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Diacylglycerol Kinase from Suspension Cultured Plant Cells[1]

Characterization and Subcellular Localization

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ABSTRACT

Diacylglycerol kinase (adenosine 5′-triphosphate:1,2-diacylglycerol 3-phosphotransferase, EC 2.7.1.107), purified from suspension cultured *Catharanthus roseus* cells (J Wissing, S Heim, KG Wagner [1989] Plant Physiol 90: 1546–1551), was further characterized and its subcellular location was investigated. The enzyme revealed a complex dependency on lipids and surfactants; its activity was stimulated by certain phospholipids, with phosphatidylinositol and phosphatidylglycerol as the most effective species, and by deoxycholate. In the presence of Triton X-100, used for its purification, a biphasic dependency upon diacylglycerol was observed and the apparent Michaelis constant values for diacylglycerol decreased with decreasing Triton concentration. The enzyme accepted both adenosine 5′-triphosphate and guanosine 5′-triphosphate as substrate and showed rather low apparent inhibition constant values for all nucleoside diphosphates tested. Diacylglycerol kinase is an intrinsic membrane protein and no activity was found in the cytosol. An investigation of different cellular membrane fractions confirmed its location in the plasma membrane.

Whereas the phosphorylation of PI[2] and its transformation by phospholipase C into inositol phosphates has been confirmed in plants (3, 11, 22, 27), the role of DG in signal transduction, *i.e.* in the stimulation of protein phosphorylation, has not been clarified in plants as of yet. Furthermore, recycling of DG by DG-kinase has not received much attention, although effective transformation of DG into PA is assumed to be an essential function in all cells (14). Recently, the purification of DG-kinase from suspension cultured *Catharanthus roseus* cells was described (33). In the present work, the subcellular localization and the further characterization of this enzyme, especially an investigation of its ATP site and the dependency on lipid substrate and phospholipid cofactors, have been performed. A preliminary account of some of this work has appeared recently (34).

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[2] Abbreviations: PI, phosphatidylinositol; BPG, bisphosphatidylglycerol (cardiolipin); DG, diacylglycerol; IMF, intracellular membrane fraction; MCF, microsomal membrane fraction; MIT, mitochondria fraction; PA, phosphatidic acid; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PS, phosphatidylserine; K_i, inhibition constant; PC, phosphatidylcholine.

MATERIALS AND METHODS

Membrane Preparations

All membrane and organelle fractions were prepared from 6-d-old suspension cultured *Catharanthus roseus* cells. The crude MCF was obtained as described previously (33) and was used for aqueous two-phase partitioning, which was performed according to the method of Larsson *et al.* (17). Whereas in the upper phase a rather pure plasma membrane fraction was obtained, the lower phase consisted of a mixture of internal membranes and cell organelles, which were further separated by centrifugation. A crude MIT was prepared by dilution of the lower phase (fivefold with 5 mM KPO_4, pH 6.8, 0.24 M sucrose) and centrifugation for 10 min at 10,000 *g*; the pellet was washed twice with the same buffer containing 0.3 M sucrose and centrifugation at 8,000*g* for 10 min. The remaining membranes, after removal of the MIT fraction, were collected by centrifugation at 44,000*g* for 60 min (IMF) as was the plasma membrane fraction from the upper phase.

Nuclear envelopes were prepared according to the method of Philipp *et al.* (21) by sonification of nuclei, obtained according to the method of Willmitzer and Wagner (32), but omitting any detergent to avoid solubilization of the membrane components. To obtain a tonoplast fraction, protoplasts were prepared from 20 g *C. roseus* cells according to the method of Ehmke *et al.* (9), suspended in 20 mL of buffer (0.4 M mannitol, 5 mM Hepes, 5 mM EDTA, 0.5% BSA, 2 mM DTT, 8% Ficoll (w/v), pH 8), and gently agitated at 30°C for 10 min for lysis. The vacuoles were filtered through a 100-μm nylon net and purified by flotation centrifugation; 20 mL of suspended vacuoles were overlaid with 10 mL of the above buffer containing 5% Ficoll and another 20 mL of the same Ficoll suspension and centrifuged at 100*g* for 20 min. The vacuoles were lysed by dilution of the 5% Ficoll phase (fourfold with 25 mM Tris-Mes, pH 6.0, and 5 mM 2-ME) and stirring on ice for 10 min; tonoplast membranes were collected by centrifugation at 22,000*g* for 20 min. All membrane fractions were stored in liquid nitrogen; the activity of the investigated enzymes did not decrease over a period of 4 weeks.

Enzyme and Analytical Assays

DG-kinase activity was determined as described previously (33). The standard assay mixture (250 μL) contained 40 mM Bis-Tris (pH 7.5), 5 mM $MgCl_2$, 0.1 mM EDTA, 1 mM spermine, 0.5 mM DTT, 1 mM sodium-deoxycholate, 0.02% Triton

X-100, 250 μM cardiolipin, 500 μM dioleoylglycerol, 1 mM ATP (about 1 μCi labeled ATP), and 25 μL of the enzyme fraction; the Triton X-100 was introduced by the purified enzyme fraction. Determination of *in situ* activities in membranes was performed, however, with endogenous lipid substrates, and dioleoylglycerol, BPG, and deoxycholate were omitted in this case; membranes were suspended in the assay buffer. The incorporation of label into PA was linear with time for up to 30 min at 25°C and with enzyme protein up to 30 μg.

1,3-β-Glucan synthase (EC 2.4.1.34) was assayed according to the method of Kauss and Jeblick (16). Cyt *c* oxidase (EC 1.9.3.1), NADPH-Cyt *c* oxidoreductase, and ATPase were assayed according to the method of Briskin *et al.* (5). DG levels of isolated membrane fractions were determined as described by Preiss *et al.* (23) using *Escherichia coli* DG-kinase (Lipidex, Westfield, NJ); the DG content was related to lipid bound phosphate (nmol DG/100 nmol lipid bound phosphate). Lipid phosphate was determined according to the method of Morrison (20), Pi according to the method of Eibl and Lands (10), and protein according to the method of Bradford (4).

Determination of K_i values was performed according to the method of Dixon and Webb (8). Substrate analogs were tested at six different concentrations in the presence of 5, 10, and 20 μM labeled ATP or GTP, respectively.

RESULTS

Dependency on Phospholipids and Surfactants

It was confirmed that the purified enzyme fraction did not form significant amounts of labeled PA without the addition of DG. Furthermore, assays with PI or PC (their heads labeled with tritium) did not reveal any water-soluble product and hence phospholipase C or D activity. Although dioleoylglycerol was used throughout as substrate for the DG-kinase assay, DGs with two saturated fatty acids, such as myristic, lauric, and caprylic acid, were also tested; however, in each case DG-kinase activity was dependent on the addition of phospholipid (BPG) and deoxycholate, *i.e.* in their absence only a low activity was observed (*e.g.* Fig. 2).

DG-kinase showed a complex dependency on lipid substrate, lipid cofactor, and surfactant; it was routinely assayed with dioleoylglycerol in the presence of 250 μM BPG, 1 mM sodium-deoxycholate, and about 0.3 mM (0.02%) Triton X-100; the latter was introduced by the purified enzyme fraction (33). Without adding phospholipids and surfactant, only about 1.5% of maximum activity was observed (*e.g.* Fig. 2); addition of either BPG or deoxycholate resulted in an approximately 10-fold increase in activity, whereas both together showed an approximately 70-fold increase.

Dependency of purified DG-kinase on phospholipids is shown in Figure 1; the assays were performed in the presence of deoxycholate and Triton X-100 as mentioned above. Interestingly, the negatively charged phospholipids showed significant lipid cofactor activity with PI as the most effective species, whereas PG was most effective at low concentrations. The positively charged phospholipids, PE and PC, showed low or insignificant effects. BPG, which was included in the standard assay at 250 μM, showed a broad plateau with about a 10-fold stimulation of the activity.

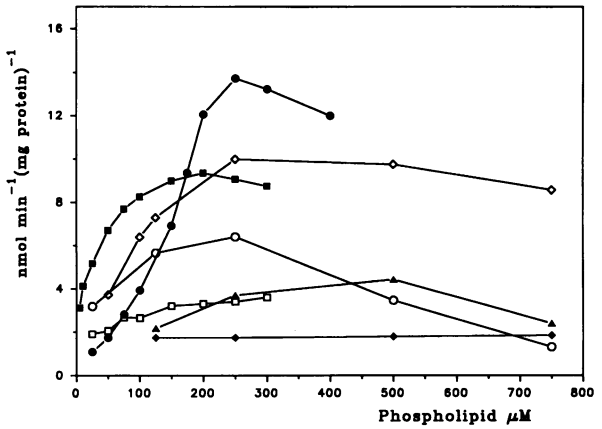

Figure 1. Dependency of purified DG-kinase activity on phospholipids. The standard assay (see "Materials and Methods") contained 1 mM deoxycholate, 0.3 mM Tritron X-100, and phospholipids as indicated. ●, PI; ■, PG; ◇, BPG; ○, PS; ▲, PE; □, PA; ♦, PC. The data are average values from three determinations; SD were below ± 5%.

Determination of K_m values for the lipid substrate DG was complicated due to the complex dependency on lipid cofactors and surfactants. Figure 2 shows the activity of the purified DG-kinase in the absence of deoxycholate and lipid cofactor; the activity was very low and showed a linear dependency on the substrate over a wide concentration range, although at DG concentrations higher than 500 μM a decrease in activity was observed. Extrapolation of the linear range resulted in an apparent K_m value of about 900 μM.

Under standard assay conditions with 1 mM deoxycholate, 0.3 mM Triton X-100 and 250 μM BPG, the dependency on the DG concentration was biphasic, showing an initial linear phase followed by a hyperbolic phase (Fig. 2). This behavior suggested a transition in the properties of the vesicles formed in the mixture of lipids and surfactants, which obviously also included the enzyme protein. Decreasing Triton concentrations shifted the transition point to lower DG concentrations, and a pure hyperbolic curve was obtained at concentrations below 0.06 mM. In this case, an apparent K_m for DG between 40 and 80 μM was estimated, although the Lineweaver-Burk plot was not strictly linear. In our previous paper (33), a K_m value of 250 μM was reported, which was obtained under the standard assay conditions, *i.e.* in the presence of 0.3 mM Triton.

Nucleotide Specificity and Apparent Kinetic Parameters

DG-kinase used ATP and GTP as substrate, showing Michaelis-Menten kinetics in both cases. The K_m values, determined in the presence of 5 mM Mg^{2+}, were 102 and 104 μM, respectively. The maximum velocity value obtained with GTP was even slightly larger than that for ATP, indicating that the enzyme did not discriminate between the two different bases with respect to both affinity and catalysis.

To investigate the nucleoside triphosphate binding site of

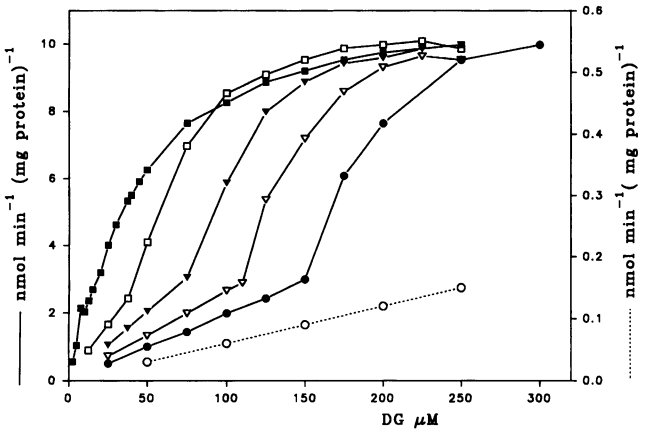

Figure 2. Effect of Triton X-100 on the activity of DG-kinase. Deoxycholate was 1 mM and BPG 250 μM (see "Materials and Methods"); Triton X-100 concentrations in mM were (■) 0.03, (□) 0.06, (▼) 0.15, (▽) 0.19, (●) 0.39. Triton was removed from the purified enzyme fraction by elution of the enzyme from a Pharmacia Mono Q column with basic buffer (33); thereafter, Triton was added as indicated. Dashed line (○) shows the activity (right scale) in the presence of 0.39 mM Triton X-100, but in the absence of BPG and deoxycholate. The data are average values from three determinations; SD were below ± 5%.

the enzyme more thoroughly, K_i values of available substrate analogs were determined by the method of Dixon and Webb (8) and listed in Table I. All nucleoside tri- and diphosphates tested were found to be competitive inhibitors (assayed in the presence of labeled ATP), whereas all ribonucleoside monophosphates showed very low affinities with no inhibitory effect at a concentration of 2 mM.

As tested with the nucleoside triphosphates, the kinase preferred the bases A and G, whereas CTP, UTP, and ITP had lower affinities. Interestingly, the nucleoside diphosphates showed low K_i values irrespective of the base, indicating that the β-phosphoryl group contributes strongly to the binding affinity. Changes at the ribose moiety were probed by showing that both the 2′deoxy- and 3′deoxy-derivatives of ATP were substrates although kinetic parameters could not be obtained, inasmuch as only a small amount of ^{32}P-labeled material (a gift of the Genetics Department of the Gesellschaft für Biotechnologische Forschung) was available. Changes in the triphosphate moiety were probed with sulfur analogs (Table I); introduction of sulfur at the γ-position of ATP did not reduce the affinity; however, catalysis of the phosphoryl transfer was obviously inhibited, as ^{35}S-labeled adenosine 5′-*O*-(3-thiotriphosphate) and guanosine 5′-*O*-(3-thiotriphosphate) did not show any incorporation into DG. The importance of the β-phosphoryl group for binding is also documented by the significantly larger K_i value of guanosine 5′-*O*-(2-thiodiphosphate) relative to GDP.

Inorganic pyrophosphate showed a rather low affinity—at 2 mM no inhibitory effect was observed—whereas Pi displayed a K_i value of about 2.5 mM, which may be of some physiological importance, inasmuch as Pi concentrations in the cytoplasm were reported to be in this range (24).

Localization of DG-Kinase

DG-kinase activity was totally associated with membranes and no activity could be detected in the cytosolic fraction (33); this was also confirmed with leaf and root tissue from *C. roseus* plants (data not shown). To elucidate the nature of the cellular membrane(s) that harbor this enzyme activity, different membrane fractions were prepared from suspension cultured *C. roseus* cells (see "Materials and Methods"). Total cellular membranes, defined as the MCF, were prepared by differential centrifugation and used as the starting material for two-phase separation, which is most suitable for the isolation of pure plasma membranes (17). In its lower phase a less defined mixture of internal membranes was obtained that was further fractionated (by differential centrifugation) into a crude MIT, which probably also contained plastids, and internal membranes (IMF). Tonoplast membranes and nuclear envelopes were prepared according to separate methods. Table II shows the specific activity of DG-kinase together with the activity of marker enzymes for the plasma membrane (1,3-β-glucan synthase), mitochondria (Cyt *c* oxidase), and ER (Cyt *c* reductase), the DG content (expressed as nmol DG/100 nmol lipid bound phosphate), and the subcellular distribution of ATPase activities (expressed as the percentage of inhibition by Na_3VO_4, KNO_3, and NaN_3, respectively).

The high specific activity of glucan synthase, the absence of Cyt *c* oxidase and reductase, and the strong inhibition of ATPase activity by vanadate were indicative of a highly purified plasma membrane fraction. Relative to the membrane weight, about 8% of the MCF could be obtained as plasma membranes by two-phase separation. The distribution of DG-kinase activity revealed a high activity in this fraction; the specific activity showed a ninefold enrichment relative to the crude MCF. The low specific activities found with the other cellular membranes could, at least in part, result from plasma membrane impurities in these fractions. This is especially apparent with the IMF and the MIT fractions, which revealed a low level of glucan synthase activity (Table II). Hence, DG-kinase is a plasma membrane-localized enzyme.

DG-kinase activity in the different membrane preparations

Table I. *K_i Values of DG-Kinase for Nucleoside Tri- and Diphosphates*

The K_i values were determined according to the method of Dixon (8) in the presence 5, 10, and 20 μM [γ^{32}-P]ATP; in the case of ATP, [γ^{32}-P]GTP was used as substrate. For each ATP concentration, enzyme activity was determined in duplicate at six different inhibitor concentrations.

Inhibitor	K_i	Inhibitor	K_i
	μM		μM
ATP[a]	102[a]	ADP	95
ATPγS	107		
GTP	81	GDP	58
		GDPβS	123
CTP	480	CDP	60
UTP	260	UDP	43
ITP	510	IDP	38

[a] Determined with [γ-^{32}P]GTP as substrate.

Table II. *Specific Activities of DG-Kinase and Marker Enzymes, and DG Content of Different Cellular Membrane Fractions*

The specific activities of DG-kinase are indicated as nmol min^{-1} (mg protein)$^{-1}$, and the other enzyme activities as μmol min^{-1} (mg protein)$^{-1}$. DG contents are expressed as mol% of phospholipids (nmol DG/100 nmol lipid-bound phosphate). Cyt *c* reductase was assayed with NADPH (5). The data for the ATPases are presented as the percent inhibition in the presence of 0.5 mM vanadate, 50 mM nitrate or 1 mM azide (5). The data are average values from three determinations; the SDS were below ± 5%.

Enzyme	Membrane Fractions					
	PM[a]	IMF	MIT	TP	NE	MCF
	nmol min^{-1} (mg protein)$^{-1}$					
DG-kinase	1.28	0.048	0.06	0.02	0.013	0.14
	μmol min^{-1} (mg protein)$^{-1}$					
1,3-β-Glucan synthase	1.12	0.09	0.034	NM[a]	NM	0.18
Cyt *c* oxidase	ND[b]	0.59	0.51	ND	0.054	0.57
Cyt *c* reductase	ND	0.10	0.05	ND	ND	0.04
ATPases	*% inhibition*					
pH 6.5, Na_3VO_4	85	11	16	11	61	NM
pH 8.0, KNO_3	3	27	23	56	9	NM
pH 8.5, NaN_3	16	31	65	37	11	NM
	nmol (100 nmol lipid bound phosphate)$^{-1}$					
DG content	4.6	1.5	2.7	0.7	2.4	4.1

[a] PM, plasma membrane fraction; TP, tonoplast membrane fraction; NE, nuclear envelopes; NM, not measured; ND, not detectable.

was tested *in situ* with endogenous lipid substrate (omitting DG, BPG, and deoxycholate in the assay); solubilization by adding deoxycholate (DG and BPG) led to lower specific activities. Obviously, the membranes contained enough available DG substrate. This was confirmed by determination of the DG pools according to Preiss *et al.* (23), which were found to be significant in all the different membrane fractions, especially in the plasma membrane (Table II).

DISCUSSION

Dependency on Lipids and Surfactants

The effects of lipids and surfactants, to be discussed below, were studied with a purified enzyme in artificial assay systems, *i.e.* under conditions strongly different from those in a native membrane. Despite these difficulties, we tried to draw some conclusions with respect to the native conditions.

Membrane proteins obviously possess a limited number of binding sites for phospholipids (28), and their occupation by various phospholipids may affect enzyme activity differently. In the case of DG-kinase from *C. roseus*, the most effective phospholipids were PI and PG and to some extent also PS, whereas the effect of BPG may not be physiologically important inasmuch as DG-kinase is a plasma membrane enzyme. The enzyme displayed a requirement for negatively charged phospholipids; it is an interesting question, whether differing concentrations of these activating phospholipids in the plasma membrane may modulate the activity of DG-kinase *in situ*. PI was also shown to be a good activator for DG-kinase from rat brain (15). However, PC, a potent activator of the cytosolic DG-kinase from pig brain (12) or the membrane localized enzyme from *E. coli* (2, 26, 31), was ineffective with the present enzyme. DG-kinases from rat brain or human white blood cells were markedly activated by PS (15, 29), which showed only a weak effect with the present plant DG-kinase.

In the case of enzymes with lipid substrates, substrate solubilization may be an additional role of phospholipids; this is especially important for DG, which alone is not able to form bilayers but disorganizes biomembranes by forming hexagonal structures (7). However, mixtures of phospholipids and DG were shown to form multilamellar membrane structures (6). Hence, PI and PG may be the natural cofactors that provide DG to the membrane-associated enzyme.

Triton X-100, at low concentrations, did not affect the activity of DG-kinase, whereas at higher levels it was found to be strongly inhibitory. It could, however, successfully be used during the different steps of purification, inasmuch as the enzyme remained fully active in the presence of this surfactant. On the other hand, deoxycholate induced a marked increase in enzyme activity with a maximum between 0.5 and 1.5 mM; above this concentration it was inhibitory. There is evidence that mixed micellar structures formed with phospholipids and Triton X-100 (ellipsoidal) are very different from those with deoxycholate (dish-like) (18, 25). DG-kinase, operating at the cytoplasmic side of the plasma membrane, may point to the inner side of DG-phospholipid bilayers, and deoxycholate may be required to disrupt these structures forming mixed micelles with DG and phospholipids (18), which are accessible to ATP.

At Triton concentrations above 0.03 mM, dependency of kinase activity upon its substrate DG became biphasic (Fig. 2), a phenomenon observed with several lipid-transforming enzymes (1). One could speculate that Triton formed mixed micelles with DG that largely excluded BPG, deoxycholate,

and the enzyme protein. Only at higher DG concentrations was DG incorporated into micelles carrying BPG, deoxycholate, and the enzyme protein. This is in accord with suggestions of Bessoule *et al.* (1) on membrane-bound enzymes on the distribution and exclusion of lipid substrates.

The ATP Site

DG-kinase did not strongly discriminate between the bases of nucleoside triphosphates, and GTP was as good a substrate as ATP, although in the presence of the prevailing cellular ATP pools (19), other nucleotides may be less likely used as substrate. ATP- and GTP-dependent DG-kinases were also described from rat brain and liver, both as cytosolic and membrane-bound enzymes (13, 15). Szopińska *et al.* (30) even reported a CTP-dependent membrane-bound enzyme from *Saccharomyces cerevisiae.* The low K_i values for nucleoside diphosphates (Table I) may suggest a physiological role during conditions when the energy charge decreases; however, at normal physiological conditions the pools of the diphosphates are lower than that of ATP, although in green tissue the diphosphate pools were found to be markedly higher than in suspension cultured cells (19).

Localization of DG-Kinase

For animal tissues, both soluble and membrane-bound DG-kinases have been described (14), whereas the present plant enzyme was found to be exclusively membrane-bound and thus is similar to the bacterial enzyme from *E. coli* (2, 31). DG-kinase from *C. roseus* was found with a high specific activity in the plasma membrane fraction, whereas other cellular membrane fractions revealed low activities that could partially result from contamination with plasma membrane vesicles. The present results suggest that DG-kinase could be used as an additional marker enzyme for plant plasma membranes.

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