The regulation of protein phosphorylation by sphingosine in A431 human epidermoid carcinoma cells was examined. Sphingosine is a competitive inhibitor of phorbol ester binding to protein kinase C (Ca<sup>2+</sup>-phospholipid-dependent enzyme) and potently inhibits phosphotransferase activity in vitro. Addition of sphingosine to intact A431 cells caused an inhibition of the phorbol ester-stimulated phosphorylation of two protein kinase C substrates, epidermal growth factor (EGF) receptor threonine 654 and transferrin receptor serine 24. We conclude that sphingosine inhibits the activity of protein kinase C in intact A431 cells. However, further experiments demonstrated that sphingosine treatment of A431 cells resulted in the regulation of the EGF receptor by a mechanism that was independent of protein kinase C. First, sphingosine caused an increase in the threonine phosphorylation of the EGF receptor on a unique tryptic peptide. Second, sphingosine caused an increase in the affinity of the EGF receptor in A431 and in Chinese hamster ovary cells expressing wild-type (Thr<sup>654</sup>) and mutated (Ala<sup>654</sup>) EGF receptors. Sphingosine was also observed to cause an increase in the number of EGF-binding sites expressed at the surface of A431 cells. Examination of the time course of sphingosine action demonstrated that the effects on EGF binding were rapid (maximal at 2 mins) and were observed prior to the stimulation of receptor phosphorylation (maximal at 20 mins). We conclude that sphingosine is a potently bioactive molecule that modulates cellular functions by: 1) inhibiting protein kinase C; 2) stimulating a protein kinase C-independent pathway of protein phosphorylation; and 3) increasing the affinity and number of cell surface EGF receptors.

The epidermal growth factor (EGF) receptor is phosphorylated at multiple sites. In addition to the autophosphorylation of the receptor on tyrosine residues, the receptor is also a substrate for phosphorylation by exogenous kinases. Treatment of human fibroblasts with EGF, PDGF, or with phorbol ester causes the phosphorylation of several sites on the EGF receptor. The phosphorylation of threonine 654 does not fully account for the transmodulation of the receptor caused by incubating cells with phorbol ester (4). However, the phosphorylation of threonine 654 does not fully account for the transmodulation of the EGF receptor caused by PDGF (5). This result suggests that the phosphorylation of additional sites on the EGF receptor may be important for PDGF action.

The functional significance of the phosphorylation of the EGF receptor at threonine 654 and at other sites has been incompletely defined. One limitation for such studies is the lack of availability of suitable probes to dissect receptor function. Sphingosine and lysosphingolipids have been demonstrated to be potent inhibitors of protein kinase C in vitro (6, 7). The use of sphingosine as an inhibitor, therefore, provides a pharmacological tool to dissect the protein kinase C-linked mechanism of EGF receptor transmodulation from alternative mechanisms. The purpose of the experiments presented here was to examine whether the addition of sphingosine to intact cells caused an inhibition of protein kinase C. The strategy that we employed was to examine the state of phosphorylation of two protein kinase C substrates, EGF receptor threonine 654 (2, 3) and transferrin receptor serine 24 (5), in A431 human epidermoid carcinoma cells. The results obtained indicate that sphingosine is an inhibitor of protein kinase C in intact A431 cells. However, additional experiments demonstrated that sphingosine regulated the EGF receptor by a mechanism that was independent of protein kinase C. These data show that sphingosine is a bioactive molecule that has pleiotropic actions on A431 cells.

**EXPERIMENTAL PROCEDURES**

**Materials**—Na<sup>251</sup>I was obtained from Amer sham Corp. [<sup>32</sup>P]Phosphate, <sup>125</sup>I-goat anti-mouse Ig, and [<sup>3</sup>H]PDBU were from Du Pont-New England Nuclear. Sphingosine, glucosylsphingosine, galactosylsphingosine, phorbol esters, protein A-Sepharose, and gangliosides (GM<sub>1</sub>, GM<sub>2</sub>, GM<sub>3</sub>) were purchased from Sigma. Restriction enzymes were from New England Biolabs. Polynucleotide kinase and Klenow were from Pharmacia LKB Biotechnologies Inc. and United States Biochemical Corp., respectively. N-Acetylsphingosine was synthesized by acetylation of sphingosine as described (5). EGF was purified and iodinated by methods described previously (10-12). Monoclonal anti-EGF receptor antibodies were obtained from Oncor and from Behring Diagnostics. Transferrin (Behring Diagnostics) was further purified by gel filtration chromatography, saturated with iron and iodinated as described (13).

**Cell Culture**—A431 human epidermoid carcinoma cells were maintained in Dulbecco's modified Eagle's medium supplemented with 5% calf serum. Chinese hamster ovary cells (CHO-K1) were maintained in Ham's F-12 supplemented with 10% fetal bovine serum. Cells were treated with sphingosine (ethanol solution), phorbol diester (dimethyl sulfoxide solution), and gangliosides (dimethyl sulfoxide solution) by direct addition to the tissue culture medium. The final volume of receptor (for review see Ref. 1). One of these sites, threonine 654 (2, 3), is a substrate for protein kinase C. Phosphorylation of this site on the EGF receptor has been reported to account for the transmodulation of the receptor caused by incubating cells with phorbol ester (4). However, the phosphorylation of threonine 654 does not fully account for the transmodulation of the EGF receptor caused by PDGF (5). This result suggests that the phosphorylation of additional sites on the EGF receptor may be important for PDGF action.
solvent was 0.1% (v/v). Control cells were treated with the solvent alone.

Plasmid Construction—The full-length cDNA for the human EGF receptor in the plasmid pMMTV-EGFR (14, 15) was obtained from Dr. G. Merlin (National Institutes of Health). The cDNA was excised as a 4-kilobase fragment using XbaI and SphI, blunt-ended receptor in the plasmid pMMTV-EGFR (14, 15) was obtained from Dr. G. Merlin (National Institutes of Health). The cDNA was subcloning into M13mp18 using methods described previously (18). cDNA (SalI fragment). The plasmid was designated pHERX. Oligonucleotide-directed mutagenesis of threonine 654 (ACG) to alanine (GCC) was carried out according to Zoller and Smith (17) after subcloning into M13mp18 using methods described previously (18).

Expression of the EGF Receptor cDNA in CHO Cells—The plasmids pHERX and pHERX (Als) were transfected together with pZipNeoSV(X) into CHO cells using the Capo4 method (16). Stable colonies resistant to G418 were isolated using cloning rings obtained were analyzed by reverse-phase HPLC using a Vydac acrylamide gel electrophoresis as previously described. The isolated phosphopeptides were eluted with a linear gradient (1%/min) of acetonitrile at a flow rate of 1 ml/min. Fractions (1 ml) were collected and the [32P]phosphopeptides were detected by measuring the associated Cerenkov radiation with a beta counter.

RESULTS

Regulation of EGF Receptor Phosphorylation by Sphingosine—A431 cells were incubated for 24 h with [32P]phosphate in order to achieve steady-state labeling of the intracellular phosphate pools. The cells were then treated for 30 min with 5 mM sphingosine at 37°C. Polycyclamid gel electrophoresis of extracts containing total cellular proteins demonstrated no significant differences between the pattern of protein phosphorylation observed in control and sphingosine-treated cells. However, immunoprecipitation of EGF receptors revealed that sphingosine caused a significant increase in the phosphorylation state of this protein (Fig. 1). This effect was specific for cells treated with sphingosine and was not observed when the cells were treated with N-acetylsphingosine (Fig. 1), a similar molecule that does not inhibit protein kinase C (7). Examination of the time course of sphingosine action indicated that it occurred relatively slowly and was maximal after approximately 20 min of treatment at 37°C (Fig. 2). The increase in EGF receptor phosphorylation caused by sphingosine was dose-dependent. The increase was observed after treatment of A431 cells with 0.5 mM sphingosine and was maximal at 5 mM sphingosine (Fig. 3). Treatment of the cells with higher concentrations of sphingosine (>25 mM) resulted in a marked decrease in the phosphorylation state of many cellular proteins, including the EGF receptor (data not shown). This general decrease in protein phosphorylation observed with high concentrations of sphingosine may reflect cytotoxic effects including cell permeabilization and the subsequent release of nucleotides into the medium (19). This action of sphingosine was not observed at lower concentrations which cause an increase in the phosphorylation state of the EGF receptor (Fig. 1).

Phosphoamino acid analysis indicated that sphingosine caused a marked increase in the level of EGF receptor [32P]phosphothreonine (Fig. 4). In further experiments the effect of sphingosine on the EGF receptor tryptic [32P]phosphopeptide map was examined. It was observed that the effect of sphingosine to increase the phosphorylation state of the EGF
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FIG. 2. Time course of sphingosine-stimulated phosphorylation of the EGF receptor. A431 cells were incubated with [32P]phosphate for 24 h and subsequently treated with 5 μM sphingosine for different times at 37 °C. The EGF receptors were then immunoprecipitated and electrophoresed on a 7% polyacrylamide gel in the presence of 0.1% NaDodSO4. The gel slices containing the receptor were excised and the associated radioactivity was measured by Cerenkov counting. The figure presents the results obtained in a single experiment. Similar results were obtained in two separate experiments.

FIG. 3. Dose response of sphingosine-stimulated phosphorylation of the EGF receptor. A431 cells were incubated with [32P]phosphate for 24 h and subsequently treated with different concentrations of sphingosine for 30 min. The EGF receptors were immunoprecipitated and electrophoresed on a 7% polyacrylamide gel in the presence of 0.1% NaDodSO4. The gel slices containing the receptor were excised and the associated radioactivity was measured by Cerenkov counting. The figure presents the results obtained in a single experiment. Similar results were obtained in three separate experiments.

The receptor could be largely accounted for by a marked increase in the level of a single [32P]phosphopeptide that eluted from the reverse-phase HPLC column at 27% acetonitrile (Fig. 4). In addition to the marked increase in threonine phosphorylation of the EGF receptor, it was also observed that sphingosine caused a small increase in serine and tyrosine phosphorylation (Fig. 4B). HPLC [32P]phosphopeptide mapping (Fig. 4A) demonstrated that the increased tyrosine phosphorylation was located at tyrosine 1173.

Regulation of EGF Binding to A431 Cells by Sphingosine—Treatment of A431 cells with sphingosine caused an increase in the binding of 125I-EGF to cell surface receptors (Fig. 5). Maximal effects of sphingosine were observed at a concentration of 5 μM (Fig. 5). The increase in binding occurred rapidly after treatment with sphingosine and was stable for 30 min following treatment. Maximal effects were observed after 2 min of treatment with sphingosine (Fig. 5). The possibility exists that the increased threonine phosphorylation of the EGF receptor in sphingosine-treated cells is causally related to the change in the affinity of the receptor. However, examination of the time course of the changes in receptor affinity and phosphorylation indicated that the increased affinity of the EGF receptor occurs rapidly and is complete prior to the onset of the increased receptor phosphorylation (Figs. 2 and 5). We conclude that the mechanism by which sphingosine increases the affinity of the EGF receptor is independent of receptor phosphorylation.

Analysis of the EGF-binding isotherm demonstrated that sphingosine increased the affinity of the EGF receptor and caused an increase in the number of EGF-binding sites detected at the cell surface (Fig. 6).
concentrations of T-EGF. After washing the cell monolayers the binding was determined in incubations with a 500-fold excess of EGF. Cells were rapidly cooled to 0 °C and incubated for 3 h with different concentrations of sphingosine and N-acetylsphingosine for different times. The specific cell-surface binding of T-EGF was observed (Fig. 6). These data indicate that sphingosine caused an increase in the affinity and number of EGF-binding sites expressed at the cell surface. Table I shows that sphingosine caused a 40% increase in the binding of two different monoclonal anti-EGF receptor antibodies to the surface of A431 cells. These results represent the means of triplicate determinations obtained in three separate experiments.

The question of whether sphingosine specifically regulated the EGF receptor was investigated by examining the binding of dimeric 125I-EGF to A431 cells by sphingosine. The binding of EGF to the cell-surface receptors of A431 cells was measured by incubation of the cells at 0 °C for 3 h with 100 pm 125I-EGF. Nonspecific binding of 125I-EGF was determined in incubations with 100 nM EGF. For the analysis of the dose response (A), A431 cells were treated with different concentrations of sphingosine and N-acetylsphingosine for 5 min at 37 °C. Analysis of the time course of action (B) was performed by incubation of the A431 cells with 5 μM sphingosine or 5 μM N-acetylsphingosine for different times. The results represent the means of triplicate determinations obtained in single experiments. Similar results were obtained in three separate experiments.

**Table I.** Regulation of the cell surface expression of the EGF receptor by sphingosine

| Anti-EGF receptor antibody binding | I | II |
|-----------------------------------|---|----|
| Control                           | 2667 ± 71 | 1742 ± 43 |
| Sphingosine                       | 3588 ± 65 | 2504 ± 85 |

The question of whether sphingosine specifically regulated the EGF receptor was investigated by examining the binding of dimeric 125I-EGF to A431 cells. In contrast to the increase in 125I-EGF binding observed in sphingosine-treated cells, a marked decrease in the binding of dimeric 125I-EGF occurred rapidly following sphingosine addition (Table I).
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**Fig. 7. Regulation of wild-type (Thr<sup>244</sup>) and mutated (Ala<sup>244</sup>) EGF receptors by sphingosine.** A431 cells (2 x 10<sup>5</sup> cells/well) and CHO cell clones (1 x 10<sup>5</sup> cells/well) were treated with and without 10 μM sphingosine for 5 min at 37 °C. The cell surface binding of 200 pm <sup>125</sup>I-EGF to cell-surface receptors at 4 °C was then measured. The results presented are the mean ± standard deviation of triplicate determinations. Similar results were obtained in three separate experiments. The CHO clones used were CHO-K1, Thr-11 and Ala-17 which express no EGF receptors (control), Thr<sup>244</sup>-EGF receptors and Ala<sup>244</sup>-EGF receptors. Respectively. Similar results were obtained using four different CHO clones expressing wild-type EGF receptors and with four different clones that express Ala<sup>244</sup>-EGF receptors.

**Fig. 8. Effect of sphingosine concentration on the growth of A431 cells.** A431 cells were seeded in 16-mm wells and grown to a density of 2 x 10<sup>5</sup> cells/well. The cells were then treated with different concentrations of sphingosine and <sup>14</sup>N-acetylsphingosine and subsequently incubated for 24 h together with 1 μCi of <sup>3</sup>H]thymidine. The incorporation of radioactive DNA is presented. The results represent the means of triplicate observations. Similar results were obtained in three separate experiments.

**Table II**

**Pharmacology of the Actions of Sphingolipids**

A431 cells were incubated for 15 min at 37 °C with 5 μM sphingolipid. The specific binding of 100 pm <sup>125</sup>I-EGF, 5 nM dimeric <sup>125</sup>I-transferrin, and 5 nM <sup>3</sup>H]PDBU were measured in separate experiments. The results are presented as the means of triplicate determinations. Similar results were obtained in four separate experiments.

|                | <sup>125</sup>I-EGF binding | Dimeric <sup>125</sup>I-transferrin binding | <sup>3</sup>H]PDBU binding |
|----------------|-----------------------------|---------------------------------------------|---------------------------|
| Control        | 2314                        | 6781                                        | 1434                      |
| Sphingosine    | 5876                        | 3104                                        | 369                       |
| N-Acetylsphingosine | 2355                      | 6847                                        | 1544                      |
| Glucosylsphingosine | 2577                     | 6514                                        | 1301                      |
| Galactosylsphingosine | 2619                     | 6651                                        | 1322                      |
| Control        | 1840                        | 8432                                        | 1566                      |
| G<sub>M</sub>1  | 1911                        | 8711                                        | 1545                      |
| G<sub>M</sub>2  | 1867                        | 8531                                        | 1589                      |
| G<sub>M</sub>3  | 1894                        | 8522                                        | 1576                      |

II). This result demonstrates that the increase in the binding of <sup>125</sup>I-EGF observed after sphingosine treatment is not a general phenomenon common to all cell surface receptors. The specificity of sphingosine to regulate the EGF receptor was further characterized by examination of the effect of treatment of A431 cells with sphingosine analogs (Table II). The increase in EGF binding caused by sphingosine was not observed when cells were treated with N-acetylsphingosine, glucosylsphingosine, galactosylsphingosine, or gangliosides (GM<sub>1</sub>, GM<sub>2</sub>, or GM<sub>3</sub>).

It has been shown that sphingosine analogs are cytotoxic when added to Chinese hamster ovary cells (23) and HL60 promyelocytic leukemia cells (24). We therefore investigated the growth of A431 cells in the presence of different concentrations of sphingosine (Fig. 8). It was observed that sphingosine, at concentrations that regulate the phosphorylation state of the EGF receptor, did not change that incorporation of <sup>3</sup>H]thymidine by logarithmically growing A431 cells. However, incubation of A431 cells with higher concentrations (>25 μM) resulted in the inhibition of <sup>3</sup>H]thymidine incorporation (Fig. 8). These results were confirmed by measuring the number of A431 cells after incubation with and without different concentrations of sphingosine for 48 h (data not shown). We conclude that the regulation of EGF receptor phosphorylation by low concentrations of sphingosine is not secondary to cytotoxic effects.

**Regulation of the EGF Receptor by Sphingosine in Phorbol Ester-treated A431 Cells—**Sphingosine inhibits protein kinase C activity and <sup>3</sup>H]PDBU binding in vitro competitively with phorbol ester and diacylglycerol (6). The data presented in Table II demonstrate that sphingosine added to the tissue culture medium inhibits the binding of <sup>3</sup>H]PDBU to A431 cells. This result strongly suggests that sphingosine is an inhibitor of protein kinase C in intact A431 cells. The stimulation of protein kinase C caused by the treatment of cells with phorbol ester (PMA) results in the inhibition of the high affinity binding of <sup>125</sup>I-EGF to A431 cells (for review see Ref. 1). The binding of <sup>125</sup>I-EGF to high affinity cell surface receptors can therefore be used as a functional assay for protein kinase C activity. Fig. 9 shows that sphingosine blocked the action of PMA to inhibit the high affinity binding of <sup>125</sup>I-EGF. This effect of sphingosine was dose-dependent and could be overcome by increasing the concentration of PMA. The competitive behavior of sphingosine with PMA is consistent with the effects of sphingosine on the binding of <sup>3</sup>H]PDBU to purified protein kinase C and on protein kinase C activity (6).

To investigate whether sphingosine inhibits protein kinase C in intact A431 cells the effects of sphingosine to regulate the phosphorylation of two protein kinase C substrates were examined. The major site of phosphorylation on the transferrin receptor (serine 24) is a protein kinase C substrate (8). Treatment of A431 cells with sphingosine caused an inhibition of the phorbol ester-stimulated phosphorylation of the transferrin receptor (Fig. 10). Tryptic [<sup>32</sup>P]phosphopeptide mapping confirmed that the action of sphingosine was to inhibit the phosphorylation of transferrin receptor serine 24 (data not shown). The state of phosphorylation of transferrin receptor serine 24 was inhibited by 74 ± 10% when cells treated with 10 nM PMA were incubated with 10 μM sphingosine (mean ± standard deviation, n = 3). In contrast to the inhibition of transferrin receptor phosphorylation caused by sphingosine, no significant effect of sphingosine to decrease
were then treated with 0.5 nM PMA for 15 min. Following this incubation the specific binding of 125I-EGF to cell-surface receptors was determined at 0 °C. B, effect of sphingosine concentration. A431 cells were treated with different concentrations of sphingosine for 15 min at 37°C. The cells were then treated with 0.5 nM PMA (C) or 5 nM PMA (D) for 15 min. Subsequently, the specific binding of 100 pm 125I-EGF to cell-surface receptors was determined at 0 °C. The results presented were obtained from single experiments and are the means of triplicate determinations. Similar results were observed in two separate experiments.

FIG. 9. Antagonism of phorbol diester action by sphingosine. The regulation of 125I-EGF binding by an activator of protein kinase C (PMA) and by sphingosine was investigated. A, effect of PMA concentration. A431 cells in 16-mm wells were incubated for 15 min with 10 μM N-acetylsphingosine (○) or with 10 μM sphingosine (●) at 37°C. The cells were then treated with different concentrations of PMA for 15 min. Following this incubation the specific binding of 100 pm 125I-EGF to cell-surface receptors was determined at 0 °C. B, effect of sphingosine concentration. A431 cells were treated with different concentrations of sphingosine for 15 min at 37°C. The cells were then treated with 0.5 nM PMA (C) or 5 nM PMA (D) for 15 min. Subsequently, the specific binding of 100 pm 125I-EGF to cell-surface receptors was determined at 0 °C. The results presented were obtained from single experiments and are the means of triplicate determinations. Similar results were observed in two separate experiments.

FIG. 10. Regulation of EGF and PMA-stimulated phosphorylation of the EGF receptor by sphingosine. A431 cells were incubated with [32P]phosphate for 24 h and subsequently incubated with and without 10 μM sphingosine for 28 min. Some of the cells were then incubated with 100 nM EGF or 100 nM PMA for 2 min. The EGF and transferrin receptors were isolated by immunoprecipitation and polyacrylamide gel electrophoresis. The figure presents the results of autoradiography of the dried gels. Similar results were obtained in four separate experiments.

the overall incorporation of [32P]phosphate into the EGF receptor of PMA-treated A431 cells was observed (Fig. 10). Further examination of the phosphorylation state of the EGF receptor by tryptic [32P]phosphopeptide mapping (Fig. 11) demonstrated that sphingosine caused a decrease in the phosphorylation of the peptide that contains the protein kinase C substrate site on the EGF receptor (threonine 654). In three experiments it was observed that 10 μM sphingosine inhibited the phosphorylation of EGF receptor threonine 654 in A431 cells incubated with 10 nM PMA by 53 ± 18% (mean ± standard deviation). These data strongly suggest that exogenously added sphingosine acts as an inhibitor of protein kinase C in intact A431 cells. The average inhibition of EGF receptor threonine 654 phosphorylation was not as great as that observed for transferrin receptor serine 24. As A431 cells express 2–3 × 106 EGF receptors and 1 × 105 transferrin receptors/cell, it is possible that the more effective decrease in transferrin receptor phosphorylation may be due to the lower level of receptor expression. It is also possible that the difference is due to the cellular localization of these receptors because sphingosine causes an increase in the cell surface expression of EGF receptors and a decreased level of expression of the transferrin receptor (Table II).

Regulation of the EGF Receptor by Sphingosine in EGF-treated A431 Cells—Treatment of A431 cells with EGF caused an increase in the phosphorylation state of the EGF receptor (Fig. 10) at many sites, including threonine 654 and tyrosine 1173 (Fig. 11). Addition of sphingosine to EGF-treated cells caused a decrease in the level of threonine 654 phosphorylation (Fig. 11). Previously, it has been proposed (25, 26) that the EGF-stimulated threonine 654 phosphorylation (27) is a mechanism of receptor desensitization because this phosphorylation is associated with a decrease in the receptor tyrosine protein kinase activity (28, 29). Inhibition of threonine 654 phosphorylation caused by sphingosine would therefore be predicted to decrease the desensitization of the EGF receptor tyrosine protein kinase. Consistent with this proposal, it was observed that sphingosine caused an increase in the EGF-stimulated phosphorylation of the receptor at tyrosine 1173, the major site of autophosphorylation (Fig. 11).

DISCUSSION

Sphingosine is a potent inhibitor of protein kinase C activity in vitro (6). The results obtained from the studies described in this report demonstrate that sphingosine is also a potent inhibitor of protein kinase C in intact A431 cells. This conclusion is based upon the following observations. First, treatment of A431 cell monolayers with sphingosine caused an inhibition of the binding of [3H]PDBU to cellular receptors (Table II). Second, sphingosine inhibited the phosphorylation of two protein kinase C substrates (EGF receptor threonine 654 and transferrin receptor serine 24) in A431 cells treated with phorbol ester (Figs. 10 and 11). Third, sphingosine blocked the regulation of the binding of 125I-EGF to high affinity sites caused by PMA (Fig. 9).

It has been demonstrated that sphingosine is an inhibitor of protein kinase C in vitro that is competitive with diacylglycerol and phorbol ester (6). Evidence for this competitive interaction in intact cells is provided by the dose-dependent inhibition of PMA regulation of the EGF receptor affinity by sphingosine (Fig. 9). Additional studies revealed that the effects of sphingosine were only partially reversed by PMA (Fig. 9). These data suggest that sphingosine regulates the EGF receptor by a mechanism that is independent of protein kinase C. Two lines of evidence support this conclusion. First, sphingosine caused an increase in the affinity of the EGF receptor in control A431 cells under conditions where the stoichiometry of threonine 654 phosphorylation is extremely low (Figs. 4 and 5). Second, the replacement of threonine 654 by an alanine residue did not significantly alter the regulation of the cell surface binding of EGF by sphingosine (Fig. 7). Together these data strongly suggest that the regulation of EGF binding to cell-surface receptors by sphingosine is not mediated by the modulation of the phosphorylation state of threonine 654. Furthermore, these data indicate that sphingosine is a potently bioactive molecule. Consequently, the use of sphingosine as a specific inhibitor to pharmacologically dissect protein kinase C-linked signaling pathways may be
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FIG. 11. Characterization of the regulation of EGF- and PMA-stimulated EGF receptor phosphorylation by [32P]phosphopeptide mapping. The effects of sphingosine on the phosphorylation state of the EGF receptors in EGF-treated and PMA-treated A431 cells were examined. [32P]Phosphate-labeled EGF receptors were prepared as described in the legend to Fig. 10. The gel slices containing the EGF receptors were excised. After elution of the receptors from the gel slices, [32P]phosphopeptides were prepared by trypsin digestion. The [32P] phosphopeptides were analyzed by reverse-phase HPLC and detected after elution from a C18 column with a linear gradient of acetonitrile by measuring the associated Cerenkov radiation. The results of [32P]phosphoamino acid analysis of fractions collected after elution from the column are presented: S, phosphoserine; T, phosphothreonine; Y, phosphotyrosine. Phosphopeptides containing known sites of phosphorylation, threonine 654 (3) and tyrosine 1173 (32), elute at 6 and 16% acetonitrile from the reverse-phase HPLC column, respectively. Similar results were obtained in four separate experiments.

limited by its other biological activities.

The effect of sphingosine to increase the affinity of the EGF receptor suggests that sphingosine causes a conformational change in the extracellular domain of the receptor. This conformational change might extend to the cytoplasmic domain of the receptor. If it does, this could account for the increase in the phosphorylation state of the receptor that occurs in sphingosine-treated cells. A conformational change may cause a unique intracellular threonine residue to be a better substrate for a protein kinase or a worse substrate for a protein phosphatase. Alternatively, it is possible that sphingosine acts by stimulating a protein kinase or by inhibiting a protein phosphatase. An objective of future experiments will be to test these hypotheses and determine the mechanism by which sphingosine stimulates protein phosphorylation in A431 cells.

Sphingosine causes the threonine phosphorylation of a unique EGF receptor tryptic peptide (Fig. 4). This residue is also phosphorylated when cells are incubated with EGF, phorbol ester, or with PDGF (3, 5), but it is not a substrate for phosphorylation by protein kinase C.2 We estimate the stoichiometry of the phosphorylation of this site to be approximately 15, 35, 90, and 90% in control, sphingosine-treated, phorbol ester-treated, and EGF-treated cells, respectively.

Sphingosine was only observed to significantly increase the phosphorylation state of this site in control cells (Fig. 4) and was not observed to increase phosphorylation in EGF-treated and phorbol ester-treated cells (Fig. 11). The lack of an effect on the phosphorylation of this site in EGF- and phorbol-stimulated cells is probably because of the high stoichiometry of phosphorylation observed under these conditions. These data demonstrate that phorbol ester mimics the effect of sphingosine to cause phosphorylation of a site on the EGF receptor that is not a protein kinase C substrate. This similarity in the actions of sphingosine and phorbol ester at this novel site of threonine phosphorylation is in marked contrast to the antagonistic interactions observed for protein kinase C regulation (6, 7) and the phosphorylation of the EGF receptor at threonine 654 (Fig. 11). The ability of phorbol ester to partially mimic sphingosine action suggests that phorbol esters may have actions that are independent of protein kinase C. Alternatively, the regulation of protein kinase C by phorbol ester and sphingosine may be different for individual protein kinase C isozymes.2

2 R. J. Davis, unpublished observations.

Recently, Ido et al. (33) reported that A431 cells express only a single protein kinase C isozyme. It is therefore unlikely that isozyme-specific effects of sphingosine can account for the contrasting actions of sphingosine to mimic some actions of PMA and to antagonize other actions of PMA.
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A small increase in the phosphorylation of tyrosine 1173 was observed in sphingosine-treated cells (Fig. 4). This effect of sphingosine to increase tyrosine phosphorylation was more marked in EGF-stimulated cells (Fig. 11). Three mechanisms can account for the action of sphingosine to increase the tyrosine phosphorylation of the EGF receptor. First, the tyrosine phosphorylation may be secondary to the inhibition of threonine 654 phosphorylation caused by sphingosine. This mechanism is plausible because it has been demonstrated that the phosphorylation of threonine 654 results in the inhibition of the receptor tyrosine kinase activity (for review see Ref. 1). Second, the increased EGF-stimulated tyrosine phosphorylation may be caused by the increased affinity and number of cell surface EGF-binding sites in sphingosine-treated A431 cells. Third, sphingosine could increase tyrosine phosphorylation of the EGF receptor by activating the receptor kinase. In uitro experiments demonstrate that the EGF receptor kinase is activated by sphingosine.

The data presented in this report indicate that sphingosine potently regulates the EGF receptor by a protein kinase C-dependent and a protein kinase C-independent pathway. These actions were not observed when the effects of other sphingolipids were examined (Table II). Thus, the affinity of the EGF receptor was not regulated by N-acetylsphingosine, glucosylsphingosine, galactosylsphingosine, or by gangliosides (GM₃, GM₁₃, and GM₁₄). We cannot conclude from this result that these sphingolipids are inactive because it is possible that these molecules are not effectively delivered to the correct physiological cell compartment when added to the tissue culture medium. Some evidence that strongly supports this hypothesis is provided by the effects of lysoglycosphingolipids to inhibit the binding of [³H]PDBU to intact A431 cells. While lysoglycosphingolipids inhibit the binding of [³H]PDBU to purified protein kinase C in uitro (6, 7) this action was not observed with intact A431 cells (Table II). We conclude that further examination of the pharmacology of the regulation of the EGF receptor by sphingolipids is warranted in order to identify the biologically active molecules.

Recently, Bremer et al. (30) reported that incubation of immunosorbent EGF receptors with 0.35 mM ganglioside GM₃ causes a 64% inhibition of the EGF-stimulated autophosphorylation of the EGF receptor. These data suggest that sphingolipids (sphingosine, gangliosides, lysosphingolipids, or breakdown products) may play a role in the regulation of EGF receptor function in uitro.

We have identified sphingosine as a biologically active molecule that inhibits protein kinase C and also regulates the cell-surface receptors for EGF by a mechanism that is independent of protein kinase C. Treatment of A431 cells with sphingosine causes an increase in the affinity and phosphorylation state of the EGF receptor. It is possible that these pharmacological actions of sphingosine reflect a physiological or pathophysiological role for this molecule in the regulation of the EGF receptor. The major pools of sphingolipids in cells are present as gangliosides, ceramides, and sphingomyelin. Sphingosine/lysosphingolipids are probably derived by sphingolipid breakdown (31). The level of long-chain sphingoid bases in A431 cells could therefore be regulated by the metabolism of any of the sphingolipid classes. Merrill et al. (24) have estimated the level of sphingosine in HL60 human promyelocytic leukemia cells to be 12 pmol/10⁶ cells. Fig. 2 shows that the maximum stimulation of EGF receptor phosphorylation occurs when 10⁻⁶ A431 cells are treated with 1 ml of 5 µM sphingosine. This is equivalent to 5 nmol/10⁶ cells and is greatly in excess of the level observed in HL60 cells. However, in this calculation we make the assumption that all of the sphingosine in the tissue culture medium is correctly delivered to the appropriate physiological compartment (without being metabolized) where it can act to regulate the EGF receptor. This is unlikely because Merrill et al. (24) have reported that the half-time for the uptake of [³H]dihydrosphingosine by HL60 cells is 0 h. This slow rate of accumulation is in marked contrast to the rapid effect of sphingosine (maximal at 2 min) to regulate the affinity of the EGF receptor (Fig. 5). These data suggest that the intracellular level of sphingosine in A431 cells necessary to regulate the EGF receptor may not be beyond the physiological range. In order to investigate the putative physiological role of sphingosine in A431 cells, it will be necessary to perform direct measurements of the level of sphingosine in cells cultured under different conditions.

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