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INTERACTION OF CYCLOPIAZONIC ACID WITH RAT SKELETAL MUSCLE SARCOPLASMIC RETICULUM VESICLES

EFFECT ON Ca^{2+} BINDING AND Ca^{2+} PERMEABILITY*

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Abstract—The interaction of cyclopiazonic acid with rat skeletal muscle sarcoplasmic reticulum (SR) vesicles was investigated in order to study the mechanism of cyclopiazonic acid inhibition of the Ca^{2+}-ATPase (Goeger *et al.*, *Biochem Pharmacol* **37**: 978–981, 1988). Cyclopiazonic acid at 25 μM prevented the binding of Ca^{2+} to the high affinity binding site of mixed (light and heavy) SR vesicles and inhibited, in a dose-dependent manner, the Ca^{2+}-dependent phosphorylation of SR vesicles by ATP. Binding of Ca^{2+} to the high affinity site of the Ca^{2+}-ATPase is necessary for both Ca^{2+} transport and for phosphorylation of the Ca^{2+}-ATPase. We conclude that inhibition of Ca^{2+} binding to the high affinity site may be responsible, at least in part, for the activity of cyclopiazonic acid. The mechanism of inhibition remains unclear. The inhibition was not reduced after dialysis and was only partially reversed by gel filtration of SR vesicles treated with cyclopiazonic acid. Neither 1 mM glutathione nor dithiothreitol pretreatment had any effect on the inhibition of the Ca^{2+}-ATPase. In addition to its inhibition of Ca^{2+} uptake and the Ca^{2+}-ATPase, cyclopiazonic acid had significant effects on Ca^{2+} efflux from both passively and actively loaded SR vesicles. Cyclopiazonic acid impeded the efflux of Ca^{2+} from passively loaded SR vesicles (in the presence of ruthenium red) when compared to either untreated vesicles or those treated with mersalyl acid, a mercurial which also inhibits the Ca^{2+}-ATPase and is known to induce Ca^{2+} release by both ruthenium red-sensitive and -insensitive pathways. Treatment of actively loaded vesicles with cyclopiazonic acid resulted in a decreased rate of Ca^{2+} efflux when compared to SR vesicles in which the Ca^{2+}-ATPase activity was inhibited by ATP depletion with hexokinase and glucose. The results are consistent with the hypothesis that, in mixed SR vesicles, cyclopiazonic acid inhibits both the Ca^{2+} pump and Ca^{2+} efflux.

Cyclopiazonic acid is an indole tetramic acid produced by certain fungi of the *Aspergillus* and *Penicillium* genera, and can be found as a natural contaminant of human foods and animal feeds [1]. Ingestion of this mycotoxin has resulted in the poisoning of both humans and animals [2–5]. Clinical symptoms of cyclopiazonic acid poisoning frequently involve the musculature [6, 7] and include muscular incoordination, hypokinesia, catelepsy, gait disturbances, tremors, opisthotonus and convulsions [8, 9]. Cyclopiazonic acid is a potent inhibitor of the Ca^{2+}-transport ATPase found in sarcoplasmic reticulum (SR)§ vesicles isolated from rat skeletal muscle [10]. Inhibition of this enzyme may be partially responsible for cyclopiazonic acid toxicity since many of the clinical symptoms also resemble the pharmacologic and toxic effects of the phenothiazine drugs which can also inhibit Ca^{2+} transport by skeletal muscle SR [11]. In addition, cyclopiazonic acid alters $^{45}Ca^{2+}$ flux in muscle cells [12] and potentiates the partitioning of the lipophilic cation, tetraphenylphosphonium, into the plasma membrane and mitochondrial fraction of cultured cells [13, 14], suggesting that the cyclopiazonic acid-induced alterations in $^{45}Ca^{2+}$ flux in cells may involve electrical alterations in biomembranes.

The present studies were initiated to investigate the interaction of cyclopiazonic acid with SR vesicles isolated from rat skeletal muscle. Because there is currently little information concerning the interaction of cyclopiazonic acid with Ca^{2+} uptake and release by SR vesicles, we chose to compare the effects of cyclopiazonic acid with mersalyl acid, a mercurial compound which is known to inhibit the Ca^{2+} pump and alter Ca^{2+} permeability of SR vesicles [15–17]. The results indicate that cyclopiazonic acid has a different mechanism of action than mersalyl acid. Cyclopiazonic acid inhibition of Ca^{2+} uptake and Ca^{2+}-dependent ATPase activity was partially reversible and did not involve sulfhydryl oxidation. Cyclopiazonic acid prevented the binding of Ca^{2+} to the high affinity site on the SR vesicles and inhibited the Ca^{2+}-dependent phosphorylation

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§ Abbreviations: SR, sarcoplasmic reticulum; MOPS, 3-[*N*-morpholino]-propanesulfonic acid; EGTA, ethylene glycol bis-(β-aminoethyl ether)*N*,*N*,*N*′,*N*′-tetraacetic acid; and DMSO, dimethyl sulfoxide.

of the SR protein in a dose-dependent manner. The interaction of cyclopiazonic acid with SR vesicles, unlike mersalyl acid, resulted in a decreased permeability of the membrane to the efflux of both passively and actively loaded Ca^{2+}.

MATERIALS AND METHODS

Materials. Crystalline Na^+-ATP from equine muscle (4 ppm Ca^{2+} and vanadate free), arsenazo III, 3-[*N*-morpholino]-propanesulfonic acid (MOPS), calcium ionophore A23187, mersalyl acid, ethylene glycol bis-(β-aminoethyl ether)*N,N,N',N'*-tetraacetic acid (EGTA), crystalline pyruvate kinase from rabbit muscle, phospho(enol)pyruvate, hexokinase from bakers yeast and histidine were purchased from the Sigma Chemical Co. (St Louis, MO); $^{45}CaCl_2$, [γ-^{32}P]ATP and $^{22}NaCl$ were from the Amersham Corp. (Arlington Heights, IL) and D-[^{14}C(U)]sorbitol was from NEN Research Products (Boston, MA). Cyclopiazonic acid, >99% pure based on its UV extinction coefficient at 284 nm, was produced and purified from cultures of *Penicillium griseofulvum* [2] or obtained from the Sigma Chemical Co.

SR vesicle preparation. A crude intermediate (light and heavy vesicles) SR fraction (10,000–45,000 *g* fraction in 10% sucrose) was prepared from skeletal muscle of the hind leg of mature male Sprague–Dawley rats using a previously described procedure [18, 19] with modifications [10].

Ca^{2+} uptake and Ca^{2+}-dependent ATPase activity. Ca^{2+} uptake was measured by either a rapid filtration method similar to that of Martonosi and Feretos [15] or using the metallochromic Ca^{2+} indicator arsenazo III by a method similar to that of Beeler and Gable [20]. Both procedures have been described previously [10]. Cyclopiazonic acid and A23187 were both dissolved in dimethyl sulfoxide (DMSO) and added to the reaction buffer at less than 0.75% (v/v) as DMSO. Mersalyl acid was dissolved directly in the buffers. Controls contained similar volumes of DMSO where appropriate. ATPase activity by SR vesicles was terminated by filtration through 0.45 μm pore size HA Millipore filters (Millipore Corp., Bedford, MA). Filtrates were analyzed for inorganic phosphate release, and the Ca^{2+}-dependent ATPase activity was determined by subtracting the inorganic phosphate released by SR vesicles incubated with 1 mM EGTA and without added Ca^{2+}.

Gel filtration and dialysis of cyclopiazonic acid treated SR vesicles. To determine whether cyclopiazonic acid inhibition of the Ca^{2+}-ATPase of SR vesicles is reversible, cyclopiazonic acid treated vesicles were subjected to either gel filtration or dialysis. Gel filtration was conducted using Bio-Gel P-60 (Bio-Rad, Rockville Centre, NY) and a column 6.5 cm × 1.0 cm i.d. equilibrated with 0.1 M KCl, 10 mM MOPS, 2 mM $MgCl_2$, and 20 μM $CaCl_2$, pH 7.0. The SR vesicles were in the same buffer but it also contained cyclopiazonic acid. Final concentrations were 6 mg SR protein/ml and cyclopiazonic acid was 50 nmol/mg protein (DMSO < 0.75%, v/v). A volume of 0.2 ml of the treated SR vesicles was chromatographed on the column using the equilibration buffer at a flow rate of approximately 9 ml/hr. Fractions were collected, and the absorbance at 280 nm and protein concentration (bicinchoninic acid method) were determined. Fractions containing SR vesicles were pooled and assayed for Ca^{2+} uptake and Ca^{2+}-dependent ATPase activity.

SR vesicles were dialyzed using Spectrapor membrane tubing (Fisher Scientific Co., Pittsburgh, PA) with a dry cylinder diameter of 6 mm and a molecular weight cutoff of 12,000–14,000 daltons. The SR vesicles were treated similarly to those in the gel filtration study with a final protein concentration of 1.5 mg/ml and DMSO < 0.2% (v/v). A volume of 1.4 ml of the treated SR vesicles was dialyzed against 250 ml of equilibration buffer used in gel filtration experiments. The SR vesicles were assayed for Ca^{2+} uptake and Ca^{2+}-dependent ATPase activity after 0, 1.5, 6 and 24 hr of dialysis. The dialysis buffer was changed after 1.5 and 6 hr of dialysis.

Isotope efflux measurements. $^{45}Ca^{2+}$ efflux was determined on passively loaded SR vesicles using methods similar to those of Meissner [21]. SR vesicles (0.15 to 0.25 mg protein/ml) were preincubated in 0.1 M KCl, 10 mM MOPS and 20 μM $CaCl_2$, pH 7.0, for 30 min at 4° and pelleted by centrifugation at 45,000 *g* for 45 min. The vesicles were then suspended in loading buffer (5 to 7.5 mg protein/ml) containing 0.1 M KCl, 10 mM MOPS and 5 mM $^{45}CaCl_2$ (20,000 cpm/nmol Ca^{2+}), pH 7.0, and incubated for 2 hr at room temperature. The passively loaded vesicles were then diluted 200-fold into iso-osmolar release buffer (0.1 M KCl, 10 mM MOPS, 5 mM $MgCl_2$, 1 mM EGTA, and 10 μM ruthenium red, pH 7.0, with continuous mixing). At selected times, aliquots were removed and filtered through 0.45 μm pore size type HA Millipore filters (Millipore Corp.) and immediately rinsed with two successive 2.0-ml volumes of release buffer. The filters were counted in 5 ml of liquid scintillation solution as previously described [10].

D-[^{14}C(U)]Sorbitol and $^{22}Na^+$ efflux were measured with a procedure similar to that used for $^{45}Ca^{2+}$ efflux but with buffer modifications. The preincubation buffer consisted of 0.1 M KCl, 10 mM MOPS, 2 mM $MgCl_2$ and 20 μM $CaCl_2$, pH 7.0, and the loading buffer was supplemented with either 5 mM D-[^{14}C(U)]sorbitol (85,000 cpm/nmol sorbitol) or $^{22}NaCl$ (14,000 cpm/nmol Na^+). The release buffers were similar to the loading buffers but in order to maintain iso-osmolarity contained, in addition, either 5 mM glucose or a 5 mM increase in KCl concentration to replace the D-[^{14}C(U)]sorbitol and $^{22}Na^+$ respectively. $^{22}Na^+$ radioactivity on filters was determined by direct counting in a Packard 500C gamma counter. Isotope efflux data were analyzed by first order regression analysis after transforming cpm to log cpm. The Y intercept was taken as isotope load at time 0.

Binding of Ca^{2+} to SR vesicles. The binding of Ca^{2+} to SR vesicles and the effect of cyclopiazonic acid on binding were determined by equilibrium dialysis. SR vesicles were diluted to 0.5 mg protein/ml with buffer containing 0.1 M KCl, 4 mM $MgCl_2$, 10 mM MOPS, pH 7.0, and various concentrations of Ca^{2+} between 1 mM and 0.1 μM. EGTA at 50 μM was used to buffer free Ca^{2+} at concentrations less than 4.6 μM using apparent stability constants of

2.514×10^6 and 40.481 for CaEGTA and MgEGTA, respectively, as determined by a computer program adapted from that of Fabiato and Fabiato [22]. Two 1-ml aliquots of the diluted SR vesicles were dialyzed for 4 hr, in 200 ml of the dilution buffer using tubing described above. One of the samples was then transferred to 25 ml of the same buffer but containing $^{45}Ca^{2+}$ (8×10^5 cpm/ml) and the other sample to a similar buffer containing 25 μM cyclopiazonic acid (DMSO < 0.1%), and both were dialyzed for an additional 20 hr. Dialysis was then terminated and radioactivity determined on triplicate 0.2-ml samples of both buffer and dialyzed SR vesicles with the difference being the Ca^{2+} bound to the SR. To minimize the error inherent to pipetting, the quantity of samples taken was determined gravimetrically to the nearest 0.1 mg. All dialyses were conducted at 4° with shaking. Protein concentration was determined by the bicinchoninic acid method.

Phosphorylation of SR vesicles with [γ-^{32}P]ATP. The effect of cyclopiazonic acid on Ca^{2+}-dependent phosphorylation of SR vesicles was determined by a method similar to that of Shoshan and MacLennan [23]. The reaction buffer contained 0.1 M KCl, 4 mM $MgCl_2$, 100 μM Ca^{2+}, 10 mM MOPS, pH 7.0, with or without various levels of cyclopiazonic acid (DMSO < 0.3%, v/v) and an ATP-regenerating system consisting of 5 mM phospho(enol)pyruvate and 23 units/ml of pyruvate kinase. SR vesicles were incubated in the buffer for 8 min before initiation of reactions by the addition of [γ-^{32}P]ATP (12,000 cpm/nmol) so that the final concentrations were 0.2 mM ATP and 0.5 mg SR protein/ml in a total volume of 2.0 ml. Reactions were terminated after 30 sec by addition of 7 ml of 5% trichloroacetic acid, 2 mM P_i and 0.1 mM ATP. The precipitated protein was pelleted by centrifugation at 10,000 *g* for 10 min, resuspended in 3.5 ml of the 5% trichloroacetic acid mixture using a bath sonicator, pelleted and washed twice. The final pellet was dissolved in 0.5 ml of 1 M NaOH by heating for 1 hr in a boiling water bath, and radioactivity was determined.

Biochemical assays. Protein was determined by either the method of Lowry *et al.* [24] or by the bicinchoninic acid method (Pierce Chemical Co., Rockford, IL) using bovine serum albumin as a standard. Inorganic phosphate was assayed by the method of Baginski *et al.* [25] as modified by Ottolenghi [26].

RESULTS

Reversibility of cyclopiazonic acid inhibition. Both gel filtration and dialysis were effective in removal of free cyclopiazonic acid from treated SR vesicles (Fig. 1). The chromatographed vesicles, as determined by protein concentration, appeared primarily in fractions 2 and 3, and these fractions were combined for analysis of Ca^{2+} uptake and Ca^{2+}-dependent ATPase activity. Cyclopiazonic acid eluted after fraction 4 and, based on an extinction coefficient of 19 mmol/l/cm at 280 nm for cyclopiazonic acid in the elution buffer, represents approximately 30 nmol, or 30% of the total cyclopiazonic acid loaded on the column. Dialysis was effective in reducing cyclopiazonic acid levels to approximately 50% of initial

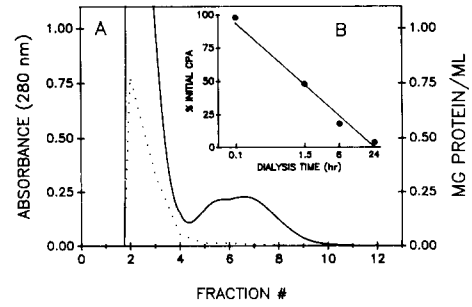

Fig. 1. Effectiveness of gel filtration and dialysis on removal of cyclopiazonic acid from rat skeletal muscle SR. (A) SR vesicles (0.2 ml, 6 mg protein/ml) treated with 50 nmol cyclopiazonic acid/mg protein were chromatographed on a Bio-Gel P-60 column (6.5 cm × 1 cm i.d.) equilibrated with 0.1 M KCl, 10 mM MOPS, 2 mM $MgCl_2$, and 20 μM $CaCl_2$ (pH 7.0) using a flow rate of approximately 9 ml/hr. Twenty drop fractions were collected (1 ml), and absorbance at 280 nm (solid line) and protein concentration (dotted line) were determined. The millimolar extinction coefficient of cyclopiazonic acid at 280 nm in this buffer was approximately 19. Fractions 2 and 3 were pooled for Ca^{2+} uptake and Ca^{2+}-dependent ATPase activity (Table 1). (B) The rate of loss of cyclopiazonic acid (CPA) (1.5 ml of a 100 μM solution in dialysis buffer) when dialyzed against 250 ml of the gel filtration buffer above was determined. At various times, absorbance at 280 nm of buffer containing cyclopiazonic acid was measured, and at 1.5 and 6 hr the dialysis buffer was changed. Dialysis time is plotted on a log scale.

levels after 1.5 hr (albeit without SR vesicles) with almost complete removal after 24 hr (Fig. 1B). However, neither gel filtration (Table 1) nor dialysis (Fig. 2) reversed the inhibitory effect of cyclopiazonic acid on Ca^{2+} uptake or ATPase activity. Gel filtration increased the Ca^{2+} uptake of cyclopiazonic acid treated vesicles from 30 to 56% and ATPase activity from 29 to 42% of DMSO treated SR vesicles subjected to gel filtration. While these increases suggest that gel filtration may partially reverse the inhibitory effects of cyclopiazonic acid they were not statistically significant (Student's *t*-test, $P > 0.05$).

It is established that there are critical sulfhydryls associated with both Ca^{2+} transport and release by SR [16, 17]. Thus, sulfhydryl reactive chemicals and oxidizing agents are potential inhibitors of transport ATPases. For this reason, we were interested to determine whether or not the inhibitory effects of cyclopiazonic acid could be prevented by thiol protecting agents. Treatment of SR vesicles with either glutathione or dithiothreitol prior to cyclopiazonic acid exposure did not produce any significant protective effect (Table 2). However, both compounds were completely effective in protecting SR vesicles against mersalyl acid, a sulfhydryl reactant. Both cyclopiazonic acid and mersalyl acid at 10 μM inhibited Ca^{2+} uptake and Ca^{2+}-dependent ATPase activity by approximately 60%, whereas neither glutathione nor dithiothreitol alone had any measurable effect on these activities (data not shown).

Effect of the sequence of cofactor addition on cyclopiazonic acid inhibition. The addition of Ca^{2+} and/or ATP prior to cyclopiazonic acid had no effect on Ca^{2+} uptake when compared to SR vesicles initially treated with cyclopiazonic acid (Fig. 3A). In all

Table 1. Effect of gel filtration on cyclopiazonic acid (CPA) inhibition of Ca^{2+} uptake and ATPase activity of rat skeletal muscle SR vesicles

	Without Bio-Gel filtration			With Bio-Gel filtration		
Expt.	Control	CPA	% Control	Control	CPA	% Control
			Ca^{2+} uptake*			
1	1076	178	16	861	403	47
2	391	189	48	398	175	44
3	422	108	26	327	252	77
			ATPase activity*			
1	1146	195	17	837	383	46
2	345	191	55	380	117	31
3	304	48	16	283	138	49

SR vesicles treated with 50 nmol cyclopiazonic acid/mg protein were chromatographed on a Bio-Gel P-60 column as described in Fig. 1A. Oxalate-assisted Ca^{2+} uptake and Ca^{2+}-dependent ATPase activity (total ATPase activity minus activity in the presence of 1 mM EGTA and without added Ca^{2+}) were assayed on SR vesicles by rapid filtration after chromatography. The mixtures consisted of 0.1 mg SR protein/ml, 0.1 M KCl, 10 mM MOPS, 2 mM $MgCl_2$, 2 mM ATP, 5 mM oxalate and 500 μM $^{45}Ca^{2+}$ (1200 cpm/nmol), pH 7.0. Reactions were initiated by ATP addition. Controls were treated in a similar manner but with DMSO in lieu of cyclopiazonic acid. The same SR vesicle preparation was used in all treatments for each experiment.

* Ca^{2+} uptake and ATPase activity are expressed in units of nmol/mg protein/min determined from the linear portion of the reactions (20–90 sec) by linear regression analysis.

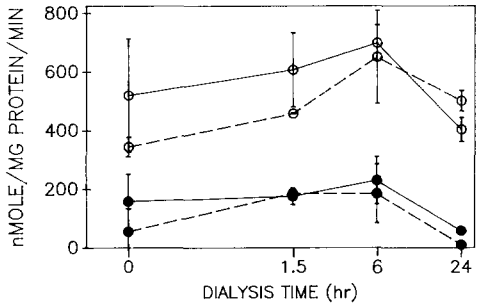

Fig. 2. Effect of dialysis on cyclopiazonic acid inhibition of Ca^{2+} uptake and ATPase activity of rat skeletal muscle SR vesicles. SR vesicles (1.5 mg protein/ml) treated with 50 nmol cyclopiazonic acid/mg protein were dialyzed (1.4 ml; N = 2) against 250 ml of 0.1 M KCl, 10 mM MOPS, 2 mM $MgCl_2$, and 20 μM $CaCl_2$ (pH 7.0). Ca^{2+} uptake and Ca^{2+}-dependent ATPase activity were assayed on SR vesicles by rapid filtration, described in Table 1, after 0, 1.5, 6 and 24 hr of dialysis. Controls were treated in a similar manner but with DMSO in lieu of cyclopiazonic acid. The dialysis buffer was changed after 1.5 and 6 hr of dialysis. Values are means ± SD where open circles represent controls and filled circles cyclopiazonic acid treated vesicles. Solid and broken lines represent Ca^{2+} uptake and ATPase activity respectively.

treatments, Ca^{2+} uptake was approximately 20% of the levels exhibited by control SR vesicles where the reactions were initiated with either Ca^{2+} or ATP addition. In comparison, addition of mersalyl acid after ATP was not as effective in inhibiting Ca^{2+} uptake as when it was added prior to ATP (Fig. 3B). Although prior addition of ATP does offer some protection against mersalyl acid inhibition, the rate of Ca^{2+} uptake (initial 90 sec) was still 65% of controls. However, mersalyl acid reduced Ca^{2+} uptake to less than 5% of controls when added prior to ATP. Ca^{2+} did not have any effect on mersalyl acid inhibition when added either before or after the inhibitor.

Cyclopiazonic acid and mersalyl acid effect on SR vesicle permeability. Addition of cyclopiazonic acid to control vesicles that had reached a steady-state level of Ca^{2+} accumulation resulted in a gradual loss of Ca^{2+} (Fig. 4). Approximately 75% of the actively accumulated Ca^{2+} was lost over a 3.5-min period. Following addition of the Ca^{2+} ionophore A23187, Ca^{2+} was released rapidly from control vesicles and cyclopiazonic acid treated vesicles. The extracellular Ca^{2+} concentration after 0.5 min in the presence of A23187 was approximately the same as the initial extracellular Ca^{2+} concentration (20 μM) prior to initiation of the reactions. The slight difference in the final Ca^{2+} concentration (Fig. 4) may indicate that the cyclopiazonic acid was contaminated with a small amount of divalent cations, that interaction of cyclopiazonic acid with SR resulted in displacement of bound Ca^{2+}, or that cyclopiazonic acid interacts with the arsenazo III chromophore. Regardless of the cause, the effect was small. At a cyclopiazonic acid concentration of 125 μM, the apparent increase in absorbance was equivalent to 5 nmol Ca^{2+}/mg protein.

Actively loaded SR vesicles, when treated with cyclopiazonic acid, exhibited a slower rate of Ca^{2+} efflux than vesicles treated with mersalyl acid (Fig. 5), a compound known to induce Ca^{2+} release via both ruthenium red-sensitive and -insensitive pathways [17]. SR vesicles were allowed to reach steady-state levels of Ca^{2+} accumulation (23.0 ± 1.2 nmol Ca^{2+}/mg protein; mean ± SD, N = 6) before being treated with a 33 μM concentration of either cyclopiazonic acid or mersalyl acid. Ca^{2+} efflux from cyclopiazonic acid treated vesicles was also slower than the efflux exhibited by vesicles treated with hexokinase in the presence of glucose (Fig. 5) which quenches the ATPase by converting ATP to ADP.

Table 2. Effects of glutathione and dithiothreitol on cyclopiazonic acid and mersalyl acid inhibition of Ca^{2+} uptake and ATPase activity of rat skeletal muscle SR vesicles

Treatment	Ca^{2+} uptake (nmol/mg protein/min)	% Controls	ATPase activity (nmol/mg protein/min)	% Controls
Control	521 ± 78	100	442 ± 85	100
CPA	217 ± 62	41 ± 7	122 ± 107	27 ± 21
CPA + GSH	244 ± 17	48 ± 9	147 ± 105	31 ± 17
CPA + DTT	253 ± 45	48 ± 2	203 ± 64	46 ± 11
Control	505 ± 114	100	482 ± 70	100
MA	173 ± 112	38 ± 31	244 ± 119	53 ± 32
MA + GSH	470 ± 133	92 ± 6	527 ± 99	108 ± 5
MA + DTT	626 ± 140	124 ± 1	547 ± 118	113 ± 8

SR vesicles (0.2 mg protein/ml) were equilibrated with 0.1 M KCl, 10 mM MOPS, 2 mM $MgCl_2$ and 20 μM $CaCl_2$ (pH 7.0) for 30 min, pelleted at 45,000 g for 45 min, and resuspended in the same buffer. Oxalate-assisted Ca^{2+} uptake and Ca^{2+}-dependent ATPase activity were assayed as described in Table 1 using the indicated treatments. Concentrations used were: 10 μM for both cyclopiazonic acid (CPA) and mersalyl acid (MA), and 1 mM for glutathione (GSH) and dithiothreitol (DTT). The SR vesicles were preincubated for 5 min at room temperature with either glutathione or dithiothreitol before addition of the indicated inhibitor. Values are the means ± SD (N = 3 for CPA or N = 2 for MA treatments).

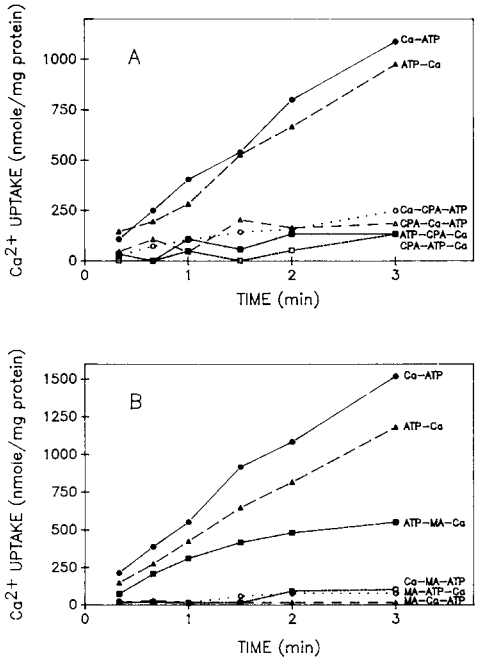

Fig. 3. Effect of the sequence of cofactor additions on cyclopiazonic acid and mersalyl acid inhibition of Ca^{2+} uptake in rat skeletal muscle SR vesicles. Oxalate-assisted Ca^{2+} uptake was measured in SR vesicles as described in Table 1 with and without a 10 μM concentration of either (A) cyclopiazonic acid (CPA) or (B) mersalyl acid (MA). The sequences of addition of $^{45}Ca^{2+}$, ATP and cyclopiazonic acid are indicated.

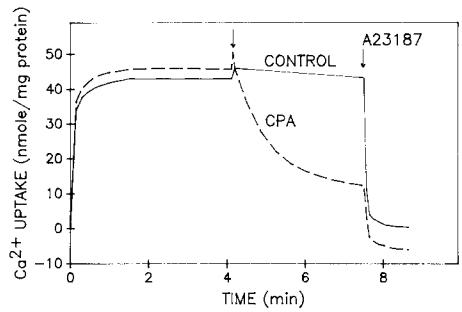

Fig. 4. Effect of cyclopiazonic acid on steady-state Ca^{2+} accumulation of rat skeletal muscle SR vesicles. SR vesicles (0.1 mg protein/ml) in 0.1 M KCl, 10 mM MOPS, 2 mM $MgCl_2$, 1 mM ATP, 20 μM Ca^{2+} and 0.1 mM arsenazo III, pH 7.0, were allowed to reach steady-state levels of Ca^{2+} accumulation. After 4 min of incubation, either 100 nmol cyclopiazonic acid (CPA) in 10 μl DMSO or DMSO only (control) was added. Two microliters of 2 mM A23187 in DMSO was added to both reactions after approximately 8 min. The total volume was 3.0 ml, and Ca^{2+} concentration was determined by monitoring change in 700 − 655 nm absorbance.

Cyclopiazonic acid also inhibited ruthenium red-insensitive efflux of $^{45}Ca^{2+}$ from passively loaded SR vesicles. Control and 10 μM cyclopiazonic acid treated SR vesicles had a $T_{1/2}$ of 4.2 and 14.7 min respectively (Fig. 6A). In contrast, 5 μM mersalyl acid, a concentration resulting in the same degree of inhibition of Ca^{2+} uptake and Ca^{2+}-independent ATPase activity as 10 μM cyclopiazonic acid, increased ruthenium red-insensitive Ca^{2+} efflux to a $T_{1/2}$ of 2.6 min. A23187 produced a rapid loss of bound Ca^{2+} to 5% of the initial levels seen in control and cyclopiazonic acid treated vesicles by 0.5 min. The conditions used for passively loading SR vesicles with Ca^{2+} did not alter the oxalate-assisted Ca^{2+} transport or Ca^{2+}-dependent ATPase activity of the vesicles (data not shown), indicating a functionally intact Ca^{2+}-ATPase.

The efflux of D-[^{14}C(U)]sorbitol from passively loaded SR vesicles (Fig. 6B) was not affected by either 10 μM cyclopiazonic acid ($T_{1/2}$, 4.5 min) or 5 μM mersalyl acid ($T_{1/2}$, 3.3 min) when compared to controls ($T_{1/2}$, 4.1 min). These rates of efflux were

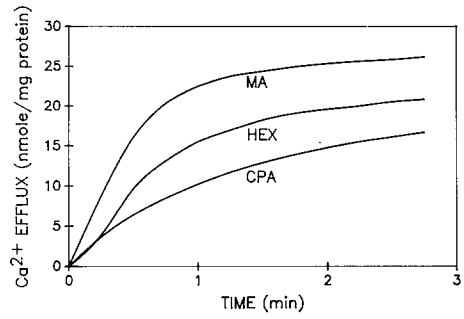

Fig. 5. Effect of cyclopiazonic acid, mersalyl acid and ATP depletion on Ca^{2+} efflux from actively loaded rat skeletal muscle SR vesicles. SR vesicles were actively loaded with Ca^{2+} using conditions described in the legend of Fig. 4 with the exception that 10 mM glucose was included in the buffer for the ATP depletion treatment. After the SR vesicles had reached steady-state Ca^{2+} accumulation levels (23.0 ± 1.2 nmol Ca^{2+}/mg protein, mean ± SD) 100 nmol of either cyclopiazonic acid (CPA) or mersalyl acid (MA) or 8 units of hexokinase (HEX) in 25 μl of buffer was added, and Ca^{2+} efflux was measured by monitoring change in arsenazo III 700 − 655 nm absorbance. Traces are the averages of duplicate determinations for each treatment.

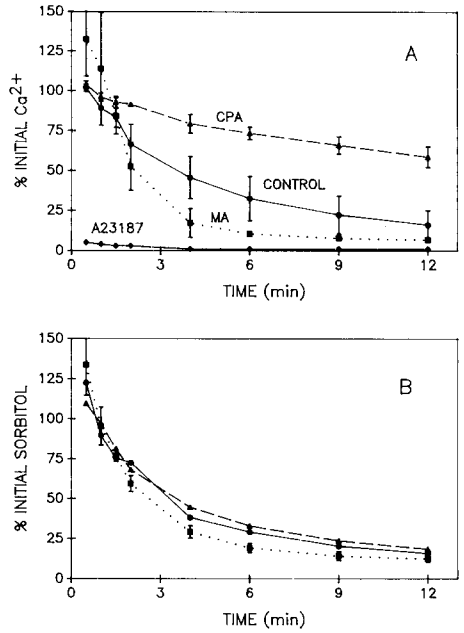

Fig. 6. Efflux of Ca^{2+} and sorbitol from passively loaded rat skeletal muscle SR vesicles and effects of cyclopiazonic acid and mersalyl acid. SR vesicles were passively loaded with Ca^{2+} (A) by incubating for 2 hr at room temperature in 0.1 M KCl, 10 mM MOPS, 5 mM $^{45}Ca^{2+}$, pH 7.0. Efflux was initiated by diluting the SR 200-fold into iso-osmolar Ca^{2+} free release buffer containing 0.1 M KCl, 10 mM MOPS, 5 mM $MgCl_2$, 1 mM EGTA, 10 μM ruthenium red and either 10 μM cyclopiazonic acid (CPA), 5 μM mersalyl acid (MA), 1 μM A23187 or DMSO only for control, as indicated in the figure. At various times, vesicles were collected by rapid filtration, and extravesicular $^{45}Ca^{2+}$ was removed by rinsing with release buffer. The remaining bound $^{45}Ca^{2+}$ was quantified by liquid scintillation counting. (B) SR vesicles were also loaded with sorbitol using conditions similar to those for Ca^{2+} but with 5 mM D-[^{14}C(U)]sorbitol in place of $^{45}Ca^{2+}$ in the loading buffer. The release buffer was also similar but without EGTA and ruthenium red. Treatment identification is the same as shown in panel A. The 100%, time 0, bound $^{45}Ca^{2+}$ and D-[^{14}C(U)]sorbitol levels were determined by extrapolation using linear regression analysis of log cpm. Each line is the mean ± SD (N = 4 for control and cyclopiazonic acid treated Ca^{2+} loaded vesicles and N = 2 for all others).

similar to the 3.8 min $T_{1/2}$ of control Ca^{2+} efflux (Fig. 6A). The rate of $^{22}Na^{+}$ efflux from all SR vesicle treatments was too rapid to be determined by the methods employed here, but was similar to rates seen for Ca^{2+} efflux from A23187-treated vesicles (Fig. 6A), indicating a rapid and complete ion efflux. Assuming initial isotopic loading levels to be similar between Ca^{2+} and Na^{+}, the Na^{+} level at 0.5 min was less than 5% of the initial loading levels. This limitation of the filtration technique for measuring efflux of rapidly penetrating univalent ions, such as Na^{+}, has been reported previously [27].

Effect of cyclopiazonic acid on Ca^{2+} binding and phosphorylation of SR. Cyclopiazonic acid (25 μM) inhibited the binding of Ca^{2+} to the SR when the free Ca^{2+} concentration was 100 μM or less (Fig. 7). Both Ca^{2+} uptake and Ca^{2+}-dependent ATPase activity were inhibited also completely by cyclopiazonic acid at this Ca^{2+} concentration (data not shown). A Scatchard plot of measurable Ca^{2+} binding (Fig. 7, inset) shows that cyclopiazonic acid inhibited the binding of Ca^{2+} to the high affinity binding site. The association constants for the high and low affinity Ca^{2+} binding sites of the untreated SR vesicles were 1.4×10^5/M and 7.3×10^3/M, respectively, whereas the maximum binding capacities were 14 nmol/mg protein and 30 nmol/mg, respectively. Cyclopiazonic acid at 1 μM, the lowest concentration tested, inhibited Ca^{2+}-dependent phosphorylation by approximately 15%, whereas 5 μM cyclopiazonic acid caused 95% inhibition (Fig. 8). When Ca^{2+}-independent phosphorylation (approximately 0.1 nmol phosphate/mg protein) is taken into account, cyclopiazonic acid at 25 μM completely inhibited phosphorylation. This cyclopiazonic acid concentration was used in the Ca^{2+} binding studies where it prevented Ca^{2+} binding to the high affinity site (Fig. 7).

DISCUSSION

The results presented here clearly demonstrate that cyclopiazonic acid inhibits Ca^{2+} binding to a high affinity site. The rat skeletal muscle SR fraction used in this study was a mixture of both light and heavy SR vesicles. Acid slab gel electrophoresis [28] of our SR vesicles indicated that the largest fraction of the total membrane protein migrated in the M_r = 100,000 range (data not shown). This is the molecular weight range of the ATPase enzyme [29]. The maximal Ca^{2+} binding capacity which we determined for our mixed SR fraction (14 nmol/mg protein) was reasonably close to that determined for rabbit SR [29] which was 8–10 nmol/mg protein. The maximal binding capacity of ATPase rich vesicles purified in sucrose gradients was calculated to be 9–11 nmol/mg protein [30]. However, our high affinity association constant was lower than that determined for rabbit SR (2.3×10^6/M) [29], and the value does not correspond well to the characterized high affinity binding site of the purified Ca^{2+} ATPase from rabbit

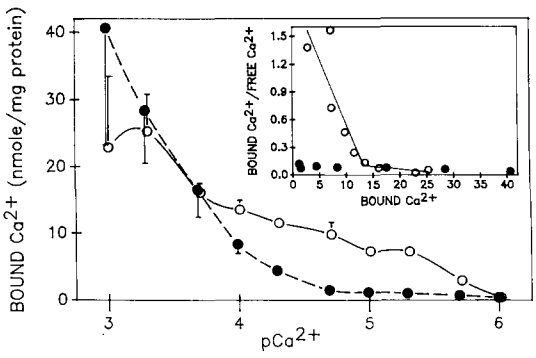

Fig. 7. Binding of Ca^{2+} to rat skeletal muscle SR vesicles with and without 25 μM cyclopiazonic acid. One milliliter of SR vesicles (0.5 mg protein/ml) was dialyzed for 4 hr against 25 ml of 0.1 M KCl, 4 mM $MgCl_2$, 10 mM MOPS, pH 7.0, and various levels of $^{45}Ca^{2+}$ with (solid circles) and without (open circles) 25 μM cyclopiazonic acid. Aliquots of both the SR vesicles and buffer were then analyzed for $^{45}Ca^{2+}$ with the difference between the two being the Ca^{2+} bound to the SR vesicles. Each point is the mean ± SD of triplicate samples taken from a single determination. Where error bars are not present, the data point has covered them completely. The inset shows a Scatchard plot of data of Ca^{2+} concentrations where binding was measurable. Bound and free Ca^{2+} are in units of nmol/mg protein and μmol, respectively.

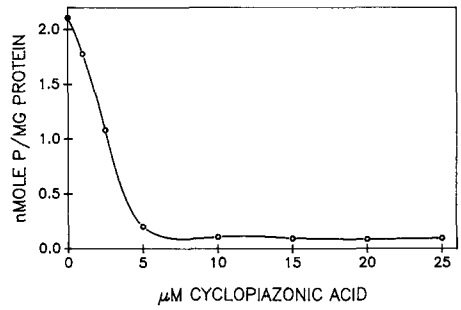

Fig. 8. Effect of cyclopiazonic acid on phosphorylation of rat skeletal muscle SR vesicles. SR vesicles (0.5 mg protein/ml) were phosphorylated with 0.2 mM [γ-^{32}P]ATP (12,000 cpm/nmol) in 0.1 KCl, 4 mM $MgCl_2$, 100 μM Ca^{2+}, 10 mM MOPS, pH 7.0, with and without various levels of cyclopiazonic acid. An ATP-regenerating system of 5 mM phospho(enol)pyruvate and 23 units/ml pyruvate kinase was also included; final volume was 2.0 ml. Reactions were initiated by ATP addition and terminated after 30 sec by addition of 7 ml of 5% trichloroacetic acid containing 2 mM P_i and 0.1 mM ATP. The 10,000 *g* pellet was washed three times and digested in 1 M NaOH by heating for 1 hr in a boiling water bath; then radioactivity and protein were determined.

skeletal muscle [21, 31, 32]. Thus, it is not possible to state conclusively that the affected high affinity Ca^{2+} binding site is that of the Ca^{2+}-ATPase. However, the fact that cyclopiazonic acid also inhibited Ca^{2+}-dependent phosphorylation of the SR vesicles is consistent with the hypothesis that it is Ca^{2+} binding to the Ca^{2+}-ATPase which is inhibited. It is generally accepted that Ca^{2+} binding to the high affinity site is obligatory to the phosphorylation of the ATPase [33]. Studies with purified light SR vesicles and purified Ca^{2+}-ATPase should resolve this question.

The inhibition of both Ca^{2+} uptake and Ca^{2+}-dependent ATPase activity by cyclopiazonic acid was not easily reversed by either dialysis (Fig. 2) or gel filtration (Table 1). Assuming that dialysis and gel filtration removed 95% of the free cyclopiazonic acid, then the free concentration would have been 2.5 nmol/mg SR protein. At this concentration we would predict only a 5% inhibition of Ca^{2+} uptake [10]. The fact that gel filtration only partially reversed the effects of cyclopiazonic acid, in some experiments, suggests that cyclopiazonic acid is either tightly bound or may partition into the lipid phase of the membrane, but is probably not covalently bound to SR vesicles. It is also evident that the mechanism of action does not involve sulfhydryl oxidation since neither glutathione nor dithiothreitol was effective in preventing the inhibitory effects of cyclopiazonic acid (Table 2). Conversely, the inhibitory effects of mersalyl acid, a Ca^{2+}-ATPase inhibitor which is known to be sulfhydryl reactive [16], were prevented by both glutathione and dithiothreitol.

The order in which cyclopiazonic acid, Ca^{2+} and ATP were added had no effect on inhibition of Ca^{2+} transport (Fig. 3A). Conversely, mersalyl acid inhibition of the Ca^{2+}-ATPase was impeded by prior addition of ATP to SR vesicles (Fig. 3B). Hasselbach and Seraydarian [16] first observed the protective effect of ATP and ADP and proposed that this resulted from protection of mersalyl acid-sensitive sulfhydryl groups located at the nucleotide binding site of the Ca^{2+}-ATPase which are essential for enzymatic activity. Although protection was not conferred by ATP in the case of cyclopiazonic acid, this would not eliminate the possibility of a direct or indirect interaction of cyclopiazonic acid at the active site. For example, previous exposure of the SR vesicles to Ca^{2+} before the addition of cyclopiazonic acid did not affect the the inhibition of Ca^{2+} uptake (Fig. 3A), although cyclopiazonic acid did inhibit Ca^{2+} binding to the high affinity site (Fig. 7). Similar results have been observed with dicyclohexylcarbodiimide, a hydrophobic carboxyl reagent that inhibits the Ca^{2+}-ATPase [34]. Like cyclopiazonic acid, dicyclohexylcarbodiimide prevents the binding of Ca^{2+} to the high affinity site. However, unlike cyclopiazonic acid, the inhibition of the Ca^{2+}-ATPase by dicyclohexylcarbodiimide is prevented by previous exposure of the ATPase to Ca^{2+}. There are several possible explanations for the absence of protection by Ca^{2+} against the cyclopiazonic acid-induced inhibition: (i) cyclopiazonic acid may act at some other portion of the ATPase, producing a conformational change in the ATPase, thus altering the binding affinity or accessibility of the site to Ca^{2+}, (ii) cyclopiazonic acid could bind tightly to some portion of the protein and block the access of Ca^{2+} to the binding site, or (iii) cyclopiazonic acid may have a higher binding affinity for some portion of the Ca^{2+} binding site and its preferential occupation of this site would inhibit Ca^{2+} binding. However, removal of free cyclopiazonic acid from treated SR vesicles did not reverse the Ca^{2+}-ATPase inhibition

(Table 1 and Fig. 2), and unless free cyclopiazonic acid is in a slow equilibrium with another bound source (i.e. the lipid phase), it is doubtful whether the last mechanism is responsible. It has been demonstrated that there are electrical changes in hydrophobic regions of SR vesicles and Ca^{2+}-ATPase associated with the binding and release of Ca^{2+} [35]. It has also been shown that cyclopiazonic acid induces electrical alterations at the cytoplasmic surface of the plasma membrane in cultured cells [14]. The relationship between these cyclopiazonic acid induced electrical alterations and inhibition of $^{45}Ca^{2+}$ flux in muscles cells [12] is being investigated currently in our laboratory.

Addition of cyclopiazonic acid initiated the efflux of actively loaded Ca^{2+} from SR vesicles (Figs. 4 and 5). The time required for 50% of the accumulated Ca^{2+} to be released was approximately 1.4 min (Fig. 4) and 1.6 min (Fig. 5) which was much slower than efflux observed with mersalyl acid and hexokinase-treated vesicles (0.25 and 0.75 min respectively; Fig. 5). Assuming that treatment with hexokinase and glucose depleted all ATP, then the rate of Ca^{2+} efflux under these conditions represents the rate of diffusion in the absence of any Ca^{2+} pump activity. The rapid rate of Ca^{2+} efflux from hexokinase-treated vesicles suggests that the vesicles were leaky to Ca^{2+} and that cyclopiazonic acid treatment made the vesicles significantly less leaky. It is unlikley that the reduced Ca^{2+} efflux, relative to hexokinase-treated vesicles, is due to a general alteration in membrane permeability, since cyclopiazonic acid did not alter the efflux of passively loaded [^{14}C(U)]sorbitol (Fig. 6B) from SR vesicles when compared to either control or mersalyl acid treated vesicles. The level of accumulated Ca^{2+} depends on the rate of Ca^{2+} uptake (which has been inhibited) and binding, minus the rate of efflux [36, 37]. It is possible that the Ca^{2+} efflux resulting from ATP depletion may be due, in part, to reversal of the pump. However, Feher and Briggs [37] found no evidence for this in cardiac SR vesicles under conditions similar to those used here. These investigators determined that the efflux observed upon addition of either EGTA or glucose plus hexokinase is a diffusional efflux which is not due to nucleotide-dependent activity of the Ca^{2+}-ATPase [38]. The increased rate of release of Ca^{2+} from mersalyl acid treated SR vesicles is well established [15], and recently both ruthenium red-sensitive and -insensitive pathways have been identified [17]. The fact that the efflux of Ca^{2+} induced by cyclopiazonic acid in the absence of ruthenium red was much slower than in actively loaded vesicles treated with mersalyl acid, a compound known to induce Ca^{2+} release via the Ca^{2+} channel, suggests that cyclopiazonic acid does not activate the ruthenium red-sensitive Ca^{2+} channel. Regardless of the mechanism, it is clear that cyclopiazonic acid modifies both Ca^{2+} pump activity and Ca^{2+} permeability of the SR.

Cyclopiazonic acid also reduced the ruthenium red-insensitive efflux of passively loaded Ca^{2+} from SR vesicles (Fig. 6A) when compared to either untreated or mersalyl acid treated vesicles. In this case, there was no ATP or ADP present for nucleotide-dependent Ca^{2+} flux, and efflux was measured in the presence of ruthenium red which blocks Ca^{2+} release via the Ca^{2+} release channel. When treated with mersalyl acid, the Ca^{2+} permeability of both passively loaded (Fig. 6A) and actively loaded (Fig. 5) SR vesicles increased. It has been demonstrated recently that both a ruthenium red-sensitive and -insensitive release is induced by mersalyl acid treatment of rabbit SR [17]. Thus, in the case of mersalyl acid, Ca^{2+} efflux can occur through alterations in ruthenium red-sensitive channels, ruthenium red-insensitive Ca^{2+} pathways, or alterations in the Ca^{2+} pump protein itself. Similarly, cyclopiazonic acid may react either with the transport protein or with other components of sarcoplasmic reticulum, causing the observed reduction in Ca^{2+} permeability of SR.

The efflux of Ca^{2+} from actively loaded SR vesicles (Fig. 5) was much faster than in passively loaded vesicles (Fig. 6A). The different rates may be partially explained by the difference in Ca^{2+} load between the two preparations. Estimates [37] of the intravesicular Ca^{2+} concentration of actively loaded SR vesicles are between 15 and 35 mM, whereas the maximal intravesicular Ca^{2+} concentration possible in SR vesicles passively loaded in a 5 mM Ca^{2+} buffer would be only 5 mM. Since the diffusional Ca^{2+} efflux is linearly related to Ca^{2+} load [37], a faster rate would be expected from the actively loaded SR vesicles. In addition, differences in the composition of the buffers between the two preparations (i.e. ruthenium red in passively loaded vesicles or ATP, ADP in actively loaded vesicles) may be responsible for differences in Ca^{2+} efflux.

The inhibitory effects of cyclopiazonic acid on the Ca^{2+}-ATPase and Ca^{2+} efflux, relative to mersalyl acid, resemble the effects of vanadate which has been shown recently to inhibit both uptake and efflux of Ca^{2+} in rabbit light SR [17]. Vanadate inhibition of the pump, however, was not sufficient to prevent heavy metal-induced Ca^{2+} release [17]. At this time it is not possible to state whether or not the effects of cyclopiazonic acid on Ca^{2+} efflux involve alterations in the Ca^{2+} pump or are due to effects on Ca^{2+} release mechanisms which do not involve the pump. It is clear that cyclopiazonic acid reduces Ca^{2+} efflux relative to the mersalyl acid and hexokinase treatments. However, it cannot be stated with certainty that cyclopiazonic acid inhibits efflux from SR. To prove inhibition it would be necessary to conduct experiments in which the ATPase was inhibited completely by only one agent; otherwise these studies would be complicated by the fact that a combination of agents would be necessary and the pump rates would not be inhibited equally by the various combinations.

In regard to the reaction scheme proposed by de Meis and Vianna [33], it appears that cyclopiazonic acid acts early in the catalytic cycle to prevent Ca^{2+} transport. Evidence in support of this is cyclopiazonic acid inhibition of Ca^{2+} binding to the high affinity site (Fig. 7) as well as the inhibition of Ca^{2+}-dependent phosphorylation by [γ-^{32}P]ATP (Fig. 8). These data are in agreement with the concept that binding of Ca^{2+} to the high affinity site is the initial step in the reaction that is necessary both for the activation of ATP hydrolysis and for the phosphorylation of the protein [31, 32].

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