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Inhibitors of the carrier-mediated influx of auxin in suspension-cultured tobacco cells

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Abstract. Active auxin transport in plant cells is catalyzed by two carriers working in opposite directions at the plasma membrane, the influx and efflux carriers. A role for the efflux carrier in polar auxin transport (PAT) in plants has been shown from studies using phytotropins. Phytotropins have been invaluable in demonstrating that PAT is essential to ensure polarized and coordinated growth and to provide plants with the capacity to respond to environmental stimuli. However, the function of the influx carrier at the whole-plant level is unknown. Our work aims to identify new auxin-transport inhibitors which could be employed to investigate its function. Thirty-five aryl and aryloxyalkylcarboxylic acids were assayed for their ability to perturb the accumulation of 2,4-dichlorophenoxyacetic acid (2,4-D) and naphthalene-1-acetic acid (1-NAA) in suspension-cultured tobacco (*Nicotiana tabacum* L.) cells. As 2,4-D and 1-NAA are preferentially transported by the influx and efflux carriers, respectively, accumulation experiments utilizing synthetic auxins provide independant information on the activities of both carriers. The majority (60%) of compounds half-inhibited the carrier-mediated influx of [^{14}C]2,4-D at concentrations of less than 10 μM. Most failed to interfere with [^{3}H]NAA efflux, at least in the short term. Even though they increasingly perturbed auxin efflux when given a prolonged treatment, several compounds were much better at discriminating between influx and efflux carrier activities than naphthalene-2-acetic acid which is commonly employed to investigate influx-carrier properties. Structure-activity relationships and factors influencing ligand specificity with regard to auxin carriers are discussed.

Key words: Auxin – Carrier (auxin) – Membrane transport – *Nicotiana* (cultured cells) – Structure-activity – Transport inhibitor (auxin)

Introduction

Auxin exchanges at the plasma membrane (PM) of plant cells via a complex pathway which associates membrane diffusion with two carriers acting in opposite directions (Lomax et al. 1995). The influx carrier catalyzes auxin entry from the surrounding medium and the efflux carrier excretes hormone to the surrounding medium. It has been proposed that the *AUX1* gene of *Arabidopsis thaliana*, which presents homologies with plant and fungal genes coding for amino acid permeases, encodes a carrier protein mediating auxin influx in root cells (Bennett et al. 1996). Mutations within the *AUX1* gene confer an auxin-insensitive root growth phenotype and abolish root gravitropic curvature. The altered root growth response is specific to auxins requiring carrier-mediated uptake, indole-3-acetic acid (IAA) and 2,4-dichlorophenoxyacetic acid (2,4-D; Yamamoto and Yamamoto 1998; Marchant et al. 1999), whereas the normal gravitropic phenotype can be restored by growing mutant seedlings in the presence of the membrane-permeable auxin naphthalene-1-acetic acid (1-NAA; Marchant et al. 1999). Genes encoding proteins with similarities to bacterial membrane transporters have been recently isolated in *Arabidopsis thaliana* (Chen et al. 1998; Gälweiler et al. 1998; Luschnig et al. 1998; Müller et al. 1998; Utsuno et al. 1998). The proteins might support directional auxin efflux in auxin-transport-competent cells, as suggested by genetic and physiological analyses, and the demonstration that members of the gene family, *AtPIN1* and *AtPIN2*, code for proteins with asymmetric cellular distribution within the elongation zones of root and shoot tissues. The function of the influx carrier has not been elucidated at the whole-plant level, whereas that of the efflux carrier is

Abbreviations: 2,4-D = 2,4-dichlorophenoxyacetic acid; 1-NAA = naphthalene-1-acetic acid; 2-NAA = naphthalene-2-acetic acid; PAT = polar auxin transport; PM = plasma membrane

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better documented. Several lines of evidence indicate that the efflux carrier mediates polar auxin transport (PAT) in plants, from the shoot apices and young leaves in which the hormone is synthesized, to the subapical tissues in which the hormone exerts its developmental effects (Lomax et al. 1995). Polar auxin transport is implicated in the establishment of morphological polarity and bilateral symmetry in developing monocot and dicot embryos (Cooke et al. 1993; Fischer and Neuhaus 1996; Fischer et al. 1997), the control of stem elongation and rhizogenesis (Davies 1995; Lomax et al. 1995), and the formation of inflorescence and floral meristems in *Arabidopsis thaliana* (Okada et al. 1991; Bennett et al. 1995; Przemeck et al. 1996; Ruegger et al. 1997). Polar auxin transport also appears essential for the transduction of the gravity signal since PAT inhibitors completely abolish the gravitropic response in shoots and roots (Lomax et al. 1995).

Most of our knowledge regarding the function of the efflux carrier and the important role of PAT in plant development and morphogenesis results from studies utilizing synthetic inhibitors, phytotropins, which block both the carrier-mediated efflux of auxin in tissue fragments, cultured cells, membrane vesicles and its polar transport in intact plants (Rubery 1990; Lomax et al. 1995). Phytotropins, such as naphthylphthalamic acid (NPA), are thought to inhibit auxin efflux through binding to a PM-associated protein, presumably distinct from the auxin-binding site at the catalytic unit of the efflux carrier (Rubery 1990; Morris et al. 1991; Lomax et al. 1995). A possible protein kinase function has been proposed for that protein because the promotive effect of NPA on the net uptake of IAA in zucchini hypocotyl segments was decreased by specific tyrosine kinase inhibitors (Bernasconi 1996). Naturally occurring flavonoids, such as quercetin, might be endogenous PAT regulators in plants since, at micromolar concentrations spanning physiological levels, they are able to inhibit auxin efflux in tissue fragments and to displace NPA from its binding protein in membrane fractions (Jacobs and Rubery 1988; Rubery 1990).

So far, the same pharmacological approach, as used to elucidate the function of the auxin efflux carrier, has not been employed to explore the role of the influx carrier at the whole-plant level. Specific inhibitors of auxin influx are missing, albeit naphthalene-2-acetic acid (2-NAA) has been employed to show the presence of influx carriers in tissue fragments (Sussman and Goldsmith 1981) and isolated membrane vesicles (Jacobs and Hertel 1978; Hertel et al. 1983; Benning 1986). However, 2-NAA also perturbs auxin efflux (Delbarre et al. 1996) and apparently displays weak auxin activity (Katekar 1979).

Our work aims to identify new auxin-transport inhibitors which could be employed to investigate the physiological function and properties of the influx carrier. We have shown that the synthetic auxins 2,4-D and 1-NAA differ by the mechanisms driving their membrane transport (Delbarre et al. 1996). Slowly diffusing 2,4-D is mostly accumulated via the influx carrier and not significantly excreted by the efflux carrier whereas the membrane-permeable auxin 1-NAA is vigorously excreted by the efflux carrier. Hence, the activities of the auxin carriers can be assayed separately by monitoring the net uptake of tracer concentrations of labeled 2,4-D and 1-NAA. Ligands interfering with the influx carrier decrease the accumulation of 2,4-D whereas those acting on the efflux carrier block the egress and consequently increase the accumulation of 1-NAA. Thirty-five compounds, mainly aryl and aryloxyalkylcarboxylic acids (see Fig. 1) selected for structural relationship with an already known substrate (2,4-D) or inhibitor (2-NAA) of the influx carrier, were

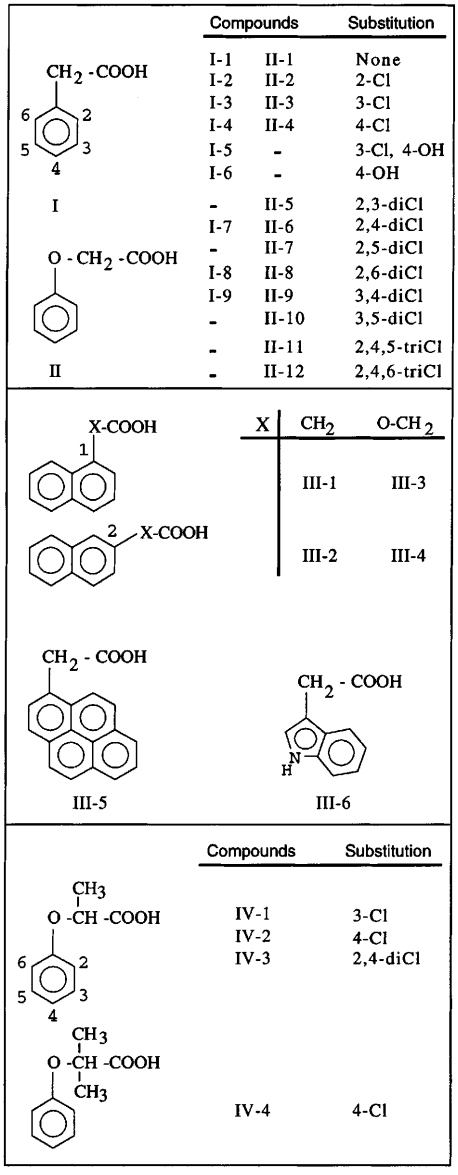

Fig. 1. Compounds tested on carrier-mediated auxin transport in cultured tobacco cells. The *roman symbols* identifying the compounds correspond to those mentioned in the text and in Tables 1–5. Phenylacetic acid (Table 1), phenoxyacetic acid (Table 2) and 2-phenoxypropionic acid (Table 4) are shown on the *upper* and *lower panels*, respectively, with the substituting groups and their positions. Compounds containing fused rings (Table 3) are shown on the *middle panel*

screened within this study. Dose-response curves, representing the capability of compounds to modify the accumulation of [^{14}C]2,4-D and [^{3}H]NAA in suspension-cultured tobacco cells, were established. Selectivity with regard to carriers was determined by comparing the external concentrations needed to half-inhibit 2,4-D influx and 1-NAA efflux. Most compounds interacted with the influx carrier in cultured cells, several with high efficiency. We discuss structure-activity relationships and factors influencing the selectivity of the compounds with regard to the influx carrier.

Materials and methods

Chemicals. Chemicals were purchased from Sigma-Aldrich and Lancaster Synthesis (Strasbourg, France) and were of analytical grade. Non-commercial compounds in the phenoxyacetic series were prepared from corresponding phenols and chloroacetic acid (Hayes and Branch 1943). The R(+) and S(−) enantiomers of IV-2 were separated by crystallization of their diastereoisomer salts with brucine and strychnine (Julia and Tchernoff 1957) and those of IV-3 by using HPLC on a chiral permethylated α-cyclodextrin column (NUCLEODEX α-PM; Macherey-Nagel, Hoerdt, France). Labeled 2,4-D ([^{14}C]2,4-D; 2.07 TBq mol^{-1}) was obtained from Amersham (Les Ulis, France). Tritiated 1-NAA ([^{3}H]NAA; 314 TBq mol^{-1}) and naphthalene-1-acetamide (1000 TBq mol^{-1}) were synthesized as described in Delbarre et al. (1994).

Plant material. Cells of *Nicotiana tabacum* L. cv. Xanthi XHFD8 (Muller et al. 1985) were grown under continuous light and 21 °C in B5 Gamborg medium (Gamborg et al. 1968) containing 1 μM 2,4-D and 60 nM kinetin. Stock suspensions were subcultured every 7 d at a density of 15 and 30 mg mL^{-1}.

Cell preparation. Exponential-phase cells from 100 mL stock suspension were filtered, resuspended in the same volume of a buffer containing 10 mM sucrose, 0.5 mM $CaSO_4$, 20 mM Mes, and adjusted to pH 5.7 by KOH (uptake buffer), and shaken for 45 min. The washing procedure was repeated once. Afterwards, the medium was decanted off, the culture topped up with fresh uptake buffer to obtain a density of 75–100 mg cells mL^{-1}, and cells were allowed to equilibrate for not less than 90 min before being used. Experiments were done in the same buffer at room temperature.

Auxin-transport assay. The method for measuring the activity of the auxin influx and efflux carriers in suspension-cultured cells was described by Delbarre et al. (1996). The cells (150–200 mg) were incubated for 30 s in the presence of [^{14}C]2,4-D (40–120 nM) or [^{3}H]NAA (10–40 nM). Afterwards, cells were separated from the medium by draining the suspension onto GFC glass fiber filters (Whatman International, Maidstone, UK) without rinsing. The radioactivity, recovered after ethanol-extraction (30 min at room temperature) of the cell pellet, corresponded to genuine [^{14}C]2,4-D and [^{3}H]NAA since auxins are not metabolized in tobacco cells during short incubations (Delbarre et al. 1996). Auxin accumulation was defined as the ratio of the radioactivity retained per unit weight of cells (becquerels per milligram) to the radioactivity per unit volume of incubation medium (becquerels per microliter). The accumulation of each auxin tracer was determined in the absence (total accumulation) and presence (non-saturable accumulation) of 50 μM of the same unlabeled auxin, the saturable transport component being calculated by subtracting the measured values from each other. The activity of the influx carrier and that of the efflux carrier were given by the intensities of the saturable accumulation components computed for [^{14}C]2,4-D and [^{3}H]NAA, respectively.

Screening of inhibitors. The saturable accumulation of [^{14}C]2,4-D and that of [^{3}H]NAA were measured, as described above, in the presence of the auxin-transport inhibitors. Inhibitors were added to the cell suspensions at the same time as or 15 min before the tracer. Residual auxin transport (R) was expressed as the ratio of the saturable accumulation of the tracer measured in treated cells to the value measured in the absence of inhibitor. Data were obtained from at least two independent experiments, each in triplicate. The concentrations required to decrease [^{14}C]2,4-D influx and [^{3}H]NAA efflux by 50% (IC_{50}) were determined by nonlinear-squares analysis of the dependance of R on the inhibitor concentration L, according to the Michaelian equation (Eq. 1)

$$R = \frac{IC_{50}}{IC_{50} + L} \quad (1)$$

Results

Assay of ligands interfering with the carrier-mediated transport of auxin in tobacco cells. We estimated the carrier-mediated influx and efflux of auxin in cultured cells through, respectively, the decrease in [^{14}C]2,4-D accumulation and the increase in [^{3}H]NAA accumulation which occurred on diluting tracer with a large excess of unlabeled auxin. Figure 2 presents a set of

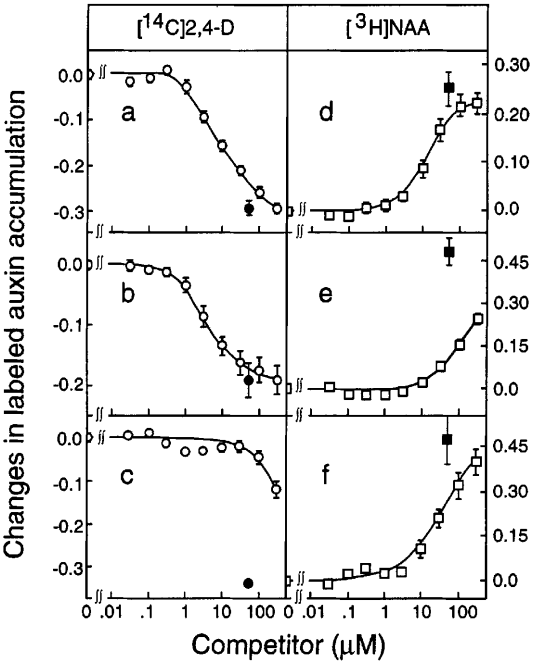

Fig. 2a–f. Selection of inhibitors of auxin influx and efflux. Suspension-cultured tobacco cells were incubated for 30 s with tracer concentrations of [^{14}C]2,4-D (**a–c**) or [^{3}H]NAA (**d–f**) and increasing concentrations of 3-chlorophenylacetic acid (I-3) (**a, d**), 4-chlorophenylacetic acid I-4 (**b, e**), and 2,6-dichlorophenylacetic acid I-8 (**c, f**), as described in *Materials and methods*. A decrease in [^{14}C]2,4-D accumulation (*left ordinate*) indicates an interaction of the assayed ligand with the influx carrier and an increase in [^{3}H]NAA accumulation (*right ordinate*) an interaction with the efflux carrier. *Closed symbols* represent the effect of saturating concentrations (50 μM) of unlabelled 2,4-D (●) and 1-NAA (■) on the accumulation of [^{14}C]2,4-D and [^{3}H]NAA, respectively. Data are means (±SE) of values measured in at least two independent experiments, each with three replicates

competition curves illustrating typical effects of ligands on the accumulation level of [^{14}C]2,4-D (Fig. 2a–c) and [^{3}H]NAA (Fig. 2d–f), in comparison with the changes provoked by a saturating concentration (50 μM) of 2,4-D or 1-NAA. Examples were obtained from experiments utilizing 3-chlorophenylacetic acid (I-3), 4-chlorophenylacetic acid (I-4), and 2,6-dichlorophenylacetic acid (I-8). Carrier-mediated [^{14}C]2,4-D entry (Fig. 2a) and [^{3}H]NAA efflux (Fig. 2d) were abolished within the same range of concentrations of I-3. Compound I-4 displayed the same efficiency as its isomer I-3 on [^{14}C]2,4-D influx (Fig. 2b) but was, at least, ten times less potent on [^{3}H]NAA efflux (Fig. 2e). The bischlorinated acid I-8 decreased auxin efflux (Fig. 2f) in the same concentration range as I-3, without modifying influx (Fig. 2c). Thus, whereas I-3 interacted with both auxin carriers with the same apparent efficiency, I-4 presented increased selectivity for the influx carrier and I-8 increased selectivity for the efflux carrier. Competition curves were established for each of the 35 compounds and employed to determine the concentrations (IC_{50}) needed to half-inhibit the carrier-mediated transport of [^{14}C]2,4-D and [^{3}H]NAA across the PM. Values of IC_{50} are listed in Tables 1 through 4.

Reduction of [^{14}C]2,4-D influx by arylalkyl and aryloxyalkylcarboxylic acids. Phenylacetic (Table 1) and phenoxyacetic (Table 2) acid derivatives, most of them containing one or several atoms of chlorine at different positions of the aromatic ring, were assayed for their effect on the carrier-mediated influx of [^{14}C]2,4-D in tobacco cells. A substantial difference in activity was observed between the unsubtituted parent acids, with phenoxyacetic acid (II-1) being more efficient than phenylacetic acid (I-1) at reducing auxin entry. However, chemical modifications provoked similar effects in both series. Substituting an atom of chlorine for a ring proton at the C-2 ortho position was scarcely favourable in phenoxyacetic acid or even unfavourable in phenylacetic acid. In contrast, substituting an atom of chlorine at the C-3 and C-4 positions substantially increased the ability of the acids to inhibit [^{14}C]2,4-D entry. Unlike unsubstituted parents, 3-chloro and 4-chlorophenylacetic and phenoxyacetic acids exhibited comparable IC_{50}

Table 1. Concentrations (IC_{50}) of phenylacetic acids half-inhibiting the carrier-mediated influx of [^{14}C]2,4-D and efflux of [^{3}H]NAA

No.	Compound	IC_{50} values (μM)[a]	
		[^{14}C]2,4-D Influx	[^{3}H]NAA Efflux
I-1	Phenylacetic acid	69 ± 9	>300
I-2	2-Chlorophenyl acetic acid	144 ± 22	93 ± 12
I-3	3-Chlorophenyl acetic acid	5.1 ± 0.6	10.8 ± 1.7
I-4	4-Chlorophenyl acetic acid	2.5 ± 0.4	154 ± 16
I-5	3-Chloro-4-hydroxyphenyl acetic acid	2.4 ± 0.2	–[b]
I-6	4-Hydroxyphenyl acetic acid	75 ± 19	–[b]
I-7	2,4-Dichlorophenyl acetic acid	3.0 ± 0.5	75 ± 21
I-8	2,6-Dichlorophenyl acetic acid	>300	21 ± 2
I-9	3,4-Dichlorophenyl acetic acid	0.4 ± 0.1	12 ± 2

[a]The concentrations (IC_{50} ± SE) of ligand decreasing [^{14}C]2,4-D influx and [^{3}H]NAA efflux by 50% were computed from data obtained in at least two experiments, each in triplicate. Saturable transport components were measured after 30 s of incubation as described in *Materials and methods*. The auxin tracer, [^{14}C]2,4-D or [^{3}H]NAA, and increasing concentrations of inhibitor were added at the same time to the cell suspensions
[b]No detectable inhibition of transport up to 300 μM

Table 2. Concentrations (IC_{50}) of phenoxyacetic acids half-inhibiting the carrier-mediated influx of [^{14}C]2,4-D and efflux of [^{3}H]NAA

No.	Compound	IC_{50} values (μM)[a]	
		[^{14}C]2,4-D Influx	[^{3}H]NAA Efflux
II-1	Phenoxyacetic acid	14.6 ± 1.9	–[b]
II-2	2-Chlorophenoxyacetic acid	9.4 ± 1.1	–[b]
II-3	3-Chlorophenoxyacetic acid	2.5 ± 0.4	245 ± 29
II-4	4-Chlorophenoxyacetic acid	1.6 ± 0.2	–[b]
II-5	2,3-Dichlorophenoxyacetic acid	1.6 ± 0.2	–[b]
II-6	2,4-Dichlorophenoxyacetic acid	3.3 ± 0.4	129 ± 23
II-7	2,5-Dichlorophenoxyacetic acid	4.6 ± 0.9	211 ± 49
II-8	2,6-Dichlorophenoxyacetic acid	–[b]	>300
II-9	3,4-Dichlorophenoxyacetic acid	0.9 ± 0.1	181 ± 28
II-10	3,5-Dichlorophenoxyacetic acid	8.5 ± 1.2	44 ± 7
II-11	2,4,5-Trichlorophenoxyacetic acid	11.1 ± 1.5	75 ± 10
II-12	2,4,6-Trichlorophenoxyacetic acid	85 ± 11	32 ± 4

[a,b]Explanations are the same as given in Table 1

values, between 1.6 and 5.1 μM. Most bischlorinated phenyl and phenoxyacetic acids showed a capability to reduce [^{14}C]2,4-D influx close to that displayed by their most-efficient monochlorinated parent. Derivatives I-9 and II-9, substituted at the C-3 and C-4 positions at once, even displayed increased activity, with 3,4-dichlorophenylacetic acid (I-9) being approximately one order of magnitude more potent than each parent, I-3 and I-4, and two orders of magnitude more potent than unsubstituted acid. Acids I-8 and II-8, substituted at both ortho positions, were devoid of activity. In comparison with their common precursor 2,4-D, trichlorophenoxyacetic acids II-11 and II-12 displayed decreased ability to reduce auxin entry. The decrease in activity was especially large in II-12 which contained two atoms of chlorine at the symetrical C-2 and C-6 positions.

The carrier-mediated influx of [^{14}C]2,4-D was inhibited by molecules containing fused aromatic rings, such as naphthaleneacetic acids, naphthoxyacetic acids, pyrene-1-acetic acid, and IAA (Table 3). There was no difference in activity between naphthoxy-1- (III-3) and naphthoxy-2-acetic (III-4) acids, both isomers half-inhibiting [^{14}C]2,4-D influx in the same micromolar range as 3- and 4-chlorophenoxyacetic acids. In contrast, the activity of the naphthaleneacetic acid isomers strongly depended on which carbon, C-1 or C-2, was occupied by the side-chain. Naphthalene-1-acetic acid (III-1) was barely more efficient than phenylacetic acid whereas 2-NAA (III-2), with an IC_{50} value of 0.6 μM, was one of the best ligands tested in this study.

The effects of IAA and 1-NAA on [^{14}C]2,4-D influx were compared with those of non-ionizable indole-3-acetamide and naphthalene-1-acetamide. Due to a poor solubility in water, naphthaleneacetamide was not employed to compete with the auxin tracer. Instead, the uptake of [^{3}H]naphthalene-1-acetamide (3 nM) was monitored in the presence of increasing concentrations of unlabeled 2,4-D. The influx of [^{14}C]2,4-D was not inhibited by indoleacetamide nor was the accumulation of [^{3}H]naphthaleneacetamide modified by 2,4-D (data not shown), suggesting that a free carboxylic group was required to confer the capability for ligands to interact with the influx carrier. One methyl group at the α-position of the side-chain created a chiral center in 2-phenoxypropionic acids (Table 4). Racemic mixtures of the chloro-substituted acids IV-1, IV-2, and IV-3 displayed an activity close to or slightly less than that of corresponding acetic acid derivatives. When IV-2 and IV-3 were split up into their enantiomers, large differences were observed between the R(+) and S(−) enantiomeric forms, with the R-form being much more efficient than its mirror image at reducing [^{14}C]2,4-D influx. The introduction of a second methyl group at the α-position of the side chain in IV-4 destroyed the activity (Table 4).

Selectivity of ligands in regard to the auxin-transport components. We compared the concentrations of arylalkyl and aryloxyalkylcarboxylic acids needed to half-inhibit the carrier-mediated influx of [^{14}C]2,4-D and efflux of [^{3}H]NAA in a 30-s treatment. As shown in

Table 3. Concentrations (IC_{50}) of polycyclic derivatives of acetic acid half-inhibiting the carrier-mediated influx of [^{14}C]2,4-D and efflux of [^{3}H]NAA

No.	Compound	IC_{50} values (μM)[a]	
		[^{14}C]2,4-D Influx	[^{3}H]NAA Efflux
III-1	Naphthalene-1-acetic acid	26 ± 3	3.2 ± 0.3
III-2	Naphthalene-2-acetic acid	0.6 ± 0.1	3.6 ± 0.6
III-3	Naphthoxy-1-acetic acid	2.2 ± 0.3	152 ± 27
III-4	Naphthoxy-2-acetic acid	1.5 ± 0.2	82 ± 20
III-5	Pyrene-1-acetic acid	12.8 ± 1.9	0.9 ± 0.2
III-6	Indole-3-acetic acid	0.6 ± 0.1	51 ± 7

[a]Explanation is the same as given in Table 1

Table 4. Concentrations (IC_{50}) of 2-phenoxypropionic acids half-inhibiting the carrier-mediated influx of [^{14}C]2,4-D and efflux of [^{3}H]NAA

No.	Compound	IC_{50} values (μM)[a]	
		[^{14}C]2,4-D Influx	[^{3}H]NAA Efflux
IV-1	(R,S)-2-(3-Chlorophenoxy)propionic acid	6.8 ± 0.9	116 ± 12
IV-2	2-(4-Chlorophenoxy)propionic acid		
	Racemic mixture	8.1 ± 1.2	263 ± 43
	R [+] Enantiomer	4.2 ± 0.7	217 ± 26
	S [−] Enantiomer	–[b]	–[b]
IV-3	2-(2,4-Dichlorophenoxy)propionic acid		
	Racemic mixture	3.1 ± 0.6	16 ± 3
	R [+] Enantiomer	2.3 ± 0.4	7.7 ± 1
	S [−] Enantiomer	66 ± 11	60 ± 13
IV-4	2-(4-Chlorophenoxy)-2-methylpropionic acid	–[b]	–[b]

[a,b]Explanations are the same as given in Table 1

Tables 1 through 4, most acids were more efficient at blocking auxin influx than efflux. Three compounds out of five tested half-reduced [^{14}C]2,4-D entry at concentrations less than 10 μM whereas only one was active on [^{3}H]NAA efflux within this concentration range. Several displayed a large differential between the concentrations half-inhibiting [^{14}C]2,4-D influx and [^{3}H]NAA efflux (e.g., I-5, II-4, II-5, II-9, III-3, ...) and, thus, could be considered as selectively interacting with the influx carrier, at least in the short term.

Some derivatives were assayed by pretreating cells for 15 min before measuring [^{3}H]NAA uptake (Table 5). In most cases, this procedure substantially decreased the concentrations needed for half-inhibiting [^{3}H]NAA efflux and even conferred activity on otherwise ineffective compounds. As an example, 2,4-D (Fig. 3a) was poor (IC_{50} = 129 μM) at inhibiting [^{3}H]NAA efflux when added at the same time as the tracer to the cell suspension. However, a 15-min preincubation shifted the dose-response curve to lower concentrations (IC_{50} = 15 μM) by approximately one order of magnitude. We utilized 2,4-D as a model to investigate whether ligands expressed an activity with respect to [^{3}H]NAA efflux that was related to their accumulation level. Figure 3b shows that 2,4-D was slowly but strongly incorporated into suspension-cultured tobacco cells. Accumulation required more than 15 min to approach the steady-state. Within this period of time, the intracellular 2,4-D concentration increased 10 fold, rising from half the external concentration after 30 s of incubation to 5 times this concentration near the equilibrium.

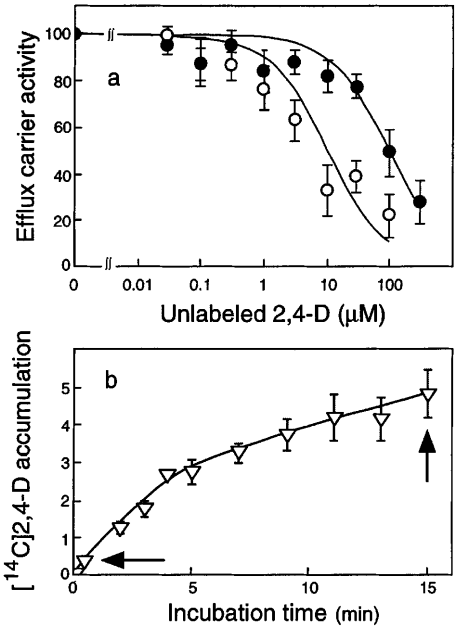

Fig. 3a,b. Relationship between the inhibition of [^{3}H]NAA efflux by 2,4-D and 2,4-D accumulation. **a** Efflux carrier activity. Suspension-cultured tobacco cells were probed for their capability to accumulate [^{3}H]NAA in 30 s, in the presence of increasing concentrations of 2,4-D added at the same time as (●) or 15 min before (○) the tracer. The efflux carrier activity was defined as the ratio of the saturable component of [^{3}H]NAA uptake measured in the presence of 2,4-D to this component measured in the absence of 2,4-D. Carrier activities are means (±SE) of values computed from two independent experiments, each with three replicates. **b** Time course of [^{14}C]2,4-D accumulation. Cells were incubated for up to 15 min in the presence of [^{14}C]2,4-D. Tobacco cells have been shown not to metabolize 2,4-D over this incubation period (Delbarre et al. 1996). At the indicated times, 2-mL aliquot fractions were filtered and the radioactivity was counted in the cell cakes. The ratios of [^{14}C]2,4-D accumulation were calculated by dividing the radioactivity accumulated in cells (becquerels per milligram) by the radioactivity in the bulk solution (becquerels per microliter). Values are means (±SE) of two series of three experiments achieved using two different batches of cells. The *arrows* point at the levels of auxin accumulation reached after 30 s and 15 min of incubation

Discussion

The capability of several aryl and aryloxyalkylcarboxylic acids to interfere with auxin membrane transport was assayed through their effect on the accumulation of [^{14}C]2,4-D and [^{3}H]NAA, in cultured tobacco cells. A majority (60%) strongly inhibited the carrier-mediated influx of [^{14}C]2,4-D. However, some also perturbed the carrier-mediated efflux of [^{3}H]NAA. The mechanism by which compounds interact with the auxin-transport components was not investigated. They might compete with labeled auxins at the auxin-binding sites on the carriers, or inhibit carrier activities through binding with

Table 5. Evolution with the incubation time of ligand concentrations (IC_{50}) half-inhibiting the carrier-mediated efflux of [^{3}H]NAA in cultured tobacco cells

No.	Compound	IC_{50} values (μM)[a]	
		30-s treatment	15-min treatment
I-4	4-Chlorophenyl acetic acid	154 ± 16	28 ± 4
I-5	3-Chloro-4-hydroxyphenyl acetic acid	–[b]	70 ± 8
I-9	3,4-Dichlorophenyl acetic acid	12 ± 2	5.2 ± 1
II-2	2-Chlorophenoxy acetic acid	–[b]	195 ± 24
II-3	3-Chlorophenoxy acetic acid	245 ± 29	166 ± 17
II-4	4-Chlorophenoxy acetic acid	–[b]	120 ± 20
II-5	2,3-Dichlorophenoxy acetic acid	–[b]	37 ± 6
II-6	2,4-Dichlorophenoxy acetic acid	129 ± 23	15 ± 2
II-9	3,4-Dichlorophenoxy acetic acid	181 ± 28	44 ± 11
III-3	Naphthoxy-1-acetic acid	152 ± 27	6.3 ± 1
IV-3	(R,S)-2-(2,4-Dichlorophenoxy)propionic acid	16 ± 3	12.1 ± 2

[a,b]Explanations are the same as given in Table 1 except that inhibitors, in the 15-min treatment experiments, were added 15 min before [^{3}H]NAA to the cell suspensions

separate control proteins, like phytotropins (Rubery 1990; Morris et al. 1991; Lomax et al. 1995), or interact with one transport component via one mechanism and with the other transport component via the second mechanism. In the absence of suitable tracers, there was no possibility of determining whether active ligands are carrier-transported across the PM.

Molecular requirements for auxin-influx inhibition. Basically, compounds perturbing auxin influx shared some structural features with auxins (Katekar 1979) and ligands of the auxin-binding protein 1 (ABP1) of maize (Edgerton et al. 1994). They contained an aromatic moiety substituted by an acidic side-chain. Fused aromatic rings and chlorine were particularly effective in increasing activity. The acidic function is probably essential for promoting inhibitory activity since indoleacetamide and naphthaleneacetamide, in contrast to their parents IAA and 1-NAA, were not substrates for the influx carrier. A large difference was observed between enantiomers of chiral phenoxypropionic acid derivatives, with the R-enantiomeric form having the highest activity. It has been postulated (Katekar 1979) that auxins are recognized by a receptor site, planar, except for the portion which accepts the oxygen atoms of the carboxylic group, and electrophilic in nature. Within this model, auxin activity depends on the interactions between the electrophilic platform at the receptor binding site and the delocalized π-electrons of the interacting ligand. Fused rings and electron-rich substituents, such as chlorine, can give rise to high activity because they increase the electron density in the ligand and the coverage between overlapping area. The carboxyl group is believed to specifically bind a particular region of the receptor, with the oxygen atoms being out of plane with the aromatic rings, possibly in a plane perpendicular to the rest of the molecule (Edgerton et al. 1994). The intermediate region between the electrophilic platform and the carboxyl acceptor at the auxin-binding site receives the auxin side-chain and can accommodate α-methylene protons or one methyl group in the appropriate configuration (R in the 2-phenoxypropionic acid series), but not two methyl groups protruding on either side of the chain.

Shape and size of the binding site of influx inhibitors. Since the compounds we tested are closely related in structure and some (2,4-D, IAA) are transported by the influx carrier (Delbarre et al. 1996), we assumed that they would block the carrier-mediated influx of [^{14}C]2,4-D by interacting with a single class of sites at the catalytic transport unit. Though retaining the same structure as described above, these sites are probably less restrictive in shape and size than ABP1 and the receptor(s) responsible for auxin action. Indeed, [^{14}C]2,4-D influx was inhibited by compounds displaying high auxin activity and low affinity for ABP1, such as 2,4-D and naphthoxy-2-acetic acid, and by compounds displaying low auxin activity and high affinity for ABP1, such as 2-NAA. The activity of pyrene-1-acetic acid (III-5) on [^{14}C]2,4-D accumulation suggests that large aromatic ligands may be accommodated by sites at the influx carrier. All the tested molecules exactly fit one region of III-5 when the carboxylic groups are aligned together. As shown in Fig. 4, the most-active ligands overlap the CD area of the pyrene tetracycle or contain one atom of chlorine at least, at the C-3 or C-4 position, projecting over this area. Less-active compounds, 1-NAA and unsubstituted or ortho-substituted phenyl and phenoxyacetic acids, only cover the upper region AB of III-5 (Fig. 4). The complete or partial inactivity of diortho-substituted compounds I-8, II-8, and II-12 can be explained by postulating specific interaction between the carboxylic group of the ligand and an acceptor group at the binding site. The bulky atoms of chlorine, at the C-2 and C-6 positions, are likely to force the ligand side-chain out of the plane of the molecule, which should hamper the proper positioning in space of the carboxylic group and consequently inhibit its attachment to the acceptor. The hydroxyl group in phenylacetic acids I-5 and I-6 protrudes onto the CD area of the pyrene model, like the atom of chlorine at the C-4 position of I-4 and I-9 (Fig. 4). However, I-5 and I-6 were much less efficient than their chlorinated analogs at decreasing [^{14}C]2,4-D influx and not appreciably different from their non-hydroxylated parents I-1 and I-3. This feature argues in favour of a strong contribution of hydrophobic interactions to the binding of inhibitors to influx-carrier proteins. Experimental and theoretical studies, dealing with the influence of substituting atoms

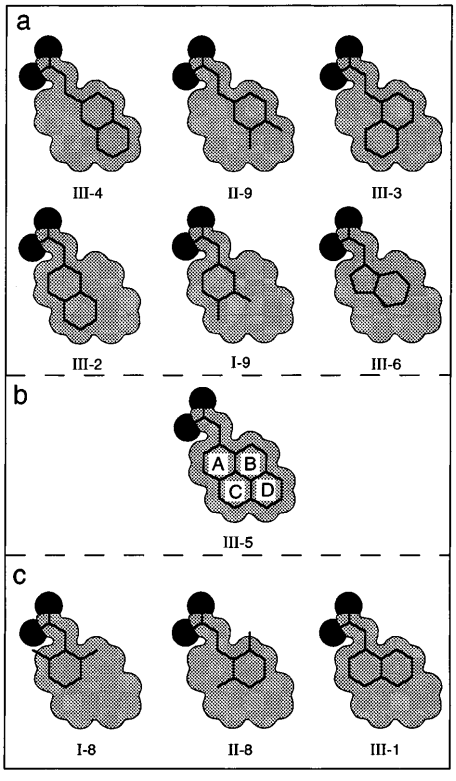

Fig. 4a-c. Structure overlaps of active (**a**) and less active (**c**) auxin-influx-carrier inhibitors with pyrene-1-acetic acid (**b**). *Dotted areas* correspond to the pyrene acetic acid structure

or groups of atoms on structure-activity relationships in various series, have demonstrated that hydroxyl and chlorine substituents have opposite effects on both the lipophilicity and electronic density of aromatic rings (Craig 1971).

Influence of accumulation on the selectivity of the auxin influx inhibitors. Data in Tables 1–4 indicate a high selectivity of most ligands with respect to the influx carrier. However, a few acids, such as naphthyl and pyrenylacetic acids (Table 3), strongly antagonized auxin efflux. We have shown previously (Delbarre et al. 1996) that 1-NAA is accumulated up to a stable concentration, close to the supplied concentration, within 1 or 2 min of incubation. Assuming that the rapidly acting efflux inhibitors possess the same accumulation properties as 1-NAA, the intracellular concentrations half-inhibiting the efflux carrier activity were probably within the range of the IC_{50} values measured in the bulk solution after 30 s of incubation. Thus, these compounds, not chemically related to known PAT inhibitors, likely display a high affinity for the efflux-carrier components. Other compounds, mainly aryloxy derivatives, had little effect on [^{3}H]NAA efflux when given at the same time as the tracer. Extending the period of time during which they were in contact with cells strongly enhanced their activity (Table 5). Kinetic experiments showed an inverse relationship between the concentration required to inhibit [^{3}H]NAA efflux and the accumulation level of 2,4-D, utilized as a model efflux inhibitor: the higher the accumulation, the lower the IC_{50} value measured in the bulk medium. The carrier-mediated efflux of [^{3}H]NAA was half-reduced by 129 μM of simultaneously added 2,4-D and 15 μM of 2,4-D added 15 min before the [^{3}H]NAA. However, between 30 s and 15 min of treatment, the intracellular 2,4-D concentration rose from 0.5 to 5 times the extracellular concentration so that, at either time, the efflux carrier was exposed to the same high level (65–75 μM) of inhibitor. We thus propose that, like 2,4-D, aryloxy derivatives display a low affinity for the efflux-carrier components and become increasingly efficient because of overaccumulation into the cell compartments.

In conclusion, we have found several aryl and aryloxyalkylcarboxylic acids that strongly inhibit the carrier-mediated influx of auxin in cultured cells. Influx inhibition was shown to depend on the free carboxylic group ending the alkyl side chain, the absolute configuration of the chain, and the prolate electron-rich platform built from substituted or fused rings. Experiments are in progress to refine our knowledge of parameters conferring their activity on inhibitors. Since several inhibitors increasingly perturbed auxin efflux when cells were given prolonged treatments, we suppose that their cellular concentration increased with time so that they finally engaged the efflux carrier at the inner face of the PM. However, some of them had no effect on PAT in *Arabidopsis* inflorescence segments (data not shown), suggesting that they did not interfere with the efflux carrier at the whole-plant level. Thus, we have available a set of inhibitors we can use to mimick a dysfunction of the influx carrier in plants. Some are more efficient at discriminating between influx and efflux carrier activities than 2-NAA, so far employed to investigate influx carrier properties. These compounds will be valuable tools for probing the function of the influx carrier in growth and development.

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