The Effects of the Herbicide Sethoxydim on Transport Processes in Sensitive and Tolerant Grass Species

I. Effects on the Electrical Membrane Potential and Alanine Uptake

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Plasmalemma, Membrane ATPase, Herbicide, Sethoxydim, Amino Acid Uptake

Sethoxydim is a postemergence herbicide used to control grass weeds. Application at concentrations higher than 0.1 mm to leaf segments of the sensitive grass species Poa pratensis and Festuca ovina and the tolerant species Poa annua and Festuca rubra, caused a reduction of the electrical membrane potential (ΔE). The depolarization was reversible and depended linearly on the herbicide concentration. The passive diffusion component of ΔE was not affected by sethoxydim indicating that the herbicide did not change passive permeability characteristics of the plasmalemma. Consequently sethoxydim reduced the active component of ΔE that depended on primary active transport processes across the plasmalemma. Moreover, sethoxydim increased the reduction of ΔE of grass leaf cells that was associated with the onset of H⁺-amino acid cotransport. Simultaneously the uptake of alanine into leaf segments was reduced. From these results it had to be concluded that the plasmalemma-bound H⁺-ATPase was inhibited by sethoxydim in sensitive and tolerant grasses. In vitro ATP hydrolysis of plasmalemma vesicles isolated from grass leaves by polymer phase partitioning, however, was not inhibited by sethoxydim. Apparently another primary active transport mechanism that may contribute to an electrochemical H⁺-gradient across the plasmalemma, i.e. a plasmalemma-bound redox system, should be the site of inhibition responsible for the membrane effects of sethoxydim observed in vivo.

Introduction

Sethoxydim (2-(1-(ethoxydiamino)butyl)-5-(2-ethyl-thio-propyl)-3-hydroxy-2-cyclohexene-1-one: BAS 9052 OH; Poast®) is a selective postemergence herbicide which shows high activity against gramineous plants and low activity against dicotyledonous and other monocotyledonous plants. Thus it can be used to control grass weeds in broadleaf crops [1]. The biochemical reactions that lead to a selective phytotoxic action of sethoxydim have not been identified unequivocally. Some evidence exists that the metabolism of sethoxydim to nontoxic compounds may be faster in tolerant than in sensitive plants [2]. Sethoxydim has been found to inhibit lipid biosynthesis in sethoxydim sensitive corn seedlings and isolated chloroplasts [3] but also in sethoxydim tolerant soybean cells [4]. There is, however, some evidence that the resistance to sethoxydim treatment may at least partially result from a lower sensitivity of lipid metabolism in tolerant plants (H. K. Lichtenthaler, personal communication; see note added in proof).

Uptake and translocation of sethoxydim are not different in sensitive and tolerant plants [5, 6]. The uptake of sethoxydim apparently is a nonspecific process involving the equilibration of the lipophilic non-dissociated weak acid sethoxydim with lipid compartments of the cell which are mainly cell membranes [6]. The uptake of sethoxydim primarily occurs via the leaves. Once in the plant sethoxydim is translocated to the meristematic zones which are thought to be the site of action, and where sethoxydim leads to chlorosis, cessation of leaf elongation and finally necrosis [7, 8].

Inhibition of lipid biosynthesis by sethoxydim and the partitioning of the lipophilic herbicide into cellular lipid phases suggest that sethoxydim may directly and/or indirectly induce a perturbation of structure and function of the plasmalemma. A differential interference with transport systems that are located at the plasmalemma in sensitive and tolerant plants could lead to more or less severe consequences for the maintenance of cell metabolism, especially in

Abbreviations: ΔE, electrical membrane potential; FW, fresh weight; Hepes, (N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid); PEG, polyethyleneglycol 4000; PVP, polyvinylpyrrolidone; seth., sethoxydim; TMA, trimethyl acetic acid; VA, valeric acid.

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metabolically highly active dividing and elongating cells.

Among the gramineous plants two species within the genera of *Poa* and *Festuca* have been found to be resistant to sethoxydim, *i.e.* *Poa annua* and *Festuca rubra* [9]. We chose to compare these two grass species to sensitive ones that are closely related, *i.e.* *Poa pratensis* and *Festuca ovina*, to examine the interference of sethoxydim with transport processes at the plasmalemma of leaf cells. We measured the electrical membrane potential ($\Delta E$), the response of $\Delta E$ during $\text{H}^+$-alanine-cotransport and $[^{14}\text{C}]$alanine uptake of grass leaf segments in the absence and presence of sethoxydim. Moreover, plasmalemma vesicles of grass leaves were isolated to test the effect of sethoxydim on the $\text{H}^+$-transporting ATPase in vitro. This allowed to study major primary active transport mechanism of plant cells independently from other simultaneous effects of the herbicide on cell metabolism.

**Materials and Methods**

**Plants**

Seeds of *Poa annua* L., *Poa pratensis* L., *Festuca rubra* L., and *Festuca ovina* L. were obtained from Nungesser, Darmstadt, FRG. Plants were cultivated in soil under greenhouse conditions. They were used for experiments when 3 to 4 weeks old. At this age the leaf length was 10 to 12 cm.

**Electrophysiological measurements and $[^{14}\text{C}]$-alanine uptake experiments**

Grass leaves were cut into segments of 3 to 5 mm length. The leaf apex and base (2 to 3 cm) were discarded. Leaf slices were washed for 16 to 24 h in the dark at room temperature in the standard experimental solution $1 \times$: 1 mM KCl, 1 mM Ca(NO$_3$)$_2$, 0.25 mM MgSO$_4$, 0.95 mM Na phosphate buffer pH 5.7 [10]. Trans-plasmalemma electrical potentials were measured as described previously [11]. Leaf segments were mounted in a plexiglas chamber of a volume of 4 ml. The chamber was continuously perfused with the standard solution $1 \times$ at a rate of 10 ml/min. Glass microelectrodes were inserted into parenchyma cells of the leaf tissue through the cut surface. The electrode tip was placed supposedly into the central vacuole giving an overall $\Delta E$ between vacuole and outside. Since the $\Delta E$ of the tonoplast is close to zero the $\Delta E$ reported was assumed to represent the $\Delta E$ of the plasmalemma [12]. Measurements were performed in the dark at room temperature.

The uptake of $[^{14}\text{C}]$-alanine (Amersham Buchler, Braunschweig, FRG) was performed at 25 °C in the dark. Leaf segments were preincubated in 1× solution in the presence of sethoxydim for 20 min. Subsequently leaf slices were transferred to 1× solution with 0.1 mM or 1 mM $[^{14}\text{C}]$alanine (3.77 x 10$^9$ Bq mol$^{-1}$). Uptake was terminated after 20 min by rapidly rinsing and then washing the leaf slices with ice-cold unlabeled experimental solution. The $^{14}$C content was determined in the leaf tissue after mounting segments on planchets in 2% gelatine solution and drying at 60 °C. For determination of pH dependence of alanine uptake 10 mM Na phosphate buffer was used. Various pH values were obtained using different ratios of primary and secondary Na phosphates. For pH values below pH 4.5 citrate buffer replaced the phosphate buffer.

**Plasmalemma isolation**

Purified vesicles were isolated from grass leaves by aqueous polymer two phase partitioning as described by Kjellbom and Larsson [13] with some modifications. Approximately 20 g fresh weight of leaves were harvested and cut into 4 mm-long segments. The segments were vacuum infiltrated with a solution consisting of 500 mM sucrose, 50 mM Hepes-KOH pH 7.6, 0.6% PVP 40 (w/v), 5 mM ascorbate and 3.6 mM cysteine. The leaf tissue was homogenized using an ultra turrax for 2–4 min at maximum speed. The homogenate was filtered through 4 layers of cheese cloth and then centrifuged at 10,000×g for 10 min. The pellet was discarded. The supernatant was centrifuged at 50,000×g for 30 min. The microsomal pellet was resuspended in 6 ml 300 mM sucrose, 5 mM Hepes-KOH pH 7.6. This resuspension was added to a two phase mixture consisting of 6.2% Dextran T 500 (w/v), 6.2% PEG 4000 (w/v), 330 mM sucrose, 10 mM Hepes-KOH pH 7.6, 3 mM KCl. The final weight of the two phase system was 8.3 g. After thorough shaking the system was centrifuged at 1500×g for 3 min to allow phase separation. Following the three batch procedure described by Kjellbom and Larsson [13] an upper PEG phase was diluted with resuspension medium. After centrifugation at 100,000×g for 60 min the pellet was resuspended again in the same medium and frozen in liquid N$_2$. The samples were stored at −70 °C.
**Determination of ATPase-activity**

Phosphate released by ATP-hydrolysis was determined according to Ames [14]. The enzymatic reaction was performed at 20 °C. The reaction time was 45 min.

**Determination of protein**

Proteins were determined by the method of Sedmark and Grossberg [15] using bovine serum albumin as a standard.

**Chemicals**

Sethoxydim was obtained from the BASF-AG, Limburgerhof, FRG. Sethoxydim was dissolved in EtOH (100 mM) and diluted to final concentrations with 1 x solution. The pH was readjusted if necessary. The EtOH concentration in the experiments did not exceed 0.8%.

All results are expressed as mean values ± S.D.

**Results**

**Effect of sethoxydim on the trans-plasmalemma electrical potential**

The steady state electrical membrane potential (∆E) of leaf cells in the dark was similar in leaf slices of *P. pratensis* and *P. annua*, i.e. in both grass species it was −145 ± 19 mV (n = 43). When sethoxydim was added to the standard perfusion solution of leaf slices to give concentrations higher than 0.1 mM ∆E began to decline within a few minutes (Fig. 1). This depolarization of the plasmalemma was reversible. After removing the herbicide from the external medium the membrane first continued to depolarize for approximately 10 min. Then the depolarization ceased and ∆E began to recover. After washing the tissue with the perfusion solution for 30 to 40 min the level of ∆E had reached a value similar to that recorded before addition of sethoxydim.

The degree of the reduction of ∆E was linearly related to the herbicide concentration in the range of 0.1 mM to 1 mM sethoxydim (Fig. 2). Fig. 2 shows the maximum depolarization that was recorded 10 min after removing and 30 min after adding sethoxydim to the perfusion solution (cf. Fig. 1) in relation to the herbicide concentration. The slopes of the two curves were 23 mV/mM and 43 mV/mM for *P. pratensis* and *P. annua*, respectively. This indicates a stronger response in the sethoxydim insensitive *P. annua* to the herbicide treatment than in the sensitive *P. pratensis*.

Sethoxydim is a weak acid with a pK of 4.62 [2]. Weak acids can affect ∆E of cell membranes by preferential permeation in the electrically neutral protonated form, dissoziation within the cell releasing protons that acidify the cytoplasm and may therefore stimulate the H⁺-transporting ATPase at the plas-
malemma [16–18]. Therefore the effects of two other weak acids, *i.e.* trimethyl acetic acid (TMA; \(pK = 5.03\)) and valeric acid (\(pK = 4.82\)), with \(pK\) values similar to the one of sethoxydim on \(\Delta E\) of *Poa* and *Festuca* leaf cells were tested. In contrast to the depolarization of the cell membrane caused by sethoxydim the treatment of leaf slices with TMA or valeric acid produced a permanent hyperpolarization (Fig. 3).

To examine if sethoxydim reduced \(\Delta E\) by affecting the energy dependent component or the passive diffusion component, the energy dependent component of \(\Delta E\) was abolished in the dark by removing the oxygen from the external medium and consequently suppressing respiration. After oxygen was excluded from the medium by bubbling the solution for at least 30 min with nitrogen, \(\Delta E\) of *Poa* leaf cells dropped to the level of \(-77 \pm 12\) mV (\(n = 13\)) (Fig. 4). The application of 0.8 mM sethoxydim did not further depolarize the membrane. After removing sethoxydim from the perfusion solution and again applying oxygen to the leaf cells the membrane repolarized to the level of \(\Delta E\) measured under standard conditions.

**Effect of sethoxydim on \(H^+\)-alanine-cotransport**

The effect of sethoxydim on \(\Delta E\) of *Poa* leaf cells indicated an action of the herbicide on the energy dependent component of \(\Delta E\). To examine this effect in more detail, we chose the \(H^+\)-amino acid cotransport mechanism as a test system to study the influence of sethoxydim on the \(H^+\)-transporting ATPase at the plasmalemma.

At the onset of the \(H^+\)-alanine-cotransport into *Poa* and *Festuca* leaf cells the plasmalemma was depolarized immediately. Fig. 5 gives an example of a typical trace of a membrane potential measurement during \(H^+\)-alanine-cotransport into leaf cells of *F. rubra*. The depolarization at the onset of alanine uptake and the repolarization at the cessation of the uptake process could be repeated several times with one cell. This depolarization of the plasmalemma originated in the increased influx of \(H^+\) during the uptake of the amino acid. To compensate the increased \(H^+\)-influx the \(H^+\)-ATPase at the plasmalemma subsequently is activated. The maximum reduction of the membrane potential is determined by the uptake rate and the rate of activation of the \(H^+\)-ATPase [19]. After preincubation of leaf slices with 0.8 mM sethoxydim for 20 min the immediate alanine induced depolarization of the membrane was higher than in the control. The increase of the depolarization of the membrane at the onset of alanine transport after sethoxydim application occurred both in herbicide sensitive and insensitive grass species (Table I). Corresponding to the effect of sethoxydim on \(\Delta E\) the reaction of the \(H^+\)-amino acid cotransport system of leaf cells to the herbicide treatment was again higher in the herbicide insensitive species *P. annua* and *F. rubra* than in the sensitive species *P. pratensis* and *F. ovina*. The effect was reversible. After washing the leaf slices with the standard solution 1 \(\times\) for at...
Fig. 5. Time course of $\Delta E$ of a leaf cell of Festuca rubra at the onset and termination of $H^+$-alanine-cotransport before and after sethoxydim (0.8 mM) was applied to the standard medium. Duration of the sethoxydim treatment: 20 min. Alanine concentration: 1 mM. Solid arrowheads indicate application, open arrowheads removal of alanine or sethoxydim as indicated. Numbers on the curve indicate $\Delta E$ (mV).

Table I. Effect of sethoxydim (0.8 mM) on the alanine-induced depolarization of the plasmalemma of leaf cells of tolerant and sensitive Poa and Festuca species, expressed in % of the control.

<table>
<thead>
<tr>
<th>Species</th>
<th>a) Treatment with sethoxydim for 20 min</th>
<th>b) Treatment with sethoxydim for 20 min followed by washing with 1 x solution for 30 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>Poa pratensis</td>
<td>111</td>
<td>76</td>
</tr>
<tr>
<td>Poa annua</td>
<td>180</td>
<td>102</td>
</tr>
<tr>
<td>Festuca ovina</td>
<td>117</td>
<td>83</td>
</tr>
<tr>
<td>Festuca rubra</td>
<td>164</td>
<td>99</td>
</tr>
</tbody>
</table>

least 30 min the rate of the alanine induced depolarization was similar to the rate observed before sethoxydim was applied (Table I).

**Effect of sethoxydim on the uptake of $[^{14}C]$alanine**

The uptake of $[^{14}C]$alanine into leaf slices of *P. pratensis* and *P. annua* was measured after exposing the leaf tissue to various concentrations of sethoxydim for 20 min in the dark. At herbicide concentrations higher than 0.5 mM the uptake of alanine into leaves of *P. pratensis* and *P. annua* was inhibited (Fig. 6). Again, the inhibition of the amino acid uptake was higher in the sethoxydim insensitive grass species than in the sensitive species. The reduction of the uptake rate was higher in both species at 0.1 mM alanine than at 1 mM alanine. At 0.8 mM sethoxydim the uptake of alanine was reduced to 52% and 71% of the controls at 0.1 mM and 1 mM alanine in *P. pratensis*, and to 35% and 55% of the controls at 0.1 mM and 1 mM alanine in *P. annua*, respectively.

The uptake of alanine into *Poa* leaf cells had a pH optimum near 4.7 (Fig. 7). The relative reduction of the alanine uptake rate by sethoxydim was independent of the pH of the medium. The inhibition of the alanine uptake into leaf slices of *P. pratensis* and *P. annua* by 0.5 mM sethoxydim was 41 ±9% ($n = 27$) and 47 ±7% ($n = 27$), respectively. The sethoxydim effect on the uptake of alanine was compared with the effect of the weak acids butyric acid (pK = 4.8) and TMA (pK = 5.0) (Fig. 8). While amino acid uptake was inhibited by sethoxydim, the...
Fig. 7. pH profile of alanine uptake into leaf cells of *Poa pratensis* and *Poa annua* in the presence (+ seth.) and absence (control) of sethoxydim (0.8 mM). Leaf segments were treated with sethoxydim for 20 min. Alanine uptake was measured for 30 min. Alanine concentration: 1 mM, number of replicates \( n = 3 \).

preincubation of the leaf tissue of *P. annua* with butyric acid or TMA highly stimulated the alanine uptake.

**Effect of sethoxydim on the ATPase activity of isolated plasmalemma**

To determine the contribution of contaminating cellular membranes to the plasmalemma prepara-

...tions of leaves of *P. pratensis*, *P. annua*, *F. ovina*, and *F. rubra* ATP hydrolysis was measured at pH 6.5 in the presence of vanadate, a specific inhibitor of plasmalemma ATPase and at pH 8.0 in the presence of \( \text{NO}_3^- \) and \( \text{N}_3^- \), inhibitors of the tonoplast ATPase and mitochondrial ATPase, respectively [20–22]. Fig. 9 shows that the plasmalemma preparations of the four grass species were highly purified. The membrane fractions showed only a low sensitivity against \( \text{NO}_3^- \) and \( \text{N}_3^- \) whereas vanadate reduced the ATP hydrolysis in all preparations by more than 70%.

The application of sethoxydim in concentrations between 0.05 mM and 1 mM had no significant effect on the ATPase activity of the isolated plasmalemma of *Poa* and *Festuca* leaves (Fig. 10).

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**Fig. 8.** Effect of sethoxydim, butyric acid and trimethylacetic acid (TMA) on the uptake of alanine into leaf cells of *Poa annua*. Leaf segments were preincubated in 0.5 mM and 0.8 mM solutions of the respective substances for 20 min. Then alanine uptake was measured for 30 min. Experiments were performed at pH 5.7, number of replicates \( n = 3 \).

**Fig. 9.** Characterization of the ATPase activities of plasmalemma vesicles isolated from leaves of *Poa pratensis*, *Poa annua*, *Festuca ovina* and *Festuca rubra*. ATP hydrolysis was determined in the presence of vanadate (\( \text{VO}_4^- \) 0.1 mM) at pH 6.5 and in the presence of \( \text{N}_3^- \) (0.07 mM) and \( \text{NO}_3^- \) (0.1 mM) at pH 8.0. Assays contained 0.01% triton. The ATPase activities are expressed in % of the respective controls without inhibitors. Specific activities at pH 6.5 (pH 8.0) were: *P. pratensis* 61.9 (11.7) \( \mu \text{mol P}_i \ h^{-1} \ mg^{-1} \) protein, *P. annua* 52.2 (10.9) \( \mu \text{mol P}_i \ h^{-1} \ mg^{-1} \) protein, *F. ovina* 47.9 (11.1) \( \mu \text{mol P}_i \ h^{-1} \ mg^{-1} \) protein, *F. rubra* 56.2 (10.7) \( \mu \text{mol P}_i \ h^{-1} \ mg^{-1} \) protein.
Fig. 10. Effect of sethoxydim on the ATPase activity of plasmalemma vesicles isolated from leaves of *Poa pratensis* (○), *Poa annua* (●), *Festuca ovina* (□) and *Festuca rubra* (■). ATP hydrolysis was determined in the presence of 0.01% triton. All assays including controls contained 0.75% EtOH. The enzyme activities are expressed in % of the control. For specific activities see legend to Fig. 9. ATPase assays were performed at pH 6.5.

**Discussion**

The application of sethoxydim to leaf segments of sensitive and tolerant grass species at concentrations higher than 0.1 mM produced a reversible depolarization of the cell membrane. This depended linearly on the herbicide concentration (Fig. 1 and 2). Sethoxydim did not affect passive diffusion processes across the plasmalemma since the passive diffusion component of \( \Delta E \) remained unchanged in the presence of sethoxydim (Fig. 4). Consequently, sethoxydim dissipated the active component of \( \Delta E \), *i.e.* the component of \( \Delta E \) that was established by primary active transport mechanisms. When we analyzed the plasmalemma depolarization that was associated with \( H^+ \)-alanine-cotransport into grass leaf cells, we found that sethoxydim treatment led to an increase of the reduction of \( \Delta E \) which occurred at the onset of alanine uptake. This effect could be reversed by washing the leaf tissue with the standard medium (Fig. 5, Table I). The increase of the \( H^+ \)-amino acid induced depolarization theoretically could be the result of either a higher \( H^+ \)-alanine uptake rate after sethoxydim treatment or an inhibition of the plasmalemma \( H^+ \)-ATPase. The ATPase is believed to be activated after the onset of \( H^+ \)-solute cotransport and runs to compensate the higher \( H^+ \)-influx during uptake of substrates. The alanine uptake rates determined directly in sensitive and tolerant *Poa* leaves after preincubation with sethoxydim showed a severe inhibition of alanine transport. Therefore, the increase of the \( H^+ \)-amino acid induced depolarization seems to indicate a sethoxydim effect on the ATPase.

The comparative studies of the effects of the weak acids TMA, butyric acid, and valeric acid on \( \Delta E \) and alanine transport revealed that the depolarization of the cell membrane and the inhibition of alanine uptake were not related to the weak acid character of the sethoxydim molecule. Only occasionally the depolarization of the plasmalemma by sethoxydim was preceeded by a transient hyperpolarization (Fig. 5). Possibly this hyperpolarizing transient of \( \Delta E \) was the result of the dissociation of sethoxydim within the cell whereas the subsequent depolarization resulted from a more complex interaction of the herbicide with plasmalemma associated transport processes.

It is unlikely that the rapid, reversible dissipation of the active component of \( \Delta E \) and the retardation of the plasmalemma \( H^+ \)-ATPase during the uptake of alanine by sethoxydim was caused by an inhibition of photosynthetic or respiratory ATP synthesis since sethoxydim has not been found to be an inhibitor of photosynthesis or respiration for several days after application [4, 23–25]. We can further exclude that the effect of sethoxydim on \( \Delta E \) and amino acid transport was caused indirectly by an inhibition of lipid metabolism changing the lipid composition of the plasmalemma since the effects occurred without any significant lag phase.

Thus, from the present results we had to conclude that sethoxydim interfered directly with active membrane transport mechanisms and that the \( H^+ \)-ATPase was one major target for its phytotoxicity. When sethoxydim was applied to plasmalemma vesicles isolated from sensitive and tolerant grass leaves, however, it did not produce an inhibition of the ATPase activity in vitro (Fig. 10).

This intriguing observation initiated the idea to look for the interference of sethoxydim with another transport mechanism possibly contributing to the generation of an electrochemical \( H^+ \)-gradient across the plasmalemma. Recently, evidence has been presented in support of an energization by a plasmalemma-bound electron transport system [26]. It is still
controversial whether the transport of electrons across the plasmalemma by this redox system is directly or indirectly associated with the transport of $\text{H}^+$ and in which way the system may be coupled to the plasmalemma $\text{H}^+\text{-ATPase}$ [27—29]. If sethoxydim indeed were interacting with a plasmalemma-bound redox system establishing in part an electrochemical $\text{H}^+\text{-gradient}$ and possibly regulating the $\text{H}^+\text{-ATPase}$, this could cause the lesions observed in our experiments. The effect of sethoxydim on the electron transport system of the plasmalemma of leaf cells of sensitive and tolerant grasses will be the objective of our succeeding publication. Presently we can not explain why the sethoxydim-tolerant grass species $P. \text{annua}$ and $F. \text{rubra}$ more strongly responded to the herbicide treatment than the sensitive species $P. \text{pratensis}$ and $F. \text{ovina}$.

From the results obtained so far, however, we can not deduce that the interaction of sethoxydim with the electrical membrane potential of leaf cells and the uptake of solutes is associated with the specific action of sethoxydim in sensitive and tolerant plants.

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