Modulation of Renal Epithelial Cell Growth by Glucosylceramide

ASSOCIATION WITH PROTEIN KINASE C, SPHINGOSINE, AND DIACYLGlycerol.*

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Two independent approaches were employed to explore the potential role of endogenous glucosylceramide or a closely related glucosphinolipid in mediating the cellular proliferation of Madin-Darby canine kidney cells. First, cultured cells were depleted of glucosphinolipids by exposure to a glucosylceramide synthase inhibitor, D-threo-1-phenyl-2-decanoylamino-3-morpholino-1-propanol. This agent markedly inhibited cell growth and DNA synthesis in a time- and concentration-dependent manner. Second, cells were grown in the presence of conduritol B epoxide, an inhibitor of glucosylceramide β-g-glucosidase. Exposure of cells to this inhibitor resulted in the time-dependent accumulation of glucosylceramide with a corresponding increase in cellular proliferation.

Alterations in protein kinase C activity were evaluated as a potential mechanism for these effects on growth. Both membrane- and cytosol-associated protein kinase C (PKC) activity declined under conditions of glucosylceramide synthase inhibition and increased under conditions of β-glucosidase inhibition. The changes in PKC activity were evident after DEAE-cellulose purification. Diacylglycerol levels increased in response to both glucosylceramide synthase and β-glucosidase inhibition. Ceramide and sphingosine levels changed only in the presence of D-threo-1-phenyl-2-decanoylamino-3-morpholino-1-propanol, increasing due to lack of conversion to glucosylceramide. However, the elevation in endogenous sphingosine was probably insufficient to account for the decrease in PKC, considering the high level of diacylglycerol in the cells. These data demonstrate an association between glucosylceramide levels, PKC activity, and cell growth.

Glycosphingolipids (GSLs)* are ubiquitous cellular mem-

brane components composed of a sphingol (a long-chain sphingoid amine), a fatty acid, and a carbohydrate. The hydrophobic moiety, ceramide, consists of the sphingol substituted at the amino group by a fatty acid in amide linkage. The carbohydrate moiety is linked to the primary hydroxyl group of the sphingol in glycosidic linkage. The simplest glucolipid, GlcCer, serves as the base for ~300 known GSLs by virtue of specific enzymes that attach additional sugars or sulfate or acetate residues. The GSLs containing neuraminic acid constitute the ganglioside family. GlcCer is made from ceramide by a glucosyltransferase (EC 2.4.1.80).

Galactosphingolipids comprise the second major class of mammalian glycolipids and are characterized by the attachment of a galactose to the C-1 of the ceramide. They occur primarily in brain, but a significant amount is found also in whole kidney (1).

Glycolipids, including GlcCer, have been shown to induce proliferation in a variety of cells. The proliferative effect has been observed in Gaucher disease, where impaired GlcCer glucosidase activity results in the accumulation of GlcCer in several tissues (2). The afflicted individuals suffer from hepatic and splenic enlargement due, in part, to cellular hyperplasia. Some of these individuals also suffer from disorders of B lymphocyte proliferation (3). In addition, injection of emulsified GlcCer into mice has been shown to induce hepatic growth (4). A model version of Gaucher's disease was induced in mice by treatment with an inhibitor of GlcCer β-glucosi-

dase, CBE, over a period of time; this led to increased growth of liver and brain (5). Other GSLs have been shown to promote proliferation and differentiation in a variety of cell types (6). Despite numerous publications describing such effects, the mechanisms responsible remain unknown.

Conversely, reduction of the level of GlcCer by a ceramide glucosyltransferase inhibitor, PDMP, has been shown to reduce cell growth in cultures (7), in the kidneys of intact mice (8), and in ascites cancer cells (9) and to block the proliferative response of lymphocytes to mitogens (10). (For a review of PDMP effects see Ref. 11.) The expression of GlcCer or a related metabolite is therefore implicated in normal and pathological cellular proliferation.

Previous studies on the growth-promoting effects of GSLs have commonly relied on the exogenous addition of these compounds to cells in culture or to whole animals to induce their effects. Such additions could act in an artifactual way by, for example, displacing a different lipid from its normal membrane site or a protein complex or by simply binding to a protein that normally does not bind to this lipid. In the present study, utilizing agents that specifically inhibit GlcCer formation or degradation, we investigated the association between endogenous GlcCer content and cellular growth and the potential role of PKC in mediating these effects. We
report evidence that the growth of MDCK cells is modulated by changes in GlcCer levels. Furthermore, the changes in cellular proliferation are associated with alterations in PKC activity.

**Experimental Procedures**

**Materials**—PDMP (12) and the glucosidase inhibitor CBE (13) were synthesized as previously described. [6-3H]Galactose (40 Ci/mmol), [9,10-3H]palmitic acid (40 or 60 Ci/mmol), and [methyl-3H]thymidine were from Amersham. [γ-32P]ATP was from ICN Biomedicals. GlcCer was isolated from the spleen of a patient with Gaucher disease (14). Galactosylceramide from bovine brain was obtained from Serdary (Port Huron, MI). MDCK cells were supplied by the American Type Culture Collection (Rockville, MD). Ganglioside standards were from Matreya, Inc. (Pleasant Gap, PA). Phorbol esters were from Fluka and Sigma.

Because serum contains sphingolipids in significant and variable quantities, the cells were maintained in serum-free media (15). For GSL depletion studies, cells were plated at a density of 5 x 10^6 cells per dish unless otherwise indicated and grown in the presence or absence of GSL metabolic inhibitors. The inhibitors were used at concentrations that do not produce cellular toxicity, as assessed by trypan blue exclusion.

**Glucolipid Analysis**—For fluororadiography of neutral and acidic GSLs, MDCK cells were metabolically labeled with [3H]galactose and [3H]palmitate. [3H]Galactose or [3H]palmitate (5 μCi/ml) was added in the presence or absence of 20 μM PDMP or CBE 24 h after plating. After 48 h of exposure to inhibitor, the cells were extracted three times with chloroform:methanol (1:2). The pooled extracts were evaporated to dryness and redissolved in chloroform:methanol:water (60:30:8) and applied to a DEAE-Sephadex column (acetate form) that had been equilibrated with chloroform:methanol:water (65:30:5) (16). Neutral lipids were eluted with the same solvent and acidic lipids were eluted with chloroform:methanol:0.3 M NaOAc (60:30:5). The solvents were evaporated under nitrogen and the lipids were subjected to silica gel chromatography. Ceramides were eluted with chloroform:methanol:water (80:20), and sphingomyelin was eluted with chloroform:methanol:water (50:50:5).

The acidic fraction was subjected to alkaline methanolysis for 1 h at 40 °C utilizing 0.5 N NaOH in methanol. This fraction was then dialyzed for 24 h against tap water and dried in vacuo. The neutral lipids were chromatographed on high performance thin layer chromatography plates with a sequential solvent system consisting of hexane:chloroform (1:1) and chloroform:methanol:HOAc (91:2:3) to separate ceramide. Sphingomyelin was separated utilizing chloroform:methanol:water (65:35:8). Galactosylceramide and GlcCer were separated with chloroform:methanol:water (65:25:4) on borate-imregnated plates. Acidic GSLs were separated with chloroform:methanol:0.2% CaCl₂ (60:40:9). Plates were exposed for 96 h to Kodak K-Omat AR film following treatment with EN'HANCE spray. Individual lipids were counted by scraping the localized spots and suspending the powder in scintillation fluid.

**Sphingolipid Analysis**—Cellular sphingolipids were quantified fluorometrically by derivatization of extracted sphingolipids following the method of Merrill et al. (17). Under this protocol, total lipids were extracted from the sample with chloroform:methanol, and acylcarnitides were cleaved by alkaline methanolysis. At the time of extraction 1 mmol of tetradecylamine (Aldrich) was added as an internal standard. The crude sphingolipid preparation was dried under nitrogen, dissolved in methanol, and derivatized with 50 μl of p-iodobenzaldehyde reagent (Sigma). Contrary to the original report of this assay (17), we observed that derivatization was complete only by 60 and not 5 min at 25 °C. Reversed-phase HPLC separation utilized a 5-μm, 25-cm x 4.6-mm C₁₈ column (Supelco) and a mobile phase of methanol:5 mM phosphoric acid (70:30). Quantitation was performed by on-line fluorometry with a Kratos 955 filter monitor.

**Ceramide and Diglyceride Analysis**—Diacylglycerol was extracted from MDCK cells using 3 ml of chloroform:methanol (1:2) and purified by thin layer chromatography. Total cellular diglyceride and ceramide were assayed by the method of Kennerly (18) as refined by Preiss and coworkers (19) utilizing partially purified diglyceride kinase from Escherichia coli (19). This assay is based on the formation of [32P]phosphatidic acid from endogenous diacylglycerol or [32P]ceramide phosphate from ceramide.

**Protein Kinase C Activity Measurements**—PKC measurements followed the protocol of Thomas et al. (20). MDCK cells were grown in 150-cm² flasks as described above and exposed to phorbol esters. Cells were washed with calcium- and magnesium-free phosphate-buffered saline and then washed twice with a buffer consisting of Tris-Cl (20 mM, pH 7.5), EDTA (2 mM), EGTA (0.5 mM), phenylmethylsulfonyl fluoride (2 mM), leupeptin (25 mg/ml), and sucrose (0.33 M). The cell suspension was homogenized in a Dounce homogenizer, and the homogenate was centrifuged at 100,000 g for 1 h. The soluble fraction was retained, and the membrane pellet was washed with the same buffer without phenylmethylsulfonyl fluoride and leupeptin. The membrane pellet was solubilized with 0.1% Triton X-100 at 4 °C for 30 min. This was then centrifuged to obtain the detergent-solubilized membrane fraction.

Cytosolic and membrane fractions were subjected to DEAE-cellulose (DE-52) chromatography in order to remove interfering materials and excess detergent (20). PKC activity was measured by the transfer of [32P] from [γ-32P]ATP to histone (Type III-S, Sigma) in 20 mM Tris-Cl (pH 7.5), 0.75 mM CaCl₂, 10 mM Mg(OAc)₂, 100 mM [γ-32P] ATP (120 cpm/pmol), 25 μg of histone, 24 μg of phosphatidylserine, and 1.6 μg of 1,2-diolein in a final volume of 250 μl. PKC activity was calculated from the difference in the amount of [32P] incorporation into histone in the presence and absence of lipid in the incubation mixture.

**Cellular Proliferation**—This was measured as the change in cellular protein (21). DNA (22), or most often as the amount of [3H]thymidine incorporation into trichloroacetic acid precipitable material. Cells were plated at a density of 0.5 x 10^6 per dish, allowed to grow for 24 h, and then exposed to inhibitors at the intervals and concentrations indicated below. [3H]Thymidine (5 μCi/ml) was added 4 h prior to the termination of the experiment. At the end of this period, the medium was removed, the cells were washed four times with phosphate-buffered saline, and 1.4 ml of 10% trichloroacetic acid was added to the dishes. The cellular material was scraped from the culture dish, transferred to a microcentrifuge tube, and centrifuged at 2000 x g. The pellet was dissolved in 1 ml of 0.2 N NaOH and aliquots were taken for scintillation counting and protein determination.

**RESULTS**

MDCK cell glucolipids were compared after exposing cells for 48 h to the GlcCer synthase inhibitor, PDMP. Under this condition, significant, time-dependent reduction in the level of GlcCer synthesis was observed as assessed by both radio-labeling with [3H]galactose and [3H]palmitate (Table I). Assuming normal metabolism of cell components, one can interpret a decrease in radioactivity as a decrease in mass. The level of the GlcCer anabolite, ganglioside G₂, (the major ganglioside in these cells), declined as well. No change was observed in the levels of galactosylceramide, confirming the now well-established specificity of the inhibitor for GlcCer.

**Table I**

| Lipid-counted | Control Cells | PDMP Cells |
|---------------|--------------|------------|
| Glucosylceramide | 2.91 ± 0.35 | 0.73 ± 0.17* |
| Galactosylceramide | 0.74 ± 0.11 | 0.74 ± 0.12 |
| Ganglioside G₂ | 8.85 ± 0.35 | 6.66 ± 0.34* |
| Ceramide | 7.9 ± 0.8 | 10.1 ± 0.8* |
| Sphingomyelin | 36.7 ± 6.3 | 64.7 ± 7.9* |

* Denotes p < 0.02 by the paired t test.
Glucosylceramide, Ceramide, and MDCK Cell Growth

Ceramide levels, as assessed by incorporation of \[^\text{[H]}\text{palmitate, were elevated. This change is apparently due to blockage of the conversion of ceramide to GlcCer; some of the accumulated ceramide was evidently converted to sphingomyelin (Table I).}

PDMP treatment produced impaired growth of MDCK cells, manifested as a time-dependent decrease in cell number, DNA, and protein (Fig. 1, A–C). 48 h after PDMP addition, cell number, DNA, and protein per dish were 59, 73, and 65% of control values. The decrease in cell number was only evident by 48 h, whereas the fall in cell protein and DNA content was apparent by 24 h. Comparable changes were observed in \[^\text{[H]}\text{thymidine incorporation (Fig. 2). After 48 h, radiolabel incorporation was 67% of control values. These data indicate that \[^\text{[H]}\text{thymidine incorporation is an appropriate marker of cellular proliferation in this system. The antiproliferative effects of PDMP were also concentration-dependent (Fig. 3), the half-maximal effect at 24 h appearing with 15 \mu M PDMP.}

In a second series of experiments, the glucosidase inhibitor CBE was used to evaluate the effect of increasing endogenous cellular GlcCer. Exposure of MDCK cells to CBE resulted in time-dependent accumulation of GlcCer as assessed by incubation with labeled sugar (Table II). No changes were observed in radiolabeling of galactosylceramide or ganglioside

**Table I**

| Sphingolipid   | Control cells | CBE cells |
|----------------|---------------|-----------|
| Glucosylceramide | 2.79 ± 0.35   | 6.58 ± 1.98* |
| Galactosylceramide | 0.74 ± 0.11   | 0.97 ± 0.26   |
| Ganglioside GM1 | 6.04 ± 0.28   | 6.04 ± 1.28   |
| Ceramide         | 25.5 ± 0.76   | 31.4 ± 0.56*  |
| Sphingomyelin    | 175 ± 4.5     | 214 ± 9.1*    |

* Denotes \( p < 0.02 \) by the paired \( t \) test.
GM:3. Evidently the rate of GlcCer galactosylation and sialylation was not enhanced by the increase in GlcCer level although the use of a different inhibitor of β-glucosidase in human fibroblasts, over a longer period, produced accumulation of a related anabolite, globoside, as well as enhanced cell growth (23). CBE treatment resulted in a modest increase in [3H]thymidine incorporation (Fig. 2). The stimulation in growth was not evident until 48 h following exposure to CBE.

Small but significant increases in [3H]palmitate incorporation into sphingomyelin and ceramide were observed as well (Table II). It is not immediately evident why decreased hydrolysis of GlcCer should lead to enhanced synthesis of ceramide, but the resultant enhanced conversion of ceramide to sphingomyelin is to be expected. Perhaps the growth stimulation due to CBE was accompanied by a generalized increase in the synthesis of many cell components.

DNA synthesis in the presence of CBE was enhanced by exposure to the inhibitor for 48 h. Cell protein 24 h after inhibitor addition was 511 ± 21 µg/dish in control cells and 503 ± 44 in CBE-treated cells. Significant differences were observed at 72 h, when the protein content was 1410 ± 56 and 1710 ± 34 µg/dish, respectively (p < 0.001 by the unpaired t test).

The incorporation of [3H]thymidine was dependent on the concentration of CBE (Fig. 4). The half-maximal stimulatory effect was obtained with 18 µM CBE. The magnitude of change in thymidine incorporation was comparable to that reported for MDCK cells stimulated by epidermal growth factor (24).

Because of the observed increases in radiolabeled ceramide under conditions of both GlcCer augmentation and depletion, the chemical levels of ceramide, diglyceride, and sphingosine were measured in cells exposed to either PDMP or CBE for 24 h. PDMP treatment (20 µM) resulted in significant increases in both ceramide (149%) and diglyceride (571%) content (Fig. 5, A and B). CBE treatment did not produce significant changes in diglyceride or ceramide.

The associated changes in cellular sphingosine content were also measured (Fig. 6). GlcCer synthase inhibition resulted in significant, concentration-dependent increases in sphingosine levels, up to 307%, while GlcCer hydrolase inhibition decreased sphingosine, although only 35%. The increase in sphingol level can be attributed to increased hydrolysis by ceramidase, due to accumulation of ceramide.

The time-dependent effect of PKC activation on proliferation was determined by exposing MDCK cells to 100 nM phorbol 12-myristate 13-acetate (Fig. 7). [3H]Thymidine incorporation was unaffected initially but significantly decreased by 18 and 24 h, 96 and 94%, respectively. The time-dependent changes in cytosol- and membrane-associated PKC activities in response to phorbol 12-myristate 13-acetate were also assessed. The phorbol ester induced a rapid translocation of PKC to the membrane fraction. Membrane-associated PKC activity was 550% of control values at 10 min and 225% at 30 min. By 24 h both membrane and cytosolic activities were almost completely absent.

PKC activity was assayed in membrane and cytosolic fractions of MDCK cells treated 24 h with either PDMP or CBE.

**Figure 4.** Concentration-dependent change in [3H]thymidine incorporation in MDCK cells following exposure to CBE. Cells were plated at a split ratio of 1:30 and grown for 72 h in the presence of varying concentrations of CBE. The data represent the mean ± S.E. of three experiments.
activity from MDCK cells exposed to PDMP

...denotes the inhibition of GlcCer synthase...treated with CBE accumulated GlcCer and displayed a marked decrease in both cellular GlcCer content...is a regulator of MDCK cell growth. Two important questions arise from these and previously published reports: one is the question as to whether GlcCer itself or a metabolite, such as lactosylceramide or ceramide, is the responsible mitogen; the other is the question of mechanism: how does the responsible lipid exert its effect?

The data relevant to the first question point to GlcCer itself or to a higher GSL, such as lactosylceramide or ganglioside G_{M_1}, rather than a hydrolysis product. We found that PDMP produced increased levels of the hydrolysis products, ceramide and sphingosine, but the mitogenic action of CBE was not reflected in corresponding decreases in the two sphingolipids. A recent report proposed that lactosylceramide, added exogenously or generated from ganglioside G_{M_1} in the plasma membrane by treatment of cells with sialidase, is responsible for the proliferation of both human fibroblasts and epidermoid carcinoma cells. However, this study could not rule out the hydrolysis product of lactosylceramide (GlcCer) as the active factor. The L-enamehtier of PDMP, when incubated with melanoma (7) and lung carcinoma cells (26), produced a marked increase in lactosylceramide level yet did not induce proliferation.

The second question, which seeks the mechanism of the proliferative relationship, is rendered particularly difficult because there are no clear mechanistic lines of chemical or physical processes that explain cell proliferation, no matter how it is induced. GlcCer, being a lipid, may be presumed to act on the physical properties of cell membranes and thereby to influence a receptor for a growth factor or some other component of the growth-stimulation cascade. A recent study, utilizing a fluorescence photobleaching technique, has shown that depletion of cellular GlcCer by PDMP led to a significant increase in the mobile fraction of lecithin and ganglioside G_{M_1}. The diffusion constants for the two (exogenously added) lipids were unaffected as were the properties of two surface glycoproteins. This may signify that GlcCer in the cell surface promotes the formation of specific lipid domains, i.e., floating islands. A related change, in the distribution of surface G_{M_1}, was induced in melanoma cells by GlcCer depletion with PDMP (7).

Another possibility, that GlcCer binds specifically to a factor in the growth-controlling cascade, is entirely plausible since it is well established that several related GSLs bind specific protein moietyes in viruses, bacteria, and toxins (see reviews in Refs. 28 and 29). Lipid molecules having structures even simpler than GlcCer are strong activators of important enzymes, such as PKC.

Our observations on the effects of PDMP and CBE on important metabolites must be considered. We have recently demonstrated enhanced bradykinin-stimulated phospholipase C activity in MDCK cells depleted of their GSLs by PDMP (30). One of the two hydrolysis products, inositol 1,4,5-trisphosphate, was elevated and we can presume that the calcium content of the depleted cells also increased. The opposite effect has been observed in CBE-treated cells. The other hydrolysis product, diacylglycerol, was found to be substantially increased in GlcCer depleted cells (Fig. 5B). This lipid is well established as an activator of protein kinase C. Our finding that PKC levels decreased in these cells can be attributed to down-regulation by the persistent presence of the diglyceride. Endogenous diglyceride has been suggested to exhibit such an effect in transformed cells (31). Decreased PKC activity was observed for both membrane and cytosolic fractions, a pattern which is most consistent with activation and subsequent down-regulation. Our trials with phorbol ester showed that the PKC of MDCK cells is readily down-regulated. Thus the effect of GlcCer deficiency on levels of phosphatidylinositol bisphosphate and diacylglycerol may act via PKC to supplement the antiproliferative action of GlcCer

**DISCUSSION**

The present study demonstrates that the growth of MDCK cells is associated with cellular GlcCer content. On the one hand, cells exposed to the GlcCer synthase inhibitor, PDMP, displayed a marked decrease in both cellular GlcCer content and proliferation, as determined by cell number, protein, DNA, and [H]thymidine incorporation. On the other hand, cells treated with CBE accumulated GlcCer and displayed enhanced proliferation. These data indicate that cellular GlcCer or a metabolically close product of GlcCer metabolism is a regulator of MDCK cell growth. Two important questions arise from these and previously published reports: one is the question as to whether GlcCer itself or a metabolite, such as lactosylceramide or ceramide, is the responsible mitogen; the other is the question of mechanism: how does the responsible lipid exert its effect?

The data relevant to the first question point to GlcCer itself or to a higher GSL, such as lactosylceramide or ganglioside G_{M_1}, rather than a hydrolysis product. We found that PDMP produced increased levels of the hydrolysis products, ceramide and sphingosine, but the mitogenic action of CBE was not reflected in corresponding decreases in the two sphingolipids. A recent report proposed that lactosylceramide, added exogenously or generated from ganglioside G_{M_1} in the plasma membrane by treatment of cells with sialidase, is responsible for the proliferation of both human fibroblasts and epidermoid carcinoma cells (25). However, this study could not rule out the hydrolysis product of lactosylceramide (GlcCer) as the active factor. The L-enamehtier of PDMP, when incubated with melanoma (7) and lung carcinoma cells (26), produced a marked increase in lactosylceramide level yet did not induce proliferation.

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**FIG. 8.** PKC activity of MDCK cytosol or membrane fractions following exposure to either 20 μM PDMP or CBE for 24 h. The data represent the mean ± S.E. for five determinations. *, denotes p < 0.05 by the paired t test versus control values.

**FIG. 9.** Concentration-dependent changes in cytosolic PKC activity from MDCK cells exposed to PDMP (○) or CBE (□) for 24 h.
deficiency. PKC activity increased in the presence of CBE and decreased in the presence of PDMP in a concentration-dependent manner, implicating the level of GlcCer in the control of PKC.

The accumulation of ceramide that we observed in PDMP-treated cells (Fig. 5A) probably also acted to elevate the level of diacylglycerol. This can occur via the phosphorylcholine transferase that converts lecithin to diacylglycerol and ceramide to sphingomyelin. Some diglyceride may also be formed via an analogous pathway involving ceramide and phosphatidylethanolamine, followed by methylation (32). Enhanced turnover of sphingomyelin was evident in the GlcCer-depleted cells (Tables I and II). This interpretation is supported by analysis of the fatty acids in the free diglyceride and sphingolipids of MDCK cells before and after exposure to PDMP; lecithin was clearly the major diglyceride source.4 Although this pathway has been suggested previously as being potentially important for the regulation of PKC (33), evidence supporting the concept has been limited.

Our finding that PDMP produced a marked elevation in cellular sphingosine, as well as N,N-dimethylsphingosine (10), raises the possibility that the sphingolipids produced are involved in the inhibition of PKC. Many papers have reported an inhibition when ceramides were exposed to relatively high concentrations of exogenous sphingolipids (34). Virtually no attempts were made to determine how much of the exogenous sphingolipid actually entered the cells and how much remained unconverted to ceramide or GSLs. Whether our observed increase in sphingosine was sufficient to produce the observed loss of PKC activity is questionable, since we found the decrease not only in cytosolic and membrane-bound PKC but also in the PKC that had been purified by ion-exchange chromatography. However, there is the possibility that the sphingosine is bound so strongly to the enzyme that it is not separated from it by ion exchange.

An important question in considering sphingolipid effects on PKC is the relative concentration of diacylglycerol present. The ester lipid, as well as diacylphorbol, seems to compete with the amino lipid so that the inhibition can be reversed by sufficient ester (35, 36). The molar ratio of the two lipids in our cells was 24:1 in the absence of PDMP and 47:1 in the presence of 50 PM PDMP. This would seem to preclude any significant role for endogenous sphingosine in PKC inhibition. However it appears that N,N-dimethylsphingosine, which accumulates in PDMP-treated cells (10) and which we could not measure with the sphingosine assay, can compete against the diacylglycerol more effectively (37).

An additional possibility is that PDMP and CBE exerted their effects through changes in cellular adhesion. Sphingolipids mediate the interactions of cells with matrix proteins, including collagen, fibronectin, and thrombospondin (7, 26, 38). They could exert their growth-modulatory effects by direct physical interactions with these proteins or through signal transducing events.

In interpreting data based on the use of any inhibitors, one must consider the possibility that the observed changes are the result of other, unknown effects of these agents on some enzyme or protein, just as one must consider the possibility of unexpected effects due to addition of a novel metabolite. The specificity of both PDMP and CBE has been assessed in several studies, and thus far no second effect has been detected. Of course, glucosphingolipids are known to be involved in many cell processes and numerous second-order effects must be expected.

In the search for mechanisms that cause cell growth or proliferation, scientists have uncovered many phenomena that seem to bear on the problem, but there is as yet no clear set of chemical or physical processes that explains the overall effects. In the case of the sphingolipids, numerous studies have shown their involvement in these processes but relatively few studies have uncovered connections with growth factors (see reviews in Refs. 28 and 39). Brener and co-workers (27) have reported alterations in tyrosine kinase activity by epidermal growth factor and platelet-derived growth factor which were stimulated by exogenous GSLs (27). More recently, interleukin 2-dependent tyrosine phosphorylation has been shown to be enhanced in a murine lymphocyte cell line depleted of GlcCer by PDMP (10).

It may be noted that the stimulatory effects of increasing GlcCer content were smaller than the inhibitory effects of decreasing GlcCer content. This may indicate that MDCK cells, in a preconfluent stage, normally contain a concentration of GlcCer that is already at the level needed for active growth.

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