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Direct Measurements of *In Situ* Interactions of Rat Brain Opioid Receptors with the Guanine Nucleotide-Binding Protein G_o

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SUMMARY

The interactions of rat brain cortical opioid receptors with the guanine nucleotide-binding protein (G protein) G_o were probed in membranes by examining the ability of selective antipeptide anti-G protein antisera to disrupt receptor-G protein interactions. This was measured both by antibody-induced alterations in the characteristics of agonist binding to μ and δ receptor binding sites and by antibody attenuation of opioid stimulation of high affinity GTPase activity. Antisera to the amino-terminal 16 amino acids (ON1), amino acids 22–35 (IM1), and the carboxyl-terminal decapeptide (OC2) of forms of $G_{o\alpha}$ were able to selectively immunoprecipitate G_o from rat cortical membranes. Both antisera OC2 and ON1 were able to immunoprecipitate $G_{o\alpha}$ quantitatively. Preincubation of rat cortical membranes with an IgG fraction isolated from antiserum OC2 was able to produce a marked reduction in the ability of the synthetic enkephalin [D-Ala^2,D-Leu^5] enkephalin (DADLE) (which interacts with δ and μ but not significantly with κ receptors) to displace specific binding of [^{3}H] diprenorphine (which binds to all of these sites), demonstrating a clear interaction of the μ and δ receptors with one or more variants of G_o. An IgG fraction from antiserum ON1 was able to mimic this effect, suggesting that the amino-terminal region of G protein α subunits also plays a role in receptor-G protein interactions. In contrast, an IgG fraction from antiserum IM1 was unable to alter the characteristics of DADLE displacement of [^{3}H] diprenorphine binding. Similarly, an antiserum (SG1) directed against the carboxyl-terminal decapeptide common to the α subunits of G_{i1} and G_{i2} was unable to reduce the affinity of DADLE binding to opioid receptors. Use of antiserum OC2 in experiments that allowed pharmacological examination of only the μ-opioid receptor provided independent evidence for the interaction of this receptor site with G_o.

Agonist interaction with opioid receptors can result in the inhibition of adenylyl cyclase and/or the regulation of a range of ion channels by causing the activation of one or more pertussis toxin-sensitive G proteins (1). At least in neuroblastoma × glioma hybrid NG108–15 cells, which are often used as a model system to examine receptor-mediated signaling from the δ-opioid receptor, agonist-mediated inhibition of adenylyl cyclase appears to be transduced specifically by G_{i2} (2). However, the opioid receptor in these cells also appears to activate G_o (3) and possibly G_{i3} (4).

The nature of the pertussis toxin-sensitive G proteins that interact with opioid receptors in brain membranes is less well established. Reconstitution experiments using G proteins and μ receptors purified by an affinity chromatography step have indicated the potential for interaction of this receptor type with both the G_i and G_o families of G proteins (5). Similar conclusions have been reached by reconstitution of μ-opioid receptors in *N*-ethylmaleimide-treated guinea pig striatal membranes with purified G_i and G_o fractions (6). This interaction of the receptor with multiple G proteins is unlikely to represent a reconstitutive artifact, because selective reconstitution of the kyotorphin receptor with G_i rather than G_o has been noted using similar protocols (7).

Agonist, but generally not antagonist, binding affinity for G protein-linked receptors is regulated by the interaction of receptor and G protein. Higher affinity for agonist is usually noted for the receptor-G protein complex than for receptor plus G protein in isolation. As such, agents that interfere with receptor-G protein coupling should produce a reduction in agonist binding affinity at the receptor. We have previously taken advantage of this phenomenon to explore the selectivity of receptor-G protein interactions in cellular membranes by using antipeptide anti-G protein α subunit antisera that are directed against synthetic peptides representing the extreme carboxyl terminus of these polypeptides (2, 8). Both genetic (9)

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ABBREVIATIONS: G protein, guanine nucleotide-binding protein; DADLE, [D-Ala^2,D-Leu^5]enkephalin; ICI 174864, *N*,*N*-diallyl-Tyr-Aib-Aib-Phe-Leu; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Gpp(NH)p, guanosine 5′-($\beta\gamma$-imido)triphosphate.

and biochemical (10) evidence has indicated that this region is a key site for interactions between G proteins and receptors.

Analysis of opioid receptor interactions with specific G proteins in native brain membranes has not been reported previously. Thus, in this study we examine *in situ* the interactions of rat brain cortical opioid receptors with the G protein G_o and assess which regions of $G_{o\alpha}$ may play a key role in this process. To perform such experiments we have analyzed the effectiveness of the synthetic enkephalin DADLE, which interacts with δ- and μ- but not κ-opioid receptor sites, to displace specific [^{3}H]diprenorphine binding from membranes of rat cerebral cortex under conditions that can interfere with receptor-G protein interactions. These conditions are either nonselective [i.e., in the presence of maximally effective concentrations of the poorly hydrolyzed GTP analogue Gpp(NH)p] or made selective by using specific antipeptide anti-G protein antisera. We further examine interactions of the μ-opioid receptor with G_o by performing such studies in the presence of the highly selective δ receptor site antagonist ICI 174864. The results indicate that both the carboxyl-terminal and amino-terminal regions of $G_{o\alpha}$ contribute to interactions of this G protein with opioid receptors in native brain membranes.

Experimental Procedures

Materials. [^{3}H]Diprenorphine (37 Ci/mmol) was from DuPont/New England Nuclear. DADLE was purchased from Sigma. The highly selective δ-opioid receptor antagonist ICI 174864 (11) was from Cambridge Research Biochemicals. Gpp(NH)p was obtained from Boehringer-Mannheim. All other reagents were of analytical grade from Sigma or BDH.

Preparation of membranes. Rat brain cortex was homogenized with 10 volumes of 10 mM Tris·HCl, 0.1 mM EDTA, pH 7.5 (buffer A). The homogenate was centrifuged at 500 × *g* for 10 min and the supernatant was further centrifuged at 48,000 × *g* for 10 min. The pellet from the second centrifugation was washed with buffer A and recentrifuged at 48,000 × *g* for 10 min. Finally the pellet was resuspended in buffer A at a protein concentration of 4 mg/ml and stored at −80°. Protein concentration was measured according to the method of Lowry *et al.* (12). NG108–15 cell membranes (2) were prepared in a similar manner.

Immunological studies. The generation and specificity of the various antisera used in this study are defined in Table 1. The G protein-directed antisera were all obtained after injection of a conjugate of the synthetic peptides and keyhole limpet hemocyanin (Calbiochem) as antigen into New Zealand White rabbits. The details of this process have been described previously (13). Immunoblotting of membrane samples was performed as in Ref. 2.

Generation of IgG fractions from anti-G protein antisera. Crude antisera were chromatographed on Protein A-Sepharose (Sigma) as described in Ref. 14. After elution of the IgG fractions at pH 4.0, the samples were dialyzed overnight against buffer A, lyophilized, and reconstituted with the same buffer before use.

TABLE 1

Peptide sequences used to generate a series of antipeptide antisera directed against forms of $G_{o\alpha}$ and $G_{i\alpha}$

The antisera, with the exception of O1B, have been described in detail previously (17, 18). Antiserum O1B does not cross-react with $G_{o2\alpha}$ but does identify at least two forms of $G_{o1\alpha}$ expressed in rat brain cortex.[1] Amino acids are represented using the one-letter code. $G_{o1\alpha}$ and $G_{o2\alpha}$ are believed to be derived by differential splicing mechanisms.

Antiserum	Peptide used	G protein sequence
OC2	ANNLRGCGLY	$G_{o1\alpha}$ and $G_{o2\alpha}$ 345–354
ON1	GCTLSAEERAALERSK	$G_{o1\alpha}$ and $G_{o2\alpha}$ 1–16
IM1	NLKEDGISAAKDV	$G_{o1\alpha}$ and $G_{o2\alpha}$ 22–35
O1B	FESKNRSPNK	$G_{o1\alpha}$ 308–317
SG1	KENLKDCGLF	TD1 α 345–354

Binding experiments. Binding experiments were performed at 30° for 30 min in 50 mM Tris·HCl, 20 mM $MgCl_2$, 100 mM NaCl, pH 7.5 (buffer B). In saturation experiments using [^{3}H]diprenorphine the concentration of ligand was varied between 0.25 and 15 nM. Nonspecific binding was defined by using parallel tubes containing either 10 μM naloxone or, in some cases, 10 μM DADLE. Nonspecific binding increased in a linear manner with ^{3}H-ligand concentration. Binding was terminated by rapid filtration through Whatman GF/C filters, followed by extensive (3 × 5 ml) washes with ice-cold 50 mM Tris·HCl, pH 7.5. Filters were maintained overnight in Hi-Safe scintillant before liquid scintillation counting. In experiments where uncoupling of opioid receptors from G proteins was performed the membranes were preincubated for 60 min at 30° with IgG fractions derived either from normal rabbit serum or from the series of polyclonal antipeptide antisera described in Table 1. Analysis of the binding data was performed using Kaleidograph software run on an Apple Macintosh Classic 2 computer. IC_{50} values were corrected for receptor occupancy using the method of Cheng and Prusoff (15). Statistical analysis of the effects of the various agents used was performed using Student's *t* test for unpaired samples.

Opioid receptor-stimulated GTPase. GTPase was assayed essentially as described by McKenzie and Milligan (2). Membranes were preincubated with either nonimmune serum or antiserum OC2, ON1, or SG1 for 60 min at 37° before assessment of DADLE stimulation of high affinity GTPase activity.

Immunoprecipitation of G proteins. Immunoprecipitation was performed essentially as described by Rothenberg and Kahn (16), with the following modifications from the published protocol. Membrane pellets (100 mg) were resuspended in 50 μl of 1% SDS and, after boiling for 3 min, 950 μl of solubilization buffer (containing 1% Triton X-100, 10 mM EDTA, 100 mM NaH_2PO_4, 10 mM NaF, 100 μM Na_3VO_4, 1 μg/ml leupeptin, 1 μg/ml aprotinin, 1 μg/ml pepstatin, 2 μg/ml trypsin inhibitor, 0.5 mM phenylmethylsulfonyl fluoride, 50 mM HEPES, pH 7.2 at 4°) were added. The samples were then incubated for 90 min on ice and centrifuged in a microcentrifuge to remove any nonsolubilized material. To the supernatant an appropriate amount of specific antipeptide antiserum was added. Nonimmune serum at the same concentration was used as control. The samples were incubated at 4° overnight, after which a 1:1 suspension of Protein A-agarose (50 μl) was added to each sample and the samples were incubated for 2 hr at 4° on a rotary mixer. The immune complex was then pelleted in a microfuge and washed with 4% Triton X-100, 100 mM NaCl, 100 mM NaF, 50 mM NaH_2PO_4, 0.3% SDS, 50 mM HEPES, pH 7.2. Finally, 50 ml of Laemmli sample buffer were added to the immune complex, and after centrifugation the supernatant was resolved by SDS-PAGE (10%, w/v, acrylamide).

Results

Antipeptide antisera IM1, OC2, and ON1 (Table 1), which were raised against peptides predicted to be common to polypeptides corresponding to products from both the G_{o1} and G_{o2} splice variants of the $G_{o\alpha}$ gene, have previously been characterized extensively (Ref. 17 and references therein). Each of these antisera identified a polypeptide of 39 kDa in immunoblots of rat brain cortical membranes resolved by 10% (w/v) acrylamide SDS-PAGE. Both OC2 and ON1 antisera were able to immunoprecipitate a 39-kDa polypeptide that could be identified as $G_{o\alpha}$ by immunoblotting of these immunoprecipitates with antiserum IM1 (Fig. 1). Antiserum IM1 was also able to immunoprecipitate a 39-kDa polypeptide that could be confirmed to be $G_{o\alpha}$ by immunoblotting of such immunoprecipitates with antiserum OC2 (data not shown). The specificity of the immunoprecipitations produced by the anti-G_o antisera was tested by immunoblotting of such immunoprecipitates with antiserum

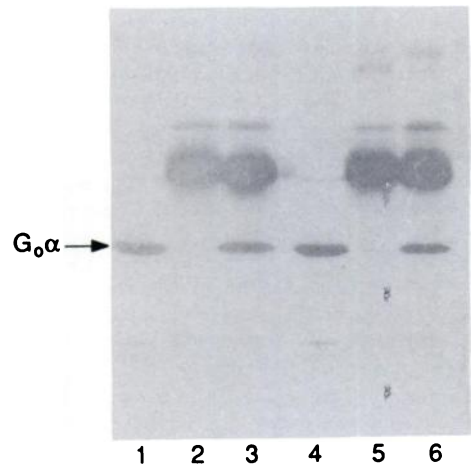

Fig. 1. Anti-$G_{o\alpha}$ antisera immunoprecipitate $G_{o\alpha}$ from rat cortical membranes. Rat cortical membranes (100 μg) were solubilized as described in Experimental Procedures (*lanes 2, 3, 5,* and *6*) and immunoprecipitated with either antiserum OC2 (*lane 3*), antiserum ON1 (*lane 6*), or normal rabbit serum (*lanes 2* and *5*) (15 μg of IgG). Both the immunoprecipitates and untreated membranes (*lanes 1* and *4*) were then resolved by SDS-PAGE (10%, w/v, acrylamide) and immunoblotted using antiserum IM1 as primary antiserum.

SG1. This antiserum identifies $G_{i1\alpha}$ and $G_{i2\alpha}$ equally (18). Apart from G_o, these are the most highly expressed pertussis toxin-sensitive G proteins in rat brain (19). SG1 failed to identify a protein or proteins of 40–41 kDa in immunoprecipitates performed with any of the three anti-G_o antisera (data not shown). Such results indicated that OC2, ON1, and IM1 each could selectively identify and interact with $G_{o\alpha}$ in rat brain membranes.

Specific [^{3}H]diprenorphine binding to μ-, δ-, and κ-opioid receptors in rat brain cortical membranes, defined as that binding displaced by 10 μM naloxone, comprised approximately 220 fmol/mg of membrane protein. Saturation analysis of specific [^{3}H]diprenorphine binding indicated a K_d of 0.8 nM for this radioligand. DADLE at a concentration of 10 μM was able to displace approximately 85% of the specific diprenorphine binding (defined in the presence of 10 μM naloxone), indicating that this percentage of the specific [^{3}H]diprenorphine binding was to a combination of μ and δ receptor sites. The IC_{50} for DADLE (corrected for receptor occupancy) was 6.0 ± 0.6 nM (mean ± standard error, four experiments) when the displacement experiments were performed in the absence of exogenous guanine nucleotides, but this increased to 100.0 ± 10.0 nM (corrected for receptor occupancy; mean ± standard error, four experiments) ($p < 0.001$) when the displacement experiments were performed in the presence of a maximally effective concentration (100 μM) of the poorly hydrolyzed GTP analogue Gpp(NH)p. Such experiments are indicative of the coupling of rat brain opioid receptors to G proteins but provide no data as to the molecular species involved.

To assess the interaction of G_o with these receptors we preincubated rat brain cortical membranes with Protein A-Sepharose-purified IgG fractions from the antisera described in Table 1 or from normal rabbit serum and we then analyzed the ability of DADLE to displace specific [^{3}H]diprenorphine binding. After preincubation with the IgG fraction from antiserum OC2, the ability of DADLE to displace specific [^{3}H]diprenorphine binding was reduced ($p < 0.001$) (IC_{50} corrected for receptor occupancy = 34.5 ± 9.8 nM, mean ± standard error, four experiments), in comparison with preincubation of the membranes with an IgG fraction from normal rabbit serum (3.9 ± 0.6 nM, mean ± standard error, nine experiments) (Fig. 2A). However, the IgG fraction of antiserum OC2 did not produce as marked a rightward shift in DADLE displacement of [^{3}H]diprenorphine binding ($p = 0.023$) as did incubation with Gpp(NH)p in the presence of IgG from normal rabbit serum (IC_{50} corrected for receptor occupancy = 110.2 ± 20.8 nM, mean ± standard error, seven experiments).

To examine whether antisera directed against other regions of $G_{o\alpha}$ would also be able to modify agonist binding affinity for rat brain opioid receptors, we performed similar preincubations of the membranes with IgG fractions from antisera ON1 and IM1. The IgG fraction from antiserum ON1 was able to produce a shift in the affinity of DADLE for displacement of [^{3}H]diprenorphine binding (IC_{50} corrected for receptor occupancy = 14.7 ± 4.5 nM, mean ± standard error, three experiments) (Fig. 2B) that was statistically significant ($p = 0.002$). However, at the maximum concentrations of antisera that we were able to test the effect of antiserum ON1 was always less than that produced by antiserum OC2 (although this did not achieve statistical significance, $p = 0.16$) or by Gpp(NH)p ($p = 0.016$) (compare Fig. 2, A and B). In contrast, preincubation of rat brain membranes with the IgG fraction from antiserum IM1 was unable to produce any significant ($p = 0.875$) reduction in affinity of DADLE for displacement of [^{3}H]diprenorphine binding (IC_{50} corrected for receptor occupancy = 4.1 ± 0.1 nM, mean ± standard error, three experiments) (Fig. 2C).

In constrast to the effectiveness of antiserum OC2 in producing a rightward shift for DADLE displacement of specific [^{3}H]diprenorphine binding, similar experiments with antiserum SG1, which identifies the equivalent epitope of both $G_{i1\alpha}$ and $G_{i2\alpha}$, were unable to produce any shift in the IC_{50} (Table 2).

Analysis of the displacement curves indicated that at 100 nM DADLE approximately 70% of the specific binding of [^{3}H]diprenorphine was displaced in the absence of Gpp(NH)p, whereas in the presence of Gpp(NH)p (100 μM) 100 nM DADLE was able to displace only approximately 20% of this specific binding (see Fig. 2). By taking the difference in observed specific binding under these conditions as a measure of the alteration in agonist affinity produced by the uncoupling of receptors and G proteins, we examined whether reduction of the amount of antiserum OC2 would restore receptor-G protein interactions and hence allow the observation of high affinity agonist binding to the receptor. This was the case, with half-maximal effects of the IgG fraction of antiserum OC2 being produced at approximately 5 μg (data not shown).

In an attempt to provide independent evidence for the functional interaction of rat brain opioid receptors with G_o, we examined the ability of antisera OC2 and ON1 to reduce DADLE stimulation of high affinity GTPase in rat brain cortical membranes (Fig. 3). Untreated membranes had a basal high affinity GTPase activity of approximately 71.7 ± 2.6 pmol/min/mg of membrane protein (mean ± standard error, four experiments), and this was stimulated by DADLE to 82.9 ± 2.6 pmol/min/mg of membrane protein. Preincubation of the membranes for 60 min markedly reduced the basal high affinity GTPase activity to 29.7 ± 0.8 pmol/min/mg of membrane protein (mean ± standard error, four experiments) and the presence of DADLE after such a preincubation stimulated this activity to 37.2 ± 1.2 pmol/min/mg of membrane protein (mean

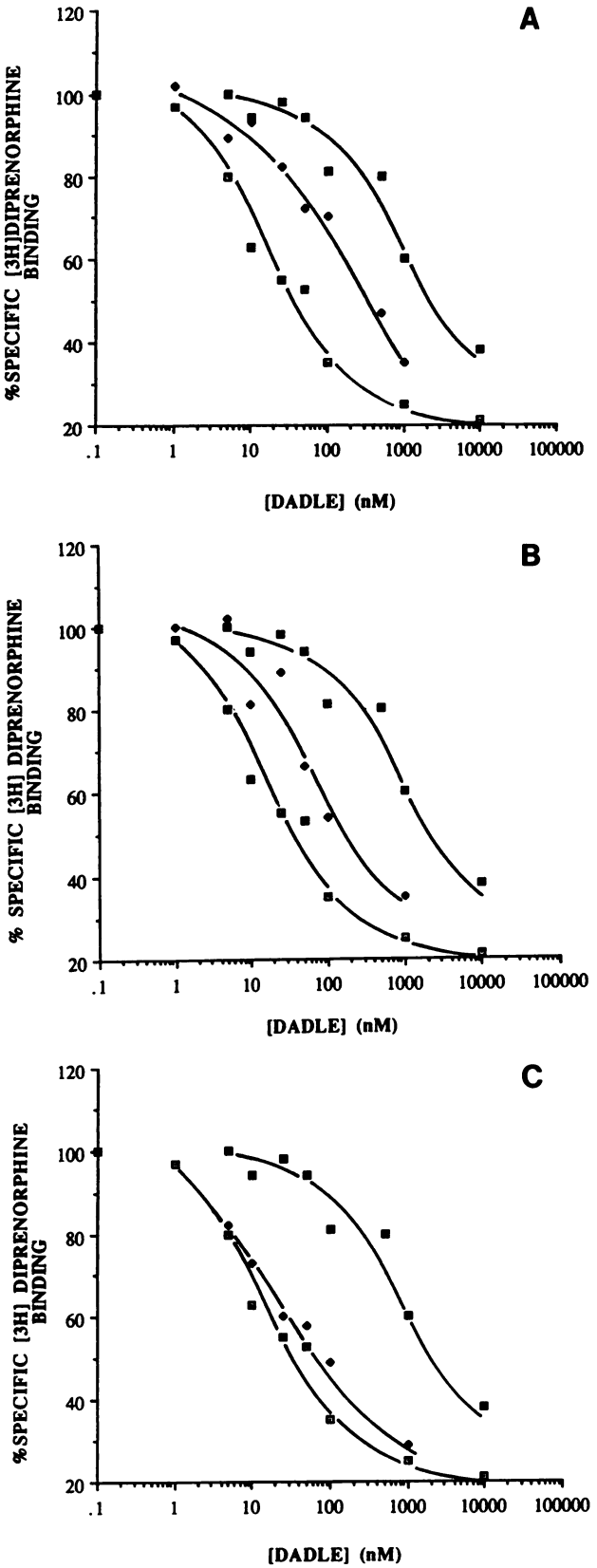

Fig. 2. Antibodies against both the carboxyl-terminal and amino-terminal regions of $G_{o\alpha}$ reduce the affinity of DADLE to displace specific [^{3}H] diprenorphine binding. A, Rat cortical membranes (70 μg) were incubated with either IgG fractions from normal rabbit serum (15 μg) (□) or antiserum OC2 (15 μg) (♦) or with 100 μM Gpp(NH)p plus the IgG fraction from normal rabbit serum (15 μg) (■) for 60 min at 30° before binding, which was performed as described in Experimental Procedures. Nonspecific binding was assessed in the presence of 10 μM naloxone. One hundred percent specific binding was 158 ± 20 fmol/mg of protein for the samples incubated with normal rabbit serum, 154 ± 23 fmol/mg of protein for the membranes that had been preincubated in the presence of OC2, and 148 ± 23 fmol/mg of protein for those membranes that had been incubated in the presence of Gpp(NH)p plus normal rabbit serum. Results were pooled from four individual experiments. Error bars have been omitted for clarity (see Table 2, however, for these numbers). B, Experiments were performed as in A, except that ON1 (♦) was used as antiserum. One hundred percent [^{3}H]diprenorphine bound under these conditions was 160 ± 7 fmol/mg of protein. Results were pooled from three individual experiments. C, Experiments were performed as in A, except that the antiserum used was IM1 (♦). One hundred percent specific [^{3}H]diprenorphine binding was 160 ± 25 fmol/mg of protein. Data were pooled from three individual experiments.

TABLE 2

Analysis of the effects of various agents on the displacement by DADLE of specific [^{3}H]diprenorphine binding in membranes of rat brain cortex (A) and statistical analysis (B)

A, All data are presented as mean ± standard error, except for data for antiserum SG1, which are presented as mean ± range. Numbers in parentheses are numbers of experiments. B, Cross-analysis of the statistical significance of the corrected IC_{50} values for DADLE displacement of [^{3}H]diprenorphine binding is shown. Values were calculated using Student's *t* test for unpaired samples.

A. Treatment	IC_{50} (corrected for receptor occupancy)
	nM
Control	3.9 ± 0.6 (*n* = 9)
Gpp(NH)p	110.2 ± 20.8 (*n* = 6)
OC2	34.5 ± 9.8 (*n* = 4)
ON1	14.7 ± 4.5 (*n* = 3(
IM1	4.1 ± 0.1 (*n* = 3)
SG1	3.6 ± 1.4 (*n* = 2)

B.	Control	Gpp(NH)p	OC2	ON1	IM1
Control		<0.001	<0.001	0.002	0.875
Gpp(NH)p	<0.001		0.023	0.016	<0.001
OC2	<0.001	0.023		0.164	0.046
ON1	0.002	0.016	0.164		0.086
IM1	0.875	<0.001	0.046	0.086	

± standard error, four experiments). Incubation with both antisera OC2 and ON1 reduced DADLE-stimulated high affinity GTPase, in comparison with incubation with nonimmune serum, but preincubation with antiserum SG1 was without effect (Fig. 3). The effects of both antisera OC2 and ON1 failed to achieve statistical significance, in comparison with nonimmune serum, however, because variability in the samples preincubted with nonimmune serum was relatively high.

To assess selectively the interaction of μ-opioid receptors with $G_{o\alpha}$, we examined the displacement of specific [^{3}H]diprenorphine binding in rat brain cortical membranes with the selective δ receptor antagonist ICI 174864 (11) and compared this with results for membranes of NG108–15 cells, which are known to express only the δ type of opioid receptor. Displacement of specific [^{3}H]diprenorphine binding from membranes of NG108–15 cells with ICI 174864 was monotonic, with an IC_{50} (corrected for receptor occupancy) of 28 nM (Fig. 4). In contrast, at concentrations up to 300 μM ICI 174864 was able to displace only approximately 40% of the specific binding of [^{3}H]diprenorphine from rat cortical membranes. This displacement was produced with high affinity (IC_{50} corrected for receptor occupancy = 20 nM) (Fig. 4), indicating that this fraction of the specific [^{3}H]diprenorphine binding was to δ receptor sites. Such data indicated that the μ receptor binding site could be examined in experiments that contained 1 μM ICI 174864 to

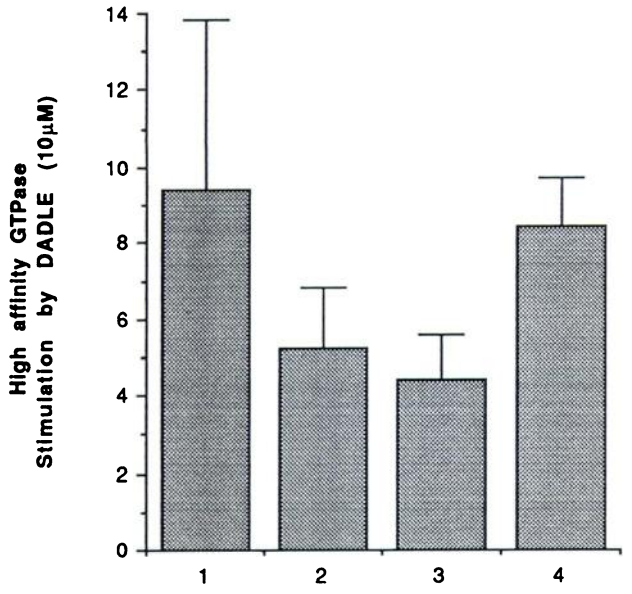

Fig. 3. Antibodies against both the carboxyl-terminal and amino-terminal regions of $G_{o\alpha}$ reduce the ability of DADLE to stimulate high affinity GTPase activity in rat brain cortex membranes. Membranes (10 μg) from rat cortex were incubated with 20 μg of nonimmune serum (*1*), antiserum OC2 (*2*), antiserum ON1 (*3*), or antiserum SG1 (*4*), as described in Experimental Procedures. Basal and DADLE (10 μM)-stimulated high affinity GTPase activities were then measured. Data are presented as DADLE-stimulated high affinity GTPase activity (pmol/min/mg of membrane protein) (mean ± standard error, four experiments) above basal activity. Basal activities in these experiments varied between 26.7 and 33.5 pmol/min/mg of membrane protein.

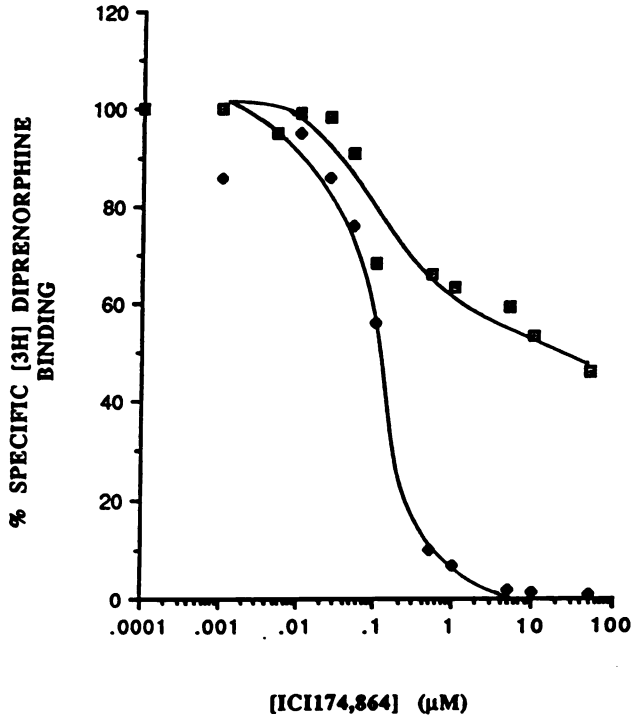

Fig. 4. Competition of ICI 174864 for specific [^{3}H]diprenorphine binding. Membranes (70 μg) from either rat cortex (□) or neuroblastoma × glioma hybrid NG108–15 cells (♦) were incubated with [^{3}H]diprenorphine (2.5 nM) and increasing concentrations of the δ receptor site-selective antagonist ICI 174864. Nonspecific binding was measured in the presence of 10 μM naloxone. In this experiment 100% [^{3}H]diprenorphine specific binding was 85 ± 11 fmol/mg of protein in NG108–15 cell membranes and 168 ± 20 fmol/mg of protein for rat cortex. The IC_{50} (corrected for receptor occupancy) for ICI 174864 was 28 nM for NG108–15 membranes and 20 nM for rat cortex.

block δ receptor binding of both [^{3}H]diprenorphine and DADLE. When the IgG fraction from antiserum OC2 was preincubated with rat cortex membranes and binding analyses were performed under conditions designed to detect only μ receptor-G protein interactions, the antiserum was able to mimic partially the effect of Gpp(NH)p and thus demonstrated a direct interaction of the μ-opioid receptor and G_o in these membranes (Fig. 5).

Discussion

Assessments of the specificity of interactions between receptors and G proteins have been performed using a variety of methods. Perhaps the most common approach has been to purify or partially purify the receptor and then reconstitute this with purified G proteins, generally in phospholipid vesicles (5, 20–22). Although valuable information has been gained by this approach, it is clear that such data can be most useful as a measure of potential interactions between a receptor and a G protein, rather than necessarily reflecting the *in situ* situation. A second, related, approach has been to reconstitute purified G protein fractions back into membranes in which the endogenous G proteins of interest have been inactivated or neutralized, for example by prior treatment of cells or membranes with pertussis toxin (23) or with low concentrations of *N*-ethylmaleimide (6). Such experiments have the distinct advantage of maintaining the lipid enviroment of the receptor, and if the added amounts of G protein are similar to endogenously expressed levels then clear information on functional contacts can be obtained. A third approach, which is currently largely restricted to analysis at the electrophysiological level, is intracellular injection of antisense oligonucleotides that bind to and cause the elimination of relevant mRNA (24). The other widely used approach to assess the specificity of receptor-G protein interactions has been to use anti-G protein antisera that are able to interfere with receptor-G protein interactions. This approach has been used to examine interactions of both β-adrenoceptors (25) and epidermal growth factor receptors (26) with G_s, both δ-opioid (2, 3) and α_2-adrenoceptors (27) with G_{i2} and G_o, a range of receptors including those for thromboxane A_2 (28) and bradykinin (29) with the G_q/G_{11} family of G proteins, and the α_{2A}-adrenoceptor with both G_{i2} and G_{i3} (14, 30). This antibody-based approach has not been reported previously for interactions between receptors and G proteins in brain. In these experiments we have analyzed the interaction of rat brain cortex opioid receptors with G_o.

Two prerequisites for an anti-G protein antiserum to be useful in this approach are that it is able to recognize the G protein *in situ* and it binds to a region of the G protein involved in G protein-receptor recognition or otherwise perturbs interaction of receptors with the G protein to an extent that can be easily measured using either biochemical or pharmacological assays. The carboxyl-terminal region of G protein α subunits has been implicated in G protein-receptor interactions both from analysis of the site of attachment of ADP-ribose to certain G protein α subunits by using pertussis toxin (10) and from analysis of the mutation in $G_{s\alpha}$ responsible for the *unc* phenotype in murine S49 lymphoma cells (9). Although a detailed

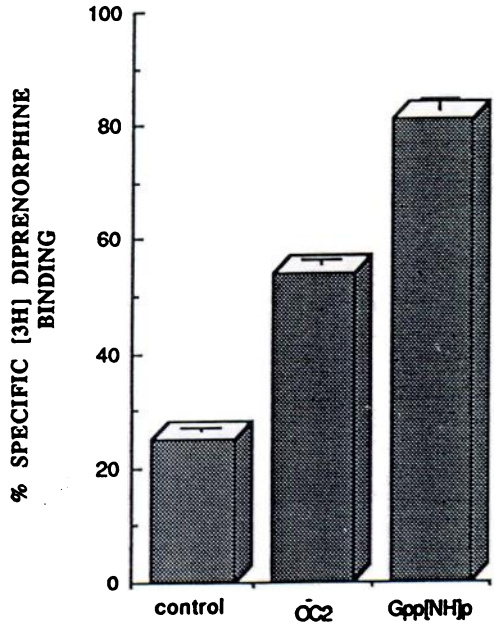

Fig. 5. μ-Opioid receptors in rat cortex interact directly with G_o. Rat cortical membranes (70 μg) were incubated with IgG fractions (15 μg) from either antiserum OC2 or normal rabbit serum (control) or with Gpp(NH)p (100 μM). Specific [^{3}H]diprenorphine binding was measured in the presence of 10 μM DADLE, to define binding to only μ and δ receptors, plus 1 μM ICI 174864, to eliminate binding of the radiolabel to δ-opioid receptors. The increase in specific binding of [^{3}H]diprenorphine (2.6 nM) after uncoupling of μ-opioid receptors from G_o was assessed in the presence of 100 nM DADLE. Similar results were produced in three independent experiments.

analysis of the role of the amino-terminal domain of G proteins in G protein-receptor interactions is lacking, it is clear that this region plays a key role in the interaction of α and $\beta\gamma$ subunits (31) and also that $\beta\gamma$ subunits are important for high affinity interactions between receptors and G proteins (32). It was thus anticipated that antisera directed against these regions would provide useful probes for receptor-G protein interactions.

Antisera directed against both the carboxyl-terminal decapeptide (OC2) and the amino-terminal hexadecapeptide (ON1) of forms of $G_{o\alpha}$ effectively immunoprecipitated a 39-kDa polypeptide that was identified by a highly specific $G_{o\alpha}$ antiserum (IM1) (Fig. 1) that is directed against amino acids 22–35 of forms of $G_{o\alpha}$. Antiserum IM1 also efficiently immunoprecipitated $G_{o\alpha}$ from rat cortical membranes, as assessed by the detection of a 39-kDa polypeptide in such immunoprecipitates by antiserum OC2 (data not shown). Such results clearly demonstrated the identification and binding of these antisera to $G_{o\alpha}$. In contrast, when we performed similar immunoprecipitation studies with an antiserum (SG1) that is directed against the carboxyl-terminal decapeptide, which is common to the α subunits of G_{i1} and G_{i2}, and that identifies both of these polypeptides in immunoblotting protocols (18) we noted that, although immunoprecipitation of these polypeptides could be seen, the fraction of the G_i forms in the rat cortical membranes that were immunoprecipitated was very small (data not shown). Thus, although we noted no reduction in the affinity of binding of DADLE to opioid receptors in these membranes after preincubation with an IgG fraction from antiserum SG1 (Table 2), this may simply be a reflection of the immunoneutralization of a very small proportion of the available G_i, rather than providing evidence for the lack of interaction of opioid receptors with G_i in rat cortical membranes. Studies of this nature that are negative can thus only be analyzed usefully after detailed consideration of potential limitations of the experimental protocol. Similar arguments relate to attempts within these studies to use an IgG fraction from antiserum O1B (Table 1). This antiserum, which is specific on immunoblots for forms of the $G_{o1\alpha}$ splice variant and does not identify $G_{o2\alpha}$ (Table 1),[1] was unable to modify the affinity of DADLE displacement of [^{3}H] diprenorphine binding. However, like antiserum SG1, it was able to immunoprecipitate only a minor fraction of $G_{o1\alpha}$ immunoreactivity from rat cortical membranes (data not shown).

Analysis of saturation curves of specific [^{3}H]diprenorphine binding to rat cortical membranes, defined by elimination in the presence of 10 μM naloxone, produced data consistent with the presence of approximately 220 fmol of receptor/mg of membrane protein. Because diprenorphine binds to μ, δ, and κ receptors, this total represents a composite of these subtypes. The enkephalin analogue DADLE interacts only weakly with κ receptors (33), and thus displacement of [^{3}H]diprenorphine with DADLE allows examination of G protein interactions with μ and δ sites. Addition of the poorly hydrolyzed GTP analogue Gpp(NH)p to binding assays substantially reduced the affinity of DADLE to displace specific [^{3}H]diprenorphine, indicative of interaction between the receptor and a G protein. Preincubation of rat cortical membranes with an IgG fraction from antiserum OC2 also reduced the affinity of DADLE displacement of [^{3}H]diprenorphine binding (Fig. 2A). However, at all concentrations of OC2 IgG that we were able to use in the assay, the effect of the antiserum was less than that produced by Gpp(NH)p (Table 2). This either might reflect the inability to immunoneutralize sufficient amounts of the membrane $G_{o\alpha}$ or, more likely, indicates that the opioid receptors are not coupled exclusively to $G_{o\alpha}$.

The IgG fraction from antiserum ON1 was also able to reduce the effectiveness of DADLE displacement of specific [^{3}H]diprenorphine binding (Fig. 2B). This effect was routinely weaker than that produced by antiserum OC2, although the difference between the antisera was not statistically significant (Table 2). These data provide additional evidence for the direct interaction of cortical opioid receptors with G_o and indicate further a role for the amino terminus of G proteins in the production of a form of the receptor-G protein complex that displays high affinity to bind agonists.

In contrast to antisera OC2 and ON1, antiserum IM1 was unable to produce a reduction in affinity of DADLE for the opioid receptors. Although at first sight such data appear contradictory to those obtained with antisera OC2 and ON1, the data are likely to indicate that binding of antiserum IM1 to G_o does not interfere with receptor-G protein coupling.

To further examine the interaction of rat brain opioid receptors with G_o, we measured the ability of antisera OC2 and ON1 to attenuate DADLE-stimulated high affinity GTPase. Preincubation of the membranes at 37° with or without the antisera led to a reduction of both basal and receptor-stimulated high affinity GTPase, as we have recorded previously for other membrane systems (34). However, after such preincubations in

[1] J. F. MacCallum, I. Mullaney, and G. Milligan, Increases in steady state levels of G_o associated with cyclic AMP-induced differentiation of neuroblastoma × glioma hybrid cells are restricted to a $G_{o1\alpha}$ splice variant, manuscript in preparation.

the presence of nonimmune serum, a small (approximately 30%) but relatively robust stimulation of the basal high affinity GTPase could be recorded in the presence of DADLE. If the preincubations were performed with either antisera OC2 or ON1, subsequent stimulation of the high affinity GTPase activity by DADLE was reduced by approximately 50%. Such data provide further evidence for the direct interaction of rat brain opioid receptors with G_o. However, due to the relatively small percentage of stimulation by DADLE over a series of experiments, the inhibitory effects of the two anti-G_o antisera failed to achieve statistical significance. As noted with the receptor binding assays, the anti-$G_{i1\alpha}$ and -$G_{i2\alpha}$ antiserum SG1 had no ability to reduce DADLE stimulation of high affinity GTPase activity.

Definition of the μ and δ receptor populations of rat cortical membranes was based on the selectivity of the δ receptor antagonist ICI 174864 (11). Displacement of [^{3}H]diprenorphine binding from membranes of NG108-15 neuroblastoma × glioma hybrid cells, which express a pure population of δ receptor sites (35), with ICI 174864 produced a monotonic isotherm with an IC_{50} (corrected for receptor occupancy) of 28 nM. Displacement of [^{3}H]diprenorphine binding from rat cortical membranes by ICI 174684 was clearly biphasic, with approximately 40% of the binding being displaced with an IC_{50} (corrected for receptor occupancy) of 20 nM and the remainder not being displaced at concentrations up to 300 μM (Fig. 4). These data indicated that the δ receptor population of these membranes comprised approximately 40% of the total opioid receptor sites. More importantly, this provided a pharmacological tool to allow specific assessment of μ receptor interactions with G_o (8, 36). Thus, we performed [^{3}H]diprenorphine binding studies in the presence of 1 μM ICI 174864, to block δ receptor sites, and 100 nM DADLE, a concentration that in the absence of added guanine nucleotides was sufficient to displace approximately 70% of the remaining specific [^{3}H]diprenorphine binding. Addition of Gpp(NH)p, by reducing the affinity of DADLE for the μ receptor, produced an increase in measured [^{3}H]diprenorphine binding. Similarly, preincubation of cortical membranes with the IgG fraction of antiserum OC2 under these pharmacologically defined conditions also produced an increase in measured [^{3}H]diprenorphine binding (Fig. 5). As with the full displacement curves (Fig. 2), the effect of antiserum OC2 was only approximately 50% of that produced by Gpp(NH)p, confirming the previous results and providing clear evidence for the *in situ* interaction of the μ-opioid receptor with G_o.

These experiments provide the first evidence for the direct *in situ* interactions of cortical opioid receptors with G_o and provide evidence for key roles for both the carboxyl-terminal and amino-terminal regions of this G protein in defining high affinity agonist binding interactions with opioid receptors. Such approaches should be amenable for use with other G protein-linked receptors in brain.

References

1. Childers, S. R. Opioid receptor-coupled second messengers systems. *Life Sci.* **48**:1991-2003 (1991).
2. McKenzie, F. R., and G. Milligan. δ-Opioid receptor mediated inhibition of adenylate cyclase is transduced specifically by the guanine nucleotide binding protein G_{i2}. *Biochem. J.* **267**:391-398 (1990).
3. Offermanns, S., G. Schultz, and W. Rosenthal. Evidence for opioid receptor mediated activation of the G proteins, G_o and G_{i2}, in membranes of neuroblastoma × glioma (NG108-15) cells. *J. Biol. Chem.* **266**:3365-3368 (1991).
4. Roerig, S. C., H. H. Loh, and P. Y. Law. Identification of three separate guanine nucleotide binding proteins that interact with the δ-opioid receptor in NG108-15 cells. *Mol. Pharmacol.* **41**:822-831 (1992).
5. Ueda, H., H. Harada, M. Noraki, T. Katada, M. Ui, M. Satoh, and H. Takagi. Reconstitution of rat brain μ-opioid receptors with purified guanine nucleotide binding regulatory proteins G_i and G_o. *Proc. Natl. Acad. Sci. USA* **85**:7013-7017 (1988).
6. Ueda, H., H. Misawa, T. Katada, M. Ui, H. Takagi, and M. Satoh. Functional reconstitution of purified G_i and G_o with μ-opioid receptors in guinea pig striatal membranes pretreated with micromolar concentrations of *N*-ethylmaleimide. *J. Neurochem.* **54**:841-848 (1990).
7. Ueda, H., Y. Yoshihara, H. Misawa, N. Fukushima, M. Ui, H. Takagi, and M. Satoh. The kyotorphin (tyrosine-arginine) receptor and a selective reconstitution with purified G_i, measured with GTPase and phospholipase C assays. *J. Biol. Chem.* **264**:3732-3741 (1989).
8. McClue, S., and G. Milligan. The α_{2B} adrenergic receptor of undifferentiated neuroblastoma × glioma hybrid NG108-15 cells interacts directly with the guanine nucleotide binding protein G_{i2}. *FEBS Lett.* **269**:430-434 (1990).
9. Sullivan, K. A., R. T. Miller, S. B. Masters, B. Beiderman, W. Heideman, and H. R. Bourne. Identification of a receptor contact site involved in receptor-G protein coupling. *Nature (Lond.)* **330**:758-759 (1987).
10. Milligan, G. Techniques used in the identification and analysis of function of pertussis toxin-sensitive guanine nucleotide binding proteins. *Biochem. J.* **255**:1-13 (1988).
11. Cotton, R., M. B. Giles, L. Miller, J. S. Shaw, and D. T. Timms. ICI 174864: a highly selective antagonist for the opioid *delta* receptor. *Eur. J. Pharmacol.* **97**:331-332 (1984).
12. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**:265-275 (1951).
13. Goldsmith, P., P. Gierschik, G. Milligan, C. G. Unson, R. Vinitsky, H. L. Malech, and A. M. Spiegel. Antibodies directed against synthetic peptides distinguish between GTP-binding proteins in neutrophil and brain. *J. Biol. Chem.* **262**:14683-14688 (1987).
14. McClue, S. J., and G. Milligan. Molecular interaction of the human α2-C10-adrenergic receptor, when expressed in Rat-1 fibroblasts, with multiple pertussis toxin-sensitive guanine nucleotide-binding proteins: studies with site-directed antisera. *Mol. Pharmacol.* **40**:627-632 (1991).
15. Cheng, Y. C., and W. H. Prusoff. Relationship between the inhibition constant (K_i) and the concentration of inhibitor which causes 50 per cent inhibition (I_{50}) of an enzyme reaction. *Biochem. Pharmacol.* **22**:3099-3108 (1973).
16. Rothenberg, P. L., and C. R. Kahn. Insulin inhibits pertussis toxin-catalysed ADP-ribosylation of G proteins: evidence for a novel interaction between insulin receptors and G proteins. *J. Biol. Chem.* **263**:15546-15552 (1988).
17. Mullaney, I., and G. Milligan. Identification of two distinct isoforms of the guanine nucleotide binding protein G_o in neuroblastoma × glioma hybrid cells: independent regulation during cyclic AMP-induced differentiation. *J. Neurochem.* **55**:1890-1898.
18. Mitchell, F. M., S. L. Griffiths, E. D. Saggerson, M. D. Houslay, J. T. Knowler, and G. Milligan. Guanine-nucleotide-binding proteins expressed in rat white adipose tissue: identification of both mRNAs and proteins corresponding to G_{i1}, G_{i2} and G_{i3}. *Biochem. J.* **262**:403-408 (1989).
19. Milligan, G., R. A. Streaty, P. Gierschik, A. M. Spiegel, and W. A. Klee. Development of opiate receptors and GTP-binding regulatory proteins in neonatal rat brain. *J. Biol. Chem.* **262**:8626-8630 (1987).
20. Kim, M. H., and R. R. Neubig. Membrane reconstitution of high affinity α_2-adrenergic agonist binding with guanine nucleotide regulatory proteins. *Biochemistry* **26**:3664-3672 (1987).
21. Cerione, R. A., J. W. Regan, H. Nakata, J. Codina, J. L. Benovic, P. Gierschik, R. L. Somers, A. M. Spiegel, L. Birnbaumer, R. J. Lefkowitz, and M. G. Caron. Functional reconstitution of the α_2-adrenergic receptor with guanine nucleotide regulatory proteins in phospholipid vesicles. *J. Biol. Chem.* **261**:3901-3909 (1986).
22. Bernstein, G., J. L. Blank, A. V. Smrcka, T. Higashijima, P. C. Sternweis, J. Exton, and E. Ross. Reconstitution of agonist stimulated phosphatidylinositol 4,5-bisphosphate hydrolysis using purified M1-muscarinic receptor, $G_{q/11}$, and phospholipase C-β1. *J. Biol. Chem.* **267**:8081-8088 (1992).
23. Milligan, G., and W. A. Klee. The inhibitory guanine nucleotide-binding protein (N_i) purified from bovine brain is a high affinity GTPase. *J. Biol. Chem.* **260**:2057-2063 (1985).
24. Kleuss, C., J. Hescheler, C. Ewel, W. Rosenthal, G. Schultz, and B. Wittig. Assignment of G protein subtypes to specific receptors inducing inhibition of calcium currents. *Nature (Lond.)* **353**:43-48 (1991).
25. Simonds, W. F., P. K. Goldsmith, C. J. Woodward, C. G. Unson, and A. M. Spiegel. Receptor and effector interactions of G_o: functional studies with antibodies to the carboxyl-terminal decapeptide. *FEBS Lett.* **249**:189-194 (1989).
26. Nair, B. G., B. Parikh, G. Milligan, and T. B. Patel. $G_{o\alpha}$ mediates epidermal growth factor elicited stimulation of rat cardiac adenylate cyclase. *J. Biol. Chem.* **265**:21317-21322 (1990).
27. Simonds, W. F., P. K. Goldsmith, J. Codina, C. G. Unson, and A. M. Spiegel. G_{i2} mediates α_2-adrenergic inhibition of adenylate cyclase in platelets membranes: *in situ* identification with G_o C-terminal antibodies. *Proc. Natl. Acad. Sci. USA* **86**:7809-7813 (1989).
28. Shenker, A., P. Goldsmith, C. G. Unson, and A. M. Spiegel. The G protein

coupled to the thromboxane A_2 receptor in human platelets is a member of the novel G_q family. *J. Biol. Chem.* **266**:9309–9313 (1991).
29. Gutowski, S., A. Smrcka, L. Nowak, D. Wu, M. Simon, and P. Sternweis. Antibodies to the αq subfamily of guanine nucleotide-binding regulatory protein α subunits attenuate activation of phosphatidylinositol 4,5-bisphosphate hydrolysis by hormones. *J. Biol. Chem.* **266**:20519–20524 (1991).
30. McClue, S. J., E. Selzer, M. Freissmuth, and G. Milligan. G_{i3} does not contribute to the inhibition of adenylate cyclase when an α_2- adrenergic receptor causes activation of both G_{i2} and G_{i3}. *Biochem. J.* **284**:565–568 (1992).
31. Schmidt, C. J., and E. J. Neer. *In vitro* synthesis of G protein βγ dimers. *J. Biol. Chem.* **266**:4538–4544 (1991).
32. Im, M. J., A. Holzhofer, H. Bottinger, T. Pfeuffer, and E. J. M. Heilmreich. Interactions of pure βγ subunits of G proteins with purified β_1-adrenoreceptor. *FEBS Lett.* **227**:225–229 (1988).
33. Zukin, R. S., M. Eghbali, D. Olive, E. M. Unterhalt, and A. Tempel. Characterization and visualization of rat and guinea pig κ opioid receptors: evidence for κ1 and κ2 opioid receptors. *Proc. Natl. Acad. Sci. USA* **85**:4061–4065 (1988).
34. McKenzie, F. R., E. C. H. Kelly, C. G. Unson, A. M. Spiegel, and G. Milligan. Antibodies which recognize the C-terminus of the inhibitory guanine-nucleotide-binding protein (G_i) demonstrate that opioid peptides and fetal calf serum stimulate the high affinity GTPase activity of two separate pertussis toxin substrates. *Biochem. J.* **249**:653–659 (1988).
35. Law, P. Y., A. K. Louie, and H. H. Loh. Effects of pertussis toxin treatment on the down-regulation of opiate receptors in neuroblastoma × glioma NG108–15 hybrid cells. *J. Biol. Chem.* **260**:14818–14823 (1985).
36. Milligan, G., F. R. McKenzie, S. J. McClue, F. M. Mitchell, and I. Mullaney. Guanine nucleotide binding proteins in neuroblastoma × glioma hybrid, NG108–15 cells: regulation of expession and function. *Int. J. Biochem.* **22**:701–707 (1990).

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