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EFFECTS OF MEMBRANE POLYUNSATURATED FATTY ACIDS ON OPIATE PEPTIDE INHIBITION OF BASAL AND PROSTAGLANDIN E_1-STIMULATED CYCLIC AMP FORMATION IN INTACT N1E-115 NEUROBLASTOMA CELLS

MARY G. MURPHY,*† CAROL M. MOAK* and BARBARA G. RAO‡
*Department of Pharmacology, Dalhousie University, and ‡Department of Biology, Mount Saint Vincent University, Halifax, Nova Scotia, Canada B3H 4H7

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Abstract—The effects of membrane polyunsaturated fatty acids (PUFA) on opiate peptide-mediated inhibition of basal and prostaglandin E_1-stimulated cyclic AMP formation were examined in intact N1E-115 neuroblastoma cells. Addition of opiate peptides such as methionine^5-enkephalin (metEnk) to control cultures and to cultures that had been supplemented for 48 hr with 50 μM linoleic acid resulted in dose-dependent decreases in cAMP formation; these decreases were blocked by naloxone. Maximum inhibition of basal cyclase activity was 50–55% in both control and PUFA-enriched cells; however, half-maximal inhibition required ten times more metEnk in supplemented cultures than in controls. This is consistent with our observation that the affinity of binding of [tyrosyl-3′,5′-^{3}H(N)](2-D-alanine-5-D-leucine)enkephalin ([^{3}H]DADLE) to intact PUFA-enriched cells was lower than that to control cells. Receptor density was not modified as a result of supplementation. Addition of prostaglandin E_1 (PGE_1) to the cells produced rapid dose-dependent increases in cAMP formation. Maximum responses were higher in PUFA-enriched than in control cells (1924 and 972 pmol cAMP formed/mg protein respectively). Also, the apparent value for EC_{50} for PGE_1 was consistently lower in supplemented cultures. MetEnk reduced PGE_1-stimulated cAMP formation by 45–55% in both control and supplemented cells, and values for IC_{50} were similar (approximately 30 nM) in both. In the presence of the opiate peptide, values for EC_{50} for PGE_1 were similar in control and PUFA-enriched cultures (0.07 and 0.09 μM respectively). The data from these studies suggest that membrane PUFA increase the efficiency of coupling of receptors that stimulate cAMP formation and decrease the efficiency of those that mediate inhibition.

Opiate peptides such as methionine^5-enkephalin (metEnk§) inhibit intracellular formation of cyclic AMP in cultured neuroblastoma [1–3] and neuroblastoma × glioma hybrid cells [3–5] by activating a homogeneous population of delta opioid receptors that are negatively coupled with adenylate cyclase (ATP pyrophosphate-lyase (cyclizing), EC 4.6.1.1). Opiate-dependent inhibition of cyclic AMP (cAMP) synthesis is mediated by the inhibitory guanine nucleotide regulatory protein, N_i, and involves opiate agonist activation of endogenous GTPase activity [6].

Functioning of opiate receptor–adenylate cyclase systems within neuromembranes appears to be very sensitive to the membrane lipid environment [7, 8]. Alterations in lipid structure by phospholipase digestion [9, 10] or by incorporation of exogenous complex lipid [11] or unsaturated fatty acids [12, 13] have been reported to have variable effects on receptor–effector interactions. Unfortunately, it has been difficult to interpret much of the data from these studies since assays have been carried out under widely varied incubation conditions, and it is becoming increasingly apparent that the choice of these conditions can alter dramatically the outcome of the study. For example, we demonstrated recently that basal accumulation of cAMP is increased significantly in clone N1E-115 neuroblastoma cells when the membrane polyunsaturated fatty acid (PUFA) content of the cells is elevated following addition of linoleic acid to the culture medium [14]. Enhanced cyclase activity is dependent upon cellular integrity, since the effect was not seen when the cells were homogenized before being assayed. Not surprisingly, McGee and Kenimer [12] also reported that PUFA enrichment does not affect basal or prostaglandin-stimulated adenylate cyclase activity in membranes from neuroblastoma × glioma hybrid cells. We have found recently that cAMP formation in the intact hybrid cells is also increased following linoleate supplementation (unpublished).

The purpose of the present study was to determine the effects of increasing membrane PUFA on opiate-dependent inhibition of basal and PGE_1-stimulated cyclic AMP formation in intact viable neuroblastoma cells. The results of the study demonstrate that PUFA enrichment has distinct effects on agonist-

† Address for correspondence: Dr. M. G. Murphy, Department of Pharmacology, Sir Charles Tupper Medical Bldg., Dalhousie University, Halifax, Nova Scotia, Canada, B3H 4H7.

§ Abbreviations: met-enkephalin (metEnk), methionine^5-enkephalin; PUFA, polyunsaturated fatty acid(s); PGE_1, prostaglandin E_1; and DADLE, [D-alanine, D-leucine]-enkephalin.

dependent inhibition and stimulation of adenylate cyclase, which suggests that there is a versatility in fatty-acid modulation of neuroreceptor function that was not appreciated previously.

MATERIALS AND METHODS

Cell culture and fatty-acid supplementation. NIE-115 neuroblastoma cells were a gift from Dr. E. Richelson. The cells were seeded at a density of 5–7 × 10^4 cells/ml into 35-mm Primaria culture dishes (Becton Dickinson, Mississauga, Canada) in Dulbecco's modified Eagle's medium (DMEM) containing 10% (v/v) fetal bovine serum (Flow Laboratories, Mississauga, Canada). Cultures were maintained in a humidified atmosphere of 5% CO_2/95% air at 37°, and subculture was carried out every 5–7 days. Only cells between passages 18 and 40 were used for this study.

The procedure for fatty-acid supplementation has been described in detail elsewhere [14]. Briefly, 24 hr following subculture, linoleic acid (Supelco Canada Ltd., Oakville, Canada) was complexed with fatty-acid-poor bovine serum albumin (BSA) and added to half of the dishes at a final concentration of 50 μM. An equal concentration of BSA was added to the control cultures. Culture was continued for 48 hr, at which time the medium was aspirated and the cells were washed with serum-free DMEM buffered at pH 7.6 with 25 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid.

Lipids were extracted, and the phospholipid fatty-acid profiles were analyzed as described previously [14].

Measurement of adenylate cyclase activity. Adenylate cyclase activity was measured as the amount (pmol/mg protein) of cAMP formed in the intact cells. Incubations were carried out in the culture dishes in 1 ml of incubation medium (HEPES-buffered, serum-free DMEM, as above) containing 0.7 mM Ro 20-1724 [4-(3-butoxy-4-methoxybenzyl)imiazolidin-2-one] (Hoffmann-LaRoche, Etobicoke, Canada) at 30° for a total incubation time of 40 min. This temperature was selected since we felt that reducing it from 37° would lower the risk of degradation of met-enkephalin and yet still support a reasonable rate of adenylate cyclase activity. Where indicated, prostaglandin E_1 (PGE_1) was added to the incubation medium in 10 μl of ethanol, 10 min prior to termination of the assay. Addition of ethanol alone did not affect cyclic AMP formation. Where indicated, met-enkephalin was added 10 min prior to the addition of PGE_1 (i.e. 20 min after beginning the assay). All assays were carried out in triplicate dishes. To terminate the reactions the medium was aspirated, and the cells were washed twice with fresh ice-cold medium. Ethanol (250 μl) was added to each dish, and the cells were disrupted by brief sonication. The dish contents were transferred with rinsing to disposable tubes, and membranes and supernatant fractions were separated by centrifugation (1000 *g*, 15 min). Pellets were digested in 1 N NaOH and analyzed for protein according to Lowry *et al.* [15] with BSA as standard. Supernatant fractions were dried under N_2, and the residues were resuspended in 50 mM sodium acetate buffer (pH 4.5) containing 4 mM EDTA; duplicate aliquots from each dish were analyzed for cyclic AMP according to Gilman [16], using a kit from Amersham (Oakville, Canada).

Opiate receptor-binding assays. The cells were harvested, washed, and suspended in 5 mM HEPES buffer, pH 7.6, containing 0.32 M sucrose, 0.5 mM $MgCl_2$ and 0.5 mM $CaCl_2$. Assays were carried out in 1.5-ml microfuge tubes in 1 ml of the above buffer that contained [tyrosyl-3',5'-^{3}H(N)](2-D-alanine-5-D-leucine) enkephalin ([^{3}H]DADLE; 46.9 Ci/mmol, New England Nuclear, Lachine, Canada) (0.5 to 20 mM), with or without met-enkephalin (10^{-5} M) as competing ligand. Incubation was initiated by addition of the cells and continued in a shaking water bath at 30° for 40 min. Bound and free [^{3}H]DADLE were separated by rapid centrifugation (10,000 *g*, 2 min); the supernatant fractions were quickly aspirated, and the pellets were washed twice with ice-cold buffer. The pellets were dissolved in 750 μl NaOH, and duplicate aliquots were counted. Specific binding (fmol/mg protein) was calculated as the amount of [^{3}H]DADLE bound to the cells in the absence of met-enkephalin minus that bound in its presence.

Fluorescence polarization measurements. Fluorescence polarization measurements were carried out as described by Crews *et al.* [17] in the laboratory of Dr. Fulton T. Crews, at the University of Florida, Gainesville, FL.

Statistical analysis. Data represent the means (±SEM) of values obtained from triplicate dishes assayed in at least two separate experiments. Data were analyzed using a Student's *t*-test, and differences were judged to be significant when $P < 0.05$.

RESULTS

Effects of linoleate supplementation on membrane PUFA content, cell proliferation, and membrane physical properties. The effects of supplementing N1E-115 neuroblastoma cells with 50 μM linoleic acid on the fatty-acid profiles of membrane phospholipids have been described in detail elsewhere [14]. Briefly, by 48 hr following addition of linoleate to the culture medium there were significant increases in the proportions of linoleic (>9-fold), eicosatrienoic (>5-fold), arachidonic (>4-fold) and docosatetraenoic (>3-fold) acids esterified to membrane phospholipid, relative to the amounts seen in control cells. Supplementation stimulates cell proliferation by approximately 20% without altering the amount of protein/cell [14], increases prostaglandin production [18], and elevates lipid peroxide levels, as judged by increased formation of malondialdehyde [19]. To determine whether PUFA enrichment altered the physical properties of the neuroblastoma membranes, we measured the fluorescence polarization (P) properties of the control and supplemented cells using the fluorescent probe, 1,6-diphenyl-1,3,5-hexatriene (DPH) [17]. As can be seen in Fig. 1, the values for fluorescence polarization in the PUFA-enriched membranes were significantly lower than those seen with the control membranes, over a temperature range from 14 to 40°.

Effects of linoleate supplementation on basal and

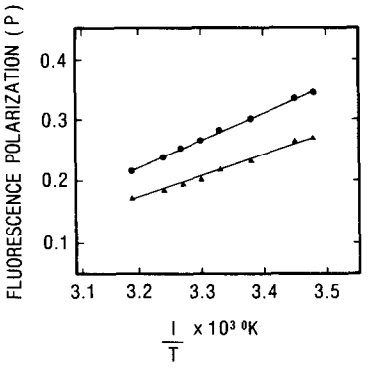

Fig. 1. Effects of supplementing N1E-115 neuroblastoma with BSA (●) or 50 μM linoleic acid/BSA (▲) on temperature-dependent changes in fluorescence polarization (P). The cells were hypotonically lysed and the membranes were isolated by differential centrifugation. The particulate fractions were suspended in phosphate-buffered saline (40 μg protein/ml) and labeled with the fluorophore 1,6-diphenyl-1,3,5-hexatriene (DPH; 1 μM, final concentration). Fluorescence polarization was measured with an Aminco-Bowman SPF-500 spectrophotofluorimeter as described by Crews *et al.* [17]. The data represent the means of values obtained from two separate membrane preparations. Individual values varied by less than 5%.

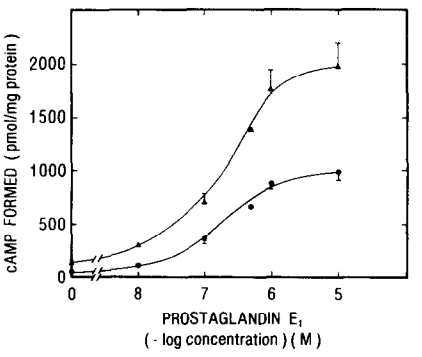

Fig. 2. Effects of prostaglandin E_1 on cyclic AMP formation in neuroblastoma cells cultured for 48 hr with BSA (controls) (●) or 50 μM linoleic acid/BSA (▲). After the cells were washed, assays were carried out *in situ* in the presence of Ro 20-1724 for 40 min at 30°, as described in the text. PGE_1 was added 10 min before the termination of the incubation. The data represent means (±SE) of values obtained from at least six separate dishes, in three independent experiments.

stimulated cyclic AMP formation. In previous studies, we demonstrated that N1E-115 neuroblastoma cells supplemented with linoleic acid for 48 hr form two to five times more cyclic AMP than do their corresponding controls during a 40-min incubation with the phosphodiesterase inhibitor, Ro 20-1724 [14]. The magnitude of PUFA-dependent enhancement varied as a function of the passage number of the cells, with the greatest differences in cyclase activities occurring in cells with low passage numbers (not shown).

Addition of PGE_1 to the incubation medium resulted in a rapid, dramatic increase in intracellular cAMP formation in both control and linoleate-supplemented cells (Fig. 2). In both, maximum response was achieved within 5 min (data not shown). Exposure of the cells to 10 μM PGE_1 for 10 min resulted in 18 ± 1- and 13 ± 3-fold increases in nucleotide levels in the control and PUFA-enriched cultures respectively. Stimulation did not require the presence of extracellular Na^+, since similar amounts of cAMP were formed when it was replaced with an equivalent amount of choline (data not shown). Kinetic analysis of data from four separate experiments with PGE_1 yielded values for maximum response of 972 ± 77 and 1924 ± 171 pmol cAMP formed/mg protein (mean ± SEM) for control and linoleate-supplemented cells respectively. Interestingly, the amount of PGE_1 required for half-maximal stimulation (EC_{50}) of adenylate cyclase activity was significantly lower in PUFA-enriched cultures than in controls (Table 1).

Opiate-dependent inhibition of basal cAMP formation. Addition of met-enkephalin to the incubation medium for the final 20 min of the 40-min assay resulted in a dose-dependent inhibition of basal cAMP formation in both control and linoleate-supplemented cultures (Fig. 3). In both, the opiate reduced cyclase activity to 50–55% of control values. However, there was an approximate 10-fold dif-

Table 1. Effects of PUFA enrichment on PGE_1-stimulated cAMP formation in intact neuroblastoma cells in the absence and presence of met-enkephalin

	Cyclic AMP formation			
	− metEnk		+ metEnk	
Culture supplement*	Maximum response (pmol cAMP/mg protein)	EC_{50} (μM)	Maximum response (pmol cAMP/mg protein)	EC_{50} (μM)
BSA (control)	972.0 ± 76.5†	0.15 ± 0.03	577.2 ± 95.5	0.07 ± 0.01‡
Linoleate/BSA	1923.8 ± 170.6	0.10 ± 0.01‡	1112.6 ± 185.4	0.09 ± 0.03

* Cells were cultured for 48 hr in the presence of BSA (0.08%) or 50 μM linoleic acid complexed to BSA, after which cAMP assays were carried out at 30° for 40 min as described in Materials and Methods.

† Data represent the means (±SEM) of values obtained from four separate saturation experiments, each of which was carried out in triplicate in the presence and absence of metEnk.

‡ $P < 0.05$, relative to values obtained with control cultures in the absence of metEnk.

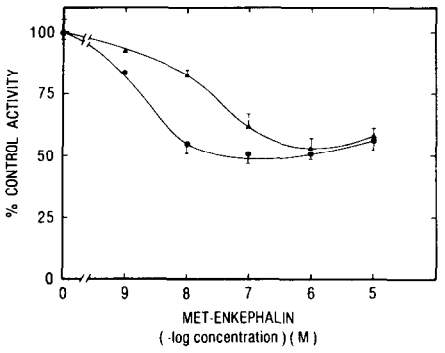

Fig. 3. Met-enkephalin-dependent inhibition of basal cyclic AMP formation in neuroblastoma cells cultured with BSA (●) or linoleate (50 μM)/BSA (▲) for 48 hr. Incubations were carried out at 30° for a total of 40 min as described in the text. After the initial 20 min, metEnk was added to the dishes at the concentrations indicated. Control values (pmol cAMP formed/mg protein, mean ± SEM) were 66 ± 4 and 142 ± 9 for control and supplemented cells respectively. Each data point represents values obtained from three to eight individual dishes, from three separate experiments.

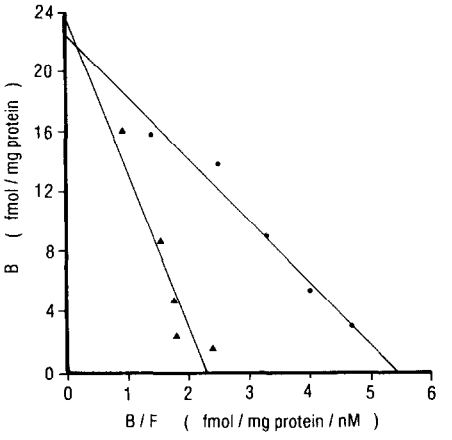

Fig. 5. Eadie–Hofstee analysis of specific binding of [^{3}H]DADLE to intact N1E-115 neuroblastoma cells that had been cultured for 48 hr in the presence of BSA (●) or linoleate (50 μM)/BSA (▲). Details for the binding assay are described in Materials and Methods. The values obtained represent the means of triplicate determinations obtained in one of three separate experiments. Linear regression analysis yielded values for B_{max} for control and supplemented cells of 22.5 and 24.0 fmol/mg protein, respectively, and for apparent K_d of 4.1 and 10.4 nM, respectively.

ference between the apparent values for K_i with the two cultures, with half-maximal inhibition in control and supplemented cells observed at 2 and 25 nM met-enkephalin respectively. Inhibition was blocked by inclusion of 10 μM naloxone in the incubation medium (data not shown).

Inhibition of PGE_1-stimulated cAMP formation. Addition of met-enkephalin to the assay dishes 10 min before PGE_1 was added resulted in a dose-dependent inhibition of adenylate cyclase activity (Fig. 4). Inhibition was not observed when Na^+ was replaced with an equimolar concentration of choline, and it was reduced significantly when naloxone (10 μM) was included in the incubation medium (data not shown). Data obtained from three separate experiments demonstrated that the magnitude of met-enkephalin-dependent inhibition was similar in control and supplemented cells. Also, equivalent amounts (approximately 30 nN) of metEnk produced half-maximal inhibition of PGE_1-stimulated cAMP formation.

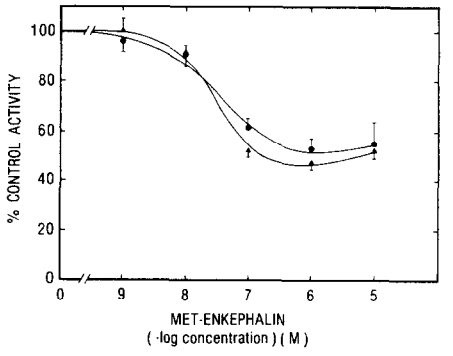

Fig. 4. Met-enkephalin-dependent inhibition of PGE_1-stimulated cAMP formation in control (●) and linoleate-supplemented (▲) neuroblastoma cells. MetEnk and PGE_1 (1 μM) were added to the incubation dishes 20 and 30 min, respectively, following initiation of the assay. Control values obtained in the absence of metEnk were 1020 and 1619 pmol cAMP/mg protein for control and PUFA-enriched cultures. The data presented are from one of three separate experiments.

We then examined the effects of met-enkephalin (1 μM) on the kinetics of activation of adenylate cyclase with increasing concentrations of PGE_1. The data in Table 1 clearly indicate that metEnk reduced the maximum response to PGE_1 under both culture conditions. In control cells, the peptide also reduced the amount of PGE_1 required to elicit half the maximum response from 0.15 to 0.07 μM. By comparison, in PUFA-enriched cultures the EC_{50} for PGE_1 was not altered significantly by the addition of met-enkephalin.

Effects of linoleate supplementation on [^{3}H]DADLE binding to intact neuroblastoma cells. To determine whether membrane PUFA affected the interaction between the opiates and their cell-surface receptors, we examined specific binding of [^{3}H]DADLE to intact control and linoleate-supplemented cells, essentially as described by Blume *et al.* [20]. Incubations were carried out in the Na^+-free incubation medium, since both total and specific binding were reduced dramatically in the presence of even small quantities of the cation. The data from a representative experiment are shown in Fig. 5. Under both culture conditions, the specific binding of [^{3}H]DADLE saturated when 20–25 fmol of the peptide were bound per mg protein. In control cells, the concentration of opiate required for half-maximal binding (K_d) ranged from 2 to 4 nM; by comparison, the apparent values for K_d in linoleate-supplemented cells were approximately 2-fold higher, ranging from 4 to 10 nM. In any given experiment, the K_d value for the PUFA-enriched cells was always higher than that for controls.

DISCUSSION

Membrane polyunsaturated fatty acids (PUFA) have the potential to modify neural receptor function by at least three distinct mechanisms [21, 22]. In neural membranes particularly, PUFA constitute a large proportion of the "boundary" lipid surrounding components of the receptor complex (e.g. recognition site, regulatory protein, catalytic unit), and their structural alteration can have specific effects on the conformation (and therefore functional properties) of these components. Second, membrane PUFA are major determinants of the physical properties of the membrane, and these will determine the efficiency of coupling of components of the receptor systems. Finally, increases in membrane PUFA may modulate receptor function via production of bioactive eicosanoids. We have not yet examined involvement of prostaglandins or leukotrienes in opiate receptor function although we have shown that they are not involved in modulation of basal activity [14].

In studies with biological membranes, it is difficult to distinguish between direct effects of PUFA on receptor components, and their indirect effects mediated through physical changes in the membrane environment. Our observation that values for fluorescence polarization were reduced significantly in membranes from linoleate-supplemented neuroblastoma cells (Fig. 1) demonstrates that acyl chain mobility, which is an indirect measure of membrane "fluidity" [23], is increased in the PUFA-enriched cells. However, the fact that PUFA alter basal adenylate cyclase activity (which would not be expected to be affected by membrane "fluidity") suggests that they also can directly influence the conformation and function of the catalytic unit. It is not unreasonable to assume that other components of the receptor complex (e.g. the nucleotide coupling proteins) could be similarly affected. We believe that the results of our studies support a concept that membrane PUFA modulate receptor function by directly affecting the structure/activity of one or more of the receptor components.

Examination of specific binding of [^{3}H]DADLE to intact N1E-115 cells demonstrated that linoleate supplementation resulted in an approximately 2-fold reduction in the apparent affinity (K_d) of the receptor for the ligand without affecting receptor density (B_{max}) (Fig. 5). These results are in direct contrast to those of McGee and Kenimer [12] and Ho and Cox [13], who reported that supplementation of NG108-15 neuroblastoma × glioma hybrid cells with linoleic acid resulted in a reduction in the number of opiate receptors but did not alter values for K_d. There are several possible explanations for the discrepancies between our findings, including possible cell-line-specific differences in these receptors. However, we believe that it is more likely that they reflect differences in assay conditions [24–26], and particularly the fact that isolated membranes were used in the above studies [12, 13], whereas we used intact cells. There are at least two possible explanations for the PUFA-dependent reduction in the apparent affinity of the neuroblastoma opiate receptor for [^{3}H]DADLE, the first of which is the fatty-acid-induced alteration in receptor conformation that was discussed above. Alternatively, or perhaps as well, the data may reflect PUFA-mediated alterations in GTP-dependent dissociation of the nucleotide regulatory protein, N_i, from the DADLE–receptor complex, a process that results in a receptor with a reduced affinity for agonist [27].

As has been demonstrated in many neuroblastoma cell lines, including N1E-115 [14, 28], addition of PGE_1 to the cells produced a dramatic increase in intracellular cAMP formation (Fig. 2). Maximum PGE_1-stimulated cyclase activities in linoleate-supplemented cultures were less than 2-fold higher than in controls, whereas the ratio of basal activities (PUFA-enriched:control cells) was consistently higher (3–5:1). We believe that the values obtained in the PUFA-enriched cultures reflect the maximum capacity of adenylate cyclase in this cell line, since the activities measured under these conditions were never exceeded. One of the interesting findings of this study was that the EC_{50} for PGE_1 for stimulation of cAMP formation was significantly lower in the cells cultured with linoleate (Table 1), which suggests that there was a PUFA-dependent increase in the efficiency of functional coupling of the PGE_1-activated stimulatory receptor with the catalytic unit. This would be consistent with the apparent increase in "fluidity" of the PUFA-enriched membrane; however, data from studies of opiate receptor function suggest that this is not the case.

Linoleate supplementation had effects on opiate receptor-mediated inhibition of adenylate cyclase activity that differed from those seen with the stimulatory agonist. First, PUFA enrichment did not alter the maximum extent to which met-enkephalin inhibited either basal (Fig. 3) or PGE_1-stimulated (Fig. 4) cAMP formation in the N1E-115 cells. Second, the concentration of opiate peptide required to elicit half-maximal inhibition of basal in the PUFA-enriched cells was approximately 10-fold higher than that needed to elicit an equivalent effect in the controls (Fig. 3), suggesting a PUFA-dependent decrease in receptor–effector coupling. This apparent decrease in effectiveness of the opiates is consistent with the reduced affinity (K_d) of the receptors on the supplemented cells for [^{3}H]DADLE as measured in the binding studies. However, the fact that linoleate supplementation reduced the apparent affinity of [^{3}H]DADLE binding by 2-fold but reduced the efficiency of opiate-mediated inhibition of cAMP formation by approximately 10-fold suggests that one or more of the post-receptor events are attenuated by increasing the proportion of unsaturated fatty acids in the membrane. Among these are the dissociation of the coupling protein N_i into its α and $\beta\gamma$ subunits (or alternatively, their reassociation), the interaction between the inhibitory $\beta\gamma$ complex and the catalytic unit, and the GTPase-catalyzed dephosphorylation of GTP. Each of these reactions is believed to play a part in receptor–effector coupling [29].

From the results obtained in this study, we propose that in the neuroblastoma cell lines, and possibly in brain, membrane PUFA modulate the functioning of receptors that stimulate adenylate cyclase activity in a manner distinct from that by which they regulate

inhibitory receptors. The fact that the effects on the two receptor systems are opposite provides evidence that modulation is not solely due to indirect, non-specific changes in membrane physical properties (e.g. membrane fluidity). Rather, it appears that regulation involves linoleate-dependent changes in the conformation and/or functional properties of one or more of the receptor components. This is particularly true of the inhibitory opiate receptor, in which functional coupling is attenuated even under growth conditions which increase the "fluidity" of the environment surrounding the receptor.

In conclusion, our observation that polyunsaturated fatty acids can affect receptor–effector interactions in a highly selective manner strongly supports the widely-held belief that these abundant membrane constituents play very important roles in the control of neural function. It is now necessary for us to define those roles more precisely.

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