Reconstitution of an electrogenic auxin transport activity mediated by *Arabidopsis thaliana* plasma membrane proteins

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Abstract Plasma membrane proteins from *Arabidopsis thaliana* leaves were reconstituted into proteoliposomes and a K^+ diffusion potential was generated. The resulting ionic fluxes, determined in the presence of the plant hormone auxin (indole-3 acetic acid), showed an additional electrogenic and saturable component, with a K_M of 6 μM. This flux was neither detected in liposomes in the presence of indole-3 acetic acid, nor in proteoliposomes in the presence of an inactive auxin analog and was completely inhibited by 3 μM naphtylphthalamic acid, a specific inhibitor of the auxin efflux carrier. The efficiency of the reconstituted carrier and the mechanism of its regulation by naphtylphthalamic acid are discussed.

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Key words: Auxin transport; Efflux carrier; Plasma membrane; Protein reconstitution; Diffusion potential; Fluorescent probe; *Arabidopsis thaliana*

1. Introduction

The plant hormone auxin (IAA) is involved in several developmental processes concerning various parts of the plant [1]. The primary sites for auxin biosynthesis are restricted to meristems and young growing organs [2], it is usually assumed that auxin moves through the plant by a cell to cell polar transport process, involving at least two plasma membrane (PM) carriers [3,4]. The influx of auxin into the cell is mediated by an electropositive active carrier co-transporting auxin and protons down their electrochemical gradient [5–7]. On the other hand, the efflux of auxin out of the cell was shown to be facilitated by an electronegative passive carrier, transporting the deprotonated auxin IAA^- down its electrochemical gradient [7]. This latter carrier is specifically inhibited by naphtylphthalamic acid (NPA) [8–10].

Genetic approaches on *Arabidopsis thaliana* mutants allowed the identification of several genes potentially involved in the auxin transport [11–16]. The product of the *Aux1* gene, a transmembrane protein related to amino acid permeases, was proposed to mediate the auxin influx [17]. On the other hand, very recent results report on the identification of a gene encoding a presumptive auxin efflux carrier [18–20].

In this context, designing auxin transport assays could be useful to ascertain and to characterize under controlled conditions the function of candidate proteins. Up to now, auxin transport assays involved exclusively the use of radiolabelled auxin. However, both the binding of protonated auxin to membranes [21] and its high passive diffusion across membranes [22] constitute serious drawbacks to measure auxin transport in the vesicles. In this work, we tried to take advantage of the electrogenicity of auxin carriers, contrasting with electrically silent passive diffusion and binding of the hormone. For this purpose, we used a recently devized procedure [23] in which the dissipation rate of a diffusion potential (E_m), generated by a K^+ gradient, is fully dependent on the size of ionic fluxes that compensate electrically the net K^+ influx (J_K) into the vesicles. In the absence of any other permeant ionic species, J_K is exclusively compensated by a net H^+ efflux (J_H) [24], but when such species (X) are present, their net flux (J_X) induces an increase of J_K ($\Delta J_K = J_X$). We show that auxin triggered a sizeable electrogenic flux (J_{IAA}) in reconstituted PM vesicles, but not in control liposomes. J_{IAA} was saturable and inhibited by NPA, demonstrating for the first time that an electrogenic activity with features of the auxin efflux carrier can be solubilized from plant PM and reconstituted.

2. Materials and methods

PM vesicles from leaves of 50 days old *Arabidopsis thaliana* plants were purified by phase partitioning between polyethylene glycol and dextran using classical procedures [25]. Reconstitution of PM proteins was performed, with slight modifications, as previously described [26]. Briefly, an aliquot of PM proteins (100 μg) was added to 200 μl of reconstitution buffer (Mes-LiOH 50 mM, pH 6.0, Li_2SO_4 50 mM, glycerol 20% w/w) containing 10% (w/w) CHAPS (3-((3-cholamidopropyl)-dimethylammonio)-1-propane sulfonate) and 1.5 mg of L-α-phosphatidylcholine (Type II-S, Sigma) liposomes. The mixture was vortexed and applied to the top of a Sephadex G-50 minicolumn, equilibrated with reconstitution buffer. After centrifugation, the cloudy eluted volume containing reconstituted PM vesicles was frozen in liquid nitrogen.

The fluorescent dye oxonol VI was used to determine ΔJ_K as detailed elsewhere [23]. Briefly, the fluorescence intensity of the dye (614/646 nm, excitation/emission) was measured with a SLM 8000 (SLM-Aminco) spectrofluorometer, using a disposable cuvette under stirring and thermostated at 30°C. An aliquot of reconstituted PM vesicles (5 μg of proteins) was added to the assay buffer (HEPES-LiOH 50 mM, pH 7.4, Li_2SO_4 50 mM) containing 100 nM oxonol VI, 20 nM valinomycin and auxin at the indicated concentration. The diffusion E_m was imposed across vesicles by adding a concentrated K^+ aliquot. At least five assays on three independent reconstitutions were performed. ΔJ_K values were analyzed using the Student test.

3. Results

The reconstitution procedure used in the present work promotes usually the inside-out insertion of PM transport systems into the lipidic membrane, because of the structural

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Abbreviations: ETH, *N,N′*-diheptyl-*N,N′*,5,5-tetramethyl-3,7-dioxanonanediamide; IAA, indole-3-acetic acid; ICA, indole-3-carboxylic acid; NPA, naphtylphthalamic acid

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asymmetry resulting from large cytoplasmic domains compared to extracellular ones [26–29]. Assuming a similar inside-out orientation for auxin carriers, experimental conditions were selected to preferentially activate the efflux carrier in reconstituted PM vesicles. For this purpose, their internal volume and external medium were strongly buffered at pH 6.0 and pH 7.4 respectively. Under these conditions, an inwardly directed electrochemical gradient of IAA^- ($pK_a = 4.7$) was generated due to the 25-fold higher concentration of IAA^- in the external medium and to the positive inside E_m. Representative fluorescence records, in the absence and in the presence of IAA, are shown in Fig. 1a. An instantaneous increase in the oxonol VI fluorescence was observed upon generation of E_m, that was followed by a slow dissipation phase, the rate of which increased in the presence of IAA. At the end of the experiment, the Li^+ ionophore ETH was added to short circuit Li^+-loaded vesicles and to determine the response of the dye at zero E_m. After data acquisition, E_m and J_{IAA} were calculated (Fig. 1b,c) as detailed elsewhere [23].

In reconstituted PM vesicles, J_{IAA} significantly increased in the presence of increasing IAA concentrations and reached a plateau in the 100 μM range, whereas no significant J_{IAA} was observed in control liposomes lacking PM proteins (Fig. 2). Non-linear fitting of data from Fig. 2 (Sigma Plot software, Jandel Scientific) according to the Michaelis-Menten equation gave K_M and J_{max} of 6 μM and 1.1×10^{-9} mol/m²/s respectively. A control experiment in the presence of ICA, an auxin-related molecule without hormonal activity, showed that at any concentration between 10 nM and 1 mM, no additional flux was observed ($J_{ICA} = 0$, not shown). Finally, the effect of NPA on J_{IAA} was investigated (Fig. 3). In reconstituted PM vesicles, J_{IAA} was progressively inhibited by NPA, the total inhibition being reached at 3 μM, whereas NPA itself had no significant effect on J_K in the absence of IAA ($\Delta J_K = J_{NPA} = 0$).

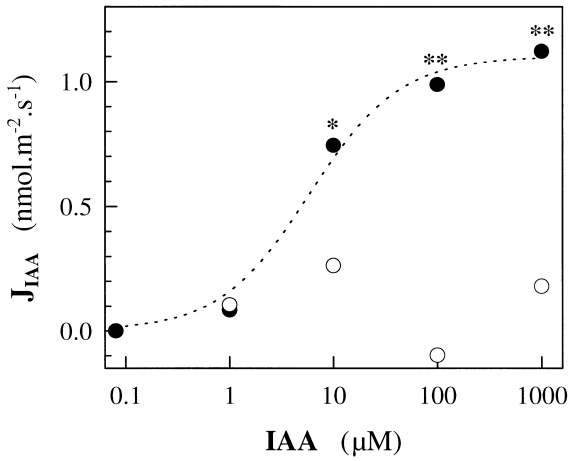

Fig. 2. The effect of increasing IAA concentrations on J_{IAA}. Full symbols, reconstituted PM vesicles; open symbols, control liposomes; dashed line, non linear fit of data according to the Michaelis-Menten equation. J_{IAA} was calculated as indicated in Fig. 1 and its statistical significance was ascertained by Student t test ($*P < 0.05$, $**P < 0.01$).

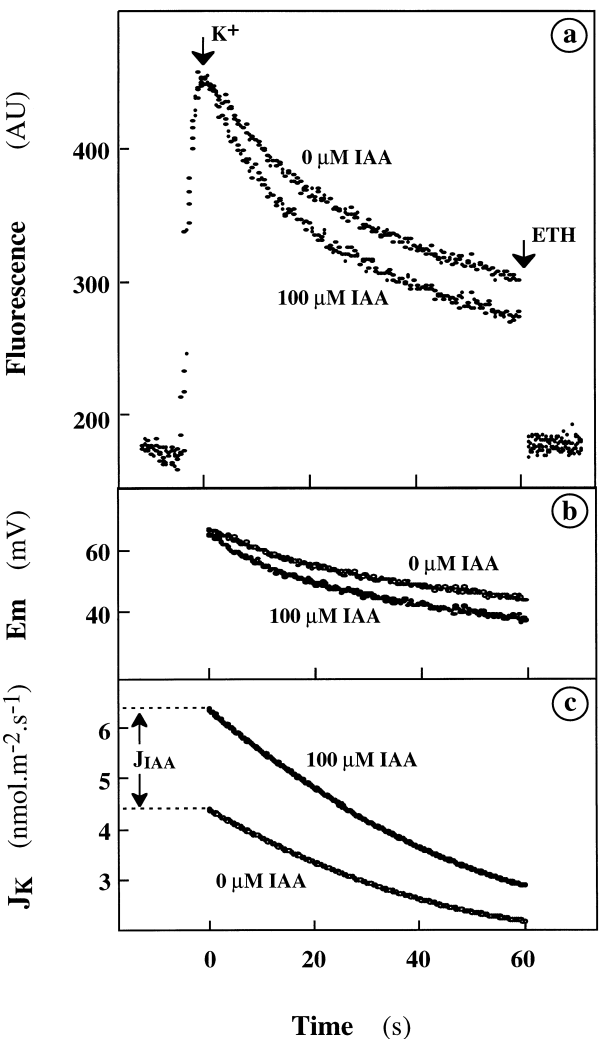

Fig. 1. Auxin effect on the dissipation of E_m generated across reconstituted PM vesicles. From oxonol VI fluorescence measurements (a), E_m (b) and J_K (c) were calculated as described in Section 2. The auxin-dependent increase in J_K (ΔJ_K) represented the net electrogenic flux of IAA (J_{IAA}).

4. Discussion

Preliminary experiments with [^{3}H]IAA showed that, due to the high solubility of the protonated hormone in the hydrophobic core of the membrane and as previously reported [21], the amount of membrane bound auxin was of the same order of magnitude as that accumulated in the vesicle lumen (data not shown). On the other hand, the protonated form of auxin diffuses very quickly across the membrane [22]. For these reasons, quantification of IAA transport using radiolabelled IAA should require both the measurement of IAA binding and correction for the passive diffusion component. Moreover, again due to the high auxin passive permeability, it is nearly impossible to avoid auxin leakage from the vesicle lumen during the separation steps required to count the radioactivity. This prompted us to devise a procedure that does not need to separate physically the vesicles from their incubation medium and that only denotes the electrogenic transport of IAA (J_{IAA}). It appeared therefore as a convenient way to overcome the above difficulties. Our results show that a significant and saturable J_{IAA} was reproducibly measured with reconstituted PM vesicles, whereas it was not detected with control liposomes. Furthermore, this electrogenic transport

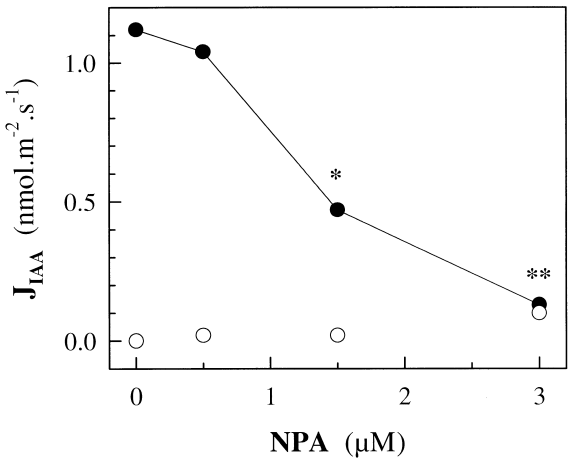

Fig. 3. The effect of NPA on J_{IAA} in reconstituted PM vesicles. J_{IAA} was calculated, as indicated in Fig. 1, in the presence of 1 mM IAA (full symbols) and statistical significance of inhibition was ascertained by Student t test ($^*P<0.05$, $^{**}P<0.01$). The effect of NPA itself on J_K in the absence of IAA was estimated in a control experiment and corresponding $\Delta J_K = J_{NPA}$ are also presented (open symbols).

was observed with the natural auxin IAA, but not with its inactive analog ICA. Finally, the K_M for IAA ($\sim$6 μM), is compatible with biologically active concentrations of auxin at the cellular level [30,31]. Taken together, these data indicate that the present approach allowed the first functional evidence of reconstituted auxin carriers.

However, as both efflux and influx auxin carriers were reported to be electrogenic [5–7], the measured J_{IAA} could denote either an entry of IAA^- into the vesicles (mediated by the inside-out reconstituted efflux carrier) or a leakage of IAA^- and $2H^+$ (mediated by the inside-out reconstituted influx carrier). Unfortunately, no selective inhibitor of the latter activity is available. Therefore, to prevent a priori this activity, a strongly buffered pH gradient, acidic inside, was imposed. Owing to the rapid diffusion of the protonated auxin, this gradient was expected to maintain a 25-fold inwardly directed gradient of IAA^-. Practically, and still assuming an inside-out sidedness, active influx carrier was estimated to operate from only 0.4 μM IAA^- in the lumen, whereas the passive efflux carrier operated from 10 μM IAA^- in the external medium. Indeed, the J_{IAA} observed in reconstituted PM vesicles was completely inhibited by 3 μM NPA, known to selectively inhibit the efflux carrier in this concentration range [32,33].

The most simple interpretation of these data is that PM from *A. thaliana* leaf cells contains an auxin transport system carrying a net negative charge in the direction of the electrochemical gradient of auxin, i.e. an IAA^- uniport. Both the behavior as uniport and the sensitivity to NPA suggest that this transport system corresponds to the auxin efflux carrier. The present results constitute therefore a first direct demonstration of the electrogenicity of this carrier [7], a question debated in previous studies performed with radiolabelled auxin [34,35]. Furthermore, another debate concerns the mechanism of inhibition of the efflux carrier by NPA. By analogy to non-competitive inhibitors of various carriers, the NPA binding site was initially thought to be located on the auxin efflux carrier itself, until some data suggested the occurrence of a separate NPA binding protein [36,37] and a possible implication of another, transducing protein [36]. In the present work, PM proteins were first solubilized and then reconstituted under experimental conditions implying a circa 15-fold surface dilution. The inhibition by NPA observed under these conditions indicates a tight interaction between the NPA binding site and the transport system.

Concerning the efficiency of the reconstituted carrier, the simultaneous availability of the observed J_{IAA} and E_m allows one to calculate, using the Goldman-Hodgkin-Katz relation, the permeability coefficient P_{IAA^-} of this facilitated transport. In the above experiments, and taking into account a circa 15-fold surface dilution of reconstituted proteins, P_{IAA^-} amounts to circa 3×10^{-8} m/s. By comparison to available data for passive diffusion of IAA^- across a lipidic membrane [22], this suggests that the efflux uniport would increase the permeability of the membrane to IAA^- by more than three orders of magnitude. This facilitation is higher than that achieved, under similar experimental conditions, by carriers of mineral anions such as nitrate (circa two orders of magnitude) [23]. Furthermore, it can be emphasized that the population of reconstituted proteins averaged both a cell type heterogeneity, since whole leaves were used, and a PM heterogeneity resulting from a particular location of proteins, as it was recently demonstrated for auxin efflux carriers [20]. Therefore, it can be speculated that the local facilitation is likely to be even higher, allowing fast regulation of the hormone content of the cell.

In conclusion, the in vitro electrophysiological approach used in this work allowed the first functional evidence of reconstituted auxin carriers. Data converge to identify the activity of an efflux uniport and reveal new features about this carrier. Similar experiments could be performed for the influx carrier, provided that specific inhibitors become available. This in vitro approach appears therefore promising to get new insights into the biochemical and biophysical properties of auxin carriers, as well as to characterize new candidates for auxin transport function.

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