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Modulation of Phytotropin Receptors by Fluoride and ATP

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Abstract: Treatment of microsomal vesicles isolated from etiolated *Pisum sativum* L. cv. Alaska epicotyl tissue with agents inhibiting protein dephosphorylation, namely NaF and/or ATP, resulted in increased binding of the phytotropin NPA to the putative auxin efflux carriers localized on the plasma membrane. The phytotropin effect was especially conspicuous if the vesicles were simultaneously treated with Triton X-100. Kinetic analysis of the binding indicated the existance of two distinct sites for NPA, each having different affinities. Increased binding of the phytotropin to the membrane where protein dephosphorylation was inhibited was attributable to the increased ligand affinity of both sites. Treatment of tissue segments with fluoride was found to enhance *in vivo* auxin transport. Implications of covalent modification of the auxin efflux carrier complex for the regulation of membrane transport of auxin molecules are discussed.
Key words: auxin transport, phytotropin binding, protein dephosphorylation.

The hormone auxin exerts its influence on almost every aspect of growth and development in plants. Transport of auxin from the site where it is synthesized to the site of its action provides means by which the level of auxin in a given cell in the transport stream is controlled. Plants have evolved to adjust their auxin transport to cope with adverse circumstances inflicted by internal and external factors. Auxin transport at the cellular level should involve at least two distinct processes associated with the plasma membrane, namely entry of auxin into, and its efflux out of the cell. Each of these processes is separately mediated by specific carriers, and it is the latter process that functions as a rate-limiting element in the auxin transport system (Hertel, 1983; Yoon and Kang, 1992). Moreover, there are indications that the auxin efflux carrier may be functionally linked to the action of auxin on cell expansion growth (Kang *et al.*, 1992).

Phytotropins such as N-1-naphthylphthalamic acid (NPA) are a group of pharmacological agents which specifically inhibit auxin transport by binding with a high affinity to the putative auxin efflux carrier localized on the plasma membrane (Kateker and Geissler, 1977 a, b). Polar auxin transport in mungbean hypocotyl segments is greatly enhanced in the presence of ether vapor, the treatment giving rise to increased affinity to NPA for the receptor (Kang, 1987). The correlation may support the notion of the phytotropin receptor having a biological function as an auxin transport carrier.

Experimental evidence to further consolidate the biological relevance of the receptor, and for possible modulation of the complex through covalent modification involving protein phosphorylation/dephosphrylation, is presented in this communication.

Materials and Methods

Materials

Subapical third internodal tissue of 6 days-old dark grown pea (*Pisum sativum* L. cv. Alaska) seedlings was used for both isolation of microsomal membrane vesicles and *in vivo* auxin transport.

Radiochemicals used were [2,3,5-^{3}H]NPA, 55 Ci/mmol, and [5-^{3}H]indole-3-acetic acid (IAA), 28 Ci/mmol, both from CEA France, Gif-sur-Yvette. Unlabelled NPA and 2-(1-pyrenoyl) benzoic acid (PBA) were synthesized by Dr. Wolfgang Michalke of University of Freiburg, Germany and his colleagues as previously described (Katekar, 1976; Katekar and Geissler 1977a, b). All other chemicals were reagent grade coming from various commercial sources.

Isolation of microsomal vesicles

Epicotyl tissue was chopped up thoroughly with a

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razor blade and ground gently with a mortar and pestle on ice with four weight equivalents of extraction medium, which consisted of 25 mM N-2'-hydroxyethylpiperazine-2-ethanolsulfonic acid (HEPES), 3 mM ethylenediaminetetraacetic acid (EDTA), 250 mM sorbitol, 25 mM 1,3-bis[tris(hydroxymethyl)-methylamino]propane (BisTrisPropane or BTP), and 1 mM KCl, at pH 7.8. One-tenth percent bovine serum albumin (BSA), 1 mM dithiothreitol (DTT), and 1 mM phenylmethylsulfonylfluoride (PMSF) were added to the extraction medium immediately before use. The crude homogenates were squeezed through a nylon cloth to remove unboken cells and cell debris, and the filtrate was centrifuged at 6000 rpm for 20 min to remove nuclei and most of the mitochondria. The supernatant was centrifuged at 43,000 rpm for 20 min with an ultracentrifuge to obtain microsomal pellets. The pellets were washed twice before resuspending with test medium (10 mM Na citrate-citric acid, 5 mM $MgCl_2$, and 250 mM sorbitol, at pH 5.5) for binding assay.

In vitro binding of phytotropins

Either a centrifugation assay or a filtration assay was employed to measure binding of [^{3}H]NPA, depending on the the kind of unlabelled ligand used to displace labelled NPA. For the centrifugation method, membrane particles resuspended in test medium, to which 0.5 nM [^{3}H]NPA and varying concentrations of unlabelled NPA were added, were equilibrated with or without test substances used at 0℃ for 30 min. The binding mixture had a volume of 500 μl. The tubes were centrifuged for 10 min at 100,000 rpm at 0℃. The supernatants were decanted, and radioactivity in the pellets was determined by scintillation counting after extraction with MeOH for 30 min. For the filtration method, after a 30 min incubation of 1 ml binding mixture, 10 ml of ice-cold test medium was poured into the mixture, and the mixture was immediately filtered through a membrane filter (0.45 μm pore size, 47 mm diameter Satorious membrane filter, Gotingen, Germany) under reduced pressure. The filter was washed rapidly with another 10 ml of the medium, air dried, and its radioactivity was counted. The centrifugation method was employed for binding work using cold NPA to displace labelled NPA, and the filtration method for that using cold PBA because of its strong lipophilic nature.

In vivo auxin transport

Agar blocks, 3 mm×3 mm×1 mm (1.5% buffered with 10 mM Na phosphate at pH 5.0), containing 38 nM [^{3}H]IAA were used as donors. Receiver blocks contained plain buffered agar and test chemicals where indicated. Individual segments were vertically placed, the basal end down, between donor and receiver blocks. At the end of a transport period, radioactivity in the receiver blocks was counted.

Results and Discussion

Localization of the NPA receptor on the plasma membrane is firmly established by the work of a number of investigators (Jacobs and Hertel, 1978; Sussman and Goldsmith, 1981; Hertel *et al.*, 1983). Plasma membrane vesicles in the microsomal fraction are supposed to have two opposing orientations, i.e. inside out and right side (outside) out vesicles. Although the former has reversed sidedness with regard to the intact cell, its presence in the assay mixture for *in vitro* binding of phytotropins imposes no serious problem because the NPA binding site on the transmembrane carrier protein is known to face the outside (Hertel, 1983), and thus labelled ligands in the assay mixture do not have access to the binding site of the inside out vesicles.

Incubation of membrane vesicles with NaF and/or ATP, especially in the presence of Triton-X 100, resulted in a marked increase in NPA binding activity (Table 1). Both NaF (Pesi and Villa-Moruzzi, 1990) and ATP (Refeno *et al.*, 1982; MacKintosh *et al.*, 1991) are known to inhibit protein serine/threonine phosphatases. Neither fluoride nor ATP, alone or in combination, in the absence of the detergent exerted appreciable effect on the binding activity. These vesicles could be kept tightly sealed for a considerable period of time under

Table 1. Specific binding of NPA to microsomal membranes incubated with NaF (10 mM) and/or ATP (1 mM) in the absence or presence of Triton-X 100 (0.02%)

Treatment	Percent specific binding[a]
None	8.23
NaF	9.58
ATP	13.48
Triton X-100	11.91
NaF, ATP	10.97
NaF, Triton X-100	34.68
ATP, Triton X-100	40.86
NaF, ATP, Triton X-100	39.16

[a]Labelled NPA at a low concentration was added to the binding mixture in the absence (sample A) and presence (sample B) of a saturating concentration (10^{-5} M) of cold NPA, and centrifuged after an equilibrium period. The difference in radioactivity between pellet A and pellet B is termed specific binding. Percent specific binding denotes percent of radioactivity "specifically" bound to the pellet.

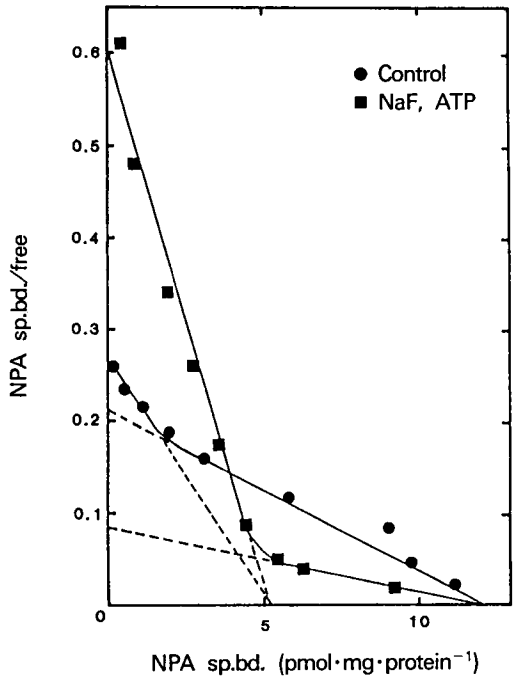

Fig. 1. Scatchard plots of NPA binding to membranes treated with or without NaF (10 mM) and ATP (1 mM) in the presence of Triton X-100 (0.02%).

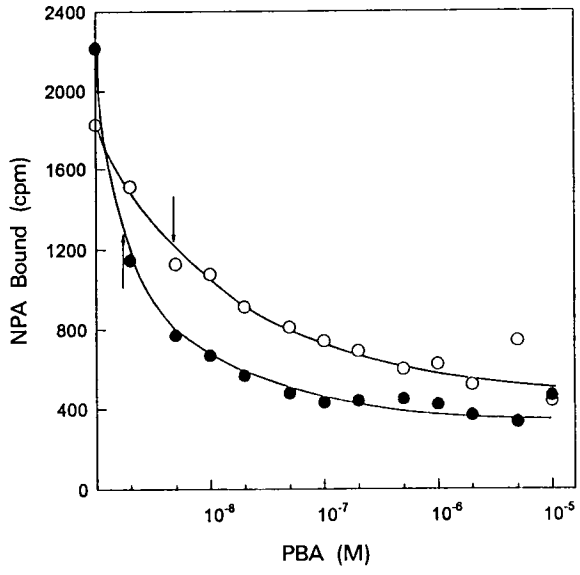

Fig. 2. Saturation kinetics of PBA displacement of NPA binding in membranes treated with (closed circles) or without (open circles) NaF and Triton X-100. The arrows indicate the KD values for the control (5×10^{-9} M) and NaF treatment (1.8×10^{-9} M), resectively.

Table 2. Effect of NaF on polar transport of labelled IAA in third internode segments in the absence or presence of NPA (0.3 uM)

Treatment	[^{3}H]IAA transported (cpm)[a]	
	−NPA	+NPA
Control	3729	2946
NaF	6523	5312

[a]Radioactivity transported through 6 mm segments for 4 h, and collected in the receiver blocks.

proper incubation conditions (Depta *et al.*, 1983) and thus the phosphatase inhibitors could have access to the intravesicular space where they could act on phosphorylated sites of either cytosolic proteins or the cytosolic side of membrane-bound proteins only in the presence of Triton-X 100. Whether the increase in NPA binding was brought about by inhibition of dephosphorylation of the NPA receptor protein or that of some other protein spatially or functionally associated with the receptor is unknown.

Kinetic analysis of binding is illustrated in Fig. 1. The Scatchard plots of NPA binding indicate that there existed two classes of binding sites, having high and low affinities, respectively. This is in accordance with what Michalke and his colleagues reported for *Cucurbita* membranes (Michalke *et al.*, 1992). Of the two kinds of binding sites, the low affinity sites were suggested to be associated with the NPA action on auxin transport (Michalke *et al.*, 1992). The data presented in Fig. 1 indicate that inhibition of protein phosphatase action led to increased affinities both at the low and the high affinity sites without the number of binding sites being affected.

PBA is another member of the phytotropin family (Katekar and Geissler, 1980; Rubery, 1990), and is known to bind to both the high and low affinity sites with a high affinity (Michalke *et al.*, 1992). The data shown in Fig. 2 illustrate saturation kinetics for displacement by cold PBA of labelled NPA occupying the sites under study. Likewise, fluoride treatment is also shown to decrease the KD value of binding for PBA.

In vivo transport of labelled IAA in internode segments was tested to see if fluoride treatment has any physiological consequences resulting from modulation of auxin efflux carriers. Treatment of tissue segments with ether vapor is known to promote auxin transport, and this might have resulted from *in vitro* action of ether on isolated membranes, where affinity to NPA is greatly increased. In accordance with the kind of correlation seen in ether treatment, fluoride treatment likewise resulted in promotion of auxin transport *in vivo* both in the absence and presence of a low concentration of NPA, where auxin transport was only partially inhibited (Table 2). Occupation of phytotropin binding sites by appropriate ligands brings about inhibition of auxin efflux and thus of auxin transport. How increased affinity at these sites could lead to enhancement of auxin transport is not clear, and seems to contradict the no-

tion that binding of ligands to the sites results in conformational changes of the auxin efflux carrier protein, which in turn lead to hindrance of auxin efflux. No plausible explanation is put forward at present. It should be noticed, however, that the protein kinase inhibitor staurosporine (Tamaoki *et al.*, 1986; Fujita-Yamaguchi and Kathuria, 1988) has an effect opposite to that of NaF/ATP on *in vivo* auxin transport (data not shown). Through photoaffinity labelling, a 23 kDa protein in the plasma membrane of maize coleoptiles was identified as a NPA receptor (Zettl *et al.*, 1992). Characterization of this protein and search for possible phosphorylation/dephosphorylation reactions of this protein in conjunction with changes in phytotropin affinity merit future investigation.

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