Phospholamban-mediated Stimulation of Ca^{2+} Uptake in Sarcoplasmic Reticulum from Normal and Failing Hearts

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Abstract

Studies in animal models have suggested that alterations affecting phospholamban-mediated stimulation of Ca^{2+} uptake by sarcoplasmic reticulum are involved in the pathophysiology of heart disease. A monoclonal antibody that binds to phospholamban and stimulates Ca^{2+} uptake was used to characterize phospholamban-mediated effects in human cardiac sarcoplasmic reticulum and to compare these effects in tissue from normal and failing hearts. Stimulation of Ca^{2+} uptake by anti-phospholamban monoclonal antibody simulated the effect of phosphorylation of phospholamban by cAMP-dependent protein kinase. Binding of anti-phospholamban antibody reduced the $K_{0.5}$ of the Ca^{2+}-transporting ATPase from 0.53 μM ($[Ca^{2+}]$) to 0.29 μM ($[Ca^{2+}]$), without affecting V_{max} or n_{Hill}. At 0.2 μM Ca^{2+}, stimulation was 1.93-fold in sarcoplasmic reticulum prepared from normal human left ventricular myocardium and 1.94-fold in sarcoplasmic reticulum prepared from the left ventricular myocardium of patients with heart failure resulting from idiopathic dilated cardiomyopathy. Stimulation of Ca^{2+} uptake in canine cardiac sarcoplasmic reticulum under identical conditions was 1.89-fold. Phospholamban-mediated stimulation of Ca^{2+} uptake in human cardiac sarcoplasmic reticulum is thus comparable in magnitude to that observed in other species and results from an increase in the apparent affinity of the Ca^{2+}-transporting ATPase for Ca^{2+}. The pathogenesis of heart failure in idiopathic dilated cardiomyopathy does not, however, appear to involve intrinsic alterations of this mechanism. (*J. Clin. Invest.* 1990. 85:1698–1702.) phospholamban • sarcoplasmic reticulum • Ca^{2+} transport • monoclonal antibodies • protein phosphorylation • heart failure

Introduction

Heart failure in humans is characterized by abnormalities of relaxation and intracellular Ca^{2+} handling that may reflect changes in the uptake of Ca^{2+} by sarcoplasmic reticulum (1, 2). We previously measured unstimulated Ca^{2+} uptake in sarcoplasmic reticulum from normal and failing human left ventricles, and found no differences in steady state kinetic parameters (3). Recent studies in animal models, however, have provided evidence that the pathophysiology of heart failure may involve changes in the regulation of Ca^{2+} uptake associated with phospholamban phosphorylation. In hyperthyroidism, a decrease in the ratio of phospholamban to Ca^{2+}-transporting ATPase in cardiac sarcoplasmic reticulum has been reported, with corresponding changes in messenger RNA levels for the two proteins (4, 5). These changes could account for the decreased sensitivity of relaxation rates to β-adrenergic stimulation in this condition (6–8). In contrast, myocardial hypertrophy induced by pressure load was accompanied by parallel decreases in messenger RNA levels for the two proteins (5). Phospholamban levels were also diminished in sarcoplasmic reticulum from rats treated with chronic infusions of isoproterenol (9). On the other hand, phospholamban-mediated stimulation of Ca^{2+} uptake has never been demonstrated in human cardiac sarcoplasmic reticulum, and in one study the magnitude of cAMP-dependent phosphate incorporation into human cardiac sarcoplasmic reticulum was only one-fourth that observed in canine preparations (10). The relative significance of phospholamban-mediated stimulation in humans is thus unclear.

In view of these considerations, we sought to measure the magnitude of phospholamban-mediated stimulation of Ca^{2+} uptake in human cardiac sarcoplasmic reticulum, and to determine whether heart failure in humans involves changes in the relative levels of the two proteins that result in changes in phospholamban-mediated stimulation of Ca^{2+} uptake. In separate studies, we have demonstrated that phospholamban is present in human cardiac sarcoplasmic reticulum, and that it is recognized by a monoclonal antibody whose binding to bovine phospholamban results in stimulation of Ca^{2+} uptake identical to that accompanying cAMP-dependent phosphorylation (Movsesian, M. A., C. Schmitz, J. Krall, J. Colyer, J. H. Wang, and K. P. Campbell. Submitted for publication.). In the studies reported here, we have used this antibody to characterize phospholamban-mediated stimulation of Ca^{2+} uptake in human left ventricular sarcoplasmic reticulum, and to compare the magnitude of this stimulation in preparations from the normal hearts of unmatched organ donors and the explanted failing hearts of transplant recipients with idiopathic dilated cardiomyopathy.

Methods

Preparation of cardiac sarcoplasmic reticulum. Human cardiac sarcoplasmic reticulum was prepared from human left ventricular free wall

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myocardial tissue as described previously (3). Normal tissue was obtained from seven kidney donors aged 8–62 yr (mean 37.3±8.5) with normal cardiac function (echocardiography) for whom no suitable recipients were available. Failing tissue was obtained from the excised hearts of 14 transplant recipients aged 21 to 61 yr (mean 48.9±4.7, P nonsignificant v. nonfailing) with idiopathic dilated cardiomyopathy. V_{max}, $K_{0.5}$ ([Ca^{2+}] at which activity was half-maximal) and n_{Hill} (Hill coefficient with respect to Ca^{2+}) for unstimulated Ca^{2+} uptake were 593±82 nmol/mg·min, 0.68±0.07 μM and 1.7±0.1 in the preparations from normal hearts and 593±36 nmol/mg·min, 0.63±0.03 and 1.6±0.1 in the preparations from failing hearts. The sarcoplasmic reticulum used in the experiments represented by Figs. 1–3 and Table I was prepared from myocardial specimens combined from three excised failing hearts. Canine cardiac sarcoplasmic reticulum was prepared as described previously (11).

Measurement of Ca^{2+} uptake. Ca^{2+} uptake in human and canine sarcoplasmic reticulum was assayed using an adaptation of our previously published methods (3, 11, 12). Uptake was measured in 0.102 M KCl, 5.05 mM $MgCl_2$, 1.0 mM EGTA (ethyleneglycol bis[β-aminoethyl ether] *N,N,N',N'*-tetraacetic acid), 5.0 mM oxalic acid, 5.0 mM NaN_3, 0.5 mM ryanodine (included to inhibit concurrent Ca^{2+}-induced Ca^{2+} efflux; see reference 13), 5.0 mM ATP (adenosine triphosphate) and 20 mM MOPS ([*N*-morpholino]propanesulfonic acid) (pH = 7.05, 37°C), in which $^{45}CaCl_2$ was present as appropriate to yield free [Ca^{2+}]'s from 0.1 μM to 5.0 μM; the free [Mg^{2+}] was 0.4 mM (14). Uptake was normalized relative to total sarcoplasmic reticulum protein determined by Bradford's method (15). V_{max}, $K_{0.5}$, and n_{Hill} were calculated from uptake rates as described previously (3).

To measure the effect of anti-phospholamban monoclonal antibody (prepared and characterized as previously reported, reference 12) on Ca^{2+} uptake, sarcoplasmic reticulum was incubated with antibody in the above reaction mixture for 5 min at 37°C before initiation of uptake by addition of ATP. To measure the effect of phosphorylation of phospholamban on Ca^{2+} uptake, phosphorylation of sarcoplasmic reticulum vesicles was allowed to proceed in the absence of oxalate as described below (using unlabeled ATP) for 2 min. Ca^{2+} uptake was then initiated by addition of a solution containing oxalate, $^{45}CaCl_2$, EGTA, KCl, $MgCl_2$, and MOPS such that the final content of the reaction mixture was identical to that described above.

Phosphorylation of phospholamban. The method is derived from procedures previously described (10). Cardiac sarcoplasmic reticulum was suspended in the absence or presence of cAMP-dependent protein kinase (prepared from bovine heart according to Peters et al., reference 16) in 5.0 mM $MgCl_2$ and 20 mM MOPS (pH 7.05, 37°C). Phosphorylation was initiated by addition of 5.0 mM [γ-^{32}P]ATP (0.1 mCi/mmol) and terminated by addition of equal volumes of a solution containing 20% glycerol, 10% β-mercaptoethanol, 4.6% SDS, 20 mM NaF, and 0.125 M Tris-HCl (pH 6.8). Aliquots were heated at 95°C for 5 min before electrophoresis through 0.1% SDS/7.5-20% polyacrylamide gels (17). Phospholamban bands were identified by autoradiography and excised. Phosphate incorporation in excised bands was determined by scintillation spectrometry and normalized to total sarcoplasmic reticulum protein per lane.

Results

Stimulation of Ca^{2+} uptake in human cardiac sarcoplasmic reticulum by anti-phospholamban monoclonal antibody. In bovine cardiac sarcoplasmic reticulum, stimulation of Ca^{2+} uptake by anti-phospholamban monoclonal antibody had been shown to be identical to stimulation by cAMP-dependent phosphorylation (12). To determine whether this were true in human preparations, we examined the effect of antiphospholamban monoclonal antibody on Ca^{2+} uptake by human cardiac sarcoplasmic reticulum (Fig. 1). At 0.2 μM Ca^{2+}, stimulation of Ca^{2+} uptake reached a level of 2.66-fold at an antibody concentration of 0.05 mg/ml (the ratio of antibody to sarcoplasmic reticulum protein was 0.5 mg/mg), with no further stimulation at higher antibody concentrations. No stimulation of uptake was seen when the antibody was denatured by heating at 95°C for 10 min before addition, and no stimulation was seen in the presence of monoclonal antibodies directed against nonsarcoplasmic reticulum proteins.

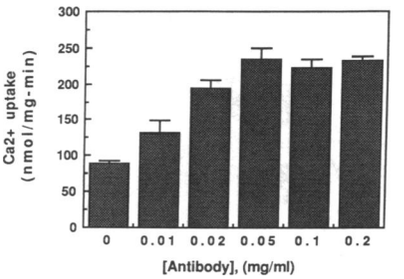

Figure 1. Stimulation of Ca^{2+} uptake by anti-phospholamban monoclonal antibody. Vesicles prepared from the left ventricular free wall tissue of three pooled failing hearts (see methods) were incubated in the presence or absence of specified concentrations of antibody, after which uptake was measured at 0.2 μM Ca^{2+}. Each value is the mean±standard error of three to six assays.

We compared these effects with those associated with phosphorylation of phospholamban by cAMP-dependent protein kinase. Phosphate incorporation into phospholamban was measured after incubation of sarcoplasmic reticulum for 2 and 5 min at concentrations of cAMP-dependent protein kinase ranging from 0.2 to 2.0 μM (Fig. 2). Phosphate incorporation reached its maximal level of 0.31±0.05 nmol/mg after 5 min incubation in the presence of 1.0 μM cAMP-dependent protein kinase. Maximal phosphate incorporation was not increased by the addition of higher concentrations of protein kinase, extension of the incubation time, or inhibition of phosphatase activity with 10 mM NaF. We then examined the effects of cAMP-dependent phosphorylation on Ca^{2+} uptake in human cardiac sarcoplasmic reticulum. Stimulation of Ca^{2+} uptake at 0.2 μM Ca^{2+} reached a level of 2.40-fold after incubation in the presence of 0.6 μM cAMP-dependent protein kinase, with no further stimulation at higher kinase concentrations. The magnitude of this stimulation was indistinguishable from that of stimulation by anti-phospholamban monoclonal antibody.

Effect of anti-phospholamban monoclonal antibody on steady state kinetics of Ca^{2+} uptake. The above experiments confirmed that, in human as in bovine cardiac sarcoplasmic reticulum, stimulation of Ca^{2+} uptake by anti-phospholamban monoclonal antibody resembles stimulation resulting from cAMP-dependent phosphorylation. We proceeded to characterize the effects of this antibody on the steady state kinetics of

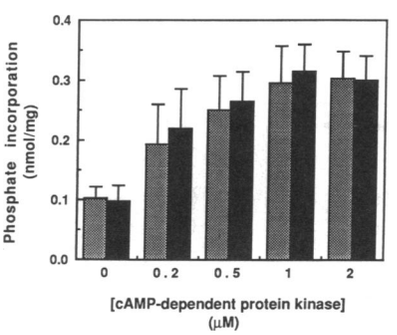

Figure 2. Phosphorylation of phospholamban by cAMP-dependent protein kinase. Phosphate incorporation was assayed at 2 min (2′, ■) and 5 min (5′, ■). Each value is the mean±standard error of four determinations.

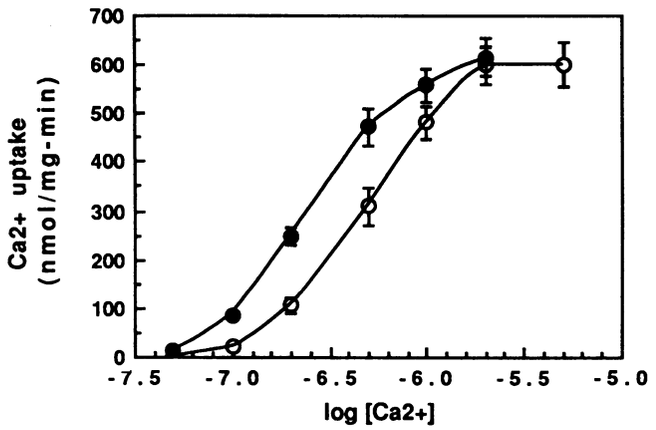

Figure 3. Phospholamban-mediated stimulation of Ca^{2+} uptake. Ca^{2+} uptake was assayed in the absence (○) and presence (●) of anti-phospholamban antibody. Each point represents the mean±standard deviation of three to six assays.

Ca^{2+} uptake by measuring Ca^{2+} uptake as a function of extravesicular [Ca^{2+}] (Fig. 3). Stimulation was profound at lower Ca^{2+} concentrations but was negligible at higher concentrations. Values for V_{max}, $K_{0.5}$, and n_{Hill} for Ca^{2+} uptake derived from these data are listed in Table I. Anti-phospholamban antibody increased the apparent affinity of the Ca^{2+}-transporting ATPase for Ca^{2+}, reducing $K_{0.5}$ from 0.53 μM to 0.29 μM, without affecting V_{max} or n_{Hill}.

For comparative purposes, we examined the effect of anti-phospholamban antibody on Ca^{2+} uptake in canine cardiac sarcoplasmic reticulum (Table II). At 0.2 μM Ca^{2+}, uptake was stimulated 1.89-fold by anti-phospholamban monoclonal antibody, whereas no stimulation was observed at 2.0 μM Ca^{2+}. The magnitude of phospholamban-mediated stimulation at 0.2 μM Ca^{2+} was comparable to that observed in humans. The lack of stimulation at 2.0 μM Ca^{2+} was consistent with an increase in Ca^{2+} affinity with no increase in V_{max}. These observations were similar to those made on human preparations.

Phospholamban-mediated stimulation of Ca^{2+} uptake in sarcoplasmic reticulum from normal and failing human hearts. Finally, we compared the effects of anti-phospholamban antibody on Ca^{2+} uptake in sarcoplasmic reticulum prepared from the normal left ventricles of unmatched human organ donors and the excised failing left ventricles of transplant recipients with class IV heart failure resulting from idiopathic dilated cardiomyopathy. As shown in Table III, inclusion of antibody stimulated Ca^{2+} uptake by an identical factor in preparations from the normal and failing hearts.

Table II. Effect of Anti-phospholamban Antibody on Ca^{2+} Uptake in Canine Cardiac Sarcoplasmic Reticulum

	Ca^{2+} Uptake (*nmol/mg · min*)		
	− Antibody	+ Antibody	Stimulation
[Ca^{2+}] (*μM*)			
0.2	24.7±2.8	46.8±2.6	1.89-fold
2.0	231.1±21.1	226.4±6.6	—

Each value represents the means±standard error of three determinations made in the absence or presence of anti-phospholamban antibody.

Discussion

In 1986, Suzuki and Wang reported the preparation of a monoclonal antibody to canine and bovine cardiac phospholamban (12). This antibody was shown to bind to a site at or near the amino acid side chain phosphorylated by cAMP-dependent and calmodulin-dependent protein kinases, and binding resulted in stimulation of Ca^{2+} uptake quantitatively identical to that accompanying cAMP-dependent phosphorylation. In the present study, we have confirmed that this antibody has similar effects in human preparations. Measurement of Ca^{2+} uptake by sarcoplasmic reticulum in the presence and absence of this antibody provides a simple assay sensitive to changes in both the relative level of phospholamban and its coupling to the Ca^{2+}-transport mechanism. The simplicity and reproducibility of this procedure made it possible for us to test our hypotheses using limited amounts of human myocardial tissue.

Our experimental conditions were designed to approximate those expected to occur physiologically. Under these conditions, phospholamban-mediated stimulation of steady state Ca^{2+} uptake in human cardiac sarcoplasmic reticulum resulted from an increase in the affinity of the Ca^{2+}-transporting ATPase for Ca^{2+}, with no significant change in maximal turnover rate or cooperativity. Stimulation of Ca^{2+} uptake was therefore profound at 0.2 μM Ca^{2+}, a value in the range expected to occur during diastole, but much less impressive at the higher Ca^{2+} concentrations expected to occur during systole (18). These observations may have important implications

Table I. Effect of Anti-phospholamban Antibody on Ca^{2+} Uptake Kinetics in Human Cardiac Sarcoplasmic Reticulum

	V_{max}	$K_{0.5}$	n_{Hill}
	nmol/mg · min	*μM*	
Antibody			
−	665	0.53	1.7
+	662	0.29	1.8

Values are derived as described in Methods from the data represented in Fig. 3.

Table III. Effect of Anti-phospholamban Antibody on Ca^{2+} Uptake in Sarcoplasmic Reticulum from Normal and Failing Human Hearts

	Ca^{2+} Uptake (*nmol/mg · min*)		
	− Antibody	+ Antibody	Stimulation
Normal (*n* = 7)	107.1±15.5	206.2±22.1	1.93-fold
Failing (*n* = 14)	126.7±14.0	246.2±32.3	1.94-fold

All uptake rates were measured at [Ca^{2+}] = 0.2 *μM*. Each value represents mean±standard error.

with respect to cell physiology in human myocardium. First, they would suggest that stimulation of Ca^{2+} uptake by phospholamban phosphorylation has little effect on the initial phase of relaxation, during which free cytosolic [Ca^{2+}] is 0.8–0.9 μM. The principal effects of phosphorylation would instead be reflected in a lowering of the free cytosolic [Ca^{2+}] during the later stages of relaxation and an increase in the amount of Ca^{2+} stored intralumenally in the sarcoplasmic reticulum (and available for subsequent release). It should be noted, however, that Ca^{2+} uptake in vivo may be limited by the increase in the free [Ca^{2+}] within the sarcoplasmic reticulum that accompanies Ca^{2+} accumulation, and that this possibility limits the extent to which measurement of steady state uptake rates can be assumed to be relevant to physiologic phenomena. (Our assays were performed in the presence of oxalate, which was included specifically to maintain a low intravesicular [Ca^{2+}] and eliminate this influence on Ca^{2+} uptake.)

It has been shown that phospholamban in its unphosphorylated conformation inhibits Ca^{2+} uptake activity, and that phosphorylation relieves this inhibition (19, 20). Recent studies suggest that neutralization of the positive charges on phospholamban's cytoplasmic amino acid side chains has similar stimulatory effects on Ca^{2+} uptake (21), and the effects of phosphorylation as well as anti-phospholamban monoclonal antibody could be compatible with such a mechanism. The nature and magnitude of phospholamban-mediated effects in human cardiac sarcoplasmic reticulum were similar to those observed in canine preparations. These results were somewhat surprising in view of a prior study in which phosphate incorporation was fourfold higher in canine than in human preparations (10), since the essentially identical stimulation we observed would suggest that relative levels and coupling of phospholamban and the Ca^{2+}-transporting ATPase are similar in the two systems. The measurements made in the prior study relied on crude membrane preparations from diverse species, and it is possible that these preparations differed with respect to relative purity and efficiency of phosphorylation.

Recent studies in animal models of thyroid disease, pressure-load hypertrophy and chronic β-adrenergic stimulation have raised the possibility that heart failure may involve abnormalities in the transcription and translation of phospholamban and the Ca^{2+}-transporting ATPase, affecting the levels of these proteins in sarcoplasmic reticulum (4, 5, 9). Were such mechanisms pathogenetic in idiopathic dilated cardiomyopathy in humans, changes in basal Ca^{2+} uptake or its phospholamban-mediated stimulation would have been predicted. Our previous studies showed no differences in basal Ca^{2+} uptake in this condition. The present experiments extend those studies, and demonstrate that phospholamban-mediated stimulation of uptake is similarly unaffected in this condition. In view of these findings, it is interesting to note that basal Ca^{2+} uptake rates are depressed in a hereditary dilated cardiomyopathy in Syrian hamsters (22). Taken together, these observations suggest that animal models of heart disease may not be representative of the pathophysiology of heart failure in humans.

It remains possible that abnormal relaxation in heart failure occurs via mechanisms involving phospholamban-mediated stimulation. The decreased β-adrenergic responsiveness observed in chronic heart failure in humans might lead in turn to decreased intracellular cAMP concentrations, decreased phosphorylation of phospholamban, decreased stimulation of Ca^{2+} uptake by sarcoplasmic reticulum, and increased end-diastolic Ca^{2+} concentrations (23). Our results do not exclude the possibility that abnormalities in other sarcoplasmic reticulum proteins involved in Ca^{2+} storage and release or in the amount of sarcoplasmic reticulum relative to contractile elements are involved in human disease. We would also emphasize that our conclusions apply only to idiopathic dilated cardiomyopathy, and should not be generalized to other forms of heart failure.

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