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REDUCTION OF OPIOID BINDING IN NEUROBLASTOMA X GLIOMA CELLS GROWN IN MEDIUM CONTAINING UNSATURATED FATTY ACIDS

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Neuroblastoma x glioma cells NG108-15 were cultured in lipid-free medium supplemented with fatty acids of various chain length and unsaturation. Binding of ^{3}H-labelled [DAla^2]-[DLeu^5]-enkephalin by membranes of cells grown in saturation fatty acids of different chain length was not significantly different from that of the control. On the other hand, a proportional decrease of binding capacity with no change in residual receptor affinity was noticed when cells were cultured in medium containing fatty acids of increasing unsaturation. This decrease was time dependent and reached a maximum at about 48 h. Binding of [^{3}H]dihydromorphine and [^{3}H]naloxone was similarly affected. In contrast, when membranes of cells grown in normal medium were preincubated up to 3 h with unsaturated fatty acid and tested for opioid binding, no significant reduction was observed. Examination of the fatty acid composition of phospholipid from cells grown in linolenate indicated that a significant alteration of the acyl composition has occurred. To evaluate the underlying cause of this type of inhibition, the effect of linolenic acid on cell growth and protein synthesis was examined. When cells were cultured in 100 μM of this fatty acid, both growth and protein synthesis were retarded by 28% and 19%, respectively. Since opiate receptors are proteineous in nature, a reduction of protein synthesis may partially account for the loss of opioid binding activity. On the other hand, an increase of membrane fluidity is known to affect a number of cellular functions, including ligand-receptor recognition. Whether this can offer a satisfactory explanation for our observations remains to be established.

Introduction

The neuroblastoma x glioma hybrid cell line NG108-15 was shown initially by Klee and Nirenberg [1] to contain a high density of opiate receptors. Subsequent experiments by the same group established that these receptors are functionally coupled to the adenylate cyclase system and that changes of adenylate cyclase activity can be correlated with the development of tolerance [2]. In view of this, the neuroblastoma x glioma hybrid cells offer an attractive alternative for study of the action of opiates under in vitro conditions.

The demonstration of the existence of opiate receptor in brain membranes has lead to a number of questions concerning the structural characteristics of these receptors. By means of chemical and enzymic studies [3–5], both lipid and protein components are thought to be required for opiate binding activity. Since most receptors are integral components of the plasma membrane, it is not unreasonable to expect that a change of the lipid composition in the membrane may perturb the

Abbreviations: $C_{14:0}$, myristic acid; $C_{16:0}$, palmitic acid; $C_{17:0}$, heptadecanoic acid; $C_{18:0}$, stearic acid; $C_{18:1}$, oleic acid; $C_{18:2}$, linoleic acid; $C_{18:3}$, linolenic acid.

binding characteristics of the ligand. In the case of the opiate receptor, manipulation of the lipid components of the brain membranes cannot be easily accomplished and in order to study such a problem the neuroblastoma x glioma hybrid cell offers an attractive solution. An initial aim of this study was to determine whether opiate receptor binding in NG108-15 cell membranes was modified by culturing the cells in defined fatty acid supplemented media.

Materials and Methods

Materials. ^{3}H-labelled [DAla^2]-[DLeu^5]-enkephalin (48 Ci/mmol), [^{3}H]dihydromorphine (48 Ci/mmol) and [^{14}C]leucine (324 mCi/mmol) were obtained from Amersham International, Amersham, U.K. [^{3}H]Naloxone (16 Ci/mmol) was from New England Nuclear, Boston, MA. Powder culture medium (Dulbecco's modified Eagle's medium, Cat. No. 430-2100) and calf serum (Cat. No. 200-6170) were purchased from Gibco, Grand Island, NY. Fatty acids were from Sigma, St. Louis, MO.

Cell culture. NG108-15 hybrid cells [1] were kindly provided by Dr. S. Udenfriend of the Roche Institute of Molecular Biology. Cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% calf serum, 100 U/ml penicillin, 0.1 mg/ml streptomycin sulfate, 0.1 mM hypoxanthine, 1 μM aminopterin and 12 μM thymidine. Cells were kept in an atmosphere of 10% CO_2 and 90% air. After reaching confluency, cells were shaken off in the growth medium and collected by centrifugation. The cell pellet collected from three Corning T-flasks (150 cm^2 surface area) was washed twice with 50 ml D_i solution (137 mM NaCl/5.4 mM KCl/0.17 mM Na_2HPO_4/5.5 mM glucose/0.22 mM KH_2PO_4) and then resuspended in 20 ml Tris-HCl/sucrose (0.3 M sucrose/0.01 M Tris-HCl (pH 7.4)/5 mM $MgCl_2$). Cells were frozen at −20°C until used. From our experience, cells frozen in this manner for up to 2 months were as effective as fresh cells in the opiate receptor binding assay.

Preparation of fatty acid supplemented medium. Calf serum was delipidated according to the method of Cham and Knowles [6] as modified by Wilkening and Nirenberg [7]. The extracted serum was dialyzed extensively against a 0.01 M phosphate-buffered saline, pH 7.4. Fatty acids dissolved in 0.1 ml absolute ethanol (100 μmol/ml) were injected into 10 ml pre-warmed (40°C) lipid-free calf serum to a final concentration of 1 mM. Due to its insolubility, stearic acid was introduced in a solution of diethyl ether. After removing the excess solvent under a gentle stream of nitrogen, the fatty acid supplemented calf serum was mixed with culture medium to a final concentration of 10% and sterilized by filtration.

Binding assay. A crude membrane preparation of the NG108-15 cells was used throughout the present study. Cells stored in Tris/sucrose solution were allowed to thaw at room temperature and then pelleted by centrifugation. The pellet was lyzed and homogenized in 0.01 M Tris-HCl at 4°C. After standing in an ice-bath for 20 min, the particulate fraction was collected by centrifugation (4°C, 10000 × g, 10 min). The pellet was washed twice in 0.01 M Tris-HCl (pH 7.4) and then resuspended in 0.05 M Tris-HCl to a final protein concentration 250–500 μg/ml.

Binding was performed in a final volume of 0.5 ml 0.05 M Tris-HCl (pH 7.4) at room temperature for 60 min as described by Dunlap et al. [8] for brain membranes. The amount of protein used per assay ranged from 100 to 200 μg. Bound and free radioactive ligands were separated by rapid filtration on Whatman GFC filters and the radioactivity retained determined by liquid scintillation counting. All bindings were performed in duplicate or triplicate and the results are presented as fmol ^{3}H-labelled ligand bound per mg cell protein. Nonspecific binding was determined from the amount of radioactivity bound in the presence of $1 \cdot 10^{-6}$ M levorphanol.

Protein determination. Protein concentration was determined by the method of Lowry et al. [9] using bovine serum albumin as the standard. Cell preparations were digested in 1 M NaOH for at least 24 h before assay.

Determination of fatty acid composition. Cells were washed four times with 0.01 M phosphate-buffered saline (pH 7.4) and then extracted with chloroform/methanol (3:2, v/v) overnight. The insoluble materials were pelleted by centrifugation and the organic phase was removed and saved in separate vials. The extract was then dried under a

gentle stream of nitrogen and the phospholipids were separated from other lipids by thin-layer chromatography (silica gel G; hexane/diethyl ether/acetic acid; 60:40:1, v/v). The fatty acid composition of the phospholipids was determined by gas-liquid chromatography. Phospholipids were first transesterified in a methanol/acid solution according to the procedure of Ways et al. [10]. The methyl esters thus obtained were chromatographed at 180°C on a column (1/8 inch × 6 feet) packed with 10% SP2300 on Supelcoport (Supelco, Bellefonte, PA). The elution profile was monitored by flame-ionization (Model 3200 chromatograph, Varian, Palo Alto, CA) and the concentration of each of the fatty acid was determined by comparison with known methyl-ester standards.

Results

Our first experiment was to evaluate if cells grown in media containing different fatty acids

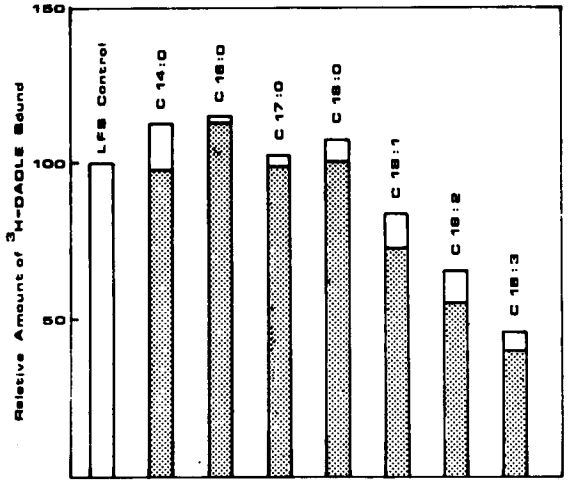

Fig. 1. Binding of ^{3}H-labelled [DAla^2]-[DLeu^5]-enkephalin (DADLE) by membranes of NG108-15 hybrid cells grown in lipid-free media supplemented with different fatty acids. 48 h before reaching confluency, the cells' regular growth medium was changed to a medium containing 10% lipid-free serum with or without fatty acid (100 μM final concentration). At the end of the incubation, cells were harvested, washed and assayed as described in Materials and Methods. Substitution of lipid-free serum for normal serum did not have any deleterious effect on cell growth (see Table III). The final concentration of ^{3}H-labelled [DAla^2]-[DLeu^5]-enkephalin for binding was 2 nM. The results from two replicate experiments are presented. □, experiment I; ▨, experiment II.

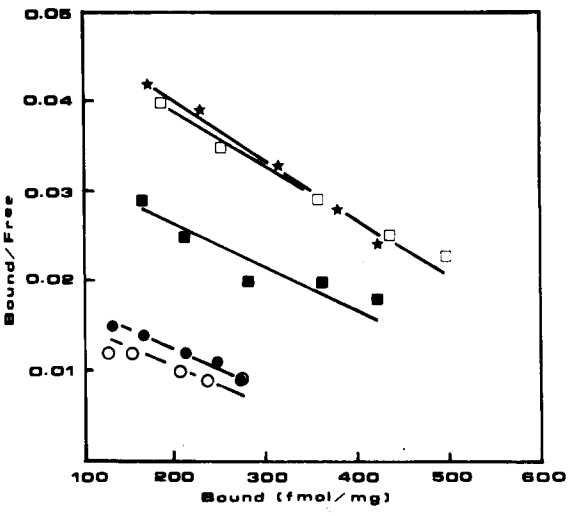

Fig. 2. Scatchard plots of ^{3}H-labelled [DAla^2]-[DLeu^5]-enkephalin binding curves of cells grown in unsaturated fatty acid supplemented media. Three replicate experiments produced similar results. ★, calf serum control; □, lipid-free serum control; ■, $C_{18:1}$; ●, $C_{18:2}$; ○, $C_{18:3}$.

will bind ^{3}H-labelled [DAla^2]-[DLeu^5]-enkephalin differently than control cells. As indicated in Fig. 1, the amount of ^{3}H-labelled [DAla^2]-[DLeu^5]-enkephalin bound by membranes of cells grown in media supplemented with saturated fatty acids (i.e., $C_{14:0}$, $C_{16:0}$, $C_{17:0}$ and $C_{18:0}$) was not remarkably different from those grown in lipid-free serum. In contrast, when fatty acids of the same chain length but of different degrees of unsaturation were used, a graded reduction of specific binding was noticed.

To evaluate further the nature of this reduction in binding, the Scatchard plots of the binding curves of cells grown in $C_{18:1}$, $C_{18:2}$, $C_{18:3}$ and control serum were compared (Fig. 2). Examination of these plots indicates that the effect of fatty acid on ^{3}H-labelled [D-Ala^2]-[DLeu^5]-enkephalin binding is primarily due to a reduction of B_{max}, while the K_D was not appreciably affected.

We have chosen ^{3}H-labelled [DAla^2]-[DLeu^5]-enkephalin as the ligand for our binding study because it has the highest affinity for the opiate receptors in the NG108-15 cells [5]. To examine whether linolenic acid ($C_{18:3}$) has a similar effect on the binding of other opiates, we repeated our experiment using [^{3}H]dihydromorphine and [^{3}H]-

TABLE I

BINDING OF ^{3}H-LABELLED [DAla^2]-[DLeu^5]-ENKEPHALIN, [^{3}H]DIHYDROMORPHINE AND [^{3}H]NALOXONE BY MEMBRANES OF NG108-15 CELLS GROWN IN A LIPID-FREE MEDIUM SUPPLEMENTED WITH LINOLENIC ACID

Binding was performed at a radioligand concentration of 2 nM. Results are means ±S.E. of three determinations.

Treatment	^{3}H-labelled compound specifically bound (fmol/mg)		
	^{3}H-labelled [DAla^2]-[DLeu^5]-enkephalin	[^{3}H]Dihydromorphine	[^{3}H]-Naloxone
$C_{18:3}$	177± 6	25±10	7±4
Lipid-free serum	340±43	39± 4	26±3

naloxone as ligands. In agreement with our previous results, the specific binding of both dihydromorphine and naloxone was significantly reduced after the cells have been cultured in a medium supplemented with linolenic acid for 48 h (Table I).

In all of the above studies we have incubated cells with fatty acid supplemented media for 48 h

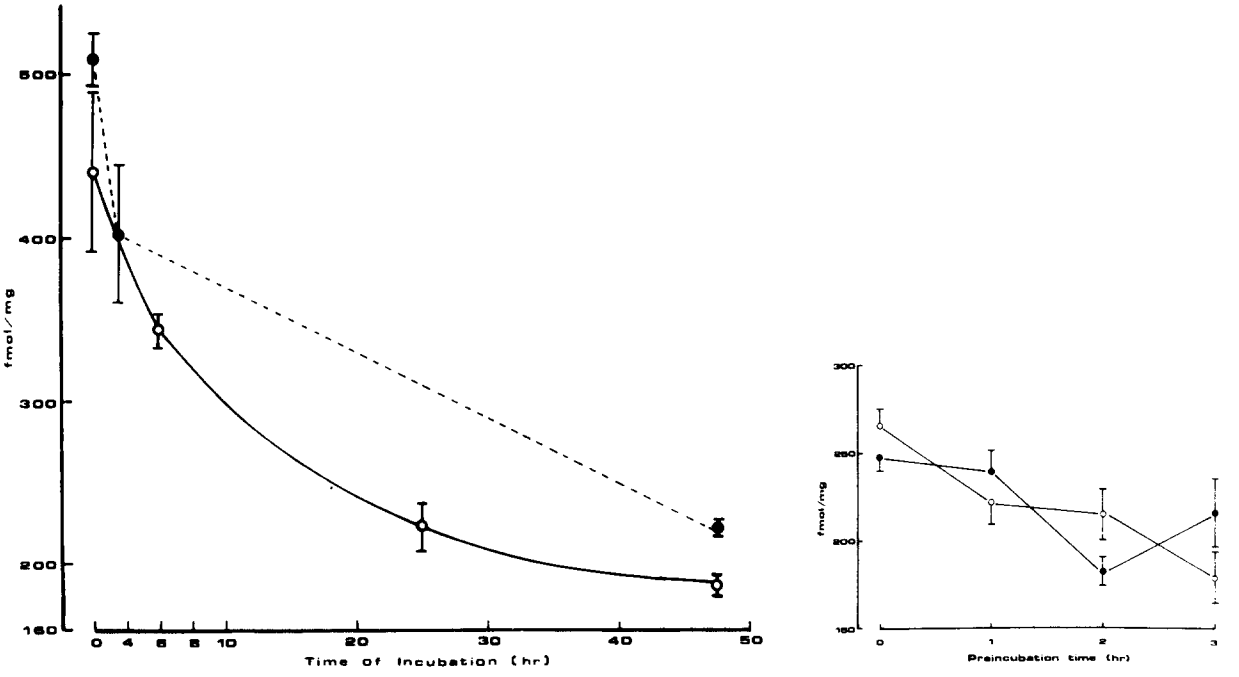

Fig. 3. Effect of time of incubation in linolenic acid supplemented medium on ^{3}H-labelled [DAla^2]-[DLeu^5]-enkephalin binding. Cells were grown in linolenic acid (100 μM) supplemented medium for the length of time indicated. After shaking off from the culture flask, cells were washed, stored and assayed as outlined in Materials and Methods. The amount of ^{3}H-labelled [DAla^2]-[DLeu^5]-enkephalin bound by cells continuously grown in lipid-free serum was within the same range as those at the zero time point. The final concentration of ^{3}H-labelled [DAla^2]-[DLeu^5]-enkephalin used in the binding assay was 2 nM. Experiment I (●), experiment II (○). Results are means ±S.E. of three determinations.

Fig. 4. Binding of ^{3}H-labelled [DAla^2]-[DLeu^5]-enkephalin by membranes of NG108-15 cells pretreated with linolenic acid. Membranes of cells grown in normal medium were preincubated at 37°C in either Dulbecco's modified Eagle's medium containing 10% lipid-free serum and 100 μM linolenic acid or 0.05 M Tris-HCl buffer (pH 7.4). At the indicated times, aliquots of the mixtures were removed and the membranes washed three times with 0.05 M Tris-HCl buffer. Binding was then performed as described in Materials and Methods. Preincubated in $C_{18:3}$ (●), preincubated in Tris-HCl buffer (○). Results are mean ±S.E. of three independent experiments. None of the differences between $C_{18:3}$ and Tris buffer at each time point is statistically significant.

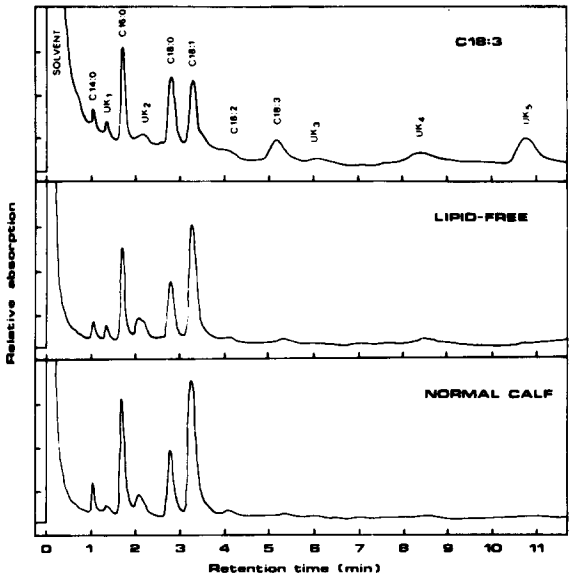

Fig. 5. Fatty acid profiles of phospholipids from fibroblasts grown in different media. Peaks whose identity was not certain are labelled as unknown (UK) and no attempt was made to quantify them.

before harvesting them for binding assay. So far we have assumed that unsaturated fatty acids are required to be incorporated into the phospholipid components of the cell membrane before they can exert their effect on opioid binding. Alternatively, unsaturated fatty acids may be adsorbed onto the membrane and thus affect the binding of [DAla^2]-[DLeu^5]-enkephalin. To resolve this, we performed two experiments. In one we incubated cells in $C_{18:3}$-supplemented medium for different lengths of time and observed their binding capacity. In another we preincubated membranes isolated from cells grown in normal medium with $C_{18:3}$ and determined if this could reduce [DAla^2]-[DLeu^5]-enkephalin binding. Since fatty acid adsorption to membrane is likely to occur much more rapidly than incorporation of the fatty acids into the membrane phospholipids, we would expect the latter experiment to yield a positive result if adsorption were the underlying cause. However, if metabolic events are required to mediate the inhibitory effect, a much slower time course of inhibition would be expected. As depicted in Fig. 3, inhibition of binding in cells grown in $C_{18:3}$-supplemented medium increased with the time of incubation, reaching a maximum only at about 48 h. On the other hand, when membranes isolated from normal cells were preincubated with $C_{18:3}$ for up to 3 h there was no appreciable decrease in binding compared with the control (Fig. 4). Taken together, the above results suggest that unsaturated fatty acids may have to be incorporated metabolically before they can exert their effect on opioid receptor binding. To evaluate this furter, we next examined the acyl composition of the phospholipids from cells grown in $C_{18:3}$-supplemented, lipid-free and normal medium. As indicated in Fig. 5 and Table II, the acyl composition of cells grown in $C_{18:3}$ medium is quite distinct from those of cells grown in either normal or lipid-free medium. Other than the expected increase of $C_{18:3}$, the amount of stearate increased by 30% while that of oleate decreased by 40%. In addition to these changes, the phospholipids of

TABLE II

ACYL COMPOSITION OF PHOSPHOLIPIDS OF CELLS GROWN IN DIFFERENT CULTURE MEDIA

Only fatty acid methyl esters that were identifiable with standard compounds are listed. As indicated in Fig. 5, there are altogether five components which could not be identified and since they did not contribute more than 5–10% of the total acyl content, they were not included in the calculated of weight percent.

Culture medium	Identifiable fatty acid components (wt.%)				
	$C_{14:0}$	$C_{16:0}$	$C_{18:0}$	$C_{18:1}$	$C_{18:3}$
$C_{18:3}$	1.5	25	30	29	15
Lipid-free	1.0	23	23	49	3
Calf-serum	2.0	24	23	49	2

TABLE III

EFFECT OF LINOLENIC ACID ON THE RATE OF GROWTH AND PROTEIN SYNTHESIS IN NG108-15 CELLS

Cells were raised in various media as described in Materials and Methods. To assess the rate of growth, cells were seeded in 75 cm^2 culture flasks to the same density at the beginning of the experiment. Cells were first allowed to grow for 48 h in normal medium and then for another 48 h in media as stated below. After incubation, cells were shaken off and their number determined. In the case of protein synthesis, 5 h before the termination of the experiment, 0.1 μCi/ml of [^{14}C]leucine was added. At the termination, cells were washed three times with 0.01 M phosphate-buffered saline and the amount of [^{14}C]leucine incorporated was determined by precipitation with 10% trichloroacetic acid. Results are means ± S.E.

Treatment	Number of experiments	Growth ($\times 10^{-6}$ cells/flask)	Protein synthesis (cpm/10^6 cells)
$C_{18:3}$	3	1.48 ± 0.01 [a]	14589 ± 2473
Lipid-free serum	3	2.05 ± 0.09 [a]	18104 ± 479
Calf serum	3	1.68 ± 0.15	19933 ± 960

[a] The difference between these two values is statistically significant by the t-test ($0.005 > P > 0.001$).

cells grown in $C_{18:3}$-supplemented medium contained an acyl group ($R_t = 10.75$ min) which was not detectable in the control cells (Fig. 5). In view of these results, incubating cells in the presence of $C_{18:3}$ probably enhances the uptake of this fatty acid as well as bringing about a profound alteration in the acyl composition of the phospholipids, viz., an increase in the number of unsaturated bonds and a decrease of oleate-to-stearate ratio.

According to the results presented in Fig. 2, the reduction of [DAla^2]-[DLeu^5]-enkephalin binding is apparently due to a decrease of binding capacity (B_{max}) rather than a decrease in affinity (K_D). This implies that there is either a masking of the existing opiate receptors or a reduction in their synthesis. To differentiate these two possibilities, we determined the rate of protein synthesis in cells grown in media supplemented with normal calf serum, lipid-free calf serum and lipid-free calf serum containing $C_{18:3}$. Compared with cells grown in plain and lipid-free serum, the rates of growth and protein synthesis in cells grown in $C_{18:3}$ medium were retarded by 28 and 19%, respectively (Table III). Although the difference in the rate of protein synthesis was only of borderline significance ($0.1 > P > 0.05$), it is probably genuine, since growth was significantly retarded.

Discussion

The present study was undertaken to evaluate the role of membrane lipids in influencing the binding of opiates to their receptors. For experimental convenience we have used the NG108-15 hybrid cells because their membrane lipid can be easily altered by varying the fatty acid composition of the culture medium [11,12]. Cells cultured in media supplemented with saturated fatty acids of various chain length were as effective as the control in the binding ^{3}H-labeled [DAla^2]-[DLeu^5]-enkephalin. On the hand, cells cultured in fatty acids of increasing unsaturation showed a proportional decrease in binding capacity and this decrease appears to require the metabolic incorporation of the unsaturated fatty acids into the membrane phospholipid.

The mechanism whereby unsaturated fatty acids affect opioid binding remains speculative at this stage. It is generally accepted that when cells are cultured in medium supplemented with unsaturated fatty acids, an increase of membrane fluidity will result [13]. Our data on the chemical analysis of the acyl composition of the phosphlipids from cells cultured in $C_{18:3}$-supplemented medium is in line with this reasoning, since the proportion of linolenate was significantly elevated. Although the structure of the opiate receptor is by-and-large unknown, recent data indicate that it is probably a membrane protein complexed with a certain type of acidic lipid [14]. Hence, according to this model, a change in membrane fluidity will affect not only the relative position of the receptor in the membrane but also the interaction of the receptor with the essential acidic lipid

component(s). In this respect, it is of interest to note that the binding of serotonin by mouse synaptic membranes is also affected by either a positive or a negative change of membrane microviscosity [15].

When the neuroblastoma x glioma hybrid cells were cultured in $C_{18:3}$-supplemented medium, a retardation of growth and protein synthesis was observed. Since we measured opioid binding as fmol per mg cell protein our result should not be affected significantly, even though the number of cells obtained from different cultures varied somewhat. The reduced rate of protein synthesis may explain, to a certain extent, the reduction of opioid binding (viz., a decrease in opioid receptor synthesis). But since the rate of protein synthesis was reduced by only 20%, it is probably too small to account for the 2-fold reduction of opioid binding observed. A more likely explanation is that treatment with unsaturated fatty acids may result in an alteration of membrane structure leading to a loss of opioid binding activity as well as a retardation of growth and protein synthesis. In support of this contention, membrane fluidity has been shown to play important roles in a number of cellular functions, including receptor-ligand recognition [15,16], membrane-bound enzyme activities [17] as well as the regulation of cell cycle [18].

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