Proc. Natl. Acad. Sci. USA
Vol. 83, pp. 9378–9382, December 1986
Biochemistry

Diacylglycerol-induced translocation of diacylglycerol kinase: Use of affinity-purified enzyme in a reconstitution system

(phospholipid metabolism/phospholipase C/protein kinase C)

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Contributed by Pedro Cuatrecasas, August 25, 1986

ABSTRACT **Diacylglycerol-induced translocation of diacylglycerol kinase (ATP:1,2-diacylglycerol 3-phosphotransferase, EC 2.7.1.107) from the soluble to the membrane-bound compartments was demonstrated both in crude tissue homogenates and in a reconstituted enzyme–membrane model system. In homogenates of either rat brain or liver, incubation with diacylglycerol or phospholipase C, but not phospholipase A_2 or phospholipase D, resulted in the translocation of diacylglycerol kinase activity from the soluble to the particulate fraction. This observation formed the basis for the first step in a two-step purification of diacylglycerol kinase. Enzyme extracted in 1 M salt from membranes of rat brain homogenates made in the presence of phospholipase C was purified further by affinity chromatography on a column containing phosphatidylserine, diacylglycerol, and cholesterol immobilized in polyacrylamide. This step yielded an enzyme preparation (step 2 enzyme) that was 500- to 750-fold purified (relative to the tissue homogenate) and required phosphatidylserine for stability. All other lipids tested failed to stabilize the enzyme. The properties of the enzyme preparation were similar to those of mammalian diacylglycerol kinases described by others. Reconstitution experiments showed that the soluble step 2 enzyme bound to inside-out vesicles of human erythrocytes only in the presence of diacylglycerol or phospholipase C but not phospholipase A_2 or D. Redistribution of the kinase from soluble to vesicle-bound forms occurred rapidly and was dependent on the concentration of phospholipase C used to treat the vesicles. Physiological concentrations of calcium (50–1000 nM) did not enhance the phospholipase C-mediated translocation of the kinase. Thus, diacylglycerol kinase can translocate from cytosol to membranes in a manner dependent on the content of membrane-bound diacylglycerol but independent of the ambient concentration of calcium.**

The recent advent of diacylglycerol as the endogenous activator of protein kinase C has propelled diacylglycerol into the limelight as an important second messenger, coupling receptor-mediated hydrolysis of phosphatidylinositols to activation of protein kinase C. It has been known for some time that diacylglycerol is produced as a result of the hydrolysis of phosphatidylinositols by phospholipase C (1–3). Many reports (4–8) indicate that protein kinase C can translocate from cytosol to membranes as a result of the formation of diacylglycerol and the increased cytosolic calcium concentration resulting from inositol trisphosphate formation. However, diacylglycerol is rapidly converted to phosphatidate by diacylglycerol kinase (ATP:1,2-diacylglycerol 3-phosphotransferase, EC 2.7.1.107) (9, 10). Like protein kinase C, diacylglycerol kinase requires phospholipid for catalytic function (11, 12). Moreover, because diacylglycerol kinase is at least partially responsible for the half-life of diacylglycerol, it may play an important role in regulating the coupling of agonist-induced generation of membrane diacylglycerol to activation of protein kinase C. In eukaryotic cells, however, diacyglycerol kinase has received little attention. Early studies (13–16) indicated that the enzyme was located in many cellular compartments, as defined by cell fractionation. Initial attempts to purify the enzyme fell short (17), and only recently was purification of the enzyme from pig brain reported (11). The purification scheme, however, required large amounts of starting material and 1 week to perform six different chromatographic steps and yielded a labile enzyme. Thus, the goals of this work were to develop a rapid method to obtain significantly purified diacylglycerol kinase, to stabilize the enzyme, and to define enzyme behavior crucial to regulation of membrane diacylglycerol levels. Here we report (*i*) the use of lipid-affinity chromatography to obtain a highly enriched preparation of the kinase in one step, (*ii*) an absolute requirement for phosphatidylserine to stabilize the highly purified enzyme, and (*iii*) the rapid translocation of the enzyme from cytosol to membrane in response to the formation of membrane diacylglycerol, as demonstrated in both crude tissue homogenates and a reconstituted enzyme–membrane model system.

MATERIALS AND METHODS

Materials. Phospholipase C (type V from *Bacillus cereus* and type XII from *Clostridium welchii*), phospholipase A_2 (from bee venom), phospholipase D (type III from peanut), and all phospholipids were from Sigma. Diacylglycerols (1,2- from pig liver lecithin) and 1,2- and 1,3-diolein (1,2- and 1,3-dioleoyl-*sn*-glycerol) were from Serdary Research Laboratories (London, ON).

Preparation of Phosphatidylserine/Diacylglycerol/Cholesterol/Polyacrylamide Affinity Column. Affinity columns were prepared essentially as described (18), using 5 mg of phosphatidylserine, 5 mg of diacylglycerol, and 25 mg of cholesterol; in some experiments 5 mg of phosphatidylcholine was substituted for phosphatidylserine. The polymerized gel was stored at 4°C and used within 1–2 days of preparation. Affinity columns were prepared by grinding the polymerized gel through a wire-mesh tea strainer (pore size 0.5 mm) six times. The resulting particles were suspended in water and allowed to settle for 5 min. The supernatant was decanted, and the pellet was resuspended in water and allowed to settle as above. This procedure was repeated four times with water and then twice with ice-cold buffer C (20 mM Tris·HCl, pH 7.6/10 mM dithiothreitol/1 mM $CaCl_2$/1 mM $MgCl_2$). The gel particles suspended in buffer C were packed into a 2-ml glass column (0.5 × 10 cm), and the column was run at 10–15 ml/hr.

Assay of Diacylglycerol Kinase. Enzyme activity was assayed by modification of the method of Kanoh *et al.* (11). The

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amount of phosphatidate produced from 1,2-diacylglycerol was determined in a reaction mixture (100 μl) containing Tris·HCl (50 mM, pH 7.6), $MgCl_2$ (10 mM), dithiothreitol (1 mM), phosphatidylserine (0.13 mM) cosonicated with diacylglycerol (1.5 mM), unlabeled ATP (0.3 mM), and [γ-^{32}P]ATP (5 nM). Reaction mixtures were incubated at 30°C for 10 min. When analyzed by thin-layer chromatography, >95% of the radioactive reaction product consisted of phosphatidate. When various phospholipids or diacylglycerols were tested in the assay, all other conditions remained as described.

Purification of Diacylglycerol Kinase from Rat Brain. All procedures were carried out at 4°C unless otherwise noted.

Step 1. Eight adult Sprague–Dawley rats were decapitated, and the brains were removed, rapidly washed, and then homogenized with buffer A (20 mM Tris·HCl, pH 7.6/10 mM dithiothreitol/1 mM $CaCl_2$/0.5 mM EDTA containing 0.2 mg of leupeptin, 4 units of type V phospholipase C, and 2 units of type XII phospholipase C per ml; 1 ml/g of tissue). The homogenate was incubated at 37°C for 15 min, EGTA (5 mM) was added, and the homogenate was incubated for 10 min at 4°C. After centrifugation at 40,000 × *g* for 25 min, the pellet was washed once with buffer B (20 mM Tris·HCl, pH 7.6/10 mM dithiothreitol/0.5 mM EDTA containing 0.2 mg of leupeptin per ml; 1 ml/g of tissue), and the diacylglycerol kinase was extracted from the pellet by homogenization with buffer B plus 1 M KCl (0.5 ml/g of tissue). The homogenate was stirred slowly at 4°C for 10 min and then centrifuged. The resulting high-salt extract was dialyzed against buffer B (without leupeptin) for 16 hr and then clarified by centrifugation at 100,000 × *g* for 30 min to yield step 1 enzyme.

Step 2. Immediately following ultracentrifugation, step 1 enzyme was applied to a phosphatidylserine/diacylglycerol/cholesterol affinity column at pH 7.6 in the presence of $CaCl_2$ (5 mM), $MgCl_2$ (5 mM), NaCl (200 mM) and phenylmethylsulfonyl fluoride (230 μM). Typically, 15–20 ml of step 1 enzyme was applied to a column continuously for 90–120 min. Columns were washed with five column volumes of wash buffer 1 (20 mM Tris·HCl, pH 7.6/10 mM dithiothreitol/150 mM NaCl/0.1 mM $CaCl_2$/0.1 mM $MgCl_2$ with 0.2 mg of leupeptin per ml), and then with three column volumes of wash buffer 2 (20 mM Tris·HCl, pH 7.6/10 mM dithiothreitol/5 mM EDTA/ 5 mM EGTA). Diacylglycerol kinase was then eluted with 4 ml of elution buffer [20 mM Tris·HCl, pH 7.6/10 mM dithiothreitol/1 M NaCl (or KCl) with 0.2 mg of leupeptin per ml]. The eluate (step 2 enzyme) was desalted and concentrated in Amicon microconcentrators, and the enzyme was stabilized by addition of phosphatidylserine at 250 μg/ml.

This purification scheme yielded 3–12 μg of protein. Typically, $NaDodSO_4$/PAGE showed four major bands, at 145, 110, 93, and 73 kDa. In some experiments, a band at 50 kDa was observed. Attempts to demonstrate catalytic activity by elution of the individual bands out of nondenaturing gels were unsuccessful, as were further purification attempts using HPLC, conventional gel filtration, or ATP-agarose affinity chromatography.

Binding of Diacylglycerol Kinase to Inside-Out Vesicles. Inside-out vesicles were prepared from human erythrocytes (19) by incubating washed "ghosts" in 1 mM Tris·HCl, pH 7.6/0.05 mM EDTA at 37°C for 45 min (8). The inside-out vesicles were washed three times with 10 mM Tris·HCl (pH 7.6) and resuspended in this buffer at 1–3 mg of protein per ml. Binding of diacylglycerol kinase to inside-out vesicles was assayed in 200-μl reaction mixtures containing Tris·HCl (10 mM, pH 7.6), $MgCl_2$ (3 mM), dithiothreitol (1 mM), bovine serum albumin (30 μg/ml), inside-out vesicles (100–300 μg of protein), EGTA (0.1–1 mM), and phospholipase C (0–2.5 units/ml). After preincubation for 5 min at 23°C, step 2 kinase (50–200 ng of protein) was added, and the mixture was incubated at 30°C for 10 min and then immediately centrifuged at 40,000 × *g* for 10 min. The supernatant was assayed for diacylglycerol kinase activity as described above. In certain experiments, $CaCl_2$ was added (in the presence of 100 μM EGTA) to give final free calcium concentrations as determined previously (20). In other experiments phospholipase A_2 or D was substituted for phospholipase C.

Certain limitations concerning the reconstitution system require mention. (*i*) Inside-out vesicles exhibited endogenous diacylglycerol kinase activity; however, binding of exogenously added diacylglycerol kinase could be evaluated easily above this background. The endogenous activity did not dissociate under the assay conditions used. (*ii*) The basal binding of the kinase to the inside-out vesicles (in the absence of phospholipase C and of calcium) varied considerably, from 10% to 65%. The majority of this background binding, rather than being nonspecific, may represent binding induced by the presence of endogenous diacylglycerol in the preparations of inside-out vesicles and of kinase.

$NaDodSO_4$/PAGE. Electrophoresis was performed as described by Laemmli (21). Protein was determined by the method of Bradford (22), using bovine serum albumin as the standard.

RESULTS

Preparation of Diacylglycerol Kinase. Step 1 kinase was further purified by chromatography on a lipid-affinity column containing phosphatidylserine/diacylglycerol/cholesterol immobilized in polyacrylamide. Typically, 25–60% of the diacylglycerol kinase activity bound to the column, and the presence of calcium was essential for binding. Addition of 1 M KCl or NaCl eluted 25–50% of the bound enzyme activity. However, in contrast to the reported elution of protein kinase C from a calcium-containing phosphatidylserine/cholesterol affinity column (18), the diacylglycerol kinase could not be eluted by addition of 10 mM EDTA or EGTA. Lower salt concentrations (300 mM) were also ineffective in eluting the kinase. Purification of the eluted step 2 enzyme was determined to be 500- to 750-fold.

To further characterize the binding of the kinase to the affinity column, the specificity of diacylglycerol kinase for immobilized phosphatidylserine and diacylglycerol was evaluated. Three columns were prepared: phosphatidylserine/diacylglycerol/cholesterol, phosphatidylserine/cholesterol, and phosphatidylcholine/cholesterol. Compared to the phosphatidylserine/diacylglycerol/cholesterol column, the phosphatidylcholine/cholesterol column bound 70% less enzyme and yielded 95% less enzyme. However, the column prepared with phosphatidylserine/cholesterol bound only 30% less enzyme and yielded only 25% less enzyme. Thus, although the presence of diacylglycerol did improve overall performance, the binding of the kinase to the phosphatidylserine/diacylglycerol/cholesterol column appeared to be governed more by the specificity of the enzyme for phosphatidylserine than by its specificity for diacylglycerol. This point is further illustrated by the finding that washing the phosphatidylserine/diacylglycerol/cholesterol column with 10 mM ATP/5 mM $MgCl_2$ failed to elute the enzyme.

Stabilization of Diacylglycerol Kinase. During a previous purification of diacylglycerol kinase, Kanoh *et al.* (11) noted the extreme lability of the isolated enzyme. Likewise, our attempts to stabilize the step 2 enzyme with dimethyl sulfoxide, polyethylene glycol, glycerol, high salt, or freezing all were unsuccessful. However, when sonicated phosphatidylserine was added to purified step 2 enzyme, 65% of the activity was retained after 7 days at 4°C. Of the five phospholipids evaluated, only phosphatidylserine was able to stabilize the activity (Fig. 1). Addition of sonicated phosphatidylcholine, phosphatidylinositol, phosphatidyleth-

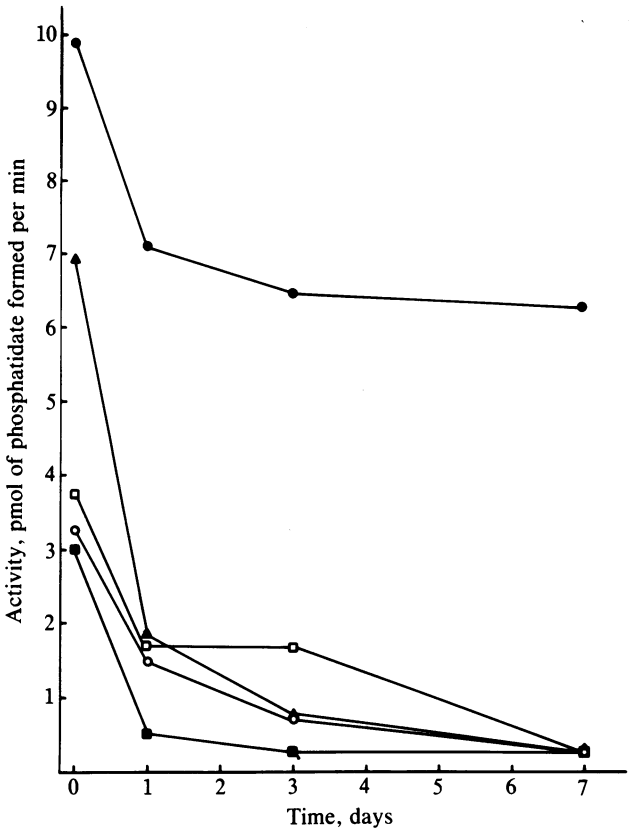

FIG. 1. Dependence of diacylglycerol kinase stability on the presence of phospholipid. Step 2 kinase was prepared and concentrated as described in *Materials and Methods*. The enzyme preparation was divided into equal aliquots and phosphatidylserine (●), phosphatidylcholine (▲), phosphatidylinositol (○), phosphatidylethanolamine (□), or sphingomyelin (■) was added (250 μg/ml). After various times of storage at 4°C, the diacylglycerol kinase activity of the aliquots was assayed in the presence of phosphatidylserine. Enzyme receiving no phospholipid addition showed nearly complete loss of catalytic activity after only 1 day of storage. The results suggest that lipids other than phosphatidylserine may not only fail to stabilize the enzyme but may also be inhibitory to the enzyme, since samples stabilized with lipids other than phosphatidylserine showed decreased catalysis at time zero. This finding is consistent with that of others (11). All values represent means of triplicate determinations, and the experiment represents one of three performed.

anolamine, and sphingomyelin to step 2 kinase resulted in total loss of activity within 1–3 days. The addition of sonicated diacylglycerol, lysophosphatidylserine and lysophosphatidylcholine were also ineffective at stabilizing the enzyme (data not shown). These results again reflect the specificity of the kinase for phosphatidylserine and provide a method for stabilization of the purified enzyme.

Characterization of the Stabilized Diacylglycerol Kinase Preparation. Step 2 kinase demonstrated kinetic properties similar to those of diacylglycerol kinase purified by others from pig brain (11, 12) and rat liver (17). Diacylglycerol kinase has been reported to be specific for 1,2-diacylglycerol (12, 15). Step 2 enzyme showed a 7- to 8-fold preference for phosphorylating 1,2-diacylglycerol over 1,3-diacylglycerol. The K_m values of step 2 kinase for 1,2-diacylglycerol and for ATP were determined to be 1 μM and 600 μM, respectively. These values are within the range reported for other preparations of diacylglycerol kinase (11, 12, 15, 17). The catalytic activity of the step 2 kinase had an absolute requirement for phospholipid, showing a 3-fold preference for phosphatidylserine over phosphatidylcholine. This finding is consistent with that of Bishop *et al.* (12), who demonstrated that pig brain diacylglycerol kinase preferred phosphatidylserine over all other individual lipid cofactors tested.

Diacylglycerol and Phospholipase C Induce Translocation of Diacylglycerol Kinase in Brain and Liver Homogenates. Protein kinase C can translocate to membranes in response to agonist-induced diacylglycerol formation (4–8), and the half-life of diacylglycerol is short, in part because of its rapid conversion to phosphatidate by diacylglycerol kinase (9, 10). Thus, for diacylglycerol kinase to convert agonist-induced diacylglycerol to phosphatidate, we hypothesized that the enzyme must be able to translocate from cytosol to membranes in response to membrane-bound diacylglycerol. To test this hypothesis, we determined the distribution of diacylglycerol kinase activity between cytosol and particulate fraction in homogenized rat brains incubated with phospholipase C, diacylglycerol, or control buffer (Table 1). Addition of diacylglycerol or phospholipase C markedly shifted the distribution of diacylglycerol kinase activity from cytosol to particulate. To determine whether this redistribution was specific for phospholipase C (specific for diacylglycerol), rat brains were homogenized, split into four aliquots, and incubated in the presence of 1 mM calcium with 5 units of phospholipase A_2, D, or C per ml or with vehicle. Following centrifugation, the pellets were washed and then extracted with 1 M KCl. The brain homogenates incubated with vehicle alone or with phospholipase A_2 or D all exhibited identical diacylglycerol kinase activities in the KCl extracts. The brain homogenate incubated with phospholipase C, however, contained nearly 3 times more diacylglycerol kinase activity associated with the particulate fraction, as reflected by the KCl extract. Virtually identical results were obtained in experiments using homogenates of liver (data not shown). These results suggest that generation of diacylglycerol by phospholipase C in brain or liver homogenates results in the translocation of diacylglycerol kinase from cytosol to the particulate fraction. This translocation provided the rationale for step 1 of the purification.

Diacylglycerol and Phospholipase C Induce Calcium-Independent Translocation of Diacylglycerol Kinase in a Reconstitution System. The use of human erythrocyte inside-out vesicles as a model system for studying protein–membrane interactions has been demonstrated (7, 8, 23). To study translocation of diacylglycerol kinase in a well-defined model system, binding of step 2 enzyme to inside-out vesicles was monitored. Kinase was added to inside-out vesicles pretreated with various concentrations of phospholipase C, and the soluble enzyme was separated from the inside-out vesicles by centrifugation. Kinase activity was lost from the supernatant in a manner dependent on the concentration of phospholipase C used to pretreat the inside-out vesicles (Fig. 2). At levels as low as 0.005 unit of phospholipase C per ml, loss of

Table 1. Diacylglycerol and phospholipase C promote the translocation of diacylglycerol kinase to membranes in brain homogenates

Addition	Activity, nmol of phosphatidate formed per min		Distribution (cytosol/pellet)
	Cytosol	Particulate	
None	42	14	75%/25%
Diacylglycerol	31	24	56%/44%
Phospholipase C	14	38	27%/73%

Six rat brains were homogenized in buffer A (without phospholipase C) at 4°C, and the homogenate split into three equal aliquots, which were incubated for 10 min at 37°C either with no addition, with diacylglycerol (40 μg/ml) or with phospholipase C (4 units of type V plus 2 units of type XII per ml). Homogenates were centrifuged at 20,000 × *g* for 30 min and the resulting supernatants and pellets were assayed for diacylglycerol kinase activity.

diacylglycerol kinase activity could be detected. Addition of exogenous diacylglycerol to inside-out vesicles also induced association (24%) of the kinase with the inside-out vesicles, although, as observed in the tissue-homogenate experiments (Table 1), this treatment was not as effective as treatment with phospholipase C.

To determine whether the induced association of diacylglycerol kinase activity with the inside-out vesicles was specific for phospholipase C (membrane-derived diacylglycerol), diacylglycerol kinase was added to vesicles pretreated either with vehicle or with 2.5 units of phospholipase A_2, D, or C per ml (Table 2). Treatment of inside-out vesicles with phospholipase A_2 or D or with vehicle did not induce loss of soluble diacylglycerol kinase activity from the supernatant. However, treatment of inside-out vesicles with phospholipase C resulted in a 32% decrease in diacylglycerol kinase activity associated with the supernatant. Control samples containing diacylglycerol kinase and phospholipase, but no inside-out vesicles, did not exhibit a decrease in soluble (supernatant) kinase activity. Additionally, all phospholipases were devoid of diacylglycerol kinase activity. In separate experiments, 81% of the kinase activity lost from the supernatant was found to be associated with the inside-out vesicles (data not shown).

To explore further the degree of similarity between diacylglycerol-induced translocation of protein kinase C and of diacylglycerol kinase, the effect of calcium on phospholipase C (2.5 units/ml)-induced translocation was investigated (Table 3). A physiological concentration of calcium (1 μM) did not significantly increase the phospholipase C-mediated association of soluble diacylglycerol kinase activity with inside-out vesicles. Note, however, that 1 mM calcium resulted in substantial association of diacylglycerol kinase activity with inside-out vesicles in the absence of phospholipase C. This observation is consistent with the binding of the kinase to phosphatidylserine/cholesterol columns in the presence of high calcium (5 mM). To ensure that the calcium-independent nature of diacylglycerol kinase translocation described in Table 3 did not result from the use of a supramaximal concentration of phospholipase C (2.5 units/ml), the experiments were repeated using a limiting concentration of the phospholipase (0.005 unit/ml; see Fig. 2). Again, the presence of physiological concentrations of calcium (50–1000 nM) failed to increase the amount of diacylglycerol kinase associated with inside-out vesicles (data not shown). These results indicate that translocation of diacylglycerol kinase from cytosol to membranes is dependent on the generation of membrane-bound diacylglycerol but independent of the concentration of calcium, within the physiological range.

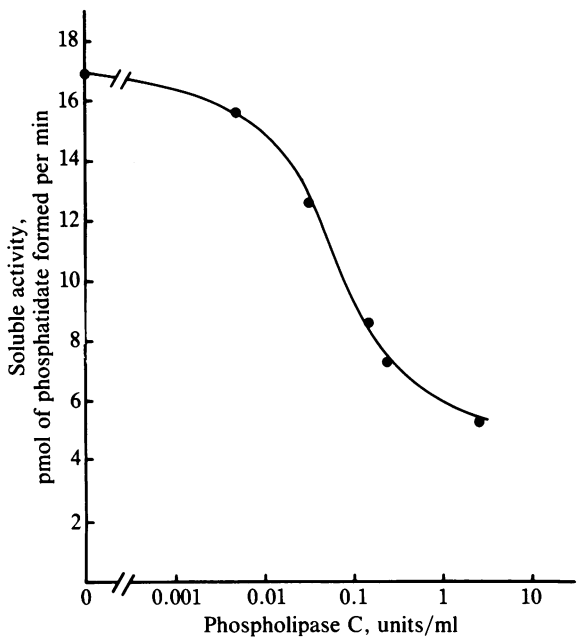

FIG. 2. Dependence of translocation of diacylglycerol kinase to inside-out vesicles on the concentration of phospholipase C. Inside-out vesicles were preincubated for 5 min at 23°C with various concentrations of phospholipase C and then incubated with step 2 kinase for 10 min at 30°C. Samples were centrifuged, and the diacylglycerol kinase activity remaining in the supernatant was determined. Experiments were performed in the presence of 1 mM EGTA. Each value represents the mean of triplicate determinations.

Table 2. Translocation of diacylglycerol kinase to inside-out vesicles is specifically dependent on the presence of phospholipase C

Addition	Soluble activity, pmol of phosphatidate formed per min	% decrease
None	10.4	0
Phospholipase A_2	9.5	9
Phospholipase D	10.2	2
Phospholipase C	7.1	32

Inside-out vesicles were preincubated for 5 min at 23°C either with 2.5 units of phospholipase A_2, D, or C per ml or with vehicle and then incubated with step 2 diacylglycerol kinase for 10 min at 30°C. Samples were centrifuged, and the diacylglycerol kinase activity remaining in the supernatant was determined. Experiments were performed in the presence of 1 mM EGTA. Values represent the means of triplicate determinations.

DISCUSSION

A two-step procedure based on the affinity of diacylglycerol kinase for diacylglycerol and phosphatidylserine has been used to obtain a highly enriched preparation of rat brain diacylglycerol kinase. Stabilization of this preparation required phosphatidylserine, as did expression of catalytic activity. In either crude tissue homogenates or a reconstituted enzyme–membrane model system, diacylglycerol-induced translocation of the kinase could be demonstrated, and this translocation appeared to be independent of calcium. In light of the participation of diacylglycerol kinase in both glycerolipid synthesis and turnover of hormone-induced

Table 3. Calcium dependence of translocation of diacylglycerol kinase to inside-out vesicles

Additions				
Vesicles	Phospholipase C	1 μM Ca^{2+}	1 mM Ca^{2+}	Soluble activity, % of maximum
−	−	−	−	96
−	+	−	−	102
+	−	−	−	100
+	+	−	−	50
+	−	+	−	88
+	+	+	−	43
+	−	−	+	74
+	+	−	+	47

Inside-out vesicles were preincubated for 5 min at 23°C in the absence or presence of phospholipase C (2.5 units/ml) and then incubated with step 2 diacylglycerol kinase for 10 min at 30°C in the absence or presence of calcium. All samples contained 100 μM EGTA; where indicated, enough $CaCl_2$ was added to achieve a final calcium concentration of 1 μM or 1 mM (20). Samples were centrifuged, and the diacylglycerol kinase activity remaining in the supernatant was determined. Values represent the means of triplicate determinations. The catalytic activity of type V phospholipase C from *B. cereus* is calcium-independent.

membrane diacylglycerol, translocation should be viewed within the framework of the subcellular organization of the enzyme.

Diacylglycerol kinase has been detected in both soluble and membrane-bound subcellular fractions, including cytosol (14, 15), microsomes (13–15), synaptosomes (14), and nuclear envelopes (16). The properties of membrane-bound and soluble kinase are reported to be similar (15), but whether both activities arise from the same enzyme species is not known. In this regard, translocation of a single species of diacylglycerol kinase between cellular compartments permits multiple sites of intracellular enzyme localization, and the data presented here make this possibility tenable. As well, polyclonal antibodies raised against a purified pig brain cytosolic diacylglycerol kinase demonstrated crossreactivity with diacylglycerol kinase from pig brain microsomes and synaptosomes (24). However, other observations suggest that there are probably a multiplicity of cellular diacylglycerol kinase species: (*i*) Antibody raised against a single species of pig brain cytosolic diacylglycerol kinase failed to precipitate 40% of the total activity in pig brain cytosol (24). (*ii*) The reported molecular mass of pig brain diacylglycerol kinase is 70 kDa (11) and that of the rat liver enzyme is 121 kDa (17). (*iii*) Although the step 2 enzyme described here was highly purified, NaDodSO$_4$/PAGE of the preparation still showed four major bands. Numerous attempts to purify this preparation to homogeneity met with little success (see *Materials and Methods*). Invariably, the four bands copurified and showed behavior inconsistent with a subunit-to-holoenzyme relationship. These observations raise the possibility that the four bands represent different species of rat brain diacylglycerol kinase.

Both protein kinase C and diacylglycerol kinase require anionic phospholipids as essential cofactors, with phosphatidylserine being the most effective (3, 12, 25). The inner surface of the plasma membrane is rich in phosphatidylserine, making the erythrocyte inside-out vesicle a good model for studying the translocation of diacylglycerol kinase and protein kinase C from cytosol to the inner aspect of the plasmalemma. In addition, both enzymes possess binding sites for diacylglycerol (diacylglycerol is a cofactor for protein kinase C and the substrate for diacylglycerol kinase). However, in contrast to protein kinase C, diacylglycerol kinase does not require physiological concentrations of calcium for either its catalytic activity or for its diacylglycerol-induced translocation from the cytosol to the membrane. This indifference to calcium may be seminal to understanding what similarities, if any, the binding sites common to both enzymes share. Recently, Bell and coworkers (26, 27) proposed a model for the binding of phosphatidylserine, diacylglycerol, and calcium by protein kinase C. The cornerstone of this model is that calcium is anchored by four phosphatidylserine head groups and diacylglycerol, forming a complex capable of activating protein kinase C. If this model proves correct, it is difficult to see how this paradigm could work for diacylglycerol kinase. Clearly, characterization of the binding domains common to both protein kinase C and diacylglycerol kinase requires further analysis. In this regard, preliminary results indicate that phorbol 12,13-dibutyrate may not effectively bind to the diacylglycerol binding site (active site) of diacylglycerol kinase, as 1 μM phorbol dibutyrate does not inhibit enzyme-mediated phosphorylation of diacylglycerol.

Competition between diacylglycerol kinase and protein kinase C for agonist-induced diacylglycerol appears likely. Both enzymes have similar affinities for diacylglycerol (25, 28), both enzymes are stereospecific for 1,2-diacyl-*sn*-glycerol (15, 29), both enzymes translocate to the cell membrane in response to the appearance of diacylglycerol (although protein kinase C requires increased cytosolic calcium to do so effectively; refs. 4–8), and both enzymes require phosphatidylserine for binding to cell membranes and for expression of catalytic activity (3). Thus, one might speculate that an additional level of complexity may exist—namely, regulation of diacylglycerol kinase by protein kinase C.

We thank Dr. Marlene Wolf for advice and assistance throughout the course of this work, Drs. Harry LeVine and Naji Sahyoun for helpful discussions, and Marian Goodwin for preparing the manuscript.

1. Berridge, M. J. & Irvine, R. F. (1984) *Nature (London)* **312,** 315–321.
2. Majerus, P. W., Neufeld, E. J. & Wilson, D. B. (1984) *Cell* **37,** 701–703.
3. Nishizuka, Y. (1986) *Science* **233,** 305–312.
4. Kraft, A. S., Anderson, W. B., Cooper, H. L. & Sando, J. J. (1982) *J. Biol. Chem.* **257,** 13193–13196.
5. Kraft, A. S. & Anderson, W. B. (1983) *Nature (London)* **301,** 621–623.
6. May, W. S., Jr., Sahyoun, N., Wolf, M. & Cuatrecasas, P. (1985) *Nature (London)* **317,** 549–551.
7. Wolf, M., Cuatrecasas, P. & Sahyoun, N. (1985) *J. Biol. Chem.* **260,** 15718–15722.
8. Wolf, M., LeVine, H., III, May, W. S., Jr., Cuatrecasas, P. & Sahyoun, N. (1985) *Nature (London)* **317,** 546–549.
9. Michell, R. H. (1975) *Biochim. Biophys. Acta* **415,** 81–147.
10. Lapetina, E. G. & Cuatrecasas, P. (1979) *Biochim. Biophys. Acta* **573,** 394–402.
11. Kanoh, H., Kondoh, H. & Ono, T. (1983) *J. Biol. Chem.* **258,** 1767–1774.
12. Bishop, W. R., Ganong, B. R. & Bell, R. M. (1986) *J. Biol. Chem.* **261,** 6993–7000.
13. Hokin, M. R. & Hokin, L. E. (1959) *J. Biol. Chem.* **234,** 1381–1386.
14. Lapetina, E. G. & Hawthorne, J. N. (1971) *Biochem. J.* **122,** 171–179.
15. Kanoh, H. & Akesson, B. (1978) *Eur. J. Biochem.* **85,** 225–232.
16. Smith, C. D. & Wells, W. W. (1983) *J. Biol. Chem.* **258,** 9368–9373.
17. Kanoh, H. & Ohno, K. (1981) *Arch. Biochem. Biophys.* **209,** 266–275.
18. Uchida, T. & Filburn, C. R. (1984) *J. Biol. Chem.* **259,** 12311–12314.
19. Steck, T. L. & Kant, J. A. (1974) *Methods Enzymol.* **31,** 172–180.
20. Fabiato, A. & Fabiato, F. (1979) *J. Physiol. (Paris)* **75,** 463–506.
21. Laemmli, U. K. (1970) *Nature (London)* **227,** 680–685.
22. Bradford, M. M. (1976) *Anal. Biochem.* **72,** 248–254.
23. Bennett, V. & Branton, D. (1977) *J. Biol. Chem.* **252,** 2753–2763.
24. Kanoh, H., Iwata, T., Ono, T. & Suzuki, T. (1986) *J. Biol. Chem.* **261,** 5597–5602.
25. Takai, Y., Kishimoto, A., Kikkawa, U., Mori, T. & Nishizuka, Y. (1979) *Biochem. Biophys. Res. Commun.* **91,** 1218–1224.
26. Bell, R. M. (1986) *Cell* **45,** 631–632.
27. Ganong, B. R., Loomis, C. R., Hannun, Y. A. & Bell, R. M. (1986) *Proc. Natl. Acad. Sci. USA* **83,** 1184–1188.
28. Sharkey, N. A., Leach, K. L. & Blumberg, P. M. (1984) *Proc. Natl. Acad. Sci. USA* **81,** 607–610.
29. Rando, R. R. & Young, N. (1984) *Biochem. Biophys. Res. Commun.* **122,** 818–823.