Regulation of Diacylglycerol Kinase Reaction in Swiss 3T3 Cells

INCREASED PHOSPHORYLATION OF ENDOGENOUS DIACYLGLYCEROL AND DECREASED PHOSPHORYLATION OF DIDECANOYLGLYCEROL IN RESPONSE TO PLATELET-DERIVED GROWTH FACTOR*

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We studied the influence of platelet-derived growth factor (PDGF) on diacylglycerol phosphorylation in Swiss 3T3 cells. Rates of incorporation of $^{32}$P into phosphatidic acid (PA) and phosphatidylinositol (PtdIns) were determined in prelabeled cells into which sn-1,2-didecanoylglycerol (diClo) had been introduced. PDGF stimulated the formation of $[^{32}]$PJA and -PtdIns from endogenous substrates but decreased the formation of $[^{32}]$PJAlo and -PtdInslo. Direct measurements of diacylglycerol phosphorylation in lysates of quiescent and stimulated cells showed that PDGF stimulated the phosphorylation of endogenous diacylglycerol 2-fold in parallel with diacylglycerol accumulation but decreased by 50% the phosphorylation of diClo. Total diacylglycerol kinase activity, measured in a mixed micellar assay, was not changed by PDGF treatment. The maximum activity of diacylglycerol kinase exceeded that needed to phosphorylate all of the endogenous diacylglycerol, suggesting that the PDGF-dependent increase in diacylglycerol mass would account for the increase in PA formation. The increased mass of diacylglycerol also could explain the inhibition of diClo phosphorylation, via substrate competition. The predominant species of endogenous diacylglycerol was 1-stearoyl-2-arachidonoyl-glycerol (18:0/20:4 diacylglycerol). In mixed micelles, the rate of phosphorylation of 18:0/20:4 diacylglycerol was 9-fold higher than that of diClo, and the 18:0/20:4 species competed with diClo for phosphorylation. Studies showed that a membrane-bound enzyme accounted for the PDGF effect on PA formation; there was no evidence for the translocation of cytosolic enzyme to the membrane. The results support these conclusions: 1) PDGF stimulates the phosphorylation of cellular diacylglycerol by promoting a transient accumulation of this lipid. 2) The stimulated phosphorylation is catalyzed by a diacylglycerol kinase that preferentially phosphorylates 18:0/20:4 diacylglycerol over diClo. 3) The diacylglycerol kinase responsible for the PDGF effect is membrane-bound.

In a variety of cell types, diacylglycerol is generated in cell membranes in response to hormones that stimulate PtdIns$^\gamma$ turnover. This endogenous, membrane-bound diacylglycerol is thought to signal cellular events by serving as an activator of protein kinase C (see Refs. 1 and 2 for review). In some cells, diacylglycerol may also be an immediate source of arachidonic acid for eicosanoid formation (3, 4). In addition, diacylglycerol provides a backbone for resynthesis of PtdIns via diacylglycerol kinase. Because changes in diacylglycerol levels are thought to regulate the activation of protein kinase C in mammalian cells, it has been suggested that the concentration of diacylglycerol in membranes may be tightly controlled.

Bishop et al. (5) have presented evidence that diacylglycerol kinase may play a central role in the attenuation of diacylglycerol second messengers.

Despite the apparent importance of diacylglycerol kinase in diacylglycerol metabolism, the eukaryotic enzyme has received relatively little attention. Diacylglycerol kinase activity is present in all tissues that have been examined (6–10) and has been found in cytosolic (11–14), microsomal (11, 12, 14–17), and nuclear fractions (18). The properties of the enzyme from different subcellular fractions were found to be similar. Diacylglycerol kinases have been purified from cytosol of pig brain (19) and liver (13) and have molecular weights of approximately 80 and 120 kDa, respectively. This indicates that mammalian cytosolic diacylglycerol kinases may differ substantially from the bacterial enzyme, which has a molecular mass of only 14 kDa (20).

Nevertheless, the eukaryotic and prokaryotic enzymes appear to have several properties in common: they require phospholipid for activation, utilize diacylglycerol either in phospholipid vesicles or detergent micelles, exhibit marked thermal instability, and are inhibited by divalent cations such as Ca$^{2+}$.

Studies of many cell systems have shown that the incorporation of $^{32}$P into PA is increased rapidly during hormone-stimulated PtdIns turnover, but the contribution of diacylglycerol kinase to total PA formation is unknown. A major question is whether the hormone-stimulated increase in diacylglycerol mass might be sufficient to increase PA formation or whether agonist-dependent activation of diacylglycerol kinase occurs by a mechanism such as phosphorylation or dephosphorylation. Recently, Kanoh and Ono (21) reported that the cytosolic enzyme from brain could be phosphorylated by an endogenous protein kinase, but the kinase was not.

The abbreviations used are: PtdIns, phosphatidylinositol; PA, phosphatidic acid; dClo, sn-1,2-didecanoylglycerol; PtdIns, didecanoylphosphatidylglycerol; dClo, 1-stearoyl-2-arachidonoylglycerol; 18:0/ 20:4 didecanoylglycerol, sn-1-stearoyl-2-arachidonoylglycerol; 18:1/18:1 diacylglycerol, sn-1,2-didecanoylglycerol; PDGF, platelet-derived growth factor; protein kinase C, Ca$^{2+}$/phospholipid-dependent enzyme; MOPS, 4-morpholinopropanesulfonic acid.

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identified and the effects of phosphorylation on enzyme activity were not tested.

To study the regulation of the diacylglycerol kinase reaction, we prepared lysates of quiescent and PDGF-stimulated Swiss 3T3 cells and measured directly the rates of phosphorylation of endogenous diacylglycerol and of a cell-permeable diacylglycerol kinase for quantitation of diacylglycerol kinase activity. We measured the activity of diacylglycerol kinase with pure diacylglycerols in a mixed micellar assay system. The results of our measurements, described in detail below, support two main conclusions: 1) PDGF stimulates the phosphorylation of endogenous diacylglycerol by promoting a transient accumulation of this lipid, and 2) the diacylglycerol kinase that catalyzes the increased phosphorylation of endogenous diacylglycerol shows a substrate selectivity for arachidonoyl-diacylglycerol, as compared with diC18:0, and is membrane-bound. In an accompanying report (22), we show the membrane-bound diacylglycerol kinase also selectively phosphorylates arachidonoyl-diacylglycerol in preference to long-chain endogenous substrates, whereas a soluble diacylglycerol kinase does not show this specificity.

**EXPERIMENTAL PROCEDURES**

**Materials**—[γ-32P]ATP (3000 Ci/mmol), [γ-32P]orthophosphate (1 mCi/ml, HCl-free), and [3H]glycerol (38 Ci/mmol) were from Du Pont-New England Nuclear.-sn-1,2-Dioleoylgluclycerol (18:1/18:1 diacylglycerol), 18:0/20:4 diacylglycerol, and pig liver phosphatidycholine were from Serdary Research Laboratories, London, Ontario, Canada. DiC18:0, pig brain phosphatidylserine, and bovine brain cardioline were from Avanti Polar Lipids, Birmingham, AL. Egg PA and bovine brain PtdIns were from Sigma. Lipids were stored under argon at -20 °C. β-Ocyl-glycerolide was from Boehringer Mannheim, W. Germany. Pre-coated cellulose plates (0.1 mm) for thin-layer chromatography were from EMI Science, Cherry Hill, N.J. Escherichia coli diacylglycerol kinase for quantitation of diacylglycerol mass as described (23) was obtained from Lipexids, Madison, WI. Partially purified PDGF was a gift from Bonnie Ashelman and Dr. Russell Ross of the University of Washington, Department of Pathology. Human plasma-derived serum was prepared as described (3). Other cell culture reagents were from GIBCO.

**Cell Culture and Labeling**—Swiss 3T3-D1 cells were maintained in culture and were made quiescent essentially as described (3, 24). For most studies, cells were plated in 100-mm dishes in 7 ml of medium containing 5% plasma-derived serum. To determine the effects of PDGF on the incorporation of 3P into PA and PtdIns, quiescent cells in 35-mm dishes were incubated with 50 μCi/dish of 32P in 1.5 ml of low (0.05 mM) phosphorous medium containing plasma-derived serum. After 3 h of labeling, diC18:0 was added in 9 μl of ethanol (final [diC18:0], 50 μM) followed immediately by 10 μl of PDGF in 10 mM acetic acid or vehicle alone (final [PDGF], 7 ng/ml). After 20 min, cells were harvested and lipids were extracted. To separate PA, and PtdIns, chromatography was carried out in the ethyl acetate solvent described above.

**RESULTS**

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experiments showed that the accumulation of diacylglycerol coincided with an increase in radiolabeling of PA from either 
$^{32}$P, [H]arachidonate, or [H]glycerol (data not shown). Since labeling of PA can occur either via diacylglycerol kinase or via de novo biosynthetic routes, the exact contribution of the diacylglycerol kinase reaction cannot be assessed from such studies. One approach to determining the role of diacylglycerol kinase in PA formation is to introduce an unusual substrate to serve as a probe. We reasoned that diClo, a synthetic diacylglycerol that has been shown to enter cells (33) and to serve as a substrate for diacylglycerol kinase in platelets (25), might be used to ask whether diacylglycerol kinase was activated in response to PDGF. Increased phosphorylation of diClo in response to agonist would be consistent with activation of the enzyme. An advantage of this approach is that the concentration of the synthetic diacylglycerol would not be affected by PDGF, so that its phosphorylation might reflect PDGF-dependent changes in diacylglycerol kinase activity independent of changes in diacylglycerol mass.

To determine the effect of PDGF on the formation of PA and PtdIns in intact cells, replicate cultures of quiescent cells were prelabeled with $^{32}$P for 3 h to label the intracellular ATP pool. DiClo was then added to all the cultures, and the cells were incubated with PDGF or vehicle. After 20 min, the cells were harvested and lipids were extracted. The phosphorylated products of the synthetic diacylglycerol, PA and PtdIns, were separated from endogenous PA and PtdIns by thin-layer chromatography on cellulose plates (Fig. 1A).

Quantitation of the results (Fig. 1B) showed that PDGF stimulated the formation of $[^{32}P]$PA and $[^{32}P]$PtdIns from endogenous sources. In contrast, there was no increase in the formation of PA or PtdIns in response to PDGF. In fact, in three separate experiments, PDGF addition to cells caused a 30-50% decrease in the conversion of diClo to PA and PtdIns. Direct measurements of the mass of intracellular diClo, using diacylglycerol kinase from E. coli for quantitation (23), showed that these results could not be explained by a decrease in the amount of diClo in response to PDGF (the mass of diClo was 1455 pmol/dish for quiescent cells and 1583 pmol/dish for PDGF-stimulated cells). The result indicated that the phosphorylation of diClo was actually decreased in response to PDGF and that the decreased phosphorylation of diClo resulted in a decreased formation of PtdIns. Although this result might indicate inhibition of diacylglycerol kinase, such inhibition would be inconsistent with the increased formation of $[^{32}P]$PA from endogenous sources. To resolve the apparent contradiction between the increased formation of $[^{32}P]$PA and the decreased formation of $[^{32}P]$PA, it was necessary to design experiments to measure directly the rate of phosphorylation of endogenous diacylglycerols in quiescent and PDGF-stimulated cells.

**Direct Measurements of Phosphorylation of Endogenous Diacylglycerol in Cell Lysates**—Because the PDGF-stimulated formation of endogenous $[^{32}P]$PA (Fig. 1) includes any PA that might be formed via de novo pathways, the exact contribution of the diacylglycerol kinase reaction cannot be assessed. To determine whether or not the formation of PA via diacylglycerol kinase was enhanced in response to PDGF, we sought to measure directly the rate of phosphorylation of endogenous diacylglycerol in lysates of quiescent and PDGF-stimulated cells. Cells were harvested at times up to 1 h after the addition of PDGF or vehicle and were lysed by freeze-thawing. Rates of phosphorylation of endogenous diacylglycerol were measured by adding MgCl$_2$ and $[^{32}P]$ATP to the lysates and incubating for 2 min at 34 °C. Fig. 2A shows that the diacylglycerol kinase activity in cell lysates utilized endogenous diacylglycerol to form $[^{32}P]$PA. The rate of formation of PA was 2-fold higher in lysates of PDGF-treated cells

We also conducted studies to determine whether diClo alone affected the incorporation of $^{32}$P into phospholipids. The addition of diClo to quiescent cells caused a 30-50% increase in incorporation of $^{32}$P into PA and an equivalent increase in the mass of endogenous diacylglycerol. Similar effects have been reported for phorbol esters in other cell systems (see Ref. 34). Although the mechanism of this effect remains unexplained, the extent of the PDGF-dependent stimulation of PA and PtdIns formation from endogenous sources was unaltered by the presence of diClo.
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Fig. 2. Effect of PDGF addition to quiescent cells on the phosphorylation of diacylglycerol by cell lysates. At the indicated times after the addition of PDGF to quiescent cultures of 3T3 cells, replicate cultures of cells were harvested and frozen. Rates of phosphorylation of endogenous and exogenous diacylglycerols were measured by the formation of \([32P]PA\) in the presence of \([\gamma-32P]ATP\) as described under "Experimental Procedures." Values are corrected to the total activity (picomoles/minute) per 100-mm dish. Each value represents the mean of three cultures ± S.D. Similar results were obtained in two separate experiments. A, phosphorylation of endogenous membrane diacylglycerol in the absence of detergent or added lipids. B, activity of diacylglycerol kinase measured with 6 mol % 18:1/18:1 diacylglycerol and 3 mol % cardiolipin in octylglucoside micelles.

Phosphorylation of Cell-associated diClO in Lysates—To determine if the decreased incorporation of \(^{32}P\) into diClO in response to PDGF (Fig. 1) was in fact due to a decrease in the rate of phosphorylation of diClO by diacylglycerol kinase, cells were incubated with diClO, treated with PDGF or vehicle for 20 min, and then harvested and frozen. The samples then were thawed, and the rate of phosphorylation of cellular diacylglycerol was measured as described above. In this experiment, the cellular diacylglycerol available for phosphorylation included the diClO that had become associated with the cells. Fig. 3 shows that, while the rate of phosphorylation of endogenous diacylglycerol was increased 2-fold in response to PDGF, the rate of phosphorylation of diClO was decreased by nearly 50%. The depression in diClO phosphorylation in response to PDGF was not due to a decrease in diClO mass, as discussed above. However, since PDGF stimulated an increase in the mass of endogenous diacylglycerol (from 1152 pmol/dish to 2086 pmol/dish in this experiment), one explanation...
for our data is that the expanded pool of endogenous diacylglycerol competes with diC10 for phosphorylation by diacylglycerol kinase. The increase in mass alone would not explain the competition because, as discussed above, the enzyme capacity appeared more than adequate to phosphorylate twice the mass of endogenous diacylglycerol. We therefore considered the alternate possibility that competition could occur if there were preferential phosphorylation of endogenous diacylglycerols as compared with diC10.

**HPLC Analysis of Endogenous Diacylglycerol Species**—Examination of the molecular species of diacylglycerols in quiescent and PDGF-treated cells (Fig. 4) showed the composition of the diacylglycerols present as substrates for diacylglycerol kinase. Cells were incubated with [3H]glycerol for 48 h, PDGF or vehicle was added, and cells were harvested after 10, 30, or 60 min. Lipid extracts were prepared and diacylglycerol was isolated by thin-layer chromatography and acetylated. The diacylglycerol derivatives were separated by high performance liquid chromatography on a reverse-phase column on which the compounds are separated by chain length and degree of unsaturation of the esterified fatty acids. Fractions were collected and were quantitated by liquid scintillation counting. In quiescent cells, the single predominant species, comprising a third of the total diacylglycerol, co-migrated with 18:0/20:4 diacylglycerol, and this species increased approximately 2-fold in response to PDGF. Other species co-migrated with diacylglycerols that contained primarily 18:1 or 18:2 in the sn-2 position. These species also accumulated in response to PDGF but to a lesser extent than the 18:0/20:4 form. An agonist-dependent accumulation of 18:0/20:4 diacylglycerol also has been observed in certain other cell types (27, 35).

**Phosphorylation of 18:0/20:4 Diacylglycerol and diC10 in Mixed Micelles**—Because 18:0/20:4 diacylglycerol was the predominant cellular species, we considered the possibility that this species might be selectively phosphorylated by diacylglycerol kinase. The utilization of this diacylglycerol as a substrate was compared with that of diC10 by measuring rates of phosphorylation of these compounds in a mixed micellar system in which octylglucoside was used to disperse the lipids. In studies of protein kinase C and the E. coli diacylglycerol kinase, Hannum et al. (30) and Walsh and Bell (29) demonstrated that the utilization of lipid substrates in mixed micelles depends not on the total concentration of lipid in the assay but rather on the molar fraction of lipid in the detergent micelles. We obtained similar results in assays of diacylglycerol kinase activity in 3T3 cells (not shown). Therefore, the phosphorylation of diacylglycerol species of different molecular weights was compared using an equal molar fraction (12 mol %), calculated as described under "Experimental Procedures." Under these conditions, rates of phosphorylation of the substrates were proportional to the amount of cell protein in the assay (Fig. 5). Based on the slopes of the fitted lines in this experiment, the rate of phosphorylation of 18:0/20:4 diacylglycerol exceeded the rate of phosphorylation of the synthetic diacylglycerol by about 8-fold, which showed that the arachidonoyl species was a substantially better substrate for diacylglycerol kinase than was diC10.

18:0/20:4 Diacylglycerol Competes with diC10 for Phosphorylation by Diacylglycerol Kinase—To determine if the preferential phosphorylation of 18:0/20:4 diacylglycerol could account for competition with diC10 for phosphorylation, mixtures were prepared in which the proportions of the two substrates were varied while the total diacylglycerol was kept constant at 12 mol %. Fig. 6 shows that, with single species present at 12 mol %, the rate of phosphorylation of diC10 was only one-fourth the rate of phosphorylation of the 18:0/20:4 species. Furthermore, as small amounts of 18:0/20:4 diacyl-
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Fig. 6. Competition between 18:0/20:4 diacylglycerol and diC10 for phosphorylation by diacylglycerol kinase. Mixtures of 18:0/20:4 diacylglycerol and diC10 were dried under nitrogen and dispersed in octylglucoside micelles. The total amount of diacylglycerol was kept constant at 12 mol %. The different diacylglycerol mixtures were incubated with cell lysate as described for Fig. 4. Lipid products were separated by thin-layer chromatography in the ethyl acetate solvent system to separate PA from PA10. Each point represents a single determination for a pool of lysates from four 100-mm dishes of cells grown to confluence in medium containing 10% calf serum. Similar results were obtained in two separate experiments.

Fig. 7. Phosphatidylserine dependence of diacylglycerol kinase with 18:0/20:4 diacylglycerol or diC10 as substrates. Lysates of whole cells were incubated for 2 min at 34 °C with [γ-32P]ATP and MgCl2 and with either 6 mol % diC10 or 6 mol % 1-stearoyl-2-arachidonoyl-glycerol in β-octylglucoside in the presence of phosphatidylserine at the indicated concentrations. Values are means of duplicate determinations.

Fig. 8. Phosphorylation of different diacylglycerol species by lysates of quiescent or PDGF-stimulated cells. A, autoradiogram showing the formation of PA from endogenous diacylglycerol and from three pure diacylglycerol species in the mixed micellar assay with lysates from PDGF-stimulated cells as the source of enzyme. Samples from the experiment described in B were spotted on a cellulose thin-layer plate, chromatographed in the ethyl acetate solvent system, and autoradiographed as described for Fig. 1A, with a 4-day exposure to film at -70 °C. Lane 1, PA formation from endogenous diacylglycerol (no added lipid or detergent); lane 2, PA formation from 6 mol % 18:1/18:1 diacylglycerol in octylglucoside; lane 3, PA formation from 6 mol % diC10 in octylglucoside; lane 4, PA formation from 6 mol % 18:0/20:4 diacylglycerol in octylglucoside. SF, solvent front; OR, origin. B, cells were harvested 20 min after the addition of PDGF or vehicle. Rates of phosphorylation of endogenous diacylglycerol (endog.) were determined as described for Fig. 2A. Rates of phosphorylation of pure 18:1/18:1 diacylglycerol, diC10, and 18:0/20:4 diacylglycerol were measured with 6 mol % diacylglycerol in the presence or absence of 6 mol % phosphatidylserine (PS), using the mixed micellar assay system. Values shown are means of duplicate determinations for three pooled cultures, corrected to total activity per 100-mm dish. Similar results were obtained in three separate experiments.
competes strongly with diC_{10} for phosphorylation by diacylglycerol kinase. The results mimicked the results obtained in intact cells in which the accumulation of membrane diacylglycerol derived from phosphoinositide breakdown (Fig. 4) was correlated with a decrease in phosphorylation of the synthetic diacylglycerol (Fig. 1B).

**Activation of Diacylglycerol Kinase by Phosphatidyserine**—Previous studies of either mammalian or bacterial diacylglycerol kinases have shown that certain phospholipids activate the enzyme (12, 19, 28, 29). We sought to determine if this was true for the 3T3 enzyme and if the substrate specificity we observed would persist with the activated enzyme. Fig. 7 shows that phosphatidyserine activated the enzyme 2- to 3-fold, with either diC_{18} or 18:0/20:4 diacylglycerol as substrate. Maximum activation was obtained with phosphatidyserine at 6 mol %. The long-chain diacylglycerol was a better substrate for diacylglycerol kinase even in the presence of optimal levels of phosphatidyserine. Similar results were obtained with cardiolipin as activator (not shown).

**Phosphorylation of Different Diacylglycerol Species by Ly- sates from Quiescent and PDGF-stimulated Cells**—Although studies of the phosphorylation of 18:1/18:1 diacylglycerol in mixed micelles (Fig. 2F) showed no apparent activation of diacylglycerol kinase in response to PDGF, the observation that diacylglycerol kinase exhibited substrate specificity suggested that subtle changes in diacylglycerol kinase properties might be revealed by studying the effect of PDGF on the phosphorylation of different diacylglycerol species. We compared rates of phosphorylation of diC_{10}, 18:1/18:1, and 18:0/20:4 diacylglycerols in mixed micelles in the presence or absence of phospholipid activator (phosphatidyserine) (Fig. 8). Fig. 8A shows that, when [32P]ATP was added to cell lysates, the only 32P-labeled phospholipid formed was PA. The addition of octylglucoside alone to cell lysates abolished the phosphorylation of endogenous diacylglycerol, probably because the endogenous substrate was diluted to a low surface concentration in the detergent micelles (not shown; see Ref. 22). Therefore, the rate of phosphorylation of pure diacylglycerol added in octylglucoside reflects only the phosphorylation of the exogenous species with no contribution from endogenous substrates. With 6 mol % of any species of added diacylglycerol, in the absence of phosphatidyserine, the rate of formation of PA was 2- to 4-fold higher than with endogenous substrates. Phosphatidyserine increased the rates of phosphorylation of each of the three diacylglycerol species (Fig. 8B). The highest activity of diacylglycerol kinase was obtained with 18:0/20:4 diacylglycerol in the presence of phosphatidyserine; this activity exceeded the activity with endogenous substrate by 6- to 10-fold, consistent with the previous conclusion that only a fraction of the enzyme activity is needed to phosphorylate all of the endogenous diacylglycerol in quiescent or PDGF-stimulated cells. The rate of phosphorylation of 18:0/20:4 diacylglycerol was greater than that of diC_{10} or of the 18:1/18:1 species, both in the presence and absence of phosphatidyserine. The observation that the rate of phosphorylation of 18:1/18:1 diacylglycerol, a naturally occurring substrate (Fig. 4), was even less than that of diC_{10} supports the idea that a principal endogenous competitor of diC_{10} phosphorylation was the 18:0/20:4 species.

\footnote{In the accompanying paper (22) we show that, of six naturally occurring long-chain diacylglycerols tested as substrates, only arachidonoyl-diacylglycerols were phosphorylated at higher rates than was diC_{10}, consistent with the conclusion that arachidonoyl-diacylglycerols are the principal endogenous competitors of diC_{10} in PDGF-stimulated cells. In the mixed micellar assay, the activity of the selective (membrane-bound) diacylglycerol kinase with the non-arachidonoyl substrates (18:1/18:1, 18:0/18:2, 16:0/18:1, or 18:0/18:1) was actually less than that with diC_{10}.}

In Fig. 8B, PDGF added to cells stimulated the formation of PA from endogenous diacylglycerol, as it did in previous experiments (Fig. 2A). This difference was abolished by incubating the lysates with saturating concentrations of 18:1/18:1 diacylglycerol, diC_{10}, or 18:0/20:4 diacylglycerol, in the presence or absence of phosphatidyserine. These results support the conclusion that the PDGF-dependent increase in the formation of PA from endogenous substrates results from the increased mass of diacylglycerol and not from an increase in diacylglycerol kinase activity.

**Participation of a Membrane Diacylglycerol Kinase in PDGF-stimulated Synthesis of PA**—The studies discussed above were performed with lysates of whole cells. Recently, Besterman et al. (36) suggested that cytosolic diacylglycerol kinase may participate in agonist-stimulated diacylglycerol phosphorylation. This conclusion was based on the observation that diacylglycerol kinase from rat brain cytosol would bind to membranes that had been treated with phospholipase C to generate diacylglycerol. We investigated the location of the PDGF-stimulated phosphorylation of endogenous diacylglycerol. Cells were harvested and freeze-thawed as described above. A portion of the whole-cell lysate was used for direct assay of endogenous phosphorylation, and the remainder was centrifuged at 100,000 × g to pellet membranes. After supernates were removed, membranes were resuspended in buffer and rates of phosphorylation of endogenous diacylglycerol were measured. The contribution of cytosolic enzyme to

![Fig. 9. Contribution of membrane diacylglycerol kinase to PDGF-dependent increase in phosphorylation of endogenous diacylglycerol. Quiescent cells were treated with PDGF or vehicle for 20 min and then harvested and frozen in 100 μl of buffer. Cells were then thawed and resuspended, and half of the lysate was used to measure endogenous diacylglycerol phosphorylation as described for Fig. 2A. The remaining 50 μl of lysate was centrifuged in an Airfuge. The cytosol was recovered, and the membrane pellet was resuspended in the original volume of buffer (50 μl) and used to measure endogenous diacylglycerol phosphorylation. \* Total cytosolic activity was measured with 4 mol % diacylglycerol and 4 mol % phosphatidyserine in octylglucoside micelles. Values (corrected to total activity per 100-mm dish) are means for nine cultures (for whole cells) or for five cultures (for membranes and cytosol) ± S.E.; each culture was assayed in duplicate, and the results from two separate experiments were pooled.}
the phosphorylation of endogenous diacylglycerol was deduced by difference between the activity in membranes and whole cells. 4 Fig. 9 shows that the enhanced phosphorylation of diacylglycerol in response to PDGF could be accounted for entirely by diacylglycerol kinase activity present in the crude membrane fraction. By difference, it was clear that a cytosolic enzyme contributed to the phosphorylation of membrane diacylglycerol in whole cells, but the apparent contribution was unaltered by PDGF addition.

Although the increased phosphorylation appeared confined to the membrane fraction, it remained possible that the crude membranes contained cytosolic enzyme that had translocated to the membrane in response to PDGF. To determine if PDGF stimulated a decrease in cytosolic activity consistent with translocation to the membrane, we used the mixed micellar assay to measure directly the total activity of the cytosolic enzyme. The results showed that the cytosolic activity did not increase upon PDGF addition. The results indicate that a membrane-bound diacylglycerol kinase may be responsible, in large part, for the increased phosphorylation of diacylglycerol in PDGF-stimulated cells.

**DISCUSSION**

Up to the present, the role of diacylglycerol kinase in agonist-stimulated formation of PA has been inferred solely from changes in the incorporation of radiolabeled precursors into PA. Our studies, in which the activity of the enzyme with endogenous substrate was measured directly in cell lysates or membranes, have shown that PDGF stimulates an increase in the formation of PA. We obtained evidence that 1) the increased formation of PA results, in large part, from the PDGF-dependent accumulation of diacylglycerol in cell membranes; 2) a membrane-bound diacylglycerol kinase is responsible for PDGF-stimulated diacylglycerol phosphorylation; and 3) the kinase activity in whole 3T3 cells exhibits substrate selectivity in that it preferentially phosphorylates 18:0/20:4 diacylglycerol as compared with diC16.

Several lines of investigation suggested that the PDGF-dependent increase in PA formation was a direct result of the increased mass of diacylglycerol. First, direct measurements of rates of phosphorylation of different species of diacylglycerols in the mixed micellar assay showed no effect of PDGF on diacylglycerol kinase activity, either in the absence or presence of phosphatidylserine. 5 In addition, comparisons of rates of phosphorylation of endogenous and exogenous diacylglycerols showed that the increased mass of diacylglycerol that occurs in response to PDGF could account for the increased phosphorylation of diacylglycerol that we measured in vitro, since it was apparent that the enzyme was not saturated with substrate under physiological conditions. Finally, the phosphorylation of a cell-permeable diacylglycerol, diC18, was decreased in response to PDGF, a result that is not consistent with activation of diacylglycerol kinase.

The decreased phosphorylation of diC16 in response to PDGF was attributable to competition with an expanded pool of endogenous diacylglycerols for phosphorylation by diacylglycerol kinase. The competition could be detected in cell lysates in which diacylglycerol kinase utilized endogenous diacylglycerols and cell-associated diC16 as substrates. The competition could not be explained by saturation of diacylglycerol kinase with PtdIns-derived diacylglycerols, because the total activity of the enzyme greatly exceeded the activity with endogenous diacylglycerol. It therefore seemed likely that the competition was due to selective phosphorylation of the long-chain endogenous diacylglycerols as compared with the medium-chain diC18. The observation that the single predominant species of diacylglycerol was 18:0/20:4 suggested that this species might be the principal competitor. Competition between pure 18:0/20:4 diacylglycerol and diC16 was verified in a mixed micellar assay system. In addition, competition between endogenous diacylglycerol and diC16 for phosphorylation was evident in intact cells in which the incorporation of 32P into PA16 and PtdIns18 was decreased in response to PDGF. These results demonstrate that a diacylglycerol kinase in 3T3 cells exhibits a pronounced substrate specificity, both in intact cells and in cell lysates. Moreover, the observation that a 50% decrease in the rate of phosphorylation of diC18 by diacylglycerol kinase resulted in a 50% decrease in the formation of [32P]PtdIns16 in intact cells suggests that the selective diacylglycerol kinase has a direct role in the resynthesis of PtdIns during PDGF-stimulated PtdIns turnover.

The selective phosphorylation of 18:0/20:4 diacylglycerol over diC16 was evident in intact cells, in measurements of rates of phosphorylation of endogenous diacylglycerols in vitro, and in the mixed micellar assay with pure diacylglycerols. The maintenance of substrate specificity in the mixed micellar assay emphasizes the validity of this assay in studying the physiologically relevant properties of diacylglycerol kinase.

The observation that the rate of phosphorylation of diC16 was inhibited by the PDGF-dependent accumulation of diacylglycerols shows that this cell-permeable diacylglycerol should not be relied upon as a probe of agonist effects on PtdIns metabolism. Instead, direct measurements of the activities of enzymes of this pathway may help to further our understanding of the mechanism by which agonists stimulate the coordinate breakdown and resynthesis of PtdIns and its phosphorylated derivatives.

The subcellular location of enzymes of the PtdIns cycle has been a subject of debate for many years. We found that the PDGF-dependent increase in diacylglycerol phosphorylation could be accounted for entirely by a diacylglycerol kinase present in the membrane fraction. Although comparisons of rates of phosphorylation of diacylglycerol in a membrane fraction with rates of phosphorylation in whole cells suggested that a cytosolic diacylglycerol kinase may have considerable activity toward membrane diacylglycerol in 3T3 cells, we found no evidence for activation of the cytosolic enzyme or translocation to the membrane in response to PDGF. These studies raise major questions about differences between the cytosolic and membrane-bound kinases and about the physiological role of the cytosolic enzyme. In addition, it will be of interest to determine the exact membrane location of the PDGF-stimulated formation of PA from diacylglycerol. Although it has been assumed widely that PtdIns-derived diacylglycerol accumulates in the plasma membrane and is converted to PA locally, direct evidence for this hypothesis is lacking.

Taken together, our results suggest that the diacylglycerol that accumulates in response to PDGF is rapidly phosphorylated by an active membrane-bound kinase, resulting in an increase in PA that is converted further to PtdIns. Since these studies also show that the PDGF-stimulated formation of PA was catalyzed by a diacylglycerol kinase that selected endog-
enous diacylglycerol over diC₀₆, it was expected that the membrane-bound enzyme would possess the substrate selectivity that we observed. In the accompanying paper (22), we present evidence that the membrane-bound and cytosolic diacylglycerol kinases have distinct properties; that only the membrane kinase is selective for arachidonoyl-diacylglycerol as compared with other naturally occurring long-chain diacylglycerols.

REFERENCES