**Short Communication**

**Phosphorylation of Membrane-Located Proteins of Soybean In Vitro and Response to Auxin**

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**ABSTRACT**

Isolated membranes of soybean incorporate $^{32}$P from $\gamma$-$^{32}$P[ATP in vitro. The incorporation was rapid and did not require added calcium. When displayed on 10% sodium dodecyl sulfate-polyacrylamide gels, several protein bands were revealed. An apparent auxin (2,4-dichlorophenoxyacetic acid) stimulation of $^{32}$P incorporation into material from membrane vesicles insoluble in trichloroacetic acid-perchloric acid may be reflected partly in enhanced incorporation into protein bands with apparent molecular weights of 45,000 and 50,000. Additionally, a low molecular weight component was sometimes observed where incorporation was stimulated 2- to 3-fold by auxin. However, protein-bound radioactivity represented only a small fraction of the total radioactivity of the acid-insoluble material. Other labeled constituents, not retained on the gels, may contribute to the apparent, rapid (10 s or less) auxin response of the isolated membranes. Stimulation of incorporation into the low molecular weight component was given by diglyceride plus calcium, constituents known to augment protein kinase activities in other systems.

**MATERIALS AND METHODS**

Soybean seeds were soaked in deionized H$_2$O for 4 to 6 h, planted in moist vermiculite, and grown 4 d in the dark. The etiolated hypocotyls were harvested and used for membrane isolations.

Hypocotyl segments consisting of approximately 2-cm sections taken 5 mm below the cotyledons were homogenized using a mechanized razor blade chopper (5) in 15 mM Tris (pH 7.2). The homogenates were filtered through Miracloth (Chicopee

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*Fig. 1. Labeling of crude TCA-PCA precipitated membrane material over 60 s (upper curves). The difference (auxin minus control) is illustrated in the lower curve. These findings were interpreted as indicative of an effect of auxin on incorporation of $^{32}$P from $\gamma$-$^{32}$P[ATP into some constituents of the membrane within the first 10 s after auxin addition. The incorporated activity was resistant to acid and heating. Auxin = 1 $\mu$g 2,4-D.
The isolated membranes were incubated in 50 mM Mes (pH 6.5), 50 mM MgCl₂, and about 10 μCi γ-[³²P]ATP for 10 to 20 s. The material insoluble in 10% TCA plus 5% PCA² was precipitated, washed, and radioactivity determined.

²Abbreviation: PCA, perchloric acid.
were fixed and dried under vacuum. Kodak RP Royal 'X-omat' film was placed over the dried gels which were then allowed to develop in a low temperature freezer.

**RESULTS**

The isolated fractions of soybean membranes yielded complex kinetics of incorporation of $^{32}$P from $\gamma$-$[^{32}$P]ATP into the TCA-PCA insoluble fraction. Although a small stimulation by auxin was observed consistently, the baseline or precipitated radioactive activity declined steadily as the ATP was hydrolyzed. Therefore, kinetics were determined at very short times of 1 min or less (Fig. 1). While not statistically significant, numerical averages of six consecutive experiments revealed a small auxin stimulation of total precipitable activity. The auxin-stimulated component appeared to rise rapidly during the first 10 s of incubation and more slowly thereafter (Fig. 1, inset).

To determine the nature of the auxin-stimulated constituents in the pellets, 10% SDS gels in combination with fluorography were used to resolve the $^{32}$P-labeled proteins (Fig. 2). About 90% of the radioactivity in the TCA pellets was trapped low mol wt material. However, stimulated by auxin was phosphorylation of two bands with apparent molecular weights corresponding to about 50,000 and 45,000 as well as a low mol wt constituent (about 20,000) just behind the dye front on the 10% gels. Under these conditions, any contaminating ATP or inorganic phosphate would have cleared the gels and entered the electrophoresis buffer.

On the premise that the activity likely was the result of a protein kinase, diglyceride (25 $\mu$g/ml dipalmitin) plus Ca (10 $\mu$M) also were tested and found to stimulate the activity (Figs. 2 and 3). Unlike the diglyceride stimulation, which appeared to require Ca for expression (Fig. 3), the 2,4-D stimulation was inhibited by Ca under the conditions depicted in Figure 3. The weak auxin analog, 2,3-D (10 $\mu$M), did not appear to stimulate. Stimulation was observed over a wide range of auxin concentrations (5 x $10^{-7}$ to $10^{-5}$ M) and also was observed in the presence of 1 $\mu$M IAA in a single trial (not shown).

Quantitatively, the auxin response in these experiments was greatest in the low mol wt constituent. This was demonstrated by determination of radioactivity from sliced gels (Fig. 4). A similar response was observed with diglyceride (Fig. 5) although of a lesser magnitude than with 2,4-D. The low mol wt constituent was more variable among experiments, and may represent a labile phosphate as this incorporation was reduced by subsequent treatment of the membranes with hydroxyamine (7) (not shown). The potentially important stimulations by 2,4-D of the 45,000 and 50,000 mol wt bands could be seen (Fig. 4, slices 4 and 5) but not satisfactorily demonstrated by the slicing method due to the small amounts of radioactivity present. Of the material applied to the gel, about 90% of the radioactivity was lost during the electrophoresis, staining, and destaining procedures associated with preparation of the gels.

**DISCUSSION**

It is widely recognized that the posttranslational phosphorylation of serine, threonine, or tyrosine residues of cellular proteins has an important role in mediating the action of various hormones and growth factors (8). This phenomenon has not been investigated extensively in plants. In vivo and in vitro phosphorylations of membrane fractions isolated from maize coleoptiles were examined by Brummer and Parish (3). More than nine bands were resolved. Two of the phosphorylated proteins ($M_r = 110,000$ and $20,000$) also were iodinated on protoplasts and were suggested to be part of the plasma membrane ATPase.

The products of the auxin-stimulated phosphorylation also are membrane associated and, like the protein kinase C of mam-
Maian cells, phosphorylating activity is enhanced by diacetyl glycerol and Ca (4), potential products of the breakdown of phosphatidylinositol. While the latter findings suggest that the activity of the enzyme in vitro might be modulated by alterations in membrane structure and phospholipid metabolism, the kinetics of protein phosphorylation seem much too rapid to result secondarily from a primary auxin effect on phosphatidylinositol breakdown. The $t_0$ of protein phosphorylation stimulated by auxin is in the order of seconds while the $t_0$ of phosphatidylinositol breakdown and Ca release from the membranes is measured in minutes. Moreover, we have been unable to demonstrate an obligatory Ca requirement for the auxin-stimulated phosphorylations.

Although Brummer and Parish (3) report the presence of a 20,000 mol wt phosphoprotein in maize coleoptiles, and an ATPase component in that mol wt range has been purified from radish seedlings by M. C. Caucchi and E. Marré (Phytochemical Society of Europe, Toulouse, France; September 13–16, 1983), the status of the low mol wt component in our studies should be regarded as problematic due to variable amounts from one membrane preparation to another.

It is not possible to compare directly results from analyses of the acid-precipitable material and the findings from the gels since only about 10% of the total radioactivity of the acid precipitates applied to the gels was recovered as materials retained on the developed gels. The remainder was material coprecipitating with protein but lost during the electrophoresis, staining, and destaining procedure. Additionally, phosphorylated constituents present as acyl phosphates, and preferentially susceptible to destruction by the alkaline pH of the Laemmli gel system used (1), would escape detection or represent a greater contribution to the total than indicated by our findings. Other possible complications that may have contributed to the findings include potential effects of auxin on protein aggregation-disaggregation or alternative dispositions of materials unrelated to the activation of specific protein kinases. The minor bands at 45,000 and 50,000 mol wt stimulated by auxin were less variable in amounts and thus may be more closely associated with some initial auxin-membrane interaction.

One possibility, presently under investigation, is that the protein kinase and/or one or both of these phosphorylated products may be an auxin binding (receptor) protein or at least a component of the putative receptor complex. Although soybean membranes do bind auxins (9), neither the receptor nor the protein kinase have been identified from soybean membranes. In a parallel series of studies, a soluble protein kinase has been purified from etiolated soybean hypocotyls. This activity is dependent upon Ca for phosphorylating activity, utilizes casein as substrate, is not stimulated by auxin, and is therefore an unlikely candidate for the activity responsible for the phosphorylation of the membrane-associated proteins.