Biochemical Pharmacology, Vol. 43, No. 8, pp. 1797–1803, 1992.
Printed in Great Britain.

0006-2952/92 $5.00 + 0.00

DISTINCT MECHANISMS OF INHIBITION OF PURIFIED CARDIAC SARCOLEMMA Ca^{2+}-ATPase BY TWO CALMODULIN ANTAGONISTS

TANIA C. PASA,* ANGELA OTERO,† HECTOR BARRABIN* and HELENA M. SCOFANO*‡

*Departamento de Bioquimica, Instituto de Ciencias Biomedicas, Centro de Ciencias da Saude, Universidade Federal do Rio de Janeiro, Cidade Universitaria, Rio da Janeiro, RJ, Brasil; and †Department of Physiology, University of Virginia, Charlottesville, VA 22901, U.S.A.

(*Received* 19 *April* 1991; *accepted* 23 *December* 1991)

Abstract—The effects of calmidazolium and compound 48/80 were studied in four different states of activation of the purified Ca^{2+}-ATPase from cardiac sarcolemma: "basal" or unactivated, activated by calmodulin, activated by phosphatidylserine, and activated by controlled trypsinization. When assayed in the presence of phosphatidylcholine as the sole phospholipid (basal state), the purified enzyme was resistant to inhibition by calmidazolium (0.1 to 3 μM). In the same range, calmidazolium inhibited the enzyme activated by controlled proteolysis as well as the calmodulin-activated enzyme regardless of the calmodulin concentration. The phosphatidylserine-activated enzyme was inhibited at higher calmidazolium concentrations due to non-specific trapping of the inhibitor by the excess of phospholipid. Adition of calmidazolium did not modify the $K_{0.5}$ for calcium activation of ATP hydrolysis by the enzyme. The inhibition by calmidazolium was counteracted by P_i. Compound 48/80 also had no effect on the enzyme when only phosphatidylcholine was present and, like calmidazolium, it inhibited the calmodulin-activated enzyme and the phosphatidylserine-activated enzyme. The apparent K_i for inhibition by compound 48/80 was dependent on the calmodulin concentration. However, the enzyme activated by controlled trypsinization was insensitive to compound 48/80. Binding of 48/80 to the enzyme in the presence of phosphatidylserine or calmodulin reversed the increased affinity for Ca^{2+} caused by these activators.

Living cells maintain a cytosolic Ca^{2+} concentration far below that existing outside the plasma membrane. A Ca^{2+} pump ATPase has now been identified in plasma membranes from a wide variety of cells [1–3]. In the plasma membranes of cardiac cells, Ca^{2+}-ATPase is one of the two principal systems responsible for Ca^{2+} extrusion, the other being a reversible Na^+–Ca^{2+} exchanger [1–3]. The sarcolemmal Ca^{2+}-ATPase of the heart has a higher affinity for Ca^{2+} and a lower maximal velocity of Ca^{2+} extrusion than the Na^+–Ca^{2+} exchanger; it has been proposed that the enzyme maintains the low intracellular Ca^{2+} levels observed during diastole [4].

Ca^{2+}-ATPase belongs to a family of tissue-specific, calmodulin-activated Ca^{2+} pumps that are coded by multiple genes [5, 6]. Calmodulin is tightly bound to plasma membranes of most cells, and the use of calmodulin antagonists to study the regulatory role of calmodulin is common. Although these antagonists bind to calmodulin in a water solution, the site of action has not been determined for any of them, nor has the mechanism of inhibition been clearly demonstrated on a biological calmodulin-ATPase system.

In this study we explored the effects of two of the most widely used calmodulin antagonists on cardiac plasma membrane Ca^{2+}-ATPase: calmidazolium, one of the most potent antagonists, and compound 48/80, one of the most specific antagonists described thus far [7–10]. For compound 48/80, mechanisms as distinct as interaction with associated polyphosphoinositides, binding to calmodulin, and binding to the enzyme have been proposed [7, 8, 11–13]. For calmidazolium, it has been shown that the drug binds to several forms of activated enzymes in the absence of calmodulin, and it is proposed that calmidazolium binds with greater affinity to the calmodulin-activated form [7–9, 14]. In the heart, calmidazolium has been reported to delay ischemic injury; this effect has been attributed to its action as a calmodulin antagonist [15]. Lamers *et al.* [16, 17] have proposed that calmidazolium interferes with the binding of calmodulin to cardiac sarcolemma Ca^{2+}-ATPase, based on a more potent inhibition when the preparation contains calmodulin than when calmodulin is removed [16, 17]. These authors used membrane preparations.

In the present study, we used purified enzyme to ensure the complete absence of endogenous activators or contaminants and to eliminate effects of these antagonists on the other protein components of the membrane. It has been proven that compound 48/80, for example, affects G proteins, which are present in the membrane of cardiac sarcolemma and inhibit the Ca^{2+}-ATPase [18, 19]. The inhibition was studied in the basal and in several activated states of the Ca^{2+}-ATPase. Our results show that calmidazolium bound at a site that was retained after enzyme trypsinization while the binding site of

‡ Corresponding author: Dr. Helena Maria Scofano, Departamento de Bioquimica, ICB, Centro de Ciencias da Saude, Universidade Federal do Rio de Janeiro, Cidade Universitaria, CEP 21910, C.P. 68041, Rio de Janeiro, RJ, Brasil.

compound 48/80 was lost by trypsinization. In addition, we show that the inhibition by compound 48/80 simulated depletion of calmodulin, whereas calmidazolium inhibited by a different mechanism.

MATERIALS AND METHODS

The reagents used were of the highest purity available. Phosphatidylcholine, phosphatidylserine, bovine brain calmodulin, trypsin (TPCK* treated, T8642) and trypsin inhibitor (T9003) were obtained from Sigma. Calmidazolium was obtained from Boehringer Mannheim and compound 48/80 from Sigma. Phosphatidylserine was clarified by sonication before use. Calmodulin–Sepharose 4B was obtained from Pharmacia. $^{32}P_i$ was obtained from the Brazilian Institute of Atomic Energy; [γ-^{32}P]ATP was prepared according to Glynn and Chappell [20] as previously described [21]. AuroDye was obtained from Hoefer Scientific Instruments.

Purification of Ca^{2+}-ATPase by calmodulin affinity chromatography. Sarcolemmal membranes were obtained from beef heart [22], and purified essentially as described by Caroni *et al.* [23], with the exception that prior to calmodulin depletion the membranes were subjected to four freeze–thaw cycles to ensure disruption of intact vesicles [22]. Phenylmethylsulfonyl fluoride (PMSF, 0.2 mM) was added during homogenization in Triton X-100 to increase the yield of active enzyme. Purified enzyme was stored in liquid nitrogen in a medium containing 0.6 M sucrose, 0.5 M KCl, 20 mM HEPES, pH 7.4, 0.1 mM $CaCl_2$, 1 mM $MgCl_2$, 0.5 mg/mL phosphatidylcholine, 0.05% (v/v) Triton X-100 and 1 mM dithioerythritol (storage buffer). The concentration of purified enzyme was determined by the colloidal gold method [24] using bovine serum albumin as a standard. Because components of the storage buffer interfere with this assay, all samples were dialyzed against water prior to protein determination.

Controlled trypsinization of the purified Ca^{2+} ATPase. The purified Ca^{2+}-ATPase (50–300 μL) eluted from the calmodulin column was digested with trypsin for 20 min on ice at a ratio of 1 μg of trypsin to 3 μg of enzyme in the storage buffer, as described by Caroni *et al.* [23]. The proteolytic reaction was arrested by adding a 50-fold molar excess of soybean trypsin inhibitor. After proteolysis, the enzyme attains maximal levels of activity and is not further activated by calmodulin.

Assay of Ca^{2+}-ATPase activity. The release of $^{32}P_i$ from [γ-^{32}P]ATP was measured as previously described [25, 26]. The purified enzyme together with phosphatidylcholine derived from the column (0.02 mg/mL) was incubated at 37° in the reaction medium described in the figure legends. When additional lipid was added to the assay medium, 1 μM A_{23187} was also present. Free Ca^{2+} concentrations were calculated as described by Fabiato and Fabiato [27] using the dissociation constant of the calcium–EGTA complex reported by Schwarzenbach *et al.* [28]. Reactions were initiated by the addition of 2 mM [γ-^{32}P]ATP and stopped after hydrolysis of 5–10% of the ATP. When inhibitors were used, the enzyme was preincubated with the compounds for 10 min at room temperature in the storage buffer before starting the reaction. Since the inhibitors were dissolved in ethanol, controls in which the Ca^{2+}-ATPase was preincubated with ethanol were performed. The ethanol concentration in the reaction mixture never exceeded 1% and had no effect on the values obtained. Results were averaged from several experiments using two to three different purified enzyme preparations with activities ranging between 2.5 and 4.0 μmol $P_i \cdot min^{-1} \cdot (mg\ protein)^{-1}$, when measured under the optimal standard assay conditions depicted in the legend of Fig. 1. Variability among experiments performed in different preparations ranged from 5 to 10%.

Polyacrylamide gel electrophoresis. Lyophilized samples were subjected to electrophoresis on polyacrylamide gels (10%) containing sodium dodecyl sulfate (SDS) [29]. The gels were stained with silver [30].

RESULTS

The purified Ca^{2+}-ATPase eluted from the calmodulin affinity column hydrolyzes ATP at a low rate and is in a state that has been termed "basal" or "unactivated". The enzyme can be activated by the addition of calmodulin ($K_{0.5}$ = 0.18 μg/mL), by the addition of phosphatidylserine ($K_{0.5}$ = 0.015 mg/mL) or, alternatively, by controlled trypsinization. When the cardiac Ca^{2+}-ATPase was activated by controlled trypsinization, maximal levels of enzyme activity were observed after 10 min of trypsinization, and the activity did not change with treatment up to 30 min. In this period of time, the 140 kDa band of purified enzyme disappeared and was replaced by a small amount of a subfragment of 81 kDa and substantial amounts of subfragments of 66, 60, 53, 49 and 33 kDa. It should be noticed that this pattern is distinct from that observed for erythrocyte Ca^{2+}-ATPase, which, due to the ease in obtaining it, has been taken as a paradigm for the plasma membrane Ca^{2+}-ATPases.

When the cardiac enzyme in the basal state was preincubated with 0.2 to 2 μM calmidazolium, no inhibition was observed (Fig. 1). The basal state was inhibited only at much higher concentrations, the threshold of inhibition being 4 μM (data not shown). Addition of 2 μg/mL calmodulin increased the rate of ATP hydrolysis 3-fold. As reported for membrane-bound Ca^{2+}-ATPase [16, 17], calmidazolium was a potent inhibitor of the purified calmodulin-activated Ca^{2+}-ATPase, with a $K_{0.5}$ of 1–2 μM under these conditions. Similar behavior was observed in the presence of either a saturating (2 μg/mL) or subsaturating (0.2 μg/mL) concentration of calmodulin.

It is evident from Fig. 1 that calmidazolium is also an effective inhibitor of the enzyme activated by limited proteolysis or by phosphatidylserine. The concentrations of calmidazolium required for 50% inhibition were similar when the enzyme was

* Abbreviations: TPCK, L-1-tosylamide-2 phenylethylchloromethyl-ketone; PMSF, phenylmethylsulfonyl fluoride; SDS, sodium dodecyl sulfate; EGTA, ethylene glycol bis (β-aminoethyl ether) *N,N,N',N'*-tetraacetic acid; BTP, 1,3-bis [tris(hydroxymethyl)methylamino]-propane); and HEPES, *N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid.

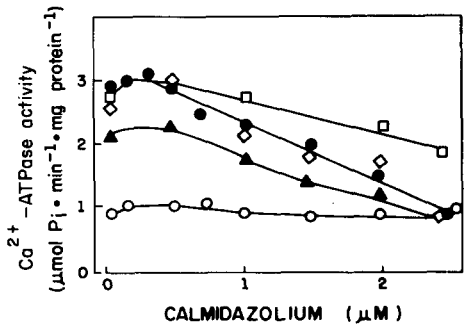

Fig. 1. Effects of calmidazolium on the different states of activation of purified sarcolemmal Ca^{2+}-ATPase. The assay medium contained 1.6 μg/mL purified enzyme, 0.02 mg/mL phosphatidylcholine, 20 mM BTP-HCl, pH 7.0, 80 mM KCl, 5 mM $MgCl_2$, 0.2 mM $CaCl_2$, EGTA to give 6 μM free calcium, the indicated calmidazolium concentrations and: (○) no additions; (●) enzyme activated by the addition of 2.0 μg/mL calmodulin; (▲) enzyme activated by the addition of 0.2 μg/mL calmodulin; (□) enzyme activated by the addition of 0.06 mg/mL phosphatidylserine; and (◇) enzyme activated by controlled proteolysis. ATPase activity was measured as described under Materials and Methods.

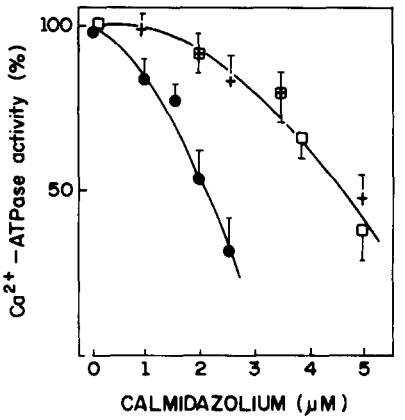

Fig. 2. Effects of lipids on the inhibition by calmidazolium of calmodulin-activated Ca^{2+}-ATPase. The same reaction medium as indicated in the legend of Fig. 1 was used with: (●) 0.02 mg/mL phosphatidylcholine and 2.0 μg/mL of calmodulin; (+) 0.08 mg/mL phosphatidylcholine and 2.0 μg/mL calmodulin; and (□) 0.02 mg/mL phosphatidylcholine, 0.06 mg/mL phosphatidylserine and no calmodulin. ATPase activity was measured as described under Materials and Methods. The maximal ATPase activity corresponds to 2.8 μmol $P_i \cdot min^{-1} \cdot mg^{-1}$ for all three curves. Values are means ± SD of four preparations.

activated by calmodulin or by controlled proteolysis. When the enzyme, which is already in phosphatidylcholine, was activated by the addition of an excess of phosphatidylserine, a higher concentration (about 4.5 μM) was required (Fig. 1). This higher value most probably reflects non-specific trapping of calmidazolium by the excess of phospholipid (phosphatidylcholine + phosphatidylserine) present in the reaction medium during activation by phosphatidylserine [14], since a higher concentration of calmidazolium was also required if excess phosphatidylcholine was added when the enzyme was activated by calmodulin rather than by phosphatidylserine (Fig. 2). At the same total phospholipid concentration, the apparent $K_{0.5}$ for inhibition by calmidazolium was in the same concentration range regardless of whether the enzyme was activated by calmodulin or by phosphatidylserine (Fig. 2).

A different pattern was observed for the inhibition of purified Ca^{2+}-ATPase by compound 48/80. The basal state was insensitive to compound 48/80 up to 100 μg/mL, whereas the enzyme activated by either calmodulin or phosphatidylserine was inhibited (Fig. 3). The concentration of compound 48/80 required for 50% inhibition of ATP hydrolysis was in the range of 15 μg/mL. In marked contrast to the results obtained here with calmidazolium and by others with the red blood cell enzyme [13], the trypsinized enzyme was completely insensitive to compound 48/80 (Fig. 3).

Another difference in the behavior of these antagonists was revealed at subsaturating calmodulin concentrations. While the apparent $K_{0.5}$ for inhibition by compound 48/80 decreased from 15 to 3 μg/mL when the concentration of calmodulin was lowered from 2 to 0.2 μg/mL, the apparent $K_{0.5}$ for inhibition

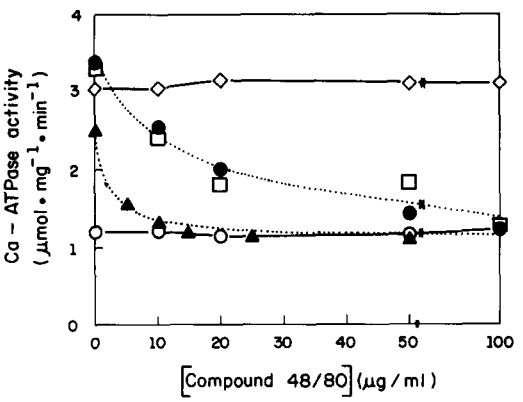

Fig. 3. Effects of compound 40/80 on the different states of activation of purified sarcolemmal Ca^{2+}-ATPase. Symbol key: (○) basal state; (●) enzyme activated by the addition of 2.0 μg/mL calmodulin; (▲) enzyme activated by the addition of 0.2 μg/mL calmodulin; (□) enzyme activated by the addition of 0.06 mg/mL phosphatidylserine; and (◇) enzyme activated by controlled proteolysis. ATPase activity was measured as described under Materials and Methods. Free calcium concentration was 6 μM. The curves for inhibition by compound 48/80 in the presence of compound 48/80 are simulations performed as described in the text, using the equation $v = 1.15 \cdot [(1/a + A/b)/(1 + I/(a \cdot K_i)) + (1/(1 + a \cdot K_i/I))]$ where $a = 1 + Cd/K_{cd}$, $b = 1 + K_{cd}/Cd$. Cd is the concentration of calmodulin, K_{cd} is the dissociation constant for calmodulin, I is the concentration of compound 48/80, K_i is the dissociation constant for compound 48/80, A is the factor of activation induced by calmodulin, and 1.15 is the experimental activity obtained in the basal state.

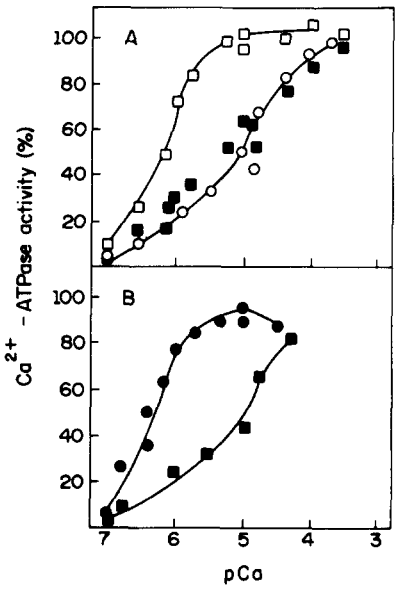

Fig. 4. Effect of compound 48/80 on the Ca^{2+} dependence of the purified Ca^{2+}-ATPase of heart sarcolemma. Purified Ca^{2+}-ATPase (1.0 μg/mL) was incubated in a reaction medium containing 0.02 mg/mL phosphatidylcholine, 20 mM BTP-HCl, pH 7.0, 80 mM KCl, 5 mM $MgCl_2$, 0.2 mM $CaCl_2$ and EGTA to give the desired free calcium concentrations and, in Fig. 4A: (○) no additions; (□) 0.06 mg/mL phosphatidylserine or (■) 0.06 mg/mL phosphatidylserine plus 50 μg/mL compound 48/80. In Fig. 4B: (●) 2 μg/mL calmodulin or (■) 2 μg/mL calmodulin plus 50 μg/mL compound 48/80. ATPase activity was initiated by the addition of 2 mM [γ-^{32}P]ATP and measured as described under Materials and Methods. Results are reported as percent of the maximal activities which were (in μmol $P_i \cdot min^{-1} \cdot mg^{-1}$) in (A): (○) 2.86; (□) 3.37; and (■) 2.82; and in (B): (●) 3.5; and (■) 3.0.

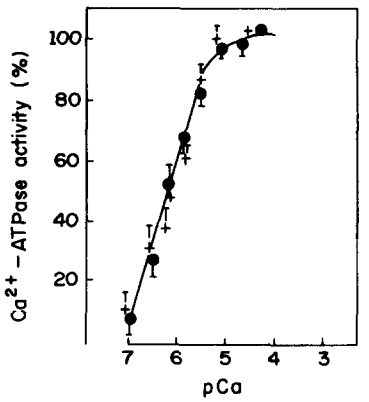

Fig. 5. Effect of calmidazolium on the Ca^{2+} dependence of the purified Ca^{2+}-ATPase of heart sarcolemma. Purified Ca^{2+}-ATPase (1.0 μg/mL) was incubated in a reaction medium containing 0.02 mg/mL phosphatidylcholine, 20 mM BTP-HCl, pH 7.0, 80 mM KCl, 5 mM $MgCl_2$, 0.2 mM $CaCl_2$, EGTA to give the desired free calcium concentrations and (●) 2 μg/mL calmodulin or (+) 2.0 μg/mL calmodulin plus 2 μM calmidazolium. ATPase activity was initiated by the addition of 2 mM [γ-^{32}P]ATP and measured as described under Materials and Methods. The means ± SD of five preparations are reported as percent of the maximal activities which were (in μmol $P_i \cdot min^{-1} \cdot mg^{-1}$): (●) 3.6, and (+) 1.6.

by calmidazolium was not affected (see Fig. 1). This result is compatible with compound 48/80 dislocating calmodulin from its binding site and could be simulated by assuming a model of simple competitive inhibition in which the free enzyme can bind calmodulin, or alternatively compound 48/80, and the activity of the enzyme inhibited by compound 48/80 is equal to the activity of the enzyme in the basal state. The curves were generated by supposing $K_{cd} = 0.18$ as previously obtained by activation by calmodulin; $A = 3.2$ at saturating calmodulin and $K_i = 1.10$ μg/mL, which gives an apparent $K_{0.5}$ for compound 48/80 of 13.3 μg/mL in the presence of 2 μg/mL calmodulin and 2.3 μg/mL in the presence of 0.2 μg/mL calmodulin. The calculated values are in good agreement with the observed values.

If either of these inhibitors acts by impairing the binding of activator (calmodulin or phosphatidylserine) to the enzyme, calcium binding to the enzyme when the inhibitor is present should be shifted from the low concentration range seen with the activated state to the high concentration range associated with the basal state [4, 23]. This was indeed the case for compound 48/80 as the inhibitor (Fig. 4). At 50 μg/mL, compound 48/80 shifted the $K_{0.5}$ for calcium activation of ATP hydrolysis in the presence of phosphatidylserine from 0.7 to 10 μM Ca^{2+} (Fig. 4A). The same effect was observed when the enzyme was activated by calmodulin (Fig. 4B). This inhibitor thus reproduces the effects of removing the activators. Similar effects on calcium binding have been reported previously for another calmodulin antagonist, trifluoperazine, and also for soluble troponin-I [4, 31]. Calmidazolium, however, did not change the $K_{0.5}$ for Ca^{2+} activation, although it decreased the rate of hydrolysis by 50% (Fig. 5). The experiments above suggest that calmidazolium does not inhibit the enzyme by removing the activators.

Because of its hydrophobic nature, calmidazolium might be expected to bind to a hydrophobic region of the enzyme. The cardiac sarcolemma Ca^{2+}-ATPase belongs to the "E_1-E_2" class of ion-transporting enzymes. Evidence has been presented that the catalytic site of these enzymes is more hydrophobic in the E_2 form, when the enzyme can be phosphorylated by P_i [32, 33]. To further clarify the inhibition by calmidazolium, we tested if this inhibition is antagonized by P_i. Figure 6 shows that P_i protected against the inhibition by calmidazolium, which is consistent with an interaction of both compounds at the hydrophobic form of the catalytic site.

DISCUSSION

It has been proposed that calmidazolium inhibits cardiac sarcolemmal Ca^{2+}-ATPase by impairing calmodulin binding [16, 17]. Our data do not support

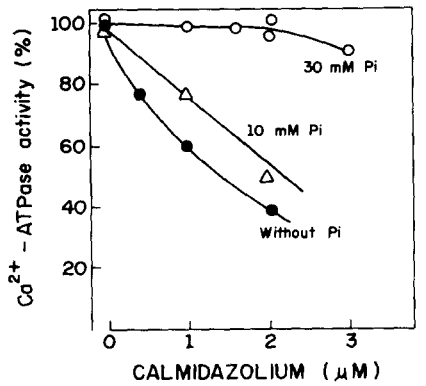

Fig. 6. Effect of P_i on the inhibition by calmidazolium. The composition of the assay medium was as described in the legend of Fig. 1 in the presence of 2.0 μg/mL calmodulin and (●) no additions; (△) 10 mM P_i plus 10 mM $MgCl_2$; and (○) 30 mM P_i plus 30 mM $MgCl_2$. Reactions were initiated by the addition of 0.015 mM [γ-^{32}P]ATP and the maximal velocities were (in μmol $P_i \cdot min^{-1} \cdot mg^{-1}$): (●) 2.55; (△) 0.85 and (○) 0.4. Mg^{2+} alone (10–30 mM) had no effect on the inhibition by calmidazolium.

such an interpretation, since calmidazolium inhibited with a similar $K_{0.5}$ regardless of whether the enzyme was activated by different amounts of calmodulin, or by phosphatidylserine, or by trypsin (Figs. 1 and 2). In addition, calmidazolium did not modify the $K_{0.5}$ for calcium activation as expected for a compound that behaves strictly as a calmodulin antagonist (Fig. 5). A possible explanation is that calmidazolium binds directly to the enzyme at a site that is exposed upon activation, regardless of the manner by which activation is achieved. This site must be situated on the active peptide portion retained after partial proteolysis (see Fig. 1). Since the inhibition by calmidazolium is counteracted by P_i, this inhibitory site may be related to the catalytic site. Thus, calmidazolium may inhibit some yet unidentified step of the reaction cycle of the enzyme in the activated state.

Lamers *et al.* [16] reported that calmidazolium decreases calmodulin binding to cardiac sarcolemma Ca^{2+}-ATPase. The authors, however, used 50 μM calmidazolium. Thus, at a concentration much higher than we have used in this study, calmidazolium also binds to calmodulin and dislocates such an activator from the enzyme.

The inhibition by calmidazolium was highly dependent on the concentration of phospholipid in the reaction medium, irrespective of the type of phospholipid (Fig. 2). This may explain the large differences in $K_{0.5}$ for calmidazolium inhibition reported by other authors [7, 9, 14], since this variable is not routinely controlled. Gietzen *et al.* [9] reported that calmidazolium inhibits the calmodulin-activated Ca^{2+}-ATPase from erythrocytes at a much lower concentration than the trypsin-activated enzyme. The authors used disrupted membranes, and attributed the low affinity of calmidazolium for the trypsin-treated ATPase to a steric problem [9]. Since the purified, solubilized cardiac Ca^{2+}-ATPase shows a different pattern of trypsinization, it may be that the binding site for calmidazolium becomes exposed in this preparation, and the steric hindrance disappears.

It is interesting to note that calmidazolium displays a dual effect on cardiac sarcolemma Ca^{2+}-ATPase. At low calmidazolium concentrations, a small activation by the compound was observed (Fig. 1) followed by an inhibition at increasing calmidazolium concentrations. A similar activating effect at low calmidazolium concentrations has been described previously for striated muscle myofilaments activated by either calmodulin, or by skeletal troponin C or by cardiac troponin C [34]. The activating effect of calmidazolium was attributed to its action as a sensitizer of the calcium-binding proteins, calmodulin or troponins, to activation by Ca^{2+}. It has also been shown that calmidazolium binds to more than one site in calmodulin and that different concentrations of calmidazolium have different effects on the structure of calmodulin [35]. The results described in this paper also suggest that calmidazolium may bind to more than one site at the system, or alternatively, to more than one intermediate at the reaction cycle, being both an activator and an inhibitor depending on the binding site. In the present work we did not study in depth the origins of the activating effect of calmidazolium. It is apparent, however, that the activating effect is not restricted to the calmodulin-regulated function, suggesting again direct binding of calmidazolium to the protein.

Figure 3 shows that, unlike calmidazolium, compound 48/80 did not inhibit the cardiac sarcolemmal enzyme after trypsinization. This observation suggests that the compound 48/80 binding site is lost by trypsinization. This does not appear to be the case for the Ca^{2+}-ATPase from erythrocytes, which continues to be inhibited after trypsinization [13]. This difference may be related to the different patterns of enzyme proteolysis. Controlled trypsinization of the erythrocyte enzyme leads to accumulation of subfragments of 81 to 76 kDa, depending on the reaction conditions [23, 36]. Controlled trypsinization of the cardiac sarcolemma Ca^{2+}-ATPase generates large amounts of subfragments of 66 kDa or less (this paper, and see Fig. 5 in Ref. 23). The binding site for compound 48/80 may be situated on the 81 or 76 kDa subfragment of the erythrocyte enzyme [13], but is lost from the fragments of 66 kDa and less.

Compound 48/80 inhibited both the calmodulin and the phosphatidylserine-activated states of the cardiac sarcolemma enzyme and in both cases shifted the $K_{0.5}$ for calcium activation to the same level observed for the unactivated state, thus suggesting that compound 48/80 inhibits by dislocating the activators. This interpretation is supported by the results displayed in Fig. 3, which shows that compound 48/80 became less effective as an inhibitor when the concentration of calmodulin was raised. In this sense, we agree with the observation of Gietzen *et al.* [8] that compound 48/80 is a powerful inhibitor of calmodulin-regulated functions.

The results presented in this work indicate that both calmidazolium and compound 48/80 bind to

the enzyme and inhibit the Ca^{2+}-activated ATPase by different mechanisms. It is likely that these compounds bind at a distinct site(s) in the enzyme. It is not known yet whether or how these compounds modify the enzyme conformation. It has been demonstrated that the pattern of trypsinization of the erythrocyte Ca^{2+}-ATPase at 37° and at a low trypsin–enzyme ratio is modified by the addition of effectors such as calmodulin and vanadate [37]. At conditions of trypsinization similar to those used in this paper (0° and higher trypsin–protein ratios), Zurini *et al.* [38] found that calmidazolium accelerates the velocity of trypsinization of the erythrocyte Ca^{2+}-ATPase. We attempted to determine whether calmidazolium or compound 48/80 could modify the conformation of the cardiac sarcolemma Ca^{2+}-ATPase in such a way that the pattern of enzyme trypsinization would be affected. Enzyme trypsinization was performed in the presence or absence of calmodulin and with calmodulin plus calmidazolium or calmodulin plus compound 48/80 both at 0° and high trypsin : protein ratios (conditions used for enzyme activation in this paper) or at 37°, at a trypsin : enzyme ratio of 1 : 15. We did not observe any obvious changes in the pattern of enzyme trypsinization with either inhibitor (data not shown) under any of these conditions. Therefore, it appears that these compounds do not alter markedly the accessibility of trypsin-sensitive regions of the enzyme, although they may well affect the conformation of the Ca^{2+}-ATPase in other domains. Nevertheless, the two distinct inhibitors bind to distinct sites and could be helpful tools in the study of structure–function relationships of domains specifically involved in crucial steps of the enzyme reaction cycle.

Acknowledgements—This work was supported by grants from the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), from Financiadora de Projectos e Pesquisa (FINEP) and from Fundação de Amparo a Pesquisa do Estado do Rio de Janeiro (FAPERJ) and by a grant-in-aid from the American Heart Association, Texas Affiliate, to A. Otero. T. C. Pasa has a fellowship from the Conselho de Aperfeiçoamento de Pessoal de Ensino Superior (CAPES). The authors wish to thank Dr. Martha Sorenson for helpful discussions. The technical assistance of Ms. Monica Freire is gratefully acknowledged.

REFERENCES

1. Schatzmann HJ, Calcium extrusion across the plasma membrane by the calcium-pump and the Ca^{2+}–Na^{+} exchange system. In: *Calcium and Cell Physiology* (Ed. Marmé D), pp. 18–52. Springer, Berlin, 1985.
2. Rega AF and Garrahan PJ, *The Ca^{2+} Pump of Plasma Membranes*, pp. 49–56. CRC Press, Boca Raton, FL, 1988.
3. Carafoli E and Zurini M, The Ca^{2+}-pumping ATPase of plasma membranes: Purification, reconstitution and properties. *Biochim Biophys Acta* **683**: 279–301, 1982.
4. Caroni P and Carafoli E, The Ca^{2+}-pumping ATPase of heart sarcolemma: Characterization, calmodulin dependence and partial purification. *J Biol Chem* **256**: 3263–3270, 1981.
5. Verma AK, Filoteo AG, Stanford DR, Wieben ED, Penniston JT, Strehler EE, Fischer R, Heim R, Vogel G, Mathews S, Strehler-Page MA, James P, Vorherr T, Krebs J and Carafoli E, Complete primary structure of a human plasma membrane Ca^{2+} pump. *J Biol Chem* **263**: 14152–14159, 1988.
6. Greeb J and Schull GE, Molecular cloning of a third isoform of the calmodulin-sensitive plasma membrane Ca^{2+}-transporting ATPase that is expressed predominantly in brain and skeletal muscle. *J Biol Chem* **264**: 18569–18576, 1989.
7. Gietzen K, Comparison of the calmodulin antagonists compound 48/80 and calmidazolium. *Biochem J* **216**: 611–616, 1983.
8. Gietzen K, Adamczyk-Engelmann P, Wuthrich A, Konstantinova A and Bader H, Compound 48/80 is a selective and powerful inhibitor of calmodulin-regulated functions. *Biochim Biophys Acta* **736**: 109–118, 1983.
9. Gietzen K, Sadorf I and Bader H, A model for the regulation of the calmodulin-dependent enzymes erythrocyte Ca^{2+}-transport ATPase and brain phosphodiesterase by activators and inhibitors. *Biochem J* **207**: 541–548, 1982.
10. Veigl ML, Klevit RE and Sedurck WD, The uses and limitations of calmodulin antagonists. *Pharmacol Ther* **44**: 181–239, 1989.
11. Missiaen L, De Smedt H, Droogmans G, Wuytack F, Raeymaekers L and Casteels R, Ruthenium red and compound 48/80 inhibit the smooth-muscle plasma-membrane Ca^{2+} pump via interaction with associated polyphosphoinositides. *Biochim Biophys Acta* **1023**: 449–454, 1990.
12. Caride AJ, Rossi JPFC, Garrahan PJ and Rega AF, Does calmodulin regulate the affinity of the human red cell Ca^{2+} pump for ATP? *Biochim Biophys Acta* **1027**: 21–24, 1990.
13. Rossi JPFC and Rega AF, A study to see whether phosphatidylserine, partial proteolysis and EGTA substitute for calmodulin during activation of the Ca^{2+}-ATPase from red cells membranes by ATP. *Biochim Biophys Acta* **996**: 153–159, 1989.
14. Adunyah ES, Niggli V and Carafoli E, The anticalmodulin drugs trifluoroperazine and R24571 remove the activation of the purified erythrocyte Ca^{2+}-ATPase by acidic phospholipids and by controlled proteolysis. *FEBS Lett* **143**: 65–68, 1982.
15. Beresewicz A, Anti-ischemic and membrane stabilizing activity of calmodulin inhibitors. *Basic Res Cardiol* **84**: 631–645, 1989.
16. Lamers JMJ, Verdouw PD and Mas-Oliva J, The effects of felodipine and bepridil on calcium-stimulated calmodulin binding and calcium pumping ATPase of cardiac sarcolemma before and after removal of endogenous calmodulin. *Mol Cell Biochem* **78**: 169–176, 1987.
17. Lamers JMJ and Stinis JT, Inhibition of Ca^{2+}-dependent protein kinase and Ca^{2+}/Mg^{2+}-ATPase in cardiac sarcolemma by the anti-calmodulin drug calmidazolium. *Cell Calcium* **4**: 281–294, 1983.
18. Mousli M, Bronner C, Landry Y, Bockaert J and Rouot B, Direct activation of GTP-binding regulatory proteins (G-proteins) by substance P and compound 48/80. *FEBS Lett* **259**: 260–262, 1990.
19. Kwo TH, Guanine nucleotide and inositol trisphosphate-induced inhibition of the Ca^{2+} pump in rat heart sarcolemmal vesicles. *Biochem Biophys Res Commun* **152**: 1111–1116, 1988.
20. Glynn JM and Chappell JB, A simple method for the preparation of ^{32}P-labelled adenosine-triphosphate of high specific activity. *Biochem J* **90**: 147–149, 1964.
21. Scofano HM, Vieyra A and de Meis L, Substrate regulation of the sarcoplasmic reticulum ATPase: Transient kinetic studies. *J Biol Chem* **254**: 10227–10231, 1979.
22. Van Alstyne E, Burch RM, Knickelbein RG, Hungerford RT, Gower EJ, Webb JG, Poe SL and Lindenmayer GE, Isolation of sealed vesicles highly

enriched with sarcolemma markers from canine ventricle. *Biochim Biophys Acta* **602**: 131–143, 1980.
23. Caroni P, Zurini M, Clark A and Carafoli E, Further characterization and reconstitution of the purified Ca^{2+}-pumping ATPase of heart sarcolemma. *J Biol Chem* **258**: 7305–7310, 1983.
24. Stoscheck CM, Protein assay sensitive at nanogram levels. *Anal Biochem* **160**: 301–305, 1987.
25. Grubmeyer C and Penefsky HS, The presence of two hydrolytic sites on beef heart mitochondrial adenosine triphosphatase. *J Biol Chem* **256**: 3718–3727, 1981.
26. Carvalho-Alves PC and Scofano HM, The hydrolytic cycle of sarcoplasmic reticulum Ca^{2+}-ATPase in the absence of calcium. *J Biol Chem* **262**: 6610–6614, 1987.
27. Fabiato A and Fabiato F, Calculator programs for computing the composition of the solution containing multiple metals and ligands used for experiments in skinned muscle cells. *J Physiol (Paris)* **75**: 463–505, 1979.
28. Schwarzenbach G, Senn H and Anderegg G, Komplexone XXIX. Ein grosser chelateffekt besonderer art. *Helv Chim Acta* **40**: 1886–1990, 1957.
29. Laemmli UK, Cleavage of structural proteins during cell assembly of the head of bacteriophage T4. *Nature* **227**: 680–685, 1970.
30. Morrissey JH, Silver stain for proteins in polyacrylamide gels: A modified procedure with enhanced uniform sensitivity. *Anal Biochem* **117**: 307–310, 1981.
31. Dixon DA and Haynes DH, Kinetic characterization of the Ca^{2+}-pumping ATPase of cardiac sarcolemma in four states of activation. *J Biol Chem* **264**: 13612–13622, 1989.
32. de Meis L, Tuena de Gómez-Puyou M and Gómez-Puyou A, Inhibition of mitochondrial F_1 ATPase and sarcoplasmic reticulum ATPase by hydrophobic molecules. *Eur J Biochem* **171**: 343–349, 1988.
33. Alves EW and de Meis L, Effect of compound 48/80 and ruthenium red on the Ca^{2+}-ATPase of sarcoplasmic reticulum. *J Biol Chem* **261**: 16854–16859, 1986.
34. El-Saleh SC and Solaro RJ, Calmidazolium, a calmodulin antagonist stimulates calcium-troponin C and calcium-calmodulin-dependent activation of striated muscle myofilaments. *J Biol Chem* **262**: 17240–17246, 1987.
35. Johnson JD, Allosteric interactions among drug binding sites on calmodulin. *Biochem Biophys Res Commun* **112**: 787–793, 1983.
36. Papp B, Sarkadi B, Enyedi A, Caride AJ, Penniston JT and Gardos G, Functional domains of the *in situ* red cell membrane calcium pump revealed by proteolysis and monoclonal antibodies: Possible sites for regulation by calpain and acidic lipids. *J Biol Chem* **264**: 4577–4582, 1989.
37. Banaim G, Zurini M and Carafoli E, Different conformational states of the purified Ca^{2+}-ATPase of the erythrocyte plasma membrane revealed by controlled trypsin proteolysis. *J Biol Chem* **259**: 8471–8477, 1984.
38. Zurini M, Krebs J, Penniston JT and Carafoli E, Controlled proteolysis of the purified Ca^{2+}-ATPase of the erythrocyte membrane: A correlation between the structure and the function of the enzyme. *J Biol Chem* **259**: 618–627, 1984.