Anti-phospholamban and protein kinase A alter the Ca^{2+} sensitivity and maximum velocity of Ca^{2+} uptake by the cardiac sarcoplasmic reticulum

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The activity of the SERCA2a Ca^{2+} pump in the sarcoplasmic reticulum (SR) of cardiac muscle is inhibited by phospholamban. When phospholamban is phosphorylated by cyclic-AMP-dependent protein kinase (PKA) this inhibition is relieved. It is generally agreed that this results in an increase in the Ca^{2+} sensitivity of the SR Ca^{2+} pump; however, some investigators have also reported an increase in the maximum velocity of the pump. We have used a sensitive fluorescence method to measure net Ca^{2+} uptake by native cardiac SR vesicles and compared the effects of a constitutively active subunit of PKA (cPKA) with those of a monoclonal antibody (A1) that binds to phospholamban and is thought to mimic the effect of phosphorylation. Both the Ca^{2+} sensitivity and the maximum velocity of uptake were increased by cPKA and by A1. The effects of cPKA and A1 on uptake velocity were only slightly additive. No changes in uptake were detected with denatured cPKA or denatured A1. These results indicate that the functional effect of phospholamban phosphorylation is to increase both the Ca^{2+} sensitivity and the maximum velocity of net Ca^{2+} uptake into the SR.

INTRODUCTION

The SERCA2a [1] Ca^{2+} pump in the membrane of the sarcoplasmic reticulum (SR) of cardiac muscle is associated with a second protein, phospholamban (see reviews in [2–4]). Phospholamban is a 6 kDa protein that forms a homopentamer [5,6] which when unphosphorylated is thought to directly interact with the pump and inhibit its ATPase activity [7–10]. Phosphorylation of phospholamban at Ser-16 by cyclic-AMP-dependent protein kinase (PKA) or cyclic-GMP-dependent protein kinase or at Thr-17 by Ca^{2+}/calmodulin-dependent protein kinase II relieves this inhibition, presumably as the result of the dissociation of phospholamban from the pump [3,4,9,11,12]. The precise nature of the interaction of phospholamban and the Ca^{2+} pump and the relationship between phosphorylation of one or more sites on the phospholamban pentamer and the functional inhibition of the Ca^{2+} pump remain the subject of much experimental interest [3,13,14].

Although it is generally agreed that regulation of SR Ca^{2+} uptake can have important physiological effects on cardiac contractility, the way or ways in which the phosphorylation of phospholamban alters Ca^{2+} uptake by the SR have remained controversial. An increase in the Ca^{2+} sensitivity of the Ca^{2+} pump associated with the phosphorylation of phospholamban has been reported by a number of laboratories; others have reported an increase in both Ca^{2+} sensitivity and the maximum velocity of the pump (see [3], [4], [15–17] and discussions in [18–21]). A change in Ca^{2+} sensitivity of the cardiac SR Ca^{2+} ATPase was also seen with monoclonal antibodies that bind to phospholamban and mimic the effect of phosphorylation [3,22–24]. It is not entirely clear why changes in the maximum velocity of Ca^{2+} uptake are not consistently seen. It has been suggested [16,21] that differences in the methods used to prepare or store cardiac SR vesicles and/or the buffers used in experiments in different laboratories could account for some of the variations in reported results. It is also possible that some of these differences may be accounted for by differences in the methods used to measure SR Ca^{2+} uptake, especially when results from methods that measure ATPase activity or experiments done on reconstituted systems are compared with results obtained from measurements of net Ca^{2+} uptake in native SR vesicles.

We have used a fluorescence method [25] to measure net Ca^{2+} uptake into cardiac SR vesicles and compared the effects of a phospholamban antibody (A1) that is thought to mimic the effects of phosphorylation [22,23] with those obtained with a constitutively active subunit of cyclic-AMP-dependent protein kinase (cPKA). Net Ca^{2+} uptake into cardiac SR vesicles was monitored with the free-acid form of Fura 2 in the extravesicular buffer. With this method, highly reproducible measurements of net Ca^{2+} uptake can be made from small samples taken from a single vesicle preparation [25–27]. A complete curve of uptake velocity as a function of the free-Ca^{2+} concentration in the extravesicular buffer can be generated from a single vesicle aliquot. From this curve, the kinetic parameters (maximum velocity, V_{max}; Ca^{2+} sensitivity; Hill coefficient, h) of uptake can be determined. Using these measurements, we studied the effects of cPKA, A1, or A1 + cPKA on the kinetics of Ca^{2+} uptake. We found that both the Ca^{2+} sensitivity and the maximum velocity of Ca^{2+} uptake were significantly altered in the presence of cPKA or A1. The magnitude of the change in maximum velocity was similar in both cases. This was also true of the change in Ca^{2+} sensitivity. The effect on maximum velocity of cPKA and A1 when added together was only slightly greater than the effects seen with either one alone. No changes in Ca^{2+} sensitivity or maximum uptake velocity were detected with either cPKA or A1 when they were denatured by prior heat treatment.

Abbreviations used: SR, sarcoplasmic reticulum; PKA, cyclic-AMP-dependent protein kinase; cPKA, constitutively active subunit of PKA; V_{max}, maximum velocity; $[Ca^{2+}]_{50\%}$, Ca^{2+} concentration at half maximum velocity; h, Hill coefficient; $[Ca^{2+}]_{free}$, free-Ca^{2+} concentration; $[Ca^{2+}]_{total}$, total-Ca^{2+} concentration; A1, monoclonal anti-phospholamban antibody.

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MATERIALS AND METHODS

Membrane vesicles

Cardiac muscle homogenates were made from canine ventricular tissue stripped of its vasculature. SR vesicle preparations were made using the method of Chamberlain et al. [28] as described previously [26]. Vesicles were stored at −80 °C in 300 mM sucrose, 100 mM KCl, 5 mM histidine, pH 7.1. Protein concentrations in the vesicle preparations were determined with a Pierce BCA protein assay (Rockford, IL, U.S.A.).

Measurement of calcium uptake

Fura 2 free-acid (2.0 μM) in an extravesicular bathing medium (uptake buffer), containing 100 mM KCl, 4 mM $MgCl_2$, 20 mM Hepes, 10 mM oxalate, 1.25 mM ATP, 1.25 mM creatine phosphate and 3.2 units/ml creatine phosphokinase at pH 7.0, was used to continuously monitor ATP-dependent Ca^{2+} uptake as described previously [25,26]. As calcium is transported into the vesicles, the free-Ca^{2+} concentration ($[Ca^{2+}]_{free}$) and total-Ca^{2+} concentration ($[Ca^{2+}]_{total}$) decline in the extravesicular solution. The instantaneous rate of Ca^{2+} uptake (uptake velocity) was calculated from the rate of decline of $[Ca^{2+}]_{total}$ (determined as described below). Fura 2 free-acid and K_2ATP were dissolved in H_2O. When used, cPKA was dissolved in kinase buffer (150 mM KCl, 30 mM $KHPO_4$, 1 mM EDTA, 1 mM dithiothreitol; stock concentration = 0.38 mg/ml). The A1 was dissolved in antibody buffer (100 mM Tris, 100 mM glycine; stock concentration = 1.6 mg/ml). Aristar grade KCl, KOH, sucrose and oxalic acid were obtained from BDH Limited (Edmonton, Alberta, Canada). Hepes and $MgCl_2$ were analytical grade obtained from Fluka (Ronkonkoma, NY, U.S.A.). The K_2ATP, $CaCl_2$, creatine phosphate and creatine phosphokinase were obtained from Sigma (St. Louis, MO, U.S.A.). Tris and glycine were obtained from Bio-Rad (Mississauga, Ontario, Canada) and fura 2 (free-acid form) was obtained from Molecular Probes (Eugene, OR, U.S.A.). The cPKA was a gift from Dr. Michael Walsh, and A1 was a gift from Dr. Jerry Wang.

Experiments were performed in 3 ml cuvettes in the sample compartment of a SPEX CMX fluorimeter (Edison, NJ, U.S.A.) with dual excitation monochrometers set at 340 and 380 nm and chopped every 0.3 s. Fluorescence emission was monitored at 510 nm; 340/380 ratios at 510 nm emission were calculated over 1 s time intervals. Background fluorescence and light scatter were measured at 510 nm before each experiment with only uptake buffer and SR vesicles (30 μg total protein) present in a cuvette. Fura 2 was then added to the cuvette and allowed to equilibrate with the buffer and vesicles for 1 min. Uptake reactions were initiated by the addition of ATP and then Ca^{2+} to the cuvette. The solution in the cuvette was constantly stirred to ensure adequate mixing. For the experiments with cPKA, it was necessary to add cPKA and ATP to cuvettes containing uptake buffer and SR vesicles so that the phosphorylation of phospholamban could occur before the addition of Ca^{2+}. To ensure that a minimum amount of uptake into the vesicles occurred during the pre-incubation with cPKA and ATP, experiments were done to determine the minimum incubation time needed (1 min) and the concentration of cPKA (22 nM) required to produce a maximum effect on Ca^{2+} uptake. In the controls for these experiments, vesicles were pre-incubated with ATP for 1 min before the addition of Ca^{2+}. For experiments done in the presence of A1, the antibody was added to cuvettes containing uptake buffer and SR vesicles 1 min before the addition of ATP and then Ca^{2+} to initiate uptake. As was the case with cPKA, the minimum incubation time (1 min) and the A1 concentration (2.35 μg/ml) required to produce the maximum effect on Ca^{2+} uptake were determined experimentally. All experiments were done at 22 °C.

Calculations

To calculate the kinetic parameters of Ca^{2+} uptake by the cardiac SR vesicles, the fura 2 340/380 fluorescence ratio (R) vs. time curves obtained experimentally were smoothed (after correction for background fluorescence and light scatter) by a moving window average calculation [26] using the equation:

$$R'_j = \frac{\sum_{i=-m}^{i=m} R_{(j+i)}}{NWIDE} \tag{1}$$

where R'_j is the smoothed 340/380 ratio value at time point (j), $NWIDE$ is the width of the smoothing window and m is given by:

$$m = \frac{(NWIDE-1)}{2} \tag{2}$$

An optimum window width of 9 (determined as described in [26]) was used in the calculation. The smoothed fura 2 fluorescence curves were used to determine the $[Ca^{2+}]_{free}$ in the cuvette at each time point (j) using the equation [29]:

$$[Ca^{2+}]_{free(j)} = K_d \beta \frac{(R'_j - R_{min})}{(R_{max} - R'_j)} \tag{3}$$

where K_d (200 nM; [30]) is the fura 2 – Ca^{2+} dissociation constant, R'_j the 340/380 fluorescence ratio at time j, R_{min} the 340/380 ratio in Ca^{2+}-free solution ([EGTA] = 25 mM in uptake buffer without oxalate), R_{max} the 340/380 ratio at saturating Ca^{2+} (2.5 mM, in uptake buffer without oxalate) and β the fluorescence measured at 380 nm excitation in the Ca^{2+}-free buffer divided by the fluorescence at 380 nm excitation in the saturating Ca^{2+} buffer (see [27]). The values for R_{min} and R_{max} were determined each day. The value for β was determined for each lot of fura 2 that was used.

The $[Ca^{2+}]_{total}$ in the extravesicular buffer as a function of time was calculated from $[Ca^{2+}]_{free}$ for each time point by solving a set of simultaneous equations for the binding of Ca^{2+}, Mg^{2+} and H^+ to fura 2, ATP, creatine phosphate and oxalate as described previously [31]. The binding constants were taken from published values [32–35]. Calcium uptake velocity (expressed as $\mu mol \cdot min^{-1} \cdot mg^{-1}$) was calculated from:

$$\text{velocity} = A \frac{-d[Ca^{2+}]_{total}}{dt} \tag{4}$$

where A (= vol/wt) is the volume of solution in the cuvette (in litres) divided by the amount of SR protein in the cuvette (in mg), and $-d[Ca^{2+}]_{total}/dt$ the negative derivative of the extravesicular $[Ca^{2+}]_{total}$ vs. time curve. The derivative at each time point was calculated by the subtraction of sequential $[Ca^{2+}]_{total}$ values (i.e. $-d[Ca^{2+}]_{total}/dt \approx -\Delta[Ca^{2+}]_{total}/\Delta t$). Velocity values obtained in this way were then plotted against the corresponding $[Ca^{2+}]_{free}$ values obtained by eqn. (3). The V_{max} of uptake was determined from the curve (for $[Ca^{2+}]_{free}$ values $\leqslant 1.5\ \mu M$) then a curve fitting program (Sigma Plot; Jandel Scientific, Corte Madera, CA, U.S.A.) was used to fit the velocity vs. $[Ca^{2+}]_{free}$ curve to determine values for h and $[Ca^{2+}]_{free}$ at half maximum velocity ($[Ca^{2+}]_{50\%}$). An evaluation of the methods used for determining the kinetics of uptake is given in Kargacin and Kargacin [26]. Values for V_{max} in the control samples from different vesicle preparations ranged from 0.35 to 1.0 $\mu mol \cdot min^{-1} \cdot mg^{-1}$. This occurred because V_{max} is dependent on the purity of a particular

Table 1 Summary of uptake kinetics

Sample	V_{max} (% of control)*	$[Ca^{2+}]_{50\%}$ (nM)*
Control ($n = 40$)	100.0 ± 1.5	472 ± 13
cPKA ($n = 11$)	142.0 ± 2.1†	325 ± 13†
Boiled cPKA ($n = 4$)	98.8 ± 6.3	510 ± 11
A1 ($n = 22$)	136.1 ± 3.9†	316 ± 14†
Boiled A1 ($n = 6$)	102.8 ± 1.6	468 ± 12
cPKA + A1 ($n = 4$)	152.4 ± 4.3†	317 ± 13†

* Errors are ±1 standard error.
† Indicates that the result is significantly different from the control value at $p \leqslant 0.01$.

vesicle preparation (i.e. the amount of pump protein relative to the total amount of protein in the preparation). For this reason, the V_{max} values for each experiment are reported below as the percentage of the average V_{max} of the corresponding controls. This allowed data from different vesicle preparations to be combined in Table 1.

RESULTS

Effects of PKA on Ca^{2+} uptake

To determine the effect of PKA (a kinase known to phosphorylate phospholamban at Ser-16) on SR function, Ca^{2+} uptake was compared in the presence and absence of the constitutively active subunit of the kinase, cPKA. Addition of the kinase increased the V_{max} and decreased the $[Ca^{2+}]_{50\%}$ of uptake. The result of one experiment done in the presence of cPKA (added in 6 μl of kinase buffer; final concentration of cPKA = 22 nM) and a control experiment done in uptake buffer alone are shown in Figure 1. A summary of the results obtained from several experiments with cPKA are shown in Table 1. In these experiments, the mean V_{max} in the presence of 22 μM cPKA increased by 142 % over control and the mean $[Ca^{2+}]_{50\%}$ was 147 nM lower than that of the mean control value of 472 nM. The Hill coefficient was reduced slightly in the presence of cPKA (h in the control experiments was 1.99 ± 0.17 and was reduced by an average of 0.12 ± 0.09); however, this appeared to be an effect of the kinase buffer since a similar shift was seen in the control experiments in which the kinase buffer without cPKA was added to the uptake buffer in the cuvette. In addition to control experiments done in uptake buffer alone, controls were done in uptake buffer with 6 μl of kinase buffer without cPKA (results not shown) and in uptake buffer with 6 μl kinase buffer containing cPKA denatured by boiling (22 nM final concentration). There were no significant differences in V_{max} or $[Ca^{2+}]_{50\%}$ in these controls when they were compared with the control experiments done in uptake buffer alone (Table 1).

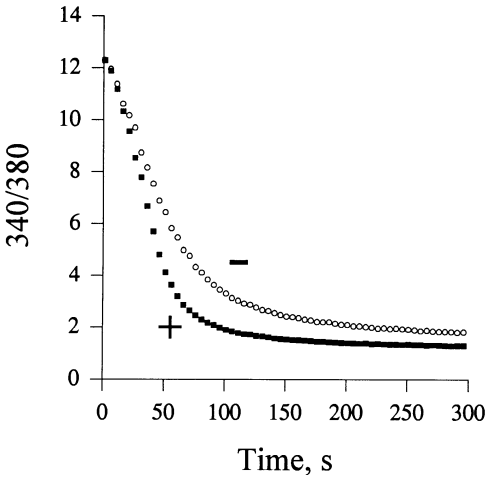

Figure 1 Effect of cPKA on Ca^{2+} uptake by cardiac SR vesicles

Fura 2 fluorescence was corrected for background fluorescence and light scatter and smoothed as described in the Materials and methods section. (○), Control trace; (■), uptake in the presence of 22 nM cPKA. Note: only every fifth data point is plotted.

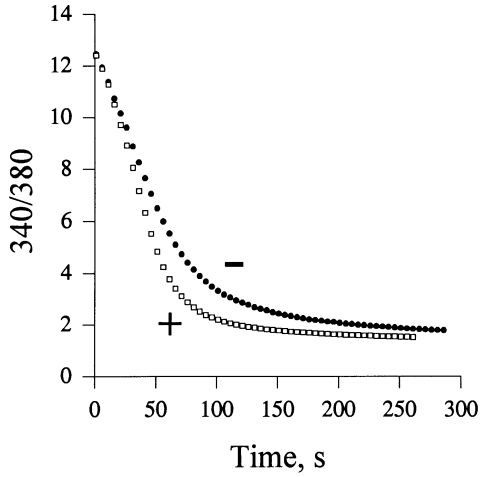

Figure 2 Effect of anti-phospholamban (A1) on cardiac SR Ca^{2+} uptake

340/380 fluorescence after smoothing and correction for background fluorescence and light scatter as described in the Materials and methods section. (●) Control trace; (□) uptake in the presence of A1 (2.35 μg/ml). Note: only every fifth data point is plotted.

Effects of anti-phospholamban on Ca^{2+} uptake

In previous work measuring SR Ca^{2+}-ATPase activity or using $^{45}Ca^{2+}$ to monitor unidirectional movement of Ca^{2+} into cardiac muscle SR vesicles, Suzuki and Wang [22] and Morris et al. [23] reported that a monoclonal antibody to phospholamban (A1) increased the Ca^{2+} sensitivity of the SR Ca^{2+} pump. No changes in V_{max} or h were noted. In the experiments described above, changes in both the V_{max} and $[Ca^{2+}]_{50\%}$ of SR Ca^{2+} uptake were detected in the presence of cPKA, a result that has not been consistently reported in the literature (see discussion in [16] and [21]). It was, therefore, of interest to re-examine the effects of A1 on Ca^{2+} uptake into cardiac SR vesicles using fura 2 to monitor uptake. The results of experiments with A1 are shown in Figure 2 and Table 1. In Figure 2, 340/380 fluorescence ratios are shown for one control experiment (uptake in the presence of uptake buffer alone) and one experiment done in the presence of A1 (2.35 μg/ml). The results of several experiments are summarized in Table 1. Significant changes were recorded in V_{max} and $[Ca^{2+}]_{50\%}$; V_{max} in the presence of A1 was 136 % of control and the mean $[Ca^{2+}]_{50\%}$ in A1 was decreased by 156 nM from the mean control value of 472 nM. As was the case for cPKA, h was not significantly different in the presence of A1. The kinetic parameters for Ca^{2+} uptake in control experiments done in uptake buffer with 3 μl of antibody buffer containing denatured A1 (Table 1) or in uptake buffer with 3 μl of antibody buffer

without A1 (results not shown) were not significantly different from than those recorded in uptake buffer alone.

Combined effects of A1 and cPKA

Both cPKA and A1 significantly increased the rate of net Ca^{2+} uptake by cardiac SR vesicles. The percentage increase in V_{max} with cPKA was similar in magnitude to that measured with A1 (Table 1). This was also true when the decrease in $[Ca^{2+}]_{50\%}$ was compared in the two cases. Since the binding of A1 to phospholamban is thought to mimic the effect of phosphorylation [22,23], one would not expect the changes in SR Ca^{2+} uptake seen with A1 and cPKA to be additive. To test this, uptake was measured in the presence of cPKA (22 nM) and A1 (2.35 μg/ml) added together. In the presence of both cPKA and A1, the increase in V_{max} appeared to be sightly greater than the increase seen with either cPKA or A1 alone; however, this difference was not significant. The decrease in $[Ca^{2+}]_{50\%}$ in the presence of both cPKA and A1 was also not different from that seen in the experiments with cPKA or A1 alone. In the presence of both cPKA and A1, V_{max} was 152% of control; the mean $[Ca^{2+}]_{50\%}$ was 155 nM less than in the control experiments (Table 1).

DISCUSSION

The results of this study show that, when net Ca^{2+} uptake into cardiac SR vesicles is continuously monitored, agents that cause or mimic phospholamban phosphorylation increase the V_{max} and decrease the $[Ca^{2+}]_{50\%}$ of the Ca^{2+} sequestration process. This result is in contrast to those reported in a number of other studies in which an increase in the Ca^{2+} sensitivity of uptake was associated with the phosphorylation of phospholamban but no detectable changes in the maximum velocity of uptake were seen, but is consistent with results reported in other studies [3,4,15–21]. There are a number of possible explanations for these discrepancies. It has been suggested that changes in the maximum activity of the SR Ca^{2+}-ATPase may not be as readily apparent in experiments conducted after long incubations in the presence of oxalate [16]. It has also been suggested [21] that in some experiments in which changes in V_{max} were reported, control experiments may not have matched those in which the effect of PKA on uptake were measured. In the experiments reported here, the same uptake buffer was used for both control experiments and experiments done with cPKA. Control experiments were also done in uptake buffer that contained kinase buffer or kinase buffer with denatured cPKA. We found no significant differences in any of the kinetic parameters of uptake when these control experiments were compared with one another (Table 1). It is also important to note that, in our experiments, A1 significantly increased the V_{max} and decreased the $[Ca^{2+}]_{50\%}$ of uptake. Changes in V_{max} or $[Ca^{2+}]_{50\%}$ were not seen in control experiments done in the presence of the antibody buffer alone or the antibody buffer containing denatured A1. In the experiments reported here, an entire Ca^{2+} uptake curve (allowing the determination of all three kinetic parameters) could be derived from a single SR vesicle aliquot. The measurements are made temporally 'on-line' over a continuous range of free-Ca^{2+} concentrations (Figures 1 and 2) and did not require the presence of EGTA. The Ca^{2+} uptake determined by the fluorescent Ca^{2+} indicator in the medium bathing the SR is a measure of the net Ca^{2+} uptake into the SR and consequently provides functional information about the SR as a whole. It is, therefore, possible that the changes in V_{max} that were seen in our experiments were the result of a significant decrease in the back flux of Ca^{2+} out of SR through a component of the SR membrane that is regulated by A1 or cPKA. It has been proposed that phospholamban may form a Ca^{2+} channel [36–38] or regulate other channels [39] in the SR membrane. The effects of A1 and cPKA could thus be on channels in the SR involved in Ca^{2+} release, on Ca^{2+} leak channels in the SR membrane, or on the regulation of channels involved in the counter-charge movement that is thought to occur during SR Ca^{2+} uptake and/or release [39]. It is more likely, however, that the change in V_{max} is owing to an effect of phospholamban on the SR Ca-ATPase itself. Starling et al. [16] and Hughes et al. [17] showed that a peptide based on the amino acid sequence of phospholamban (residues 1–25) decreased the maximum rate of ATP hydrolysis by the skeletal muscle form of the SR Ca-ATPase and the rate of Ca^{2+} accumulation by the SR. The magnitudes of the effects reported by these investigators are similar to the percentage change in V_{max} seen in our experiments. The results of our experiments are consistent with the hypothesis that the effects of cPKA and A1 are mediated through the same mechanism since the changes in V_{max} and $[Ca^{2+}]_{50\%}$ observed with A1 and cPKA were of similar magnitude but were not additive when the two were applied together. This interpretation is in agreement with the work of Suzuki and Wang [22] and Morris et al. [23], indicating that A1 mimics the effect of phospholamban phosphorylation by altering the interaction of phospholamban with the cardiac SR Ca^{2+}-ATPase. It was also noted in these studies that A1 inhibited the phosphorylation of phospholamban by PKA.

Studies of net Ca^{2+} uptake, such as the ones reported here, provide information about how the SR as a whole responds to regulatory stimuli. Experimental work that focuses on the ATPase activity of the SERCA2a Ca^{2+} pump and that done with reconstituted systems, in which only one or two components of the SR are studied in isolation, can provide information about specific elements involved in the regulation of the SR. The continued use of both approaches should provide a better understanding of the role of the SR in normal cardiac function, its regulation and the changes in function and regulation that occur in disease.

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