Glucosphingolipid Dependence of Hormone-stimulated Inositol Trisphosphate Formation*

(Received for publication, April 3, 1990)

James A. Shayman‡, Shahybar Mahdiyoun‡, Issayri Rehmukh‡, Florence Barcleoni, Jin-Ichi Inokuchi*, and Norman S. Radin†**

From the Department of Internal Medicine and the [Mental Health Research Institute, University of Michigan Medical Center, Ann Arbor, Michigan 48109-0676 and the Faculty of Pharmaceutical Sciences, Fukuoka University, Fukuoka, Japan]

The modulatory role of endogenous cellular glucosphingolipids in bradykinin-stimulated InsP₃ formation by MDCK cells was evaluated utilizing the glucosylceramide synthase inhibitor, threo-1-phenyl-2-decanoylamino-3-morpholino-1-propanol (PDMP). Bradykinin-stimulated InsP₃ formation in intact cells and in isolated plasma membranes was significantly enhanced when cells were first depleted of their glucosphingolipids. The effect of glucosphingolipid depletion on phospholipase C activity was dependent on the duration of exposure to the inhibitor and the cellular level of glucosylceramide. Inclusion of glucosylceramide in the culture medium prevented the stimulatory effect of PDMP on InsP₃ formation. It is concluded that membrane glucosphingolipids may regulate phospholipase C activity.

Glycosphingolipids are ubiquitous and structurally diverse compounds. Glucosylceramide is a fundamental glucosphin-golipid made from ceramide and UDP-glucose by a glucosyltransferase. It has been stated that there are at least 300 glycosphingolipids, including gangliosides, most of them formed from glucosylceramide by the attachment of additional sugars and sulfate. Known properties of these lipids include binding to viruses (1), toxins (2, 3), bacteria (4), and matrix proteins (5), the regulation of ionic transport (6), and immunoprotection (7). Additionally, these compounds have been implicated in a variety of cellular growth and differentiation phenomena (8) including cancer (9). Tyrosine kinase activities stimulated by platelet-derived growth factor and epidermal growth factor have been shown to be modulated by structurally distinct gangliosides (10). Protein kinase C, like tyrosine kinases, has recently been shown to be modulated by the addition to cell cultures of sphingolipids, particularly the free base, sphingosine (11). It remains to be seen whether endogenous sphingolipids exert similar effects. Methods for evaluating whether endogenous sphingolipids are capable of modifying receptor-stimulated generation of second messengers have previously been limited. The development of a specific glucosylceramide synthase inhibitor, PDMP, provides a tool for decreasing cellular glucosphingolipid content (12). This paper describes the use of PDMP to study the role of glucosphingolipids in hormone-stimulated formation of InsP₃.

EXPERIMENTAL PROCEDURES

Materials—PDMP was synthesized as previously described (12). [3H]Galactose (30 Ci/mmol), [3H]palmitate (52 Ci/mmol), and myo-[2-3H]inositol 1,4,5-trisphosphate (45 Ci/mmol) were from Amersham Corp. Glucosylceramide was isolated from the spleen of a patient with Gaucher disease (13). Galactosylceramide from bovine brain was obtained from Serday (Port Huron, MI). Madin-Darby canine kidney (MDCK) cells were supplied by the American Type Culture Collection (Rockville, MD). On the basis of glycolipid composition (14) and the absence of dome formation in cultures, these appear to be strain I. GT-1 S was from Boehringer Mannheim, and ganglioside standards were from Matreya, Inc. (Pleasant Gap, PA).

Glycosphingolipid Extraction and Isolation—MDCK cells (0.5 × 10⁶) were split at a ratio of 1:10 and grown in 75-cm² flasks in defined (serum-free) medium according to the formulation of Taub et al. (15). [3H]Galactose (5 μCi/ml) or [3H]palmitate (5 μCi/ml) with or without 20 μM PDMP was added 24 h after plating. After another 24 h, the cells were scraped off the flask with a rubber policeman and extracted three times with 3 ml of chloroform:methanol (1:2). The pooled extracts were evaporated to dryness. The residues were suspended in 1 ml of chloroform:methanol:water (30:60:8) and applied to a DEAE-Sephadex column (acetate form, equilibrated with chloroform: methanol:water 40:60:8). Neutral lipids were eluted with 30 ml of the same solvent, and acidic lipids were eluted with 30 ml of chloroform, methanol, 0.3 M sodium acetate (30:60:8). The eluates were evaporated under nitrogen and subjected to alkaline methanolysis by adding 3 ml of 0.5 N NaOH in methanol and heating for 60 min at 40 °C. The samples were dialyzed against tap water at 4 °C for 24 h and dried in vacuo. The neutral lipid fraction was then suspended in 1 ml of chloroform:methanol (98:2) and subjected to silicic acid chromatography with 0.5 g of Unisil (200–325 mesh, Clarkson Chemical, Williamsport, PA). Ceramides were eluted with 35 ml of chloroform:methanol (98:2), cerebrosides were eluted with 35 ml of chloroform:methanol (90:10), and sphingomyelin was eluted with 35 ml of chloroform:methanol (90:50:5). The acidic lipid fraction from the DEAE-Sephadex column was also subjected to alkaline methanolysis and dialysis.

Thin Layer Chromatography—Samples were analyzed by high performance thin layer chromatography (HPTLC plates from E. Merck, Darmstadt). Galactosyl- and glucosylceramides were separated on plates which were pretreated with 2.5% borax in methanol/water (1:1) and air-dried. The solvent system consisted of chloroform:methanol:water (65:25:4). The acidic glycosphingolipids were separated with chloroform, methanol, 0.2% CaCl₂ (60:40:9). Ceramide (isolated from [3H]palmitate-labeled cells) was developed with hexane:chloroform (1:1) followed by chloroform:methanol:acetic acid (91:2:3). Sphingomyelin was detected with 0.5 g of Unisil (200–325 mesh) and air-dried. The solvent system utilized was chloroform:methanol:water (60:35:8). Plates were exposed for 96 h to Kodak X-Omat AR film following treatment with ENHANCE spray. Charring was done with the copper sulfate-phosphoric acid reagent (17).

Plasma Membrane Preparation—Crude plasma membranes were prepared by the method of Roth, 1976.

* This work was supported by National Institutes of Health Grants KO8 DK0184 (to J. A. S.) and NS03192-31 (to N. S. R.) and by a Merit Review Research Award from the Veterans Administration (to J. A. S.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ To whom correspondence should be addressed.

** Jacob Javits Investigator Awardee.
isolated following exposure to hypotonic buffer by the protocol of Hepler and Harden (18). Prior to the exposure to agonist, the membranes were pelleted at 12,000 x g. Protein was determined utilizing the fluorescein reagent (19).

InsP<sub>3</sub> Formation—Hormone-stimulated InsP<sub>3</sub> formation was measured in intact MDCK cells as previously detailed (20). For these studies, membranes were suspended in a buffer consisting of 10 mM Hepes, 2 mM EGTA, 424 mM CaCl<sub>2</sub>, 0.91 mM MgSO<sub>4</sub>, 115 mM KCl, and 5 mM KH<sub>2</sub>PO<sub>4</sub>, pH 7.0. Thereafter additions consisting of the same buffer, containing 1.0 mM MgSO<sub>4</sub>, 1.0 mM GTPγS, 0.1 mM bradykinin, or GTPγS and bradykinin together, were made. After 10 min 6% trichloracetic acid was added, and the resultant precipitate was sedimented by centrifugation at 5000 x g. The supernatant was treated with triclyethylamine-Freon to extract the acid. InsP<sub>3</sub> formation was measured by a competitive binding assay utilizing high specific activity <sup>3</sup>H-labeled InsP<sub>3</sub> (21). The formation of InsP<sub>3</sub> in these cells has been shown to be regulated by a guanine nucleotide regulatory protein (22). The formation of InsP<sub>3</sub> in these experiments was measured utilizing membrane preparations from MDCK cells (20).

RESULTS AND DISCUSSION

Madin-Darby canine kidney cells generate InsP<sub>3</sub> and an increase in intracellular calcium in response to bradykinin (22). The formation of InsP<sub>3</sub> in these cells has been shown to be regulated by a guanine nucleotide regulatory protein (22). These cells can be grown in defined (serum-free) media, and the formation of InsP<sub>3</sub> was measured in intact MDCK cells as previously described (22). Prior to the exposure to agonist, the membranes were pelleted at 12,000 x g. Protein was determined utilizing the fluorescein reagent (19). InsP<sub>3</sub> formation was measured by a competitive binding assay utilizing high specific activity <sup>3</sup>H-labeled InsP<sub>3</sub> (21). The formation of InsP<sub>3</sub> in these cells has been shown to be regulated by a guanine nucleotide regulatory protein (22). These cells can be grown in defined (serum-free) media, and the formation of InsP<sub>3</sub> was measured in intact MDCK cells as previously described (22).

When cells were stimulated with 10<sup>-7</sup> M bradykinin, maximal InsP<sub>3</sub> formation was observed at 15 s (data not shown). Cells which had been exposed to 20 μM PDMP for 24 h and then stimulated with 10<sup>-7</sup> M bradykinin also showed maximal production at 15 s. Multiple determinations of InsP<sub>3</sub> accumulation 15 s after stimulation demonstrated a statistically significant increase in the product by the glucosphingolipid-depleted cells (Fig. 3).

The stimulatory effects of glucosphingolipid depletion were further assessed in membranes isolated from MDCK cells following 24 h of exposure of the cells to 20 μM PDMP. The membranes were incubated for 10 min with buffer, 10<sup>-6</sup> M
GTP-γS, 10^{-7} M bradykinin, or GTP-γS and bradykinin together. The formation of InsP_3 was stimulated in the control membranes by both agonists, particularly the combination of the two (Table II). The stimulatory effects of GTP-γS and bradykinin were enhanced in glucosphingolipid-depleted membranes. This observation is consistent with the interpretation that enhanced InsP_3 levels are due to an increase in phospholipase C activity and not simply the result of impaired metabolism of InsP_3.

This effect was not the result of enhanced phosphatidylinositol 4,5-bisphosphate formation since levels of this lipid were unchanged in control and glucosphingolipid-depleted cells. For these experiments MDCK cells were grown in the presence of 20 μM PDMP for 24 h. Phospholipids were extracted by the addition of chloroform, methanol, 1 HCl (1:2:1) to scraped cells. The aqueous phase was extracted with chloroform:methanol (2:1), and the lower phases were pooled and evaporated under nitrogen. Phosphatidylinositol 4,5-bisphosphate was separated by HPTLC (23) and quantitated by phosphate analysis (24). Under these conditions control levels were 1.42 ± 0.16 nmol/mg protein; PDMP cells contained 3.57 ± 0.18 nmol/mg protein (n = 10, p = 0.57 by the unpaired t test).

The enhanced formation of InsP_3 was dependent on the duration of exposure of the cells to the glucosylceramide synthase inhibitor. Increased formation of hormone-stimulated InsP_3 paralleled changes in glucosylceramide mass (Fig. 4). The close temporal association between decreased glucosylceramide and increased InsP_3 suggests that glucosylceramide itself or a closely related sphingolipid mediates the observed changes. Finally, addition of glucosylceramide to the culture medium prevented the stimulatory effect of glucolipid depletion (Table III). Galactosylceramide, which is also absorbed by the cells and hydrolyzed to ceramide, fatty acid, and sphingosine, did not block the stimulatory action of PDMP. This experiment confirms the assumption that the PDMP effect on phospholipase C is due to a cellular deficiency in glucosylceramide.

In many tissues the concentration of phospholipase C is sufficiently high, if assayed under ideal conditions in vitro, to cause the rapid hydrolysis of the entire pool of cellular inositol lipids within seconds. On this basis it has been postulated that there exists an endogenous negative regulator of phospholipase C (25). Our experiments support the hypothesis that glucosphingolipids may serve as such regulators. Specifically, the findings suggest that cellular glucosphingolipids, when present at normal levels in MDCK cells, regulate the hormone-stimulated formation of InsP_3.

### Table II

**InsP_3 content in plasma membranes**

InsP_3 content (pmol/mg protein) was measured in plasma membranes isolated from MDCK cells that had been incubated under control conditions or in contact with 20 μM PDMP for 24 h. Significance was determined by ANOVA using repeated measures. Parentheses indicate the percent change from control "no addition" values.

| Cells                | Exp. 1 | Exp. 2 | Exp. 3 | Exp. 4 |
|----------------------|--------|--------|--------|--------|
| Control              | 3.57   | 5.00   | 8.42   | 2.17   |
| No addition          | 4.57 (28) | 11.2 (124) | 10.7 (27) | 9.09 (319) |
| Bradykinin           | 4.66 (31) | 13.5 (170) | 16.3 (94)  | 11.3 (421) |
| GTP-γS + bradykinin  | 5.92 (66) | 17.2 (244) | 17.4 (107) | 17.4 (762) |

* p < 0.05 versus parallel control conditions.

### Table III

**Prevention of PDMP effect by glucosylceramide**

InsP_3 content (pmol/mg protein) was measured in MDCK cells treated with D-PDMP as described in Table II. The cells were simultaneously incubated for 24 h with PDMP and liposomes made from either phosphatidylycholine alone (lines 1 and 2) or from phosphatidylycholine plus glucosyl- or galactosylceramide. Cerebroside incorporation was documented with [3H]glucosyl- and [3H]galactosylceramide containing liposomes in parallel incubations under identical conditions. The liposomes consisted of egg phosphatidylcholine (3 mg) and cerebroside (1 mg) in 1 ml of 20 mM Tris-Cl, pH 7.4. Sufficient liposomes were added to produce a final concentration of 1 μM cerebroside. Plasma membranes were then isolated and InsP_3 formation determined as in Table II. The data are expressed as pmol of InsP_3/mg of protein and represent the mean ± S.E. of six individual determinations.

| Incubation conditions | No addition | GTP-γS + bradykinin |
|-----------------------|-------------|---------------------|
| Control medium        | 2.6 ± 0.96  | 7.8 ± 0.28          |
| PDMP                  | 10.2 ± 0.1  | 22.0 ± 4.2          |
| PDMP + glucosylceramide| 3.6 ± 1.11  | 10.3 ± 1.27         |
| PDMP + galactosylceramide| 10.4 ± 1.2  | 22.3 ± 3.8          |
Several potential mechanisms may explain the enhanced generation of InsP₃ under glucosphingolipid-depleted conditions. First, alterations in the plasma membrane sphingolipid content may directly affect the activity of phospholipase C. The activity of phospholipase C in hydrolyzing phosphatidylcholine in artificial membranes, for example, was observed to be dependent on the content of ceramide (26). Second, enhanced phospholipase C activity may be the indirect result of inhibition of protein kinase C. The activation of protein kinase C has recently been shown to produce a decrease in phospholipase C activity in several cell types (27). Free sphingosine and N-deacylated glycolipids inhibit protein kinase C and may accumulate under conditions of glucosylceramide synthesis inhibition. Third, the γ-isoenzyme of phospholipase C is a substrate for epidermal growth factor- and platelet-derived growth factor-mediated tyrosine kinase activity (28). These kinase activities have been demonstrated to be modulated by the exogenous addition of a variety of sphingolipids (10). Thus the observed changes in phospholipase C activity may be secondary to the changes in tyrosine kinase activity.

In summary, these data demonstrate the modulation of hormone-stimulated InsP₃ formation by endogenous glycosphingolipids. Although the mechanisms whereby glucosylceramide levels regulate bradykinin-stimulated phospholipase C activity remain to be elucidated, glycosphingolipid metabolism appears to play an important role in the regulation of InsP₃ generation.

REFERENCES
1. Bock, K., Karlsson, K. A., Stromberg, N., and Teneberg, S. (1988) Adv. Exp. Med. Biol. 228, 153-186
2. Spiegel, S., Fishman, P. H., and Weber, R. J. (1985) Science 230, 1285-1287
3. Lingwood, C. A., Law, H., Richardson, S., Petric, M., Brunton, J. L., DeCrandos, S., and Karmali, M. (1987) J. Biol. Chem. 262, 8834-8839
4. Karlsson, K. A. (1986) Chem. Phys. Lipids 42, 153-172
5. Roberts, D. D., and Ginsburg, V. (1966) Arch. Biochem. Biophys. 67, 405-415
6. Spiegel, S., Handler, J. S., and Fishman, P. H. (1986) J. Biol. Chem. 261, 15755-15760
7. Ladish, S., Kitada, S., and Hays, E. F. (1987) J. Clin. Invest. 79, 1879-1882
8. Hakomori, S. (1984) Am. J. Clin. Pathol. 82, 635-648
9. Radin, N. S., and Inokuchi, J. (1988) Biochem. Pharmacol. 37, 2879-2886
10. Bremer, E. G., Schlessinger, J., and Hakomori, S. (1986) J. Biol. Chem. 261, 2434-2440
11. Hannun, Y. A., and Bell, R. M. (1987) Science 235, 670-674
12. Inokuchi, J., and Radin, N. S. (1987) J. Lipid Res. 28, 598-571
13. Radin, N. S. (1976) J. Lipid Res. 17, 290-293
14. Hansson, G. C., Simons, K., and van Meer, G. (1986) EMBO J. 5, 483-489
15. Taub, M., Chuman, L., Saier, M. H., Jr., and Sato, G. (1979) Proc. Natl. Acad. Sci. U. S. A. 76, 3338-3342
16. Lasset, R. W., and Yu, R. K. (1982) Methods Enzymol. 83, 139-191
17. Touchstone, J. C., Levin, S. S., Dobbins, M. F., Matthews, L., Beore, P. C., and Gabbe, S. G. (1983) Clin. Chem. 29, 1951-1964
18. Hepler, J. R., and Harden, T. K. (1986) Biochem. J. 239, 141-146
19. Bohen, P., Stein, S., Dairman, W., and Udenfriend, S. (1973) Arch. Biochem. Biophys. 155, 213-220
20. Shyams, J. A., and Wu, D. (1966) Adv. Physiol. 258, F1287
21. Palmer, S., Hughes, K. T., Lee, D. Y., and Wakelam, M. J. O. (1989) Cell. Signalling 1, 147-156
22. Portilla, D., Morrissey, J., and Morrison, A. R. (1988) J. Clin. Invest. 81, 1896-1902
23. Jolles, J., Zwiers, H., Dekker, A., Witte, K. W. A., and Gispen, W. H. (1981) Biochem. J. 194, 283-291
24. Vaukovskyy, V. E., Kostetsky, E. Y., and Vassendin, I. M. (1975) J. Chromatogr. 114, 129-141
25. Rhee, S. G., Suh, P. G., Rya, S. H., and Lee, S. Y. (1989) Science 244, 544-550
26. Deruel, R. A., Geurts Van Kessel, W. S. M., Zwaal, R. F. A., Roelofsen, B., and Van Deenen, L. L. M. (1975) Biochim. Biophys. Acta 406, 97-107
27. Labarca, R., Janowsky, A., Patel, J., and Paul, S. M. (1984) Biochem. Biophys. Res. Commun. 123, 703-709
28. Wahl, M. L., Daniel, T. O., and Carpenter, G. (1988) Science 241, 968-970
29. McMaster, M. C., Jr., and Radin, N. S. (1977) J. Labelled Compd. & Radiopharm. 13, 353-357
30. Hajra, A. K., Bowen, D. M., Kishimoto, Y., and Radin, N. S. (1966) J. Lipid Res. 7, 379-386