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Potentiation of Opiate Action in Neuroblastoma N18TG2 Cells by Lipid Incorporation

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SUMMARY

The effect of cerebroside sulfate, phosphatidylserine, and other phospholipids on opiate receptor function in neuroblastoma N18TG2 cells was studied by incorporation of lipids into the membrane bilayer of viable cells. A concentration- and time-dependent incorporation of sulfatide by N18TG2 cells was observed. The incorporated lipid was not metabolized during the incubation period of up to 48 hr at 37°. Optimal conditions for lipid incorporation were determined to be 4 days after the cell seeding and in 1% fetal calf serum. The incorporated lipid was established to be associated with the plasma membrane fraction of the crude cell homogenate. Furthermore, increases in V_{max} but not K_m values of the adenylate cyclase for Mg^{2+}, ATP, and prostaglandin E_1 were observed in neuroblastoma N18TG2 cells exposed to cerebroside sulfate for 4–6 hr. The incorporation of cerebroside sulfate or phosphatidylserine by N18TG2 cells did not increase the number of opiate binding sites in this cell line as determined by [^{3}H]naloxone, [^{3}H]etorphine, or ^{3}H-labeled D-Ala^2-Met^5-enkephalinamide binding. Although there was an increase in the affinity of [^{3}H]naloxone binding, linear correlation between the amount of cerebroside sulfate incorporated and the quantity of binding increase was not observed. However, augmentation of both the potencies and the efficacies (maximal inhibitory level) of morphine and enkephalin to regulate adenylate cyclase activity was observed after sulfatide incorporation. At the maximal concentration of cerebroside sulfate used (67 μM) the opiate receptor activity in N18TG2 cells approached that of NG108-15 cells. Identical treatment of N18TG2 cells with cerebroside or psychosine sulfate did not produce any potentiation of the opiate inhibition of adenylate cyclase. Of all of the phospholipids tested—phosphatidylserine, phosphatidylinositol, and phosphatidylcholine—only phosphatidylcholine produced a potentiation of the opiate effect. Both synthetic dipalmitoyl phosphatidylcholine or brain phosphatidylcholine could elicit the potentiation.

INTRODUCTION

In the preceding paper (1), the presence of opiate activity in neuroblastoma N18TG2 cells was demonstrated. Contrary to earlier reports by Klee and Nirenberg (2) and Blosser *et al.* (3), we have demonstrated the existence of a high-affinity, steroespecific opiate receptor in plasma membrane of N18TG2 cells (4). Furthermore, both basal and PGE_1[3]-stimulated increases in adenylate cyclase activity were observed to be inhibited by Met^5-enkephalin in the whole cells and membrane preparations of N18TG2 cells. A linear correlation between the potencies of various opiate agonists to inhibit adenylate cyclase activity in N18TG2 and NG108-15 cells existed, and the opiate inhibition in these two cell lines responded similarly to the guanine nucleotide GTP and monovalent cations, in particular, sodium ions.

However, there was one major difference between the opiate inhibition of adenylate cyclase activity in N18TG2 cells and that in NG108-15 cells. Namely, all of the opiate agonists tested have a significantly lower efficacy level (the maximal percentage of the basal and PGE_1-stimulated adenylate cyclase activity being inhibited) in the N18TG2 cells than in the NG108-15 cells. Such a difference in efficacy levels could be due to differences in (a) receptor affinity and/or density; (b) density of the N-component, the GTP-binding regulator component of adenylate cyclase; and/or (c) the coupling mechanisms between receptor and adenylate cyclase molecules. It is unequivocal that the densities of opiate receptors are not

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[3] The abbreviations used are: PGE_1, prostaglandin E_1; PS, phosphatidylserine; CS, cerebroside sulfate; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; IBMX, 3-isobutyl-1-methylxanthine; ZK62711, 4-(3-cyclopentyloxy-4-methoxyphenyl)-2-pyrrolidone; TCA, trichloroacetic acid; SLV, sonicated lipid vesicles; Gpp(NH)p, guanyl-5'-yl-imidodiphosphate; PE, phosphatidylethanolamine; PC, phosphatidylcholine; PI, phosphatidylinositol.

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identical in cell lines N18TG2 and NG108-15 (1, 4). Whether the density of the N-components in these two cell lines is the same requires future investigation. The data obtained from receptor inactivation (5) suggested that factors other than receptor density could regulate the opiate receptor activity in these neuroblastoma cell lines.

There are numerous factors which can modulate receptor coupling with adenylate cyclase. The membrane lipids have been demonstrated to affect the ligand-receptor interaction of many receptors, e.g., the nicotinic receptor (6, 7), the tetradotoxin binding sites (8), *beta*-adrenergic receptor (9), and human growth hormone binding sites (10). Furthermore, membrane lipids also affect the coupling of the ligand-receptor complex with the adenylate cyclase molecule. Glucagon receptor-mediated (11–13) or *beta*-adrenergic receptor-mediated (14–18) stimulation of adenylate cyclase activity in various target cellular membranes was affected by the membrane lipid alterations. Thus, by either altering the receptor-agonist interaction or coupling the receptor with the adenylate cyclase, membrane lipid could modulate the receptor activity.

Although in their studies the acute opiate effect was not altered, Wilkening and Nirenberg (19) reported that the chronic effect of opiate in neuroblastoma × glioma NG108-15 hybrid cells was affected by unsaturated fatty acids. With the ultrasensitivity of opiate receptor binding toward phospholipase A_2 (20) and phospholipase C (21), and evidence for the possible involvement of PS (21–23) or CS (24–26) in opiate receptor-ligand interaction, membrane lipids, especially the acidic lipids, might play a pivotal role in opiate regulation of adenylate cyclase activity in NG108-15 and N18TG2 cell lines. Thus, in the present communication, the lipid effect on opiate activity was studied. Lipids were incorporated into the cellular membrane of N18TG2 cells, and the subsequent effect on opiate receptor binding and opiate inhibition of adenylate cyclase activity was investigated.

METHODS

Cell lines. The initial stock cultures of mouse neuroblastoma N18TG2, rat glioma C6BU1, and neuroblastoma × glioma NG108-15 hybrid cells were generous gifts from Dr. B. Hamprecht (Physiologischchemisches Institut des Universität, Wurzburg, Federal Republic of Germany). The growth preparation of the cell lines for experimentation was as described previously (4). The viability of the cell lines was monitored by the nigrosin dye exclusion method (27).

Measurement of adenylate cyclase activity. Adenylate cyclase activity in membrane preparations was determined by measuring the production of cyclic AMP from [α-^{32}P]ATP, as described in the preceding paper (1). The intracellular cyclic AMP level in viable cells was monitored by a modified protein binding assay as reported by Tovey *et al.* (28). The method of incubation with intact cells was analogous to that reported earlier (4). Briefly stated, 4 days before the experiments, neuroblastoma N18TG2 cells (10^5) were inoculated onto 60 × 15 mm pretreated sterile tissue culture plates containing 5 ml of growth medium. The day before the experiments, the medium was removed and 5 ml of DMEM plus 0.1 mM 6-thioguanine and 1% FCS were added. At the start of incubations, the growth medium was removed and the cells were washed twice with 5 ml of incubation medium (1). Subsequently, 5 ml of incubation medium were added, followed by the addition of the phosphodiesterase inhibitors IBMX (0.5 mM) and ZK62711 (0.1 mM). After incubation at 37° and 10% CO_2 for 30 min, reactions were initiated by the addition of 50 μl of PGE_1 (1 mM in 25% ethanol), 100 μl of opiates (in saline), or diluents. Incubations were carried out for 10 min at 37° and 10% CO_2. Afterward, the medium was removed and 2 ml of 8% TCA with ^{3}H-labeled cyclic AMP (2000 cpm) were added. The TCA was then removed by ether extraction, and the intracellular cyclic AMP level was determined after partial purification of the cyclic nucleotide in the lyophilized extract with Dowex-1 column chromatography; the subsequent protein binding assay was carried out as described by Tovey *et al.* (28).

Opiate receptor binding assay. Opiate receptor binding assays were carried out with the pellets obtained by centrifugation at 22,000 × *g* for 20 min or with washed intact cells as described previously (4). The membranes or cells were incubated at 24° for 1 hr with the radioactive ligands, and incubations were terminated by collecting the membranes or cells on Whatman GFB glass-fiber filters. Excess nonspecifically bound radioactivity was removed by washing the filters 3 times with 5 ml of 25 mM 4(2-hydroxyethyl)-1-piperazineethanesulfonic acid (pH 7.7). Specific binding was determined by measuring the difference in the amount of radioactivity bound to membrane in the absence and in the presence of 5 μM levorphanol.

Lipid incorporation. Neuroblastoma N18TG2 cells were treated with various acidic and neutral lipids under growth conditions for various periods of time. Lipids were added to N18TG2 growth medium containing 1% fetal calf serum as SLV. SLV were prepared by first drying a chloroform-methanol (2:1) lipid solution at the bottom of a 30-ml Corex tube with a stream of N_2. The lipids were then resuspended in saline in a final concentration of 1–2 mg/ml. The SLV were then prepared by sonicating 4 ml of lipid suspension with a Heath sonicator W220-F fitted with a standard tip at the maximal output of the probe for 10 min at 4°. After sonication, the lipid suspension was translucent, and minute amounts of lipid vesicles settled after 24 hr at 0°. The SLV were used immediately after preparation.

Other biochemical methods. The lipid composition of the membrane and the amount of lipid incorporated into membrane were determined by extracting polar lipids according to the method of Bligh and Dyer (29). Individual lipids were resolved from one another by thin-layer chromatography on silica gel G plates developed in solvent mixtures as described by Rouser (30). Concentrations of phospholipids were determined by hydrolyzing the organic phosphate with 70% perchloric acid at 160° for 2 hr, and the phosphate content was measured by the method of Ames (31). Sulfatide concentrations were determined by the azure A partitioning method of Kean (32). Protein concentrations were measured by first solubilizing the membrane protein in a solution of 1 N NaOH-1% sodium dodecyl sulfate-water (1:1:1) at 60° for 15 min; protein was then determined by the method of Lowry *et al.* (33).

Materials. ^{3}H-Labeled cyclic AMP (40 Ci/mmole), [^{3}H]naloxone (19 Ci/mmole), and ^{3}H-labeled D-Ala^2-Met^5-enkephalinamide (46 Ci/mmole) were purchased from New England Nuclear Corporation (Boston, Mass.). [^{3}H]Etorphine (42 Ci/mmole) and [α-^{32}P]ATP (10–40 Ci/mmole) were supplied by Amersham/Searle (Arlington Heights, Ill.). [^{3}H]CS (3 Ci/mmole) was prepared by Dr. C. T. Peng (School of Pharmacy, University of California, San Francisco) at Lawrence Laboratory (Berkeley, Calif.) and was purified by thin-layer chromatography. All nonradioactive reagents were supplied by Sigma Chemical Company (St. Louis, Mo.). Precoated silica gel G plates were obtained from EM Laboratory, Inc. (Elmsford, N.Y.). All purified or synthetic lipids were obtained from Supelco, Inc. (Bellefonte Park, Pa.). Partially purified binding protein components of the protein kinase from beef muscles were generous gifts from Dr. C. Grämsch (Max Planck Institüt für Psychiatrie, München, Federal Republic of Germany). ZK62711 was a generous gift from Schering (Berlin, Federal Republic of Germany).

RESULTS

Incorporation of lipid by neuroblastoma N18TG2. As shown in Fig. 1, there was a sulfatide concentration-dependent increase in the amount of lipid retained by the cells after 2 hr of exposure to sonicated vesicles. Over 10% of sulfatide added to N18TG2 cells in the 60-mm plates was incorporated by the cells under the experimental conditions. With the highest concentration of CS added, 400 μg/5 ml of medium in the 60-mm plates, the amount of lipid retained by neuroblastoma N18TG2 cells did not reach saturation. The incorporation of the sulfatide was time-dependent (Fig. 2). After subtracting the amount of CS adsorbed by the cells at zero time, a 6-fold increase in CS incorporated by N18TG2 cells after 25 hr was observed as compared with that after 30 min. The increase in CS incorporated exceeded the generation time of N18TG2, which was determined to be 24 hr in 10% FCS.

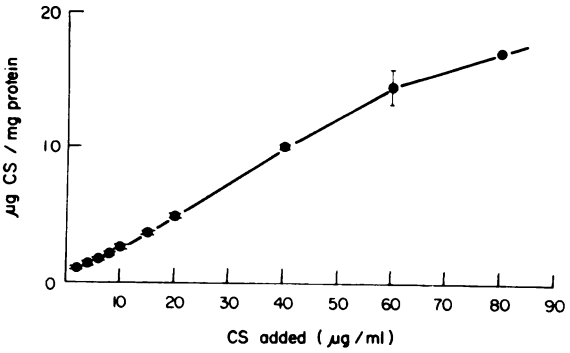

FIG. 1. *CS concentration-dependent sulfatide incorporation by neuroblastoma N18TG2 cells*

N18TG2 cells were cultured in 60-mm Petri dishes in DMEM + 0.1 mM 6-thioguanine + 10% FCS as described previously (4). On day 4 of culturing, the medium was changed to DMEM + 0.1 mM 6-thioguanine + 1% FCS. [^{3}H]Sulfatide, specific radioactivity 1.4 × 10^5 cpm/mg of lipid (tritium counting efficiency 30%), was added to the growth medium. After exposing the cells to sulfatide for 2 hr, the medium was removed and the cells were killed by the addition of 2 ml of 10% TCA. Excess sulfatide was removed by twice washing cells adhering to the surface of the Petri dishes with 5 ml of 10% TCA. The cells were then redissolved in 2 ml of 1 N NaOH by heating at 60° for 15 min. Aliquots were then removed for protein determination and measurement of radioactivity. The values represent the average from three separate plates.

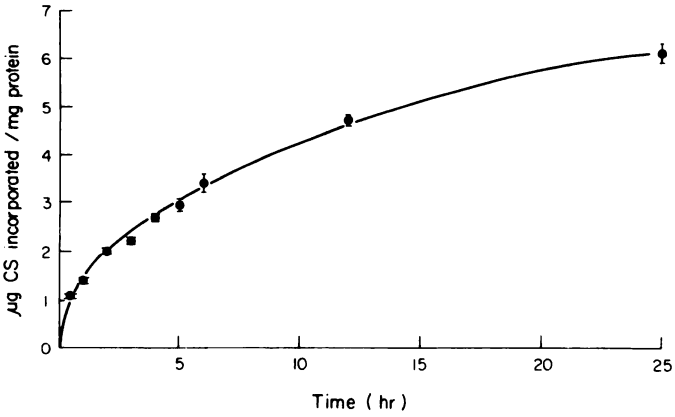

FIG. 2. *Time course of sulfatide incorporation*

The growth of neuroblastoma N18TG2 cells and the treatment with [^{3}H]CS were as described in the legend to Fig. 1. In order to ensure the same number of cells at the termination of the experiment, CS was added to neuroblastoma cells at various periods of time. All time points were terminated simultaneously. The cells were killed with 10% TCA, and excess sulfatide removed by TCA washes as described in Fig. 1. The values represent the average from three separate plates subtracted from the amount of CS adsorbed by the cells at zero time.

The amount of CS incorporated was reported to be dependent on the age of the culture (34); the amount of lipid incorporated was inversely proportional to the number of cells in culture plates. It was observed also that the amount of sulfatide being incorporated by N18TG2 cells was dependent on the FCS concentrations in the medium. When the concentration of FCS in the medium was increased from 1% to 10%, there was an exponential decrease in the amount of CS incorporated.

The integrity of the CS incorporated was not altered by neuroblastoma cells during incubation at 37°. After N18TG2 cells were exposed to 40 μM CS for 6 hr, membrane lipids were extracted and the mixtures were separated by thin-layer chromatography. As shown in Fig. 3a, the CS incorporated by N18TG2 cells under these conditions had mobility similar to that of standard lipid on silica gel G plates as visualized by the iodine vapor technique. Both the standard and extracted sulfatide produced doublets, which represented the hydroxy and the nonhydroxy form of the sulfatide. When the radioactivity of 0.5-cm fractions of the chromatograms was determined, it was observed that the thin-layer chromatographic pattern of the sulfatide incorporated by N18TG2 cells was similar to that of standard CS (Fig. 3b). The R_F values of incorporated CS were analogous to standard sulfatide in all of the solvent mixtures used. Comparable observations were obtained with the CS incorporated by the N18TG2 cells after 48 hr.

When the crude homogenate of N18TG2 cells was fractionated, the incorporated CS was located at the plasma membrane of the cell. Seventy per cent of the radioactive CS incorporated by N18TG2 cells after 6 hr was associated with the P_2 fraction (22,000 × g for 20 min), the subcellular fraction which contained the highest activity of opiate receptor binding (4), and all of the

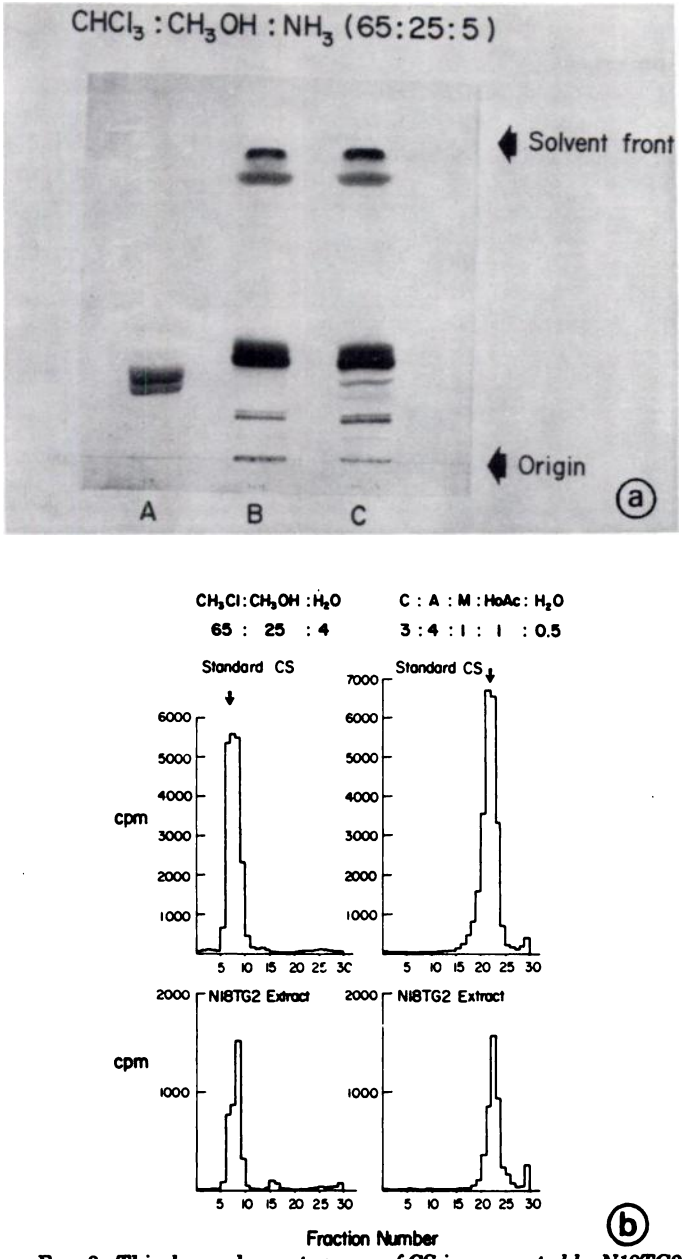

FIG. 3. *Thin-layer chromatograms of CS incorporated by N18TG2 cells*

Neuroblastoma N18TG2 cells were treated for 6 hr with 40 μM [^{3}H]CS in 60-mm plates containing growth medium + 1% FCS. Afterward, polar lipids were extracted as described under Methods.

a. The lipid extract was resolved by thin-layer chromatography in silica gel G developed in a solvent mixture of chloroform/methanol/ammonia (65:25:5). The lipids were visualized by exposing the developed plates in an iodine vapor chamber. *Column A* presents the mobility of standard bovine brain CS. *Columns B* and *C* represent N18TG2 extract and CS-treated N18TG2 extract, respectively.

b. The chromatograms of standard [^{3}H]CS and CS extracted from N18TG2 cells were developed in solvent mixtures of chloroform/methanol/water (65:25:4) and chloroform/acetone/methanol/acetic acid/water (3:4:1:1:0.5). The chromatograms were divided into 0.5-cm fractions and the radioactivity of each fraction was determined. *Arrows* indicate the location of CS as visualized with iodine vapor.

adenylate cyclase activity. When the plasma membrane of neuroblastoma N18TG2 cells was isolated according to the method of Mathews *et al.* (35), the sulfatide radioactivity was recovered in the sucrose gradient fractions containing opiate receptor binding activity and adenylate cyclase activity (Table 1). Therefore, using opiate receptor binding and adenylate cyclase activity as markers for plasma membrane, it was demonstrated that sulfatide was incorporated into the plasma membrane of neuroblastoma N18TG2 cells.

Effect of sulfatide incorporation and adenylate cyclase activity. Since sulfatide has been demonstrated to be incorporated into the plasma membrane of neuroblastoma N18TG2 cells, the consequence of lipid incorporation on adenylate cyclase activity was investigated. As summarized in Fig. 4, after N18TG2 cells were treated with 10 μM CS for 8 hr, significant increases in both the basal intracellular cyclic AMP level and PGE_1-stimulated level were observed. The basal and the PGE_1-stimulated adenylate cyclase activities were increased 4.3-fold and 2-fold from the control level, respectively. This increase in adenylate cyclase activity was dependent on the CS concentration. When 5 μM CS was added to the medium, basal and PGE_1-stimulated adenylate cyclase activities were increased 1.4-fold and 1.3-fold from the control level, respectively.

The sulfatide-induced increase in adenylate cyclase activity in neuroblastoma N18TG2 cells was not observed when the SLV of CS were added to the enzyme assay mixtures. Addition of various concentrations of CS vesicles from 1 to 100 μM to the adenylate cyclase assay mixture prior to the initiation of the reaction did not augment the basal adenylate cyclase activity or the enzymatic activity in the presence of PGE_1-stimulated or Gpp(NH)p.

From analysis of the kinetic parameters of adenylate cyclase activity after sulfatide treatment, it was observed that the increase in enzymatic activity after lipid treatment was due to the increase in V_{max} values and not in K_m values. Linear regression analysis of the Eadie-Hofstee plot of the PGE_1-sensitive adenylate cyclase activities in control and sulfatide-treated membranes at various concentrations of ATP revealed an increase of V_{max} values without any alteration of the slope (Fig. 5). The V_{max} and K_m values of the enzyme after sulfatide treatment with various substrates are summarized in Table 2. Interestingly, the Gpp(NH)p-sensitive adenylate cyclase activity was not affected significantly by the sulfatide treatment.

Opiate receptor binding in neuroblastoma cells treated with lipids. When neuroblastoma N18TG2 cells were exposed to various concentrations of sulfatide for 6 hr, a sulfatide concentration-dependent increase in the amount of [^{3}H]naloxone specifically bound to the N18TG2 cells was observed (Fig. 6). However, the amount of [^{3}H]naloxone binding increase was measured in femtomoles per milligram of protein whereas the amount of CS being incorporated by the cells was measured in nanomoles per milligram of protein. Moreover, the increase in [^{3}H]naloxone binding reached a maximal level, 50 fmoles/mg of protein, with respect to the amount of CS incorporated. These data suggested that the incorporated CS produced an increase in the affinity of the opiate receptor and not in the number of binding sites for [^{3}H]naloxone. Scatchard analysis (36) of the [^{3}H]naloxone binding data revealed an increase in K_{diss} values with no apparent alteration in B_{max} values after CS

TABLE 1

Subcellular distribution of incorporated sulfatide in neuroblastoma N18TG2 cells

Neuroblastoma N18TG2 cells were treated with sulfatide (37 μM) for 6 hr as described under Methods. The plasma membranes of treated cells were prepared by the Zn^{2+} ion method of Mathews *et al.* (35). The opiate receptor binding activity and adenylate cyclase activity of individual fractions were determined as described under Methods. The final concentration of ^{3}H-labeled D-Ala^2-Met^5-enkephalinamide in reaction was 1 nM. The amount of sulfatide incorporated was monitored with [^{3}H]sulfatide, specific radioactivity 2300 cpm/nmole. The values in parentheses represent the percentage of total amount in crude homogenate.

	Protein	Sulfatide	^{3}H-labeled D-Ala^2-Met^5-enkephalinamide specific binding	Adenylate cyclase activity
	mg	*μmoles*	*fmoles*	*pmoles/min*
Crude homogenate	66.0 (100)	0.185 (100)	2945.6 (100)	57.9 ± 0.35 (100)
First sucrose gradient (8000 × *g*, 10 min)				
20–35%	4.6 (7.0)	0.013 (7.1)	206.0 (7.0)	4.62 ± 0.32 (8.0)
35–40%	4.4 (6.7)	0.011 (5.9)	227.4 (7.7)	1.89 ± 0.57 (3.3)
40–65%	35.3 (53.5)	0.085 (45.9)	2636.9 (89.5)	39.9 ± 0.64 (69.0)
Pellet	5.1 (7.7)	0.010 (5.6)	334.0 (11.0)	2.64 (4.6)
Second sucrose gradient (90,000 × *g*, 60 min)				
50–65%	15.5 (44.0)	0.037 (43.8)	605.4 (23.0)	16.4 ± 0.35 (41.1)
Pellet	11.7 (33.2)	0.026 (30.1)	203.6 (7.7)	4.59 ± 0.15 (11.5)

treatment (Fig. 7). The K_{diss} values of the control and CS-treated cells were 11.6 and 5.7 nM, respectively. The B_{max} values for the control and CS-treated cells were determined to be 185 fmoles/mg of protein and 206 fmoles/mg of protein, respectively.

The effect of CS incorporation on the agonist high-affinity binding was studied with [^{3}H]etorphine and ^{3}H-labeled D-Ala^2-Met^5-enkephalinamide. As shown in Fig. 8, Scatchard analysis of [^{3}H]etorphine binding to the control and CS-treated cell membrane preparations revealed similar K_{diss} and B_{max} values. There was no apparent increase in [^{3}H]etorphine binding sites in the N18TG2 cells treated with varying concentrations of sulfatide. The increase in the incubation temperature to 37°, which was above the phase transition temperature of the membrane, did not alter the [^{3}H]etorphine binding to the membrane after CS treatment. Sulfatide treatment did not alter ^{3}H-labeled D-Ala^2-Met^5-enkephalinamide binding also. Therefore, the etorphine and enkephalin binding sites in the neuroblastoma N18TG2 cells were not affected by the incorporation of CS.

When the PS content in neuroblastoma cells was increased by treating the cells with varying concentrations of PS in a procedure identical with that for CS treatment, there was no apparent increase in ^{3}H-labeled D-Ala^2-Met^5-enkephalinamide or [^{3}H]etorphine specific binding. Scatchard analysis of the binding data with the [^{3}H] enkephalin analogue revealed similar K_{diss} and B_{max} values for the control and PS-treated (40 μM for 6 hr) membranes. Therefore, PS, similar to the action of CS, did not alter the ^{3}H-labeled agonist binding.

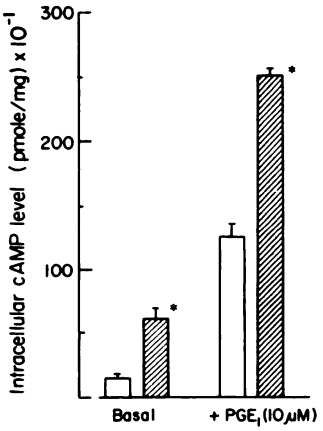

FIG. 4. *Increase in neuroblastoma N18TG2 adenylate cyclase activity by CS incorporation*

N18TG2 cells, on day 4 of culturing, were treated with 10 μM CS for 4 hr in 60-mm plates. The medium was then removed and the intracellular cyclic AMP level in the presence or absence of 10 μM PGE_1 was measured as described under Methods. *Open bars* and *hatched bars* represent intracellular cyclic AMP levels after 10-min incubations in the control and CS-treated cells, respectively. The values represent averaged cyclic AMP determinations from three individual plates. *Asterisks* signify p values < 0.01.

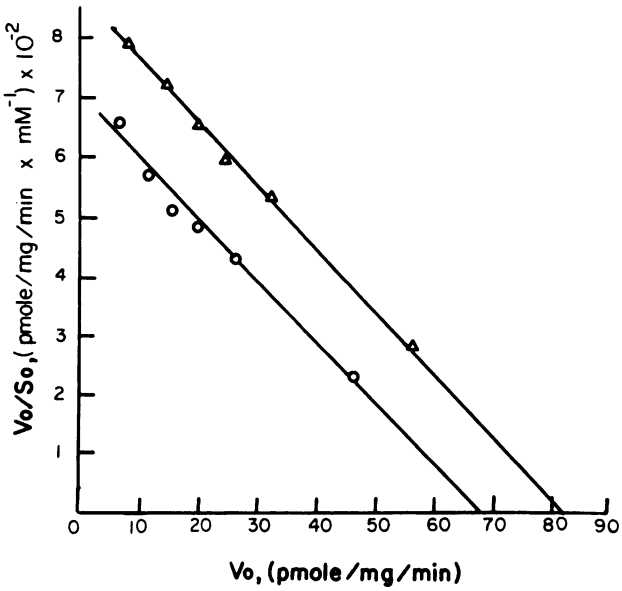

FIG. 5. *Eadie-Hofstee plot of PGE_1-sensitive adenylate cyclase activity in control (○—○) and CS-treated (△—△) N18TG2 membrane preparations*

N18TG2 cells were treated with 20 μM CS for 6 hr. Adenylate cyclase activity in the presence of various concentrations of ATP was measured. The final concentration of PGE_1 was 25 μM.

TABLE 2

Effect of sulfatide incorporation on the kinetic parameters of neuroblastoma N18TG2 PGE_1-sensitive adenylate cyclase activity

Adenylate cyclase activity in membrane preparations of N18TG2 cells treated with 20 μM sulfatide for 6 hr was compared with that of control membranes. When the kinetic parameters of Mg^{2+} were determined, the assays contained 0.1 mM ATP and 25 M PGE_1. In the case of ATP, the assays contained 5 mM Mg^{2+} and 25 μM PGE_1. For PGE_1 and Gpp(NH)p, the concentrations of ATP and Mg^{2+} in the assays were 0.1 mM and 5 mM, respectively. The values in parentheses represent percentage increase in the enzyme activity in sulfatide-treated membranes. * denotes $p \leq 0.025$ using the unpaired t-test. $N = 3$ in all experiments.

	K_m		V_{max}	
	Control	+ Sulfatide	Control	+ Sulfatide
	μM		pmoles/mg/min	
Mg^{2+}	350 ± 20	400 ± 10	24.3 ± 0.1	29.1 ± 0.3* (19.7%)
ATP	95 ± 0.3	93 ± 0.5	66.7 ± 1.1	81.2 ± 1.3* (21.7%)
+PGE_1	0.14	0.11	46.2 ± 0.5	55.7 ± 0.9* (20.6)
+Gpp(NH)p	4.3 ± 1.1	6.0 ± 0.5	20.9 ±0.9	19.8 ± 0.5

Effect of sulfatide incorporation on opiate inhibition of adenylate cyclase activity. In the reported membrane effect on receptor-mediated regulation of adenylate cyclase activity, either the membrane lipids could specifically restore the receptor activity after the phospholipase treatment (11, 12) or alteration of the membrane microenvironment could influence coupling efficiency (14, 15) or the nature (13) of coupling between receptor and adenylate cyclase. In our studies with opiate inhibition of adenylate cyclase activity in neuroblastoma × glioma NG108-15 hybrid cells, opiate activity was attenuated by phospholipase C hydrolysis of polar head groups (36a). Therefore, it is likely that alteration of membrane lipid composition in the neuroblastoma N18TG2 membrane could modify opiate inhibition of adenylate cyclase activity in this cell line.

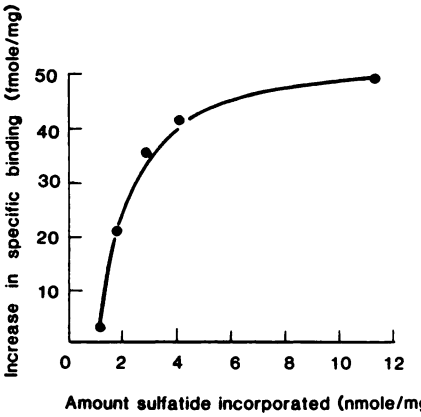

FIG. 6. *Correlation between amount of CS incorporated and increase in [^{3}H]naloxone binding*

Neuroblastoma N18TG2 cells cultured in 60-mm plates were treated with various concentrations of CS as described in Fig. 1. After exposure to CS for 6 hr, [^{3}H]naloxone (10 nM) binding to whole cells was then carried out as described in ref. 4. The specific binding of [^{3}H]naloxone to control cells was determined to be 110 fmoles/mg of protein. The amount of CS incorporated was determined using [^{3}H]CS as described in Fig. 1 in a parallel set of plates.

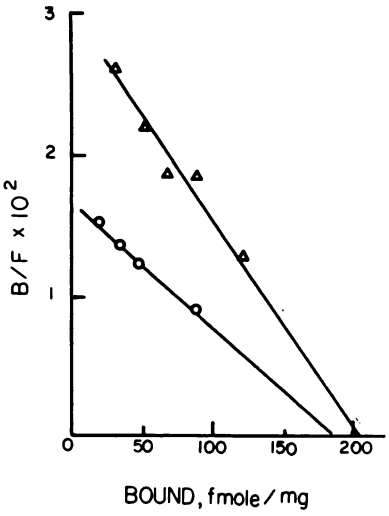

FIG. 7. *Scatchard analysis of [^{3}H]naloxone specific binding to neuroblastoma N18TG2 control cells (○—○) and cells treated with 56 μM CS for 6 hr (△—△)*

[^{3}H]naloxone binding to whole cells was measured as described in ref. 4.

As reported previously (2, 4), morphine sulfate did not inhibit either the basal or the PGE_1-stimulated adenylate cyclase activity in N18TG2 cells. Maximally, a 10% inhibition of the control level of intracellular cyclic AMP was observed at 20 μM morphine (Fig. 9). However, when neuroblastoma N18TG2 cells were exposed to 10 μM CS for 4 hr, a morphine concentration-dependent inhibition of the basal level (Fig. 9a) and a PGE_1-stimulated increase (Fig. 9b) in the intracellular cyclic AMP level of sulfatide-treated cells were observed. The maximal opiate inhibitory level (efficacy) was increased from 10% to greater than 70% also.

As summarized in Table 3, IC_{50} values of morphine sulfate to inhibit the PGE_1-stimulated increase in intracellular cyclic AMP levels decreased with respect to the concentration of CS present during the 4-hr pretreatment

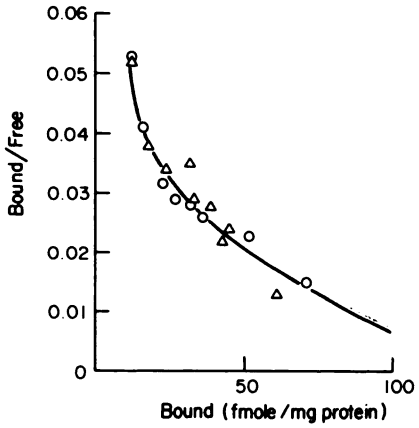

FIG. 8. *Scatchard analysis of [^{3}H]etorphine binding to neuroblastoma N18TG2 cells*

[^{3}H]Etorphine specific binding to membrane preparations of control N18TG2 cells (○—○) or cells treated with 80 μM CS for 6 hr in T-75-cm^2 flasks (△—△) was carried out as described in text. K_{diss} values of 0.3 nM and 2.4 nM and B_{max} values of 32 fmoles/mg of protein and 110 fmoles/mg of protein were obtained from regression analysis of the plot.

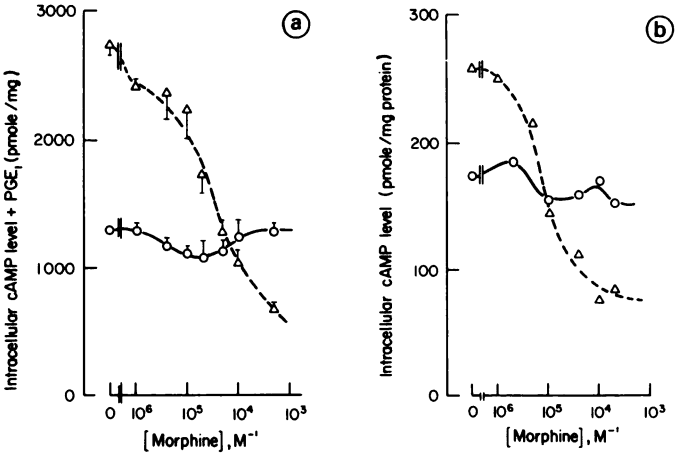

FIG. 9. *CS potentiation of morphine inhibition of intracellular cyclic AMP production in neuroblastoma N18TG2 cells*

N18TG2 cells were cultured for 4 days in 60-mm plates as described in text. On the day prior to experiments, the growth medium was completely changed into DMEM + 6-thioguanine + 1% FCS. On the morning of the experiments, cells were then treated with 10 μM CS-sonicated vesicles for 4 hr. Measurements of (a) basal and (b) 10 μM PGE_1-stimulated increase in the intracellular cyclic AMP level were carried out as described under Methods. Incubation time was 10 min. The inhibition of cyclic AMP production by various concentrations of morphine sulfate (1–500 μM) was determined. The symbols △- - -△ and ○—○, respectively, represent the adenylate cyclase activity in CS-treated and control cells in the presence of various concentrations of morphine. The values represent the averaged cyclic AMP determinations from three individual plates.

with lipid. At the highest concentration of CS used, 67 μM, the IC_{50} value of morphine to inhibit the adenylate cyclase activity in N18TG2 cells was found to be similar to that in the neuroblastoma × glioma NG108-15 cells, which was determined to be 500 nM (37).

The sulfatide potentiation of the opiate effect was not limited to the opiate alkaloid, morphine. Pretreatment of N18TG2 cells with varying concentrations of CS produced an analogous potentiation effect on enkephalin

TABLE 3

Sulfatide potentiation of morphine inhibition of the PGE_1-stimulated increase in the intracellular cyclic AMP level of N18TG2 cells

Culturing of neuroblastoma N18TG2 cells in 60-mm Petri dishes and preparation of cells for experimentation were as described under Methods. CS was added to the growth medium + 1% FCS as sonicated lipid vesicles form as described under Methods. The N18TG2 cells were then exposed to lipid for 6 hr and the morphine effect on the PGE_1-stimulated increase in the intracellular cyclic AMP level was measured. Morphine concentrations of 0.1 μM–500 μM were used in these experiments. The IC_{50} values were obtained by linear regression analysis of the log-logit plots of the inhibition data. Three individual 60-mm Petri dishes were used for each morphine concentration. NS indicates that a statistically insignificant inhibition was observed.

CS concentration	IC_{50}	Maximal inhibition
μM	μM	%
0	—	NS
10	25.1	51.2
20	7.6	68.5
45	0.74	75.5
67	0.58	89.7

TABLE 4

Sulfatide potentiation of Met^5-enkephalin inhibition of PGE_1-stimulated increase in intracellular cyclic AMP concentration levels of N18TG2 cells

The culturing and treatment of the neuroblastoma N18TG2 cells with CS were as described in the legend to Table 3. The concentrations of Met^5-enkephalin used in the present studies were 0.1 nM–1 μM.

CS concentration	IC_{50}	Maximal inhibition
μM	nM	%
0	97.3	58.5
5	14.1	55.6
20	4.5	64.8
30	1.2	87.2

activity (Table 4). Similar to the sulfatide potentiation of morphine effect, both potency and efficacy of enkephalin were increased by various concentrations of sulfatide. At the highest concentration of CS used, 30 μM, the IC_{50} value of enkephalin in N18TG2 cells was similar to that of the NG108-15 cells, which was reported to be 3 nM (37).

Parallel potention of opiate alkaloid activity in membrane preparation of the sulfatide-treated N18TG2 cells was observed. As shown in Fig. 10, in membrane preparation of the control cells, levorphanol possessed no measurable inhibitory activity on PGE_1-stimulated adenylate cyclase activity. However, when neuroblastoma N18TG2 cells were treated with 75 μM CS for 6 hr, a levorphanol concentration-dependent inhibition of the enzymatic activity was observed (Fig. 10). Although the magnitude of the levorphanol inhibition was minute (less than 15%) because there was no measurable levorphanol inhibition in the control membrane, the increase in efficacy level after CS treatment was significant ($p \leq 0.05$). The apparent IC_{50} value of levorphanol to inhibit PGE_1-

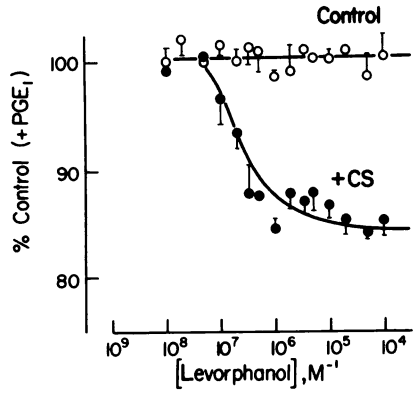

FIG. 10. *Potentiation of levorphanol inhibition of adenylate cyclase activity in neuroblastoma N18TG2 cells by CS incorporation*

N18TG2 cells were treated with 75 μM CS for 6 hr. Membrane fractions were then prepared as described under Methods. The adenylate cyclase activity of membrane preparations at various concentrations of levorphanol was determined. Tris salts of all reagents were used in the present assays. The symbols ○—○ and ●—● represent the percentage of adenylate cyclase activity in the control and CS-treated membranes, respectively, being inhibited by various concentrations of levorphanol. The specific activities of adenylate cyclase in the presence of 25 μM PGE_1 were determined to be 56.9 ± 0.8 and 67.9 ± 0.4 pmoles/mg per minute for the control and CS-treated membranes, respectively ($N = 3$).

stimulated increase in adenylate cyclase activity in the absence of sodium salts was determined to be 400 nM. This amount of levorphanol needed to produce 50% inhibition of adenylate cyclase activity in the CS-treated N18TG2 membrane compared favorably with that in the NG108-15 membrane preparations, which was determined to be 320 nM in the absence of sodium salts [figure 7 in the preceding paper (1)].

Potentiation of enkephalin activity in the membrane preparations was observed with the CS-treated cells also. However, in order to obtain a greater difference in control and lipid-treated groups, N18TG2 cells were treated with CS for a longer duration. Met^5-enkephalin inhibition of adenylate cyclase activity in the pellets obtained by centrifugation at 22,000 × *g* for 20 min was potentiated by treating the N18TG2 cells with 75 μM CS for 48 hr. The amount of Met^5-enkephalin required to elicit 50% of maximal inhibitory level in sulfatide-treated membranes decreased from a control value of 21.3 nM to 2.1 nM. The maximal enkephalin inhibitory level was increased to 40% from the 15% inhibitory level in control membranes also. Both potency and efficacy of enkephalin in CS-treated N18TG2 membranes compared favorably with those of the NG108-15 membranes.

The opiate activity in the CS-treated NG18TG2 cells was reversed by naloxone. As shown in Fig. 11, pretreatment of the N18TG2 cells with 20 μM CS for 6 hr produced a morphine-dependent inhibition of both basal and PGE_1-stimulated increase in intracellular cyclic AMP levels. Twenty-five micromolar morphine sulfate inhibited basal and PGE_1-stimulated increases in adenylate cyclase activity by 45% and 32%, respectively. This morphine inhibition in the CS-treated cells was completely reversed by 10 μM naloxone (Fig. 11). Naloxone also antagonized the Met^5-enkephalin inhibition. The IC_{50} value of the Met^5-enkephalin inhibition of PGE_1-stimulated increase in the intracellular cyclic AMP levels in the CS-treated N18TG2 cells was increased from 4.5 nM (Table 4) to 1.4 μM by 10 μM naloxone. The slopes of the log-logit plots of enkephalin inhibition in the presence or absence of naloxone were identical.

The increase in opiate activity in sulfatide-treated N18TG2 cells was not observed with neuroblastoma N18 cells or glioma C6BU-1 cells which did not possess any opiate binding activity (4). CS incorporation into N18 or C6BU1 did not produce any measurable opiate inhibition of the intracellular cyclic AMP level in these two cell lines.

Lipid specificity. Requirement of the complete CS molecule in the potentiation of opiate activity was demonstrated. As shown in Fig. 12, when neuroblastoma N18TG2 cells were pretreated with equal molar concentrations of CS, psychosine, or cerebroside SLV for 6 hr, potentiation of opiate inhibition was observed only with the CS-treated cells. In these experiments, the IC_{50} of morphine in CS-treated cells was determined to be 20 μM, which was comparable to that obtained in the experiments summarized in Table 3.

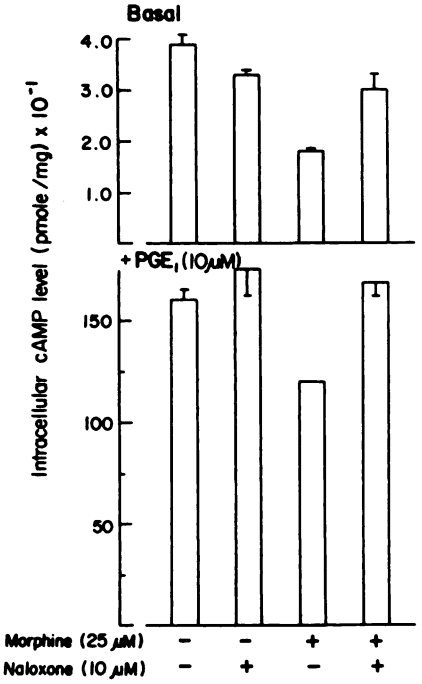

FIG. 11. *Naloxone reversal of morphine inhibition of adenylate cyclase activity in N18TG2 cells*

Neuroblastoma N18TG2 cells, cultured in 60-mm plates for 4 days, were treated with 20 μM CS for 4 hr. The basal and 10 μM PGE_1-stimulated increase in the intracellular cyclic AMP level was measured in the presence or absence of 25 μM morphine or 10 μM naloxone, or both, as described under Methods. The values represent averaged cyclic AMP determinations from three separate plates.

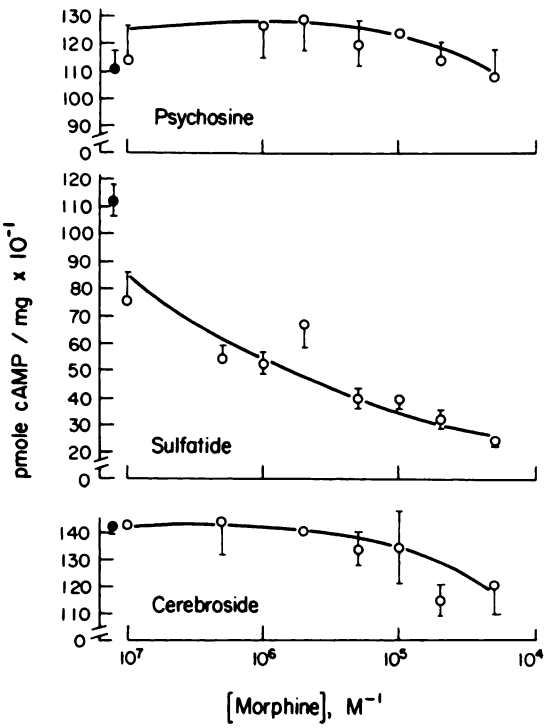

FIG. 12. *Effect of cerebroside and psychosine on morphine inhibition in neuroblastoma N18TG2 cells*

Neuroblastoma N18TG2 cells, cultured in 60-mm plates for 4 days, were treated with equal molar concentrations (10 μM) of psychosine, CS, or cerebroside for 4 hr. The intracellular cyclic AMP level in the presence of 10 μM PGE_1 at various concentrations of morphine were determined after the treatment. The symbol ● represents the intracellular cyclic AMP level in the absence of morphine. The values represent averaged cyclic AMP determinations from three individual plates.

TABLE 5

Incorporation of various phospholipids and their effect on morphine inhibition of adenylate cyclase activity in neuroblastoma N18TG2 cells

Neuroblastoma N18TG2 cells, cultured in 60-mm plates, were exposed to various lipids for 6 hr on day 4 of culturing as described under methods. Incubations with PGE_1 and morphine were as described in the text. The intracellular cyclic AMP level was measured by the protein kinase method. Values in parentheses represent the percentage of adenylate cyclase activity being inhibited by morphine. The averages represent cyclic AMP determinations from three individual plates.

Lipids	Intracellular cyclic AMP level	
	Control	+ 20 μM Morphine
	pmole/mg protein	
None	1447 ± 21	1398 ± 23
+CS (10 μM)	2204 ± 193	998 ± 84 (54.7%)
+PC (14 μM)	1738 ± 39	1872 ± 88
(56 μM)	1844 ± 124	1189 ± 80 (35.5%)
+PI (10 μM)	943 ± 32	1200 ± 123
+PS (10 μM)	1528 ± 153	1986 ± 112
(20 μM)	1905 ± 140	1878 (1.4%)

With all of the phospholipids tested, only PC demonstrated a potentiation of opiate inhibition of the PGE_1-stimulated increase in intracellular cyclic AMP levels (Table 5). At the higher concentration of PC (56 μM) the IC_{50} of morphine was determined to be 4.7 μM. The IC_{50} value of morphine in N18TG2 cells treated with comparable concentrations of CS was observed to be 0.7 μM (Table 3). There was no apparent dependence on the fatty acid composition of PC in the lipid potentiation of morphine effect. Both the synthetic dipalmitoyl PC and the PC extracted from bovine brain potentiated the morphine effect to a similar extent (data not shown). ^{3}H-labeled D-Ala^2-Met^5-enkephalinamide binding at 24° or 37° was not altered by the PC incorporation into N18TG2 membranes.

DISCUSSION

In the current studies, by exposing viable neuroblastoma N18TG2 cells to various polar lipid sonicated vesicles, a large quantity of lipids was incorporated into the cellular membrane of N18TG2 cells. [^{3}H]Sulfatide was incorporated in a concentration- and time-dependent manner. There was no apparent metabolism of the incorporated sulfatide by N18TG2 cells during the incubation period (Fig. 3). It was concluded that CS was incorporated by N18TG2 cells from observations that (a) the time-dependent increase in CS incorporation was greater than the rate of cell division, (b) the incorporated lipid increased membrane-bound adenylate cyclase activity, and (c) this augmentation of adenylate cyclase activity was not observed with the addition of CS-sonicated vesicles to adenylate cyclase reaction mixtures. Therefore, it was unlikely that sulfatide was adsorbed by the plasma membrane of N18TG2 cells via charge-charge or hydrophobic interaction.

Analogous to observations that phospholipids could enhance adenylate cyclase activity (11–13), incorporated CS did alter the membrane-bound enzyme activity; the V_{max} and not the K_m of adenylate cyclase activity was altered. However, opiate binding capacity in the CS-treated membrane remained similar to that of control. This was in contrast to the studies carried out in organic solvent-aqueous partitioning systems (22, 24, 25). In those studies, opiate alkaloids possessed high affinity for the acidic lipids CS and PS. The opiate ligand's affinity for acidic lipids in conjunction with observations on the sensitivity of receptor binding toward specific lipid degradative enzymes (20, 21, 26) suggested the possible involvement of acidic lipids in the recognition of opiate ligands. The possible involvement of membrane lipids was further substantiated by the recent successful [^{125}I] diazosulfanilic acid labeling of membrane polar lipids which were stereospecifically protected by opiate ligands from the nonradioactive diazosulfanilic acid (38). Possibly, polar lipid could be the putative lipid component of the protein-lipid model of the opiate receptor, as postulated by Lee and Smith (39). But in the current experiments, with a large quantity of CS or PS being incorporated by neuroblastoma N18TG2 cells, a concomitant increase in the number of binding sites was not observed. At best, only [^{3}H]naloxone binding affinity ($K_{diss} \geq 10$ nM) was increased by the incorporated lipids (Figs. 6 and 7). In contrast to the report by Abood and Takeda (23) in which brain synaptic membrane was used, incorporation of the acidic lipid PS did not produce any significant increase in opiate agonist binding as determined by ^{3}H-labeled D-Ala^2-enkephalinamide and [^{3}H]etorphine binding. The incorporation of polar lipids CS, PS, and PC did not produce any alteration of the number of opiate binding sites in the membrane of N18TG2 cells. Therefore, the results of current experiments clearly indicated that polar lipids per se could not be the recognition sites of the opiate ligands. This conclusion was further substantiated by experiments in which lipids incorporated by glioma C6BU1, the other parent cell line of neuroblastoma × glioma NG108-15 which contained no measurable opiate binding activity, did not produce any opiate binding activity (data not shown).

Although the incorporated CS did not increase the number of opiate receptor binding sites, sulfatide potentiated opiate inhibition of adenylate cyclase activity. Since CS potentiation was observed in the opiate inhibition of both basal and PGE_1-stimulated adenylate cyclase activity (Fig. 9), the lipid effect could not be due to the inhibition of PGE_1 receptor adenylate cyclase coupling. In the present studies, as in the previous reports (2, 4), morphine possessed little to no measurable inhibition of adenylate cyclase activity in neuroblastoma N18TG2 cells. The absence of morphine effect was not due to the inability of opiate receptors to couple to adenylate cyclase. Other opiate alkaloids and opioid peptides exhibited significant inhibitory activity in N18TG2 cells [see preceding paper (1)]. The current studies demonstrated that the inability of morphine to elicit significant inhibition of adenylate cyclase activity could not be due to the absence of insufficient opiate binding sites. The ability of prior treatment of sulfatide or PC to potentiate the opiate effect suggested that, by lipid treatment, the opiate receptor in the N18TG2 cells could be converted to a form which could better regulate adenylate cyclase activity.

The exact mechanism by which sulfatide and PC potentiated opiate receptor activity is unknown. It is possible that the addition of polar lipids, hence altering the

ratio of cholesterol to phospholipid, could change the fluidity of the membrane. There is no question that membrane fluidity could influence receptor activity. It has been reported with *beta*-adrenergic receptor in turkey erythrocytes that the increase in membrane fluidity resulted in the potentiation of *beta*-adrenergic receptor-mediated activation of adenylate cyclase (14–17). These observations supported the mobile receptor hypothesis. Although it can be demonstrated that opiate receptor in neuroblastoma NG108-15 and neuroblastoma N4TG1 cells can cluster (40), and hence its mobility in membrane bilayer is suggested, experimental data in support of membrane fluidity alteration of operate receptor activity have not been obtained. Wilkening and Nirenberg (19) reported the dependence of chronic opiate effect on unsaturated fatty acids. But in the same sets of experiments, the acute opiate effect, inhibition of adenylate cyclase activity, was not affected by the added unsaturated fatty acids. Sulfhydryl reagents, which inhibited clustering, did not inhibit opiate regulation of adenylate cyclase activity in N4TG1 (40). Hence, the lateral movement of the opiate receptor in the membrane bilayer might not be the rate-limiting factor in opiate inhibition of adenylate cyclase activity. On the contrary, the increase in membrane fluidity using standard techniques—fatty acid insertion or raising the incubation temperature to that which is above the phase transition temperature of the membrane—attenuated opiate inhibition of adenylate cyclase activity in NG108-15 cells.[4] Thus, most likely, the mechanism of CS or PC potentiation of the opiate effect in N18TG2 cells was not due to the increase of membrane fluidity.

There are data which suggest involvement of polar head groups of phospholipids in receptor action. The glucagon and *beta*-adrenergic receptor stimulation of adenylate cyclase activity was regulated by polar head groups in addition to membrane fluidity (14, 16, 17). Because of the ability of CS and not cerebroside to potentiate the opiate effect, analogous polar head group regulation of opiate inhibition of adenylate cyclase activity could be occurring in neuroblastoma cell lines. By removing polar head groups of phospholipids in NG108-15 cells with phospholipase C (*Clostridium welchii*), opiate inhibition of adenylate cyclase activity was attenuated.[4] Similar treatment with phospholipase A_2, which hydrolyzed the β-fatty acids, did not produce a decrease of opiate activity. These data clearly suggested the importance of the lipid's polar head groups in the microenvironment of the opiate receptor for its function.

Then, what are the possible mechanisms in which CS and PC polar head groups could influence opiate receptor activity? There are distinct differences in physical properties of the lipids sulfatide and PC. Potentiation of the opiate effect by these two lipids could not be due to the insertion of either lipid into membrane and subsequently producing an identical microenvironment for the receptor. Conceivably, these two lipids could mediate their potentiating effect via another membrane component. Two such possible components are the membrane-bound transmethylases I and II, i.e., the enzymes which methylated the PE with *S*-adenosylmethionine and converted the PE molecules in the inner bilayer to PC, which is in the outer bilayer. The transmethylases have been implicated in *beta*-adrenergic receptor action (18). Whether the transmethylases are the focal points of lipid action requires further experimentation.

[4] P. Y. Law and H. H. Loh, unpublished observations.

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