Biochemistry

 Volume 33, Number 16 April 26, 1994

Articles

Mechanism of Inhibition of the Ca^{2+}-ATPase by Spermine and Other Polycationic Compounds†

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Received November 29, 1993; Revised Manuscript Received February 24, 1994®

ABSTRACT: The ATPase activity of the Ca^{2+}-ATPase of skeletal muscle sarcoplasmic reticulum is inhibited by a variety of polyamines, including spermine, spermidine, and poly(arginine). The effects of spermine on the ATPase are highly specific. It has no effect on the affinity of the ATPase for Ca^{2+} or ATP, and no effect on the rate of phosphorylation by ATP. When the ATPase is phosphorylated with P_i in the presence of dimethyl sulfoxide at pH 6.0, and then dephosphorylation is induced by dilution in buffer at pH 7.5 in the absence of dimethyl sulfoxide, spermine is found to have no effect on the rate of dephosphorylation. If the ATPase is phosphorylated with [γ-^{32}P]ATP and the rate of loss of radiolabeled phosphoenzyme is measured following the addition of unlabeled ATP, spermine is found to decrease the rate of loss of radiolabel, consistent with an effect of spermine on the rate of the $Ca_2E1P \rightarrow E2P$ step. Direct measurement confirms that spermine decreases the rate of dissociation of Ca^{2+} from the phosphorylated ATPase ($Ca_2E1P \rightarrow E2P$), with the decrease in the rate of this step explaining the inhibition of ATPase activity. Spermine also increases the equilibrium constant E1/E2 and inhibits phosphorylation of the ATPase by P_i by competition with the Mg^{2+} essential for the reaction. It is suggested that spermine could bind to the site on the Ca^{2+}-ATPase that interacts with phospholamban.

The activity of the Ca^{2+}-ATPase of cardiac sarcoplasmic reticulum (SR[1]) is modulated by interaction with phospholamban. Phospholamban consists of a membrane-embedded hydrophobic segment and a hydrophilic segment exposed on the cytoplasmic side of the SR (Simmerman et al., 1986; Fujii et al., 1987). Binding of the hydrophilic segment of phospholamban to the ATPase reduces the maximum rate of ATP hydrolysis with no effect on the Ca^{2+} affinity, whereas binding of the hydrophobic segment of phospholamban to the ATPase decreases the affinity of the ATPase for Ca^{2+} with no effect on the maximum rate (Sasaki et al., 1992). The hydrophilic segment of phospholamban is highly basic. Phosphorylation of phospholamban reduces its interaction with the ATPase, suggesting the importance of the net positive charge on phospholamban (Colyer & Wang, 1991). Although skeletal muscle SR does not contain phospholamban, it has been shown that if the purified Ca^{2+}-ATPase from skeletal muscle SR is reconstituted with phospholamban, then inhibition is observed as for the Ca^{2+}-ATPase from cardiac SR (Szymanska et al., 1990; Vorherr et al., 1992).

Inhibition of the Ca^{2+}-ATPase from cardiac SR has been demonstrated with a variety of polycationic species, including spermine and spermidine (Xu & Kirchberger, 1989). Spermidine and ruthenium red ($[(NH_3)_5Ru–O–Ru(NH_3)_4–O–Ru(NH_3)_5]^{6+}$) inhibit P_i phosphorylation of the Ca^{2+}-ATPase from skeletal muscle by competition with the Mg^{2+} essential for the reaction (de Meis, 1991; de Meis et al., 1991). Binding of ruthenium red is not, however, competitive with the binding of ATP, and binding results in quenching of the fluorescence

† We thank the SERC and the Wessex Medical Trust for financial support.

® Abstract published in *Advance ACS Abstracts,* April 1, 1994.

[1] Abbreviations: DPH, diphenylhexatriene; SR, sarcoplasmic reticulum; NBD, nitro-1,2,3-benzoxadiazole; di(C18:1)PC, dioleoylphosphatidylcholine; IU, international units.

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of the tryptophan residues located in trans-membrane regions of the ATPase rather than those thought to be located in cytoplasmic domains of the ATPase, where the ATP binding site is located (Moutin et al., 1992). It has been suggested that ruthenium red binds to the specific Ca^{2+} binding sites on the ATPase, as well as to nonspecific cationic sites, with millimolar affinities for Mg^{2+} and Ca^{2+} (Moutin et al., 1992), although it has also been concluded that ruthenium red fails to bind to the specific Ca^{2+} binding sites (Corbalan-Garcia et al., 1992). Ruthenium red has no effect on the rate of ATP hydrolysis (Alves & de Meis, 1986; Moutin et al., 1992), although it has been reported to increase the rate of phosphorylation of the ATPase by ATP (Meszaros & Ikemoto, 1985).

Here we report on the effects of spermine on the function of the Ca^{2+}-ATPase of skeletal muscle and show that inhibition follows from a decrease in the rate of the Ca^{2+} transport step.

MATERIALS AND METHODS

Spermine was obtained from Fluka; spermidine, protamine, protamine sulfate, poly(L-arginine), cadaverine, and putrescine were from Sigma; Arg-Arg-Arg was from Bachem; and Joro spider toxin was from Research Biochemicals Inc. Sarcoplasmic reticulum from rabbit skeletal muscle and the purified Ca^{2+}-ATPase were prepared as described in Michelangeli et al. (1991). ATPase activity was determined at 25 °C by using a coupled enzyme assay in a medium containing, unless otherwise specified, 40 mM Hepes/KOH (pH 7.2), 100 mM KCl, 5 mM $MgSO_4$, 2.1 mM ATP, 1.1 mM EGTA, 0.53 mM phosphoenolpyruvate, 0.15 mM NADH, pyruvate kinase (7.5 IU), and lactate dehydrogenase (18 IU) in a total volume of 2.5 mL. The reaction was initiated by the addition of an aliquot of a 25 mM $CaCl_2$ solution to a cuvette containing the ATPase and the other reagents to give a maximally stimulating concentration of Ca^{2+} (free Ca^{2+} concentration of ca. 10 μM). Maximal ATPase activities measured under these conditions at 25 °C were found to vary between preparations, typically between 3 and 6 IU/mg of protein, as reported previously (Michelangeli et al., 1990a). Concentrations of ATPase were estimated using the extinction coefficient (1.2 L $g^{-1}cm^{-1}$ for a solution in 1% SDS) given by Hardwicke and Green (1974). Free concentrations of Ca^{2+} were calculated using the binding constants for Ca^{2+}, Mg^{2+}, and H^+ to EGTA given by Godt (1974).

Steady-state measurements of phosphorylation by [^{32}P]P_i were carried out in 150 mM Mes/Tris (pH 6.3) containing 5 mM EGTA and the required concentrations of P_i and Mg^{2+}, at 25 °C and a protein concentration of 0.1 mg/mL. Samples were incubated for 10 s and then quenched with 12% trichloroacetic acid/0.2 M phosphoric acid. The precipitate was collected on Whatman GF/C filters, washed three times with quenching solution, and then counted.

The time dependence of phosphorylation of the ATPase by [γ-^{32}P]ATP at 25 °C was determined using a Biologic QFM-5 system as described in Starling et al. (1994) (see the legend to Figure 8). The time dependence of dephosphorylation of the ATPase phosphorylated with [γ-^{32}P]ATP following mixing with ATP or ADP were also determined using the Biologic QFM-5 system (Starling et al., 1994). The time dependence of dephosphorylation of the ATPase phosphorylated with [^{32}P]P_i was determined as described by Henao et al. (1991). The ATPase (4 mg/mL) was incubated in 12.5 mM Mes/Tris (pH 6.0) containing 10 mM EGTA, 1 mM [^{32}P]P_i, 20 mM $MgSO_4$, and 14% (v/v) dimethyl sulfoxide. One volume of this suspension was mixed with 16 vol of 100 mM Tris/Mes (pH 7.5) containing 100 mM KCl, 4.1 mM $MgSO_4$, 5.3 mM ATP, and 10.6 mM spermine, followed by quenching as described above. Nonspecific [^{32}P]P_i binding was assessed by manually quenching the ATPase, in the absence and presence of spermine, before the addition of [^{32}P]P_i.

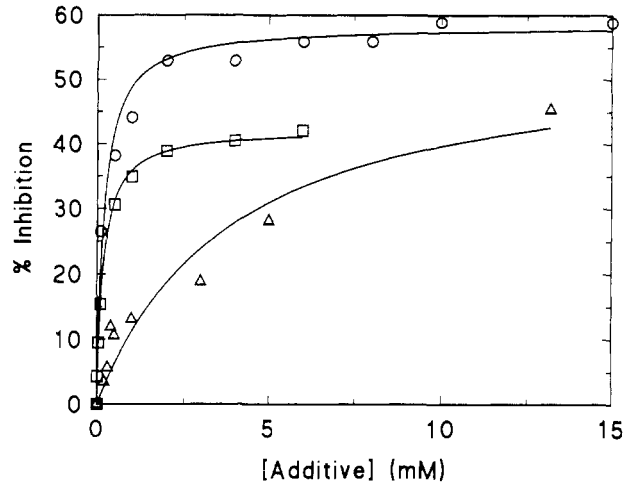


FIGURE 1: Effects of spermine (○), spermidine (□), and Arg-Arg-Arg (△) on the ATPase activity of uncoupled SR vesicles (0.036 μM ATPase) measured at pH 7.2 and 25 °C in 100 mM KCl, 2.1 mM ATP, and 10 μM Ca^{2+} in the presence of 4.0 μg/mL A23187. Results are expressed as the percent inhibition of activity measured in the absence of inhibitor (3.4 IU/mg of protein).

Steady-state measurements of Ca^{2+} release from ATPase on the addition of ATP were made by recording the change in absorbance of antipyrylazo III at 720–790 nm using an Aminco DW2000 dual wavelength spectrophotometer (Starling et al., 1994). ATPase (0.3 mg/mL) was added to buffer (40 mM Mes/Tris (pH 6.0) and 20 mM $MgSO_4$) containing antipyrylazo III (100 μM), and the optical signal was calibrated by additions of a concentrated stock solution of $CaCl_2$ to a final Ca^{2+} concentration of 50 μM. An aliquot of a concentrated stock solution of ATP was then added to give a final ATP concentration of 40 μM, and the level of Ca^{2+} released at steady state was calculated from the change in optical signal. The time dependence of phosphorylation-induced Ca^{2+} release from the ATPase was determined using a Biologic rapid filtration device at room temperature (typically 20 °C). SR vesicles in buffer (0.2 mg of protein/mL; 150 mM Mes/Tris (pH 6.0), 20 mM $MgSO_4$, 100 μM $^{45}CaCl_2$, and 500 μM [^{3}H]sucrose) containing 4% (w/w protein) A23187 were loaded onto a Millipore HAWP 0.45-μm filter and then rapidly perfused with the same buffer containing 2 mM ATP and 100 μM $^{40}CaCl_2$. The filter was then counted in Optiphase HiSafe 3. The amount of Ca^{2+} bound to the ATPase was calculated as previously described (Starling et al., 1993).

The ATPase was labeled with NBD as described (Wictome et al., 1992a). Measurements of NBD and tryptophan fluorescence were performed at 25 °C using an SLM Amino 8000C fluorimeter with excitation and emission wavelengths of 430 and 520 nm and 290 and 340 nm, respectively. Fluorescence polarizations of DPH were measured at a molar ratio of DPH:dioleoylphosphatidylcholine of 1:100, with excitation and emission wavelengths of 348 and 426 nm respectively, corrected for instrumental polarization.

RESULTS

Inhibition of Steady-State Activity by Polycationic Species. As shown in Figure 1, hydrolysis of ATP by the Ca^{2+}-ATPase at pH 7.4 and 25 °C in the presence of 100 mM KCl is inhibited

Table 1: Inhibition of ATPase Activity by Polycations[a]

polycation	maximum inhibition (%)	IC_{50} (μM)
spermine	60	200
N^1-acetylspermine	29	780
spermidine	42	180
N^1-acetylspermidine	0	*b*
N^4-benzylspermidine	0	*b*
protamine	58	18
protamine sulfate	41	19
Arg-Arg-Arg	55	3900
poly(L-Arg)[c]	41	0.2
poly(L-Lys)[d]	17	8.5
cadaverine	0	*b*
putrescine	0	*b*
Joro spider toxin	0	*e*
agmatine	0	*b*

[a] ATPase activities were measured at pH 7.2 in 100 mM KCl, 5 mM Mg^{2+}, and 2.1 mM ATP. [b] No inhibition was observed at concentrations up to 10 mM. [c] Average molecular weight, 10 000 (range 5–15000). [d] Average molecular weight, 22 500 (range 15–30000). [e] No inhibition was observed at concentrations up to 10 μM.

by spermine or spermidine, with inhibition reaching maximum levels of 60 and 42%, respectively (Table 1). Inhibition was less marked at 37 °C than at 25 °C, with 10 mM spermine producing only 26% inhibition at 37 °C. At pH 6.0 in the absence of K^+, 10 mM spermine had no detectable effect on the rate of ATP hydrolysis (data not shown). Ca^{2+} uptake by sealed SR vesicles was followed spectrophotometrically by using the dye murexide to monitor the external Ca^{2+} concentration (McWhirter et al., 1987). Maximal Ca^{2+} uptake at pH 6.3 was reduced 63% by 10 mM spermine (data not shown). Table 1 lists maximal levels of inhibition of ATPase activity and concentrations giving 50% inhibition for a variety of cationic species.

Effects of Spermine on the Affinity of the ATPase for Ca^{2+}. Binding of Ca^{2+} to high-affinity sites on the ATPase can be monitored by observation of the resulting changes in tryptophan fluorescence intensity (Dupont & Leigh, 1978; Fernandez-Belda et al., 1984; Orlowski & Champeil, 1991a; Henderson et al., 1994). As shown in Figure 2A, the change in fluorescence intensity on the removal of Ca^{2+} is smaller in the presence of 10 mM spermine than in its absence, but the concentration of Ca^{2+} resulting in half-maximal changes is unaltered (pCa value of 6.45). The effect of spermine on the Ca^{2+} dependence of ATPase activity at pH 7.2 and 25 °C is shown in Figure 2B. Spermine had no significant effect on the apparent affinity for Ca^{2+} in this experiment, either at sites resulting in the stimulation of activity or at inhibitory sites seen at high concentrations of Ca^{2+}.

At high pH values, Mg^{2+} has been shown to inhibit ATPase activity, probably by binding to Ca^{2+} sites on the phosphorylated ATPase (Bishop & Al-Shawi, 1988; Michelangeli et al., 1990a). As shown in Figure 3, at pH 8.0 in the presence of 2.1 mM ATP, maximal ATPase activity is observed at ca. 2 mM Mg^{2+}. In the presence of 10 mM spermine, activity is decreased by ca. 50% at concentrations of Mg^{2+} below 3 mM, with less inhibition at higher Mg^{2+} concentrations.

Effect of Spermine on the E1–E2 Equilibrium of the ATPase. The fluorescence of the ATPase labeled with nitro-1,2,3-benzoxadiazole chloride (NBD-Cl) has been shown to be sensitive to the E2–E1 transition of the ATPase, with E2 and E1 being states of low and high fluorescence intensity, respectively (Wakabayashi et al., 1990; Wictome et al., 1992b; Henderson et al., 1994). The E2–E1 equilibrium has been shown to be sensitive to pH, with low pH favoring the E2 state (Wakabayashi et al., 1990; Henderson et al., 1994). As shown

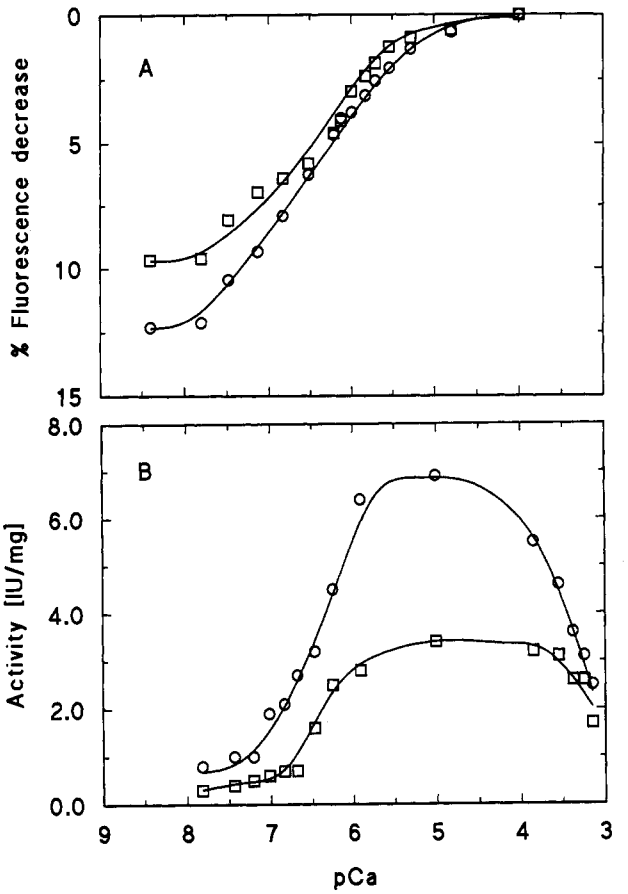


FIGURE 2: Effects of spermine on Ca^{2+} binding to the ATPase. (A) Changes in the tryptophan fluorescence intensity of the ATPase in 20 mM Hepes/KOH (pH 7.2) and 100 mM KCl, measured following the addition of EGTA to give the final free pCa values in the absence (○) or presence of 10 mM spermine (□). Fluorescence was excited at 295 nm and observed at 325 nm. (B) Ca^{2+} dependence of the ATPase activity (IU/mg of protein) of uncoupled SR vesicles, measured at pH 7.2 and 25 °C in the presence of 2.1 mM ATP, 100 mM KCl, 0.036 μM ATPase, and 4.0 μg/mL A23187 for ATPase alone (○) and in the presence of 5.0 mM spermine (□).

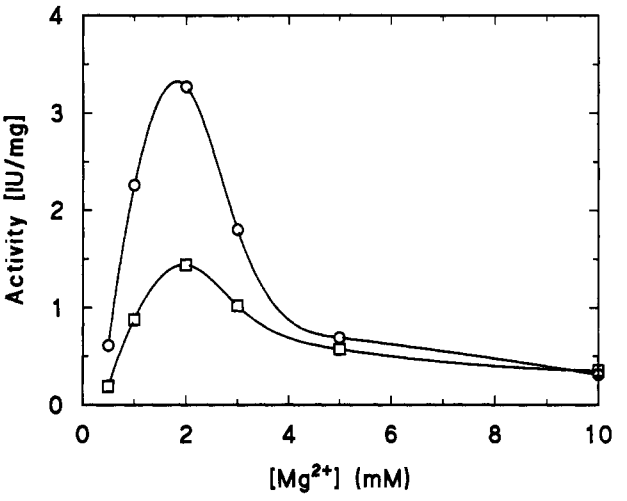


FIGURE 3: Effects of spermine on the Mg^{2+} dependence of the ATPase activity (IU/mg of protein) of uncoupled SR vesicles, measured at pH 8.0 and 25 °C in 50 mM Tris/Hepes containing 2.1 mM ATP, 100 mM KCl, 4.0 μg/mL A23187, and 0.036 μM ATPase for ATPase alone (○) and in the presence of 10 mM spermine (□).

in Figure 4, the addition of spermine to NBD-labeled ATPase in the absence of Ca^{2+} results in an increase in fluorescence intensity, which is particularly marked at intermediate pH values. It has been shown that the effect of pH on the

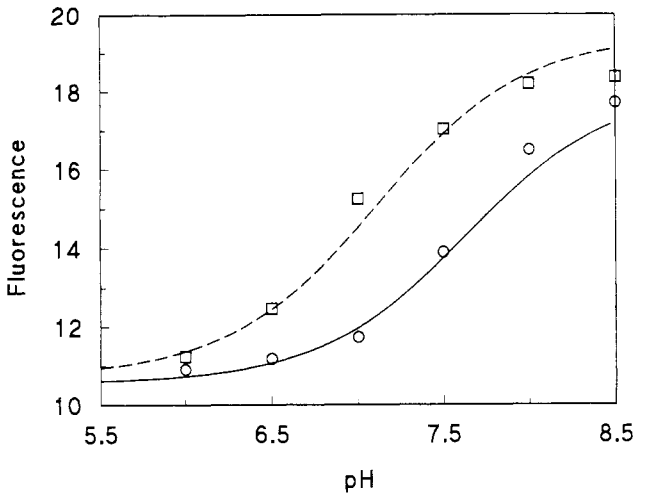


FIGURE 4: Effect of spermine on the fluorescence intensity of NBD-labeled ATPase. The fluorescence intensities of NBD-labeled ATPase in the absence (○) or presence (□) of 10 mM spermine are shown. The solid and broken lines are simulations of the data in the absence and presence of spermine, respectively, calculated using the parameters in Henderson et al. (1994) with values for the equilibrium constant E1/E2 (K_1) of 4.0 and 16.0 in the absence and presence of spermine, respectively. Buffers were as follows: pH 6.0, 130 mM Mes/50 mM Tris; pH 6.5, 164 mM Mes/82 mM Tris; pH 7.0, 150 mM Mops/80 mM Tris; pH 7.5, 140 mM Mops/82 mM Tris; pH 8.0, 100 mM Tris/27 mM Mes; pH 8.5, 100 mM Tris/27 mM Mes. All contained 0.3 mM EGTA.

fluorescence intensity of NBD-labeled ATPase is consistent with Scheme 1, with values for K_{H6}, K_{H7}, and K_1 of 5×10^5 M^{-1}, 3.0×10^8 M^{-1}, and 4.0, respectively (Henderson et al., 1994), and fluorescence intensities of 20.0 and 10.5 for the E1 and E2 states, respectively. As shown in Figure 4, the fluorescence data obtained in the presence of 10 mM spermine fit to the same model, but with a value for K_1 of 16.0.

Effects of Spermine on Phosphorylation and Dephosphorylation of the ATPase. Incubation of the ATPase with P_i in the presence of Mg^{2+} and the absence of Ca^{2+} at acidic pH leads to phosphorylation of the ATPase (de Meis, 1981). As shown in Figure 5A, the level of phosphorylation observed at low concentrations of Mg^{2+} is reduced in the presence of spermine, although effects are small at high concentrations of Mg^{2+} (Figure 5B). In the absence of spermine, the level of phosphorylation of the ATPase fits to Scheme 2 with the parameters given in Froud and Lee (1986) (K_{15} = 120.0 and K_{17} = 130.0). In the presence of spermine the data can be fit within experimental error, assuming a reduction in the binding constants K_{16} and K_{17} by a factor of 2.5 (Figure 5). Figure 6 shows the concentration dependence of the effect of spermine at 5 mM Mg^{2+} and 1 mM P_i. As shown, a maximum inhibition of 56% is observed, with a K_i for spermine of 0.1 mM.

Possible effects of spermine on the rate of dephosphorylation of the ATPase were studied by first phosphorylating the ATPase with [^{32}P]P_i at pH 6.0 in the absence of Ca^{2+} and the presence of 14% dimethyl sulfoxide, conditions under which high levels of phosphorylation are observed (de Meis, 1981), and then mixing with an excess of pH 7.5 medium containing KCl and ATP (Figure 7A). Spermine had no significant effect on the rate of dephosphorylation when present in the final dephosphorylation buffer, with dephosphorylation fitting to single-exponential processes with rate constants of 9.5 ± 2.3 and 8.6 ± 1.7 s^{-1} in the absence and presence of 10 mM spermine, respectively. Binding of spermine to the ATPase was shown to be fast compared to the rate of dephosphorylation of the ATPase, since the same rate of ATP-induced loss of

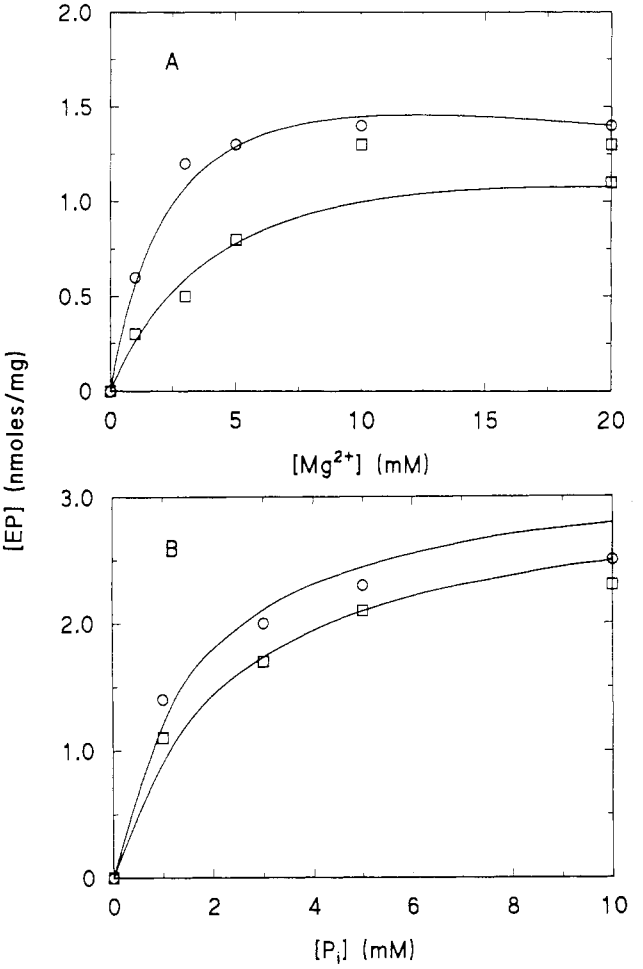
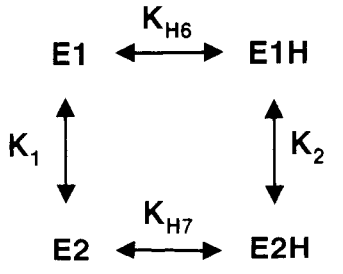


FIGURE 5: Effect of spermine on the level of phosphorylation of the ATPase by phosphate. The ATPase (0.1 mg/mL) was incubated in 150 mM Mes/Tris (pH 6.3), 5 mM EGTA, and (A) 1 mM P_i and the given concentration of Mg^{2+} and (B) 20 mM Mg^{2+} in the presence of the given concentration of P_i, in the absence (○) or presence (□) of 10 mM spermine. The lines are simulations calculated as described in the text, assuming a maximal level of phosphorylation of 4.0 nmol of EP/mg of protein with Mg^{2+} binding constants of K_{15} = 120.0 and K_{17} = 130.0 in the absence of spermine and of 48.0 and 52.0, respectively, in the presence of spermine.

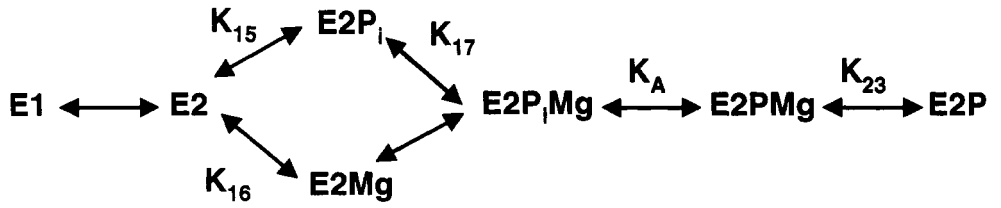
Scheme 1



$^{45}Ca^{2+}$ from the ATPase was observed in the experiment described in Figure 10, whether or not the ATPase was preincubated with spermine (data not shown).

The effect of spermine on the rate of phosphorylation of the ATPase following mixing of the ATPase, incubated in the presence of Ca^{2+}, with [γ-^{32}P]ATP is shown in Figure 8. Spermine at 5 mM had no significant effect on either the rate or the maximum extent of phosphorylation observed with 25 μM ATP, the data fitting to single exponentials with rates and amplitudes of 45.7 ± 9.0 s^{-1} and 2.70 ± 0.17 nmol of EP/mg of protein, respectively, in the absence of spermine and 44.9 ± 9.3 s^{-1} and 2.52 ± 0.17 nmol of EP/mg of protein,

Scheme 2



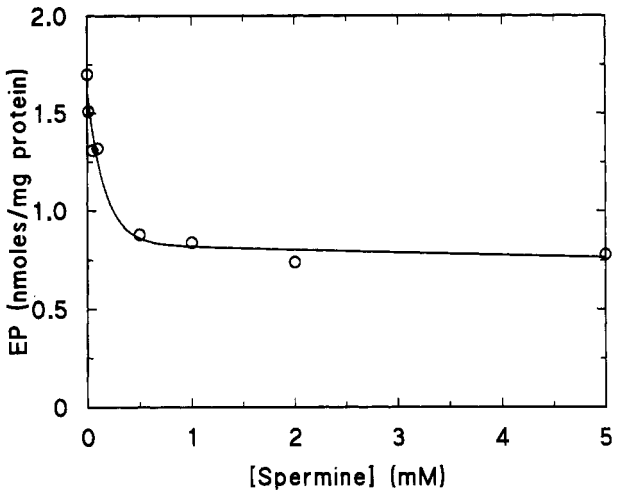


FIGURE 6: Concentration dependence of the effect of spermine on the phosphorylation of ATPase by phosphate. The experiment was performed in the presence of 5 mM Mg^{2+} and 1 mM P_i at the given concentrations of spermine, as described in the legend to Figure 5. The line shows a fit to a simple inhibition curve with a maximum inhibition of 56% and a K_i value for spermine of 0.1 mM.

respectively, in the presence of 10 mM spermine. With 5 μM ATP, however, a significantly higher level of phosphorylation is observed in the presence of spermine, but with no effect on the rate of phosphorylation (Figure 8). Again, the data fit to single exponentials with rates and amplitudes of 10.9 ± 2.7 s^{-1} and 2.35 ± 0.26 nmol of EP/mg of protein, respectively, in the absence of spermine and 11.7 ± 1.1 s^{-1} and 0.78 ± 0.09 nmol of EP/mg of protein, respectively, in the presence of spermine.

Table 2 shows the maximal level of phosphorylation of the ATPase by [γ-^{32}P]ATP under conditions (1 mM Ca^{2+}) where the rate of dephosphorylation would be expected to be low. As shown, under these conditions spermine had no significant effect on the observed level of phosphoenzyme formation. At low concentrations of ATP (20 μM) and low concentrations of Ca^{2+} (0.1 mM), an increased level of phosphoenzyme is observed in the presence of spermine that is attributable to significant hydrolysis of the added ATP, which will be slower in the presence of spermine.

The rate of dephosphorylation of the ATPase phosphorylated with [γ-^{32}P]ATP in the presence of Ca^{2+} can be determined by dilution with an excess of nonradioactive ATP. As shown in Figure 7B, the rate of dephosphorylation decreases from 11.8 ± 0.8 s^{-1} in the absence of spermine to 6.4 ± 1.3 s^{-1} in the presence of 10 mM spermine. The effect of spermine on dephosphorylation of ATPase with ADP is shown in Figure 9. The ATPase in sealed SR vesicles was phosphorylated with 50 μM [γ-^{32}P]ATP for 10 s in the presence of Ca^{2+} and then mixed in a 1:1 ratio with buffer containing ADP and spermine to give final concentrations of ADP and spermine of 1.5 and 10 mM, respectively, and a final Ca^{2+} concentration of 44 μM. As shown, under these conditions a rapid initial decrease in EP is followed by a slower phase, which fits to a single-exponential process over the time period 20–100 ms;

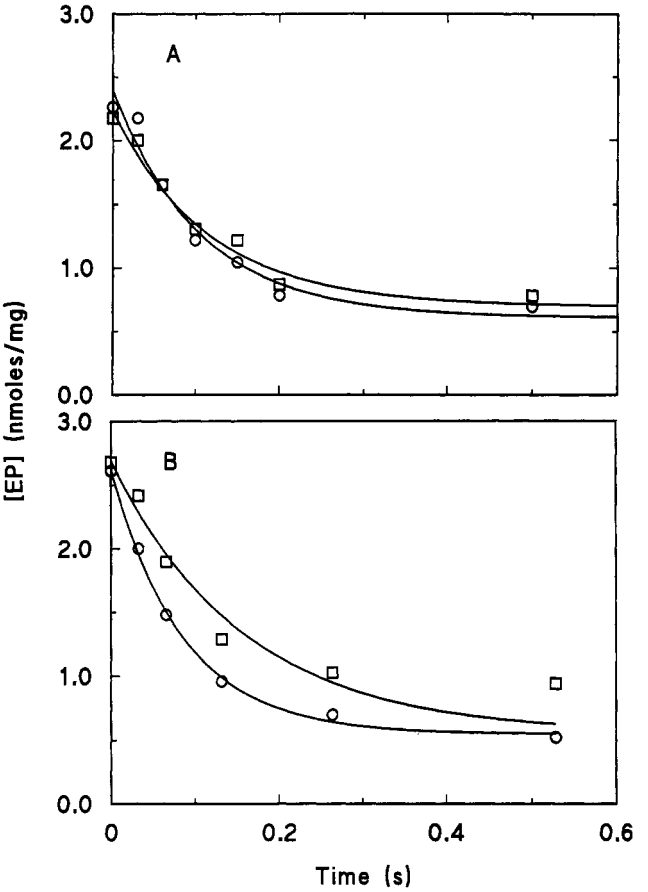


FIGURE 7: Effects of spermine on the rate of dephosphorylation of the ATPase at 25 °C following phosphorylation with P_i in the absence of Ca^{2+} (A) or with ATP in the presence of Ca^{2+} (B). In A, the enzyme syringe contained uncoupled SR (4.0 mg/mL) in 12.5 mM Mes/Tris (pH 6.0) containing 0.16 mg/mL A23187, 10 mM EGTA, 1 mM [^{32}P]P_i, 20 mM Mg^{2+}, and 14% (v/v) dimethyl sulfoxide. The second syringe contained 100 mM Tris/Mes (pH 7.5) containing 100 mM KCl, 4.1 mM Mg^{2+}, 5.3 mM ATP, and either no spermine (○) or 10.6 mM spermine (□). The contents of the enzyme syringe were mixed in a 1:16 volume ratio with the dephosphorylation mixture, and the reaction was quenched at the given times with 12% trichloroacetic acid/0.2 M phosphoric acid. The concentration of spermine in the final dephosphorylation medium was 10 mM. The lines represent fits to single exponentials, with the parameters given in the text and nonspecific binding of 0.07 ± 0.02 and 0.08 ± 0.01 nmol of EP/mg of protein in the absence and presence of spermine, respectively. In B, the enzyme syringe contained ATPase (0.2 mg/mL) in 20 mM Hepes/Tris (pH 7.2), 5 mM Mg^{2+}, 100 mM KCl, and 100 μM Ca^{2+}. This was mixed in a 1:1 ratio with a solution containing 50 μM [γ-^{32}P]ATP in the same buffer. The mixture was incubated for 200 ms and then mixed in a 1:1 ratio with the same buffer containing 2.5 mM unlabeled ATP and either no spermine (○) or 20 mM spermine (□). The reaction was quenched at the given times with 12% trichloroacetic acid/0.2 M phosphoric acid. The lines represent fits to single exponentials with the parameters given in the text.

the rates of the slow phase were 12.9 ± 5.9 and 8.5 ± 1.4 s^{-1} in the absence and presence of spermine, respectively, with the fraction of EP reacting in the fast phase being 0.56 in the absence or presence of spermine.

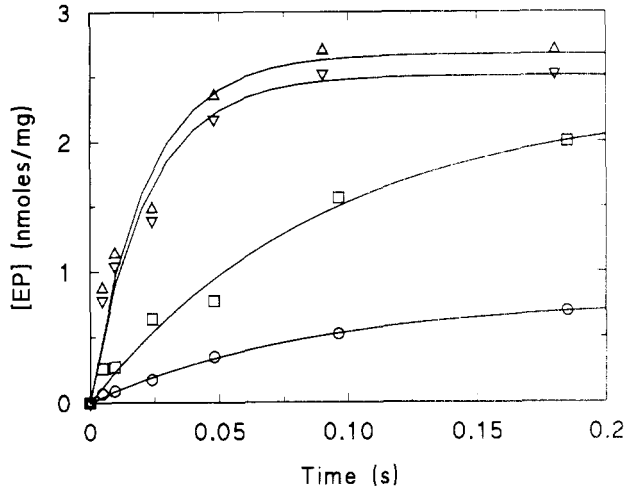


FIGURE 8: Rate of phosphorylation of the ATPase by [γ-^{32}P]ATP in the absence (△, ○) or presence (▽, □) of 10 mM spermine. The ATPase (0.2 mg/mL) incubated in buffer (pH 7.2, 20 mM Hepes/Tris, 5 mM Mg^{2+}, 100 mM KCl, and 100 μM Ca^{2+}) was mixed in a 1:1 ratio with the same buffer containing 50 μM [γ-^{32}P]ATP (△, ▽) or 10 μM [γ-^{32}P]ATP (○, □) to give a final ATP concentrations of 25 (△, ▽) or 5 (○, □) μM, respectively. The lines represent fits to single exponentials with the parameters given in the text.

Table 2: Phosphorylation of the ATPase with [γ-^{32}P]ATP[a]

concn of ATP (μM)	concn of Ca^{2+} (mM)	level of phosphorylation (nmol of EP/mg of protein)	
		ATPase	ATPase + 10 mM spermine
20	0.1	1.2	2.5
100	0.1	3.6	3.4
20	1	2.6	2.7
100	1	3.3	3.7

[a] The ATPase (0.1 mg/mL) was incubated with the given concentrations of [γ-^{32}P]ATP and Ca^{2+} for 15 s in a medium containing 40 mM Hepes/Tris (pH 7.2), 100 mM KCl, and 5 mM $MgSO_4$ at 25 °C before quenching the reaction.

Effect of Spermine on the Rate of Dissociation of Ca^{2+} from the Phosphorylated ATPase. Orlowski and Champeil (1991b) have shown that the rate of Ca^{2+} dissociation from the phosphorylated ATPase can be measured by preequilibrating the ATPase in leaky vesicles with $^{45}Ca^{2+}$ and then perfusing them on Millipore filters with $^{40}Ca^{2+}$ and ATP; the experiment relies on the rate of phosphorylation of ATPase being much faster than the rate of dissociation of $^{45}Ca^{2+}$ from the unphosphorylated ATPase (Orlowski & Champeil, 1991b). This is demonstrated by experiments with sealed vesicles. As shown in Figure 10, if SR vesicles incubated with $^{45}Ca^{2+}$ are perfused with ATP and $^{40}Ca^{2+}$ in the absence of the Ca^{2+} ionophore A23187, then very little loss of $^{45}Ca^{2+}$ is observed due to transport of the $^{45}Ca^{2+}$ initially bound into the lumen of the SR; this experiment demonstrates that phosphorylation of the ATPase by ATP and transport of Ca^{2+} are much faster than the dissociation of $^{45}Ca^{2+}$ from the unphosphorylated ATPase. An identical slow rate of dissociation of $^{45}Ca^{2+}$ was observed when the experiment was repeated in the presence of 10 mM spermine (data not shown) In the presence of A23187, a much faster rate of dissociation of $^{45}Ca^{2+}$ is observed, the rate being slower in the presence of spermine. The data fit to single-exponential processes with rate constants of 12.7 ± 1.4 and 3.5 ± 0.5 s^{-1} in the absence and presence of 10 mM spermine, respectively. At pH 7.2, faster rates of $^{45}Ca^{2+}$ are observed (Starling et al., 1994), with the dissociation of $^{45}Ca^{2+}$ again being slower in the presence of spermine; the

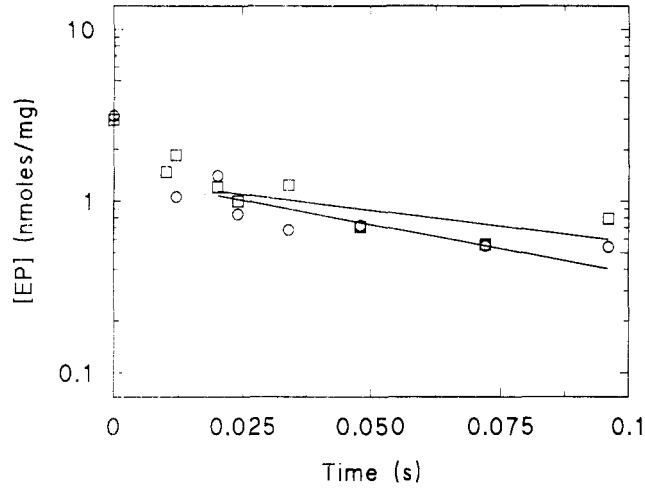


FIGURE 9: Effect of spermine on the rate of dephosphorylation of the ATPase caused by addition of ADP. SR vesicles (0.2 mg/mL) were incubated in 50 mM Mops (pH 7.2) containing 5 mM $MgSO_4$, 100 mM KCl, 50 μM $CaCl_2$, and 50 μM [γ-^{32}P]ATP for ca. 1 min and then mixed in a 1:1 ratio with the same buffer containing 5 mM $MgSO_4$, 100 mM KCl, 10.08 mM $CaCl_2$, 10.0 mM EGTA, 3 mM ADP, and either no spermine (○) or 10 mM spermine (□), followed by quenching. The solid lines represent fits to single exponentials with the parameters given in the text, and the dashed lines show extrapolations to time zero giving the rapidly reacting fractions.

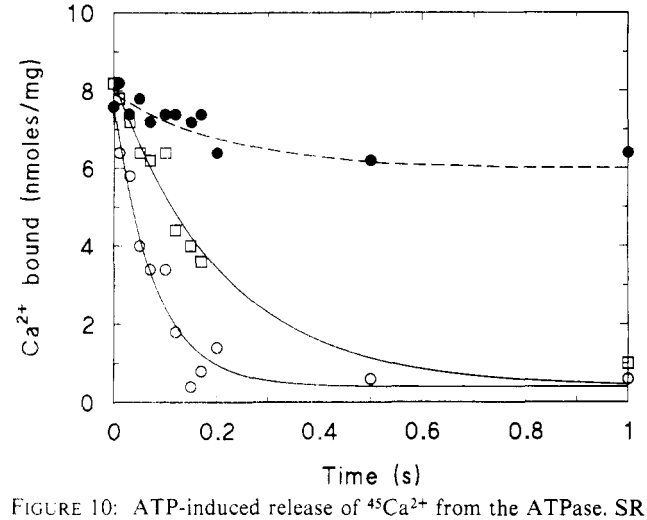


FIGURE 10: ATP-induced release of $^{45}Ca^{2+}$ from the ATPase. SR vesicles (0.2 mg/mL) were first equilibrated in pH 6.0 buffer (150 mM Mes/Tris and 20 mM Mg^{2+}) containing 100 μM $^{45}Ca^{2+}$ and 0.5 mM [^{3}H]sucrose in the absence (●) or presence (○, □) of 4% (w/w protein) A23187, and then 0.1 mg of protein was adsorbed onto Millipore filters. The loaded filter was perfused for the given times with the same buffer containing 100 μM unlabeled Ca^{2+}, 0.5 mM [^{3}H]sucrose, and 2 mM ATP in the absence (○, ●) or presence (□) of 10 mM spermine. The solid lines represent fits to single-exponential decays with the parameters given in the text.

data fit to rates of 21.4 ± 1.8 s^{-1} in the absence of spermine and 9.8 ± 1.7 s^{-1} in the presence of 10 mM spermine (data not shown).

Effects of spermine on $^{45}Ca^{2+}$ binding to the phosphorylated ATPase in steady-state experiments are shown in Table 3. The ATPase was incubated with 100 μM $^{45}Ca^{2+}$ in buffer containing 5 mM Mg^{2+} and 100 mM KCl at pH 7.2 and then perfused with the same medium containing 100 μM $^{45}Ca^{2+}$ and 0.5 mM ATP. A steady-state release of 2.3 nmol of Ca^{2+}/mg of protein was observed in the absence of spermine, but no steady-state release of Ca^{2+} was observed in the presence of 10 mM spermine.

Effects of Spermine on Lipid Bilayers. Possible effects of spermine on the lipid bilayer component of the membrane

Table 3: Effect of Spermine on the Steady-State Release of Ca^{2+} from the ATPase in the Presence of ATP[a]

system	Ca^{2+} bound (nmol/mg of protein)
ATPase	10.1
ATPase + 0.5 mM ATP	7.8
ATPase + 10 mM spermine	10.4
ATPase + 0.5 mM ATP + 10 mM spermine	10.5

[a] ATPase (mg of protein) was incubated at pH 7.2 in 5 mM Mg^{2+}, 100 mM KCl, and 100 μM $^{45}Ca^{2+}$ in the presence or absence of 10 mM spermine, adsorbed onto Millipore filters, and then washed with the same buffer containing 0.5 mM ATP. $^{45}Ca^{2+}$ bound to the ATPase was determined as described in Materials and Methods.

were monitored using the fluorescence polarization of DPH incorporated into bilayers of dioleoylphosphatidylcholine. In the absence of spermine, the polarization, P, was determined to be 0.144 at 25 °C. A polarization value of 0.140 was determined in the presence of between 1 and 10 mM spermine, indicating no significant effect of spermine on fluidity as detected by DPH polarization.

DISCUSSION

Slow-twitch muscles such as the heart express an isoform (SERCA2) of the Ca^{2+}-ATPase whose activity is regulated by phospholamban. Phospholamban is a membrane protein with a single hydrophobic domain at the C-terminus (residues 31–52) and a hydrophilic N-terminal domain (residues 1–30) containing residues whose phosphorylation reduces interaction with the Ca^{2+}-ATPase (Simmerman et al., 1986; Fujii et al., 1987; Colyer & Wang, 1991). Binding of a peptide corresponding to residues 1–31 of phospholamban to the Ca^{2+}-ATPase in heart led to a reduction in v_{max} for the ATPase with no effect on the affinity for Ca^{2+}, whereas binding of a peptide corresponding to residues 28–47 led to a reduction in the affinity of the ATPase for Ca^{2+} without any effect on v_{max} (Sasaki et al., 1992). Fast-twitch skeletal muscle contains no phospholamban, but coexpression of phospholamban and the fast-twitch isoform of the Ca^{2+}-ATPase (SERCA1) in COS cells showed that phospholamban is capable of regulating SERCA1 in identical fashion to SERCA2 (Toyofuku et al., 1993).

The region of the cytoplasmic domain of phospholamban critical for its interaction with Ca^{2+}-ATPase lies between residues 7 and 16, probably involving the three Arg residues in the sequence RXXXRR (Morris et al., 1991). An important role for charge interactions would be consistent with the observation that high ionic strength or phosphorylation of phospholamban eliminates its regulation of the Ca^{2+}-ATPase (Chiesi & Schwaller, 1989). Cross-linking experiments have suggested that phospholamban interacts with the Ca^{2+}-ATPases at a region just C-terminal from the residue (Asp-351) phosphorylated by ATP, and using ^{125}I-labeled phospholamban, radioactivity was recovered in Lys-397 and Lys-400 (James et al., 1989; Vorherr et al., 1992). These two residues occur in a region conserved in the SERCA1 and SERCA2 isoforms of the Ca^{2+}-ATPase, but not in the SERCA3 isoform (Burk et al., 1989) or in more distantly related Ca^{2+}-ATPases such as those in *Plasmodium* (Kimura et al., 1993), *Artemia* (Palmero & Sastre, 1989), or plants (Wimmers et al., 1992). Phospholamban has been shown not to affect the Ca^{2+} affinity of the SERCA3 isoform of the Ca^{2+}-ATPase, and the construction of chimeric Ca^{2+}-ATPases between SERCA2 and SERCA3 has shown that the region between residues 336 and 412 is essential for interaction with phospholamban (Toyofuku et al., 1993). Presumably, binding of the positively charged phospholamban would be to the negatively charged residues found in this region of the ATPase (e.g., Glu-392, Glu-394 and Asp-399). An antipeptide antibody raised to residues 381–400 bound to the native ATPase, suggesting surface exposure for this region (Matthews et al., 1989; Mata et al., 1992).

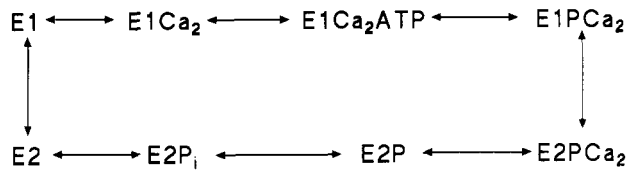
A number of polycationic compounds have been observed to inhibit Ca^{2+} uptake by cardiac SR, including poly(L-arginine), poly(L-lysine), spermine, spermidine, histone, and polymyxin B (Xu & Kirchberger, 1989). Histone and polymyxin B have also been reported to inhibit Ca^{2+} uptake and ATP hydrolysis by skeletal muscle SR vesicles (Xu & Kirchberger, 1989). However, effects are selective in that ruthenium red with six positive charges has no effect either on Ca^{2+} accumulation by SR vesicles or on the rate of Ca^{2+} binding, even though it does bind to the Ca^{2+}-ATPase (Corbalan-Garcia et al., 1992; Moutin et al., 1992). Examination of the effects of polycationic compounds on the kinetics of Ca^{2+}-ATPase may suggest a mechanism for regulation of the ATPase by phospholamban.

The physiological significance of inhibition of the cardiac ATPase by spermidine is unclear. Spermidine and other polyamines such as spermine and putrescine are found in many animal tissues, including skeletal muscle (Kaminska et al., 1982). Spermine has been shown to increase the accumulation of Ca^{2+} by mitochondria (Lenzen et al., 1992), and intracellular polyamines have been shown to reduce the Con A-induced increase in cytoplasmic free Ca^{2+} observed in spleen cells (Thomas et al., 1993). Spermine and ruthenium red have also been shown to interact with the ryanodine-sensitive Ca^{2+} channel in SR (Zarka & Shoshan-Barmatz, 1992; Antoniu et al., 1985; Chamberlain et al., 1984).

As shown in Figure 1, hydrolysis of ATP by the Ca^{2+}-ATPase of skeletal muscle sarcoplasmic reticulum is inhibited by spermine (four amino groups) and spermidine (three amino groups) with maximal inhibitions of 60 and 42%, respectively. The concentrations of spermine and spermidine giving 50% inhibition (IC_{50}) are 200 and 180 μM, respectively. No inhibition was observed with either putrescine or cadaverine (two amino groups), suggesting that a minimum of three positive charges is required for inhibition. This is consistent with the observation that no inhibition was observed with either N^1-acetylspermidine or N^4-benzylspermidine (Table 1). The level of inhibition observed with N^1-acetylspermine was less than that observed with spermidine, and the IC_{50} value was higher for N^1-acetylspermine than for spermidine. Thus, although three positive charges are sufficient for binding and inhibition, a primary amino group may be more favorable for binding than a secondary amino group. No inhibition was observed with agmatine [$H_2N(CH_2)_4NHC(=NH)NH_2$], showing that inhibition was not simply dependent on the number of nitrogen centers. Joro spider toxin, a polycationic neurotoxin with very high affinity for the L-glutamate receptor (Jackson & Usherwood, 1988), had no effect on Ca^{2+}-ATPase at concentrations up to 30 μM.

As shown in Table 1, Arg-Arg-Arg, although an inhibitor of the Ca^{2+}-ATPase, is only effective at very high concentrations in contrast to poly(L-Arg), which has an IC_{50} value of 0.2 μM. Inhibition is also observed with protamine, a peptide rich in Arg residues. However, poly(L-Lys) causes a maximum of 17% inhibition, with an IC_{50} value 40 times higher than that observed for poly(L-Arg). It is likely that the binding site for poly(L-Arg), corresponds to that for phospholamban and that the binding site is a region of high negative charge on the surface of the ATPase.

Scheme 3



The mechanism of the Ca^{2+}-ATPase can be described in terms of the E2–E1 model developed from the Post–Albers scheme for (Na-K)-ATPase (de Meis & Vianna, 1979) (Scheme 3). In the E1 conformation, the ATPase has two outward-facing binding sites of high affinity. Following the binding of MgATP the ATPase is phosphorylated to give Ca_2E1P, which can undergo a change in conformation to a state (Ca_2E2P) in which the two Ca^{2+} binding sites are of low affinity and are inward facing. Following the loss of Ca^{2+} to the inside of the SR, the ATPase can dephosphorylate and recycle to E1.

Although spermine decreased ATPase activity (Figure 1), it had no significant effect on the Ca^{2+} dependence of ATPase activity (Figure 2B) or on the Ca^{2+} dependence of Trp fluorescence (Figure 2A). In this it was similar to the hydrophilic domain of phospholamban (Sasaki et al., 1992).

High concentrations of Ca^{2+} inhibit ATPase activity, attributed partly to the binding of Ca^{2+} to the phosphorylated ATPase, resulting in a decrease in the rate of dephosphorylation, and partly to the formation of CaATP, which phosphorylates the ATPase to a form with a slow rate of dephosphorylation (Shigekawa et al., 1983; Yamada et al., 1986; Michelangeli et al., 1990a). As shown in Figure 2B, the Ca^{2+} dependence of inhibition of the ATPase is unaffected by spermine, suggesting that spermine has no effect on the affinity of the phosphorylated ATPase for Ca^{2+}. Inhibition of ATPase activity observed at high concentrations of Mg^{2+} at pH 8.0 has been attributed to the binding of Mg^{2+} to the Ca^{2+} binding sites on the phosphorylated ATPase with subsequent inhibition of dephosphorylation (Bishop & Al-Shawi, 1988; Michelangeli et al., 1990a). As shown in Figure 3, the Mg^{2+} dependence of inhibition is unaffected by spermine, again suggesting that spermine has no effect on the affinity of the phosphorylated ATPase for Ca^{2+}.

It has been shown that changes in the E2–E1 equilibrium of the ATPase can be detected from changes in the fluorescence intensity of NBD-labeled ATPase (Wakabayashi et al., 1990; Wictome et al., 1992b; Henderson et al., 1994). The equilibrium is pH-sensitive and fits to Scheme 2 (Wakabayashi et al., 1990; Henderson et al., 1994). As shown in Figure 4, spermine shifts the E2–E1 equilibrium of the ATPase toward E1, with the data fitting a 4-fold increase in the equilibrium constant K_1 at 10 mM spermine with no change in the proton binding constants. It has been suggested that the decreased Ca^{2+} affinity for the ATPase seen on binding phospholamban could follow from a shift in the E2–E1 equilibrium toward E2 (Toyofuku et al., 1993; Tada et al., 1988). If this were the case, then the shift would presumably follow from interaction of the hydrophobic domain of phospholamban with the ATPase.

The dependence of ATPase activity on the concentration of ATP is complex, attributable to the binding of ATP at the catalytic site with micromolar affinity and binding to a stimulatory site with millimolar affinity (Gould et al., 1986). The presence of 10 mM spermine had no significant effect on the ATP dependence of ATPase activity (data not shown), suggesting that spermine did not affect the affinity of the ATPase for ATP. Possible effects of competition between Mg^{2+} and spermine for binding to ATP were studied. Inhibition of ATPase activity by 10 mM spermine at 0.1 or 1 mM ATP was found to be independent of Mg^{2+} concentration over the range 1–10 mM (data not shown). Thus, binding of spermine to ATP does not appear to be an important factor in inhibition, presumably because the spermine–ATP binding constant is less than that for the Mg^{2+}-ATP complex (Nakai & Glinsmann, 1977). The binding constant for spermidine and ATP is about 10-fold less than that for spermine with ATP (Nakai & Glinsmann, 1977), and the similar effects of spermine and spermidine on the activity of the ATPase again suggest that complex formation of the polyamines with ATP is unimportant.

As shown in Table 2, spermine had no effect on the level of phosphorylation observed at high (100 μM) concentrations of ATP, demonstrating that inhibition of ATPase activity occurs at some stage following phosphorylation and preceding dephosphorylation. In the presence of low concentrations of Ca^{2+} (0.1 mM) and ATP (20 μM), the presence of spermine leads to higher levels of phosphoenzyme formation; this can be attributed to significant hydrolysis of the added ATP during the time course of the experiment under these conditions.

As shown in Figure 8, spermine had no effect on the rate of phosphorylation at either 25 or 5 μM ATP. The rate of phosphorylation observed at 5 μM ATP is slower than that observed at 25 μM ATP, because at the lower concentration the rate of binding of ATP to the ATPase becomes a slow step [see Michelangeli et al. (1991)]. The data therefore suggest that the on-rate for ATP binding and the rate of the phosphorylation step are both unaffected by spermine. We attribute the higher level of phosphoenzyme observed with 5 μM ATP in the presence of spermine than in its absence (Figure 8) to the decreased rate of the $Ca_2E1P \rightarrow E2P$ step (see below); simulations show that increasing the rate of this step leads to a decreased level of phosphoenzyme (Michelangeli et al., 1990b; Starling et al., 1994).

Spermine had no significant effect on the rate of dephosphorylation of E2P (Figure 7A). It did, however, reduce the level of phosphoenzyme formation from P_i (Figure 5). The effect was dependent on the concentration of Mg^{2+} and could be simulated assuming a reduction in the affinity of the ATPase for Mg^{2+} with no effect on the equilibrium constant for the phosphorylation step $E2P_iMg \rightleftharpoons E2PMg$. de Meis (1991) has reported that spermidine also decreases the level of P_i phosphorylation of the ATPase by competition with Mg^{2+} binding. The concentration of spermine causing a 50% reduction in the level of phosphorylation at 5 mM Mg^{2+} and 1 mM P_i was 0.1 mM (Figure 6), which is comparable to the value causing 50% maximal inhibition of ATPase activity (0.2 mM, Table 1).

Addition of ADP to the phosphorylated ATPase in Ca^{2+}-loaded SR vesicles leads to biphasic dephosphorylation. The magnitude of the slow phase of dephosphorylation was found to decrease with increasing concentrations of ADP, which is consistent with the rapid formation of enzyme-bound ATP followed by a slow dissociation of the bound ATP (Stahl & Jencks, 1987):

$$Ca_2EP + ADP \rightleftharpoons Ca_2EPADP \rightleftharpoons Ca_2EATP \rightleftharpoons Ca_2E + ATP$$

The results are inconsistent with biphasic dephosphorylation being due to two forms of the phosphorylated ATPase: one sensitive to ADP and one insensitive to ADP, with the ADP-insensitive form being responsible for the slow phase of dephosphorylation. In this case, the amount of the ADP-

insensitive form would be independent of the concentration of added ADP so that the magnitude of the slow phase of dephosphorylation would be independent of ADP concentration (Stahl & Jencks, 1987). By applying the quasi-equilibrium approach (Pickart & Jencks, 1982), this model predicts a burst size α given by

$$\alpha = [1 + K_{int}(1 + K_d^{ADP}/[ADP])]^{-1}$$

and a rate constant k for the slow phase given by

$$k = \alpha k_{off}$$

where K_d^{ADP} is the dissociation constant for ADP, K_{int} is the equilibrium constant for phosphorylation on the ATPase, and k_{off} is the rate constant for dissociation of ATP. Froehlich and Heller (1985) and Fernandez-Belda and Inesi (1986) have observed significant dephosphorylation to P_i in the slow phase, so that it is possible that the slow step also contains a significant contribution from the E2P → E2 + P_i step.

As shown in Figure 9, spermine had no effect on the burst size, so that neither K_{int} nor K_d^{ADP} is likely to be affected by spermine. Rates of the slow phase of dephosphorylation were not significantly different in the absence or presence of spermine, so that the binding of spermine has no effect on the rate of dissociation of ATP.

The inhibitory effect of spermine on the ATPase follows largely from its effect on the rate of dissociation of Ca^{2+} from the phosphorylated ATPase. In the experiment shown in Figure 10, SR vesicles were incubated in the presence of the Ca^{2+} ionophore A23187 with 100 μM $^{45}Ca^{2+}$ and then perfused with 100 μM $^{40}Ca^{2+}$ and 2 mM ATP. Under the conditions of the experiment, the rate of phosphorylation of the ATPase was much faster than the observed rate of dissociation of $^{45}Ca^{2+}$ (see Figure 8 at 25 μM ATP), and the rate of dissociation of $^{45}Ca^{2+}$ from the unphosphorylated ATPase was slow, as shown by the slow rate of loss of $^{45}Ca^{2+}$ in the corresponding experiment with sealed vesicles in the absence of A23187 (Figure 10). Thus, the rate of dissociation of $^{45}Ca^{2+}$ from the ATPase reflects the rate of dissociation of Ca^{2+} from the phosphorylated ATPase at the luminal membrane surface. As shown, the rate decreased by a factor of ca. 3 in the presence of 10 mM spermine. The rate of this same step is also decreased by poly(L-Arg) and by a peptide corresponding to the hydrophilic domain of phospholamban (residues 1–26) (G. Hughes, J. M. East, and A. G. Lee, unpublished observations).

Dephosphorylation in the presence of Ca^{2+} can be followed by phosphorylation with [γ-^{32}P]ATP, followed by mixing with unlabeled ATP. As shown in Figure 7B, the presence of 10 mM spermine decreased the rate of dephosphorylation from 11.8 ± 0.8 to 6.4 ± 1.3 s^{-1}. Comparison with the data shown in Figure 10 would suggest that this follows from a decrease in the rate of the Ca_2E1P → E2P step and that this is rate controlling for the overall change Ca_2E1P → E2.

A decrease in the rate of the Ca_2E1P → E2P step would be expected to lead to a buildup of Ca^{2+}-bound forms of ATPase at steady state in the presence of ATP. This is observed in experiments in which the ATPase is incubated with $^{45}Ca^{2+}$, adsorbed onto Millipore filters, and then perfused with $^{45}Ca^{2+}$ and ATP. As shown in Table 3, at pH 7.2 perfusion with ATP normally leads to the release of 2.3 nmol of Ca^{2+}/mg of protein, which is attributable to the formation of some E2P at steady state under these conditions. In the presence of spermine, no significant release of $^{45}Ca^{2+}$ was observed, showing that at steady state in the presence of spermine the ATPase exists fully in Ca^{2+}-bound forms. Steady-state release of Ca^{2+} can also be detected spectrophotometrically, using antipyrylazo III to measure the Ca^{2+} concentration in the medium. At pH 6.0, addition of 100 μM ATP was found to lead to the release of 6.7 nmol of Ca^{2+}/mg of protein in the absence of spermine and 2.7 nmol of Ca^{2+}/mg of protein in the presence of 10 mM spermine (data not shown). These experiments are therefore consistent with a decrease in the rate of the Ca_2E1P → E2P step. The 4-fold increase in the equilibrium constant E1/E2 and the 3-fold decrease in the rate of the Ca_2E1P → E2P step are consistent with stronger binding of spermine to the E1 conformation of the ATPase than to the E2 conformation.

Spermine has been shown to affect a wide variety of Ca^{2+}-dependent processes in the cell (Schuber, 1989). It has been shown to bind to phospholipid bilayers (Schuber, 1989), but has no effect on the motional properties of a phospholipid as monitored by the fluorescence polarization of DPH (see Results). Effects of spermine on the Ca^{2+}-ATPase are therefore likely to follow from direct interaction with the ATPase. The observation that spermine affects only specific steps in the reaction sequence of the ATPase suggests that the inhibitory effect of spermine is likely to follow from binding to a restricted number of sites on the Ca^{2+}-ATPase. Similarity with the effects of phospholamban suggests that the site could be the site at which the hydrophilic region of phospholamban binds to the Ca^{2+}-ATPase. Spermine and spermidine are found in skeletal muscle (Kaminska et al., 1982), and levels can reach 10 mM in some cells (Schuber, 1989). Since IC_{50} values are ca. 0.2 mM (Table 1), it is possible that polyamines cause inhibition of the Ca^{2+}-ATPase under some physiological conditions.

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