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Activation of the plant plasma membrane H$^+$-ATPase. Is there a direct interaction between lysophosphatidylcholine and the C-terminal part of the enzyme?

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Abstract The antagonistic effects of the fungal toxin beticolin-1 and of L-α-lysophosphatidylcholine (lysoPC) were investigated on the plasma membrane H$^+$-ATPase of the plant *Arabidopsis thaliana* (isoform 2) expressed in yeast, using both wild-type enzyme (AHA2) and C-terminal truncated enzyme (aha2Δ92). Phosphohydrolytic activities of both enzymes were inhibited by beticolin-1, with very similar 50% inhibitory concentrations, indicating that the toxin action does not involve the C-terminal located autoinhibitory domain of the proton pump. Egg lysoPC, a compound that activates the H$^+$-ATPase by a mechanism involving the C-terminal part of the protein, was found to be able to reverse the inhibition of AHA2 by beticolin-1. The lack of effect of other detergents and the comparison of different carbon chain length lysoPCs show that the capacity to reverse the enzyme inhibition is clearly related to their ability to activate the pump. Long chain length lysoPC was also shown to reverse the inhibition of aha2Δ92 by beticolin-1, which strongly suggests that lysoPC binds to the H$^+$-ATPase on site(s) not located on its autoinhibitory domain.

Key words: Autoinhibitory domain; Beticolin; Plasma membrane H$^+$-ATPase activation; L-α-Lysophosphatidylcholine

1. Introduction

By pumping protons from the cytoplasm to the cell exterior, the plasma membrane H$^+$-ATPase of higher plant cells creates an electrochemical gradient across the plasma membrane that is thought to be the driving force for nutrient uptake. This enzyme is also believed to be involved in the extensive apoplast acidification that leads to cell wall loosening, a prerequisite for cell growth (review, [1]) and the resulting cytoplasm alkalinization may be the triggering factor for cell division [2]. Because of its central role in the physiology of plant cells, this enzyme is likely to be regulated by various factors including plant hormones, light and fungal toxins (review, [3]).

It is now well known that the hydrophilic C-terminal region of the enzyme contains an autoinhibitory domain that is involved in regulation of both phosphohydrolysis and proton pumping [4,5]. Removal of this autoinhibitory domain with trypsin [4,6,7] or making deletions at the gene level [8] results in an activated H$^+$-ATPase that exhibits a higher V_{max}, a lower K_m for ATP and a pH optimum shift to a more alkaline value. Similar changes of the enzyme properties are observed after treatment of isolated plasma membrane vesicles with L-α-lysophosphatidylcholine (lysoPC) which is known to be an activator of the plant plasma membrane H$^+$-ATPase [9,10], and believed to displace the C-terminal part of the proton pump [5].

Although it is clear that the C-terminal autoinhibitory domain is implied in the activation of the H$^+$-ATPase by lysoPC, it remains unknown whether the effector indeed binds to the C-terminus or binds elsewhere on the protein. In the latter case, lysoPC may activate the H$^+$-ATPase by inducing a conformational change of the protein that displaces the C-terminus. Many proteins contain regulatory domains with autoinhibitory sequences. The constraint exerted on the protein can be released by regulatory molecules, but most often by binding of the modulator directly to the regulatory sequences (an example is the binding of calmodulin to the C-terminus of the plasma membrane Ca^{2+}-ATPase [11]). In this report, beticolin-1, a fungal toxin which has been shown to inhibit the purified H$^+$-ATPase [12]), and lysoPC have been used. We studied their effects, alone or in combination, on both wild-type and C-terminus truncated H$^+$-ATPase to provide evidence that this enzyme can be regulated by compounds binding to site(s) distant from its regulatory domain.

2. Materials and methods

2.1. Yeast culture

Saccharomyces cerevisiae RS72 [13] transformed with the plasmid pMP136 containing the cDNA of AHA2 (isoform 2 of *Arabidopsis thaliana* plasma membrane H$^+$-ATPase, wild-type) or ahaΔ92 (AHA2 with a deletion corresponding to a lack of the 92 C-terminal amino acids [14]) were used for this study. Cells were grown for 24 h at 30°C on a synthetic liquid medium containing 2% glucose to allow expression of plasmid-borne plant H$^+$-ATPase, 0.7% yeast nitrogen base without amino acids, 0.2 mM adenine and 0.4 mM histidine.

2.2. Membrane preparation

Endoplasmic reticulum (ER) of yeast was purified by differential and sucrose gradient centrifugations as described by Regenberg et al. [8]. Microsomes were resuspended in 1 ml of STED 20 buffer (20% [w/w] sucrose, 10 mM Tris-HCl pH 7.5, and 1 mM EDTA), layered onto a 12 ml sucrose step gradient (2.5 ml each of 50, 42, 33 and 29% [w/w] sucrose in 10 mM Tris-HCl pH 7.5, 1 mM EDTA) and centrifuged for 16 h at 30000 rpm (SW 41 rotor, Beckman). ER membranes were collected from the 29/33% interface, diluted fourfold with STED 10, pelleted at 50000 rpm (70 TI rotor, Beckman) for 45

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Abbreviations: AHA2, *A. thaliana* wild-type plasma membrane H$^+$-ATPase isoform 2; aha2Δ92, *A. thaliana* plasma membrane H$^+$-ATPase with the truncation of 92 C-terminal amino acids; lysoPC, L-α-lysophosphatidylcholine

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min and resuspended in STED 20 supplied with 1 mM PMSF and 0.1 mg/ml chymostatin. The membrane fraction was frozen in liquid nitrogen and stored at −80°C. ER was preferred to plasma membrane because it is essentially latency-free.

2.3 ATPase activity measurement

Phosphohydrolytic activity of the H^+-ATPase was monitored by quantifying the released Pi according to Baginsky et al [15] with 2 μg of membrane protein, at 30°C. The basic assay medium (300 μl) contained 20 mM Mes-KOH pH 6.5, 50 mM KNO_3, 5 mM NaN_3, 0.2 % sodium molybdate, 5 mM $MgSO_4$, 2 units pyruvate kinase, 2.4 mM phosphoenol pyruvate and 1.5 mM ATP. Membranes were preincubated for 30 min with beticolin-1, the reaction was started by addition of ATP and allowed to proceed for 30 min. Controls were performed to check that beticolin-1 has no effect on pyruvate kinase activity.

2.4 Protein determination

Protein concentration was determined by the method of Bradford [16] with the Bio-Rad protein assay reagent and bovine gamma globulin as a standard.

2.5 Partition coefficient determinations

The partition coefficient of beticolin-1 onto liposomes or detergent micelles was determined on the basis of fluorescence increase (excitation and emission wavelengths 340 and 513 nm, respectively) after their binding to the lipid bilayers or detergent micelles, as described by Mikès et al. [17]. The relationship between fluorescence variations and the partition coefficient is given by the following equation:

$$P = \frac{F \cdot V}{(F_b - F) \cdot M}$$

where P is the partition coefficient, F the fluorescence, F_b the fluorescence of a bound compound at infinite phospholipid or detergent concentration, M the amount of phospholipid or detergent, and V the volume of the aqueous phase.

2.6 Chemicals

Lysophospholipids and other detergents (Sigma or Aldrich) were dissolved in EtOH:water 4:96 (v/v, 2 mg/ml stock solution) and sonicated under N_2 to clarity. Beticolin-1 was purified as described by Milat and Blein [18] and dissolved in DMSO/water 2:98 (v/v, 0.1 mg/ml stock solution). All other products were reagent grade.

3. Results

Plant H^+-ATPase heterologously expressed in yeast is normally expressed to high level in a fully functional form in the ER membranes [8,19,20]. It has been shown that upon isolation of these ER membranes, membrane vesicles form with the catalytic site of the H^+-ATPase exposed to the exterior of the vesicle [20,21]. This makes it possible to study the effects of detergents on the ATP hydrolytic activity directly, without interference of the effects of the unmasking of latent ATPase activity.

Table 1
Beticolin-1 partition coefficient determinations

Compound	Beticolin-1 partition coefficient (ml/mg)
Egg lysoPC	347 ± 16
Brij 58	332 ± 21
β-D-Dodecylmaltoside	346 ± 13
Soybean asolectin liposomes	401 ± 22

Beticolin-1 (2 μM) was titrated with liposomes or detergent (0.02–0.4 mg/ml), and fluorescence changes of beticolin-1 were followed with an excitation wavelength set at 340 nm and an emission wavelength set at 513 nm. The data are the mean ± S.D. of three independent experiments.

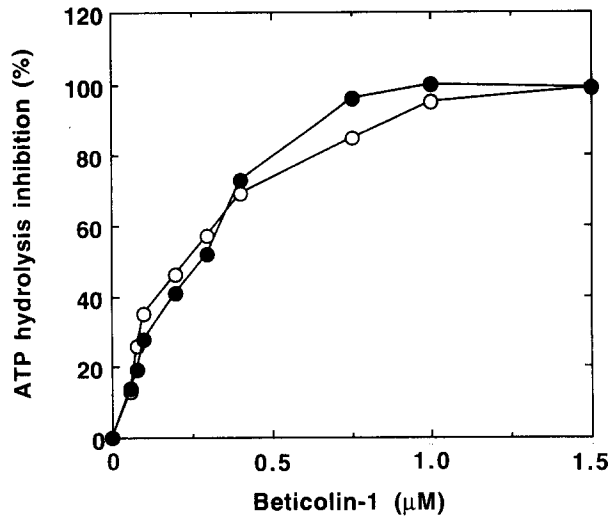

Fig. 1. Inhibition of AHA2 (○) and aha2Δ92 (●) ATP hydrolytic activity by beticolin-1. Membranes (2 μg) were preincubated for 30 min with various amounts of beticolin-1 at room temperature, as described in Section 2. The reaction was started by the addition of ATP and allowed to proceed for 30 min at 30°C. The data are representative of three independent experiments, and the standard deviations were always less than 10%.

3.1. Effect of beticolin-1 on wild-type and truncated H^+-ATPase

Incubation of 2 μg of ER proteins with various amounts of beticolin-1 led to a dose-dependent decrease of ATP hydrolysis both with membranes containing AHA2 (wild-type enzyme) or aha2Δ92 (truncated enzyme) (Fig. 1). The 50% inhibitory concentrations were very similar for both enzymes: 0.25 ± 0.03 and 0.30 ± 0.04 μM for AHA2 and aha2Δ92, respectively.

3.2. Effect of detergents and liposomes on AHA2 H^+-ATPase inhibition by beticolin-1

Effects of two detergents that do not activate the ATPase activity (β-D-dodecylmaltoside and Brij 58 [9,22]), of egg lysoPC and of soybean liposomes were tested on the inhibition of AHA2 by 2.0 μM of beticolin-1, a toxin concentration that ensures a total inhibition of the enzyme activity. Titration of ER membranes from yeast expressing AHA2 H^+-ATPase with Brij 58, dodecylmaltoside or soybean liposomes has no effect on the inhibition of ATPase activity by beticolin-1 (Fig. 2), except a slight decrease in the inhibition percentage observed with liposomes for concentrations exceeding 40 μg/ml. On the contrary, egg lysoPC, a compound known to activate the plasma membrane H^+-ATPase [10,23], was found to be able to abolish the inhibition of AHA2 activity by beticolin-1. A full restoration of the hydrolysis activity in the presence of 2 μM of beticolin-1 was obtained with 60 μg/ml egg lysoPC (Fig. 2). Moreover, ATP hydrolysis could be fully inhibited again when 60 μg/ml egg lysoPC was present by adding higher concentrations of beticolin-1 (not shown). With such a concentration of egg lysoPC (60 μg/ml) the 50% inhibitory concentration of beticolin-1 was displaced in a similar way for both wild and truncated enzymes: from 0.25 μM for AHA2 and 0.30 μM for aha2Δ92 to 17 ± 1 μM for both enzymes. This suggests that the two compounds have antagonistic effects on the enzyme.

The determination of the partition coefficients of beticolin-1

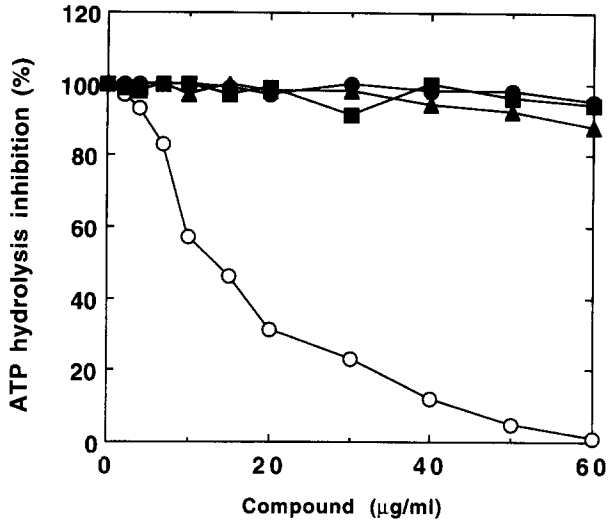

Fig. 2. Effect of detergents and liposomes on the inhibition of AHA2 ATP hydrolysis activity inhibition by beticolin-1. Membranes (2 μg) were preincubated for 30 min with 2 μM beticolin-1 at room temperature, in the medium described in Section 2 supplemented with increasing amounts of egg lysoPC (○), β-D-dodecylmaltoside (●), Brij 58 (■) or soybean asolectin liposomes (▲). The reaction was started with ATP and allowed to proceed for 30 min at 30°C. The data are representative of three independent experiments, and the standard deviations were always less than 14%.

onto liposomes and onto micelles of detergents (Table 1) ruled out the possibility of a particularly high affinity of the toxin for egg lysoPC: the values of the partition coefficient were similar for the three detergents and significantly higher for liposomes.

3.3. *Effect of different carbon chain length lysoPC on AHA2 H^+-ATPase inhibition by beticolin-1*

In order to test the hypothesis that the effect of egg lysoPC on AHA2 inhibition by beticolin-1 is linked to its ability to activate the enzyme, we tested the effects of different lysoPCs with increasing chain lengths: $C_{10:0}$, $C_{12:0}$, $C_{14:0}$, $C_{16:0}$, $C_{18:0}$ and $C_{18:1}$ lysoPC. First, their ability to activate the ATPase activity of the wild-type enzyme was examined. Compared to a control without detergent (100%, corresponds to an activity of 0.86 μmol Pi min^{-1} mg^{-1} protein), long chain lysoPCs were found to increase ATP hydrolysis (Fig. 3) with the following order of efficiency: $C_{18:1}$ (187% of control), $C_{16:0}$ (177%), $C_{18:0}$ (169%). No or very low activation was found with short length lysoPC (106 and 102% of control for $C_{10:0}$ and $C_{12:0}$ lysoPC, respectively). $C_{14:0}$ lysoPC had an intermediate effect (134% of control). This order of efficiency is in agreement with previous reports [9]. Second, we tested the ability of those different compounds to reverse the inhibition of AHA2 ATP hydrolysis activity induced by beticolin-1. A good correlation was found between ability to stimulate ATPase activity and reversion of this inhibition: long chain lysoPCs ($C_{16:0}$, $C_{18:1}$ and $C_{18:0}$ lysoPC) were very effective to restore the ATPase activity, whereas $C_{10:0}$ and $C_{12:0}$ lysoPC have no effect (in the range 0–150 μM) on the inhibition of AHA2, and $C_{14:0}$ lysoPC has an intermediate effect (Fig. 4).

3.4. *Effect of $C_{16:0}$ lysoPC on the inhibition of AHA2 and aha2Δ92 H^+-ATPase by beticolin-1*

Titration of ER membranes with increasing concentrations of $C_{16:0}$ lysoPC (0–150 μM), in the presence of 2 μM beticolin-1, led to a restoration of ATP hydrolysis activity, both on wild-type and truncated enzyme (Fig. 5). For the wild-type enzyme, ATP hydrolysis inhibition decreased rapidly between 0 and 100 μM $C_{16:0}$ lysoPC and a full restoration of the ATPase activity was obtained with 120 μM. For the truncated enzyme, ATP hydrolysis inhibition did not significantly change between 0 and 25 μM $C_{16:0}$ lysoPC and rapidly decreased for concentrations higher than 25 μM, to obtain 77% of restoration of the ATPase activity at 150 μM $C_{16:0}$ lysoPC. The profiles of the two curves, however, looked very similar.

4. Discussion

The only demonstrated regulatory mechanism of the H^+-ATPase at the molecular level, at least in vitro, is the modulation of its activity by an autoinhibitory domain located in the C-terminal part of the protein [5]. This domain is involved in the action of different effectors of the H^+-ATPase activity [6,7,24]. In this report, we showed that egg lysoPC, a compound known to activate the H^+-ATPase by a process involving the C-terminal part of the protein [5], was able to restore the activity of AHA2 fully inhibited by 2 μM beticolin-1 (Fig. 2). The lack of effect of the other detergents we tested, Brij 58 and β-D-dodecylmaltoside, and of soybean liposomes suggested that the reversion of the inhibition was a specific effect of lysoPC. Moreover, the β-D-dodecylmaltoside/protein ratios used in Fig. 2 (ranging from 0 to 30) were sufficient to solubilize the H^+-ATPase [22], and no restoration of the enzyme activity was observed. This ruled out the possibility that the reversion by egg lysoPC of the ATPase activity inhibition could be due to a simple detergent effect. Brij 58 and β-D-dodecylmaltoside do not activate the ATPase activity [9,22],

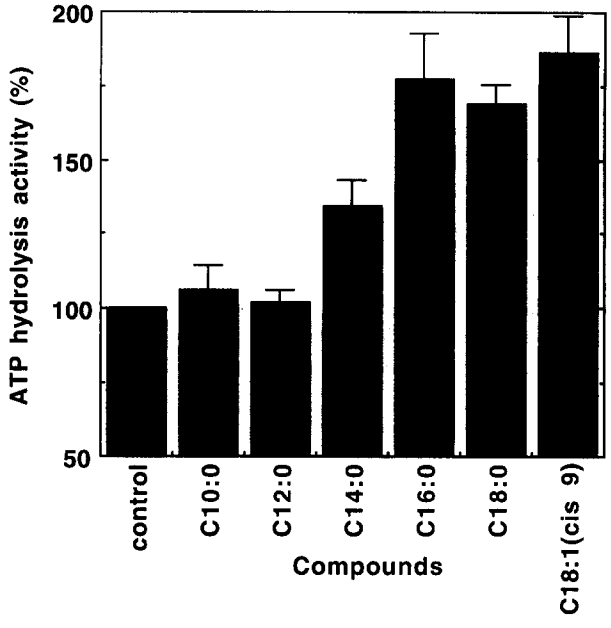

Fig. 3. Activation of AHA2 ATP hydrolysis activity by different carbon chain length lysoPCs. Membranes (2 μg) were incubated in the media described in Section 2 supplemented with 150 μM of the indicated lysoPC. In the control, no lysophospholipids were added (100% corresponds to an activity of 0.86 μmol Pi min^{-1} mg^{-1} protein, no latency was observed). The reaction was started with ATP and allowed to proceed for 30 min at 30°C. The data are the mean ± S.D. of three independent experiments.

and a possible hypothesis was that the capacity of egg lysoPC to reverse the inhibition was linked to its ability to activate the proton pump. The test of lysoPC derivatives with increasing carbon chain length, that have differential stimulatory effect on the ATPase activity supports this hypothesis (Figs. 3 and 4). The specific reversion of the inhibitory effect of beticolin-1 by the lysoPC derivatives that activate the H^+-ATPase could be due to a competition for the binding to the same site(s) on the enzyme. Alternatively, both effectors could bind to separate sites on the protein, the binding of one compound excluding the binding of the second one, via conformational changes. The current results make it impossible to choose between these two hypotheses. However, interesting features are (i) both wild-type and truncated enzyme were inhibited by the same range of beticolin-1 concentrations, ruling out the hypothesis that beticolin-1 could bind to the C-terminal part of the enzyme or inhibit its activity by a mechanism involving the autoinhibitory domain (Fig. 1) and (ii) the inhibition of the ATPase activity due to beticolin-1 can be reversed by lysoPC whether the C-terminal regulatory part of the enzyme is present or not (Fig. 5). Indeed, in the truncated enzyme there are still about 20 amino acids left of the C-terminus. However, this part is not involved in activation of the enzyme [8]

Beticolin-1, because of its inhibitory action, is likely to bind directly to the plasma membrane H^+-ATPase probably to hydrophobic parts of the protein (e.g. transmembrane segments), although it cannot be totally excluded that beticolin-1 could bind to the lipids that strongly bind to the enzyme. The fact that lysoPC reverses the action of beticolin-1 could be taken as evidence for a direct binding of lysoPC to the H^+-ATPase. Indeed, because it is a detergent, lysoPC is expected to distribute into the membrane. In addition to having detergent properties, lysoPC in some way releases the constraint exerted on the enzyme by the C-terminal regulatory domain. The problem is whether this effect requires binding of lysoPC to some other site(s), e.g. the C-terminal regulatory domain of H^+-ATPase, in addition to the binding of the detergent to the membrane embedded part of the enzyme. Four lines of evidence argue against this hypothesis: (i) beticolin-1 obviously does not bind to the C-terminus; (ii) lysoPC is able to abolish the inhibition caused by beticolin-1, and this seems to be caused by the displacement of beticolin-1 from its hydrophobic binding sites; (iii) the binding of lysoPC to beticolin-1 binding site(s) seems to be specific and not related to its function as a detergent; (iv) the efficiency with which lysoPC of different chain lengths reverse the beticolin-1 effect shows the same specificity as the activation by lysoPC of the H^+-ATPase. Taken together, these arguments suggest that lysoPC and beticolin-1 could bind to identical site(s) of the protein, or sites in the close vicinity of each other, and buried into the membrane. The fact that the binding of lysoPC influences the interaction between the inhibitory C-terminal domain and the rest of the enzyme is best explained by assuming that the binding of lysoPC to site(s) buried into the membrane induces a conformational change of the enzyme involving a rearrangement of the C-terminal regulatory domain not situated in the membrane.

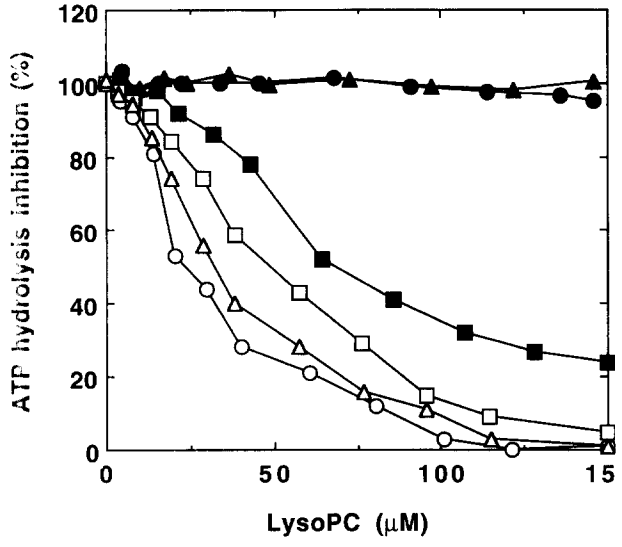

Fig. 4. Effect of different carbon chain length lysoPCs on the inhibition of AHA2 ATP hydrolytic activity by beticolin-1. Membranes (2 μg) were preincubated for 30 min with 2 μM beticolin-1 at room temperature, in the medium described in Section 2 supplemented with increasing concentrations of lysoPC. ▲: $C_{10:0}$ lysoPC; ●: C12:0 lysoPC; ■: $C_{14:0}$ lysoPC; ○: C16:0 lysoPC; □: C18:0 lysoPC; △: C18:1 lysoPC. For each lysoPC concentration, the inhibition percentage value was calculated with reference to the activity of the enzyme without the toxin and with the appropriate amount of lysoPC. The reaction was started with ATP and allowed to proceed for 30 min at 30°C. The data are representative of three independent experiments and the standard deviations were always less than 14%.

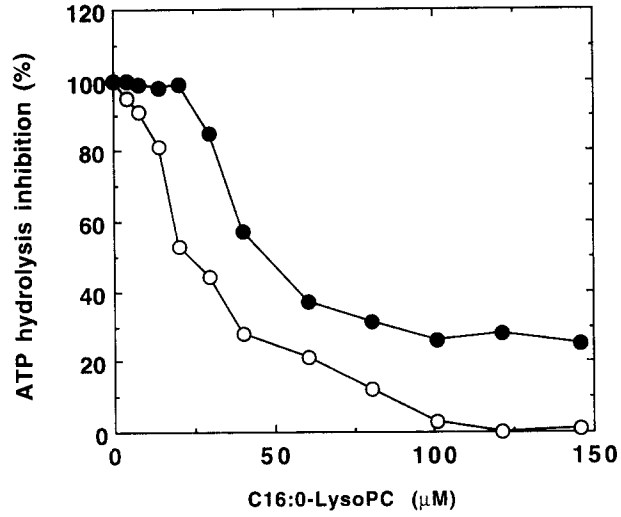

Fig. 5. Effect of $C_{16:0}$ lysoPC on AHA2 (○) and aha2Δ92 (●) ATP hydrolysis inhibition by beticolin-1. Membranes (2 μg) were preincubated for 30 min with 2 μM beticolin-1 at room temperature, in the medium described in Section 2 supplemented with increasing concentrations of $C_{16:0}$ lysoPC. For each lysoPC concentration, the inhibition percentage value was calculated with reference to the activity of the enzyme without the toxin and with the appropriate amount of lysoPC. The reaction was started with ATP and allowed to proceed for 30 min at 30°C. The data are representative of three independent experiments, and the standard deviations were always less than 16%.

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