Benzylaminopurine-induced coupling between calmodulin and Ca-ATPase in wheat root microsomal membranes

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The properties of the Ca-ATPase prepared from roots of wheat seedlings treated with benzylaminopurine were studied. The affinity of the ATPase towards Ca^{2+}, plant or erythrocyte calmodulin increased after the hormonal treatment. It seems that in the membrane calmodulin-binding sites were induced by benzylaminopurine, contributing to an increased affinity of the ATPase. The lower Ca-content of the hormone-treated plants suggests that in vivo the Ca-ATPase is involved in a Ca-extrusion process.

Wheat root *Benzylaminopurine* *Ca-ATPase* *Ca-content*
Wheat root calmodulin *Erythrocyte calmodulin*

1. INTRODUCTION

Recently, increased attention is paid to the influences of hormones on transport of metabolites and ions in plants [1–6]. In connection with the action of cytokinins, the involvement of a Ca-dependent protein has been supposed [7]. Calcium itself is a potent regulator of many cellular processes and it may act through calmodulin [8]. This possibility has been proved decisively only for the ATP-dependent Ca-transport in microsomal vesicles [9,10] and for the phosphorylation of NAD by NAD kinase [11,12]. In general, however, the steps of the molecular events between the binding of the hormone and the resulting changes in the enzyme activity are still unclear.

Here we report on the effects of the synthetic cytokinin, benzylaminopurine on the calmodulin-activation of the Ca-ATPase and on the altered Ca-levels in the roots of wheat seedlings.

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Abbreviation: BA, 6-benzylaminopurine

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2. MATERIALS AND METHODS

2.1. *Growth of plants*

Winter wheat seedlings (*Triticum aestivum* L. cv. Martonvásári [8]) were grown in water culture in a phytotron as in [13]. Hormonal treatment was carried out starting from the 7th day by the addition of 6-benzylaminopurine (Calbiochem) at a final concentration of 10^{-8} M into the nutrient solution. The growth solution was changed twice a week. The 13–15 days old roots of seedlings were used for preparation of microsomal membranes.

2.2. *Preparation of the plasmalemma-enriched microsomal fraction*

The plasmalemma-enriched microsomal fraction was prepared with minor modifications by the method in [14] as described in [15]. Essentially this method was used by authors in [9] in studying calmodulin activation of Ca-uptake. The 10 000–30 000 × *g* pellet was taken up in Tris–MES buffer containing 20–50 mM EDTA and was dialyzed 3-times overnight against the 100-fold volume of the buffer without EDTA.

2.3. *Preparation of calmodulin from wheat root*

Calmodulin from roots was prepared as in [9].

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For the preparation, the 30 000 × *g* supernatant above the microsomal fraction was used. The supernatant was heated to 85°C for 2 min, cooled and filtered. EGTA (0.1 mM) and 2 g purified DEAE-cellulose were added to 100 ml of the supernatant and stirred for 1 h. The DEAE-cellulose was sedimented by centrifugation and resuspended in the homogenising buffer containing 1 M NaCl. The resulting supernatant was then dialyzed against 25 mM Tris-MES buffer (pH 7.5) to remove NaCl. This preparation was well comparable with the calmodulin preparation in [9] and was used as wheat root calmodulin.

2.4. *Preparation of calmodulin from human erythrocytes*

Red blood cell calmodulin was prepared as in [16]. The fresh blood was washed 4-times in 172 mM Tris-HCl (pH 7.6) at 4°C by centrifugation at 4000 × *g* for 5 min. The cells were lyzed in 14 volumes of ice-cold distilled water and the membranes were collected by centrifugation at 23 000 × *g* for 10 min. The supernatant was mixed with 5 g purified DEAE-cellulose/liter, stirred for 1 h and filtered. From the resulting cake, a column was formed and eluted with increasing concentrations of NaCl (0.15, 0.3 and 0.6 M) in 10 mM imidazole-HCl buffer, pH 6.5. The protein-containing fractions of the 0.6 M NaCl-eluted material were collected and dialyzed against 40 volumes of distilled water. This preparation was used as erythrocyte calmodulin. According to the original description [16] the purification of this preparation is 1750-fold.

2.5 *Determination of ATP-ase activity*

The ATPase activity of the microsomal fraction was determined as P_i-liberated in triplicate samples [15]. The 1-ml reaction mixture contained 33 mM Tris-MES (pH 6.0), 3 mM ATP, and where stated, 1 mM Ca^{2+}, 10–170 μg erythrocyte calmodulin or 5–100 μg plant calmodulin. Protein was measured as described in [17].

2.6. *Plant analysis*

Minerals in the plant material were determined by atomic absorption spectrophotometry as in [13].

3. RESULTS

Fig. 1 shows the pH dependence of the Ca- and Mg-ATPase activities in the plasmalemma-enriched microsomal fraction prepared from the BA-treated (A) and from the control (B) plant roots. The ATPase activity of the hormone-treated plants increased 3-fold in comparison with that of control plants. The different pH optima in the presence of Ca or Mg suggest that two independent enzymes are present. The hormonal treatment changed the kinetic characteristics of the Ca-ATPase (table 1), since the affinity towards Ca increased 6-fold and the V_{max} by about 70%.

Between the action of BA and the increased Ca-ATPase activity the involvement of a Ca-binding activator could be supposed. To check this possibility, the effects of calmodulin preparations originating either from the supernatant of root microsomal fraction or from human erythrocytes were tested (table 2 and fig. 2). It is seen that plant calmodulin did not stimulate the ATPase from control plants, neither in the presence nor in the absence of Ca (table 2) while it did stimulate the

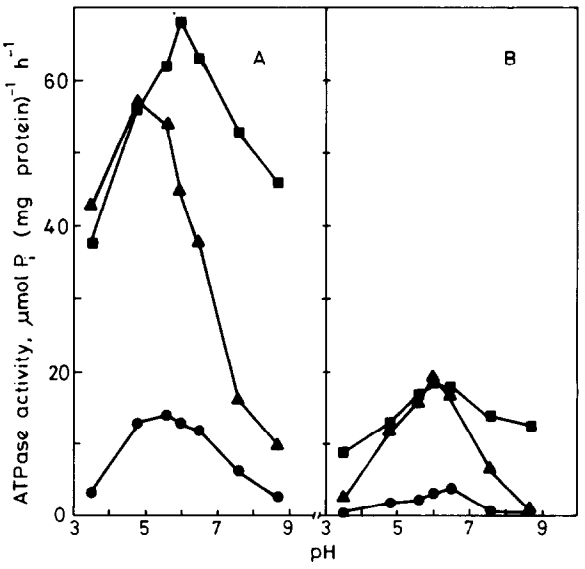

Fig. 1. The effect of in vivo pretreatment with benzylaminopurine on the Ca- and Mg-ATPase activities in the plasmalemma-enriched microsomal fraction prepared from wheat root. Standard errors of measurements lie within the symbols: (A) hormone-treated plants; (B) control plants; (●) basic activity; (▲) Mg-ATPase activity; (■) Ca-ATPase activity.

Table 1

K_m and V_{max}-values obtained from the Lineweaver-Burk plots of the Ca-stimulated ATPase activity from control and BA-pretreated plants

	V_{max} μmol P_i.(mg protein)$^{-1}$.h^{-1}	K_m μmol Ca.dm^{-3}	(r)
Control	49.3	54.4	0.974
Treated	82.4	9.59	0.997

(r) denotes the correlation coefficient calculated

Table 2

The effect of plant and erythrocyte calmodulin on the Ca-ATPase activity from control plant roots

	Ca-ATPase activity μmol P_i.(mg protein)$^{-1}$.h^{-1}		
	(−Ca)	(+Ca)	(n)
Plant calmodulin	9.6 ± 1.7	40.4 ± 2.6	8
Erythrocyte calmodulin	9.6 ± 0.7	43.8 ± 2.1	6

(−Ca) and (+Ca) denote the absence and presence of Ca^{2+} during ATPase assay; (n) means the number of ATPase assays with different calmodulin amounts (see fig. 3)

ATPase from the BA-treated plants (fig. 2). Calmodulin preparations from the control and BA-treated plants were equally effective in stimulating Ca-ATPase from the BA-treated plants (only control is shown). The crossed experiment using erythrocyte calmodulin and plant membrane preparation provided similar results (fig. 2).

These data strongly suggest that the treatment of plants with BA affects in vivo Ca transport. In fig. 3 the Ca-contents of the BA-treated and of the control plants are shown. The hormonal treatment decreased Ca-contents both in roots and shoots indicating that the Ca-ATPase participates in Ca extrusion.

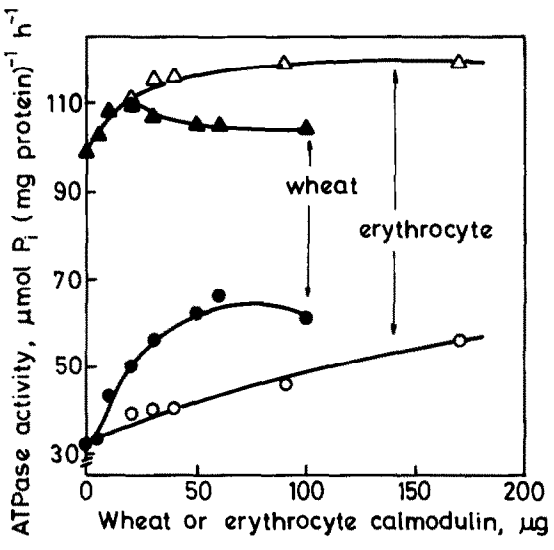

Fig. 2. The effect of calmodulin prepared from the roots of control plants and from erythrocytes on the Ca-ATPase activity of the BA-treated plants. The ATPase assay was carried out in the absence (○,●) and presence (△,▲) of 1 mM Ca^{2+}.

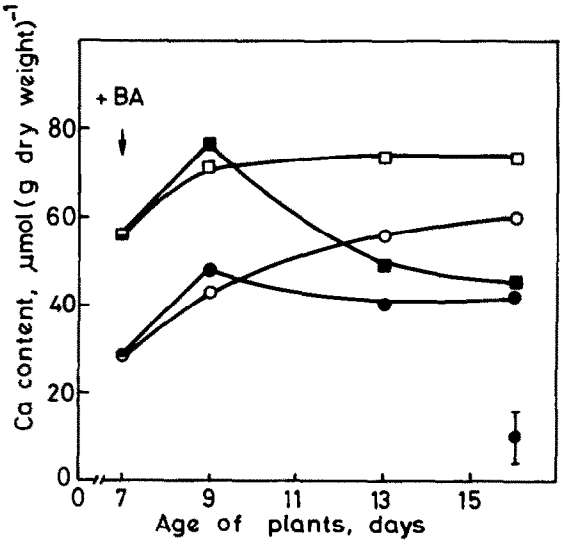

Fig. 3. Changes in the Ca-content of roots (○,●) and shoots (□,■) of wheat plants grown in the absence (○,□) or in the presence (●,■) of benzylaminopurine in the complete nutrient solution. BA-treatment started on the 7th day (+BA). In the right bottom, the ± standard deviation of Ca determination is given.

4. DISCUSSION

Alterations in the properties of the Ca-ATPase of the plasmalemma-enriched microsomal fraction as brought about by the treatment of roots with BA suggest that a Ca-dependent step is involved in the cytokinin action. The idea of the hormonal regulation of transport processes is supported by many data. For instance, treatment with cytokinins changed K/Na selectively [18,19] and altered the transport of K and Na [20]. In our experiments plant analysis also revealed alterations in the K/Na ratio. These results together with those concerning the Mg-ATPase will be dealt with elsewhere.

Changes in the properties of transport ATPase in auxin-pretreated rice seedlings were observed in [21], when the stimulation of K-influx and the ATPase activity by Ca apparently disappeared. Later [9] it was shown that calmodulin was involved in the ATP-dependent Ca-uptake in microsomes. Similarly, the participation of calmodulin in a cytikinin-dependent enzyme induction was suggested [7]. A clear sequence of reactions between the hormonal action and the Ca-binding or the transport step, however, cannot be drawn. One of the reasons for this is that our knowledge about plant calmodulin is rather scarce [8] in comparison with the animal systems [22].

We have shown that as a response to hormonal treatment:

(i) The ion-dependent basic ATPase activity increased;
(ii) The affinity of the ATPase towards Ca increased;
(iii) Calmodulin, prepared from either wheat roots of erythrocytes, stimulated Ca-ATPase activity;
(iv) Calmodulin from both hormone-treated and control plants were effective, while membranes only from the hormone-treated plants were responsive;
(v) Seedlings contained less Ca.

The increased basic activity may be due to the activation of the ion-independent kinase and phosphatase cascade which are activable by cAMP or cytokinins [10,23]. The resuls concerning the calmodulin stimulation of the Ca-ATPase suggest that new calmodulin-binding sites were induced by the cytokinin in the membrane. The relatively long lag period (fig. 3) for the manifestation of the hormonal effect indicates an action on gene or ribosomal level [21,24,25]. The calmodulin-binding sites or receptors can become a part of the ATPase complex, in accordance with an earlier scheme of the transport-ATPase [15]. Thus, the cytokinin-induced changes can lead to a more efficient Ca-extrusion system, maintaining the cells in juvenile form.

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