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

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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 catalyzes the transfer of sulfate from PAPS 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 have previously been demonstrated in renal cell carcinoma as described previously (22) and cultured in Dulbecco’s modified essential 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%.

MATERIALS AND METHODS

Materials. [35S]PAPS (1.5 Ci/mmol), [35S]sodium sulfate (250 to 1000 mCi/mmol), and [3H]PDBu (20 Ci/mmol) were purchased from New England Nuclear; unlabeled PAPS, OAG, PDBu, and PMA were from Sigma; O-Methyl-PMA was from Chemicals for Cancer Research; the protein kinase inhibitors H-7 and HA-1004 were from Seikagaku Kogyo; staurosporine was from Kyowa Hakko; and dexamethasone was from Wako Chemicals. DEA/E-Sephadex A-25 and Sephadex G-25 were the products of Pharmacia-LKB. Teleocidin was generously donated by H. Fujiki (National Cancer Center Research Institute, Tokyo, Japan). GalCer and LacCer were purified in this laboratory from bovine brain and horse erythrocytes, respectively. Other reagents were of analytical grade.

Cell Culture. SMKT-R3 cells were established from human renal cell carcinoma as described previously (22) and cultured in Dulbecco’s modified essential 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%.

Auxs of Glycolipid Sulfotransferase and Arylsulfatase A Activities. SMKT-R3 cell monolayers were washed with Tris-buffered saline [10 mM Tris (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-nitrocatechol sulfate 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 50 mM Tris-HCl (pH 7.5) containing 5 mM EDTA, 5 mM EGTA, 1 mM dithiothreitol, 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.
Assay of \[^{3}H\]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 \[^{3}H\]PDBu in the presence (nonspecific binding) or absence (total binding) of 10 μM unlabelled 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 \[^{3}H\]PDBu binding to the cells was defined as the difference between the amounts of \[^{3}H\]PDBu bound in the presence and absence of unlabelled PDBu.

Metabolic Labeling of SMKT-R3 Cells. SMKT-R3 cells (3 × 10^5 cells) were incubated with or without 10 nM TPA for 12 h, followed by the addition of sodium \[^{35}S\]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:water:2% CaCl₂ (60:35:8). 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 were 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.

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
regulated, 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 glucocorticoid receptor and the transcription factor AP-1 (fosl1jun) can reciprocally repress one another's transcriptional activation by protein-protein interactions (31–33). In order to examine whether a TPA-responsive element is involved in the expression of glycolipid sulfotransferase, SMKT-R3 cells were cultured in the presence of dexamethasone. Fig. 6 shows the effects of increasing concentrations of dexamethasone on the sulfotransferase activity levels. Indeed, dexamethasone induced a dose-dependent reduction, but partially 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.

Fig. 3. Effect of cycloheximide on glycolipid sulfotransferase and arylsulfatase A activities. SMKT-R3 cells were treated with or without cycloheximide (25 μg/ml) 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.

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>
</tr>
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</table>

*Mean ± SD of three separate experiments performed in triplicate.

Numbers in parentheses, percentage of control value.

Fig. 4. Effects of H-7 and HA-1004 on glycolipid sulfotransferase and arylsulfatase A activities. SMKT-R3 cells were incubated without or with various concentrations of staurosorine for 12 h and assayed for enzyme activities. Columns, mean of three different experiments performed in triplicate and expressed as the percentage 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 staurosorine on glycolipid sulfotransferase and arylsulfatase A activities. SMKT-R3 cells were incubated without or with various concentrations of staurosorine for 12 h and assayed for enzyme activities. Columns, mean of three separate experiments performed in triplicate and expressed as percentages of the enzyme activities in the absence of staurosorine (vehicle alone); bars, SD. The symbols are the same as in Fig. 1.
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 $[^{35}S]$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.

Fig. 7. Effect of TPA on sulfolipid synthesis. SMKT-R3 cells ($3 \times 10^6$ cells) were incubated with (Lanes 3 and 5) or without (Lanes 2 and 4) 10 nM TPA for 12 h, followed by labeling metabolically with sodium $[^{35}S]$sulfate for 24 h as described in "Materials and Methods." The acidic glycolipids of the cells were extracted and chromatographed on an HPTLC plate. The labeled glycolipids were detected by autoradiography of the plate and scanned for radioactivity. Sulfolipid standards were stained with an orcinol reagent (Lane 1). Asterisks indicate sulfolipids whose structures are unknown (Lanes 2 and 3).
with OAG. This result is consistent with the previous observations that cell-permeant diacylglycerol analogues, such as OAG and 1,2-dioctanoyl-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 isoquinolinesulfonamide 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. 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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