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Phytotropins

III. NAPHTHYLPHTHALAMIC ACID BINDING SITES ON MAIZE COLEOPTILE MEMBRANES AS POSSIBLE RECEPTOR SITES FOR PHYTOTROPIN ACTION

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ABSTRACT

Certain members of the phytotropin class of auxin transport inhibitors are shown to bind with high affinity to the known naphthylphthalamic acid binding sites in maize (*Zea mays*) coleoptiles. The binding site is, thus, a phytotropin binding site. In general, the degree of binding correlates with the phytotropin structure activity rules and with physiological activities of model compounds. It is argued that the binding site may be a receptor, and it also may be the receptor involved in the control of the auxin transport process. The possibility is raised that the binding sites may be intrinsic receptors for endoanalog(s) of the phytotropins.

Recently, a class of auxin transport inhibitors, the phytotropins, has been defined by common chemical (15) and biological properties (16), with members of the class appearing to achieve their effects by a common mode of action (16). The chemical requirements, as represented in Table I, are that active compounds should possess a carboxylic acid function (or one which can become available by hydrolysis) which is attached to an aromatic ring which is connected at the *ortho* position to a second aromatic ring. The aromatic rings may be separated by a conjugated or planar system of atoms. There is also a spatial requirement which confers high activity when the distance between the centers of the two extreme aromatic rings is at least 7.3 Å. The biological properties include the ability to inhibit the polar transport of auxin, abolish the apical dominance effect, and prevent the geo- and phototropic responses.

N-1-Naphthylphthalamic acid (structure III, Table I) has been shown to be a member of this class (16) and is known to be a potent inhibitor of auxin transport and geotropic curvature (22). NPA[1] has further been shown to bind to fractions containing plasma membrane vesicles from maize coleoptiles (20, 31, 32). These initial findings were confirmed using density gradient procedures and a wider number of markers (8, 23, 24). Thus, the presence of an NPA binding site has been demonstrated and it has also been shown to have a relatively low affinity for the known phytohormones (31, 32).

Drug receptors, that is, receptors for xenobiotic substances which achieve a physiological effect, were postulated (or at least implied) long ago in animal physiology (6, 18), and it now would seem appropriate to extend the concept to the phytotropin area. Thus, it is suggested that NPA binding sites may possibly represent receptor sites for phytotropin action. It has been found (R. Hertel, personal communication) that CPD can bind to the NPA binding site, while Sussman and Goldsmith (30) have found that fluoresceins can also compete. In general, however, the means available for assessing whether a binding site may function as a receptor is to compare specificity of binding with the specificity of the physiological response toward a series of analogs. The specificity of the NPA binding site from maize coleoptile membrane vesicles toward NPA and a series of model compounds was examined by measuring the ability of the compounds to compete for the NPA-binding site and comparing this with estimates of physiological activity. Three physiological processes which phytotropins are known to affect, namely auxin transport, root geotropism, and phototropism, were estimated in experiments on cress seedlings and bean petioles and from information in the literature.

MATERIALS AND METHODS

Chemicals. [2,3,4,5,^{3}H]-*N*-Naphthylphthalamic acid (16 Ci/mmol) was obtained from CEA (Gif-sur-Yvette, France). Unlabeled NPA was prepared by the method of Meyer and Wolfsleben (21). The *iso* indolones VII and VIII, CPP V and phenacylphthalide (7), CPD IV and phenacylidenephthalide VI (3, 4), 2-(1-naphthoyl) benzoic acid (11), PBA (14), and 2-(4-chlorophenyl)benzoic acid (10) were synthesized by published methods. Other chemicals were obtained from commercial sources, mainly Aldrich Chemical Company.

Plant Material for Binding Assay. Maize seeds (*Zea mays* cv. INRA 258) were allowed to germinate on damp filter paper in the dark at 25°C. After two days, embryos were exposed for 2 h to red light (MAZDA fluorescent tubes with a ROHM filter ROT 501). The intensity of light received by the embryos was 2,300 ergs·cm^{-2}·s^{-1}. After 5 days, coleoptiles were separated from primary leaves and kept chilled on ice. Harvesting and all subsequent procedures were performed in daylight.

Isolation of Membranes. The membrane preparation was performed at 0 to 4°C. Coleoptiles (100 g, fresh weight) were homogenized in a mortar in 100 ml of medium containing 0.5 M D-mannitol, 1 mM EDTA, 5 mM 2-mercaptoethanol, 0.5% BSA, and 0.1 M Tris-HCl (pH 7.6). The homogenate was filtered through a nylon cloth having a pore diameter of 50 μm. During grinding, the pH of the medium was maintained at 7.6 with 1 M Tris base. The filtrate was first centrifuged at 12,000*g* for 15 min to eliminate unbroken cells, cell wall fragments, starch, nuclei, and most of the mitochondria. The resulting supernatant was centrifuged at

[1] Abbreviations: CPD, 1-(2-carboxyphenyl)-3-phenylpropane-1,3-dione, IV; CPP, 5-(2′-carboxyphenyl)-3-phenylpyrazole, V; DPX1840: 3,3a-dihydro-2-(4′-methoxyphenyl)-8H-pyrazolo(5-1a) isoindol-8-one, VIII; NPA, *N*-(naphth-1-yl)phthalamic acid, III; PBA, 2-(1-pyrenoyl)benzoic acid, II.

100,000*g* for 60 min. The membrane pellet so obtained was then resuspended in 100 ml of 0.1 M Tris-HCl (pH 7.6) and centrifuged at 100,000*g* for 60 min. The membrane pellet was finally resuspended in the binding medium consisting of 0.5 M sucrose, 5 mM $MgSO_4$, and 20 mM citric acid-sodium citrate (pH 5.5), to about 0.6 mg protein · ml^{-1}.

Binding Assay. This assay is based on the centrifugation method for particle-associated ligand-binding sites, widely used for membrane-bound hormone receptors (9). Experimental conditions for the assay were mainly derived from the works of Ray *et al.* (25, 26) on auxin binding sites.

Binding experiments were performed at 0 to 4°C in 1 ml polycarbonate tubes. [^{3}H]NPA (0.17 μCi) in 20 μl ethanol was added to 1-ml samples of membrane suspension in binding medium to give a final ligand concentration of 10^{-8} M. Various concentrations of unlabeled NPA or compounds to be tested were then added in 20-μl ethanolic solutions. Control samples were obtained by the addition of 20 μl ethanol. After 30 min (the time shown to be necessary for the NPA-NPA binding site complex to reach equilibrium [34]), the tubes were centrifuged for 30 min at 35,000*g*. After pelleting, the supernatants were discarded and the tube walls carefully wiped with paper towels. Membrane pellets were allowed to dissolve overnight in 300 μl 10 mM Tris-HCl (pH 8), containing 4% Triton X-100. The tube contents were transferred to scintillation vials, and the tubes were rinsed with 200 μl Triton X-100 solution. Ethanol (500 μl) and Bray's solution (10 ml) were then added, and radioactivity was measured, using a Packard liquid scintillation spectrometer.

[^{3}H]NPA binding (cpm/pellet) measured in the presence of a high concentration of unlabeled NPA (10^{-4} M) is termed nonspecific binding (5, 9). It comprises low affinity or partition binding of the labeled ligand to the membrane and also of the unbound labeled ligand molecules trapped inside the membrane vesicles and the pellet. It reflects the existence of a nonsaturable component of ligand binding to the membrane.

After substraction of nonspecific binding from [^{3}H]NPA binding (cpm/pellet) measured in the absence of unlabeled NPA, specific binding is obtained (5, 9). Specific binding reflects the existence of a saturable component of ligand binding to the membrane. This saturable binding has been shown to be reversible and of high affinity (32, 34) and is expected to represent the first step of the molecular mechanism of ligand action *in vivo*. Under the described test conditions, the average ratio of specific binding to nonspecific binding is 3.7.

Displacement Curves of [^{3}H]NPA Binding by Structural Analogs. Twenty-one compounds have been tested for interaction with NPA binding sites of corn coleoptile membranes by competition with 10^{-8} M [^{3}H]NPA. With the exception of carboxy-fluorenone, each compound was tested in at least two separate experiments. Displacement curves were established, plotting [^{3}H]NPA binding (cpm/pellet) *versus* the negative logarithm of the concentration of tested compounds. In each assay series, a displacement curve of [^{3}H]NPA binding by unlabeled NPA was established for the determination of nonspecific and specific binding. The displacement curves of all the tested compounds (except Ia and IIb, which are inactive) have a shape similar to that of the displacement curve for unlabeled NPA (data not shown). At sufficiently high concentrations of any of the more effective compounds, [^{3}H]NPA binding is reduced to the level of nonspecific binding. This indicates that the compounds affect specific but not nonspecific binding.

Measure of Apparent Dissociation Constants of Binding of Tested Compounds. The concentration of a given compound which reduces one-half of the specifically bound radioactivity is a measure of the apparent dissociation constant of this compound toward the NPA binding sites and is termed K_D app. Results were generally expressed as the negative logarithm of K_D app, also termed pK_D app (see Tables I, II, and III).

***In Vivo* Assays.** The assays for auxin transport activity (15), root geotropism on cress seedlings (12), and phototropic response on cress seedlings (11), have been described previously.

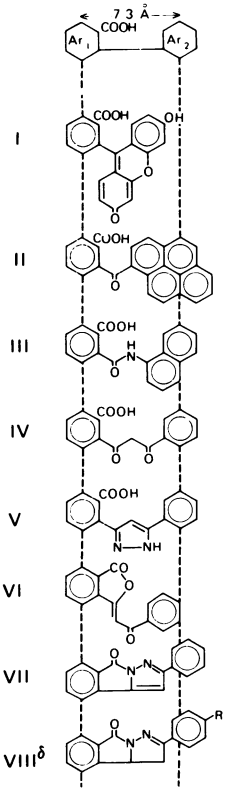
RESULTS

Representatives of the various chemical types of phytotropins (16) are shown in Table I. These include the fluoresceins I and the aroylbenzoic acids, represented by PBA II, with NPA III representing the arylphthalamic acids, CPD IV the propanediones, and CPP V the carboxyphenylpyrazoles. The pyrazoloisoindolone VIII, R = H is an analog of DPX1840 VIII; R = OCH_3 (1). Compounds with high activities in inhibiting auxin transport, geotropism, and phototropism (II–VII) have high binding affinities. Also, the isoindolone VIII appears to have a lower binding affinity than might be expected from its *in vivo* activity. However, it has been shown that the active form of the analogous DPX1840 corresponds to the pyrazole V (2), so that an oxidation step as well as hydrolysis is required to convert it to this form, and there may be differences in this regard between the *in vitro* and *in vivo* systems.

If the NPA binding site is, in fact, a general phytotropin binding site, then affinities for the site should be related to the chemical parameters deduced for phytotropin activity, as outlined above. Selected compounds were, therefore, examined to determine whether the requirements for phytotropin activity could be the same as those for binding affinity.

Table I

Apparent Dissociation Constants of Binding of Some Chemical Types of Phytotropins and Comparison with Biological Activities.



	pK_D● app.	Auxin* transport	Root† geotropism	Stem‡ phototropism
I	5.1	95	10^{-4}	10^{-3}
II	7.2	24	10^{-9}	3×10^{-7}
III	7.2	30	10^{-6}	10^{-5}
IV	6.75	32	3×10^{-8}	10^{-6}
V	7.3	16	10^{-7}	3×10^{-7}
VI	6.75	61	3×10^{-8}	10^{-6}
VII	6.75	25	3×10^{-8}	10^{-6}
VIII$^{\delta}$	3.9	67	10^{-5}	3×10^{-4}

* % transmission at 10^{-7} M
† lowest concentration at which root georesponse is abolished
‡ lowest concentration at which phototropic response is lost
δ results shown are for R = H
● pK_D app: Molar concentration (K_D app.) giving 50% reduction of specific (^{3}H)–NPA binding expressed as its negative logarithm.

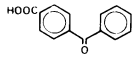
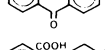
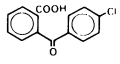
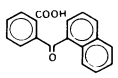
The requirement for a second aromatic ring in the Ar_2 portion of the molecule, together with stereospecific requirements of *ortho* substitution and chain length of Ar_2, were examined using the chemically similar aroylbenzoic acids. The compounds used are shown in Table II. A second aromatic ring is required for phytotropin activity, and there would appear to be a similar requirement for binding activity (compare IIa with II, IV, and IIc). Chemically, IIb and IIc are essentially identical, yet IIb is inactive in all assays, while IIc elicits significant activity. We conclude that, as in phytotropin action, there may be a stereospecific requirement for binding, in that the Ar_2 group cannot be *para*-substituted on Ar_1 with respect to the carboxyl group. The effect of increasing the length of Ar_2 can be seen by comparing IIb to IIe in Table II together with II in Table I. Again, the compounds are chemically similar, and both binding affinity and activity are increased by increasing the length of Ar_2. When the molecule is longer than the postulated minimum size for high activity (as in II), binding is very strong. Again, requirements for *in vitro* binding appear to be similar to those required for physiological activity. The results presented in Table III are also consistent with this conclusion. An increase of the chain length of Ar_2 by a chlorine atom increased binding activity by 1 order of magnitude and significantly increased phytotropin action (compare IX and IXa, Table III). Further stereochemical factors were explored by comparison of IX with IXb in Table III. The latter compound can be regarded as a bridged *meta*-substituted phenyl benzoic acid. It can be seen that the two compounds have similar binding affinity and auxin transport inhibiting activity, but only the *ortho*-substituted and conformationally mobile IX has activity in the geotropic and phototropic assays. This result appears anomalous, suggesting that further examination is needed and will require the synthesis of additional model compounds.

Similarly, the carboxyfluorenone, IIg, also has a fixed conformation. It has a bridging ketone group, making it electronically similar to IIc, while its overall length is essentially the same as that of IX. Although its binding affinity is of the same order of magnitude as that of these latter two compounds, it is less active than IX. The possibility is, thus, raised that the phytotropins may have to be conformationally mobile to have significant physiological activity, but further research is required to confirm this point. A possible conformational requirement for inhibition of geotropism has been suggested previously (17).

The importance of a carboxylic group for binding is shown by the much lower affinities of IVa and IVb in Table III as compared with IV in Table I. In the first compound, the carboxyl is replaced by a hydroxyl group, while in the second, no acidic group is present. The requirements for binding again are similar to those required for biological activity. A requirement for conjugation or planarity of the Ar_2 portion of the molecule is shown by comparison of Ia and VIa (Table III) with I and VI (Table I), respectively. Compounds VII and VIII (Table I) can also be compared. Both binding and activity of the less conjugated molecules are markedly reduced. It should be pointed out, however, that all but compound I of those used to assess the conjugation requirement are either lactones or amides rather than free acids, so that other explanations for differences in activity are possible. For example, there may be differences in hydrolysis rates of the molecules between the *in vitro* and *in vivo* assays.

Table II

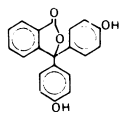
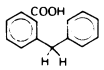
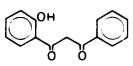
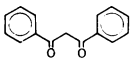
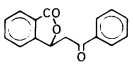
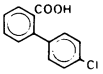
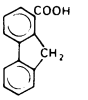
Effect of Increasing Molecular Length on tne Apparent Dissociation Constants of Binding and Comparison with Biological Activities.

		pK_D app.	Auxin* transport	Root geotropism	Stem phototropism
IIa		2.75	>90†	$>10^{-3}$	$>10^{-3}$
IIb		inactive	>90†	$>10^{-3}$	$>10^{-3}$
IIc		3.25	68	10^{-4}	10^{-3}
IId		4.7	21	3×10^{-6}	3×10^{-5}
IIe		4.8	15	10^{-6}	3×10^{-4}

* % transmission at 10^{-5} M
† 67% at 10^{-4} M
‡ 39% at 10^{-3} M

Table III

Effect of Compounds Lacking a Requirement for High Activity on the Apparent Dissociation Constants of Binding and Comparison with Biological Activities.

		pK_D app.	Auxin† transport	Root geotropism	Stem phototropism
Ia		inactive	83	$>10^{-3}$	$>10^{-3}$
IIf		3.2	18	10^{-3}	10^{-3}
IIg		3.2	42	10^{-3}	10^{-3}
IVa		3.9	>90‡	$>10^{-3}$	$>10^{-3}$
IVb		3.5	65	$>10^{-3}$	$>10^{-3}$
VIa		4.15	5	10^{-5}	3×10^{-4}
IX		3.8	16	3×10^{-5}	3×10^{-4}
IXa		4.8	12	3×10^{-6}	10^{-4}
IXb		3.9	23	10^{-3}	10^{-3}

† % transmission at 10^{-4} M
‡ 53% at 10^{-3} M

DISCUSSION

A representative of each of the various chemical types of phytotropins can bind to the NPA site, with compounds II to VII binding strongly (Table I). Also, there is a positive correlation between binding affinities and the structure-activity rules, such that compounds which fulfill all of the rules tend to bind strongly, while those which do not have a reduced affinity. The site, therefore, exhibits a chemical specificity as well as a stereochemical specificity, to the extent that *ortho* substitution and a minimum length are required for high affinity. There may be conformational requirements, also, but the question is by no means settled (17). It is concluded that the NPA binding site exhibits specificity towards

the phytotropins and can be described as a phytotropin-binding site.

The correlation between binding affinity and physiological activity exists, even though the plant species used to assess these two aspects are different. Additional evidence for the correlation is the finding that several fluoresceins (analogs of structure I) have affinities which correspond to their effect on auxin uptake in corn coleoptile tissue (30). From this, we conclude that there is a similarity in chemical and stereochemical specificity between the binding site and the presumed receptors which give rise to physiological activity in the plant material tested.

The morphactins are also known to interact with the site (33), and it has been shown that the phytotropin class is potentially a large one (16). The range of chemicals which can interact with the site may, therefore, be capable of further definition. The reason that both phytotropins and the chemically different morphactins can compete for the same site is unclear. It can be pointed out, however, that the morphactins can be chemically converted to 2-phenylbenzoic acids (*e.g.* IX) (10), which have been shown here to be phytotropins.

Whereas the relationship between binding and activity is not strictly proportional in all cases, it is suggested that the demonstrated affinity-activity relationships are of the kind encountered (27, 35) in animal pharmacology as well as those observed for the hormonal binding of auxins (26). Reasons for variations could be minor differences in specificity between the binding site isolated from maize and the receptors giving rise to activity in the different plant material tested as well as differences in efficacy, uptake, sequestration, conjugation, and metabolism due to secondary chemical differences between the molecules. It is suggested that the observed relationship between binding and activity can be accounted for by assuming that the compounds tested are agonists of about equal efficacy (capacity to elicit a response when bound to the recetpor site [27]). Then, high affinities should give rise to correspondingly high activities. It is, therefore, possible that the phytotropin-binding site may be a receptor.

Criteria generally used to determine whether ligand binding to a membrane fraction is, in fact, occurring at receptors include factors such as specificity, stereospecificity, and appropriate kinetics (19). It is suggested that these factors are present here and that the phytotropin-binding site may, therefore, be an intrinsic drug receptor which could give rise to phytotropin action. That the site may be a receptor is implicit in the work of Thomson and Leopold (33), and it is explicitly regarded as a receptor by Sussman and Gardner (28). The determination of whether this is so, however, will depend upon the establishment of a direct cause and effect relationship between binding to the site and a physiological response.

In this regard, the recent work of Sussman and Goldsmith (29, 30) is of interest. These authors assessed the effect of NPA and other compounds on auxin uptake in the same tissue from which the binding site is obtained, namely, maize coleoptiles. Here also, NPA was regarded as achieving its effect at a specific receptor. This was because the rapidity and specificity of the effect of NPA on auxin flux is such that localization of the receptor on the plasma membrane would be expected, and the binding site is, in fact, found in a plasma membrane fraction. In addition, it must interact with the auxin carrier, which would also be on the plasma membrane (29, 30; M. R. Sussman, personal communication). A cause and effect relationship is, thus, indicated, although the evidence remains as yet circumstantial.

The phytotropins are all known to be auxin-transport inhibitors (15), and their solubilized receptor(s) have been shown to have weak affinities for the auxins α-NAA, IAA, and the weak auxin β-NAA, although the membrane bound receptors do not show such affinities (28, 31, 32). On this evidence, the possibility was raised that the receptor may be one conformation of an auxin binding site involved in polar auxin transport (28). While this is a possibility, the affinities of these auxin analogs ($K_D \simeq 10^{-5}$ M) are two orders of magnitude less than those for NPA ($K_D \simeq 10^{-7}$ M). On this model, because phytotropins have specific and different structure-activity requirements from those deduced for auxin activity (13, 15), the auxin binding site involved in polar auxin transport would have quite different specificity from those involved in auxin-induced growth. On the other hand, it is shown here that the common chemical and structural properties of the phytotropins are essential requirements for both auxin transport inhibition in plants and in *in vitro* binding to membrane-bound phytotropin-binding sites. The auxin molecule does not possess these properties and has a very poor affinity for the phytotropin binding site. Further, at concentrations as high as 100 μM, auxin does not detectably displace NPA taken up by corn coleoptile tissues (30). These facts would be in favor of an auxin transport system composed of an auxin carrier with a specific recognition site for auxin (effector unit) together with a related phytotropin-specific-binding site (regulatory unit). From the above considerations, we conclude that the phytotropin-binding site may well be an intrinsic drug receptor involved in the polar transport of auxins.

It can be argued that there would be no survival benefit for plants to have recognition sites for synthetic chemicals, so that intrinsic receptors should have natural intrinsic agonists. By such reasoning, recent extensive work on morphine receptors in animals has led to the discovery of endomorphines or endorphins. By analogy, the interesting possibility is raised that endotropins may also exist, and further elucidation of the nature, structure, and function of phytotropin receptors may be of importance to the understanding of hormone-controlled mechanisms of plant growth and development.

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