Relationship between Phospholamban and Nucleotide Activation of Cardiac Sarcoplasmic Reticulum Ca^{2+} Adenosinetriphosphatase

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ABSTRACT: A strong connection with nucleotide activation of Ca^{2+}ATPase and phospholamban inhibition has been found. Phospholamban decreases the number of activatable Ca^{2+}ATPase without affecting substrate affinity or the ability of nucleotide to serve its dual modulatory roles, i.e., catalytic and regulatory. Low concentrations of certain nucleotide mimetics, quercetin, tannin, and ellagic acid, with structural similarity to adenine can unmask phospholamban's inhibitory effect while concurrently acting as competitive inhibitors of nucleotide binding. Micromolar concentrations of tannin ($EC_{50} \approx 0.3$ μM) and ellagic acid ($EC_{50} \approx 3$ μM) stimulated Ca^{2+} uptake and calcium-activated ATP hydrolysis at submicromolar Ca^{2+} in isolated cardiac sarcoplasmic reticulum (SR). Stimulation of Ca^{2+}ATPase was followed by pronounced inhibiton at only slightly higher tannin concentrations ($IC_{50} \approx 3$ μM), whereas inhibitory effects by ellagic acid were observed at much greater concentrations ($IC_{50} > 300$ μM) than the EC_{50}. A complex relationship between compound, SR protein, and MgATP concentration is a major determining factor in the observed effects. Stimulation was only observed under conditions of phospholamban regulation, while the inhibitory effects were observed in cardiac SR at micromolar Ca^{2+} and in skeletal muscle SR, which lacks phospholamban. Maximal stimulation of Ca^{2+}ATPase was identical to that observed with the anti-phospholamban monoclonal antibody 1D11. Both compounds appear to relieve the Ca^{2+}ATPase from phospholamban inhibition, thereby increasing the calcium sensitivity of the Ca^{2+}ATPase like that observed with phosphorylation of phospholamban or treatment with monoclonal antibody 1D11. Tannin, even under stimulatory conditions, is a competitive inhibitor of MgATP with a linear Dixon plot. The subsequent inhibitory action of higher tannin concentrations results from competition of tannin with the nucleotide binding site of the Ca^{2+}ATPase. In contrast, ellagic acid produced a curvilinear Dixon plot suggesting partial inhibition of nucleotide activation. The data suggest that nucleotide activation of Ca^{2+}ATPase is functionally coupled to the phospholamban interaction site. These compounds through their interaction with the adenine binding domain of the nucleotide binding site prevent or dissociate phospholamban regulation. Clearly, this portion of Ca^{2+}ATPase needs further study to elucidate its role in phospholamban inhibition.

Relaxation of cardiac and skeletal muscle requires the active removal of Ca^{2+} from the cytosol into the sarcoplasmic reticulum (SR).[1] The SR membrane contains a high number of calcium pumps (Ca^{2+}ATPase) that hydrolyze ATP to move Ca^{2+} against a large concentration gradient. The rate of Ca^{2+} uptake is dependent upon the load of Ca^{2+} that needs to be removed and may reflect the aggregation state of the enzyme (*1*). In cardiac SR, the rate of Ca^{2+}ATPase activity is regulated by an endogenous inhibitor, phospholamban (PLB), which reduces the calcium sensitivity of the enzyme. While PLB does not affect the binding affinity of Ca^{2+} (*2*) or ATP (*3*, *4*), it decreases the responsiveness of the enzyme to substrate activation. Although the precise mechanism of Ca^{2+} transport is not understood, most models invoke two conformational states (see ref *5* for review) and PLB is proposed to slow or alter the rate-limiting transition step between two enzyme conformations, thereby slowing the kinetics of active enzyme. Like most enzymes (*6*), Ca^{2+}-ATPase exhibits allosteric regulation and oligomerization/dissociation may lead to different conformational states. A recruitment hypothesis for PLB regulation (*4*) proposes that PLB regulates the number of activatable enzymes, possibly by stabilizing inactive aggregates, and does not alter the kinetics of active enzyme.

A direct interaction between nonphosphorylated PLB and Ca^{2+}ATPase has been inferred (*7*−*18*), while PLB phosphorylation (*19*), anti-PLB monoclonal antibody (*19*, *20*), or low molecular weight compounds including highly charged molecules such as heparin, long-chain aliphatic compounds such as gingerol (*21*), $C_{12}E_8$ (*22*, *23*), or Plakortones (*24*) may disrupt the interaction between PLB and Ca^{2+}ATPase.

A functional relationship between the phospholamban binding site and the nucleotide site of Ca^{2+}ATPase has been suggested (*3*, *4*). The phosphorylation domain and the nucleotide/hinge domain of the Ca^{2+}ATPase and the cytoplasmic N-terminus of PLB have been shown to be required

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[1] Abbreviations: DMSO, dimethyl sulfoxide; mAb, monoclonal antibody; P_i, inorganic phosphate; PLB, phospholamban; SR, sarcoplasmic reticulum.

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for a PLB-regulated Ca^{2+}ATPase complex (*14*−*17*). The putative PLB binding domain of Ca^{2+}ATPase, first identified in chemical cross-linking experiments (*10*) and later localized to a short amino acid segment ^{401}KDDKPV (*17*), lies close to the catalytically active ^{351}Asp high-energy phosphate acceptor. However, the portion of the nucleotide/hinge domain required for PLB inhibition has not been delineated. Selective trypsin cleavage of the cytosolic N-terminus of PLB produced marked changes in the MgATP activation of Ca^{2+}-ATPase (*3*). Experiments using the anti-PLB monoclonal antibody 1D11 (directed against the N-terminus of PLB, amino acids 7−17) showed that PLB was a noncompetitive inhibitor of nucleotide activation (*4*).

Plant-derived polyphenolic compounds often exhibit potent and selective inhibition of a wide variety of nucleotide-requiring enzymes. Some of these compounds have now been identified as activators of cardiac SR Ca^{2+}ATPase and reverse the phospholamban-induced decrease in Ca^{2+} sensitivity. Quercetin, a bioflavanoid, has been characterized for its complex inhibitory effects on skeletal muscle SR Ca^{2+}-ATPase (*25*) and its biphasic effects on cardiac SR Ca^{2+}-ATPase (*4*). Direct interaction with the nucleotide binding site of Ca^{2+}ATPase was demonstrated. No evidence for a direct interaction with phospholamban was found. Tannin, a polyphenolic plant extract, has properties similar to quercetin, but it was purported to also directly interact with the hydrophilic domain of PLB to cause its stimulatory effect on cardiac SR Ca^{2+}ATPase (*26*). Similarly, another plant extract, ellagic acid (*27*), has been suggested to interact with PLB to stimulate cardiac SR Ca^{2+}ATPase activity and calcium uptake. Both tannin (*28*) and ellagic acid (*29*) are potent inhibitors of gastric H^+K^+ATPase with IC_{50} values of 29 nM and 2.1 μM, respectively, and they were competitive with respect to ATP. The present work extends the connection between the nucleotide binding site of Ca^{2+}-ATPase and its importance for PLB regulation by presenting evidence that tannin and ellagic acid are competitive inhibitors of nucleotide activation and obscure PLB regulation.

EXPERIMENTAL PROCEDURES

Materials. $^{45}CaCl_2$ was purchased from DuPont−New England Nuclear. Ellagic acid, tannin, quercetin, and phosphoenol pyruvate were purchased from Sigma. Compounds were prepared as 10 mM stocks dissolved in DMSO. Pyruvate kinase was from Boehringer-Mannheim and A23187 was from Calbiochem. Stimulatory anti-PLB mAb 1D11 was prepared as described (*20*). Hydrophilic amino-terminal phospholamban peptides (PLB 1−25 and PLB 1−30) were synthesized on an Applied Biosystems 430A synthesizer.

Cardiac and Skeletal Muscle Sarcoplasmic Reticulum. Sarcoplasmic reticulum vesicles were prepared from canine heart (as described in ref *30*) and rabbit skeletal muscle (as described in ref *4*).

Calcium Uptake Measurement. The initial rate of calcium uptake into SR vesicles at 37 °C was determined. Cardiac SR (100 μg/mL) was incubated in uptake buffer [109.7 mM KCl, 1.09 mM $MgCl_2$, 5 mM potassium oxalate, 5 mM NaN_3, 0.5 mM EGTA [ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid], and 20 mM imidazole, pH 7.1] containing varying amounts of $^{45}CaCl_2$. For each experiment, appropriate solvent controls, DMSO up to 2%, Tris-buffered saline (20 mM Tris-HCl, pH 7.4, and 150 mM NaCl), or mAb 1D11 in Tris-buffered saline were performed. Free Ca^{2+} concentrations were calculated (*31*). Uptake was initiated by the addition of 3.75 mM MgATP. At serial times (15−20 s), 50 μL aliquots were vacuum-filtered through 0.45 μm HAWP (Millipore) filter plates, followed by two washes with 0.2 mL of ice-cold 150 mM KCl. Radioactivity trapped inside SR vesicles at each time point was determined by Topcount (Packard) liquid scintillation counting. Uptake rates for each free Ca^{2+} were determined by linear regression of radioactivity over time. Data for pCa curves were analyzed by nonlinear regression using the Sigma Plot Scientific Graph System (Jandel Scientific) as described (*15*).

ATPase Activity Microtiter Plate Assay. The malachite green/acid−molybdate assay for inorganic phosphate described (*32*) was adapted to 96-well plates. Reactions were performed at room temperature in a total volume of 100 μL with final concentrations of 109.7 mM KCl, 0.89 mM $MgCl_2$, 50 mM imidazole (pH 7.1), 0.5 mM EGTA, 5 mM NaN_3, 4 μM A23187, and 0.5 mM MgATP with cardiac (1 μg) or skeletal muscle sarcoplasmic reticulum (0.25 μg). Zero calcium samples including solvent (5% DMSO) or compound were always paired with test sets for subtraction of basal ATPase activity. Free Ca^{2+} concentration was calculated as above. After the linearity of the enzyme activity over time was established, a single end-point time was selected for the reaction. For MgATP concentration dependency curves, the assay was performed with 100 mM KCl, 0.89 mM $MgCl_2$, 0.5 mM EGTA, 20 mM MOPS (pH 7.1), 5.0 mM NaN_3, 4 μM A23187 containing varying amounts of MgATP, and an ATP-regenerating system comprising of 0.4 μg/mL pyruvate kinase and 0.5 mM phosphoenol pyruvate was included in the buffer. For pyrophosphate concentration dependency curves, a fixed MgATP concentration of 100 μM plus the ATP-regenerating system was used with increasing amounts of pyrophosphate added.

RESULTS

Phospholamban Inhibition of Nucleotide Activation. PLB inhibition alters the MgATP dependency of Ca^{2+}ATPase and the effects are reversed by addition of anti-PLB mAb 1D11 or saturating free [Ca^{2+}] (Figure 1A). As we previously reported (*4*), PLB is a noncompetitive inhibitor of nucleotide activation and alters the V_{max} without affecting nucleotide binding affinity. It is clear from the biphasic curve at pCa 6.75 that the two modes of MgATP activation are still present and the regulatory effect of MgATP at concentrations greater than 1 mM is unaffected by PLB. Two PLB effects are readily apparent; first, the rate of ATP hydrolysis at each MgATP concentration is diminished, suggestive of a decreased number of activatable enzymes, and second, the plateau phase lacks the slow upward rise possibly suggesting protein−protein interaction during activation. To confirm that the regulatory mode of MgATP activation is intact during PLB inhibition, the catalytic site was saturated with 100 μM MgATP and pyrophosphate was used to mimic the regulatory action (Figure 1B). Indeed, the regulatory nucleotide site was unaffected and PLB does not discriminate between the two modes of ATP activation. Furthermore, although the PLB binding domain has been mapped near the high-energy phosphate acceptor, the inhibitory effects do not appear to be directly linked to this area. In addition, we could not

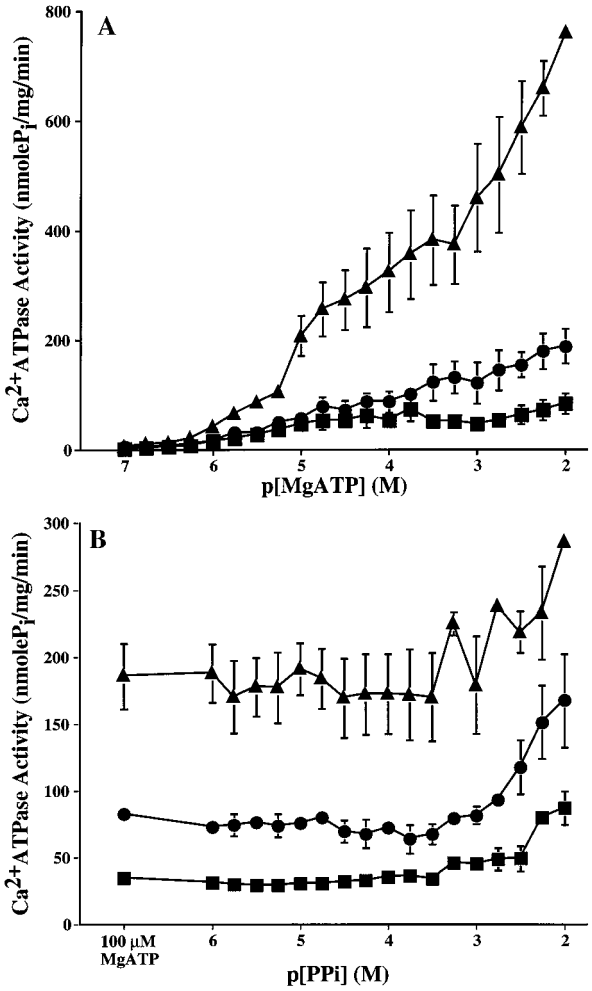

FIGURE 1: MgATP dependency (panel A) and PP_i dependence (panel B) of cardiac sarcoplasmic reticulum Ca^{2+}ATPase activity at pCa 6.75 (■), pCa 6.75 + mAb 1D11 (●), and pCa 5.0 (▲). Calcium-stimulated ATP hydrolysis was measured at 25 °C using 10 μg/mL cardiac SR, an ATP-regenerating system, and varying concentrations of MgATP ($n = 5$) or a fixed concentration of 100 μM MgATP plus varying concentrations of PP_i ($n = 3$).

achieve mAb 1D11 stimulation of Ca^{2+}ATPase when acetyl phosphate was used as the substrate (data not shown). Therefore, PLB inhibition must require additional components possibly within the adenine binding pocket.

Stimulation of Cardiac SR Ca^{2+}ATPase. Low micromolar concentrations of ellagic acid and tannin markedly increased the rate of calcium uptake (Figure 2) into cardiac SR vesicles (100 μg/mL) at a submaximal free Ca^{2+} of 0.1 μM. The maximum stimulation was equivalent to the effect of saturating anti-PLB mAb 1D11 treatment, which typically increased the rate of calcium uptake by 800−1200% of the control rate. The stimulatory effect of ellagic acid peaked at 10 μM and remained over a wide range of concentrations. Half-maximal stimulation (EC_{50}) for ellagic acid was approximately 3 μM. Ellagic acid did not produce any significant effect on calcium uptake into skeletal muscle SR at the same free Ca^{2+} (0.1 μM), although a modest inhibition of about 15% occurred at 500 μM. In contrast, the stimulatory effect of tannin had an EC_{50} around 1 μM and peaked at 3 μM. The stimulatory effect diminished rapidly as the tannin concentration was increased until complete inhibitory effects (relative to the control activity) were observed at 31 μM. Half-maximal inhibition (IC_{50}) of maximal activity occurred

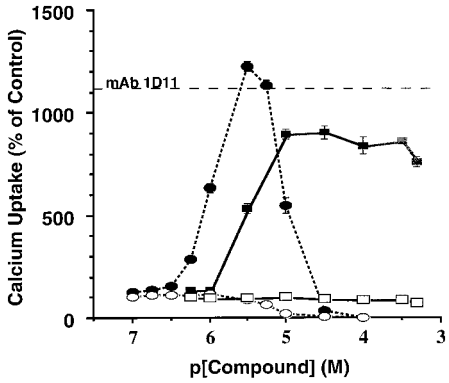

FIGURE 2: Concentration dependence of the effects of ellagic acid (■, □) and tannin (●, ○) on calcium uptake into cardiac (■, ●) and skeletal muscle (□, ○) SR. The rates of calcium uptake at pCa 7.0 (0.10 μM Ca^{2+}), 3.75 mM MgATP, 100 μg/mL SR protein, and 1% DMSO were measured at 25 °C. Shown are the mean and standard error of triplicate measurements of canine cardiac SR and rabbit skeletal muscle SR. Basal calcium uptake rates were 6.35 nmol mg^{-1} min^{-1} for cardiac SR and 526 nmol mg^{-1} min^{-1} for skeletal muscle SR. The data were normalized to each of these rates.

at 10 μM. In skeletal muscle SR at 0.1 μM free Ca^{2+}, only the inhibitory portion of the tannin concentration−response curve was present with an IC_{50} of 3 μM. Tannin's biphasic concentration−response profile observed in cardiac SR at low free Ca^{2+} and monophasic inhibitory effect on skeletal muscle SR calcium uptake is similar to the results obtained with quercetin (*4*).

Saturating amounts of Ca^{2+} (>1 μM) can completely overcome the inhibitory effect of PLB on Ca^{2+}ATPase activity in cardiac SR. At high free Ca^{2+} (pCa 5.5), neither ellagic acid nor tannin produced a stimulatory effect on calcium uptake into cardiac SR (*33*). In fact, the results were comparable to those obtained with skeletal muscle SR shown in Figure 2. Ellagic acid had virtually no effect and at 500 μM produced a mild (10−15%) inhibition of the maximal activity. Tannin produced only inhibitory effects with an IC_{50} of 5 μM with complete inhibition at concentrations above 10 μM.

As shown with quercetin (*4*), a stoichiometry between tannin or ellagic acid, ATP, and SR protein seems to be required for optimal effect. Concentration−response curves were constructed at different SR protein concentrations for both ellagic acid and tannin by measuring calcium uptake into cardiac SR at pCa 7.0. A correlation between the SR protein concentration and the effect of compound at a given concentration is evident, but the selectivity ratio (IC_{50}/EC_{50}) remains constant (Table 1). Analogous results were obtained when ATP hydrolysis was measured. There was a leftward shift in the concentration−response curves (Figure 3) due to the fact that, in this assay, the SR protein concentration is $^1/_{10}$ that used in the calcium uptake (Figure 2) and the MgATP concentration (0.5 mM) is substantially lower. Ellagic acid and tannin at both free [Ca^{2+}] produced stimulatory effects equivalent to that of mAb 1D11. The EC_{50} for ellagic acid (0.7 μM) and tannin (0.2 μM) were identical for both low and high [Ca^{2+}]. Similarly, the free [Ca^{2+}] did not affect the IC_{50} of 2 μM for tannin nor the selectivity ratio of 10. A significant reduction (up to 60%) in ellagic acid's stimulatory effect on cardiac SR Ca^{2+}ATPase actvivity occurred at high concentrations (>100 μM) and this was seen

Table 1: Protein Dependency of the Effects of Ellagic Acid and Tannin on Calcium Uptake into Cardiac Sarcoplasmic Reticulum at PCa 7.0[a]

protein (μg/mL)	EC_{50} (μM)	IC_{50} (μM)	ratio
	Ellagic Acid		
10	2.0	>200	>100
25	2.0	>200	>100
50	3.75	>500	>100
100	5.5	>1000	>100
	Tannin		
10	0.316	3.16	10
25	0.316	3.16	10
50	0.713	7.19	10
100	1.400	14.5	10

[a] Concentration−response curves were constructed as shown in Figure 1 with different concentrations of cardiac sarcoplasmic reticulum membranes. The EC_{50} and IC_{50} values were determined with the maximal rate, which was equivalent to anti-phospholamban mAb 1D11 treatment. It is assumed that higher concentrations of ellagic acid would produce complete inhibition if it remained in solution.

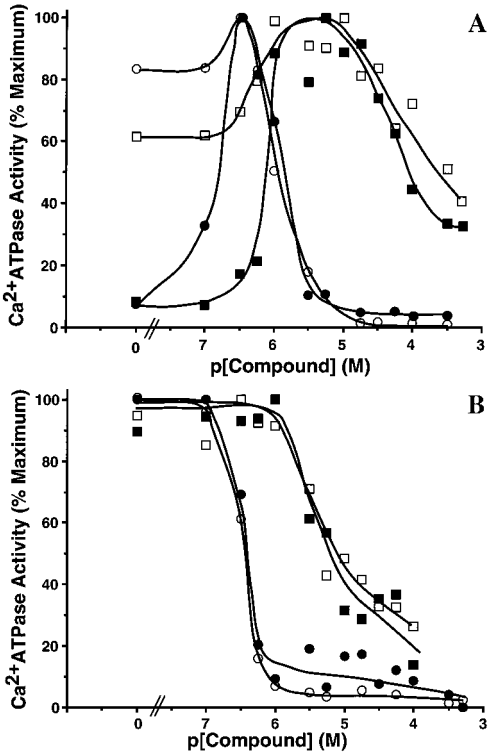

FIGURE 3: Concentration dependence of the effects of ellagic acid (■, □) and tannin (●, ○) on cardiac (panel A) and skeletal muscle (panel B) SR Ca^{2+}ATPase activity at pCa 7.0 (■, ●) and pCa 5.5 (□, ○). Calcium-stimulated ATP hydrolysis at low (pCa 7.0) and high (pCa 5.5) free Ca^{2+} was measured at 25 °C with 0.5 mM MgATP, 10 μg/mL cardiac SR or 2.5 μg/mL skeletal muscle SR, and 5% DMSO. The means of triplicate measurements are shown. The data were normalized to the maximum rate measured for each curve.

at both low and high free $[Ca^{2+}]$. The activity curves at both $[Ca^{2+}]$ were almost superimposable. With 2.5 μg/mL skeletal muscle SR, both compounds produced only inhibitory effects which were similar at pCa 7.0 and pCa 5.5. Ellagic acid produced up to a 70% inhibition of basal ATP hydrolysis rate again occurring at both low and high pCa. Because of solubility problems with ellagic acid, it was not possible to test Ca^{2+}ATPase activity at higher compound concentrations. However, assuming complete inhibition with higher ellagic acid concentrations, IC_{50} values of 0.5 μM tannin and 316 μM ellagic acid were obtained.

Table 2: Effects of Ellagic Acid and Tannin on Calcium Sensitivity of Cardiac Sarcoplasmic Reticulum[a]

treatment	V_{max} (nmol of P_i mg^{-1} min^{-1})	$K_{m(Ca)}$ (μM)	$K_{0.5}$ (μM)
control	423.1 ± 8.4	0.515 ± 0.021	6.29 ± 0.02
mAb 1D11	406.7 ± 7.5	0.248 ± 0.012	6.61 ± 0.02
10 μM ellagic acid	414.2 ± 18.3	0.263 ± 0.022	6.58 ± 0.03
3.16 μM tannin	423.3 ± 19.0	0.284 ± 0.022	6.55 ± 0.03

[a] Calcium titration curves of cardiac SR Ca^{2+}ATPase activity were constructed in the presence of the conditions listed. All curves were produced in the presence of 5% DMSO.

Ellagic acid, tannin, and quercetin, well-known protein kinase inhibitors, are unique in their ability to stimulate cardiac SR Ca^{2+}ATPase activity. The effects of some commercially available kinase inhibitors (Calbiochem signal transduction catalog) on cardiac SR Ca^{2+}ATPase activity were tested. Potent inhibitors (IC_{50}) include damnacanthal (2 μM), H-89 (5 μM), KN-93 (10 μM), staurosporine (20 μM), pseudohypericin (25 μM), bisindoylmaleimide (40 μM), KT5926 or KT5823 (50 μM), and tyrphostin AG 370 (50 μM). Other compounds exhibited no inhibitory effects up to 100 μM; erbstatin analogue, and the following tyrphostins A23, A47, A63, B56, AG 879, and AG 1288. None of these compounds demonstrated the ability to stimulate cardiac SR Ca^{2+}ATPase activity. IC_{50} values for the cAMP-dependent protein kinase inhibitor H-89 of 8.1 and 7.2 μM for cardiac and skeletal muscle SR Ca^{2+}ATPase, respectively, have been reported (*34*).

Loss of Phospholamban Regulation. Optimal stimulatory concentrations of ellagic acid or tannin reduced the $K_{m(Ca)}$ (increased the apparent calcium sensitivity) of cardiac SR Ca^{2+}ATPase activity to the same amount as treatment with mAb 1D11 (Mayer et al., 1996) and there was no effect on the V_{max} (Table 2). Furthermore, combining saturating amounts of mAb 1D11 with either compound did not produce an additive increase in $K_{0.5(Ca)}$ (data not shown). Thus, mAb 1D11 and the two compounds are indistinguishable in their effects on calcium sensitivity and appear to act through a common mechanism.

Ellagic acid and tannin appear to mimic mAb 1D11 in relieving the cardiac SR Ca^{2+}ATPase from the endogenous PLB inhibition. If subsaturating amounts of mAb 1D11 were used, then the compounds can still stimulate Ca^{2+}ATPase activity. Concentration−response curves on cardiac SR Ca^{2+}-ATPase activity were constructed at pCa 7.0 (Figure 4). The maximal rate reaches the same level as treatment with saturating amounts (10 μg/mL) of mAb 1D11 and the optimal concentration of compound is not decreased. Thus, mAb 1D11 and either compound show additivity. The presence of mAb 1D11 does not affect the inhibitory properties of either compound, suggesting that the inhibitory effects are mediated by interactions with Ca^{2+}ATPase directly.

A direct interaction of stimulatory concentrations of tannin and ellagic acid with PLB was checked. The effect of short PLB-derived peptides (PLB 1−25 and PLB 1−30) on compound-induced stimulation of cardiac SR Ca^{2+}ATPase activity was assayed in the calcium uptake assay (Figure 5). PLB 1−25 at a concentration of 50 μM had no effect on the basal rate of calcium uptake at pCa 7.0. As can be seen in

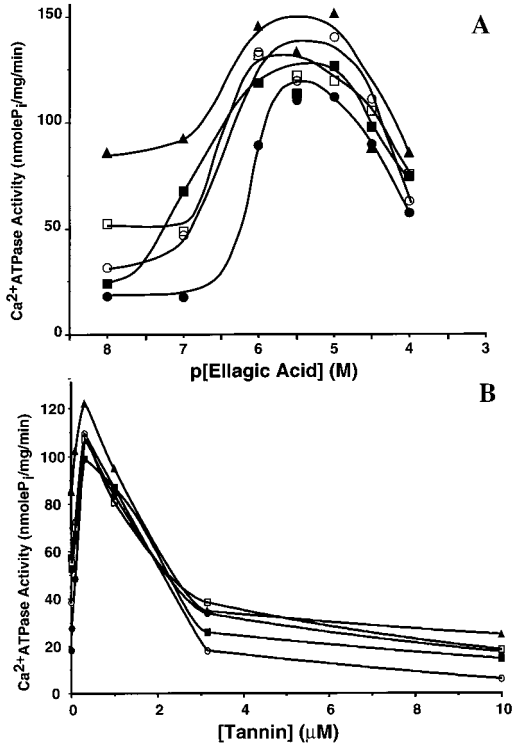

FIGURE 4: Concentration dependence of the effects of ellagic acid (panel A) and tannin (panel B) on cardiac SR Ca^{2+}ATPase in the presence of increasing amounts of mAb 1D11: 0 μg/mL (●), 1 μg/mL (○), 1.25 μg/mL (■), 2 μg/mL (□), and 5 μg/mL (▲). The rate of ATP hydrolysis in the presence of 10 μg/mL mAb 1D11 was 132 nmol of P_i mg^{-1} min^{-1} for the ellagic acid experiments and 110 nmol of P_i mg^{-1} min^{-1} for the tannin experiments. Ca^{2+}-stimulated ATP hydrolysis was measured in triplicate as described for Figure 3.

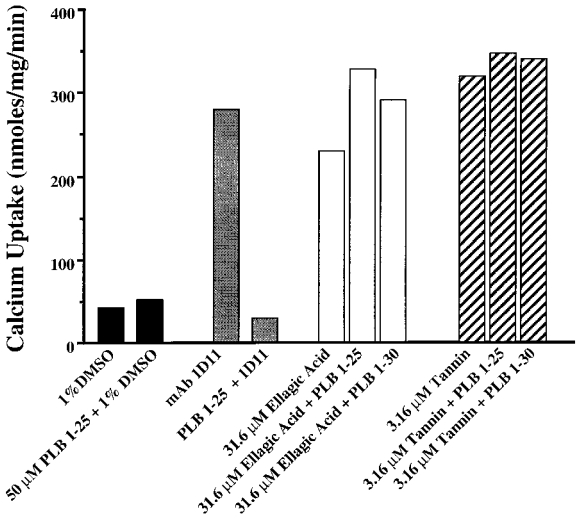

FIGURE 5: Effects of phospholamban peptides on reversing the stimulation produced by mAb 1D11, ellagic acid, and tannin. The histogram shows the average ($n = 3$) rate of calcium uptake into cardiac SR at pCa 7.0 under the following conditions. The solid column shows the DMSO alone control and DMSO plus 50 μM PLB 1−25 control rates. The shaded columns depict the effect of mAb 1D11 and its reversal with 50 μM PLB 1−25 peptide. The open columns show the stimulation with 32 μM ellagic acid and the combinations of ellagic acid and 50 μM PLB 1−25 or PLB 1−30, respectively. The hatched columns show similar data for 3 μM tannin and the two PLB peptides.

the histogram (Figure 5), 50 μM PLB 1−25 peptide completely reversed the stimulation of calcium uptake by mAb 1D11. In contrast, PLB 1−25 and PLB 1−30 were ineffective at reversing the stimulation of calcium uptake by either ellagic acid or tannin. Therefore, a specific interaction with the hydrophilic domain of PLB is not evident.

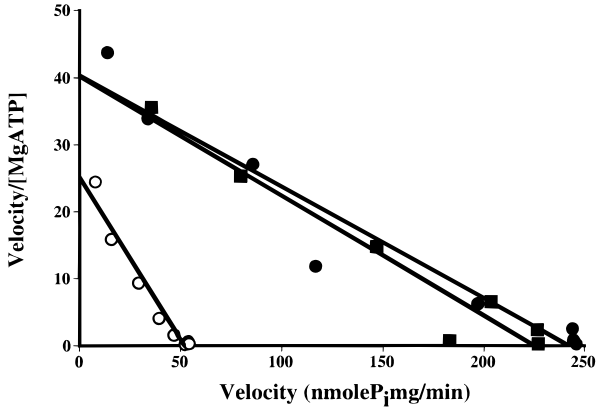

FIGURE 6: Eadie−Scatchard plots of V/[ATP] vs V comparing the MgATP dependency of control (○) to the effect of 10 μM ellagic acid (●) and 0.3 μM tannin (■) on the $V_{max(MgATP)}$ and the $K_{m(MgATP)}$. Lines were fit by linear regression with correlation coefficients (r^2) greater than 0.93.

Nucleotide Binding Site of Ca^{2+}ATPase. Polyphenolic compounds have been well-documented as kinase inhibitors, specifically as inhibitors of nucleotide activation. The MgATP dependency of cardiac SR Ca^{2+}ATPase under conditions of ellagic acid and tannin stimulation was characterized and compared with that of normal cardiac SR Ca^{2+}ATPase activity. Complete MgATP dependency curves at pCa 7.0 were constructed with MgATP concentrations ranging from 0.1 to 1000 μM and an ATP-regenerating system. Optimal conditions for compound stimulation were selected. The data were transformed into Eadie−Scatchard format (V/[ATP] vs V) and the plots are shown in Figure 6. Values for the V_{max} derived from the x-intercept were 51.6 nmol of P_i mg^{-1} min^{-1} for the control, while rates of 241.6 and 224.7 nmol P_i mg^{-1} min^{-1} were derived in the presence of 10 μM ellagic acid and 0.32 μM tannin, respectively. The apparent $K_{m(ATP)}$ from the negative inverse of the slope ($m = -1/K_m$) were 2.06 μM (control), 5.99 μM (ellagic acid), and 5.59 μM (tannin). As reported previously (*4*), mAb 1D11 treatment increased the V_{max} without affecting the $K_{m(ATP)}$. Hence, mAb 1D11 is a noncompetitive activator of cardiac SR Ca^{2+}ATPase, or alternatively, PLB is a noncompetitive inhibitor of cardiac SR Ca^{2+}ATPase. Both ellagic acid and tannin increase the V_{max} like mAb 1D11, while at the same time, the apparent $K_{m(ATP)}$ is increased. Under these optimal stimulating conditions, ellagic acid and tannin act as a competitive inhibitors for MgATP activation of cardiac SR Ca^{2+}ATPase. However, stimulation is observed because they mimic mAb 1D11 by removing the inhibition by PLB.

Additional MgATP dependency curves were generated in the presence of varying concentrations of ellagic acid or tannin including both stimulatory and inhibitory concentrations of compound (data not shown). Eadie−Scatchard plots were constructed for each compound concentration and the values of apparent $K_{m(ATP)}$ and V_{max} were determined. Dixon plots (Figure 7) of apparent $K_{m(ATP)}$ vs each inhibitor concentration were constructed. Tannin yielded a linear Dixon plot with a K_i value of 2 μM. This concentration is

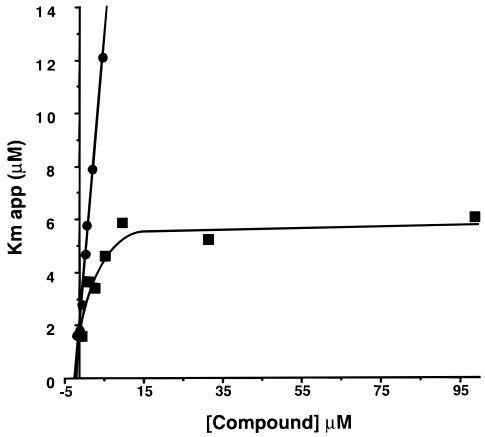

FIGURE 7: Dixon plot for a competitive inhibitor of MgATP activation of Ca^{2+}ATPase activity. The effect of ellagic acid concentrations (■) ranging from 5 to 100 μM or tannin concentrations (●) ranging from 0.1 to 10 μM on the MgATP concentration dependence of Ca^{2+}ATPase was measured in triplicate. For each compound concentration, Eadie−Scatchard plots were constructed and the $K_{m(ATP)app}$ was determined. These values were then plotted versus the compound concentration. The *x*-intercept equals $-K_i$ and the *y*-intercept is the $K_{m(MgATP)}$ for control cardiac SR. For tannin, the linear regression fit was $y = 2.5371 + 1.7392x$ and the correlation coefficient $r^2 = 0.939$.

close to the optimal stimulatory concentration of tannin. Tannin is similar to quercetin which also yielded a linear Dixon plot and had a $K_i = 26$ μM and an optimal stimulatory concentration of 25 μM. In contrast, ellagic acid yielded a curvilinear Dixon plot indicative of partial inhibition of the nucleotide binding site. Ellagic acid yielded a similar inhibitory relationship with the tyrosine-specific protein kinase $pp60^{src}$ (*35*). This partial inhibition may explain the high selectivity ratio of ellagic acid on cardiac SR Ca^{2+}-ATPase at low free Ca^{2+}.

DISCUSSION

The results demonstrate that ellagic acid and tannin interact with the nucleotide binding site on cardiac SR Ca^{2+}ATPase that dissociates it from the inhibitory action of phospholamban, leading to an apparent stimulation of the enzymatic activity. The compounds do not have a direct stimulatory effect on Ca^{2+}ATPase, since when assayed in the absence of phospholamban regulation, e.g., treatment with mAb 1D11, the stimulatory effect is lost. No evidence for a direct interaction with phospholamban was found. Examination of the effect of ellagic acid and tannin on the MgATP dependency of Ca^{2+}ATPase revealed competitive interaction with the nucleotide binding site even under conditions where enzyme activity was stimulated. The K_i values suggest an intermediate binding affinity similar to that reported for ATP binding ($K_d = 20$ μM) in the absence of Mg or at acid pH (*36*). This competition with the activating ATP substrate can account for inhibitory effects at higher concentrations. The partial inhibitory effects of ellagic acid may account for the large difference in the concentrations required for stimulation and inhibition.

Tannins are potent inhibitors of protein kinases, the effects of a variety of tannin components on several protein kinases were characterized (*37*). Inhibition of cAMP-dependent protein kinase occurred with an $IC_{50} = 0.2-1.7$ μM and inhibition of other protein kinases had similar potencies (IC_{50}); wheat embryo Ca^{2+}-dependent protein kinase (1.8 μM), protein kinase C (26 μM), and myosin light chain kinase (56 μM). Tannin inhibited PLB phosphorylation by cAMP-dependent protein kinase with an $IC_{50} = 3$ μM, but it could only partially inhibit troponin I phosphorylation (*26*). The reason for the discrepancy between the two phosphorylation substrates is not clear but may be related to the substrate to enzyme stoichiometry. Similar IC_{50} values for inhibition of cardiac (5 μM) and skeletal muscle (2 μM) SR Ca^{2+}ATPase (50 μg/mL SR protein) were reported. The results (Figures 2 and 3) demonstrate the complex relationship between compound, SR protein, and MgATP concentration for observing stimulation or inhibition and the determination of EC_{50} and IC_{50}. In contrast to the earlier study, where a partial reversal of tannin-induced stimulation was achieved with the PLB 1−20 peptide, we could not observe any reversal effect with either PLB peptide 1−25 or 1−30. Since tannin and ellagic acid are potent inhibitors of cAMP-dependent protein kinase, we did not attempt to assess their additivity with phospholamban phosphorylation. Instead, we did observe additivity between tannin and submaximal amounts of anti-PLB mAb 1D11, which suggests that tannin does not alter the hydrophilic portion of PLB with respect to antibody recognition. Several polyphenolic compounds related to quercetin failed to inhibit antibody binding to the hydrophilic portion of PLB (amino acids 7−17) in a competitive enzyme-linked immunosorbent assay (*4*). Thus, we do not see evidence of a direct interaction between tannin and phospholamban as previously suggested (*26*).

Ellagic acid is a potent inhibitor of numerous protein kinases, so a detailed structure−activity search was performed (*35*) with a range of selectivities between serine/threonine and tyrosine kinases. Ellagic acid inhibited tyrosine protein kinase $pp60^{src}$ and cAMP-dependent protein kinase with similar potencies, $IC_{50} = 0.3$ and 0.6 μM, respectively. As with tannin, no evidence of a direct interaction of ellagic acid with the hydrophilic domain of PLB was found. Also, additivity between ellagic acid and mAb 1D11 was present when submaximal amounts were combined, as previously reported (*27*), but no addivity was observed with saturating levels of each treatment. Again, no evidence for a direct interaction between ellagic acid and phospholamban was found.

Ca^{2+}ATPase has a complex nucleotide dependency with strong negative cooperativity at low ATP concentrations that diminishes at high millimolar ATP concentrations (*38*). The cause of this negative cooperativity is highly controversial. Half-the-site reactivity requiring at least two interacting enzymes, an extreme circumstance of negative cooperativity, has been hypothesized (*39*−*41*). In this mechanism, a reaction at one subunit induces a structural change in the neighboring subunit to reduce the substrate binding affinity and reactivity; thus, only half the sites can perform catalysis. This mechanism aptly fits the observed 2:1 stoichiometry between FITC labeling (*42*) to measured phosphoenzyme level, as well as the 1:1 stoichiometry between rabbit skeletal muscle (heat-stable) and scallop (heat-labile) Ca^{2+}ATPase for heat inactivation (*43*). Another outcome of this mechanism is the ability of substrate binding to the low-affinity site to accelerate the turnover rate of the catalytically active subunit. Two modes of nucleotide activation can be ascribed; a high-affinity, catalytic site ($K_d \approx 2-4$ μM) and a low-

affinity, regulatory site ($K_d \approx 1$ mM). The catalytic site requires the formation of phosphoenzyme upon ATP hydrolysis followed by calcium translocation. The regulatory site is noncatalytic, but the presence of substrate serves to accelerate the turnover of the phosphoenzyme. Therefore, a single nucleotide binding site can serve two functions.

Ellagic acid, tannin, and quercetin bind to the nucleotide binding domain (presumably in the adenine binding pocket) and do not, per se, discriminate between the two modes as MgATP does. These compounds lack a high-energy phosphate required for catalysis, and since the regulatory mode appears to involve the terminal two phosphates of MgATP, these compounds cannot serve a regulatory function. As alluded (*3*), phospholamban appears to mask the regulatory nucleotide site, thereby preventing the acceleration of enzyme turnover. However, as Figure 1 clearly demonstrates, the regulatory nucleotide site is still functional. One would predict that these compounds would cause the dissociation of an unactivatable Ca^{2+}ATPase complex into smaller activatable units. Long-chain aliphatic compounds such as $C_{12}E_8$ at low concentrations have been reported to unmask the regulatory nucleotide site of NaK-ATPase (*44*). Not surprisingly, low concentrations of $C_{12}E_8$ lead to the loss of PLB inhibition (*22*, *23*), but $C_{12}E_8$ (like mAb 1D11) does not affect nucleotide binding affinity (our unpublished results). This is consistent with the PLB mutagenesis work (*16*, *17*) that some hydrophobic interactions are involved with PLB inhibition. In addition, low concentrations of $C_{12}E_8$ lead to dissociation of Ca^{2+}ATPase aggregates (*23*), thereby allowing more coupling of Ca^{2+}ATPase units and increased enzymatic activity. A strong connection between compounds that affect the cooperativity between nucleotide sites and the loss of phospholamban's inhibitory effects is evident. These data support the recruitment hypothesis (*4*) and the suggestion (*1*) that phospholamban's primary effect is to keep a reserve aggregated pool of enzyme from being activated. However, these reserve enzymes can still bind nucleotide and calcium, which leads to dissociation and activation. The oligomeric conditions of both molecules must be considered when probing this important regulatory mechanism.

In summary, the results with ellagic acid and tannin strengthen the hypothesis of a functional link between the nucleotide binding site and the PLB binding site of Ca^{2+}-ATPase (*3*, *4*). Under conditions where stimulation of cardiac Ca^{2+}ATPase activity occurs, these compounds, as well as quercetin, increase the apparent $K_{m(ATP)}$ and yield linear Dixon plots, indicating competitive interaction. Further, the biphasic concentration−response curves suggest a common site for stimulation and inhibition of cardiac SR Ca^{2+}ATPase. Because the inhibitory portion exists in the absence of PLB (skeletal muscle SR) and in the absence of PLB regulation in cardiac SR (high free Ca^{2+} or presence of mAb 1D11), competition with ATP binding likely accounts for the inhibition of Ca^{2+}ATPase. PLB is a noncompetitive inhibitor of Ca^{2+}ATPase (Figure 6) and decreases the number of activatable enzymes (V_{max}). In a complementary study, mild trypsinization to cleave the hydrophilic domain of PLB (*3*) increased the V_{max}. Thus, as has been shown, the removal of PLB regulation should not affect nucleotide binding affinity (*2*, *3*) or inactivation by phenylglyoxyl (*3*). Instead, PLB has been shown to increase the population of large inactive Ca^{2+}-ATPase aggregates (*1*) that can still bind nucleotide but are precluded from entering the catalytic cycle. It is postulated that quercetin, ellagic acid, and tannin dissociate Ca^{2+}ATPase aggregates into smaller active complexes. To test this hypothesis will require a molecular tag to be placed in a location other than the nucleotide binding site, as presently utilized.

We conclude that PLB inhibition and nucleotide activation of Ca^{2+}ATPase are interrelated. The precise mechanism of coupling calcium translocation and ATP hydrolysis is unknown, but oligomerization (dimerization?), as has been suggested (*41*), may be part of the process. Likewise, the basis of the biphasic nucleotide activation curve with catalytic and regulatory components remains controversial. The data in this study support the existence of two independent but interacting sites (*45*), most likely a half-site reactivity mechanism.

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