Cyclopiazonic Acid Is a Specific Inhibitor of the Ca$^{2+}$-ATPase of Sarcoplasmic Reticulum*

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The mycotoxin, cyclopiazonic acid (CPA), inhibits the Ca$^{2+}$-stimulated ATPase (EC 3.6.1.38) and Ca$^{2+}$ transport activity of sarcoplasmic reticulum (Goeger, D. E., Riley, R. T., Dorner, J. W., and Cole, R. J. (1988) Biochem. Pharmacol. 37, 978-981). We found that at low ATP concentrations (0.5-2 μM) the inhibition of ATPase activity was essentially complete at a CPA concentration of 6-8 nmol/mg protein, indicating stoichiometric reaction of CPA with the Ca$^{2+}$-ATPase. Cyclopiazonic acid caused similar inhibition of the Ca$^{2+}$-stimulated ATP hydrolysis in intact sarcoplasmic reticulum and in a purified preparation of Ca$^{2+}$-ATPase. Cyclopiazonic acid also inhibited the Ca$^{2+}$-dependent acetylphosphate, p-nitrophenylphosphate and carboxyphosphate hydrolysis by sarcoplasmic reticulum. ATP protected the enzyme in a competitive manner against inhibition by CPA, while a 10$^5$-fold change in free Ca$^{2+}$ concentration had only moderate effect on the extent of inhibition. CPA did not influence the crystallization of Ca$^{2+}$-ATPase by vanadate or the reaction of fluorescein-5'-isothiocyanate with the Ca$^{2+}$-ATPase, but it completely blocked at concentrations as low as 1-2 mol of CPA/mol of ATPase the fluorescence changes induced by Ca$^{2+}$ and [ethylenebis(oxyethylenenitrilo)]tetraacetic acid (EGTA) in FITC-labeled sarcoplasmic reticulum and inhibited the cleavage of Ca$^{2+}$-ATPase by trypsin at the T$_{2}$ cleavage site in the presence of EGTA. These observations suggest that CPA interferes with the ATP-induced conformational changes related to Ca$^{2+}$ transport. The effect of CPA on the sarcoplasmic reticulum Ca$^{2+}$-ATPase appears to be fairly specific, since the kidney and brain Na$^{+}$,K$^{+}$-ATPase (EC 3.6.1.37), the gastric H$^{+}$,K$^{+}$-ATPase (EC 3.6.1.36), the mitochondrial F$_{1}$-ATPase (EC 3.6.1.34), the Ca$^{2+}$-ATPase of erythrocytes, and the Mg$^{2+}$-activated ATPase of T-tubules and surface membranes of rat skeletal muscle were not inhibited by CPA, even at concentrations as high as 1000 nmol/mg protein.

Fungal toxins are common contaminants in agricultural products that present potential health risks to farm animals and humans. Cyclopiazonic acid (CPA), 1 an indole tetramic acid metabolite of Aspergillus and Penicillium, is one such mycotoxin that produces toxic effects in muscle (1, 2). Goeger and his colleagues (3) found that CPA is a potent inhibitor of the Ca$^{2+}$ uptake and ATPase activity of rat skeletal muscle sarcoplasmic reticulum (SR) and suggested that this inhibitory action may be involved in CPA toxicity.

The Ca$^{2+}$-ATPase is a major protein component of the SR membrane that regulates the contraction-relaxation cycle in muscle by transport of Ca$^{2+}$ from the cytoplasm into the lumen of the SR, coupled to the hydrolysis of ATP. The primary sequence of the Ca$^{2+}$-ATPase has been determined (4), two stable conformations of the enzyme have been crystallized (5-9), and mapping of the various functional domains has begun (10). The kinetic mechanism of Ca$^{2+}$-dependent ATP hydrolysis was determined in considerable detail (11) but so far no specific, high affinity inhibitor of the enzyme has been identified.

We further examined the reaction of CPA with the Ca$^{2+}$-ATPase of sarcoplasmic reticulum by kinetic analysis of its effects on the hydrolysis of ATP, acetylphosphate, p-nitrophenylphosphate, and carboxyphosphate and by testing its influence on the conformational dynamics of the Ca$^{2+}$-ATPase. We find that CPA specifically interferes with the conformational transitions of the Ca$^{2+}$-ATPase without significant effect on the hydrolysis of ATP by the Na$^{+}$,K$^{+}$-ATPase, the H$^{+}$,K$^{+}$-ATPase, the mitochondrial F$_{1}$-ATPase, the erythrocyte Ca$^{2+}$-ATPase, and the Mg$^{2+}$-activated ATPase of muscle cell surface membranes. Therefore, CPA may serve as a specific high affinity inhibitor of the sarcoplasmic reticulum Ca$^{2+}$ pump. A preliminary report of some of these results has been presented (12).

EXPERIMENTAL PROCEDURES

Materials

Cyclopiazonic acid, acetylphosphate, p-nitrophenylphosphate, di-sodium ATP, Tris-ATP, EGTA, Tris, NADH, imidazole, phosphoenolpyruvate kinase (rabbit muscle), lactate dehydrogenase (rabbit muscle), trypsin (bovine pancreas), trypsin inhibitor (soybean), acrylamide, bisacrylamide, ammonium persulphate, albumin (bovine serum), Tris-maleate, MOPS, and Mes$_{2}$O$_{2}$ were supplied by Sigma. Sodium vanadate, hydroxylamine hydrochloride and 2-mercaptoethanol were purchased from Fisher. Sodium dodecyl sulfate was obtained from Polysciences, Inc., Washington, PA; TEMED from Eastman Organic Chemicals, Rochester, NY; Coomassie Brilliant Blue R-250 from Bio-Rad; and molecular weight markers from Pharmacia LKB Biotechnology Inc. Fluorescein-5'-isothiocyanate was purchased from Molec-

1 The abbreviations are: CPA, cyclopiazonic acid; SR, sarcoplasmic reticulum; FITC, fluorescein-5'-isothiocyanate; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; MOPS, 4-morpholinopropane sulfonic acid; TEMED, N,N',N'-tetramethylethylenediamine; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.
Cyclopiazonic Acid Effect on Ca\textsuperscript{2+}-ATPase

17817

ular Probes, Inc., Eugene, OR, Ca\textsuperscript{2+} ionophore A23187 and carbamyolphosphate were from Behring Diagnostics. All other chemicals were of analytical grade.

Isolation of Transport ATPases

SR vesicles were obtained by differential centrifugation of homogenates of predominantly white, rabbit skeletal muscle (13, 14). The purified Ca\textsuperscript{2+}-ATPase was prepared according to Messner et al. (15). A rat skeletal muscle surface membrane and T-tubule preparation was isolated, and its Mg\textsuperscript{2+}-ATPase activity was assayed according to Beeler et al. (16, 17). The preparation was kindly provided to us by Dr. Troy J. Beeler of the Dept. of Biochemistry, Uniformed Services University of the Health Sciences, Bethesda.

ATPases prepared from pig gastric mucosa as described by Ray et al. (18) was kindly supplied by Dr. Tushar K. Ray of the Department of Surgery, SUNY Health Science Center at Syracuse. Na\textsuperscript{+},K\textsuperscript{-}ATPase purified from dog kidney according to Jorgensen (19), was given to us by Dr. Joseph D. Robinson, Jr., of the Department of Pharmacology, SUNY Health Science Center at Syracuse. The Na\textsuperscript{+},K\textsuperscript{-}ATPase and Mg\textsuperscript{2+}-ATPase activities were also tested in rabbit rabbit microsomes isolated according to Hart and Titos (20).

Mitochondrial F\textsubscript{1}-ATPase prepared from beef heart according to Penefsky (21) was provided by Dr. Harvey S. Penefsky of the Department of Biochemistry and Molecular Biology, SUNY Health Science Center at Syracuse. The human erythrocyte surface membrane Ca\textsuperscript{2+}-ATPase was purified according to Niggli et al. (22) and was kindly sent to us by Dr. John T. Pennistor, Department of Biochemistry and Molecular Biology, Mayo Clinic, Rochester, Minnesota.

Assay of the Sarcoplasmic Reticulum and Erythrocyte Surface Membrane Ca\textsuperscript{2+}-ATPases

The rate of Ca\textsuperscript{2+}-dependent ATP hydrolysis was determined at various concentrations of Ca\textsuperscript{2+}, ATP, and CPA, either by a coupled-enzyme system (14, 23) or by analysis of inorganic phosphate (P\textsubscript{i}) release according to the method of Pázsí and Subbarow (24).

The composition of the final reaction mixture for the coupled enzyme assay was 0.1 M KCl, 20 mM Tris-HCl, pH 7.5, 5 mM MgCl\textsubscript{2}, 0.7 mM CaCl\textsubscript{2}, 0.5 mM Mg\textsubscript{ATP}, 5 mM EGTA, 0.42 mM phosphoenolpyruvate, 0.2 mM NADH, 18 IU/ml of lactate dehydrogenase, 1-5 pg of protein/ml, 1-2 mM ATP, 0.7 mM CaCl\textsubscript{2}, and 0-2 mM CPA. The rate of hydrolysis of p-nitrophenylphosphate by SR was analyzed as described by Varga et al. (14).

The Mg\textsuperscript{2+}-activated ATPase of Skeletal Muscle Surface Membrane—The Mg\textsuperscript{2+}-stimulated ATPase activity was measured at 25 °C in an assay medium of 0.05 M KCl, 0.05 M imidazole, pH 7.0, 5 mM MgCl\textsubscript{2} protein concentration was 1 pg/ml. In parallel assays, the EGTA was replaced by 0.1 mM CaCl\textsubscript{2} to assess the possible contribution of the sarcoplasmic reticulum Ca\textsuperscript{2+}-ATPase. The ATPase activity was preincubated with 0, 100, 1,000, and 10,000 nM of CPA/mg protein for 5 min prior to the addition to the assay system.

The Hydrolysis of Substrate Analogos by the SR Ca\textsuperscript{2+}-ATPase

The acetylphosphatase and carboxamidophosphate activities were measured as described by Pucell and Martonosi (26). The acetylphosphate was determined by the method of Lipmann and Tuttle (27) and acamidophosphate according to Spector et al. (28). The hydrolysis of p-nitrophenylphosphate by SR was analyzed as described by Varga et al. (14).

RESULTS AND DISCUSSION

The Effects of Cyclopiazonic Acid on the ATPase Activity of Sarcoplasmic Reticulum

With increasing concentration of cyclopiazonic acid (Fig. 1) we found progressive inhibition of the Ca\textsuperscript{2+}-dependent ATPase activity of sarcoplasmic reticulum (Fig. 2), in substantial agreement with earlier observations of Goeger and his colleagues (3). At ATP concentrations (0.5-2.0 mM) significant inhibition of the Ca\textsuperscript{2+}-ATPase was obtained at CPA concentrations as low as 5-10 nM/mg of sarcoplasmic reticulum protein, indicating nearly stoichiometric reaction of the CPA with the Ca\textsuperscript{2+}-ATPase; the inhibition became complete at ~25 nM of CPA/mg SR protein.

The dependence of the activity of Ca\textsuperscript{2+}-ATPase on ATP concentration is biphasic (30, 31). This results in a convex curvature of the double reciprocal plot of the steady state

Protein was determined according to Lowry et al. (25).

Assays of H\textsuperscript{+},K\textsuperscript{-}ATPase, Na\textsuperscript{+},K\textsuperscript{-}ATPase, and Mitochondrial F\textsubscript{1}-ATPase

The phosphohydrolase activities of H\textsuperscript{+},K\textsuperscript{-}ATPase, Na\textsuperscript{+},K\textsuperscript{-}ATPase, and mitochondrial F\textsubscript{1}-ATPase were measured at 25 °C in coupled-enzyme assay systems of surface composition. The final composition of the reaction systems for the various enzymes was as follows.

H\textsuperscript{+},K\textsuperscript{-}ATPase—20 mM HEFES/Tris, pH 6.8, 2 mM MgCl\textsubscript{2}, 0.2-2 mM Tris-ATP, 0.42 mM phosphoenolpyruvate, 0.2 mM NADH, 7.5 IU/ml of pyruvate kinase and 18 IU/ml of lactate dehydrogenase, with or without 120 mM NaCl. The final concentration of enzyme was 5 μg/ml. The Na\textsuperscript{+},K\textsuperscript{-}ATPase activity was calculated by subtracting the activity obtained in the absence of Na\textsuperscript{+} from the activity measured in the complete assay system. In the analysis of the Na\textsuperscript{+},K\textsuperscript{-}ATPase activity of brain microsomes, the contribution of Na\textsuperscript{+},K\textsuperscript{-}ATPase to the total Mg\textsuperscript{2+}-stimulated ATPase activity was also assessed by testing the inhibition caused by 1.0 mM ouabain.
ATPase activity at high ATP concentration (31), yielding two apparent \( K_a \) values of 2-3 and 500 \( \mu M \), respectively. The inhibition of ATPase activity by CPA was particularly pronounced at low ATP concentration, causing sharp departure from linearity in the double reciprocal plot of ATPase hydrolysis (Fig. 2, inset). Increasing the concentration of ATP to 5 mM counteracted the effect of CPA, restoring the ATPase activity to near control levels (Fig. 2), suggesting competition. Dixon plots of the inhibitory effect of CPA at ATP concentrations of 0.5-5000 \( \mu M \) (Fig. 3) confirm the competitive relationship between CPA and ATP, with some indication of an irreversible component of inhibition at low ATP concentration. Due to the high affinity of the enzyme for CPA, the free CPA concentration for half-maximal inhibition by CPA could not be determined accurately, but it is estimated to be in the range of 10-20 \( nM \). The intercept in the upper left quadrant of the Dixon plot corresponds to \( \approx 6 \) nmol of CPA/mg SR protein, suggesting a nearly stoichiometric reaction of the CPA with the \( Ca^{2+} \)-ATPase. The inhibition of ATPase persisted after washing of the microsomes by repeated centrifugation, consistent either with tight binding of CPA to the plasmic reticulum.

The \( Ca^{2+} \) transport ATPase of SR is activated by \( Ca^{2+} \) at micromolar concentrations, approaching maximum activity at \( \approx 10^{-8} M \) free \([Ca^{2+}]\) (31). The activity declines at millimolar \( Ca^{2+} \) concentrations due to inhibition of \( Ca^{2+} \) transport by high intravesicular \( Ca^{2+} \). The inhibitory effect of CPA on the \( Ca^{2+} \)-ATPase was only moderately affected by changes in the \( Ca^{2+} \) concentration between 1-100 \( \mu M \) (Fig. 4).

The inhibitory effect of CPA on the \( Ca^{2+} \)-stimulated ATPase activity of purified \( Ca^{2+} \)-ATPase was similar to that seen in the native sarcoplasmic reticulum membrane. The specific activity of the purified \( Ca^{2+} \)-ATPase was 5.14 \( \mu mol \cdot mg^{-1} \cdot min^{-1} \) and it was reduced to 1.68 \( \mu mol \cdot mg^{-1} \cdot min^{-1} \) in the presence of 100 nmol of CPA/mg protein in an assay system containing 5 mM ATP. Essentially similar inhibition was obtained after reconstitution of \( Ca^{2+} \)-ATPase into vesicular structures by removal of detergent through dialysis.

The Effect of CPA on the Hydrolysis of Acetylphosphate, \( p \)-Nitrophenylphosphate, and Carbamylphosphate by the Sarcoplasmic Reticulum \( Ca^{2+} \)-ATPase

The apparent competition between CPA and ATP points to the possible involvement of the nucleotide-binding domain in the inhibition of ATPase activity by CPA. This possibility was tested by measuring the effect of CPA on the hydrolysis of acetylphosphate, \( p \)-nitrophenylphosphate, and carbamylphosphate by the sarcoplasmic reticulum. Although they lack the nucleotide ring, all three substrate analogs are cleaved by the \( Ca^{2+} \)-ATPase of SR and can serve as energy donors for \( Ca^{2+} \) transport (26, 31). As shown in Table I, the \( Ca^{2+} \)-dependent hydrolysis of acetylphosphate and \( p \)-nitrophenylphosphate was nearly completely inhibited by 40-100 nmol of CPA/mg SR protein, while the \( Ca^{2+} \)-insensitive component of the hydrolysis was relatively unaffected. At low CPA concentration (10 nmol/mg protein) significantly greater inhibition of the \( p \)-nitrophenylphosphatase activity was observed at low (\( \approx 10 \mu M \)) than at high (0.5 mM) \( Ca^{2+} \) concentration; this may be due to the formation of \( Ca^{2+} \)-CPA, since the difference diminished and eventually disappeared at higher CPA concentrations.

In contrast to ATP, acetylphosphate or \( p \)-nitrophenylphosphate, only about half of the carbamylphosphate hydrolysis is \( Ca^{2+} \) sensitive under the conditions shown in Table I. While the total carbamylphosphatase activity was apparently unaffected by CPA, the \( Ca^{2+} \)-insensitive component of carbamylphosphate hydrolysis was significantly elevated (Table I); the net result is an inhibition of the \( Ca^{2+} \)-stimulated carbamylphosphatase by CPA that is similar in magnitude to that
phate (Table I).

Control values, ATPase activity was measured by the release of inorganic phosphate for the Ca\(^{2+}\)-EGTA complex. The concentration of ATP (\(\mu\)M) was as follows: 0, 0.5; \(\bigcirc\), 10.0; \(\Delta\), 50.0; \(\boldsymbol{\Delta}\), 100.0; \(\boldsymbol{\Delta}\), 500; +, 5000. Each data point represents the mean of duplicate assays from two to seven experiments on seven different sarcoplasmic reticulum preparations.

These observations indicate that the inhibitory effect of CPA, unlike that of fluorescein-5'-isothiocyanate, is not confined to nucleoside triphosphate substrates and therefore not likely to involve the nucleotide binding domain of the Ca\(^{2+}\)-ATPase.

**The Effect of CPA on the Conformational Transitions of the Sarcoplasmic Reticulum Ca\(^{2+}\)-ATPase**

CPA Effect on the Fluorescence of FITC-labeled Ca\(^{2+}\)-ATPase—FITC reacts with lysine 515 in the nucleotide-binding domain of the Ca\(^{2+}\)-ATPase, causing inhibition of ATPase activity; ATP protects the enzyme against reaction with FITC (32). The fluorescence of FITC-labeled ATPase decreases upon saturation of the high affinity Ca\(^{2+}\) sites of the Ca\(^{2+}\)-ATPase by Ca\(^{2+}\) (33) or by lanthanides (34), and increases in the presence of EGTA and vanadate. These changes in fluorescence intensity are taken to indicate a shift in the conformational equilibrium of Ca\(^{2+}\)-ATPase in favor of the E\(_1\) state in the presence of Ca\(^{2+}\) or La\(^{3+}\), and in favor of the E\(_2\) state in the presence of EGTA and vanadate. The transition between the E\(_1\) and E\(_2\) states is an essential feature of Ca\(^{2+}\) transport (31).

Cyclopiazonic acid (6–120 nmol/mg protein) had no effect on the reaction of the Ca\(^{2+}\)-ATPase with FITC but completely inhibited the changes in the fluorescence intensity of FITC-labeled Ca\(^{2+}\)-ATPase caused by the addition of either Ca\(^{2+}\) or EGTA (Fig. 5). This effect was observed at concentrations as low as 6 nmol of CPA/mg protein, i.e. at 1:1 molar ratio with the Ca\(^{2+}\)-ATPase (Fig. 5). These observations imply that CPA inhibits the conformational transition of the enzyme between the E\(_1\) and the E\(_2\) states. Therefore, the inhibition of ATPase activity by CPA may be related to the inhibition of the conformational changes associated with ATP hydrolysis and Ca\(^{2+}\) transport. The polarization of fluorescence of FITC-labeled ATPase is relatively high \((P \sim 0.345–0.351)\) indicating significant immobilization of the covalently bound FITC on the Ca\(^{2+}\)-ATPase (35). The polarization of fluorescence was not affected significantly by 1–100 nmol of CPA/mg protein in the presence of either 0.1 mM Ca\(^{2+}\) (E\(_1\)-state), or in a solution of 0.1 mM EGTA and 0.5 mM Na\(^{3+}\) orthovanadate containing mono- and oligovanadate anions (E\(_2\)-V state) (not shown).

The Effect of CPA on the Tryptic Proteolysis of Ca\(^{2+}\)-ATPase—The primary cleavage of the Ca\(^{2+}\)-ATPase by trypsin at the T\(_1\) site (arginine 505) produces two large fragments (A and B) of nearly equal size (36). The secondary cleavage of fragment A by trypsin at the T\(_2\) site (arginine 198) yields the A\(_1\) and A\(_2\) subfragments. Subsequent slower cleavage of the A\(_1\) and A\(_2\) fragments produces a spectrum of smaller polypeptides. The cleavage at the T\(_3\) site is not particularly sensitive to the conformational change of the Ca\(^{2+}\)-ATPase, but the cleavage at the T\(_3\) site is completely blocked by vanadate in the presence of EGTA (E\(_2\)-V state). This inhibition can be reversed by Ca\(^{2+}\) that converts the enzyme into the E\(_2\) state (6, 7).

Cyclopiazonic acid (100 nmol/mg protein) had no effect on the cleavage of the Ca\(^{2+}\)-ATPase at the T\(_2\) site. The rate of the subsequent cleavage of the A\(_2\) fragment at the T\(_2\) site was significantly reduced by CPA in the presence of 1 mM EGTA (i.e. \(\sim 10^{-5} \text{ m Ca}^{2+}\)) (Fig. 6, C and D), suggesting that a change in the conformation of the Ca\(^{2+}\)-ATPase caused by CPA limited the access of trypsin to the T\(_2\) cleavage site. Saturation of the high affinity binding sites of the Ca\(^{2+}\)-ATPase by \(\sim 10^{-5} \text{ m Ca}^{2+}\) did not influence the inhibitory effect of CPA on the T\(_2\) cleavage (not shown), but at high Ca\(^{2+}\) concentration (20 mM), the effect of CPA was no longer observed and the cleavage at the T\(_2\) site proceeded at a fast rate with the formation of the A\(_1\) and A\(_2\) fragments (Fig. 6, A...
Cyclopiazonic Acid Effect on Ca\textsuperscript{2+}-ATPase

**TABLE I**

Inhibition of acetylphosphate, p-nitrophenylphosphate, and carbamylphosphate hydrolysis by CPA

The acetylphosphatase and carbamylphosphatase activities were measured at 25 °C in a medium containing 0.1 M KCl, 10 mM imidazole, pH 7.0, 5 mM MgCl\textsubscript{2}, 2 mM EGTA, 0.25–0.5 mg of sarcoplasmic reticulum protein/ml, and either 0.1 mM EGTA (A) or 0.1 mM CaCl\textsubscript{2} (B). The reaction was started after 5 min preincubation with 0, 40, or 100 nmol of CPA/mg protein by the addition of 5 mM acetylphosphate or carbamylphosphate, respectively. The changes in acetylphosphate concentration were measured according to Lipmann and Tuttle (27). The liberation of inorganic phosphate from carbamylphosphate was measured according to Spector et al. (28). The liberation of p-nitrophenol from p-nitrophenylphosphate was followed spectrophotometrically at 420 nm as described earlier (14). The data are presented as the mean ± S.E. of 3-15 measurements made on 9 different sarcoplasmic reticulum preparations. The number of independent measurements made on each preparation varied. In the control samples (top line) each value represents the mean of 4-15 measurements; in the presence of 40 and 100 nmol of CPA/mg protein (middle and bottom lines) the number of independent assays was 3-6 and 3-9, respectively. The difference between the activities measured with and without Ca\textsuperscript{2+} (B-A) yields the Ca\textsuperscript{2+} dependent hydrolysis rate (C). Column D gives the % inhibition of the Ca\textsuperscript{2+}-dependent hydrolysis by cyclopiazonic acid.

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<tr>
<th>CPA nmoI/mg</th>
<th>Acetylphosphate μmol/mg/min</th>
<th>%</th>
<th>Carbamylphosphate μmol/mg/min</th>
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<th>p-Nitrophenylphosphate nmol/mg/min</th>
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<td>40</td>
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<td>100</td>
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<td>0.07</td>
<td>78</td>
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Fig. 5. The effect of CPA on the fluorescence response of FITC-labeled Ca\textsuperscript{2+}-ATPase to Ca\textsuperscript{2+} and EGTA. The Ca\textsuperscript{2+}-ATPase was labeled with FITC and the changes in fluorescence intensity caused by Ca\textsuperscript{2+} and EGTA were measured as described under "Experimental Procedures." The final concentrations of Ca\textsuperscript{2+} and EGTA are given in parentheses. A: B: no CPA; C, D: 6 nmol CPA/mg protein. CPA blocked the fluorescence intensity changes whether EGTA was added before (A and C) or after (B and D) the addition of Ca\textsuperscript{2+}. Ordinate bars indicate 1% change in fluorescence intensity.

In the presence of 1 mM EGTA and 5 mM Na\textsuperscript{+}-orthovanadate, the enzyme is converted into the E\textsubscript{2}-V state with complete inhibition of the T\textsubscript{2} cleavage, as observed earlier (6, 7); under these conditions CPA had no further effect (Fig. 6, E and F). The pattern of the cleavage of the B fragment, monitored by fluorescence after labeling the enzyme with FITC (37), was not significantly altered by CPA (not shown).

These observations suggest that CPA, similarly to vanadate, stabilizes a conformation of the Ca\textsuperscript{2+}-ATPase in which the tryptic cleavage of the A fragment into the A\textsubscript{1} and A\textsubscript{2} subfragments is inhibited.

Comparison of the Effects of CPA and Vanadate on the Crystallization and Pressure Sensitivity of Ca\textsuperscript{2+}-ATPase—Mono-, oligo-, and decavanadate anions in the presence of EGTA promote the formation of two-dimensional crystals of the Ca\textsuperscript{2+}-ATPase by stabilizing the E\textsubscript{2}-V conformation (8, 9) and impart increased stability on the Ca\textsuperscript{2+}-ATPase during exposure to 1500–2000 atm pressure (14).

In contrast to vanadate, cyclopiazonic acid (20–200 nmol/mg protein) did not stimulate the formation of two-dimensional Ca\textsuperscript{2+}-ATPase crystals and did not protect or sensitize the Ca\textsuperscript{2+}-ATPase against pressure induced denaturation. Therefore, the mechanism of action of CPA does not involve the stabilization of the enzyme in a conformation similar to the state stabilized by vanadate. Neither did CPA (20–200 nmol/mg protein) disrupt the vanadate-induced crystals of Ca\textsuperscript{2+}-ATPase, that would be expected if CPA would shift the enzyme into a stable E\textsubscript{1}-like conformation (7).

In summary, the studies with various conformational indicators suggest that CPA stabilizes the Ca\textsuperscript{2+}-ATPase in an
FIG. 6. The effect of CPA on the trypic digestion pattern of SR microsomes. FITC-labeled SR vesicles (37) (2 mg protein/ml) were digested with trypsin (50 pg/ml) at 25 °C in a medium of 0.1 M KCl, 5 mM MgCl₂, 10 mM imidazole, pH 7.0, containing either 20 mM Ca²⁺ (panels A and B), or 1 mM EGTA (panels C and D), or 1 mM EGTA and 5 mM sodium vanadate (panels E and F) for times ranging from 0.5 to 120 min, as indicated on the bottom line. The digestion was carried out in the absence (left panels, A, C, and E) or in the presence of 100 nmol of CPA/mg protein (right panels, R, D, and F). The digestion was stopped by the addition of soybean trypsin inhibitor (100 pg/ml). To the zero time samples (O) trypsin and trypsin inhibitor were added together. The samples were solubilized in a solution of 5% sodium dodecyl sulfate, 10 mM Tris-HCl, pH 8.0, 1% 2-mercaptoethanol, 10% (v/v) glycerol, boiled at 100 °C for 10 min, and aliquots of 70 pg of protein were applied for electrophoresis on sodium dodecyl sulfate gradient gels (6-18%) as described in Ref. 6. The far left column indicates the positions of molecular weight markers in kilodalton (kDa). The far right column identifies band positions as follows: ATPase, Ca²⁺-ATPase; A, B, A₁, A₂, B₁, and B₂, the corresponding proteolytic fragments of the Ca²⁺-ATPase; T, trypsin; TI, trypsin inhibitor. The reaction of the enzyme with FITC had no influence on the course of trypic digestion (37).

enzymatically inactive conformation that differs both from the E₁ state stabilized by Ca²⁺ and from the E₂ state stabilized by EGTA and vanadate.

The Specificity of CPA Effect on the Ca²⁺-ATPase of Sarcoplasmic Reticulum

CPA inhibits the phosphorylation of Ca²⁺-ATPase by ATP (38). Inhibition of some reaction step leading to enzyme phosphorylation is probably also involved in the inhibition by CPA of the hydrolysis of acetylphosphate, carbamylphosphate, and p-nitrophenylphosphate. Since phosphorylation of an active site aspartyl residue is a common feature of the P type ion transport ATPases, the effect of CPA on other ion transport enzymes was also explored.

The hydrolysis of ATP by the kidney Na⁺,K⁺-ATPase, the gastric H⁺,K⁺-ATPase, the Ca²⁺ transport ATPase of erythrocytes, and by the beef heart mitochondrial F₁, ATPase was not inhibited significantly by 100–1000 nmol of CPA/mg protein at either low (0.2–0.6 mM) or high (2–6 mM) ATP concentrations (Table II). The sarcoplasmic reticulum Ca²⁺-ATPase tested under the specific conditions used for each of these enzymes was inhibited by 100 nmol of CPA to the extent of 59–89%.

For comparison with native sarcoplasmic reticulum, we also investigated the effect of CPA on the Na⁺,K⁺-ATPase activity in a native membrane, without prior purification. The Na⁺,K⁺-ATPase of brain microsomes (20) was 0.37 μmol-mg⁻¹-min⁻¹ in the absence of CPA, representing 79% of the total ATPase activity; the corresponding values were 0.38 and 0.43 μmol-mg⁻¹-min⁻¹ in the presence of 100 and 1000 nmol of CPA, respectively, indicating no inhibition by CPA. The Mg²⁺-stimulated, Na⁺- and ouabain-independent component of ATP hydrolysis in brain microsomes was 0.10 μmol-mg⁻¹-min⁻¹ in the absence of CPA and remained essentially unchanged (0.12 μmol-mg⁻¹-min⁻¹) in the presence of 1000 nmol/mg CPA.

Cyclopiazonic acid was also essentially without effect, even at concentrations as high as 10 μmol/mg protein on the Mg²⁺-stimulated ATPase of T-tubules and surface membranes (Table III), assayed at pH 6.8 and 7.8, in the presence of 4 mM EGTA, that lowered the Ca²⁺ concentration to ≈10⁻⁸ M. Under these conditions the Mg²⁺-stimulated, Ca²⁺-independent ATPase activity of the surface membrane preparation represented more than 80% of the total activity measured in the presence of 5 mM MgSO₄ and 0.1 mM CaCl₂. By contrast,
in the sarcoplasmic reticulum vesicles the Ca\(^{2+}\)-independent ("basal") ATPase activity is only about 5% of the total activity. The small Ca\(^{2+}\)-activated component of ATP hydrolysis in the surface membrane preparations was inhibited by CPA and may represent slight admixture of sarcoplasmic reticulum elements. The Ca\(^{2+}\)-independent basal ATPase activity of sarcoplasmic reticulum was resistant to cyclopiazonic acid, consistent with its proposed identity with the Mg\(^{2+}\)-ATPase of the surface membranes and T-tubules (16, 31, 39). Contaminating surface membrane vesicles may also account for the Ca\(^{2+}\)-independent component of acetylphosphatase and \(p\)-nitrophenylphosphate hydrolysis that was also unaffected by cyclopiazonic acid (Table I). The purified preparation of Ca\(^{2+}\)-ATPase obtained as described by Meissner et al. (15) did not contain a detectable Ca\(^{2+}\)-insensitive component, and the inhibition of the Ca\(^{2+}\)-stimulated ATPase by CPA was somewhat greater than in native sarcoplasmic reticulum vesicles.

These observations establish cyclopiazonic acid as a highly selective, high affinity inhibitor of the Ca\(^{2+}\)-ATPase of sarcoplasmic reticulum. The mechanism of inhibition by CPA is likely to involve a feature of the sarcoplasmic reticulum Ca\(^{2+}\) pump that is distinct from the other P and F type ATPases tested in this study. Covalent labeling of the Ca\(^{2+}\)-ATPase with a radioactive form of cyclopiazonic acid may help in the identification of the region of the molecule where CPA exerts its striking effect on the conformational transitions related to Ca\(^{2+}\) transport. CPA may also be useful to assess the contribution of sarcoplasmic reticulum Ca\(^{2+}\)-ATPase to the Ca\(^{2+}\) regulation and ATPase activity of intact and permeabilized living cells and membrane preparations that contain a mixture of different ATPases.

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Note Added in Proof—Cyclopiazonic acid also inhibits the Ca\(^{2+}\)-ATPase of rabbit cardiac sarcoplasmic reticulum. Therefore, the structural features involved in the inhibition are shared by the fast

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**Table II**

Effect of CPA on the hydrolysis of ATP by the Na\(^{+}\), K\(^{+}\)-ATPase, H\(^{+}\), K\(^{+}\)-ATPase, mitochondrial F\(_{1}\)-ATPase, and the Ca\(^{2+}\)-ATPase of erythrocytes

The ATPase activities were tested by the coupled-enzyme assay at the indicated ATP and CPA concentrations, as described under "Experimental Procedures." Each value is the average of 2-10 independent measurements. The Ca\(^{2+}\)-dependent ATPase activity of the sarcoplasmic reticulum Ca\(^{2+}\)-ATPase determined in parallel assays, under the assay conditions used for the various enzymes, was inhibited by 59-89% in the presence of 100 nmol of Ca\(^{2+}\)/mg protein. The specific activities of the sarcoplasmic reticulum Ca\(^{2+}\)-ATPase tested in the absence of CPA ranged from 1.4 to 1.8 \(\mu\)mol-ATPase activity was only about 0.75 \(\mu\)mol-ATPase activity was only about 0.6 ATP/mg ATP. The Ca\(^{2+}\) concentration, when indicated, was 100 nmol/mg protein for the sarcoplasmic reticulum and 1000 nmol/mg protein for surface membrane preparations. CPA (1000 nmol/mg protein) completely abolished the Ca\(^{2+}\)-dependent ATPase activity of sarcoplasmic reticulum, leaving the Ca\(^{2+}\)-independent ATPase component unaffected (not shown). The Ca\(^{2+}\)-independent ATPase activity of the surface membranes was also tested with 100 and 10,000 nmol of CPA/mg protein, and at 0.2 and 0.02 mM ATP concentration, without significant inhibition (not shown).

**Table III**

Comparison of the effect of CPA on the phosphohydrolase activities of sarcoplasmic reticulum and surface membrane preparations

The hydrolysis of ATP (2 mM) was measured at pH 6.8 or 7.8, at 25 °C, either in the presence of 0.1 mM CaCl\(_{2}\) (total activity) or in the presence of 4 mM EGTA (Ca\(^{2+}\)-independent activity) using the coupled-enzyme assay system, as described under "Experimental Procedures." The Ca\(^{2+}\)-dependent ATPase activity was calculated as the difference between the total and the Ca\(^{2+}\)-independent hydrolysis rates. The CPA concentration, when indicated, was 100 nmol/mg protein for the sarcoplasmic reticulum and 1000 nmol/mg protein for surface membrane preparations. CPA (1000 nmol/mg protein) completely abolished the Ca\(^{2+}\)-dependent ATPase activity of sarcoplasmic reticulum, leaving the Ca\(^{2+}\)-independent ATPase component unaffected (not shown). The Ca\(^{2+}\)-independent ATPase activity of the surface membranes was also tested with 100 and 10,000 nmol of CPA/mg protein, and at 0.2 and 0.02 mM ATP concentration, without significant inhibition (not shown).
and slow isoenzymes of the Ca\(^{2+}\)-ATPase but are distinct from the other P and F type ATPases tested in this study.

REFERENCES