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Interaction of δ-Opioid Receptors with Multiple G Proteins: A Non-relationship between Agonist Potency to Inhibit Adenylyl Cyclase and to Activate G Proteins

P. L. PRATHER, H. H. LOH, and P. Y. LAW

Department of Pharmacology, School of Medicine, University of Minnesota, Minneapolis, Minnesota 55455

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SUMMARY

The purpose of the present investigation was to determine whether the coupling of δ-opioid receptors to multiple G proteins in NG108–15 neuroblastoma × glioma cells is a characteristic limited to only this cell line (because of the high density of δ-opioid receptors) and to ascertain whether there is any correlation between δ-opioid agonist potency to inhibit adenylyl cyclase and to activate G proteins. Interactions between receptors and G proteins were investigated using agonist-stimulated incorporation of the photoreactive GTP analog azidoanilido[α-^{32}P]GTP ([α-^{32}P]AA-GTP) into G protein α subunits, with subsequent separation by urea/sodium dodecyl sulfate-polyacrylamide gel electrophoresis. In NG108–15, NS20Y, and N1E115 cell membranes, four α subunits ($G_{i2\alpha}$, one isoform of $G_{i3\alpha}$, and both isoforms of $G_{o\alpha}$) in the 39–41-kDa region were labeled with [α-^{32}P]AA-GTP. The δ-opioid agonist [D-Ala^2,D-Leu^5]-enkephalin (DADLE) produced a dose-dependent, naloxone-reversible increase of [α-^{32}P]AA-GTP incorporation into all four α subunit subtypes, in all cell lines tested. In addition, with the single exception of $G_{i3\alpha}$ in NG108–15 cells, the maximal increases in incorporation of the photoaffinity label into all G_α subunits induced by DADLE were similar. The B_{max} values determined for δ-opioid receptors in NG108–15, NS20Y, and N1E115 cell membranes were 570, 370, and 120 fmol/mg of protein, respectively. Finally, although the IC_{50} values to inhibit intracellular cAMP production and affinity for DADLE were similar across the three cell lines, the ED_{50} values to produce labeling of the G_α subunits between cell lines differed by >100-fold. In fact, only in NS20Y cells were the IC_{50} and ED_{50} values comparable. Firstly, these results suggest that simultaneous coupling of the δ-opioid receptor to multiple G protein α subunits occurs in a variety of cell lines that express a range of receptor densities. Secondly, the magnitudes with which δ-opioid receptors interact with available G_α subunits in response to agonist are approximately the same. Finally, there appears to be no relationship between the potency of agonists to inhibit adenylyl cyclase and that required for activation of G proteins.

Signal transduction of information across cellular membranes by opioid receptors and many other types of receptors is mediated through coupling to G proteins (1). Recent evidence indicates that a single receptor type can interact with several G proteins (2, 3), which in turn can couple to more than one effector (4, 5). Due to this complexity, receptor/G protein interactions are often investigated in cellular models. The NG108–15 neuroblastoma × glioma hybrid cell line provides such a model because it contains a homogeneous population of δ-opioid receptors (6), as well as five subtypes of G protein α subunits ($G_{i2\alpha}$ and two isoforms of both $G_{o\alpha}$ and $G_{i3\alpha}$) (2). In addition, the δ-opioid receptor in this cell line has been shown to couple to adenylyl cyclase and calcium channels through $G_{i2\alpha}$ (4) and one isoform of G_o (5), respectively. Furthermore, it has been demonstrated recently that the δ-opioid receptor in NG108–15 cells also interacts with one isoform of $G_{i3\alpha}$, although the effector to which it is coupled is not yet known (2).

Recent studies, however, have indicated that receptor/G protein interactions are influenced by a variety of factors that should be considered before it is assumed that a receptor can couple to several G proteins simultaneously. For example, studies with the α_2-adrenergic receptor have revealed that, when high levels of the receptor are expressed, agonists not only can inhibit but also can stimulate cAMP production at high concentrations (7). This indicates that, when receptor densities are high, the α_2-adrenergic receptor could couple not only with $G_{i\alpha}$ but also with $G_{s\alpha}$. In addition, differential coupling of μ- and δ-opioid receptors to G protein α subtypes has recently

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ABBREVIATIONS: [α-^{32}P]AA-GTP, azidoanilido-[α-^{32}P]GTP; DADLE, [D-Ala^2,D-Leu^5]-enkaphalin; DAMGO, [D-Ala^2,*N*-methyl-Phe^4,Gly-ol^5]-enkephalin; DPDPE, [D-Pen^2,D-Pen^5]-enkephalin; DSLET, [D-Ser,Leu^5-enkephalyl]-Thr; PLO17, [*N*-methyl-Phe^3,D-Pro^4]-morphiceptin; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; TBS, Tris-buffered saline; GTPγS, guanosine-5′-*O*-(3-thio)triphosphate; App(NH)p, adenosine-5′-(2,3-imido)triphosphate; ECL, enhanced chemiluminescence.

been reported in a cell line that contains both opioid receptor types (3). Furthermore, it was shown that the δ-opioid receptor preferentially coupled to $G_{i1\alpha}$, while interacting only weakly with $G_{i2\alpha}$ and $G_{i3\alpha}$. These examples introduce the possibility that the coupling patterns of different receptors with their respective G proteins may depend on multiple factors, such as receptor densities, the presence of other receptor types that might share the same G proteins, and the cell line being investigated.

The purpose of the present investigation was to determine whether the coupling of δ-opioid receptors to multiple G proteins in NG108–15 neuroblastoma × glioma cells is a characteristic limited to only this cell line (because of the high density of δ-opioid receptors) and to ascertain whether there is any correlation between δ-opioid agonist potency to inhibit adenylyl cyclase and to activate G proteins. To investigate the interaction between receptors and G proteins, we used agonist-stimulated incorporation of the photoreactive GTP analog [α-^{32}P]AA-GTP into G protein α subunits, with subsequent separation by urea/SDS-PAGE. The ability of the δ-opioid agonist DADLE to induce photolabeling and to inhibit adenylyl cyclase was compared across three neuroblastoma cell lines (NG108–15, NS20Y, and N1E115) that contain homogeneous populations of δ-opioid receptors expressed across a wide range of densities.

Experimental Procedures

Materials. [^{32}P]NAD$^+$ (30 Ci/mmol), [α-^{32}P]GTP (3000 Ci/mmol), and all antisera (AS/7, EC/2, and GC/2) were purchased from DuPont/New England Nuclear (Boston, MA). [^{3}H]Adenine (27 Ci/mmol), [^{3}H]diprenorphine (36 Ci/mmol), and [α-^{32}P]ATP (17 Ci/mmol) were purchased from Amersham (Arlington Heights, IL). [^{32}P]cAMP was prepared by enzymatic conversion of [α-^{32}P]ATP using NG108–15 membranes. Pertussis toxin was obtained from List Biologicals (Campbell, CA). All reagents used in the present study were purchased from Sigma Chemical Co. (St. Louis, MO). ECL reagents were obtained from Amersham (Arlington Heights, IL). Naloxone was obtained from Endo Chemical Co. (Garden City, NY). PLO17, DPDPE, DADLE, DAMGO, and DSLET were obtained from Peninsula Laboratories, Inc. (Belmont, CA).

Cell culture. Neuroblastoma NS20Y and N1E115 cells were cultured at 37° in a humidified atmosphere of 10% CO_2/90% air, in Dulbecco's modified Eagle's medium supplemented with 5% fetal calf serum. Neuroblastoma × glioma NG108–15 cells were cultured similarly, except that 0.1 mM hypoxanthine, 10 μM aminopterin, and 17 μM thymidine were added to the growth medium. For some studies, cells were incubated with 100 ng/ml pertussis toxin or 100 nM DADLE for 24 hr before assay.

Membrane preparation. Immediately after control or chronic drug treatment, cells were harvested by detachment from the growth surface using 0.1% phosphate-buffered saline supplemented with 0.4% EDTA. Bound opioid ligands were removed by washing the pelleted cells twice with 50 ml of Krebs-Ringer-HEPES buffer (110 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1.8 mM $CaCl_2$, 25 mM glucose, 55 mM sucrose, 10 mM HEPES, pH 7.4) and incubating them at 37° for 10 min. P_2/P_3 membrane pellets were then prepared by centrifugation of membrane pellets, minus the nuclei preparations, at 100,000 × *g* for 60 min. Concentrations of protein in membrane preparations were then determined by the method of Lowry *et al.* (8).

Synthesis and purification of [α-^{32}P]AA-GTP. The method for synthesis and purification of [α-^{32}P]AA-GTP was a modification of that described by Offermanns *et al.* (9). Briefly, [α-^{32}P]GTP (1 mCi) was lyophilized in a silanized reaction vial. The sample was then dissolved in 60 μl of solution 1 [0.1 M 2-(*N*-morpholino)ethanesulfonic acid, pH 5.6, 15.5 μM *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride]. After 10 min of stirring, 30 μl of solution 2 (28.2 μM 4-azidoaniline in peroxide-free 1,4-dioxane) were added. The mixture was stirred continuously for 4 hr at room temperature (22°) in darkness. After synthesis, the reaction mixture was stored at −80° until purification.

[α-^{32}P]AA-GTP was purified by high performance liquid chromatography on a C_{18} column. The column was equilibrated with 97.2% solvent A (0.1 M triethylamine in water) and 2.8% solvent B (0.1 M triethylamine in absolute ethanol), at a flow rate of 0.7 ml/min. The reaction mixture (90 μl) was thawed and diluted to 250 μl with solvent A. The [α-^{32}P]AA-GTP was separated by a discontinuous gradient from 2.8 to 90% solvent B. Five-minute fractions (3.5 ml) were collected and 10-μl aliquots of each were counted for radioactivity. Two major radioactive peaks were collected, with retention times of 6 and 23 min, for [α-^{32}P]GTP and [α-^{32}P]AA-GTP, respectively. The retention times for these peaks corresponded to those determined previously for nonradioactive GTP and α-AA-GTP, and both major peaks were also able to displace [^{35}S]GTPγS binding. The peak containing [α-^{32}P]AA-GTP was divided into aliquots (containing 10 μCi each), lyophilized, and stored at −80° until use.

Photoaffinity labeling of G proteins with [α-^{32}P]AA-GTP. Photoaffinity labeling was performed using slight modifications of the method of Offermanns *et al.* (9). Plasma membranes (50 μg/assay tube) were incubated for 6 min at 30° in the presence or absence of agonist, in 100 μl of buffer I [30 mM HEPES, pH 7.4, 0.1 mM EDTA, 5 mM $MgCl_2$, 100 mM NaCl, 30 μM GDP, 1 mM benzamidine, 0.1 mM App(NH)p]. After agonist incubation, [α-^{32}P]AA-GTP (1 μCi/assay) was added and samples were incubated for an additional 6 min at 30°. The reaction was terminated by placing samples on ice. Membranes were then collected by centrifugation at 12,000 × *g* for 10 min and resuspended in 100 μl of buffer II [30 mM HEPES, pH 7.4, 0.1 mM EDTA, 5 mM $MgCl_2$, 100 mM NaCl, 2 mM dithiothreitol, 1 mM benzamidine, 0.1 mM App(NH)p]. Resuspended pellets (droplets) were then transferred onto Parafilm with a micropipette, maintained at 4°, and irradiated with 240 mJ from a UV lamp (254 nm, 150 W) at a distance of 15 cm. Samples were then centrifuged as before, resuspended in 50 μl of sample buffer (0.065 mMTris·HCl, pH 6.8, 2% SDS, 10% glycerol, 5% 2-mercaptoethanol), and heated at 90° for 2 min.

SDS-PAGE and autoradiography. Photolabeled membrane proteins were separated by SDS-PAGE, according to the method of Laemmli (10), on 20-cm separating gels containing 10% (w/v) acrylamide and 6 M urea. [α-^{32}P]AA-GTP-labeled G proteins were visualized autoradiographically with Kodak film and quantified by densitometry using the Image software program on a Macintosh II computer.

Immunoblotting. The ECL method of immunoblotting was used. Briefly, SDS-PAGE gels were transferred to Hybond-ECL nitrocellulose membranes and incubated overnight with 10% milk in blotting buffer (TBS-0.1%) (25 mM Tris·HCl, pH 7.6, 0.9% NaCl, 0.1% Tween-20). Blots were then washed three times (5 min each) with TBS-0.1% and incubated with primary antibody (1/1000) for 1 hr, with shaking. Antisera used were AS/7 (anti-$G_{i1\alpha}$ and -$G_{i2\alpha}$), EC/2 (anti-$G_{i3\alpha}$), and GC/2 (anti-$G_{o1\alpha}$ and -$G_{o2\alpha}$). Primary antibody was removed and blots were washed as described previously. Secondary antibody (donkey anti-rabbit immunoglobulin-horseradish peroxidase, 1/5000) was then added and incubated for 30 min, with shaking. Secondary antibody was removed and blots were washed with three 5-min washes with TBS-0.3%, followed by three 5-min washes with TBS-0.1%. Blots were then incubated for 1 min with equal volumes of ECL detection reagents 1 and 2, wrapped in Saran Wrap, and exposed to Hybond-ECL X-ray film for periods varying between 30 sec and 10 min.

Measurement of cAMP levels. The conversion of the [^{3}H]adenine-labeled ATP pools to cAMP was used as a measure of opioid ligand effects on cAMP levels, as described previously (6). Briefly, measurements were made with cells seeded into 17-mm (24-well) plates and cultured to 100% confluency for 2 days. On the day of the assay, medium was removed and replaced with incubation mixture (warmed

to 37°) (Dulbecco's modified Eagle's medium containing 0.09% NaCl, 500 μM 3-isobutyl-1-methylxanthine, and 2 μCi/well [^{3}H]adenine) for 1 hr. At the time of the assay, plates were placed in an ice-water bath for 5 min. The incubation mixture was removed and replaced with ice-cold assay mixture (Krebs-Ringer-HEPES buffer containing 500 μM 3-isobutyl-1-methylxanthine and 10 μM forskolin) with the opioid peptide to be tested. The plates were then incubated at 30° for 20 min and placed back in the ice-water bath for 5 min. After termination of incubations with 50 μl of 3.3 N perchloric acid and subsequent addition of [α-^{32}P]cAMP as an internal standard, radioactive cAMP was separated from other ^{3}H-labeled nucleotides by a double-column chromatographic method (11). Seven milliliters of scintillation fluid were then added and samples were immediately counted in a Beckman LS2800 scintillation counter.

Opioid receptor binding. Opioid binding was performed in 25 mM HEPES, pH 7.7, containing 5 mM $MgCl_2$. Membrane proteins (250–300 μg) from NG108–15, NS20Y, or N1E115 neuroblastoma cells were incubated with various agents at 24° for 90 min, in a final volume of 1 ml. Incubations were begun with addition of 100 μl of membrane suspension and were terminated by filtration through Whatman GF/B filters. To remove excess radioactivity, filters were then washed twice with 5 ml of ice-cold 25 mM HEPES, pH 7.7. Radioactivity on the filters was then determined by liquid scintillation counting, 12 hr after addition of 7 ml of scintillation fluid. Specific binding of opioid ligands was defined as the difference between the averages of triplicate samples in the absence and presence of 10 μM DADLE. In the saturation binding of [^{3}H]diprenorphine, the nonspecific binding of the tritiated ligand was determined with the addition of 10 μM DADLE. For both competition and saturation binding experiments, a minimum of 15 different concentrations of opioid ligands were used.

Data analysis. Measurements of [α-^{32}P]AA-GTP incorporation were expressed as mean density and in some experiments were converted to percentage of control (i.e., condition receiving no drug treatment). All measurements of intracellular [^{3}H]cAMP values were expressed as percentage of control, whereas specific binding data were measured as femtomoles/milligram of protein. Mean density and percentage of control data were analyzed by one-way analysis of variance, with subsequent comparisons between individual treatment groups by *post hoc* contrasts. The best fit curve, as determined by SigmaPlot (version 4.14), was used to calculate all IC_{50} and EC_{50} values. Opioid receptor binding data were analyzed with the LIGAND program (12), providing estimates of the B_{max} and K_d values.

Results and Discussion

Identity of G protein α subunits incorporating the photoaffinity label [α-^{32}P]AA-GTP. The photoreactive GTP analog [α-^{32}P]AA-GTP labeled four protein bands in the 39–41-kDa region of NG108–15, NS20Y, and N1E115 membranes after separation on a 6 M urea/10% acrylamide SDS-PAGE gel and subsequent exposure for autoradiography (Fig. 1). The incorporation of radioactivity was antagonized by increasing concentrations of GDP, nonradioactive α-AA-GTP, GTP, and the stable GTP analog GTPγS (data not shown). The identity of these bands was determined by immunoblotting (Western analysis) using antisera EC/2 ($G_{i3\alpha}$ selective), GC/2 ($G_{o1\alpha}$ and $G_{o2\alpha}$ selective), and AS/7 ($G_{i1\alpha}$ and $G_{i2\alpha}$ selective). Identical electrophoretic mobility with immunopositive protein bands identified the [α-^{32}P]AA-GTP-labeled bands, from higher to lower molecular weight (i.e., from top to bottom), as $G_{i3\alpha}$, $G_{o1\alpha}$, $G_{i2\alpha}$, and $G_{o2\alpha}$, respectively (data not shown). An additional isoform of $G_{i3\alpha}$ was identified by antiserum EC/2 but was not labeled by [α-^{32}P]AA-GTP.

These results (both identity and pattern) are identical to those obtained previously in this laboratory using incorporation of [^{32}P]ADP-ribose into G_α subunits by pertussis toxin in NG108–15 cells (2). However, an earlier study also in NG108–15 cells (13), without using pertussis toxin ADP-ribosylation, was able to resolve only three of the five G_α subunits identified in the present study. To circumvent this problem, a combination of [α-^{32}P]AA-GTP photoaffinity labeling followed by immunoprecipitation of selective G_α subunits to identify opioid/G protein coupling in human SHSY5Y neuroblastoma cells was implemented (3). Although attractive, this method is labor intensive and thus agonist-induced incorporation at only single concentrations is usually reported, which does not allow for ED_{50} calculations or adequate comparisons of potency and maximal agonist-induced effects.

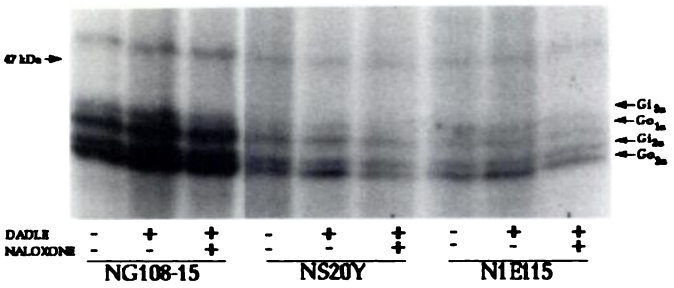

Fig. 1. Comparison of DADLE-induced [α-^{32}P]AA-GTP labeling of 39–41-kDa G_α subunits in NG108–15, NS20Y, and N1E115 cells. Membranes (50 μg) were photolabeled with [α-^{32}P]AA-GTP (1 μCi) in the presence of no drug (*lanes 1, 4,* and *7*), DADLE (1 μM) (*lanes 2, 5,* and *8*), or DADLE (1 μM) plus naloxone (100 μM) (*lanes 3, 6,* and *9*), as described in Experimental Procedures. G protein α subunits were separated by urea/SDS-PAGE and exposed for autoradiography.

Evidence that the increase in labeling of G protein α subunits by [α-^{32}P]AA-GTP in the presence of opioid agonist is a function of opioid receptor activation. In the three cell lines tested, the δ-opioid agonist DADLE (1 μM) produced an increase in [α-^{32}P]AA-GTP incorporation into all four G_α subunit subtypes ($G_{i3\alpha}$, $G_{o1\alpha}$, $G_{i2\alpha}$, and $G_{o2\alpha}$) (Fig. 1). Furthermore, the increase in photoaffinity labeling produced in all four subunits by DADLE was also dose dependent (Fig. 2) and could be antagonized by the addition of naloxone (100 μM) (Fig. 1). Although DADLE has affinity for both δ- and μ-opioid receptors, the effect observed was due to interaction with the δ-opioid receptor, because all of the cell lines tested contain a homogeneous population of δ-opioid receptors (6, 14, 15). However, to determine whether the increase in labeling produced by DADLE exhibited characteristics of classical δ-opioid receptor activation, a series of selective and nonselective opioid agonists were tested in NG108–15 membranes for their ability to induce increases in labeling (Table 1). Because our purpose in this experiment was not to determine agonist-induced labeling in any specific G_α subunit, the mean densities of all four G_α subunits in the 39–41-kDa region were considered together. The δ-selective opioid agonists DPDPE (1 μM) and DSLET (1 μM) produced increases of labeling to 180 and 210% of control values, respectively. As in Fig. 1, DADLE (1 μM) produced an increase in incorporation to 170% of control levels. Furthermore, the nonselective (δ, μ, and κ) agonists etorphine (1 μM) and bremazocine (1 μM) also increased labeling of G_α subunits to 200 and 170% of control incorporation, respectively. In contrast to the large increases in [α-^{32}P]AA-GTP incorporation produced by opioid agonists with δ affinity, μ-opioid agonists PLO17 (10 μM) and DAMGO (1 μM) induced only slight elevations in labeling (110 and 140% of control values, respectively). The increase in incorporation produced by the μ-opioid

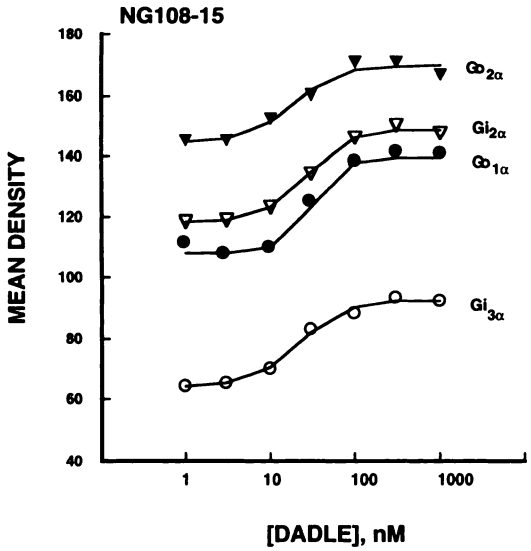

Fig. 2. DADLE concentration-dependent labeling of various G protein α subunits by [α-^{32}P]AA-GTP in NG108–15 cells. Membranes (50 μg) were photolabeled with [α-^{32}P]AA-GTP (1 μCi) in the presence of increasing concentrations (1–1000 nM) of the δ-opioid agonist DADLE, as described in Experimental Procedures. G protein α subunits were subsequently separated by urea/SDS-PAGE and exposed for autoradiography. Densitometric evaluation of each specific G protein α subunit is shown. The results are representative of five experiments performed separately with three different membrane preparations.

TABLE 1

Comparison of a series of selective and nonselective opioid agonists for their ability to induce increases in incorporation of [α-^{32}P]AA-GTP into the 39-41-kDa G_α subunits

NG108-15 cells were treated for 24 hr with no drug, DADLE (100 nM), or pertussis toxin (100 ng/ml). Membranes were then prepared and photolabeled (50 μg) with [α-^{32}P]AA-GTP (1 μCi) in the presence of various opioid agonists, as described in Experimental Procedures. G protein α subunits were separated by urea/SDS-PAGE and exposed for autoradiography. The combined mean density of all G_α subunits was calculated as the percentage of [α-^{32}P]AA-GTP incorporation with respect to that with control membranes (i.e., in the absence of drug).

Membrane pretreatment	Opioid tested	Receptor specificity	Mean density (39-41-kDa G proteins)
			% of control
None	None		100
None	DPDPE	δ	180
None	DSLET	δ	210
None	DADLE	δ, μ	170
None	Etorphine	δ, μ, κ	200
None	Bremazocine	δ, μ, κ	170
None	PLO17	μ	110
None	DAMGO	μ	140
None	U50-488H	κ	130
DADLE (100 nM, 24 hr)	DADLE	δ, μ	102 ± 7
Pertussis toxin (100 ng/ml, 24 hr)	DADLE	δ, μ	94 ± 5

agonist DAMGO (140%), although less than that observed for δ-opioid agonists, was unanticipated. However, DAMGO has been shown to inhibit adenylyl cyclase activity in NG108–15 cells (14), which indicates that in this cell line DAMGO does possess some δ-opioid activity. As with the μ agonists, the κ-opioid agonist U50–488H (1 μM) increased incorporation to only 130% of that in control membranes. Finally, DADLE failed to produce any increase in incorporation of [α-^{32}P]AA-GTP in membranes prepared from cells that had been treated with DADLE (100 nM) or pertussis toxin (100 ng/ml) for 24 hr. These data all support the conclusion that δ-opioid agonists are indeed coupled to G_α subunits through δ-opioid receptor activation.

Comparison of the maximal increase in DADLE-induced [α-^{32}P]AA-GTP incorporation into G protein α subunits. In NG108–15 membranes, DADLE produced a significantly ($p < 0.01$) greater increase in incorporation of the photoaffinity label into $G_{i3\alpha}$ (215%), relative to the other G protein α subunits, i.e., $G_{o1\alpha}$ (163%), $G_{i2\alpha}$ (167%), or $G_{o2\alpha}$ (161%) (Fig. 3, *top*). The maximal increase of photoaffinity incorporation produced by DADLE in NS20Y cells was not significantly different among any of the G_α subunits (Fig. 3, *middle*). In this cell line, DADLE produced labeling in G_α subunits that ranged from 145% for $G_{o2\alpha}$ to 186% for $G_{i3\alpha}$. Although it appeared in N1E115 cells that DADLE produced a greater maximal incorporation of [α-^{32}P]AA-GTP into $G_{o2\alpha}$ (168%), compared with the other G_α subunits (~135%), no significant differences were detected (Fig. 3, *bottom*).

Findings from the present experiment are different from those reported by the first study that used agonist-induced [α-^{32}P]AA-GTP labeling to examine δ-opioid receptor/G protein interactions in NG108–15 cells (13). In that study, $G_{o\alpha}$ was slightly more stimulated by DADLE than was $G_{i2\alpha}$, whereas the photolabeling of a second, unidentified, $G_{i\alpha}$ could not be quantified. A second study in NG108–15 cells, using cholera toxin-induced [^{32}P]ADP-ribosylation, did, however, show approximately 15% and 30% greater maximal [^{32}P]ADP-ribosylation induced by DADLE with $G_{i3\alpha}$, relative to $G_{i2\alpha}$ and $G_{o\alpha}$, respectively (2). Furthermore, studies in human neuroblastoma SHSY5Y cells revealed that the δ-opioid receptor preferentially coupled to $G_{i1\alpha}$, while interacting only weakly with $G_{i2\alpha}$ and $G_{i3\alpha}$ (3). These results all indicate that the δ-opioid receptor preferentially couples to selective G_α subunits. However, findings from the present study, with one exception of $G_{i3\alpha}$ in NG108–15 cells, indicate that the magnitudes with which δ-opioid receptors interact with available G_α subunits in response to agonist are approximately the same.

Comparison between δ-opioid agonist potency to inhibit adenylyl cyclase and to activate G proteins. DADLE inhibited forskolin-stimulated cAMP production in NG108–15, NS20Y, and N1E115 cells with similar IC_{50} values of 0.46, 1.77, and 0.34 nM, respectively (Table 2). These values are similar to those reported in previous studies using DADLE in NG108–15 (16) and NS20Y cells (14) and Met^5-enkephalin in N1E115 cells (15). Although not a direct measure, the observed opioid-mediated reduction in cAMP accumulation reflects an inhibition of the membrane-bound enzyme adenylyl cyclase. This is indicated because addition of the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine in the incubation mixtures rules out any effect opioids might have on cAMP degradation and, furthermore, opioids have also been shown to inhibit basal and prostaglandin E_1-stimulated adenylyl cyclase activity in membrane preparations (17, 18).

As with the IC_{50} values, the affinities of DADLE for the δ receptors in these cell lines were also similar, i.e., 0.76, 2.59, and 5.0 nM in NS20Y (14), NG108 (6), and N1E115 cells, respectively. Receptor binding was conducted in the presence of Mg^{2+}, with which all δ-opioid receptors have been shown to exist in a high affinity state (16). This high affinity state represents a complex composed of δ-opioid receptors coupled to G proteins. Further support for this hypothesis is demon-

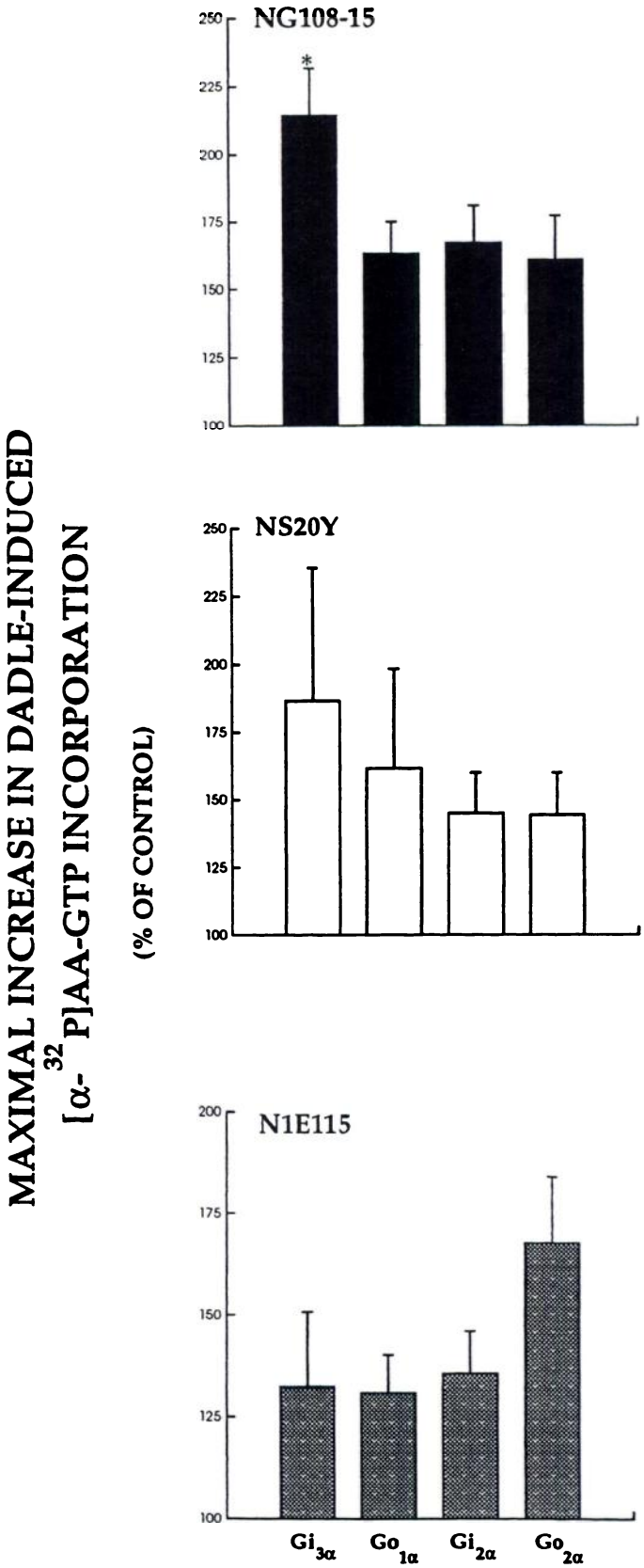

Fig. 3. Comparison of the maximal increases in DADLE-induced [α-^{32}P]AA-GTP incorporation into G protein α subunits. Membranes (50 μg) from NG108–15, N1E115, and NS20 cells were photolabeled with [α-^{32}P]AA-GTP (1 μCi) in the presence of increasing concentrations of the δ-opioid agonist DADLE to obtain dose-effect curves similar to those illustrated in Fig. 2, as described in Experimental Procedures. G protein α subunits were separated by urea/SDS-PAGE and exposed for autoradiography. Using the best fit for a sigmoidal curve, as determined by using the SigmaPlot (version 4.14) program with a Macintosh-LC personal computer, the maximal increase in photoaffinity labeling was calculated for each G_α subunit in each cell line as a percentage increase with respect to the labeling of control membranes.

strated by the marked reduction of the percentage of receptors in this high affinity state after chronic treatment with either opioid agonists or pertussis toxin (19). Generation of this high affinity state by agonists, during which receptor/G protein coupling occurs, should reflect effector function, and it is therefore not surprising that the affinity of DADLE for the δ-opioid receptor is also similar to the IC_{50} values discussed previously. In contrast to receptor affinity, the density of δ-opioid receptors differed between cell lines, with N1E115 cells having the lowest density (B_{max} of 120 fmol/mg of protein). NS20 (14) and NG108–15 (6) cells have increasing densities of δ-opioid receptors (B_{max} values of 370 and 570 fmol/mg of protein, respectively).

In contrast to the relatively similar IC_{50} and affinity values for DADLE across the three cell lines, the ED_{50} to induce photoaffinity labeling of G protein α subunits differed by >100-fold between the three cell lines. In NG108–15 cells, $G_{i3\alpha}$, $G_{o1\alpha}$, and $G_{i2\alpha}$ had similar ED_{50} values of 29–30 nM. However, the amount of DADLE needed to induce half-maximal labeling of $G_{o2\alpha}$ (12 nM) was significantly lower ($p < 0.05$) than that required for the other three G_α subunits. Roerig *et al.* (2), using cholera toxin-catalyzed [^{32}P]ADP-ribosylation by DADLE in NG108–15 cells, obtained similar ED_{50} values, ranging from 12 to 54 nM. The ED_{50} values obtained in the present study are also in agreement with those of 10–100 nM found by Offermanns *et al.* (13). The ED_{50} values to induce photoaffinity labeling of all four G proteins in NS20Y cells were not significantly different, ranging from 0.54 ($G_{o2\alpha}$) to 1.0 nM ($G_{i3\alpha}$). Furthermore, these values were >10-fold less than those observed in NG108–15 cells. In N1E115 cells, the ED_{50} for incorporation of [α-^{32}P]AA-GTP into $G_{o2\alpha}$ (0.071 nM) was significantly greater ($p < 0.05$) than that for $G_{o1\alpha}$ (0.012 nM) but not those for $G_{i3\alpha}$ (0.026 nM) or $G_{i2\alpha}$ (0.021 nM). In addition, it is interesting to note that the ED_{50} values in this cell line were >10- and >100-fold less than those determined in NS20Y and NG108–15 cells, respectively. Unlike the large difference in the potency of DADLE to induce photoaffinity labeling of G_α subunits across cell lines, the ED_{50} values for incorporation of [α-^{32}P]AA-GTP into specific G_α subunits within each cell line were not different. Therefore, unlike previous studies that suggested some selectivity of δ-opioid receptor/G protein coupling (2, 3, 13), data obtained in the present study indicate no difference in the efficacy or the potency of the δ-opioid agonist DADLE to induce photoaffinity labeling of available G protein α subunits.

There is evidence to suggest that, as the density of α_2-adrenergic receptors is increased in transfected CHO cells, the receptors begin to couple to G proteins, which they normally do not do (7). However, data from the present study show that, in three neuroblastoma cell lines with densities of δ-opioid receptors varying from rather low (B_{max} = 120 fmol/mg of protein) to high (570 fmol/mg of protein), the same set of G_α subunits are coupled to the δ-opioid receptor.

It is also interesting to note that, although the IC_{50} values to inhibit intracellular cAMP production and affinities for DA-

TABLE 2

Comparison of DADLE-induced inhibition of adenylyl cyclase activity, affinity for the receptor, and [α-^{32}P]AA-GTP labeling of G_α subunits in NG108-15, NS20Y, and N1E115 cells

IC_{50} values for inhibition of adenylyl cyclase by DADLE and K_d and B_{max} values were determined as described in Experimental Procedures. For photoaffinity labeling of G_α subunits, membranes (50 μg) from NG108-15, N1E115, and NS20 cells were photolabeled with [α-^{32}P]AA-GTP (1 μCi) in the presence of increasing concentrations (0.01 nM to 1 μM) of the δ-opioid agonist DADLE as described in Experimental Procedures. G protein α subunits were separated by urea/SDS-PAGE and exposed for autoradiography. Densitometric evaluation of each specific G protein α subunit is shown.

	NG108-15	NS20Y	N1E115
IC_{50} to inhibit adenylyl cyclase (nM)	0.46 ± 0.23	1.77 ± 0.22	0.34 ± 0.11
DADLE affinity for receptor, K_d (nM)	2.59 ± 0.18[a]	0.76 ± 0.19[b]	5.0 ± 1.3
B_{max} (fmol/mg of protein)	570 ± 5.5[a]	370 ± 50[b]	120 ± 8.0
ED_{50} to induce G protein labeling (nM)[c]			
$G_{i3\alpha}$	30.3 ± 6.8	1.0 ± 0.87	0.026 ± 0.011
$G_{o1\alpha}$	29.9 ± 2.1	0.58 ± 0.21	0.012 ± 0.007
$G_{i2\alpha}$	29.2 ± 5.9	0.82 ± 0.16	0.021 ± 0.006
$G_{o2\alpha}$	12.4 ± 2.6[d]	0.54 ± 0.24	0.071 ± 0.055[d]

[a] Obtained from Law et al. (6).
[b] Obtained from Law and Bergsbaken (14).
[c] ED_{50} values for stimulation of [α-^{32}P]AA-GTP incorporation were calculated by the curve fit function of the SigmaPlot version 4.14 program, using a Macintosh-LC personal computer. The results are expressed as mean ± standard error and are representative of five, three, or five experiments performed separately with different membrane preparations of NG108-15, N1E115, or NS20 cells, respectively.
[d] Significant difference level of $p < 0.05$.

DLE were similar across the three cell lines, the ED_{50} values to produce labeling of the G_α subunits differed by >100-fold between cell lines. This is surprising, because inhibition of adenylyl cyclase requires the formation of receptor/G protein complex, which represents the high affinity state of the receptor, and therefore one would expect the concentration of agonist required to induce labeling of G_α subunits to be equal to the affinity of the agonist for the receptor/G protein complex. Furthermore, the amount of DADLE required to produce 50% maximal inhibition of adenylyl cyclase in NG108–15 cells was much lower than the concentration required to induce half-maximal labeling of any G_α subunit; in NS20Y cells these two sets of values were similar, whereas in N1E115 cells concentrations of DADLE required to induce half-maximal labeling were much lower than those required for inhibition. Therefore, there appears to be a difference in the ability of the δ-opioid receptor to regulate second messenger activity that is not due to DADLE affinity for the receptor. In NG108–15 cells maximal inhibition of adenylyl cyclase could be obtained before full activation of G proteins, whereas in N1E115 cells the reverse was observed. In other words, in N1E115 cells there is no measurable DADLE inhibition of adenylyl cyclase even with maximal agonist effect on GTP association with G proteins. It appears likely that other cellular factors are involved in this particular receptor/effector coupling, which may modify the signal after receptor/G protein interaction.

There are several possible factors that could contribute to the observed differences between the ED_{50} values for G protein labeling and the measures of receptor affinity and effector function. One such consideration is the effect of differences in the constituents of the buffers used for the binding and response assays. For example, GDP, GTP, and Na^+ were not added to the binding assay, whereas these agents were added to the buffer used for photoaffinity labeling and were present at physiological concentrations in the adenylyl cyclase assay. This is an important issue because both guanine nucleotides and Na^+ have been shown to reduce the affinity of opioid receptors for their agonists (20). In assays using both membrane preparations (16) and whole cells (21), the δ-opioid receptor has been demonstrated to exist in multiple affinity states. A high affinity state is thought to result from the formation of ternary complexes between ligand binding sites and G proteins, whereas uncoupling of receptors from G proteins reflects conversion to a low affinity state (22). The affinity for agonists in such preparations is often determined by binding studies using a one-site model, with the resultant averaged K_d values actually reflecting the proportion of receptors existing in these various affinity states. Therefore, any situation that promotes dissociation of receptors from heterotrimeric G proteins, such as addition of sodium ions and guanine nucleotides (16) or chronic treatment with agonists or pertussis toxin (19), produces a reduction in receptor affinity, measured by the one-site model as an increase in the proportion of receptors existing in the low affinity state. However, the agonist K_d value for the high affinity state under these conditions is not altered. Therefore, estimation of the receptor agonist high affinity state is more accurately determined either by using a two-site model or by using conditions in which all receptors exist in a single affinity state. One such method involves the addition of magnesium to receptor binding buffers, which produces a condition in which all receptors exist in the high affinity state (16). Consequently, the K_d determined under these conditions reflects the high affinity state of the receptor. Because the purpose of the present investigation was to study receptor/G protein interactions, ED_{50} values for G protein labeling and IC_{50} values for inhibition of adenylyl cyclase were most appropriately compared for the high affinity state of the receptor. For this reason, receptor binding was conducted in the presence of magnesium.

It could be suggested that the presence of guanine nucleotides and Na^+ in the buffers used to measure photoaffinity labeling and adenylyl cyclase activity may have contributed to the unexpected results in the present study, due to their effects on the affinity of δ-opioid agonists for their receptors. This, however, seems unlikely, because the ultimate ability of an agonist to exchange GTP for GDP on the G_α subunit (photoaffinity labeling), as well as to produce inhibition of adenylyl cyclase, depends upon the generation of the high affinity state of the receptor, which requires guanine nucleotides and Na^+. In other words, formation of the high affinity state of the receptor is a prerequisite for observation of these effects, and thus comparison of values obtained while the receptor is in this state seems appropriate.

There are other, more plausible, factors that could also produce such alterations in receptor signal transduction and offer explanations for our current observations and the disparity between receptor/G protein and receptor/effector coupling. Such factors include the regulation of GTP hydrolysis by G_α subunits. For example, effectors with which G_α subunits interact after activation by δ-opioid receptors may be acting as GTPase-activating proteins (23). In response to muscarinic m1 receptor stimulation, GTP hydrolysis by the G_α subunit G_q was increased as much as 50-fold over steady state turnover rates by addition of the effector phospholipase C-β1 (24). This was also observed in the visual system, in which addition of the effector phosphodiesterase accelerated the hydrolysis of GTP by transducin (G_t) 4-fold after activation by photoexcited rhodopsin (25). Additionally, the stimulation of K^+ channels by muscarinic receptors in cardiac tissue can be inhibited by the interaction of the low molecular weight G protein *ras* p21 and a GTPase-activating protein, which produces an uncoupling of these receptors from the G_α subunit G_k (26).

After receptor/G protein interaction, signal transduction could also be modified by the participation of βγ subunits. Not only can βγ subunits regulate effectors directly (27), but they can also increase the affinity of G_α subunits for GDP by 100-fold (28). Hence, these effects could greatly influence the regulation of the association and dissociation of heterotrimers and consequently the activity of G proteins. Furthermore, there is evidence for selectivity of the formation of different β/γ subunit complexes (29, 30) and evidence that different β and γ subunits are involved in the coupling of somatostatin and muscarinic receptors to voltage-sensitive Ca^{2+} channels in GH_3 cells (31). In any case, based on the results presented, the ability of agonists to stimulate exchange of GTP for GDP on G_α subunits may not be reflective of receptor/effector coupling.

In summary, the present study indicates, firstly, that the coupling of δ-opioid receptors to multiple G proteins simultaneously is a property that is not limited to the NG108–15 cell line but occurs in a variety of cell lines that express a range of receptor densities. Secondly, the magnitudes with which δ-opioid receptors interact with available G_α subunits in response to agonist are approximately the same. Finally, there appears to be no relationship between the potency of agonists to inhibit adenylyl cyclase and that required for activation of G proteins. Therefore, it appears likely that other cellular factors are involved in this particular receptor/effector coupling.

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Send reprint requests to: Paul L. Prather, Department of Pharmacology, School of Medicine, University of Minnesota, 3–249 Millard Hall, 435 Delaware Street, Minneapolis, MN 55455.