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Stimulation of phospholipase A_2 by auxin in microsomes from suspension-cultured soybean cells is receptor-mediated and influenced by nucleotides

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Abstract. The molecular mechanism of membrane-associated reactions induced by auxin was investigated in membranes isolated from cultured cells of soybean (*Glycine max* L.). Auxins increased the activity of phospholipase A_2 in microsomes isolated from suspension-cultured soybean cells. The reaction was measured as the accumulation of radioactive lysophosphatidylcholine hydrolyzed from radioactive phosphatidylcholine in membranes which had been prelabelled with [^{14}-C]choline in vivo. Stimulation by auxin was detectable after 1 min and was auxin-specific in that weak auxins had little effect. Auxin concentrations as low as $2 \cdot 10^{-8}$ M and up to $2 \cdot 10^{-3}$ M α-naphthaleneacetic acid already stimulated the phospholipase A_2 activity. Guanosine and adenosine diphosphate at 100 μM, if applied during homogenization of cells, completely abolished the stimulation of phospholipase A_2 by auxin and, when applied after homogenization, had no effect. Guanosine and adenosine 5′-O-thiotriphosphate, uridine 5′-diphosphate, and uridine 5′-triphosphate, all at 100 μM, had no effect in either treatment, suggesting that only nucleotides entrapped in the vesicles could exert an effect. The effect of auxin on phospholipase A_2 had an optimum at pH 5.5 and was abolished completely by an antibody against the membrane-associated auxin-binding protein from maize coleoptiles, applied after homogenization. This antibody recognized a 22-kDa polypeptide in highly purified plasma membranes from cultured soybean cells. This suggests a receptor function for this auxin-binding protein and a role for a cytosolic nucleotide-binding protein in the activation of phospholipase A_2 by auxin. It is concluded that phospholipase A_2 has a function in plant signal transduction.

Key words: Auxin-binding protein – *Glycine max* L. – Guanosine nucleotides – Phospholipase A_2 – Signal transduction

Introduction

Growth media for cultured plant cells contain auxin, and cultured plant cells usually completely depend on auxin to sustain growth. Recent observations also show that membranes from cultured plant cells bind and respond to auxin (LaSchiavo et al. 1991) so that they can be expected to exhibit basic membrane-associated responses which may occur in many plant tissues. We have investigated the response of cultured soybean cells and of membranes isolated from them, and of rapidly growing zucchini hypocotyl tissue and found in both a rapid activation of phospholipase A_2 by auxin (Scherer and André 1989; André and Scherer 1991). The use of cultured plant cells as a source to isolate membranes enabled us to further analyse details of the auxin activation of phospholipase A_2 (PLA_2) in plant membranes.

Phospholipase A_2 is part of the signal-transduction pathways in animal cells (Burch et al. 1986; Jelsema 1987; Jelsema and Axelrod 1987; Burch et al. 1988; Brooks et al. 1989; Murayama et al. 1990; Narasimhan et al. 1990; Silk et al. 1990; Teitelbaum et al. 1990). In animals the physiologically important metabolite is arachidonic acid generated by PLA_2 which is a precursor of prostaglandins and leucotrienes which have many physiological roles, e.g. in inflammatory reactions (Chang et al. 1987; Samuelson and Funk 1989). For plants we have postulated that lysophospholipids can act as second-messenger-like lipid mediators since these lipids and the structurally similar platelet-activating factor activate a membrane-associated protein kinase (Martiny-Baron and Scherer 1988; Martiny-Baron and Scherer 1989) and H^+-ATPase (Scherer 1985; Martiny-Baron and Scherer 1989; Palmgren and Sommarin 1989) so that in principle, a chain of reactions could lead from auxin to the plasma-membrane H^+-ATPase, involving the membrane-associated auxin receptor, PLA_2 and a lipid-

Abbreviations: ABP = auxin-binding protein; ATP γ S = adenosine 5′-O-thiotriphosphate; 2,4-D = 2,4-dichlorophenoxyacetic acid; GTP γ S = guanosine 5′-O-(thiotriphosphate); IgG = immunoglobulin G; LPC = lysophosphatidylcholine; α-, β-NAA = α, β-naphthaleneacetic acid; PLA_2 = phospholipase A_2

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activated protein kinase (Scherer 1990; Scherer et al. 1990; André and Scherer 1991).

Moreover, recent experiments have shown that a fatty acid, linolenic acid, is the precursor to a plant hormone, jasmonic acid, with a biosynthetic pathway reminiscent of leukotrienes (Anderson 1989), and that both this fatty acid and jasmonic acid have striking biological effects in wounding responses (Farmer and Ryan 1992), in tendril coiling (Falkenstein et al. 1991) and in accumulation of secondary plant metabolites (Gundlach et al. 1992). The fatty-acid-releasing enzyme has not yet been identified but, conceivably, it could be a PLA_2 so that the similarities to animal systems would extend even further. Since cultured soybean cells are a convenient experimental system we present here some properties of the auxin-dependent PLA_2 reaction in membranes isolated from them. We found that it is receptor-mediated and influenced by nucleotides, suggesting that a nucleotide-sensitive protein might take part in the signal-transduction pathway proposed by us.

Materials and methods

Preparation of radioactive membranes. Soybean (*Glycine* max. L.) cells were grown in suspension culture as previously described (Scherer and Nickel 1988) in Gamborg B5 medium. Five days after inoculation, 50 ml of cell suspension was transferred into 200 ml of fresh auxin-free medium and used for experiments after 4 d. Cells were transferred into fresh auxin-free medium and 0.19–0.37 MBq [^{14}C]choline (1.85–2.2 GBq · mmol^{-1}; Amersham, Braunschweig, FRG) was added. After 3 h the medium was removed by gentle suction. The cells were transferred in homogenization buffer consisting of 0.5 M sucrose, 1 mM ethylenetetraacetic acid (EDTA), 1 mM dithioerythritol and 15 mM tris(hydroxymethyl)aminomethane-HCl (Trizma; pH 7.5), and homogenized for 10 min on ice with a mortar and pestle. The homogenate was filtered through Miracloth (Calbiochem, La Jolla, Calif., USA), centrifuged for 10 min at 5000 · g and the resulting supernatant centrifuged again for 30 min at 50000 · *g*. The pellet was resuspended in 1.5 ml incubation buffer consisting of 250 mM sucrose, 200 mM N-morpholinoethanesulfonic acid(Mes)/Na^+ pH 5.5 (or of other desired pH values), and 1.5 mM $MgCl_2$. Other compounds were added as indicated in the figure legends.

Phospholipase A_2 assay. To 80 µl of microsomal fraction (containing 80 µg protein), 20 µl of incubation buffer, containing auxin and other additions, or incubation buffer containing the same amount of ethanol alone (controls), was added. In experiments with antibody to the membrane fraction the antibody was added and the reaction started after 5 min preincubation on ice by addition of auxin. The final ethanol content was always 1% and the incubation temperature was 25° C. The assays were stopped at the desired time with 0.4 ml chloroform:methanol (1:2, v/v) and the lipid was extracted (Bligh and Dyer 1959). The whole chloroform phase was submitted to thin-layer chromatography in a solvent system of chloroform:methanol:acetone:acetic acid:water 106:28:40:20:10 (by vol.) on silica gel plates. Chromatograms were stained with iodine vapour and relevant lipids were identified by co-chromatography of standards. The silica gel was scraped off after the complete evaporation of iodine and the radioactivity was counted in a liquid scintillation counter. Experiments were either performed with different membrane preparations and one out of a set of two to four experiments is shown (Fig. 1, 2, 4, 7, 10) or repeats with the same or several preparations were made and the statistical treatments are indicated in the legends or by the figures themselves (Fig. 3, 5, 6, 8, 9).

Preparation of membranes by free-flow electrophoresis. Plasma membranes were prepared by two-phase partitioning and subsequent free-flow electrophoresis (Scherer et al. 1992).

Immunoblotting. Membrane protein was solubilized by 2 min boiling in 125 mM Tris-HCl pH 6.8, 8% (w/v) sodium dodecyl sulfate, 20% (v/v) glycerol, and 0.01% (w/v) bromophenol blue and subjected to sodium dodecyl sulfate gel electrophoresis in a 12.5% gel 10 × 10 cm^2. Immunoblotting was done for 1 h at 10 V (constant voltage) onto nitrocellulose sheets. The nitrocellulose was blocked for 2 h with 0.5% hemoglobin in TBS (50 mM tris(hydroxyaminomethyl)aminomethane-HCl, pH 8.0 in 150 mM NaCl) and then incubated overnight at 4° C and for 2 h at room temperature with primary antibody in TBS with 0.5% hemoglobin, followed by four washing steps in TBS: once in TBS (5 min), twice in TBS with 0.5% Triton X-100 (5 min each) and in TBS with 0.5% hemoglobin (5 min). Incubation with phosphatase-coupled anti-rabbit immunoglobulin-G (IgG) antibody was for 2 h in TBS with 0.5% hemoglobin followed by the same four washing steps as above in TBS, then once in borate buffer (60 mM borate, 5 mM $MgSO_4$, pH 9.5 (NaOH), and colour was developped in Fast Blue B and β-naphthyl acid phosphate (0.25 mg · ml^{-1} each) in borate buffer.

Results

In order to investigate the PLA_2 response to hormones in vitro, cultured soybean cells were labelled with [^{14}C]choline and microsomes, which contained phosphatidylcholine (PC) and lysophosphatidylcholine (LPC) as the only radioactive lipids (Scherer and André 1989), were isolated from them. Phospholipase A_2 activity can be determined by the accumulation of radioactive LPC (Fig. 1). In order to avoid pipetting errors it is more accurate to express the amount of LPC as a percentage of the total radioactive lipid. However, in all experiments an accumulation of radioactive LPC with time was found (not shown) even though there was some influence of phospholipase D (Fig. 2b) which can hydrolyze both PC and LPC (Yang et al. 1967; Witt et al. 1987). In the experiments shown here, phospholipase D had no appar-

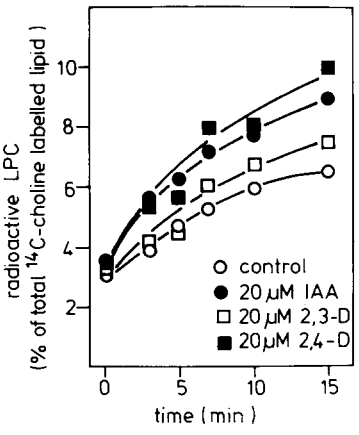

Fig. 1. Hormone specificity of the stimulation by auxins of PLA_2 in microsome prepared from cultured soybean cells. Hormones were added to the PLA_2 assays at a concentration of 20 µM to test the hormone specificity. Both IAA and 2,4-D are active auxins; 2,3-D is a weakly active auxin in growth tests (Penny and Penny 1978; André and Scherer 1991). For comparison of α-NAA and β-NAA see Fig. 2a

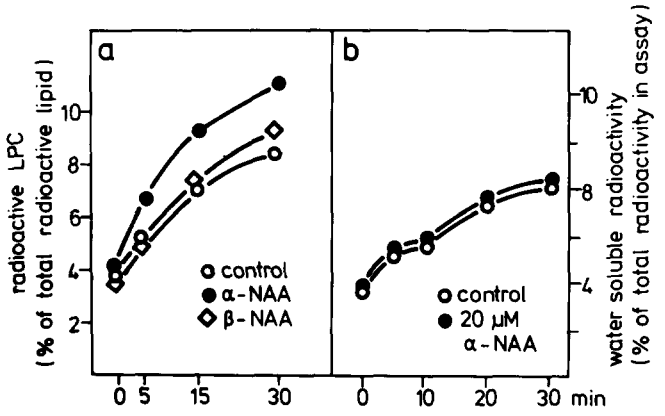

Fig. 2. a Comparison of the activation of PLA_2 by α-NAA and β-NAA (each at 20 μm) in microsomes from cultured soybean cells. ○–○, control; ●–●, α-NAA; ◇–◇, β-NAA. **b** Absence of activation of phospholipase D or phospholipase C by 20 μM α-NAA. The reaction was measured as release of labelled polar compounds into the aqueous phase of the lipid extract. ○–○, control; ●–●, α-NAA

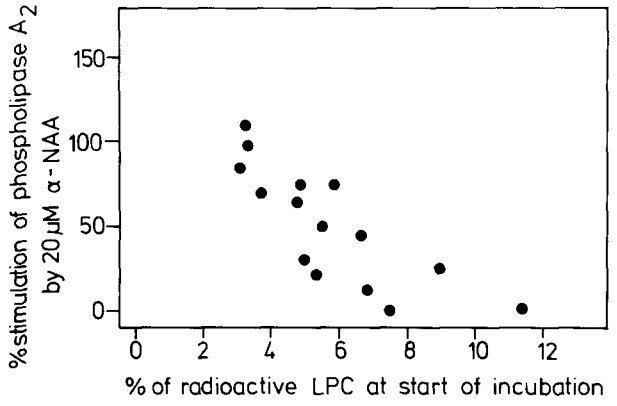

Fig. 3. Dependence of the percentage stimulation of PLA_2 activity on the percentage of radioactive LPC at the beginning of the assay. All experiments in which a 10-min datum point was taken are included in this graph. Control PLA_2 activity was set as 100% and each dot represents a single experiment. When the data were plotted as maximal percentage activation, more experiments could be included but the graph was essentially the same (not shown)

ent differential effect on the hydrolysis of PC or LPC. An even greater difficulty is the presence of at least three or more different PLA_2 activities (Hirayama et al. 1975; Huang 1987; Brglez et al. 1992; Kim et al. 1992), only one of which is probably the hormone-sensitive PLA_2. Hence, the true extent of stimulation by hormone cannot, at present, be determined in a realistic manner (see also Fig. 3) because the fraction of hormone-sensitive PLA_2 in the control assays cannot be determined. Values given, therefore, are relative values, mostly for a single experiment, but trends were always similar. Because of the rapid degradation of membrane lipids, no membrane purification was attempted.

The native auxin, indol-3-ylacetic acid (IAA), stimulated LPC accumulation in prelabeled microsomes by 80% compared to the control after 7 min treatment (Fig. 1). The active analogue, 2,4-dichlorophenoxyacetic acid (2,4–D), stimulated LPC accumulation by 100% whereas the weakly active auxin analogue, 2,3-dichlorophenoxyacetic acid (2,3–D), stimulated only by 37% (Fig. 1a). Typically, after a linear rise for about 10–15 min the velocity of LPC accumulation decreased, perhaps, because of degradation of LPC by phospholipase D and-or phospholipase C (Scherer and Morré 1978a), or degradative processes other than those induced by phospholipases. Rapid unspecific degradation of phospholipids in plants is also observed with PLA_2 (Scherer and Morré 1978b) and could explain the high apparent basal activity of PLA_2 in control assays without auxins, resulting in a low percentage of stimulation. Another active auxin analogue, α-naphthaleneacetic acid (α-NAA), also stimulated PLA_2 (Figs. 2–6) and β-naphthaleneacetic acid (β-NAA) the inactive stereoisomer, was inactive (Fig. 2a). This hormone specificity reflects the in-vivo auxin specificity of PLA_2 stimulation in soybean cells (Scherer and André 1989; Scherer 1990) and of growth stimulatory activity in zucchini hypocotyls (André and Scherer 1991). The stimulation of PLA_2 by auxin was rapid (Fig. 1; see also Fig. 2a; Fig. 5a, b; Fig. 7) and after 3 min of incubation with 2,4–D or IAA the amount of LPC was increased over the controls. In vivo, PLA_2 stimulation is also seen after 1 min (Scherer 1990) so that, in principle, this reaction is likely to precede growth stimulation by auxin which typically has a lag time of 10–15 min (Penny and Penny 1978). The sometimes longer time spans needed to observe PLA_2 activation (Fig. 8) are interpreted to be due to interfering unspecific degradative processes, as also discussed for Fig. 3.

When phospholipase D or, indistinguishably, phospholipase C in combination with phosphatidic-acid phosphatase activity, was measured as the release of radioactive choline metabolites into the water phase of lipid extracts, phospholipase D or an equivalent activity

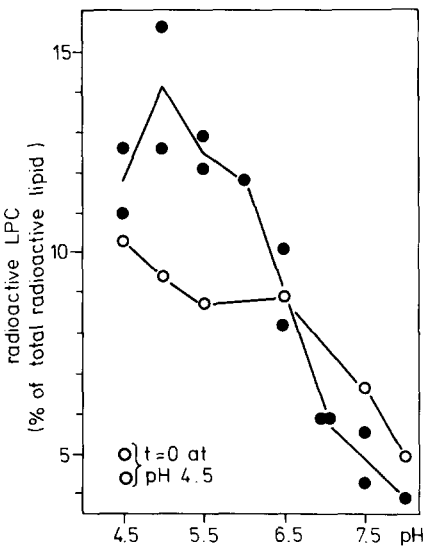

Fig. 4. Dependence of PLA_2 stimulation by 20 μM α-NAA on the pH of the assay medium. Both the pH dependence of the control without hormone (○–○) and the pH dependence of the hormone stimulation (●–●) is shown. The heterogeneous nature of the pH dependence of PLA_2 activity is evident

was quite active but no auxin sensitivity was found (Fig. 2b). In an average of four experiments the influence of 20 μM α-NAA on the release of polar radioactive choline metabolites into the aqueous phase was $-4\% \pm 16\%$ (SD).

An inherent property of the assay is that we cannot start the reaction at a defined percentage of radioactive LPC because this is determined after the assay. However, when the stimulation of PLA_2 by α-NAA was plotted as a function of the initial percentage of radioactive LPC (Fig. 3) it clearly became less, on average, as the LPC content of the membranes increased, which we interpret as indicative of unknown degradative processes.

The pH dependence of the assay showed that the PLA_2 activity probably was heterogeneous, as also described by Kim et al. (1992), and the stimulation by auxin was found preferentially at acidic pH values at around pH 5.5 (Fig. 4).

The stimulation of PLA_2 by auxin was dependent on the concentration of α-NAA (Fig. 5) with an increase in PLA_2 activity from $2 \cdot 10^{-8}$ M to $2 \cdot 10^{-4}$ M hormone and a decrease at an auxin concentration of $2 \cdot 10^{-3}$ M α-NAA. This concentration dependency of the stimulation of PLA_2 activity is similar to the concentration dependency of the growth-stimulatory effect of α-NAA (Schneider and Wightman 1978; André and Scherer 1991).

In order to test whether GTP-binding proteins (G proteins) might be involved in plant signal transduction via PLA_2, we tested the influence of nucleotides on the stimulatory effect of auxin on PLA_2 activity. As nucleotides cannot be expected to diffuse through the membrane, we tested the effects of nucleotides added after vesicle preparation or during homogenization in order to trap the nucleotides in the vesicles. Different effects were

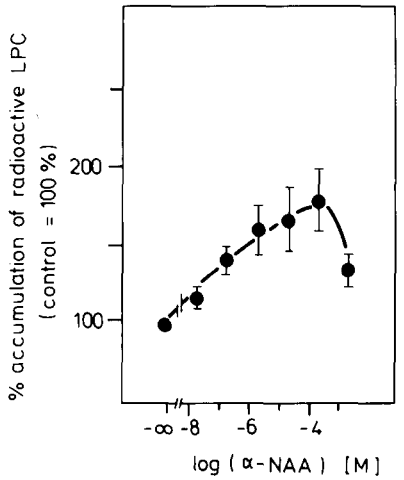

Fig. 5. Dependence of PLA_2 stimulation on the concentration of α-NAA. Average of the stimulations of PLA_2 by different α-NAA concentrations as determined by six experiments with two different membrane preparations from cultured soybean cells. To allow comparison, data are expressed as stimulation ($\pm$SD, $n=6$) above that of the controls, which was set as 100% (for $t=0$). The reaction proceeded for 30 min and the LPC content was 2.5% at the beginning and 4.7% at the end of the control reaction. Maximal stimulation was at $2 \cdot 10^{-4}$ M with 90.5%

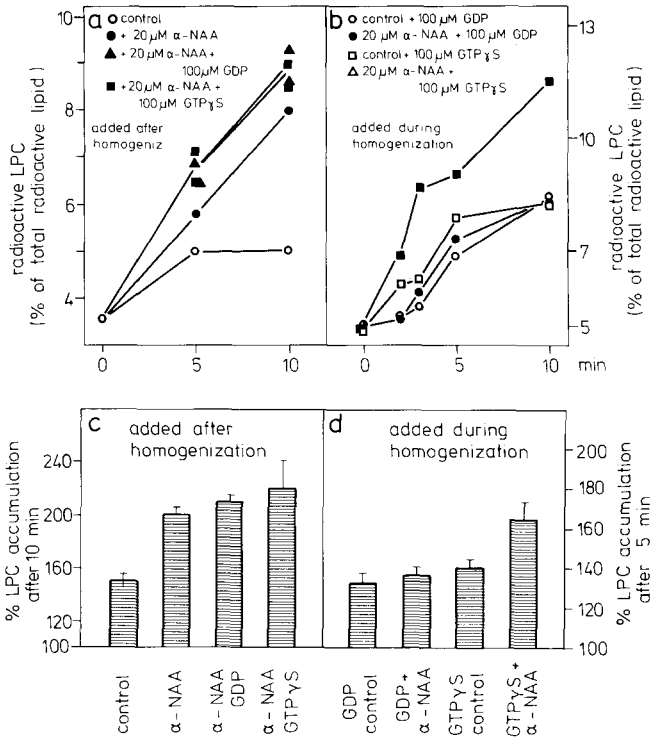

Fig. 6a–d. Effects of guanosine nucleotides on the hormonal stimulation of PLA_2. **a** After preparation of microsomal vesicles from cultured soybean cells, either 100 μM GDP or 100 μM GTPγS was added directly to the assay vesicles which were from the same preparation. **c** Activation of PLA_2 by 20 μM α-NAA. The value of LPC content at $t=0$ was set as 100% and all values were calculated relative to this to allow comparisons. Values for $t=5$ min are shown. *Error bars:* SD, $n=3$. **b** Either 100 μM GDP or 100 μM GTPγS was added prior to homogenization to the buffer in which two equal aliquots of [^{14}C]choline-prelabelled cells from the same batch were homogenized. The microsomes of both aliquots were prepared in parallel. **d** The LPC content at $t=0$ was set as 100% and all other values were calculated relative to this to allow comparison of experiments done with three different preparations each for **c** and **d**. Values for 10 min are shown. *Error bars:* SD, $n=3$

found in both treatments (Fig. 6, 8). When guanosine 5′-O-(thiotriphosphate) (GTPγS), a non-hydrolyzable analogue of GTP, or GDP were added after the homogenization of [^{14}C]choline-labelled cells no significant effect of either GTPγS or GDP was found (Fig. 6a, c). When these nucleotides were added prior to homogenization, so that the nucleotides should be enclosed in membrane vesicles during their preparation, GDP prevented the stimulatory effect of auxin on the PLA_2 activity almost completely (Fig. 6b, d). In the presence of hormone, GTPγS enclosed in the vesicles allowed full stimulation of PLA_2. From this we conclude that the nucleotide-sensitive protein must reside inside the vesicles. This was further supported by a control experiment in which the nucleotides were added after homogenization but together with the detergent digitonin (Fig. 7). In this case, GDP was active in preventing the PLA_2 stimulation by auxin.

In order to test the nucleotide specificity of this effect similar experiments were carried out with ADP and adenosine 5′-O-thiotriphosphate (ATPγS), and with UDP and UTP (Fig. 8). Surprisingly, ADP, when added

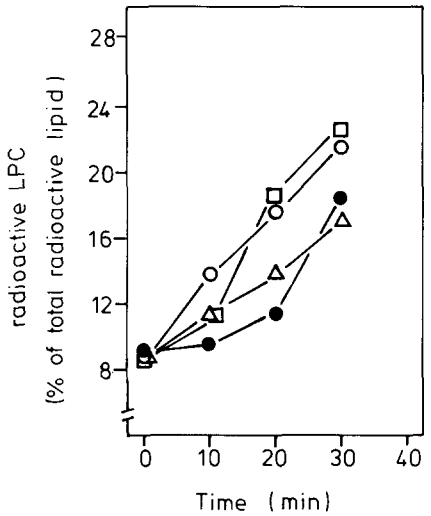

Fig. 7. Effect of guanosine nucleotides on PLA_2 stimulation by α-NAA in the presence of the detergent digitonin (0.05%; w/vol). Nucleotides were added at 100 μM after vesicle isolation prior to the test. In contrast to non-permeabilized vesicles (Fig. 6a), GDP suppressed the hormonal activation of PLA_2. ●–●, control; ○–○, 20 μM α-NAA alone; □–□, 20 μM α-NAA plus 100 μM GTPγS; △–△, 20 μM α-NAA plus 100 μM GDP

during homogenization, had an effect similar to GDP whereas UDP clearly did not. Both ATPγS and UTP had no apparent effect. This result, and the observation that GTPγS alone cannot significantly stimulate PLA_2, precludes to some extent the assumption that typical trimeric GTP-binding proteins are involved in this type of nucleotide sensitivity.

Both the orientation of the known membrane-associated auxin-binding protein (ABP) from maize and its participation in the auxin-dependent PLA_2 reaction were tested with antibodies against this ABP (Löbler and Klämbt 1985; Tillmann et al. 1989). Increasing concentrations of a polyclonal anti-ABP antibody (total IgG fraction) were used (Fig. 9). More than 90% inhibition was found at a concentration of 2.1 mg · ml^{-1} anti-ABP IgG. In the control, 2.1 mg · ml^{-1} IgG from non-immunized rabbits inhibited only by 16%.

Since we found a clear optimum for the stimulation of PLA_2 by auxin at pH 5.5 (Fig. 4), which is the pH optimum of auxin binding (Löbler and Klämbt 1985), we take both experiments as indicating the participation of the ABP in the stimulation of PLA_2 by auxin and its function as an auxin receptor (Barbier-Brygoo et al. 1989; Klämbt 1991; Napier and Venis 1991).

In order to identify the ABP in the microsomes of the cultured soybean microsomes they were purified by the two-phase partitioning method and subjected to free-flow electrophoresis. Two pooled fractions, a plasma-membrane fraction and a microsomal fraction containing ER, Golgi membranes and tonoplast, were obtained, (Fig. 10a; for marker studies see Scherer et al. 1992). Both fractions were probed with the polyclonal antibody used for the PLA_2 experiments and showed a signal in a Western blot, the plasma membrane fraction at 22 kDa, the microsomal fraction at 20.5 kDa (Fig. 10b). Molecu-

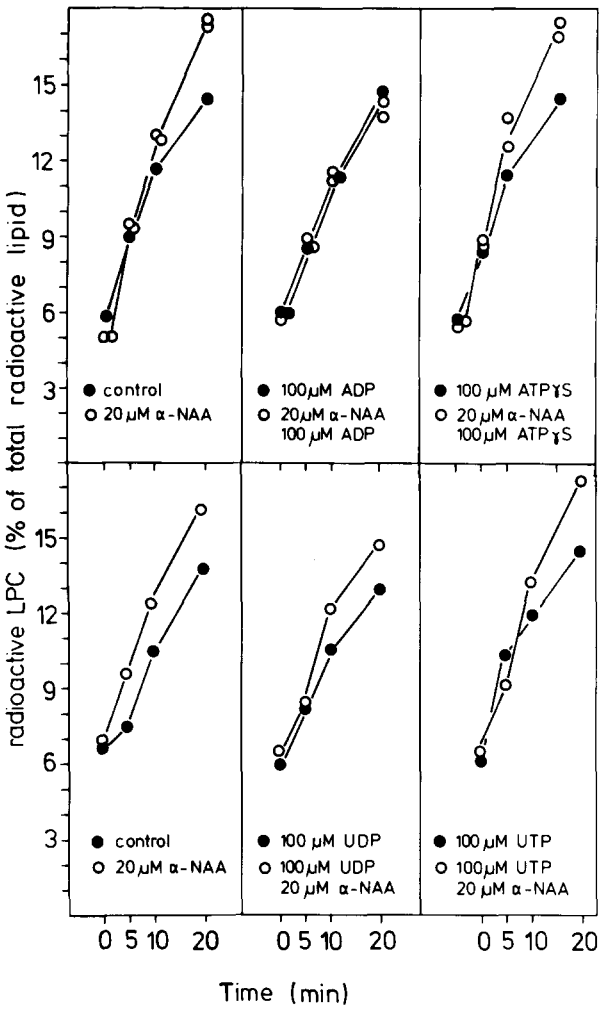

Fig. 8a–d. Effects of ADP or ATPγS and UDP or UTP on PLA_2 stimulation by 20 μM α-NAA. Nucleotides were added prior to homogenization at 100 μM each. **a** Control with 20 μM α-NAA alone for the experiments shown in panels **b** and **c**. **b** Homogenization of cells with 100 μM ADP suppressed the PLA_2 stimulation. **c** Homogenization with 100 μM ATPγS did not affect the PLA_2 stimulation. **d** Control with 20 μM α-NAA alone for the experiments shown in panels **e** and **f**. **e** Homogenization of cells with 100 μM UDP did not suppress tha PLA_2 stimulation as much as 100 μM UTP shown in **f**

lar isoforms of the ABP have been described, but their functional significance is not clear (Tillmann et al. 1989; Napier and Venis 1990; Feldwisch et al. 1992). Several cross-reacting bands were also apparent in the 66-kDa region, both in the Western blot of the soybean membranes and, relatively weaker, in Western blots of the crude maize ABP. It was often found that monoclonal or polyclonal antibodies prepared against maize ABP stained bands unspecifically in the 66-kDa region of Western blots when membrane fraction were used for gel electrophoresis, and these stained bands were only absent in blots from highly purified ABP (Napier et al. 1988; Tillmann et al. 1989; Napier and Venis 1990; Feldwisch et al. 1992). The ABP from soybean may be only distantly related to the maize ABP, as was found for the *Arabidopsis* and strawberry ABPs (McDonald et al. 1991;

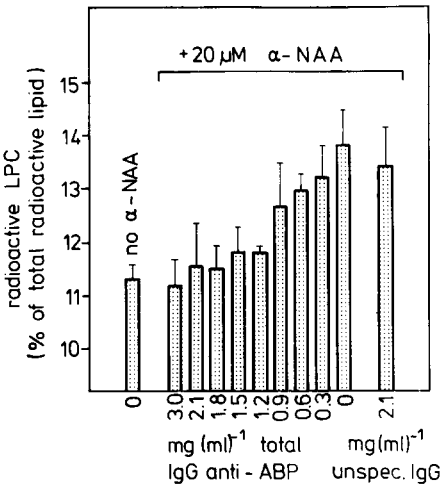

Fig. 9. Effect of rabbit polyclonal antibodies (total IgG fraction prepared by ammonium sulfate fractionation) against auxin-binding protein (ABP) from maize coleoptile (Tillmann et al. 1989) on auxin-stimulated PLA_2 activity in microsomes from cultured soybean cells. The total IgG fraction prepared by ammonium sulfate fractionation was used. A control without hormone and without antibody was made *(left bar)* and two controls with 20 µM α-NAA, without antibody and with unspecific preimmune IgG, were made *(two right-hand bars)*. Increasing antibody concentrations were inhibitory to the PLA_2 stimulation. Incubation time was 20 min. Values are averages from triplicate determinations (± SD) done with one membrane preparation

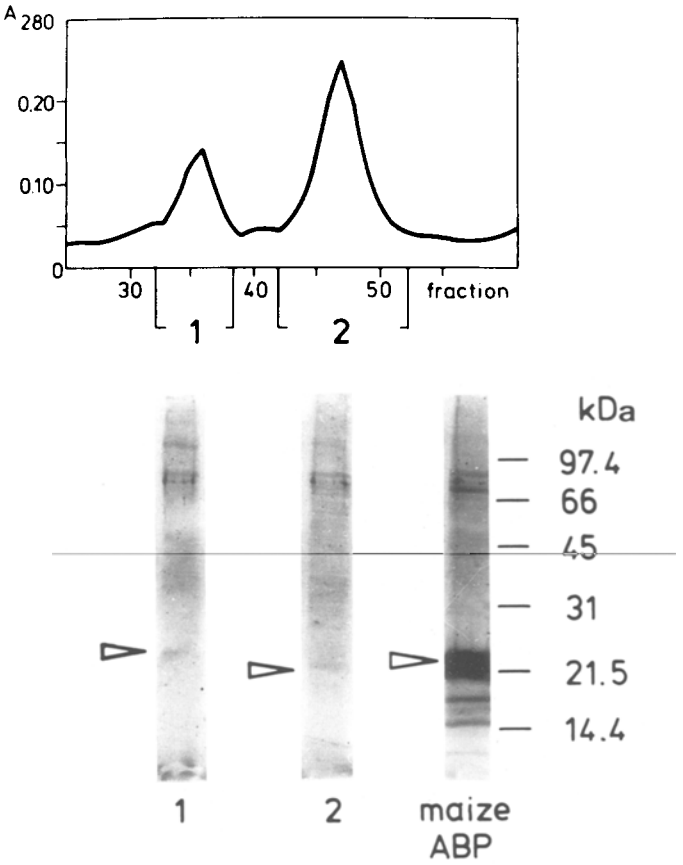

Fig. 10a, b. Probing of purified membranes from cultured soybean cells with a polyclonal rabbit antibody against maize ABP. **a** The free-flow-electrophoresis profile of a plasma-membrane fraction prepared by two-phase partitioning is shown (see Scherer et al. 1992). Plasma membranes constitute the most cathodic fraction 1 (pooled as indicated by *bracket 1*) and the other membranes (ER, Golgi, tonoplast) constitute the anodic fraction 2 (pooled as indicated by *bracket 2*). **b** Western blot of fractions 1 and 2 (50 µg protein per lane) and a crude maize ABP preparation probed with the polyclonal antibody against maize ABP. The purified plasma membrane *(lane 1)* gave a signal at 22 kDa and the contaminants *(lane 2)* a signal at 20.5 kDa *(arrowheads)*

Feldwisch et al. 1992), so that a weak signal in the 20- to 22-kDa region of the blot would result, and the unspecific signals could be relatively stronger than those from maize preparations (Fig. 10b).

Discussion

The response of PLA_2 to auxin in isolated membranes from cultured soybean cells reported here was rapid, dependent on the auxin concentration, auxin-specific, and pH-dependent. We found an equally rapid response of PLA_2 to auxin in vivo in cultured soybean cells (Scherer and Andre 1989; Scherer 1990). The concentration dependence for the auxin α-NAA and the auxin specificity of the PLA_2 stimulation in vitro were more distinct than in vivo (Scherer and André 1989; Scherer 1990) and resembled the auxin sensitivity and specificity of growth tests (Schneider and Wightman 1978; André and Scherer 1991).

The instability of the lipid composition of isolated plant membranes (Scherer and Morré 1978a, b), due to the presence of phospholipase D activity and multiple PLA_2 activities (Brglez et al. 1992; Cho and Boss 1992; Kim et al. 1992), and the deleterious consequences for the PLA_2 response (Fig. 3) is a serious obstacle to further analysis. Both unspecific vacuolar PLA_2 (Kim et al. 1992) and phospholipase D (Yang et al. 1967; Witt et al. 1987) are very active at the optimal assay pH of pH 5.5 so that a membrane purification was not attempted.

The experiments with added anti-ABP antibody and with nucleotides prior or after homogenization allow conclusions about the membrane topology of these key components but no clear decision about the membrane compartment of the hormone-sensitive PLA_2. Due to this uncertainty, also no clear decision can be made whether or not outside-out plasma membrane vesicles or inside-out ER vesicles could be the hormone-sensitive type of vesicles. Since the antibody recognizes the ABP, this must be located at the outside of the respective vesicle whereas the nucleotide-sensitive site is located on the inside of the vesicle. The ABP is a glycoprotein with no apparent membrane-spanning domain (Hesse et al. 1989; Tillmann et al. 1989) so that it must be located at the extracytosolic leaflet of the respective vesicle, and the nucleotide-sensitive site and the PLA_2 must be located on the inside and, hence, at the cytosolic leaflet. Also, no residual antibody-insensitive, hormone-sensitive PLA_2 was found (Fig. 9a; André and Scherer 1991) but, if the binding site was inside the vesicle, one would expect that auxin would permeate the membrane and trigger the reaction. In addition to this, one has to consider that

vesicles, independent of origin, could loose key components if isolated in the cytosolic-side-out orientation so that they would be auxin-insensitive. The experiment in Fig. 10a shows that both on the plasma membranes and on other membranes, presumably on the ER as in other systems (Ray 1977; Ray et al. 1977; Jones et al. 1989), an ABP serologically related to the maize ABP is present in soybean cells so that both plasma membrane and ER could be a compartment for the PLA_2 response. These uncertainties preclude conclusions about the type of membrane that harbours the hormone-sensitive PLA_2 although, of all vesicle types, outside-out plasma-membrane vesicles would most easily fulfill this condition. The isolation of the hormone-responsive membrane(s) will, however, only be possible with selective inhibition of hormone-insensitive PLA_2 and of other membrane-degrading enzymes. Then this open question can be solved and, moreover, the function of the apparently different ABPs can be further resolved.

The nature of the nucleotide-sensitive site or protein can only be speculated about. Though tempting as an hypothesis, our experiments do not necessarily indicate a trimeric G protein (Ma et al. 1990) to be this nucleotide-sensitive site in the PLA_2 response. Firstly, in this case, one would expect GTPγS alone to activate PLA_2, as has been observed in animal systems (Murayama et al. 1990; Narasimhan et al. 1990; Teitelbaum 1990) and, secondly, one would not expect an effect of ADP since in plant membranes adenine nucleotides do not compete for GTP-binding sites very efficiently (Blum et al. 1988; Droeback et al. 1988; Jacobs et al. 1990; Zbell et al. 1989, 1990a; Perdue and Lomax 1992). Only in membranes from *Neurospora* has binding of both GTP and ATP been observed, but the functional context remained unclear (Hasanuma 1991).

A relationship to other signal-transduction pathways in eucaryotic cells is clearly indicated by our results. In animal cells, where a PLA_2-signalling pathway is established (Burch et al. 1986, 1988; Jelsema 1987; Jelsema and Axelrod 1987; Brooks et al. 1989; Silk et al. 1989; Murayama et al. 1990; Narasimhan et al. 1990; Teitelbaum et al. 1990), arachidonic acid is liberated as a metabolite which is then turned-over into several biologically active metabolites (Samuelson and Funk 1989). Very recently, it has been found that linolenic acid is liberated in plants by signals typical of wounding, such as cutting and insect bites (Farmer and Ryan 1992), and touching of tendrils (Falkenstein et al. 1991), and is then turned-over to the plant hormone jasmonic acid. The similarity is obvious but the relevant (phospho)lipase A has not yet been identified. Moreover, the peptide mastoparan, an activator of G proteins and-or PLA_2 activity in animal systems (Higashijima et al. 1988), also activated PLA_2 and growth in plant systems (Scherer 1992).

We think that the activation of PLA_2 by auxin is intimately involved in the activation of the plasma membrane H^+-ATPase (Scherer and Nickel 1988; Scherer 1990; Nickel et al. 1991) by either binding of the liberated lysophospholipids to the H^+-ATPase or by activating a membrane-associated protein kinase or, perhaps, even by both (Schaller and Sussman 1988; Martiny-Baron and Scherer 1989; Palmgren 1991). Evidence for this latter possibility has already been obtained in earlier experiments on the activation of ATPase by auxin (Scherer 1981, 1984) as well as in more recent ones (Santoni et al. 1990, 1991). Patch-clamp studies with maize protoplasts showed that a rather direct pathway for the activation of plasma-membrane H^+-ATPase must exist, which is observable after only a 1-min lag phase and involves the known maize ABP (Rück et al. 1992). We also interpret the stimulation of proton extrusion by the lysophospholipid-like platelet-activating factor in cultured soybean cells as an auxin-mimicking effect, indicating a second-messenger-like biological activity for lysophospholipids (Scherer and Nickel 1988; Nickel et al. 1991). Since the activity of the plasma-membrane H^+-ATPase can be increased by several mechanisms (Palmgren 1991), other types of regulation of its activity, e.g. by exocytosis, must be envisaged (Hager et al. 1991). Regulation of the essential transport activity of the H^+-ATPase may be one of the functions of auxin in sustaining growth of cultured plant cells.

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