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The Effects of Exposure to Unsaturated Fatty Acids on Opiate Receptors, Prostaglandin E_1 Receptors, and Adenylate Cyclase Activity of Neuroblastoma × Glioma Hybrid Cells

RICHARD MCGEE, JR.,[1] AND JAMES G. KENIMER[2]

Department of Pharmacology, Georgetown University Schools of Medicine and Dentistry, Washingon, D. C. 20007, and Division of Drug Biology, Bureau of Drugs, The Food and Drug Administration, Washington, D. C. 20204

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SUMMARY

The effects of alterations in membrane fatty acid composition on receptors for opiates and prostaglandin E_1 (PGE_1) and on the activity of adenylate cyclase were studied in clonal neuroblastoma × glioma hybrid cells (NG108-15) following exposure to unsaturated fatty acids. Decreases in binding of [^{3}H]etorphine and [^{3}H]PGE_1 were observed in cells exposed to arachidonate (20:4), linoleate (18:2), and oleate (18:1). The observed decreases in binding (as much as 80% decrease under some conditions) were primarily associated with changes in the number of binding sites. The effects of 20:4 and 18:2 were more rapid and greater than those of 18:1. The decreases in binding approximately paralleled the time (6–72 hr) and concentration-dependent enrichment of the membranes with the exogenously supplied fatty acids. However, when 20:4-treated cells were switched to normal culture medium the number of binding sites returned to control values much more rapidly than did the phospholipid fatty acid composition. This observation suggests that decreases in receptor number were a result of changes occurring during the pretreatment with fatty acids (e.g., a decrease in receptor synthesis, a decrease in receptor insertion into the membranes, or increased removal of the receptors from the membranes); the apparent number of receptors was not determined by the membrane fatty acid composition at the time binding was measured. In contrast, the adenylate cyclase activity of the cells was unaffected by even very large changes in phospholipid fatty acid composition. Likewise, the regulatory response to chronic exposure of the cells to PGE_1 (decreases in both PGE_1 receptors and adenylate cyclase activity) was not affected by simultaneous exposure to PGE_1 and 20:4. Thus, the number of opiate and PGE_1 receptors was selectively altered by exposure of the cells to unsaturated fatty acids.

INTRODUCTION

Many different approaches have been used for studying the interaction between membrane-bound receptors and the lipids in which they reside (for a review see ref. 1). One approach has been to study the effects of lipolytic enzymes, such as phospholipases, on receptor/ligand interactions. In general, phospholipase treatment interferes with receptor/ligand interactions. For example, Abood *et al.* (2, 3) have observed that the specific binding of opiates to neural membranes is decreased by treatment with phospholipase A_2. The effects of phospholipase could be prevented by the presence of BSA,[3] presumably because of its ability to bind fatty acids and lysophospholipids formed as a result of enzymatic hydrolysis. Additional experiments suggested a specific requirement for acidic phospholipids such as phosphatidylserine in the activity of the opiate receptor (2, 3). Similar studies demonstrated a loss of muscarinic cholinergic receptor binding upon treatment with phospholipases, which could be restored by the addition of acidic phospholipids (4). The binding of glucagon to its receptor also was decreased by phospholipase A_2 treatment but not until 85% of the membrane phospholipids had been hydrolyzed (5). The *beta*-receptor of frog erythrocytes is sensitive to phospholipase treatment, as evidenced by decreased ligand binding and agonist-activated adenylate cyclase activity (6). Interestingly, similar changes also were induced by the membrane-perturbing antibiotic, amphotericin B. Finally, the acetylcholine receptor of *Torpedo californica* has been shown to be sensitive to phospholipase treatment, as evidenced by a decrease in agonist-induced Na^+ flux into membrane vesicles (7). However,

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[1] Georgetown University Schools of Medicine and Dentistry.

[2] Food and Drug Administration.

[3] The abbreviations used are: BSA, bovine serum albumin; PGE_1, prostaglandin E_1; GLC, gas-liquid chromatography; MOPS, morpholinopropanesulfonate.

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this decrease in receptor function was not a result of a decrease in the apparent number of binding sites but appeared to represent a conversion of the receptors to a high affinity, desensitized state.

A second approach to studying membrane protein/lipid interactions is to induce membrane lipid changes in tissue culture cells by exposing them to various lipids and membrane precursors. This technique has been applied primarily to studies of enzyme activities, such as receptor-mediated adenylate cyclase activity, without extensive characterization of receptor/ligand interactions. An example of this approach is the work of Englehard *et al.* (8), in which supplementation of fibroblasts (LM cells) with polyunsaturated fatty acids and/or ethanolamine caused an elevation of both basal and PGE_1-stimulated adenylate cyclase activity. These results do not appear to describe a universal phenomenon, however, because a decrease in adenylate cyclase activity was observed in chicken embryo fibroblasts supplemented with polyunsaturated fatty acids (9). Using a different approach, Bakardjieva *et al.* (10) have studied the effects of fusing Chiang liver cells with liposomes of varying composition. They observed that lipids such as dimyristoyl- and dioleoylphosphatidylcholine, which fluidized the cell membranes, caused losses of both *beta*-receptor binding and agonist-induced adenylate cyclase activity.

In applying membrane modification techniques to neuronal cells, we recently have shown that the membrane fatty acid composition of a neuroblastoma × glioma hybrid cell, NG108-15 (which displays many neuronal characteristics in culture (11, 12)), can be extensively manipulated simply by exposing the cells to various fatty acids (13). Initial experiments have shown that exposure of the cells to arachidonic acid causes a decrease in the rate of rise of action potentials generated by the cells without significant changes in the other electrical properties of the cells (14). We now present a characterization of the effects of exposure of the cells to unsaturated fatty acids on receptor/ligand interactions for opiate and PGE_1 receptors, and on the activity of adenylate cyclase.

MATERIALS AND METHODS

Tissue culture methods. The basic methods for growing the cells have been described (13, 15). Briefly stated, the cells were grown in Dulbecco's modified Eagle's medium containing 100 μM hypoxanthine, 1 μM aminopterin, 16 μM thymidine, and 5% fetal bovine serum (K. C. Biologicals, Lenexa, Kan). Cells were maintained at 37° in a humidified atmosphere of 90% air/10% CO_2 in 75-cm^2 flasks. Cells were fed as needed when the pH had dropped from 7.4 to about 7.0.

Addition of fatty acids to culture medium. Fatty acids (NuChek Prep, Elysian, Minn.) were added to culture media as complexes with BSA which had been delipidated by the method of Chen (16) as previously described (13). Fatty acid/BSA complexes were made by adding a warm solution of the potassium salt of the fatty acid to a rapidly stirred solution of 0.5 mM BSA. The solution then was readjusted to pH 7.4 and stored sterile at 4° until used in experiments. The fatty acid/BSA molar ratio was 4.0 in all cases, a ratio at which only a very low concentration of the fatty acid is unbound (free) in solution. The stock fatty acid/BSA solutions were added directly to the culture medium at the time of feeding the cells to achieve the indicated total fatty acid concentrations. Equivalent concentrations of BSA without fatty acid were added to control cultures.

Extraction and quantitation of lipids from cells. Cells were removed from culture vessels by gentle expulsion of culture medium onto the monolayer. They were collected by centrifugation, resuspended in serum-free culture medium, and again collected by centrifugation. Lipids were extracted from the cell pellet with $CHCl_3/CH_3OH$ (2:1, v/v), and precipitated protein was removed by centrifugation. The $CHCl_3$ and CH_3OH phases were separated by addition of 0.22 volume of 0.1 M KCl followed by centrifugation. The $CHCl_3$ phase was recovered and evaporated to dryness with a stream of N_2. Neutral and polar lipids were separated on small columns (0.5 × 1.5 cm) of Unisil (Clarkson Chemical Company, Williamsport, Pa.), activated at 110° for 30 min. Neutral lipids were eluted with $CHCl_3$ and phospholipids with CH_3OH. The phospholipid fraction then was saponified, and fatty acids were converted to methyl esters as previously described (13). The methyl esters were separated by gas-liquid chromatography (GLC) using a Hewlett-Packard Model 5710 gas-liquid chromatograph equipped with a 6-foot column of 10% SP 2330 on Chromosorb WAW (Supelco, Bellefonte, Pa.). Peaks were identified by comparison with authentic standards, and peak areas were determined with a digital integrator. Duplicate GLC analyses were made for each sample, and peak areas obtained always agreed within ±5% of the mean. Likewise, values obtained from two or more separate flasks of cells analyzed in parallel always agreed within ±5% of the mean.

Homogenization of cells and isolation of cell membranes. Washed cells were exposed to ice-cold 5 mM MOPS·HCl (2 ml/10 mg of cell protein) for 5 min to cause hypotonic swelling of the cells. An equal volume of cold 640 mM sucrose then was added and the cells were ruptured using 10–15 strokes of a Dounce homogenizer. Unbroken cells and nuclei were removed by centrifugation for 10 min at 600 × *g*. The supernatant was used directly in some experiments for both determination of PGE_1 and etorphine binding, and for measurement of adenylate cyclase activity. In other experiments, a crude membrane fraction was prepared by centrifugation of the 600 × *g* supernatant at 125,000 × *g* for 30 min. The membranes then were resuspended in 50 mM MOPS/10 mM $MgCl_2$ (pH 7.5). Both the 600 × *g* supernatant and the crude membrane fractions were frozen in aliquots and stored at −80° for up to 1 month without significant changes in either receptor binding or adenylate cyclase activity. Protein concentrations were determined by the method of Lowry *et al.* (17), using BSA as the standard.

Adenylate cyclase assay. Adenylate cyclase activity was determined by a modification (18) of method C of Solomon *et al.* (19). Each 100 μl of reaction mixture contained 50 mM MOPS·HCl (pH 7.5); 5 mM $MgCl_2$; 20 mM creatine phosphate, disodium salt; 10 units (71 μg of protein) of creatine phosphokinase; 1 mM [^{32}P]ATP, tetrasodium salt (2 μCi); 0.5 mM cyclic AMP; [G-^{3}H]cyclic AMP (approximately 10,000 cpm); 0.5 mM Ro 20-1724; 0.25% ethanol; and 50–200 μg of NG108-15 homogenate

protein. Reaction mixtures were incubated for 6 min at 37° unless otherwise indicated. Under these conditions [^{32}P]cyclic AMP synthesis was proportional to time of incubation for at least 20 min. Reactions were terminated by the addition of 0.9 ml of cold 6% trichloroacetic acid. Tubes were centrifuged at 1800 × *g* for 20 min, and each supernatant solution was added to a Dowex AG50W-X4 column. The cyclic AMP fraction from the column was eluted onto an alumina column and eluted from the alumina with 4 ml of 0.1 mM imidazole-HCl (pH 7.5) into a counting vial. Values reported are the means of three or more replicate determinations ± standard error of the mean. Radioactive compounds were obtained from New England Nuclear Corporation (Boston, Mass.).

Binding assays. The binding of [15,16-^{3}H]etorphine and [5,6(*n*)-^{3}H]PGE$_1$ (both isotopes were obtained from Amersham/Searle, Arlington Heights, Ill.) was determined using a filtration assay. Whole-cell homogenates or washed membranes were incubated at 37° for 10 min ([^{3}H]etorphine) or 15 min ([^{3}H]PGE$_1$) with the concentrations of [^{3}H]etorphine and [^{3}H]PGE$_1$ indicated in the tables and figures. Each assay contained 50 mM MOPS·HCl (pH 7.5), 10 mM $MgCl_2$, and 0.15–0.30 mg of cell protein in a total volume of 150 μl. At the completion of the incubations the reaction mixtures were rapidly diluted by the addition of 2 ml of ice-cold 25 mM Tris·HCl (pH 7.5), and immediately filtered under reduced pressure through Whatman GF/F glass-fiber filters. Each filter was successively washed with three 5-ml aliquots of ice-cold buffer, dried under a heat lamp, and counted in a liquid scintillation spectrometer. The amount of nonspecific binding was determined in the presence of 1.0 μM or 10 μM nonradioactive etorphine or PGE$_1$, respectively. Using these assay conditions, binding was at equilibrium with respect to time and linear with respect to the amount of protein (20). Triplicate determinations were made for both total and nonspecific binding, and Scatchard analysis of binding was performed using the LIGAND computer program written by P. J. Munson, National Institutes of Health.

RESULTS

Effects of exposure of cells to unsaturated fatty acids on receptor binding. Our original studies had shown that long-term exposure (5 days) of NG108-15 cells to unsaturated fatty acids produced large changes in the phospholipid fatty acid composition of the cells (13). As shown in Table 1, changes also were seen even after only 6 hr of exposure to polyunsaturated fatty acids and increased during the next 30 hr. In general, the fatty acid compositions reflected incorporation of the exogenously supplied fatty acids and their elongation/desaturation products. Both 18:2 and 20:4 primarily caused losses in 16:1 and 18:1. After 36 hr of exposure to 20:4 an increase in 16:0 was observed, a change which became much more pronounced with longer exposure to 20:4 (13).

When the membranes of the fatty acid-treated cells were examined for their ability to bind [^{3}H]etorphine and [^{3}H]PGE$_1$, time-dependent decreases in binding were observed. A 30-min exposure to 20:4 [sufficient time for an equilibrium to be reached between extracellular and intracellular unesterified fatty acids (21)] had little if any effect on either etorphine or PGE$_1$ binding (data not shown). However, within 6 hr both 18:2 and 20:4 had caused measurable losses of PGE$_1$ binding, and after 36 hr a 70% loss of binding was seen (Table 2). Changes in etorphine binding occurred more slowly, not being observed until after 10 hr in the presence of 18:2 and after 36 hr with 20:4. On the basis of additional experiments, changes in PGE$_1$ binding were near-maximal at 36–48 hr, but changes in etorphine binding were not maximal until at least 48–72 hr (data not shown).

By varying the molar ratio of 20:4 to BSA in the culture medium, and thus the total concentration of 20:4, the concentration dependence for receptor changes was examined. As seen in Table 3, concentration-dependent changes in etorphine binding, PGE$_1$ binding, and enrichment with 20:4 and 22:4 were observed. The amount of binding in this experiment was considerably higher than in Table 2 for two reasons. First, binding was performed with isolated, washed membranes instead of a whole homogenate; second, higher ligand concentrations were used to approximate maximal binding more closely. The data in Table 3 demonstrate that the changes in receptor binding parallel the concentration-dependent utilization of 20:4. In addition they establish that similar changes are observed with both whole homogenates and isolated membranes.

TABLE 1

Time dependence for changes in phospholipid fatty acid composition upon exposure to exogenous fatty acids

Cells were grown in the presence or absence of 125 μ M linoleate (18:2) or arachidonate (20:4) for the indicated periods of time. Total cell phospholipids were isolated and their fatty acid compositions determined as described under Materials and Methods. Values represent means of two GLC analyses.

Growth condition	Time	Phospholipid fatty acid								
		16:0	16:1	18:0	18:1	18:2	20:3	20:4	22:4	Others
	hr	*% of total*								
Control		19.6	7.7	12.0	44.6	2.3	0.4	5.4	1.0	7.0
18:2	6	17.2	5.7	11.2	35.4	13.5	1.5	7.4	2.1	6.0
	10	19.4	4.8	12.5	27.6	14.4	4.2	10.4	2.2	4.5
	36	18.9	3.2	12.3	19.9	16.9	3.8	15.4	5.0	4.6
20:4	6	18.4	5.3	11.3	35.0	1.3	0.7	15.8	5.0	7.2
	10	20.0	5.0	11.5	32.0	1.0	0.4	18.0	6.5	5.6
	36	23.2	3.1	11.2	20.1	0.2	0.4	21.8	18.0	2.4

TABLE 2

Time dependence for decrease in [^{3}H]etorphine and [^{3}H]PGE$_1$ binding upon exposure to linoleate (18:2) and arachidonate (20:4)

Cells were grown in the presence or absence of 125 μM 18:2 or 125 μM 20:4 for the indicated periods of time. Binding of [^{3}H]etorphine (0.7 nM) or [^{3}H]PGE$_1$ (20 nM) was determined using whole homogenates as described under Materials and Methods. Values represent means ± standard error of the mean of triplicate assays.

Growth condition	Time	Receptor binding	
		[^{3}H]Etorphine	[^{3}H]PGE$_1$
	hr	*fmoles/mg protein*	
Control	6	183 ± 12	146 ± 5
18:2	6	172 ± 6	129 ± 12
20:4	6	174 ± 9	94 ± 2
Control	10	185 ± 2	160 ± 10
18:2	10	145 ± 6	82 ± 15
20:4	10	180 ± 7	93 ± 5
Control	36	125 ± 2	164 ± 16
18:2	36	75 ± 2	47 ± 6
20:4	36	49 ± 3	44 ± 8

TABLE 3

Effect of exposure of cells to various concentrations of arachidonate on PGE$_1$ binding

Cells were grown for 24 hr in the presence of the indicated concentrations of 20:4 (BSA concentration held constant at 32 μM). Binding of [^{3}H]PGE$_1$ (20 nM) and [^{3}H]etorphine (1.0 nM) to washed membranes then was determined. The phospholipid content of 20:4 and 22:4 was determined by GLC analysis as described under Materials and Methods.

Concentration of 20:4	Receptor binding		Phospholipid content of	
	[^{3}H]PGE$_1$	[^{3}H]Etorphine	20:4	22:4
μM	*fmoles/mg protein*		*% of total fatty acids*	
0	541 ± 14	467 ± 13	5.4	1.3
12	416 ± 10	335 ± 16	13.9	4.9
64	307 ± 25	301 ± 7	20.6	17.0
125	212 ± 4	222 ± 4	22.6	20.1

Since 20:4 is the direct precursor for prostaglandins of the 2-series, the possibility existed that all or some of the changes in receptor binding were a secondary result of increased prostaglandin synthesis. [Previous studies have shown that exposure of NG108-15 cells to PGE$_1$ caused a loss of PGE$_1$ receptors and a decrease in adenylate cyclase activity (20, 22).] To test this possibility, a high concentration of the cyclooxygenase inhibitor ibuprofen (0.5 mM) was added to cultures along with 20:4 for 24 hr. The inhibitor did not prevent the decreases in etorphine or PGE$_1$ binding (data not shown). This observation, coupled with the experiments involving 18:1 (see below) appear to rule out an involvement of prostaglandins in the phenomena being studied.

Most of our experiments have focused on supplementation with polyunsaturated fatty acids because they create the largest changes in phospholipid composition. However, exposure of the cells to oleate (18:1) caused a time-dependent enrichment with 18:1 at the expense of 16:0 and 16:1 (Table 4). Exposure of the cells to 18:1 also caused a decrease in the binding of etorphine and PGE$_1$ to washed membranes. However, the effects of 18:1 on binding were generally slower to develop and smaller in magnitude (Table 5). Only small changes in PGE$_1$ binding were observed at 10 hr, but by 44 hr greater than 50% of the PGE$_1$ binding had been lost. Etorphine binding was totally unaffected until after 44 hr of treatment, and only a small decrease was observed at this time. Thus, all three of the unsaturated fatty acids tested caused decreases in both etorphine and PGE$_1$ binding, but the time courses and magnitude of change appeared to be different.

Direct effects of arachidonate on receptor binding. The time dependencies for decreases in receptor binding described above appeared to rule out the possibility that free (unesterified) fatty acids were directly inhibiting binding. However, since other reports of direct effects of free fatty acids on membrane functions have appeared, it was important to examine this question in detail. To test this possibility, the K$^+$ salt of 20:4 was added directly to isolated membranes from control cells. The membranes then were washed by centrifugation in the presence or absence of excess BSA, a procedure which has been shown to remove fatty acids from membranes (23). As shown by the data in Table 6, direct addition of 20:4 to the membranes had no effect on etorphine binding. In contrast, exposure of membranes to 20:4 inhibited the binding of PGE$_1$. We have not determined whether this represents an effect of 20:4 on the receptor/ligand interaction or a simple competition of the large excess of 20:4 with [^{3}H]PGE$_1$ for the receptor site. In either case, washing of the membranes with BSA-containing buffer completely restored PGE$_1$ binding.

Since washing of the membranes with BSA-containing

TABLE 4

Time dependence for changes in phospholipid fatty acid composition upon exposure to exogenous oleate (18:1)

Cells were grown in the presence or absence of 125 μM 18:1 for the times indicated. Total cell phospholipids were extracted and their fatty acid compositions determined as described under Materials and Methods. Values represent means of two GLC analyses.

Growth	Time	Phospholipid fatty acid								
		16:0	16:1	18:0	18:1	18:2	20:3	20:4	22:4	Others
	hr	*% of total*								
Control		20.5	8.9	11.4	44.3	2.5	—	4.3	0.5	7.6
18:1	10	18.5	7.0	11.0	51.3	1.9	—	4.1	0.3	6.0
	20	15.6	6.3	10.5	54.1	1.6	—	3.8	0.8	7.3
	44	13.0	3.7	9.3	61.2	0.8	—	4.3	1.2	6.4

TABLE 5

Effects of exposure of cells to oleate (18:1) on etorphine and PGE_1 binding

Cells were grown in the presence or absence of 125 μM 18:1 for the indicated periods of time. Specific binding of [^{3}H]etorphine and [^{3}H]PGE_1 were determined using washed membranes and 8.8 nM [^{3}H]etorphine or 60 nM [^{3}H]PGE_1. Values represent means ± standard error of the mean of six assays each of total and nonspecific binding.

Growth condition	Time	Receptor binding	
		[^{3}H]Etorphine	[^{3}H]PGE_1
	hr	*fmoles/mg protein*	
Control	10	503 ± 5	619 ± 10
18:1	10	514 ± 14	521 ± 25
Control	20	726 ± 20	820 ± 16
18:1	20	736 ± 20	663 ± 17
Control	44	614 ± 26	724 ± 3
18:1	44	370 ± 13	347 ± 30

buffer was able to reverse any direct effects of exogenous 20:4, we studied the ability of BSA to restore receptor binding to membranes from 20:4-treated cells. As seen in Table 6, washing of the membranes from 20:4-treated cells with BSA-containing buffer had no effect on etorphine binding. However, washing of the membranes from 20:4-treated cells with BSA partially restored the binding of PGE_1, although clearly not to control levels. This observation, along with the more rapid loss of PGE_1 binding, suggests that some of the decrease in PGE_1 binding is a direct effect of excess 20:4, whereas a second component of the effect is not free fatty acid-mediated. In contrast, none of the apparent decrease in etorphine binding was the direct effect of exogenous fatty acids present in the membranes at the time binding was determined.

TABLE 6

Effects of exposing isolated membranes to albumin and arachidonate (20:4) on receptor binding

Cells were grown in the presence or absence of 125 μM 20:4 for 48 hr and then were washed and homogenized as described under Materials and Methods. After removal of nuclei and unbroken cells, membranes were isolated by centrifugation at 105,000 × *g* for 30 min. Membranes were washed by resuspension in buffer with or without 50 μM albumin and isolated by recentrifugation. To examine the direct effects of exposing isolated membranes to 20:4, membranes from control cells were incubated at 37° for 15 min in the presence and absence of 50 μM 20:4 (K^+ salt). The membranes then were diluted with cold buffer with or without a 50-fold molar excess of BSA and reisolated by centrifugation at 105,000 × *g* for 30 min. Values represent the mean specific binding ± standard error of the mean (SEM) of three assays each of total and nonspecific binding (SEM was calculated as the sum of the SEM for the two sets of assays). Ligand concentrations were 0.6 nM for [^{3}H]etorphine and 25 nM for [^{3}H]PGE_1.

Membrane treatment	Receptor binding	
	[^{3}H]Etorphine	[^{3}H]PGE_1
	fmoles/mg protein	
Control cells		
Whole homogenate	140 ± 4	177 ± 3
Isolated membranes	328 ± 4	361 ± 19
Membranes treated without 20:4		
Washed (−) BSA	352 ± 5	382 ± 10
Washed (+) BSA	308 ± 9	353 ± 22
Membranes treated with 20:4		
Washed (−) BSA	329 ± 3	217 ± 22
Washed (+) BSA	349 ± 14	422 ± 29
20:4-Treated cells		
Whole homogenate	38 ± 4	55 ± 6
Isolated membranes	99 ± 3	97 ± 21
Membranes treated without 20:4		
Washed (−) BSA	103 ± 5	112 ± 18
Washed (+) BSA	125 ± 12	175 ± 27

Effects of fatty acids on the binding constants for etorphine and PGE_1 binding. Up to this point binding at only single ligand concentrations was determined. To determine whether changes in receptor binding represented changes in K_D or B_{max}, equilibrium displacement studies were performed and the data were analyzed by the method of Scatchard. Figure 1 presents an example of the data obtained, and Table 7 provides a tabulation of calculated values for K_D and B_{max}. Exposure of the cells to 20:4 caused a substantial decrease in the B_{max} for etorphine binding with little if any change in K_D. In other experiments, decreases in B_{max} as large as 80% were observed with no reproducible changes in K_D. When studying PGE_1 binding, a relatively short exposure to 20:4 was used because longer treatments reduced specific binding below the level which could be measured accurately. As can be seen, treatment with 20:4 primarily affected the B_{max} for PGE_1 binding, determined either in isolated membranes or membranes washed with BSA. When cells were exposed to 20:4 for longer periods of time, inconsistent results were obtained. In all cases, B_{max} was decreased, but in several cases K_D was elevated as much as 2-fold. Thus, the changes in etorphine binding appear to represent relatively simple changes in the number of receptors, whereas changes in PGE_1 binding may reflect more complicated phenomena.

Long-term reversibility of decreases in etorphine binding. It is important to emphasize that studies of the type being reported here deal with complex metabolic situations in which any immediate extrapolation to cause/effect relationships are difficult. For example, even though the time and concentration dependencies for changes in phospholipid fatty acid composition and receptor binding appeared to be rather parallel, this in no way proved a direct relationship between steady-state fatty acid composition and apparent number of binding sites. As a first step in testing this possible cause/effect relationship we examined the reversibility of the apparent decrease in receptor number upon removal of 20:4 from the culture medium. Both [^{3}H]etorphine and [^{3}H]PGE_1 binding returned toward control values during a 48- to 72-hr time period following removal of 20:4 from the culture medium. The speed and extent to which binding reappeared depended on the concentration of 20:4 used, the duration of exposure, and the duration of time following removal of the fatty acid. However, as shown by a representative experiment (Table 8), the apparent number of binding sites for [^{3}H]etorphine had returned to near-control values at a time when the phospholipid fatty acid composition (as represented by the content of 20:4 and 22:4) had hardly begun returning toward control values. Thus, an apparent correlation between steady-state phospholipid fatty acid composi-

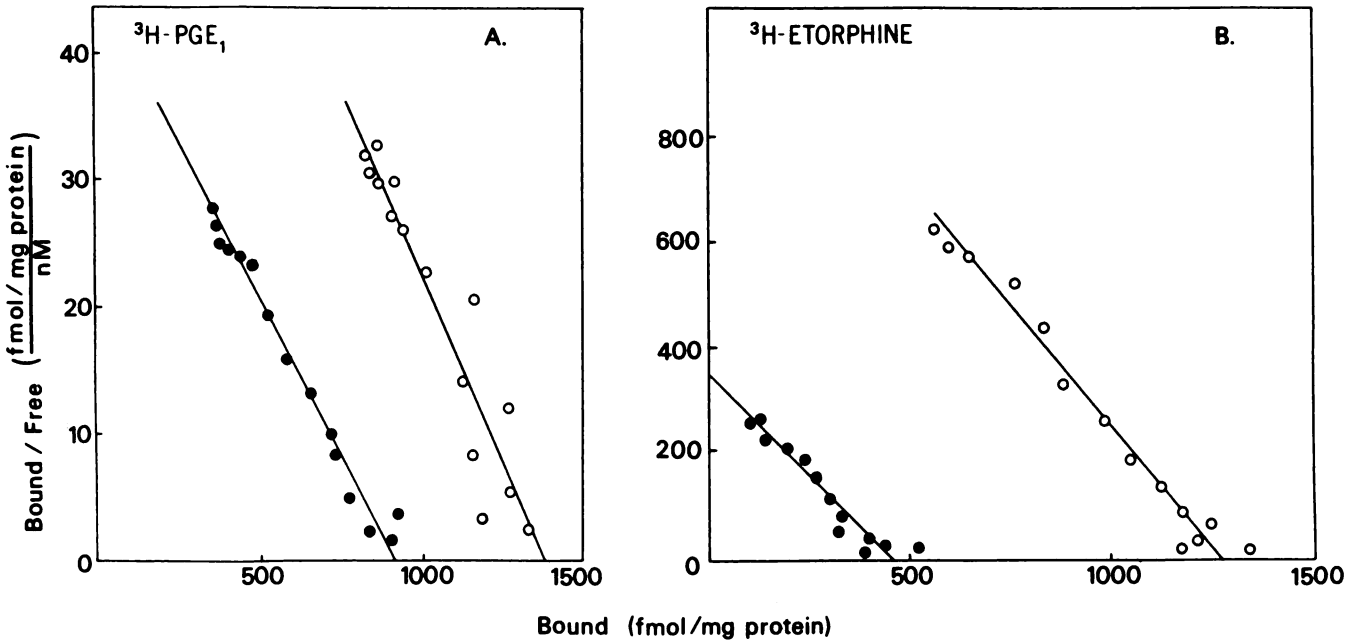

FIG. 1. *Effects of arachidonate (20:4) on the equilibrium binding of [^{3}H]PGE$_1$ and [^{3}H]etorphine to membranes from NG108-15 cells*
Binding of [^{3}H]PGE$_1$ and [^{3}H]etorphine to buffer-washed membranes (see Table 6) was determined using a fixed concentration of radioactive ligand and increasing concentrations of nonradioactive ligand. ○, Control cells; ●, 20:4-treated cells. Each point represents a single assay. Lines were determined using Scatchard analysis and the LIGAND curve-fitting program. The best fit lines consisted of a single class of saturable binding sites and a nonspecific binding component. A. Cells treated with 64 μM 20:4 for 24 hr, 13.9 nM [^{3}H]PGE$_1$. B. Cells treated with 125 μM 20:4 for 4 days, 0.7 nM [^{3}H]etorphine.

tion and receptor binding no longer held. Several possible interpretations of these observations are presented under Discussion.

Effects of exposure to unsaturated fatty acids on adenylate cyclase. The receptors for PGE$_1$ and etorphine have been shown to activate and inhibit adenylate cyclase in NG108-15 cells, respectively. Activation of these receptors also has been shown to be capable of triggering compensatory processes which work toward re-establishment of a "normal" cyclic AMP concentration in the cells (22, 24). Thus, it became important to determine whether the apparent changes in PGE$_1$ and etorphine receptor number were transmitted to changes in adenylate cyclase. As shown in Table 9, basal activity was altered very little, and maximal PGE$_1$-stimulated activity was, if anything, elevated in membranes from 18:1 and 20:4-treated cells. Thus, we can rule out the possibility that the fatty acids were causing a generalized loss of membrane proteins. The ability of a maximal concentration of etorphine to antagonize PGE$_1$ activation of adenylate cyclase also was not appreciably altered. These observations were suprising in light of the marked decrease in the numbers of receptors for these two compounds. The most likely interpretation is that even in fatty acid-treated cells the number of receptors present still ex-

TABLE 7

Effect of exposure to arachidonate (20:4) on equilibrium binding characteristics of [^{3}H]etorphine and [^{3}H]PGE$_1$

Cells were grown for the indicated periods of time in the presence or absence of 20:4. Membranes were isolated and then washed either with buffer or BSA-containing buffer as described in the legend to Table 6. Values for K_D and B_{max} were determined as described in the legend to Fig. 1.

Growth condition	Membrane wash	K_D	B_{max}
		nM	*fmoles/mg protein*
Expt. 1			
[^{3}H]Etorphine binding			
Control	Buffer	1.2	1280
	BSA	1.5	1040
20:4 (125 μM, 4 days)	Buffer	1.3	450
	BSA	2.0	520
Expt. 2			
[^{3}H]PGE$_1$ binding			
Control	Buffer	17.5	1380
	BSA	13.3	1210
20:4 (64 μM, 24 hr)	Buffer	20.1	920
	BSA	14.7	860

TABLE 8

Return of receptor binding sites upon removal of arachidonate (20:4) from culture medium

Cells were grown for 108 hr in normal culture medium, for 108 hr in culture medium containing 125 μM 20:4, or for 60 hr with 125 μM 20:4 followed by 48 hr in normal culture medium (all normal medium supplemented with 32 μM BSA). Cells were harvested, washed, homogenized, and analyzed for etorphine binding using whole cell homogenates as described in the legend to Table 7.

Cells	[^{3}H]Etorphine binding		Phospholipid content	
	K_D	B_{max}	20:4	22:4
	nM	*fmoles/mg*	%	
Control	1.4	780	4.7	0.7
20:4-Treated	1.6	230	22.4	23.4
20:4-Treated followed by 48-hr reversal	1.9	690	21.0	15.6

TABLE 9

Effects of growth of cells with oleate (18:1) and arachidonate (20:4) on adenylate cyclase activity

Cells were grown for 4 days in the presence or absence of 125 μM 18:1 or 20:4. After washing and homogenization, adenylate cyclase activity was determined either without further additions (basal activity) or in the presence of PGE_1 (10 μM), PGE_1 plus etorphine (1 μM), or 2-chloroadenosine (50 μM). Values represent means ± standard error of the mean of eight assays.

Cell treatment	Addition to enzyme assay	Adenylate cyclase activity
		pmoles/min/mg protein
Control	None	31 ± 3
	PGE_1	171 ± 9
	PGE_1 + etorphine	128 ± 5
	2-Chloroadenosine	72 ± 3
18:1-Treated	None	33 ± 2
	PGE_1	215 ± 11
	PGE_1 + etorphine	137 ± 6
	2-Chloroadenosine	66 ± 2
20:4-Treated	None	36 ± 2
	PGE_1	214 ± 9
	PGE_1 + etorphine	166 ± 7
	2-Chloroadenosine	53 ± 2

ceeded that necessary to activate maximally or inhibit maximally the enzyme.

In contrast to the small elevation of PGE_1-stimulated cyclase, adenosine-activated adenylate cyclase activity was slightly decreased. The small decrease was observed in several different experiments but was not investigated further.

Effect of arachidonate on PGE_1-induced decreases in PGE_1 receptors and adenylate cyclase activity. As mentioned above, exposure of NG108-15 cells to PGE_1 causes a decrease in PGE_1 receptors and adenylate cyclase activity (20, 22). To determine whether the effects of the unsaturated fatty acids were related to this regulatory phenomenon, cells were exposed to PGE_1 in the presence and absence of 20:4 (Table 10). As expected, PGE_1 caused a decrease in [^{3}H]PGE_1 binding and in both basal and PGE_1-activated adenylate cyclase activity. The presence of 20:4 had no effect on this loss of enzymatic activity,

TABLE 10

Effects of arachidonate (20:4) on PGE_1-induced decreases in PGE_1 receptors and adenylate cyclase activity

Cells were exposed to 20:4 (125 μM) and/or PGE_1 (10 μM) for 24 hr as indicated. Control cultures without 20:4 received the equivalent concentration of BSA. PGE_1 binding was determined with whole homogenates using 20 nM [^{3}H]PGE_1. Adenylate cyclase activity was determined in the presence and absence of 10 μM PGE_1.

Growth condition	[^{3}H]PGE_1 binding	Adenylate cyclase activity	
		Basal	PGE_1-stimulated
	fmoles/mg protein	*pmoles/min/mg protein*	
Control	138.0 ± 2.6	49.0 ± 4.7	182.0 ± 10.6
PGE_1	18.9 ± 3.7	32.9 ± 1.5	83.4 ± 19.9
20:4	80.0 ± 7.4	50.9 ± 2.0	194.1 ± 15.1
PGE_1 + 20:4	10.9 ± 3.5	32.2 ± 5.0	92.6 ± 5.0

indicating that the fatty acid in no way interfered with the desensitization process.

DISCUSSION

It appears from the results of these studies that exposure of NG108-15 cells to unsaturated fatty acids causes a decrease in the number of receptors for etorphine and PGE_1. However, it will be necessary to prepare antibodies to the receptors in order to rule out unequivocally the possibility that the receptors are still present but are converted to a form undetected by the binding assay. Working from the hypothesis that the number of receptors actually has decreased, several interesting aspects of this phenomenon can be considered. The effects of the fatty acids do not reflect a toxic action because they are not toxic to the cells (13). Changes in receptor binding also do not represent direct, immediate effects of the fatty acids, as shown by using both intact cells and isolated membranes. Thus, the changes in receptor number correlate much better with the time- and concentration-dependent utilization of the exogenous fatty acids.

At this stage of our studies, several different explanations for decreases in receptor binding must be considered. The simplest explanation would be that the apparent number of receptor sites reflects the steady-state phospholipid fatty acid composition of the cells at the time when binding is measured. This possibility seems unlikely for several reasons. First, the phospholipid fatty acid compositions induced by 18:1, 18:2, and 20:4 are very different, making it difficult to envision some specific composition necessary for binding. Second, upon removal of exogenous fatty acids from the culture medium, receptor binding (especially etorphine binding) returns toward control values much more rapidly than does the phospholipid fatty acid composition. It is possible that receptor binding would show a closer correlation with the fatty acid composition of some specific phospholipid species. Our previous experiments have shown that the fatty acid composition of phosphatidylcholine, phosphatidylethanolamine, and phosphatidylinositol is substantially altered in cells exposed to unsaturated fatty acids (13). However, we have not as yet determined whether one of these species returns to control values more rapidly than the others upon removal of exogenous fatty acids.

A second possible explanation for the decrease in receptor binding is that this change reflects some secondary steady-state change in membrane lipids induced by exposure to unsaturated fatty acids. For example, exposure to 18:2 and 20:4 has been shown to induce a decrease in the cholesterol/phospholipid molar ratio of NG108-15 membranes (13). However, initial experiments indicate that this change in membrane lipids persists even after 20:4 has been removed from the culture medium for 40 hr.[4]

In aggregate, the evidence does not strongly support the conclusion that receptor binding reflects a steady-state membrane lipid composition. However, it is important to emphasize that our membrane lipid analyses thus far have been made only with a total membrane fraction. Although experiments with other cells have shown that the phospholipids of plasma and of microsomal and mi-

[4] R. McGee, unpublished observation.

tochondrial membranes are altered to similar extents by exposure to unsaturated fatty acids (e.g., ref. 25), it is important to determine whether this also is true for NG108-15 cells. It could be that cytoplasmic membrane phospholipids are turning over more rapidly than the average cellular membrane pool and that this is the reason the correlation between binding and lipid composition breaks down upon removal of the fatty acids from the culture medium.

The alternative explanation for our results is that decreases in receptor binding reflect events occurring during the time in which the cells are exposed to the fatty acids. The number of receptors in the cell membrane at any given time reflects a composite of the rates of receptor synthesis as well as the rates of receptor insertion into and removal from the membranes. Our observations could suggest that the presence of the fatty acids causes a decrease in receptor synthesis or insertion, or an increase in the rate of removal of the receptors from the membrane. Most of the data would be consistent with this hypothesis, suggesting that the magnitude of the change being produced is proportional to the concentration and duration of exposure to unsaturated fatty acids. Interestingly, the changes in etorphine and PGE_1 binding do not follow the same time courses, and could reflect different controls over the number of each receptor in the membrane. Evidence already has been obtained that these two receptors are regulated by different control mechanisms in NG108-15 cells. Exposure of cells to PGE_1 causes a loss of PGE_1 receptors as well as a decrease in basal and PGE_1-stimulated adenylate cyclase (20, 22). In contrast, exposure to etorphine causes an increase in adenylate cyclase activity without any accompanying change in opiate receptor number (24, 26).

If our observations do reflect changes in the rates of turnover of PGE_1 and opiate receptors, several observations can be made. First, not all of the unsaturated fatty acids induce the same changes; 18:1 causes a slower and smaller decrease in both receptors than do 18:2 and 20:4. It would be of interest to determine whether saturated fatty acids, such as palmitate (16:0), also cause changes in receptor binding. Unfortunately, 16:0 is toxic to NG108-15 cells, and any effect or lack of effect would be difficult to interpret. Second, any fatty acid-induced changes in rates of receptor turnover do not reflect generalized changes in membrane protein turnover. As shown by our data, adenylate cyclase activity is not decreased upon exposure to unsaturated fatty acids. One could speculate that the difference between receptors and adenylate cyclase is that the former are oriented toward the outside of the cell, whereas the latter is oriented toward the inside. Third, the mechanisms involving the effects of fatty acid on PGE_1 receptor number do not interfere with the mechanisms regulating cellular adaptation to chronic PGE_1 receptor occupancy. Simultaneous exposure of the cells to PGE_1 and 20:4 did not diminish the down-regulation of either PGE_1 receptor or adenylate cyclase activity associated with receptor occupancy. Further extension of these ideas requires more definitive measurements of receptor turnover.

The apparent decrease in number of both PGE_1 and etorphine receptors under conditions which should be fluidizing the cell membranes is consistent with the observations of other investigators who have studied the effects of phospholipases (2, 3, 5, 6). It also is consistent with the liposome fusion studies of Bakardjieva *et al.* (10). However, from all of these studies it is impossible to determine whether receptors really have been lost from the membranes, or, as recently suggested (27), have moved to a region of the membrane where receptor/ligand interactions no longer are measurable. The use of either antibodies to the receptors or quantitative solubilization of the receptors is required to resolve this issue.

Our observations that adenylate cyclase activity is only slightly elevated by the unsaturated fatty acids contrasts with the large increases observed in LM cells (8) and the decreases observed in chicken embryo fibroblasts (9). Thus, as yet, no clear conclusion is possible regarding the general regulation of adenylate cyclase activity by membrane lipid modification.

We now have studied the effects of exogenous unsaturated fatty acids on several different cellular activities in neuronal tissue culture cells. The changes observed appear to represent a general, but not universal, disruptive or inhibitory effect. In NG108-15 cells, treatments similar to those reported here cause a decrease in the rate of rise of action potentials generated by the cells (14). This could be explained as being the result of either impairment of Na^+ channel function or a decrease in the number of Na^+ channels. In another neuronal cell line, the pheochromocytoma clone PC12, exposure to unsaturated fatty acids causes a loss of carbamylcholine-stimulated and veratridine-stimulated exocytosis of norepinephrine (28). However, no change in K^+-stimulated release of norepinephrine from PC12 cells was observed, suggesting that the activities of the acetylcholine receptor and Na^+ channel were altered, but not the membrane potential-dependent Ca^{2+} channel. All of these observations allow the conclusion that simply changing the amount and nature of the free fatty acids in culture medium can have pronounced effects on cellular functions. However, many more experiments will have to be performed before mechanistic interpretations, as well as assessment of the physiological importance of these observations, are possible.

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Send reprint requests to: Dr. Richard McGee, Jr., Department of Pharmacology, Georgetown University, 3900 Reservoir Road, Washington, D. C. 20007.