Cyclopiazonic Acid Effect on Ca^{2+}-Dependent Conformational States of the Sarcoplasmic Reticulum ATPase. Implication for the Enzyme Turnover†

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ABSTRACT: The affinity of sarcoplasmic reticulum Ca^{2+}-ATPase for cyclopiazonic acid is dependent on the conformational state of the enzyme. It is high in the absence of Ca^{2+} but low in its presence. When Ca^{2+} was added to the enzyme in the presence of equimolar toxin, the apparent rate constant for Ca^{2+} binding was 0.6 min^{-1} when measured at 37 °C. The apparent equilibrium constant for Ca^{2+} dissociation increased from 0.2 to 0.6 μM at neutral pH, and from 5.9 to 37 μM at pH 6.0. The apparent equilibrium constant for Ca^{2+} dissociation increased progressively as the amount of toxin increased above an equimolar level. Cyclopiazonic acid decreased phosphorylation by ATP and Ca^{2+} when the enzyme in the absence of Ca^{2+} was incubated in the presence of toxin, although no effect was observed after a preliminary incubation with Ca^{2+} at 37 °C. Cyclopiazonic acid incubated with the enzyme in the presence of Ca^{2+} could be eliminated with a Sephadex column. However, the toxin could not be removed when it was incubated with the enzyme in the absence of Ca^{2+}. In the latter case, cyclopiazonic acid was eliminated when the enzyme in the presence of toxin was incubated with Ca^{2+} at 37 °C. Under turnover conditions and in the presence of 10 μM ATP, the toxin−enzyme interaction can be characterized by an apparent K_d of 7 nM. With an ATP concentration of 1 mM, the enzyme was inhibited completely at a toxin/enzyme molar ratio of ∼10. Furthermore, enzyme activity was observed to recover at a toxin/enzyme molar ratio of 1 when the Ca^{2+} concentration was raised, which is consistent with the competitive character of cyclopiazonic acid and Ca^{2+}. It is concluded that ATP and Ca^{2+} can protect against cyclopiazonic acid inhibition.

Cyclopiazonic acid (CPA),[1] a secondary metabolite produced by certain fungi of the *Penicillium* and *Aspergillus* genera (*1*), represents a toxicological problem for humans and animals since it may be found as a contaminant in processed food, grain, and poultry (*2*−*4*). The first clinical symptoms related to the ingestion of CPA were associated with several neurological and muscular disorders (*5, 6*). Subsequent biochemical characterization revealed that CPA inhibits the Ca^{2+}-ATPase activity of isolated SR vesicles (*7*) and that this inhibition occurs at equimolar levels of toxin, there being no significant effect on other cation transport ATPases (*8*).

With regard to the inhibition mechanism involved, it has been found that CPA at a toxin/enzyme molar ratio of 12.5 inhibits Ca^{2+} binding and ATP-dependent phosphorylation of the enzyme (*9*). Likewise, the use of FITC as fluorescence probe indicated that CPA stabilizes the E_2 conformation of the enzyme, preventing the $E_2 \rightarrow E_1Ca_2$ transition (*8, 10*). We have recently shown (*11*) that equimolar amounts of CPA lower the binding affinity of ATP, although CPA and ATP do not compete for the same binding site, and that the enzyme is not protected by Ca^{2+}, when CPA is incubated with E_1Ca_2, if enzyme turnover is induced. There are also some contradictory data in the literature regarding the putative reversibility of CPA, since it has been reported as being irreversible (*8*) or partially reversible (*9*). A perusal of the experimental data concerning the effect of CPA on the Ca^{2+} sites of the enzyme reveals that the information available is sketchy. Many of the experiments were carried out under different assay conditions, precluding comprehensive data analysis and an assessment of their functional relevance. We therefore thought it was advisable to perform a more detailed study under well-controlled assay conditions in order to improve our knowledge of the CPA action mechanism.

In this study we have examined some critical aspects related to the CPA−enzyme interaction in connection with the Ca^{2+}-dependent conformational states of the enzyme (E_1-

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[1] Abbreviations: CPA, cyclopiazonic acid; SR, sarcoplasmic reticulum; ER, endoplasmic reticulum; Mops, 4-morpholinepropanesulfonic acid; Mes, 4-morpholineethanesulfonic acid; EGTA, [ethylenebis-(oxyethylenenitrilo)]tetraacetic acid; A23187, calcimycin; FITC, fluorescein 5′-isothiocyanate; pCa, negative logarithm of the molar free Ca^{2+} concentration; EP, phosphorylated intermediate of the enzyme; E_1, enzyme conformation with high affinity for Ca^{2+}; E_2, enzyme conformation with low affinity for Ca^{2+}.

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Ca_2 and E_2) and the enzyme turnover. We studied Ca^{2+} binding in the absence of ATP, both by radioactive tracer $^{45}Ca^{2+}$ and by the fluorescence signal of FITC bound to the protein, to obtain information on the enzyme affinity for Ca^{2+} and the time course of the $E_2 \rightarrow E_1Ca_2$ transition. Measurements of EP accumulation after the simultaneous addition of [γ-^{32}P]ATP and Ca^{2+} were useful when evaluating the enzyme capacity to be phosphorylated by nucleotide, while the Ca^{2+}-ATPase activity data after gel filtration of the samples provided information on the nature of the CPA binding. A kinetic titration was also applied to evaluate the K_d for the CPA−enzyme complex. Ca^{2+}-ATPase activity experiments performed in the presence of 1 mM ATP were useful for understanding the role of saturating ATP and Ca^{2+} concentrations during enzyme turnover in the presence of CPA. A comparison of the CPA inhibition data with those obtained in the presence of thapsigargin (*12*−*14*) or 2,5-di-*tert*-butyl-1,4-benzohydroquinone (*15*) reveals clear similarities in the action mechanism of these structurally unrelated compounds that act as highly specific inhibitors of SR/ER Ca^{2+}-ATPases.

MATERIALS AND METHODS

SR Vesicles. Fast-twitch skeletal muscle microsomes were obtained from the hind limbs of adult New Zealand rabbits according to the Eletr and Inesi method (*16*). Isolated membranes were finally resuspended in 10 mM Mops, pH 7.0, and 30% sucrose at a protein concentration of 10−15 mg/mL, and stored at −80 °C until use. Typically, the yield was approximately 1 mg of SR protein per gram of homogenized muscle.

pCa Values. Free Ca^{2+} concentrations expressed as pCa were established by including appropriate concentrations of $CaCl_2$ and EGTA according to a computer program (*17*). The absolute stability constant for the EGTA−Ca^{2+} complex was taken from Schwartzenbach et al. (*18*), and the p*K* values for the EGTA protonation were those reported by Blinks et al. (*19*).

Radiometric Measurements of Ca^{2+} Binding. The equilibrium and kinetics of Ca^{2+} binding in the absence of ATP were measured by using a double radioactive labeling procedure (*20*). SR vesicles were initially pre-equilibrated with the proper medium as described below. Aliquots of the protein suspension containing 0.5 mL, equivalent to 0.1 mg of SR protein, were used for each experimental point. Microsomal vesicles were separated from the incubation media by manual filtration through 0.45 μm nitrocellulose filters (Millipore, HAWP). The filters were not subjected to any additional washing step after sample filtration. Specific conditions for this assay were as follows.

(i) Effect of CPA on E_1Ca_2. The enzyme suspension (0.2 mg/mL SR protein) containing 20 mM Mops, pH 7.0, 80 mM KCl, 3 mM $MgCl_2$, 1 mM [^{3}H]glucose, 0.1 mM EGTA, and 0.106 mM [^{45}Ca]$CaCl_2$ (pCa 5) was preincubated for 10 min at 22 °C with different CPA concentrations. Thereafter, protein aliquots were subjected to vacuum filtration, and the filters were counted by the liquid scintillation technique.

(ii) Effect of Ca^{2+} on E_2CPA. The initial incubation medium containing 20 mM Mops, pH 7.0, 80 mM KCl, 3 mM $MgCl_2$, 1 mM [^{3}H]glucose, 0.1 mM EGTA, and 0.2 mg/mL SR protein was supplemented with different CPA concentrations, and the incubation was maintained at 22 °C for 10 min. Then, 0.106 mM [^{45}Ca]$CaCl_2$ was added to give a final pCa of 5, and incubation was prolonged at 0, 22, or 37 °C for an additional 10 min. Aliquots of the protein suspension were filtered, and the radioactivity retained on the filters was counted. In some experiments, the time course of Ca^{2+} binding in the presence of equimolar CPA (0.2 mg/mL SR protein and 0.8 μM CPA) was also evaluated at 0, 22, or 37 °C by filtration of samples at different times after the addition of Ca^{2+}.

FITC Labeling and Measurements. Enzyme derivatization by FITC was performed for 20 min in the dark at 25 °C, essentially as described previously (*21*). The reaction medium consisted of 50 mM Tris-HCl, pH 8.8, 80 mM KCl, 5 mM $MgCl_2$, 0.2 M sucrose, 2 mg/mL SR vesicles, and 30 μM FITC. Unreacted label was removed by centrifugation for 1 min in a benchtop centrifuge through a 2 mL Sephadex G-50 column (*22*) pre-equilibrated in a medium of 20 mM Mops, pH 7.0, 80 mM KCl, and 5 mM $MgCl_2$. Before the sample was added, the column was centrifuged for 1 min to remove excess buffer. The labeling stoichiometry was checked by absorbance measurements in the presence of 1% sodium dodecyl sulfate and 0.1 N NaOH, ϵ_{495} being 80 000 M^{-1} cm^{-1} (*23*). FITC−ATPase fluorescence was measured at 37 °C under continuous stirring in a high-sensitivity optical system from Bio-Logic Co. (Claix, France). Samples were irradiated at 490 nm with a 150-W mercury−xenon arc lamp, and the emitted light was passed through a cutoff filter with 50% transmission at 515 nm (OG-515 from Ealing Electro-optics, Holliston, MA). The quenching of fluorescence after the addition of Ca^{2+} was expressed in percentage as $\Delta F/F$.

(i) Kinetics of the $E_2 \rightarrow E_1Ca_2$ Transition. FITC-labeled SR vesicles (0.1 mg/mL) were first equilibrated for at least 10 min at 37 °C in the presence of 20 mM Mops, pH 7.0, 80 mM KCl, 3 mM $MgCl_2$, 0.1 mM EGTA, and 0.4 μM CPA (CPA/enzyme molar ratio = 1). The fluorescence signal was monitored as a function of time after addition of a certain Ca^{2+} concentration (59.2 μM, 70.2 μM, 105.7 μM, 0.2 mM, or 1.1 mM) to give final pCa values of 6.2, 6.0, 5.0, 4.0, or 3.0, respectively.

(ii) Equilibrium Levels of E_1Ca_2 vs pCa. The incubation medium consisted of 20 mM buffer [Mops (pH 7.0) or Mes (pH 6.0)], 80 mM KCl, 3 mM $MgCl_2$, 0.1 mM EGTA, 0.1 mg/mL FITC-labeled SR, and the indicated concentration of CPA. The decrease in fluorescence was continuously recorded after successive additions of a 100 mM $CaCl_2$ solution.

Phosphorylation by Adding ATP and Ca^{2+}. Levels of EP were measured at 22 °C by manual mixing under vortexing of a Ca^{2+}-free enzyme suspension (0.5 mL) containing 20 mM Mops, pH 7.0, 80 mM KCl, 3 mM $MgCl_2$, 0.1 mM EGTA, 0.2 mg/mL SR protein, 15 μM A23187, and up to 0.8 μM CPA when indicated, with 0.02 mL of phosphorylating medium containing 20 mM Mops, pH 7.0, 80 mM KCl, 3 mM $MgCl_2$, 0.1 mM EGTA, 3.12 mM $CaCl_2$, and 1.3 mM [γ-^{32}P]ATP. Concentrations after mixing were 0.12 mM Ca^{2+} (final pCa 4.66) and 50 μM radioactive ATP. The phosphorylation reaction was stopped after 1 s by adding 1 volume (0.5 mL) of ice-cold 0.25 M perchloric acid and 2 mM sodium phosphate as a carrier. Quenched samples were

filtered under vacuum using HAWP Millipore filters (0.45 μm pore size), and the denatured protein was rinsed five times with 5 mL each of ice-cold 0.125 M perchloric acid and 1 mM sodium phosphate. Filters were solubilized in scintillation cocktail before radioactive counting. Alternatively, the enzyme suspension (E_2 form) containing a certain CPA concentration was subjected to incubation for 10 min at 37 °C in the presence of 0.12 mM $CaCl_2$. Thereafter, the phosphorylation reaction was performed at 22 °C by adding 0.02 mL of radioactive medium containing 20 mM Mops, pH 7.0, 80 mM KCl, 3 mM $MgCl_2$, 0.1 mM EGTA, 0.12 mM $CaCl_2$, and 1.3 mM [γ-^{32}P]ATP.

Ca^{2+}-ATPase Activity. Initial rates of ATP hydrolysis were measured at 25 °C in the time scale of minutes by following the liberation of inorganic phosphate (*24*). The enzyme activity was constant over the period of measurement. To evaluate enzyme activity after chromatographic treatment, the reaction medium contained 20 mM Mops, pH 7.0, 80 mM KCl, 3 mM $MgCl_2$, 0.1 mM EGTA, 0.12 mM $CaCl_2$, 0.02 mg/mL SR protein, 1.5 μM A23187, 2 mM phosphoenolpyruvate, 6 units/mL pyruvate kinase, and 50 μM ATP. The effect of CPA and Ca^{2+} on enzyme turnover was studied in the presence of 0.02 mg/mL SR protein and 1 mM ATP. A complete description of the reaction media is given in the legends of Figures 8 and 9. In all cases, the Ca^{2+}-independent activity measured in the presence of excess EGTA (1 mM) was subtracted.

CPA−Enzyme Interaction. (i) Interaction of CPA with E_1Ca_2. SR vesicles (0.2 mg/mL) in a medium containing 20 mM Mops, pH 7.0, 80 mM KCl, 3 mM $MgCl_2$, 0.1 mM EGTA, and 0.12 mM $CaCl_2$ were incubated for 10 min at 22 °C in the presence of up to 0.8 μM CPA. Microsomal vesicles (0.8 mL) were centrifuged for 1 min through Sephadex G-50 columns (*22*), pre-equilibrated with 20 mM Mops, pH 7.0, 80 mM KCl, 3 mM $MgCl_2$, 0.1 mM EGTA, and 0.12 mM $CaCl_2$. Samples collected from the columns were diluted to 0.02 mg of protein/mL and used to evaluate the Ca^{2+}-ATPase activity.

(ii) Interaction of CPA with E_2. The same experimental protocol was applied but in the absence of Ca^{2+}. Enzyme incubation was performed for 10 min at 22 °C in a medium containing 20 mM Mops, pH 7.0, 80 mM KCl, 3 mM $MgCl_2$, 0.1 mM EGTA, 0.2 mg/mL SR protein, and a certain CPA concentration. The chromatography column was pre-equilibrated in a Ca^{2+}-free medium containing 20 mM Mops, pH 7.0, 80 mM KCl, 3 mM $MgCl_2$, and 0.1 mM EGTA.

(iii) Interaction of CPA with E_2 and Subsequent Ca^{2+} Addition. The initial incubation was performed for 10 min at 22 °C in the presence of 20 mM Mops, pH 7.0, 80 mM KCl, 3 mM $MgCl_2$, 0.1 mM EGTA, 0.2 mg/mL SR protein, and a certain CPA concentration. After addition of 0.12 mM $CaCl_2$, the incubation was prolonged for 10 min at 37 °C. Microsomes (0.8 mL) were then centrifuged through the Sephadex column in the presence of Ca^{2+} and processed as for the E_1Ca_2 samples.

Dissociation Constant for CPA. The rate of ATP hydrolysis as a function of CPA concentration was measured at five different enzyme concentrations. Experiments were performed at 25 °C in a medium containing 20 mM Mops, pH 7.0, 80 mM KCl, 3 mM $MgCl_2$, 0.1 mM EGTA, 0.12 mM $CaCl_2$, 4% A23187, 2 mM phosphoenolpyruvate, 5 units/mL pyruvate kinase, different CPA concentrations, and 10 μM ATP. The membrane protein concentration was varied between 0.003 and 0.03 mg/mL to yield a certain enzyme concentration in the range of 12−120 nM. For each enzyme concentration, the $K_{1/2}$ value, i.e., the CPA concentration giving half-maximal inhibition, was determined. The apparent dissociation constant was calculated using the following equation: $K_{1/2} = (K_d)^{1/n} + [Ca^{2+}\text{-ATPase}](n/2)$. For $n = 1$, K_d would be the intercept in a plot of $K_{1/2}$ vs [Ca^{2+}-ATPase].

Materials and Other Procedures. $^{45}CaCl_2$ and [^{3}H]glucose were purchased from DuPont NEN. They were used at a specific activity of ∼10 000 cpm/nmol. [γ-^{32}P]ATP was obtained from Amersham Corp. and used at ∼20 000 cpm/nmol. CPA from *Penicillium cyclopium* (catalog no. S-1530), FITC Isomer I (F-7250), and the liquid scintillation cocktail (S-4023) were products from Sigma Chemical Co. The Ca^{2+} ionophore A23187 was from Boehringer Mannheim. FITC was dissolved in dimethylformamide, and the Ca^{2+} ionophore was prepared as ethanolic solution. CPA concentrations given throughout the text correspond to amounts of CPA added that are different from free CPA concentrations in solution due to the high-affinity toxin−enzyme interaction. The microsomal protein concentration was estimated by the Lowry et al. procedure (*25*) using bovine serum albumin as a standard. The protein concentration of different SR preparations was slightly adjusted to obtain a CPA/enzyme molar ratio of 1:1 as defined in ref *11*. Initial species E_1Ca_2 or E_2CPA was formed by pre-equilibrating SR vesicles with Ca^{2+} or CPA in the absence of Ca^{2+}, respectively, for at least 10 min at 22 °C.

Data Presentation. The experimental values in this report correspond to an average of at least three independent measurements performed in duplicate and the use of more than one membrane preparation. Standard deviations of mean values (plus or minus) are given when indicated. The fluorescence traces are representative of at least five different measurements.

RESULTS

The sensitivity of SR Ca^{2+}-ATPase to CPA was initially studied by measuring the Ca^{2+} binding capacity of the enzyme at equilibrium. Addition of CPA at a toxin/enzyme molar ratio ≤1 to the enzyme in the presence of 10 μM Ca^{2+} (E_1Ca_2) did not modify maximal Ca^{2+} binding (∼7.5 nmol/mg of protein) when measured after 10 min incubation at 22 °C (Figure 1A, open circles). A CPA concentration of 0.8 μM added to 0.2 mg/mL SR protein corresponds to a toxin/enzyme molar ratio of 1, taking into consideration a CPA binding stoichiometry of 4 nmol/mg of SR protein (*11*). When CPA above an equimolar level was added to E_1Ca_2, Ca^{2+} binding to the enzyme was protected by increasing the Ca^{2+} concentration (data not shown). The effect of equimolar CPA on E_2 was also evaluated by the same procedure. SR vesicles in the absence of Ca^{2+} were first equilibrated for 10 min at 22 °C with CPA at a toxin/enzyme molar ratio of ≤1 and then exposed to saturating [^{45}Ca]Ca^{2+} for another 10 min at 22 °C. Under these conditions, the high-affinity Ca^{2+} binding did not reach maximal levels, the partial inhibition observed being dependent on the amount of CPA present (Figure 1A, solid squares). Further experiments indicated that the final level of Ca^{2+} binding was dependent

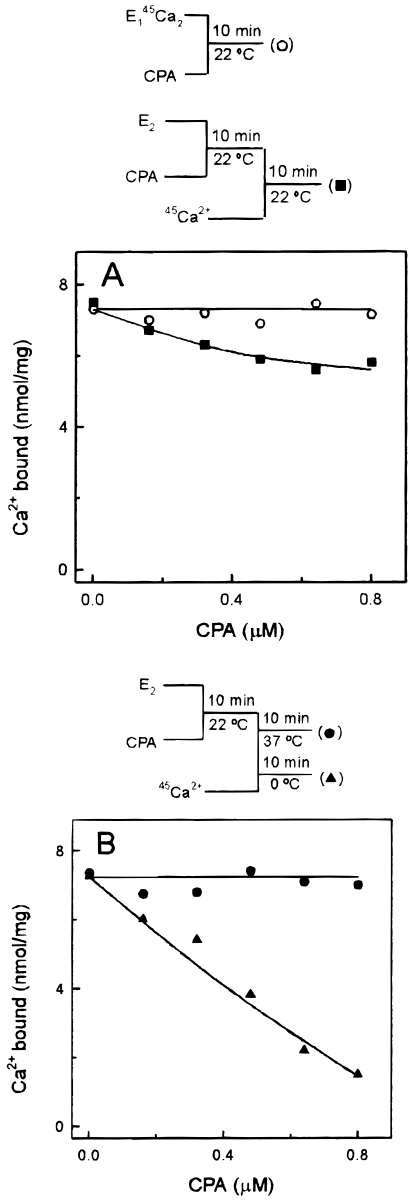


FIGURE 1: Equilibrium Ca^{2+} binding to the Ca^{2+}-ATPase protein as a function of the CPA concentration added. SR vesicles at 0.2 mg of protein/mL (0.8 μM ATPase) were suspended in a final incubation medium containing 20 mM Mops, pH 7.0, 80 mM KCl, 3 mM $MgCl_2$, 1 mM [^{3}H]glucose, 0.1 mM EGTA, 0.106 mM [^{45}Ca]$CaCl_2$ (final pCa 5), and a certain CPA concentration. Aliquots of 0.5 mL were filtered in each case to evaluate the radioactive tracers retained by the filters. The initial enzymatic form, the sequence of additions, and the incubation time and temperature are given in the corresponding diagrams (A and B).

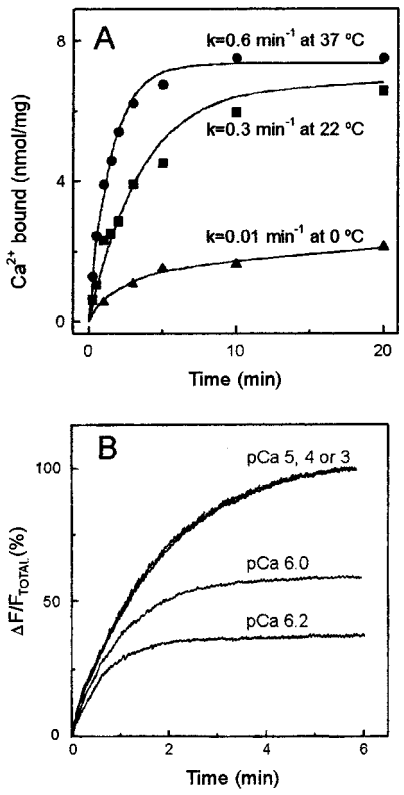


FIGURE 2: Effect of equimolar CPA on the kinetics of the $E_2 \rightarrow E_1Ca_2$ interconversion measured by radioactive tracer (A) or fluorescence quenching of FITC bound to the enzyme (B). The time course of Ca^{2+} binding in panel A was measured by adding 0.106 mM [^{45}Ca]Ca^{2+} to the incubation medium containing 20 mM Mops, pH 7.0, 80 mM KCl, 3 mM $MgCl_2$, 1 mM [^{3}H]glucose, 0.1 mM EGTA, 0.8 μM CPA, and 0.2 mg/mL SR protein (0.8 μM ATPase). Data points were collected at 0 °C (▲), 22 °C (■), or 37 °C (●). The initial incubation medium in panel B consisted of 20 mM Mops, pH 7.0, 80 mM KCl, 3 mM $MgCl_2$, 0.1 mM EGTA, 0.1 mg/mL FITC-labeled microsomes (0.4 μM ATPase), and 0.4 μM CPA. The fluorescence signal (490 nm/OG-515 cutoff filter) was monitored at 37 °C after addition of the free Ca^{2+} concentrations stated on the experimental traces.

on the incubation time and temperature in the presence of Ca^{2+}. Thus, the inhibition was almost complete when the 10 min incubation in the presence of Ca^{2+} was performed at 0 °C (Figure 1B, solid triangles). However, Ca^{2+} binding was maximal after 10 min incubation at 37 °C in the presence of Ca^{2+} (Figure 1B, solid circles). No effect on Ca^{2+} binding was noticed after further incubation at a different temperature.

The observed effects of Ca^{2+} when added to E_2 in the presence of CPA prompted us to study the kinetics of the $E_2 \rightarrow E_1Ca_2$ interconversion. The time course of Ca^{2+} binding measured by radioactive tracer was very slow (k_{app} = 0.3 min^{-1}) when the experiments were performed at 22 °C, and a saturating Ca^{2+} concentration was added to E_2 in the presence of equimolar CPA (Figure 2A, solid squares). However, the Ca^{2+} transition in the presence of equimolar CPA and saturating Ca^{2+} showed an apparent rate constant of 0.6 min^{-1} when measured at 37 °C (solid circles), but was only 0.01 min^{-1} when the experiments were carried out at 0 °C (solid triangles). The fluorescence intensity of FITC-labeled ATPase is an alternative procedure for studying Ca^{2+} binding. The fluorescence signal decreases when Ca^{2+} is added to saturate the high-affinity sites, and increases when the Ca^{2+} concentration is lowered by the addition of EGTA (*21*, *26*). Accordingly, SR vesicles were first derivatized with FITC and then incubated at neutral pH with an equimolar amount of CPA in the presence of EGTA, i.e., E_2CPA. The subsequent addition of Ca^{2+} to give a certain free concentration was accompanied by a specific time-dependent decrease in fluorescence. The family of curves presented in Figure 2B were obtained at 37 °C. As can be seen, the initial rate and amplitude increased as the Ca^{2+} concentration was raised, although the overall apparent rate constant decreased. The fluorescence change in the presence of equimolar CPA and saturating Ca^{2+}, when measured at 37 °C, showed an apparent rate constant of 0.6 min^{-1} (it was completed in ~6 min), confirming that such a fluores-

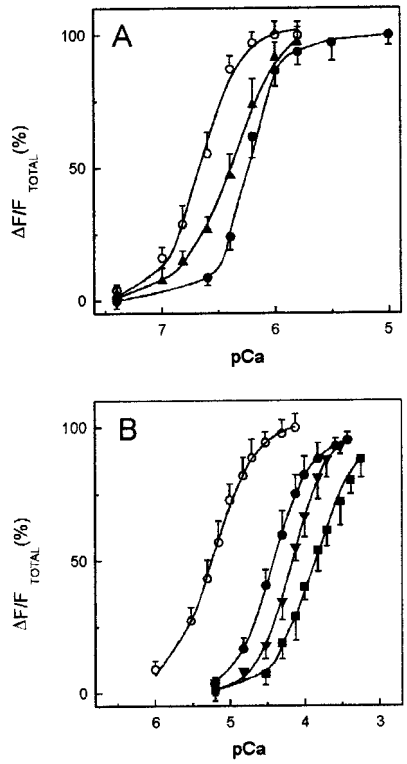


FIGURE 3: CPA effect on the titration of Ca^{2+} binding sites measured by FITC fluorescence quenching. FITC-labeled SR vesicles at 0.1 mg/mL (0.4 μM ATPase) were first equilibrated for at least 10 min at 37 °C in the presence of 20 mM Mops (pH 7.0) or Mes (pH 6.0), 80 mM KCl, 3 mM $MgCl_2$, 0.1 mM EGTA, and a certain CPA concentration. The fluorescence signal was continuously recorded before and after addition of $CaCl_2$ to yield different pCa values. Fluorescence transitions required several minutes to be completed. Experiments were carried out at pH 7.0 and a CPA/enzyme molar ratio of ≤ 1 (A) or at pH 6.0 and a CPA/enzyme molar ratio of ≥ 1 (B). CPA concentrations used were 0.2 μM (▲), 0.4 μM (●), 0.8 μM (▼), or 1.6 μM (■). Data points in the absence of CPA (○).

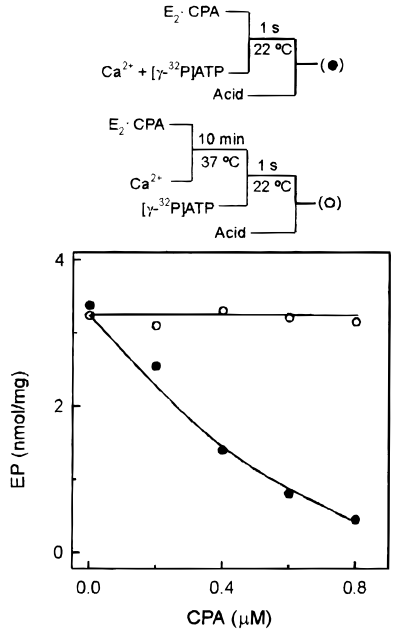


FIGURE 4: CPA effect on functional states of the enzyme as determined by ATP phosphorylation. Treatment of samples as outlined in flowcharts. SR vesicles at 0.2 mg of protein/mL (0.8 μM ATPase) were initially equilibrated for at least 10 min at 22 °C in a medium containing 20 mM Mops, pH 7.0, 80 mM KCl, 3 mM $MgCl_2$, 0.1 mM EGTA, 15 μM A23187, and a certain CPA concentration up to 0.8 μM. Maximal levels of EP were measured after mixing 0.5 mL of enzyme suspension with 0.02 mL of [γ-^{32}P]-ATP medium. Enzyme phosphorylation was maintained in the presence of 50 μM radioactive ATP and 20 μM free Ca^{2+} for 1 s at 22 °C (●). E_2CPA was incubated with Ca^{2+} for 10 min at 37 °C prior to phosphorylation when indicated (○). See Materials and Methods for further details.

cence transition was related to the Ca^{2+} binding process. It should be noted that the Ca^{2+}-dependent transition measured at 22−25 °C but in the absence of CPA takes place in less than 1 s (*27*−*29*).

The titration of high-affinity Ca^{2+} sites can be studied by sequential additions of Ca^{2+} to FITC-derivatized SR vesicles in the presence of EGTA (*30*). Our measurements were performed at 37 °C, and caution was taken to ensure that any fluorescence change was completed each time Ca^{2+} was added, since CPA decreases the rate of the $E_2 \rightarrow E_1Ca_2$ transition. The Ca^{2+} binding isotherm that can be measured in SR vesicles at pH 7.0 by the FITC fluorescence probe showed a saturable and positive cooperativity curve (Figure 3A, open circles), as previously reported (*30*). The apparent K_d in the presence of 3 mM Mg^{2+} that can be deduced from these data was 0.2 μM. Preincubation of FITC-treated SR vesicles in the presence of EGTA (E_2 form of the enzyme) with 0.2 μM CPA before the sequential addition of Ca^{2+} was accompanied by an increase in the half-maximal concentration for saturating the Ca^{2+} binding sites and a decrease in the cooperativity parameter (Figure 3A, solid triangles). It was expected that half of the enzyme molecules would be saturated initially by 0.2 μM CPA since the protein concentration in this assay was 0.1 mg/mL. Furthermore, the Ca^{2+} binding isotherm recovered the cooperativity of the control curve and exhibited an apparent K_d of 0.6 μM when the experiments were performed in the presence of 0.4 μM CPA (Figure 3A, solid circles). Under these conditions, all the enzyme molecules would be initially saturated by CPA.

It is well-known that the enzyme affinity for Ca^{2+} is pH dependent (*31*, *32*), and so Ca^{2+} titration curves were obtained at pH 6.0. The enzyme affinity for Ca^{2+} in the absence of CPA was lower at pH 6.0 than at pH 7.0, with an apparent K_d of 5.9 μM (Figure 3B, open circles). Moreover, the K_d of the enzyme for Ca^{2+}, as measured in the presence of 0.4 μM CPA, was 37 μM (Figure 3B, solid circles), which means that the toxin effect on Ca^{2+} binding affinity is higher as pH decreases. It is interesting to note that increasing the CPA concentration to 0.8 (solid inverted triangles) or 1.6 μM (solid squares), representing CPA/enzyme molar ratios of > 1, brought about a progressive increase in the apparent K_d for Ca^{2+}.

The specificity of the E_1 form to be phosphorylated by ATP is a useful tool for studying the selective effect of CPA on the Ca^{2+}-dependent enzyme conformations. In a first set of experiments, E_2CPA at a CPA/enzyme molar ratio of ≤ 1 was mixed with a medium containing [γ-^{32}P]ATP and Ca^{2+}. The phosphorylation reaction was stopped after 1 s at 22 °C by acid quenching. As can be seen in Figure 4, preincubation of E_2 with CPA precluded enzyme phosphorylation in a manner that was dependent on the amount of CPA present (solid circles). In another set of experiments, E_2CPA was supplemented with saturating Ca^{2+} and maintained for 10 min at 37 °C before the phosphorylation process. Notably,

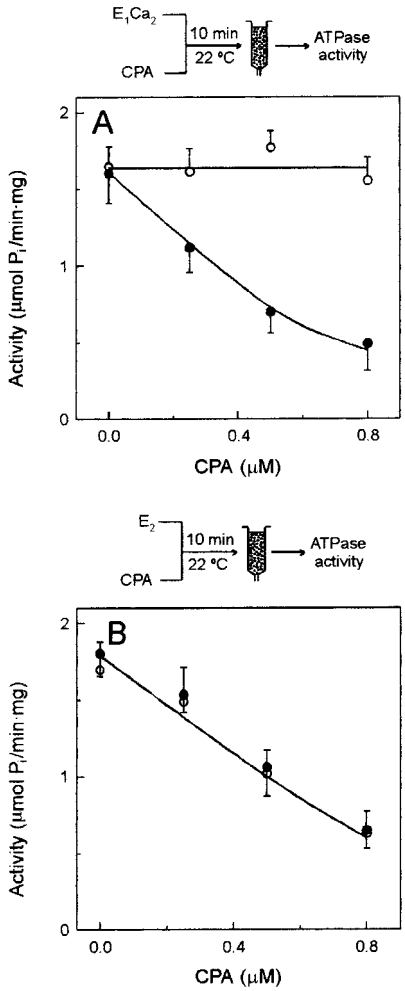


FIGURE 5: Effect of CPA on E_1Ca_2 or E_2 states after gel filtration of samples. (A) The enzyme in the presence of 22 μM free Ca^{2+} (20 mM Mops, pH 7.0, 80 mM KCl, 3 mM $MgCl_2$, 0.1 mM EGTA, 0.12 mM $CaCl_2$, and 0.2 mg of SR protein/mL, equivalent to 0.8 μM ATPase) was incubated for 10 min at 22 °C in the presence of up to 0.8 μM CPA. (B) The enzyme in the absence of Ca^{2+} (20 mM Mops, pH 7.0, 80 mM KCl, 3 mM $MgCl_2$, 0.1 mM EGTA, and 0.2 mg of SR protein/mL) was incubated for 10 min at 22 °C in the presence of CPA. In all cases, aliquots of 0.8 mL were applied to 2 mL Sephadex G-50 columns pre-equilibrated in the presence (A) or in the absence of Ca^{2+} (B) and centrifuged for 1 min at low speed. Samples from the eluate were used to determine Ca^{2+}-ATPase activity. Open circles correspond to samples treated and subjected to gel filtration as depicted in the diagrams. Solid circles correspond to samples that were not subjected to gel filtration (control).

E_2CPA samples subjected to Ca^{2+} incubation at 37 °C displayed full capacity to be phosphorylated by ATP (open circles). Incubation for 10 min at 37 °C in the absence of Ca^{2+} did not permit such phosphorylation when the reaction was initiated from E_2CPA.

The Ca^{2+} effect when incubated with E_2CPA on the enzyme capacity to be phosphorylated by ATP could be associated with toxin displacement. This possibility was explored by gel filtration experiments after incubating samples under well-controlled conditions. When E_1Ca_2 was incubated for 10 min at 22 °C with CPA at a toxin/enzyme molar ratio of ≤ 1, there was a progressive inhibitory effect of the Ca^{2+}-ATPase activity as the amount of toxin increased (Figure 5A, solid circles). However, the inhibition was no longer observed once the E_1Ca_2 samples containing CPA

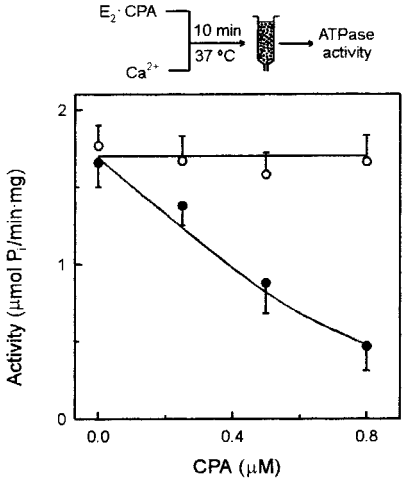


FIGURE 6: Effect of Ca^{2+} incubation and Sephadex column on E_2-CPA. SR vesicles at 0.2 mg/mL (0.8 μM ATPase) were incubated for 10 min at 22 °C in the presence of 20 mM Mops, pH 7.0, 80 mM KCl, 3 mM $MgCl_2$, 0.1 mM EGTA, and a certain CPA concentration. After addition of 0.12 mM $CaCl_2$ (22 μM free Ca^{2+}), the incubation was prolonged for 10 min at 37 °C. Aliquots of 0.8 mL were subjected to gel filtration/centrifugation through a Sephadex G-50 column pre-equilibrated with a Ca^{2+}-containing buffer. Samples collected from the columns were used to evaluate enzyme activity. Open circles stand for samples treated as described in the diagram. Solid circles stand for samples not subjected to the chromatographic treatment (control).

were passed through the column (Figure 5A, open circles). It is expected that the Sephadex column removes loosely bound and free CPA, and therefore the expression of Ca^{2+}-ATPase activity would indicate absence of toxin. By contrast, when E_2 was incubated for 10 min at 22 °C with CPA at a toxin/enzyme molar ratio of ≤ 1, enzyme inhibition was observed whether samples were subjected to gel filtration or not (Figure 5B, open and solid triangles, respectively). In another set of experiments, E_2CPA was also formed at a toxin/enzyme molar ratio of ≤ 1 and then supplemented with saturating Ca^{2+}. The Ca^{2+} incubation was prolonged for 10 min at 37 °C, and samples were subsequently subjected to chromatography/centrifugation as before. After this treatment, the enzymatic activity was completely recovered after gel filtration (Figure 6, open circles). Samples preincubated with Ca^{2+} at 37 °C that were not passed through the column (solid circles) were sensitive to CPA inhibition.

The CPA−enzyme interaction was also examined by kinetic titration in order to evaluate the enzyme affinity for CPA. Enzyme activity data were generated in the presence of 10 μM ATP and an ATP-regenerating system. The dependence of enzyme inhibition on CPA concentration displayed monophasic decay for each enzyme concentration (Figure 7A), although different CPA concentrations were needed to achieve half-maximal inhibition ($K_{1/2}$). As the enzyme concentration approached the K_d for CPA, complete inhibition required toxin concentrations above an equimolar level. By plotting $K_{1/2}$ values vs Ca^{2+}-ATPase concentrations, we obtained an apparent K_d of 7 nM (Figure 7B).

It is important to establish whether the observed effects of CPA on the Ca^{2+}-dependent conformations of the enzyme are relevant to the catalytic cycle. To address this issue, the steady-state Ca^{2+}-ATPase activity was measured in the presence of 0.02 mg SR protein/mL and 1 mM ATP. In some experiments (Figure 8) the free Ca^{2+} concentration was

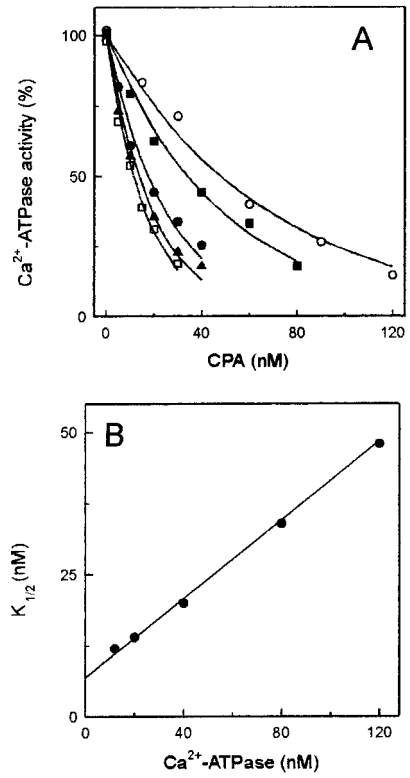


FIGURE 7: Effect of enzyme concentration on Ca^{2+}-ATPase inhibition by CPA. (A) The hydrolytic activity was measured at 25 °C in a medium containing 20 mM Mops, pH 7.0, 80 mM KCl, 3 mM $MgCl_2$, 0.1 mM EGTA, 0.12 mM $CaCl_2$, 4% A23187, 2 mM phosphoenolpyruvate, 5 units/mL pyruvate kinase, different CPA concentrations, and 10 μM ATP. For each set of experiments the membrane protein concentration was varied between 0.003 and 0.03 mg/mL to yield the following Ca^{2+}-ATPase concentrations: 12 nM (□), 20 nM (▲), 40 nM (●), 80 nM (■), and 120 nM (○). (B) CPA concentrations giving half-maximal inhibition ($K_{1/2}$ values) were plotted as a function of the enzyme concentration. The dissociation constant for CPA (K_d = 7 nM) can be obtained from the intercept.

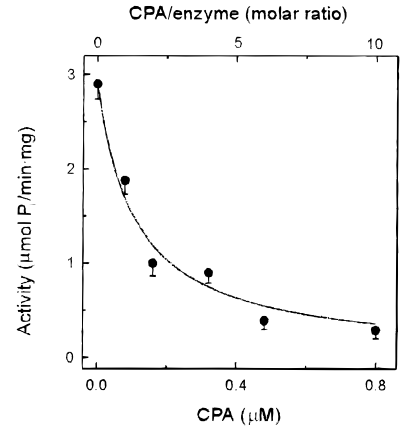


FIGURE 8: Inhibitory effect of CPA on the enzyme turnover measured in the presence of 1 mM ATP. Steady-state Ca^{2+}-ATPase activity was evaluated at 25 °C in a medium containing 20 mM Mops, pH 7.0, 80 mM KCl, 5 mM $MgCl_2$, 1 mM EGTA, 0.967 mM $CaCl_2$ (10 μM free Ca^{2+}), 0.02 mg/mL SR protein (i.e., 0.08 μM ATPase), 1.5 μM A23187, and a specified CPA concentration. The reaction was started by adding 1 mM ATP. The hyperbolic dependence was fit to the equation $y = 2.904/(1 + x/0.111)$.

fixed at 10 μM, and the CPA concentration added was varied. The data show a hyperbolic decrease of enzyme activity as the amount of CPA increases. The activity tended to decrease even in the presence of low amounts of CPA, although the ATP binding site would be saturated by 1 mM ATP (*11*). Under these conditions, the CPA effect on enzyme turnover is not restricted to the equimolar level. It is clear that complete enzyme inhibition requires a CPA/enzyme molar ratio of ~10.

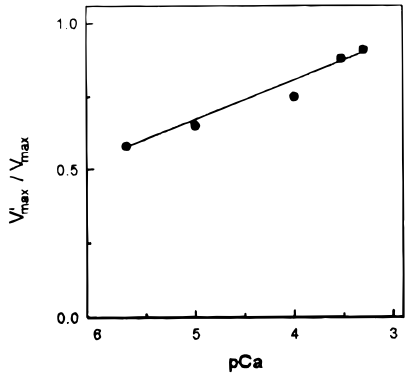


FIGURE 9: Activating effect of Ca^{2+} on the enzyme activity measured in the presence of equimolar CPA and 1 mM ATP. The effect of pCa on Ca^{2+}-ATPase activity was measured at 25 °C. The reaction medium for obtaining the V'_{max} values consisted of 20 mM Mops, pH 7.0, 80 mM KCl, 5 mM $MgCl_2$, 0.02 mg/mL SR protein (0.08 μM ATPase), 1.5 μM A23187, 1 mM EGTA, a $CaCl_2$ concentration between 0.81 and 1.55 mM (to yield a free concentration in the range 2−500 μM), 0.08 μM CPA, and 1 mM ATP. The V_{max} values were obtained under the same experimental conditions but in the absence of CPA.

We also examined the dependence of Ca^{2+} on Ca^{2+}-ATPase activity in the presence of 1 mM ATP and 0.08 μM added CPA, equivalent to a CPA/enzyme molar ratio of 1. These experiments provided the maximal hydrolytic rate at each Ca^{2+} concentration (V'_{max}). Parallel experiments performed with the same Ca^{2+} concentrations but in the absence of CPA allowed us to obtain the corresponding V_{max} values. Figure 9 shows a plot of V'_{max}/V_{max} vs pCa, demonstrating that the enzyme inhibition promoted by equimolar CPA in the presence of 1 mM ATP can be overcome by increasing the Ca^{2+} concentration in the reaction medium.

DISCUSSION

Our aim in the present study has been to uncover the effect of CPA on the E_1Ca_2 and E_2 conformations and the implication of this effect for enzyme turnover. The fact that CPA at a toxin/enzyme molar ratio of ≤1 has no effect on Ca^{2+} binding when added to E_1Ca_2 (Figure 1A) is an indication that Ca^{2+} may prevent the interaction of CPA with the enzyme. Therefore, the interaction of E_1Ca_2 with CPA can be defined as low affinity in contrast to that of E_2 with CPA as high affinity. The final level of Ca^{2+} binding was dependent on the incubation conditions when Ca^{2+} was added to E_2CPA at a toxin/enzyme molar ratio of ≤1. Our data indicate that the extent of Ca^{2+} binding is dependent on the $E_2CPA + Ca^{2+} \rightarrow E_1Ca_2 + CPA$ transition. Accordingly, the partial inhibition observed when the Ca^{2+} incubation was carried out for 10 min at 22 °C (Figure 1A) suggests that CPA did not completely dissociate from the enzyme. The lack of effect on Ca^{2+} binding when Ca^{2+} was incubated for 10 min at 37 °C (Figure 1B) is an indication that E_2CPA was completely transformed into E_1Ca_2. We also found some CPA dissociation in the presence of Ca^{2+} after incubation for 10 min at 0 °C (Figure 1B). The inhibition was

practically equimolar when measured at 0 °C but 1 min after addition of Ca^{2+} to E_2CPA (see solid circle at 1 min in Figure 2A).

Binding of Ca^{2+} to the enzyme takes place in the minute time scale at 37 °C when added to E_2CPA (Figure 2). This suggests that CPA dissociation from E_2 is a rate-limiting step since the Ca^{2+}-dependent isomeric transition of the enzyme occurs in the millisecond time scale (*27*−*29*). Therefore, the rate of the $E_2 \rightarrow E_1Ca_2$ interconversion developed in the presence of CPA is critically dependent on the incubation time and temperature. This explains the previously reported inhibition of the $E_2 \rightarrow E_1Ca_2$ transition (*8*) and the absence of clear-cut results when the CPA effect on Ca^{2+} binding was studied under poorly controlled conditions (*9*).

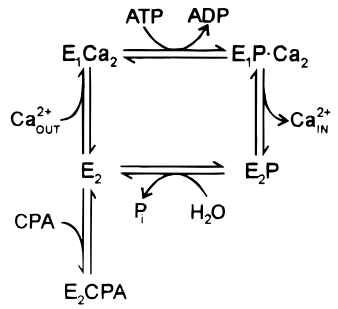
Equimolar amounts of CPA decrease the enzyme apparent affinity for Ca^{2+} binding by ∼0.45 pCa unit at pH 7.0 and by ∼0.8 unit at pH 6.0 (Figure 3). This enhanced effect of CPA at pH 6.0 can be explained by the prevalence of E_2 conformations at acidic pH (*32*). A decrease in the rate of the $E_2 \rightarrow E_1Ca_2$ transition and in the enzyme affinity for Ca^{2+} have also been observed for other highly specific inhibitors such as thapsigargin and 2,5-di-*tert*-butyl-1,4-benzohydroquinone (*15, 33, 34*).

The Ca^{2+} titration curves obtained as a function of pCa at pH 6.0 clearly indicate that CPA above the equimolar level produces a progressive shift in the apparent K_d for Ca^{2+} binding (Figure 3B). This competitive behavior, which was more difficult to observe at pH 7.0 (data not shown), can be explained if CPA and Ca^{2+} bind to two different species, i.e., CPA to E_2 and Ca^{2+} to E_1, as has been proposed for the thapsigargin action (*13, 14*). Similar studies performed at pH 7.2 in the presence of thapsigargin (*34*) or 2,5-di-*tert*-butyl-1,4-benzohydroquinone (*35*) reported a saturating shift of the Ca^{2+} binding curve when these compounds were used above an equimolar level. The authors concluded that inhibitors can bind to the enzyme in the presence or absence of Ca^{2+}. This conclusion is in disagreement with the proposed model of thapsigargin inhibition (*13, 14, 36*) and the present observations on Ca^{2+} binding (Figure 3B), although the experimental discrepancy may be attributed to the pH used.

The selective effect of CPA on E_2 can also be demonstrated by phosphorylation experiments. Our data indicate that E_1Ca_2 + CPA may be directly phosphorylated by ATP (*11*), whereas full phosphorylation of the enzyme requires dissociation of the toxin, i.e., a Ca^{2+} incubation at 37 °C when the reaction was initiated from E_2CPA (Figure 4).

The presence of equimolar CPA in the incubation medium does not affect Ca^{2+} binding (Figure 1A) or phosphorylation by ATP (*11*) when added to E_1Ca_2 although, Ca^{2+}-ATPase activity may be inhibited by enzyme turnover (*11*). Therefore, the enzyme activity measured after chromatographic treatment is a useful criterion for analyzing the CPA−enzyme interaction. A Sephadex column containing Ca^{2+} protected enzyme activity from inhibition when ATP was added to E_1Ca_2 in the presence of CPA (Figure 5A). Since the chromatographic column removes loosely bound and free CPA, it can be deduced that CPA was not tightly bound to E_1Ca_2. On the other hand, the inhibition observed when samples were not passed through the column can be attributed to the CPA effect on E_2 generated during the enzyme turnover (*11*). The fact that 85−90% of the activity was recovered when E_1Ca_2 + equimolar CPA was sedimented by centrifugation and the supernatant removed by aspiration suggests that CPA was not bound to E_1Ca_2 (data not shown). The chromatographic column containing Ca^{2+} was also able to eliminate CPA when the E_2CPA species was allowed to transform into E_1Ca_2 + CPA (Figure 6). In contrast, the Sephadex column equilibrated in the absence of Ca^{2+} did not eliminate enzyme inhibition of the E_2CPA samples (Figure 5B), suggesting that CPA was tightly bound to E_2. The fact that no recovery of activity was observed in the minute time scale, even though passage through the column would permit CPA dissociation, is consistent with a very low dissociation rate of toxin from E_2CPA and the apparent K_d for the CPA−enzyme complex calculated in Figure 7. Here again, our observations are difficult to explain if we assume an equal binding affinity of CPA to both enzyme conformations as proposed for other specific inhibitors (*34, 35*). The inhibition by CPA was previously reported to persist after repeated microsome washing (*8*), although no experimental conditions with regards to CPA/enzyme molar ratios and incubation conditions were indicated.

Scheme 1: Ca^{2+}-ATPase Reaction Cycle in the Presence of CPA[a]



[a] Addition of CPA inhibits steady-state enzyme activity through the inactive route E_2 + CPA → E_2CPA. An increase in the Ca^{2+} or ATP concentration relieves the inhibition by favoring the catalytic transition E_2 + Ca^{2+} → E_1Ca_2.

Although complete enzyme inhibition at low ATP concentrations requires a CPA/enzyme molar ratio of 1 (*11*), the CPA/enzyme molar ratio was ∼10 when measured in the presence of 1 mM ATP (Figure 8). We know that CPA decreases the binding affinity of ATP by approximately 1 order of magnitude (K_d ∼ 28 μM) without affecting the maximal binding capacity of the enzyme (*11*). It is therefore to be expected that the catalytic site should be saturated by 1 mM ATP when CPA inhibition takes place. We also found, under steady-state conditions, that the enzyme inhibition by equimolar CPA in the presence of saturating ATP can be reversed by the addition of Ca^{2+} (Figure 9).

The present observations can be explained by the reaction cycle depicted in Scheme 1. When enzyme cycling occurs in the presence of 1 mM ATP, the addition of CPA results in enzyme inhibition due to accumulation of the inactive species, E_2CPA. Under these conditions, complete inhibition of the enzyme requires a CPA/enzyme molar ratio of ∼10. This may be attributed to the activating effect of millimolar ATP on the hydrolytic cleavage of EP (*37*−*39*) and the subsequent stabilization of E_1Ca_2 with respect to E_2 species. High ATP concentrations protect enzyme activity, and so complete inhibition requires the presence of several CPA molecules. Furthermore, the partial enzyme inhibition

induced by equimolar CPA in the presence of 1 mM ATP can be overcome by adding Ca^{2+}, i.e., pulling forward the catalytic cycle by favoring the formation of E_1Ca_2 as opposed to the CPA-sensitive E_2 form.

In conclusion, the stabilization of E_1Ca_2 species under turnover conditions by adding Ca^{2+} or millimolar ATP may protect against CPA inhibition. As a result, the degree of inhibition would be critically dependent on Ca^{2+} and ATP concentrations in addition to the CPA/enzyme molar ratio due to the high affinity character of the toxin.

Inhibition by CPA affects both the catalytic and transport domains and is specific for SR/ER Ca^{2+}-ATPase, as occurs with thapsigargin. Therefore, it is expected that the interaction site should be located in a region of the enzyme showing little homology with other P-type ATPases. In this regard, chimeric constructs have indicated that transmembrane segment M3 is essential for thapsigargin sensitivity (*40*).

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