Calmodulin Stimulates both Adenosine 5′-Triphosphate Hydrolysis and Synthesis Catalyzed by a Cardiac Calcium Ion Dependent Adenosinetriphosphatase†

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ABSTRACT: A Ca^{2+}-dependent ATPase purified from a rabbit heart membrane preparation was compared to the Ca^{2+}-dependent ATPase purified from skeletal muscle sarcoplasmic reticulum. The two ATPases display an identical electrophoretic pattern and an identical Ca^{2+}-concentration dependence. However, only the cardiac preparation exhibits a 2–3-fold activation by calmodulin. This effect is best observed when the molar concentrations of calmodulin and ATPase are equivalent and in the presence of high Ca^{2+} ($\sim 10^{-5}$ M) and ATP ($\sim 10^{-3}$ M) concentrations. It is demonstrated for the first time that calmodulin stimulates the rate of ATP synthesis, as revealed by an increased production of P_i and a faster ATP $\rightleftharpoons$ P_i exchange, as well as the rate of ATP hydrolysis. It is also demonstrated that calmodulin activation is expressed with purified and detergent-solubilized enzyme in addition to membrane-bound systems. These findings indicate that the effect of calmodulin is an acceleration of the enzyme turnover, due to direct interaction of calmodulin with the enzyme.

In analogy to the effect on the Ca^{2+}-dependent ATPase and Ca^{2+} transport of red cell membranes (Jarrett & Penniston, 1978; Vincenzi & Larsen, 1980; Rega & Garrahan, 1980), calmodulin has been reported to stimulate the Ca^{2+}-ATPase and Ca^{2+}-transport activities of sarcolemmal (SL) and sarcoplasmic reticulum (SR) membranes obtained from cardiac muscle (Caroni & Carafoli, 1981; Lamers et al., 1981; Le Peuch et al., 1979; Katz & Remtulla, 1978; Kirchberger & Antonetz, 1982). Whether due to direct interaction of calmodulin with ATPase or mediated by phosphorylation of "phospholamban", the calmodulin effect has been described as an increase in ATP utilization leading to higher P_i production and/or Ca^{2+} transport. We are describing here a series of experiments of demonstrating calmodulin stimulation of ATP $\rightleftharpoons$ P_i exchange as well as ATP hydrolysis. This indicates that the effect of calmodulin is expressed kinetically by an increased turnover of the enzyme in both forward and reverse directions.

Materials and Methods

Ca^{2+}-dependent ATPase was derived from membrane vesicles that were obtained by homogenization and differential centrifugation of pooled rabbit hearts according to the procedure of Van Altstyne et al. (1980). The final pellets of membrane vesicles were resuspended in 10 mM tris(hydroxymethyl)aminomethane (Tris) (pH 7.4) and 100 mM KCl, frozen in liquid nitrogen, and stored at −80 °C. For preparation of Ca^{2+}-ATPase, several samples of membrane vesicles were thawed, pooled, and solubilized in Triton X-100 and subjected to a preparation method similar to that described by Trifaro and Warner. To this effect, 80–100 mg of sarcolemmal vesicles (8 mg/mL) was dissolved in 5 mL of a solution to yield the following final concentrations: 0.2% Triton X-100, 0.5 M sucrose, 1 mM $MgCl_2$, and 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes), pH 7.0. The mixture was incubated at 4 °C for 1 h with mild continuous stirring. After this incubation, the mixture was diluted (1:1) with 10 mM Hepes and centrifuged at 40000*g* for 60 min. The supernatant was mixed with a solution of 0.2 mg/mL asolectin (final concentration) previously dispersed and sonicated in 50 mM KCl and 20 mM Hepes (pH 7.0) in a LKB Soniprep at maximum power. This solution, containing approximately 30–40 mg of protein, was eluted through a DEAE-Sephadex A-25 (40–120 μm) or a DEAE-cellulose column. For this purpose, 5 g of DEAE-Sephadex A-25 was swollen for 48 h at 25 °C and equilibrated in a buffer containing 5 mM Hepes, pH 6.5. The gel was packed in a 30-cm LKB glass column and equilibrated by perfusion through the column with 200–250 mL of 5 mM Hepes, pH 6.5, 0.1% Triton X-100, and 0.2 mg/mL of dispersed asolectin (buffer A). A total of 30–40 mg of solubilized protein was then applied on top of the column and passed through the column at 4 °C at a constant flow rate (15 mL/h). The eluted protein was monitored at 280 nm by means of a LKB Uvicord II system. Fractions of 5 mL were collected and assayed for protein content and ATPase activity. Following elution of the first peak, a stepwise ionic strength gradient was utilized (0.2, 0.4, 0.6, and 0.8 M NaCl in starting buffer) for further protein elution.

The peak that was found to have the highest ATPase activity was concentrated 2-fold by using an Amicon microconcentration unit and applied on top of an ATP affinity column. For this second chromatographic procedure, activated Sepharose 4B was coupled to adipic acid dihydrazide and peroxidized ATP following the method of Lamed et al. (1973). Five milliliters of Sepharose–adipic acid–ATP was then set up in a 15-cm LKB column and equilibrated with 50 mL of buffer A. Ten milliliters of concentrated protein containing 1.2 mg/mL was passed through the column at a flow rate of 4–5 mL/h. When no further protein could be eluted as determined by the use of an Uvicord II reading at 280 nm, elution of the bound protein was achieved with a pulse of 2 mM tetrasodium pyrophosphate prepared in buffer A. During this step, the flow rate was increased to 15–20 mL/h. This last fraction was concentrated by using an Amicon microconcentration unit and used for studies of enzyme activity.

The yield of membrane vesicles was approximately 0.1 g of protein per 100 g of wet tissue. The yield of purified

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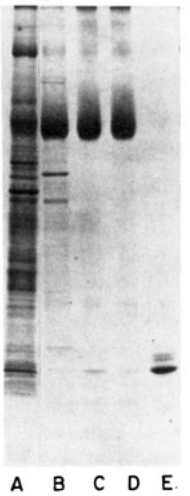

FIGURE 1: Electrophoretic analysis of various preparations: (A) cardiac membrane vesicles; (B) skeletal muscle SR vesicles; (C) ATPase purified from cardiac membrane vesicles; (D) ATPase purified from skeletal muscle SR vesicles; (E) calmodulin standard. Approximately 20 μg of protein dissolved in 1% SDS was placed in each gel, which was run according to Laemmli (1970).

ATPase from membrane vesicles was approximately 15%. Parallel experiments were conducted with Ca^{2+}-ATPase purified from rabbit skeletal muscle SR, according to the procedure of Mac Lennan (1970).

ATP hydrolysis was measured as release of $[^{32}P]P_i$ from [γ-^{32}P]ATP, and ATP synthesis was monitored as ATP $\rightleftharpoons$ P_i exchange by determining the incorporation of $[^{32}P]P_i$ into ATP in the presence of ADP. $[^{32}P]P_i$ was extracted from the reaction mixture into an organic phase as previously described (Carvalho et al., 1976), and samples of organic and aqueous phases were withdrawn for determination of $[^{32}P]P_i$ and [γ-^{32}P]ATP by scintillation spectrometry. Electrophoretic analysis of protein samples dissolved in 1% sodium dodecyl sulfate (SDS) was conducted on acrylmaide gels by the Laemmli (1970) method.

Results and Discussion

Electrophoretic Pattern. The electrophoretic protein pattern of the cardiac membranes used for preparation of Ca^{2+}-ATPase is much more complex than that of skeletal SR vesicles (Figure 1A,B). This is due to the presence of a large percentage of SL membranes in the cardiac preparation (Van Alstyme, 1980). Yet, the ATPase purified from these membranes exhibits the same electrophoretic mobility as that of skeletal SR ATPase (Figure 1C,D). The electrophoretic gels also show that both the enzymes obtained from cardiac and skeletal muscle are fairly pure with a prominent component of approximately M_r 115000. It is noteworthy that the cardiac enzyme (Figure 1C) retains a faint band migrating with the same mobility as that of calmodulin (Figure 1C,E).

Hydrolytic Activity. The cardiac ATPase sustains a low level of enzymatic activity in the absence of Ca^{2+} whereby ATP is hydrolyzed at a rate of 0.1–0.3 μmol of P_i (mg of protein)$^{-1}$ min^{-1}. The enzyme is activated by Ca^{2+} in a concentration range between 2×10^{-8} and 2×10^{-7} M (Figure 2), yielding a maximal velocity of 13 μmol of P_i (mg of protein)$^{-1}$ min^{-1}. The Ca^{2+} activation isotherm has a cooperative appearance, with a Hill coefficient approximating 2.

In the presence of Ca^{2+}, the cardiac ATPase is further activated by calmodulin, with a maximal (3-fold) activation observed in the presence of 3×10^{-5} M Ca^{2+}. No calmodulin effect is observed in the absence of Ca^{2+}. On the other hand, it is readily apparent that the Ca^{2+} concentration dependence

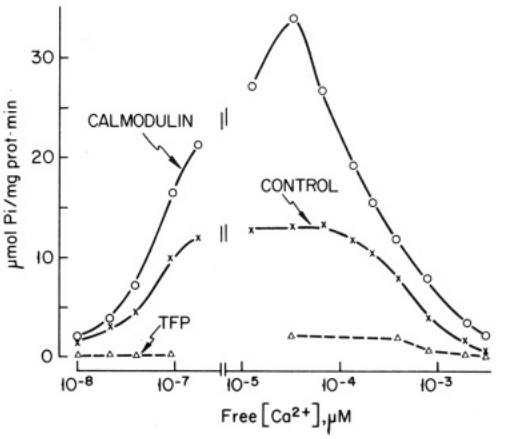

FIGURE 2: Activation of ATP hydrolysis by Ca^{2+} and calmodulin. The assay medium contained 50 mM Tris–maleate buffer (pH 7.4), 10 mM $MgCl_2$, 6 mM [γ-^{32}P]ATP, 0.2 mM ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid (EGTA), and different $CaCl_2$ concentrations to give the free calcium concentrations shown in the figure. This was calculated as described by Fabiato & Fabiato (1979). The reaction was started by the addition of cardiac ATPase (7.5 μg of protein/mL) and quenched after 4-min incubation at 35 °C by the addition of 3 volumes of a 10% (w/v) ice-cold trichloroacetic acid (TCA) solution. (×) Control without other additions; (○) plus 1 μg of calmodulin/mL; (△) plus fluoroperazine, to a final concentration in the medium of 30 μM.

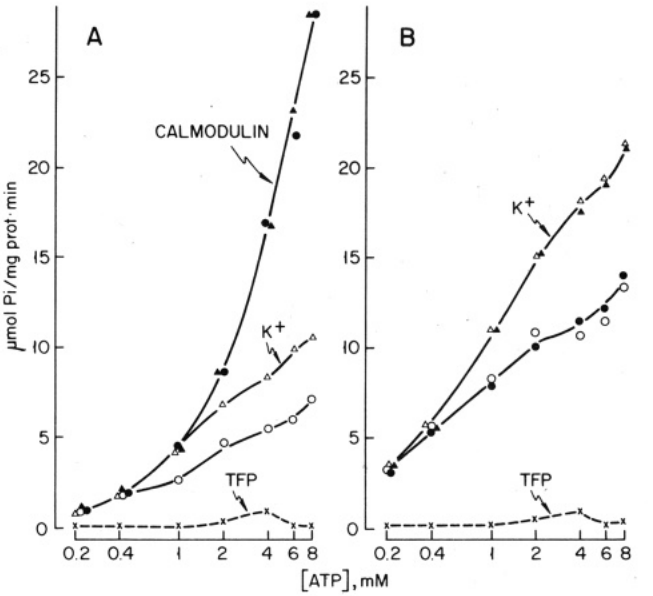

FIGURE 3: ATP dependence. The assay medium contained 50 mM Tris–maleate buffer (pH 7.4), 10 mM $MgCl_2$, 0.1 mM $CaCl_2$, [γ-^{32}P]ATP as in the figure, and either cardiac ATPase (A) or skeletal muscle ATPase (B) at 7.5 μg/mL. The reaction was started by the addition of ATPase and arrested after 8 min at 35 °C by the addition of 3 volumes of ice-cold TCA solution. (○) Control without other additions; (△) plus 100 mM KCl; (●) plus 1 μg of calmodulin/mL; (▲) plus 100 mM KCl and 1 μg of calmodulin/mL; (×) plus 1 μg of calmodulin/mL and 30 μM trifluoroperazine.

of the calmodulin effect is distinct from the direct ATPase activation by Ca^{2+} (Figure 2).

The cardiac ATPase is inhibited by Ca^{2+} at concentrations higher than 10^{-4} M, both in the absence and in the presence of calmodulin. Trifluoroperazine, a phenothiazine drug often used as a calmodulin antagonist, in our experiments strongly inhibited both cardiac and skeletal muscle ATPases, independent of the presence of calmodulin (Figures 2 and 3).

It is shown in Figure 3A that the cardiac ATPase requires substrate concentrations in the 10^{-4}–10^{-3} M range and is stimulated by K^+. Calmodulin activation occurs only in the

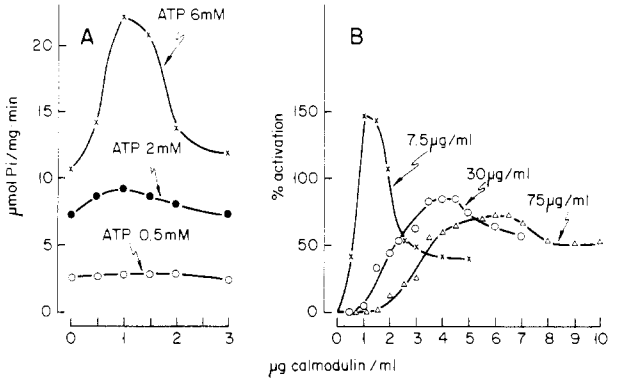

FIGURE 4: Dependence of hydrolytic activity on ATPase to calmodulin ratio. (A) The assay medium contained 50 mM Tris–maleate buffer (pH 7.4), 100 mM KCl, 10 mM $MgCl_2$, 0.1 mM $CaCl_2$, 7.5 μg of cardiac ATPase protein/mL, and (×) 6, (●) 2, or (○) 0.5 mM [γ-^{32}P]ATP. The reaction was performed at 35 °C. (B) The assay medium was the same as in (A) except that the [γ-^{32}P]ATP concentration was 6 mM and the cardiac ATPase concentration was (×) 7.5, (○) 30, and (△) 75 μg of protein/mL.

presence of high (2–8 mM) concentrations of ATP and is independent of K^+ (Figures 3 and 4). It is noteworthy that the skeletal muscle SR ATPase is not affected at all by calmodulin, even though displaying Ca^{2+}, ATP, and K^+ dependences that are quite similar to those of cardiac SR (Figure 3B).

In a study of the protein concentration dependence of the calmodulin effect, we found that highest activation occurs when the calmodulin ATPase molar ratio is approximately 1 (Figure 4) and in the presence of high ATP concentrations. In fact, in the presence of 6 mM ATP, the highest activity [23 μmol of P_i (mg of protein)$^{-1}$ min^{-1}] is obtained when cardiac ATPase and calmodulin are added to the medium at 7.5 and 1.5 μg/mL concentrations, respectively. When one considers the molecular weight of the two proteins, these concentration yield a molar ratio approximating 1. Different ATPase to calmodulin ratios, as well as more concentrated ATPase concentrations even at an optimal ATPase to calmodulin ratio, yield lower activation coefficients (Figure 4B).

Reversal of Catalytic Cycle. As previously established for SR ATPase (de Meis & Vianna, 1979), inhibition of the Ca^{2+}-dependent ATPase by high (>10^{-4} M) Ca^{2+} concentrations may be attributed to calcium occupancy of binding sites in a low-affinity state. Since Ca^{2+} dissociation from these sites is an obligatory step in the reaction cycle of ATPases that are coupled to Ca^{2+} transport, a Ca^{2+} concentration higher than the dissociation constant of the low-affinity sites prevents progression of the enzyme cycle in the forward direction. In the same conditions, the activity of the cycle in the reverse direction is increased, as demonstrated by the occurrence of a rapid ATP ⇌ P_i exchange (Figure 5). In this respect, cardiac and skeletal muscle ATPases display an identical behavior, with the exception of calmodulin activation. It is shown in Figure 5 that calmodulin activates the ATP ⇌ P_i exchange of cardiac ATPase, while it is totally ineffective on the skeletal muscle ATPase. The rate of ATP ⇌ P_i exchange is highest at 3 mM Ca^{2+} concentration (Figure 5). This Ca^{2+} concentration dependence of ATP ⇌ P_i exchange remains the same (10^{-4}–10^{-3} M) in the absence and in the presence of calmodulin (Figure 5). This in contrast with the different Ca^{2+} concentration dependences of hydrolytic activity in the absence and in the presence of calmodulin (Figure 2) and suggests that the affinity of the calmodulin calcium sites is lower than the ATPase activating (high-affinity state) sites and higher than the ATPase inhibitory (low-affinity state) sites.

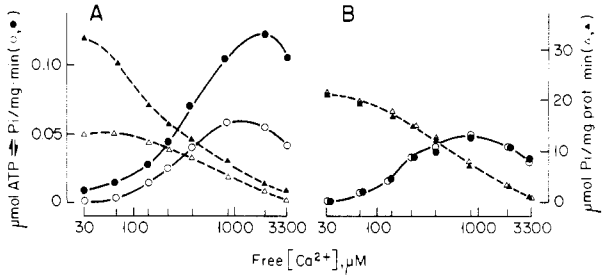

FIGURE 5: Activation of ATP ⇌ P_i exchange by calmodulin. The assay medium contained 50 mM Tris–maleate buffer, 10 mM $MgCl_2$, 8 mM ATP, 0.2 mM ADP, 4 mM P_i, and 0.1, 0.2, 0.4, 0.6, 1, 2, or 4 mM $CaCl_2$. The free Ca^{2+} in the medium was 0.032, 0.066, 0.137, 0.212, 0.376, 0.847, 2.0, and 3.3 mM. For the ATPase activity, [γ-^{32}P]ATP and nonradioactive P_i were used (△, ▲). For ATP ⇌ P_i exchange, nonradioactive ATP and [^{32}P]P_i were used (○, ●). In (A) cardiac ATPase was used, and in (B) skeletal muscle ATPase was used. In both cases, the enzyme concentration was 7.5 μg/mL. The incubation time at 35 °C was 10 min. (△, ○) Control without calmodulin; (▲, ●) with calmodulin, 1 μg/mL.

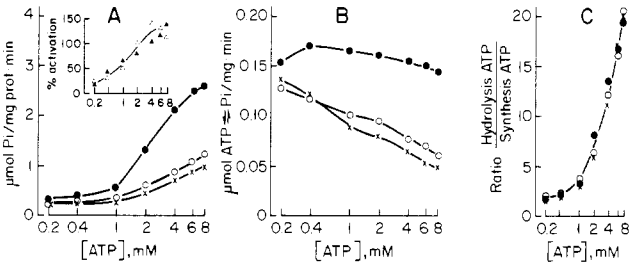

FIGURE 6: ATP dependence of ATP ⇌ P_i exchange. The assay medium contained 50 mM Tris–maleate buffer (pH 7.4), 10 mM $MgCl_2$, 4 mM $CaCl_2$, 0.2 mM ADP, 4 mM P_i, and the ATP concentration shown in the figure. The enzyme concentration was 7.5 μg/mL. (A) ATP hydrolysis; [γ-^{32}P]ATP and nonradioactive P_i were used. (B) ATP synthesis; nonradioactive ATP and [^{32}P]P_i were used. (C) Ratio between velocity of ATP hydrolysis and of ATP synthesis calculated from the values of (A) and (B). (×) Skeletal muscle ATPase; (○) cardiac ATPase; (●) cardiac ATPase plus 1 μg/mL calmodulin. Inset in (A) shows activation by calmodulin of ATP hydrolysis (△) and ATP synthesis (▲) obtained with the use of cardiac ATPase.

In the presence of constant P_i, ADP, and Ca^{2+} concentrations, an increase of the ATP concentration favors progression of the ATPase cycle in the forward direction (hydrolytic activity), while ATP ⇌ P_i exchange is reduced. The same effect is observed under conditions of calmodulin activation (Figure 6). In fact, the ratio between ATP hydrolysis and ATP synthesis can be varied with an identical pattern independent of whether cardiac (in the presence or in the absence of calmodulin activation) or skeletal muscle ATPase is used (Figure 6C).

In analogy to ATP hydrolysis, the ATP ⇌ P_i exchange catalyzed by cardiac ATPase is activated optimally when ATPase and calmodulin are present at a molar ratio equal to 1 (Figure 7). The exchange activity of skeletal muscle ATPase is not activated by calmodulin at any concentration ratio. We also found that trifluoroperazine inhibits the ATP ⇌ P_i exchange activity (Figure 8) of both cardiac and skeletal muscle ATPases, in the presence or in the absence of calmodulin.

Conclusions

A novel finding obtained during our experimentation is that calmodulin activates both forward and reverse directions of the enzyme cycle catalyzed by a Ca^{2+}-dependent ATPase. Therefore, the effect of calmodulin consists of a general in-

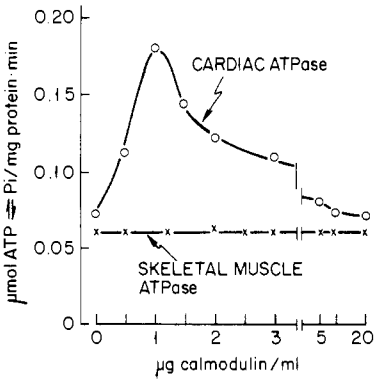

FIGURE 7: Dependence of ATP $\rightleftharpoons$ P_i exchange on ATPase to calmodulin ratio. The assay medium contained 100 mM Tris–maleate buffer (pH 7.4), 100 mM KCl, 10 mM $MgCl_2$, 4 mM [^{32}P]P_i, 6 mM ATP, 0.2 mM ADP, 4 mM $CaCl_2$, and the calmodulin concentrations shown in the figure. (O) Cardiac ATPase, 7.5 μg of protein/mL; (×) skeletal muscle ATPase, 7.5 μg of protein/mL.

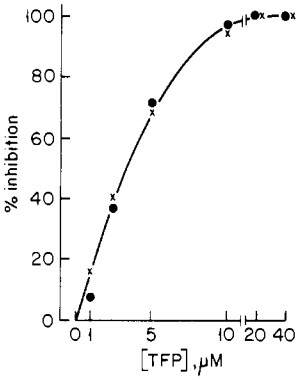

FIGURE 8: Inhibition of ATP $\rightleftharpoons$ P_i exchange by trifluoroperazine. The assay medium contained 50 mM Tris–maleate buffer (pH 7.4), 10 mM $MgCl_2$, 6 mM ATP, 0.2 mM ADP, 4 mM [^{32}P]P_i, and 100 mM KCl. The enzyme concentration was 7.5 μg or protein/mL. (●) Cardiac ATPase; (×) skeletal muscle ATPase.

crease in enzyme turnover. Optimization of the calmodulin effect requires approximately equivalent molar concentrations of calmodulin and ATPase, suggesting that interaction of the two proteins plays a role in the activation mechanism. Other requirements for the calmodulin effect include high concentrations of Ca^{2+} (10^{-5} M) and ATP ($\sim 10^{-3}$ M).

We also found that calmodulin activation occurs with a preparation of purified ATPase in a nonmembranous, detergent-solubilized form. Therefore, calmodulin activation cannot be attributed to alteration of membrane permeability but rather to a direct effect on the enzyme.

Another interesting finding is related to the specificity of the cardiac and skeletal muscle ATPase preparations, inasmuch as only the former is sensitive to calmodulin, even though the electrophoretic behavior and the Ca^{2+}, ATP, and K^+ dependences of the two preparations appear identical. Future work must be directed to detailed studies of the purification methods to clarify whether this difference is due to perturbations and cofactors lost during the preparation or is due to the intrinsic character of the two proteins.

Finally, we found that trifluoroperazine, an agent frequently used to prevent calmodulin activation, inhibits hydrolytic and ATP $\rightleftharpoons$ P_i exchange activities of both the cardiac and the skeletal muscle ATPase preparations, independent of calmodulin activation. This is consistent with a direct perturbation produced by trifluoroperazine on membranous ATPases (Ho et al., 1983).

Registry No. ATPase, 9000-83-3; ATP, 56-65-5.

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