Cyclopiazonic acid reduces the coupling factor of the Ca^{2+}-ATPase acting on Ca^{2+} binding

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Received 30 June 2004; revised 6 August 2004; accepted 30 August 2004

Available online 11 September 2004

Edited by Peter Brzezinski

Abstract The mycotoxin cyclopiazonic acid (CPA) is a potent inhibitor of the sarcoplasmic reticulum Ca^{2+}-ATPase. The compound decreases the affinity of the Ca^{2+}-ATPase for Ca^{2+} and reduces the maximum specific activity of the enzyme. Furthermore, CPA abolishes the cooperativity of Ca^{2+} transport, showing a Ca^{2+}/ATP ratio ~1 at any extent of Ca^{2+} saturation. There is also an effect on the Ca^{2+}-binding mechanism, where the addition of CPA results in binding of only half-maximal amount of Ca^{2+} observed in its absence. The experimental data suggest that in the presence of CPA, only a single Ca^{2+} ion binds to the Ca^{2+}-ATPase.

Keywords: Ca^{2+}-ATPase; Sarcoplasmic reticulum; Inhibition; CPA; Ca^{2+}-binding; Coupling factor

1. Introduction

Sarcoplasmic reticulum (SR) Ca^{2+}-ATPase is a 110-kDa membrane protein that consists of 10 transmembrane helices (M1–M10), three cytoplasmic domains and small luminal loops. SR Ca^{2+}-ATPase is a calcium pump transporting two mol of Ca^{2+} across the SR membrane by hydrolytic coupling with one mol of ATP. According to the E_1/E_2 theory [1], the enzyme with bound Ca^{2+} (E_1) is autophosphorylated by ATP to form E_1P. This phosphorylation causes the bound Ca^{2+} ions to be occluded at the transport sites and the subsequent conformational transition to the E_2P form releases Ca^{2+} into the lumen. Finally, dephosphorylation takes place and returns the enzyme into the unphosphorylated and Ca^{2+}-unbound E_2 form.

Cyclopiazonic acid (CPA), a mycotoxin produced by certain strains of *Penicillium cyclopium* and *Aspergillus flavus* [2], is a specific and potent inhibitor of sarco-endoplasmic reticulum Ca^{2+}-ATPase [3–5]. The specificity of CPA for SR Ca^{2+}-ATPase and not for other cation ATPases was also established [4]. The affinity of SR Ca^{2+}-ATPase for CPA is dependent on the conformational state of the enzyme, being high in the absence of Ca^{2+} but low in its presence [5], because CPA binds to the Ca^{2+}-ATPase E_2 intermediate and block enzyme turnover [6]. ATP protected the enzyme against inhibition by CPA, while Ca^{2+} had only moderate effect on the extent of inhibition [4]. Thus, CPA decreases enzyme affinity for ATP but do not compete for the same binding site [6], because the CPA binding domain is in the S3 stalk segment (cytoplasmic prolongation of M3) of the Ca^{2+}-ATPase [7].

The present work shows that binding of CPA to the Ca^{2+}-ATPase results in an uncoupling of ATPase activity by changing the extent of Ca^{2+} binding, from the usual two Ca^{2+} ions per ATPase molecule to one.

2. Materials and methods

2.1. Materials

[^{3}H]Glucose and $^{45}CaCl_2$ were obtained from DuPont NEM. A stock solution of CPA from *Penicillium cyclopium* (Sigma) was prepared in ethanol. The volume of ethanol added did not exceed 1% of the total volume.

2.2. Sample preparation

A microsomal fraction of SR membrane enriched in Ca^{2+}-ATPase protein was isolated from rabbit leg white muscle as previously described [8]. The protein content was estimated by the method of Lowry et al. [9] using bovine serum albumin (BSA) as a standard.

2.3. Free Ca^{2+} concentration

Reaction media with different free Ca^{2+} concentration were prepared with the aid of a Ca^{2+}-EGTA buffer according to a computer program [10] that takes into consideration the association constant for the Ca^{2+}-EGTA complex [11] and the equilibrium constants for the EGTA protonation [12].

2.4. ATPase activity

The rate of ATP hydrolysis was measured at room temperature following the liberation of inorganic phosphate (Pi) [13]. ATPase activity was assayed in a reaction mixture containing 0.05 mg of SR/ml, 20 mM Mops (pH 7), 80 mM KCl, 5 mM $MgCl_2$, 5 mM potassium oxalate, 1 mM EGTA, 0.967 mM $CaCl_2$ (pCa 5) and different CPA concentrations. After 5 min of incubation, the reaction was started by the addition of 1 mM ATP. The rates of Pi release were calculated from the initial phase of time course plots. Ca^{2+}-independent ATPase activity was assayed in the presence of 2 mM EGTA and no added Ca^{2+}. The Ca^{2+}-dependent ATPase activity was estimated by subtracting the Ca^{2+}-independent ATPase from the total activity. The Ca^{2+} dependence of the ATPase activity was measured in presence of 1 mM EGTA and different Ca^{2+} concentrations to yield the desired free concentration.

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Abbreviations: CPA, cyclopiazonic acid; SR, sarcoplasmic reticulum; Pi, inorganic phosphate; pCa, negative logarithm of the molar free Ca^{2+} concentration

0014-5793/$22.00
doi:10.1016/j.febslet.2004.08.064

2.5. Ca^{2+} transport

The active transport of Ca^{2+} was measured at room temperature by the radioactive tracer method. The incubation medium included 0.05 mg of SR/ml, 20 mM Mops (pH 7), 80 mM KCl, 5 mM $MgCl_2$, 5 mM potassium oxalate, 1 mM EGTA, different $^{45}CaCl_2$ (~4000 cpm/nmol), 0 or 0.75 μM CPA and 1 mM ATP. The Ca^{2+} accumulated inside the vesicles was determined by filtering aliquots of 1 ml (0.05 mg of protein) on HAWP Millipore filters (0.45 μm) at serial time intervals. The filters were rinsed with 3 ml of medium containing 20 mM Mops (pH 7), 80 mM KCl, 5 mM $MgCl_2$ and 1 mM $LaCl_3$ before counting the $^{45}Ca^{2+}$ retained on the filters. The rates of Ca^{2+} transport were calculated from the initial phase of time course plots.

2.6. Ca^{2+} binding

The high affinity Ca^{2+} binding was measured by the double-labeling filtration technique [14]. The enzyme (0.3 mg of SR/ml) was incubated at room temperature in a medium consisting of 20 mM Mops (pH 7), 80 mM KCl, 5 mM $MgCl_2$, 69.4 μM EGTA, 1 mM [^{3}H]Glucose (~1000 cpm/nmol), different concentrations of CPA and 100 μM $^{45}CaCl_2$ (~5000 cpm/nmol; pCa 4.5). After equilibration for 5 min, aliquots of 0.2 ml (0.06 mg of protein) were placed onto filters (Millipore HAWP 0.45 μm) previously soaked in unlabeled medium and subjected to vacuum. Counting of radioactive tracers in the incubation medium and the filters allowed to determine the Ca^{2+} bound by subtracting the non-specific free Ca^{2+} trapped in each filter (^{3}H labeling).

2.7. Measurement of parameters

The curves were fitted to the Hill equation expressed as a function of the $K_{0.5}$:

$$Y = N \times Ca^h/(K_{0.5}^h + Ca^h)$$

Y being the experimental values, N the maximum Ca^{2+}-ATPase or Ca^{2+} transport activities, $K_{0.5}$ the Ca^{2+} concentration needed for half-activity or apparent affinity constant and h the Hill coefficient.

2.8. Data presentation

The experimental values represent the average of at least three independent experiments. The standard deviations of the mean values are given when indicated.

3. Results

3.1. Ca^{2+}-ATPase inhibition

CPA dependence of Ca^{2+}-ATPase activity. The effect of CPA was initially assessed by measuring the hydrolytic capacity of the Ca^{2+}-transporting ATPase. Thus, SR vesicles in the presence of 5 mM potassium oxalate and increasing concentrations of CPA were preincubated under optimal conditions to measure the Ca^{2+}-dependent ATPase activity. As depicted in Fig. 1, the inhibitory effect elicited by CPA was dependent on the concentration used as has been shown previously [3–5,7,15]. These data provide half-inhibition value of approximately 0.2 μM. Thus, assuming from the maximal EP level that 1 mg of SR protein contains ~4 nmol of Ca^{2+}-ATPase active sites (data not shown) [16], it can be deduced a drug:enzyme molar ratio ~1:1 at 0.2 μM.

Ca^{2+}-dependence of hydrolytic and transport activities. Steady-state experiments on ATP hydrolysis and Ca^{2+} transport were suitable to provide information on the coupling phenomenon.

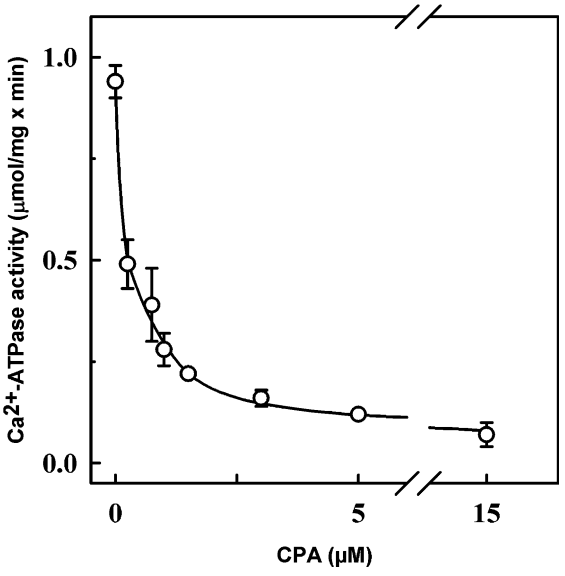

Fig. 1. Inhibition of the steady-state ATPase activity of Ca^{2+}-ATPase by CPA. The Ca^{2+}-ATPase activity as a function of CPA concentrations at pCa 5.

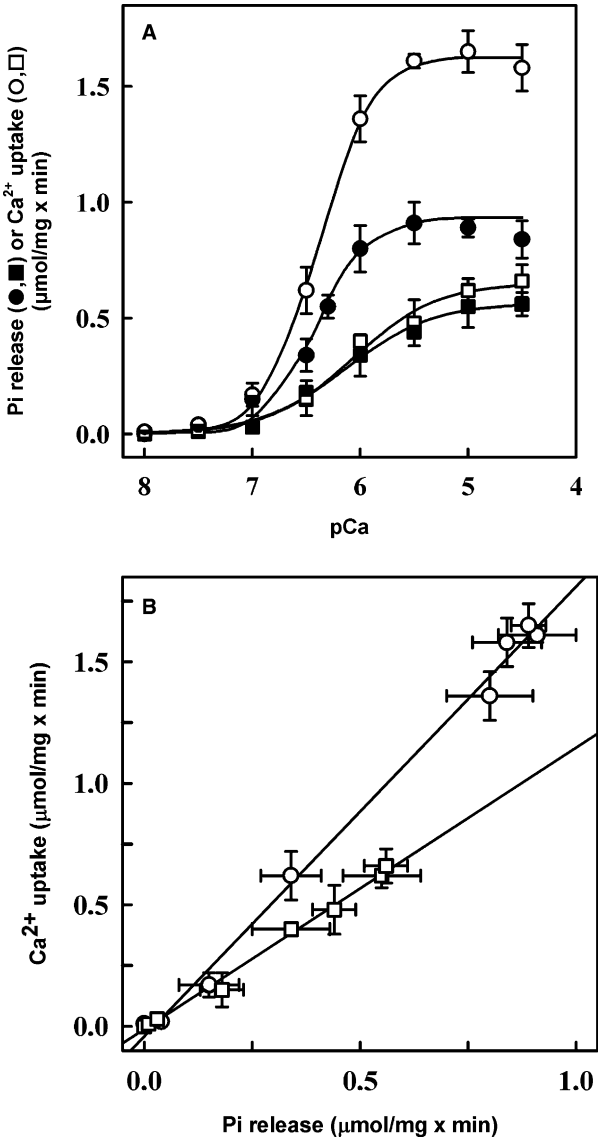

Fig. 2. Steady-state experiment of Ca^{2+} transport and ATP hydrolysis. (A) Ca^{2+} dependence on the Ca^{2+}-ATPase activity (●, ■) or Ca^{2+} transport (○, □) of Ca^{2+}-ATPase measured in the absence (circles) or presence of 0.75 μM (squares) CPA. (B) Plot of Ca^{2+} transport versus Pi release measured at different pCa to calculate the coupling factor at 0 (○) or 0.75 μM (□) CPA.

Table 1
Parameters and coupling values describing the Ca^{2+}-ATPase activity and Ca^{2+}-uptake in the presence and in the absence of 0.75 μM CPA

	Pi release (●, ■)			Ca^{2+} uptake (○, □)			Ca^{2+}/Pi coupling
	$K_{0.5}$ (μM)	N (nmol/mg min)	h	$K_{0.5}$ (μM)	N (nmol/mg min)	h	
Control	0.39	0.93	1.9	0.41	1.63	1.8	1.9
0.75 μM CPA	0.74	0.57	1.1	0.86	0.65	1.1	1.1

The data from Fig. 2 were fitted to Hill equation.

The presence of CPA reduces the apparent affinity ($K_{0.5}$) of the enzyme for Ca^{2+} from 0.39 μM in the absence of CPA to 0.74 μM at 0.75 μM CPA (Fig. 2A); therefore, higher Ca^{2+} concentration are needed to express the same enzymatic activity. In addition, lower maximum ATPase activities were observed at higher CPA concentrations. Thus, the maximum specific activity of Ca^{2+}-ATPase (N) was 0.93 μmol/mg min and it was reduced to 0.57 μmol/mg min in presence of 0.75 μM CPA. Meanwhile, the Hill coefficients (h) decreased from 1.9 to 1.1 (Table 1).

The effect of CPA on the Ca^{2+}-transporting activity is also shown in Fig. 2A. In the absence of CPA, the data could be fitted assuming a $K_{0.5}$, N and h values of 0.41 μM, 1.63 μmol/mg min and 1.8, respectively, and in presence of 0.75 μM CPA, 0.86 μM, 0.65 μmol/mg min and 1.1, respectively (Table 1). Therefore, under steady-state conditions, the ATPase inhibition by CPA is not reversed by addition of higher Ca^{2+} concentration (Fig. 2A).

The stoichiometric ratio between Ca^{2+} transport and Pi release in the presence of different free Ca^{2+} concentrations can be obtained from Fig. 2B. These experiments are suitable to provide information on the coupling phenomenon because the passive permeability of the vesicles to Ca^{2+} after the CPA treatment is not increased (data not shown) [17]. The coupling between both processes is given by the slope of the straight lines, which is ~1.9 in control and ~1.1 in the presence of CPA. These data clearly show that in control, the Ca^{2+}:ATP coupling remains unaltered (~2) and in presence of CPA it is reduced (~1) within the Ca^{2+} concentration range used to saturate the binding sites (Table 1).

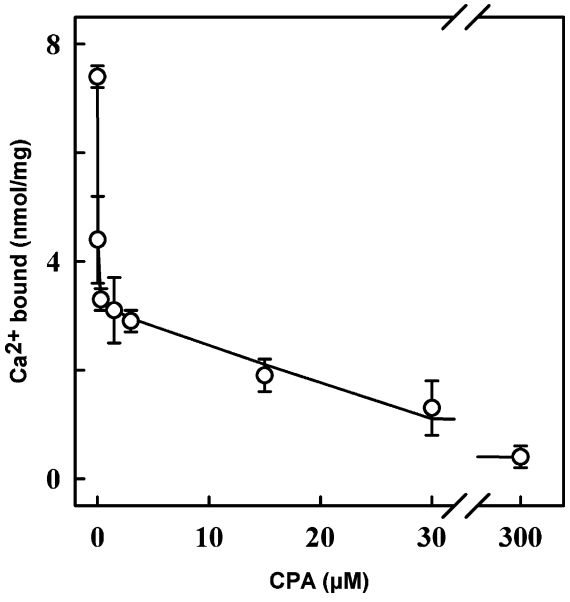

Fig. 3. Effect of CPA on Ca^{2+} binding by Ca^{2+}-ATPase. Binding of $^{45}Ca^{2+}$ to Ca^{2+}-ATPase as a function of CPA concentration at pCa 4.5.

3.2. Ca^{2+} binding

In a different set of experiments, the binding of Ca^{2+} to the Ca^{2+}-ATPase protein under equilibrium conditions was investigated. The incubation medium, with a free Ca^{2+} concentration of ~32 μM (pCa 4.5), was supplemented with 1 mM [^{3}H]glucose in order to evaluate the Ca^{2+} actually bound to the protein after the filtration. Thereby, Fig. 3 shows the effect of CPA on the level of $^{45}Ca^{2+}$ bound to the Ca^{2+}-ATPase. Addition of CPA at a toxin/enzyme molar ratio $\leqslant 1$ to the enzyme reduced the maximal Ca^{2+} binding (~7.5 nmol/mg of protein) to the half (~3.2 nmol/mg of protein). When CPA above an equimolar level was added, the effect was less sharp. Thus, the Ca^{2+} binding capacity tended to decrease and the inhibition was almost complete when the concentration of CPA was increased up to 300 μM.

4. Discussion

In the present work, the effect of CPA on Ca^{2+}-binding to the SR Ca^{2+}-ATPase has been investigated, because most of Ca^{2+}-ATPase inhibitors act or affect the Ca^{2+}-binding mechanism [16,18]. CPA concentration dependently inhibits the activity of the SR Ca^{2+}-ATPase (Fig. 1), as has been shown before [3–5,7,15], and the calculated $K_{0.5}$ (~0.2 μM) and molar ratio (~1) are in agreement with those previously published results [5,7,15].

The most important result presented in this study is the demonstration that CPA acts reducing the stoichiometric ratio between Ca^{2+} transport and Pi release of the Ca^{2+}-ATPase of SR, that is, CPA uncouples ATPase activity. In vesicles derived from rabbit white muscle, the hydrolysis of one ATP molecule leads to the translocation of two Ca^{2+} ions across the membrane. This was determined by measuring the ratio between the initial rates of Ca^{2+} uptake and ATP hydrolysis (Fig. 2). In the presence of CPA, the uncoupled hydrolysis of Ca^{2+}-ATPase promotes a decrease on the Ca^{2+}/ATP ratio to values ~1. The data of Fig. 2 and Table 1 show that CPA reduces the Ca^{2+}/ATP ratio at all Ca^{2+} concentrations tested. This conclusion is also supported by the Ca^{2+}-binding experiments (Fig. 3). Thereby, the stoichiometry of the high affinity binding sites was determined and was found to be ~7.5 nmol/mg of protein in the absence of CPA (control), which corresponds to two Ca^{2+} sites per ATPase monomer, when com-

pared with the maximum phosphorylation (data not shown, [16]). In the presence of CPA at molar ratio ~1, the data were ~3.4 nmol/mg of protein, which corresponds to one Ca^{2+} site per ATPase monomer. In agreement with these data, the Hill coefficient indicates positive cooperativity in the absence of CPA (with two Ca^{2+} binding sites) and non-cooperativity in the presence of CPA (with only one site). Therefore, it seems clear that one Ca^{2+}-binding site is destroyed. However, this finding was not obtained clearly in a previous work where the Ca^{2+} binding to the Ca^{2+}-ATPase was also measured after incubation with CPA (see Fig. 1 in [5]), although the experiments were carried out under different conditions.

Previous observations have suggested that CPA interferes with Ca^{2+} binding and with the Ca^{2+}-induced changes in the conformation of Ca^{2+}-ATPase [4,17,19].

The mechanism of action of CPA is quite similar to thapsigargin (TG), the most specific, potent and the best characterized inhibitor of SR Ca^{2+}-ATPase [20]. It has been shown that binding of TG to the Ca^{2+}-ATPase also results in a change in extent of Ca^{2+} binding, from the usual two Ca^{2+} ions per ATPase molecule to one [21].

The two Ca^{2+} binding sites (I and II) are surrounded by helices M4, M5, M6 and M8 [22], and the N-terminal region of M3 helix plays an important role in controlling the Ca^{2+} entry pathway [23]. The CPA-binding domain resides at the membrane interface and interacts with S3 stalk segment (cytoplasmic prolongation of M3) of the Ca^{2+}-ATPase [7], which can explain the effect on the Ca^{2+}-binding sites. Thereby, changes in the packing of the transmembrane α-helices result in changes at the Ca^{2+}-binding site [24] and large-scale rearrangements of transmembrane helices take place during Ca^{2+}-bound and Ca^{2+}-free structures transition. As a result, the number of oxygen atoms that can coordinate to Ca^{2+} decreases [22] and site I appears to be more intact in the Ca^{2+}-free form than site II. It was suggested that in the process of Ca^{2+}-binding, Ca^{2+} ions pass through site II to reach site I, and that this would only be possible if site II were not properly formed in the absence of Ca^{2+} at site I. Comparison of the Ca^{2+}-free and Ca^{2+}-bound structures suggests that a single binding site for Ca^{2+} could exist in the Ca^{2+}-free structure [25]. The Ca^{2+}-free structure is the equivalent to E_2 conformation to which the CPA is bound [6]. Therefore, CPA may act stabilizing this conformational state of the Ca^{2+}-ATPase that can bind only one Ca^{2+}, thus altering the binding affinity and accessibility of the site to Ca^{2+}, or could block the access of Ca^{2+} to one of the binding sites. However, this is only a speculative model and other possibilities remain open.

The effect of CPA on Ca^{2+}-binding of SR Ca^{2+}-ATPase described here is additional to the mechanism involved in the inhibition that has been deeply described before [5,6].

Acknowledgements: I thank Alicia Torrado for valuable comments on the manuscript.

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