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Effects of Ca^{2+} on Amino Acid Transport and Accumulation in Roots of *Phaseolus vulgaris*[1]

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ABSTRACT

Ca^{2+} stimulates the uptake of α-aminoisobutyric acid (AIB) into excised or intact *Phaseolus vulgaris* L. roots by a factor of two. In roots depleted of Ca^{2+} by preincubation with ethylenediaminetetraacetate, ethyleneglycol-bis(β-aminoethyl ether)-*N,N'*-tetraacetic acid, or streptomycin, the stimulatory effect is 7- to 10-fold. In the presence of Ca^{2+}, roots accumulate AIB more than 100-fold; Ca^{2+}-depleted roots only equilibrate with AIB. Radioautography shows [^{14}C]AIB to be present in all cells after 90 min. Although Ca^{2+}-depleted roots lose accumulated [^{14}C]AIB about 10 times faster than roots supplied with Ca^{2+}, this increased efflux is not the main cause for the decrease in net uptake observed. The latter is rather due to a less negative membrane potential $\Delta\psi$ in Ca^{2+} depleted roots (−120 mV → −50 mV). The basic feature explaining all the results of Ca^{2+} deficiency is an increase in general membrane permeability. No indication of a specific regulatory function of Ca^{2+} in membrane transport of roots has been obtained.

It has long been known that Ca^{2+} constitutes an essential element for higher plants (reviewed in Refs. 3 and 21). Research concerning the role of Ca^{2+} in plants may be summarized by the statement that Ca^{2+} certainly affects membrane transport phenomena, and, in addition, it probably fulfills essential intracellular—possibly regulatory—functions (3, 21).

In the past, an increased ion leakage of roots lacking proper Ca^{2+} supply had been noticed (17) and an increased efflux of organic and inorganic substrates from various plant cells under such conditions has repeatedly been described (4, 7, 15). Also the uptake of ions and organic solutes is decreased in Ca^{2+}-depleted cells or tissues (4, 7, 15, 20). However, the molecular mechanism for all these membrane associated phenomena is not clear (3). Thus, questions such as the following have remained largely unanswered: to what extent are general permeability changes or rather direct effects of Ca^{2+} on special transport proteins involved? Is a decreased net uptake of solutes mainly due to their increased efflux? To what extent is a decreased solute accumulation caused by changes in influx or efflux or by both? Does the effect of Ca^{2+} on membrane "energization," which has been described (5), influence solute uptake?

We have studied the influence of Ca^{2+} and of Ca^{2+} depletion on the amino acid uptake of excised roots of *Phaseolus vulgaris*. Influx, efflux, and accumulation of the alanine analog AIB[2] have been investigated in detail. It will be shown that AIB within 90 min was accumulated more or less equally in all cortex and central cylinder cells. In Ca^{2+}-depleted roots the cells only equilibrated with AIB. Ca^{2+} depletion increased the leakage of accumulated AIB. This increased efflux is only partly responsible, however, for the observed low rate of AIB net uptake. The latter effect correlates well with a corresponding depolarization of the membrane potential.

MATERIALS AND METHODS

Plant Material. Seeds of *Phaseolus vulgaris* L. 'Wachs Beste von Allen' were purchased from a local seed merchant. The seeds were sterilized in a solution of 2.5% NaOCl and 0.05% SDS for 20 min and then washed 5 times with sterile H_2O. Germination occurred at 28°C and 300 lux in large sterile Petri dishes containing cellulose and filter papers which were moistened with distilled H_2O.

Chemicals. All chemicals if not indicated otherwise were obtained from Merck (Darmstadt). Amino acids, SDS, NEM, PPO, DDSA, and DMP-30 were purchased from Serva (Heidelberg). Streptomycin as its sulfate was supplied by Sigma (München). Quickszint 212 and Tissue Solubilizer TS-1 were obtained from Zinsser (Frankfurt), Epon 812 and Araldite from Fluka (Neu-Ulm) and x-ray film emulsion L4 from Ilford (Dietzenbach). [^{14}C]AIB (58 mCi/mmol), [^{14}C]adenine (62 mCi/mmol) and [^{14}C]3-*O*-methylglucose (295 mCi/mmol) were purchased from Amersham Buchler (Braunschweig), [^{45}Ca]Cl_2 (15.86 mCi/mg) from New England Nuclear (Dreieich).

Uptake Experiments. Two-centimeter-long root-tip segments of 5-d-old seedlings were excised under sterile conditions. Thirty segments were placed into a 25-ml Erlenmeyer flask containing 5 ml of 10 mM K-phosphate, pH 5.2, together with 0.1 mM [^{14}C] AIB (0.5 mCi/mmol) and further additions as indicated. The flasks were shaken in a water bath at 28°C; 100 μl aliquots of the medium were taken at various times, and the radioactivity was determined. After adding 5 ml of scintillation cocktail the samples were counted in a scintillation counter. At the end of the experiment roots were blotted dry on filter paper and fresh weight was determined. In some cases, the radioactivity in the roots was directly determined by adding 0.7 ml Tissue Solubilizer TS-1 to four root segments. After 24 h at room temperature, 10 ml of toluene cocktail were added and the samples counted. The uptake rates determined were the same with each method.

Efflux Experiments. Forty root-tip segments were preloaded for 3 h with [^{14}C]AIB in the presence of 10 mM Ca^{2+} in the medium as described for uptake experiments. After the loading period roots were washed twice in 10 ml ice-cold K-phosphate (10 mM, pH 5.2) for 3 min and then transferred into Erlenmeyer flasks containing 5 ml 10 mM K-phosphate and further additions as indicated. Efflux of [^{14}C]AIB was determined by counting the radioactivity in 100 μl aliquots withdrawn from the medium as described for uptake experiments. All uptake and efflux experiments were repeated at least three times.

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[2] Abbreviations: AIB, α-aminoisobutyric acid; DDSA, dodecyl succinic acid anhydride; DMP-30, 2,4,6-tris(dimethylaminomethyl)phenole; NEM, *N*-ethylmaleimide; 3-OMG, 3-*O*-methylglucose; PPO, 2,5 diphenyloxazole.

Microautoradiography. For autoradiography root-tip segments were preloaded with [^{14}C]AIB for 90 min as described for uptake experiments, except that specific radioactivity was higher and amounted to 12.5 mCi/mmol in the presence of Ca^{2+} and to 37.5 mCi/mmol for Ca^{2+}-depleted roots. Further procedures were carried out as described by Fritz (2). After freeze-drying at temperatures of −70°C at the condensor and −60°C at the trays, tissue samples were fixed with vapor of paraformaldehyde and then infiltrated with ether under high pressure over night. For embedment in plastic, Epon-Araldite was used and the steps in detail were as follows: 4 h in plastic:ether (1:1, w/v), 4 h in plastic:ether 1:3, w/v + 0.25% DMP-30 (hardener) and 24 h in 100% plastic + 0.25% DMP-30, all at 26°C; then 24 h in fresh 100% plastic + 0.25% DMP-30 at 50°C and finally 3 d in fresh 100% plastic + 0.25 DMP-30 in little aluminum troughs at 60°C. After polymerization and sectioning coating with photoemulsion and staining was carried out exactly as described (2).

^{45}Ca Uptake. Forty root tip segments were shaken in a 25-ml Erlenmeyer flask with 5 ml, 10 mM K-phosphate, pH 5.2, containing 0, 2 mM [^{45}Ca]Cl_2 (0.15 mCi/mmol). Radioactivity in the root segments was determined as described for [^{14}C]AIB uptake by dissolving the roots in TS-1.

Measurement of the Membrane Potential. $\Delta\Psi$ of cortical root cells was determined with a glass microelectrode filled with 0.5 M KCl as described by Felle (1). Root tip segments were placed in a chamber which was continuously perfused with the test solution at a flow rate of 10 ml/min and which allowed horizontal approach with the electrode. Before the electrical measurements the root segments were adapted in the chamber with 5 mM Tris-Mes buffer pH 7.3 containing 1 mM KCl, 1 mM NaCl, 0.1 mM $CaCl_2$ for about 1 h.

RESULTS

Effect of Ca^{2+} and Ca^{2+} Depletion on AIB Uptake. Uptake of the nonmetabolizable amino acid analog [^{14}C]AIB from phosphate buffered (pH 5.2) solutions into excised 2 cm root tips of bean seedlings was linear for at least 3 h (Fig. 1). The rate of uptake was increased more than twofold in the presence of 10 mM Ca^{2+}; this Ca^{2+} effect was not time-dependent (Fig. 1). Since almost the same stimulation by Ca^{2+} was observed when intact roots were immersed into the AIB solution (data not shown), the Ca^{2+} effect could not have been due to wound healing at the cut end.

Although the beans had been germinated and the seedlings grown for 5 d in deionized H_2O, the positive effect of Ca^{2+} on uptake was expected to be even stronger if the roots were depleted of Ca^{2+} more effectively. This was achieved by the addition of Ca^{2+} chelators (0.7 mM EDTA or EGTA) or by streptomycin, which has been claimed to exchange with Ca^{2+} when bound to cell surfaces by its positive charge (8, 9). To check the effectiveness of these treatments, excised roots were incubated with $^{45}Ca^{2+}$. Radioactivity was found to be rapidly associated with the roots and to double within 1 to 2 h; then a plateau was reached (Fig. 2). Sixty to 70% of this radioactivity was rapidly lost due to the addition of 0.7 mM EDTA or streptomycin (Fig. 2). When streptomycin or EDTA was present from the beginning of the experiment, the amount of ^{45}Ca associated with the roots after 2 h equaled the amount not released by EDTA or streptomycin from parallel samples (Fig. 2).

The uptake of AIB into Ca^{2+}-depleted roots was measured by following incorporation of radioactivity in the presence of 0.7 mM EDTA or streptomycin or into roots, which were pretreated with EDTA for 15 min only. The same results were obtained in both ways.

When pretreated with chelators and measured in the absence of Ca^{2+} the rate of AIB uptake was indeed drastically reduced (0.04 μmol/h·g as compared to 0.16). In the presence of Ca^{2+} the corresponding control rate was established again; thus in Ca^{2+}-depleted, excised bean roots, the uptake of AIB was stimulated 7- to 10-fold by Ca^{2+} (Fig. 1). Results very similar to those in Figure 1 were obtained when Ca^{2+} depletion was carried out with EDTA or EGTA. Again this large effect of Ca^{2+} was also obtained when intact roots were Ca^{2+}-depleted by chelators or streptomycin. Under all conditions Mg^{2+} and Mn^{2+} stimulated uptake less than 50% of the stimulation with Ca^{2+} (data not shown).

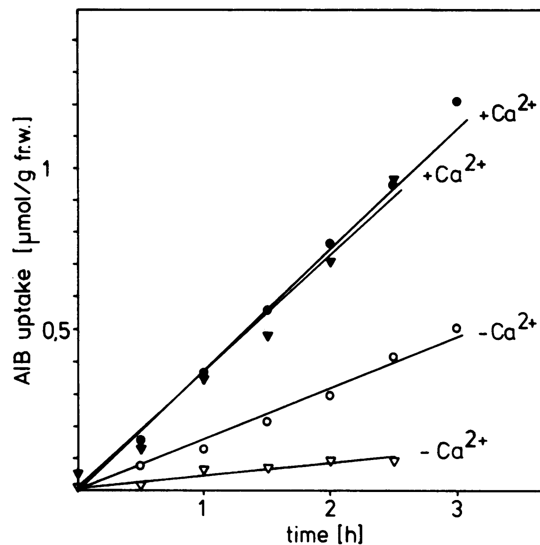

FIG. 1. Uptake of AIB by excised root tip segments. Thirty root segments were incubated in 5 ml K-phosphate (10 mM, pH 5.2) together with 0, 1 mM [^{14}C]AIB (0.5 mCi/mmol) at 28°C. Uptake was calculated from the decrease in radioactivity of the medium. Where indicated 10 mM Ca^{2+} were present. (○, ●) No further additions, (▽, ▼) 0.7 mM streptomycin present.

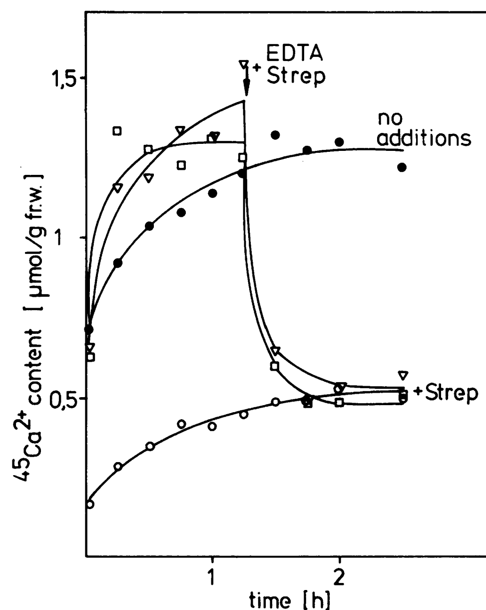

FIG. 2. Absorption of $^{45}Ca^{2+}$ by excised root tip segments. Forty root segments were incubated in 5 ml K-phosphate (10 mM, pH 5.2) together with 0.2 mM [^{45}Ca]Cl_2 (0.15 mCi/mmol) at 28°C. $^{45}Ca^{2+}$ content was determined by counting radioactivity in dissolved root segments. (○) 0.7 mM streptomycin added at the beginning of the experiment. (▽) No additions at the start of the experiment; 0.7 mM EDTA added at the time indicated by the arrow. (□) No additions at the start of the experiment; 0.7 mM streptomycin added at the time indicated by the arrow.

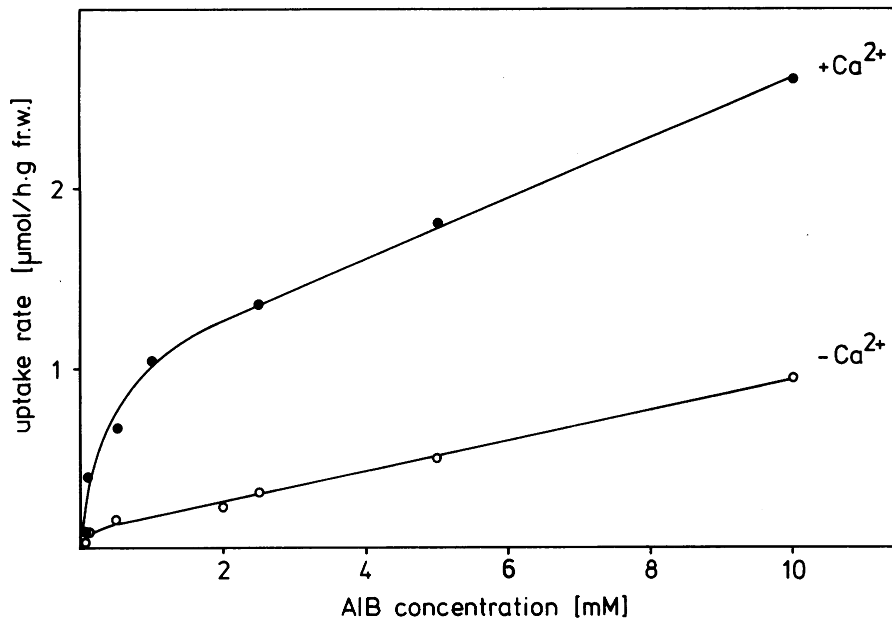

FIG. 3. Uptake kinetics of AIB by excised root tip segments deprived of Ca^{2+} with streptomycin. Conditions of uptake were the same as in Figure 1. (○) No Ca^{2+} added; 0.7 mM streptomycin present. (●) 10 mM Ca^{2+} added; 0.7 mM streptomycin present.

This clear, highly significant and well reproducible phenomenon demonstrated in Figure 1 prompted us to study this system in greater detail.

Ca^{2+} Affects the Velocity of AIB Uptake. To find out whether Ca^{2+} influences the V_{max} or the K_m of the amino acid transport system responsible for the uptake of AIB, the experiment of Figure 3 was carried out. The uptake kinetics were biphasic both in the presence and absence of Ca^{2+}. Ca^{2+} affected almost exclusively the saturable component at low AIB concentration, the V_{max} of which was 0.92 µmol/h·g in the presence and 0.09 µmol/h·g in the absence of Ca^{2+} (obtained by extrapolation of the linear portion to the vertical axis). A K_m of 0.2 mM was obtained in the presence of Ca^{2+}; it amounted to 0.15 to 0.3 mM with Ca^{2+}-depleted roots (Lineweaver-Burk plot of three independent experiments; the values up to 2 mM with an additional concentration at 0.02 mM, not included in Fig. 3, were plotted). The slope of the nonsaturable component was approximately doubled in the presence of Ca^{2+} (Fig. 3); this positive effect on the diffusional component of net uptake is most likely due to an inhibition of efflux by Ca^{2+} (see below).

Effect of Ca^{2+} on AIB Accumulation. When excised roots were incubated with [^{14}C]AIB in the presence of Ca^{2+} for more than 10 h, all the AIB was taken up from the medium. At time points for which it still was reasonable to estimate accumulation ratios, values of more than hundred fold were obtained (Fig. 4). These are lower estimates, since they are based on the whole root volume without correcting for free space. Even so, it seems obvious that AIB is actively transported into root cells. This does not seem to be the case in Ca^{2+} depleted cells (Fig. 4, insert), where an accumulation ratio of only one is approached after 3 h.

A possible alternative explanation for the difference observed plus or minus Ca^{2+}, however, could be the following: in the absence of Ca^{2+} AIB may be accumulated only in a very few cells (*e.g.* in the rhizodermis), whereas in the presence of Ca^{2+} additional cell layers located further inside may have become accessible to AIB. To test this possibility and to learn in which cells AIB is accumulated, radioautography of roots that had accumulated [^{14}C]AIB for 90 min was carried out. As can be seen in Figure 5 silver grains originating from [^{14}C]AIB are present at very similar frequencies in almost all cells of the cross-section of Ca^{2+}-depleted roots. The distribution pattern of radioactive AIB in roots not deprived of Ca^{2+} was the same (data not shown). Thus, in Ca^{2+}-depleted roots, no cells are able to actively accumulate AIB, whereas more or less all root cells seem to be able to do so in the presence of sufficient Ca^{2+}.

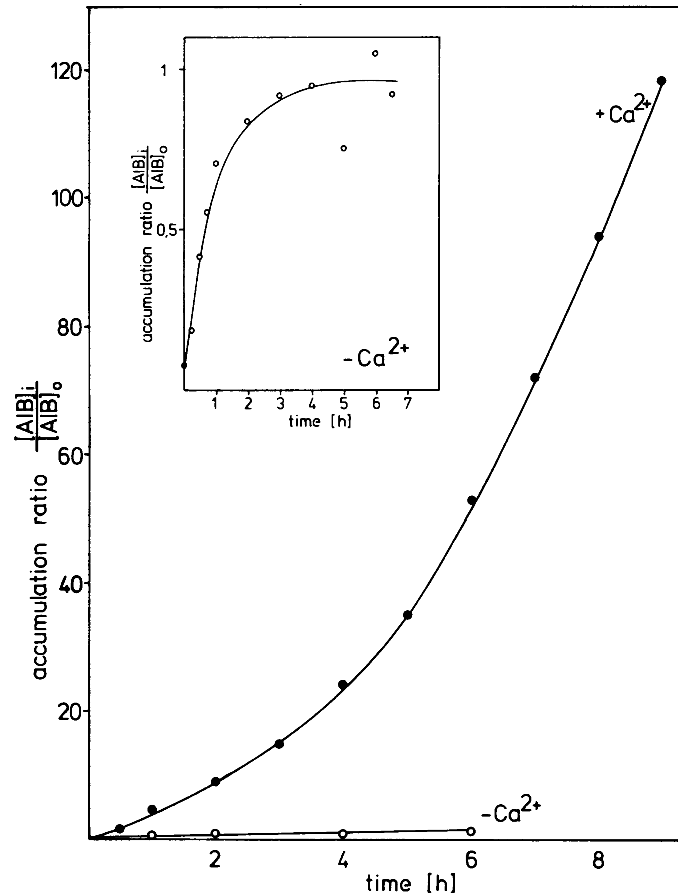

FIG. 4. Accumulation ratios of AIB in excised root tip segments. Conditions for uptake were the same as in Figure 1. Accumulation ratios were calculated after determination of [^{14}C]AIB concentrations in root segments and in the medium. (○) No Ca^{2+} added; 0.7 mM streptomycin present. (●) 10 mM Ca^{2+} added; 0.7 mM streptomycin present.

Does Ca^{2+} Affect AIB Efflux? The rate of AIB uptake was determined by following net uptake, *i.e.* total AIB influx minus AIB efflux. An inhibition of net uptake by Ca^{2+} depletion could,

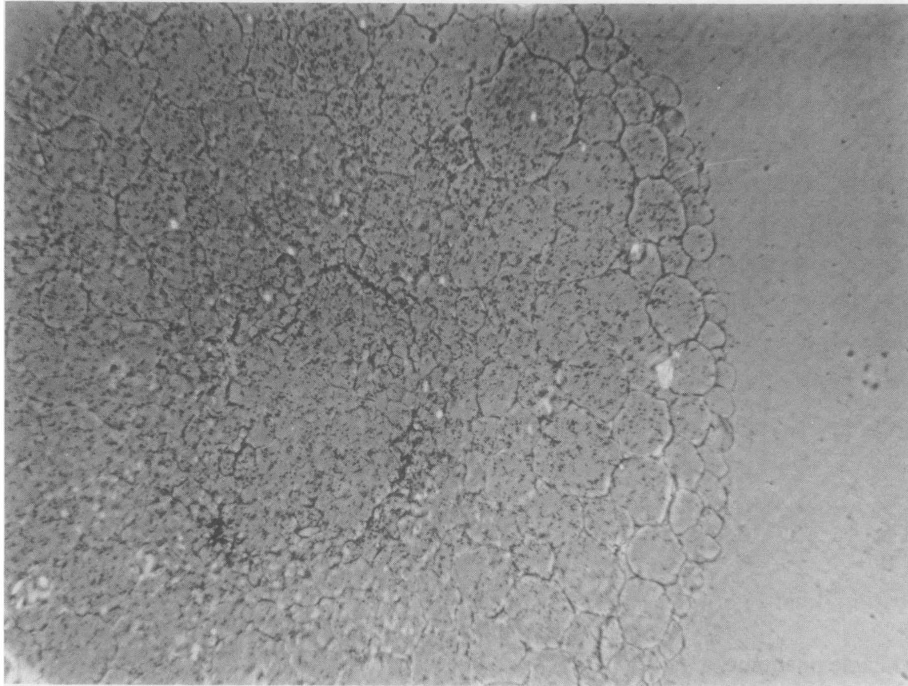

FIG. 5. Microradioautography of a root cross-section. The root segment was incubated for 90 min with [^{14}C]AIB in the presence of 0.7 mM streptomycin and then treated as described in "Materials and Methods."

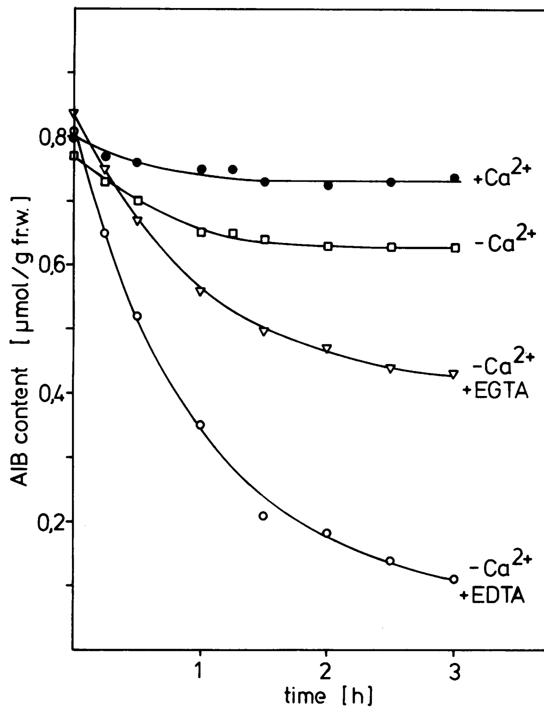

FIG. 6. Efflux of AIB from excised root tip segments. Forty root segments were preloaded with [^{14}C]AIB (0.5 mCi/mmol) for 3 h in the presence of 10 mM Ca^{2+} at 28°C. Afterward efflux was determined from the increase in radioactivity of the medium. (●) 10 mM Ca^{2+} added; 0.7 mM EDTA (or EGTA or streptomycin) were present. (□) No Ca^{2+} added; no chelators. (○) No Ca^{2+} added; 0.7 mM EDTA present. (▽) No Ca^{2+} added; 0.7 mM EGTA present.

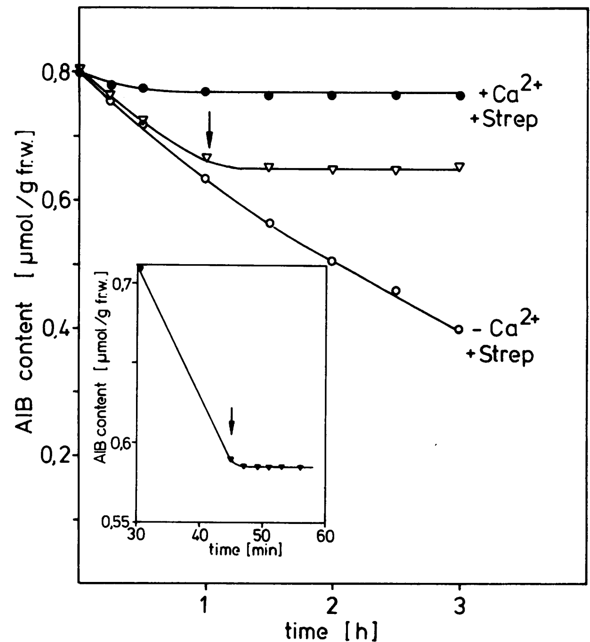

FIG. 7. Efflux of AIB from excised root tip segments. Conditions were the same as in Figure 6. (●) 10 mM Ca^{2+} added; 0.7 mM streptomycin present. (○) no Ca^{2+} added; 0.7 mM streptomycin present. (▽) Addition of 10 mM Ca^{2+} indicated by the arrow; 0.7 mM streptomycin present. Insert: inhibition of AIB efflux from root segments depleted of Ca^{2+} with 0.7 mM EDTA. Addition of 10 mM Ca^{2+} indicated by the arrow.

therefore, have been brought about—fully or partly—by an increased AIB efflux. Also the large difference in the accumulation ratio could be caused by the reduced influx or by an increased efflux, or by both.

When excised roots were preloaded in the presence of Ca^{2+} with [^{14}C]AIB for 3 h, then briefly washed and reincubated either in the same medium without [^{14}C]AIB or in buffer plus Ca^{2+}-chelators, a strong inhibitory effect of Ca^{2+} on AIB efflux was observed. As shown in Figure 6, the AIB efflux increased 9.2-fold with EDTA and 5.6-fold with EGTA as compared to the Ca^{2+} control (1 h values). The efflux induced by streptomycin shows a somewhat different time course, its stimulation of efflux being the least at shorter times (5.1-fold after 1 h). In the presence of Ca^{2+} chelators the initial AIB efflux seems to follow a simple first order reaction. In the presence of external nonradioactive

AIB (10 mM) the rate of [^{14}C]AIB efflux increased by 18% in Ca^{2+}-depleted roots and by less than 50% in the presence of Ca^{2+}. The observation, that efflux is not inhibited by NEM which completely blocks AIB uptake (data not shown), is suggestive—although certainly no proof—of a noncarrier mediated efflux. This assumption is strengthened by the observation that the classical overshoot eperiment of carrier mediated passive transport (6, 12) cannot be carried out with AIB and roots (data not shown). Finally, similar efflux behavior has been observed with 3-OMG and with adenine, and has been reported in the literature for a number of ions as well as organic solutes (3, 15, 19), which suggests a nonspecific membrane leak.

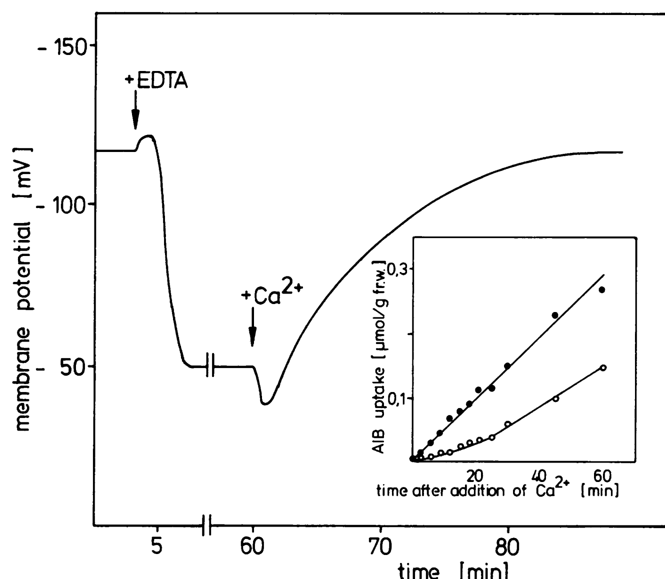

FIG. 8. Membrane potential of cortical cells of an excised root tip segment determined with a microelectrode. Arrows indicate addition of 0.7 mM EDTA and 10 mM Ca^{2+}, respectively, to the buffer K-phosphate, 10 mM, pH 5.2). The insert shows the recovery of AIB uptake in the presence of 10 mM Ca^{2+} by root segments which were deprived of Ca^{2+} with 0.7 mM EDTA. (●) Control, not Ca^{2+} depleted. (○) Ca^{2+} depleted for 45 min before uptake experiment.

An interesting feature of the high rate of efflux of Ca^{2+}-depleted roots is its complete reversal within a short time after the readdition of Ca^{2+} (Fig. 7). Indeed, when this reversion was studied with very short time intervals, it could be shown that AIB efflux stopped within 3 min after Ca^{2+} addition (Fig. 7, insert).

As pointed out above, the plasmalemma of Ca^{2+}-depleted roots has obviously a significantly increased permeability. However, in the case of AIB transport into and out of root cells this increased efflux cannot account for the strong inhibition of net uptake (Fig. 1), since the AIB-efflux in Ca^{2+}-depleted roots amounts to 0.07 μmol/h·g at an inside concentration of 0.1 mM and thus still is a factor of 5 to 8 smaller than the corresponding rate of AIB influx in the presence of Ca^{2+}. It is clear, therefore, that Ca^{2+} also directly affects AIB influx.

Effect of Ca^{2+} Depletion on Membrane Potential. As with sugars (16) most amino acids (1, 10) are cotransported together with protons during active uptake into plant cells. A change in proton motive force could, therefore, possibly cause the reduced rate of net uptake of [^{14}C]AIB in Ca^{2+}-depleted roots. Bean root cortex cells possess a $\Delta\Psi$ of about -120 mV (Fig. 8) which becomes less negative (-50 mV) within 5 min after the excised root is flooded with 0.7 mM EDTA. Addition of 10 mM Ca^{2+} reverses this depolarization, however it takes 20 min until the initial $\Delta\Psi$ is regained (Fig. 8). The length of time taken for AIB uptake to proceed with a linear rate again after Ca^{2+} readdition was investigated. As shown in Figure 8 (insert) the time scale closely resembled the recovery in $\Delta\Psi$, although the rate of AIB uptake recovered to only 70% after 45 min of EDTA pretreatment.

DISCUSSION

The results reported herein demonstrate the requirement for divalent cations, whereby Ca^{2+} is more than twice as effective as Mn^{2+} or Mg^{2+} for optimal net uptake and accumulation of the amino acid analog AIB into bean root cells. This uptake proceeds via a general amino acid permease, since the amino acids alanine > leucine > glycine = isoleucine > proline strongly inhibit AIB uptake, whereas arginine and lysine show no effect (data not shown). Whether in the presence or absence of Ca^{2+}, AIB is accumulated more or less in all root cells is shown by radioautography (Fig. 5). Although Ca^{2+} depletion of the roots seems to lead to an increased membrane permeability of all cells that had

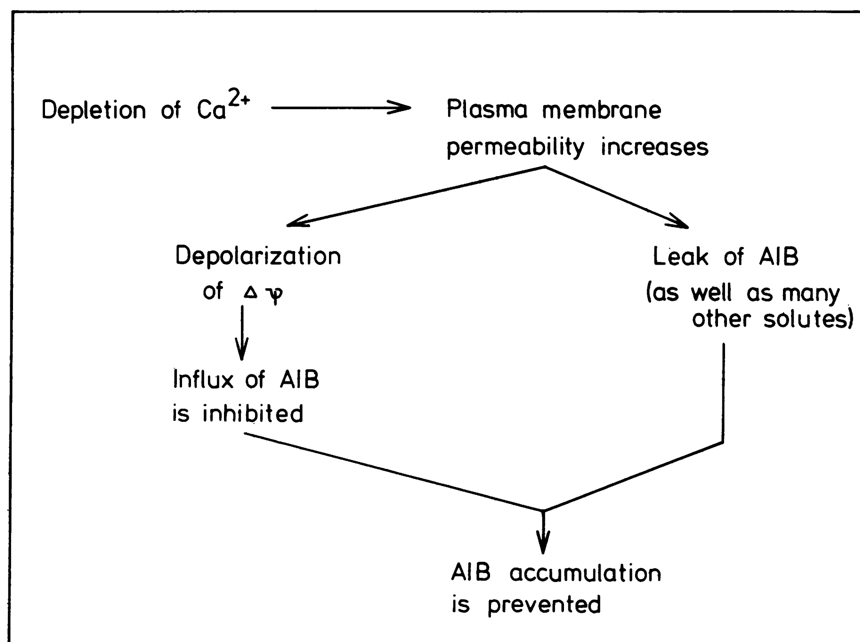

FIG. 9. Scheme: action of Ca^{2+} on AIB uptake, efflux and accumulation.

accumulated [^{14}C]AIB, this leak of [^{14}C]AIB as such is not the main cause for the observed inhibition of net uptake and accumulation. Thus, if a first order reaction for efflux is assumed—as pointed out in the results section—a rate constant of 0.78 h^{-1} can be calculated from the data of Figure 6 for EDTA-treated roots as compared with 0.07 h^{-1} for roots supplied with Ca^{2+}. At a cellular AIB concentration of 0.1 mM, the efflux would amount to 0.07 μmol/h·g, which is less than 20% of the rate of net uptake of [^{14}C]AIB at 0.1 mM in roots optimally supplied with Ca^{2+}. If unidirectional influx of AIB were not affected by Ca^{2+} depletion, these roots should still accumulate AIB considerably; however, their accumulation ratio does not exceed 1 (Fig. 4).

A comprehensive explanation for all the phenomena described, therefore, is the following (Fig. 9). The increased general leakiness of the plasmalemma due to the lack of Ca^{2+} will decrease all ion gradients (including $\Delta[H^+]$) as well as the membrane potential difference $\Delta\Psi$ (Fig. 8). As a consequence, active transport of [^{14}C]AIB will be severely inhibited. This together with a strong leak also for AIB itself will prevent any accumulation of the nonmetabolizable amino acid analog. Addition of Ca^{2+} to these roots, rapidly decreases membrane permeability. The membrane potential as well as the active uptake of AIB recover more slowly (Fig. 8). This time response seems slow if H^+-ATPase simply had to reenergize a membrane which before was deenergized because of its leakiness. The slow response, however, could be a consequence of partial injury of the cells due to their extended loss of cellular material.

Divalent cations and especially Ca^{2+} have repeatedly been shown to decrease the fluidity of phospholipid bilayers (13) and to reduce the surface pressure of phospholipid monolayers (11). A tighter packing of phospholipids in the presence of Ca^{2+} is, therefore, most likely responsible for the decrease in general permeability, which has also been shown with liposomes (18). From studies with vesicles it has been reported (14) that it is outside Ca^{2+} which tightens the membranes.

The results with bean roots described here do not indicate a specific regulatory role of Ca^{2+} in transport reactions.

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