Presence of Two Different Membrane-bound, KCl-stimulated Adenosine Triphosphatase Activities in Maize Roots

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ABSTRACT

Recent publications have indicated that a KCl-stimulated ATPase from cereal roots is specifically associated with plasmalemma-enriched membrane fractions. However, in previous work we found that relatively high specific activities of this enzyme were also associated with a membrane fraction which did not contain plasmalemma. In an attempt to clarify this discrepancy, we have investigated the effect of density gradient composition upon the association of the enzyme with different membrane fractions isolated from the roots of Zea mays L. (WF9 × M14).

When roots were fractionated on a single, discontinuous sucrose density gradient the KCl-stimulated ATPase activity was concentrated in membrane fractions of relatively high density (σ = 1.17–1.22) which were enriched in plasmalemma. When fractions were isolated on both Ficoll and sucrose density gradients, KCl-stimulated ATPase activity was again found associated with plasmalemma and also in a second membrane fraction which did not contain plasmalemma. This fraction was found at relatively low densities (σ = 1.08) on the Ficoll gradient. The precise identity of the membrane in this fraction could not be determined.

A preliminary investigation into the properties of the two KCl-stimulated ATPase activities indicated that both had acid pH maxima and both displayed similar responses to changes in KCl concentration.

KCl-stimulated ATPase from oat roots was associated with a fraction enriched in plasmalemma (6). This fraction was found at a relatively high density (σ = 1.18) on a discontinuous sucrose density gradient. The maximum KCl-stimulated ATPase activity occurred at pH 6 and, at that pH, the kinetics of ion stimulation closely resembled the kinetics of ion uptake by oat roots (12).

In earlier work (23, 24) we found that the highest specific activity of KCl-stimulated ATPase from maize roots was associated with a membrane fraction which was found at a relatively low density (σ = 1.07) on a Ficoll gradient. The membranes in this fraction did not stain with PACP (22, 23) and therefore were not plasmalemma, although some KCl-stimulated ATPase activity was also found associated with a plasmalemma-enriched fraction. The properties of the ATPase found in the light Ficoll fraction implicated it in ion transport (24). Leonard et al. (10) also found a small peak of KCl-stimulated ATPase activity from oat roots at low buoyant densities but regarded it as less significant than the activity associated with the plasmalemma.

Because there were obvious discrepancies in the reported distributions of KCl-stimulated ATPase activities in membrane fractions from cereal roots, we felt that the problem merited further investigation. One possible explanation for the anomalies lies in the different centrifugation systems employed. Williamson and Wyn Jones (24) used a Ficoll gradient followed by a sucrose gradient, whereas Hodges and colleagues (6, 10, 12) employed only sucrose gradients. The observation that the composition of density gradients can markedly affect the buoyant density of membrane vesicles (2, 15, 20) may be relevant. We report on an investigation into the distribution of ATPase activities in membrane fractions from maize roots prepared by two different centrifugation systems. One used a single sucrose density gradient, while the other employed both Ficoll and sucrose gradients. The results, in confirmation of previous suggestions (22, 24) indicate that at least two membrane-bound, KCl-stimulated ATPase activities are present, only one of which is associated with a plasmalemma-enriched fraction. A summary of these results has been published elsewhere (9).

MATERIALS AND METHODS

Plant Material. Seeds of Zea mays L. (WF9 × M14, Crow Hybrid Corn Co., Milford, Ill.) were surface-sterilized for 15 min in vacuo in a solution of sodium hypochlorite containing 1 to 2% available chlorine. After rinsing, the seeds were placed in sterile Petri dishes lined with filter paper and containing sterile 0.5 mM CaCl₂. The seeds were germinated and grown in a darkened growth cabinet for 5 days at 30 C.

Membrane Fractionation. All operations were carried out
at 2–4 °C. Root tips (1 cm) were excised into deionized water and rinsed several times. The rinsed tips were chopped into approximately 1-mm lengths and suspended in homogenizing medium (0.3 M sucrose, 4 mM 2-mercaptoethanol, 25 mM tris-HCl, pH 7.8) to a final ratio of 1 g of tissue to 1 ml of medium. Chopped roots were homogenized using a Polytron homogenizer (Northern Media Supply Co., Ltd., Hull, Yorkshire, U.K.) operated at 9,000 rpm for 2 min. The homogenate was filtered through Miracloth (Calbiochem) and centrifuged at 6,000g for 10 min (6 × 16.5 ml swinging bucket rotor; MSE superspeed "65" centrifuge, M.S.E., Manor Royal, Crawley, Sussex, U.K.). The supernatant was further fractionated by density gradient centrifugation (Fig. 1). All sucrose solutions were buffered with 25 mM tris-HCl, pH 7.2, containing 4 mM 2-mercaptoethanol. All Ficoll solutions (Pharmacia Fine Chemicals) contained 0.3 M sucrose, 25 mM tris-HCl, pH 7.2, and 4 mM 2-mercaptoethanol. Fractions were removed from the gradients with a Pasteur pipette, diluted with 25 mM tris-HCl, 4 mM 2-mercaptoethanol, pH 7.2, pelleted at 70,000g for 1.5 hr and resuspended in 0.3 M sucrose, 4 mM 2-mercaptoethanol, 25 mM tris-HCl, pH 7.2.

**ATPase Assay.** Assays were performed in duplicate in disposable plastic tubes (GL4; Henlys Medical, London) at 30°C in a final volume of 1 ml. ATP (B. D. H. Biochemicals, Poole, Dorset, U.K.) was added as the tris salt prepared from the disodium salt using Dowex 50 ion-exchange resin. The standard assay medium contained 0.25 mM sucrose, 50 mM tris, 20 mM maleic acid, 2 mM MgSO₄, 2 mM tris-ATP, KCl at the required concentration (when added) and was adjusted to pH 5.5 with acetic acid, unless otherwise indicated. The reaction was started by the addition of a suitable aliquot of membrane suspension containing 15 to 30 μg of protein. Zero time samples and no enzyme blanks were included to correct for turbidity, endogenous phosphate, and nonenzymic ATP hydrolysis. After 1 hr the reaction was stopped by the addition of 1 ml of 10% (w/v) trichloroacetic acid, and the phosphate release was measured.

**Phosphate Assay.** Phosphate was determined by a modification of the procedure of Buell et al. (1) as previously described (24), except that the concentration of ammonium molybdate was increased to 50 mM. Nonenzymic hydrolysis of ATP was found to be dependent upon ammonium molybdate in the presence of 2 mM ATP (7). Increasing the ammonium molybdate concentration to 50 mM was found to reduce variation between replicates to less than 5%. Because of the nonenzymic hydrolysis all steps were carried out with strict attention to time.

**Definition of ATPase Activities.** Mg²⁺-ATPase refers to the ATPase activity measured in the presence of 2 mM MgSO₄. KCl-stimulated ATPase refers to the stimulation of the Mg²⁺-ATPase activity by KCl. This measurement was calculated as the difference between the activity in the presence of MgSO₄ plus KCl and the activity in the presence of MgSO₄ alone.

**NPPase Assay.** NPPase was assayed under the same conditions as ATPase using 2 mM tris-NPP as the substrate. Reaction was stopped by the addition of 2 ml of 0.2 N NaOH. Absorbance at 410 nm was determined and p-nitrophenol release was calculated from a standard curve of p-nitrophenol in alkaline conditions at this wavelength.

**Sucinate-INT Reductase Assay.** This enzyme was assayed according to the method of Morré (16).

**Protein Assay.** Protein was assayed according to the method of Lowry et al. (14). Interference from sucrose was determined from a reagent blank containing an aliquot of resuspension medium. Bovine serum albumen (fraction V, Sigma) was used as a standard.

**Electron Microscopy.** Whole root segments or membrane fractions were fixed for 1 to 2 hr in 2% buffered glutaraldehyde (0.1 M sodium cacodylate, pH 7) at 4°C, unless otherwise stated. The material was rinsed with three changes of buffer and postfixed for 1 to 2 hr with 1% (w/v) OsO₄ in 0.1 M sodium cacodylate pH 7 at room temperature. Specimens were rinsed in three changes of buffer, dehydrated through a graded acetone series, and embedded in Epon. The sections were cut on an Ultratome III ultramicrotome (LKB Producter AB, Stockholm, Sweden) and were collected on plastic-coated grids. Sections were stained with either lead citrate (18) or PACP (19). Since the periodic acid used in the PACP

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**Fig. 1.** Two alternative density gradient centrifugation systems used to isolate membrane fractions from maize roots. Maize root tips were chopped and homogenized in a Polytron homogenizer at 9,000 rpm for 2 min. The homogenate was filtered through Miracloth and centrifuged at 70,000g for 1.5 hr.
The 6,000g supernatant from a maize root homogenate was fractionated on a single, discontinuous sucrose gradient. The fraction nomenclature is described in Figure 1. ATPase and NPPase were assayed at pH 5.5 and, when added, KCl was present at a final concentration of 60 mM.

### Table I. Enzyme Activities of Subcellular Fractions of Maize Roots Prepared by Fractionation Scheme A

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Specific Activity of Enzyme</th>
<th>Mg(^{2+})-ATPase</th>
<th>KCl-stimulated ATPase</th>
<th>NPPase</th>
<th>Mg(^{2+}) + KCl NPPase</th>
<th>INTase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Homogenate</td>
<td></td>
<td>3.9</td>
<td>0.4</td>
<td>2.8</td>
<td>2.8</td>
<td>0.2</td>
</tr>
<tr>
<td>6,000g pellet</td>
<td></td>
<td>3.8</td>
<td>0.1</td>
<td>1.0</td>
<td>1.0</td>
<td>0.8</td>
</tr>
<tr>
<td>70,000g supernatant</td>
<td></td>
<td>4.4</td>
<td>0.6</td>
<td>3.8</td>
<td>3.8</td>
<td>0.1</td>
</tr>
<tr>
<td>A(_V)</td>
<td></td>
<td>4.4</td>
<td>0.6</td>
<td>4.5</td>
<td>4.5</td>
<td>0.2</td>
</tr>
<tr>
<td>A(_A)</td>
<td></td>
<td>0.4</td>
<td>0</td>
<td>3.7</td>
<td>4.0</td>
<td>0.2</td>
</tr>
<tr>
<td>A(_B)</td>
<td></td>
<td>4.9</td>
<td>0.2</td>
<td>3.2</td>
<td>3.2</td>
<td>0.2</td>
</tr>
<tr>
<td>A(_C)</td>
<td></td>
<td>3.0</td>
<td>1.4</td>
<td>2.3</td>
<td>2.4</td>
<td>0.2</td>
</tr>
<tr>
<td>A(_D)</td>
<td></td>
<td>2.7</td>
<td>0.8</td>
<td>2.9</td>
<td>2.5</td>
<td>0.4</td>
</tr>
</tbody>
</table>

### RESULTS AND DISCUSSION

The 6,000g supernatant from a maize root homogenate was fractionated on a discontinuous sucrose gradient (Scheme A, Fig. 1). The sucrose bands employed were of a density similar to those used by others for the isolation of plasmalemma-enriched fractions (5, 6). The distribution of enzyme activities in fractions prepared according to Scheme A are given in Table I. The highest specific activity of Mg\(^{2+}\)-ATPase at pH 5.5 was found in the A\(_D\) fraction. A high activity was also found in the 70,000g supernatant, probably due to the presence of nonspecific acid phosphatase. Indeed, NPPase activity was associated with all fractions but was not KCl-stimulated. The highest KCl-stimulated ATPase activities at pH 5.5 were found in the A\(_C\) and A\(_B\) fractions. A high concentration of succinate-INT reductase, a mitochondrial marker (16), was also associated with the A\(_B\) fraction but, in general, KCl-stimulated ATPase and succinate-INT reductase did not show parallel distributions. Although the specific activities of the staining procedure reacts with copper, sections to be stained by this method were collected on gold grids.

**Fig. 2.** Electron micrographs of maize root cortical cells fixed for 1 hr at room temperature in 1% formaldehyde + 2% glutaraldehyde buffered with 0.1 M sodium cacodylate pH 7. Postfixation was in 1% OsO\(_4\) in 0.1 M sodium cacodylate buffer pH 7. A: Stained with lead citrate; B: stained with PACP. Abbreviations: CW: cell wall; ER: endoplasmic reticulum; GA: golgi apparatus; M: mitochondrion; PM: plasmalemma; T: tonoplast; V: unidentified vesicle.

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KCl-stimulated ATPase are lower than those found in oat root membrane fractions (6, 10, 12), they are comparable to previously reported values for the activity of this enzyme in maize root membrane fractions (4, 11, 17, 24).

Membrane fractions were examined electron microscopically following both lead citrate and PACP staining. The specificity of PACP as a stain for plasmalemma in maize roots was initially confirmed (Fig. 2). The \( A_v \), and \( A_d \) and \( A_b \) fractions consisted of vesicles of smooth membrane which did not stain significantly with PACP (7). The heavier \( A_c \) and \( A_o \) fractions contained smooth membrane vesicles and mitochondria (Fig. 3, A and B). PACP staining showed that approximately 30 to 50% of the membranous material in the \( A_c \) fraction, and 50 to 70% of that present in the \( A_o \) fraction was derived from plasmalemma (Fig. 3, C and D). Other smooth membrane was also present, particularly in the \( A_c \) fraction.

These results indicated that both KCl-stimulated ATPase activity and the plasmalemma were concentrated at relatively high densities (\( \sigma = 1.17 \) and 1.22) on the sucrose gradient and are in reasonable agreement with those of Hodges et al. (6) who used a single sucrose density gradient to fractionate oat root membranes. However, they were clearly different from our previous observations with maize root membrane fractions, prepared on both Ficoll and sucrose gradients (23, 24), because relatively little KCl-stimulated ATPase activity occurred in the light, nonplasmalemma fractions. An experiment was therefore designed to determine the effect of Ficoll on the distribution of ATPase activities by fractionating a single batch of maize roots using both Schemes A and B (Fig. 1).

The distribution of enzyme activities between membrane fractions prepared according to Scheme A confirmed the data given above (Table II). When an equal portion of the same homogenate was fractionated by Scheme B, relatively high specific activities of KCl-stimulated ATPase were found in the \( B_c \) and \( B_b \) fractions at densities equivalent to the \( A_c \) and \( A_o \) fractions. There was no evidence of membranous material at the sucrose interfaces equivalent to the \( A_v \), \( A_d \), and \( A_b \) fractions. Instead, membranous material was found on the initial 0 to 17% Ficoll gradient. These membrane fractions had

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**Fig. 3.** Electron micrographs of subcellular fractions from maize roots. A: \( A_c \) fraction stained with lead citrate; B: \( A_d \) fraction stained with lead citrate; C: \( A_c \) fraction stained with PACP; D: \( A_o \) fraction stained with PACP. (See Fig. 2 legend for abbreviations used.)
higher KCl-stimulated ATPase activities than the light membrane fractions found on the sucrose gradient in Scheme A (Table II). This result implied that material normally found in the A and A₀ fractions, when using Scheme A, had been retained on the Ficoll gradient together with the material usually recovered in the lighter sucrose bands.

The fractions prepared by Scheme B were also examined electron microscopically. The B₂ fraction contained smooth membranes and mitochondria (Fig. 4A) and PACP staining showed that much of the former was plasmalemma (Fig. 4B). In contrast, the F₂ fraction contained vesiculated smooth membrane (Fig. 4C) which did not stain with PACP (Fig. 4D). These results suggest that maize roots contain at least two KCl-stimulated ATPase activities which can be separated on a combined sucrose and Ficoll density gradient but not on a sucrose-only density gradient. Only one of these activities is associated with a plasmalemma-enriched fraction. Part of the KCl-stimulated ATPase activity associated with the light vesicular membrane fractions from the Ficoll gradient may be recovered at higher densities on sucrose gradients and contaminated plasmalemma-enriched fractions. Changes in the apparent buoyant density of membrane-bound vesicles on sucrose and Ficoll gradients have been noted by others (2, 15, 20). Steck et al. (20) proposed that the final equilibrium position of a membrane vesicle in a density gradient is a function of both the fixed charges within the vesicle and the osmotic activity of the gradient medium. Other explanations have also been suggested (15).

Since the A₀ and B₂ fractions contained both plasmalemma and mitochondria, further evidence was sought to determine whether the KCl-stimulated ATPase activity was specifically associated with one or other of those components. An A₀ fraction was prepared (Fig. 1), diluted with 25 mM tris-HCl, 4 mM 2-mercaptoethanol, pH 7.2, and then layered over a cushion of 17% Ficoll in 0.3 mM sucrose, 4 mM 2-mercaptoethanol, 25 mM tris-HCl, pH 7.2. After centrifugation at 70,000g for 1.5 hr, this gave two further fractions, FA₀₁ and FA₀₂, which were assayed for KCl-stimulated ATPase activity and examined for plasmalemma and mitochondria content by electron microscopy. The FA₀₁ fraction, which remained above the 17% Ficoll cushion, was enriched in KCl-stimulated ATPase activity and plasmalemma. The FA₀₂ fraction, recovered as a pellet below the 17% Ficoll, was mainly mitochondria and had little KCl-stimulated ATPase activity (Table III; Fig. 4, E and F). It should be noted that the separation achieved with this system was probably due to interference by the overlay/Ficoll interface rather than a separation based upon differences in buoyant density. In another experiment, the layers visible in a pellet of A₀ fraction were resuspended separately. The upper pellet was enriched in KCl-stimulated ATPase and plasmalemma. The lower pellet contained mitochondria with little KCl-stimulated ATPase activity at pH 5.5. Thus one of the KCl-stimulated ATPase activities isolated from maize roots is associated with plasmalemma in agreement with the conclusions of Hodges et al. (6).

The identity of the membranes in the F₁ fraction which were also associated with a high specific activity of KCl-stimulated ATPase is not known. Since they did not stain with PACP, they are unlikely to be plasmalemma. The properties of the ATPase differed in several respects from that associated with the plasmalemma (7, 9). The vesicles in this fraction are, therefore, probably derived from smooth intracellular membranes. However, in the absence of unequivocal markers for these membranes further identification of this fraction is impossible.

In an attempt to partially characterize the F₁ and B₂ ATPase activities some of their properties were investigated. The Mg²⁺-ATPase activity of the B₂ fraction was observed over a wide range with maximum activity occurring at pH 6.5 to 7 (Fig. 5). The KCl-stimulated ATPase activity of this fraction was observed up to pH 6.5 and had a pH optimum of 5. This value is lower than the value reported for the KCl-stimulated ATPase activity associated with a plasmalemma-enriched fraction from oat roots (12). Mg²⁺-ATPase and KCl-stimulated ATPase activities of the F₁ fraction both had pH optima of 5.5 (Fig. 6). However, in contrast to the B₂ fraction, KCl-stimulated ATPase activity was observed over the entire pH range tested. The pH response curves of both fractions resembled closely those of two similar ATPase activities previously studied in maize roots (22, 24).

The stimulation of the ATPase activities by KCl was determined over a wide range of KCl concentrations. Both ATPase activities exhibited biphasic kinetics of stimulation (Figs. 7 and 8). One phase was observed below 1 mM KCl and saturated at 0.1 mM KCl. The second phase was activated at KCl concentrations greater than 1 mM and saturated at approximately 20 mM KCl. Similar concentration-dependent isotherms have been observed for the uptake of a number of ions by cereal roots (3, 8, 12, 21) and were also found to be characteristics of the KCl-stimulated ATPase associated with a plasmalemma-rich fraction from oat roots (12).

On the basis of the similarity between the kinetics of KCl-activation of the plasmalemma-associated ATPase activity of oat roots and the kinetics of ion uptake by excised oat roots, it has been speculated (12) that this enzyme might be involved in the transport of ions across the plasmalemma. The discovery of two different KCl-stimulated ATPase activities with activation kinetics similar to those of ion uptake raises the possibility that both play some role in the transport of ions. Indeed, it has been possible to correlate the KCl-stimulated ATPase activity of the A₀ fraction with an increase in the amount of KCl-stimulated ATPase activity.
Fig. 4. Electron micrographs of subcellular fractions from maize roots. A: B₀ fraction stained with lead citrate; B: B₁ fraction stained with PACP; C: F₁ fraction stained with lead citrate; D: F₁ fraction stained with PACP; E: F₂₁ fraction stained with PACP; F: F₂₂ fraction stained with lead citrate. (See Fig. 2 legend for abbreviations used.)
Table III. Redistribution of ATPase Activities of A₀ Fraction after Refractionation on 17% Ficoll Cushion

An A₀ fraction was prepared as indicated in Figure 1. The fraction was diluted and layered over a cushion of 17% Ficoll. After centrifugation at 70,000g for 1.5 hr, material was recovered in two fractions, one above and one below the cushion. These fractions, FAD₁ and FAD₂, respectively, were removed and assayed for ATPase activities at pH 5.5. When added, KCl was at a concentration of 60 mM.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>ATPase Activity</th>
<th>Mg²⁺-ATPase</th>
<th>KCl-stimulated ATPase</th>
</tr>
</thead>
<tbody>
<tr>
<td>A₀</td>
<td></td>
<td>2.3</td>
<td>1.5</td>
</tr>
<tr>
<td>FAD₁</td>
<td></td>
<td>2.1</td>
<td>1.5</td>
</tr>
<tr>
<td>FAD₂</td>
<td></td>
<td>1.2</td>
<td>0.5</td>
</tr>
</tbody>
</table>

**Fig. 5.** Effect of pH on the ATPase activities of the B₀ fraction. The pH was adjusted to the required value with acetic acid. Activity in the presence of 2mM MgSO₄ (○); activity in the presence of 2mM MgSO₄ + 60 mM KCl (●); KCl-stimulated activity (▲).

**Fig. 6.** Effect of pH on the ATPase activities of the F₂ fraction. The pH was adjusted to the required value with acetic acid. Activity in the presence of 2mM MgSO₄ (○); activity in the presence of 2mM MgSO₄ + 60mM KCl (●); KCl-stimulated activity (▲).

**Fig. 7.** KCl-stimulated ATPase activity of the B₀ fraction at various KCl concentrations (0.03–60 mM).

**Fig. 8.** KCl-stimulated ATPase activity of the F₂ fraction at various KCl concentrations (0.03–60 mM).

ATPase activities of the two membrane fractions with ion fluxes in whole root tissue (7; Leigh and Wyn Jones, in preparation). However, the correlations are difficult to interpret in an unequivocal manner and, while the evidence appears to indicate that both may be involved in ion uptake, no firm proof for this suggestion has been found.

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LITERATURE CITED