A HIGH AFFINITY RECEPTOR FOR INDOLEACETIC ACID IN CULTURED TOBACCO PITH EXPLANTS

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1. Introduction

The mechanism of action of auxins in long-term responses of plant tissue to these hormones is still unknown. However, it is very likely that the first event in the chain of processes leading to, for instance, cell division or differentiation, is coupling of the auxin with a cellular binding site. It is therefore logical to start an investigation on the mechanism of action of indoleacetic acid (IAA) with a study of its primary binding site(s) or receptor(s). Such a receptor should meet criteria derived from physiological effects. It should have a high affinity (association constant $K_a = 10^7 - 10^8\ M^{-1}$) and a low capacity; the auxins which are bound should probably show structure–binding relations similar to the structure–activity relations in physiological responses.

The localization and characteristics of the receptor depend, of course, very much on its function in the cell. The work presented here is based on the assumption that this function is performed in the nucleus. Therefore, we mainly investigated the cytosol fraction and not the membrane fraction of our extracts, for localization of the receptor in the plasma membrane would probably mean that a second messenger is involved in its function. In this paper we present evidence that a receptor for IAA with the expected properties does exist, and is localized in the cytosol fraction of tissue cultures derived from tobacco stem pith.

Investigations concerning the function of this receptor in the cell are in progress.

2. Material and methods

2.1. *Plants*

Tobacco plants (*Nicotiana tabacum* var. White Burley) were grown at 24°C in a greenhouse with continuous additional light. They were used 8–10 weeks after sowing when they were 50–60 cm tall.

2.2. *Explants*

Fresh explants of pith tissue were punched out of stem pieces at 4°C and were immediately used for protein extraction. The whole stem was used.

For culturing, defoliated stems were rinsed with water followed by ethanol, and surface-sterilized for 15 min in a commercial hypochlorite solution. After two 10 min rinses in sterile distilled water, about 2 mm thick explants, varying in diameter from 4–10 mm, were cut and placed in 15 cm Petri dishes with 100 ml of solid Murashige and Skoog medium [1] containing kinetin (0.2 mg/l) and potassium-IAA or naphtylacetic acid (2.4 mg/l). 40–60 explants (depending on diameter) were placed in one dish, after which the dishes were sealed with parafilm. The explants were cultured for 5–7 days in the greenhouse (24°C, continuous light).

2.3. *Protein extraction*

Fresh or cultured material (about 100 g) was homogenized for 30 sec in a Waring blendor with 100 ml of buffer (60 mM Tris–HCl, pH 7.8; 3 mM 2-mercaptoethanol and 0.3 mM EDTA). Homogenization was completed in a glass homogenizer accord-

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ing to Potter-Elvehjem (3 strokes at 1500 rev/min).

After filtration through 4 layers of nylon cloth (30 mesh) the homogenate was centrifuged at 65 000 *g* for 2 h. The clear supernatant (200 ml) was dialysed overnight against TME buffer (20 mM Tris–HCl, pH 7.8; 1 mM 2-mercaptoethanol and 0.1 mM EDTA) in a stirred ultrafiltration cell with an Amicon PM10 membrane. The extract was concentrated 10 times, centrifuged at 2900 *g* for 15 min and immediately used in a binding assay. All procedures were carried out at 0–4°C.

2.4. *Binding assay*

1 ml of extract was added to 1 ml of TME buffer containing different amounts of unlabelled IAA and [^{3}H]IAA (spec. act. 29 Ci/mmol; Schwarz/Mann, New York). For each different auxin concentration a blanc was added with 1 ml of TME buffer instead of 1 ml of extract. After a 20 min incubation at 30°C the (polypropylene) tubes were transferred to an ice-bath and cooled for 30 min. In between a 50 μl sample was taken to check the amount of added labelled auxin. Then 1 ml of a dextran-coated charcoal suspension (0.5 l g of Dextran T70 and 5.1 g of Norit A in 100 ml of TME buffer) was added. After thoroughly mixing the tubes were placed on a shaker in the cold room for 60 min. They were centrifuged twice at 2900 *g* for 20 min to remove the charcoal. Then a 0.8 ml sample was added to 8 ml of a Toluene-Triton X 100 scintillation liquid and counted in an LKB-Wallac scintillation counter.

2.5. *Protein determination*

The protein concentration in the extracts was determined with the biuret method according to Gornall et al. [2].

3. Results

Calculations based on estimated association constants ($K_a = 10^6$ 10^8 M^{-1}) and estimated numbers of binding sites (0.5 – 5 pmol/ml) revealed that a possible binding of auxin would not be detectable with equilibrium techniques, since this binding would be of the same magnitude as experimental deviations. We therefore used a non-equilibrium technique, viz., the dextran-coated charcoal (DCC) method [3].

Before we could use this method we had to establish that IAA does bind to DCC. We therefore added a DCC suspension to a solution containing 10^6 cpm [^{3}H]IAA/ml. After the time indicated in table 1 a sample was taken and centrifuged at 2900 *g* for 10 min, and the radioactivity in the supernatant was determined. From table 1 it is clear that after 30 min only 0.15% of the original activity was not bound to the charcoal. This amount could not be removed by prolonged treatment with DCC or addition of more DCC. To all subsequent experiments blanks were added to correct for this amount of non-bound IAA.

In order to obtain data on the association constant and the number of binding sites, binding experiments have to be performed at different hormone concentrations. Usually this is done by raising the concentration of unlabelled hormone, keeping the concentration of labelled hormone constant. Our calculations revealed that in our case the radioactivity of the samples with high IAA concentration would be very low, so we also raised the concentration of labelled IAA according to the scheme presented in table 2.

The data from the binding assays are presented as Scatchard plots [4]. For non-cultured tissue the result was an almost horizontal line (fig.1). This means that in extracts from normal tobacco pith tissue only a 'low affinity, high capacity' (or non-specific) binding is present, whereas the concentration of specific binding sites might be too low to be detected. The result for cultured tobacco pith explants is shown in fig.2. Apart from the 'low affinity, high capacity' binding the presence of a 'high affinity, low capacity' (or specific) binding component is evident. After subtraction of the low affinity binding from the total binding, the associa-

Table 1
Binding of ^{3}H-IAA to dextran-coated charcoal

Time (min)	cpm per ml supernatant
0	4020
15	2300
30	1660
45	1400
60	1580

For explanation see text.

Table 2
Quantities of IAA used in binding-assays

[^{3}H]IAA (pmol)	IAA (pmol)	total IAA (pmol)	final concentration (M)
10	–	10	5×10^{-9}
15	–	15	7.5×10^{-9}
20	–	20	1×10^{-8}
25	–	25	1.25×10^{-8}
50	–	50	2.5×10^{-8}
50	50	100	5×10^{-8}
50	100	150	7.5×10^{-8}
50	250	300	1.5×10^{-7}
50	450	500	2.5×10^{-7}
100	900	1000	5×10^{-7}
100	1900	2000	1×10^{-6}

tion constant could be calculated. For different experiments the K_a varied between 0.7×10^8 M^{-1} and 1.5×10^8 M^{-1} (see table 3). The mean value of K_a is 1×10^8 M^{-1}.

The real value is probably somewhat higher because we did not correct for the dissociation occurring during the incubation with DCC. The concentration of binding sites was very low: about 0.05 pmol/ml of extract was found for all experiments. Pronase treatment of the extract completely destroyed the high affinity binding. It is therefore very likely that this specific binding component is a protein.

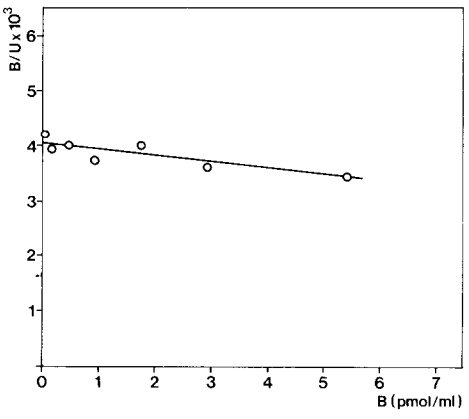

Fig.1. Scatchard plot ((bound/free) × 10^3 vs. bound (pmol)) of IAA binding by extracts from non-cultured tobacco pith tissue. Only a low affinity binding, represented by an almost horizontal line, can be seen.

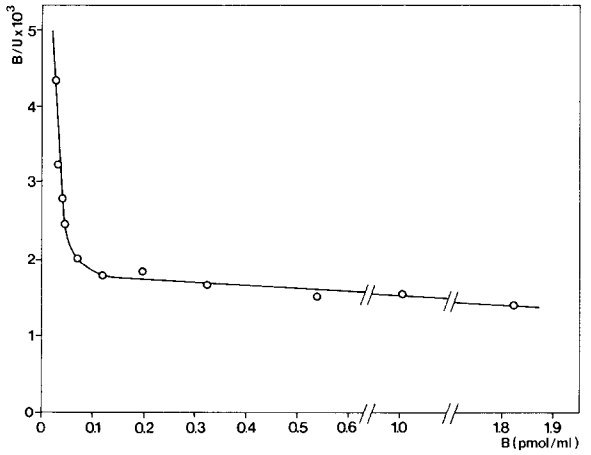

Fig.2. Scatchard plot of IAA binding by extracts from cultured tobacco pith explants. The steeply descending part of the curve represents the kinetics of the high affinity binding. The protein content of the extracts was 5 mg/ml. The concentration of binding sites was 0.01 pmol/mg of protein.

Table 3
K_a values determined in different experiments

Auxin in culture medium	K_a
IAA	1×10^8
IAA	0.8×10^8
IAA	1.1×10^8
IAA	0.7×10^8
NAA	1.5×10^8

4. Discussion

Our results clearly indicate the presence of a receptor for IAA in the cytosol of cultured tobacco pith explants (experiments with resuspended membrane fractions showed no signs of high affinity binding). The specificity of this receptor remains to be determined. We are now performing experiments with different auxins and non-auxins as competitors for [^{3}H]IAA in the binding assays. However, since the association constant is high (10^8 M^{-1}), the binding will probably appear to be very specific. In addition table 3 shows that a receptor with the same association constant appears in the cytosol of tissue cultures grown in the presence of NAA.

That this non-membrane bound receptor has up to now escaped the attention of other investigators is mainly due to the following facts: (1) most investigators did not perform experiments on saturation kinetics. (2) If they did (cf. Hertel et al. [5]) they used 10^{-7} M as the lowest auxin concentration, while at this concentration our receptor is already completely saturated (and, therefore, is considered to be non-specific).

It is, of course, very tempting to speculate about the function of the found receptor protein. Because it is not localized in the plasma membrane it cannot play a part in the fast auxin responses thought to occur at this site. However, its localization is very well suited to mediate IAA-influences on transcription. The existence of proteins able to alter transcription in conjunction with IAA has already been established (Matthysse and Phillips [6], Mondal et al. [7]). Therefore we are now trying to purify the receptor in order to determine a possible influence on transcription. In addition we are trying to exactly localize the receptor (cytoplasm or nucleus) and to investigate the possibility of an auxin-induced translocation.

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