Use of a fluorescent analog of CDP-DAG in human skin fibroblasts: characterization of metabolism, distribution, and application to studies of phosphatidylinositol turnover

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Abstract We studied the uptake, metabolism, and distribution of a fluorescent analog of CDP-diacylglycerol [cytidine diphosphate-1, 2-oleoyl, {N-(4-nitrobenzo-2-oxa-1,3-diazole) aminocaproyl} diacylglycerol; CDP-NBD-DAG]. When cells were incubated with CDP-NBD-DAG for 60 min at 11°C and washed, the fluorescent lipid was localized to the plasma membrane. However, upon warming to 37°C, the fluorescent lipid redistributed into various intracellular membranes and was metabolized primarily to fluorescent analogs of DAG and phosphatidylcholine (PC), although small amounts of fluorescent phosphatidic acid and phosphatidylinositol (PI) were also formed. The incorporation of $^{32}P_i$ into some of the fluorescent lipids was also determined in order to assess their turnover. Stimulation of cells with platelet-derived growth factor enhanced the synthesis of fluorescent PI relative to unstimulated cells by ~68%, while the synthesis of fluorescent PC was unaffected. In addition, the incorporation of $^{32}P_i$ into fluorescent PI was enhanced. Stimulation of cells with interleukin-1β enhanced the synthesis of both fluorescent PI (~88%) and PC (~250%) compared to non-stimulated cells, but with less incorporation of $^{32}P_i$ into fluorescent PI. Finally, incubation of CDP-NBD-DAG-treated cells with inhibitors of phosphatidic acid phosphohydrolase and DAG kinase resulted in a dramatic increase in the amount of fluorescent PI formed (~64% of all the CDP-NBD-DAG metabolites). We conclude that CDP-NBD-DAG can be used for the de novo synthesis of fluorescent PI, and in combination with ^{32}P labeling, provides a convenient method for studying PI turnover.—**Salman, M., and R. E. Pagano.** Use of a fluorescent analog of CDP-DAG in human skin fibroblasts: characterization of metabolism, distribution, and application to studies of phosphatidylinositol turnover. *J. Lipid Res.* 1997. **38:** 482–490.

Supplementary key words *IL-1β • PDGF • fluorescent lipid analogs • NBD • phospholipid metabolism*

Fluorescent lipid analogs have been useful for studying membrane lipid traffic in animal cells (reviewed in refs. 1–3). In this approach, one of the naturally occurring fatty acids of a lipid is replaced with a short-chain fluorescent fatty acid. Most of the resulting fluorescent lipid analogs can be readily integrated into cellular membranes by spontaneous lipid transfer from exogenous sources. The intracellular distribution of the labeled molecules can then be observed in living cells by high resolution fluorescence microscopy, and temporal changes in the distribution of a given lipid (and its metabolites) can be correlated with changes in its metabolism. From such studies, it is possible to define the pathways of transport of a particular lipid analog and to study the underlying mechanism(s) of that transport. This approach has been used to study the uptake and metabolism of fluorescent analogs of phosphatidylcholine [PC; (4, 5)], phosphatidylserine and phosphatidylethanolamine (6, 7), phosphatidylinositol [PI; (8, 9)], phosphatidic acid [PA; (10–12)], ceramide (13, 14), and various other sphingolipids (reviewed in ref. 3).

In the present study we used this approach to study a fluorescent analog of CDP-DAG, a key intermediate in PI biosynthesis. We developed a method for introducing this analog into living cells and studied its distribution and metabolism over time. Our results suggest that this approach may be particularly useful for evaluating

Abbreviations: CDP-NDB-DAG, cytidine diphosphate-1,2-[oleoyl, (N-(4-nitrobenzo-2-oxa-1,3-diazole)aminocaproyl)] diacylglycerol; DAG, diacylglycerol; HMEM, 10 mM 4-(2-hydroxyethyl)-1-piperazineethane sulfonic acid-buffered MEM, pH 7.4, without indicator; HPS, 10 mM 4-(2-hydroxyethyl)-1-piperazine sulfonic acid-buffered Puck's saline with 1 mM calcium and magnesium; IL-1β, interleukin 1β; NBD, 4-nitrobenzo-2-oxa-1,3-diazole; NBD-PI, 1,2-[oleoyl, N-(6-[7-nitrobenz-2-oxa-1,3-diazo-4-yl] aminocaproyl)]-phosphatidylinositol; PA, phosphatidic acid; PAP, phosphatidic acid phosphohydrolase; PI, phosphatidylinositol; PC, phosphatidylcholine; PDGF, platelet-derived growth factor; PS, phosphatidylserine; PG, phosphatidylglycerol; TAG, triacylglycerol.

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the effects of stimulation on the metabolism, turnover, and distribution of PI analogs in living cells.

EXPERIMENTAL PROCEDURES

Materials

CDP-(1-2-oleoyl, C_6-NBD)-DAG (CDP-NBD-DAG) was synthesized (15, 16) by Avanti Polar Lipids, Inc. (Alabaster, AL) and was a mixture of isomers. Approximately 80% of the product contained the fluorescent fatty acid in the *sn*-2 position while ~20% was in the *sn*-1 position. NBD-PI was synthesized from CDP-NBD-DAG as described (9, 16). Lipids were stored at −20°C and repurified when necessary. PDGF (BB form) and IL-1β (human) were from Promega (Madison, WI). Propranolol, RHC 80267, R 59022, and oleoylacetylglycerol were from Sigma (St. Louis, MO). $^{32}P_i$ was from Amersham (Arlington Heights, IL).

Cell culture

Normal (GM5659) human skin fibroblasts were obtained from the Coriell Institute, Human Genetic Mutant Cell Repository (Camden, NJ) and grown as described (17). All experiments were performed using cultures below passage 18. For microscopy experiments, cultures were seeded in 35-mm dishes containing 25 mm diameter acid-etched glass cover slips; for metabolic studies, cultures were grown on 60-mm diameter tissue culture dishes.

Incubation of cells with CDP-NBD-DAG

Monolayer cultures were washed twice with ice-cold HMEM and incubated for 60 min at 11°C with an aqueous dispersion of CDP-NBD-DAG prepared by vortex mixing of the dried fluorescent lipid in 10 mM 4-(2-hydroxyethyl)-1-piperazineethane sulfonic acid-buffered Puck's saline with 1 mM each calcium and magnesium (HPS buffer). (There was no loss of material when the dispersion was placed in a dialysis bag with a molecular weight cutoff of 10,000 and dialyzed against HPS overnight.) The final concentration of the NBD-lipid in HPS was 10 μM and the total incubation volume was 2.5 ml per 60-mm culture dish and 0.7 ml per 35-mm culture dish. After these incubations, the cultures were washed 2 times with cold HMEM, and warmed to 37°C for 2–60 min. In some experiments, the cultures were then chilled and incubated (3 changes; 10 min each at 11°C) with 2% defatted BSA (DF-BSA) in HMEM ("back-exchanged") to remove fluorescent lipid from the plasma membrane (17, 18). These cells were then either examined under the fluorescence microscope or the cellular lipids were extracted and analyzed (see below).

Radiolabeling of human skin fibroblasts

Cell cultures were incubated for the designated times at 37°C in EMEM containing 1% FBS and 30 μCi of carrier-free $^{32}P_i$, or 7 μCi of [1-^{14}C]palmitic (50–62 mCi/mmol), or [1-^{14}C]oleic (50–62 mCi/mmol) acids (added from an ethanol stock solution) per 60-mm dish. In the latter case, the final concentration of ethanol was ≤0.2%.

Lipid extraction and analysis

Monolayer cultures incubated with fluorescent lipids were washed with HMEM and scraped with a Teflon policeman into 2 ml of PBS. The culture dish was washed with an additional 2 ml of PBS which was combined with the cell suspension. The cells were then extracted by the procedure of Bligh and Dyer (19), using 0.9% NaCl and 0.01 N HCl in the aqueous phase. Lipid extracts were separated by TLC on Silica Gel 60 plates (Merck, Darmstadt, Germany) using one or more of the following solvent systems: *a*) C–M–28% NH_4OH 65:25:5 (v/v/v); *b*) C–M–28% NH_4OH 65:35:5 (v/v/v); *c*) C–petroleum ether–M–acetic acid–boric acid 40:30:20:10:1.8 (v/v/v/v/w); or *d*) C–acetone–M–acetic acid–H_2O 3:4:1:1:0.5 (v/v/v/v). For two-dimensional analysis, the TLC plate was developed in system *a*), then dried for at least 1 h and developed in the second dimension using solvent system *d*). To resolve neutral lipids, a two-step developing system was used (20). First, plates were developed in benzene–diethyl ether–ethanol–acetic acid 50:40:2:0.2 (v/v/v) 120 mm from the origin. The plates were then dried and developed in the same direction, 180 mm from the origin, using hexane–diethyl ether 94:6 (v/v). TLC plates were photographed under UV light and individual spots were identified by comparison with fluorescent standards and quantified by image analysis as described (18).

In experiments using $^{32}P_i$, TLC plates were covered with X-ray film for autoradiography or the radioactive spots were quantified using a phosphoimager (Molecular Analyst GS-363, Bio-Rad) with Molecular Analyst Software. Control experiments using several different TLC solvent systems established that the ^{32}P-label associated with an NBD-lipid was not due to a radiolabeled endogenous lipid with a similar R_f. The specific activity of a ^{32}P-labeled NBD-lipid was calculated from its ^{32}P-cpm and the amount of NBD-lipid present. This latter value was determined by quantitative fluorescence measurements of the NBD-lipid on the TLC plate compared to values obtained using known amounts of NBD-lipid standards.

Miscellaneous procedures

The concentrations of lipid stock solutions were determined by analysis of lipid phosphorus (21). The fluorescence of cell lipid extracts was normalized per culture dish or per total cellular protein measured by the Bradford procedure (22). Fluorescence microscopy was performed as described (23).

RESULTS

Uptake and distribution of CDP-NBD-DAG in human skin fibroblasts

When CDP-NBD-DAG was prepared as an aqueous dispersion in HPS and incubated with human skin fibroblasts for 60 min at 11°C, strong labeling of the plasma membrane and weak labeling of intracellular membranes including the Golgi apparatus was observed (**Fig. 1A**). When these cells were back-exchanged, all the plasma membrane fluorescence was removed and the fluorescently labeled Golgi apparatus could readily be seen (Fig. 1B). Surprisingly, when the cells were warmed to 37°C for several minutes prior to back-exchange, the ER, Golgi apparatus, nuclear envelope, and other intracellular membranes were brightly labeled (Fig. 1C). Although cells were incubated with 10 μM CDP-NBD-DAG in these experiments, virtually identical labeling patterns were obtained using a range of concentrations from 0.5 to 25 μM (data not shown). CDP-NBD-DAG had no effect on cell viability as assessed by trypan blue staining or on cell growth (data not shown).

Metabolism of CDP-NBD-DAG

The changes in intracellular distribution shown in Fig. 1 were accompanied by the metabolism of CDP-NBD-DAG. Immediately after incubation at 11°C, cells contained large amounts of fluorescent CDP-DAG and DAG, and small amounts of NBD-PA, and NBD-PC. However, upon warming the cells to 37°C, the amounts of fluorescent products changed markedly (**Fig. 2**). Namely, increasing amounts of NBD-PC, as well as small amounts of NBD-PI and NBD-PE, were formed over time. The identity of each NBD-lipid product was verified by comparing its R_f to that obtained for authentic NBD-lipid standards in four different TLC solvent systems (data not shown). Analysis of the products of CDP-NBD-DAG metabolism in a neutral TLC solvent system also revealed the presence of small amounts of NBD-TAG (data not shown).

We next examined the effects of several inhibitors of endogenous lipid synthesis on the metabolism of CDP-NBD-DAG (**Fig. 3**). When propranolol, an inhibitor of phosphatidic acid phosphohydrolase (24), was used, both NBD-PA (35 ± 6 vs. 10 ± 2 pmol/dish) and NBD-PI (45 ± 11 vs. 25 ± 5 pmol/dish) were increased rela-

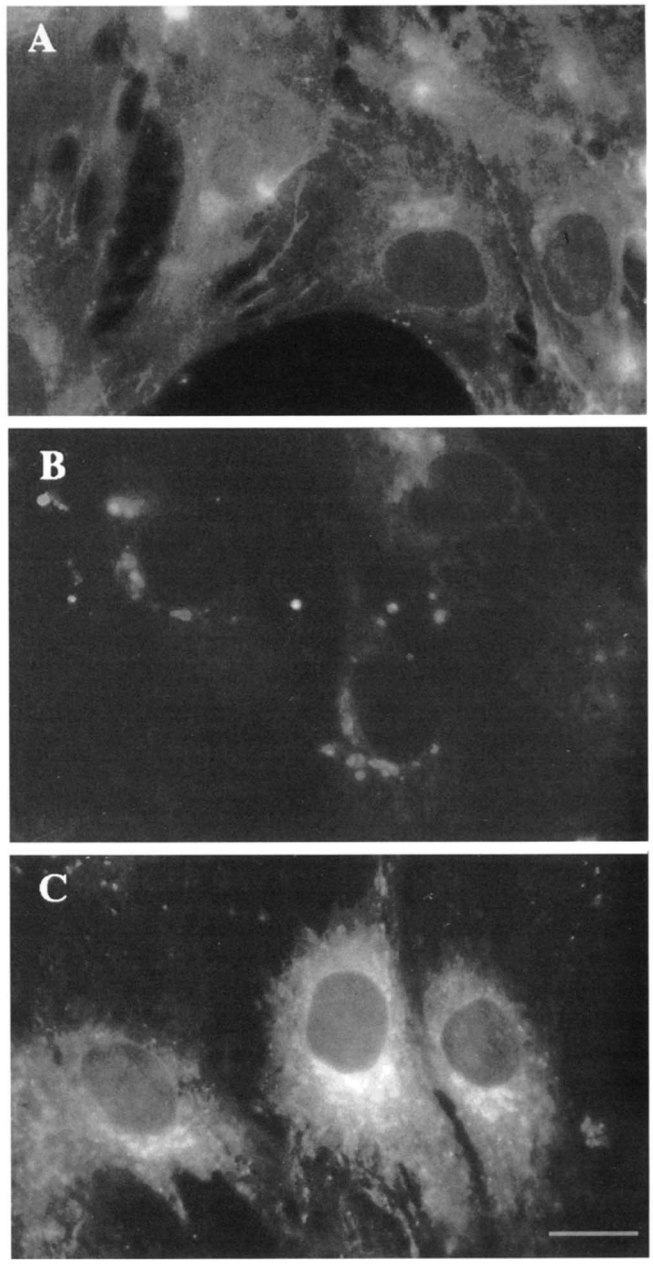

Fig. 1. Fluorescence micrographs of human skin fibroblasts after incubation with CDP-NBD-DAG. Cells were incubated with an aqueous dispersion of CDP-NBD-DAG for 60 min at 11°C and washed. They were then (A) photographed immediately; (B) incubated with 2% DF-BSA at 11°C ("back-exchanged") to remove plasma membrane fluorescence prior to photography; or (C) warmed to 37°C for 10 min prior to back-exchange and photography. All micrographs were exposed and printed identically. Bar, 20 μM.

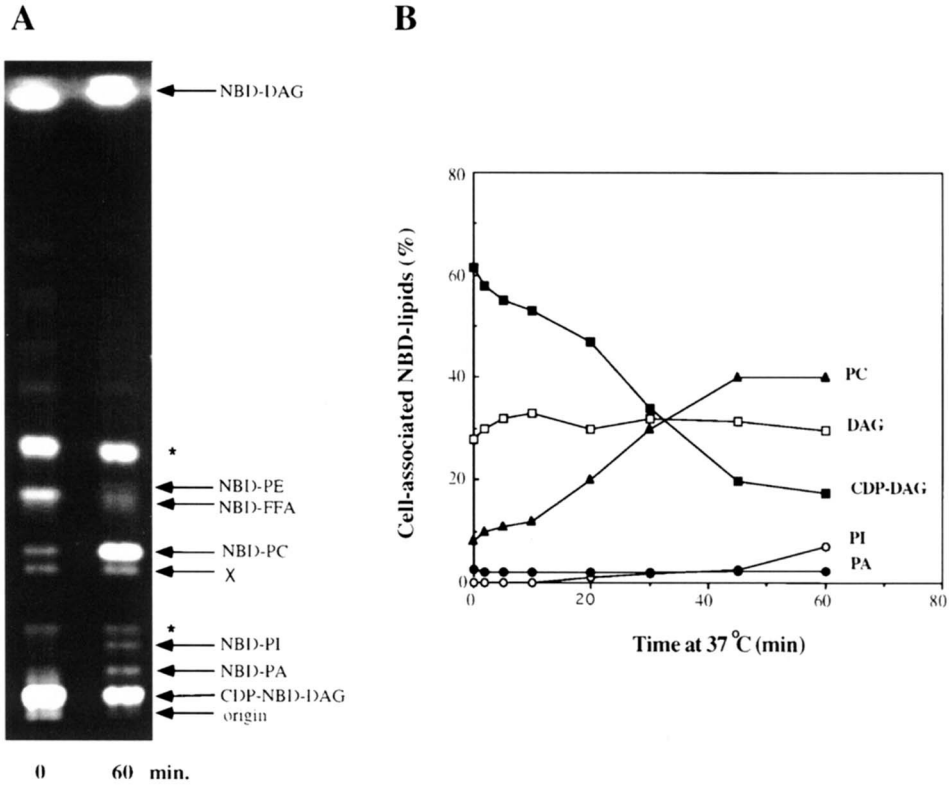

Fig. 2. Metabolism of CDP-NBD-DAG in human skin fibroblasts. Monolayer cultures were incubated with CDP-NBD-DAG for 60 min at 11°C, washed, and warmed to 37°C for the indicated times. The cell-associated NBD-lipids were extracted, analyzed by TLC using C–M–28% NH_4OH 65:25:5 (v/v/v) as the solvent system, and the NBD-lipid species were then quantified (see Experimental Procedures). (A) TLC of lipid extracts obtained after 0 and 60 min at 37°C. The positions of authentic NBD-lipid standards are indicated by the arrows. The two bands marked by asterisks were also present in lipid extracts from control cells that were not incubated with fluorescent lipid. (B) Time course of metabolism of CDP-NBD-DAG: (■) CDP-NBD-DAG; (□) NBD-DAG; (▲) NBD-PC; (●) NBD-PA; (○) NBD-PI.

tive to control cultures, whereas the amount of NBD-PC (40 ± 10 vs. 150 ± 23 pmol/dish) and NBD-DAG (12 ± 7 vs. 130 ± 32 pmol/dish) were significantly decreased. Thus, propranolol enhanced the metabolism of fluorescent substrate along the CDP-DAG pathway and decreased its metabolism along the DAG pathway. When RHC 80267, a selective inhibitor of DAG lipase (25, 26) was used, increased amounts of fluorescent DAG (380 ± 58 vs. 130 ± 32 pmol/dish) and NBD-PI (47 ± 9 vs. 25 ± 5 pmol/dish) were found relative to control cultures. In addition, the amount of cell-associated NBD-PA was elevated (20 ± 5 vs. 10 ± 2 pmol/dish). Finally, we examined the effect of R 59022, an inhibitor of DAG kinase (27, 28) and found, surprisingly, that the amount of NBD-PI formed increased to about 45% of the NBD metabolites formed. When propanolol was used together with R 59022, the synthesis of NBD-PC was strongly inhibited, the amount of NBD-PA increased about 5-fold compared to control cells, and NBD-PI became the major product, constituting ~56% of the total fluorescence and ~70% of the polar NBD-metabolites (Fig. 3).

We also examined the effect of R 59022 on endogenous PI synthesis in human skin fibroblasts. Cells were incubated with $^{32}P_i$ in HMEM for 60 min at 37°C. Under these labeling conditions, only radiolabeled PI and PA were seen, presumably because of their high turnover rates, while little or no labeling of PC, PE, and PS was seen. Addition of R 59022 to the medium resulted in a concentration-dependent increase in the incorporation of $^{32}P_i$ into PI (**Fig. 4**). Addition of the cell-permeable DAG analog, oleylacetylglycerol (29), to the medium also enhanced the incorporation of $^{32}P_i$ into PI (about 85% relative to control cells; data not shown).

Effect of cytokines on the incorporation of $^{32}P_i$ into the metabolites CDP-NBD-DAG

We used PDGF and IL-1β to stimulate the cells and study the effects of stimulation by cytokines on the me-

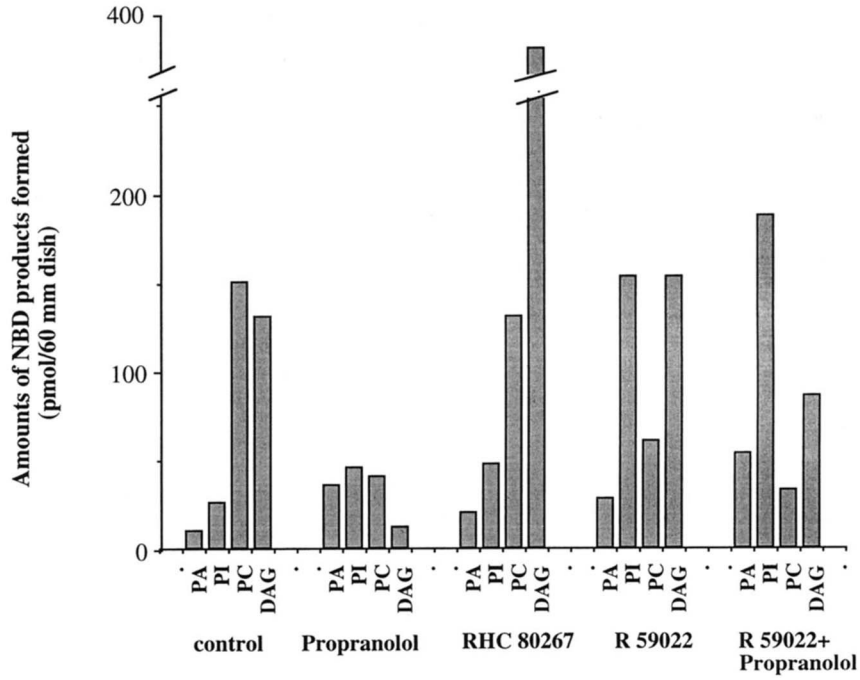

Fig. 3. Effect of inhibitors on the metabolism of CDP-NBD-DAG in human skin fibroblasts. Monolayer cultures of human skin fibroblasts were preincubated in HMEM with propranolol (100 μM), RHC 80267 (25 μM), R 59022 (5 μM), or with both R 59022 (5 μM) and propranolol (100 μM) for 20 min at 37°C, after which CDP-NBD-DAG in HPS was added and the cells were further incubated for 60 min at 11°C. The cells were then washed and warmed for 45 min at 37°C. All incubations and washes were carried out in buffers containing the inhibitors. Cell-associated NBD-lipids were extracted, analyzed by TLC, and quantified as described in Experimental Procedures. Data are expressed as pmol NBD-lipid found per 60-mm culture dish of cells and represent the average of three experiments.

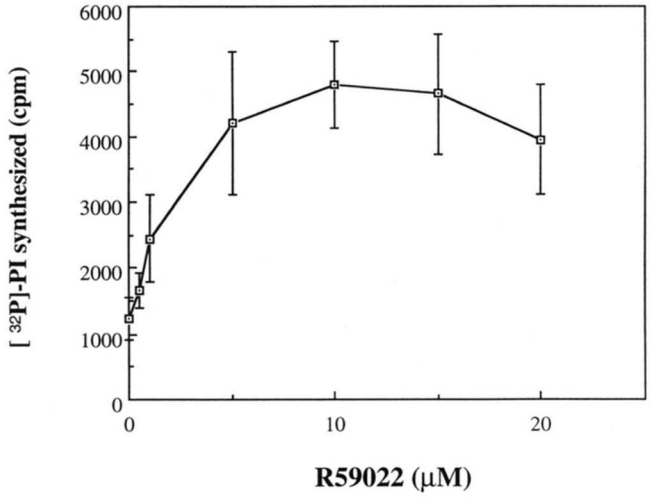

Fig. 4. Enhancement of endogenous PI synthesis by an inhibitor of DAG kinase. Monolayer cultures of human skin fibroblasts were washed 3 times with HMEM, preincubated with R 59022 (0.5–20 μM) for 20 min at 37°C, and $^{32}P_i$ was then added to the culture dishes. The cells were then incubated for 60 min at 37°C. Lipids were extracted, analyzed by two-dimensional TLC, and the amount of radioactivity in the PI fraction was quantified as described in Experimental Procedures. Data represent the mean ± SD.

tabolism of CDP-NBD-DAG and the incorporation of $^{32}P_i$ into the NBD-metabolites. Cells were prelabeled with $^{32}P_i$ for 3 h in medium containing a low concentration of serum (1%) and stimulated with PDGF or IL-1β for 1 h prior to the addition of CDP-NBD-DAG. The amounts and specific activities of the NBD-PC, -PE, and -PI that were formed were determined in control and stimulated cells. As shown in **Table 1,** stimulation by PDGF resulted in increased synthesis of NBD-PI (+68%; 42 vs. 25 pmol) and NBD-PE (+50%; 12 vs. 8 pmol), while the amount of NBD-PC formed was not affected. The specific activity of NBD-PI was also increased, suggesting that both the de novo synthesis of NBD-PI and its turnover (as judged by incorporation of $^{32}P_i$) were increased by PDGF stimulation.

Treatment of cells with IL-1β, another potent stimulator of fibroblasts, also enhanced the synthesis of NBD-PI, -PC, and -PE. However, in this case, the specific activity of NBD-PI decreased by about 44%, suggesting that the increased synthesis of NBD-PI during IL-1β stimulation was not accompanied by increased turnover. Both PDGF and IL-1β increased the incorporation of $^{32}P_i$ into NBD-PC (Table 1). Similar results of cytokine stimula-

TABLE 1. Effect of cytokines on the incorporation of ^{32}P into the metabolites of CDP-NBD-DAG

Treatment	NBD-Metabolite	Amount Formed	Specific Activity	% Change
		pmol/dish	*cpm/nmol lipid-P*	
None	NBD-PC	150 ± 23	3700 ± 570	0
	NBD-PE	8 ± 2	4750 ± 410	0
	NBD-PI	25 ± 5	8040 ± 805	0
+PDGF	NBD-PC	148 ± 16	5510 ± 405	+49
	NBD-PE	12 ± 5	4370 ± 320	−8
	NBD-PI	42 ± 10	14230 ± 1280	+77
+IL-1β	NBD-PC	375 ± 52	5250 ± 700	+42
	NBD-PE	14 ± 1	2280 ± 630	−52
	NBD-PI	47 ± 9	5306 ± 320	−44

Cells were labeled with $^{32}P_i$ for 3 h in EMEM+1% FBS. During the last hour, 10 ng/ml PDGF or IL-1β was added to the medium. The cells were then washed, incubated with CDP-NBD-DAG for 60 min at 11°C, washed, and incubated for 45 min at 37°C prior to lipid extraction and analysis by two-dimensional TLC. Specific activities were determined as described in Experimental Procedures. The data are average of 4 experiments ± SD.

tion on $^{32}P_i$ incorporation into endogenous PI and PC were also seen (data not shown).

Incubation of [^{14}C]palmitate- and [^{14}C]oleate-labeled cells with CDP-NBD-DAG

To learn whether transacylation reactions (i.e., the exchange of the NBD-FA with endogenous fatty acids) contributed to the formation of the radiolabeled NBD-metabolites, cells were incubated overnight with radioactive palmitic or oleic acid, washed, and then incubated with CDP-NBD-DAG. (In these experiments, the amount of free fatty acid in the cells did not exceed 1% of the total label after the overnight incubation.) No radiolabeled NBD metabolites were detected using cells prelabeled with these fatty acids, except for small amounts of radioactive NBD-TAG (data not shown).

DISCUSSION

In this paper we present a simple method for the introduction of a fluorescent analog of CDP-DAG into the cytoplasm of intact human skin fibroblasts and report for the first time the de novo synthesis of NBD-PI from this fluorescent precursor. We also demonstrate that CDP-NBD-DAG was a substrate for the de novo synthesis of other NBD-lipids and that the amounts and turnover of NBD-PI were enhanced when cells were stimulated with the cytokine, PDGF.

Introduction of CDP-NBD-DAG into intact cells

In a previous study CDP-NBD-DAG was synthesized and its properties in artificial lipid vesicles and in living cells were studied (16). It was shown that the lipid rapidly equilibrates ($t_{1/2} < 20$ sec) between labeled and unlabeled populations of liposomes, but that it is not capable of spontaneous transbilayer movement. It was also found that when lipid vesicles containing CDP-NBD-DAG were incubated with cultured fibroblasts for up to 90 min at 2°C, intense plasma membrane labeling resulted but ~75% of the cell-associated fluorescence could be accounted for by vesicles adsorbed to the cell surface.

In the present study we were interested in introducing CDP-NBD-DAG into the cytoplasm of intact cells so that it might enter the pool of cellular CDP-DAG and be metabolized. We used an aqueous dispersion of CDP-NBD-DAG, prepared by vortex mixing the dried lipid in a simple buffer, and found strong labeling of intracellular membranes such as the nuclear envelope, ER, and Golgi apparatus (Fig. 1C). The physiochemical properties of the aqueous dispersion of CDP-NBD-DAG were not studied, but as no bilayer-forming lipids were added to the system and as there was no loss of material during dialysis, it is assumed that the lipid is present as micelles in equilibrium with a low concentration of monomers in the aqueous phase. The mechanism by which the fluorescent lipid enters the cell is also unknown. A punctate pattern of intracellular fluorescence that is characteristic of entry by endocytosis (18, 30) was not seen, suggesting that the uptake mechanism involved transbilayer movement of the CDP-NBD-DAG at the plasma membrane followed by spontaneous diffusion to label other intracellular membranes (1, 7, 17). As CDP-NBD-DAG does not undergo spontaneous transbilayer movement in liposomes, we speculate that its transbilayer movement at the plasma membrane is somehow enhanced, perhaps because the aqueous dispersion has detergent-like properties that facilitate penetration of the CDP-NBD-DAG across the membrane. Regardless of the mechanism, it is important to emphasize that incubation with CDP-NBD-DAG did not visibly damage the plasma membrane of cells or alter cell viability.

Metabolism of CDP-NBD-DAG

CDP-NBD-DAG was readily metabolized by cells at 37°C to a number of NBD-glycerolipids as expected from the known pathways of glycerolipid metabolism shown in **Fig. 5.** In particular, large amounts of fluorescent DAG and PC were formed, as well as small amounts of fluorescent PI and PA. No fluorescently labeled PIP, phosphatidylglycerol, phosphatidylserine, or cardiolipin was detected. We used several different inhibitors of glycerolipid metabolism (refer to Fig. 5) and showed that when different enzymes in the metabolic pathway were blocked, the fluorescent metabolites that

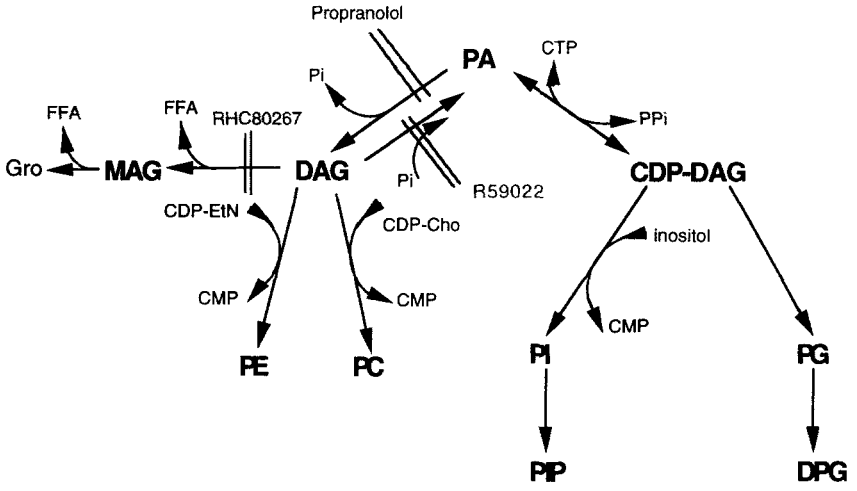

Fig. 5. Expected pathways of phospholipid metabolism and the targets of various inhibitors. CTP, cytidinetriphosphate; CMP, cytidinemonophosphate; CDP-EtN, cytidinediphosphate-ethanolamine; CDP-Cho, CDP-choline. Parallel lines show the targets of inhibitors of phosphatidic acid phosphohydrolase (propranolol), DAG lipase (RHC 80267), and DAG kinase (R59022) which were used in the present study.

were formed were affected in a predictable manner. For example, inhibition of PA phosphohydrolase by propranolol resulted in increased metabolism of CDP-NBD-DAG to NBD-PI and significantly decreased metabolism along the DAG pathway. Similarly, inhibition of DAG lipase by RHC 80267 resulted in increased metabolism along the CDP-DAG pathway, possibly because the degradation of CDP-NBD-DAG to NBD-PA, and further to NBD-DAG, was inhibited by the large increase in the amount of DAG (product inhibition). Interestingly, upon addition of the DAG kinase inhibitor to the cells, we observed both the stimulation of NBD-PI (Fig. 3) and endogenous PI synthesis (Fig. 4). We speculate that this most likely results from an increase in certain molecular species of DAG which act as a "second messenger" to promote phosphoinositide synthesis. This is supported by our studies with the cell-permeable analog of DAG, oleoyl acetyl glycerol, which enhanced incorporation of $^{32}P_i$ into endogenous PI (data not shown).

While the profile of fluorescent metabolites obtained from CDP-NBD-DAG was consistent with the pathways of glycerolipid synthesis and catabolism, we were surprised to find radiolabeled NBD-PI when cells prelabeled with $^{32}P_i$ were used. The radioactivity associated with NBD-PI was not present in trace amounts, but was judged to be substantial based on the specific activity of this lipid and comparison to the values obtained for the de novo synthesized NBD-PC and -PE (refer to Table 1). This result was unexpected as CDP-DAG is the immediate precursor of PI and no radioactivity was present in the fluorescent analog of CDP-NBD-DAG. In control experiments, we were able to eliminate transacylation reactions as a source of NBD-labeling of endogenous lipids from CDP-NBD-DAG (see Results). Furthermore, previous studies from this laboratory have shown that C_6-NBD-FA cannot be utilized in the de novo synthesis of fluorescent lipids in cultured fibroblasts (10, 12), presumably because it is not a substrate for acyl-CoA synthase. Thus we conclude that ^{32}P-labeling of NBD-PI can only occur from its breakdown to NBD-PA (and/or NBD-DAG) and resynthesis of NBD-PI through "turnover."

Stimulation of cells with cytokines and the effect on lipid metabolism

PDGF is a potent stimulator in different cell lines, including fibroblasts (31–35). We found that stimulation of human skin fibroblasts with PDGF resulted in increased NBD-PI synthesis and turnover (Table 1). This is in agreement with the existing data on the effects of PDGF on PI metabolism in which both increased synthesis and hydrolysis of phosphoinositides has been documented (32–34).

IL-1β is another cytokine implicated in the stimulation of fibroblast cell lines (36). However, in the case of IL-1β and IL-1α, cell stimulation is achieved primarily via PLC activity linked to PC (37) and PLA_2-mediated hydrolysis of PC (38). Also, IL-1 promotes the remodeling of phospholipids (39). Evidently, in the case of IL-1, the signal transduction mechanism is not via phosphoinositide hydrolysis, but rather via the hydrolysis of different molecular species of PC to DAG and arachidonic acid. Our data using CDP-NBD-DAG and IL-1β are in agreement with these findings. Namely, we observed increased synthesis and turnover of NBD-PC. In contrast, NBD-PI turnover was not stimulated, although its synthesis was increased (Table 1).

Based on the present studies we suggest that CDP-

NBD-DAG may be used to study the overall changes in lipid metabolism in mammalian cells after treatment with various agents including cytokines. Importantly, in combination with $^{32}P_i$ labeling, the use of CDP-NBD-DAG provides a novel method for obtaining information about the turnover of phosphoinositides under various conditions. Finally, we note that when cells were treated with inhibitors of both PA phosphohydrolyase and DAG kinase, NBD-PI became the major polar NBD-lipid metabolite formed from CDP-NBD-DAG. Thus, it may be possible to "load" a cell with NBD-PI and then study the effects of various agents on the subcellular distribution of this lipid in the living cell by fluorescence microscopy. Given the importance of phosphoinositides in signal transduction and other cellular processes, such as regulation of the cell cycle, this approach can potentially yield important new information about the relationship between ligand action and cell-mediated response.

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