0026-895X/92/050822-10$03.00/0

MOLECULAR PHARMACOLOGY, 41:822-831

Identification of Three Separate Guanine Nucleotide-Binding Proteins that Interact with the δ-Opioid Receptor in NG108–15 Neuroblastoma × Glioma Hybrid Cells

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Received July 24, 1991; Accepted February 21, 1992

SUMMARY

Five separate guanine nucleotide-binding proteins (G proteins) were immunologically identified in membranes from neuroblastoma × glioma NG108–15 hybrid cells. These α subunit proteins were $G_{i2\alpha}$, two isoforms of $G_{i3\alpha}$, and two isoforms of $G_{o\alpha}$. The G proteins that interacted with δ-opioid receptors in these membranes were identified using cholera toxin (CTX)-induced ADP-ribosylation and antisera selective for various G protein α subunits. In the presence of δ-opioid agonists, CTX induced the incorporation of [^{32}P]ADP-ribose into three pertussis toxin substrates. Using antisera generated against peptide sequences from G_α subunits, these three pertussis toxin substrates were identified as $G_{i2\alpha}$, $G_{o2\alpha}$, and one isoform of $G_{i3\alpha}$, which has yet to be identified. This CTX-induced labeling was demonstrated to be mediated via the δ-opioid receptor in these hybrid cells by the observation that δ agonists D-Ala^2-D-Leu^5-enkephalin (DADLE) and D-Pen^2-D-Pen^5-enkephalin, as well as the nonselective agonists etorphine and bremazocine, were active, but the μ agonist PL017 and the κ agonist U-50–488H did not show this activity. This incorporation into all three substrates induced by DADLE was dose dependent, with EC_{50} (95% confidence interval) values ranging from 12 (3–52) to 183 (65–520) nM, which compared with the K_d value of 10 ± 1.5 nM for this agonist, a dose that produces maximal inhibition of adenylate cyclase activity. Furthermore, pretreatment of the cells with pertussis toxin or treatment of the membranes with the antagonist naloxone blocked the incorporation induced by DADLE. Incorporation of [^{32}P]ADP-ribose into all three substrates decreased 35–83% in membranes in which the receptors had been down-regulated by chronic treatment of the cells with DADLE. Thus, a single opioid receptor type can interact with three separate G proteins.

Hormone receptor control of adenylate cyclase activity has been proposed to be mediated by a family of G proteins. These G proteins are heterotrimers consisting of α, β, and γ subunits, and it is the α subunits that confer receptor and effector specificity (1). Bacterial toxins from *Bordetella pertussis* (PTX) and *Vibrio cholerae* (CTX) have been used to implicate involvement of G proteins in receptor-mediated agonist-induced activities. These toxins catalyze the ADP-ribosylation of G protein α subunits, which inhibits the response to agonists. PTX acts on G proteins that are involved in inhibition of adenylate cyclase activity (G_i), as well as other G proteins (G_o), whereas CTX interacts with G proteins that are implicated in stimulation of adenylate cyclase activity (G_s).

In neuroblastoma × glioma NG108–15 hybrid cells, activation of the opioid receptor results in a decrease in intracellular cAMP (2). The opioid receptor in these cells has been identified as a homogeneous population of the δ type, with DADLE as the prototypic agonist (3, 4). Opioid-induced inhibition of adenylate cyclase is believed to be mediated by G_i, because this inhibition is attenuated by PTX pretreatment (5). The presence of both G_i and G_o proteins has been observed in NG108–15 cells (6) and, recently, two distinct isoforms of G_o have been identified (7). Using selective antisera, the transduction of opioid-mediated inhibition of adenylate cyclase activity in NG108–15 cells was recently attributed to G_{i2} (8).

In addition to inhibition of adenylate cyclase activity, DADLE has been shown to inhibit a voltage-dependent calcium current in differentiated NG108–15 cells. This effect is blocked by PTX pretreatment and is proposed to be mediated by G_o (9). However, it is not known which of the recently identified isoforms of G_o is involved in this receptor-mediated event.

Other intracellular activities, such as arachidonic acid release and stimulation of the phospholipase C pathway, have also been linked to PTX-sensitive G proteins (10) but not, as yet, to opioid receptor activation. Opioids have been shown to

This work was supported by Grants DA05370 (S.C.R.), DA00564 (H.H.L.), and DA05945 (P.Y.L.). from the National Institutes on Drug Abuse.

ABBREVIATIONS: G protein, guanine nucleotide-binding protein; CTX, cholera toxin; DADLE, D-Ala^2-D-Leu^5-enkephalin; PTX, pertussis toxin; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; DPDPE, D-Pen^2-D-Pen^5-enkephalin; GTPγS, guanosine-5′-*O*-(3-thio)triphosphate.

accelerate Na^+/H^+ exchange in NG108–15 cells (11) and to stimulate potassium channels in locus coeruleus neurons (12), but involvement of G proteins in these activities is unclear. Recent preliminary work from our laboratories has described five distinct PTX substrates in NG108–15 cells (13), but which of these are involved in opioid-induced activity has not been defined. Thus, a first step in linking opiates to G protein-mediated events is to identify which G proteins interact with the opioid receptor.

One method of identifying the G protein that might interact with the opioid receptor in NG108–15 cells is to utilize the properties of PTX- and CTX-catalyzed ADP-ribosylation of the G_α subunits. It has been reported that PTX preferentially catalyzes ADP-ribosylation of the G protein heterotrimer (14). Receptor-mediated dissociation of the G protein heterotrimer after agonist association could thus result in the reduction of ADP-ribosylation of the α subunits. Such an approach has been used successfully to identify the G protein involved in thrombin activation of platelets (15) and insulin-like growth factor II activation of a calcium channel in fibroblasts (16).

Another method of identifying the G protein involved in opioid receptor action is to take advantage of one of the properties of CTX, which is to interact preferentially with the free α subunits (17). In the cloned PTX substrates, the $G_{i\alpha}$ and the $G_{o\alpha}$ subunits, there is an internal arginine moiety at a position similar to Arg^{202}, which is the site for ADP-ribosylation of $G_s\alpha$ subunits (18), the usual substrates for this toxin. This internal arginine is the substrate for ADP-ribosylation by CTX. However, the $G_{i\alpha}$ and $G_{o\alpha}$ subunits are not ADP-ribosylated in the presence of CTX unless an agonist is present. Agonist interaction with a receptor induces not only dissociation of the G protein α and $\beta\gamma$ subunits, but also some change that allows the free α subunit to be ADP-ribosylated by CTX. This approach has been used to identify the G proteins that interact with the formyl peptide receptor in HL-60 cells (19). Milligan and McKenzie (17) and Klinz and Costa (20) have also utilized this approach to demonstrate DADLE concentration-dependent ADP-ribosylation of the PTX substrates in NG108–15 cells. However, these investigators did not determine which PTX substrates were responsible for this observation.

Recently, we have been able to resolve clearly five PTX substrates in NG108–15 cell membranes (13), by using a urea gradient SDS-PAGE system (21, 22). The present study used CTX-induced ADP-ribosylation in the presence of opioid agonists and separation of the ribosylated proteins in the urea gradient SDS-PAGE system to identify which G proteins were interacting with the opioid receptor in NG108–15 cells.

Materials and Methods

Neuroblastoma × glioma NG108–15 hybrid cells were cultured as previously described (23). Cells were cultured in Dulbecco's modified Eagle's medium containing 0.1 mM hypoxanthine, 10 μM aminopterin, and 17 μM thymidine, plus 5% fetal calf serum, in a humidified atmosphere of 10% CO_2 and 90% air. In most cases, experiments were performed using confluent cultures, of passage 20–30. For chronic drug treatment, cells were incubated for 18 hr in the presence of 100 nM DADLE, harvested in phosphate-buffered saline (0.1 N NaCl in 0.01 M $NaPO_4$, pH 7.5) with 0.04% EDTA as usual, and stored at −70° until use. For some studies, cells were incubated with 25 ng/ml PTX for 14 hr before harvesting.

Membranes were prepared by homogenization of the cells in 0.32 M sucrose, 2 mM EGTA, 20 mM HEPES, pH 7.7, using a Dounce homogenizer (24). Homogenates were centrifuged at 1000 × g for 10 min, and supernatants were recentrifuged for 60 min at 100,000 × g. Pellets were resuspended in 25 mM HEPES, pH 7.7, to give a final protein concentration of 10 mg/ml. Aliquots of membrane preparations were stored at −70°.

Membrane proteins were ADP-ribosylated with either PTX or CTX. When PTX was used, the assay mixture contained 100 μg of protein, 0.25% Lubrol, 50 mM Tris·HCl, pH 8.0, 20 mM thymidine, 1 mM ATP, 5 μM GTP, 20 mM arginine, 50 mM NaCl, 4 μM $MgCl_2$, 100 mM dithiothreitol, 1 μg of PTX, and 3–5 μCi of [α-^{32}P]NAD^+, in a final volume of 0.1 ml. For CTX-catalyzed ADP-ribosylation, the toxin (5 μg) was preactivated for 2 hr at 30°, with 50 mM dithiothreitol, and then added to a solution containing 100 μg of protein, 250 mM KPO_4, pH 7.0, 20 mM thymidine, 1 mM ATP, 20 mM arginine, 4 mM $MgCl_2$, and 3–5 μCi of [α-^{32}P]NAD^+ (final concentrations), in a final volume of 0.1 ml. In some experiments, 5 μM GTP was added. When opioids were included in the experiments, the mixture was incubated for 5 min at 30° without toxin, toxin was added, and the incubation was continued for 2 hr at 30°. After incubation with PTX, 1 ml of ice-cold acetone was added to the mixtures and proteins were pelleted by centrifugation in a microfuge at 4°. In the CTX experiments, 1 ml of ice-cold 20 mM HEPES, pH 7.7, was added and solutions were centrifuged. In all experiments, protein pellets were resuspended in 0.065 mM Tris·HCl, pH 6.8, 2% SDS, 10% glycerol, 5% 2-mercaptoethanol, heated at 95° for 2 min, and subjected to SDS-PAGE in either 10% acrylamide or 9% acrylamide, in a 4–8 M urea gradient. Gels were either dried and exposed to X-ray film for autoradiography or transferred to Immobilon-P membranes for autoradiography and immunoblotting.

Autoradiographs were digitized and the intensity of bands was quantified using the Image software program, on a MacIntosh II. For concentration-response analysis, EC_{50} values and 95% confidence intervals were calculated using the graded dose-response method of Tallarida and Murray (25). Each autoradiogram was evaluated separately, as was each identified G protein. Density of the gel lanes in which no agonist was present was subtracted from the density of lanes in which agonist was added. The density of the selected protein band at the highest agonist dose used was assigned as 100%, and the density of each band of similar electrophoretic mobility at a lower dose was calculated as a percentage of this maximum.

Immunoblotting was performed as previously described (26), with overnight incubation with the primary antisera in a 1/1000 dilution. Antisera used were AS/7 (selective for carboxyl-terminal sequences of $G_{i1\alpha}$ and $G_{i2\alpha}$ (27), EC/2 (selective for carboxyl-terminal sequence of $G_{i3\alpha}$, with cross-reactivity to $G_{o\alpha}$ (28), and GC/2 (selective for amino-terminal sequence of $G_{o\alpha}$) (29). These antisera were purchased from E. I. DuPont de Nemours & Co. The antiserum selective for an internal sequence of $G_{i2\alpha}$ (J-883) was a gift from Dr. Susanne Mumby (30). Affinity-purified antibody selective for amino acid residues 19–39 in the recently identified $G_{o2\alpha}$ (AS 201) (31) was provided courtesy of Drs. W. Rosenthal and G. Schultz. The secondary antibody used was coupled to alkaline phosphatase (goat anti-rabbit IgG alkaline phosphatase conjugate; Bio-Rad Laboratories). Color was developed using the 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium phosphatase substrate system (Kirkegaard & Perry Laboratories, Inc.).

To facilitate identification of the [^{32}P]ADP-ribosylated proteins, autoradiographic markers were attached to the Immobilon P transfers before exposure to X-ray film. After film exposure and development, the transfers were subjected to immunoblotting with the various antisera, and immunoreactive proteins were visualized either colormetrically (with the alkaline phosphatase-coupled secondary antibody) or autoradiographically (with the ^{35}S-coupled secondary antibody). The first X-ray film (from the ^{32}P) could then be aligned exactly with the color-developed transfer or the second X-ray film (from the ^{35}S), so that the bands could be unequivically identified.

Results

To verify that DADLE induces the ADP-ribosylation of PTX substrates in the presence of CTX, as reported by others (17,

20), NG108–15 membranes from cells treated with PTX or not treated were ADP-ribosylated with either PTX and CTX, in the presence of [α-^{32}P]NAD$^+$. Separation of radiolabeled proteins by SDS-PAGE produced results typified by the autoradiogram shown in Fig. 1. In the absence of added toxins (Fig. 1, *lanes 1* and *4*), no radioactively labeled protein bands with molecular mass values between 38 and 50 kDa (the approximate molecular mass range of G_i, G_o, and G_s α subunits) were observed in membranes from untreated (Fig. 1, *lane 1*) or PTX-pretreated (Fig. 1, *lane 4*) cells. In membranes from untreated (control) cells, PTX catalyzed the incorporation of radioactivity into a single band, which migrated as a 39–41-kDa protein (Fig. 1, *lane 8*). This band represented the G proteins that contain 354 or 355 amino acid residues and a cysteine residue at position −4 from the carboxyl terminus (the site of ADP-ribosylation). Pretreatment of the cells with PTX eliminated the incorporation of radioactivity into this protein (Fig. 1, *lane 7*).

Most of the radioactivity after CTX-induced ADP-ribosylation of membranes from control cells was associated with bands that migrated as 45–52-kDa proteins (Fig. 1, *lanes 2* and *3*). These protein bands represented the $G_{s\alpha}$ subunits, which are the usual substrates for ADP-ribosylation of the internal arginine residue in the presence of CTX. Addition of 1 μM DADLE to the incubation mixture that contained CTX (Fig. 1, *lane 3*) resulted in an increase in [^{32}P]ADP-ribose incorporation into the 39–41-kDa protein, which appeared to have the same electrophoretic mobility as the PTX substrate (Fig. 1, *lane 8*). Pretreatment of the cells with PTX eliminated the increase in incorporation of radioactivity induced by DADLE in this protein in the presence of CTX (Fig. 1, *lanes 5* versus *6*). These results implied that the CTX substrate that incorporated radioactivity in the presence of the δ-opioid agonist was identical to the PTX substrate.

To improve the resolution of the PTX substrates, the radiolabeled proteins were separated by urea gradient SDS-PAGE, and a representative autoradiogram in shown in Fig. 2A. The single PTX substrate seen in Fig. 1, *lane 8*, was resolved into five distinct bands. These bands were assigned numbers 1–5, in order, from the top of the gel to the bottom. The greatest amount of ^{32}P radioactivity was incorporated into bands 1 and 4, which showed approximately equal incorporation. Bands 2, 3, and 5 incorporated less radioactivity than did bands 1 and 4.

In order to identify the various PTX-sensitive protein bands, antisera directed against selected peptide sequences in the α subunits were utilized. Previous reports (30, 32) had shown that PTX treatment induces a change in electrophoretic mobility of α subunits; thus, the NG108–15 membrane proteins that incorporated [^{32}P]ADP-ribose were identified using antisera directed toward peptides from different G_α subunit proteins, in the presence or absence of PTX, and the results from a representative experiment are shown in Fig. 2B. The Immobilon P immunoblot shown in Fig. 2B, *lanes 1–3*, shows the proteins that were immunopositive with the GC/2 antiserum (directed against $G_{o\alpha}$), using the ^{35}S-radiolabeled secondary antibody. The upper five bands in Fig. 2B, *lane 1*, are the five protein bands that incorporated radioactivity in the presence of PTX (Fig. 2A). The appearance of the "sixth" band in Fig. 2B, *lane 1*, was due to incomplete ADP-ribosylation and the interaction of the unribosylated G_α with the ^{35}S-labeled secondary antiserum. Note that the mobility of this band corresponds to the faster migrating protein in Fig. 2B, *lane 2*, which represents membranes not treated with PTX. The ^{35}S-labeled secondary antibody was used for these lanes, because the two G_o isoforms are expressed less than the other α subunits in the NG108–15 membranes and the radiolabeled antibody allows a more sensitive method of detection. Use of the alkaline phosphatase-coupled secondary antibody gave the same results (data not shown). The two bands in Fig. 2B, *lane 2*, which resulted from membranes not incubated with PTX, shifted in mobility in the presence of PTX (Fig. 2B, *lane 3*). The two ADP-ribosylated protein bands in Fig. 2B, *lane 3* (PTX treated), had electrophoretic mobilities identical to those of bands 3 and 5 in Fig. 2B, *lane 1* (also PTX treated). Thus, these two PTX substrates were identified as probable isoforms of $G_{o\alpha}$. Densitometric analysis of nine different lanes from six different experiments was performed, and the results are summarized in Table 1. It is of interest to note that, although these two bands demonstrated similar intensity of ADP-ribosylation, the primary antibody interacted quite differently with the two proteins. The faster migrating form, termed G_{o2}, showed more immunoreactivity than the slower migrating form (G_{o1}), as seen in Table 1. Calculation of this difference showed that the G_{o1} form showed 46.5 ± 2.9% of the immunoreactivity of the G_{o2} form. Whether this difference in immunoreactivity reflected different amounts of the two proteins or represented slight differences in carboxyl-terminal sequence remains to be demonstrated.

The identities of the other G_α subunits were obtained using the alkaline phosphatase-coupled secondary antibody (Fig. 2B, *lanes 4–9*). The single immunopositive protein band that appeared in Fig. 2B, *lanes 4* and *5*, was identified as either $G_{i1\alpha}$ or $G_{i2\alpha}$, because the AS/7 antiserum interacts with both of these G_α subunits. The mobility of the band in Fig. 2B, *lane 5* (PTX treated), was identical to that of ^{32}P-radiolabeled band 4 in Fig. 2B, *lane 1* (also PTX treated). Identical electrophoretic mobil-

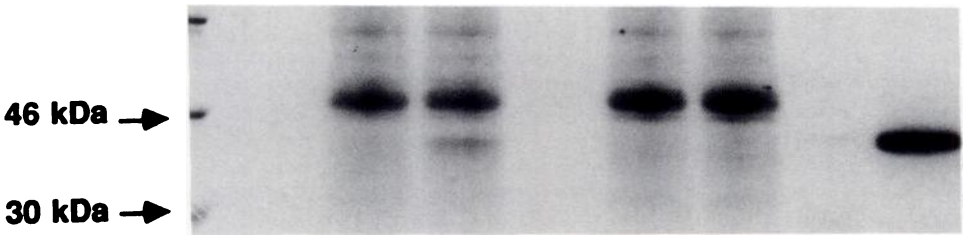

Fig. 1. Autoradiograms of SDS-PAGE separations of NG108–15 membranes from cells pretreated or not treated with PTX. Molecular weight standards are in the *far left lane*. Gel was 10% acrylamide. *Lanes 1–3* and *8*, membranes from untreated cells; *lane 1*, not incubated with toxin; *lane 2*, incubated with CTX alone; *lane 3*, incubated with CTX plus 1 μM DADLE; *lane 8*, incubated with PTX. *Lanes 4–7*, membranes from cells pretreated with PTX; *lane 4*, no toxin; *lane 5*, CTX alone; *lane 6*, CTX plus 1 μM DADLE; *lane 7*, PTX.

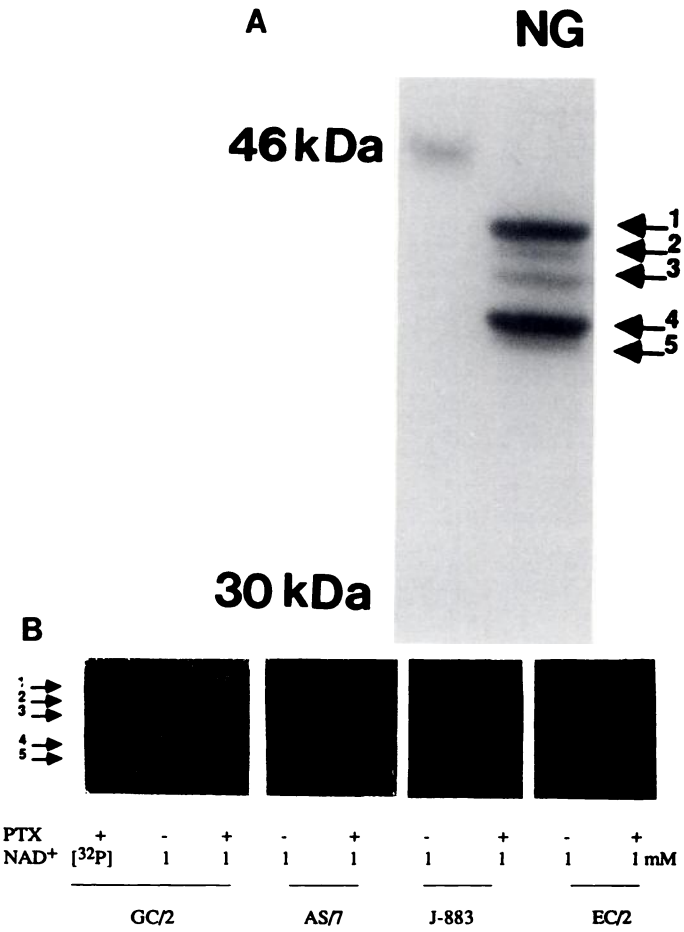

Fig. 2. Separation of the PTX substrates in urea gradient SDS-PAGE. A, Separation of PTX substrates into five distinct protein bands; *left*, lane is molecular weight standards; *right*, NG108-15 cell membranes. B, Identification of the five PTX substrates in NG108–15 hybrid cell membranes. Membranes were incubated with either [α-^{32}P]NAD$^+$ (*lane 1*) or NAD$^+$ (*lanes 2–9*), in the presence (*lanes 1, 3, 5, 7,* and *9*) or absence (*lanes 2, 4, 6,* and *8*) of PTX. Proteins were separated in the urea gradient SDS-PAGE system and transferred to an Immobilon P membrane. The Immobilon P membrane was cut into strips and incubated with the following antisera: GC/2 (*lanes 1–3*), AS/7 (*lanes 3–5*), J-883 (*lanes 6* and *7*), and EC/2 (*lanes 8* and *9*). *Lanes 1–3* were developed using the ^{35}S-labeled secondary antibody, and *lanes 4–9* were developed using the alkaline phosphatase-conjugated secondary antibody.

ity was also observed for the single J-883 antiserum-immunopositive protein band in Fig. 2B, *lanes 6* and *7*. Because the J-883 antiserum was directed against a peptide sequence unique to $G_{i2\alpha}$, band 4 in Fig. 2B, *lane 1*, and in Fig. 2A was positively identified as $G_{i2\alpha}$. The mobility of this protein recognized by both antisera was decreased in the presence of PTX (Fig. 2B, *lanes 4* versus *5* and *6* versus *7*).

Immunoblotting using the EC/2 antiserum (developed against the $G_{i3\alpha}$ carboxyl terminus) produced results shown in Fig. 2B, *lanes 8* and *9*. Fig. 2B, *lane 8*, shows two immunopositive bands of about equal intensity. After treatment of the NG108–15 membranes with PTX and 1 mM NAD$^+$, the mobilities of both of these bands shifted (Fig. 2B, *lane 9*), as expected from previous results with the other antisera (Fig. 2B, *lanes 2* versus *3*, *4* versus *5*, and *6* versus *7*). The mobilities of the protein bands after PTX treatment corresponded to mobilities of bands 1 and 2 in Fig. 2B, *lane 1*, and Fig. 2A. Thus, both these proteins were identified as $G_{i3\alpha}$. Band 1 incorporated [^{32}P]

TABLE 1

Mean densities of G_o α subunits that incorporate [^{32}P]ADP-ribose in the presence of PTX

Densities were determined from densitometric analysis of autoradiograms, using the Image software, on a Macintosh computer. Values represent mean density of the individual bands after background values were subtracted.

	Density		*t* value	Significance
	$G_{o1\alpha}$ (band 3)	$G_{o2\alpha}$ (band 5)		
[^{32}P]ADP-ribosylation				
	81	74	0.24	NS[a]
	104	72		
	56	62		
	25	36		
	77	86		
	170	181		
	149	143		
	29	35		
	45	37		
^{35}S-Antibody				
	5.0	12.0	3.907	$p < 0.05$
	18.0	42.0		
	30.8	64.7		
	25.6	47.5		

[a] NS, not significant.

ADP-ribose to a greater extent than did band 2 and also demonstrated more recognition by the EC/2 antiserum. From these results, one might conclude that protein in band 1 occurred in greater amount in these membranes than did band 2 protein. However, this is unlikely, because before PTX-catalyzed ADP-ribosylation EC/2 antiserum recognized these two proteins equally (Fig. 2B, *lane 8*). Thus, it is likely that there are two distinct proteins in NG108–15 membranes that have similar immunoreactivity toward EC/2 antiserum but different antiserum reactivity after ADP-ribosylation with PTX.

The single band at 39–40 kDa in CTX-treated membranes, which intensified in the presence of DADLE (Fig. 1, *lane 3*), was also separated using the urea gradient SDS-PAGE system. Results shown in the autoradiogram in Fig. 3 (*lanes 1* and *2*) show that this single band appeared to separate into three distinct bands. These bands were numbered 1, 2, and 3, from the slowest to fastest electrophoretic migration. Band 1 was the most intense of these three, and it was present in a lesser amount in the absence of DADLE (Fig. 3, *lane 1*). In agreement with results shown in Fig. 1, the intensity of band 1 (Fig. 3, *lane 1*) increased in the presence of 1 μM DADLE (Fig. 3, *lane 2*). Bands 2 and 3 appeared only in the presence of the opioid. A fourth band, which appeared just below band 3, was observed in the absence of toxin (data not shown) and may be a self-ribosylation product.

Tentative identification of the bands that increased in intensity in the presence of DADLE was made using selective antisera, and results are shown in Fig. 3. The immunoblots shown in Fig. 3, *lanes 3–5*, were cut from the Immobilon P transfer membrane from the same gel and incubated with the indicated antisera. Bands were visualized using the alkaline phosphatase-coupled secondary antibody and colorometric development. Protein bands shown in Fig. 3, *lane 3*, were immunopositive for the antiserum EC/2, which is selective for $G_{i3\alpha}$ subunit protein. This antiserum showed a positive interaction with two distinct proteins, as observed previously (Fig. 2B). The upper EC/2-positive band had the same electrophoretic mobility as band 1, which showed increased incorporation of

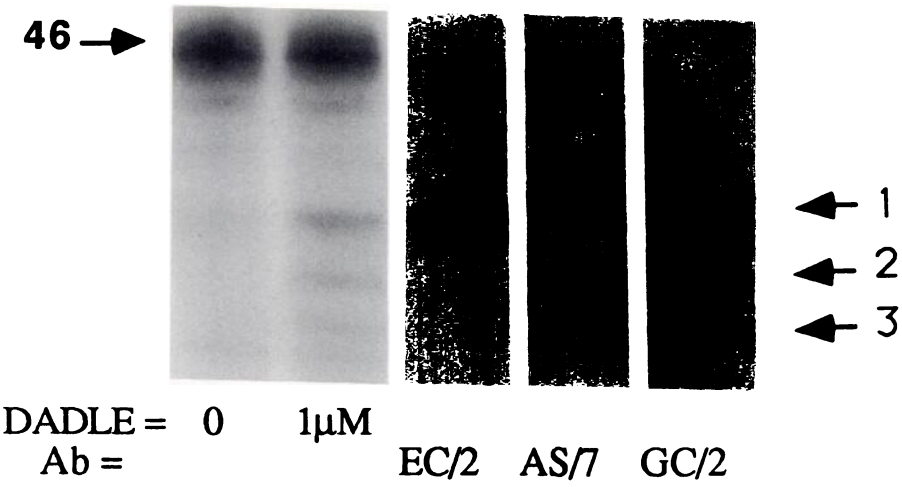

Fig. 3. Identification of protein bands in which ^{32}P incorporation was catalyzed by CTX in the presence of 1 μM DADLE. *Arrow on left*, location of 46-kDa standard. *Lanes 1* and *2*, autoradiogram of ^{32}P incorporation, as also shown in Fig. 1B. *Lanes 3–5*, immunoblots developed colorometrically using the alkaline phosphatase-conjugated primary antiserum and the indicated secondary antisera. *Lane 3*, EC/2 for $G_{i3\alpha}$; *lane 4*, AS/7 for $G_{i1,2\alpha}$; *lane 5*, GC/2 for $G_{o\alpha}$. *Arrows on right*, positions of CTX substrates that incorporate radioactivity in the presence of DADLE (*lane 2*), as indicated in Fig. 1B.

[^{32}P]ADP-ribose in the presence of DADLE (Fig. 3, *lane 2*). A single immunopositive band was observed after immunoblotting with the AS/7 antiserum, which interacts with G_{i1} and G_{i2} α subunits (Fig. 3, *lane 4*). This band was previously identified as $G_{i2\alpha}$ in these NG108–15 cell membranes (Fig. 2B) and had the same mobility as DADLE-induced radiolabeled band 2 in Fig. 3, *lane 2*. Two separate proteins showed a positive interaction with the GC/2 antiserum (for $G_{o\alpha}$), as shown in Fig. 3, *lane 5*. This result agrees with previous findings of two separate $G_{o\alpha}$ isoforms in these cells (33). The lower band had the same mobility as DADLE-induced band 3 observed in Fig. 3, *lane 2*. Thus, the three bands that interacted with the δ-opioid receptor were identified as one isoform of $G_{i3\alpha}$ (band 1), $G_{i2\alpha}$ (band 2), and one isoform of $G_{o\alpha}$ (band 3).

Recently, the isoform of $G_{o\alpha}$ termed $G_{o2\alpha}$ was identified by molecular cloning (34). An antibody generated against a peptide sequence that is encoded by the $G_{o2\alpha}$ cDNA recognized PTX substrates of 39–40 kDa (34). This affinity-purified antibody, AS 201, was used to identify the subtype of $G_{o\alpha}$ that interacted with the δ-opioid receptor. Fig. 4 shows an immunoblot of NG108–15 membranes, using the AS 201 and GC/2 antisera. The faster migrating band, which was immunopositive for GC/2, was also immunopositive with AS 201. This α subunit was also the one that incorporated radioactivity in the presence of

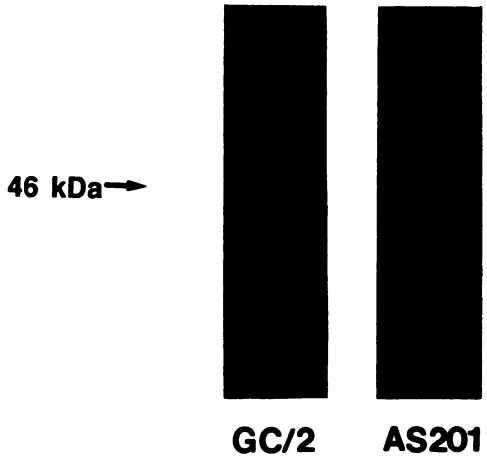

Fig. 4. Identification of $G_{o\alpha}$ isoform in NG108–15 membranes. Immunoblot from urea gradient SDS-PAGE, using antisera GC/2 (*lane 1*) or AS 201 (*lane 2*). The position of the 46-kDa molecular mass marker is indicated on the *left*.

DADLE and CTX (Fig. 3). Thus, the δ-opioid receptor interacted with the $G_{o2\alpha}$ subtype.

In order to demonstrate that the CTX-induced ADP-ribosylation is mediated via the δ-opioid receptor, a concentration-response relationship was determined. Incubation of the NG108–15 membranes with increasing concentrations of DADLE in the presence of CTX resulted in an increase in the [^{32}P]ADP-ribose incorporation into all three bands, as shown in Fig. 5A. Each DADLE-induced band was quantified using densitometric analysis. The three separate protein bands were referred to as identified in Fig. 3, i.e., band 1 is $G_{i3\alpha}$, band 2 is $G_{i2\alpha}$, and band 3 is $G_{o2\alpha}$. The concentration-response relationship between DADLE dose and mean density of the separate bands, from a representative experiment, is shown graphically in Fig. 5B. All three proteins showed increased [^{32}P]ADP-ribose incorporation, as indicated by increases in mean density in the presence of increasing amounts of the opioid. $G_{o2\alpha}$, which showed the least radioactivity incorporation, also did not show as great an increase in mean density as did the two G_i α subunit proteins. In the experiment shown in Fig. 5A, GTP was added to the reaction mixture. A previous report has suggested that CTX-induced ADP-ribosylation of PTX substrates occurs only in the absence of added guanine nucleotide (17). Other investigators have either added a nonhydrolyzable GTP analog (19) or not added GTP (20) when using CTX and agonist to induce ADP-ribosylation of PTX substrates. In the present studies, the effect of adding GTP was evaluated, because the opioid agonist stimulated CTX-induced ADP-ribosylation in both the presence and the absence of the added guanine nucleotide. Three experiments were performed under each condition (either with or without added GTP), the autoradiograms were evaluated with densitometric analysis, and EC_{50} values were calculated. The EC_{50} (95% confidence intervals) values (in nM) in the absence of added GTP were $G_{i3\alpha}$ = 120 (50–290), $G_{i2\alpha}$ = 183 (65–520), and $G_{o2\alpha}$ = 56 (9–363). In the presence of added GTP the EC_{50} values (in nM) were $G_{i3\alpha}$ = 12 (3–52), $G_{i2\alpha}$ = 54 (22–130), and $G_{o2\alpha}$ = 48 (4–576). Although there appeared to be some variability among these values, they were not significantly different from one another, an indication that each of the three G α subunits was equally capable of interacting with the opioid receptor. In addition, GTP was not required for the receptor/G protein interaction, and its presence did not affect the interaction.

To confirm further that the CTX ADP-ribosylation of the

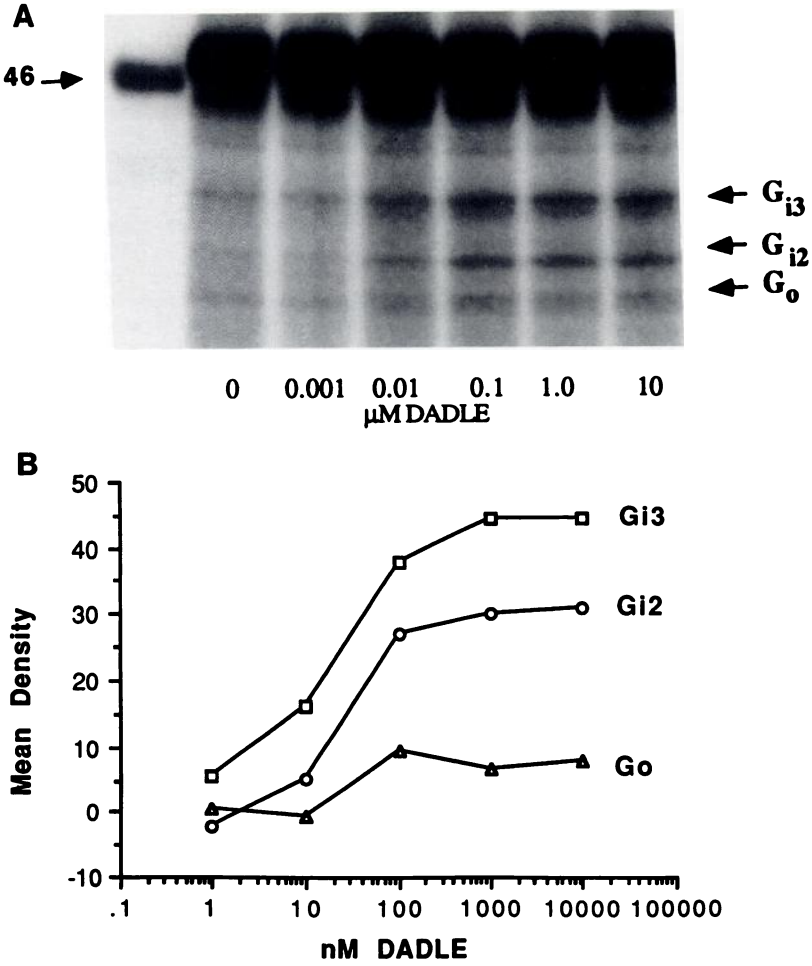

Fig. 5. Concentration response for DADLE-induced, CTX-catalyzed ^{32}P incorporation into G proteins. A, Autoradiogram of urea gradient SDS-PAGE, in the presence of increasing (0–10 mM) DADLE. *Arrows on right*, positions of G protein α subunits that were identified in Fig. 2 (1 = G_{i3}; 2 = G_{i2}; 3 = G_o). B, Mean density of each band ($G_{i3\alpha}$, $G_{i2\alpha}$, and $G_{o\alpha}$) from gel represented in A, as determined by densitometry. Mean density values from the no-drug lane were subtracted from values obtained after incubation with the indicated DADLE concentrations.

G_α subunits is due to activation of the δ-opioid receptor, NG108–15 membranes were preincubated with opioid agonists that were selective for various receptor types or with the antagonist naloxone. The δ-selective agonists DADLE and DPDPE, the μ agonist PL017, the κ agonist U50–488H, the nonselective agonist etorphine, and bremazocine (which also acts as a nonselective agonist in these cells)[1] were incubated for 5 min with membranes in the presence of [α-^{32}P]NAD$^+$, CTX was added, and incubation was continued for 2 hr. Various concentrations of agonist were used, depending on the predetermined affinity of that agonist for opioid binding sites in these membranes. Concentrations used were 1 μM for DADLE, DPDPE, etorphine, and bremazocine, whose respective K_d values were 10 ± 1.5, 1.43 ± 0.7, 22.2 ± 2.3, and 13.5 ± 2.5 nM, and 10 μM for PL017 and U50–488H, whose K_d values were >10 μM (4).[1] A concentration of 100 μM naloxone was used either by itself or with 1 μM DADLE. Results from one experiment are shown in the autoradiogram in Fig. 6. Fig. 6, *lane 1*, shows the condition without CTX, and Fig. 6, *lane 2*, shows membrane plus CTX but without opioid. The δ agonists DADLE and DPDPE, as well as the nonselective agonists etorphine and bremazocine, produced increases in radioactivity incorporation, as shown previously (Figs. 1–3). The μ agonist PL017 and the κ agonist U50–488H did not induce radioactivity incorporation into these proteins. Naloxone, a nonselective opioid antagonist, also had no effect and blocked the effect of DADLE. Thus, results expected for an opioid receptor-selective effect were achieved; δ and nonselective agonists showed activity, whereas the μ and κ agonists did not.

Chronic treatment of NG108–15 cells with DADLE has been shown to result in down-regulation of opioid receptors from the plasma membrane to the lysosomal compartment (35). If the increase in radioactivity induced by DADLE in the presence of CTX and [^{32}P]NAD$^+$ is indicative of receptor/G protein interaction, then a decrease in membrane receptors might be expected to decrease the DADLE-induced increase in radioactivity incorporation into the G protein α subunits, as seen in the experiments described above. Accordingly, NG108–15 cells were treated for 18 hr with 100 nM DADLE, and membranes prepared from these cells were incubated with 1 μM DADLE in the presence of [^{32}P]NAD$^+$ and CTX. Autoradiograms from four separate experiments were analyzed densitometrically, and results are shown in Fig. 7. Incorporation of radioactivity into the $G_{i3\alpha}$ and $G_{i2\alpha}$ subunits was significantly decreased in the chronically treated membranes, compared with the controls, but a significant decrease was not shown for the $G_{o\alpha}$. The association between the opioid receptor and the $G_{o2\alpha}$ protein appeared to be the weakest of the opioid/G protein interactions, because the amount of radioactivity incorporated into this band was always less than in the other two (Figs. 1–3). Changes in this weak interaction may be inherently difficult to detect using this method.

[1] P.Y. Law, unpublished observations.

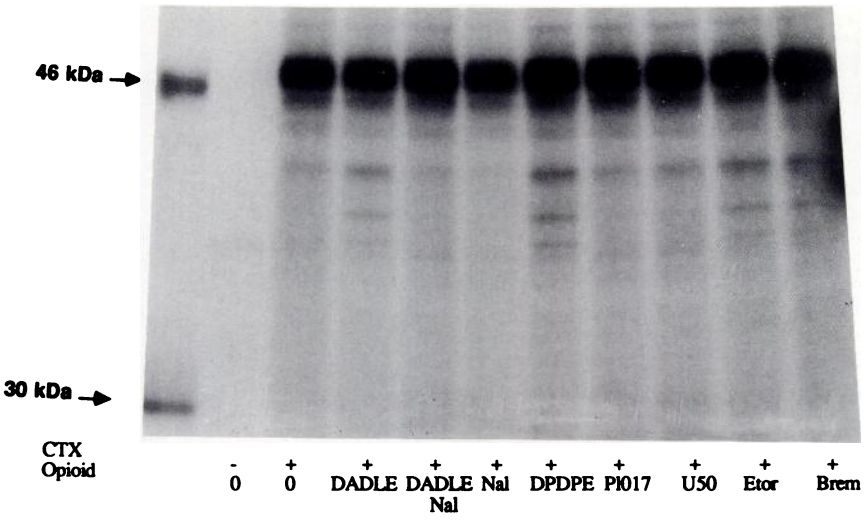

Fig. 6. Effect of various opioid agonists or naloxone on ^{32}P incorporation into proteins, catalyzed by CTX. *Left lane*, molecular weight standards. *Lane 1*, no CTX and no opioid. *Lanes 2–10*, addition of CTX. *Lanes 3* and *4*, 1 μM DADLE. *Lanes 4* and *5*, 100 μM naloxone. *Lane 6*, 1 μM DPDPE. *Lane 7*, 10 μM PL017; *lane 8*, 10 μM U50–488H; *lane 9*, 1 μM etorphine; *lane 10*, 1 μM bremazocine. *Arrows on right*, positions of the identified G_α subunits, as in Fig. 5.

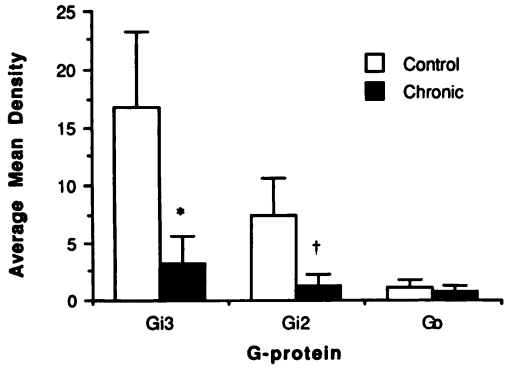

Fig. 7. Effect of chronic DADLE treatment of NG108–15 cells on ^{32}P incorporation into membrane proteins, catalyzed by CTX in the presence of 1 μM DADLE. Average mean density (three experiments) from densitometric analysis of proteins, $G_{i3\alpha}$, $G_{i2\alpha}$, and $G_{o\alpha}$, that incorporated ^{32}P in the presence of DADLE. *Error bars*, standard error. □, Control membranes; ■, membranes from cells incubated for 24 hr with 100 mM DADLE. *, Significantly different from control ($p < 0.05$); †, $p < 0.1$.

Discussion

In the present report, evidence is presented that indicates that the δ-opioid receptor in NG108–15 cells interacted with three separate G proteins. In the presence of δ-opioid agonists and [α-^{32}P]NAD$^+$, CTX catalyzed the incorporation of [^{32}P] ADP-ribose into membrane-associated PTX-sensitive G_α subunits. Separation of the radiolabeled proteins using a urea gradient SDS-PAGE system allowed distinct resolution in one dimension of three separate G_α subunits that showed agonist-dependent ^{32}P incorporation. The DADLE-induced incorporation was concentration dependent, in a range that included the K_d value, and could be blocked by the antagonist naloxone. δ but not μ or κ receptor-selective agonists produced this effect. Chronic treatment of the cells with DADLE, which decreases the number of receptors in the membrane, also decreased the incorporation of [^{32}P]ADP-ribose into the G_α subunits. Immunoblotting with antisera generated against selected peptide sequences in cloned G_α subunits permitted identification of these α subunit proteins as $G_{i2\alpha}$, one isoform of $G_{o\alpha}$, and one isoform of $G_{i3\alpha}$ (Fig. 2).

The observation of two distinct protein bands that were immunopositive for the $G_{i3\alpha}$ antiserum (EC/2) was unexpected, because isoforms of this α subunit have not been previously reported. These two isoforms were clearly resolved in the urea gradient SDS-PAGE system and appeared to be about equally expressed in the NG108–15 membranes. However, they were not equally ADP-ribosylated in the presence of PTX, and only one of them appeared to interact with the δ-opioid receptor. Thus, these $G_{i3\alpha}$ isoforms appear to have distinct characteristics, and further studies on these proteins are in progress.

Using a similar agonist-induced CTX-catalyzed ADP-ribosylation procedure, other investigators (17, 20) have previously demonstrated that the δ agonist DADLE induces concentration-dependent, naloxone-reversible, ADP-ribosylation of the PTX substrates in NG108–15 membranes, but these investigators did not demonstrate involvement of multiple G proteins. Evidence from several laboratories indicates that activation of δ receptors in these cells results in more than one PTX-sensitive intracellular event (5, 9, 36); thus, the present finding of δ receptor interaction with more than a single G protein appears inevitable. Interaction of a single receptor with two G proteins has been shown, using a similar protocol, in at least two other systems. The *N*-formyl-methionyl-leucyl-phenylalanine peptide receptor has been shown to interact with two separate G_i proteins, G_{i2} and G_{i3}, in HL-60 cells (19), and Milligan *et al.* (37) have recently reported that the α_2-C10 adrenergic receptor interacts with G_{i2} and G_{i3} after being transfected into rat fibroblasts.

The mechanism by which certain agonists induce CTX-mediated ADP-ribosylation of G proteins is not well understood. The amino acid sequences of all the PTX substrates include an internal arginine residue, which is the site for CTX-mediated ADP-ribosylation. However, these proteins are not usually CTX substrates. It is possible that the agonist induces some change in the receptor that, in turn, induces a conformational change in the G protein α subunit that exposes the CTX-sensitive arginine. The arginine then becomes an available substrate for ADP-ribosylation. In any case, use of CTX to identify receptor-associated PTX substrates is a unique procedure that can be used in many biological systems.

One potential disadvantage of using CTX to identify receptor-coupled G proteins is that it can only identify those G proteins that contain the target internal arginine residue. However, all the currently known G protein α subunits do contain

this site (38), even those that have not yet been associated with specific receptors (39). Another noticeable disadvantage of this method is the lack of sensitivity. A considerable amount of cell protein is expressed as G protein, and these proteins may be coupled with many different membrane receptors. If the receptor of interest in a particular membrane does not occur in sufficient numbers, the G proteins that incorporate [^{32}P]ADP-ribose in the presence of CTX are difficult to detect by autoradiography, because few free α subunits will be available to act as CTX substrates. In identification of the specific G proteins that interact with one selected receptor population of many populations, only a limited number of G protein α subunits will incorporate the radionucleotide. Thus, the sensitivity is limited by the number of receptors. Our initial attempts to use PTX-catalyzed ADP-ribosylation to identify the G proteins, as others have reported (15, 16), probably were unsuccessful for this same reason. That is, if there are few activated receptors to induce dissociation of the G protein α and $\beta\gamma$ subunits, a small decrease in radioactivity incorporation in the presence of agonist would be difficult to discern from the intense radiolabeling catalyzed by PTX in the absence of agonist. Use of CTX provided a more sensitive method than use of PTX, but even more sensitive methods may be developed in the future.

An alternative method of identifying which G proteins interact with a selected receptor involves reconstitution experiments in which the purified or recombinant form of a selected G_α subunit protein is added to cells that have been previously treated with PTX. This method was used by Hescheler *et al.* (9) to indicate that G_o interacts with opioid receptors to produce a decrease in inward Ca^{2+} current. However, this method does not show that the particular α subunit is the physiological mediator of a selected receptor function. The advantage of the CTX method is that the G protein and receptor remain associated in the membrane preparation and addition of agonist produces the observed change in CTX-catalyzed ADP-ribosylation. Thus, the interaction of the receptor with G protein(s) can be evaluated.

A recent report using photoaffinity labeling techniques has linked the δ-opioid receptor in NG108–15 cells with G_{i2} and G_o proteins (40). This technique, which utilizes [α-^{32}P]GTP azidoanilide, is based on guanine nucleotide exchange, which follows agonist-induced receptor activation. Results from the present studies supported the proposed interactions with G_{i2} and G_o, derived from the photoaffinity studies, and also suggested that the opioid receptor interacted with one isoform of G_o but not the other. Because this receptor-linked isoform is the faster migrating one, it appeared to be the more basic isoform (G_o2) of the two G_o forms observed by Mullaney and Milligan (33). However, no association of opioid receptor with G_{i3} has been suggested previously, even though the receptor-linked isoform of $G_{i3\alpha}$ appears in approximately equal amounts as $G_{i2\alpha}$ in these cells (Fig. 2). This receptor/G_{i3} interaction may not have been observed earlier because the PTX substrates have been difficult to resolve on one-dimensional gel systems. Incorporation of photoaffinity labeling techniques with urea gradient SDS-PAGE may allow more sensitive detection as well as enhanced resolution of G proteins that interact with receptors.

Although a previous report has shown that the agonist-induced CTX-catalyzed ADP-ribosylation occurs only in the absence of added guanine nucleotide (17), other investigators added (19) or did not add (20) guanine nucleotide to achieve similar results. The present studies suggested that the reaction occurs both in the presence and in the absence of added GTP. The free G_α α subunit appears to be the preferred substrate for CTX (14); thus, the presence of GTP might be expected to increase the toxin-induced ADP-ribosylation. However, it is possible that the nucleotide-free α subunit is necessary for the agonist-induced CTX-catalyzed ADP-ribosylation (17). Perhaps the protocol used in the present studies, i.e., preincubation of agonist with or without GTP before addition of CTX, is responsible for the results showing ADP-ribosylation of the PTX substrate in both the presence and the absence of added GTP.

The concentration dependence of incorporation of radioactivity into all three G_α subunits promoted by DADLE was demonstrated by results depicted in Fig. 5. The observation that the EC_{50} values for radioactivity incorporation were similar for all three G_α subunits suggested that the receptor/G protein association was similar for $G_{i3\alpha}$, $G_{i2\alpha}$, and $G_{o2\alpha}$. However, there was differential incorporation of radioactivity into these three proteins; the greatest amount of radioactivity was always incorporated into the $G_{i3\alpha}$ isoform, and the least into $G_{o2\alpha}$. Results from PTX-catalyzed ADP-ribosylation indicated that both the $G_{o\alpha}$ isoforms were expressed less abundantly than were the slower migrating $G_{i3\alpha}$ isoform or $G_{i2\alpha}$ (Fig. 1), which were the major PTX substrates. The occurance of less $G_{o\alpha}$ would account for the least radioactivity incorporation observed between the opioid receptor and this G protein. However, because the $G_{i3\alpha}$ isoform and $G_{i2\alpha}$ showed about equal expression, there appeared to be some difference in the manner in which the receptor interacted with these two proteins; the interaction with the $G_{i3\alpha}$ isoform resulted in greater incorporation of radioactivity.

The opioid receptor characteristics of the increased ^{32}P incorporation into specific G_α subunits were determined by the use of the antagonist naloxone and various receptor-selective opioid agonists (Fig. 6). Naloxone reversed the effects of DADLE, confirming the opioid character of the DADLE activity. Naloxone alone did not increase radioactivity incorporation into α subunits, indicating that the antagonist did not induce the same receptor change as the agonist and could not promote a proposed conformational change in the associated G proteins that would expose the arginine for subsequent ADP-ribosylation. The δ agonists DADLE and DPDPE, as well as the nonselective agonists etorphine and bremazocine, have similar affinities for opioid receptors in NG108–15 cells (4).[1] These agonists all stimulated CTX-mediated incorporation of ^{32}P into the three separate G protein α subunits. As expected for a δ-mediated system, the μ agonist PL017 and the κ agonist U50–488H did not show this effect.

Chronic treatment of NG108–15 cells with DADLE results in a reduction of opioid receptors in the cell membrane, without a concominent decrease in PTX substrate content (36). Results in the present study that show a decrease in agonist-induced radioactivity incorporated into all three G_α subunits in membranes chronically treated with DADLE imply that fewer opioid receptors were in the membrane to promote CTX-catalyzed ADP-ribosylation. Because the amount of PTX substrate in these membranes was unchanged, these results support the conclusion that receptor/agonist interaction was required for increased ^{32}P incorporation into specific α subunits.

The intracellular functions mediated by these various G proteins in NG108–15 cells remain to be identified. Involvement of G_{i2} in opioid-induced inhibition of adenylyl cyclase activity was implied by McKenzie and Milligan (8), who showed inhibition of DADLE activity with AS/7 antiserum (directed against $G_{i1,2\alpha}$). Earlier studies have indicated that opioid/G_o interactions mediate activity of a voltage-sensitive calcium channel in differentiated NG108–15 cells (9). Only one G_o protein had been characterized at the time of that study; thus, whether one or both of the isoforms are involved in this activity was unresolved. Recently, both G_o subtypes were shown to be involved in inhibition of voltage-dependent Ca^{2+} channels in rat pituitary GH_3 cells (41). These two subtypes were associated with two different receptor types, muscarinic and somatostatin, which produce the same action on the channels. Based on electrophoretic mobility, the G_o subtype that is associated with the δ receptor appears to be the G_{o2} type (faster migrating form) described by Mullaney and Milligan (33). Studies using an antibody against $G_{o2\alpha}$ (AS 201) (31) in NG108–15 membranes showed a single immunopositive band, which migrated with the G_o α subunit associated with the δ receptor.

The strong association of the opioid receptor with the $G_{i3\alpha}$ isoform shown in the present study has not been previously shown, and the function mediated by this G protein is not clear. By comparing amino acid sequences, Codina *et al.* (42) have suggested that $G_{i3\alpha}$ may be G_k, which is involved in stimulation of potassium channels. However, patch-clamp studies with GTPγS-activated α subunits isolated from recombinant subunits expressed in *Escherichia coli* or from natural sources have shown that G_{i1} and G_{i2} have activity to stimulate potassium channels equal to that of G_{i3} (43). Because undifferentiated NG108–15 cells do not express active potassium channels, the function of G_{i3} in this system is unknown.

The finding that one opioid receptor type can interact with more than one form of G protein is important, because activation of one type of receptor has been shown to activate more than one second messenger system (44–46). In these examples, one second messenger system is preferentially stimulated or inhibited, but under certain conditions a second effector system is also affected. Alternatively, one receptor may be coupled with G proteins that activate opposing effects on the same effector system (47). These various effector systems may be activated by specific G proteins that are selective for designated effects, or a single effector system could be controlled by several G proteins (48, 49). Such diversity in effects of receptor activation allows fine tuning of systems that rely upon selective G protein activation within a single cell. Which final effect results from the agonist/receptor interaction is, thus, dependent on a multitude of intracellular interactions.

In conclusion, δ-opioid receptors in NG108–15 cells were demonstrated to be associated with $G_{i2\alpha}$, one isoform of $G_{i3\alpha}$, and $G_{o2\alpha}$. These associations were concentration dependent, reversed by antagonist, and decreased in cells in which receptors had been down-regulated. Which intracellular events are mediated by which G proteins remains to be characterized, but these studies, along with many others, have suggested that opioids are involved in control of more than a single effector system.

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