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Cercospora beticola toxins. Part XVII. The role of the beticolin/Mg^{2+} complexes in their biological activity Study of plasma membrane H^+-ATPase, vacuolar H^+-PPase, alkaline and acid phosphatases

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Abstract

Beticolin-1 and beticolin-2, yellow toxins produced by the phytopathogenic fungus *Cercospora beticola*, inhibit the plasma membrane H^+-ATPase. Firstly, since beticolins are able to form complexes with Mg^{2+}, the role of the beticolin/Mg^{2+} complexes in the inhibition of the plasma membrane proton pump has been investigated. Calculations indicate that beticolins could exist under several forms, in the H^+-ATPase assay mixture, both free or complexed with Mg^{2+}. However, the percentage inhibition of the H^+-ATPase activity is correlated to the concentration of one single form of beticolin, the dimeric neutral complex $Mg_2H_2B_2$, which appears to be the active form involved in the H^+-ATPase inhibition. Secondly, since previous data suggested that beticolins could also be active against other Mg^{2+}-dependent enzymes, we tested beticolin-1 on the vacuolar H^+-PPase, which requires Mg^{2+} as co-substrate, and on the alkaline and acid phosphatases, which do not use Mg^{2+} as co-substrate. Only vacuolar H^+-PPase is sensitive to beticolin-1, which suggests that beticolins are specific to enzymes that use a complex of Mg^{2+} as the substrate. The same $Mg_2H_2B_2$ complex which is responsible of the plasma membrane H^+-ATPase inhibition appears to be also involved in the inhibition of the vacuolar H^+-PPase.

Keywords: Beticolin; ATPase, H^+-; Pyrophosphatase, H^+-; Inhibition; Magnesium ion complex

1. Introduction

The plasma membrane (PM) H^+-ATPase (EC 3.6.1.35) plays a major role in plant cell physiology. Coupling ATP hydrolysis to proton transport from the cytoplasm to the cell exterior, this enzyme generates a proton motive force, which then drives proton-

Abbreviations: BTP, 1,3-bis[tris(hydroxymethyl)methylamino]propane; H^+-ATPase, adenosine triphosphatase proton pump; H^+-PPase, pyrophosphatase proton pump; $Mg_2H_2B_2$, neutral dimeric beticolin/Mg^{2+} complex; PM, plasma membrane.

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coupled symporters and antiporters present on the plant plasma membrane [1]. Because of the important role of this enzyme, its activity is regulated by various factors including phytohormones such as auxin [2] and is also the target of fungal toxins such as fusicoccin [3,4] and bacterial toxins such as syringomicin [5].

Beticolins are yellow, non host-specific toxins produced by the phytopathogenic fungus *Cercospora beticola*. CBT (*Cercospora beticola* toxin, which corresponds to beticolin-1) was shown to induce loss of ions and to inhibit H^+-extrusion in corn roots [6]. Those results suggested that the PM H^+-ATPase could be inhibited by beticolins. We recently demonstrated, using purified PM H^+-ATPase, that this enzyme is a target for these toxins [7]. The dissociation properties of two beticolins, beticolin-1 and beticolin-2 and their ability to form complexes with Mg^{2+} have been studied [8]. Beticolin-1 and beticolin-2 loose three protons when the pH increases from 3.0 to 12.0 and form four different complexes with Mg^{2+}; beticolin-1 is a more efficient chelating agent than beticolin-2. Moreover, at the physiological pH range (6.0 to 8.0), in the presence of Mg^{2+}, the major form of beticolin-1 and beticolin-2 is the dimeric neutral complex ($Mg_2H_2B_2$). However, other beticolin forms could also exist at this pH range.

The first aim of the work was to identify the form of beticolin responsible for the inhibition of the plasma membrane H^+-ATPase. We used the SOLCON software to determine the concentrations of the different forms of beticolin-1 (either free or complexed with Mg^{2+}) at different ATP and Mg^{2+} concentrations. These calculations allowed us to determine whether the inhibition by beticolin-1 of the solubilized, highly purified H^+-ATPase could be correlated to the concentrations of one or several forms of the toxin. In addition, the efficacy of beticolin-1 and beticolin-2, compounds which form complexes with Mg^{2+} with different stability constants, has been compared on the H^+-ATPase hydrolysis activity. The second aim of this work was to investigate whether the beticolins were specific inhibitors of the plasma membrane H^+-ATPase since preliminary results suggested that these toxins could inhibit other enzymes that require Mg^{2+} (Milat, M.L., unpublished data). Thus, the effect of beticolin-1 was also tested on the solubilized vacuolar H^+-PPase (EC 3.6.1.1) that uses a complex of Mg^{2+} and PP_i as substrate [9–11] and on two other enzymes that hydrolyse free ATP (the acid and alkaline phosphatases, EC 3.1.3.2 and EC 3.1.3.1, respectively) and do not require magnesium as the co-substrate [12,13].

2. Materials and methods

2.1. Plant materials

Corn seeds (*Zea mays* L.) were surface sterilised for 20 min with calcium hypochlorite (20 g/l), rinsed with distilled water and germinated on stainless steel screens above distilled water in the dark, at 25°C, for 7 days. Seeds of mung bean (*Vigna radiata* L.) were germinated and grown on vermiculite in the dark at 25°C for 4 days.

2.2. Plasma membrane preparation

All steps were performed at 4°C. Plasma membranes were obtained from maize root microsomes by phase partition as described previously [7].

2.3. Plasma membrane H^+-ATPase purification

The purified plasma membrane H^+-ATPase was obtained according to Grouzis et al. [14]. Briefly, plasma membranes were suspended in a buffer A [10 mM Tris/Mes (pH 7.3), 250 mM sucrose, 1 mM ATP, 1 mM EDTA, 1 mM DTT, 1 mM PMSF, 20% (w/v) glycerol], containing 500 mM KBr and 0.25% Triton X-100, gently stirred for 20 min at 4°C and centrifuged at $120000 \times g$, for 40 min. The pellet was washed with 20 ml of the same buffer without Triton X-100 and centrifuged at $120000 \times g$, for 40 min. The resulting membrane fraction was diluted with buffer A and L-α-lysophosphatidylcholine was added to a final concentration of 2 mg/ml. After 15 min of incubation at room temperature, the sample was centrifuged for 40 min at $120000 \times g$. The supernatant was layered onto a linear glycerol gradient (25–50% (v/v)) and centrifuged for 15 h at $150000 \times g$ in a vertical rotor. Fractions of 1.5 ml were collected from the bottom of the gradient and

the fractions with the highest ATPase specific activity were pooled and stored at −80°C.

2.4. Tonoplast preparation

All steps were performed at 4°C. Tonoplast vesicles were prepared essentially as described by Rea et al. [15]. Mung beans hypocotyls were blended in the homogenisation medium [10% (w/v) glycerol, 5 mM Tris/EGTA, 1% (w/v) ascorbic acid, 1.5% (w/v) polyvinylpolypyrolidone (M_r 40000) and 50 mM Tris/Mes (pH 6.5)) in a tissue/medium ratio of 1:3 (w/v)]. The homogenate was filtered through 6 layers of muslin and centrifuged at 10000 × *g* for 10 min. The supernatant was centrifuged at 100000 × *g*. The resulting pellet (microsomes) was resuspended in buffer B [10% (v/v) glycerol, 1 mM Tris/EGTA, 2 mM DTT and 5 mM Tris/Mes (pH 7.6)], layered onto 10–23% (w/v) sucrose step gradients and centrifuged 100000 × *g* for 2 h. The tonoplast vesicles were carefully removed from the 10–23% interface, diluted in the buffer B and sedimented at 100000 × *g* for 45 min. The resulting pellet was resuspended in the same buffer, frozen in liquid N_2 and stored at −80°C.

2.5. Vacuolar H^+-PPase solubilization

The solubilization follows the procedure described by Britten et al. [16]. The tonoplast vesicles were equilibrated for 30 min in ice-cold buffer C [20% (w/v) glycerol, 1 mM Tris/EGTA, 2 mM $MgSO_4$, 1 mM DTT and 20 mM Tris/acetate (pH 7.5)] and centrifuged at 100000 × *g* for 40 min. The pellet was resuspended in buffer C containing 50 mM KCl and sodium deoxycholate at a final concentration of 2 mg per mg of membrane protein. After a 10 min period of gentle stirring in ice, the suspension was pelleted at 150000 × *g* for 40 min. The supernatant was removed and the pellet resuspended in buffer D [40 mM CHAPS, 20% (w/v) glycerol, 4 mM $MgSO_4$, 1 mM Tris/EDTA, 1 mM DTT, 50 mM KCl, 10 mM Tris/Mes (pH 8.0)], gently stirred for 15 min at room temperature and 20 min on ice. The suspension was centrifuged at 150000 × *g* for 50 min. The supernatant was collected, frozen in liquid N_2 and stored at −80°C.

2.6. Measurement of ATPase, acid phosphatase and alkaline phosphatase activities

Phosphohydrolase activities of the solubilized, purified plasma membrane H^+-ATPase and the two phosphatases (acid and alkaline) were monitored by quantifying the release of inorganic phosphate, according to the method of Ames [17], in the appropriate media.

2.6.1. H^+-ATPase assay

Unless otherwise stated, the standard assay medium (0.5 ml) contained, 25 mM Tris/Mes (pH 6.5), 50 mM KCl, ATP (0.04, 1.5 and 3 mM, depending on the experimental conditions required), 2 mM phospho*enol*pyruvate, 6 units pyruvate kinase and 0.65 μg of purified enzyme. The reaction was started by the addition of $MgSO_4$ (variable amounts, indicated in the figure legends), and allowed to proceed for 45 min at 25°C. Controls were performed to verify that beticolin-1 and beticolin-2 had no effect on pyruvate kinase activity.

2.6.2. Alkaline phosphatase assay

The standard assay medium (0.5 ml) contained 40 mM Hepes/BTP pH 8.0, 0.3 μg alkaline phosphatase (Sigma type VII-L) and 3 mM $MgSO_4$. The reaction was started by the addition of 3 mM ATP and allowed to proceed for 10 min at 25°C.

2.6.3. Acid phosphatase assay

The standard assay medium (0.5 ml) contained 30 mM Tris/Mes (pH 6.5), 10 mM KCl, 5 μg acid phosphatase (Sigma type IV-S) and 0 or 6 mM $MgSO_4$. In the experiments performed without $MgSO_4$, 2 mM EDTA was added to the reaction mixture. The reaction was started by the addition of 6 mM ATP and allowed to proceed for 30 min at 25°C.

2.7. Measurement of H^+-PPase activity

H^+-PPase activity was determined by measuring the released P_i, divided by 2, according to the method of Leigh et al. [11]. The reaction medium (0.5 ml) contained 20 mM Hepes/BTP (pH 8.0), 50 mM KCl, 0.3 mM PP_i/BTP (pH 8.0), 0.1 mM EDTA/BTP and 1.25 μg of solubilized enzyme. The reaction was

started by addition of $MgSO_4$ (variable amounts, indicated in the figure legends), and allowed to proceed for 1 h at 25°C.

2.8. Chemicals

Beticolins were purified as described by Milat and Blein [18]. The toxins were dissolved in DMSO/H_2O (2:98, v/v) to a 0.1 mg/ml stock solution. All other chemicals were reagent grade. All the solutions were made using ultra-pure water produced by a Millipore Milli-Q plus system.

2.9. Calculation of complex concentrations

The concentrations of the different ligand-metal complexes present in the enzymatic assays were calculated using the computer program SOLCON, written by D.C.S. White (University of York) and Y.E. Goldman (University of Pennsylvania). The dissociation constants of the beticolins complexes are from Mikès et al. [8], and those of the other complexes from Martell and Smith [19].

Dissociation constants of beticolin-1 complexes: $[H^+][B^{3-}]/[HB^{2-}]$, $8.71 \cdot 10^{-13}$ M; $[H^+]^2[B^{3-}]/[H_2B^-]$, $4.17 \cdot 10^{-19}$ M^2; $[H^+]^3[B^{3-}]/[H_3B]$, $5.01 \cdot 10^{-24}$ M^3; $[Mg^{2+}]^2[B^{3-}]^2/[Mg_2B_2^{2-}]$, $1.38 \cdot 10^{-17}$ M^3; $[Mg^{2+}]^2[H^+][B^{3-}]^2/[Mg_2HB_2^-]$, $9.77 \cdot 10^{-29}$ M^4; $[Mg^{2+}]^2[H^+]^2[B^{3-}]^2/[Mg_2H_2B_2]$, $4.57 \cdot 10^{-39}$ M^5; $[Mg^{2+}]^2[H^+]^3[B^{3-}]^2/[Mg_2H_3B_2^+]$, $1.32 \cdot 10^{-44}$ M^6.

Dissociation constants of beticolin-2 complexes: $[H^+][B^{3-}]/[HB^{2-}]$, $6.16 \cdot 10^{-13}$ M; $[H^+]^2[B^{3-}]/[H_2B^-]$, $2.19 \cdot 10^{-19}$ M^2; $[H^+]^3[B^{3-}]/[H_3B]$, $1.44 \cdot 10^{-24}$ M^3; $[Mg^{2+}]^2[B^{3-}]^2/[Mg_2B_2^{2-}]$, $2.09 \cdot 10^{-16}$ M^3; $[Mg^{2+}]^2[H^+][B^{3-}]^2/[Mg_2HB_2^-]$, $1.86 \cdot 10^{-27}$ M^4; $[Mg^{2+}]^2[H^+]^2[B^{3-}]^2/[Mg_2H_2B_2]$, $1.12 \cdot 10^{-37}$ M^5; $[Mg^{2+}]^2[H^+]^3[B^{3-}]^2/[Mg_2H_3B_2^+]$, $8.31 \cdot 10^{-43}$ M^6.

Dissociation constants of other complexes: $[H^+][ATP^{4-}]/[HATP^{3-}]$, $2.91 \cdot 10^{-7}$ M; $[H^+]^2[ATP^{4-}]/[H_2ATP^{2-}]$, $2.45 \cdot 10^{-11}$ M^2; $[K^+][ATP^{4-}]/[KATP^{3-}]$, $1.25 \cdot 10^{-1}$ M; $[Na^+][ATP^{4-}]/[NaATP^{3-}]$, $6.31 \cdot 10^{-2}$ M; $[Mg^{2+}][H^+][ATP^{4-}]/[MgHATP^-]$, $1.93 \cdot 10^{-9}$ M^2; $[Mg^{2+}][ATP^{4-}]/[MgATP^{2-}]$, $3.05 \cdot 10^{-5}$ M; $[H^+][PP_i^{4-}]/[HPP_i^{3-}]$, $1.12 \cdot 10^{-9}$ M; $[H^+]^2[PP_i^{4-}]/[H_2PP_i^{2-}]$, $8.51 \cdot 10^{-16}$ M^2; $[Mg^{2+}][PP_i^{4-}]/[MgPP_i^{2-}]$, $3.63 \cdot 10^{-6}$ M; $[Mg^{2+}]^2[PP_i^{4-}]/[Mg_2PP_i]$, $1.66 \cdot 10^{-8}$ M^2; $[Mg^{2+}][H^+][PP_i^{4-}]/[MgHPP_i^-]$, $8.91 \cdot 10^{-3}$ M^2; $[K^+][PP_i^{4-}]/[KPP_i^{3-}]$, $3.98 \cdot 10^{-2}$ M; $[K^+][H^+][PP_i^{4-}]/[KHPP_i^{2-}]$, $1.78 \cdot 10^{-10}$ M^2; $[H^+][EDTA^{4-}]/[HEDTA^{3-}]$, $5.50 \cdot 10^{-11}$ M; $[H^+]^2[EDTA^{4-}]/[H_2EDTA^{2-}]$, $3.80 \cdot 10^{-17}$ M^2; $[H^+]^3[EDTA^{4-}]/[H_3EDTA^-]$, $8.13 \cdot 10^{-20}$ M^3; $[H^+]^4[EDTA^{4-}]/[H_4EDTA]$, $8.32 \cdot 10^{-22}$ M^4; $[Mg^{2+}][EDTA^{4-}]/[MgEDTA^{2-}]$, $2.04 \cdot 10^{-9}$ M; $[Mg^{2+}][H^+][EDTA^{4-}]/[MgHEDTA^-]$, $2.88 \cdot 10^{-13}$ M^2; $[K^+][EDTA^{4-}]/[KEDTA^{3-}]$, $1.58 \cdot 10^{-1}$ M; $[Mg^{2+}][SO_4^{2-}]/[MgSO_4]$, $5.62 \cdot 10^{-3}$ M; $[K^+][SO_4^{2-}]/[KSO_4^-]$, $1.26 \cdot 10^{-1}$ M.

3. Results

3.1. Effect on the PM H^+ – ATPase activity

When the total Mg^{2+} concentration was increased from 0.04 to 3 mM, in the presence of fixed concentrations of ATP (0.04 mM) and total beticolin-1 (3.1

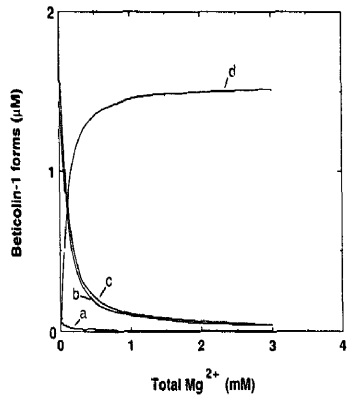

Fig. 1. Concentration of the different beticolin-1 forms, as a function of Mg^{2+} concentration. The calculations have been made for a reaction mixture containing 25 mM Tris/Mes (pH 6.5), 50 mM KCl, 0.04 mM ATP, 3.1 μM of beticolin-1, 2 mM phospho*enol*pyruvate, 6 units pyruvate kinase and various amounts of $MgSO_4$. Line a: H_3B, line b: H_2B^-, line c: HB^{2-}, line d: $Mg_2H_2B_2$. The concentrations of the three other forms of beticolin-1 ($Mg_2B_2^{2-}$, $Mg_2HB_2^-$ and $Mg_2H_3B_2^+$), are negligible (< 0.1 nM) and are not presented.

μM) at pH 6.5, the SOLCON calculations indicated that the concentrations of the non-complexed toxin forms (H_3B, H_2B^- and HB^{2-}) rapidly decreased whereas that of the neutral complex ($Mg_2H_2B_2$) increased (Fig. 1). The concentrations of the other beticolin-1/Mg^{2+} complex species were negligible (less than 0.1 nM when 3 mM Mg^{2+} are present in the assay mixture). This is in agreement with the studies of Mikès et al. [8]. When the same computations were made at three different ATP concentrations (0.04, 1.5 and 3 mM), with the same beticolin-1 concentration (3.1 μM), the calculations predict three different patterns of concentration increase for the $Mg_2H_2B_2$ complex (Fig. 2A). This reflects the ability of the ATP to trap magnesium ions, a feature which influences the formation of the beticolin/Mg^{2+} complexes. In the presence of 3 mM ATP and Mg^{2+} concentrations lower than 0.16 mM, the amount of beticolin-1 which is complexed with Mg^{2+} is negligible (less than 0.1 nM).

When the effects of beticolin-1 on the activity of solubilized PM H^+-ATPase were measured under conditions shown in Fig. 2A the degree of inhibition increased with the increase in total Mg^{2+} concentration but the effect depended on the total ATP concentration (Fig. 2B). The similarity between the increase in the $Mg_2H_2B_2$ concentration (Fig. 2A) and the inhibition of the PM H^+-ATPase variation (Fig. 2B) suggests that the neutral beticolin/Mg^{2+} complex is responsible for the inhibition. When the percentage

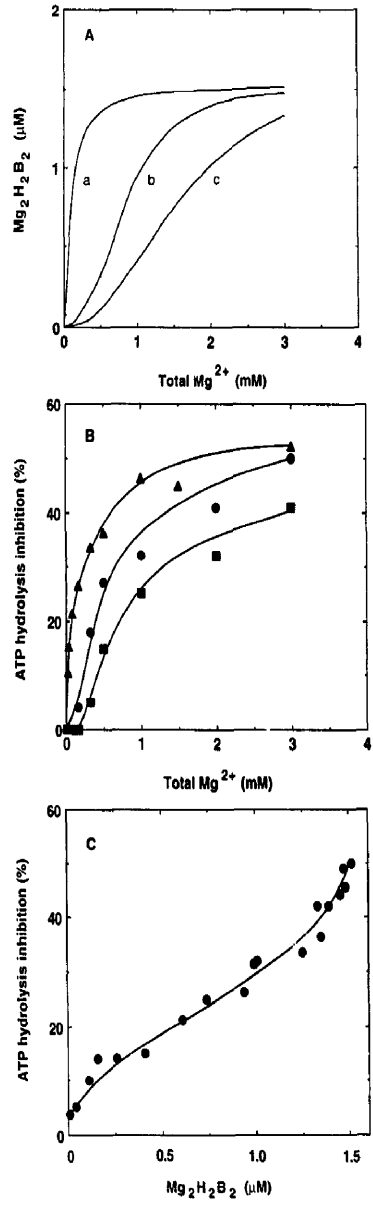

Fig. 2. Effect of Mg^{2+} concentration on the formation of the neutral magnesium complex of beticolin-1 and on inhibition of the H^+-ATPase by beticolin-1. (A) Calculated $Mg_2H_2B_2$ concentration in the H^+-ATPase assay mixture. The computations were made for a reaction mixture (0.5 ml) containing 25 mM Tris/Mes (pH 6.5), 50 mM KCl, 0.04 (line a), 1.5 (line b) or 3 (line c) mM ATP, 3.1 μM of beticolin-1, 2 mM phospho-*enol*pyruvate, 6 units pyruvate kinase and various amounts of $MgSO_4$, at 25°C. (B) Percent inhibition of ATP hydrolysis. Purified H^+-ATPase (0.65 μg) was incubated in the mixture described in (A), in presence or in absence of 3.1 μM of beticolin-1. The reaction was started by addition of various amounts of $MgSO_4$ (0.04 to 3 mM) and allowed to proceed for 45 min at 25°C (Maximal specific activity of the control: 2.6 μmol P_i min^{-1} mg^{-1} protein). ATP concentrations: 0.04 (▲), 1.5 (●) and 3 (■) mM. The experiments were performed at least three times and the figure shows a typical result. (C) Inhibition of H^+-ATPase hydrolytic activity as a function of the concentration of the $Mg_2H_2B_2$ neutral complex.

inhibition was plotted versus the $Mg_2H_2B_2$ concentration, a positive correlation between the two parameters was obtained (Fig. 2C). This strongly suggests that the neutral complex is indeed the active form of beticolin-1 which inhibits the PM H^+-ATPase.

A comparison of beticolin-1 and beticolin-2 further suggested that the $Mg_2H_2B_2$ complex is responsible for inhibition of the PM H^+-ATPase. As expected from the difference in dissociation constants [8], the SOLCON calculations indicated that less of the neutral complex was formed by beticolin-2 compared with beticolin-1 under the same conditions (data not shown). Similarly, beticolin-2 was less inhibitory to the PM H^+-ATPase than beticolin-1 (Fig. 3).

The formation of the neutral magnesium complex of beticolin-1 increases when the pH raises from 5.5 to 7.5 (Fig. 4A). The inhibition of the PM H^+-ATPase hydrolytic activity also increases over the same pH range (Fig. 3B), and a positive correlation exists between the $Mg_2H_2B_2$ concentration and the inhibition of enzyme activity (Fig. 4B, inset).

3.2. *Effect of beticolin-1 on acid and alkaline phosphatase activities*

A 30 min incubation of acid phosphatase with beticolin-1 (1.5 to 70 μM) had no effect on enzyme activity, in the absence or in the presence of 4 mM Mg^{2+} (specific activity 1.01 and 0.96 μmol P_i min^{-1} mg^{-1} protein, respectively). A similar experiment showed that alkaline phosphatase assayed in presence of 4 mM of Mg^{2+} (specific activity 15.30 μmol P_i

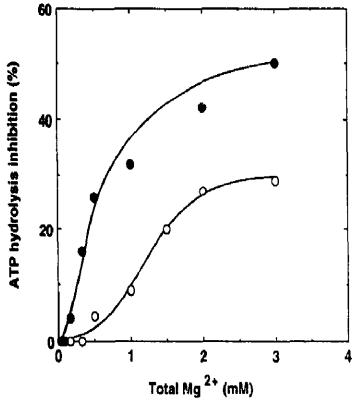

Fig. 3. Comparison of beticolin-1 and beticolin-2 inhibitory effects on the purified H^+-ATPase at different Mg^{2+} concentrations. Experimental conditions are as described in Fig. 2, with an ATP concentration of 1.5 mM. Concentrations of beticolin-1 (●) and beticolin-2 (○) were 3.2 μM. The experiments were performed at least three times and the figure shows a typical result.

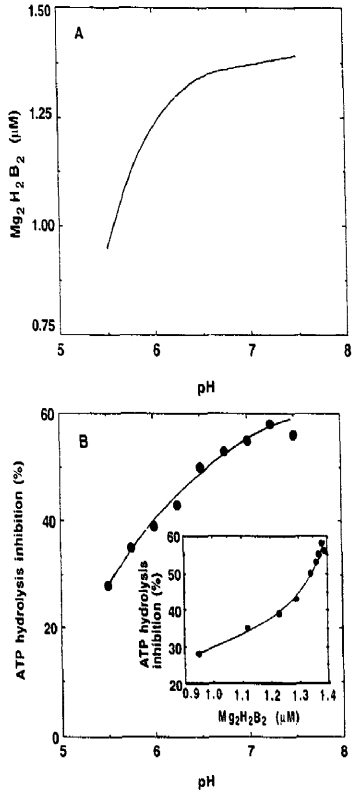

Fig. 4. Effect of pH on H^+-ATPase inhibition by beticolin-1. (A) Calculation of the $Mg_2H_2B_2$ neutral complex concentration, as a function of pH. Computations have been made for a reaction mixture (0.5 ml) containing 25 mM Tris/Mes, 50 mM KCl, 1.5 mM ATP, 3 mM $MgSO_4$, 3.1 μM of beticolin-1, 2 mM phospho*enol*pyruvate, 6 units pyruvate kinase and various amounts of $MgSO_4$, at 25°C and at various pH (5.5 to 7.5). (B) Percent inhibition of ATP hydrolysis as a function of pH. Purified H^+-ATPase (0.65 μg) was incubated in the mixture described in (A), in presence or in absence of 3.1 μM of beticolin-1. The reaction was started by addition of various amounts of $MgSO_4$ (0.04 to 3 mM) and allowed to proceed for 45 min at 25°C. (Inset) ATP hydrolysis percentage inhibition as a function of $Mg_2H_2B_2$ concentration. The experiments were performed at least three times and the figure shows a typical result.

min^{-1} mg^{-1} protein) was also unaffected by beticolin-1.

3.3. Effect of beticolin-1 on the activity of the solubilized vacuolar H^+-PPase

Incubation of 1.25 μg solubilized H^+-PPase with beticolin-1 for 1 hour resulted in a dose-dependent inhibition of activity (data not shown). A 50% inhibition was obtained with 2.8 μM beticolin-1. Thus, like the PM H^+-ATPase, the H^+-PPase is a target for beticolin-1, in vitro.

SOLCON calculations indicated that when the Mg^{2+} concentration increases from 0.16 to 3 mM, in the presence of 3.1 μM beticolin-1, 0.1 mM EDTA and 0.3 mM PP_i, at pH 8.0, the concentration of the unchelated form of beticolin-1 (H_3B, H_2B^- and HB^{2-}) rapidly decreases, whereas the concentration of the neutral complex $Mg_2H_2B_2$ rapidly increases (data not shown, but very similar to Fig. 1). The concentrations of the other complexes were negligible (less than 0.1 nM for $Mg_2B_2^{2-}$, $Mg_2H_3B_2^+$) or very low (0.01 μM for $Mg_2HB_2^-$), even at 3 mM Mg^{2+}. When PP_i hydrolysis is performed under these conditions, the solubilized vacuolar H^+-PPase displays the classical, non-Michaelian, Mg^{2+} activation kinetics [20], in the presence or in the absence of beticolin-1 (data not shown). At the same time, the inhibition of enzyme activity by beticolin-1 increases when the magnesium concentration increases from 0.16 to 1.3 mM, i.e. conditions in which the $Mg_2H_2B_2$ complex concentration also increases (Fig. 5). Moreover, the plot of the inhibition of PP_i hydrolysis activity versus $Mg_2H_2B_2$ (Fig. 5, inset) shows that the two parameters are correlated.

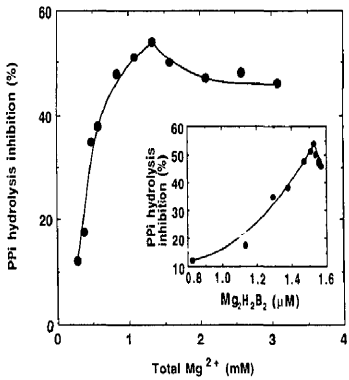

Fig. 5. Effect of various Mg^{2+} concentrations on the inhibition of the solubilized H^+-PPase by beticolin-1. Solubilized H^+-PPase (1.25 μg protein) was incubated in a reaction mixture (20 mM Hepes/BTP pH 8.0, 50 mM KCl, 0.1 mM EDTA, 0.3 mM PP_i/BTP, in the presence or in the absence of 3.1 μM beticolin-1. The reaction was started by addition of various amounts of $MgSO_4$ (0.16 to 3 mM) and allowed to proceed for 1 h, at 25°C (Maximal specific activity of the control: 0.6 μmol PP_i hydrolyzed min^{-1} mg^{-1} protein). (Inset) Inhibition of PP_i hydrolysis as a function of $Mg_2H_2B_2$ concentration. The experiments were performed at least three times and the figure shows a typical result.

4. Discussion

The inhibition of the solubilized PM H^+-ATPase activity by beticolin-1 is clearly related to the concentration of the $Mg_2H_2B_2$ complex in the assay mixture (Fig. 2C). When the concentration of the complex is negligible (less than 0.1 nM), no inhibition of the PM H^+-ATPase is observed (Fig. 2A and B, curves with 3 mM ATP and Mg^{2+} concentrations below 0.16 mM). This strongly supports the hypothesis that this complex is inhibiting the PM H^+-ATPase, in vitro. The comparison between the effects of beticolin-1 and beticolin-2 is also consistent with this suggestion. The calculations indicate that over a Mg^{2+} concentration range between 0.04 and 3 mM, the concentration of the neutral complex is always lower for beticolin-2 than for beticolin-1 and the experiments show that the inhibition of ATP hydrolysis by beticolin-2 is always less than by beticolin-1. Only few data concerning the cytosolic free Mg^{2+} concentration are available in the literature. The only measurement in plants suggests a concentration of 0.4 mM [21], and in animal cells the values range between 0.4 and 3.5 mM [22,23]. However, even a cytosolic free Mg^{2+} concentration of 0.4 mM would allow the formation of the $Mg_2H_2B_2$ complex in the plant cell, suggesting that inhibition of the PM H^+-ATPase could also occur in the whole cell. Experiments studying the plasma membrane depolarisation effect of the beticolins on tobacco cell suspensions are now in progress in order to investigate this hypothesis.

When the pH rises from 5.5 to 7.5, the increase in the inhibition of the PM H^+-ATPase induced by beticolin-1 can also be related to an increase in the $Mg_2H_2B_2$ complex concentration (Fig. 4A and B). However, the inhibition percentage continues to increase over pH values ranging between 6.5 and 7.5 (Fig. 4B), whereas the concentration of the complex does not change significantly. This suggests that the concentration of the $Mg_2H_2B_2$ complex is not the only parameter to consider to explain the pH effect. Possibly, the interaction between the beticolin-1/Mg^{2+} complex and the PM H^+-ATPase involves an ionisable group with a pK around 6.5. This group could correspond to the imidazole group of a histidine residue that would need to be protonated to interact with the $Mg_2H_2B_2$ complex. The involvement of such a group has been evoked for the modulation of the PM H^+-ATPase activity by its C-terminal domain [24]. An alternative hypothesis is that the pH changes could affect the enzyme conformation and increase the accessibility of the $Mg_2H_2B_2$/PM H^+-ATPase interaction site.

It appears that the formation of the same neutral $Mg_2H_2B_2$ complex is also involved in the inhibition of the vacuolar H^+-PPase by beticolin-1. The effect of the toxin is maximal when 1.3 mM Mg^{2+} is present in the assay medium (Fig. 5). Under these conditions, calculations indicate that the concentration of the $Mg_2H_2B_2$ complex is close to its maximum. Although free Mg^{2+} is an activator of the enzyme [10], the inhibition of the H^+-PPase activity cannot be due to trapping of the free Mg^{2+} by the toxin: the presence of 3.1 μM of beticolin-1, does not significatively change the concentration of free Mg^{2+}, particularly at a total Mg^{2+} concentration of 1.3 mM. The slight diminution of the inhibition observed at Mg^{2+} concentrations above 1.3 mM could be due to an increase in the concentration of an other complex, that restricts the binding of the beticolin-1/Mg^{2+} complex. Mg_2PP_i, which is believed to be both substrate and noncompetitive inhibitor of the enzyme [11], could be a such complex: it is the only chemical species whose concentration continues to increase when the Mg^{2+} concentration exceeds 1.3 mM (data not shown).

The fact that beticolin-1 inhibits both the PM H^+-ATPase and the vacuolar H^+-PPase suggests that beticolins could be specific for enzymes that use Mg^{2+} as a co-substrate, and not to enzymes which hydrolyse ATP. This is supported by the lack of beticolin-1 activity on the alkaline phosphatase, which is activated by magnesium but does not require it as co-substrate, or on the acid phosphatase which has no requirement for magnesium [12,13]. Moreover, recent work reporting the effect of beticolin-1 on the vacuolar H^+-ATPase (EC 3.6.1.3) from *Commelina communis* L. protoplasts [25] supports this hypothesis. If such a specificity is a reality, it allows us to assume that beticolins could also affect the mitochondrial ATPase, and other magnesium dependent cation translocases both in vegetal and animal cells.

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