Involvement of Protein Kinase C in the Regulation of Glycolipid Sulfotransferase Activity Levels in Renal Cell Carcinoma Cells

Takahiko Kobayashi, Koichi Honke, Shinsei Gasa, Makoto Sugiura, Tamotsu Miyazaki, Ineo Ishizuka, and Akira Makita

Biochemistry and Virology Laboratory, Cancer Institute, and the Third Department of Internal Medicine, Hokkaido University School of Medicine, Sapporo, and Department of Biochemistry, Teikyo University, Tokyo, Japan

ABSTRACT

Accumulation of sulfolipids associated with elevated levels of glycolipid sulfotransferase activities has previously been demonstrated in renal cell carcinoma cells. To investigate the role of protein kinase C in the synthesis of sulfolipids, the effects of 12-O-tetradecanoylphorbol-13-acetate and protein kinase C inhibitors on glycolipid sulfotransferase activity levels were examined in a human renal cell carcinoma cell line, SMKT-R3. Continuous treatment of the cells with 12-O-tetradecanoylphorbol-13-acetate caused a dose- and time-dependent reduction of the sulfotransferase activity levels. Similarly, protein kinase C inhibitors, 1-(5-isoquinolinesulfonyl)-2-methylpiperazine dihydrochloride and staurosporine, reduced the enzyme activities in a dose-dependent manner. These observations suggest that the glycolipid sulfotransferase activity levels are regulated by protein kinase C in SMKT-R3 cells. Furthermore, long-term 12-O-tetradecanoylphorbol-13-acetate treatment resulted in a reduction of sulfolipid synthesis and a decrease of the expression of sulfolipids on the cell surface. Taken together, it is suggested that protein kinase C is involved in the synthesis of sulfolipids through the regulation of the glycolipid sulfotransferase activity levels in renal cell carcinoma cells.

INTRODUCTION

Glycolipids have been known to undergo marked cancer-associated changes (1). In previous studies on human cancer tissues, sulfolipids were found to increase in lung adenocarcinoma (2-4), gastric cancer (5), colon adenocarcinoma (6), ovarian cystoadenocarcinoma (7), and renal cell carcinoma (8, 9). Especially, the increment of the sulfolipid content in renal cell carcinoma was considerable and associated with enhanced activities of glycolipid sulfotransferase (EC 2.8.2.11) in the tissue (8), which catalyze the transfer of sulfate from PAPS2 to galactose on GalCer and LacCer to form sulfolipids SM4 and SM3, respectively (10, 11). Furthermore, the levels of the sulfotransferase activity appeared to be elevated in sera from patients with renal cell carcinoma (12). However, the elevation mechanisms of the sulfotransferase activities has previously been demonstrated in renal cell carcinoma as described previously (22) and cultured in Dulbecco’s modified essential minimal medium supplemented with 10% fetal bovine serum. Details on sulfolipids and glycolipid sulfotransferase activities in SMKT-R3 cells are described previously (21). Cell viability was estimated by the trypan blue exclusion test and was always greater than 95%.

ASSAY OF GLYCOLIPID SULFOTRANSFERASE AND ARYL SULFATASE ACTIVITIES

SMKT-R3 cell monolayers were washed with Tris-buffered saline (10 mm Tris-HCl (pH 7.5): 150 mm NaCl) and harvested by scraping with a rubber policeman. Then the cell suspensions were centrifuged and washed 3 times with Tris-buffered saline. The cell pellets were resuspended in Tris-buffered saline containing 0.1% Lubrol PX and sonicated on ice. Glycolipid sulfotransferase activities of the cell homogenate, the protein concentration of which was adjusted to approximately 1 mg/ml, were assayed using GalCer and LacCer individually as substrates according to a previously reported method (23). Arylsulfatase A activity of the cell homogenate was assayed using p-nitroacetyl sulfamate as a substrate by the method of Baum et al. (24). To enable comparisons between the separate experiments, the measured enzyme activity was expressed as a percentage of the mean of control values within each experiment. When SMKT-R3 cells were cultured in the absence of some reagents as untreated control, the specific activities (mean ± SD) of glycolipid sulfotransferase toward GalCer and LacCer and of arylsulfatase A were 9320 ± 680 pmol/h/mg of protein, 3140 ± 270 pmol/h/mg of protein, and 210 ± 14 nmol/h/mg of protein, respectively.

ASSAY OF PROTEIN KINASE C ACTIVITY

The cell pellets, which were prepared as above, were resuspended and lysed by sonication in 30 mm Tris-HCl (pH 7.5) containing 5 mm EDTA, 5 mm EGTA, 1 mm diithiothreitol, 1 mm phenylmethylsulfonyl fluoride, and 20 µg/ml of leupeptin. The cell lysate was centrifuged for 1 h at 100,000 × g. The supernatant was examined for protein kinase C activity of the cytosol fraction using an Amersham protein kinase C enzyme assay system according to the manufacturer’s instructions.

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1 To whom requests for reprints should be addressed, at Biochemistry Laboratory, Cancer Institute, Hokkaido University School of Medicine, Kita-ku N15 W7, Sapporo 060, Japan.

2 The abbreviations used are: PAPS, 3′-phosphoadenosine-5′-phosphosulfate; GalCer, galactosylceramide; LacCer, lactosylceramide; SB2, bis-sulfoglycolactosylceramide; SM2, gangliosylceramide 3′-sulfate; SM3, lactosylceramide 3′-sulfate; SM4, galactosylceramide 3′-sulfate; HPTLC, high-performance thin-layer chromatography; TPA, 12-O-tetradecanoylphorbol-13-acetate; PDBu, phorbol 12,13-dibutyrate; OAG, 1-oleoyl-2-acetyl-sn-glycerol; H-7, l-(5-isoquinolinesulfonyl)-2-methylpiperazine dihydrochloride; HA-1004, N-(2-guanidinomethyl)-5-isoquinolinesulfonamide hydrochloride.
**REGULATION OF GLYCOLIPID SULFOTRANSFERASE ACTIVITY**

**Assay of [3H]PDBu Binding to Intact Cells.** Cells grown to subconfluence in 35-mm-diameter wells (Corning) were washed twice with binding solution (serum-free Dulbecco’s modified minimal essential medium containing 1 mg/ml of bovine serum albumin) followed by incubation of the cells at 37°C for 20 min in binding medium containing 10 nM [3H]PDBu in the presence (nonspecific binding) or absence (total binding) of 10 µM unlabeled PDBu. After washing with ice-cold phosphate-buffered saline 3 times, cells were lysed in 500 µl of 1 M NaOH, and cell-bound radioactivity was measured by scintillation counting. Specific [3H]PDBu binding to the cells was defined as the difference between the amounts of [3H]PDBu bound in the absence and presence of unlabeled PDBu.

**Metabolic Labeling of SMKT-R3 Cells.** SMKT-R3 cells (3 × 10⁶ cells) were incubated with or without 10 nM TPA for 12 h, followed by the addition of sodium [35S]sulfate to the medium (the final concentration, 5 µCi/ml), and then metabolically labeled for a further 24 h. The cells were harvested as above, extracted with 2 ml of a mixture of chloroform:methanol:water (60:35:8, the ratio of the solvent mixture is expressed by volume), and then reextracted with 2 ml of a mixture of chloroform:methanol:water (30:60:8). The two extracts were combined, subjected to mild alkaline hydrolysis to destroy ester lipids, and neutralized with acetic acid. After evaporation of the solvent, the total lipid extract was desalted with a Sephadex G-25 column (25). The eluate was concentrated and applied on a DEAE-Sephadex A-25 (acetate form) column. After washing with chloroform:methanol:water (30:60:8), the acidic glycolipid fraction of the cells was eluted with chloroform:methanol:CH₃COONH₄ (30:60:8) and desalted as above. The acidic glycolipids were chromatographed on a precoated Silica Gel 60 HPTLC plate (Merck) using the solvent system chloroform:methanol:0.2% CaCl₂ (60:35:7). The labeled glycolipids were detected by autoradiography of the plate and scanned for radioactivity as described previously (23).

**Cytofluorometric Analysis.** SMKT-R3 cells were incubated for 24 h in the culture medium with or without 10 nM TPA. The cells were harvested, washed, and stained by the indirect immunofluorescence method; the cells were reacted with an anti-sulfolipid monoclonal antibody, Sulph I (26), as the first antibody and subsequently with a fluorescein isothiocyanate-conjugated F(ab')₂ fragment of rabbit anti-mouse immunoglobulins (Dako) as the second antibody. Fluorescence profiles were determined with a FACScan (Becton Dickinson).

**RESULTS**

**Effect of TPA on Glycolipid Sulfotransferase Activities.** To determine whether protein kinase C participates in the expression of glycolipid sulfotransferase activities in renal cell carcinoma cells, the effects of TPA were investigated. When SMKT-R3 cells were cultured in the presence of TPA for 48 h, the activity levels of the sulfotransferases for two substrates were reduced in a dose-dependent manner in the range of 1 nM to 100 nM TPA, while that of arylsulfatase A, which catalyzes hydrolysis of sulfolipids, was not affected (Fig. 1). Half-maximal reduction of the sulfotransferase activities was achieved at approximately 1 nM TPA. The time course study of the effect of TPA is shown in Fig. 2. On incubation with 10 nM TPA, the sulfotransferase activity levels decreased in a time-dependent manner. Thus, TPA treatment was found to reduce glycolipid sulfotransferase activity levels in SMKT-R3 cells, and the diminution patterns of the sulfotransferase activity levels for GalCer and LacCer were similar. To confirm these observations, the effect of other TPA-type tumor promoters was examined. As shown in Table 1, 4-O-methyl-PMA, which is an inactive analogue of TPA, had no effect on the activity levels of the sulfotransferases. On the other hand, teleocidin, which activates protein kinase C-like TPA (27), decreased the sulfotransferase activity levels. The degree of attenuation by teleocidin was similar to that by TPA and was dose dependent (data not shown). However, none of them had any effect on the activity level of arylsulfatase A. To investigate the effect of protein synthesis on the sulfotransferase activity levels in SMKT-R3 cells, the cells were incubated in the presence of a protein synthesis inhibitor, cycloheximide. As shown in Fig. 3, treatment of the cells with cycloheximide for 12 h reduced the sulfotransferase activities to approximately 10% of that measured in untreated cells. On the other hand, the activity level of arylsulfatase A was not affected within 12 h, in agreement with a report that the half-life of arylsulfatase A was calculated as 65 days in human fibroblasts (28). Since most sulfotransferase activity disappeared within 12 h after the inhibition of protein synthesis in the cells, we chose 12 h for incubation with various reagents and used 10 nM as the TPA dose to minimize its cytotoxicity effect in the subsequent experiments, unless otherwise stated.

**Protein Kinase C in Renal Cell Carcinoma Cells.** To determine whether the reduction of the sulfotransferase activity levels by TPA treatment resulted from down-regulation of protein kinase C, the **Fig. 1.** Dose-dependent effect of TPA on glycolipid sulfotransferase and arylsulfatase A activities. SMKT-R3 cells were incubated with the indicated doses of TPA for 48 h and assayed for sulfotransferase activities toward GalCer (●) and LacCer (■) and for arylsulfatase A activity (□). Columns, mean from three separate experiments performed in triplicate and expressed as the percentage of the enzyme activities in the absence of TPA (vehicle alone); bars, SD.

**Fig. 2.** Time course of TPA effects on glycolipid sulfotransferase activities. SMKT-R3 cells were incubated for various times without (control) or with 10 nM TPA and assayed for sulfotransferase activities toward GalCer (●) and LacCer (□). Points, mean of three different experiments done in triplicate and plotted as the percentage of the value for control cells; bars, SD.

**Table 1.** Effect of tumor promoters on glycolipid sulfotransferase and arylsulfatase A activities

<table>
<thead>
<tr>
<th>Tumor Promoter</th>
<th>GalCer Sulfotransferase</th>
<th>LacCer Sulfotransferase</th>
<th>Arylsulfatase A</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>TPA</td>
<td>34</td>
<td>40</td>
<td>103</td>
</tr>
<tr>
<td>Teleocidin</td>
<td>37</td>
<td>34</td>
<td>96</td>
</tr>
<tr>
<td>4-O-Methyl-PMA</td>
<td>98</td>
<td>97</td>
<td>104</td>
</tr>
</tbody>
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on the sulfotransferases. Thus, these observations suggest that down
of the sulfotransferase did not inhibit the activities (data not shown),

dent decrease of the sulfotransferase activity levels (Fig. 5). However,
catalytic domain of protein kinase C (30). also showed a dose-depen
ing that these protein kinase C modulators did not act directly
addition of TPA or the protein kinase C inhibitors to the assay mixture
rosporine, which has been characterized as an inhibitor acting on the
approximately 100 U.M. In contrast, treatment with a control reagent.

Effect of Protein Kinase C Modulators on Glycolipid Sulfo
transferase Activities. To confirm the involvement of protein ki
activity and specific binding of [3H]PDBu to SMKT-R3 cells were
examined after incubation with or without TPA. As shown in
Table 2, TPA diminished the kinase activity level of the cells by
94.3%, compared with the value of the untreated control. Furthermore,
the treatment with TPA caused a 93.7% decrease in specific binding of
[3H]PDBu to the cells in agreement with the reduction of the kinase
activity. Thus, it was ascertained that the continuous treatment with
TPA brought about down-regulation of protein kinase C in SMKT-R3
cells.

Effect of Protein Kinase C Modulators on Glycolipid Sulfo
transferase Activities. To confirm the involvement of protein ki
nase C in the expression of the sulfotransferase and to determine
which transient activation or down-regulation of the kinase by TPA
caued depletion of the sulfotransferase activity levels, additional
experiments were carried out. Long-term treatment (12 h) of the cells
with a permeant diacylglycerol, OAG, in the range of 1 to 100 μg/ml
had no significant effect upon the activities of the sulfotransferase
(data not shown). Furthermore, the effects of protein kinase C inhibi
tors were examined. H-7, which inhibits ATP from binding to protein
kinase C (29), reduced the sulfotransferase activity levels in a dose-
dependent fashion (Fig. 4). Half-maximal inhibition was achieved at
approximately 100 μM. In contrast, treatment with a control reagent,
HA-1004, which is the weakest inhibitor for protein kinase C among
isoquinolinesulfonamide derivatives (29), had no effect (Fig. 4). Stauro
sporine, which has been characterized as an inhibitor acting on the
catalytic domain of protein kinase C (30), also showed a dose-depen
dent decrease of the sulfotransferase activity levels (Fig. 5). However,
addition of TPA or the protein kinase C inhibitors to the assay mixture
of the sulfotransferase did not inhibit the activities (data not shown),
indicating that these protein kinase C modulators did not act directly
on the sulfotransferases. Thus, these observations suggest that down-
regulation, but not transient activation, of protein kinase C caused the
considerable reduction of sulfotransferase activity levels in SMKT-R3
cells.

Effect of a Glucocorticoid Hormone on Glycolipid Sulfotransferase Activities. Recently, it has been demonstrated that the gluco
corticoid receptor and the transcription factor AP-1 (fos/jun) can reci
crocally repress one another's transcriptional activation by pro	ein-protein interactions (31–33)). In order to examine whether a TPA
responsive element is involved in the expression of the activities of
glycolipid sulfotransferase, SMKT-R3 cells were cultured in the pres
ence of dexamethasone. Fig. 6 shows the effects of increasing con
centrations of dexamethasone on the sulfotransferase activity levels.
Indeed, dexamethasone induced a dose-dependent reduction, but par
tially decreased the sulfotransferase activity levels in SMKT-R3 cells,
while it repressed collagenase gene expression almost completely at
similar doses (31–33)). These results suggest that a TPA-responsive
element participates in the expression of the sulfotransferase activities
in SMKT-R3 cells to some extent and that other transcription factors
may be also involved in it.

Table 2. Down-regulation of protein kinase C in SMKT-R3 cells

<table>
<thead>
<tr>
<th>Pretreatment</th>
<th>Kinase activity (pmol/min/mg of protein)</th>
<th>[3H]PDBu binding (dpm/10^5 cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TPA (-)</td>
<td>586 ± 145 (100)</td>
<td>7055 ± 259.0 (100)</td>
</tr>
<tr>
<td>TPA (+)</td>
<td>33.3 ± 10.2 (5.7)</td>
<td>500 ± 37.5 (6.3)</td>
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</table>

* Mean ± SD of three separate experiments performed in triplicate.
* Numbers in parentheses, percentage of control value.

Fig. 3. Effect of cycloheximide on glycolipid sulfotransferase and arylsulfatase A activities. SMKT-R3 cells were cultured in the absence or presence of 10 μM TPA for 12 h and assayed for enzyme activities. Columns, mean from three separate experiments performed in triplicate and expressed as percentages of the enzyme activities in the absence of cycloheximide (vehicle alone); bars, SD. The symbols are the same as in Fig. 1.

Fig. 4. Effects of H-7 and HA-1004 on glycolipid sulfotransferase and arylsulfatase A activities. SMKT-R3 cells were incubated with different doses of H-7 or with HA-1004 (200 μM) for 12 h and assayed for enzyme activities. Columns, mean of three different experiments performed in triplicate and expressed as percentages of the control with neither H-7 nor HA-1004 (vehicle alone); bars, SD. The symbols are the same as in Fig. 1.

Fig. 5. Effect of staurosporine on glycolipid sulfotransferase and arylsulfatase A activities. SMKT-R3 cells were incubated without or with various concentrations of staurosporine for 12 h and assayed for enzyme activities. Columns, mean of three separate experiments carried out in triplicate and expressed as percentages of the enzyme activities in the absence of staurosporine (vehicle alone); bars, SD. The symbols are the same as in Fig. 1.
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Fig. 6. Effect of dexamethasone on glycolipid sulfotransferase and arylsulfatase A activities. SMKT-R3 cells were cultured in the absence or in the presence of varying concentrations of dexamethasone for 12 h and assayed for enzyme activities. Columns, mean from five separate experiments done in triplicate and expressed as percentages of the enzyme activities in the absence of dexamethasone (vehicle alone); bars, SD. The symbols are the same as in Fig. 1.

Effect of TPA on Synthesis of Sulfolipids in Renal Cell Carcinoma Cells. To determine whether TPA treatment actually reduces the synthesis of sulfolipids in SMKT-R3 cells, metabolic labeling with [35S]sulfate was performed. Four kinds of sulfolipids were detected by autoradiography of the thin-layer chromatogram of the acidic glycolipid fraction from SMKT-R3 cells (Fig. 7, Lane 2). In addition to the sulfolipids corresponding to authentic SM4, SM3, and SM2, another more slowly migrating sulfolipid (Fig. 7, Lanes 2 and 3, asterisks), which remains to be characterized, was also found. These sulfolipids appeared as doublets, probably due to heterogeneity of the lipid moiety. On the addition of TPA to the culture medium, the radioactivity incorporated into the sulfolipids was considerably diminished (Fig. 7, Lanes 3 and 5). Furthermore, the total amount of sulfolipids on the cell surface was examined by flow cytometry with an anti-sulfolipid monoclonal antibody, Sulph I (26). As shown in Fig. 8, incubation of the cells with TPA resulted in significant reduction of the sulfolipid expression on the cell surface in accordance with the result of metabolic labeling.

DISCUSSION

In the present study, we have demonstrated the participation of protein kinase C in the expression of sulfolipids through the regulation of glycolipid sulfotransferase activities in renal cell carcinoma cells.

Here, it was shown that the reduction of the activity levels of glycolipid sulfotransferases was associated with down-regulation of protein kinase C, rather than direct activation of the kinase. The effects of TPA on the sulfotransferase activity levels in SMKT-R3 cells were similar to those of protein kinase C inhibitors. However, the marked effects of TPA on the sulfotransferase activity levels were not seen...
with OAG. This result is consistent with the previous observations that cell-permeant diacylglycerol analogues, such as OAG and 1,2-diacyl-sn-glycerol, were unable to induce down-regulation of protein kinase C, although they could activate the kinase (34, 35). Alternatively, the differential effects of TPA and OAG on the sulfotransferase activity levels may be due to the ability of the two activators to bind and activate different isozymic forms of protein kinase C (36, 37), though the isozymes expressed in SMKT-R3 cells remain to be determined.

Protein kinase C inhibitors, including H-7 and staurosporine, have been widely used in studies of many cellular phenomena involving protein kinase C. In our study, both H-7 and staurosporine reduced the sulfotransferase activity levels in SMKT-R3 cells. Staurosporine was somewhat less potent than H-7, although the concentrations of these reagents used were similar to those observed in other reported protein kinase C-dependent systems. The degree of the effects of the two inhibitors may be dependent on the sensitivity of the isozymes of protein kinase C or on the used cell specificity. The possible effects of presently used protein kinase C inhibitors on other kinases could not be ruled out completely, since these inhibitors may also inhibit cyclic nucleotide-dependent protein kinases and myosin light chain kinase (29). To attribute the effect of H-7 to protein kinase C, we used HA-1004 as a control for H-7. The inhibitory activity of HA-1004 against protein kinase C is the weakest among isoxquinolinesulfonamide derivatives, whereas it is similar against other kinases (29). Therefore, the observed distinction of the effects between H-7 and HA-1004 can be ascribed to the inhibitory activity against protein kinase C.

Although our observations suggest that protein kinase C regulates the sulfotransferase activity levels in renal cell carcinoma cells, details of the regulatory mechanism remain to be determined. Purification and complementary DNA cloning of human glycolipid sulfotransferase have not been reported, though the purification of the enzymes from rat kidney (38) and testis (39) was reported. To attribute the effect of H-7 to protein kinase C, we used HA-1004 as a control for H-7. The inhibitory activity of HA-1004 against protein kinase C is the weakest among isoxquinolinesulfonamide derivatives, whereas it is similar against other kinases (29). Therefore, the observed distinction of the effects between H-7 and HA-1004 can be ascribed to the inhibitory activity against protein kinase C.

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