Glucose-independent inhibition of yeast plasma-membrane H⁺-ATPase by calmodulin antagonists

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Glucose metabolism causes activation of the yeast plasma-membrane H⁺-ATPase. The molecular mechanism of this regulation is not known, but it is probably mediated by phosphorylation of the enzyme. The involvement in this process of several kinases has been suggested but their actual role has not been proved. The physiological role of a calmodulin-dependent protein kinase in glucose-induced activation was investigated by studying the effect of specific calmodulin antagonists on the glucose-induced ATPase kinetic changes in wild-type and two mutant strains affected in the glucose regulation of the enzyme. Pre-incubation of the cells with calmidazolium or compound 48/80 impeded the increase in ATPase activity by reducing the $V_{\max}$ of the enzyme without modifying the apparent affinity for ATP in the three strains. In one mutant, *pma1-T912A*, the putative calmodulin-dependent protein kinase-phosphorylatable Thr-912 was eliminated, and in the other, *pma1-P536L*, H⁺-ATPase was

constitutively activated, suggesting that the antagonistic effect was not mediated by a calmodulin-dependent protein kinase and not related to glucose regulation. This was corroborated when the *in vitro* effect of the calmodulin antagonists on H⁺-ATPase activity was tested. Purified plasma membranes from glucose-starved or glucose-fermenting cells from both *pma1-P890X*, another constitutively activated ATPase mutant, and wild-type strains were preincubated with calmidazolium or melittin. In all cases, ATP hydrolysis was inhibited with an IC_{50} of $\approx 1 \mu\text{M}$. This inhibition was reversed by calmodulin. Analysis of the calmodulin-binding pattern in the plasma-membrane fraction eliminates ATPase as the calmodulin target protein. We conclude that H⁺-ATPase inhibition by calmodulin antagonists is mediated by an as yet unidentified calmodulin-dependent membrane protein.

INTRODUCTION

The plasma-membrane H⁺-ATPase (Pma1) of yeast cells belongs to the family of P-type ATPases [1,2]. It hydrolyses ATP and transports protons from the cytosol to the extracellular medium. The electrochemical gradient it creates is the driving force for nutrient uptake. The enzyme also plays an essential role in the maintenance of intracellular pH (for reviews see [3–7]). The crucial physiological role of the ATPase requires its strict regulation. It is known that H⁺-ATPase activity is regulated in response to growth conditions [8–11], and glucose is the external signal that has been most extensively investigated. It has a dual role in regulating both the expression and catalytic activity of the plasma-membrane H⁺-ATPase. On the one hand, glucose metabolism increases ATPase gene (PMA1) transcription [12] and, on the other, it induces a modification of the enzyme's kinetic properties [8], resulting in an overall stimulation of ATPase activity. The molecular mechanism of glucose-induced activation is not known, but it is based on the elimination of an inhibitory interaction of the C-terminus with the active site of the enzyme [13,14], probably mediated by Ser/Thr phosphorylation of the enzyme [15]. In fact, several C-terminal amino acids important in H⁺-ATPase regulation have been identified using site-directed mutagenesis [14,16]. The residues that are essential for glucose activation define the potential phosphorylation sites for casein kinase II and calmodulin-dependent protein kinase II [17] which are necessary for the glucose-induced K_m and $V_{\max}$ changes respectively [14,16]. The physiological role of casein kinase II in

glucose-induced activation of the H⁺-ATPase is not known, although there is evidence that Ser-899, the phosphorylatable residue in the consensus site for this kinase, could be phosphorylated during glucose activation of the enzyme [16]. Involvement of a calmodulin-dependent protein kinase in glucose activation is less clear, since there is no evidence that Thr-912, the phosphorylatable residue in the ATPase consensus sequence for calmodulin-dependent protein kinase II, is phosphorylated [16]. Nevertheless, results obtained *in vivo* using several kinase inhibitors [18] indicate that glucose-induced activation of the enzyme could be mediated by a calmodulin-dependent protein kinase. To unravel the role of calmodulin-dependent protein kinase in glucose activation of H⁺-ATPase, we studied the effect of calmodulin antagonists on the glucose-induced activation of glucose-regulation mutants. We describe potent inhibitory effects of these compounds that affect the $V_{\max}$ of the enzyme, and we show, *in vivo* and *in vitro*, that these effects are not related to glucose regulation of the enzyme. The inhibition affects the basal activity of the enzyme and can be reversed by calmodulin, suggesting modulation of the H⁺-ATPase activity through a calmodulin-binding protein.

MATERIALS AND METHODS

Yeast strain and growth medium

The strain used was *Saccharomyces cerevisiae* RS-72 transformed with pSB32, an autonomous and centromeric plasmid with the

Abbreviations used: DSS, suberic acid bis-(*N*-hydroxysuccinimide ester); P536L, mutant of yeast plasma-membrane H⁺-ATPase with Leu substituted for Pro-536; P890X, mutant of yeast plasma-membrane H⁺-ATPase with an opal stop codon substituted for Pro-890; T912A, mutant of yeast plasma-membrane H⁺-ATPase with Ala substituted for Thr-912.

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LEU2 gene for transformant selection [19], containing a 5 kb *Hind*III fragment that includes either the wild-type or the selected mutant allele of the *pma1* gene. RS-72 is a derivative of BWG1-7A (*MATa*, *ade1-100*, *his4-519*, *leu2-3,112* and *ura3-52*) [20] in which the wild-type promoter of PMA1 has been replaced by the galactokinase gene (*gal1*) promoter [21]. Cells were grown in synthetic medium containing 2% glucose or galactose, 0.7% yeast nitrogen base without amino acids (Difco), 0.2 mM adenine and 0.4 mM histidine.

Chemicals

Calmidazolium, compound 48/80, melittin, calmodulin and suberic acid bis-(*N*-hydroxysuccinimide ester) (DSS) were obtained from Sigma Chemical Co. 125 I-calmodulin was from DuPont. All other chemicals used in this work were of analytical grade.

Expression of mutant ATPases

Mutants were grown to a final A_{660} of 3 (about 10 mg fresh weight/ml) in galactose medium, in which both the wild-type (chromosomal) and mutant (plasmid) ATPases are expressed. To express the mutant enzyme only, the galactose-grown cells were diluted 100-fold in glucose medium and harvested after 24 h. In these conditions, cultures expressing active ATPase reach an A_{660} of about 1.5 [21,22].

ATPase activation, homogenization and membrane preparation conditions

Yeast cells were harvested by centrifugation, washed twice with deionized water and resuspended at 70 mg fresh weight/ml in water to eliminate glucose activation [8]. For experiments with inhibitors, small aliquots of the compound dissolved in methanol were added to the cell suspension and the final methanol concentration was kept constant at 2% (v/v). The results of control experiments with 0 or 2% methanol were identical. After 30 min incubation at 30 °C, 5 ml samples were taken (glucose-starved cells), frozen in liquid nitrogen and stored at -70 °C until use. Then 2% glucose was added to the rest of the suspension for glucose activation. Samples of volume 5 ml were taken after 8 min and frozen as above. Homogenization and membrane preparation were as previously described [8].

ATPase activity

ATP hydrolysis was assayed with 2 mM ATP at 30 °C and pH 6.5, unless otherwise specified, in buffer containing 5 mM NaN_3 to inhibit mitochondrial ATPase, 0.2 mM ammonium molybdate to inhibit acid phosphatase and 50 mM potassium nitrate to inhibit vacuolar ATPase. Residual activity represented specific ATP hydrolysis by the plasma-membrane H^+ -ATPase, as it was more than 90% inhibited by 1 mM diethylstilboestrol [23].

To measure the effect of inhibitors on ATPase activity, the assay buffer contained 0.1 mM CaCl_2 , and all enzyme assays were performed at a protein concentration of 20 $\mu\text{g}/\text{ml}$ unless otherwise stated. Before initiating the reaction by ATP addition, the plasma membranes were preincubated with the drug for 5 min in the assay medium, followed by an additional 5 min in the absence or presence of calmodulin when indicated. Calmidazolium and compound 48/80 were dissolved in methanol. The final methanol concentration in the assay medium including the controls was always < 4% (v/v).

125 I-Calmodulin cross-link

125 I-Calmodulin cross-linkage to plasma-membrane proteins was performed essentially as described [24]. Membrane fractions (80 μg of protein) were incubated at 37 °C for 30 min in 100 μl of 50 mM Hepes (sodium salt), pH 7.4, containing 1 mM PMSF, 200 μM CaCl_2 or 2 mM EGTA and 100 nCi of 125 I-calmodulin (90–110 $\mu\text{Ci}/\mu\text{g}$). Samples were subsequently incubated at 0 °C for 20 min in the presence of a 3 mM concentration of the cross-linking agent DSS, and thereafter 250 mM glycine was added to terminate the reaction. Controls were performed with an excess (1.2 μM) of unlabelled calmodulin to displace the bound 125 I-calmodulin before addition to DSS. The samples were precipitated with 15% (w/v) trichloroacetic acid, centrifuged in a microcentrifuge and the pellets processed for SDS/PAGE and autoradiography or Western blotting as described below.

Protein analysis

Protein concentration was measured by the Bradford method [25], using the Bio-Rad protein assay reagent and bovine IgG as standard. SDS/PAGE was performed as described [26] in 6% polyacrylamide gels. Samples were prepared as described [27]. The gels were stained with Coomassie Brilliant Blue R-250, and vacuum dried at 70 °C on Whatman 3MM filter paper. Autoradiographs were obtained using an intensifying screen by exposure of X-ray-sensitive Hyperfilm-MP (Amersham) at -20 °C. For Western-blot analysis, proteins were transferred to Immobilon P membranes (Millipore). Transfer buffer was 50 mM Tris/38 mM glycine/20% methanol. Transfer was carried out for 1.5 h at a constant current of 2 mA/cm². Immunodetection of ATPase was carried out using monoclonal antibody AT1-1-A4/1-A6 [28] diluted 1:6000. A second antibody coupled to alkaline phosphatase (Bio-Rad) was used as described [29].

RESULTS

Effect of calmodulin antagonists on glucose-induced activation of H^+ -ATPase

One way to study the physiological role of the calmodulin-dependent protein kinase in plasma-membrane H^+ -ATPase activation by glucose is selective inhibition of its activity using specific calmodulin antagonists. Calmidazolium and compound 48/80 were selected on the basis of their wide use as calmodulin-specific inhibitors [30–32]. Table 1 shows that the *in vivo* activation of the plasma-membrane H^+ -ATPase by glucose was progressively inhibited when cells were treated with increasing inhibitor concentrations before addition of glucose. Activation by glucose was virtually abolished at 400 μM calmidazolium or 400 $\mu\text{g}/\text{ml}$ compound 48/80. These inhibitor concentrations were used in subsequent *in vivo* experiments. The inhibitor concentration is higher than that used for specific enzyme inhibition *in vitro*, as the cationic nature of these inhibitors limits their entry into the phospholipid phase of the membrane [33]. The results point to involvement of calmodulin-dependent protein kinase in glucose-induced activation of the H^+ -ATPase and are consistent with previous results [18].

Effect of calmodulin antagonists on the glucose-induced changes in H^+ -ATPase kinetic parameters

Glucose treatment of wild-type cells induces important changes in the kinetic characteristics of plasma-membrane ATPase. Among them are a decrease in the K_m for ATP and an increase

Table 1 Inhibition of glucose-induced H⁺-ATPase activation by calmodulin antagonists

Cells were cultured, collected and treated with various concentrations of calmidazolium or compound 48/80 and glucose as outlined in the Materials and methods section. The ATPase assay was carried out with 2 mM ATP at pH 6.5 in purified plasma membranes as described in the Materials and methods section. ATPase activity of glucose-starved cells was 0.12 $\mu\text{mol}/\text{min}$ per mg. The concentration for calmidazolium was in μM and for compound 48/80 in $\mu\text{g}/\text{ml}$. Results shown correspond to the mean of two experiments differing by less than 10%.

Antagonist concentration	ATPase activity of glucose-fermenting cells ($\mu\text{mol}/\text{min}$ per mg)	
	+ Calmidazolium	+ Compound 48/80
0	1.87	1.90
25	1.57	1.64
50	1.05	1.12
100	0.84	0.92
150	0.62	0.56
200	0.52	0.50
400	0.17	0.24

in V_{max} [8]. Some *pmal* mutants that are affected in their glucose regulation have been described [13,14,16] in which these glucose-induced kinetic parameters are altered in different ways. To specify further the extent to which the calmodulin antagonists affect glucose-induced activation of the ATPase, we measured their effect on the activity and kinetic properties of the enzyme in two such mutants. In one, *pmal-T912A*, elimination of the putative calmodulin-dependent protein kinase-phosphorylatable Thr-912, localized in the regulatory domain, results in an enzyme that is defective in the V_{max} increase but which exhibits the typical glucose-triggered K_{m} decrease. In the other mutant, *pmal-P536L*, the replacement of Pro-536 with leucine in the proposed ATP-binding domain probably eliminates the inhibitory interaction of the C-terminus with that domain resulting in a constitutively activated ATPase (K_{m} and V_{max}), even in the absence of glucose metabolism. Preincubation with calmidazolium or compound 48/80 abolishes glucose activation in wild-type and mutant enzymes (Table 2). The effect of calmidazolium on the kinetic parameter changes induced by glucose is shown in Table 3. The K_{m} decrease is produced in glucose-fermenting cells of the wild-type and *pmal-T912A* strains, whether they are treated with

Table 2 Effect of calmodulin antagonists on glucose-induced activation of H⁺-ATPase mutants affected in their glucose regulation

Cells were cultured, collected and treated with glucose as described in the Materials and methods section. Cells treated with inhibitors were preincubated with 400 μM calmidazolium or 400 $\mu\text{g}/\text{ml}$ compound 48/80 before the addition of glucose. The ATPase assay was carried out with 2 mM ATP at pH 6.5 in purified plasma membranes, as described in the Materials and methods section. GS, glucose-starved cells; GF, glucose-fermenting cells. The results shown are means of two experiments differing by less than 10%.

Mutation in the ATPase allele expressed	ATPase activity ($\mu\text{mol}/\text{min}$ per mg)			
	GS	GF	GF + calmidazolium	GF + compound 48/80
None (wild-type)	0.07	1.41	0.08	0.12
T912A	0.04	0.18	0.05	0.08
P536L	1.76	1.95	0.28	0.24

Table 3 Effect of calmidazolium on the kinetic properties of mutant ATPases affected in their glucose regulation

ATPase activity was assayed at pH 6.5 with ATP concentrations from 0.8 to 6 mM. The apparent K_{m} (mM) and V_{max} ($\mu\text{mol}/\text{min}$ per mg of protein) were extrapolated from double-reciprocal plots fitted by using a standard linear regression program ($P = 0.97\text{--}0.99$). Similar values (within 10%) were obtained with two different independently isolated purified plasma-membrane preparations. GS, glucose-starved cells; GF, glucose-fermenting cells; GFC, calmidazolium-treated glucose-fermenting cells. Cells were grown, collected and treated with calmidazolium and glucose as described in the Materials and methods section.

Mutation in the ATPase allele expressed	K_{m}			V_{max}		
	GS	GF	GFC	GS	GF	GFC
None (wild-type)	4.0	1.4	1.3	0.3	3.0	0.1
T912A	4.0	1.4	1.2	0.1	0.3	0.1
P536L	1.3	1.0	1.1	2.8	3.0	0.4

calmidazolium or not. This glucose-induced decrease in K_{m} is not produced in the *pmal-P536L* mutant, as it was already low [16], nor is this behaviour modified by calmidazolium. On the other hand, the glucose-induced V_{max} increase is not produced in the wild-type strain, nor is the small increase in *pmal-T912A* observed. Even the glucose-independent constitutive effect of the P536L mutation is abolished in the presence of calmidazolium.

The results indicate that the calmodulin antagonists affect the V_{max} of the enzyme, whereas the K_{m} remains unaltered. This effect is unrelated to glucose activation since the calmodulin antagonists inhibit a mutant, *pmal-P536L*, the ATPase of which is completely independent of glucose metabolism. The results also suggest that the inhibitory effect of calmidazolium and compound 48/80 is not mediated by elimination of calmodulin-dependent phosphorylation of the ATPase, since these antagonists inhibit a mutant, *pmal-T912A*, in which the putative phosphorylatable residue has been eliminated.

Effect of calmodulin and calmidazolium on *in vitro* H⁺-ATPase activity

If a calmodulin-dependent protein kinase is not involved in the inhibition of the ATPase activity caused by calmodulin antagonists, it is feasible that another calmodulin-specific receptor system could be implicated. Several enzymes are known that possess an autoinhibitory domain that is removed on binding of calmodulin to the enzyme itself [34], thereby activating the enzyme. The basal activity of these enzymes is stimulated *in vitro* when the enzyme assay is performed in the presence of calmodulin. This calmodulin-stimulated activity can also be inhibited *in vitro* by calmodulin antagonists. We have considered these possibilities. The ATPase activity in purified plasma membranes from glucose-starved or glucose-fermenting cells was not stimulated in the presence of calmodulin, not even in membranes obtained in the presence of EGTA to eliminate the Ca²⁺-dependent membrane-bound calmodulin pool (results not shown). This would suggest that either calmodulin is not a direct ATPase activator or cytosolic factors required for *in vivo* activation are lost during isolation of plasma membranes. Lack of activation could also be explained by the existence of significant amounts of calmodulin tightly bound to the plasma-membrane fraction not solubilized during plasma-membrane isolation. Complete solubilization of endogenous calmodulin would require drastic treatments [35] which would abolish H⁺-ATPase activity.

When purified plasma membranes from glucose-starved or glucose-fermenting cells were preincubated with calmidazolium,

Figure 1 Concentration-dependence of *in vitro* H⁺-ATPase inhibition by calmidazolium

ATPase activity was measured in the presence of several concentrations of calmidazolium, as described in the Materials and methods section, using 10 (●), 20 (○) and 40 (▲) μg/ml purified plasma membranes from glucose-fermenting cells. The ATPase activity of membranes not preincubated with the inhibitor, taken as 100%, was 1.87 ± 0.07 μmol/min per mg. Results are the means from three experiments. Inset, dependence of IC_{50} on plasma-membrane concentration.

inhibition of the H⁺-ATPase activity was observed. In Figure 1, the concentration-dependence of the inhibition caused by the calmodulin antagonist is shown at several plasma-membrane concentrations. The IC_{50} value for calmidazolium was different depending on the plasma-membrane concentration and varied from 1.1 to 3.0 μM at the protein concentrations assayed (Figure 1, inset).

Specificity of the inhibition of H⁺-ATPase activity by calmidazolium

Membrane-dependence suggested that much of the added inhibitor was binding to the membranes. Since calmodulin

antagonists can be considered to be cationic amphiphiles at physiological pH, interaction with the lipid portion of the membrane may take place. In fact, phospholipid-mediated effects caused by calmidazolium have been previously reported, based on 1:1 stoichiometric binding of inhibitor to phospholipid [36]. To dismiss this possibility, we estimated the stoichiometry of calmidazolium binding. Approximate values of the inhibition constant (K_i) and binding stoichiometry for calmidazolium can be obtained by a plot of IC_{50} against plasma-membrane concentration, where K_i is the y intercept and $0.5 B$ the slope, where B is the binding stoichiometry in nmol/mg of protein [36]. A K_i of 0.4 μM and B of 150 nmol/mg were calculated from the data of Figure 1 (inset). The inhibitor does not appear to bind to membrane phospholipids with a 1:1 stoichiometry, since the calculated amount of calmidazolium bound is lower than the phospholipid content of the yeast plasma membrane [37]. Nevertheless, because of the dependence of the inhibition on plasma-membrane concentration, equal amounts of membranes were used in all experiments.

To assess the specificity of the inhibition of H⁺-ATPase activity, the effect of calmidazolium was compared with that of other calmodulin antagonists. Melittin is a high-affinity calmodulin-binding peptide [38] which inhibits calmodulin through a mechanism different from that of calmidazolium [39,40]. In Figure 2, the concentration-dependence of the inhibition caused by both calmodulin antagonists is shown. The IC_{50} values for calmidazolium and melittin, 1.4 μM and 1.1 μM respectively, were increased to 7.6 μM and 6.7 μM respectively in the presence of calmodulin. Calmidazolium- and melittin-caused inhibition of H⁺-ATPase activity occurs in purified plasma membranes from both glucose-starved and glucose-fermenting cells not only from wild-type, but also from *pma1-P890X* cells (results not shown). The P890X mutation generates a C-terminus deletion that produces an enzyme that is permanently in the activated state, independent of glucose fermentation [13,16].

Binding of calmodulin to plasma-membrane proteins

The above results do not reveal the calmodulin target for stimulation of H⁺-ATPase activity. It could be either a membrane component tightly coupled to the H⁺-ATPase or the enzyme itself. The calmodulin-binding domains of the target-binding proteins show extreme sequence variability, although they share

Figure 2 Effect of calmodulin on the *in vitro* inhibition of ATPase activity by calmidazolium and melittin

ATPase activities were measured in purified plasma membranes from glucose-fermenting cells, as described in the Materials and methods section, at a protein concentration of 20 μg/ml. The membranes were preincubated with various concentrations of calmidazolium or melittin in the presence (○) or absence (●) of calmodulin (CaM). The ATPase activity of membranes not preincubated with calmodulin antagonists, taken as 100%, was 2.32 ± 0.1 μmol/min per mg. The results are means from three experiments.

Figure 3 Detection of calmodulin-binding proteins in the plasma membrane by ¹²⁵I-calmodulin cross-linking

Aliquots of 80 μ g of plasma membranes purified from glucose-treated cells were used in the ¹²⁵I-calmodulin cross-linking reaction, which was performed in medium containing Ca²⁺ or EGTA in the presence or absence of unlabelled calmodulin, as described in the Materials and methods section. The autoradiograph of the proteins cross-linked to ¹²⁵I-calmodulin, after separation by SDS/PAGE, is shown in the first four lanes of the panel. The right line shows a Western blot of a protein sample not treated with the cross-linking agent and probed with anti-Pma1 monoclonal antibody. Molecular-mass standards are indicated in kDa on the left.

an amphiphilic structure [41]. Yeast H⁺-ATPase has no obvious region with structural features of calmodulin-binding domains. Nevertheless, we tested the possibility that the inhibitory effect of the antagonists was mediated via elimination of calmodulin binding to the ATPase. Figure 3 shows the pattern of calmodulin-binding proteins of the plasma-membrane fraction from glucose-fermenting cells obtained by ¹²⁵I-calmodulin cross-linking (similar results were obtained with membranes from glucose-starved cells; not shown). Calmodulin actually bound to several plasma-membrane proteins in a Ca²⁺-dependent manner, and the binding of labelled calmodulin to these proteins was completely blocked by unlabelled calmodulin. However, no 116 kDa band corresponding to the calmodulin-ATPase complex was detected, indicating that the target for calmodulin binding is not the ATPase, but may rather be a membrane component.

DISCUSSION

The results presented here indicate that plasma-membrane H⁺-ATPase activity is greatly reduced by calmodulin antagonists. The inhibitors diminish the $V_{\max}$ of H⁺-ATPase-catalysed ATP hydrolysis, although the apparent affinity for MgATP is not appreciably modified. Previous experiments using calmidazolium assigned a role in glucose-induced activation of H⁺-ATPase to one or more calmodulin-dependent kinases [18]. Our results show that the calmodulin antagonists affect the basal activity of the enzyme and that their inhibition is unrelated to activation by glucose, as shown by the response to the antagonists of several ATPase mutants affected in their glucose regulation. Thr-912 is the phosphorylatable residue located in the potential phosphorylation site for calmodulin-dependent protein kinase II, which is presumably involved in glucose regulation of the ATPase. A mutant lacking this residue should therefore not be affected by calmodulin antagonists. However, the $V_{\max}$ of this mutant ATPase was reduced on calmidazolium treatment. Inhibition of mutant *pma1-T912A* together with the inhibition of a mutant whose ATPase is completely independent of glucose metabolism, *pma1-P536L*, demonstrate that a role in the ac-

tivation of H⁺-ATPase by glucose cannot be assigned to a calmodulin-dependent protein kinase. Furthermore, inhibition of ATPase activity in purified plasma membranes excludes an effect of calmodulin antagonists via a calmodulin-dependent protein kinase and suggests that a membrane component is necessary for H⁺-ATPase activity as the calmodulin target. A role for a calmodulin-dependent protein kinase in glucose activation of the enzyme cannot be definitely ruled out, as it could be masked by the inhibition of the $V_{\max}$ caused by these antagonists.

Many calmodulin inhibitors described are more or less non-specific, in the sense that they are able to produce direct effects on calmodulin effector enzymes as well as phospholipid-mediated effects when used at high concentrations (50–75 μ M) [35,42]. The possibility that the action observed of calmodulin antagonists on H⁺-ATPase is non-specific seems unlikely, since this inhibition is produced in a concentration range that inhibits calmodulin-mediated reactions in other systems (IC_{50} from 1 to 3 μ M) [43,44]. Moreover, that this effect could be mediated by antagonist interaction with calmodulin is indicated by the displacement of the IC_{50} values in the presence of calmodulin. Further evidence for the specificity of the calmodulin antagonists was obtained from the similar response produced when calmodulin antagonists with different modes of action were tested. Calmodulin antagonists exert their inhibitory action on calmodulin-dependent reactions by binding to the calmodulin molecule. Calmidazolium binds to calmodulin predominantly via hydrophobic interactions [39], whereas not only hydrophobic but also electrostatic interaction contributes to melittin-calmodulin binding [40].

Phospholipid-mediated effects by calmodulin antagonists have been previously reported, based on a 1:1 stoichiometric binding of inhibitor to phospholipid [36]. That this does not seem to be the case here is indicated by the fact that the phospholipid content of the yeast plasma membrane is higher than the apparent binding stoichiometry found for calmidazolium [37]. However, the dependence of the inhibition on membrane concentration indicates a large amount of bound calmidazolium. This could reflect the existence in the plasma membrane of several calmodulin-specific receptor systems (Figure 3) or even the interaction of part of the added inhibitor with the phospholipid portion of the plasma membrane. Therefore the results are compatible with the possibility that, of the large number of calmidazolium molecules that bind, only a small number is responsible for the specific inhibition of H⁺-ATPase activity.

Calmodulin regulates the activity of mammalian Ca²⁺-ATPase by interaction with the C-terminal domain [45]. The ¹²⁵I-calmodulin-binding protein pattern in the plasma-membrane fraction eliminates H⁺-ATPase as the calmodulin effector enzyme, and points to a membrane component being indispensable for its activity. Many membrane proteins exist as part of homo-oligomeric or hetero-oligomeric structures. In fact, there is a 115 kDa glycoprotein that remains tightly associated with H⁺-ATPase during purification of the enzyme [46], although it is not essential for its activity [27]. It has recently been shown that two small proteolipids are at least partially associated with H⁺-ATPase in the plasma membrane [47]. A regulatory function for these proteolipids has been proposed, since a strain lacking both exhibited a lower $V_{\max}$ for plasma-membrane H⁺-ATPase activity and a wild-type K_m for MgATP. Both proteolipids are predicted to be amphipathic proteins [47,48] with a very basic hydrophilic C-terminal domain, which shows, in the α -helical wheel representation, the characteristic structural features of calmodulin-binding domains [49]. It is tempting to speculate that the calmodulin antagonists reduce the $V_{\max}$ of H⁺-ATPase by acting

on these proteolipids in a calmodulin-mediated effect. Calmodulin-mediated regulation could represent an additional regulatory mechanism of this essential enzyme in yeast, the activity of which is under strict genetic and environmental control.

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