Inhibition of Anion Transport in Corn Root Protoplasts

WILLY LIN
Central Research and Development Department, Experimental Station, E. I. du Pont de Nemours and Company, Wilmington, Delaware 19801

ABSTRACT
The effects of several amino-reactive disulfonic stilbene derivatives and N-(4-azido-2-nitrophenyl)-2-aminoethylsulfonate on Cl−, SO42−, and inorganic phosphate (Pi) uptake in protoplasts isolated from corn root tissue were studied. 4-Acetamido-4′-isothiocyanato-2,2′-stilbenedisulfonic acid, 4,4′-diisothiocyanato-2,2′-stilbenedisulfonic acid, 4,4′-diamino-2,2′-stilbenedisulfonic acid, and NAP-taurine inhibited Cl− and SO42− but not Pi and K+ uptake in corn root protoplasts; whereas mersalyl inhibited Pi but not Cl− or SO42− uptake. The rate of uptake of all anions decreased with increasing external pH. In addition, these reagents markedly inhibited plasmalemma ATPase activity isolated from corn root tissue. Excised root segments were less sensitive to Cl− and SO42− transport inhibitors.

MATERIALS AND METHODS
A modification of our previously reported method (13) was used to isolate protoplasts from young corn roots. As outlined in Figure 1, 0.1% pectolysin Y-23 (16) substituted for pectinase and hemi-cellulase in the digestion enzyme mixture. Also, the tissue incubation time in the digestion mixture was shortened to 1.5 to 2 h and the precentrifugation steps were omitted. The yield of 106 protoplasts/g root tissue and viability of the protoplasts were similar to those previously described (13). Transport rates for Pi and K+ were 2-fold higher and linear for longer periods of time (10–20 min longer) than in the previously described technique (Ref. 13; Table I and Fig. 2). To avoid possible adverse effects on the surface proteins of the protoplast by proteases from the cell wall digestion enzyme mixture (15), 0.05% BSA and 10 mM DIFP, a specific inhibitor for fungal serine proteases (15), were added to the digestion enzyme mixture.

The previously described rapid separation technique (13) was used to measure ion influx into protoplasts. About 0.5 million protoplasts were used in each measurement. Carrier-free 35Cl−, 32SO42−, H218OPO43−, and 86Rb+ (New England Nuclear) in 1 mM KCl, K2SO4, KH2PO4, and KCl were added in 1 mM Hepes (pH 6.0), 0.2 mM CaCl2, and 0.65 mM mannitol to measure Cl−, SO42−, Pi, and K+ influx, respectively. NaOH or H2PO4− were used to adjust the pH of the absorption media in the pH studies. Except for the time course experiments, a 15-min radioactive exposure period was employed. Previously described methods were used to measure the ion influx into 4 h-washed excised root segments at 0.2 mM salt concentration (12) and plasmalemma ATPase activity (14). All experiments were duplicated.

Pectolyase Y-23 was purchased from Seishin Pharmaceutical Co., Japan, Cellylysin and DIFP from Calbiochem, SITS, DIDS, and NAP-taurine from Pierce Chemical Co., DADS from Eastman Kodak Co., and mersalyl from Sigma. All other chemicals were ACS reagent grades.

RESULTS AND DISCUSSION
The inhibition of Cl− and SO42− uptake in red blood cells by membrane impermeable amino-reactive disulfonic stilbene derivatives (SITS, DIDS, and DADS) was proposed to result from the covalent binding of these chemicals to the outer peptide chain of the band 3 protein, the major anion transport carrier (10, 24). Figure 2 shows SITS and DIDS also rapidly and completely inhibited the Cl− and SO42− accumulation in corn root protoplasts, whereas, in excised root segments, higher concentrations of these chemicals and a 10 to 15 min lag were required to produce limited inhibition. This suggests that an amino-reactive protein may also be involved in Cl− and SO42− in plant cells. The concentration dependency of the SITS and DIDS inhibition of chloride and sulfate transport into protoplasts and root segments is shown in Figure 3. The maximum inhibition of Cl− uptake by SITS and DIDS in protoplasts was 80%, whereas in root segments higher than 95% inhibition was observed at higher SITS or DIDS con-

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2 Abbreviations: SITS, 4-acetamido-4′-isothiocyanato-2,2′-stilbenedisulfonic acid; DIDS, 4,4′-diisothiocyanato-2,2′-stilbenedisulfonic acid; DADS, 4,4′-diamino-2,2′-stilbenedisulfonic acid; NAP-taurine, N-(4-azido-2-nitrophenyl)-2-aminoethylsulfonate; FCCP, (p-trifluoromethoxy) carbonyl cyanide phenylhydrazone; DIFP, disopropylfluorophosphate.
centrations. Similar results were found for $\text{SO}_4^{2-}$ uptake in protoplasts versus segments. The requirement of higher reagent concentrations for the inhibition of $\text{Cl}^-$ and $\text{SO}_4^{2-}$ transport in root segments is probably due to either decreased rate of penetration of the reagent to the membrane or reaction of the reagent with cell wall constituents.

Accumulation of $\text{Cl}^-$ and $\text{SO}_4^{2-}$ ions leveled off after 30 min of incubation (Fig. 2). When 1 mM glucose was added in the uptake medium, ion accumulation was extended by 10 to 15 min before leveling off but without any change in the initial rate of accumulation. The presence of 1 mM glucose in the medium did not alter the inhibitory effect of the anion transport inhibitors tested. The observation that glucose extends the accumulation of ions by 10 to 15 min into protoplasts suggests that $\text{O}_2$ is not limiting under the transport assay condition.

In bacterial and red blood cells (2–5, 10, 23), SITS, DIDS, and DADS were found to be specific inhibitors for $\text{Cl}^-$ and $\text{SO}_4^{2-}$ transport. Importantly, a similar specificity of inhibition by these chemicals was observed in protoplasts isolated from corn roots (Table I) in that they markedly inhibited $\text{Cl}^-$ and $\text{SO}_4^{2-}$ uptake, while $\text{Pi}$ and $\text{K}^+$ uptake was essentially unaffected. The degree of inhibition of $\text{Cl}^-$ uptake was similar to that caused by the uncoupler FCCP. A similar but smaller inhibition of $\text{SO}_4^{2-}$ uptake was found in the same experiment. The fact that $\text{Pi}$ and $\text{K}^+$ were not affected by these chemicals suggests that the protoplasts suffered no general damage during the uptake period. The failure of the uncoupler to inhibit anion uptake strongly in protoplasts may be due to a higher passive influx of ions into the protoplasts.

The specificity of SITS and DIDS inhibition for $\text{Cl}^-$ uptake is further demonstrated in Figure 4. Very little inhibition of $\text{Pi}$ uptake was observed by either of these chemicals.

### Table 1: Effects of Chemical Modifiers on Anion Transport in Corn Root Protoplasts and Segments

<table>
<thead>
<tr>
<th></th>
<th>Cl⁻</th>
<th>SO₄²⁻</th>
<th>Pi</th>
<th>K⁺</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Protoplasts</td>
<td>Segments</td>
<td>Protoplasts</td>
<td>Segments</td>
</tr>
<tr>
<td></td>
<td>nmol/10⁶</td>
<td>µmol/10⁶</td>
<td>nmol/10⁶</td>
<td>µmol/10⁶</td>
</tr>
<tr>
<td>Control</td>
<td>9.96</td>
<td>1.74</td>
<td>1.54</td>
<td>0.28</td>
</tr>
<tr>
<td>SITS, 0.1 mM</td>
<td>4.84</td>
<td>1.84</td>
<td>0.59</td>
<td>0.26</td>
</tr>
<tr>
<td>DIDS, 0.1 mM</td>
<td>2.11</td>
<td>1.75</td>
<td>1.08</td>
<td>0.23</td>
</tr>
<tr>
<td>NAP-taurine, 0.1 mM</td>
<td>3.79</td>
<td>1.81</td>
<td>1.01</td>
<td>0.23</td>
</tr>
<tr>
<td>DADS, 0.1 mM</td>
<td>4.85</td>
<td>1.93</td>
<td>0.99</td>
<td>0.24</td>
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<tr>
<td>Mersalyl, 25 µM</td>
<td>9.86</td>
<td>1.28</td>
<td>1.28</td>
<td>1.28</td>
</tr>
<tr>
<td>FCCP, 10 µM</td>
<td>5.21</td>
<td>0.07</td>
<td>0.78</td>
<td>0.05</td>
</tr>
</tbody>
</table>

Fig. 1. An improved procedure for the isolation of protoplasts from corn roots. 0.05% BSA and 10 mM DIFP were added in enzyme solution and osmoticum was included throughout the Ficoll step gradient.

Fig. 2. The effect of SITS (A and △) and DIDS (C and ○) on $\text{Cl}^-$ (lower panel) and $\text{SO}_4^{2-}$ (upper panel) uptake in isolated corn root protoplasts (A, △, ○) and excised root segments (△, ○, ○). SITS or DIDS was added at the time indicated by arrows to protoplasts (0.1 mM) or segments (0.5 mM). ○ and ○ are the controls.

Fig. 3. Function of SITS or DIDS concentrations on $\text{Cl}^-$ uptake in isolated corn root protoplasts and excise root segments (SITS and DIDS gave identical results). Control rate for protoplasts were 10.15 nmol/10⁶ protoplast-h and 1.82 µmol/g-h for segments.
uptake can be found in the concentration range tested, suggesting different transport systems for Pi and Cl⁻ or SO₄²⁻. The inhibition of K⁺ influx by high concentrations of DIDS or SITS (>0.2 mM) may be an indirect effect on K⁺ transport since at these concentrations Cl⁻ transport was inhibited to a maximal level, which would tend to increase the cytoplasmic pH (20, 21) and possibly affect K⁺ transport.

Excised root segments were insensitive to the specific anion transport inhibitors at low concentrations (Table I, Fig. 3), indicating that removal of cell wall is desirable in studying the effects of these compounds. The water-soluble sulfhydryl-binding mercurial, mersalyl, inhibited Pi uptake, by protoplasts more effectively than in root segments (12), presumably through its inhibition of the Pi/OH⁻ antiporter, but Cl⁻ and SO₄²⁻ transport was essentially unaffected. These results support the conclusion that the Cl⁻ and SO₄²⁻ transport system(s) is distinct from that for Pi. At present, the reason for the strong stimulatory effect of DADS on Pi uptake in the protoplast suspension is unknown.

Previous studies with corn root segments (12) and protoplasts (13) suggested that Pi was taken up through a Pi/Cl⁻ transport system. It was shown that Pi uptake decreased with increasing external pH. Figure 5 shows a similar pH dependency for Cl⁻ and SO₄²⁻ influx. The data may be consistent with an OH⁻ exchanging or H⁺ co-transporting system being involved in the Cl⁻ and SO₄²⁻ transport. In other tissues, H⁺ and OH⁻ or HCO₃⁻ have been suggested as counter-ions for Cl⁻ uptake (9, 21, 22).

An ATPase mediated electronegic Cl⁻ transport mechanism has been proposed in both animal (22) and plant (9, 18, 19, 21) tissues. All chemical modifiers which inhibited anion uptake also inhibited the KCl-stimulated plasmalemma ATPase activity at pH 6.5 (Table II) which is regarded as that ATPase component associated with active transport (7). FCCP increased ATPase activity probably through an uncoupling effect.

Microsomal (Na⁺ + K⁺)-ATPase activity in both turtle bladders and eel electric organs has been reported to be strongly inhibited by SITS (5). Since K⁺ uptake, which is generally thought to be directly coupled to the plasmalemma ATPase activity in higher plant tissues (12, 19), was not inhibited by the anion transport inhibitors (Table I), the inhibition of plasmalemma ATPase by these chemicals was unexpected. There are three possible reasons for this apparent disparity. First, the current adapted technique (7) for the isolation and determination of the plasmalemma ATPase from plant tissue may not be able to separate different ATPases (if there are any) which might be responsible for different ion transport in the plasmalemma. Consequently, the ATPase affected by anion transport inhibitors (Table II) might be directly involved in the Cl⁻ and SO₄²⁻ transport and may not be the same as that for K⁺ uptake. Secondly, the isolation of the plasmalemma may have caused a rearrangement of the membrane and exposed the ATPase to the external solution, thereby resulting in an in vitro inhibition of ATPase activity by these chemicals. A similar result was found in a membrane-impermeable Pi/OH⁻ antiporter inhibitor, mersalyl, which did not affect the K⁺ uptake but strongly inhibited plasmalemma ATPase activity (Table II). Lastly, the inhibition of ATPase activity observed here may be a secondary effect of these inhibitors which is separable from the Cl⁻ and SO₄²⁻ transport system. Further studies with the photoaffinity and radiolabeled anion transport inhibitors should provide some insight into the transport mechanism, especially the involvement of the membrane bound ATPase on anion uptake.

**CONCLUSIONS**

The present study shows that isolated protoplasts are quite sensitive to several anion transport inhibitors which have been used with red blood cells, bacteria, and fungi, and suggests the

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**Table II. Effect of Chemical Modifiers on Plasmalemma ATPase Activity in Corn Root Tissues**

Plasmalemma fraction (20–25 µg protein) was used in the assay. Reaction time was 20 min.

<table>
<thead>
<tr>
<th>ATPase Activity</th>
<th>Δ KCl</th>
</tr>
</thead>
<tbody>
<tr>
<td>µmol Pi/mg protein h⁻¹</td>
<td>µmol Pi/mg protein h⁻¹</td>
</tr>
<tr>
<td>+KCl</td>
<td>−KCl</td>
</tr>
<tr>
<td>Control</td>
<td>14.78</td>
</tr>
<tr>
<td>SITS, 0.1 mM</td>
<td>9.61</td>
</tr>
<tr>
<td>DIDS, 0.1 mM</td>
<td>6.80</td>
</tr>
<tr>
<td>NAP-taurine, 0.1 mM</td>
<td>9.31</td>
</tr>
<tr>
<td>DADS, 0.1 mM</td>
<td>11.38</td>
</tr>
<tr>
<td>Mersalyl, 25 µM</td>
<td>9.31</td>
</tr>
<tr>
<td>FCCP, 10 µM</td>
<td>19.51</td>
</tr>
</tbody>
</table>

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**Fig. 4.** Function of SITS or DIDS concentrations on Cl⁻, K⁺, and Pi uptake in isolated corn root protoplasts (SITS and DIDS gave identical results). Control rates were 10.15, 28.02, and 4.98 nmol/10⁶ protoplast·h for Cl⁻, K⁺, and Pi, respectively.

**Fig. 5.** The effect of pH on Cl⁻ (upper panel) and SO₄²⁻ (lower panel) uptake in isolated corn root protoplasts (---) and excised root segments (—).
presence of comparable transport proteins in the plant cell plasmalemma. Transport of $\text{Cl}^-$ or $\text{SO}_4^{2-}$ is indicated to be similar to anion carriers, but the phosphate carrier is distinct. All the inhibitors affect the $K^+$-stimulated ATPase of isolated plasmalemma, but the data do not permit the conclusion that anion transport is directly coupled to ATP hydrolysis. The anion transport inhibitors are only effective on root segments at higher concentrations, possibly due to binding and inactivation of the inhibitors in the cell wall.

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