Inhibition of Adenosine Triphosphatase Activity from a Plasma Membrane Fraction of *Acer pseudoplatanus* Cells by 2,2,2-Trichloroethyl 3,4-Dichlorocarbanilate\(^1,2\)

Received for publication September 3, 1985 and in revised form November 20, 1985

JEAN-PIERRE BLEIN*, XAVIER DE CHERADE, MICHEL BERGON, JEAN-PIERRE CALMON, AND RENÉ SCALLA

Laboratoire des Herbicides et Autres Produits Phytosanitaires, I.N.R.A., B.V 1540, 21034 Dijon Cedex (France) (J.P.B., R.S.) and Laboratoire de Chimie Organique et Biologique, E.N.S.A., 145 av de Muret, Toulouse Cedex (France) (X.d.C., M.B., J.P.C.)

ABSTRACT

2,2,2-Trichloroethyl 3,4-dichlorocarbanilate (SW26) is toxic for *Acer pseudoplatanus* cell cultures. It inhibited the cellular proton extrusion and depolarized the plasmalemma. In *vitro*, it inhibited the plasma membrane ATPase. SW 26 was also inhibitory to membrane ATPases of other origins—plant (maize shoot), fungus (*Schizosaccharomyces pombe*), and animal (dog kidney)—with about the same efficiency (7.5 micromolar < \( I_0 < \) 22 micromolar). It did not inhibit the oligomycin-sensitive ATPase from purified plant mitochondria, nor molybdate-sensitive soluble phosphatases. SW26 was more specific for plasma membrane ATPases than diethylstilbestrol or vanadate. A Lineweaver-Burk plot analysis showed that inhibition kinetics were purely noncompetitive (\( K_I = 14.7 \) micromolar) below 20 micromolar. Above this concentration, the inhibition pattern was not consistent with Michaelis-Menten kinetics, and a Hill plot representation revealed a positive cooperativity.

to accumulate protons at the expense of ATP, and possessing the functional characteristics generally reported for plant plasma membrane ATPases (11, 20, 24).

In the present paper we study some properties of 2,2,2-trichloroethyl 3,4-dichlorocarbanilate. This compound is a derivative of the herbicide SWEP (3,4-dichlorocarbanilate), which can be classified in the anilide or carbamate families, and is a weak photosynthesis inhibitor (13). We have found that SW26\(^3\) is toxic for nonphotosynthetetic sycamore cell cultures; it inhibited the extrusion of protons from the cells and depolarized their plasma membrane. Furthermore, this compound was able to inhibit the ATPase activity of a microsomal fraction enriched in plasma membrane.

MATERIALS AND METHODS

**In Vitro Determinations.** Determination of cell growth, pH of the extracellular medium, transmembrane electric potential difference (calculated from the distribution of tetraphenyl phosphonium bromide) and rubidium uptake, have been previously described (5).

**In Vitro Determinations.** Preparation of the sycamore microsomal fraction has already been reported (6). Soluble phosphatases were assayed in the 80,000g supernatant, after sedimentation of the microsomal fraction. *S. pombe* ATPase was a gift of Prof. A. Goffeau, and dog kidney ATPase was purchased from Sigma (grade IV, ouabain sensitive). Submitochondrial particles were prepared from purified *Acer pseudoplatanus* mitochondria isolated on a Percoll gradient according to Gauvrit (15). After sonication, the preparation was centrifuged (4,000g, 10 min) to eliminate intact mitochondria. SMP were then pelleted (13,000g, 30 min), resuspended in 10 mM Tris/Mes (pH 7.3), 1 mM EDTA, 1 mM ATP, 20% glycerol, and finally stored at \(-80^\circ\)C.

**Determination of ATPase Activity.** Assays were carried out at 38°C in 1 ml of 60 mM Tris/Mes (pH 6.5 for plasma membrane ATPase and soluble phosphatases, and pH 9.0 for SMP ATPase assay), 50 mM KCl, 3 mM MgSO\(_4\), and 3 mM ATP (Tris salt). Each assay contained 20 to 40 μg membrane protein. The reaction was stopped by the addition of 3 ml of 7% SDS (10). Pi was measured as described by Pullman and Peneffsky (26). For inhibition studies, oligomycin, DES and other chemicals were dissolved in DMSO and appropriate controls were run. ATPase activities were linear for at least 30 min in our assay conditions. Substrate blanks were subtracted to calculate all enzyme activities.

---

\(^1\) Supported by the Institut National de la Recherche Agronomique (Ministère de l'Agriculture and Ministère de la Recherche et de la Technologie); The Institut National Polytechnique de Toulouse (Ministère de l'Education Nationale); and the Société Nationale des Poudres et Explosifs.

\(^2\) All requests about 2,2,2-trichloroethyl 3,4-dichlorocarbanilate should be sent to Dr. Blein or Dr. Scalla.

\(^3\) DES, diethylstilbestrol; SW26, 2,2,2-trichloroethyl 3,4-dichlorocarbanilate; SMP, submitochondrial particles; \( I_0 \), 50% inhibition.
INHIBITION OF ATPase ACTIVITY BY SW26

Table I. Action of Inhibitors on Sycamore Cell Plasma Membrane ATPase

<table>
<thead>
<tr>
<th>Inhibitor (100 μM)</th>
<th>Plasma mem. Subunit</th>
<th>Soluble</th>
<th>Control ATP Hydrolysis Activity μmol P1 released min⁻¹ mg⁻¹ protein</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ATPase</td>
<td>ATPase</td>
<td>% control</td>
</tr>
<tr>
<td>NaN₃</td>
<td>99</td>
<td>60</td>
<td>97</td>
</tr>
<tr>
<td>Oligomycin (5 μg/ml)</td>
<td>101</td>
<td>10</td>
<td>104</td>
</tr>
<tr>
<td>Molybdate</td>
<td>98</td>
<td>101</td>
<td>20</td>
</tr>
<tr>
<td>DES</td>
<td>37</td>
<td>50</td>
<td>99</td>
</tr>
<tr>
<td>Vanadate</td>
<td>6</td>
<td>85</td>
<td>35</td>
</tr>
<tr>
<td>SW26</td>
<td>8</td>
<td>103</td>
<td>102</td>
</tr>
</tbody>
</table>

Table II. Effect of SW26 on Plasma Membrane ATPases from Different Origins

Experimental conditions were the same as in Table I, except with dog kidney ATPase assay for which the monovalent cations used were 20 mm KCl, 100 mm NaCl.

<table>
<thead>
<tr>
<th>Inhibitor (100 μM)</th>
<th>Sycamore Cells</th>
<th>Maize Hypocotyl</th>
<th>Yeast</th>
<th>Dog Kidney</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control ATP Hydrolysis Activity μmol P1 released min⁻¹ mg⁻¹ protein</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NaN₃</td>
<td>0.76</td>
<td>0.70</td>
<td>5.50</td>
<td>1.15</td>
</tr>
<tr>
<td>Oligomycin (Na)</td>
<td></td>
<td>4.30</td>
<td>22.00</td>
<td>3.00</td>
</tr>
<tr>
<td>DES</td>
<td>82.00</td>
<td>65.00</td>
<td>25.00</td>
<td>14.00</td>
</tr>
<tr>
<td>Ouabain</td>
<td>NE*</td>
<td>NE</td>
<td>NE</td>
<td>NE</td>
</tr>
<tr>
<td>SW26</td>
<td>14.50</td>
<td>8.50</td>
<td>22.00</td>
<td>7.50</td>
</tr>
</tbody>
</table>

* NE, no effect.

RESULTS

At a concentration of 100 μM, SW26 was lethal for sycamore cells; at lower concentration, it inhibited the growth of the cultures, with an IC₅₀ of 14.5 μM (Fig. 1A). SW26 inhibited the acidification of culture medium induced by fusicoccin, known to stimulate the extrusion of protons (22) (Fig. 1B). In addition, SW26 was able to collapse the trans-plasma membrane electric potential difference with an IC₅₀ of 30 μM (Fig. 1C), and also to inhibit the uptake of rubidium after a lag (Fig. 1D). These observations suggest that the plasma membrane ATPase could be a target for SW26.

To test this possibility, a microsomal fraction was prepared from sycamore cells according to a previously published method (6). The ATPase activity of this fraction possessed the characteristics generally reported for plant plasma membrane ATPases (28), and in particular was sensitive to DES and vanadate, but insensitive to molybdate, azide and oligomycin (Table I). As expected, plasma membrane ATPase was strongly inhibited by SW26 (Table I). By contrast, the mitochondrial ATPase present in submitochondrial preparation, and characterized by its sensitivity toward azide and oligomycin, was unaffected by SW26 (Table I). Moreover, SW26 was inactive on soluble phosphatases, which were inhibited by molybdate and vanadate (Table I).

SW26 was also a potent inhibitor of maize plasma membrane ATPase (Table II). This inhibition was not restricted to higher plants; the corresponding enzyme from yeast (Schizosaccharomyces pombe), which possesses the same characteristics as sycamore and maize enzymes (6, 8, 17), as well as the dog kidney ATPase, characterized by its sensitivity to ouabain, were also inhibited by SW26 (Table II). Except for maize hypocotyl
Fig. 2. Effect of SW26 concentration on ATPase activity of the plasma membrane fraction of sycamore cells: Lineweaver-Burk and Dixon plots of data. The assay mixtures contained 60 mM Tris/Mes, pH 6.5, 3 mM MgSO₄, 50 mM KCl, variable ATP concentrations, and 20 to 40 μg protein. Kinetic constants were calculated by linear regression analysis. Vₐ, ATPase activity (μmol Pi released min⁻¹ mg⁻¹ protein). A, (a) Control; (b) SW26 (5 μM); and (c) SW26 (10 μM). Km was 0.51 ± 0.04 mM. B, Plot of 1/V versus SW26 concentration. ATP concentrations were respectively: (a) 0.03 mM, (b) 0.45 mM, (c) 0.75 mM, (d) 1.125 mM, and (e) 3.0 mM. Kᵢ = 14.7 ± 2.6 μM.

Fig. 3. Hill plot representation of the inhibitory effect of SW26. The assay mixtures contained 60 mM Tris/Mes at pH 6.5, 3 mM MgSO₄, 50 mM KCl, and variable ATP and SW26 concentrations. A, ATP 0.3 mM; V₀, control reaction rate; Vₐ, reaction rate in the presence of SW26. B, Dependence of ATP concentrations, of the slope of Hill plot for SW26 < 20.8 μM (O) or > 20.8 μM (●).

Fig. 4. Effect of SW26 on H⁺ translocating activity of inside-out sealed vesicles of the plasma membrane fraction of Acer cells. The assay mixture contained 60 mM Tris/Mes at pH 6.5, 3 mM MgSO₄, 50 mM KCl, 3 mM ATP, 7.5 μM acridine orange and about 100 μg protein. NH₄Cl (40 mM) and SW26 (25 μM) were added as indicated by arrows.

ATPase, inhibition by SW26 ranked between vanadate and DES (Table II).

Inhibition of sycamore plasma membrane ATPase showed simple Michaelis-Menten kinetics for Mg-ATP hydrolysis, with inhibitor concentrations not exceeding 20 μM. Linear regression analysis of Lineweaver-Burk plots (Fig. 2), indicated pure non-competitive inhibition kinetics; only the maximum velocity was affected while affinity remained constant (Kᵢ = 0.51 ± 0.04 mM). In these conditions, the Kᵢ for ATP was 14.7 ± 2.6 μM. Moreover, as expected for noncompetitive inhibition, Iₕ₀ concentration was equal to Kᵢ and was identical for all substrate concentrations (Iₕ₀ = 14.5 ± 1.7 μM), as deduced from data from Figure 2.

Above 20 μM, inhibition exerted by SW26 no longer followed Michaelis-Menten kinetics. Figure 3A represents a typical Hill plot showing a break for log(I) = 1.32 (I = 21.11 μM). The average of the data from 6 experiments using different ATP concentrations, indicated a break for a mean SW26 concentration of 20.83 ± 1.92 μM. Below this concentration, the slope was equal to 1.15 ± 0.07, in agreement with the purely noncompetitive kinetics already demonstrated (Fig. 3B, open circles). Above an inhibitor concentration of 20 μM, a positive cooperativity was indicated by an increase of the slope, well above unity (Fig. 3B, closed circles).

Besides its effect on the phosphohydrolase activity, the action of SW26 on the transport of protons into vesicles in the microsomal fraction was also examined. Figure 4 shows that sycamore cell microsomal preparations were able to accumulate protons in the interior of closed vesicles, as revealed by fluorescence quenching of acridine orange. Addition of SW26 after reaching the steady state equilibrium partially reversed the quenching until a new stable level was established. Half reversion was observed for a concentration of SW26 of 2 μM (Fig. 5).
DISCUSSION

The above results show that SW26 is lethal for sycamore cells. This compound inhibited proton pumping activity of the cells, reduced the electric potential gradient across cell membranes, and inhibited the uptake of rubidium. About the latter effect, it must be kept in mind that we have found a similar lag with DES, a well-known inhibitor of plasma membrane ATPase (5). It thus appears that, in that respect, sycamore cells do not behave in the same way as oat roots, in which rubidium absorption is inhibited by DES within 3 min (1). However, taken as a whole, all the above effects could be explained by an inhibition of plasma membrane H*-ATPase.

Indeed, SW26 was a potent inhibitor of plasma membrane ATPase activity in the microsomal fraction, and its effects can be demonstrated both for the phosphorylase and the proton transport activities in the microsomal fraction. The inhibiting power of SW26 is of the same order as that of vanadate, and is generally higher than DES. About the inhibition of the proton-pumping activity, Figure 4 shows that fluorescence quench reversal was quasi-instantaneous, and the rapidity of this effect suggests that some uncoupling. However, at the concentrations used, SW26 did not uncouple oxidative phosphorylation in purified sycamore cell and potato tuber mitochondria (C Gauvrit, JP Blein, unpublished data). At this time, it can be only concluded that, in addition to its effect on the phosphorylase activity, SW26 apparently changes ionic equilibria in our vesicle preparations.

SW26 appeared specific for plasma membrane ATPase, since it was inactive on mitochondrial ATPase, or on nonspecific phosphatases. It is thus more specific than DES, which inhibited the mitochondrial ATPase, and vanadate, which inhibited nonspecific phosphatases (see also ref. 14). Moreover, the inhibiting properties of SW26 extend to fungal or animal enzymes, and consequently this compound appears as a general inhibitor of plasma membrane ATPases. In the future, it will be very interesting to examine its effect on the tonoplast ATPase to evaluate its degree of specificity.

Our kinetic studies showed that, at concentrations not exceeding 20 µM, SW26 is a noncompetitive inhibitor of sycamore cell plasma membrane ATPase. This same mode of inhibition was also demonstrated for vanadate on corn root ATPase (14), and for DES on oat root ATPase (2). Above 20 µM, however, double reciprocal plots were no longer linear, and Hill plots revealed a positive cooperativity. In that respect, SW26 can be compared to DES: on the basis of Dixon plot kinetics, Balke and Hodges (2) have suggested that this inhibitor has two or more binding sites on the enzyme. Whether or not that is true for SW26 remains to be examined.

Acknowledgments—The authors wish to express their gratitude to Mrs. M. R. Allard for her valuable technical assistance, Professor A. Goffta for the gift of S. pombe ATPase, and Professor J. Guern for helpful discussions.

LITERATURE CITED

7. BUCHHOLZ DL, TL LAVY 1979 Alachlor and trifluralin effects on nutrient uptake in oats and soybeans. Agron J 71: 24-26
10. DULLEY J 1975 Determination of inorganic phosphate in the presence of detergents or protein, Anal Biochem 67: 91-96
17. GOPPEAU A, CW SLAYMAN The proton-translocating ATPase of the fungal plasma membrane. Biochim Biophys Acta 639: 197-223
30. ZIPPEL K, M MADER, M BOPP 1977 The effect of some herbicides on ion accumulation in tissue culture of Anagallis arvensis. Angew Bot 51: 77-87