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Diacylglycerol Levels Unchanged during Auxin-Stimulated Growth of Excised Hypocotyl Segments of Soybean[1]

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ABSTRACT

Diacylglycerol contents of excised soybean (*Glycine max* L.) hypocotyl segments, incubated for 4 hours in the presence or absence of a growth promoting concentration of 2,4-dichlorophenoxyacetic acid (2,4-D) were monitored by three different methods as a sensitive measure of the action *in vivo* of C-type phospholipases. By all three methods, steady state levels of diacylglycerols representing about 3% of the total lipids or about 7% of the neutral lipids, depending on method of assay, declined 18% over 4 hours of incubation as determined by extraction of total lipids and analysis by thin layer chromatography and densitometry. The average decline with 2,4-D-treated segments was less but the difference from controls was not significant. In those experiments where a small effect of 2,4-D was noted, the fraction showing an elevated diacylglycerol level in response to 2,4-D, after separation into membrane and supernatant fractions, was the supernatant and not the membranes. Results were confirmed from analyses of total fatty acids in each of the major lipid fractions and from diacylglycerol assays by conversion into phosphatidic acid upon incubation with [γ-^{32}P]ATP and purified diacylglycerol phosphokinase from *Escherichia coli*. In the presence of 2,4-D, the diacylglycerol content of the membranes was unchanged compared to membranes from control segments. As with the densitometric method, the small 2,4-D induced increase in diacylglycerols, when observed, was insignificant and in the supernatant. The only membrane-associated lipid fraction consistently showing a response to 2,4-D was the fraction containing sterols esterified with fatty acids. Either total microsomes or purified plasma membranes when incubated for 10 to 20 minutes with 1 micromolar 2,4-D showed no accelerated formation of diacylglycerols compared to membranes not incubated. The results do not support operation during auxin growth of the animal paradigm where diacylglycerol activation of C-type protein kinases occurs in response to activated phospholipase C breakdown of phosphoinositides.

The hydrolysis of phospholipids catalyzed by various lipases yield substances that may serve important roles as second messengers. These include inositol phosphates (1), calcium ions (24), and diacylglycerols (1, 16). Under appropriate conditions, these second messengers may serve to amplify or translate hormonal stimuli as part of a response cascade potentially important to growth control. One class of products of phospholipase action, the diacylglycerols, is usually present only in low levels in biological membranes. Increases in steady state levels of diacylglycerols in response to a particular effector might not only signal the possible *in situ* involvement of a phospholipase in the response cascade but may be important as regulators of other enzyme activities. In mammalian cells, protein kinases of the C type are activated specifically by certain phospholipids and diacylglycerols in combination (16).

In this study, steady state levels of diacylglycerols in auxin-treated hypocotyl segments were compared to levels present in segments incubated without auxin. Membrane diacylglycerols were largely unaltered with 2,4-D treatment. Therefore, steady state stimulation of elongation growth of soybean hypocotyls is not readily explicable in terms of the signal-response paradigm involving phospholipid metabolism and diacylglycerol activation of membrane-associated C-type protein kinases.

MATERIALS AND METHODS

Plant Material

Seeds of soybean (*Glycine max* (L.) Merr., cv Wayne) were soaked in deionized water 4 to 6 h, planted in moist vermiculite, and grown 4 to 5 d in darkness. Two cm long segments, cut 5 mm below the cotyledons, were harvested under normal laboratory conditions and used for isolation of membranes.

Studies *in Vivo*

Excised intact hypocotyl segments 1 cm long were incubated with or without 1 μM 2,4-D in water at 25°C and in darkness for the times indicated. The solutions (5 mL) were contained in 1 × 10 cm covered Petri dishes.

Membrane Isolations

Six g of hypocotyl segments were homogenized for 60 to 90 s with a mechanized razor blade chopper (12) or chilled mortar and pestle in 5 mL of ice-cold medium (0.30 M sucrose, 10 mM KCl, 1.0 mM $MgCl_2$, 25 mM Tris/Mes [pH 7.5]). The homogenate was filtered through one layer of Miracloth (Chicopee Mills, NY), and the residue was washed once with 5 mL of homogenization medium. The combined filtrates were centrifuged for 10 min at 10,000g_{max} (Sorvall HB-4 rotor) and the resulting supernatant was centrifuged for 30 min at

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60,000g_{max} (Beckman SW-28 rotor). The resulting microsomal pellet was resuspended in assay medium (0.25 M sucrose, 10 mM KCl, 25 mM Tris/MES [pH 8.0]).

Proteins were determined by the method of Lowry *et al.* (10) using bovine serum albumin as the standard.

Lipid Extraction and Separation

Lipids were extracted by the Folch *et al.* (6) method. Component lipids were separated by TLC (silica gel G, EM Science 60 F-254, layer thickness 0.25 mm) using heptane-ether-acetic acid (75:25:4). In some experiments lipids were visualized with cupric acetate (5) and quantitated by image analysis using a LeMont model DV 4400 Oasys image analysis system equipped with an IMP program.

Fatty Acid Analyses

Acyl lipids were *trans*-esterified with 5% H_2SO_4 in methanol (v/v) for 4 h at 80°C. GLC was used to analyze the resulting methyl esters (DANI 3800 GLC equipped with *WCOT* fused silica capillary column, 50 m × 0.23 mm i.d., flame ionization detector). The stationary phase was CP Sil88 (Chrompack, film thickness 0.20 μm). Nitrogen was used as carrier gas at a flow rate of 1.1 ml/min (split 1:100). The column temperature was 200°C. Identification and quantitative determinations were carried out using standards as described (7). The data are mean values of two determinations.

Measurement of Diacylglycerols Using Diacylglycerol Kinase

Diacylglycerols present in the lipid extracts were quantitated by an enzymic assay which employed *Escherichia coli* *sn*-1,2-diacylglycerol kinase (19) and [γ-^{32}P]ATP under conditions were diacylglycerols are converted quantitatively to [^{32}P]-phosphatidic acid. The phosphatidic acid formed was isolated as described (25) and standardized against known quantities of added diacylglycerol.

RESULTS

Lipid extracts of hypocotyl segments of soybean, when separated by TLC to display both neutral and phospholipids (Fig. 1) revealed a clear diacylglycerol region identified by comparison with authentic diacylglycerol standards. In unincubated segments, diacylglycerols amounted to 7% of the total neutral lipids by densitometry (Table I; Fig. 2) or 3% of the total lipids based on analyses of fatty acids (Table II). With incubation of tissue segments, the proportion of diacylglycerols declined (Fig. 3; Tables I–III). The decline on average was numerically less with segments incubated with 2,4-D but the differences, even after 4 h of incubation, were small and insignificant (Table I). One possibility to be investigated was that of phospholipase C activation in a particular membrane fraction. In order to examine the subcellular location of diacylglycerols, homogenates were first centrifuged to yield total microsomes and a microsome-free supernatant fraction essentially devoid of membranes. The homogenate revealed more diacylglycerol with 2,4-D treatment for 4 h than in its absence (Fig. 4). However, with microsomes, no difference was apparent either visually (Fig. 4) or by densitometry (not shown). The 2,4-D increase was primarily in the supernatant fraction.

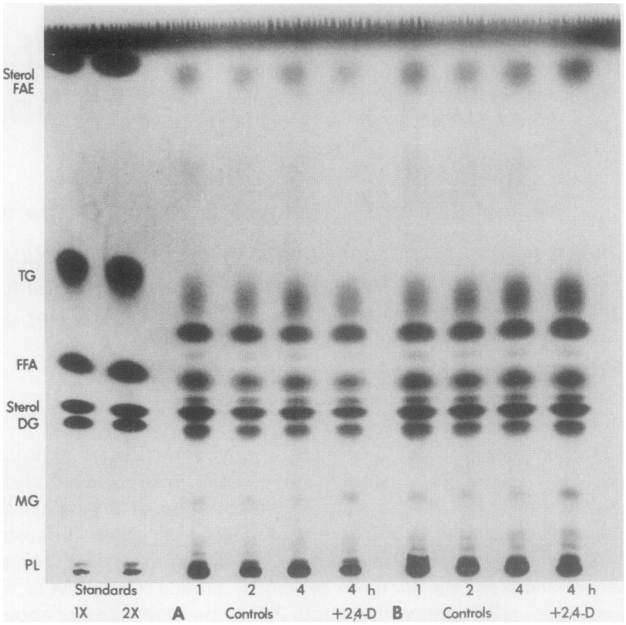

Figure 1. Thin layer chromatographic separation of lipid extracts of hypocotyls incubated for 1, 2, and 4 h in the absence of 2,4-D and for 4 h in its presence. Lipid standards were 10 (1×) and 20 (2×) μg: A, Lipid extracts corresponding to about 300 mg fresh weight of hypocotyl segments; B, lipid extracts corresponding to about 600 mg fresh weight of hypocotyls.

When quantitated on the basis of analyses of the total fatty acids (Table II), membrane-located diacylglycerols again declined with tissue incubation but the proportions were unchanged as a result of 2,4-D treatment. The supernatant contained 25% or more of the diacylglycerols and free fatty acids and about 65% of the triacylglycerols but only 6% of the phospholipids. Diacylglycerols of the supernatant fraction were increased by treatment of the tissue with 2,4-D.

As a final method of diacylglycerol estimation, membrane and supernatant fractions were incubated with [γ-^{32}P]ATP in the presence of *E. coli* diacylglycerolkinase to quantitatively convert diacylglycerols to radiolabeled phosphatidic acid. With replicate determinations within an experiment and with replicate experiments, no differences in diacylglycerol contents of the membrane fractions were seen as a result of 2,4-D treatment of tissue segments. A small and insignificant increase in diacylglycerols of the supernatant fraction as a result of the 2,4-D treatment was observed in experiment II but not in experiment III.

As a control on possible rapid effects and/or a direct response of the plasma membrane-associated C-type phospholipase to 2,4-D, diacylglycerol contents of isolated membranes (total microsomes) incubated directly with auxin after isolation of the membranes also were determined (Table 1). Again the change in diacylglycerols was insignificant. Similar results were obtained with total microsomes or plasma membranes prepared by aqueous two phase partition with the

Table I. *Diacylglycerol Contents of Hypocotyl Segments and of Isolated Membranes*

Hypocotyl segments (incubated 4 h) or isolated membranes (heavy microsomes incubated for 15 min) (both with and without 1 μM 2,4-D) were analyzed for content of diacylglycerol by densitometry following extraction and TLC with visualization using the cupric acetate reagent.

Incubation	Treatment	Experiments	Diacylglycerols
		n	*% of neutral lipids*
Hypocotyl segments *in situ*	Control (0 h)	8	6.9 ± 0.4
	Control (4 h)	8	4.8 ± 0.3
	1 μM 2,4-D (4 h)	8	5.3 ± 0.4
Isolated membranes *in vitro*	Control (0 min)	3	6.2 ± 0.3
	Control (15 min)	4	5.5 ± 0.3
	1 μM 2,4-D (15 min)	4	5.7 ± 0.2

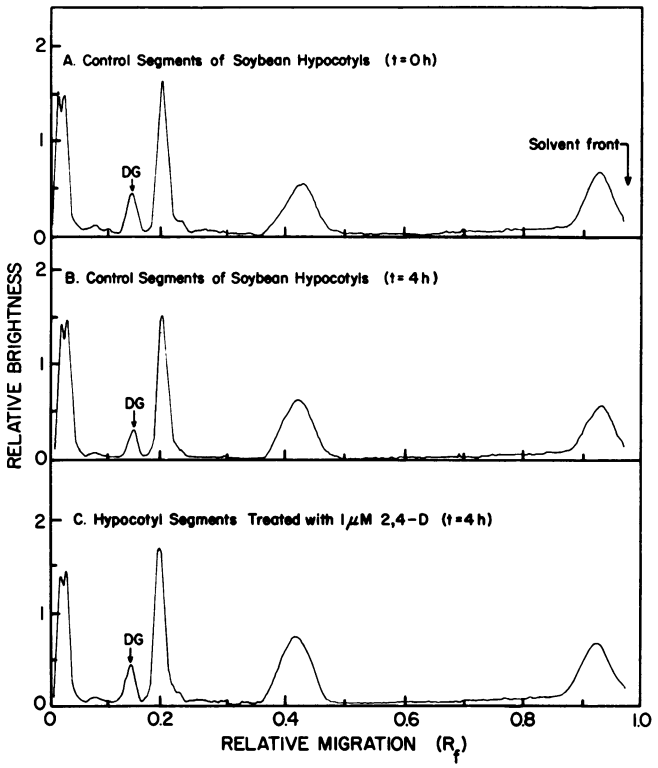

Figure 2. Image analyzer tracings of TLC plates used in the quantitation of diacylglycerols. A, Lipid extract from control segments of soybean hypocotyls not incubated ($t = 0$); B, lipid extract from control segments of soybean hypocotyls incubated 4 h in distilled water ($t = 4$). C, Lipid extract from tissue segments incubated 4 h with 1 μM 2,4-D in distilled water. DG, diacylglycerols. The first major peak just past the origin is phospholipid. All other peaks are neutral lipids (see Fig. 1).

diacylglycerol estimation by incubation with [γ-^{32}P]ATP and diacylglycerolkinase. After a 10 min incubation of freshly isolated plasma membranes, for example, the diacylglycerol content without 2,4-D was 45 ± 4 nmol of diacylglycerol/mg protein (initial value of 48 ± 5 nmol/mg protein) compared to 45 ± 5 nmol of diacylglycerol/mg protein) for a 10 min incubation with 1 μM 2,4-D. Results with 1 μM indole-3-acetic acid were similar.

Since emphasis was on diacylglycerols and neutral lipids, individual phospholipids were not resolved by the separation techniques employed. Among neutral lipids other than diacylglycerols, a reproducible alteration noted with auxin treatment was a decrease in the percentage of sterol fatty acids associated with membranes upon treatment of the hypocotyl sections with 2,4-D (Table II). The percentage of free fatty acids of the supernatant fraction was increased correspondingly.

DISCUSSION

The action of a number of animal effectors including growth factors and hormones (1, 16) may involve the formation of diacylglycerols from phosphoinositides through the action of an inositol phosphate-specific phospholipase C. These diacylglycerols are considered of themselves to serve important functions as activators of C-type protein kinases involved in the signal response cascade of growth control (20).

In plants, phospholipid breakdown and altered phosphatidylinositol metabolism have been observed with isolated membrane vesicles (12, 21) as has the occurrence (2, 14, 21) and formation (22) of inositol phosphatides in plants. However, evidence for the *in situ* operation of an auxin-responsive pathway of phosphoinositide breakdown is altogether lacking as is information concerning the possible involvement of a hormone-responsive C-type phospholipase (18).

Diacylglycerols, because of the low natural abundance, provide a sensitive indicator for effector stimulated phospholipid breakdown *in situ* (9, 26). Thus, the present evidence for a lack of response to auxin treatment of the steady state level of diacylglycerols does not support the operation in auxin control of growth of the animal paradigm where diacylglycerols activate a membrane-associated C-type protein kinase in response to accelerated phosphoinositide turnover. Alternatively, the lipid changes in response to auxin might result from other types of phospholipases (*e.g.* phospholipase D- or an A-type phospholipase). Thus far, we have been unable to demonstrate a direct effect of auxin on phosphoinositide- or polyphosphoinositide-specific phospholipase C despite their existence in both soluble form and in association with the plasma membrane of soybean hypocotyls (11, 18).

When separated into membrane-associated (microsome) and supernatant fractions, only 5% of the phospholipids and 63% of the triacylglycerols were found in the supernatant, presumably largely as lipid droplets or spherosomes that float upon centrifugation to enter the supernatant fraction (8). This

Table II. *Lipid Content of Soybean Fractions Based on Analysis of Total Fatty Acids*

Hypocotyl segments were incubated 4 h with or without 1 μM 2,4-D and the homogenized segments were separated into microsomes and a supernatant fraction. Acyl lipids were extracted, resolved on thin layer plates, eluted and *trans*-esterified. The resulting methyl esters were analyzed by GLC. The experiments were done twice. Percentages of total are given in parentheses.

Lipid Constituent	Microsomes		Supernatant	
	Control	2,4-D	Control	2,4-D
	nmol/g fresh wt (mol %)			
Phospholipids	1321 (87)	1245 (88)	79 (41)	77 (37)
Diacylglycerols	53 (3)	44 (3)	17 (9)	26 (13)
Free fatty acids	64 (4)	56 (4)	33 (17)	39 (19)
Triacylglycerols	36 (2)	31 (2)	62 (32)	66 (32)
Sterol fatty acid esters	49 (4)	36 (3)		

Table III. *Diacylglycerol Content of Soybean Fractions Based on Enzyme Assay Employing E. coli sn-1,2-Diacylglycerolkinase and [γ-^{32}P]ATP under Conditions where sn-1,2-Diacylglycerols Were Quantitatively Converted to [^{32}P]Phosphatidic Acid*

Hypocotyl segments were incubated for 0 or 4 h ± 1 μM 2,4-D and the homogenates separated into total membrane and a supernatant fraction. Determinations of experiment (Expt.) I were in duplicate, those of experiment II in triplicate and those of experiment III in quadruplicate. One μM IAA (indole-3-acetic acid) included in experiments I and II gave results similar to 2,4-D.

Treatment	Total Membranes				Supernatant			
	Expt. I	Expt. II	Expt. III	Average	Expt. I	Expt. II	Expt. III	Average
	diacylglycerol, nmol/g fresh wt							
Initial	30.7 ± 2.9	22.6 ± 2.9	28.6 ± 1.3	27.3 ± 4.2		16.0 ± 3.1	15.7 ± 0.7	15.9 ± 0.1
Control (t = 4 h)	26.0 ± 0.1	22.8 ± 4.6	25.6 ± 3.1	24.8 ± 1.7		11.7 ± 2.0	13.4 ± 0.4	12.5 ± 0.8
1 μM 2,4-D (t = 4 h)	25.6 ± 0.1	23.5 ± 1.1	26.0 ± 1.7	25.0 ± 1.3		14.2 ± 1.9	13.4 ± 0.3	13.8 ± 0.4

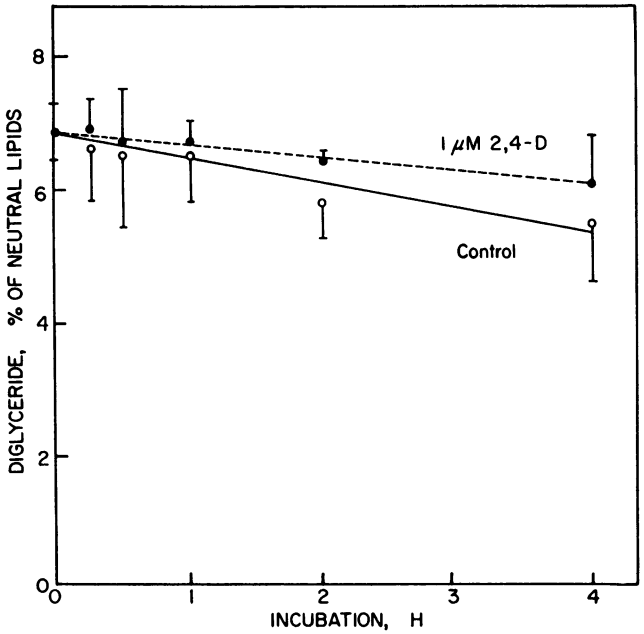

Figure 3. Diacylglycerol content of hypocotyl segments incubated for varying times without (control) and with 1 μM 2,4-D. Results are means of 3 to 6 determinations for each time point ± standard deviations. Analysis was by densitometry following extraction and TLC with visualization using the cupric acetate reagent.

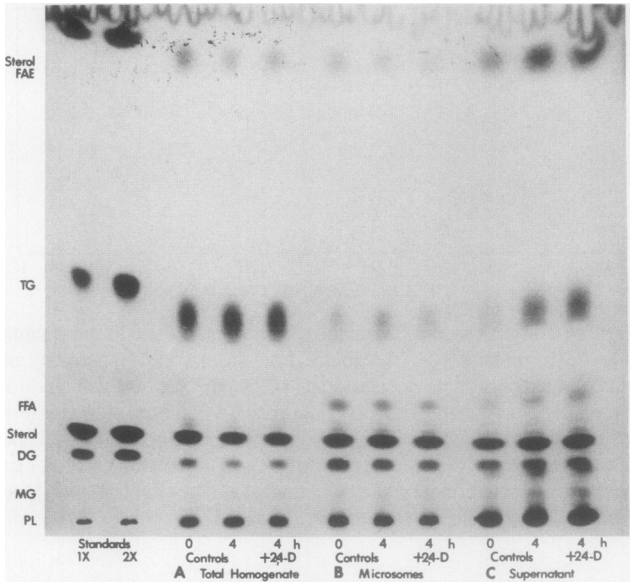

Figure 4. Thin layer chromatographic separation comparing lipid extracts of hypocotyls incubated for 0 and 4 h without and for 4 h with 1 μM 2,4-D. The homogenates were divided and a portion extracted directly (A) and a portion separated by centrifugation into microsomes (B) and supernatant (C) prior to analysis by densitometry following thin layer chromatography with visualization using the cupric acetate reagent.

contrasts with diacylglycerols where about 25% are found in the supernatant fraction suggesting a preferential association with membranes but a presence in lipid droplets as well. Even if the small alterations in diacylglycerol levels sometimes observed with auxin treatment were significant, their restriction to the supernatant fraction would be difficult to reconcile with any known regulatory role. A small, unspecific stimulation of protein kinase activity by diacylglycerol and calcium in both crude membranes (13) and in partially purified preparations (4, 23) has been reported but a membrane located, diacylglycerol-stimulated C-type protein kinase remains to be reported for plants. Activation of protein kinase C by diacylglycerol is considered normally to be a consequence of diacylglycerol increasing the affinity of the enzyme for Ca^{2+} to reduce the amount of Ca^{2+} needed to stimulate activity (3, 15, 17). While it is conceivable that diacylglycerol could act through some interaction with a soluble protein kinase, this type of regulatory mechanism seems less consequential than a mechanism based on activation of a membrane-bound kinase. With soybean hypocotyls, evidence for a C-type protein kinase is lacking and membrane-associated diacylglycerols were mostly reduced or unchanged by auxin treatment of hypocotyl segments. Thus, while the animal paradigm cannot be ruled out as a mediator of auxin control of elongation growth in etiolated soybean seedlings, the possibility remains that plants may have evolved second messenger systems for growth control distinct from those of animal cells. Alternatively, diacylglycerol increases may occur as part of the signal transduction cascade but either may be transient (lasting seconds, for example) or sufficiently small so as to be undetected against a background decrease representing less than 1% (30% of 3% over 8 h) of the total lipids.

LITERATURE CITED

1. **Berridge MJ** (1987) Inositol triphosphate and diacylglycerol: Two interacting second messengers. Annu Rev Biochem **56:** 156–193
2. **Boss WJ, Massell MO** (1985) Polyphosphoinositides are present in plant tissue culture cells. Biochem Biophys Res Commun **132:** 1018–1023
3. **Donnelly TE, Jensen R** (1983) Effects of fluphenazine on the stimulation of calcium-sensitive, phospholipid-dependent protein kinase by 12-*o*-tetradecanoyl phorbol-13-acetate. Life Sci **3:** 2247–2253
4. **Elliott DC, Skinner JD** (1986) Calcium-dependent, phospholipid-activated protein kinase in plants. Phytochemistry **25:** 39–44
5. **Fewster ME, Burns BJ, Mead JF** (1969) Quantitative densitometric thin-layer chromatography of lipids using copper acetate reagent. J Chromatogr **43:** 120–126
6. **Folch J, Lees M, Sloane Stanley GH** (1957) A simple method for the isolation and purification of total lipides from animal tissues. J Biol Chem **226:** 497–509
7. **Hartmann E, Beutelmann P, Vandekerkhove O, Euler R, Kohn G** (1986) Moss cell cultures as sources of arachidonic and eicosapentaenoic acids. FEBS Lett **198:** 51–55
8. **Jelsema CL, Morre DJ, Ruddat M, Turner C** (1977) Isolation and characterization of the lipoprotein reserve bodies (spherosomes) from aleurone layers of wheat. Bot Gaz **138:** 138–149
9. **Lacal JC, de la Pena P, Moscat J, Garcia-Barreno P, Anderson PS, Aaronson SA** (1987) Rapid stimulation of diacylglycerol production in Xenopus oocytes by microinjection of H-ras p21. Science **238:** 533–536
10. **Lowry OH, Rosebrough NJ, Farr AL, Randall RJ** (1951) Protein measurement with the Folin phenol reagent. J Biol Chem **193:** 265–275
11. **Melin P-M, Sommarin M, Sandelius AS, Jergil B** (1987) Identification of Ca^{2+}-stimulated polyphosphoinositide phospholipase C in isolated plant plasma membrane. FEBS Lett **223:** 87–91
12. **Morré DJ, Gripshover B, Monroe A, Morré JT** (1984) Phosphatidylinositol turnover in isolated soybean membranes stimulated by the synthetic growth hormone, 2,4-dichlorophenoxyacetic acid. J Biol Chem **259:** 15364–15368
13. **Morré DJ, Morré JT, Varnold RL** (1984) Phosphorylation of membrane located proteins of soybean *in vitro* and response to auxin. Plant Physiol **75:** 265–268
14. **Morse MJ, Crain RC, Satter RL** (1987) Phosphatidylinositol cycle metabolites in *Samanea saman* pulvini. Plant Physiol **83:** 640–644
15. **Niedel JE, Blackshear PJ** (1986) Protein kinase C. *In* JW Putway, Jr, ed, Phosphoinositides and Receptor Mechanism. Alan R Liss, New York, pp 47–88
16. **Nishizuka Y** (1984) Studies and perspective of protein kinase C. Science **225:** 1365–1368
17. **O'Brien CA, Arthur WL, Weinstein IB** (1987) The activation of protein kinase C by the polyphosphoinositol 4-phosphate. FEBS Lett **214:** 339–342
18. **Pfaffmann H, Hartmann E, Brightman AO, Morré DJ** (1987) Phosphatidylinositol-specific phospholipase C of plant stems: membrane associated activity concentrated in plasma membranes. Plant Physiol **85:** 1151–1155
19. **Preiss J, Loomis CR, Bishop WR, Stein R, Niedel JE, Bell RM** (1986) Quantitative measurement of *sn*-1,2-diacylglycerols present in platelets, hepatocytes and *ras*- and *sis*-transformed normal rat kidney cells. J Biol Chem **261:** 8597–8600
20. **Ranjeva R, Boudet AM** (1987) Phosphorylation of proteins in plants: regulatory effects of potential involvement in stimulus-response coupling. Annu Rev Plant Physiol **38:** 73–94
21. **Sandelius AS, Morré DJ** (1987) Characteristics of phosphatidylinositol exchange activity of soybean microsomes. Plant Physiol **84:** 1022–1027
22. **Sandelius AS, Sommarin M** (1986) Phosphorylation of phosphatidylinositols in isolated plant membranes. FEBS Lett **201:** 282–286
23. **Schäfer A, Bygrave F, Matzenauer S, Marone D** (1985) Identification of a calcium- and phospholipid-dependent protein kinase in plant tissue. FEBS Lett **187:** 25–28
24. **Schumaker KS, Sze H** (1987) Inositol 1,4.5-triphosphate releases Ca^{2+} from vacuolar membrane vesicles of oat roots. J Biol Chem **262:** 3944–3946
25. **Sommarin M, Sandelius AS** (1988) Phosphatidylinositol and phosphatidyl-inositolphosphate kinases in plant plasma membranes. Biochim Biophys Acta **958:** 268–278
26. **Wolfman A, Macara IG** (1987) Elevated levels of diacylglycerol and decreased phorbol ester sensitivity in ras-transformed fibroblasts. Nature **325:** 359–361