Attenuation of sn-1,2-Diacylglycerol Second Messengers

METABOLISM OF EXOGENOUS DIACYLGlycerols BY HUMAN PLATELETS*

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The metabolism of exogenous [3H]diacylglycerols by intact human platelets was studied in order to examine: 1) the metabolic fate of these second messengers in an intact cell, 2) the effect of diacylglycerol kinase and diacylglycerol lipase inhibitors on this metabolism, 3) the effect of agonist stimulation on metabolism, and 4) the dependence of metabolism on diacylglycerol chain length. When 2.5 μM [3H]dioctanoylglycerol (dIC8) was added to 10^8 platelets it was rapidly metabolized; 80% was converted to various products in 2.5 min. Initially, 40% was recovered as [3H]-labeled phospholipid (predominantly phosphatidic acid) reflecting the action of diacylglycerol kinase, 20% was recovered as [3H]glycerol due to the action of diacylglycerol and monoaoylglycerol lipases, and small amounts were recovered as triacylglycerol and monoaoylglycerol. Thrombin stimulation of platelets did not affect the rate or pathway of metabolism. Pretreatment of platelets with the diacylglycerol kinase inhibitors, diC18, ethylene glycol or 1-monooleoylglycerol, inhibited [3H]-labeled phospholipid production 47% and 75%, respectively, and resulted in a longer lived diC18 signal. The diacylglycerol lipase inhibitor, RHC 80267, inhibited the production of water-soluble metabolites 75%. Despite inhibition of the lipase, the overall metabolism of exogenous [3H]dC8 occurred at a similar rate as in control platelets due to an increased flux towards phospholipid. The ability of exogenous diacylglycerols to be metabolized by diacylglycerol kinase correlated well with their ability to activate protein kinase C in platelets. [3H]Dibutyroylglycerol, didodecanoylglycerol, and dietradecanoyle triglycerol, were not metabolized by this route. These diacylglycerols were still metabolized via the lipase pathway. The results indicate that platelets possess potent attenuation systems to defend against the accumulation of diacylglycerol second messengers, and that the primary metabolic fate of cell-permeable, exogenous diacylglycerols is conversion to phosphatidic acid.

sn-1,2-Diacylglycerols, produced by the phosphodiesteratic cleavage of polyphosphoinositides, function as intracellular second messengers through the activation of protein kinase C. A central role for this protein kinase in the responses elicited by agonists in a variety of cell types has been demonstrated (1-3). Cell-permeable diacylglycerols, 1-oleyl-2-acetylglycerol, and diacylglycerols with symmetric short acyl chains (e.g. dioctanoylglycerol) have proven especially valuable in defining these roles (reviewed in Ref. 3).

The formation of diacylglycerols induced by agonists has been followed in cells labeled with biosynthetic precursors of phospholipid such as arachidonic acid (e.g. Refs. 4 and 5). Recently, methods for quantitation of sn-1,2-diacylglycerols in crude lipid extracts have been developed (6-8). These methods employed high performance liquid chromatography with refractive index detection (7) or quantitative conversion of diacylglycerol to [32P]phosphatidic acid using Escherichia coli diacylglycerol kinase (6, 8). The labeling studies and mass measurements demonstrated that the increase in diacylglycerol levels was transient, indicating that these second messengers were rapidly metabolized following their agonist-stimulated formation. In platelets stimulated with thrombin or other agonists, a 2-3-fold increase in diacylglycerol levels was observed, reaching a maximum by 0.5 min after stimulation, and declining to basal levels by 5 min (8). In Swiss 3T3 cells stimulated with platelet-derived growth factor a similar increase in diacylglycerol levels was observed. In this case the response was slower, diacylglycerol levels peaked 10 min after stimulation and declined to basal levels over the next 50 min (5).

Two pathways have been implicated in the attenuation of diacylglycerol second messengers, phosphorylation by diacylglycerol kinase (9, 10) and degradation by the sequential action of a diacylglycerol and monoaoylglycerol lipase (11, 12). The relative role of these two pathways in diacylglycerol removal in stimulated platelets has been a subject of controversy (9, 12).

Recently a series of diacylglycerol analogs were tested as inhibitors of pig brain diacylglycerol kinase in vitro (13). Dioctanoylethylene glycol, a diacylglycerol kinase inhibitor in vitro, also inhibited [32P]phosphatidic acid formation in thrombin-stimulated human platelets. This inhibition resulted in a slightly longer lived diacylglycerol signal compared to that in control platelets. The diacylglycerol produced was still metabolized rapidly, suggesting that the lipase pathway can function effectively to attenuate diacylglycerol signals (13).

In this report, short-chain sn-1,2-diacylglycerols containing radiolabel in their glycerol backbone were prepared. These were utilized to follow the metabolic fate of diacylglycerol in intact human platelets. This approach allowed a direct measurement of their metabolism and an assessment of the relative contributions of the diacylglycerol metabolic pathways. The effect of inhibitors of diacylglycerol kinase and diacylglycerol lipase on these pathways were examined.

The effect of acyl chain length on the metabolism of exogenous diacylglycerol was also investigated. Previous studies demonstrated that dioctanoylglycerol (dC8)1 added exoge-

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1 The abbreviations used are: dCn, dioctanoylglycerol; dCn, dibutyroylglycerol; dCn, dioleinoylglycerol; dCn, dioleinoylglycerol; dCn, dioleinoylglycerol; dCn, dioleinoylglycerol; dCn, dioleinoylglycerol; dCn, dioleinoylglycerol; dCn, dioleinoylglycerol; dCn, dioleinoylglycerol; dCn, dioleinoylglycerol.
nously to human platelets was a potent activator of protein kinase C, as assessed by the phosphorylation of a 40-kDa protein (14). DiC₈ and diC₁₀ were also effective, while very short-chain diacylglycerols (diC₂) or very long-chain diacylglycerols (diC₁₆) were ineffective. Similar findings have been made in a variety of biological systems (reviewed in Ref. 3).

The metabolism of some of these diacylglycerols was examined in platelets and compared with the ability of these agents to activate protein kinase C.

**EXPERIMENTAL PROCEDURES**

**Materials**—[1,2,3-³H]Glycerol (200 mCi/mmol) was purchased from New England Nuclear. Calf intestinal phosphatase, monooctanoylglycerol, and trioctanoylglycerol were from Sigma. Diocanoylphosphatidylcholine and dioctanoylglycerol were from Avanti Polar Lipids Inc. Other chain length unlabeled diacylglycerol and di-C₆thyleeneglycerol were prepared as described previously (15). Capric (C₁₄), lauric (C₁₂), and myristic (C₁₄) anhydrides were products of Serday Research Laboratories, while butyric (C₄) anhydride, octanoyl (C₈) chloride, and hexanoyl (C₆) chloride were from Aldrich. Bio-Sil A for silicic acid chromatography was obtained from Bio-Rad. RHC 80267 was a kind gift of Dr. Charles A. Sutherland, Rorer Group Inc.

**Platelet Preparation**—Human platelets were prepared from freshly drawn human blood essentially as described by Siess et al. (4) except that incubation at 37 °C for 75 min was performed in the absence of prostaglandin I₂ after washing the platelets in Tyrodes buffer containing 5% platelet-poor plasma.

**³H]Diacylglycerol Synthesis—sn-1,2,3-³H]Glycerol 3-phosphate was prepared enzymatically by the method of Chang and Kennedy (16) and converted to its pyridinium salt by passage over pyridinium Dowex 50 W. Acylations were performed using the appropriate fatty acid anhydride or chloride as described previously (17). After discharging any mixed anhydride, the phosphatidic acid was purified by silicic acid chromatography as described (17). Removal of dimethylaminopyridine catalyst was achieved by passage over Dowex 50W-H⁺.

The resulting [³H]phosphatidic acid was converted to [³H]diacylglycerol using calf intestinal phosphatase. Phosphatidic acid was taken to dryness under N₂ and re suspended in 0.1 ml of phosphatase buffer (50 mM Tris·HCl, pH 9.0, 1 mM MgCl₂, 0.1 mM EDTA, 1 mM spermidine). Phosphatase (100 units) was added and the reaction was allowed to proceed for 60 min at room temperature. Due to the limited solubility in the reaction mixture, the efficiency of the reaction declined with chain length; about 50% of diC₆-diC₁₀ phosphatidic acid was cleaved, while only about 15% of the diC₁₂ compound was dephosphorylated. Reactions were terminated with 3 ml of methanol/chloroform (2:1) and extracted by the method of Bligh and Dyer (18) using H₂O as upper phase. The lower phase was dried under N₂, redissolved in CHCl₃, and chromatographed on silicic acid. sn-1,2-diC₈glycerol was eluted with CHCl₃, phosphatidic acid was eluted with chloroform/methanol (1:1) and recycled, as desired.

The final [³H]diacylglycerol had a specific activity of 200 mCi/mmol and was brought to the desired specific activity by addition of unlabeled sn-1,2-diC₈glycerol.

**Metabolism of [³H]Diacylglycerol—Platelets were resuspended in Tyrodes buffer (usually 1 × 10⁸ cells/ml) and [³H]diacylglycerol was added as an ethanol solution (final concentration of ethanol 0.5%). The concentration of exogenous phosphatidic acid was generally 0.2-2.5 μM as indicated (2 × 10⁻⁹-10⁻⁸ cm/ml). For experiments in which the effect of various inhibitors was tested, these were added 10 min prior to the addition of [³H]diacylglycerol. Reactions were carried out at 37 °C.

Reactions were terminated by transferring 0.4-ml samples to 3.0 ml of methanol/chloroform (2:1). Ex Extractions were performed by the method of Bligh and Dyer (18) using 1% HClO₄ as upper phase. Some experiments were performed using the isolated platelet incubator media by centrifugation for 0.5 min at 13,000 × g in an Eppendorf microfuge; the platelet pellet and supernatant were extracted. The upper phases from the extraction were carefully removed; 1.0-ml fractions were taken for liquid scintillation counting into 10 ml of Aquasol, New England Nuclear) and the remainder was saved for further analy. A sample of the lower phase (1.5 ml) was dried under N₂ and redissolved in 0.1 ml of chloroform. Samples were taken for scintillation counting and/or thin layer chromatography.

**RESULTS**

**Metabolic Fate of [³H]Diocanoylglycerol—**Initially we chose to follow the fate of diC₈ in intact platelets since this molecule is the most efficient of the short-chain diacylglycerols at activating protein kinase C and eliciting biological responses in platelets and other cell types (3). DiC₈ causes enhanced phosphorylation of a 40-kDa protein in human platelets (14). [³H]DiC₈ (2.5 μM) was rapidly metabolized upon addition to platelets; 80% was converted to various products in the first 2.5 min (Fig. 1). Most of the label was initially recovered in phospholipid (40% of the input at 2.5 min) with

About 20% of the added [³H]diC₈ appeared to be metabolically inert. Addition of sn-1,2-diC₈ to the platelet incubation mixture was followed by some accumulation of 1,3-diC₈. The presence of 1,3-diC₈ accounted for about 25% of this non-metabolized material. It is possible that some of the diC₈ phase separated in the reaction mixture and therefore was inaccessible to the platelet metabolic machinery.
the remainder being metabolized to water-soluble products (19%), triacylglycerol (13%), and monoacylglycerol (5%) (Fig. 1). At later times, a slight decline in phospholipid labeling was observed with a concomitant increase in water-soluble counts.

The distribution of the [3H]phospholipid counts is shown in Fig. 2. At early times, most of the label was in diC₈phosphatidic acid. This product is consistent with rapid phosphorylation of diC₈ by diacylglycerol kinase. The [3H]phosphatidic acid was subsequently metabolized. Production of [3H]diC₈phosphatidylinositol demonstrated the functioning of the phosphatidylinositol cycle. [3H] label also appeared in phosphatidylichenole and other phospholipid species.

Greater than 95% of the water-soluble [3H]-labeled products were identified as free [3H]glycerol using paper chromatography indicating complete deacylation of diC₈ by diacylglycerol and monoacylglycerol lipase. Little accumulation of a monoacylglycerol intermediate was detected.

Under the conditions employed, metabolism to phospholipid was consistently about 2-fold greater than the contribution of the lipase pathway, suggesting that diacylglycerol kinase is the predominant pathway of diacylglycerol signal attenuation. Production of [3H]triacylglycerol was apparently due to the action of a diacylglycerol acyltransferase.

Water-soluble metabolites accumulated briefly within the platelets (42% cell-associated at 5 min) and were then released to the reaction media with time (90% released at 30 min). Greater than 80% of the chloroform-soluble label was cell associated, including intact diC₈ and its metabolites. The chloroform-soluble counts not associated with the platelets were almost entirely nonmetabolized diC₈, and represented about 16% of the input at 15 min.

Platelet and Concentration Dependence of [3H]diC₈ Metabolism—The dependence of [3H]diC₈ metabolism (2.5 μM diC₈, 1 min reaction) on the amount of platelets employed is shown in Fig. 3. Production of water-soluble metabolites was linear with the amount of platelets up to 10⁸/ml, while near maximal formation of [3H]phospholipid was observed using 10⁹/ml. At 10⁸ platelets/ml metabolism to phospholipid was 7.5-fold greater than to water-soluble products (0.6 nmol/min/10⁸ platelets and 0.08 nmol/min/10⁹ platelets, respectively).

Metabolism of [3H]diC₈ by all pathways was proportional with the concentration of diC₈ used up to 10 μM (Fig. 4). At higher concentrations, [3H]phospholipid production showed apparent saturation.

Effect of Diacylglycerol Kinase and Lipase Inhibitors on [3H] DiC₈ Metabolism—Since [3H]diC₈ was metabolized by both the diacylglycerol kinase and lipase pathways, the effects of inhibitors of these enzymes on the metabolic fate of diC₈ was investigated. Among various diacylglycerol analogs tested previously, diC₈cetylethylglycerol and 1-monooctanoylglycerol were the most potent inhibitors of diacylglycerol kinase in vitro (13). Inhibition of diacylglycerol kinase by diC₈cetylethylglycerol was also observed in thrombin-stimulated human platelets (13), where it inhibited 32P incorporation into phosphatidic acid.

DiC₈cetylethylglycerol inhibited the conversion of exogenous [3H]diC₈ to [3H]phospholipid by platelets (Fig. 5). The data shown were obtained 5 min after addition of [3H]diC₈ (2.5 μM) to 10⁷ platelets/ml. In the absence of diC₈cetylethylglycerol 20% of the added [3H]diC₈ remained intact after 5 min, with 45% of the [3H] label recovered as phospholipid and 20% as water-soluble metabolites. Increasing concentrations of diC₈cetylethylglycerol inhibited [3H] incorporation into phospholipid. The extent of inhibition observed in the presence of 200 μM diC₈cetylethylglycerol was 47.3 ± 4.9% (n = 5). DiC₈cetylethylglycerol had no effect on the production of water-
Platelets pretreated with 100 to 44.6% in control platelets. Maximal inhibition of about platelets treated with 100 with control platelets.

dicethyleneglycol (Fig. 6), with 65% inhibition observed in

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5). 40% of the [3H]diC8 remained intact when platelets were

concentration of 1-monooleoylglycerol added as an ethanol solution

glycerol.

15.3% of the 3H label was recovered in phospholipid compared

olized slower in platelets treated with diC8ethyleneglycol (Fig. 6), presumably through inhibition of the diacylglycerol lipase pathway as suggested previously (13). The combined inhibitory effects of 1-monooleoylglycerol resulted in considerably slower metabolism of [3H]diC8 by platelets (Fig. 6). 45% of

1-Monooleoylglycerol inhibited [3H]diC8 metabolism (measured at 5 min) to phospholipid more potently than did diC8ethyleneglycol (Fig. 6), with 65% inhibition observed in platelets pretreated with 100 µM 1-monooleoylglycerol. In platelets treated with 100 µM 1-monooleoylglycerol only 15.3% of the 3H label was recovered in phospholipid compared to 44.6% in control platelets. Maximal inhibition of about 75% was seen using 500 µM 1-monooleoylglycerol. This finding is consistent with the observed inhibition of diacylglycerol kinase by 1-monooleoylglycerol in vitro (13).

1-Monooleoylglycerol also inhibited conversion of [3H]diC8 to water-soluble metabolites (30% inhibition at 200 µM, Fig. 6), presumably through inhibition of the diacylglycerol lipase pathway as suggested previously (13). The combined inhibitory effects of 1-monooleoylglycerol resulted in considerably slower metabolism of [3H]diC8 by platelets (Fig. 6). 45% of

the [3H]diC8 remained intact after 5 min in platelets treated with 100 µM 1-monooleoylglycerol, while only 20% remained unmetabolized in control platelets. In addition, increased amounts of label were recovered as triacylglycerol in platelets pretreated with 1-monooleoylglycerol.

RHC 80267 has been reported to be a specific inhibitor of diacylglycerol lipase from rat and dog platelets (19). It also inhibits human platelet diacylglycerol lipase in vitro (20).

Intact human platelets pretreated with RHC 80267 converted much less [3H]diC8 to water-soluble metabolites (77% inhibition at 125 µM, Fig. 7). In spite of the inhibition of the lipase pathway, [3H]diC8 was still metabolized rapidly, due to an increased production of [3H]phospholipid (Fig. 7).

When platelets were pretreated with combinations of diacylglycerol kinase and lipase inhibitors the overall rate of metabolism of exogenous [3H]diC8 was slowed substantially (data not shown). Following a 2.5-min incubation 90.5% of exogenous [3H]diC8 was metabolized in control platelets, 50% was metabolized in platelets treated with 200 µM diC8ethyleneglycol and 125 µM RHC 80267, and only 32.9% was metabolized in platelets treated with 200 µM 1-monooleoylglycerol and 125 µM RHC 80267. No further increase in the flux towards triacylglycerol was observed under these conditions.

Metabolism of Other Chain Length Diacylglycerols—Previously, Lapetina et al. (14) demonstrated that addition of exogenous diC6, diC8, or diC10 to human platelets led to activation of protein kinase C and phosphorylation of 40-kDa protein. Exogenous diC6 or diC11 were inactive. These findings were confirmed and extended using the [3H]diacylglycerols prepared in the current work. 40-kDa phosphorylation was examined 0.5 min following the addition of 5 µM exogenous [3H]diacylglycerol. [3H]diC8 was ineffective at eliciting 40-kDa phosphorylation. [3H]diC6 and diC10 were equally effective, while [3H]diC12 showed low activity (30% of diC8). DiC14 was inactive (data not shown).

Fig. 8A shows the overall rate of metabolism of various exogenous diacylglycerols. DiC10 (0.5 µM) was initially metabolized at a similar rate to diC8 (about 50% in 1 min), and only slightly slower at later times. In contrast, diC12 (0.5 µM) and diC14 (0.5 µM) were metabolized very slowly (Fig. 8A). The products of this metabolism were further examined. About 25% of exogenous diC8 was metabolized to water-soluble products in 5 min, while diC12 and diC14 were metabolized slower (about 5–10% in 5 min) (Fig. 8B). Surprisingly, diC10
Diacylglycerol Metabolism in Platelets

FIG. 8. Effect of diacylglycerol chain length and thrombin stimulation on metabolism. [3H]Diacylglycerol of the indicated chain length was added to platelets (10^9 cells/ml) to a concentration of 0.5 μM. Samples were extracted and analyzed as described. When the effects of thrombin were examined, human thrombin was added to 1 unit/ml immediately prior to addition of [3H]diC. Panel A, percent of [3H]diacylglycerol remaining intact; Panel B, percent of 3H recovered as water-soluble metabolites; Panel C: percent of 3H recovered as phospholipid. The abbreviation used is: DAG, diacylglycerol.

The use of cell-permeable, radio-labeled diacylglycerols has allowed the metabolic fate of exogenous diacylglycerols in human platelets to be directly investigated. The data presented demonstrate a role for both diacylglycerol kinase and diacylglycerol lipase in the removal of this second messenger in human platelets. The data also suggest that acylation to triacylglycerol is an additional, although minor, pathway of diacylglycerol signal attenuation.

Conversion of exogenous [3H]diC to phospholipid was the predominant pathway for its metabolism by platelets. Using 10^9 platelets/ml, the flux through the diacylglycerol kinase pathway was about 2-fold greater than that through the lipase pathway, while at lower platelet levels up to 7.5-fold more 3H chain diacylglycerols in the aqueous reaction mixture and, therefore, their inaccessibility to the metabolic enzymes. It is possible that the limited degradation of longer chain diacylglycerols via the lipase pathway may be due to an outward facing orientation of the lipase enzymes, making it unnecessary for the substrates to enter the cells.

The metabolism of shorter chain diacylglycerols has also been examined (Fig. 9). [3H]DiC was converted to water-soluble products at the same rate as [3H]diC. Some accumulation of monoC was also apparent. This metabolism of [3H] diC by the lipase pathway was inhibited by RHC 80267 (data not shown). No [3H]phosphatidic acid was detected in either the organic or aqueous phase of the extractions from these reactions, indicating that no metabolism to phospholipid occurred. This was likely due to the inability of diC to partition into the platelet membrane, making it inaccessible to diacylglycerol kinase and unable to activate protein kinase C.

DISCUSSION

Metabolic Fate of DiC—The use of cell-permeable, radio-labeled diacylglycerols has allowed the metabolic fate of exogenous diacylglycerols in human platelets to be directly investigated. The data presented demonstrate a role for both diacylglycerol kinase and diacylglycerol lipase in the removal of this second messenger in human platelets. The data also suggest that acylation to triacylglycerol is an additional, although minor, pathway of diacylglycerol signal attenuation.

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was recovered as phospholipid than as water-soluble metabolites.

Other attempts to address the relative importance of these two pathways of diacylglycerol signal attenuation have provided conflicting results. Studies of diacylglycerol metabolism utilizing biosynthetic precursors are complicated by the lack of specific labels for diacylglycerol. Previous studies using [14C]arachidonic acid-labeled platelets have led to the conclusion that diacylglycerol kinase was the predominant pathway of attenuation (9, 24, 25). Lapetina et al. (9) utilized platelets in which phosphatidylinositol degradation by phospholipase A2 was blocked with quinacrine. Under these conditions, the label incorporated into phosphatidic acid quantitatively accounted for the loss of label from phosphatidylinositol. This suggested that all of the diacylglycerol generated in response to thrombin was converted to phosphatidic acid.

On the other hand, Majerus' group has argued that diacylglycerol lipase is the predominant pathway of both diacylglycerol attenuation and arachidonic acid release in thrombin-stimulated platelets (11, 26). This conclusion was supported, in part, by the demonstration of a transient accumulation of [3H]arachidonoylmonoacylglycerol following thrombin stimulation (26). The metabolism of diacylglycerols by platelets has also been approached in vitro. Platelet membranes were shown to possess a diacylglycerol lipase which removes the sn-1 fatty acid, and a monoacylglycerol lipase (26). Mauro et al. (27) examined the metabolic fate of long-chain diacylglycerols using [3H]oleic acid labeled diacylglycerol and a platelet plasma membrane fraction. About 50% of the long-chain diacylglycerols were degraded by the lipase pathway. However, inclusion of Mg2+-ATP in their reaction mixture led to enhanced phosphatidic acid production. This approach is not useful for understanding how diacylglycerols are handled in the intact cell since not all diacylglycerol utilizing enzymes are membrane bound. For example, when platelets in Tyrode's buffer were broken by freeze-thaw and sonication we found greater than 80% of the diacylglycerol kinase activity was in the 100,000 × g supernatant.

The approach described in this report has circumvented the problems of these previous studies. First, the label employed is initially present only in the molecule of interest, diacylglycerol. Second, the platelets are intact and responsive to agonists. In addition, the levels of diacylglycerol employed (500-2500 pmol/10^9 platelets) are similar to the maximal concentration reached in agonist-stimulated platelets (300 pmol/10^9 platelets) (8). The difference in acyl chain composition between diC8 and endogenously generated diacylglycerols may, however, affect metabolism. Development of methods for the introduction of long-chain [3H]glycerol-labeled diacylglycerols (e.g. 1-stearoyl-2-arachidonyl) into platelets would be worthwhile.

Effects of Inhibitors—Inhibition of exogenous diC8 metabolism to phospholipid by diC8ethyleneglycol is consistent with the effects of this compound on diacylglycerol kinase in vitro and on 32P incorporation into phosphatidic acid in thrombin-stimulated platelets (13). Treatment of platelets with diC8ethyleneglycol slowed the metabolism of exogenous diC8 (this work) and slightly prolonged the life of endogenously generated diacylglycerol second messengers (13).

1-Monooleoylglycerol inhibition of [3H]phospholipid formation from exogenous diC8 is also consistent with its in vitro effect on diacylglycerol kinase (13). 1-Monooleoylglycerol also inhibited (less potently) the production of water-soluble metabolites. The combined effect of 1-monooleoylglycerol on both the diacylglycerol kinase and lipase pathways slowed the metabolism of exogenous [3H]diC8. These effects may also account for the greatly elevated diacylglycerol levels previously observed in both thrombin-stimulated and unstimulated 1-monooleoylglycerol-treated platelets (13). In unstimulated platelets, 1-monooleoylglycerol treatment elevated diacylglycerol levels 3-4-fold, with only slight activation of protein kinase C, suggesting that this diacylglycerol may reside in a nonsignaling pool (13). In thrombin-stimulated platelets, 1-monooleoylglycerol treatment resulted in diacylglycerol levels nearly 10 times the basal level in control platelets. These elevated diacylglycerol levels masked the inhibition of diacylglycerol kinase when assessed by 32P incorporation into phosphatidic acid (13).

Previous studies employing RHC 80267 have been inconclusive. Chau and Tai (28) reported that RHC 80267 abolished the transient rise in monoacylglycerol following thrombin stimulation of human platelets. However, Bross et al. (20) reported that this drug was inactive in intact human platelets. The present work shows that RHC 80267 is active in intact platelets. RHC 80267 blocked the production of water-soluble metabolites from [3H]diC8 over the same concentration range reported to be effective in vitro on diacylglycerol lipase (19). Unfortunately, the usefulness of this compound is limited by its pleiotropic effects. RHC 80267 also inhibited the production of endogenous diacylglycerol in thrombin-stimulated platelets (data not shown). Inhibition of cyclooxygenase and phospholipase C by this compound has been reported (29). When the diacylglycerol lipase was inhibited with RHC 80267 there was little difference in the overall rate of [3H]diC8 metabolism due to a compensatory increase in the flux towards phospholipid.

Inhibitors of diacylglycerol second messenger attenuation may prove to be useful tools for exploring the activation of protein kinase C in vivo. Preliminary studies suggest that reduced diC8 metabolism may lower the concentration of this compound needed for activation of protein kinase C. 1 μM diC8 resulted in 70% of maximal 40-kDa phosphorylation in control platelets, while this concentration resulted in 91% and 77% maximal 40-kDa phosphorylation in 1-monooleoylglycerol- and diC8ethyleneglycol-treated platelets, respectively.

Chain Length Dependence—The studies employing various chain length diacylglycerols have furthered our understanding of the behavior of these compounds when added to platelets. Neither diC12 nor diC4 were metabolized by the diacylglycerol kinase pathway. Access to this enzyme would require partitioning into the platelet plasma membrane and transmembrane movement to the cytoplasmic surface. These same events are required for activation of protein kinase C (3). It is likely that, due to its high water solubility, diC4 does not favorably partition into the platelet membrane, and that long-chain diacylglycerols, due to their limited water solubility, phase separate in the reaction mixture. Therefore, neither gain access to diacylglycerol kinase or to suitable sites for protein kinase C activation. The residual metabolism of these compounds by the lipase pathway raises the possibility that the enzymes of this pathway may be able to act on extracellular diacylglycerol.

The observation that diC8 was metabolized by diacylglycerol lipase is an important one. The use of diC8 will provide a means to study the lipase under conditions where protein kinase C is not activated, and where the substrate is not metabolized by other routes.

The rapid metabolism of exogenous diacylglycerol observed here is consistent with the transient nature of the diacylglycerol signal generated in platelets in response to agonists (8,
13. No evidence for enhanced diacylglycerol metabolism following agonist stimulation was observed. These results suggest that platelets have potent defense mechanisms preventing the accumulation of diacylglycerol second messengers. The metabolism of exogenous diacylglycerols in other cell types will be of interest in relation to protein kinase C and pathways of diacylglycerol signal attenuation.

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