly and stored in liquid nitrogen. ATP hydrolysis and calcium uptake activities declined only a few percent over the course of eight weeks when frozen and stored as described, if aliquots were thawed slowly on ice. Thawing and refreezing of the same sample caused a loss of functional activities at a greater rate. Most experiments were carried out within four weeks of preparation.

Trypsin Digestion

Proteolytic digestion with trypsin was carried out essentially according to the method of Ikemoto et al., 1971. SR was homogenized at 5 mg/ml in a medium of 0.02 M Tris-Cl, 0.1 M KCl, 1.0 M sucrose, pH 7.0, and trypsin was added to yield final doses as indicated in the given experiment. Digestion was at 22 °C and at the appropriate times aliquots were withdrawn and the reaction terminated by quenching into 0.56 volume of soybean trypsin inhibitor in 0.02 M Tris-Cl, 0.1 M KCl, pH 7.0, at a weight ratio of inhibitor/trypsin of 4:1 and placed on ice for direct use in assays.

Quantitation of Tryptic Fragments

Aliquots of tryptically digested SR were diluted fourfold with 0.02 M Tris-Cl, 0.1 M KCl, pH 7.0 (Tris-KCl) in order to reduce the sucrose concentration to 0.25 M and centrifuged at $105{,}000 \times g$ for 45 min. The pellets were homogenized in 0.02 M Tris-Cl, 0.1 M NaCl and centrifuged as before and finally suspended at 5 mg/ml in 0.25 M sucrose, 0.01 M Tris-Cl, pH 7.5. The samples were subjected to polyacrylamide gel electrophoresis using 10% acrylamide (0.27% bis), 0.1 M Na phosphate, 0.1% SDS according to the method of Weber and Osborn (1969). The gels were stained and fixed overnight in 0.1%. Coomassie blue: 45% methanol: 10% acetic acid, destained by diffusion in 10% acetic acid and scanned at 550 nm in a Gilford spectrophotometer. The areas under the peaks corresponding to each of the fragments were determined by planimetry and protein values given as weight percent.

Calcium Uptake Assay

Initial rates of calcium uptake were measured by the Millipore filtration technique (Martonosi & Feretos, 1964) in a medium consisting of 0.2 mg/ml SR in 25 mM HEPES-K^+, pH 7.0, 100 mM KCl, 5 mM $MgCl_2$, 0.1 mM $^{45}CaCl_2$ (5×10^3 cpm/nmol) in a total volume of 0.6 ml at 22 °C. The uptake reaction was initiated by addition of Na_2 ATP to a final concentration of 5 mM. At 0.25, 0.50, and 1.0 min after ATP addition, 0.5 ml of the reaction mixture was filtered through a 0.45 Millipore filter (HAWP) and washed with 5.0 ml of the same medium without SR, $CaCl_2$ and ATP. The filters were dried in an oven at 65 °C, placed in 5.0 ml of Insta-Gel cocktail and counted in a Beckman LS-150 scintillation counter. Passive calcium-binding by the vesicles was determined as above in the absence of ATP and subtracted from the values above to obtain ATP-dependent calcium uptake, expressed as nmoles Ca^{2+}/mg min.

In some experiments, calcium transport rates were measured in the above reaction medium plus 1.0 mM K-oxalate at a protein concentration of 10 μg/ml.

ATP Hydrolysis Assay

ATP hydrolysis activity was determined in 0.3 ml of the same medium as that for calcium uptake, using $^{40}CaCl_2$ and initiation with 5 mM [γ-^{32}P]ATP (5×10^5 cpm/μmol).

The reaction was terminated at 0.25, 0.50 and 1.0 min by the addition of 0.1 ml ice-cold 30% trichloroacetic acid and the ^{32}P-phosphomolybdate complex extracted into a butyl acetate phase essentially according to the method of Sanui (1974) and determined by liquid scintillation counting. Calcium-independent ATPase activity was determined as above in the presence of 1 mM EGTA and no added calcium and subtracted from the total activity to yield Ca^{2+}-simulated ATPase activity in units of μmoles Pi/mg min.

Phosphorylation of ($Ca^{2+} + Mg^{2+}$)-ATPase

Measurement of the steady-state level of the phosphoprotein intermediate was carried out in 1.0 ml of 10 mM HEPES-K^+, 50 mM KCl, 2.2 mM $MgCl_2$, 0.085 mM $CaCl_2$ added and 1.0 mg SR at 0 °C. The reaction was initiated by the addition of 1.0 mM [γ-^{32}P]ATP (104 cpm/nmol) to a final concentration of 0.1 mM and terminated at 6 sec by the addition of 5.0 ml of 4% trichoroacetic acid and incubated overnight in 0.5 ml of 1.0 N NaOH. An aliquot of the solubilized pellet was taken for determination of protein by the method of Lowry, Rosebrough, Farr and Randall (1951); another for EP determination by liquid scintillation counting.

Passive Efflux

The passive permeability of SR to Ca^{2+} was determined by incubation of vesicles at 5–10 mg/ml 25 mM HEPES-K^+, 100 mM KCl, 5 mM $MgCl_2$, 1–10 mM $^{45}CaCl_2$ (10^4 cpm/nmol Ca^{2+}) at 0 °C for 20–30 hr. The loaded vesicles were allowed to come to room temperature, then diluted 25- to 100-fold into the same medium plus concentrations of EGTA from 5–15 mM (efflux medium) and at various times 1-ml aliquots were passed through Millipore filters (0.45 μ, HA) and processed as for the calcium uptake assay. Rate constants were determined by linear regression of semilogarithmic plots of [cpm(t) − cpm (∞)]/[cpm (0) − cpm (∞)] versus time after dilution. In some experiments digestion was performed after passive loading of vesicles. Control vesicles were incubated at 5 mg/ml in 1 M sucrose, 100 mM KCl, 25 mM HEPES-K^+, pH 7.0, and 1 mM $^{45}CaCl_2$ (10^4 cpm/nmol Ca^{2+}) at 0 °C for 24 h. The loaded vesicles were allowed to come to room temperature, then digested in the same medium with trypsin as described above. The reaction was quenched by addition of trypsin inhibitor in 25 mM HEPES, 100 mM KCl, pH 7.0, containing 1 mM $^{45}CaCl_2$ of the same specific activity as that used for loading. The vesicles were diluted 50-fold into the efflux medium and efflux rates determined as above.

Determination of "Tightly Bound" Nucleotides

Control and trypsin-treated SR were suspended at 3–5 mg/ml in 0.25 M sucrose, 10 mM Tris-acetate, 2 mM EDTA, pH 7.5, and cen-

trifuged at 166,500 × *g* for 30 min at 4 °C. The pelleted vesicles were homogenized in the same medium and centrifuged as before. This wash procedure was performed a total of five times. The final pellet was homogenized at 10 mg/ml in 0.25 M sucrose, 10 mM Tris-acetate, 2 mM EDTA, pH 7.5.

The nucleotides were liberated by precipitation of the protein with the addition of ice-cold 10% perchloric acid to a final concentration of 4%. The denatured samples were placed on ice for 5 min and the precipitate removed by centrifugation at 16,000 × *g* for 10 min at 4 °C. The supernatants were neutralized at 0 °C with cold 0.1 M-Tris-SO_4, 10% KOH to pH 7–7.5 and placed on ice for 15 min. The precipitated $KClO_4$ was removed by centrifugation at 32,000 × *g* for 2 min and the supernatant placed on ice for assay of nucleotides. A neutralized solution blank was prepared as above in the absence of protein.

Nucleotides were determined fluorometrically using coupled enzyme systems by the methods of Greengard (1965) for ATP and Garrett and Penefsky (1975) for ADP. The recovery of ATP was 65–95% and of ADP was 80–95% when known amounts of nucleotide were added immediately after perchloric acid addition in the presence or absence of protein and the sample values were corrected for this recovery factor.

Protein Determinations

Protein was determined by the method of Lowry et al. (1951) using bovine serum albumin as standard. At intermediate stages of the preparation, protein was determined by absorbance at 280 nm 1% SDS using $E_{1\,cm}^{1\%} = 10.0$ (Thorley-Lawson & Green, 1977).

Results

Kinetics of Trypsin Digestion of Sarcoplasmic Reticulum

The kinetics of the digestion reaction were examined at various doses of trypsin/SR (wt/wt) from 1:5 to 1:1500 in the presence of 1 M sucrose, 0.1 M KCl. Figure 1 shows SDS polyacrylamide gels of control SR and the pattern of digestion with time. As shown by Stewart and MacLennan (1974) and Thorley-Lawson and Green (1975), upon exposure to trypsin, the ATPase is cleaved very rapidly to A and B fragments. At longer times the A fragment is split to A_1 and A_2 peptides. The molecular weights of the fragments are A, 57,900; B, 48,600; A_1, 31,900; A_2, 20,600 as determined from plots of R_f on polyacrylamide gel *vs.* log molecular weight of known peptides (*data not shown*). At later times of digestion, the A_1 and A_2 peptides are degraded to low molecular weight fragments which migrate near the dye front of the gel. At this point, ATPase activity declines. However, at low doses of trypsin to SR, the TD 2 cleavage does not go to completion. This is not due to the presence of digestion-resistant A fragment, such as in inside-out vesicles, since at very high doses of trypsin the A fragment can be completely digested (*data not shown*). The lack of completion of TD 2 at low doses probably represents autodigestion of trypsin. In light of the above discussion, it is virtually impossible to obtain a preparation which contains only A_1 and A_2 peptides, with no appearance of low molecular weight products. Thus correlation of functional activities with peptide structure requires a kinetic analysis of fragment production and disappearance.

Effect of Fragmentation on ATP Hydrolysis Activity

The initial event, TD 1 cleavage of the native ATPase to A and B fragments, has variable effects on the ATP hydrolytic activity of SR. At very early times, before the A fragment reaches its maximum value,

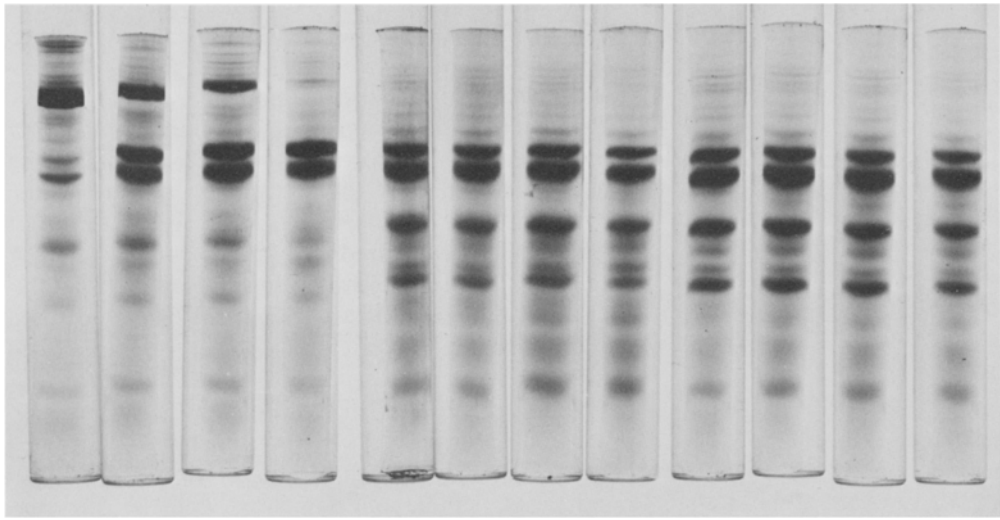

Fig. 1. Polyacrylamide gels of undigested SR (left) and the time course of trypsin digestion (increasing time from left to right). Gels are 10% acrylamide run according to Weber and Osborn (1969). SR was digested under the conditions described in Materials and Methods at a dose of trypsin/SR of 1:20, times from 0–20 min

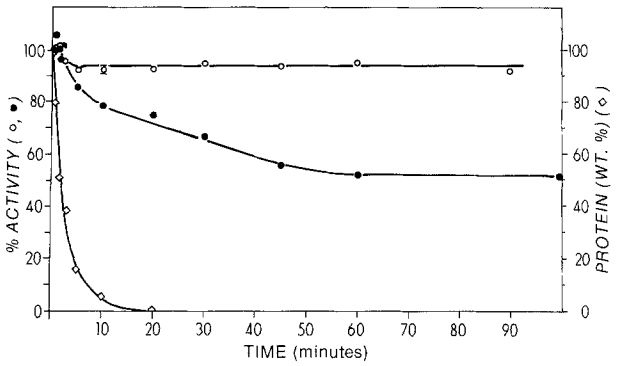

Fig. 2. Time course of disappearance of the ATPase enzyme with trypsin digestion at a trypsin/SR dose of 1:1500 under conditions described in Materials and Methods. Ca^{2+}-stimulated ATP hydrolysis and calcium uptake rates assayed as described. ◇, 100K dalton peptide; ○, ATP hydrolysis; ●, calcium uptake

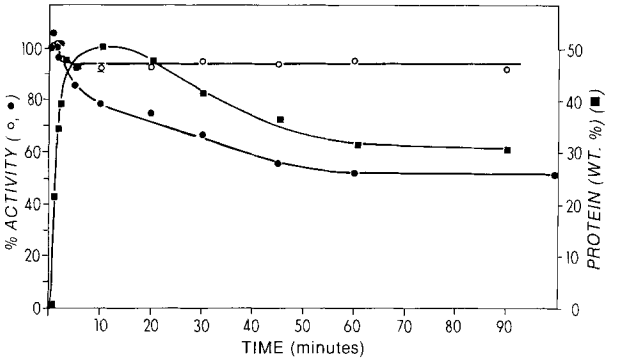

Fig. 3. Time course of the A fragment with trypsin digestion for the experiment depicted in Fig. 2. ■, A fragment; other symbols as in Fig. 2

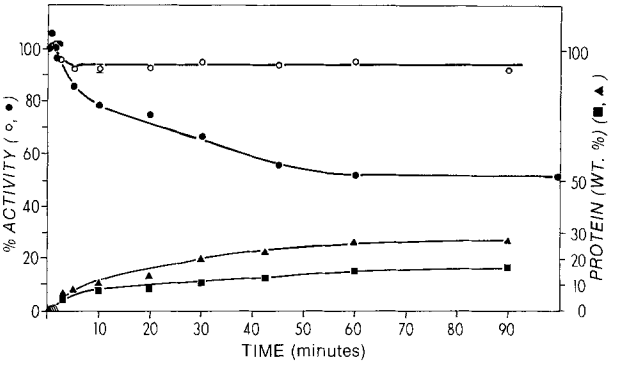

Fig. 4. The appearance of the A_1 and A_2 fragments for the experiment depicted in Figs. 2 and 3. ▲, A_1 fragment; ■, A_2 fragment; other symbols as above

hydrolytic activity is enhanced to as much as 110% of the control value in some experiments, while it is inhibited to as low as 83% in others. This initial effect is not reproducible and varies among preparations. In either case, hydrolytic activity returns to the control value at the point where the A fragment reaches its maximum extent and remains essentially at the level of the undigested control. In the experiment depicted in Fig. 2–4 the initial stimulation is slight and within experimental error. This very early phenomenon may represent some transient rearrangement of the interaction of the ATPase protein with the membranes or with adjacent ATPase units with simultaneous processes of stimulation and inhibition.

The total and Ca^{2+}-dependent ATPase activities are subsequently unaffected at further extents of TD 1 and TD 2, indicating that digestion of the native enzyme does not affect the nucleotide hydrolysis site. Further, the Ca^{2+} dependence of the hydrolytic activity persists even at extreme extents of fragmentation, none of the Ca^{2+}-dependent activity being converted to basal ATPase.

Prolonged exposure to trypsin at high doses in which degradation of the A_1 and A_2 peptides occurs results in the eventual decline of ATPase activity, though this could not be quantitated directly with the loss of A_1 and A_2 for the reasons cited above in Kinetic Results.

Inhibition of Calcium Uptake by Tryptic Cleavage

The decline in the calcium uptake rate versus the time of digestion for SR subjected to a low dose of trypsin (Figs. 2 and 3) indicates that the loss of calcium uptake is not corrected with the TD 1 cleavage of the native enzyme to A and B fragments, *viz.*, at 5 min of digestion, the intact enzyme has declined to $<15\%$ of its initial value, yet uptake remains at 96% of the control level. At this low dose of trypsin, there is a definite lag in the production of the A_1 and A_2 peptides via TD 2 cleavage of fragment A (Fig. 4). This lag phase clearly indicates that even at almost total extents of TD 1 cleavage, Ca^{2+} transport remains unimpaired. Upon digestion of the A fragment to A_1 and A_2 peptides, calcium uptake ability is lost. At high doses of trypsin, there is no lag in the production of the A_1 and A_2 peptides from fragment A and calcium uptake is inhibited even at very early times. The disappearance of fragment A follows a time course which closely parallels the inhibition of uptake. This was consistently demonstrated whether the initial rates of uptake were measured in the presence or absence of 1 mM oxalate.

The digestion of the A fragment was analyzed as a first-order process and rate constants determined for the initial phase of the decline of the native enzyme and the A peptide and compared with the rate constants for inactivation of calcium uptake. The rate constants calculated by least-squares analysis are presented in Table 1 for a range of trypsin/SR doses.

Table 1. Comparison of rates of trypsin cleavage of intact enzyme and A fragment with inactivation rate of calcium transport[a]

Dose trypsin/ SR (wt/wt)	k_{enz}	k_A	k_u
1:5	15.33	3.10	3.32
1:75	2.64	0.18	0.13
1:200	–	0.019	0.018
1:500	0.547	0.027	0.029
1:1300	0.253	0.010	0.008
1:1500	0.307	0.0097	0.0085

[a] SR vesicles were digested with various doses of trypsin under the conditions describe in Materials and Methods. First-order rate constants determined from semi-logarithmic plots of weight percent protein and calcium uptake rate versus time of digestion. k_{enz}, rate constant for disappearance of 100K dalton peptide; K_A, A fragment disappearance; k_u, inactivation of calcium uptake; all values in units of min^{-1}.

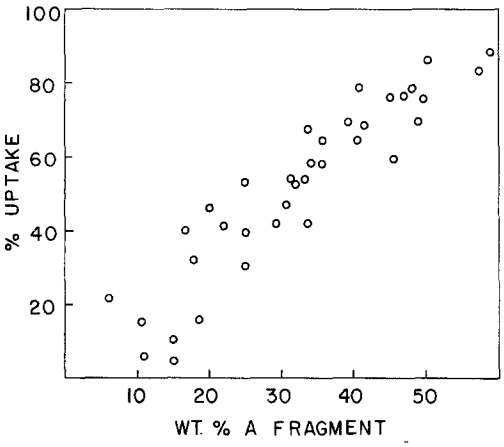

Fig. 5. Correlation of the residual rate of Ca^{2+} uptake and the amount of the A fragment. The data are taken from the time courses of six separate experiments, with a range of trypsin/SR doses from 1:20 to 1:1500. Rates of Ca^{2+} uptake and amount of A fragment determined as in Materials and Methods. Correlation coefficient, $r=0.92$, $p>0.99$

The rate of inactivation of calcium uptake is independent of the TD 1 cleavage of the intact enzyme, but is clearly correlated with the decline of the A fragment, demonstrating that the TD 2 cleavage of the A fragment to A_1 and A_2 peptides results in the uncoupling of ATP hydrolysis from calcium transport. Figure 5 demonstrates the correlation between the inhibition of uptake rate and the amount of the A fragment, $r=0.92$ ($p<0.01$).

Permeability of Digested Vesicles to Calcium

The nature of this uncoupling of hydrolysis from transport was examined in order to determine whether trypsin digestion disrupts the membrane permeability barrier to calcium. Vesicles were digested and passively loaded with calcium as described in Materials and Methods. The passive efflux of calcium in the presence of EGTA is essentially identical for undigested control and the TD 2 vesicles (Fig. 6). The efflux from both control and digested vesicles is biphasic. The rate constants for the rapid and slower phases are 0.17–0.19 min^{-1} and 0.046–0.050 min^{-1}, respectively, for both the digested and control vesicles. The rate constant for the rapid phase of efflux is far too small to have any effect on the measured uptake rates, as pointed out by Scales and Inesi (1974). In seven different SR preparations with at least three determinations for each of the control and TD 2 efflux rates, the probability that the rate constants for the rapid phase are equal is greater than 95% as determined by *t*-tests of the hypothesis that $K_{TD2}=k_{control}$. The Ca^{2+} capacity of the digested vesicles was always >90% of the control value, as determined by extrapolation of semi-logarithmic plots of Ca^{2+} efflux to the ordinate.

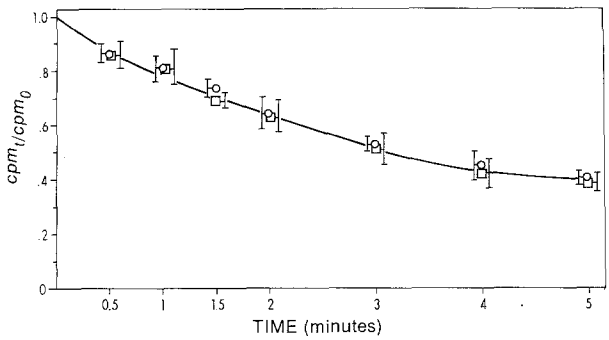

Fig. 6. Passive calcium efflux of control and digested vesicles. Vesicles were digested with trypsin at a ratio of trypsin/SR of 1:100 for passively loaded overnight and efflux assayed as described in Materials and Methods. ○, control SR; □, digested SR

Steady-State Phosphoenzyme

Both Ca^{2+}-dependent and total ATP hydrolysis rates are unaffected by TD 2 in the experiments reported here, while Ca^{2+} uptake is inhibited. Since the formation of the phosphorylated intermediate is directly coupled with the Ca^{2+} translocation process (Yamada & Tonomura, 1972), the steady-state levels of phosphoprotein were measured in control, TD 1 and TD 2 preparations (Table 2). In all experiments, the TD 1 preparations exhibit slightly higher EP levels than the control values. The EP levels of the TD 2 preparation are lower than TD 1 and are similar, but always slightly higher than the control. Increased EP in TD 2 is presumably due to the fact that TD 2 preparations always contain some enzyme in the A+B form due to the constraints imposed by the kinetics of digestion as discussed above. The lack of inhibition of EP levels by trypsin digestion provides further support for the conclusion that TD 2 does

Table 2. Steady-state levels of phosphoenzyme for control, TD 1 and TD 2 SR[a]

Control	TD 1	TD 2
1.84 ± 0.066	1.89 ± 0.057	1.79 ± 0.091

[a] EP formation was measured in 1.0 ml of 10 mM HEPES-K^+, pH 7.0, 50 mM KCl, 2.2 mM $MgCl_2$, 0.085 mM $CaCl_2$ and 1.0 mg SR at 0 °C. Reaction initiated by addition of [γ-^{32}P]ATP to a final concentration of 0.1 mM, terminated by addition of TCA and EP determined as described. Values represent the average of five preparations of each sample, with at least four determinations on each, given as nmol EP/mg ± SEM

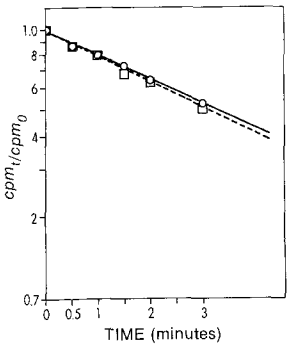

Fig. 7. Time course of the effect of trypsin digestion on "tightly bound" ADP, A fragment and calcium uptake rate. ▲, ADP; ■, A fragment; ●, calcium uptake

not impair the nucleotide hydrolysis site and indicates that the ATPase turnover, v/EP, is not altered by TD 2.

"Tightly Bound" Nucleotides

Measurement of tightly bound nucleotides reveals that SR contains 2.7 nmoles ATP per mg SR protein. "Tightly bound" is used here in analogy with the mitochondrial F_1-ATPase to indicate that the nucleotides are not removed by repeated washing in 10 mM Tris-acetate, 0.25 M sucrose, 2 mM EDTA, pH 7.5. The ATP content varies considerably among different preparations, as low as 1.5 nmole/mg in one. The ATP content varies during the course of trypsin digestion, but remains approximately at the control (*data not shown*). Thus ATP content is not affected by any of the structural or functional changes due to uncoupling of transport from hydrolysis. Bound ADP, on the other hand, has control values of 2.6 nmoles/mg SR. The tightly bound ADP is lost concomitant with the TD 2 cleavage, as seen in Fig. 7. There is a 10–20% decline in ADP at the early state of digestion in some cases. The loss of bound ADP parallels the degradation of the A fragment to A_1 and A_2 peptides and thus with the loss of calcium uptake. Semilogarithmic plots of the decline of bound ADP and inhibition of calcium uptake versus time of digestion, show very similar rate constants for the two processes. To summarize the effects of TD 2, Table 3 compares the rate constants for disappearance of the A fragment, inhibition of calcium uptake and loss of tightly bound ADP, which clearly indicates the correlation between these three processes.

Table 3. Correlation of A fragment cleavage, loss of tightly bound ADP and inactivation of calcium transport by trypsin digestion of SR[a]

Exp. No.	k_A	k_u	k_{ADP}
1	0.029	0.032	0.029
2	0.010	0.009	0.012

[a] First-order rate constants determined by exponential least-squares regression. k_A, A fragment disappearance; k_{ADP}, loss of tightly ADP; k_u, inactivation of calcium uptake rate, in units of min^{-1}. The doses of trypsin SR were 1:500 and 1:1500 for Experiments 1 and 2, respectively.

Discussion

The kinetic analysis of trypsin fragmentation supports previous evidence (Stewart & MacLennan, 1974; Thorley-Lawson & Green, 1975) that the 102,000 dalton ATPase enzyme is stoichiometrically split to A (57,900 daltons) and B (48,600 daltons) fragments in the first event upon trypsin exposure, designated here as TD 1. In the second phase, denoted TD 2, the A fragment is cleaved to A_1 (31,900 daltons) and A_2 (20,600 daltons) peptides. The B fragment produced as a result of TD 1 does not appear to possess exposed trypsin-sensitive sites under the conditions of sucrose protection employed. These findings are consistent with the results of Thorley-Lawson and Green (1973, 1975) and Stewart et al. (1976).

Examination of the functional activities of the enzyme during the time course of trypsin digestion indicates that the ATP hydrolysis activity is unaffected by either TD 1 or TD 2. The total ATPase activity is maintained even at considerable extents of digestion in which the enzyme is predominantly in the $A_1 + A_2$ form. The hydrolysis activity retains its strict dependency on calcium after both TD 1 and TD 2, thus the high affinity calcium sites are still associated functionally with the nucleotide hydrolysis site.

Calcium transport shows very different characteristics from those of hydrolysis upon fragmentation. Uptake activity is maintained in the TD 1 state, consistent with the findings of Stewart and MacLennan (1974) and MacLennan, Khanna and Stewart (1976). As seen in Figs. 3 and 5, inhibition of calcium trans-

port occurs upon cleavage of the A fragment to A_1 and A_2 peptides. Kinetic analysis demonstrates clearly that the inhibition of uptake is correlated with the disappearance of the A fragment and not with the first cleavage of the enzyme. Further, the similarity of the rate constants of the two processes shows that TD 2 causes total inactivation of the transport mechanism.

A number of procedures and agents have been described which inhibit calcium transport without affecting ATPase activity. These include treatment with diethyl ether (Inesi, Goodman & Watanabe, 1967), phospholipase A digestion (Fiehn & Hasselbach, 1970) and incubation with EDTA at pH 9.0 (Duggan & Martonosi, 1970). Each of these procedures results in disruption of the membrane permeability barrier and hence loss of transport ability. It should be noted in these studies, enhanced "leakiness"! of the vesicles led to a stimulation of the steady state ATPase activity in every case. Trypsin digestion does not stimulate the steady-state ATPase activity, indicating that the inhibition of transport is not the result of increased leakiness. Direct measurements of efflux after passive loading with $^{45}CaCl_2$ show that TD 2 vesicles do not have increased efflux rates compared with control SR. This demonstrates that the mode of inhibition of calcium transport by TD 2 is not disruption of the membrane permeability barrier for calcium, but rather a direct impairment of the influx process. Berman, McIntosh and Kench (1977) have shown that acid treatment of SR results in loss of Ca^2 uptake with no effect on hydrolysis. Hidalgo, Petrucci and Vergara (1981) have demonstrated that uncoupling of transport from hydrolysis can be effected by labelling the amino groups of phosphatidylethanolamine with fluorescamine. Inesi and Asai (1968) have reported that trypsin digestion of SR causes a release of calcium, but digestion was not performed under the conditions of sucrose protection used here, which may account for the discrepancy with the present results.

"Tightly bound" nucleotides have been shown to play an essential role in energy coupling in the mitochondrial F_1-ATPase and chloroplast CF_1-ATPase complexes (Harris & Slater, 1975). While recent studies have indicated that tightly bound ADP is not the acceptor for Pi in the ATP synthesis reaction, it is clear that tightly bound ADP is required for coupling ability in the F_1-ATPase (is required for coupling ability in the F_1-ATPase (Leimgruber & Senior, 1976). In analogy with these energy transducing systems, the SR ATPase contains 2.7 nmol ATP and 2.6 nmol ADP which are tightly bound by the criteria discussed above. "Tightly bound" is an operational definition used in studies with the F_1-ATPase to indicate that the nucleotides are not removed by column chromatography in low ionic strength buffer in the presence of EDTA or by repeated ammonium sulfate precipitation, release occurring only by denaturation of the protein with, e.g., perchloric acid.

In these experiments with SR, "tightly bound" is used to indicate that the nucleotides are not removed by repeated washes in 0.25 M sucrose, 10 mM Tris-acetate, 2 mM EDTA, pH 7.5. The ATP content varies considerably among the preparations analyzed and remains unaffected by trypsin digestion even at considerable extents of TD 2. Tightly bound ADP content is more consistent among preparations and is released from the SR at the point of TD 2 cleavage. While there is a small decline in the ADP content of 10–20% in the early phase of digestion, the loss of ADP clearly parallels the disappearance of the A fragment and production of the A_1 and A_2 peptides and the inhibition of calcium uptake as a result of TD 2. This implies that the tightly bound ADP is either directly involved in the energy coupling process or at least that the structure of this ADP binding site is dependent on the integrity of the coupled state of the enzyme. Since the ATPase enzyme comprises 65–70% of the total protein in the SR preparations employed here, 1 mg of SR protein contains 6.5–7 nmol of ATPase (assuming $M_r = 100{,}000$). Then 2.6 nmol ADP per mg SR protein yields ~ 0.4 mol ADP per mol ATPase. This suggests that tightly bound ADP is involved in the interaction between ATPase units. Anderem, McIntosh and Berman (1979) have reported that acid inactivation of SR inhibits Ca^2 uptake and destroys the ability to reload bound nucleotides.

Steady-state phosphoenzyme is unaffected by TD 2, thus the ATP hydrolysis site is not perturbed even by extensive digestion. Further, since EP formation is tightly coupled to the transport reaction in the undigested enzyme, this indicates that the ATP hydrolysis reaction is functioning with the normal reaction steps. The fact that the two processes are tightly coupled in the intact state and yet inactivation of transport can occur with no apparent change in hydrolysis is consistent with the concept that the two functions are localized in different portions of the enzyme structure (Shamoo & Goldstein, 1977).

These results are consistent with the gated pore mechanism for SR active transport (Shamoo & Ryan, 1975; Shamoo & Goldstein, 1977). The effect of TD 2 in this scheme would then be to disrupt the association between the energy-transducing portion at the exterior of the membrane (A_1) and the ion-translocating structure (A_2). In the TD 2 state, calcium moves through the A_1 portion in normal fashion, but cannot

cross the membrane since the linkage between the hydrolytic and the ionophorous domains is disrupted and thus Ca^{2+} diffuses to the membrane exterior.

The digestion of the ATPase peptide is sequential, i.e., there is no TD 2 cleavage until the native enzyme is split to A and B fragments, indicating that TD 1 cleavage exposes the second trypsin-sensitive site, which is normally buried. The ion translocating structure would then be situated just inside the lipid bilayer at the cytoplasmic face of the sarcoplasmic reticulum membrane.

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