Attenuation of sn-1,2-Diacylglycerol Second Messengers by Diacylglycerol Kinase

INHIBITION BY DIACYLGlycerOL ANALOGS IN VITRO AND IN HUMAN PLATELETS*

W. Robert Bishop, Barry R. Ganong, and Robert M. Bell

From the Department of Biochemistry, Duke University Medical Center, Durham, North Carolina 27710

Many cells respond to a variety of extracellular stimuli by activation of a phospholipase C which catalyzes the phosphodiesteric cleavage of phosphatidylinositol 4,5-bisphosphate (1, 2). The products of this reaction, inositol trisphosphate and sn-1,2-diacylglycerol, both function as intracellular second messengers. Inositol trisphosphate functions in the mobilization of intracellular Ca²⁺ stores (1), whereas diacylglycerol activates protein kinase C (3-6). Protein kinase C plays a central role in signal transduction, cellular regulation, tumour promotion, and perhaps oncogenesis (1, 4, 5, 7). Activation of this kinase using cell-permeable diacylglycerols has been useful in further defining its role in a variety of cells (8-13). The diacylglycerol signal produced in response to extracellular stimuli is transient, and may be removed via several pathways. Diacylglycerol kinase has been suggested to play an essential role in this process in platelets (14, 15) and other cell types (16, 17). The phosphatidic acid thus formed is believed to be recycled back to phosphatidylinositol 4,5-bisphosphate via a sequence of reactions known as the phosphatidylinositol cycle (reviewed in Ref. 5).

The best-characterized mammalian diacylglycerol kinases are those from pig brain (18) and rat liver (19). Kanoh et al. (18, 20) have described a membrane-bound and a soluble form of the enzyme in both tissues. These two forms of the enzyme have similar properties (20). The soluble pig brain enzyme has been purified to homogeneity and studied in vitro (18, 21).

We have investigated the ability of a number of diacylglycerol analogs to act as substrates or inhibitors of diacylglycerol kinase. These analogs were constructed with constant acyl chain length (Cs), whereas the headgroup region was varied to explore the specificity of the kinase. This acyl chain length was chosen because these compounds are cell-permeable (8-13). These same analogs have been tested as effectors of protein kinase C (22). For screening of these compounds we chose a partially purified preparation of the soluble pig brain enzyme. We report here a modified assay for this enzyme employing diacylglycerol substrates co-sonicated with various phospholipids. Several of the diacylglycerol analogs were substrates and several were inhibitors. The effects of diacylglycerol kinase inhibitors on diacylglycerol formation and metabolism in human platelets were investigated.

**EXPERIMENTAL PROCEDURES**

Materials—sn-1,2-Dioleoylglycerol (diC18:1) was prepared by phospholipase C digestion of dioleoylphosphatidylcholine as previously described (23). All phospholipids and diC₈ were obtained from Avanti Polar Lipids. 1-Monooleoylglycerol and 2-monooleoylglycerol were from Serdary Research Laboratories. DiC₄ analogs were prepared as previously reported (22). All other reagents were of the highest quality commercially available.

Purification of Pig Brain Diacylglycerol Kinase—Diacylglycerol kinase was purified through G-150 column chromatography (Step 4) as described by Kanoh et al. (18). Purification was monitored using the deoxycyclate assay of Kanoh et al. (18) except assays were performed at 25 °C and products were extracted by the method of Bligh and Dyer (24) using 1% HClO₄, as upper phase. Under these conditions activity was proportional with the amount of protein employed, and with time over 15 min. This extraction procedure produced results equivalent to those obtained using butanol.

Specific activities of the partially purified preparations ranged from 35 to 40 nmol/min/mg of protein using diC₁₂₈ as substrate, repre
senting a 25–30-fold purification over brain cytosol. The activities observed at all stages of purification were 5–6-fold lower than those previously reported (18). This was due, in part, to the lower assay temperature employed.

**Assay of Diacylglycerol Kinase**—Enzyme assays were performed essentially as described (18) with the modifications noted above. Assays were usually performed for 5 min using 6.5 μg of protein. Assays in the presence of phospholipids were performed by mixing the diacylglycerol substrate with the appropriate phospholipid in CHCl₃, drying under N₂, and resuspension in 2 x assay buffer by vortexing and sonication. Diacylglycerol analogs were also added in CHCl₃ prior to drying. Phorbol esters, 4β-phorbol 12β-myrystate 13-acetate and phorbol 12,13-dibutyrate, were added to the reaction mixture as dimethyl sulfoxide or ethanol solutions, respectively.

**RESULTS AND DISCUSSION**

**Rationale**—The objective of this work was to screen a series of diacylglycerol analogs for their ability to act as substrates or inhibitors of diacylglycerol kinase. This should facilitate the identification of biologically useful inhibitors of diacylglycerol kinase which will further understanding of diacylglycerol metabolism in vitro. To accomplish this it was necessary to develop suitable in vitro assays for this enzyme. These assays were then employed for preliminary screening. Analogues that were found to inhibit pig brain diacylglycerol kinase in vitro were then tested in platelets where they had marked effects on diacylglycerol metabolism. Fig. 1 shows the structures of several diacylglycerol analogs which were substrates or inhibitors of the kinase.

**In Vitro Assays of Pig Brain Diacylglycerol Kinase**—Co-sonicated mixtures of diacylglycerol and various phospholipids were good substrates for the partially purified pig brain kinase in vitro (Table I). These results are unlike those of Kanoh et al. (18). When diC₁₂ and phospholipid were sonicated separately and mixed prior to assay, little or no activity was detected (Table I). This is likely due to inefficient dispersal of the diC₁₂, since separate sonication of the more water-soluble compound diC₈ yielded good activity.

A variety of anionic phospholipids were activators of this enzyme, as were mixtures of phospholipids containing anionic species, such as PE/PG/CL (6:1:1) (Table I). Activities greater than observed with the deoxycholate assay were obtained with PS alone, PG alone, CL alone, or a mixture of PE/PG/CL. In contrast, little activity was observed with the zwitterionic phospholipids, phosphatidylcholine or PE. These results differ somewhat from those of Kanoh et al. (18) who found optimal stimulation by phosphatidylcholine, and little or no stimulation by PG and CL. These differences do not appear to be due to the state of purity of the enzyme used, since similar results were obtained by Kanoh et al. (18) using homogeneous or partially purified (Step 2) enzyme. Cofactor dependence may be modulated by degree of sonic dispersion, temperature, or other factors.

The properties of the diacylglycerol kinase assay were further characterized using phospholipid co-sonicated with diC₁₂ or diC₈ as substrate (Table II). The apparent Kᵅ for

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**SUBSTRATES**

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**INHIBITORS**

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Fig. 1. Substrates and inhibitors of diacylglycerol kinase. The structures of several of the diacylglycerol analogs employed are shown. DiC₁₀, diC₁₂-2-amide, and 2-monooleoylglycerol were substrates for partially purified pig brain diacylglycerol kinase. DiC₈-ethyleneglycerol and 1-monooleoylglycerol were the most potent inhibitors of the kinase in vitro. Structures of other analogs and synthetic methods have been previously published (22).
Effect of co-sonicated and separately sonicated phospholipids on diacylglycerol kinase activity

All activators were present at 1 mM, except for mixtures of PE/PG/CL where the concentrations were 1 mM, 0.16 mM, 0.16 mM, and PS, which was used at 0.5 mM. The molecular species of phospholipid employed were: dioleoyl phosphatidylcholine (PC), PG, and PS; E. coli PE; and bovine heart CL. Substrates were present at 1 mM for diC18:1 or 0.5 mM for diC12. Assays were performed for 3 min in the presence of 6.5 µg of protein, using either co-sonicated mixtures of diacylglycerol and phospholipid or following mixing of separately sonicated preparations as indicated.

| Activator                        | Substrate          | diC18:1 | diC12:1 |
|----------------------------------|---------------------|---------|---------|
| None                             |                      | 0.07    | 0.09    |
| Deoxycholate                     |                     | 0.87    | 0.75    |
| PC (co-sonicated)                |                     | 0.92    | 0.13    |
| (separate)                       |                     | 0.94    | 0.11    |
| PE/PG/CL (co-sonicated)          |                     | 1.30    | 1.01    |
| (separate)                       |                     | 1.33    | 0.05    |
| PS (co-sonicated)                |                     | 1.79    | ND*     |
| (separate)                       |                     | 1.82    | ND      |
| PG (co-sonicated)                |                     | 1.62    | ND      |
| CL (co-sonicated)                |                     | 1.07    | ND      |
| PE (co-sonicated)                |                     | 0.04    | ND      |

*ND, not determined.

**TABLE II**

Kinetic properties of diacylglycerol kinase in the presence of various activators

Various concentrations of diacylglycerol were co-sonicated with 1 mM deoxycholate; 1 mM PE, 0.16 mM PG, 0.16 mM CL; or 0.1 mM PS. Standard reaction conditions were employed (3 min, 6.5 µg of protein). Kinetic parameters were calculated from double-reciprocal plots of the data. Using PS as activator, kinase activity declined when greater than 0.2 mM diC18:1 was employed.

| Activator            | Substrate | diC18:1 | diC12:1 |
|----------------------|-----------|---------|---------|
|                       | apparent  | apparent | Vmax   | Vmax   |
| diC18:1              | µM      | nmol/min/mg | µM    | nmol/min/mg |
| Deoxycholate         | 135      | 37.4    | 63.0    | 40.0    |
| PE/PG/CL             | 110      | 49.2    | 24.5    | 58.5    |
| PS                   | 46       | 42.6    | 38.3    | 71.8    |

diC18:1 was similar using deoxycholate or PE/PG/CL as activator, whereas a lower apparent Km was observed using PS.

With all activators examined, diC12:1 was a better substrate, as evidenced by the lower apparent Km and the higher reaction velocities (Table II). The apparent Km for diC12:1 was 1.5-2-fold lower with phospholipid activators than with deoxycholate.

To understand further the role of phospholipids in the activation of diacylglycerol kinase, the amount of phospholipid present was varied at fixed concentrations of diC18 (Fig. 2). When PS was used as activator, optimal activity occurred with a molar ratio of 0.5-1 PS/1 diC18. This was true at 30 µM diC18 and 500 µM diC12 (Fig. 2A). When a greater molar excess of PS was used, a decline in activity occurred.

At 30 µM diC12, optimal activity was reached at 0.1 mM PE, 0.016 mM PG, 0.016 mM CL, representing a molar ratio of 3.3 PE/0.53 PG/0.53 CL/1 diC12 (Fig. 2B). Similar molar ratios were required for maximal activity at 500 µM diC18 (Fig. 2B).

These results suggest that optimal activity of diacylglycerol kinase is obtained over a narrow phospholipid/diacylglycerol range, and that 1 mol of anionic lipid/mmol of diacylglycerol is sufficient for maximal activity. The physical properties of such phospholipid/diacylglycerol mixtures are not well defined. The presence of 10 mM Mg2+ in these assays adds to the physical uncertainty of substrates and activators. Induction of nonlamellar phases by high concentrations of diacylglycerol has been reported (31). Changes in the physical state of PS vesicles (i.e. vesicle fusion) induced by divalent cations is well documented (32, 33). To circumvent these problems, we attempted to develop a mixed micellar assay for diacylglycerol kinase using a variety of detergent and phospholipid mixtures. None of these supported kinase activity. The inability to measure activity of the rat liver enzyme in nonionic detergents has been previously reported (19). Therefore, investigations on the effect of diacylglycerol analogs on kinase activity were performed using both PS and PE/PG/CL as activators.

**Phosphorylation of Diacylglycerol Analogs by Pig Brain Diacylglycerol Kinase**—All of the diacylglycerol analogs previously described (22) which contain a free OH group were tested as substrates of pig brain diacylglycerol kinase. Among the analogs designed to test the positional requirement of the hydroxyl group, neither diC18-butanetriol nor hexanetriol which displace the hydroxyl group 1 and 3 methylenes, respectively, showed any activity. 1,3-DiC18 had about 5% of the activity seen with 1,2-diC18. This activity is undoubtedly due to acyl group migration and therefore, contamination of the 1,3 isomer with small amounts of 1,2. Specificity for the sn-1,2 isomer has been reported for the rat liver enzyme (20).

Among the analogs designed to test the requirement for O-ester carbonyls, most were not substrates, including diC12-ether, diC12-thioether, monoC18-propanediol, and monoC18-ethylene glycol. Only the diC12-2-amide was a good substrate for the kinase (Fig. 3). DiC12-2-amide had an apparent Km of 90 µM, and a maximal velocity of 31 nmol/min/mg (compared to 58.5 nmol/min/mg for diC12). Although the kinase tolerated an amide linkage in the 2 position, only slight activity was observed using diC12-1-amide.
Diacylglycerol Kinase Inhibitors

2-Monooleoylglycerol was a substrate for diacylglycerol kinase, with an apparent $K_v$ of 82 μM and a $V_{max}$ of 9.6 nmol/min/mg (Fig. 3). 1-Monooleoylglycerol was not a substrate. In addition, ceramide and phorbol esters (4β-phorbol 12β-myristate 13α-acetate and phorbol 12,13-dibutyrate) were not substrates. These results indicate that diacylglycerol kinase from pig brain is quite specific for sn-1,2-diacylglycerol, both in regard to the O-ester carbonyl linkage and in regard to the position of the hydroxyl group relative to the esters.

Inhibition of Diacylglycerol Kinase by Diacylglycerol Analogs—Diacylglycerol analogs were tested as inhibitors of pig brain diacylglycerol kinase, and these results are summarized in Table III. In contrast to the specificity observed when analogs were tested as substrates, virtually all of the analogs were inhibitory in assays using PE/PG/CL as activator. With the exception of diC₈-ethylene glycol and 1- monooleoylglycerol, only slight inhibitions were observed when analogs were present at 0.1 mM, whereas at 0.5 mM many analogs exerted considerable inhibitory effects. Among the 3-hydroxy analogs, diC₈-ethylene glycol, diC₈-glyceramide, and diC₈-butanetriol were the most potent inhibitors. Among the O-ester analogs, 1-monooleoylglycerol and diC₈-ether were most potent.

Analogs were also tested in assays using PS as activator (Table III). At the lower concentration of PS (0.1 mM) the extent of inhibition was similar to that seen in the PE/PG/CL assays. However, when the PS concentration was raised to 0.5 mM, inhibition by a number of analogs (e.g. diC₈-ether, diC₈-butanetriol, and deoxydiC₈) was alleviated. This suggests that inhibition by these analogs may have been due to changes in the physical state of the diacylglycerol/analogue/phospholipid mixture. In contrast, inhibition by the most potent inhibitors, diC₈-ethylene glycol and 1-monooleoylglycerol was not affected by elevating the PS level. We chose, therefore, to focus our attention on these compounds.

Double-reciprocal plots indicated that inhibition by diC₈-ethylene glycol or 1-monooleoylglycerol was competitive with respect to diC₈ (Fig. 4). The $K_i$ values observed were 68 μM for diC₈-ethylene glycol and 91 μM for 1-monooleoylglycerol. A similar extent of inhibition occurred when diC₈ was employed as substrate (data not shown).

Effects of Diacylglycerol Analogs on Diacylglycerol Formation and Turnover in Thrombin-stimulated Platelets—To extend the in vitro results, diacylglycerol kinase inhibitors were tested for their effects on diacylglycerol production and turnover in thrombin-stimulated human platelets.

**Table III**

| 3-OH analog          | % inhibition |
|----------------------|-------------|
| DiC₈-ethylene glycol | 79.2        |
| DeoxydiC₈            | 5.9         |
| ChlorodiC₈           | 20.9        |
| ThiodiC₈             | 0.0         |
| DiC₈-glyceramide     | 51.4        |
| DiC₈-methylether     | 14.0        |
| DiC₈-butanetriol     | 21.7        |
| DiC₈-hexanetriol     | 20.9        |

**Table III continued**

| O-Ester analog       | % inhibition |
|----------------------|-------------|
| DiC₈-ether           | 14.8        |
| DiC₈-thioether       | ND          |
| DiC₈-1-amide         | 0.0         |
| MonoC₈-propanediol   | 0.0         |
| MonoC₈-ethyleneglycol| 0.0         |
| 1-Monooleoylglycerol | 85.7        |
| Ceramide             | ND          |
| PDBu                 | 6.0         |
| PMA                  | 57.6        |

* ND, not determined.

**Fig. 4. Double-reciprocal plots of inhibition by diC₈-ethylene glycol and 1-monooleoylglycerol.** Various concentrations of diC₈ were co-sonicated with PE/PG/CL (final concentrations: 1 mM, 0.16 mM, and 0.16 mM, respectively) in the presence or absence of the indicated concentration of diC₈-ethylene glycol (A) or 1-monooleoylglycerol (B). Standard assay conditions were used (3 min, 7 μg of protein). The specific activity of the enzyme preparation employed in the experiment in panel A was 28.0 nmol/min/mg.
metabolism in thrombin-stimulated platelets. Addition of thrombin to platelets caused a rapid, transient rise in diacylglycerol levels, as determined by mass measurements (2-3-fold within 15 sec) (e.g. Fig. 5A). [32P]PA levels also increased upon thrombin stimulation (4-5-fold, maximal by 2 min) (e.g. Fig. 5A; Refs. 15, 34), reflecting successive action of phospholipase C and diacylglycerol kinase. The effects of analogs on diacylglycerol formation and [32P]PA production were examined.

DiCs-ethylene Glycol—Pretreatment of platelets with diCs-ethylene glycol resulted in decreased basal and stimulated levels of [32P]PA, consistent with inhibition of diacylglycerol kinase (Fig. 5A). DiCs-ethylene glycol at 30 mM only partially reduced thrombin-stimulated levels of [32P]PA, whereas 100 or 500 mM diCs-ethylene glycol dramatically decreased [32P]PA formation. The extent of inhibition varied somewhat between platelet preparations, with 100 mM diCs-ethylene glycol resulting in complete inhibition in platelets from three donors and about 70% inhibition in two other preparations. 50 mM diCs-ethylene glycol resulted in about 50% inhibition in all cases (e.g. Fig. 6).

Proximal portions of the thrombin-stimulated phosphatidylinositol cycle (i.e. phospholipase C activation) were not affected by diCs-ethylene glycol treatment, since a rapid rise in diacylglycerol levels occurred similar to that seen in control platelets (Fig. 5B). The diacylglycerol signal generated in diCs-ethylene glycol-treated platelets was longer-lived than in controls, 5 min after stimulation, diacylglycerol levels had declined markedly, but basal levels were not achieved. Even though inhibition of the kinase was apparent, the diacylglycerol generated was still metabolized. The small elevation of basal diacylglycerol levels in diCs-ethylene glycol-treated platelets may be due to a decreased ability to metabolize diacylglycerol via the kinase pathway.

Other DiCs Analogs—The effects of several other diCs analogs on [32P]PA production were tested. None of these analogs affected the initial thrombin-induced rise in [32P]PA (Fig. 7A). No difference from control platelets was seen following pretreatment with diCs-ether (Fig. 7A), or diCs-hexamethy ether analogs (data not shown).

In the case of the 3-hydroxy analogs tested, [32P]PA levels were found to decline more rapidly than in control platelets. This was observed with the deoxy-, chloro-, and diCs-O-methyl ether analogs. The reason for this is not clear, but the possibility that these compounds stimulate some other metabolic fate of PA cannot be excluded. As shown in Fig. 7B, pretreatment with 0.5 mM deoxydiCs had no effect on diacylglycerol production.

I-Monooleoylglycerol—Despite in vitro inhibition of pig brain diacylglycerol kinase by 1-monooleoylglycerol, this compound affected the initial thrombin-induced rise in [32P]PA (Fig. 7A). The results in platelets following treatment with diCs-glyceramide (data not shown).

The in vivo inhibition of [32P]PA formation was observed in two experiments; however, this was not consistently seen.
pound did not inhibit thrombin-induced [32P]PA formation in platelets (Fig. 8A). In fact, in platelets treated with 100–400 μM 1-monooleoylglycerol [32P]PA levels were elevated (up to 2-fold) above stimulated controls.

The effect of 1-monooleoylglycerol on diacylglycerol levels in platelets was also examined (Fig. 8B). Basal levels of diacylglycerol were elevated following a 10-min preincubation with 1-monooleoylglycerol (42 pmol/5 × 10^8 platelets in controls, 125 pmol following treatment with 30 μM, and 155 pmol following treatment with 400 μM). These elevated levels were stable over the 5-min time course examined (Fig. 8B). Diacylglycerol accumulation could be due to inhibition of diacylglycerol utilization or possibly conversion of exogenous monoacylglycerol to diacylglycerol via the action of a monoacylglycerol acyltransferase.

The diacylglycerol levels reached following 1-monooleoylglycerol treatment are comparable to those seen following thrombin stimulation of control platelets. This finding led us to investigate the effect of 1-monooleoylglycerol treatment on protein kinase C activation, as measured by phosphorylation of a 40-kDa protein (35). 1-Monooleoylglycerol treatment (400 μM) only weakly stimulated 40-kDa phosphorylation in comparison to stimulation with thrombin or diCs (Fig. 9). This suggests that much of the diacylglycerol formed in response to monoacylglycerol treatment is in a compartment where it cannot activate protein kinase C. Importantly, 1-monooleoylglycerol treatment did not impair the ability of platelets to phosphorylate 40 kDa in response to thrombin (Fig. 9). Pre-treatment of platelets with diCs-ethylene glycol or diCs-glyceramide did not affect 40-kDa phosphorylation (Fig. 9).

When 1-monooleoylglycerol-treated platelets were stimulated with thrombin, the level of diacylglycerol rose even further (Fig. 8B). Platelets treated with 30 μM 1-monooleoylglycerol demonstrated a rapid rise (1.6-fold) in diacylglycerol upon stimulation, and this level remained elevated for 5 min. Diacylglycerol levels in platelets treated with 400 μM 1-monooleoylglycerol continued to rise for 5 min following stimulation (to 405 pmol/5 × 10^8 platelets). The level reached is nearly 10 times the basal level in control platelets. These persistent elevated levels of diacylglycerol suggest an inability of monooleoylglycerol-treated platelets to metabolize the diacylglycerol signal. It is possible that this reflects an inhibition of diacylglycerol lipase (see “Discussion”) by monoacylglycerol. It is also likely that inhibition of diacylglycerol kinase (as measured by [32P]PA production) was overcome by the elevated levels of diacylglycerol in these platelets.

FIG. 9. Phosphorylation of 40-kDa protein in the presence of diacylglycerol analogs. Phosphorylation of 40-kDa protein was analyzed in 32P-labeled platelets by sodium dodecyl sulfate-polyacrylamide gel electrophoresis as described under “Experimental Procedures.” Lanes 1 and 2, unstimulated; lane 3, 5 μM diC6Ca, 0.5 min; lane 4, 1 unit/ml thrombin, 0.5 min; lane 5, 400 μM 1-monooleoylglycerol, 10 min; lane 6, 400 μM 1-monooleoylglycerol, 10 min, then 1 unit/ml thrombin, 0.5 min; lane 7, 100 μM diC6-ethylene glycol, 10 min; lane 8, 100 μM diC6-ethylene glycol, 10 min, then 1 unit/ml thrombin, 0.5 min; lane 9, 100 μM diC6-glyceramide, 10 min; lane 10, 100 μM diC6-glyceramide, 10 min, then 1 unit/ml thrombin, 0.5 min. Phosphorylation of 20-kDa protein is due to the action of a calmodulin-dependent kinase, and is observed upon stimulation of platelets with thrombin, but not with exogenous diacylglycerols (5).

CONCLUDING DISCUSSION

In Vitro Properties of Diacylglycerol Kinase—The important role that diacylglycerol kinase plays in the attenuation of diacylglycerol signals in agonist-stimulated cells (14–17) makes an understanding of its regulation essential. Our in vitro studies on pig brain diacylglycerol kinase have defined further its requirements for phospholipid cofactors and its substrate specificity. In addition we have begun to screen for potentially interesting and biologically useful inhibitors of this enzyme.

Co-sonicated mixtures of diacylglycerol and phospholipid should provide a substrate closely resembling the physiological one. Using this approach, a variety of anionic phospholipids were found to be activators of the kinase, whereas zwitterionic phospholipids did not support activity. These results suggest that diacylglycerol kinase may require anionic phospholipids as essential cofactors. Such a surface would be present on the inner surface of the plasma membrane which is rich in PS. Alternatively, surfaces containing anionic phos-
pholipids may affect activity by physically modulating diacylglycerol accessibility.

Substrate Specificity of Diacylglycerol Kinase—Our data indicate that pig brain diacylglycerol kinase has high specificity for sn-1,2-diacylglycerol. DiC\(_2\)-amide was the only analog tested which was phosphorylated at a significant rate. 2-Monooleoylglycerol and diC\(_1\)-amide were also substrates for the kinase, but low maximal velocities were observed. Phosphorylation of 2-monoacylglycerol, but not 1-monoacylglycerol, has also been demonstrated by Kanoh’s group.\(^4\)

Testing of diC\(_2\) analogs as substrates of diacylglycerol kinase may aid in the identification of cell-activated promoters of protein kinase C which will not be attenuated. For example, diC\(_2\)-butanetriol has been found to activate protein kinase C (22) but is not a substrate for diacylglycerol kinase, and therefore should be longer lived in vitro than diC\(_2\). Such compounds will be useful for in vitro studies on protein kinase C.

In Vitro Inhibition of Diacylglycerol Kinase—Inhibitors of diacylglycerol kinase will be valuable tools in studying the role of this enzyme in vivo. Although most of the analogs tested were inhibitory using PE/PG/CL or 0.1 mM PS as activators, inhibition by a number of these was overcome by raising the PS concentration. This suggests that inhibition was due to alterations in the physical state of the diacylglycerol/phospholipid mixtures. The concentration of diC\(_2\) employed (30 \(\mu\)M) represents 2.3 mol % in the PE/PG/CL assay and 30 mol % in the 0.1 mM PS assay. At 0.5 mM, the mole % of diacylglycerol analog becomes very high and may induce such perturbations. The most potent inhibitors, however, exhibited the same inhibition at 0.1 or 0.5 mM PS, suggesting that inhibition was due to interaction with the kinase; these were competitive inhibitors with respect to diacylglycerol (Fig. 4).

Effects of Analogs on Human Platelet Diacylglycerol Metabolism—DiC\(_2\)-ethylene glycol also inhibited diacylglycerol kinase in human platelets, as indicated by the decreased basal and stimulated levels of \(^{32}\)P]PA. This effect was exerted without any suppression of diacylglycerol formation. DiC\(_2\)-ethylene glycol may prove a useful tool for investigation of diacylglycerol kinase in other cells.

1-Monooleoylglycerol inhibition of the pig brain kinase was not reflected in platelets when \(^{32}\)P]PA production was measured. Pretreatment with this compound, however, had profound effects on diacylglycerol levels in both unstimulated and stimulated platelets. The elevation of diacylglycerol levels induced by 1 monooleoylglycerol alone resulted in only slight activation of protein kinase C. This observation indicates that distinct intracellular pools of diacylglycerol exist. Diacylglycerol generated by acylation of monoacylglycerol may enter an internal membrane pool where it can be used in phospholipid biosynthesis, and where it cannot activate protein kinase C.

The levels of diacylglycerol attained upon thrombin stimulation of 1 monooleoylglycerol-treated platelets were extremely high. These elevated diacylglycerol concentrations probably masked inhibition of the kinase when assessed by \(^{32}\)P]PA formation.

The possibility that diacylglycerol generated by phospholipase C action is metabolized by alternate pathways has not been excluded. Human platelets are known to contain a diacylglycerol lipase specific for the sn-1 position (36). The 2 monoacylglycerol generated is metabolized further by a lipase activity capable of using 1- or 2 monoacylglycerol (36). Evidence that this pathway functions in stimulated platelets (37) and 3T3 cells (38) has been presented. Other workers, however, have suggested that this pathway does not play an important role in metabolism of diacylglycerol in stimulated platelets (39, 40).

Our data are consistent with a role for diacylglycerol lipase in stimulated platelets. This could account for (i) the ability of platelets to metabolize thrombin-induced diacylglycerol in spite of apparent inhibition of the kinase by diC\(_2\)-ethylene glycol, and (ii) the effects of 1 monooleoylglycerol on diacylglycerol levels in stimulated platelets. This compound, at high concentrations, could compete for the monoacylglycerol lipase, preventing flux of endogenously generated diacylglycerol through this pathway. The results seen with 1 monooleoylglycerol treatment are similar to those obtained using platelets treated with indomethacin, a compound reported to inhibit lipase activity in vitro (27).

Further analysis of diacylglycerol metabolism in platelets would profit from potent and specific inhibitors of both the diacylglycerol kinase and diacylglycerol lipase. These, in conjunction with mass quantitation of diacylglycerol levels as used in the current work, could provide further insight into the regulation of diacylglycerol second messengers.

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