Evidence for an Effect of Phospholamban on the Regulatory Role of ATP in Calcium Uptake by the Calcium Pump of the Cardiac Sarcoplasmic Reticulum†

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ABSTRACT: The purpose of this study was to investigate the functional relationship between phospholamban and the nucleotide site of the calcium pump protein of the cardiac sarcoplasmic reticulum. We used control and trypsin-treated cardiac microsomes in which cleavage of the inhibitory cytoplasmic domain of phospholamban is associated with an activation of the calcium pump similar to that produced by protein kinase A catalyzed phospholamban phosphorylation. Phenylglyoxal was shown to inactivate the calcium pump in a pseudo-first-order reaction by binding to a single Arg at the nucleotide binding site. No differences upon trypsin treatment of microsomes were observed in the kinetics of phenylglyoxal inactivation or the ability of millimolar ATP to protect against inactivation. In subsequent kinetic studies, Ca-uptake rates measured at saturating Ca^{2+} and 5 μM–1 mM $MgATP^{2-}$ were increased 15–32% by trypsin treatment in each of three different microsome preparations. Double-reciprocal plots of the data showed marked downward curvature indicating an acceleratory effect associated with ligand binding to a lower affinity site. At 0.32 μM Ca^{2+}, Ca-uptake rates were lower than at 11 μM Ca^{2+} but were stimulated to a greater extent by trypsin treatment; control microsomes showed reduced evidence of apparent negative cooperativity. At 0–2 μM $MgATP^{2-}$ and saturating Ca^{2+}, there was a 50% increase in $V_{max(app)}$ when the Hill coefficient (N) was 1. At 0–10 μM $MgATP^{2-}$, second-site binding was evident. At both 0–10 μM and 5 μM–1 mM $MgATP^{2-}$, trypsin-treated microsomes showed greater activation of Ca uptake attributable to second-site binding than did control microsomes. Trypsin treatment produced a significant decrease in turbidity in cardiac microsome suspensions. Our data suggest that cleavage of the cytoplasmic segment of phospholamban from the membrane produces marked changes in the physical and functional properties of cardiac SR membranes that may be associated with a shift in the E* ↔ E equilibrium toward E. The data, furthermore, are consistent with effects of phospholamban at two or more sites in a branched reaction pathway of the catalytic cycle, one of which is likely to interfere with the acceleratory effect of ATP at concentrations above those necessary for binding to the high-affinity catalytic site.

The calcium pump protein of the sarcoplasmic reticulum (SR)[1] of striated muscle plays a major role in reducing cytoplasmic Ca^{2+} during muscle relaxation. In cardiac muscle, calcium pump activity is regulated through phosphorylation/dephosphorylation of phospholamban, a 27-kDa protein associated with the SR membrane (Tada et al., 1989). Phospholamban phosphorylation by protein kinase A is correlated with an increase in the rate of calcium transport across SR membranes (Kirchberger et al., 1974) and cardiac muscle relaxation (Lindemann et al., 1983) in response to β-adrenergic agonists.

The regulation of the calcium pump by phospholamban involves an inhibition of pump activity when phospholamban is in the unphosphorylated state and the relief of the inhibition upon phosphorylation (Inui et al., 1986; Kirchberger et al., 1986; Suzuki & Wang, 1986), which is associated with a decrease in the apparent K_m of the pump for Ca^{2+}. Close proximity of the cytoplasmic domain of phospholamban to the calcium pump protein at a location C-terminal to its phosphorylation site has been demonstrated in cross-linking studies (James et al., 1989). Because of progressively reduced cross-linking of phospholamban to the calcium pump protein at increasing Ca^{2+} concentration, it was suggested that phospholamban may slow the E_2 to E_1 (low Ca^{2+} affinity to high Ca^{2+} affinity) conversion of the calcium pump, thereby accounting for the increase in the apparent K_m of the pump for Ca^{2+}. Significant increases in V_{max} have also been reported when phospholamban is phosphorylated by protein kinase A (Tada et al., 1979; Kirchberger et al., 1986), and recently, an effect of a synthetic peptide resembling the cytoplasmic domain of phospholamban was reported to decrease V_{max} at saturating Ca^{2+} but was without effect on the apparent K_m of the Ca^{2+}-ATPase for Ca^{2+} (Sasaki et al., 1992).

In the present study, we initially used phenylglyoxal, an arginine reactive agent considered to bind covalently at the nucleotide site of various enzymes (Borders & Riordan, 1975; Murphy, 1976; Kasher et al., 1986), to evaluate the possibility of a regulation by phospholamban involving the nucleotide site of the cardiac SR Ca^{2+}-ATPase protein. We carried out our study with control and trypsin-treated microsomes, which have been shown previously to exhibit increased rates of Ca uptake as a result of cleavage of the inhibitory cytoplasmic domain of phospholamban and to be functionally similar to microsomes that had been phosphorylated by protein kinase A (Kirchberger et al., 1986). Trypsin treatment of rabbit fast skeletal muscle microsomes, which lack phospholamban, produced no change in the Ca-uptake rate in our previous study (Kirchberger & Tada, 1976), and it has been shown repeatedly by others (Inesi & Scales, 1975; Scott & Shamoo,

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[1] Abbreviations: SR, sarcoplasmic reticulum; EGTA, [ethylenebis-(oxyethylenenitrilo)]tetraacetic acid; AMPPCP, adenylyl methylenediphosphonate.

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1982; Dux et al., 1985) that mild trypsin treatment of fast skeletal muscle microsomes resulting in cleavage at a single site, T_1 (Brandl et al., 1986), does not affect ATP-supported calcium transport; loss of enzyme activity occurs only after hydrolysis at a second site, T_2. Mild trypsin treatment of smooth muscle microsomes also produces an increase in Ca uptake associated with cleavage of phospholamban (Raeymaekers et al., 1990).

We present evidence to suggest control by phospholamban of the regulatory role of nucleotide in calcium pump activation. ATP concentrations above those known to saturate the catalytic nucleotide site of the SR Ca^{2+}-ATPase produce further increases in enzyme activity (McIntosh & Boyer, 1983; Cable et al., 1985; Bishop et al., 1987). It is suggested, moreover, that phospholamban's well-known effect on $K_{Ca^{2+}}$ is dependent on an effect of phospholamban on the allosteric regulation of the calcium pump protein by nucleotide.

EXPERIMENTAL PROCEDURES

Materials. Canine cardiac microsomes were obtained as described previously (Kirchberger at al., 1986). (Ca^{2+} + Mg^{2+})-ATPase was purified from cardiac microsomes (Xu & Kirchberger, 1989). Both types of preparations were stored in liquid nitrogen prior to use. Rabbit muscle pyruvate kinase and phosphoenolpyruvate (trisodium salt) were obtained from Sigma Chemical Co. Phenylglyoxal was obtained from both Sigma and Aldrich Chemicals. Bovine pancreatic trypsin and soybean trypsin inhibitor were obtained from Cooper Biomedical.

Assay of Ca Uptake and (Ca^{2+} + Mg^{2+})-ATPase Activity in Microsomes Treated with Phenylglyoxal. To determine the effect of phenylglyoxal on Ca uptake, microsomes (1.0 mg/mL) were incubated in a reaction medium consisting of 40 mM histidine hydrochloride, pH 6.8 at 25 °C, 0.12 M KCl, 5 mM NaN_3, and various concentrations of phenylglyoxal. The pH of the phenylglyoxal stock solution was adjusted to 6.8 with KOH prior to addition to the reaction medium. To start the Ca-uptake reaction, aliquots of treated microsomes (0.01 mg/mL, final concentration) were added to a standard medium containing 40 mM histidine hydrochloride, pH 6.8 at 25 °C, 0.12 M KCl, 5 mM NaN_3, 2.5 mM oxalate, 1 mM $MgCl_2$, 1 mM ATP, and a ^{45}Ca-labeled $CaCl_2$–EGTA buffer (Kirchberger et al., 1986) containing 125 μM $CaCl_2$ and different concentrations of EGTA to give the Ca^{2+} concentrations indicated in the text. The specific radioactivity was 0.18–1.45 μCi/μmol of Ca. At 2 and 4 min of incubation, aliquots of the reaction mixture were filtered and processed as described previously (Kirchberger et al., 1986) in order to obtain initial rates of Ca uptake. For assay of (Ca^{2+} + Mg^{2+})-ATPase activity, microsomes were pretreated as before. Aliquots of the pretreated microsomes were transferred to an ATPase reaction mixture identical to the Ca-uptake reaction mixture except that nonradiolabeled calcium and [γ-^{32}P]ATP were used and the final volume was 280 μL. Reactions were stopped after 1, 2, 3, and 5 min by adding aliquots of incubation mixture to tubes containing 25 μL of a solution of 25% trichloroacetic acid and 0.1 mM KH_2PO_4. [^{32}P]P_i liberation was detected by a method based on the extraction of a phosphomolybdate complex in an organic solvent layer (Kirchberger & Antonetz, 1982). When purified cardiac Ca-ATPase was used, the protein concentrations during phenylglyoxal treatment and the ATPase reaction were 0.1 and 0.005 mg/mL, respectively. Protein concentrations of microsomes were determined by the biuret procedure except that the Peterson modification of the Lowry method (Peterson, 1977) was used for purified (Ca^{2+} + Mg^{2+})-ATPase preparations. Bovine serum albumin was the protein standard.

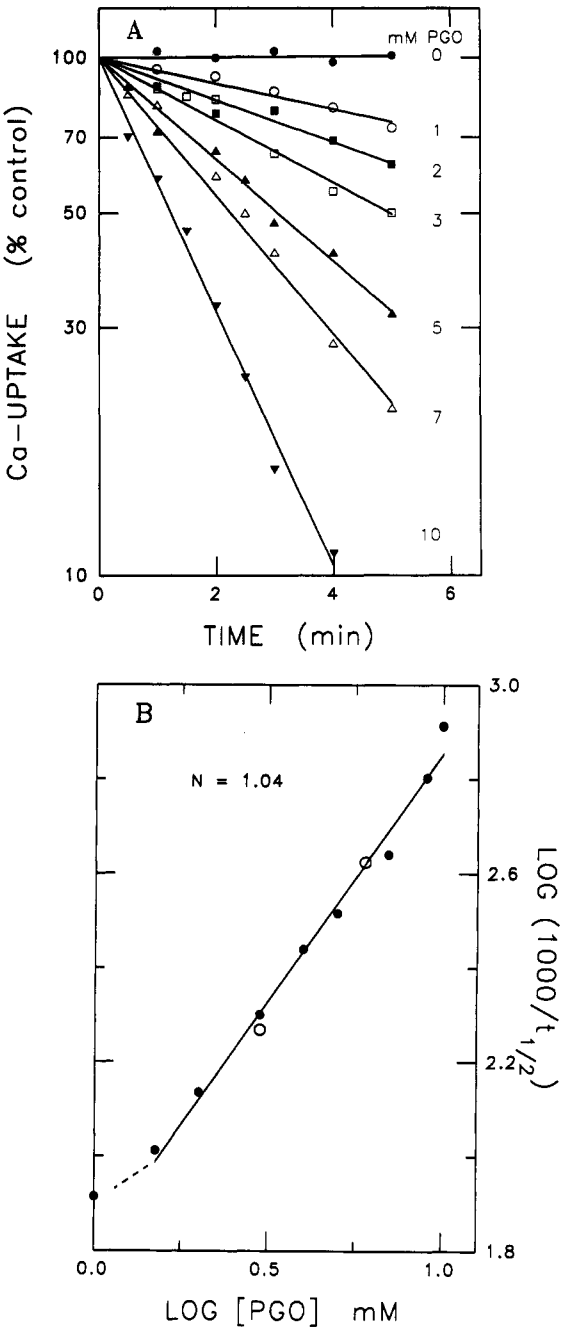


FIGURE 1: Kinetics of the inactivation of cardiac microsomal Ca uptake by phenylglyoxal. (A) Cardiac microsomes were incubated with 0–10.0 mM phenylglyoxal (PGO), and at the indicated times, aliquots were added to a standard Ca-uptake reaction mixture containing 0.32 μM Ca^{2+}. 100% represents the extrapolated rate of Ca uptake at zero time in the absence of phenylglyoxal. (B) The reciprocal of the half-time of the inactivation reaction ($t_{1/2}$) was plotted as log(1000/$t_{1/2}$) versus log [phenylglyoxal] in order to obtain the reaction order (N) with respect to phenylglyoxal concentration from the slope of the line according to the analysis of Levy et al. (1963). Trypsin-treated cardiac microsomes (○) were tested under the same conditions as untreated microsomes (●).

Trypsin Treatment of Microsomes. Cardiac microsomes (0.5 or 1.0 mg/mL in experiments with phenylglyoxal) were treated with 0.01 mg/mL trypsin at 25 °C for 2 min as described previously (Kirchberger et al., 1986) with minor modifications. Control microsomes were treated similarly except that 0.12 mg/mL trypsin inhibitor was present in the reaction medium prior to the addition of microsomes to start

Table I: Half-Times of the Inactivation of Various Forms of the Cardiac SR Calcium Pump Protein by Phenylglyoxal[a]

form	$t_{1/2}$ (min) at phenylglyoxal concn (mM) of 3	6
control microsomes: Ca uptake	5.0	2.4
control microsomes: ATPase act.	5.3	2.9
purified ATPase act.	5.3	2.4
trypsin-treated microsomes: Ca uptake	5.4	2.4

[a] Ca uptake and Ca^{2+}-ATPase activity were measured at 0.32 μM Ca^{2+} under standard assay conditions.

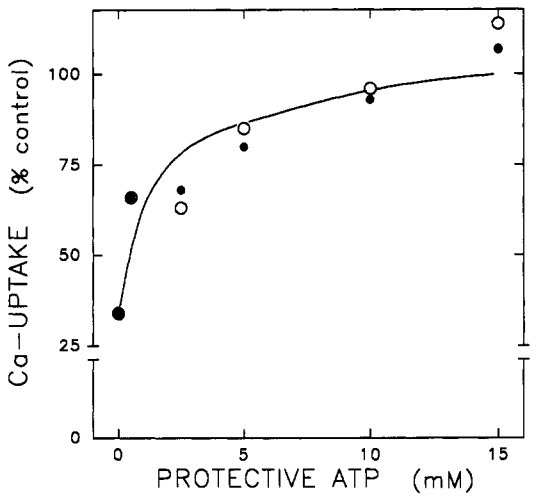


FIGURE 2: Protection of Ca-uptake activity by ATP against phenylglyoxal inactivation. Microsomes were pretreated with trypsin (○) or with trypsin and trypsin inhibitor (controls) (●) as described under Experimental Procedures. They were then incubated at a concentration of 0.6 mg/mL in 0 or 5 mM phenylglyoxal and the indicated concentrations of ATP and equimolar $MgCl_2$. After 5 min, aliquots of reaction mixture were transferred to a standard medium for assaying Ca uptake at 11 μM Ca^{2+}. 100% represents the rate of Ca uptake by control and trypsin-treated microsomes pretreated at 0 mM phenylglyoxal: 0.22 and 0.26 μmol·mg^{-1}·min^{-1}, respectively. The experiment was repeated 3 times with similar results.

the incubation instead of being added to stop the proteolytic digestion. After a further 2-min incubation after the addition of the trypsin inhibitor, the microsomes were chilled to 0–4 °C and used immediately to assay Ca uptake except when used for the study with phenylglyoxal in which case they were stored in liquid nitrogen.

Assay of Ca Uptake at Different Concentrations of ATP. Control and trypsin-treated microsomes (0.01 mg/mL) were incubated in 40 mM histidine hydrochloride, pH 6.8 at 25 °C, 0.12 M KCl, 5 mM NaN_3, 2.5 mM oxalate–Tris, 0.5 mM $MgCl_2$, various concentrations of equimolar ATP and $MgCl_2$, a Ca^{2+}–EGTA buffer system containing ^{45}Ca [(20–40) × 10^3 cpm/nmol], and an ATP-regenerating system consisting of 0.2 mg/mL pyruvate kinase and 4 mM phosphoenolpyruvate. $MgATP^{2-}$ concentrations were calculated as described by Katz et al. (1970). Microsomes were added to the temperature-equilibrated reaction medium, and after 2 min, the Ca-uptake reaction was begun by the addition of the Ca^{2+} buffer. Aliquots of the reaction mixture were filtered at 2 and 4 min or at 3 and 6 min, as appropriate, in order to obtain linearity of Ca uptake with time. The samples were processed as described above. Ca-uptake rates were corrected for counts associated with the microsomes in control reactions carried out at 0 mM $MgATP^{2-}$.

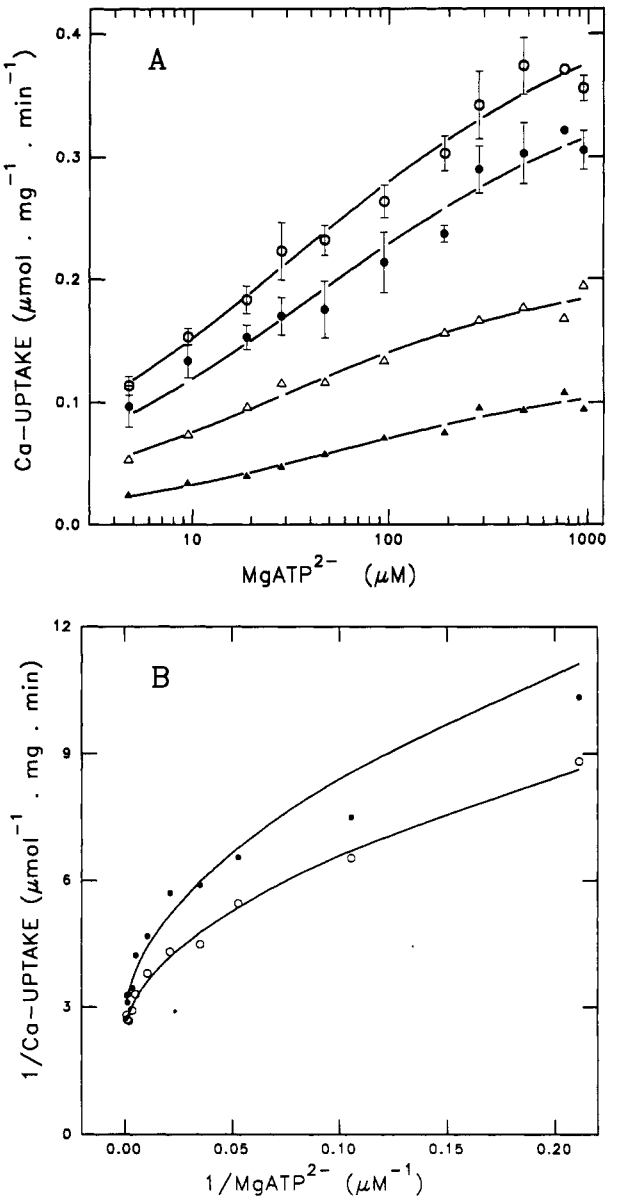


FIGURE 3: Effect of trypsin treatment of cardiac microsomes on the [$MgATP^{2-}$] dependence of Ca uptake measured at 5 μM–1 mM nucleotide. (A) Direct plot. Ca uptake was measured at 11 (●, ○) and 0.32 μM (▲, △) Ca^{2+} in control (●, ▲) and trypsin-treated (○, △) microsomes. Data points for 11 μM Ca^{2+} are means ■ SE of three experiments with different microsome preparations. Data points for 0.32 μM Ca^{2+} are the averages of two experiments with two of the three microsome preparations used before. Mean rates of Ca uptake by control and trypsin-treated microsomes, obtained at the indicated Ca^{2+} concentrations and $MgATP^{2-}$ concentrations in the range of 5 μM–1 mM, were fit (unweighted) to the equation $V = V_{max}/[1 + (K_m/[MgATP^{2-}])^N]$ where N is the Hill number. (B) Double-reciprocal plot of the data obtained at 11 μM Ca^{2+}. Kinetic parameters are shown in Table II.

RESULTS

Inactivation of Microsomal Ca Uptake and Ca^{2+}-ATPase Activity by Phenylglyoxal. A concentration- and time-dependent inhibition of Ca uptake was obtained when cardiac microsomes were incubated in the presence of phenylglyoxal (Figure 1). No change in Ca-uptake activity was detected in control microsomes incubated for up to 5 min in the absence of phenylglyoxal. Inactivation followed pseudo-first-order kinetics at concentrations ranging from 1.5 to 10 mM as seen in Figure 1B. A slope of 1.0 seen in the plot shown in Figure 1B indicates that pump inactivation is the result of modification

Table II: Effect of Trypsin Treatment of Cardiac Microsomes on the Apparent Kinetic Parameters of Ca Uptake Measured at 5 μM–1 mM $MgATP^{2+}$ [a]

parameter	control microsomes	trypsin-treated microsomes
$V_{max(app)}$ (μmol·mg^{-1}·min^{-1})	0.38 ± 0.05	0.44 ± 0.04
$K_{m(app)}$ (μM)	45 ± 27	34 ± 14
Hill coefficient (N)	0.52	0.52 ± 0.01

[a] The parameters ± SE shown in the table were obtained by separate unweighted fits to each of the two data sets obtained at 11 μM Ca^{2+} and shown in Figure 3A. Each data set consisted of 10 points each. The N value obtained for the trypsin-treated microsomes was used as a constraint in the fit for the control microsomes.

of a single Arg residue. Although two molecules of phenylglyoxal are known to combine with an Arg residue (Takahashi, 1968), the binding of the second molecule produces inactivation in a pseudo-first-order reaction once the first molecule has bound. Very low concentrations of phenylglyoxal do not allow binding of a second molecule of phenylglyoxal; hence, the slope of the line in Figure 1B deviates from linearity at concentrations less than 1.5 mM.

The half-times for phenylglyoxal inactivation of the Ca^{2+}-ATPase activity in cardiac microsomes and purified Ca^{2+}-ATPase were similar to those obtained in the Ca-uptake assay (Table I), evidence of formation of a phenylglyoxal–Arg adduct involving the calcium pump protein. Also similar were the half-times of phenylglyoxal inactivation of Ca uptake in trypsin-treated microsomes (Table I and Figure 1B).

On the basis of the assumption that the nucleotide binding site of the cardiac calcium pump protein contains an "essential" Arg as do a number of other ATPases that share homologous regions of their amino acid sequences (Kasher et al., 1986), we tested whether ATP, in the presence of $MgCl_2$, could protect against calcium pump inactivation by phenylglyoxal in cardiac microsomes. Progressively higher Ca-uptake rates were seen with increased ATP concentration, and almost complete protection against inactivation by 5 mM phenylglyoxal was obtained at 11 mM MgATP (data not shown). Trypsin treatment of microsomes produced no detectable change in the sensitivity of phenylglyoxal inactivation of Ca uptake to protection by ATP (Figure 2). A 10–15% increase above the 100% value observed in Figure 2 at the highest concentration of ATP tested is attributable to a protective effect of ATP on the Ca pump, as determined in separate experiments in which microsomes were incubated under similar conditions in the absence of phenylglyoxal. Under the conditions used for trypsin treatment, 5% or less phospholamban remains associated with the microsomes (Kirchberger et al., 1986). Cleavage of the cytoplasmic portion of phospholamban from the membrane was also apparent from a Western blot (not shown) using a polyclonal antibody against a synthetic peptide whose amino acid sequence corresponds to residues 2–30 of phospholamban (Kasinathan et al., 1988).

Kinetics of Ca Uptake at Different Concentrations of MgATP. In order to further compare their nucleotide sites, control and trypsin-treated microsomes were used to assay Ca uptake at 5 μM–1 mM $MgATP^{2-}$ in the presence of 11 μM (saturating) Ca^{2+} (Figure 3A). Trypsin treatment produced a significant increase in Ca uptake ranging from 15 to 32% at all $MgATP^{2-}$ concentrations in this range. A double-reciprocal plot of the data shows downward curvature and a Hill coefficient of 0.52 for trypsin-treated microsomes (Table II), suggesting negative cooperativity or a decrease in ligand binding affinity as a function of ligand saturation (Levitzki & Koshland, 1985). However, in SR Ca^{2+}-ATPase activity, apparent negative cooperativity has been attributed to the existence of a second ATP binding site with lower affinity than that of the catalytic site but which is associated with a marked increase in enzyme activity (McIntosh & Boyer, 1985; Cable et al., 1985; Bishop et al., 1987). Differing K_m values of two interacting sites in an enzyme may produce breaks in a double-reciprocal plot; kinetic parameters can be determined from linear portions of the plot provided they extend over a wide enough concentration range. In Figure 3B, no clearly linear regions were identifiable. The apparent kinetic parameters shown in Table II (and Table III at 0–10 μM $MgATP^{2-}$) are those of the system rather than those associated with a single nucleotide site and hence reflect site interaction. Deviation of data points above and below the fitted lines is apparent in at least three of the four data sets in the lower half of the concentration range shown in Figure 3A and the coresponding area of the plot shown in Figure 3B when N was fixed at 0.52. The fact that this deviation occurs at the same $MgATP^{2-}$ concentrations in all four data sets suggests that the affinity of $MgATP^{2-}$ does not change with trypsin treatment. Increased deviation of the control data points at 11 μM Ca^{2+} is seen in Figure 3A. This kinetic pattern may be attributable to a regulation of enzyme velocity at a second ATP binding site. The contribution of this site appears to be least evident in control microsomes at low [Ca^{2+}], where the deviation of data points from the fitted curve is negligible. This pattern is seen also in the data obtained at 0–10 μM $MgATP^{2-}$, as shown in Figure 4. An additional acceleratory effect at the high end of the concentration range seen in Figure 3A remains a possibility but will not be considered other than to point out that further acceleration of enzyme activity has been reported to occur at 0.05–5 mM ATP in skeletal muscle SR (McIntosh & Boyer, 1983).

Ca-uptake rates at 0.32 μM Ca^{2+} were lower whereas the stimulation upon trypsin treatment was markedly increased at subsaturating Ca^{2+}. The simulation of Ca uptake at 0.32 μM Ca^{2+} by trypsin was maximally 5-fold greater than at 11 μM Ca^{2+} (Figures 3 and 4).

We then sought to estimate the apparent K_m and V_{max} for the catalytic nucleotide site. At 0–10 μM $MgATP^{2-}$, N was already less than 1 (Table III); hence, the $K_{m(app)}$ overestimates

Table III: Effect of Trypsin Treatment of Cardiac Microsomes on the Apparent Kinetic Parameters of Ca Uptake Measured at Low $MgATP^{2-}$ Concentration[a]

	[$MgATP^{2-}$] (μM)					
	control microsomes (C)		trypsin-treated microsomes (T)		T/C ratio	
parameter	0–2	0–10	0–2	0–10	0–2	0–10
$V_{max(app)}$ (μmol·mg^{-1}·min^{-1})	0.11 ± 0.01	0.19 ± 0.02	0.17 ± 0.02	0.26 ± 0.02	1.5	1.4
$K_{m(app)}$ (μM)	1.2 ± 0.5	3.5 ± 0.3	1.8 ± 0.3	3.8 ± 0.8	1.5	1.1
Hill coefficient (N)	1	0.9	0.99 ± 0.3	0.90 ± 0.06		

[a] The parameters ± SE were obtained by separate fits to each of the two data sets obtained at 11 μM Ca^{2+} and shown in Figure 4. The data sets consist of 7 and 10 points each in the 0–2 and 0–10 μM $MgATP^{2-}$ concentration ranges, respectively. N values obtained with the trypsin-treated microsomes were used as a constraint in the fit of the control data.

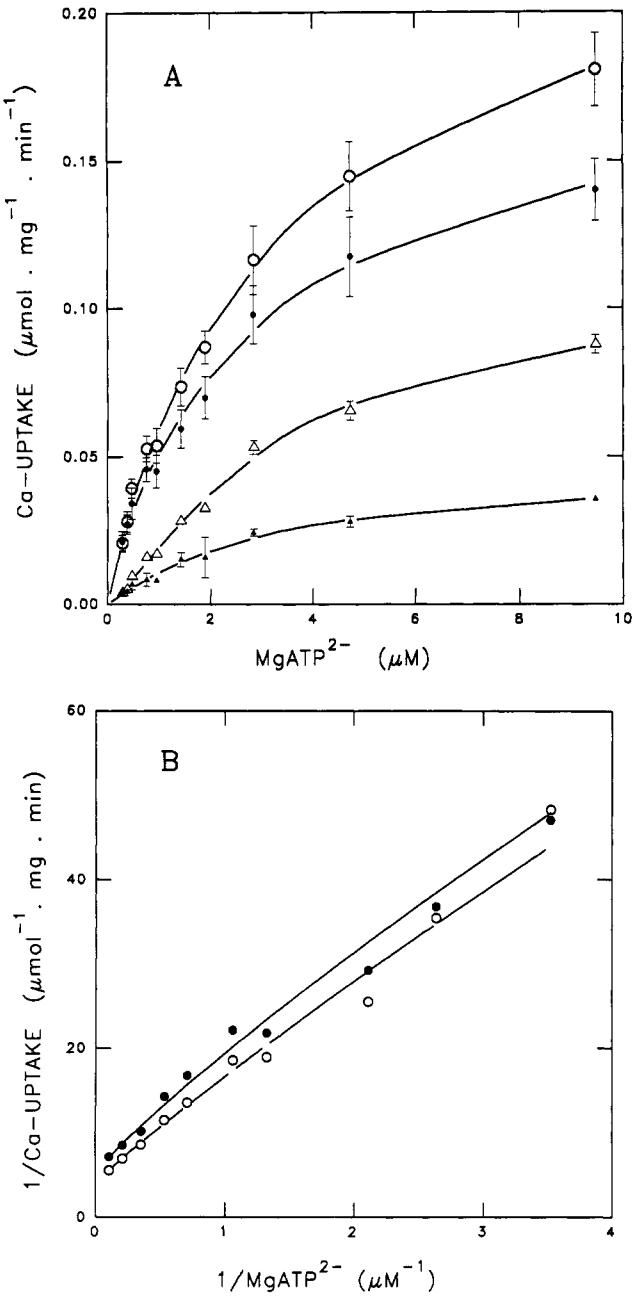


FIGURE 4: Effect of trypsin treatment of cardiac microsomes on the $MgATP^{2-}$ concentration dependence of Ca uptake measured at 0–10 μM nucleotide. The symbols are the same as in Figure 3. (A) Direct plot. Mean rates of Ca uptake by control and trypsin-treated microsomes ± SE, obtained at 11 μM Ca^{2+} and at each $MgATP^{2-}$ concentration in the ranges of 0–2 and 0–10 μM, were fit (unweighted) to the equation given in the legend of Figure 3. Data points are the means ● SE of the same three microsome preparations that were used in Figure 3 and include an additional experiment at 0.32 μM Ca^{2+}. (B) Double-reciprocal plot of the data obtained at 11 μM Ca^{2+}. Kinetic parameters are shown in Table III.

the K_m of the catalytic site. The reduced N values are seen in the slight downward curvature of the double-reciprocal plot of the data obtained at 11 μM Ca^{2+} (Figure 4B). Narrowing the ATP concentration range to 0–2 μM resulted in an N of 1 for the trypsin-treated microsomes and an apparent K_m of 1.8 μM, which compares favorably with the values of 1.1–2.1 μM reported by Shigekawa et al. (1976) under different conditions for control (untreated) cardiac microsomes. Fixing N to 1 resulted in an improved fit of the data for control microsomes. $V_{max(app)}$ increased by 50% while $K_{m(app)}$ appeared to decrease although the two parameters showed dependency. A comparison of $V_{max(app)}$ in the range of 0–10 μM $MgATP^{2-}$ in the absence of constraints during the fitting process shows a 29% increase as a result of trypsin treatment. A comparison of the actual data points obtained toward the millimolar concentration range of $MgATP^{2-}$ in Figure 3 showed an increase in the Ca-uptake rate of 15–20% at saturating Ca^{2+}. The decrease in the difference in the Ca-uptake rate as a result of trypsin treatment when comparing $V_{max(app)}$ at 0–2 μM $MgATP^{2-}$ (when N is 1) and at the highest $MgATP^{2-}$ concentration range tested suggests that different steps in the reaction cycle are being affected by trypsin treatment.

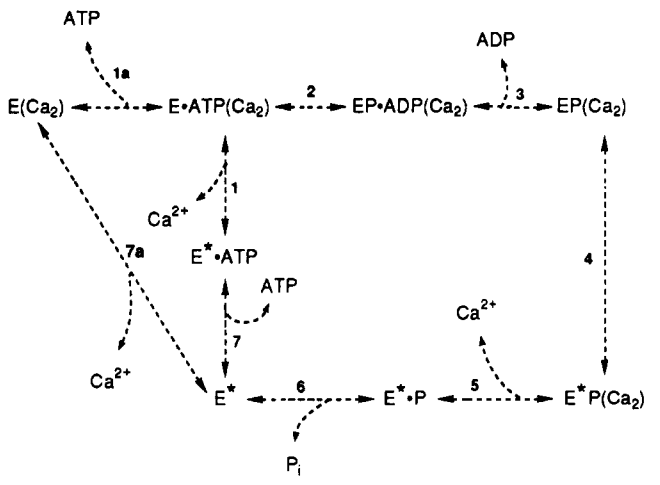
Effect of Trypsin Treatment on the Turbidity of Microsomal Suspensions. During the course of the kinetic measurements described above, we noticed a visible decrease in the turbidity of suspensions of trypsin-treated microsomes compared to control microsomes that differed only in the order of the addition of the trypsin inhibitor. Measurement of the absorbance at 600 nm provides an estimate of turbidity or light scattering in the absence of absorbance bands; no absorbance band was seen at or near this wavelength. The mean absorbance ± SE of control microsome preparations was 0.180 ± 0.021 (12%) and 0.082 ± 0.005 (6%) for the trypsin-treated microsomes. Variability among suspensions of control microsomes was twice that of the trypsin-treated microsome suspensions.

DISCUSSION

Downward curvature of double-reciprocal plots of Ca uptake against $MgATP^{2-}$ concentration and differences in these data between control and trypsin-treated microsomes reflect binding of ATP to a low-affinity site and suggest a role of phospholamban in the controlling enzyme activity associated with this site. Kinetics characteristic of apparent negative cooperativity with respect to nucleotide have been reported by Cable and Briggs (1988) for the cardiac SR calcium pump. However, on the basis of their analysis of binding of nucleotide to SR membranes involving the use of the ATP analog AMPPCP, these investigators eliminated the possibilities (a) of allosteric regulation of the Ca^{2+}-ATPase through negative cooperativity in substrate binding or (b) of half-of-the-sites reactivity involving oligomer formation. Measurement of AMPPCP binding to microsomal vesicles indicated a single class of binding sites whose affinity was high (K_D = 6.42 μM) in cardiac SR and low (K_D = 40.3 μM) in fast skeletal muscle SR. The difference in affinity was ascribed to a difference in the primary structure of the two enzymes and may be related to an extra amino acid sequence to which phospholamban may be cross-linked in the cardiac SR protein in close proximity to the phosphorylated aspartyl residue (James et al., 1989). Cable and Briggs (1988) concluded that allosteric regulation in SR membranes occurs in an enzyme monomer through a branched kinetic pathway. Our present results may be interpreted in terms of the hypothetical reaction scheme shown in Scheme I and are based on a model proposed by Cable and Briggs (1988) for the catalytic cycle of the Ca^{2+}-ATPase in cardiac SR including a branched kinetic pathway to account for allosteric regulation in the micromolar range of [ATP].

In the intact cell containing millimolar concentrations of ATP, ATP will enter the cycle at step 7 rather than step 1a in Scheme I. Therefore, in the presence of Ca^{2+}, an E·ATP-(Ca_2) complex is formed (step 1). ATP is hydrolyzed in step 2, and ADP is released from the enzyme in step 3, resulting in the formation of $EP(Ca_2)$, also known as E~P(Ca). The energy in $EP(Ca_2)$ is transformed into the vectorial transfer

Scheme I



of Ca^{2+} (Pethithory & Jencks, 1986) in step 4, which is associated with marked changes in the properties of the enzyme. The active site of the enzyme including the phosphorylated aspartic acid residue is now in a more hydrophobic environment (De Meis, 1981). Its lower Ca^{2+} affinity results in the release of Ca^{2+} into the lumen of the SR. Step 6, the departure of P_i from the enzyme, was proposed by Cable and Briggs to overcome (in cardiac SR) the rate-limiting E* to E (or E_2 to E_1) conversion, made possible by the higher rate constant of step 1 than of step 7a. Our results are consistent with an effect of unphosphorylated phospholamban to shift the equilibrium from E to E*. This would affect steps 7a and 7 as discussed further below.

At low Ca^{2+} concentrations when Ca uptake rate is limited by Ca^{2+}, the stimulation by phospholamban phosphorylation (Tada et al., 1988) or trypsin treatment of cardiac microsomes (Kirchberger et al., 1986) is greater. This may be related to ATP binding at the active site, which has been shown to accelerate the binding of Ca^{2+} to the fast skeletal muscle enzyme, causing the steady-state level of $EPCa_2$ to increase in the range of 5–10 μM ATP (Inesi & al., 1980; Stahl & Jencks, 1984; McIntosh & Davidson, 1984). Rate constants for the conformational change associated with Ca^{2+} binding are 4–6-fold higher in the presence of ATP than in its absence (Stahl & Jencks, 1984). The proposed inhibition by phospholamban at step 7 is consistent with a marked increase in the initial rate of EP formation at 5–10 μM ATP in cardiac microsomes in which phospholamban has been phosphorylated compared to control (unphosphorylated) microsomes (Tada et al., 1980).

In skeletal muscle SR in which trypsin treatment produces no stimulation of Ca transport and phospholamban is absent (Kirchberger & Tada, 1982; Kirchberger et al., 1986), we found no evidence of a break in a double-reciprocal plot of Ca-uptake rates measured at 0–10 μM $MgATP^{2-}$ (data not shown). This is in accord with the conclusion by Cable and Briggs (1988) that ATP binding to a second site occurs at a different step in the catalytic cycle in skeletal SR. A much lower K_m for AMPPCP was found with cardiac microsomes than in skeletal microsomes (see above). A difference in the K_m of the catalytic site (1–2 μM) and that of ATP binding at step 7 in cardiac SR would promote the appearance of a break in a double-reciprocal plot or produce deviation of the data points from the fitted lines in Figure 4A at 3 μM $MgATP^{2-}$. An acceleratory effect of ATP in this range would also be more apparent in trypsin-treated microsomes where the effect would be unimpeded if unphosphorylated phospholamban were to shift the E* ↔ E equilibrium in favor of the E* conformation. It has also been shown more recently that binding of ATP to a single site in E_2 in fast skeletal muscle SR accelerates the E_2 to E_1 conversion, thus increasing the rate of Ca^{2+} binding (Wakabayashi & Shigekawa, 1990).

The presence of unphosphorylated phospholamban appears to affect a number of membrane properties in addition to its effects on Ca uptake. During the course of our study, we observed that control microsomes that are stored in liquid nitrogen lose Ca-uptake activity at a greater rate than stored trypsin-treated microsomes. The apparently greater stability of the trypsin-treated microsomes may be the dominant factor in the smaller standard error values seen of some of the parameters in Tables II and III with trypsin-treated microsomes. McIntosh and Berman (1978) have reported an irreversible loss of calcium transport activity in the absence of Ca^{2+} stabilization of the pump and suggested that the high Ca^{2+} affinity state is more stable. The calcium pump protein in trypsin-treated microsomes may be in physical surroundings that favor the high-affinity state.

Decreased aggregability of microsomal vesicles subjected to mild trypsin treatment under our standard conditions provides one possible explanation (Meissner, 1988) for the decreased turbidity of these microsome suspensions. Voss et al. (1991) have suggested that the cytoplasmic domain of phospholamban, by virutue of its amphipathic nature, like mellitin, may cause aggregation of monomers of ATPase in the membrane. Aggregation of the ATPase monomers by mellitin was concluded on the basis of mellitin's ability to restrict microsecond rotational motion utilizing time-resolved phosphorescence and fluorescence spectroscopy. The decrease in turbidity seen in our trypsin-treated microsomes is somewhat comparable to effects of detergents on SR vesicles. At low $C_{12}E_8$ concentrations, Ludi et al. (1982) observed an increase in the viscosity of microsomal suspensions, decreases in Ca^{2+}-ATPase activity and Ca^{2+} affinity, and a change in the kinetics of enzyme activity suggestive of an interaction of two binding sites for the ligand. A subsequent study (Andersen et al., 1983) showed that low concentrations of the nonionic detergent $C_{12}E_8$ abolish the modulatory effect of ATP provided Ca^{2+} is present and are associated with an *increase* in the E* to E transition that may be related to an increased affinity of the ATPase for ATP in the presence of the detergent. Since enzyme velocity was decreased, an additional unidentified step was considered to be affected by $C_{12}E_8$. Like $C_{12}E_8$, phospholamban appears to produce a decreased modulatory effect of ATP in our control microsomes compared to trypsin-treated microsomes (Figures 3 and 4) although the means by which the effect is produced appear to differ. Nevertheless, the presence of the amphipathic, highly basic cytoplasmic domain of phospholamban in our control cardiac SR membranes may be the major factor in the higher turbidity of these vesicles besides increased aggregability. Another explanation for the turbidity change in trypsin-treated membranes besides may be rearrangement of phospholamban or the Ca pump in the lipid bilayer causing a change in vesicle diameter.

A shift from the E_2 to E_1 conformation has been proposed to occur as a result of treatment of skeletal muscle microsomes with trypsin under considerably harsher conditions (Andersen & Jorgensen, 1985) than were used in the present study where no effects on Ca uptake were seen in skeletal muscle microsomes. A greater susceptibility of microsomes in the E_2 conformation to proteolytic attack in the absence of protective ATP was suggested. The proposed phospholamban-dependent

shift toward the E* (E_2) conformation of the cardiac SR calcium pump may be a factor in the greater variability of some of our kinetic data and turbidity measurements with respect to control microsomes. Moreover, such a shift in equilibrium during steady state at saturating [Ca^{2+}] could the basis for the marked decrease in $V_{max(app)}$ associated with high-affinity binding of ATP to the catalytic site. Phospholamban phosphorylation is known to produce effects at multiple steps in the catalytic cycle of Ca^{2+}-ATPase (Tada et al., 1988). Multiple sites of phospholamban action in the catalytic cycle (e.g., EP decomposition) may serve to prevent these sites from becoming rate-limiting at higher rates of enzyme activity. Multiple conformational changes may occur during the ATPase catalytic cycle (Plethithory & Jencks, 1986; Jencks, 1989) so that conformational flexibility may well be hindered by the highly basic and hydrophobic (amphipathic) cytoplasmic segment of phospholamban at one or more steps in the cycle.

Regulation of the cardiac SR calcium pump by phospholamban partially resembles the regulation of the plasma membrane calcium pump by an autoinhibitory domain, which is controlled by calmodulin (Falchetto et al., 1991; Chiesi et al., 1991; Vorherr et al., 1992). The cytoplasmic domain of phospholamban shows structural homology to the autoinhibitory domain, and proteolytic cleavage of the latter domain by trypsin results in plasma membrane calcium pump activation. A synthetic 28-residue peptide corresponding to the autoinhibitory domain produces a decrease in V_{max} and an increase in K_{Ca} when tested with proteolytically treated plasma membrane calcium pump preparations. The same peptide produces marked inhibition of Ca uptake by fast skeletal muscle microsomes when tested at 0.3 μM Ca^{2+} and becomes less inhibitory at increasing Ca^{2+} concentrations. Evidence for an effect of calmodulin on regulatory nucleotide binding by the Ca^{2+}-ATPase of plasma membranes has been reported (Rossi et al., 1985).

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