Vol. 121, No. 1, 1984 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
May 31, 1984 Pages 386-391

1,2-DIACYLGLYCEROLS DO NOT POTENTIATE THE ACTION OF PHOSPHOLIPASES A_2 AND C IN HUMAN PLATELETS

S. P. Watson*, B. R. Ganong†, R. M. Bell†, and E. G. Lapetina*

*Department of Molecular Biology, The Wellcome Research Laboratories, 3030 Cornwallis Road, Research Triangle Park, North Carolina 27709
†Department of Biochemistry, Duke University Medical Center, Durham, North Carolina 27710

Received April 19, 1984

SUMMARY. 1,2-Diacylglycerol has recently been reported to potentiate the ability of phospholipases A and C to hydrolyze phospholipids in a cell-free system. The present study has been undertaken to investigate whether 1,2-diacylglycerol can also perform this function in intact cells using the platelet as a test system. Exogenous 1-oleoyl-2-acetyl-glycerol (OAG) and 1,2-didecanoylglycerol, at concentrations sufficient to produce maximal phosphorylation of a 40,000 dalton protein, caused no significant formation of [^{3}H]inositol phosphates and [^{32}P]phosphatidic acid (products of phospholipase C activation) or [^{14}C]arachidonic acid metabolites and lysophosphatidyl[^{3}H]inositol (products of phospholipase A_2 activation). These data therefore imply that 1,2-diacylglycerols do not potentiate the actions of phospholipases A_2 and C in intact platelets at concentrations that are physiologically relevant.

Activation of platelets by a large number of physiological stimuli, such as collagen, thrombin and platelet activating factor, is associated with the rapid breakdown of membrane inositol phospholipids via the action of phospholipase C (1), thus resulting in the transient accumulation of 1,2-diacylglycerol (2,3). 1,2-Diacylglycerol appears to function as an important second messenger agent in the platelet through the activation of protein kinase C, which has been demonstrated to phosphorylate a 40,000 dalton protein (3,4). In addition, Dawson *et al.* (5) and Hofmann and Majerus (6) demonstrated that 1,2-diacylglycerols potentiate the ability of phospholipases A and C to hydrolyze phospholipids in both solution (5,6) and microsomes (6), and the possibility therefore arises that this action may be of importance *in vivo*. The present study has investigated this hypothesis using the human platelet as a test system.

Abbreviations: OAG, 1-oleoyl-2-acetyl-glycerol

0006-291X/84 $1.50

METHODS

Platelets were isolated from human volunteers on the day of the experiment as previously reported (7). They were suspended in a modified Tyrode-HEPES buffer (composition in mM: NaCl 134, $NaHCO_3$ 12, KCl 2.9, Na_2HPO_4 0.34, $MgCl_2$ 1, HEPES 5, glucose 5 and EGTA 1 buffered to pH 7.4) and labeled with either [^{32}P]orthophosphate (0.5 mCi/ml for 60 min), [^{14}C]arachidonic acid (2 µCi/ml for 90 min) or [^{3}H]inositol (100 µCi/ml for 3 h). Platelets were then washed and resuspended at a final concentration of 8 x 10^8/ml. Aliquots (1 ml) were then incubated in a Chronolog aggregometer at 37°C with stirring for 1 min and OAG, 1,2-didecanoylglycerol or thrombin added. The use of thrombin serves as a positive control since it has been observed to activate both phospholipases C and A_2 (1). The reaction was stopped at various times by addition of 3.76 ml chloroform:methanol:HCl (100:200:2), and chloroform (1.2 ml) and water (1.2 ml) subsequently added. Phases were separated by centrifugation. The upper phase contained the water soluble inositol phosphates and these were separated and quantitated as described by Berridge *et al.* (8). The lower lipid containing phase was dried under nitrogen, resuspended in chloroform, and applied either to oxalate-impregnated silica gel plates developed in chloroform: methanol:ammonia:water (90:70:5:15) for analysis of [^{3}H]inositol phospholipids (9), or silica gel plates developed in ethylacetate: isooctane:acetic acid:water (9:5:2:10) for analysis of [^{14}C]arachidonic acid metabolites and [^{32}P]phosphatidic acid (10). Spots were visualized by autoradiography, scraped relative to standards and counted by liquid scintillometry. Protein phosphorylation studies were performed as previously described (7).

Myo-[2-^{3}H]Inositol was purchased from Amersham International and 1,2-didecanoylglycerol was from Serdary. OAG was prepared from dioleoylphosphatidylcholine (Ganong and Bell, manuscript in preparation). In brief, dioleoylphosphatidylcholine was hydrolyzed by phospholipase A_2, and the product acetylated with acetic anhydride. 1-Oleoyl-2-acetyl-phosphatidylcholine was then purified and converted to OAG by phospholipase C. The structure of the purified OAG was established by mass spectroscopy. 1,2-Diacylglycerols were dissolved in ethanol before use. Other reagents were from previously described sources (7,8).

RESULTS

1,2-Didecanoylglycerol (Fig. 1) and OAG (not shown) were found to produce both dose- and time-dependent phosphorylation of a 40,000 dalton protein and, to a lesser extent, phosphorylation of a 20,000 dalton protein related to the myosin light chain. Similar results have been previously reported for OAG (3). Maximal phosphorylation of the 40,000 dalton protein was produced within 1 min by 10 µM of 1,2-didecanoylglycerol (Fig. 1) and 50 µM OAG (not shown). The phosphorylations were accompanied by a much slower onset and more variable shape change response in the platelets.

The action of phospholipase C on inositol phospholipids produces 1,2-diacylglycerol and a water soluble inositol phosphate, the nature of which is dependent on the inositol phospholipid that is degraded. In intact

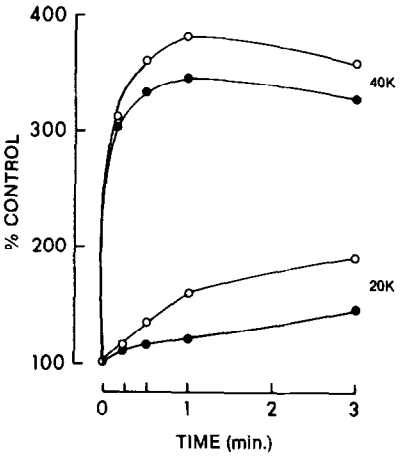

Figure 1 Effect of 1,2-didecanoylglycerol on the [^{32}P]phosphorylation of a 40,000 dalton (40K) and 20,000 dalton (20K) protein in platelets. Platelets were incubated with 1,2-didecanoylglycerol for various times and proteins were analyzed as described in "Methods." (o)-10 μM 1,2-didecanoylglycerol, (•)-50 μM 1,2-didecanoylglycerol.

platelets 1,2-diacylglycerol is then rapidly phosphorylated to phosphatidic acid, which therefore serves as a marker of phospholipase C activity (10). Table 1 shows that OAG (50 μM), at a concentration sufficient to produce maximal phosphorylation of the 40,000 dalton protein, caused no significant formation of [^{3}H]inositol phosphates, even in the presence of Li^{+} (10 mM) which blocks hydrolysis of inositol 1-phosphate to free inositol (8). Further, 1,2-didecanoylglycerol (50 μM), at a concentration sufficient to produce maximal phosphorylation of the 40,000 dalton protein, did not cause significant formation of [^{32}P]phosphatidic acid derived from inositol phospholipids, although it was rapidly converted to 1,2-didecanoyl-

Table 1. Effect of 1-oleoyl-2-acetyl-glycerol (OAG) on [^{3}H]inositol phosphates in human platelets.

Additions	n	[^{3}H]Inositol-monophosphate	[^{3}H]Inositol-bisphosphate	[^{3}H]Inositol-trisphosphate
OAG (50 μM)	5	102.7 ± 7.0	108.5 ± 5.5	99.2 ± 13.3
Thrombin (1 U/ml)	3	294 ± 85	675 ± 223	299 ± 72

Platelets were incubated with either OAG or thrombin in the presence of LiCl (10 mM) for 2 min, and [^{3}H]inositol phosphates subsequently extracted and analyzed as described in "Methods." Results are expressed as the mean ± S.E.M. percentage change relative to basals. Thrombin serves as a positive control since it has been demonstrated to activate phospholipase C in platelets (1).

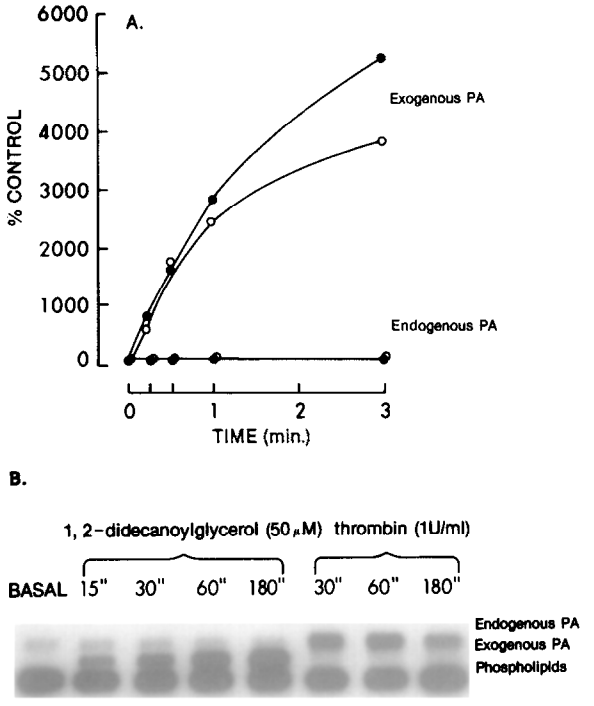

Figure 2 Conversion of 1,2-didecanoylglycerol but not endogenous 1,2-diacylglycerol to [^{32}P]phosphatidic acid in human platelets. Platelets were incubated with 1,2-didecanoylglycerol for various times and [^{32}P]phosphatidic acid extracted and analyzed as described in "Methods." *A.* Phosphorylation of 1,2-didecanoylglycerol to 1,2-didecanoyl-[^{32}P]phosphatidic acid (exogenous phosphatidic acid) with no formation of endogenous [^{32}P]phosphatidic acid. *B.* Autoradiogram showing separation of exogenous and endogenous phosphatidic acid. Thin-layer silica gel plates were developed in ethylacetate: isooctane:acetic acid:water (9:5:2:10). Platelets stimulated with thrombin (1 U/ml) are included for illustration of [^{32}P]phosphorylation of endogenously produced 1,2-diacylglycerol.

[^{32}P]phosphatidic acid (Fig. 2). The identification and separation of phosphatidic acid derived from endogenous 1,2-diacylglycerol and exogenously added 1,2-diacylglycerol (1,2-didecanoylglycerol) were possible because of their different fatty acid compositions; the endogenous species contains predominantly stearic acid in position 1 and arachidonic acid in position 2 (11). Thus, neither OAG or 1,2-didecanoylglycerol stimulated platelet phospholipase C activity.

The action of phospholipase A_2 on phospholipids produces arachidonic acid and the corresponding lysophospholipids. Arachidonic acid is rapidly converted within platelets to cyclooxygenase and lipoxygenase products (12). Table 2 demonstrates that OAG (50 μM), at a concentration sufficient to

Table 2. Effect of 1-oleoyl-2-acetyl-glycerol (OAG) on [^{14}C]arachidonic acid metabolites in human platelets.

Additions	n	HHT	HETE
OAG (50 μM)	2	102.5 ± 7.0	87.5 ± 11.0
Thrombin (1 U/ml)	1	708	1220

Platelets prelabeled with [^{14}C]arachidonic acid were incubated with OAG or thrombin for 2 min, and [^{14}C]arachidonic acid metabolites subsequently extracted and analyzed as described in "Methods." Results are expressed as the mean ± S.E.M. percentage change relative to basals. Thrombin liberates [^{14}C]arachidonic acid from platelet phospholipids which is rapidly converted to 12-hydroxy-5,8,10,14-eicosatetraenoic acid (HETE), a product of lipoxygenase activity, and 12-hydroxy-5,8,10-heptadecatrienoic acid (HHT), a product of cyclooxygenase activity (1). Free [^{14}C]arachidonic acid was not observed.

produce maximal phosphorylation of the 40,000 dalton protein, caused no significant formation of metabolites of either the cyclooxygenase or lipoxygenase pathways. Similar results were observed for 1,2-didecanoylglycerol (50 μM) (not shown). Further, OAG (50 μM) caused no significant formation of lysophosphatidyl[^{3}H]inositol (not shown). Thus, neither OAG or 1,2-didecanoylglycerol stimulated platelet phospholipase A_2 activity.

In marked contrast, thrombin (1 U/ml), which is a potent stimulant of phospholipases C and A_2 in platelets (1), caused significant formation of [^{3}H]inositol phosphates (Table 1), [^{32}P]phosphatidic acid (Fig. 2), [^{14}C]arachidonic acid metabolites (Table 2) and lysophosphatidyl[^{3}H]inositol (not shown).

DISCUSSION

Dawson *et al.* (5) and Hofmann and Majerus (6) showed that in cell-free systems various 1,2-diacylglycerols potentiate the action of a number of phospholipases. It was suggested that this effect may result from 1,2-diacylglycerol disruption of normal bilayer structure. This mechanism was supported by the observation that 1,2-diacylglycerol does not potentiate phospholipase attack of phosphatidylethanolamine which assumes a hexagonal II structure rather than a bilayer (5).

The present study has shown, however, that neither OAG or 1,2-didecanoylglycerol potentiate phospholipases C or A_2 in human platelets

when administered in concentrations sufficient to produce maximal phosphorylation of a 40,000 dalton protein. In contrast, Dawson *et al.* (5) observed that 1,2-dioleoylglycerol and 1,2-diacylglycerol, prepared from egg phosphatidylcholine, potentiated the hydrolysis of phosphatidylinositol by human platelet phospholipase C in a cell-free system. It is possible that higher concentrations of 1,2-diacylglycerols than used in this study may potentiate phospholipases in platelets. However, this action would appear to be of little physiological significance because the concentrations of 1,2-diacylglycerol required to produce maximal phosphorylation of the 40,000 dalton protein are only reached under normal circumstances by exposure to relatively high concentrations of inositol phospholipid mobilizing agents. Yet, the present study clearly demonstrates that 1,2-didecanoylglycerol, at a concentration five times greater than that needed to produce maximal phosphorylation of the 40,000 dalton protein, does not potentiate phospholipases C and A_2. The possibility remains that other 1,2-diacylglycerols than those employed here may be more effective.

In conclusion, the present study has failed to find support for the argument that the ability of 1,2-diacylglycerol to potentiate phospholipases *in vitro* may be of importance *in vivo* using the human platelet as a test system.

ACKNOWLEDGMENTS

The careful technical assistance of Bryan Reep is gratefully acknowledged, and we thank Tonya Hales for her expert typing preparation of this manuscript.

REFERENCES

1. Lapetina, E.G., and Siess, W. (1983) *Life Sci.* **33**, 1011-1018.
2. Rittenhouse-Simmons, S. (1979) *J. Clin. Invest.* **63**, 580-587.
3. Nishizuka, Y. (1983) *Phil. Trans. R. Soc. Lond. B* **302**, 101-112.
4. Rink, T.J., Sanchez, A., and Hallam, T.J. (1983) *Nature* **305**, 317-319.
5. Dawson, R.M.C., Hemington, N.L., and Irvine, R.F. (1983) *Biochem. Biophys. Res. Commun.* **117**, 196-201.
6. Hofmann, S.L., and Majerus, P.W. (1982) *J. Biol. Chem.* **257**, 14359-14364.
7. Lapetina, E.G., and Siegel, F.L. (1983) *J. Biol. Chem.* **258**, 7241-7244.
8. Berridge, M.J., Downes, C.P., and Hanley, M.R. (1982) *Biochem. J.* **206**, 587-595.
9. Billah, M.M., and Lapetina, E.G. (1982) *J. Biol. Chem.* **257**, 12705-12708.
10. Lapetina, E.G., and Cuatrecasas, P. (1979) *Biochim. Biophys. Acta* **573**, 394-402.
11. Broekman, M.J., Ward, J.W., and Marcus, A.J. (1981) *J. Biol. Chem.* **256**, 8271-8274.