Auxin Transport in Suspension-Cultured Soybean Root Cells

Matthew T. Loper and Roger M. Spanswick*

Section of Plant Biology, Division of Biological Sciences, Plant Science Building, Cornell University, Ithaca, New York 14853

ABSTRACT

To test the hypothesis that the carrier-mediated component of the indoleacetic acid (IAA) influx involves an electrogenic proton/IAA anion symport, the effects on the IAA influx of salts expected to depolarize the membrane potential were examined in suspension-cultured soybean (Glycine max [L.] Merr.) root cells. Although KCl does inhibit carrier-mediated uptake, the effect is specific to the anion at low concentrations and not due to more general processes such as changes in ionic or osmotic strength. Other anions such as bromide, iodide, and fluoride inhibit the carrier more strongly. Because potassium iminodiacetate, which is also expected to depolarize the membrane potential, has no inhibitory effect on the IAA influx, there is no evidence for the involvement of the membrane-mediated potential in uptake. It is therefore most likely that in soybean cells, if carrier-mediated uptake occurs via a proton symport, the \text{H}^+:\text{IAA}^{-} stoichiometry is 1:1. At concentrations greater than 70 millimolar, sorbitol, a nonionic osmoticum, inhibits carrier-mediated IAA uptake. The effects of specific anions and osmotic potential on the uptake carrier necessitates the reevaluation of other auxin transport studies in which KCl was routinely used as an agent with which to depolarize the membrane potential.

Based on the observation that carrier-mediated influx appeared to work against an auxin anion electrochemical gradient, Rubery (24) suggested that the uptake carrier might operate by a proton/anion symport mechanism. Hertel (11) investigated accumulation of IAA in vesicles prepared from hypocotyls of \textit{Cucurbita pepo} \textbf{L}. in relation to imposed ion gradients. Under conditions favoring a large membrane potential (inside negative relative to the outside), IAA accumulation was greatly enhanced. To explain these data, Hertel proposed IAA uptake to occur via an electrogenic 2\text{H}^+/\text{anion} mechanism. This was supported by Lomax et al. (16) who utilized electron spin resonance techniques to quantify pH gradients with the same zucchini vesicle system in relation to auxin accumulation. They found that the observed auxin accumulation could not be explained by the equilibration of IAA by a weak acid process (equivalent to a 1\text{H}^+/\text{IAA}^{-} mechanism) but, rather, more closely fit a 2\text{H}^+/\text{IAA}^{-} process.

Recently, several detailed studies dealing chiefly with vesicle preparations have attempted to quantify not only pH but electrical gradients as manipulated by various ionophore treatments. Sabater and Rubery (27) utilized \textsuperscript{86}Rb and [\textsuperscript{14}C]SCN\textsuperscript{-} to show that electric potential gradients could indeed be established in either direction in zucchini vesicles by manipulation of KCl concentration gradients across the membrane in the presence of valinomycin. They then demonstrated that an interior negative membrane potential stimulated IAA uptake and that this effect could be reversed by using KCl to reduce the membrane potential (28), again consistent with an electrogenic 2\text{H}^+/\text{IAA}^{-} mechanism. Probably the most definitive work on this topic was done by Clark and Goldsmith (3, 4) who investigated the effects of vesicle volume, ionic strength, and proton and electrical gradients on auxin accumulation in zucchini vesicles. They found that, whereas accumulation of the pH gradient probe butyric acid, a nonauxin with physical properties (lipophilic, \(pK_a = 4.81\) at 20°C) very similar to IAA, was strongly dependent on vesicle volume, accumulation of IAA was relatively independent of volume. In a later paper, they showed that addition of valinomycin failed to stimulate IAA uptake into vesicles that had lost most of their \[^{\text{3}}\text{H}]\text{IAA} after the addition of 0.1 \mu M nigericin to collapse the pH gradient (see Fig. 6, ref. 4). Under these conditions valinomycin still hyperpolarizes the membrane potential quite effectively. These results, in addition to the

\footnote{1}This material is based on work supported by the National Science Foundation under award No. DCB-8716363.

\footnote{2}Abbreviations: TIBA, triiodobenzoic acid; FCCP, carbonyl cyanide \(p\)-(trifluoromethoxy)phenylhydrazone; DIDS, 4,4'-diisothiocyanato-2,2'-stilbenedisulfonic acid.
Consisted of experiments, the modified basal medium (17). Uptake Assays were set to determine the total influx, that in the presence of unlabeled IAA represents the passive diffusion component of influx, and the difference of the two values is taken to represent saturable influx (17). Each calculated value represents the mean of at least three replicates ± the SE of the difference of the means, except where noted in the figure legends. All other pertinent experimental details are included in the figure legends.

Effects of Ionic Strength

effects of ionic strength, led them to conclude that, although voltage-dependent IAA uptake cannot be ruled out, saturable binding may be the main factor contributing to changes in apparent uptake in membrane vesicles.

Thus, some controversy exists concerning the driving forces that govern auxin uptake. We have investigated the effect of salts on carrier-mediated auxin uptake by soybean root cells (17), in which the influx can be measured independently of the internal binding that may affect the (net) uptake measured in isolated membrane vesicles (3). The results indicate that the anion of the salt may have a significant effect on carrier-mediated auxin transport that is not related to the effect of the salt on the membrane potential.

MATERIALS AND METHODS

Plant Material

Suspension-cultures from soybean (Glycine max [L.] Merr. cv Mandarin) were grown and harvested as described previously (17).

Uptake Assays

The procedures and solutions used in the uptake assays are the same as described previously (17) with the following modifications: the resuspension medium was replaced by a modified basal medium which was used for the washing procedures and, with additions as specified for particular experiments, for preincubations and uptake measurements. It consisted of 20 g/L sucrose, 1 mM CaCl₂, 0.1 mM KNO₃, and 40 mM Mes/1,3-bis[tris(hydroxymethyl)methylamino]propane, pH 5.5. Initial uptake rates were measured utilizing 60-s uptake periods (subtracting a zero-time point as background) in the presence of 10 μM TIBA. The uptake rate was determined in the absence and presence of 30 μM unlabeled IAA in each case. Uptake of 0.28 μM [¹⁴C]IAA in the absence of unlabeled IAA represents the total influx, that in the presence of unlabeled IAA represents the passive diffusion component of influx, and the difference of the two values is taken to represent saturable influx (17). Each calculated value represents the mean of at least three replicates ± the SE of the difference of the means, except where noted in the figure legends.

RESULTS

Effects of Modifiers of the Electrical and pH Gradients

An analysis of the effects on the passive and saturable components of IAA uptake by treatments designed to alter the electrical and/or pH gradients across the plasma membrane is shown in Figure 1. Cells preincubated in low K⁺ basal medium containing 0.1 mM KNO₃ were exposed to 35 mM KCl, 10 μM fusicoccin, or 10 μM FCCP (see figure legend). Addition of 35 mM KCl (expected to depolarize the membrane potential) significantly inhibited saturable uptake, whereas passive diffusion was unaffected. Fusicoccin (expected to induce hyperpolarization of the membrane potential) had no effect on either component of influx. The greatest effect was seen in the presence of the protonophore FCCP, which would probably alter the pH gradient. FCCP greatly inhibited total uptake as a result of a large inhibition of saturable uptake.

The role that potassium plays in the inhibition of saturable uptake was investigated in the experiments shown in Figures 2 and 3. Addition of 10 μM valinomycin to increase the membrane's permeability to potassium was only slightly more inhibitory to saturable uptake than KCl alone (Fig. 2). In contrast, replacing potassium with the nonpermeant choline ion resulted in a significantly greater effect (Fig. 3). Replacing chloride with other halide salts of potassium reveals a pattern
that appears to be more dependent on the anion than on potassium itself. The same concentrations of KBr, KI, and KF give even greater degrees of inhibition than KCl (Fig. 4). The fluoride salt almost totally abolishes saturable uptake of IAA. Unlike the other salts, KI and KF also exert an effect on passive diffusion. This may reflect a more general effect on membrane permeability in addition to a specific effect on the carrier.

**Ionic and Osmotic Strength**

The general phenomena associated with the addition of a salt to a solution, such as an increase in osmotic potential and ionic strength, were investigated next. Sorbitol, a nonionic sugar alcohol, was used to mimic the same osmotic concentration produced by a salt (Fig. 5A). At 70 mM (equivalent to 35 mM KCl), sorbitol had no effect on IAA influx. The same result was seen with 70 mM mannitol (data not shown). However, when higher concentrations of sorbitol were used, the IAA influx was progressively depressed, appearing to reach a minimum of about 40%. The inhibition was mainly expressed in the saturable component, although at 160 mM sorbitol passive diffusion was reduced by nearly 50%. Interestingly enough, above 70 millimolar, equivalent osmotic concentrations of KCl gave consistently less inhibition than sorbitol (Fig. 5B). In addition, KCl inhibited only the saturable component; passive diffusion was relatively unchanged or even slightly stimulated.

The monobasic sodium salt of iminodiacetate was used to observe the effects of ionic strength on IAA influx (Fig. 6). The plasma membrane is generally thought to have a low...
permeability to both the sodium and iminodiacetate ions, and thus they should not directly alter the membrane potential. At the same concentration used in the other salt treatments (35 mM), sodium iminodiacetate had no significant effect on either diffusion or saturable uptake. When the monobasic potassium salt of iminodiacetate was used in place of the corresponding sodium salt, there was a slight stimulation of total uptake which was reflected primarily in diffusion. However, the addition of 35 mM NaCl significantly inhibited carrier-mediated uptake but not passive diffusion. These results suggest that, at a salt concentration of 35 mM, the carrier displays a salt sensitivity that is dependent on the anion and not the cation. At concentrations greater than this, the influence of osmotic potential becomes the predominant factor influencing carrier-mediated uptake.

It is possible that the anion effect observed could be nonspecific, affecting metabolism and/or other processes involved in maintaining cell homeostasis. However, omission of the 10-min preincubation period to minimize this effect produced the same pattern of inhibition by KCl, KBr, and KF (data not shown), although to a reduced extent.

Effects of Chemical Modifying Reagents

The chemical modifiers phenylglyoxal and DIDS were used to test for the presence of specific amino acid residues at the active site of the uptake carrier (Fig. 7). Incubation with 15 mM phenylglyoxal (specific for arginine residues) for 10 min essentially abolished carrier-mediated uptake, whereas 100 μM DIDS (which affects lysine and histidine and cysteine to a lesser extent) reduced carrier activity by <30%. In both cases, passive diffusion was unaffected. A protection experiment was then performed to determine whether phenylglyoxal interacts directly with the active site of the carrier. Thirty seconds before exposure to phenylglyoxal, unlabeled IAA (final concentration, 30 μM) or water was added to the cells. A 9-min preincubation in the presence of 10 mM phenylglyoxal was then performed. The cells were rinsed twice with basal medium and the uptake assay was initiated. IAA was unable to prevent inhibition of carrier activity by phenylglyoxal, as clearly shown in Figure 8. In fact, the preincubation process itself inhibited the carrier by almost 50%. Apparently not all of the unlabeled IAA added before the uptake assay had been rinsed out.

DISCUSSION

It is well established that the IAA influx at the plasma membrane consists of two components, a carrier-mediated flux and a passive diffusion of the lipophilic associated form of the acid (17, 26). In theory, the diffusional influx will depend only on the product of the membrane permeability and the external concentration of the neutral molecule. Although the pH gradient across the membrane will affect the long-term accumulation of IAA by the phenomenon of ion trapping (22, 26), it will not affect the influx, measured as the initial rate of uptake under conditions in which the carrier-mediated component is eliminated, given that the external pH is held constant. The observation that the passive influx of IAA is affected very little by a wide range of treatments (Figs. 1, 3, 6, and 7), with the exception of the more reactive halogen ions (Fig. 4), indicates that the permeability coefficient of the associated IAA molecule is also unaffected by these treatments.

If, as has been postulated (11, 24), the carrier-mediated uptake of IAA is via an H+/IAA− symport mechanism, an alteration of the transmembrane pH gradient, and the electrical gradient if the H+/IAA stoichiometry is >1.0, may affect the initial rate of uptake. Indeed, this appears to be the case for soybean root cells. After 30 min preincubation in the presence of 10 μM FCCP, the saturable component of uptake is strongly inhibited (Fig. 1), whereas passive diffusion is affected only slightly. Martin and Pilet (18) found the same result with maize roots. Other groups reported similar effects (3, 12, 16) with membrane vesicles.

It is interesting that fusicipocin fails to alter IAA uptake.
Figure 8. Effects of IAA preincubation on (phenylglyoxal) inhibition of IAA influx. A 9.5-min serial preincubation with or without 30 μM unlabeled IAA and 10 mM PGO was performed as described in "Results." The experimental conditions of the influx assays and derivation of total influx, passive diffusion, and saturable influx are exactly as described in Figure 1. The values are given relative to the total amount of IAA taken up in the control, which represents 100%. All values are the average of three replicates from a single experiment.

Others have found fusicoccin to enhance uptake (10), although it appears to have no effect (9) or may even inhibit (10) IAA transport. Fusicoccin has been shown to both hyperpolarize the membrane potential (6) and stimulate proton extrusion (5) quite effectively. The pH of the cytoplasm changes little during proton extrusion (23), but that of the cell wall space decreases significantly (13). The net effect of hyperpolarization of the membrane potential and acidification of the cell wall is an increase in the proton-motive force which may stimulate an H⁺/IAA⁻ symport. It is possible that the cells are insensitive to fusicoccin or that the initial hyperpolarization that occurs when the cells are placed in low potassium basal medium increases the rate of carrier-mediated transport (assuming an electrogentic process) to a maximum and any further hyperpolarization has no effect. Furthermore, the decrease in cell wall pH in suspension cells incubated in a buffered medium will be negligible compared to that which may occur in a tissue.

At first sight, the effect of 35 mM KCl on carrier-mediated uptake appears to support an electrogentic mechanism and is in agreement with findings using membrane vesicles (1, 11, 28). Measurements of membrane potentials on soybean cells derived from cotyledons (21) indicate that 10 mM KCl produces a depolarization. However, if the effect of KCl on IAA uptake in soybean cells is examined more closely, it is clear that it is not specific for potassium. The failure of valinomycin to enhance significantly the inhibition by 35 mM KCl implies that the potassium ion is not the main antagonist in this phenomenon. (However, it is possible that steric hindrances prevent penetration of valinomycin, \( M_f \), 1111, through the cell wall [2].) Replacing the potassium ion with the nonpermeant choline ion gives a larger inhibition of saturable uptake. Exchanging the chloride ion with the more reactive halide ions such as bromide, iodide, and fluoride increasingly inhibits carrier-mediated uptake even though potassium is present at the same concentration. Indirect effects of these other ions may contribute to the inhibition as suggested by the reduction in passive diffusion in some cases (fluoride and iodide), but, in the presence of chloride salts and potassium bromide, the inhibition appears to be specific. Some indirect effects may include the alteration of membrane properties (which might affect carrier conformation), reduction of ATP levels (fluoride is a strong inhibitor of glycolysis), or a direct inhibition of the plasma membrane ATPase (7, 14). The latter two effects would result in a reduction in proton-motive force, but the change would be significant only after a few minutes of exposure. However, inhibition was observed during a 1-min uptake period without a preincubation (see "Results").

The increasing inhibition of saturable uptake observed at external KCl concentrations >35 mM is probably due to plasmolysis of the cells, because the same effect is seen with sorbitol >70 mM. In fact, at 115 mM sorbitol, which corresponds to a total osmotic concentration of approximately 217 milliosmolar when the osmolarity of the modified basal medium is taken into account, the cells were visibly flaccid. The fact that equal osmotic concentrations of sorbitol are more inhibitory than KCl between 70 and 115 mM may be because potassium and choline ions are able to enter the cell to some extent, thus reducing the amount of water lost compared to the case of the relatively impermeant sorbitol.

A general effect resulting from the addition of elevated salt concentrations is an increase in ionic strength. The lack of inhibition by the potassium and sodium salts of the monovalent iminodiacetate ion (a nonpermeant anion) suggests that ionic strength is not a factor (Fig. 6). This contrasts with the conclusions of Clark and Goldsmith (4) who had similar findings in that inhibition by increasing potassium salt concentrations could be matched by sodium or choline salts. They concluded that ionic strength exerts an effect on internal binding of IAA. In another experiment they monitored the time course of \(^{3}H\)IAA (10 nM) uptake by Cucurbita vesicles which were preloaded with 50 mM K$_2$SO$_4$. It was found that after 5 min of uptake the addition of 1 μM valinomycin, which was shown to produce a significant hyperpolarization of the membrane potential, resulted in a transient stimulation of IAA uptake. This stimulation disappeared within 10 min with no change in the hyperpolarization of the membrane potential. Such an increase could support an electrogentic mechanism. However, why this effect is only a transient one is puzzling and appears to contradict other findings in which, under similar conditions, a net stimulation is seen after 40 min or more (1, 11, 28). These results, in addition to the previously mentioned failure of an electrical gradient to stimulate accumulation in the absence of a pH gradient, do not favor an electrogentic mechanism. At any rate, the effect of KCl on saturable uptake simply as a modifier of the membrane potential is clearly in doubt. Obviously, other factors must be taken into account.

The most significant finding is that potassium iminodiacetate, which is probably effective in depolarizing the membrane potential, has no effect on saturable uptake. In addition, salts such as NaCl and choline chloride, which exert little or no effect on the membrane potential, inhibit uptake. Thus, there appears to be a specific inhibition of the carrier-mediated IAA by halogen anions. This is not unprecedented in that Lien and Rognes (15) observed inhibition by Cl⁻ of amino acid uptake into barley leaf tissue. Also, an apparent Cl⁻ effect on
auxin accumulation by zucchini membrane vesicles has been observed (T Lomax, personal communication).

Phenylglyoxal has been used in different systems such as the anion transporter of red blood cell ghosts (29) to study the possible role arginine residues play at anion-binding sites. The large effect of phenylglyoxal on carrier-mediated uptake in soybean cells, which is unaffected by the presence of the substrate IAA, suggests two possible modes of action. One is that phenylglyoxal binds directly to the carrier at a site distinct from the IAA-binding site, effecting (or preventing) a conformational change in the carrier and thus impairing IAA transport. The other possibility is that phenylglyoxal binds to the ATPase, impairing its ability to maintain the proton gradient and thus indirectly inhibiting carrier-mediated transport. In corn roots (19, 20) phenylglyoxal inhibits NO₃⁻ transport and the associated electrical transients. However, it also depolarizes the membrane potential in a manner consistent with inhibition of the electrogenic H⁺-ATPase. In red beet, phenylglyoxal has been used to implicate an arginine residue at the active site of the plasma membrane ATPase (8). Distinguishing between direct and indirect effects of phenylglyoxal requires a more detailed analysis.

In summary, the auxin uptake carrier in soybean root cells has been shown to be strongly dependent on pH (17), possibly operating via a one- or two-proton per auxin anion symport. No evidence has been found that supports the electrogenic, two-proton mechanism over the electroneutral, one-proton process. However, it is clear that the carrier is sensitive both to specific anions and to the osmotic potential. Thus, the utilization of potassium salts to manipulate the membrane potential and study its relationship to the uptake mechanism must be performed with the knowledge that other secondary, or even primary, effects must be taken into account. The carrier's sensitivity to anions and osmotic potential may be useful for investigating the biochemical mechanisms of carrier-mediated auxin transport.

ACKNOWLEDGMENT

We thank Dr. Terri Lomax for helpful comments on the results.

LITERATURE CITED

27. Sabater M, Rubery PH (1987) Auxin carriers in Cucurbita vesicles. II. Evidence that carrier-mediated routes of both indole-3-acetic acid influx and efflux are electroimpelled. Planta 171: 507–513