J. Biochem. **143,** 253–259 (2008)
doi:10.1093/jb/mvm218

Heparin Stimulates a Plasma Membrane Ca^{2+}-ATPase of *Arabidopsis thaliana*

Silvia Meneghelli[†], Laura Luoni[†] and Maria Ida De Michelis*

Dipartimento di Biologia 'L. Gorini', Università di Milano, CNR Istituto di Biofisica - Sezione di Milano, via G. Celoria 26, 20133 Milano, Italy

Received August 3, 2007; accepted November 1, 2007; published online November 15, 2007

We have studied the effect of heparin, a glycosaminoglycan widely used in releasing tags from fusion proteins, on isoform 8 of *Arabidopsis thaliana* PM Ca^{2+}-ATPase (ACA8) expressed in *Saccharomyces cerevisiae* strain K616. Heparin stimulates hydrolytic activity of ACA8 with an estimated $K_{0.5}$ value for the complex of $15 \pm 1\ \mu g\ ml^{-1}$, which is unaffected by free [Ca^{2+}]. Heparin increases V_{max} up to 3-fold while it does not significantly affect the apparent K_m for free Ca^{2+} and for the nucleoside triphosphate substrate. The heparin effect is not additive with that of exogenous calmodulin and heparin is ineffective on a mutant devoid of the N-terminal auto-inhibitory domain (*Δ74–ACA8*). Altogether, these results indicate that heparin activation is due to partial suppression of the auto-inhibitory function of ACA8 N-terminus. Pull-down assays using heparin–agarose gel show that heparin directly interacts with ACA8. Binding to the heparin–agarose gel occurs also with a peptide reproducing ACA8 sequence ^{1}M-I^{116}. Several single-point mutations within ACA8 sequence A56–T63 significantly alter the enzyme response to heparin, suggesting that heparin interaction with this site may be involved in ACA8 activation. These results highlight a new difference between the plant PM Ca^{2+}-ATPase and its animal counterpart, which is inhibited by heparin.

Key words: *Arabidopsis thaliana*, Ca^{2+}-ATPase, calmodulin, heparin, plasma membrane.

Abbreviations: ACA8, auto inhibited Ca^{2+}-ATPase isoform 8; Brij 58, polyoxyethilene 20 cethyl ether; BTP, bis tris propane (1,3-bis[tris(hydroxymethyl)methylamino]propane); CaM, calmodulin; ER, endoplasmic reticulum; GST, glutathione *S*-transferase; NTA, nitrilotriacetic acid; PM, plasma membrane; PMCA, animal plasma membrane Ca^{2+}-ATPase.

Calcium plays a crucial role in plant physiology by acting as a second messenger of a number of endogenous and environmental signals. Fine-tuning of cytoplasmic free Ca^{2+} concentration in plant cells is achieved through regulation of a number of passive and active Ca^{2+} transport proteins localized both at plasma membrane (PM) and at intracellular membrane systems such as tonoplast and endoplasmic reticulum (*1*). The plant PM Ca^{2+}-ATPase, which catalyses Ca^{2+} extrusion from the cytoplasm to the apoplast in exchange with H^+, plays a central role in cytosolic Ca^{2+} homoeostasis, in particular by re-establishing the low basal free Ca^{2+} concentration after a stimulus-induced Ca^{2+} influx from the extra cellular environment (*2–5*).

ACA8, the first cloned *Arabidopsis thaliana* PM Ca^{2+}-ATPase, is a type IIB P-type ATPase characterized by the direct interaction with calmodulin (CaM), the best-known cytoplasmic sensor of Ca^{2+}, at the regulatory CaM-binding domain localized in the cytosolic N-terminus (*6–11*). CaM-binding activates the enzyme by suppressing the action of an auto-inhibitory domain, largely overlapping the CaM-binding site (*9–15*).

The localization of CaM-binding domain at the N-terminal end of the pump is a typical feature of plant type IIB Ca^{2+}-ATPases, independent of their localization at the PM or at endomembranes (*8*), and constitutes a major difference between type IIB Ca^{2+}-ATPases of plants and those of mammals: in fact, animal type IIB Ca^{2+}-ATPases (PMCA) have the auto-inhibitory CaM-binding domain at the C-terminus (*16*, *17*).

Heparin is a highly sulphated, animal linear polysaccharide that binds many proteins with high affinity and is involved in different physiological events, including modulation of intracellular Ca^{2+} concentration (*18*). Recently, it was shown that heparin inhibits the activity of a PMCA from porcine brain synaptosome. This effect is evident both in the presence and in the absence of the pump activator CaM and is rescued by addition of acidic phospholipids (*19*).

In this work, we studied the effect of heparin on ACA8 and on its constitutively active mutant *Δ74-ACA8*, in which the first 74aa containing the auto-inhibitory CaM-binding domain has been deleted (*20*). Both the full-length and the truncated mutant were expressed in *Saccharomyces cerevisiae* strain K616; this yeast strain is the ideal host for functional expression of heterologous P-type ATPases because it lacks the main endogenous

*To whom correspondence should be addressed. Tel: 39 02 50314822, Fax: 39 02 50314815,
E-mail: mariaida.demichelis@unimi.it
[†]Equal contributors to this work.

active Ca^{2+} systems, *PMR1* and *PMC1* (*8*, *20*). In contrast with what was reported for the animal enzyme, our results show that heparin stimulates the basal activity of the full-length form of ACA8, partially counteracting the auto-inhibitory action of the N-terminal domain, which contains a heparin-binding site.

MATERIALS AND METHODS

Yeast Strains, Transformation and Growth Media—*Saccharomyces cerevisiae* strain K616 (*MATa pmr1::HIS3 pmc1::TRP1 cnb1::LEU2, ade2, ura3*) was used for expression of *ACA8*, *Δ74-ACA8* and the specified single point *ACA8* mutants. Yeast cells were transformed as described by Bonza *et al*. (*20*). The transformants were selected for uracil prototrophy on synthetic complete medium lacking uracil (SC-URA) as described by Bonza *et al*. (*20*).

Isolation of Yeast Microsomes and ER Membrane Fraction—Plant pumps were expressed in yeast grown in SC-URA medium containing 2% (w/v) galactose, 1% (w/v) raffinose, 50 mM succinic acid/Tris (pH 5.5), 0.7% (w/v) yeast nitrogen base and 10 mM $CaCl_2$, for 24 h at 30°C. Microsomes were harvested as described by Bonza *et al*. (*20*).

To analyse the activity of *ACA8* single-point mutants, yeast microsomes were extensively washed with 10% (w/w) glycerol, 10 mM benzamidine, 5 mM EDTA, 1 μg ml^{-1} pepstatin, 1 μg ml^{-1} chymostatin, 5 μg ml^{-1} leupeptin and 25 mM 3-[*N*-morpholino]propane sulphonic acid-KOH, pH 7. The pellet obtained after centrifugation for 45 min at 48,000*g* and 4°C was re-suspended at about 5–6 mg protein ml^{-1} in the same solution used for the washing step, except for EDTA, and kept at –80°C.

To isolate yeast ER fraction, microsomes were fractionated by sucrose gradient centrifugation (*20*). Microsomes re-suspended in 10% (w/w) sucrose, 10 mM Tris–HCl pH 7.5, 5 mM EDTA and 5 μg ml^{-1} leupeptin were loaded onto a discontinuous sucrose gradient consisting of 5 ml 18% (w/w), 3.5 ml 33% (w/w) and 2 ml 45% (w/w) sucrose in 10 mM Tris–HCl pH 7.5, 5 mM EDTA and 5 μg ml^{-1} leupeptin. After over night centrifugation at 210,000*g* and 4°C, the 18–33% interface (ER-enriched fraction) was collected and extensively washed with 10% (w/w) glycerol, 10 mM benzamidine, 1 μg ml^{-1} pepstatin, 1 μg ml^{-1} chymostatin, 5 μg ml^{-1} leupeptin and 25 mM 3-[*N*-morpholino]propane sulphonic acid-KOH, pH 7. The pellet obtained after centrifugation for 45 min at 48,000*g* and 4°C was re-suspended at about 6–12 mg protein ml^{-1} in the same solution used for the washing step. Protein concentration was determined using the Bio-Rad assay (Bio-Rad, Hercules, CA, USA; cat. No. 500-001).

Assays of Ca^{2+}-ATPase Activity—Ca^{2+}-ATPase activity in yeast microsomes (*ca*. 4 μg of protein per sample) or ER-enriched fraction (*ca*. 2–5 μg of protein per sample) was measured as Ca^{2+}-dependent MgITP hydrolysis as described by Bonza *et al*. (*7*). The assay medium contained 40 mM BTP-HEPES, pH 7, 50 mM KNO_3, 1 μM A_{23187}, 0.1 mg ml^{-1} Brij58, 5 mM $(NH_4)_2SO_4$, 0.25 mM NH_4 molybdate, 1 μg ml^{-1} oligomycin, 2 mM phosphoenolpyruvate, 10 units ml^{-1} pyruvate kinase and $MgSO_4$ and ITP at final concentrations of 3 mM and 1 mM, respectively, unless otherwise specified. Free Ca^{2+} concentration was buffered at 40 μM, unless otherwise specified, with 1 mM EGTA. Membrane proteins were pre-incubated in assay buffer plus or minus heparin and CaM as specified in the legends, for 20 min at 4°C before starting the reaction by addition of MgITP. Samples were incubated at 25°C for 60 min. Ca^{2+}-dependent hydrolytic activity was determined as the difference between the activities measured in the presence and in the absence of Ca^{2+}; the activity measured in the absence of Ca^{2+} did not exceed 120 nmol mg^{-1} min^{-1} in microsomes and 25 nmol mg^{-1} min^{-1} in the ER fraction and was unaffected by heparin. Under the above-described conditions, the reaction catalysed by ACA8 is linear for up to 90 min (data not shown). All the assays were performed with three replicates and repeated at least twice, with similar results.

Binding Assays—Proteins of the yeast ER-enriched fraction were solubilized in active form with *n*-dodecyl β-D-maltoside (1 mg detergent ml^{-1}: 1 mg protein ml^{-1}) for 30 min at 25°C in a medium (loading buffer) containing 20 mM 3-[*N*-morpholino]propane sulphonic acid-KOH, pH 7.5, 1 mM benzamidine, 2 mM DTT, 1.5 mM ITP, 1 mM $MgSO_4$, 5 μg ml^{-1} leupeptin, 1 mM $CaCl_2$ and 0.25 M NaBr. After a centrifugation step at 20,000*g* for 60 min at 4°C, the supernatant was diluted nine times with loading buffer without detergent and loaded onto the heparin–agarose gel (0.3–1 ml per 1 ml of packed resin; SIGMA, St. Louis, MO, USA, cat. No. H3025). The incubation was performed for 2 h at 4°C under gentle rotation; then, after flow-through collection and two washing steps with loading buffer without detergent, the proteins linked to heparin–agarose gel were released by incubating the heparin–agarose gel in SDS-solubilization buffer. The collected fractions were analysed by SDS–PAGE and immunoblotting as described below. In binding assays performed in the absence of Ca^{2+}, $CaCl_2$ was replaced with 1 mM EDTA in the loading buffer. Peptide 6His-^{1}M-I^{116}, reproducing *At*-ACA8 N-terminus (*10*), was diluted 50 times with a modified loading buffer (no detergent and ITP and 0.05 M NaBr) and loaded (*ca*. 0.3 nmol) onto the heparin–agarose gel (*ca*. 1 ml of packed resin). Binding assay procedure was performed as described above. Experiments were performed at least twice, with similar results.

Electrophoresis and Immunoblotting Analysis—Fractions collected from the binding-assays were pre-treated, solubilized and subjected to SDS–PAGE as described by Bonza *et al*. (*6*). After running, the pre-cast Tris–glycine polyacrylamide gels (8% or 18%, Anamed, Darmstadt, Germany; cat. No. TG08110 or TG18110) were blotted as described by Luoni *et al*. (*3*). Immunodecoration with polyclonal antibodies against the sequence ^{17}V-T^{31} of ACA8 was performed as reported by Bonza *et al*. (*7*). Immunodecoration with polyclonal antibodies against the sequence ^{268}E-W^{348} of ACA8 was performed as described by Luoni *et al*. (*10*).

RESULTS

Effect of Heparin on ACA8 Hydrolytic Activity—In order to study the effect of heparin on a plant PM Ca^{2+}-ATPase, we expressed the isoform 8 of *A. thaliana*

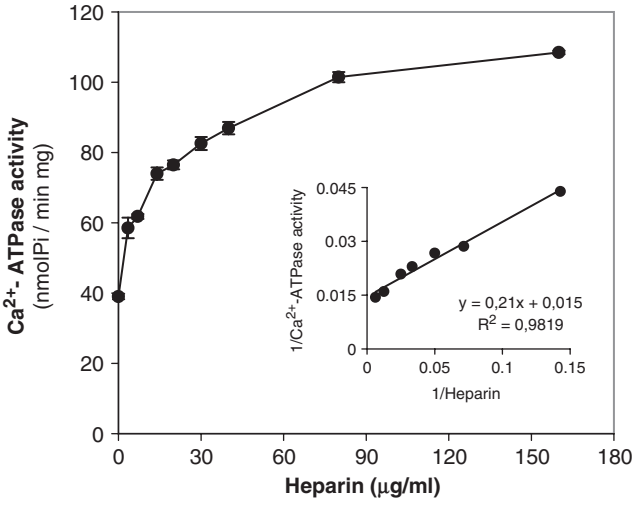

Fig. 1. **Activation of ACA8 by heparin.** Ca^{2+}-ATPase activity of the ER-enriched fraction from *S. cerevisiae* expressing ACA8 was assayed as described in MATERIALS AND METHODS section, in the presence of the specified heparin concentrations (3.5–160 µg ml^{-1}). Results (±SE, $n = 3$) are from one experiment representative of eight giving similar results. The inset shows the Lineweaver–Burk plot of the reported data, used to evaluate the $K_{0.5}$ and V_{max} values.

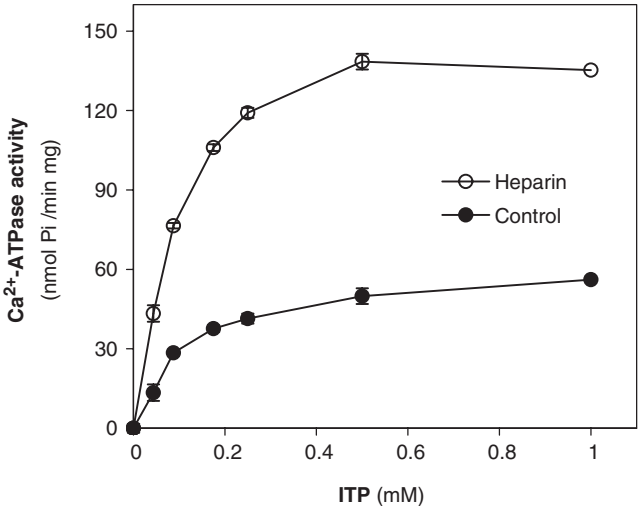

Fig. 2. **Effect of heparin on ACA8 activity as a function of ITP concentration.** Ca^{2+}-ATPase activity of the ER-enriched fraction from *S. cerevisiae* expressing ACA8 was assayed as described in MATERIALS AND METHODS section in the absence (closed circles) or presence (open circles) of 100 µg ml^{-1} heparin. ITP concentration was varied between 0.05 and 1 mM, as indicated, in the presence of a constant excess of 2 mM $MgSO_4$. Results (±SE, $n = 3$) are from one experiment representative of two giving similar results.

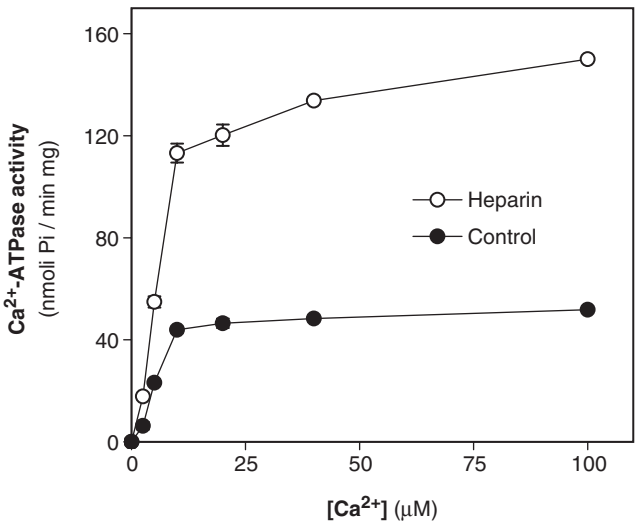

Fig. 3. **Effect of heparin on ACA8 activity as a function of free Ca^{2+} concentration.** Ca^{2+}-ATPase activity of the ER-enriched fraction from *S. cerevisiae* expressing ACA8 was assayed as described in MATERIALS AND METHODS section in the absence (closed circles) or presence (open circles) of 100 µg ml^{-1} heparin. Free Ca^{2+} concentration was buffered at 2.5–100 µM with 1 mM EGTA. Results (±SE, $n = 3$) are from one experiment representative of two giving similar results.

enzyme (ACA8) in *S. cerevisiae* strain K616; this yeast strain is the ideal host for functional expression of heterologous P-type ATPases because it lacks the main endogenous active Ca^{2+} systems, *PMR1* and *PMC1* (*8*). We isolated the yeast ER-fraction that is enriched in ACA8 by discontinuous sucrose-gradient (*20*). As shown in Fig. 1, heparin activates the Ca^{2+}-ATPase in a concentration-dependent manner described by a typical saturation curve. Analysis of eight independent experiments, giving similar results, indicated that the heparin effect is semi-saturated ($K_{0.5}$) by 15 ± 1 µg ml^{-1} of heparin and that the average value for maximal stimulation is between 150 and 200%. Furthermore, free Ca^{2+} concentration did not affect heparin affinity: in fact, at three different free Ca^{2+} concentrations tested (5, 20, 40 µM), heparin affinity for the Ca^{2+}-ATPase did not show any remarkable change (data not shown).

Analysis of heparin effect on the dependence of ACA8 hydrolytic activity on ITP and free Ca^{2+} concentration was performed at the heparin concentration of 100 µg ml^{-1}. Figure 2 shows the saturation kinetics of the pump for ITP in the absence and in the presence of heparin. The enzyme affinity for this substrate showed no significant change in presence of heparin (apparent $K_m = 0.12 \pm 0.01$ mM) with respect to the control (apparent $K_m = 0.11 \pm 0.01$ mM). In contrast, heparin strongly increased the turn over of the enzyme ($V_{max} = 173 \pm 6$ nmol Pi mg^{-1} min^{-1}, $n = 3$) with respect to the control ($V_{max} = 61 \pm 1$ nmol Pi mg^{-1} min^{-1}, $n = 3$).

Figure 3 shows the effect of heparin on Ca^{2+}-ATPase activity as a function of free Ca^{2+} concentration: the stimulation of enzyme activity by heparin was virtually constant at all the Ca^{2+} concentrations tested. Analysis of the kinetic parameters of the pump demonstrated that the most relevant effect of heparin was to increase the V_{max} value from 54 ± 1 nmol Pi mg^{-1} min^{-1} ($n = 3$) to 161 ± 2 nmol Pi mg^{-1} min^{-1} ($n = 3$); the slight increase of apparent K_m for Ca^{2+} measured in presence of heparin (from 4 ± 1 µM to 6 ± 1 µM) was not highly significant.

A preliminary analysis of the mechanism of heparin-induced activation of ACA8 was performed by investigating the relationship between heparin-effect and

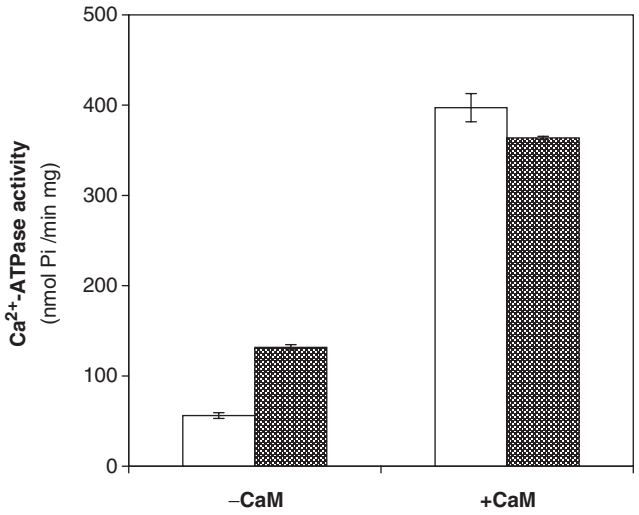

Fig. 4. **Effect of heparin on ACA8 activity measured in the absence or in presence of CaM.** Ca^{2+}-ATPase activity of the ER-enriched fraction from *S. cerevisiae* expressing ACA8 was assayed as described in MATERIALS AND METHODS section with (black bars) or without (white bars) 100 μg ml^{-1} heparin and with or without 1 μM CaM. Results (±SE, $n = 3$) are from one experiment representative of four giving similar results.

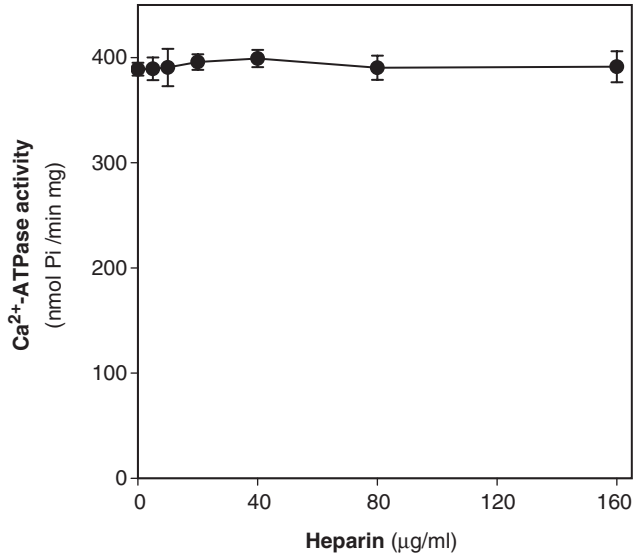

Fig. 5. **Effect of heparin on the activity of the Δ*74-ACA8* mutant.** Ca^{2+}-ATPase activity of the ER-enriched fraction from *S. cerevisiae* expressing △*74-ACA8* was assayed as described in MATERIALS AND METHODS section. Results (±SE, $n = 3$) are from one experiment representative of two giving similar results.

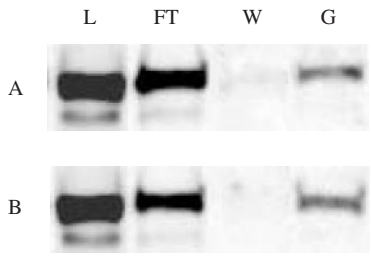

Fig. 6. **Binding of ACA8 to heparin–agarose gel.** Proteins of ER fraction from *S. cerevisiae* expressing ACA8 were solubilized in native form with *n*-dodecyl β-D-maltoside and loaded on heparin–agarose gel as described in MATERIALS AND METHODS section. After SDS–PAGE on 8% (w/v) polyacrylamide gel, proteins were blotted and immunodecorated with an antiserum raised against ACA8 sequence ^{268}E-W^{348}. Binding assay was performed in the presence (1 mM $CaCl_2$, panel A) or absence of Ca^{2+} (1 mM EDTA, panel B). L: ER-solubilized proteins (2 μg) loaded on heparin–agarose gel; FT: flow trough fraction from 2 μg loaded proteins; W: last wash fraction from 2 μg loaded proteins; G: proteins associated with the heparin–agarose gel from 2 μg loaded proteins. Results are representative of four experiments giving similar results.

CaM-stimulation of enzyme activity. Hydrolytic activity of the enzyme was measured in the presence and absence of 1 μM CaM and in the presence and absence of 100 μg ml^{-1} heparin. As shown in Fig. 4, the Ca^{2+}-ATPase activity was stimulated about 10 times by CaM; the addition of heparin increased the basal activity but did not significantly affect the activity of the enzyme measured in presence of CaM. Consequently, in the presence of heparin CaM-stimulation was reduced about 3-fold. Coherently with its effect on the hydrolytic activity of ACA8, heparin stimulated also ATP-dependent Ca^{2+} uptake, but only in the absence of CaM (data not shown). These results imply that the effect of heparin is not additive with that of CaM suggesting that the mechanism of heparin activation may be similar to that of CaM stimulation and could involve the inhibition of the N-terminal auto-inhibitory function.

To further investigate the involvement of the N-terminal auto-inhibitory function in heparin effect, the hydrolytic activity of the N-terminal deleted ACA8 mutant △*74-ACA8*, expressed in *S. cerevisiae* (*20*), was measured in the presence of increasing concentrations of heparin (5–160 μg ml^{-1}). As shown in Fig. 5, heparin did not produce any significant variation of the activity of △*74-ACA8* at all the concentrations tested, thus supporting the idea that heparin activates ACA8 by partially suppressing the autoinhibitory action of the enzyme N-terminus.

Interaction of ACA8 with Heparin—To test whether heparin and ACA8 actually interacted, binding assays were performed by incubating heparin–agarose gel with the proteins from the ER-fraction of yeast expressing ACA8, solubilized in active form as described in MATERIALS AND METHODS section. After flow-trough collection and two washes with the loading buffer, the proteins linked to heparin–agarose gel were released by incubating the heparin–agarose gel in SDS solubilization buffer. Comparable amounts of the relevant fractions were separated by SDS–PAGE, blotted and immunodecorated with an antiserum raised against ACA8 sequence ^{268}E-W^{348} (*10*). Figure 6, panel A, shows that the ACA8 signal was clearly lower in the flow-through fraction than in the loaded sample; in the second wash fraction, ACA8 signal was virtually undetectable whereas it was evident in the fraction containing the heparin–agarose gel associated proteins. Taken together, these data show that in the applied conditions ACA8 binds to heparin–agarose gel; the absence of ACA8 signal in the second

Fig. 7. **Binding to heparin–agarose gel of peptide 6His-^{1}M-I^{116} reproducing the cytosolic N-terminus of *At*–ACA8.** Peptide 6His-^{1}M-I^{116}, expressed in *E. coli* and purified by affinity chromatography (*ca*. 0.3 nmol) was loaded onto the heparin–agarose gel as described in MATERIALS AND METHODS section; after SDS–PAGE on 18% (w/v) polyacrylamide gel, proteins were blotted and immunodecorated with an antiserum raised against the portion ^{17}V-T^{31} of ACA8. L: sample loaded on heparin–agarose gel (0.12 μg of 6His-^{1}M-I^{116}); FT: flow trough fraction from 0.12 μg loaded 6His-^{1}M-I^{116}; W: last wash fraction from 0.12 μg loaded 6His-^{1}M-I^{116}; G: proteins associated with the heparin–agarose gel from 0.12 μg loaded 6His-^{1}M-I^{116}. Results are representative of two experiments giving similar results.

wash of the heparin–agarose gel indicates that the ACA8–heparin interaction is fairly stable.

To further investigate if this interaction was affected by Ca^{2+}, binding assay was performed in the absence of Ca^{2+} (Fig. 6, panel B). This assay demonstrates that, in agreement with activity data, the interaction of heparin with ACA8 is not affected by free Ca^{2+} concentration.

It has been proposed that a common structural theme in the wide range of proteins interacting with heparin may define the specific molecular contacts. The motifs (XBBXBX) and (XBBBBXXBX), where B designates a basic amino acid and X any other amino acid, have been identified by linear alignment of a broad collection of known heparin-binding sequences (*18*, *19*, *21*). Moreover, a distinct common spatial pattern in the distribution of the basic amino acids emerged: in sequences that fold in α-helices, two basic residues were separated by a constant distance of 20 Å and facing in opposite directions; in addition, the α-helical-binding sites showed a clear amphipathic nature with basic residues on one side and hydrophobic residues on the other side (*21*). Analysing ACA8 amino acid sequence, we found that the cytosolic N-terminal region contained the portion ^{57}SRRFRY^{62} consistent with the heparin-binding motif (XBBXBX). To test whether heparin could bind to this portion of ACA8, the peptide 6His-^{1}M-I^{116}, corresponding to the first 116 amino acids of ACA8, was expressed in *E. coli* and purified by Ni–NTA agarose gel as described by Luoni *et al*. (*10*). Binding assay was performed by incubating peptide 6His-^{1}M-I^{116} with the heparin–agarose gel as described in MATERIALS AND METHODS section. After solubilization and SDS–PAGE, the relevant binding-assay fractions were subjected to immunoblotting. As shown in Fig. 7, heparin was able to interact with the N-terminal region of ACA8. This result suggests that the portion ^{57}SRRFRY^{62} at the N-terminus of ACA8, consistent with a known canonical motif, could actually function as a heparin-binding site.

To test whether heparin binding to such a site is responsible for ACA8 activation, we analysed the effect of heparin on the activity of *ACA8* mutants produced by single-point mutation of aa 56 to 63. Mutants *R58A*, *F60A* and *Y62A* are deregulated (*9*) and scarcely

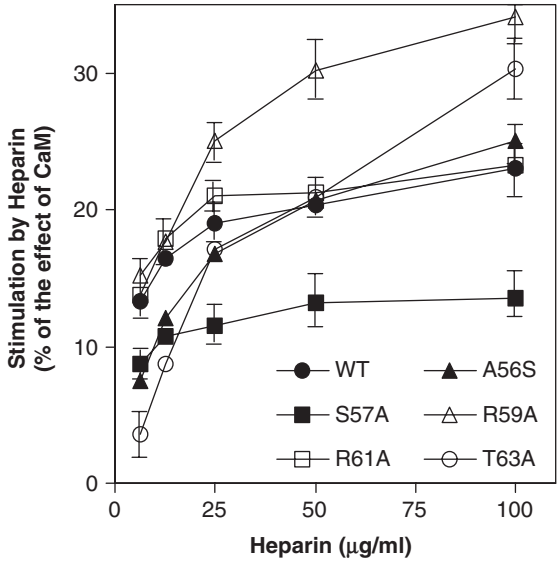

Fig. 8. **Activation of *ACA8* single-point mutants by heparin.** Ca^{2+}-ATPase activity of the microsomal fraction from *S. cerevisiae* expressing the specified *ACA8* mutants was assayed as described in MATERIALS AND METHODS section, in the presence of the specified heparin concentrations (6–100 μg ml^{-1}). Results (±SE, $n = 9$) are from three experiments, each with three replicates. Specific activities measured in the presence of CaM ranged between 120 and 240 nmol min^{-1} mg^{-1} protein and stimulation by CaM ranged between 130 and 400%.

activated by CaM: accordingly, these mutants were scarcely activated by heparin (data not shown). None of the other mutations suppressed the activating effect of heparin, but several mutations altered ACA8 response to heparin: in particular, as shown in Fig. 8, *ACA8* mutants *A56S*, *R59A* and *T63A* had a lower apparent affinity for heparin than the wild type, while mutant *S57A* was less activated than the wild type.

DISCUSSION

It is well known that animal and plant PM Ca^{2+}-ATPases share structural and functional features (*12*, *13*, *16*, *17*, *22–28*); nevertheless, remarkable differences exist between animal and plant isoforms. At the structural level, the most relevant difference concerns the localization of the auto-inhibitory CaM-binding domain: whereas the mammalian enzyme (PMCA) has the auto-inhibitory CaM-binding domain at the extended C-terminus, the plant PM Ca^{2+}-ATPase has this regulatory domain at the N-terminal end of the molecule (*7–11*, *16*, *17*, *26*, *28*, *29*).

In this work, a new peculiarity concerning the effect of heparin is highlighted: heparin inhibits PMCA activity independently of the presence or the absence of CaM and of the tryptic cleavage of the CaM-binding domain; the heparin effect on mammalian enzyme is associated with a decrease in ATP affinity of the enzyme whereas Ca^{2+} affinity shows no significant change (*19*, *30*). Conversely, isoform 8 of *A. thaliana* PM Ca^{2+}-ATPase, ACA8, is activated by heparin.

Heparin does not change the affinity of the enzyme for the substrates, Ca^{2+} and ITP, but increases the enzyme turn over up to 3-fold. Heparin-activation of ACA8 is not

additive with CaM-stimulation and heparin does not affect the activation status of the △*74-ACA8* mutant, which lacks the N-terminal auto-inhibitory CaM-binding domain (*7*, *9–11*, *20*). All together, these results indicate that the mechanism of heparin-activation can be similar to that of CaM-stimulation involving the partial suppression of the N-terminal auto-inhibitory function.

Both ACA8 and peptide 6His-^{1}M-I^{116}, corresponding to the cytosolic N-terminal region of ACA8, are able to bind heparin–agarose gel. The response to heparin is significantly altered in some mutants produced by single-point mutation of ACA8 sequence A56–T63 that contains a canonical heparin-binding motif (XBBXBX) (*18*, *19*, *21*). Further work is needed to unequivocally identify heparin-binding site(s) in ACA8 and to determine its/their affinity and specificity. However, the reported results suggest that heparin interaction with the N-terminal region containing the autoinihibitory and CaM-binding domain (*7*, *9–11*) may be involved in ACA8 activation. Heparin binding to this domain may hamper the auto-inhibitory interaction between the two intramolecular partners (*10*) thus leading to enzyme activation.

Heparin binding to the N-terminal auto-inhibitory domain might also provide a structural basis for the different response to heparin of ACA8 and PMCA. In PMCA, preliminary results indicate that the sequence KVPKKEKSVL that contains a canonical heparin-binding motif may be the heparin interaction site responsible for inhibitory effect; this sequence corresponds to a portion of the actuator domain localized between the second and the third transmembrane segments and involved in phospholipids activation (*19*), which is not conserved in the plant enzyme.

The effect of heparin on unexpected targets, such as for example ACA8, must be considered with particular care when this linear polysaccharide is used while releasing tags from fusion proteins. Indeed, while binding of peptide ^{268}E-W^{348} to ACA8 N-terminus (*10*) could be reproduced with a heparin-free peptide, its stimulating effect on *A. thaliana* PM Ca^{2+}-ATPase activity, reported in (*10*), was largely due to heparin carried over from cleavage of the GST tag (unpublished data from authors laboratory).

A further output of this work regards the possible use of heparin–agarose gel for affinity purification: the ACA8–heparin interaction may provide a new tool for enzyme purification. In particular, heparin affinity chromatography may be applied to purify mutants of plant PM Ca^{2+}-ATPase that have lost the ability to bind CaM (*9*).

Heparin is not found in plant cells but this system contains a lot of heparin-like molecules including acidic and sulphated polysaccharides. These polyanions serve a number of structural and protective roles in plants; for example, sulphated fucans in the body wall of sea cucumber and algae are involved in maintenance of the wall integrity and in the cell wall organization by cross-linkage of alginate and cellulose (*31*, *32*) and oligogalacturonides are involved in the early response to fungal attack (*33*, *34*). However, while in animals sulphated polysaccharides are found in various intracellular compartments (*18*), in plants these molecules are localized mainly in the apoplast and their presence in the cytoplasm has not been demonstrated, so that it is difficult to assign a physiological role to the possible regulation of plant PM Ca^{2+}-ATPase activity by heparin-like molecules. Thus, at present, in plants heparin is mainly a useful tool for studies concerning structural–functional relationship of proteins.

This project was supported by the Italian Ministry for University and Research in the PRIN 2005 frame. Plasmids encoding *ACA8* single-point mutants were kindly provided by Dr Lone Bækgaard, KVL, Copenhagen (Denmark).

REFERENCES

1. Hetherington, A.M. and Brownlee, C. (2004) The generation of Ca^{2+} signals in Plants. *Annu. Rev. Plant Biol.* **55**, 401–427
2. Rasi-Caldogno, F., Pugliarello, M.C., and De Michelis, M.I. (1987) The Ca^{2+} transport ATPase of plant plasma membrane catalyzes a nH^{+}/Ca^{2+} exchange. *Plant Physiol.* **83**, 994–1000
3. Luoni, L., Bonza, M.C., and De Michelis, M.I. (2000) H^{+}/Ca^{2+} exchange driven by the plasma membrane Ca^{2+}-ATPase of *Arabidopsis thaliana* reconstituted in proteoliposomes after calmodulin-affinity purification. *FEBS Lett.* **482**, 225–230
4. Beffagna, N., Romani, G., and Sforza, M.C. (2000) H^{+} fluxes at plasmalemma level: in vivo evidence for a significant contribution of the Ca^{2+}-ATPase and for the involvement of its activity in the abscisic acid-induced changes in *Egeria densa* leaves. *Plant Biol.* **2**, 168–175
5. Romani, G., Bonza, M.C., Filippini, I., Cerana, M., Beffagna, N., and De Michelis, M.I. (2005) Involvement of the plasma membrane Ca^{2+}–ATPase in the short term response of *Arabidopsis thaliana* cultured cells to oligogalacturonides. *Plant Biol.* **6**, 192–200
6. Bonza, M.C., Carnelli, A., De Michelis, M.I., and Rasi-Caldogno, F. (1998) Purification of the plasma membrane Ca^{2+}-ATPase from radish seedlings by calmodulin-agarose affinity chromatography. *Plant Physiol.* **116**, 845–851
7. Bonza, M.C., Morandini, P., Luoni, L., Geisler, M., Palmgren, M.G., and De Michelis, M.I. (2000) *At*-ACA8 encodes a plasma membrane-localized Calcium-ATPase of *Arabidopsis thaliana* with a calmodulin binding domain at the N-terminus. *Plant Physiol.* **123**, 1495–1505
8. Geisler, M., Axelsen, K., Harper, J.F., and Palmgren, M.G. (2000) Molecular aspects of higher plant P-type Ca^{2+}-ATPases. *Biochem. Biophys. Acta* **1465**, 52–78
9. Bækgaard, L., Luoni, L., De Michelis, M.I., and Palmgren, M.G. (2006) The plant plasma membrane Ca^{2+} pump ACA8 contains overlapping as well as physically separated autoinhibitory and calmodulin-binding domains. *J. Biol. Chem.* **281**, 1058–1065
10. Luoni, L., Meneghelli, S., Bonza, M.C., and De Michelis, M.I. (2004) Auto-inhibition of *Arabidopsis thaliana* plasma membrane Ca^{2+}–ATPase involves an interaction of the N-terminus with the small cytoplasmic loop. *FEBS Lett.* **574**, 20–24
11. Luoni, L., Bonza, M.C., and De Michelis, M.I. (2006) Calmodulin/Ca^{2+}-ATPase interaction at the *Arabidopsis thaliana* plasma membrane is dependent on calmodulin isoform showing isoform-specific Ca^{2+} dependencies. *Physiol. Plant* **126**, 175–186
12. Rasi-Caldogno, F., Carnelli, A., and De Michelis, M.I. (1993) Controlled proteolysis activates the plasma membrane Ca2+ pump of higher plants. *Plant Physiol.* **103**, 385–390
13. Rasi-Caldogno, F., Carnelli, A., and De Michelis, M.I. (1995) Identification of the plasma membrane Ca^{2+}-ATPase and of its autoinhibitory domain. *Plant Physiol.* **108**, 105–113

14. Olbe, M., Widell, S., and Sommarin, M. (1997) Active calcium transporters in isolated membranes of wheat root cells. *J. Exp. Bot.* **48**, 1767–1777
15. Olbe, M. and Sommarin, M. (1998) The spinach plasma membrane Ca^{2+} pump is a 120 kDa polypeptide regulated by calmodulin binding to a terminal region. *Physiol. Plant* **103**, 35–44
16. Carafoli, E. (1994) Biogenesis: plasma membrane calcium ATPase: 15 years of work on the purified enzyme. *FASEB J.* **8**, 993–1002
17. Brandt, P.C. and Vanaman, T.C. (1998) Calmodulin and ion flux regulation? in *Calmodulin and Signal Transduction* (Van Eldik, L. and Watterson, D.M., eds.) pp. 397–471 Brace & Company Publishers, USA Academic Press, San Diego
18. Jackson, R.L., Busch, S.J., and Cardin, A.D. (1991) Glycosaminoclycans: molecular properties, protein interactions, and role in physiological processes. *Physiol. Rev.* **71**, 481–539
19. Zhao, Y. and Zhang, X. (2003) Heparin inhibits the reconstituted plasma membrane Ca^{2+}-ATPase from porcine brain synaptosome. *Glycoconjugate J.* **19**, 373–378
20. Bonza, M.C., Luoni, L., and De Michelis, M.I. (2004) Functional expression in yeast of an N-deleted form of At-ACA8, a plasma membrane Ca^{2+}-ATPase of *Arabidopsis thaliana*, and characterization of a hyperactive mutant. *Planta* **218**, 814–823
21. Margalit, H., Fischer, N., and Ben-Sasson, S.A. (1993) Comparative analysis of structurally defined heparin binding sequences reveals a distinct spatial distribution of basic residues. *J. Biol. Chem.* **268**, 19228–19231
22. Rasi-Caldogno, F., Pugliarello, M.C., Olivari, C., De Michelis, M.I., Gambarini, G., Colombo, P., and Tosi, G. (1990) The plasma membrane Ca^{2+}-pump of plant cell: a radiation inactivation study. *Bot. Acta* **103**, 39–41
23. Rasi-Caldogno, F., Carnelli, A., and De Michelis, M.I. (1992) Plasma membrane Ca^{2+}–ATPase of radish seedlings. II. Regulation by calmodulin. *Plant Physiol.* **98**, 1202–1206
24. Carafoli, E. (1991) Calcium pump of the plasma membrane. *Physiol. Rev.* **71**, 129–153
25. Axelsen, K.B. and Palmgren, M.G. (1998) Evolution of substrate specificities in the P-type ATPases superfamily. *J. Mol. Evol.* **46**, 84–101
26. Penniston, J.T. and Enyedi, A. (1998) Modulation of the plasma membrane Ca^{2+} pump. *J. Membr. Biol.* **165**, 101–109
27. Bonza, M.C., Luoni, L., and De Michelis, M.I. (2001) Stimulation of plant plasma membrane Ca^{2+}-ATPase activity by acidic phospholipids. *Physiol. Plant* **112**, 315–320
28. Sze, H., Liang, F., Hwang, I., Curran, A.C., and Harper, J.F. (2000) Diversity and regulation of plant Ca^{2+} pumps: insights from expression in yeast. *Ann. Rev. Plant Physiol. Plant Mol. Biol.* **51**, 433–462
29. Chung, W.S., Lee, S.H., Kim, J.C., Heo, W.D., Kim, M.C., Park, C.Y., Park, H.C., Lim, C.O., Kim, W.B., Harper, J.F., and Cho, M.J. (2000) Identification of a calmodulin-regulated soybean Ca^{2+}-ATPase (SCA1) that is located in the plasma membrane. *Plant Cell* **12**, 1393–1407
30. Felix, C.F., Oliveira, V.H., Moreira, O.C., Mignaco, J.A., Barrabin, H., and Scofano, H.M. (2007) Inhibition of plasma membrane Ca^{2+}-ATPase by heparin is modulated by potassium. *Int. J. Biochem. Cell Biol.* **39**, 586–596
31. Berteau, O. and Mulloy, B. (2003) Sulfated fucans, fresh perspectives: structures, functions, and biological properties of sulfated fucans and an overview of enzymes active toward this class of polysaccharide. *Glycobiology* **13**, 29R–40R
32. Gunay, N.S. and Linhardt, R.J. (1999) Heparinoids: structure, biological activities and therapeutic applications. *Plant. Med.* **65**, 301–306
33. Cervone, F., Costorne, R., Leckie, F.D., and De Lorenzo, G. (1997) Perception of fungal elicitors and signal transduction? in *Signal Transduction in Plants* (Aducci, P., ed.) pp. 153–177 Birkhauser, Basel, Switzerland
34. De Lorenzo, G., D'Ovidio, R., and Cervone, F. (2001) The role of polygalacturonase-inhibiting proteins (PGIPs) in defense against pathogenic fungi. *Annu. Rev. Phytopathol.* **39**, 3131–335