Although a well ascertained evidence proves that the activity of the plant plasma membrane H^+-ATPase is regulated by 14-3-3 proteins, information about physiological factors modulating the phosphorylation-dependent association between 14-3-3 proteins and the proton pump is largely incomplete. In this paper we show that the 5'-AMP-mimetic, 5-aminoimidazole-4-carboxamide ribonucleoside (AICAR), inhibits the fusicoccin-promoted proton extrusion in maize roots. We also demonstrate that 5'-AMP inhibits the association of 14-3-3 proteins with the C-terminal domain of the H^+-ATPase in an overlay assay as well as the 14-3-3-dependent stimulation of the Arabidopsis thaliana H^+-ATPase AHA1 isoform expressed in yeast membranes. Finally, by means of affinity chromatography with immobilized 5'-AMP and trinitrophenyl-AMP fluorescence analysis, we demonstrate that the 14-3-3 isoform GF14-6 from maize is able to bind 5'-AMP. The possible role of 5'-AMP as a general regulator of 14-3-3 functions in the plant cell is discussed.

14-3-3 proteins are conserved acidic proteins occurring, in a number of isoforms, in all eukaryotic organisms, in which they function as regulators of signaling pathways (1, 2). In plants these proteins seem to have evolved peculiar functions, among which are the regulation of key enzymes of primary metabolism such as nitrate reductase and sucrose-phosphate synthase (3). The common theme in 14-3-3 action is its ability to associate with target proteins through binding to phosphorylated consensus motifs (4, 5).

The plasma membrane H^+-ATPase is the pivotal enzyme for energization of secondary active transport in the plant cell, generating the electrochemical gradient that provides the driving force for a number of key physiological processes such as stomata opening, phloem loading, and root ion uptake (6). As far as the molecular bases of H^+-ATPase regulation, some light has been shed by investigating the mode of action of the fungal toxin fusicoccin (FC).1 In fact, it has been ascertained that FC promotes the irreversible association of stimulatory 14-3-3 proteins with the C-terminal autoinhibitory domain of the H^+-ATPase (7–11). Very recently it has been shown that blue light activates the H^+-ATPase of atomata guard cells by promoting 14-3-3 association (12). This finding indicates that 14-3-3 proteins are physiological regulators of the H^+-ATPase and raises a question about the mechanisms controlling 14-3-3 association under natural conditions. Although evidence for a phosphorylation-mediated regulation has been reported (12–14), it is very likely that to integrate a number of stimuli and to achieve fine regulation, multiple mechanisms may occur in the plant cell. Circumstantial evidence of the presence of plant extracts of endogenous ligands able to inhibit the FC-stimulated H^+-ATPase activity have been reported (15). Recently, it has been shown that 5'-AMP is able to inhibit 14-3-3 association with the nitrate reductase (16). In this paper we demonstrate that 5'-AMP is able to interfere with 14-3-3 binding to the proton pump and consequently to hamper 14-3-3 stimulation, therefore representing an endogenous modulator of the plasma membrane H^+-ATPase.

**EXPERIMENTAL PROCEDURES**

**Chemicals—**FC was prepared according to Ballio et al. (17); [γ-32P]ATP (specific activity 110 TBq/mmol) and thrombin were from Amersham Pharmacia Biotech. Protein kinase A, catalytic subunit, 5'-AMP agaroase, 5-aminoimidazole-4-carboxamide ribonucleoside (AICAR), and AMP were from Sigma. 2'-O-(trinitrophenyl)-AMP (TNP-AMP) was from Molecular Probes (Eugene, Oregon). The peptide biotinyl-LKGLIDIDTIQQNYTpV (where Tp stands for phosphothreonine) was synthesized by NeoSystems (Strasbourg, France). Chemicals for gel electrophoresis were from Bio-Rad. All other reagents were of analytical grade.

**Plant Material—**Maize seeds (Zea mays L. cv. Santos) from Dekalb (Mestre, Italy) were germinated and grown in the dark for 6 days as already described (18). Roots were suspended in 0.5 mM CaCl2 for 30 min and then cut into segments 1 cm long. 500-mg roots were dispersed in 5 ml of 0.5 mM potassium phosphate buffer, pH 6.5, containing 5 mM KCl and incubated at 27 °C for the addition of 10 μM FC and 10 μM AICAR. The pH of the incubation medium was measured every 30 min with a Hanna HI8520 pH meter equipped with a Hanna HI1083 glass microelectrode (Padova, Italy).

**Purification of ER from Yeast Expressing AHA1—**Plasma membrane H^+-ATPase isoform AHA1 (Arabidopsis thaliana H^+-ATPase isoform 1) was expressed in Saccharomyces cerevisiae as described previously (19). After cell homogenization, the membranes were purified by differential centrifugation, and the ER, containing most of the AHA1, was isolated by sucrose gradient centrifugation (20).

**Isolation of Maize Plasma Membranes—**Two-phase partitioned plasma membranes from maize roots were obtained as described previously (18).

**Expression of the C-terminal Domain of the H^+-ATPase—**The last 103 amino acids of the MHA2 isoform (Maize H^+-ATPase isoform 2) were expressed in Escherichia coli as a fusion protein with glutathione S-transferase (GST) as described in Ref. 9. SDS-Polyacrylamide Gel Electrophoresis and Overlay Assay—SDS-polyacrylamide gel electrophoresis was performed as described by Laemmli (21) in a Mini Protein apparatus (Bio-Rad). The cDNA of the 14-3-3 isoform GF14-6 from maize, cloned into a pGEX-2TK vector, was
expressed in E. coli as described previously (9). The expression system produces a GST-fused 14-3-3 containing a cAMP-dependent protein kinase phosphorylation site and a thrombin site between the two polypeptides. The 32P-labeled GF14-6 was obtained as already described (9). The specific activity of 32P-labeled 14-3-3 was 3.5 MBq/mg.

The overlay assay was carried out according to Pullone et al. (9) with minor modifications. Briefly, two-phase partitioned plasma membranes (10 μg of protein) or the GST-C-terminal domain of the MHA2 (0.5 μg) were subjected to SDS-polyacrylamide gel electrophoresis and blotted onto nitrocellulose membrane using a semidy LKB apparatus (2 h, 0.8 mA cm–2). The membrane was blocked with 5% fatty acid-free milk in buffer H (25 mM Hepes-OH, 75 mM KCl, 5 mM MgCl2, 1 mM dithiothreitol, 0.1 mM EDTA, and 0.05% Tween 20, pH 7.5) and then incubated overnight at 4 °C in the same buffer containing 2% fatty acid-free milk, the 32P-labeled GF14-6 (8.3 kBq/ml), and where indicated, 10 μM FC and 100 μM 5'AMP. After incubation, the membrane was washed three times with buffer H, dried, and subjected to autoradiography at –80 °C.

Binding of GF14-6 to Resin-bound Phosphopeptide—The peptide biotinyl-LKGLDIDTIQQNYTpV, where Tp represents phosphothreonine, 0.1 mg/ml, and BSA was used at 0.1 mg/ml.

Fluorescence Spectroscopy—Fluorescence emission spectrum of TNP-AMP was determined according to Serrano (22). The fluorescence emission spectrum of TNP-AMP was determined as described above with addition of 0.1 mg/ml BSA.

RESULTS

AICAR Inhibits FC-induced Proton Extrusion in Maize Roots—Administration of AICAR to intact cells causes the accumulation inside the cell of its phosphorylated form, 5-aminoimidazole-4-carboxamide ribonucleoside monophosphate, a compound mimicking the effect of 5'-AMP (24, 25). To ascertain whether 5'-AMP could interfere with the FC-mediated stimulation of the H+-ATPase, segments of maize roots were incubated with FC in the presence or absence of AICAR. The results are reported in Fig. 1. As expected, 10 μM FC induced a pH decrease of the incubation medium of about 2 units compared with the control, whereas AICAR was almost ineffective. Incubation of roots with 10 mM AICAR in the presence of FC resulted in a strong (∼75%) inhibition of the proton extrusion.

5'-AMP Inhibits 14-3-3-induced Stimulation of AHA1 in vitro—The effect of 5'-AMP on the 14-3-3 in vitro induced activation of the H+-ATPase was tested by using purified ER vesicles of S. cerevisiae expressing the AHA1 isoform of A. thaliana. In this system a significant and reproducible stimulation of H+-ATPase can be obtained by exogenous 14-3-3 proteins, provided that FC is also added. No stimulation of the H+-ATPase activity can be observed in the presence of 14-3-3 but in the absence of FC (10).

As shown in Fig. 2, the administration of 5 μM FC to a mixture containing ER vesicles and the 14-3-3 isoform GF14-6 nearly doubled the ATPase activity of AHA1. This stimulation was reduced progressively by increasing amounts of 5'-AMP ranging from 10 to 500 μM (open circles). 5'-AMP additions in the absence of FC (filled squares) were completely ineffective, thus indicating that the inhibitory effect of 5'-AMP was caused by a reduced capability of 14-3-3 proteins to stimulate the H+-ATPase rather than to a direct effect on the proton pump.

5'-AMP Inhibits 14-3-3 Binding to the H+-ATPase—The effect of 5'-AMP on the association of 14-3-3 proteins with the proton pump was investigated by means of an overlay assay. In this system the 32P-labeled GF14-6-14-3-3 maize isoform was used as probe, and the H+-ATPase from maize roots or the GST-fused C-terminal domain of the MHA2 isoform was used as bait. The results of autoradiography are reported in Fig. 3A. As expected, the 14-3-3 bound to the H+-ATPase; the association was strongly increased by FC, whereas interaction with the C terminus occurred only in the presence of the toxin (9). The addition of 100 μM 5'-AMP in the incubation medium
significantly reduced both the association of the 14-3-3 with the H\textsuperscript{+}-H\textsubscript{11001}-ATPase and the C terminus even when the interaction was stabilized by FC. These data were confirmed by testing the capability of GF14-6 to bind a biotinyl-peptide reproducing the last 15 amino acids of the H\textsuperscript{+}-H\textsubscript{11001}-ATPase isoform MHA2, which contains the phosphorylated 14-3-3 binding site (11). The peptide was immobilized onto a streptavidin-agarose resin and incubated with\textsuperscript{32}P-labeled GF14-6 in the absence or presence of 5\textsuperscript{-}AMP and/or FC. The results are shown in Fig. 3B. 1 mM 5\textsuperscript{-}AMP resulted in a 40\% reduction of GF14-6 binding to the peptide. The inhibitory effect of 5\textsuperscript{-}AMP was also detectable at 100 \mu M 5\textsuperscript{-}AMP (10\% of inhibition, data not shown). As expected, FC brought about a strong increase of binding (112\%); interestingly, 5\textsuperscript{-}AMP was able to partially counteract (57\%) the FC action.

GF14-6 binds 5\textsuperscript{-}AMP—To test whether the GF14-6 isoform was able to directly bind 5\textsuperscript{-}AMP, two different methods were used.

**Fig. 3. Effect of 5\textsuperscript{-}AMP on the interaction between GF14-6 and H\textsuperscript{+}-ATPase.** A, autoradiography of the overlay assay. 10 \mu g of purified plasma membranes from maize roots (lanes 1) or 0.5 \mu g of affinity-purified GST-C terminus of MHA2 H\textsuperscript{+}-ATPase (lanes 2) were subjected to SDS-polyacrylamide gel electrophoresis and blotted onto nitrocellulose membrane. The membrane was then incubated with \textsuperscript{32}P-labeled GF14-6. The 100-kDa band was identified as the H\textsuperscript{+}-ATPase by immunodecoration with anti-H\textsuperscript{+}-ATPase antibodies (data not shown). Where indicated, 10 \mu M FC and 100 \mu M 5\textsuperscript{-}AMP were added in the incubation medium. The experiment was repeated three times, and a representative one is shown. B, 5\textsuperscript{-}AMP effect on the binding of GF14-6 to a phosphopeptide reproducing the 14-3-3 binding site of the MHA2 H\textsuperscript{+}-ATPase. 0.5 nmol of biotinyl peptide were immobilized onto streptavidin-agarose beads and incubated with 3.5 kBq of \textsuperscript{32}P-labeled GF14-6. Where indicated, 10 \mu M FC and 1 mM 5\textsuperscript{-}AMP were added. The data are the mean of three replicates.
used. In the first method TNP-AMP, an analogue of 5′-AMP, the fluorescence of which increases upon binding to proteins (26), was utilized. As shown in Fig. 4A, the relative intensity of the fluorescence peak at 545 nm of TNP-AMP was increased when the GF14-6 was added to the incubation medium. On the contrary, addition of an equimolar amount of BSA did not affect the fluorescence level, thus confirming the specificity of the binding. In the second method, the ability of GF14-6 to bind to a 5′-AMP-derivatized agarose matrix was tested. As reported in Fig. 4B, upon incubation of the 5′-AMP-agarose matrix with 32P-labeled GF14-6, a significant amount of radioactivity was bound by the resin. The specificity of the interaction was tested by the addition of a saturating concentration of 5′-AMP to the incubation mixture.

**DISCUSSION**

In the last few years a large body of evidence has been accumulated that 14-3-3 proteins serve as modulators of multiple cellular processes through the association with a number of proteins. It has been firmly ascertained that the main mechanism regulating 14-3-3 association is represented by phosphorylation of serine or threonine residues within consensus motifs. On the other hand, the complexity of functions of 14-3-3 proteins renders conceivable that multiple mechanisms can finely modulate the phosphorylation-dependent binding. Although it has been shown that factors such as Mg2+ levels or pH can influence association of 14-3-3 proteins to their partners (9, 27), information on the occurrence of physiological compounds able to modulate the 14-3-3 action is still largely undetermined.

In this paper we show that the endogenous metabolite 5′-AMP is able to hamper the association of 14-3-3 proteins with the H+-ATPase. This compound is also able to inhibit the 14-3-3-promoted stimulation of the plasma membrane H+-ATPase in vitro and the FC-induced proton extrusion in vivo. Our finding is worth noting because it represents the first evidence of the occurrence in the plant cells of a physiological metabolite able to regulate the activity of the plasma membrane H+-ATPase.

Moreover, we show that the 5′-AMP effect depends on its binding to 14-3-3 proteins. This result is in accordance with data obtained by Athwal et al. (16), who reported on the presence of a possible 5′-AMP-binding site on the 14-3-3 Arabidopsis isoform GF14α. Sequence analysis indicates that a putative nucleotide-binding site is present between helices 4 and 5 of both GF14-6 and GF14α. Interestingly, this motif seems to be conserved in all animal and plant 14-3-3 isoforms, thus suggesting that the ability to bind 5′-AMP may be a general feature of 14-3-3 proteins.

Our results are consistent with the occurrence of multiple mechanisms for the control of a central enzyme such as H+-ATPase. In fact, it seems that the activation of H+-ATPase, which depends on its interaction with 14-3-3 proteins, may be regulated, besides the phosphorylation/dephosphorylation of the enzyme (14), by 5′-AMP binding to 14-3-3 proteins. Moreover, our findings also suggest that 5′-AMP can be a general modulator of 14-3-3-regulated processes in plant cells. Although the physiological relevance of this observation is unclear, activities of 14-3-3-regulated enzymes may be linked by 5′-AMP to the energy charge of the cell. Interestingly, it has been reported that 5′-AMP levels vary in response to some stress such as drought or anoxia (29), conditions known to affect the activity of nitrate reductase (28), H+-ATPase (6), and other 14-3-3-regulated enzymes of primary metabolism (30).

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Adenosine 5'-Monophosphate Inhibits the Association of 14-3-3 Proteins with the Plant Plasma Membrane H^+-ATPase

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