STUDIES OF THE MU-OPIOID RECEPTOR/G-PROTEIN COMPLEX AFFINITY CO-PURIFIED AND MEMBRANE PREPARATIONS FROM 7315c CELLS

1989

GOSSE

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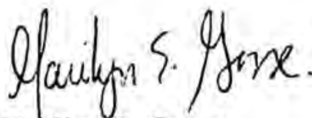

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ABSTRACT

Title of Dissertation: Studies of the Mu-Opioid Receptor/G-Protein Complex in Affinity Co-Purified and Membrane Preparations from 7315c Cells.

Marilyn E. Gosse — Doctor of Philosophy, 1989

Dissertation directed by: Thomas E. Cote, Assistant Professor, Department of Pharmacology

The 7315c cell is a prolactin secreting tumor that expresses a homogeneous population of mu-opioid receptors. Morphine, an opioid agonist, inhibits prolactin secretion, in part, by inhibiting adenylyl cyclase activity via activation of the GTP-binding protein, Gi. Using membranes prepared from the 7315c cell, the influence of the mu-opioid receptor on the affinity of the GTP-binding protein for guanine nucleotides was studied in an adenylyl cyclase system. Morphine was shown to decrease the affinity of Gi for GDP, the inactive guanine nucleotide, but to have no effect on the affinity of Gi for GTP, the active guanine nucleotide. When the receptor was functionally uncoupled from Gi by pertussis toxin, Gi had an enhanced affinity for GDP. These results suggest that the unoccupied receptor exerts a tonic, inhibitory influence on the affinity of Gi for GDP; activation of the mu-opioid receptor by an agonist decreases further the affinity of Gi for GDP, thereby ensuring that GTP, but not GDP, will interact with Gi.

Further studies of the receptor protein interaction with its G-protein included co-purification of these two proteins from the 7315c cell membrane and identification of the receptor-coupled GTP-binding protein. The mu-opioid receptor was affinity purified 2919 ± 128-fold

with a recovery of 5.6 ± 0.9% on an opioid agonist affinity resin. The affinities of the purified and membrane-associated mu-opioid receptors for [^{3}H]naloxone, an opioid receptor antagonist, were similar. By cross-linking ^{125}I-β-endorphin to the mu-opioid receptor, the molecular weight of both membrane-associated and affinity purified mu-opioid receptor was determined to be 64 kD. The ratio of recovered co-purified receptor to G-protein was approximately 3 to 1. The receptor purified G-protein was found to be a 40 to 41 kD pertussis toxin substrate. The receptor/G-protein complex was calculated to represent approximately 10% of the protein recovered from the affinity chromatography. Western blot analysis of the mu-opioid receptor purified G-proteins has identified these G-proteins to be G_{i1} and G_o. These studies have demonstrated for the first time that the mu-opioid receptor interacts with these GTP-binding proteins and sheds light on the process of opioid mediated signal transduction.

STUDIES OF THE INTERACTION OF THE MU-OPIOID RECEPTOR/G-PROTEIN COMPLEX IN AFFINITY CO-PURIFIED AND MEMBRANE PREPARATIONS FROM 7315c CELLS

by

Marilyn E. Gosse

Dissertation submitted to the Faculty of the Department of Pharmacology
Graduate Program of the Uniformed Services of the
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DEDICATION

To My Loving Husband, Fabian.

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Abbreviations and Definitions

AppNHp, 5'Adenylylimidodiphosphate; Bmax, the receptor number as determined by Scatchard Analysis of saturation binding data; Brown's cAMP Binding Protein, a Bovine adrenal extract that contains cAMP-dependent Protein Kinase; Buffers, see below; BSA, Bovine Serum Albumin; cDNA, complementary DNA; CHAPS, 3-[3-chloramidopropyl)dimethyl-ammonio]-1-propanesulfonate (cmc, 4-7 mM); cmc, critical micelle concentration; <u>cyc</u>$^-$ mutants, mutant S49 lymphoma cell that lacks the stimulatory GTP-binding protein; Dithiothreitol, a reducing agent used to prevent the formation of disulfide bonds; DSS, disuccinimidyl suberate; EBSS/BSA, Earles Balanced Salt Solution containing 0.25% Bovine Serum Albumin; EC_{50}, the concentration of the drug mediating 50% of the total response; EDC, 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide; G proteins, GTP-binding regulatory proteins: G/F, the stimulatory GTP-binding protein also called G_s; G_s, G protein that mediates stimulation of adenylyl cyclase activity; G_i, G protein that mediates inhibition of adenylyl cyclase activity; $G_{\alpha s}$, the subunit of G_s that binds and hydrolyzes GTP; $G_{\alpha i}$, the subunit of G_i that binds and hydrolyzes GTP; $G_{\beta\gamma}$, subunits common to both G_s and G_i; GDPβS, guanosine-5'-O-(2-thiodiphosphate); Gpp(CH_2)p, guanylyl(β, γ-methylene)diphosphate; GppNHp, 5'-guanylylimidodiphosphate; GTPγS, guanosine-5'-O-(3-thiotriphosphate); GTPase activity, the hydrolysis of GTP to GDP; HEPES, (4-[2-hydroxymethyl]-1-piperazine-ethanesulfonic acid; IAP, Islet-Activating Protein a toxin known to ADP-ribosylate the α-subunit of G_i (also known as pertussis toxin); IC_{50}, the concentration of the drug mediating 50% inhibition of the response, $IC_{50} = Ki(1 + S/Km)$ where Ki is the

affinity, S is the concentration of the drug, and Km is the concentration of of half-maximal inhibition by the competing drug; Kd, the affinity binding constant; PGE_1, prostaglandin E_1; PLA_2, the enzyme mediating the hydrolysis of phospholipids to release arachodonic acid and phophatidic acid, Polygram 300, polyethyleneimine chromatography plates; Protease inhibitors, see below; PKA, cAMP-dependent protein kinase; NG108-15, a neuroblastoma x glioma cell line; 7315c, a lactotroph tumor cell that has mu-opioid receptor-mediated inhibition of both prolactin secretion and adenylyl cyclase activity; TRIS, Tris(HydroxylMethyl)AminoMethane; UNC, a mutant S49 lymphoma cell that has a mutation on G_s that prevents G_s from interacting with the β-adrenergic receptor.

BUFFERS

SDS-PAGE sample buffer	4% SDS, 0.05 M Tris-HCl (pH 6.8), and 50 µl β-mercaptoethanol, final volume 0.5 ml
Homogenizing Buffer	2 mM TRIS-HCl (pH 7.4), 1 mM $MgSO_4$, 2 mM EGTA, 250 mM Sucrose
Binding Buffer	25 mM K^+ HEPES (pH 7.4), 1 mM $MgSO_4$, 2 mM EGTA
Freezing Buffer	6 mM TRIS-HCl (pH 7.4), 1 mM $MgSO_4$, 4 mM EGTA, 10% Glycerol
Buffer A	50 mM K^+ HEPES (pH 7.4), 1 mM $MgSO_4$, 2 mM EGTA, 30 mM NaCl,0.1 mM Phenyl Methyl Sulfonyl Flouride (PMSF), 1 µM leupeptin, and 1 mM dithiothreitol
Buffer B	50 mM K^+ HEPES (pH 7.4), 1 mM $MgSO_4$, 2 mM EGTA, 100 mM NaCl

Buffer C	50 mM K^+ HEPES (pH 7.4), 2 mM EDTA, 5 mM $MgSO_4$, 100 mM NaCl, 0.1 mM PMSF, 1 µM leupeptin, 1 mM dithiothreitol, 100 µM 1,10-O-phenanthroline, and 20 µM bestatin
Buffer D	50 mM K^+ HEPES (pH 7.4), 2 mM EDTA, 5 mM $MgSO_4$, 100 mM NaCl, 0.1 mM PMSF, 1 µM leupeptin, 1 mM dithiothreitol, 100 µM 1,10-O-phenanthroline, and 20 µM bestatin, and 1 mM CHAPS
Buffer E	50 mM K^+ HEPES (pH 7.4), 2 mM EDTA, 5 mM $MgSO_4$, 100 mM NaCl
Buffer F	50 mM Na^+HEPES, pH 7.4, 2 mM EDTA, 5 mM $MgSO_4$
Buffer G	50 mM K^+ HEPES (pH 7.4), 2 mM EDTA, 50 mM $MgSO_4$, 100 mM NaCl, 0.1% Lubrol PX, 0.1 mM PMSF, 1 µM leupeptin, and 1 mM dithiothreitol
Buffer H	50 mM Tris-HCl (pH 8.0), 1 mM EDTA, 6 mM $MgSO_4$, 0.1% Lubrol PX, 0.1 mM PMSF, 1 µM leupeptin, and 1 mM dithiothreitol

Protease Inhibitors

Bestatin	amino terminal peptidase inhibitor
Leupeptin	thiol protease inhibitor
Phenyl Methyl Sulfonyl Fluoride	(PMSF) serine protease inhibitor
1,10-O-Phenanthroline	metal chelator
Ethylenediamine-tetraacetic acid	(EDTA) metal chelator

Antisera to the α-Subunits of GTP-binding Proteins

Code	α-subunit selectivity
LD	α_{i2}
AS	α_{i1}/α_{i2}
SQ	α_{i3}, carboxy terminal
EC	α_{i3}, internal sequence
GC	α_o
RM	α_s
MS	β

Introduction

In 1958 Sutherland and Rall first described the epinephrine-stimulated and NaF-stimulated formation of a heat stable factor, 3',5'-cyclic adenosine monophosphate (cAMP), in cardiac and skeletal muscle tissues. Not long after, they named the plasma membrane enzyme catalyzing the conversion of ATP to cAMP, adenylyl cyclase. Once formed, cAMP acts as the intracellular second messenger promoting its signal through a protein phosphorylation cascade. It is now known that there are three plasma membrane components that comprise this second messenger generating system: 1) a membrane bound receptor that is exposed to the extracellular environment and available for physiologic noncovalent interaction with a ligand molecule, 2) a guanosine triphosphate (GTP) binding protein, and 3) the enzyme adenylyl cyclase.

Early models of the adenylyl cyclase membrane system assumed a receptor-catalytic unit to stimulate the production of cAMP (Robison et al., 1967), but it was shown quite quickly that the receptor is a separate plasma membrane protein from the adenylyl cyclase (catalytic unit). Evidence for this statement came from both the binding data and the fusion studies in the β-adrenergic receptor system. The binding of [^{3}H]antagonists to the β-adrenergic receptor was shown to be saturable and competitively inhibited by agonists (Mukherjee et al. 1975; Levitski et al. 1974), however this information did not define the receptor as a separate entity from the adenylyl cyclase catalytic subunit. Fusion studies provided definitive answers. Cell membranes from Friend erythroleukemic cells that have adenylyl cyclase activity that is insensitive to β-adrenergic agonists were shown to be fused with the

membranes from turkey erythrocytes that have β-adrenergic receptors but inactive adenylyl cyclase activity due to treatment with N-methyl maleimide. The resulting hybrid cell had a β-adrenergic receptor functionally linked to adenylyl cyclase (Orly and Schramm, 1976). Thus the β-adrenergic receptor could be transferred to and activate the adenylyl cyclase catalytic units from different membrane preparations.

The first evidence for the involvement of guanine nucleotides in the adenylyl cyclase system was put forth by Rodbell and his colleagues in 1971. They demonstrated in liver membranes that GTP was necessary for the activation of adenylyl cyclase (Rodbell et al., 1971b), and also that GTP and a nonhydrolyzable analog of GTP, Gpp(CH_2)p, could diminish the binding affinity of ^{125}I-glucagon from a high affinity state to one of lower affinity (Rodbell et al., 1971a). GDP was shown to be unable to sustain stimulation of adenylyl cyclase activity (Salomon et al., 1975). Using a similar experimental design, guanine nucleotide analogs were tested for their effects on agonist and antagonist binding as well the GTP requirement for the production of cAMP in other receptor systems (Rodbell et al., 1975).

The role of guanine nucleotide binding proteins in the formation of cAMP was studied by measuring the activation of isoproterenol-stimulated adenylyl cyclase activity by metabolically stable analogs of GTP in pigeon erythrocyctes (Pfeuffer and Helmreich, 1975). The analogs were more effective at enhancing agonist stimulation of cyclase than was GTP. Moreover, these analogs could stimulate cyclase in the absence of GTP. The order of analog effectiveness was GTPγS > Gpp(NH)p >> GTP. To compare this functional data with the binding properties of the analogs, [^{3}H]Gpp(CH_2)p and [^{14}C]Gpp(NH)p were incubated with the pigeon

erythrocyte membranes, and the kinetic parameters of the binding were determined. The affinity of [^{3}H]Gpp(CH_2)p for its binding sites compared well to its affinity in activating cyclase. Using the ligand [^{14}C]Gpp(NH)p and GTP-Sepharose chromatography, it was possible to separate a GTP-binding protein from the fraction forming cAMP. This was the first suggestion that there was a distinct GTP-binding regulatory protein separate from the receptor and the catalytic unit. Further work by Pfeuffer in 1977 reinforced this concept. The experiment was designed as follows: pigeon erythrocyte membranes were incubated with a photoreactive [^{32}P]GTP derivative and then solubilized with the detergent, lubrol-PX. The solubilized proteins covalently labeled with GTP were analyzed by gel electrophoresis. Two GTP-binding proteins having molecular weights of 42 and 23 kdal were detected. In addition, using GTP-Sepharose beads, these proteins that bind GTP were separated from adenylyl cyclase. Adenylyl cyclase activity could be measured only when the GTP-binding protein was recombined with the eluant (proteins that did not bind the to the GTP-bead). These studies provided the evidence that there is a separate GTP-binding protein from the receptor and catalytic subunits (Pfeuffer and Helmreich, 1975; Pfeuffer, 1977).

Although the GTP-binding protein was identified, its function in the generation of cAMP remained unclear. In 1976, Cassel and Selinger first reported a specific GTPase activity (the hydrolysis of GTP to GDP) in turkey erythrocyte membranes which was stimulated by isoproterenol. Furthermore, Cassel and Selinger in 1978 reported that isoproterenol enhanced the [^{3}H]GDP release from turkey erythrocyte membranes that had been incubated with [^{3}H]GTP. Therefore, it was hypothesized that the stimulatory GTP-binding protein was responsible for the hydrolysis of

the GTP to GDP. These data also explained guanine nucleotide activation of cyclase activity seen above and suggested that the resident guanine nucleotide bound to the G-protein in its inactive state was GDP.

Prior to the understanding of the role of guanine nucleotides in the adenylyl cyclase system, cholera toxin was known to increase the intracellular concentration of cAMP (Kimberg et al., 1971). Additionally, treatment of cells with cholera toxin abolished both the GTP-dependence for stimulation of the production of cAMP and GTPase activity (Cassel and Selinger, 1977). In 1978, Cassel and Pfeuffer showed that the modification of the adenylyl cyclase system by cholera toxin was a covalent ADP-ribosylation of a membrane-bound protein. They used [^{32}P]Nicotinamide Adenine Dinucleotide (NAD) as the donor ADP-ribose to demonstrate a 42 kdal protein incorporated the ^{32}P. Since it was known that the stimulatory G-protein had GTP hydrolyzing activity and was susceptible to covalent modification by cholera toxin in the presence of [^{32}P]NAD, the sedimentation pattern of the [^{32}P]labeled GTP-binding protein in the presence or absence of GTPγS was followed (Pfeuffer, 1979). The sedimentation pattern of the GTPγS bound form indicated a lower molecular weight, and it co-sedimented with the catalytic unit; this fraction contained cyclase activity. The GDP bound form sedimented higher on the gradient and no cyclase activity was detectable in this fraction. These findings suggested that there is more than one component of this protein and that the active form was the GTPγS bound form.

A model for the agonist activation of adenylyl cyclase via a GTP-binding protein was proposed: a GTP-binding protein (G protein) has GDP bound until a ligand interacts with the receptor/G-protein complex. The

agonist then enhances the replacement of the GDP by GTP at the nucleotide binding site. The now active G protein can activate the catalytic subunit. Upon hydrolysis of the GTP to GDP, the G protein becomes inactive and ceases to activate the catalytic subunit. Analogs of GTP that are resistant to GTPase activity would cause persistent activation of the G-protein (Cassel and Selinger, 1977; Pfeuffer, 1979).

Studies of the isolated enzyme unit, adenylyl cylase, were hindered by the instability of this protein when removed from the intact membrane. Detergent solubilized and purified catalytic units have been reported (Neer, 1974; Pfeuffer and Metzger, 1982; Pfeuffer et al., 1985). However, it was shown that if membranes were incubated for 20 min at 37° C adenylyl cyclase activity was lost (Ross and Gilman, 1977). Clearly, the lability of the catalytic unit limits the experimental design of reconstitution studies of the membrane components for this system.

Functional studies had shown that at maximally effective concentrations, two or more agonists failed to produce additive stimulation of adenylyl cyclase activity (Birnbaumer et al., 1970). These studies suggested that only one form of adenylyl cyclase existed, i.e. isoenzymatic forms did not exist. Bourne and his colleagues used a genetic approach to study the adenylyl cyclase catalytic unit. They created mutant cells deficient in the cyclase enzymatic activity (Bourne et al., 1975). The S49 lymphoma cell line was chosen because these cells die when the intracellular concentration of cAMP is persistently elevated, e.g. when these cells are in the constant presence of β-adrenergic agonists, dibutyryl cAMP (a form of cAMP resistant to phosphodiesterase activity), or cholera toxin. The mutants survived

exposure to adrenergic agonist treatment, indicating that adenylyl cyclase activity was not a protein absolutely required for the life of the cell. The created mutants did not respond to stimulation by isoproterenol in the presence or absence of GTP, NaF, or by cholera toxin treatment. Thus, since neither agonist nor NaF stimulated adenylyl cyclase activity, and since the basal concentrations of cAMP were barely detectable, the implication was that the mutant did not contain adenylyl cyclase. These mutant cells were named S49 cyc⁻ because at that time, knowledge of this system suggested that this cell did not have the adenylyl cyclase enzymatic activity. However, this conclusion was erroneous, yet fortuitous. Reconstitution of β-adrenergic stimulation of adenylyl cyclase activity occurred when S49 wild type cell extracts that had been incubated at 37° C for 20 min (to inactivate the catalytic unit) were incubated with S49 cyc⁻ cell membranes (Ross et al., 1978). This experiment showed that the S49 cyc⁻ mutants are not deficient in the catalytic subunit. However, the results suggested that there were other factors involved that were in the treated wild type membranes but were absent in the S49 cyc⁻ membranes. The factor or factors were named G/F (G, for requirement for guanine nucleotide and F, for NaF stimulation) regulatory components. Therefore, G/F was a separate entity from either the receptor or adenylyl cyclase and was involved in the signal transduction process (Ross and Gilman, 1977; Ross et al., 1978).

Another cell line that proved useful in understanding of the hormone signal transduction mechanism of action was the UNC cell line (UNC, uncoupled; Haga et al., 1977). This mutant cell line was developed using S49 lymphoma cells grown in media containing terbutaline

(a β-adrenergic agonist) and was found to be sensitive to choleră toxin, NaF, and GppNHp, but insensitive to isoproterenol or prostaglandin E1 in the presence of GTP. Additionally, when isoproterenol displacement of ^{125}I-IHYP (iodohydroxybenzylpindolol, a β-adrenergic antagonist) binding was performed in the absence and presence of GTP, agonist affinity was diminished in the presence of GTP in wild type membranes. In the UNC cell membranes the agonist affinity was lower (the ability of the agonist to compete for the receptor was diminished) and the affinity was not further diminished in the presence of GTP . Based on the knowledge gained from the cyc^- mutants, it was proposed that stimulatory receptors in UNC mutants are uncoupled from the G/F but G/F is coupled to adenylyl cyclase in UNC cells.

In 1980, Northup et al. purified the stimulatory GTP-binding (G/F) protein from rabbit liver membranes. The presence of the GTP-binding protein (G protein) was detected throughout the purification procedure by virtue of the ability of cholera toxin to [^{32}P]ADP-ribosylate the stimulatory regulatory protein and the ability to detect G/F activity when extracts were able to reconstitute adenylyl cyclase activity in S49 cyc^- mutant cells. Gel electrophoresis of the purified G-protein depicted subunits having 52, 45, and 35 kDa molecular masses, and autoradiography of the gels showed that only the 52 and 45 kdal subunits were ADP-ribosylated by cholera toxin in the presence of [^{32}P]NAD (Northup et al. 1980; Hanski et al. 1981). After further purification, the 45 (or 52) and 35 kDa subunits were named α and β, respectively (Northup et al, 1983a; Northup et al., 1983b). The G/F factor was then named the stimulatory GTP-binding protein, or G_s. In addition, using the S49 cyc^- cell line previously shown not to contain the stimulatory

GTP-binding protein, Northup and his colleagues demonstrated a concentration-dependent reconstituted cyclase activity in these cells with the purified G_s. Neither α nor β-subunits alone could reconstitute adenylyl cyclase activity in the cyc$^-$ cell line (Northup et al, 1983a; Northup et al., 1983b). Further investigation showed that a third subunit, γ, an 8-10 kDa protein (Hildebrandt et al., 1984a), often missed in the electrophoretic identification, was part of G_s. Thus, G_s is a heterotrimer protein complex composed of an α subunit of 45 kDa that binds GTP (Northup et al., 1982) and is sugested to have GTP hydrolyzing activity (Codina et al., 1984a, Birnbaumer et al., 1985; Gilman, 1987), a β subunit of 35 kDa, and a γ subunit of 8 kDa.

Molecular biological techniques have allowed the cloning and sequence determination of the G_s heterotrimer subunits. The cDNAs for the α subunit of G_s (α_s) (Robishaw et al., 1986a; Sullivan et al., 1986, Itoh et al., 1986) as well as for β (Fong et al., 1987) and γ (Hurley et al., 1984) subunits are known. The cDNA for the α_s subunit has been shown to encode a 377 amino acid protein (45 kDa). Two cDNAs from a bovine adrenal cDNA library were shown to encode both forms (45 and 52 kDa) of α_s because antibodies to the purified α_s subunits specifically recognized synthetic peptides generated from the cDNA sequence (Robishaw et al., 1986b). The cDNA for the short (42 kDa) and long (52 kDa) forms were expressed in COS-M6 cells (Robishaw et al., 1986b) or *Escherichia Coli* (Graziano et al., 1987); these cDNAs were shown to direct the translation of two distinct α_s proteins. Furthermore, the recombinant α_s proteins (when added with purified $\beta\gamma$ subunits) were able to reconstitute isoproterenol- and GTPγS-stimulated adenylyl cyclase activity in S49 cyc$^-$ mutants. Both receptor and GTPγS-

induced stimulation occurred but the stimulation of cyclase activity was considerably less than purified native α_S proteins. The two forms of the α_S proteins were named α_S-short and α_S-long for the 45 and 52 kDa proteins, respectively. In a similar study, a λgt10 library was screened with oligonucleotide probes for recombinants that coded for α_S subunits. Four separate clones were isolated and named α_s-1, α_s-2, α_s-3, and α_s-4. It was proposed that these subtypes arose from separate mRNAs based on S1-nuclease protection analysis (Bray et al., 1986). A third report confirmed the existence of two forms of mRNA molecules for the α_S-1 and α_S-4 forms (Mattera et al., 1987). Based on the sequence analysis of these cDNAs, α_S-1 and α_S-4 correspond to the previously reported α_S-short and α_S-long. These reports documented that the cDNA sequence difference between the two α_s subunits (45 and 52 kDa) is a contiguous 42 nucleotide (coding 14 amino acids) insert at position 72. This amino acid insert α_s-long (52 kDa) may account, in part, for the detected difference in migration on polyacrylamide gels. The two protein sequences are otherwise identical and the 3' untranslated region was found to be identical in both mRNA molecules. This information strongly suggested that the two mRNAs arose from one gene. In support of this hypothesis, it was shown that S49 cyc$^-$ mutants (cells lacking Gs) do not to express either α_S-short and α_S-long. Since frequent mutations in two separate genes for related gene products would be unlikely it was proposed that the α_S-short and α_S-long subunit mRNAs were generated from alternate splicing of the α_S mRNA.

The gene for the α_S subunit of the G_s protein has been isolated and shown to have 13 exons and 12 introns (Kozasa et al., 1988). Southern blot analysis indicated that the human peripheral leukocyte

genome contains only one $G_{s\alpha}$ gene. In addition, based on the organization of the third exon and the suggested splice sites of the third intron, it was suggested that the four separate mRNA molecules reported earlier could be generated, thus, corraborating the theory that differential splicing of the immature mRNA molecule could generate the short and long forms of the α_s protein. The 5' flanking sequence was shown to lack typical TATA or CAAT box sequences but to contain a "GC" rich region.

All four splice variants of αs were tested in <u>cyc</u>$^-$ mutants and were shown to stimulate adenylyl cyclase activity when activated by isoproterenol or GTPγS (Robishaw et al., 1986b; Graziano et al., 1987; Mattera et al., 1989). Recently, all four forms of αs were also able to stimulate Ca^{++} channel activity (Mattera et al., 1989a). These results suggested that stimulatory receptor interaction with Gs and subsequent α_s subunit activation may result in stimulation of more than one effector system.

The cholera toxin ADP-ribosylation site has been shown to be the arginine residue #182 (Robishaw et al., 1986a; Sullivan et al., 1986, Itoh et al., 1986). The guanine nucleotide binding site, effector interaction site, and receptor interaction site of α_s have been postulated (Masters et al., 1986) based on the x-ray crystallographic studies of a soluble GTP-binding protein, EF-Tu (Jurnak, 1985). The α_s subunit has not been crystallized, therefore the actual protein conformation remains unknown.

Recently, cDNA libraries from wild type and <u>UNC</u> S49 mutants have been prepared. Through sequence analysis of α_s from these libraries, Bourne and his colleagues were able to detect a substitution of a

guanosine for a cytosine at base position number 1115. This point mutation would result in a substitution of a proline for an arginine at amino acid #372 (six residues from the carboxyl terminus) in the <u>UNC</u> α_s protein (Sullivan et al., 1987). The entire cDNA from both wild type and <u>UNC</u> was not sequenced. To confirm that this point mutation results in the <u>UNC</u> phenotype, a chimeric α_s cDNA was constructed. This construct was identical to the "wild type" cDNA for α_s except for the point mutation at position number 1115. Both the normal and chimeric cDNAs were expressed in S49 <u>cyc</u>$^-$ cells. The UNC phenotype occurred only in those cells having the chimeric DNA. The point mutation resulting in the expression of a proline at position #372 in the α_s protein results in the <u>UNC</u> phenotype. Therefore, the C-terminal of α_s is critically important for the interaction of α_s with the β-receptor. In another study (Masters et al., 1988) 40% of the carboxyl terminal of α_s and 60% of the amino terminal of the α-subunit of another GTP-binding protein, G_i (to be discussed below) was used to construct a chimeric cDNA. This chimeric Gs_α/Gi_α cDNA was expressed in S49 <u>cyc</u>$^-$ mutant cells and was shown to reconstitute receptor mediated stimulation of adenylyl cyclase activity. This further supports the notion that the carboxyl terminal of α_s is important for the interaction with the stimulatory receptor and furthermore suggests the α_s protein has a role in the interaction with the effector protein.

Purified β subunits have been shown to have two molecular weights of 35 and 36 kDa (Sternweis et al., 1981). Both β subunits have been shown to be tightly associated with the γ subunit. Both 35 and 36 kDa proteins have been cloned (Fong et al., 1987). The two cDNAs encode for proteins having 340 amino acids and were found to be >90% homologous.

Differences were found at 31 amino acid positions. These two proteins differ in their two-dimensional electrophoretic mobility patterns (Wookalis and Manning, 1987). One of the cDNAs encoding a β subunit has been expressed in COS-M6 cells. This cDNA directs the synthesis of the 35 kdal protein (Gao et al., 1987). In contrast to the α_s subunit, the β subunits seem to arise from separate genes and map to different chromosomes in the human genome (Amatruda et al., 1988). Since the heterotrimer, $\alpha\beta\gamma$ subunits, forms the active signal transducer protein, it will be interesting to see if the expression of one subunit regulates the transcription or translation of another subunit.

The exact mechanism of action of these β subunits is under investigation. One theory is that the $\beta\gamma$ subunits inhibit α_s activation, i.e. $\beta\gamma$ maintains α_s in the GDP-bound state (Gilman, 1987). However the $\beta\gamma$ subunits have been shown to have unique activity. For example, $\beta\gamma$ binds to and inhibits Ca^{++}-calmodulin stimulated cyclase activity (Katada et al., 1987). Phospholipase A_2 activity in bovine rod outer segments have been shown to be stimulated by $\beta\gamma$ subunits (Jelsema and Axelrod, 1987). In addition, the $\beta\gamma$ subunits have been reported to regulate K^+ channel gating in guinea pig atrial cells (Logothetis et al., 1987). These studies are contrary to the dogma that the α subunit alone directly regulates effector activity. However, $\beta\gamma$ subunit regulation of effector activity may account for the myriad of intracellular response to receptor activation by an agonist.

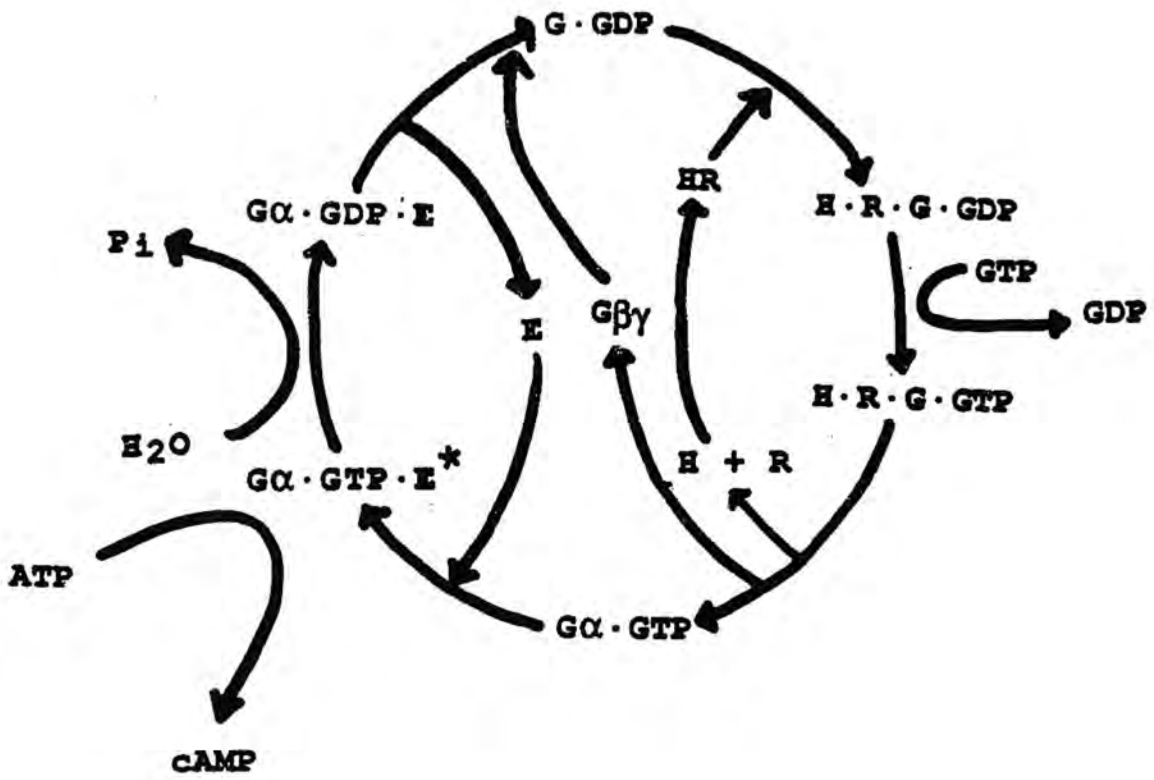
The γ subunit has been cloned and has a 74 amino acid sequence (Hurley et al., 1984). Two-dimensional gel electrophoresis of the purified γ subunits from different tissues sources resulted in different

electrophoretic patterns (Hildebrandt et al., 1985). This evidence suggests multiple forms of γ subunits may exist.

The purification and cloning of G_S allowed for the study of the mechanism of activation of this G-protein, i.e. what are the roles of the three subunits of this transducer protein. It had been shown by Pfeuffer in 1979 that the activation of G_s by a nonhydrolyzable analog of GTP resulted in differential centrifugation patterns of the GTPγS-activated and inactivated G protein complex. These studies provided the first evidence that the subunits (α, β, and γ) that make up G_S may dissociate from each other when GTPγS binds to α_S. Birnbaumer and his colleagues confirmed centrifugational sedimentation rates of the GTPγS-activated and inactivated purified G_S (Birnbaumer et al., 1985). Recently, different antibodies raised against the α_S subunit have been shown to identify both the $\beta\gamma$ associated or unassociated protein. Using these discriminating antibodies, it was shown that the adrenergic agonist activation of the β-adrenergic receptors caused the dissociation of the α subunit from the $\beta\gamma$ subunits. In addition, binding of the nonhydrolyzable analogs of GTP to α_S also caused the subunit dissociation (Ransnas and Insel, 1989). Incorporating the discussed data both Birnbaumer and Gilman proposed a model (figure 1) of activation of adenylyl cyclase activity. When GDP is bound to the G protein, the interaction between the receptor and the G-protein is such that the receptor has low affinity for the agonist. When an agonist binds to the receptor, GDP is replaced released. GTP can now bind to G_S. The brief period following the release of GDP when there is no guanine nucleotide bound to Gs, high affinity agonist binding occurs. However, when GTP or one of its analogs become bound, the G protein

Figure 1. Hormone-Induced Activation of Adenylyl Cyclase via GTP-binding Proteins.

The free hormone, H, interacts with its receptor, R. The HR complex has a higher affinity for the GTP-binding protein, G. The G protein is inactive when GDP is bound. The H·R·G·GDP complex enhances the release of GDP for GTP at the GTP-binding site (the α-subunit) of the G protein. When GTP is bound, the affinity of HR for the G protein, and the H for R is diminished. The active G protein, G·GTP, undergoes subunit dissociation; G_α·GTP dissociates from Gβγ. The G_α·GTP activates the enzyme, (E*) which converts ATP to cAMP. The α-subunit hydrolyzes GTP to GDP, the enzyme unit is inactivated, and Gβγ reassociates with G_α forming the heterotrimer G protein with GDP bound (G·GDP) (adapted from Gilman, 1986).



G · GDP
HR
H · R · G · GDP
Gα · GDP · E
Pi
GTP
E
Gβγ
GDP
H · R · G · GTP
H2O
H + R
Gα · GTP · E*
ATP
Gα · GTP
cAMP

dissociates from the receptor, and the receptor is shifted from the transitory high affinity to a lower affinity state. The α-GTP subunit dissociates from the $\beta\gamma$ subunits and activates the catalytic subunit. Activation of cyclase by α-GTP is terminated by the hydrolysis of GTP to GDP at the level of α_s. The α_s·GDP subunit then reassociate with $\beta\gamma$. An antagonist will bind with only one affinity to the receptor; antagonist binding is unaffected by guanine nucleotides suggesting an inability of antagonist to induce release of GDP from the G protein (Birnbaumer et al., 1985; Gilman, 1986).

As was described above, G_s has been thoroughly investigated in intact and reconstituted membrane systems. This model of the mechanism of signal transduction via G_s is now well accepted. In contrast, receptor-mediated inhibition or attenuation of the formation of cAMP was not well understood. It was not clear whether an inhibitory receptor interferred with the ability of a stimulatory receptor to activate G_s or whether an inhibitory receptor acted through its own inhibitory GTP-binding proteins to inhibit adenylyl cyclase activity directly. Part of the difficulty stemmed from the fact that inhibition of basal adenylyl cyclase activity is difficult to detect. Stimulatory agonists and suspected inhibitory agonists failed to potentiate or attenuate, respectively, NaF stimulated activity (Cote et al., 1982). However, Rodbell and his colleagues in 1977 (Yamamura et al., 1977) described the inhibition of adenylyl cyclase activity and suggested distinct GTP-binding proteins mediate the two signals.

Further study of the inhibition of adenylyl cyclase activity utilized methods other than attenuation of receptor-mediated stimulation of adenylyl cyclase activity. The first method of stimulation of

cyclase, not requiring the activation of a stimulatory receptor, was the direct activation of G_S by cholera toxin. As mentioned previously, this modification of the α subunit of the Gs protein inhibits GTPase activity associated with G_S and thus results in the persistent activation of the stimulatory subunit when bound by GTP. Reports using this technique demonstrated both agonist-induced and GppNHp-induced inhibition of adenylyl cyclase activity (Cote et al., 1982). The other stimulator of cyclase activity that aided in determining the mechanism of action of inhibitory receptors was the diterpene, forskolin (Seamon et al., 1981). Although the mechanism of action of forskolin is still not fully understood, the current hypothesis is that this molecule activates cyclase directly. Thus, forskolin has been useful in the understanding of the inhibition of adenylyl cyclase. Both techniques have provided a means to study receptor-mediated inhibition of cAMP production. These studies strongly suggested that a GTP-binding protein distinct from G_S mediated the effect of inhibitory receptors.

Based on our knowledge of the stimulatory receptor signal transduction through G_S, the study of the receptor-mediated inhibition of cyclase followed a similiar direction, at a highly accelerated rate. Receptor-mediated inhibition of adenylyl cyclase activity has been shown for A1-adenosine (Londos et al., 1978), D2-dopamine (Cote et al., 1982), muscarinic cholinergic (Murad et al., 1962), α-2 adrenergic (Aktories and Jakobs, 1981), and opioid receptor systems (Cooper et al; 1982; Sharma et al., 1975; Traber et al., 1975). These agonists have been shown to inhibit basal adenylyl cyclase activity in a GTP-dependent manner. Analogs of GTP were shown to inhibit cyclase activity in the absence of agonist (Cote et al., 1982; Cooper et al., 1982). GTP and

its analogs were shown to decrease the agonist affinity for the inhibitory receptor but do not affect antagonist affinity (U'Prichard and Snyder, 1978; Cote et al., 1984). Hydrolysis of GTP to GDP was shown to be stimulated in the presence of an inhibitory agonist (Koski and Klee, 1981; Atkories and Jakobs, 1981). In addition, inhibitory agonists were shown to stimulate the release of [^{3}H]GDP from cell membranes (Michel and Lefkowitz, 1982). The idea of separate and distinct GTP-binding proteins mediating either stimulatory or inhibitory signals was first proposed by Rodbell in 1980.

The first physical evidence of a separate GTP-binding protein was reported by Ui and his colleagues. They discovered that one of the toxins produced by *Bordatella Pertussis* produced a persistent stimulation of insulin secretion from islet cells of the pancreas (Katada and Ui, 1979b). The toxin became known as Islet Activating Protein, IAP, and now it is commonly referred to as pertussis toxin. Subsequent studies have shown that IAP treatment produced a slight stimulation of cyclase activity in some systems (Katada and Ui, 1981), but more importantly the toxin-treatment abolished the ability of an agonist acting via an inhibitory receptor, to inhibit cyclase activity but had no effect on GppNHp-induced inhibition of cyclase activity (Katada and Ui, 1981; Cote et al., 1984). It was shown that the pertussis toxin treatment resulted in the ADP-ribosylation of a 41 kDa protein (Katada and Ui, 1982) that was distinct from the cholera toxin substrate (Bokoch et al., 1983). [^{32}P]NAD labeling provided the first physical evidence for the existence of an inhibitory GTP-binding protein. Isolation and purification of the pertussis toxin substrate, G_i, confirmed the αβγ heterotrimer subunit structure (Bokoch et al.,

1984b, Codina et al.,1983). In addition, it was shown that the α subunit (α_i), a 41 kDa protein, is susceptible to ADP-ribosylation by pertussis toxin (Bokoch et al., 1983), is the subunit responsible for GTP binding (Bokoch et al., 1984), and is suggested to be responsible for the hydrolysis of GTP to GDP (Birnbaumer et al., 1985; Milligan and Klee, 1985). Furthermore, it has been shown that activation of purified G_i by GTPγS results in the dissociation of α_i-GTPγS from $\beta\gamma$ subunits (Katada et al., 1984b). The proposed mechanism of activation of G_i follows closely the proposed mechanism of activation of G_s. As shown in figure 2, in the presence of inhibitory agonist, the exchange of GDP for GTP occurs. Upon binding of GTP, the α_i-GTP dissociates from the $\beta\gamma$ subunits. Once GTP is hydrolyzed to GDP, the subunits reassociate and the cycle is complete. Interestingly, $\beta\gamma$ subunits from Gi can reassociate with other α subunits in addition to α_i (Kanaho et al., 1984). Functional reconstitution studies later suggested the possible underlying mechanisms of action of the G_i subunits.

The purification of G_i (41 kDa protein) has aided in the study of the mechanism of inhibition of adenylyl cyclase activity. Reconstitution studies that employed the purified components of Gi demonstrated that the αi·GTPγS inhibited adenylyl cyclase activity in wild type and <u>cyc</u>$^-$ mutants, but that the $\beta\gamma$ subunits inhibited adenylyl cyclase activity only in the wild type S49 lymphoma cells (Katada et al., 1984c). These data suggested that Gi·GTPγS could inhibit foskolin stimulated cyclase activity only if Gs subunits were present (Cerione et al., 1986b); $\beta\gamma$ subunits of Gi seemed to inhibit adenylyl cyclase activation of cyclase activity by Gs (Katada et al., 1984a). These results have led to the formation of two theories for the mechanism of

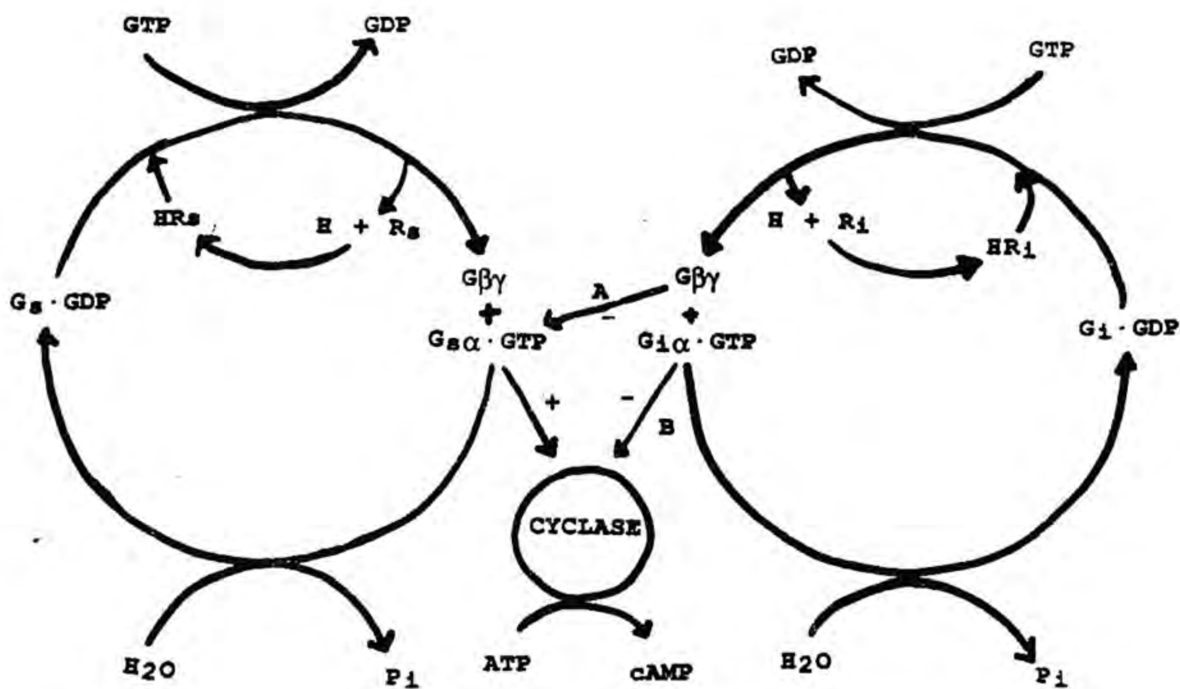
G_i-mediated inhibition of cyclase activity (figure 2). Both theories have incorporated the reconstitution data and the subunit dissociation models for G_s and G_i. One theory is that the dissociation of $\beta\gamma$ from G_i provides an available pool of free $\beta\gamma$ that interacts and reassociates with the α subunit of the G_s, thus preventing the exchange of GTP for GDP and inhibiting the activity of Gs (figure 2, A) (Northup et al., 1983a; Cerione et al., 1986b). The second theory is that the GTP liganded α-subunit from G_i directly inhibits cyclase (figure 2, B) (Hildebrandt et al., 1984b). These mechanisms may not be mutually exclusive.

It has been demonstrated that other GTP-binding proteins susceptible to ADP-ribosylation by pertussis toxin have α-subunits of 39 and 40 kDa (Sternweis and Robishaw, 1984; Neer et al., 1984). Very recently, it has been reported that the 40, 41 kDa α_i proteins could be purified, and selective antibodies could be raised to each of these α_i-subunits (Mumby et al., 1988). Two of these pertussis toxin substrates, α_{39} and α_{41}, have been shown to be myristoylated (Buss et al., 1987). This modification may serve to anchor these α subunits to the plasma membrane.

The functional role of each of these pertussis toxin substrates is currently under investigation. A pertussis toxin sensitive G-protein involved in receptor mediated stimulation of phospholipase C activity in HL-60 cells has been identified (Ohta et al., 1985). In 3T3 cells a pertussis toxin sensitive G-protein appears to be involved in cell proliferation (Murayama and Ui, 1987). It has been proposed that the 39 kDa α subunit may be involved in neuronal Ca^{++} channel gating (Yatani et al., 1987b; Heschler et al., 1987), as well as K^+ channel gating in

Figure 2. Model for the Inhibition of Adenylyl Cyclase Activity by α_i or $\beta\gamma$ Subunits of GTP-binding Proteins.

The hormone, H, interacts with either the stimulatory of inhibitory receptor, Rs or Ri, respectively. The HR_s or HR_i complex then interacts with their respective GTP-binding proteins, G_s or G_i. The HR·G protein interaction enhances the replacement of GTP for GDP at the α-subunit of Gs or Gi, respectively. Once GTP is bound to the α-subunit, the $G_{s\alpha}$·GTP and the $G_{i\alpha}$·GTP dissociate from their respective $\beta\gamma$ subunits. The $G_{s\alpha}$·GTP subunit stimulates adenylyl cyclase to convert ATP to cAMP. One hypothesis of the inhibition of adenylyl cyclase is that the inhibitory hormone receptor interaction is to effectively increase the available free $G\beta\gamma$ subunits; thus there is a greater pool of $\beta\gamma$ subunits to reassociate with $G_{s\alpha}$ (A). The other hypothesis is that $G_{i\alpha}$·GTP interacts directly with the adenylyl cyclase catalytic subunit to inhibit the conversion of ATP to cAMP (B) (adapted from Gilman, 1987).



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hippocampal pyramidal neurons (Van Dongen et al., 1988). The 40 and 41 kDa proteins are thought to mediate inhibition of adenylyl cyclase activity (Hildebrandt et al., 1984) as discussed, and may also be involved in potassium channel gating (Yatani et al., 1987a; Yatani et al., 1987c; Codina et al., 1987).

There is a controversy over whether the α_{41} subunit or the $\beta\gamma$ subunits activate K^+ channel gating in atrial tissue. Recently, it was shown that maximal activation of K^+ channel gating by $\beta\gamma$ occured at concentrations of 10 nM as opposed to a concentration of 10 pM for α_{41} (Logothetis et al., 1988). These results suggest that although both subunits can affect channel activity, the α_{41} subunit is the physiological mediator of K^+ channel activity. Subsequently, it was shown the $\beta\gamma$ subunits at lower concentrations, i.e. lower than 10 nM, could activate phospholipase A_2 actitivy (Kim et al., 1989); the metabolic by-products of the activation of this enzyme, arachidonic acid and leukotrienes, were responsible for the activation of the K^+ channel gating (Kurachi et al., 1989). Therefore, although it now appears that the $\beta\gamma$ subunits by themselves are not sole activators of the K^+ channel in atrial tissue, these subunits may indirectly affect channel gating. These studies have shed light on the mechanism of muscarinic agonist mediated hyperpolarization of the atrial tissue via a pertussis toxin sensitive GTP-binding protein.

The α_i (41 kDa) and α_o (39 kDa) have also been cloned (Sullivan et al., 1986; Itoh et al., 1986; Lavu et al., 1987). The cDNA for αi encodes a 355 amino acid protein. The sequence data has shown that cysteine #352 is the pertussis toxin ADP-ribosylation site. The amino acids involved in the α_i and α_s protein interaction with the

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stimulatory or inhibitory receptor proteins, respectively, have also been shown to be located near the carboxy terminal of these subunits. As described, the genetic analysis of the UNC cells (Sullivan et al., 1987) and the pertussis toxin-induced abolishment of inhibitory receptor interaction with Gi both suggest that the C-terminal of α_i is important in coupling α_i to an inhibitory receptor (Cote et al., 1984; Katada and Ui, 1981). Comparison of the sequences of α_i and α_s has not provided adequate sequence homology to define the $\beta\gamma$ interaction site of α_i or α_s. By screening cDNA libraries, three separate α_i subunit sequences termed α_i1, α_i2, and α_i3 were defined (Jones and Reed, 1987; Nukada et al., 1986; Itoh et al., 1986; Itoh et al., 1988). Although sequence homology exists among the three cloned α_i's, the sequences differ and allow speculations suggesting that these sequences may be the 39, 40, and 41 kDa proteins. Unique decapeptide sequences of each of the three α_i subunits were used to generate antibodies. These antibodies were then used to localize and identify the individual α_i subunit proteins obtained from various sources (Goldsmith et al., 1988). It was reported that the pertussis sensitive G-protein $G\alpha_{i40}$ was $G\alpha_{i2}$ while $G\alpha_{i41}$ in brain was principally $G\alpha_{i1}$. Although the identification and localization of these subunits is fairly clear, the exact function of these subunits is not well understood.

An attempt to gain insight into the effector specificity of the three α_i subunits (pertussis toxin sensitive substrates), the potential regulation of atrial K^+ channel gating by each of the three α_is was tested. The α subunit that was originally shown to activate K^+ channel activity was purified from human erythrocytes to homogeneity, sequenced, and shown to be α_{i3} (Codina et al., 1988). The cDNA for α_{i3} was

expressed in *Eschericia Coli* using a T7 promoter based expression vector system. This recombinant α_{i3} subunit also activated K^+ channel gating in atrial cell patches (Mattera et al., 1989b). However, recombinant α_{i1} and α_{i2} subunits also stimulated K^+ channel gating. In these experiments the recombinant and purified α subunit proteins all hyperpolarized the atrial cell tissue with similar EC_{50} values. (Yatani et al., 1988). Therefore, as in the case of α_s, different α_i subunits exist but seem to alter the same effectors. The hypothesis of one receptor interacting with one type of GTP-binding protein has been shown both for α_s and α_i to be a simplistic model. Evidence is mounting that one receptor may interact with several subtypes of a particular GTP-binding protein.

The three distinct $G_i\alpha$ subunits are products of three distinct genes. All three genes have been isolated from the human genome (Itoh et al., 1988). Southern blot analysis determined that the three unique α_i subunits are found in single copies in the human genome. All three genes have eight exons and seven introns. Comparison of the cDNA and gene sequences for all three α_i subunits have shown identical exon-intron organization. The 5' flanking sequences vary among the three $G_{i\alpha}$ genes. For example, the $Gi_{\alpha 1}$ gene 5' flanking sequence does not have a typical TATAA or CAAT box, but does have 2 regions of GC rich sequences (GC box). Interestingly, a steriod response element-like sequence (half of the pallindromic steroid response element dyad; TGTTCT) is found 784 nucleotides upstream of the transcriptional start site. $Gi_{\alpha 2}$ has two CAAT boxes at -262 and -411, seven GC boxes, and no typical TATAA boxes. One interesting segment of the 5' flanking sequence of the $Gi_{\alpha 2}$ gene (-162 to -189) has an 86% homology to a 5' flanking sequence (-510 to -

537) of the c-Harvey ras proto-oncogene. This homologous region may implicate the expression of α_{i2} during cell proliferation. The 5' flanking sequence to the $Gi_{\alpha 3}$ gene has a typical TATAA-like sequence, three GC boxes, but no typical CAAT box. The three α_i subunit genes have one common characteristic; all have GC boxes upstream from the ATG codon. Since it has been suggested that $Gi_{\alpha 1}$ and $Gi_{\alpha 2}$ are expressed ubiquituously and constitutively, these GC boxes may imply that these GTP-binding proteins are members of commonly referred "housekeeping genes".

It is the goal of this project to study the interaction of an inhibitory receptor (mu-opioid receptor) with pertussis toxin sensitive GTP-binding proteins. Generally, the model of the stimulatory receptor-G_s interaction is assumed to depict the mechanism for the inhibitory receptor-G_i interaction. It has been assumed, but not shown, (1) that GDP cannot support receptor-mediated inhibition of cyclase activity; (2) that the receptor-G_i interaction affects the guanine nucleotide affinity at Gi; and (3) that agonist activation of an inhibitory receptor will cause the receptor to form a stable complex with G_i; the stability of this complex should allow us to co-purify the receptor and G_i. The co-purification of the mu-opioid receptor with its GTP-binding protein will allow identification of the G-proteins which interact with this receptor and thus mediate the intracellular signal transduction. The inhibitory receptor-G protein interaction will first be studied using the opioid receptor-mediated inhibition of adenylyl cyclase activity as a model.

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Previous studies of the opioid receptor were performed in brain tissue. Great difficulty has been encountered because of the heterogeneous population of opioid receptor subtypes in brain tissues. In 1975, opioid-mediated inhibition of PGE_1 stimulated adenylyl cyclase activity was described in neuroblastoma x glioma cell lines (Sharma et al., 1975; Traber et al., 1975). The morphine-mediated inhibition of adenylyl cyclase could be reversed with naloxone (20 μM). Ligand binding studies indicated that the neuroblastoma x glioma (NG-108-15) cells have delta preferring opioid receptors on the plasma membrane (Puttfarcken et al., 1986; Chang et al., 1978). Another cell that was shown to have opioid-mediated inhibition of adenylyl cyclase activity and prolactin secretion is the 7315c tumor cell (Frey and Kebabian, 1984). The 7315 tumor cell was originally isolated from the anterior pituitary gland of the rat (Bates et al., 1966). In addition, it has been shown that guanine nucleotides diminish agonist, but not antagonist affinity for the mu-opioid receptor on the 7315c cell (Werling et al., 1988; Aub et al., 1987). Opioid-mediated inhibition of adenylyl cyclase was shown to require GTP and to be affected by sodium in the 7315c tumor cells (Frey and Kebabian, 1984; Puttfarken et al., 1986). These studies have led to questions regarding the interaction of the ligand with opioid receptors, the interaction between the opioid receptor and G proteins, as well as the exact mechanism of opioids inhibition of adenylyl cyclase.

The present studies are aimed at expanding our knowledge of the mu-opioid receptor interaction with Gi in the 7315c tumor cell. The studies will include testing the ability of guanine nucleotides to sustain mu-opioid agonist inhibition of adenylyl cyclase activity and

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the ability of the mu-opioid receptor to influence the relative affinity of guanine nucleotides at Gi. The results may explain the overall mechanism of mu-opioid receptor-mediated inhibition of adenylyl cyclase activity. The influence of guanine nucleotides on the affinity of the receptor for mu-opioid agonist agonist will be evaluated. Since high and low affinity states have been described in the absence and presence of guanine nucleotides, respectively, it will be of interest to determine the functional role of affinity states in the presence of varying concentrations of GTP as measured by agonist-induced inhibition of adenylyl cyclase activity.

The studies described above will provide evidence for the intact membrane mu-opioid receptor/G-protein interaction. These components can then be co-purified from the intact membrane, away from other extraneous proteins, and the interactions of this complex could be studied free of interference. Other groups have utilized inhibitory receptors and Gi and Go purified separately from different tissues, and reconstituted them together in phospholipid vesicles (Haga et al., 1986; Cerione et al., 1986a; Ueda et al., 1988). The interaction of the receptor with Gi may be best studied when the two interacting proteins are co-purified from the same tissue. Furthermore, the co-purification of the receptor/G-protein complex would allow for the accurate identification of which of the pertussis toxin substrates actually interacts with the inhibitory receptor in the intact cell membrane. Inhibitory receptors have been co-solubilized with Gi (Smith and Limbird, 1981; Kilpatrick and Caron, 1983; Michel et al., 1981; Demoliou-Mason and Barnard, 1986). Two reports have been described for the co-purification of an inhibitory receptor coupled to inhibitory GTP-binding proteins (Tota et al., 1987;

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Senogles et al., 1987). In these reports, the receptor-G_i complex was co-purified using affinity columns, and the reconstitution of the receptor-G_i complex was subsequently studied.

The mu-opioid receptor has been solubilized (Demoliou-Mason and Barnard, 1984; Ruegg et al., 1980; Bidlack and Abood, 1980; Howells et al., 1982; Chow and Zukin, 1983; Simonds et al., 1980; Mollereau et al., 1988) and purified (Ruegg et al. 1981; Maneckjee et al., 1985; Gioannini et al., 1984; Cho et al., 1986; Simonds et al., 1985) from brain tissues. In general, the interest was centered on the mu-opioid ligand interaction and its differences from the other opioid receptor subtypes. The affinity purified mu-opioid receptor from rat brain has been reconstituted and shown to interact with both purified Gi and Go, indicating that mu-opioid receptors may couple to one or both types of pertussis toxin sensitive substrates (Ueda et al. 1988). Therefore, this study will define the GTP-binding proteins which interact with the mu-opioid receptor in the intact membrane which to date has not been achieved. In this way, the mechanism of mu-opioid agonist mediated intracellular signal transduction will be better understood.

The co-purification of mu-opioid receptor/G-protein complexes from 7315c tumor cells will take advantage of the following: (1) the 7315c tumor cell has been shown to have a homogeneous population of mu-opioid receptors (Frey and Kebabian, 1984; Puttfarcken et al., 1987); (2) these cells have been shown to have the 41 kDa pertussis toxin sensitive Gi (Aub et al., 1986); (3) the model of the inhibitory receptor-Gi interaction indicates the formation of a stable agonist/receptor/G_i complex in the absence of guanine nucleotides (Birnbaumer et al., 1985; Birnbaumer et al., 1987; Gilman, 1987); and (4) the 7315c tumor cell can

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be grown in large quantities in the intraperitoneal cavity of the rat. Therefore, the 7315c tumor cell provides a source of mu-opioid receptors that are coupled to Gi, and the model suggests a "locked-in" agonist-receptor/Gi interaction that will allow the solubilization, purification, and reconstitution of the mu-opioid/G-protein complexes.

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METHODS

7315c tumor cell preparation and propagation

The 7315c tumor was propagated by injecting 2 x 10^6 cells into the intraperitoneal cavity of female buffalo rats; approximately three weeks post injection the tumor (3-5 grams) was removed. The tumor was quickly washed with Earles Balanced salt solution containing 2.5% Sodium Bicarbonate and 0.25% Bovine Serum Albumin (EBSS/BSA). At this point obvious vasculature, clots, and fat tissues were removed. The cells were dispersed by chopping the tumor with a razor blade. Finally, dispersed tumor was poured through 8 layers of gauze to remove any large tumor particles. The cells were then separated from cellular debris by centrifugation, 200 x g. The cells forming the pellet were layered on a EBSS/4% BSA solution and centrifuged at 200 x g. This step allows for the separation of tumor cells from blood cells and cellular debris. The EBSS/4% BSA supernatant was discarded. The cells forming the pellet were washed in fresh EBSS/BSA, and counted using a light microscope and a hemocytometer.

Preparation of membranes

The cells were resuspended in 2 mM Tris-HCl (pH 7.4), 2mM EGTA, 1 mM $MgSO_4$, 250 mM sucrose (homogenizing buffer) and homogenized with a Brinkman Polytron (setting 3-4, for 30 seconds). This suspension was

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subjected to centrifugal force (1000 rpm (200 x g)) for 10 minutes. The resultant supernatant was retained and further spun at 15,000 rpm, 27,000 x g, for 30 minutes. The pellet was resuspended in 6 mM Tris-HCl (pH 7.4), 4 mM EGTA, 1 mM $MgSO_4$, and 10% glycerol (freezing buffer). Aliquots (4, 1.6, or 0.8 mg protein per ml) were then stored in liquid nitrogen.

Incubation of 7315c Tumor cells with Pertussis Toxin

Cells were resuspended in Eagles Minimum Essential Medium supplemented with penicillin (10,000 units/ml) and streptomycin (100 µg/ml) at a concentration of 2 x 10^6 cells/ ml. The final concentration of pertussis toxin was 30 ng/ml. This concentration of pertussis toxin was shown to ADP-ribosylate >70% of the available Gi and completely abolish opioid receptor-mediated inhibition of adenylyl cyclase activity (Aub et al., 1986). The cells were then placed in an incubator in an atmosphere of 95% air and 5% CO_2 with constant humidity at 37° C. After 3 hrs of incubation the cells were removed via centrifugal force (1000 rpm, 200 x g) and resuspended in homogenizing buffer. Membranes were then prepared as described above.

Adenylate Cyclase Activity

The adenylyl cyclase activity was measured by the method of Brown et al. (Brown et al., 1971). Briefly, the assay system for the determination of adenylyl cyclase activity contains 80 mM Tris-HCl (pH 7.4), 10 mM theophylline (a phosphodiesterase inhibitor), 1 mM $MgSO_4$,

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0.8 mM EGTA, 30 mM NaCl, and 0.25 mM AppNHp or ATP (as the substrate for cyclase), the indicated drugs, and 15-30 µg of protein. The components were added to each assay tube (final volume, 0.06 ml) on ice; the reaction was initiated by the addition of 7315c tumor cell membranes and placing the tubes in a water bath at 30° C for 10 min. The assay was terminated by placing the tubes in boiling water for 1 min. Approximately 80,000 cpm/100 µl of [^{3}H]cAMP (approximately 3 pmoles) in 100 µl of 75 mM citrate-phosphate buffer (pH 5.0) and 20 µl of the Brown's cAMP binding protein were added to each tube. These tubes were allowed to incubate on ice for at least one hour. The assay was terminated by the addition of 100 µl of an activated charcoal solution (5 g Norit A, 2 g bovine serum albumin, 0.5 ml Antifoam A, and 50 ml H_2O). The tubes were quickly centrifuged at 4,000 rpm (1500 x g) for 10 min, and 180 µl of the supernatant containing [^{3}H]cAMP bound to the cAMP dependent protein kinase was removed and subjected to liquid scintillation spectroscopy. Under these assay conditions cyclase activity was 2.4 ± 0.2 and 40 ± 3 pmol/mg protein·min (n=45) in the absence or presence of 10 µM forskolin. The cyclase activity was not significantly different in the experiments comparing control and pertussis toxin treated tissues.

Assay of the Conversion of GDP to GTP

Membranes prepared from 7315c tumor cells were incubated in the adenylyl cyclase experimental assay mixture supplemented with 20,000 dpms of [^{3}H]GDP and other drugs as indicated in the figure legend. The reaction was terminated by placing the assay tubes on ice. Polygram Gel

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300 polyethyleneimine chromatography plates (Brinkman Instruments, Westbury, N.Y.) were used in the analysis of [^{3}H]GTP and [^{3}H]GDP (White, 1975). Plates were first washed by ascending development with 0.5 M formic acid with absorbent paper applied to the edge of the plate to remove impurities; the plates were then dried overnight. GDP and GTP standards (20 μl, 100 mM) and samples were spotted on the plate. The plate was soaked in cold 100% methanol for 30 min, air dried, and developed in a solvent system containing (v/v, 7:3) 0.5 M formic acid:4 M LiCl. After the solvent front had reached the top, the plate was air dried and then soaked in 100% methanol for 30 min. The plate was cut into sections based on the location of the standards as determined by UV inspection. The sections were soaked in 1 ml of 0.5 M NH_4HCO_3 (pH 8.6) for 30 min, and analyzed for tritium content using liquid scintillation spectroscopy.

Ligand Binding Experiments

All ligand binding experiments were performed by using membranes prepared from 7315c tumor cells as described above. Briefly, the membranes were thawed, washed, and resuspended in a solution containing 25 mM K^+ HEPES (pH 7.4), 2 mM EGTA, 1 mM $MgSO_4$ (binding buffer). Two different protocols were used to determine receptor affinity for the indicated [^{3}H]ligands. In the first assay the washed membranes were incubated at 37° C for 30 min in the presence of the indicated concentrations of [^{3}H]ligands and in the absence or presence of the indicated drugs (final volume 0.5 ml). The assay was terminated by the addition of 3 ml ice cold buffer to the assay tube followed by rapid

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filtration on Whatman GF/B filters under reduced pressure, and two rinses of 5 mls of buffer. The filters were analyzed for tritium content using liquid scintillation spectrometry.

In the second protocol membranes were resuspended in binding buffer that had been supplemented with 10 nM [^{3}H]etorphine with or without 100 µM morphine (to determine nonspecific binding) and were allowed to incubate for 30 min at 37° C. The membranes were cooled on ice for 10 min, and the volume was increased 10 fold with binding buffer. After centrifugation (27,000 x g) for 30 min, the membrane pellet was resuspended using a Brinkman Polytron (setting 2-3 for 10 seconds) in binding buffer. These prelabeled membranes were then allowed to incubate for 30 min at 37 C in the absence or presence of the indicated drugs. This assay was terminated by glass fiber rapid filtration as described above.

Phospholipid Vesicle Formation and Reconstituted Ligand Binding Studies

Co-solubilization of mu-opioid receptors with their GTP-binding proteins followed the protocol described in Appendix I with the following modifications. Frozen intact 7315c tumor cell membranes were quickly thawed, diluted 50-fold with 50 mM K^+ HEPES (pH 7.4), 1 mM $MgSO_4$, 2 mM EGTA, 30 mM NaCl, 0.1 mM Phenyl Methyl Sulfonyl Flouride (PMSF), 1 µM leupeptin, and 1 mM dithiothreitol (Buffer A) supplemented with either 10 µM morphine or 1 µM naloxone, and were centrifuged at 48,000 x g for 15 min. The membrane pellets were homogenized in buffer A supplemented with either 10 µM morphine of 1 µM naloxone. The homogenate was incubated 30 min at 37° C to allow for agonist or

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antagonist receptor occupation. The membranes were chilled on ice for 10 min and centrifuged at 48,000 x g for 15 min. Pellets of membranes prebound with morphine or naloxone were resuspended in buffer A supplemented with either 10 μM morphine or 1 μM naloxone and 10 mM 3-[3-chloramidopropyl)dimethyl-ammonio]-1-propanesulfonate (CHAPS) by homogenization with a Brinkman Polytron (setting 2 for 15 sec). These solutions were incubated with oscillation for 30 min at 4 C. The membrane suspensions were centrifuged at 48,000 x g for 30 min. Each supernatant was diluted by a factor of 10 with buffer A supplemented with either 10 μM morphine or 1 μM naloxone. The 10 mM CHAPS solution was then diluted with Buffer A supplemented with either 10 μM morphine of 1 μM naloxone to achieve a final concentration of 2 mM CHAPS, i.e. this concentration is below the critical micelle concentration (cmc) value for CHAPS (cmc, 4-7 mM). The volume was then reduced 20-fold by using a Millipore CX-30 ultrafiltration device. Phospholipid vesicle formation and receptor/G-protein complex reconstitution was as described (Cerione et al., 1984). Briefly, a detergent-phospholipid solution containing 2.4 mM cholate, 3.6 mM deoxycholate in 50 mM K^+ HEPES (pH 7.4), 1 mM $MgSO_4$, 2 mM EGTA, 100 mM NaCl (Buffer B), and 100 mg Brain extract type IV (final volume, 1 ml) was sonicated in a bath type sonicator (Heat Systems, Inc.) filled with ice water. A probe sonicator was not used to avoid introduction of titanium particles in the phospholipid mixture (Barrow and Lentz, 1980). Dispersion of the phospholipids was accomplished using repeated 100 watt bursts, 10 seconds each, until the lipids were dispersed (approximately 4 min). The solubilized receptors (730 μl) were then combined with the sonicated phospholipid solution (200 μl), octyl-β-D-glucoside (50 μl), and bovine

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serum albumin (20 µl, 100 mg/ml solution). This solution (final volume, 1 ml) was allowed to incubate for 30 min at 4 C. The sample was then applied to a 0.7 x 30 cm Sephadex G50 column preequilibrated with buffer B. The void volume of this column, as determined by blue dextran (m.w. is approximately 2,000,000) was 4 to 5 ml. Elution with buffer B allowed removal of the excess bile salts, octyl-β-D-glucoside, CHAPS, and free ligand from the phospholipid mixture (Enoch and Strittmatter, 1979). The turbid eluates were combined (the 4th and 5th ml). This phospholipid solution was diluted 2-fold with buffer A and 250 µl aliquots of this solution were then used in a binding assay. The vesicles were allowed to incubate with [^{3}H]etorphine and the indicated drugs (final volume, 0.5 ml) overnight (>18 hrs.) at 4 C. Nonspecific binding was measured by the inclusion of 10 µM naloxone during the assay. Separation of bound from free ligand was accomplished by the use of Sephadex G50 gel filtration chromatography (0.7 x 14 cm column, preequilibrated with Buffer A). Using buffer A, blue dextran eluted in the 2nd ml; free [^{3}H]etorphine eluted in the 5th ml. The assay mixture was added to the column, and each tube was rinsed with 0.5 ml Buffer A (final volume loaded onto column, 1 ml). Fractions containing the vesicles (2nd ml) were collected and counted using liquid scintillation spectroscopy.

Preparation of DADLE-AH Sepharose 4B

[D-ala^2, D-leu^5]-enkephalin (DADLE) was selected as the opioid agonist to construct an affinity column to co-purify the mu-opioid receptor/G-protein complex because it has been shown to inhibit adenylyl cyclase (IC_{50}, 9 nM) and prolactin secretion (IC_{50}, 120 nM) in 7315c

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cells (Frey and Kebabian, 1984), and because unlike DAGO (a mu-selective opioid agonist) DADLE has a free carboxyl terminal available for the carbodiimide mediated coupling to the AH Sepharose 4B (figure 20, page 99). To prepare the DADLE affinity resin one gram of AH-Sepharose 4B was swollen and washed in 0.5 M NaCl. Swollen AH-Sepharose 4B was incubated with 5.7 mg DADLE (10 μM final concentration), and 1 mM 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC). The solution was gently mixed for 24 hours; pH was maintained at 4.5. The DADLE-AH Sepharose 4B was then washed once with buffers containing 0.1 M sodium acetate (pH 4.0), 0.5 M NaCl and 0.1 M sodium bicarbonate (pH 8.3), 0.5 M NaCl. The resin was stored in 1 M NaCl with 0.1% Thimersol at 4 C. Human β-endorphin was also covalently attached to AH-Sepharose 4B following the above procedure under similar conditions. Using this protocol, the carboxyl terminal of DADLE or β-endorphin was coupled via an amide bond to the AH-Sepharose 4B. The coupling efficiency of this reaction is shown in table 5 (page 100). The amount of enkephalin bound to the AH-Sepharose 4B was 61.4 ± 5.1 nmoles per ml of gel representing a 2.4 ± 0.2% coupling efficiency.

Affinity Co-Purification of the Mu-Opioid Receptor/G-protein Complex

Membranes from 7315c tumor cells were prepared and stored in liquid nitrogen as described above. The membranes were washed and resuspended with 50 mM K^+HEPES, 2 mM EDTA, 5 mM $MgSO_4$, 100 mM NaCl, 1 μM leupeptin, 1 mM dithiothreitol, 0.1 mM PMSF, 100 μM 1,10-O-phenanthroline, and 20 μM bestatin (Buffer C). The membranes were incubated with 1 ml of the DADLE-AH Sepharose 4B (61.4 ± 5.1 nmoles of DADLE) in a 1.5 x 5 cm Biorad Econo-Column for 1 hr with gentle mixing

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at 20 C. The detergent, CHAPS, was added to the membrane-affinity resin mixture (final concentration, 10 mM); the mixture was further incubated for 30 min at 4 C. All subsequent procedures were performed at 4 C. The affinity resin was washed in the column with 300 mls Buffer C supplemented with 2 mM CHAPS follwed by 50 mls of Buffer C supplemented with 1 mM CHAPS (Buffer D). No protein was detected from the 1 mM CHAPS elution (absorbance at 280 nM <0.001). The receptor and G-protein were eluted with five mls Buffer D supplemented with 10 μM morphine and 100 μM GTP. The eluted solution was subjected to ultrafiltration using Millipore CX30 ultrafiltration units to reduce the volume to 1 ml. This procedure for the affinity purification of the mu-opioid receptor from 7315c cell membranes is depicted in figure 21 (page 102).

Reconstitution of the Purified Mu-Opioid Receptor

The affinity purified receptor/G-protein complexes were reconstituted into phospholipid vesicles as described for the co-solubilized receptor/G-protein complex. BSA was omitted in the reconstitution mixture as indicated in the figure legends.

Binding assay for the Affinity Purified Mu-Opioid Receptor/G-Protein Complex

The reconstituted receptor/G-protein complex was incubated in Buffer C with the indicated [^{3}H]ligands alone or in the presence of the indicated drugs for 18 hrs at 4 C. Nonspecific binding was determined in the presence of 10 μM naloxone. Separation of bound from free ligand was performed by Sephadex G-50 gel filtration, 0.7 x 14 cm columns preequilibrated with 50 mM K^+HEPES pH 7.4, 2 mM EDTA, 5 mM $MgSO_4$, and

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100 mM NaCl (Buffer E), essentially as described above. The protein bound [^{3}H]ligands in eluted fractions were collected and analyzed by liquid scintillation spectrometry.

Determination of the Molecular Weight of the Mu-Opioid Receptors

Reconstituted affinity purified receptors were incubated with 2 nM ^{125}I-β-endorphin in the presence and absence of 10 μM naloxone for 18 hrs at 4 C. The free ^{125}I-β-endorphin was separated from the bound as above, except the Sephadex G50 columns were pre-equilibrated with 50 mM Na^{+}HEPES, pH 7.4, 2 mM EDTA, 5 mM $MgSO_4$ (Buffer F). The eluted fraction containing the affinity purified receptor ^{125}I-β-endorphin complex was incubated with 0.1 mM disuccinimidyl suberate (DSS) for 15 min at 4C; the reaction was terminated by the addition of 0.1 volume of 100 mM Tris·HCl (pH 7.4), 1 mM EDTA. The samples were dried under a stream of nitrogen gas and resuspended in 250 μl of 2x SDS-PAGE sample buffer (4% SDS, 0.05 M Tris-HCl (pH 6.8), and 50 μl β-mercaptoethanol, final volume 0.5 ml).

Membrane-associated mu-opioid receptors were incubated with 2 nM ^{125}I-β-endorphin in the presence or absence of 10 μM naloxone for 1 hour at 20 C. The samples were diluted 20-fold with ice-cold buffer F and centrifuged at 26,000 x g for 20 min. The resulting pellet was resuspended in Buffer F and was incubated in the presence of 1 mM DSS on ice for 15 min. The crosslinking reaction was stopped as for the purified receptors. The samples were diluted 20-fold with Buffer F and were centrifuged at 26,000 x g for 20 min . The resulting pellet was resuspended in 250 μl of SDS-PAGE sample buffer.

Crosslinked mu-opioid receptors were subjected to SDS-gel electrophoresis separation on 10 or 12% gels. The electrophoresis was

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performed overnight at constant voltage (30 volts). Each gel was stained, dried, and exposed to Kodak X-OMAT AR film for the time indicated in the figure legend.

[^{35}S]GTPγS Binding to the Receptor Purified G-Protein

The material eluted from the opioid affinity column with morphine and GTP was dialyzed exhaustively against 50 mM K^+HEPES, 2 mM EDTA, 50 mM $MgCl_2$, 100 mM NaCl, 0.1% Lubrol PX, 1 μM leupeptin, 1 mM DTT, 0.1 mM PMSF (Buffer G). [^{35}S]GTPγS Binding was determined essentially as described by Northup et al. (Northup et al., 1982). The material was then incubated with 1 μM [^{35}S]GTPγS (specific activity 2538 ± 481 dpm/pmole) [^{35}S]GTPγS. Non-specific binding was determined in the presence of 10 μM GTPγS. The samples were incubated for 1 hour at 30C. Separation of bound from free was by Sephadex G50 gel filtration (0.7 x 14 cm). The void volume fractions containing the G-protein was collected, and the [^{35}S] content was determined by liquid scintillation spectrometry.

Pertussis Toxin Mediated ADP-Ribosylation of the Receptor Purified G-Protein

The mu-opioid receptor/G-protein complex was affinity purified using β-endorphin-AH Sepharose as described above. Pertussis toxin treatment of the eluted proteins of the affinity column and the membrane-associated receptors was as described (Birnbaumer et al., 1986). Briefly, intact membranes or an aliqout of the affinity purified receptor/G-protein complex were incubated in the presence of 10 mM thymidine, 1 mM ATP, 0.1 mM GTP, 1 mM EDTA, and 25 mM Tris-HCl pH 7.0,

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and 3.0 μCi [^{32}P]NAD, in the absence or presence of pertussis toxin and as described in the legend to figure 29. The samples were incubated at 37 C for 30 min. 10 μg of BSA was added to each sample; TCA was added to attain a final concnetraion of 10%. Each tube was then incubated for 1 hour on ice. Following microcentrifugation each pellet was washed with 1 ml of 5% TCA, and microfuged again. Each pellet was resuspended in 40 μl of 1 N NaOH and 40 μl of 2x SDS-PAGE sample buffer and was subjected to electrophoresis at 35 volts, overnight. The dried gel was exposed to Kodak X-OMAT AR film for the time indicated in the figure legend.

Western Analysis of the Receptor Purified G-Protein

7315c cell membranes were washed and resuspended in Buffer F. The mu-opioid receptor/G-protein complex was affinity purified as described above. The receptor/G-protein complex was eluted from the affinity resin using 10 μM morphine and 100 μM GTP. The volume of the eluted material was reduced to 1 ml, and dialyzed overnight in 50 mM Tris-HCl, pH 8.0, 1 mM EDTA, 6 mM MgSO4, 0.1% lubrol PX, 0.1 mM PMSF, 1 μM leupeptin, and 1 mM dithiothreitol (Buffer H). Dialyzed samples were frozen in liquid nitrogen. Ten samples of purified material were thawed, combined, and dried under reduced pressure and centrifugation (15,000 rpm). Each dried sample of affinity purified material and washed membranes were resuspended in SDS-PAGE sample buffer. Both affinity purified and membrane samples were subjected to electrophoresis on 10% "low" bis-acrylamide (final 0.13 g/100 ml) gels. Western blotting was performed as described (Gierschik et al., 1985). Briefly, the proteins were transferred to nitrocellulose paper under constant

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current (150 mA) for 12 hr. After the transfer, the paper was incubated in 10 mM Tris-HCl (pH 7.5) and 100 mM NaCl supplemented with 3% gelatin to block nonspecific protein binding. The paper was cut into strips and incubated for 4-24 hours with 1-5 μg/ml of the affinity antiserum or with a 1:250 dilution of the antisera for the β-subunit. For detection of immunoreactivity of the membrane associated G-proteins, the paper was washed extensively and incubated with second antibody (1 μg of peroxidase-conjugated goat IgG per ml) for 2 hr at room temperature. After extensive washing, the paper was stained in 8.3 mM Tris-HCl (pH 7.5), 415 mM NaCl, 20% methanol, 0.015% H_2O_2, and 0.5 mg 4-chloro-1-naphthol per ml for 10 min at room temperature. For detection of immunoreactivity of the affinity purified G-proteins, each paper strip was exposed to the appropriate antisera for 4-24 hrs, washed and then incubated with ^{125}I-protein A in 50 ml of Tris-buffered saline supplemented with 1% gelatin for 45 min at room temperature. Each paper strip was washed extensively with Tris-buffered saline supplemented with 0.05% Tween 20 and dried. Autoradiography was performed using Kodak XAR film for the time indicated in the figure legend.

Determination of Protein

Concentration of protein in 7315c cell membranes was determined by the Bradford assay (Bradford, 1976); the protein in the affinity purified material was determined by the Amido Black assay (Schaffner and Weissmann, 1973).

Statistics

Unless stated otherwise, all data is depicted as the mean ± S.E.M.

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RESULTS

I. Studies of the agonist induced changes in the interaction of the inhibitory G-protein for guanine nucleotides.

Rationale: The current model for receptor-mediated stimulation of adenylyl cyclase suggests that agonist-induced activation of the stimulatory receptor results in an enhanced interaction of receptor with stimulatory G-protein, Gs. This interaction modifies the protein conformation of Gs so that exchange of inactive for active guanine nucleotide occurs. GTP, once bound, activates Gs and Gs·GTP stimulates adenylyl cyclase. This model has been generally assumed to hold for the interaction of an receptor with Gi although there is no experimental evidence to substantiate this assumption. Therefore, using the model of mu-opioid receptor mediated inhibition of adenylyl cyclase activity in 7315c cell membranes, the following experiments were designed to determine the effect of morphine, an opioid agonist, acting at the mu-opioid receptor to affect the exchange of guanine nucleotides at the inhibitory GTP-binding protein.

Use of AppNHPp as a Substrate to Measure Inhibition of Adenylyl Cyclase Activity.

AppNHp was used as a substrate for adenylyl cyclase (Salomon et al., 1975). Basal and forskolin-stimulated adenylyl cyclase activity were linear for up to 12 min at tissue concentrations between 1 and 4 mg protein/ml. Under these assay conditions GDP was not phosphorylated to

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GTP (table 1). In a previous study, GTP was shown to cause a concentration-dependent inhibition of adenylyl cyclase activity in the presence of morphine when ATP was used as the substrate for cyclase; the IC_{50} was determined to be approximately 1 μM (Frey and Kebabian, 1984). Using AppNHp as the substrate, the IC_{50} of GTP was determined to be 0.69 ± 0.14 μM (figure 3, panel A). In contrast, GDP was unable to support morphine-induced inhibition of adenylyl cyclase activity (figure 3, panel B). However GDP was able to shift the concentration-response curve of GTP to the right; a 7.1 ± 1.2 fold higher concentration of GTP ($p < 0.05$ vs no GDP) was required to inhibit adenylyl cyclase activty when 100 μM GDP was included in the assay (figure 4, panel A). In the presence of morphine and 10 μM GTP, GDP caused a concentration-dependent blockade of the morphine-induced GTP-dependent inhibition of adenylyl cyclase (figure 4, panel B). Half-maximal blockade occurred at a GDP concentration of 81 ± 10 μM. These data are consistent with the hypothesis that the hydrolysis of GTP to GDP at Gi terminates the activation of Gi by GTP.

Agonist Effect on Guanine Nucleotide Potency at Gi

It has been proposed that the primary effect of an agonist is to enhance the exchange of GDP for GTP at the G protein (Rodbell, 1980; Birnbaumer et al., 1985; Gilman, 1987). Therefore, it was of interest to determine if stimulation of the mu-opioid receptor could influence the interaction of either GTP or GDP with Gi as assessed by changes in adenylyl cyclase activity. It has been shown previously that G_i can be

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Table 1

PEI Chromatography

Determination of the Conversion of GDP to GTP in the Presence of AppNHp.

	% Conversion to $[^3H]$GTP			
	Control	10 μM GDP	100 μM GDP	100 μM GDP + 10 μM Morphine
Membranes	6.5 ± 3.6	4.8 ± 3.3	3.3 ± 1.8	7.2 ± 3.1
Buffer	1.7 ± 0.2	2.1 ± 0.6	1.6 ± 0.3	1.6 ± 0.3

7315c cell membranes or buffer were incubated in the adenylyl cyclase assay system containing 0.25 mM AppNHp. All samples contained approximately 20,000 cpm (23.3 nM) $[^3H]$GDP. The samples were incubated for 10 min 30 C and were placed on ice. Separation of the $[^3H]$GDP from $[^3H]$GTP was performed using polyethyleneimine thin layer chromatography. Sections of the developed plate containing the $[^3H]$GDP and $[^3H]$GTP were removed and counted for tritium content by liquid scintillation spectroscopy.

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Figure 3. GTP, but not GDP, is Able to Support Morphine-Induced Inhibition of Adenylyl Cyclase Activity.

7315c cell membranes were incucbated with AppNHp and 10μM forskolin. In the absence of morphine neither GTP nor GDP was able to inhibit cyclase activity (panel A, B, ○). In the presence of 10 μM morphine (●) GTP supported inhibition (panel A) whereas GDP was unable to support inhibition (●) (panel B). GTP-dependent inhibition by morphine had an IC_{50} of 0.69 ± 0.14 μM. Figure shown is representative of data (n=3).

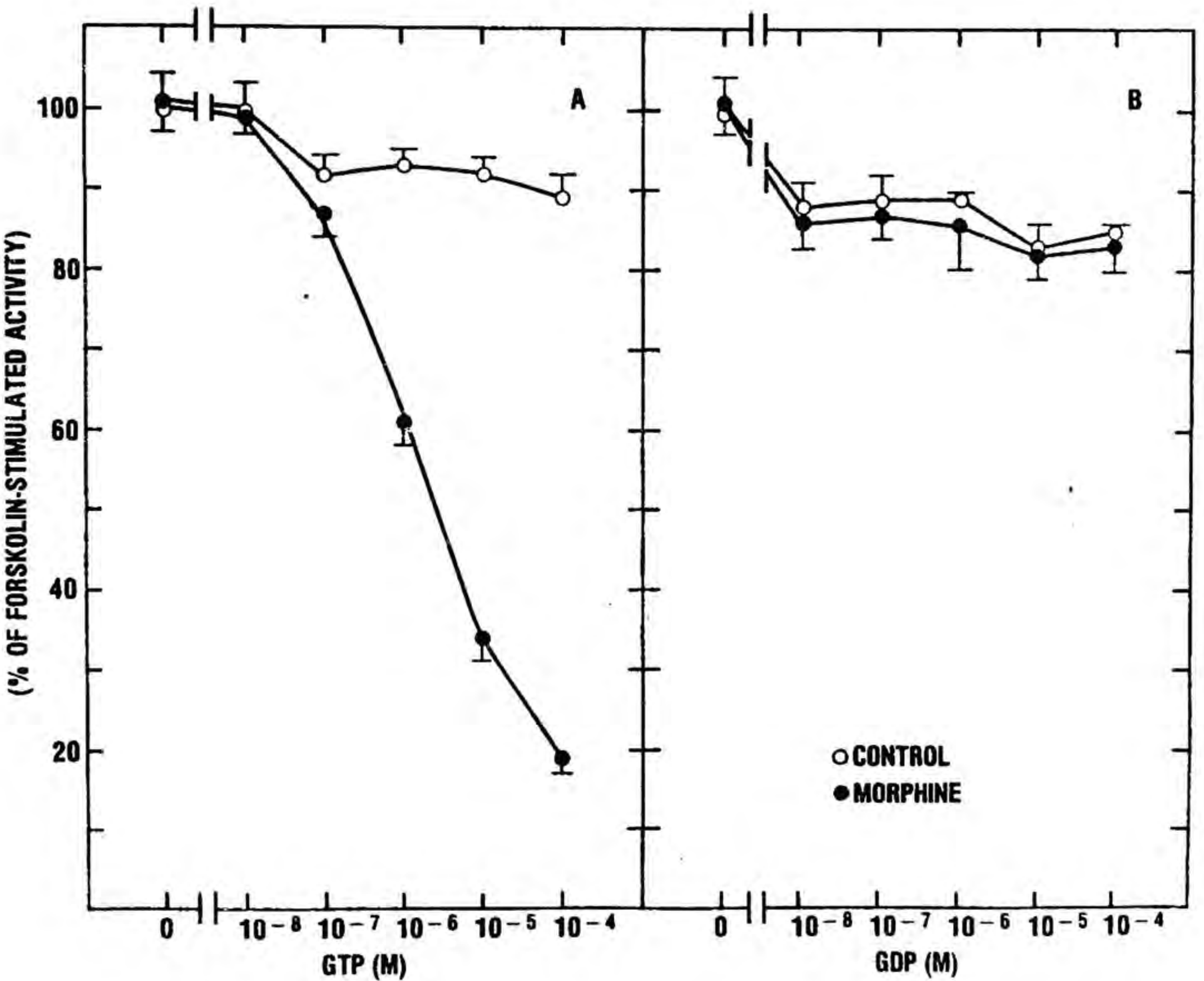

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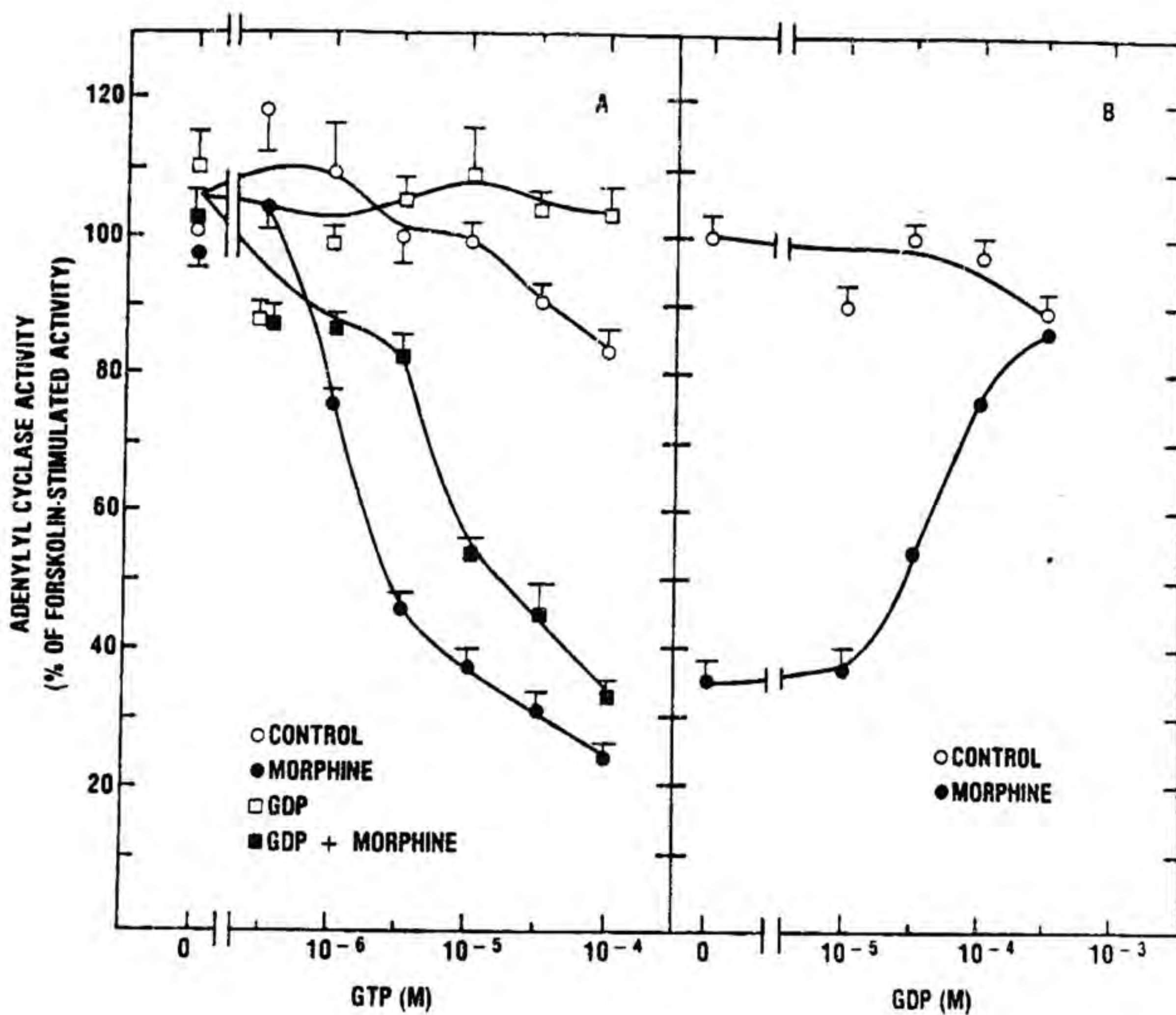
Figure 4. GDP can Competitively Block Morphine-Induced GTP-Dependent Inhibition of Adenylyl Cyclase Activity.

7315c cell membranes were incucbated with AppNHp and 10 μM forskolin. Panel A: GTP was unable to support inhibition of adenylyl cyclase activity in the absence of additions (○), or in the presence of 100 μM GDP (□). GTP in the presence of 10 μM morphine (●) supported inhibition of cyclase activity on a concentration-dependent manner. In the presence of 10 μM morphine plus 100 μM GDP (■), GTP was less potent at sustaining concentration-dependent inhibition of cyclase activity. Panel B: In the presence of 10 μM morphine and 10 μM GTP (●), GDP blocked the agonist-dependent inhibition in a concentration-dependent manner. The EC_{50} for this blockade was 81 ± 10 μM. Figure shown is representative of data (n=3).



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activated by a nonhydrolyzable analog of GTP in the absence of an inhibitory agonist (Coté et al., 1982; Aub et al, 1986). As is shown in figure 5 (inset), GTPγS inhibited forskolin-stimulated adenylyl cyclase activity with IC_{50}s of 45 ± 2.9 and 42 ± 6.5 in the absence and in the presence of 10 μM morphine, respectively. Thus, stimulation of the mu-opioid receptor had no significant effect on the activation of G_i by GTPγS.

Although morphine failed to affect the ability of GTPγS to inhibit forskolin-stimulated adenylyl cyclase activity, morphine diminished profoundly the ability of GDP to block the inhibitory effect of GTPγS (figure 5, GDP vs GDP plus morphine, $p < 0.05$). GTPγS inhibited adenylyl cyclase activity with IC_{50}s of 0.02 ± 0.01 μM, 0.18 ± 0.04 μM, and 2.19 ± 0.47 μM, in the absence of other drugs, in the presence of a combination of 100 μM GDP and morphine, and in the presence of 100 μM GDP, respectively. Morphine had a similar effect on the abililty of GppNHp, another nonhydrolyzable analog of GTP, to inhibit forskolin stimulated adenylyl cyclase activity (figure 6); GppNHp inhibited adenylyl cyclase activity with IC_{50}'s of 0.13 ± 0.04 μM, 2.0 ± 1.5 μM and 19 ± 5.9 μM, in the absence of drugs, in the presence of a combination with 100 μM GDP and morphine, and in the presence of 100 μM GDP, respectively.

The ability of GDP to cause a concentration-dependent blockade of the GTPγS-induced inhibition of forskolin-stimulated adenylyl cyclase activity was also investigated in the absence and presence of morphine; the EC_{50}s of the GDP were 16 ± 2.6 μM and 170 ± 32 μM in the absence and in the presence of morphine, respectively (figure 7, panel A; $p < 0.05$).

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Figure 5. Morphine does not Affect the Potency of GTPγS, an Active Guanine Nucleotide, but does Affect the potency of GDP, the Inactive Guanine Nucleotide, in Affecting Adenylyl Cyclase Activity.

7315c cell membranes were incubated in the presence of AppNHp and 10 μM forskolin. GTPγS can inhibit forskolin-stimulated adenylyl cyclase activity in the absence of agonist (○); the measured IC_{50} was 0.02 ± 0.01 μM. In the presence of 10 μM morphine plus 10 μM GDP (■) or in the presence of 10 μM GDP alone (△); the measured IC_{50}s were 0.18 ± 0.04 and 2.19 ± 0.47 μM, respectively. Inset: GTPγS can inhibit forskolin-stimulated adenylyl cyclase cyclase activity in the absence of agonist (○); the measured IC_{50} was 45 ± 2.9 nM. GTPγS in the presence 10 μM morphine (●) inhibited forskolin-stimulated cyclase activity with a measured IC_{50} of 42 ± 6.5 nM. Figure shown is representative of data (n=3).

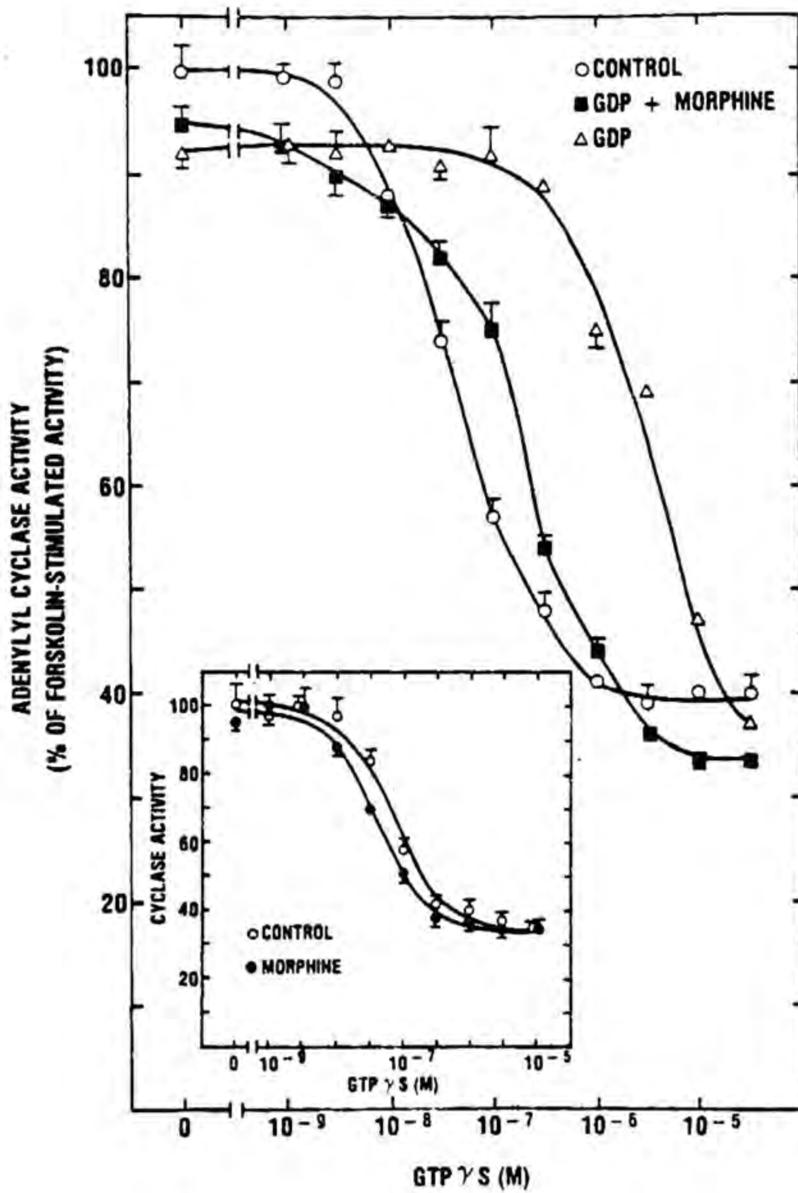

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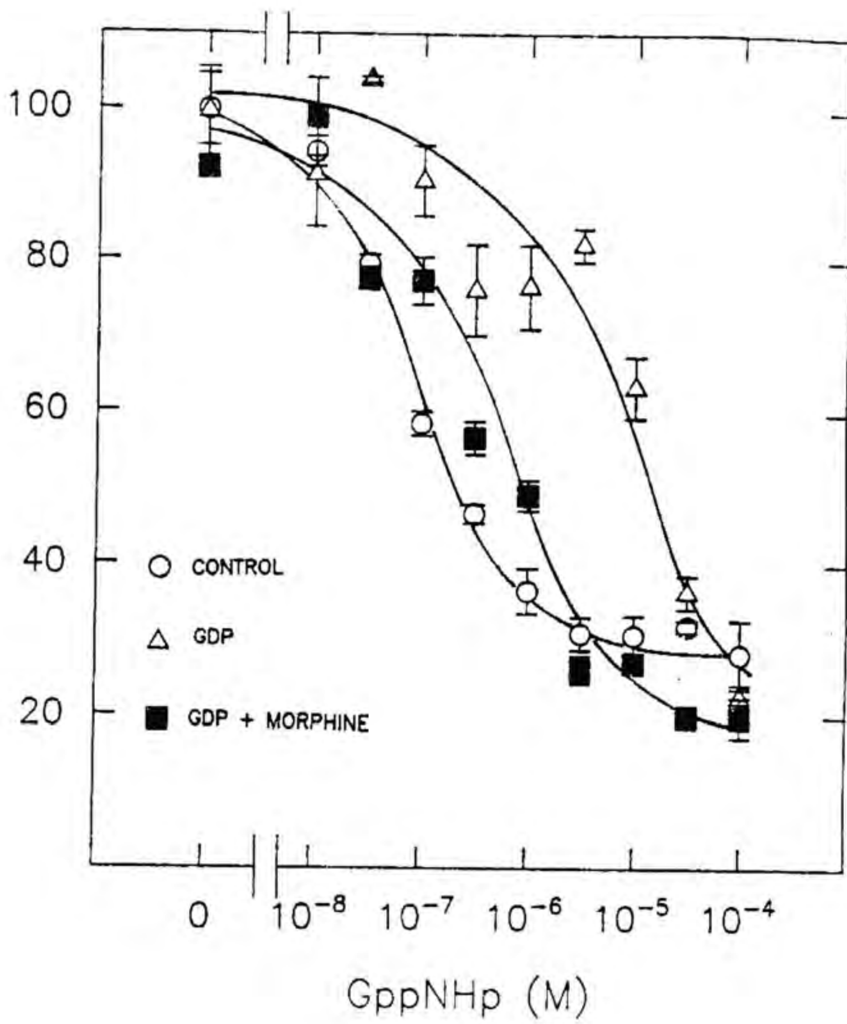
Figure 6. Morphine does not Affect the Potency of GppNHp, an Active Guanine Nucleotide, but does Affect the Potency of GDP, in Affecting Adenylyl Cyclase Activity.

7315c cell membranes were incubated in the presence of AppNHp and 10 μM forskolin. GppNHp inhibited forskolin-stimulated cyclase activity in the absence of drug (○) with a measured IC_{50} of 0.13 ± 0.04 μM. The measured IC_{50}s were 2.0 ± 1.5 μM in the presence of 10 μM GDP plus 10 μM morphine (■) and 19 ± 5.9 μM in the presence of 10 μM GDP alone (△). Figure shown is representative of data (n=3).



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GDPβS, an analog of GDP that is a poor substrate for phosphorylation, was also tested for its ability to block the inhibitory effect of GTPγS (figure 7, panel B); the EC_{50}s of GDPβS were 50.7 ± 5.5 μM and 290 ± 85 μM in the absence and in the presence of morphine, respectively ($p < 0.05$). Thus, stimulation of the mu-opioid receptor diminished approximately 10-fold the potency of GDP in blocking the interaction of GTPγS with G_i. The ability of GDP (10μM) to block GTPγS-induced inhibition of adenylyl cyclase activity was linear during the first 12 min of the assay (figure 7, inset).

Effects of Pertussis Toxin Treatment on the Potency of Guanine Nucleotides at G_i.

Since agonist activation of the mu-opioid receptor caused a 10-fold decrease in the ability of GDP to interact with G_i, we speculated that the opioid receptor itself (unoccupied by an agonist) may have a negative influence in the interaction of GDP and Gi. Previous studies have suggested that pertussis toxin, via ADP-ribosylation of the α-subunit of G_i, uncouples G_i from its interaction with the inhibitory receptor but does not prevent guanine nucleotides from interacting with G_i or G_i from inhibiting adenylyl cyclase activity (Cote et al, 1984; Aub et al., 1986). Therefore, pertussis toxin was tested for its ability to affect the interaction of GDP with G_i. As has been shown previously, treatment of 7315c tumor cells for 3 h with pertussis toxin abolished the ability of morphine to inhibit adenylyl cyclase activity (Aub et al, 1986). Pertussis toxin treatment also diminished by

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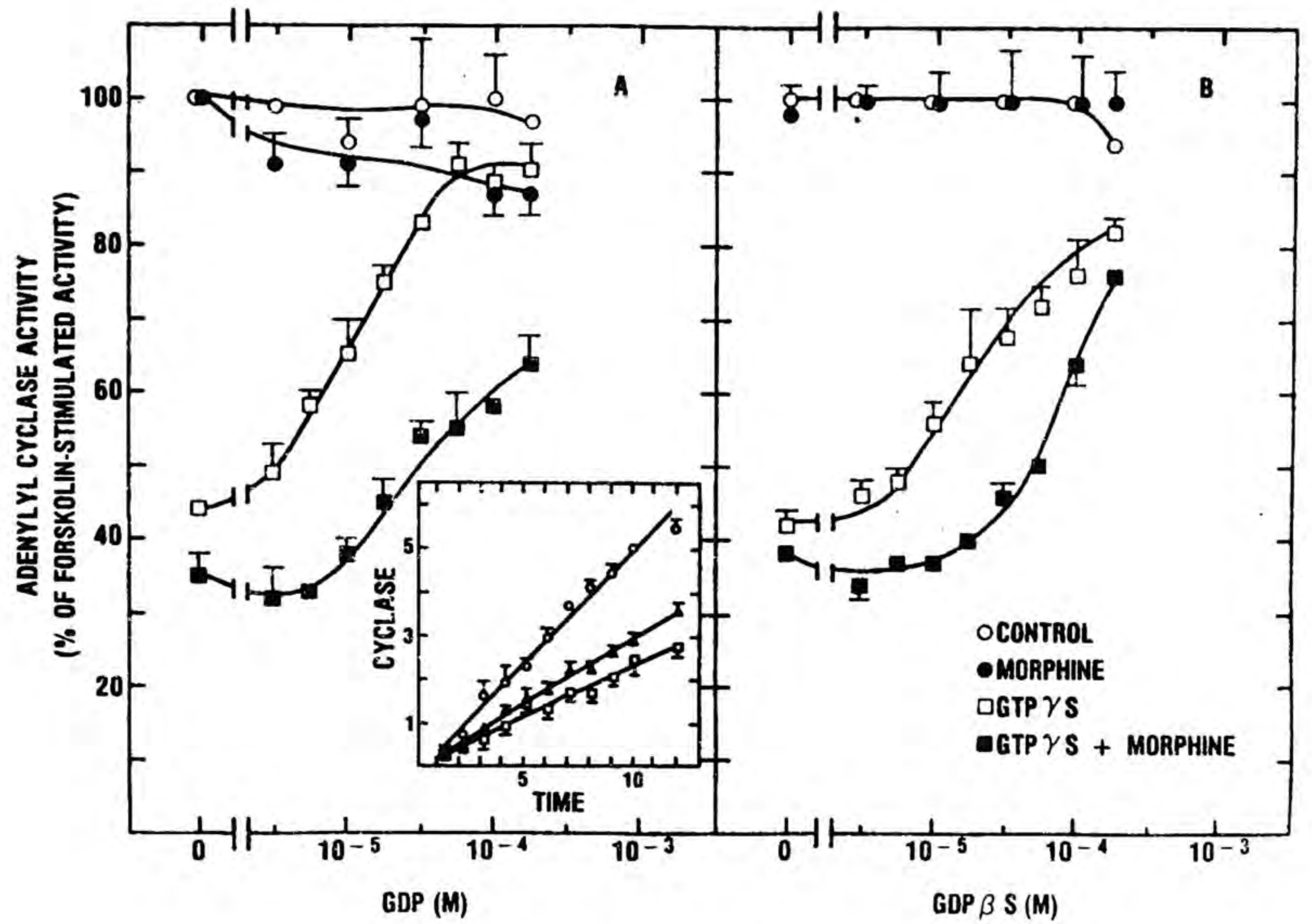
Figure 7. Morphine diminished the Ability of GDP or GDPβS to Block GTPγS-Induced Inhibition of Adenylyl Cyclase.

7315c cell membranes were incubated in the presence of AppNHp and 10 μM forskolin. Panel A: GDP was able to block the GTPγS-induced inhibition of forskolin-stimulated adenylyl cyclase activity (□,■). In the presence of 0.3 μM GTPγS the blockade by GDP had EC_{50}s of 16.0 ± 2.6 μM alone (□) and 170 ± 32 μM in the presence of 10 μM morphine (■). Panel B: GDPβS also blocked GTPγS-induced inhibition of forskolin-stimulated cyclase activity (□,■). In the presence of 0.3 μM GTPγS the blockade by GDPβS had EC_{50}s of 50.7 ± 5.5 μM alone (□) and 290 ± 85 μM in the presence of 10 μM morphine (■). In the absence of GTPγS, both GDP and GDPβS in the absence (○) or presence of morphine (●) were unable to support inhibition of adenylyl cyclase activity. Inset: 10 μM Forskolin-stimulated cyclase activity alone (○), in the presence of 0.3 μM GTPγS (□), and in the presence of 10 μM GDP and 0.3 μM GTPγS (▲) were linear with respect to time over the incubation period described in Methods. Figures shown are representative of the data (n=3).



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approximately 2-fold the IC_{50} for GTPγS in inhibiting adenylyl cyclase activity (figure 8, panel A). In membranes from cells incubated for 3 h in the absence of pertussis toxin, GTPγS inhibited adenylyl cyclase activity with an IC_{50} of 19 ± 1.5 nM; in membranes prepared from cells incubated for 3 h in the presence of pertussis toxin (30 ng/ml), GTPγS inhibited adenylyl cyclase with an IC_{50} of 44 ± 1.6 nM (figure 9 A; $p < 0.05$ vs pertussis toxin treated). Pertussis toxin also diminished the efficacy of the GTPγS in inhibiting adenylyl cyclase. In membranes from cells incubated for 3 h in the absence of pertussis toxin, GTPγS inhibited forskolin-stimulated adenylyl cyclase activity by 81 ± 1.1 %; in membranes from cells incubated for 3 h in the presence of pertussis toxin, GTPγS inhibited forskolin-stimulted adenylyl cyclase activity by 54 ± 4.4 %. In the same membranes, GDP was tested for its ability to block the inhibitory effect of GTPγS. The EC_{50}'s of GDP in blocking the inhibitory effect of 0.3 μM GTPγS were 11 ± 4 μM and 0.81 ± 0.2 μM in control and pertussis toxin-treated membranes, respectively (figure 8, panel B; $p < 0.05$).

The time course of the inhibitory effect of GTPγS on forskolin-stimulated adenylyl cyclase activity was investigated (figure 9, panel A). In the absence of GTPγS forskolin caused a linear stimulation of adenylyl cyclase activity; cAMP was formed at the rate of 64 ± 6.1 pmoles cAMP $\cdot$ mg protein^{-1} $\cdot$ min^{-1}. The inclusion of GTPγS diminished the rate of formation of cAMP. During the first three min of the assay, cAMP was formed at a rate of approximately 41 ± 10 pmoles cAMP $\cdot$ mg protein^{-1} $\cdot$ min^{-1}; between min 3 and 10, cAMP was formed at a rate of approximately 22 ± 7.5 pmoles cAMP $\cdot$ mg protein^{-1} $\cdot$ min^{-1}. The time

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Figure 8. GTPγS Inhibited Adenylyl Cyclase Activity in the Absence of Agonist in Both Control and Pertussis Toxin-Treated Membranes; Toxin-Treatment Enhanced the Association of GDP for Gi.

7315c cells were incubated in the absence or presence of 30 ng/ml pertussis toxin. Membranes of control (circles) and toxin-treated (squares) cells were incubated in the presence of 0.25 mM AppNHp and 10 μM forskolin. Panel A: GTPγS inhibited forskolin-stimulated adenylyl cyclase activity in the absence of agonist in control membranes (●); the measured IC_{50} was 19 ± 1.5 nM. In toxin-treated membranes, GTPγS inhibited forskolin-stimulated cyclase activity with a measured IC_{50} of 44 ± 1.6 nM (■). Panel B: GDP was able to competitively block the GTPγS-induced inhibition of forskolin-stimulated adenylyl cyclase activity (●, ■). In the presence of 0.3 μM GTPγS, the EC_{50}s of the blockade by GDP were 11.0 ± 4 μM in control membranes (●) and 0.81 ± 0.2 μM in toxin-treated membranes (■). GDP, in the absence of GTPγS, was unable to support inhibition of adenylyl cyclase activity in control or toxin-treated membranes (○, □). Figures shown are representative of the data (n=3).

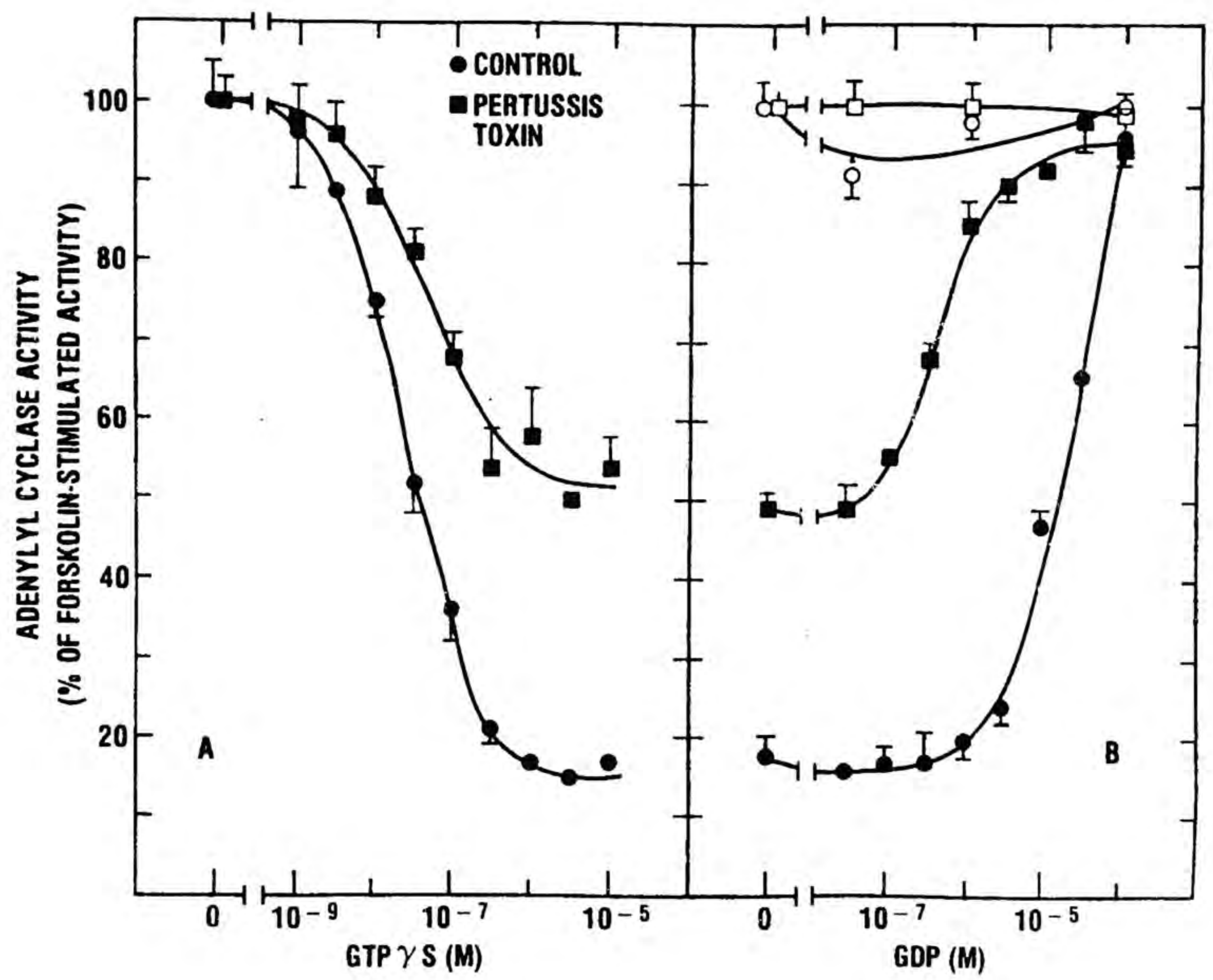
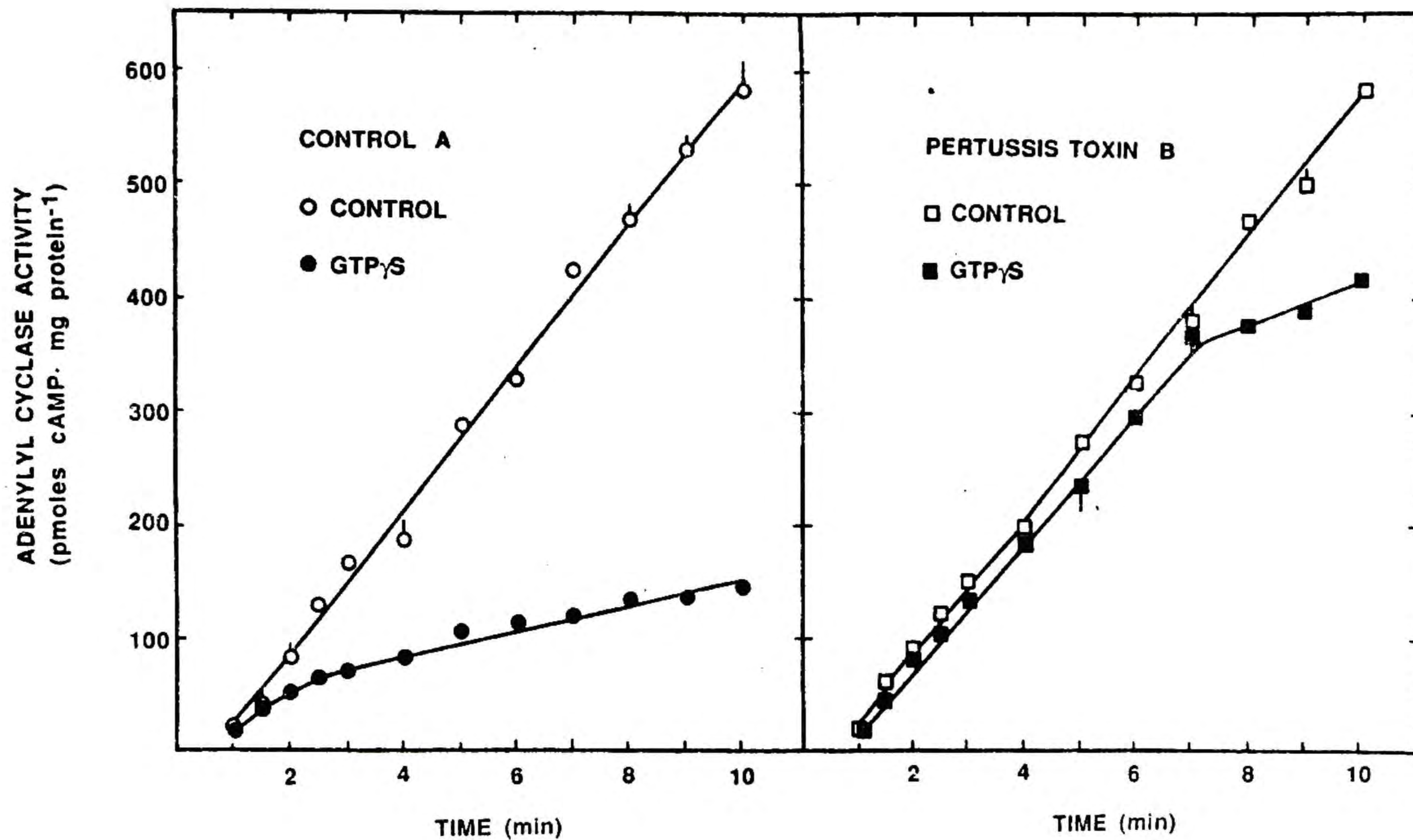

ADENYLYL CYCLASE ACTIVITY
(% OF FORSKOLIN-STIMULATED ACTIVITY)
100
80
60
40
20
CONTROL
PERTUSSIS TOXIN
A
B
0
10 − 9
10 − 7
10 − 5
GTP γ S (M)
0
10 − 7
10 − 5
GDP (M)

Figure 9. Pertussis Toxin-Treatment Enhanced the Lag of GTPγS-Induced Inhibition of Adenylyl Cyclase Activity.

7315c cells were incubated in the absence or presence of 30 ng/ml pertussis toxin. Membranes of control (panel A) and toxin-treated (panel B) cells were incubated in the presence of 0.25 mM AppNHp plus either 10 μM forskolin (○,□) or 10 μM forskolin and 0.3 μM GTPγS (●,■), and measured for adenylyl cyclase. Panel A: In control membranes, adenylyl cyclase activity was linear in the presence of forskolin alone, and after an initial lag, in the presence of forskolin and GTPγS. At 10 min, in the presence of GTPγS, forskolin-stimulated adenylyl cyclase activity was inhibited by 71%. Panel B: In pertussis toxin-treated membranes, adenylyl cyclase activity was linear in the presence of forskolin alone, and after an exaggerated lag, in the presence of forskolin and GTPγS. At 10 min, in the presence of GTPγS, forskolin-stimulated adenylyl cyclase activity was inhibited by 38%.



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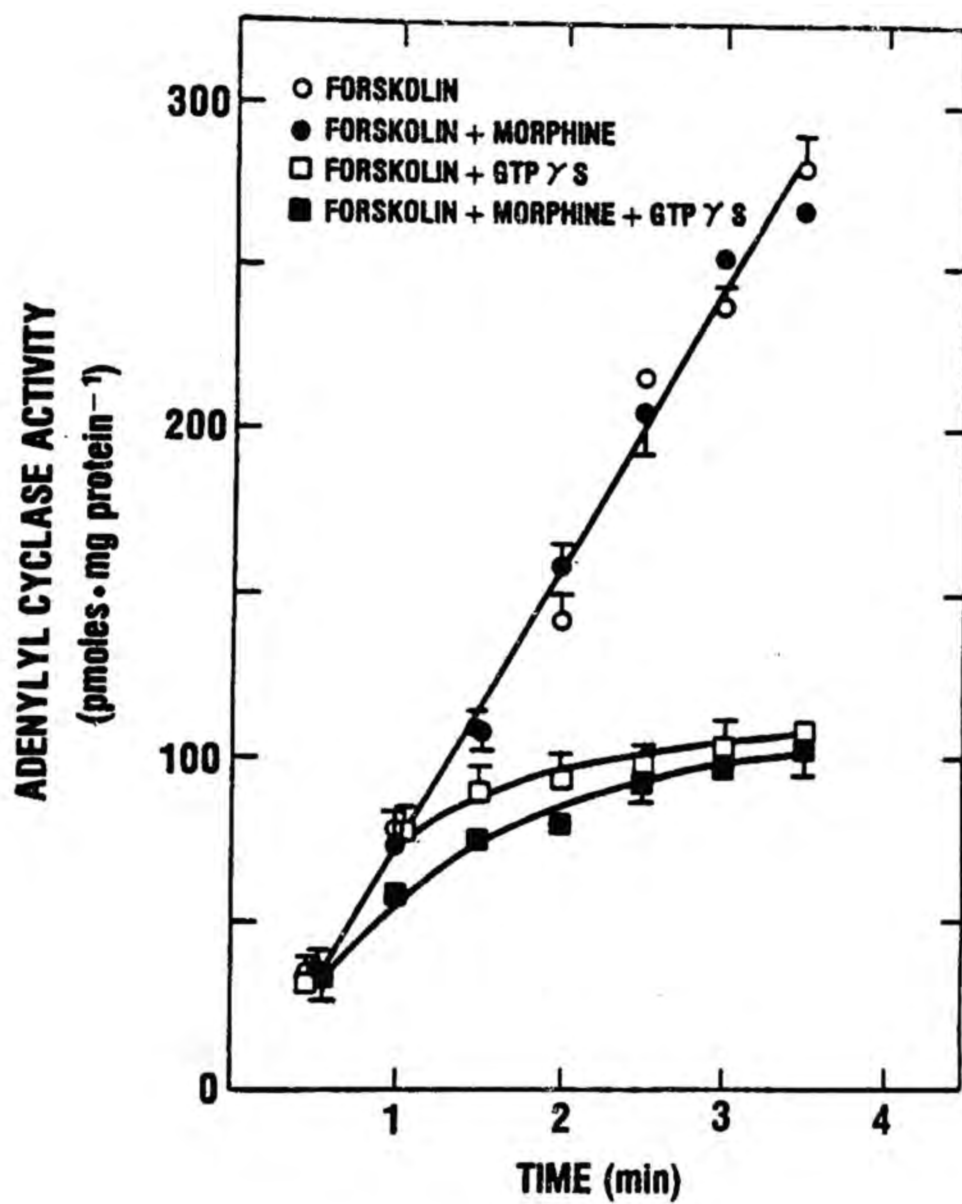
course of the inhibitory effect on forskolin-stimulated adenylyl cyclase activity was investigated in pertussis toxin-treated membranes (figure 9, panel B). In the absence of GTPγS, forskolin caused a linear stimulation of adenylyl cyclase activity; cAMP was formed at a rate of 55 ± 2.7 pmoles cAMP · mg protein^{-1} · min^{-1}. In the presence of GTPγS, there was a prolonged lag before GTPγS caused a susbstantial inhibition of adenylyl cyclase activity. During the first 7 min of the assay, cAMP was formed at the rate of 44 ± 2.6 pmoles cAMP · mg protein^{-1} · min^{-1} in the presence of GTPγS; from min 7 through 10, cAMP was formed at the rate of 17 ± 1.8 pmoles cAMP · mg protein^{-1} · min^{-1}. Thus, in pertussis toxin-treated membranes, there was a prolonged lag in the GTPγS-induced inhibition of adenylyl cyclase activity. This lag accounts for the diminished efficacy of GTPγS in inhibiting adenylyl cyclase activity in pertussis toxin-treated membranes and is consistent with the proposal that GDP is bound with greater avidity to G_i in pertussis toxin-treated membranes than in control membranes.

Morphine was tested for its ability to affect the lag in the GTPγS-induced inhibition of adenylyl cylcase activity (figure 10). In these experiments, ATP was used as the substrate for adenylyl cyclase to enhance the level of cAMP formed at the early time points. In this study, morphine had no effect on the formation of cAMP in the presence of forskolin, but significantly enhanced the inhibition of adenylyl cyclase activity by GTPγS at 1 min.

Figure 10. Morphine Diminishes the Lag in the GTPγS-Induced Inhibition of Adenylyl Cyclase Activity.

7315c cell membranes were preincubated for 2 min in the absence (○,□) or presence (●,■) of 10 μM morphine. The adenylyl cylase assay was initiated by the addition of either 0.25 mM ATP and 10 μM forskolin (circles) or a combination of 0.25 mM ATP, 10 μM forskolin, and 10 μM GTPγS (squares). Triplicate samples of each assay condition were placed in a 100 C water bath at the indicated time points. Data represent the mean ± SEM (n=3) of a single time experiment representative of three.



FORSKOLIN
FORSKOLIN + MORPHINE
FORSKOLIN + GTP γ S
FORSKOLIN + MORPHINE + GTP γ S
ADENYLYL CYCLASE ACTIVITY
(pmoles • mg protein⁻¹)
300
200
100
0
1
2
3
4
TIME (min)

II. Studies of the Mu-Opioid Receptor Interaction with the Inhibitory G-Protein in 7315c Cell Membranes.

Rationale: Characterization of the mu-opioid receptor/G-protein interaction in 7315c cell membranes is a necessary preliminary step in the experimental plan to co-solubilize and co-purify these two protiens. The characterization of the receptor/G-protein interaction can be assessed in membranes by demonstrating the effects of guanine nucleotides on receptor affinity for agonists. Additionally, since GTP is required for sustaining receptor mediated inhibition of adenylyl cyclase activity, these studies are aimed at determining the correlation of the potency of GTP to diminish agonist affinity with the potency of GTP to sustain morphine-induced inhibition of cyclase activity.

The Affinity of Etorphine for the Mu-Opioid Receptor is Higher than its IC_{50} of Inhibition of Cyclase Activity.

[^{3}H]Etorphine has been shown to bind to mu-opioid receptors on the 7315c membranes in a time and tissue linear fashion (Puttfarcken et al., 1986). Therefore, [^{3}H]etorphine was allowed to incubate in the absence or presence of 100 µM morphine, for 30 min at 37 C. Saturation binding demonstrated, specific, one site ligand binding (figure 11). The affinity was analyzed via the computer program LIGAND (Munson and Rodbard, 1980); the Kd was 0.53 ± 0.25 nM and the Bmax was 57.5 ± 20 fmoles (mg protein)$^{-1}$ (n=3). The affinity of [^{3}H]etorphine for the mu-opioid receptor was approximately 10 times higher than the IC_{50} value of etorphine-mediated inhibition of adenylyl cyclase activity (figure 12).

Figure 11. Saturation Binding of [^{3}H]Etorphine to 7315c Cell Membranes.

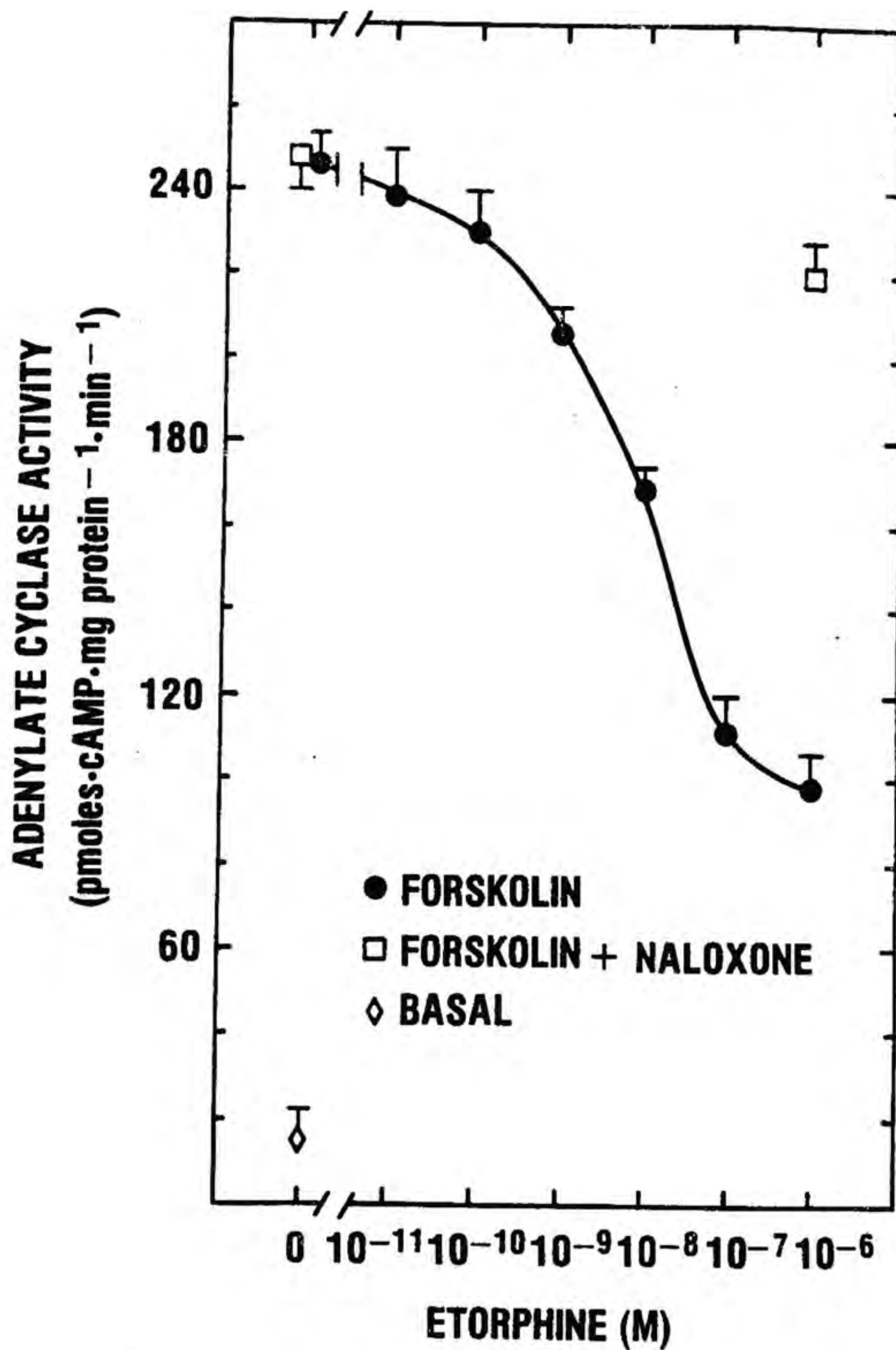
Panel A: 7315c cell membranes were incubated in the presence of the indicated concentrations of [^{3}H]etorphine (total binding, ●). Nonspecific binding was determined in the presence of 100 μM morphine (○). Nonlinear parametric analysis of the saturation curve data (Munson and Rodbard, 1980) resulted in a one-site model with a calculated Kd of 0.53 ± 0.25 nM and the receptor number was determined to be 57.5 ± 20 fmoles/mg protein. Panel B: Specific binding (□) is shown. Inset: Scatchard analysis of the saturation data is shown. This experiment was conducted in the absence of guanine nucleotides.

[³H] ETORPHINE BINDING (dpm)
7000
5000
3000
1000
2
4
6
8
10
[³H] ETORPHINE (nM)
● TOTAL
○ NONSPECIFIC

SPECIFIC [³H] ETORPHINE BINDING (dpm)
2400
1800
1200
600
2
4
6
8
10
[³H] ETORPHINE (nM)
B/F
100
60
20
40
80
BOUND (pM)

Figure 12. Etorphine Inhibits Adenylyl Cyclase Activity With an IC_{50} Similar to the Binding Affinity of [^{3}H]Etorphine.

7315c cell membranes were assayed in the presence of 0.25 mM ATP, 10 μM GTP (basal, ◇). Etorphine was tested in the presence of 10 μM forskolin (●) or in the presence of a combination of forskolin and 10 μM naloxone (□). Data represent the mean ± SEM from three separate experiments.



ADENYLATE CYCLASE ACTIVITY
(pmoles·cAMP·mg protein − 1·min − 1)
240
180
120
60
FORSKOLIN
FORSKOLIN + NALOXONE
BASAL
0 10−11 10−10 10−9 10−8 10−7 10−6
ETORPHINE (M)

The IC_{50} for etorphine in the adenylyl cyclase assay was 5.1 ± 0.7 nM. The inhibition of adenylyl cyclase activity was blocked when performed in the presence of 10 μM naloxone.

Guanine Nucleotide Effects on Mu-Opioid Agonist Affinity

Mu-opioid receptors on 7315c membranes were prelabeled with $[^3H]$etorphine and incubated with 100 μM naloxone (to prevent $[^3H]$etorphine reassociation) to measure agonist off-rate as described in Methods. GTP enhanced the dissociation of prebound $[^3H]$etorphine from 7315c membranes (figure 13). In the absence of GTP, 30% of $[^3H]$etorphine dissociated from membranes receptors in 15 min at 37 C. An additional 35% of the $[^3H]$etorphine dissociated in the presence of 100 μM GTP during a 15 min incubation. The half-time for the effect of GTP was 9.3 ± 1.8 min. The enhancement of the off-rate of $[^3H]$etorphine by GTP was consistent with the decreased opioid receptor affinity in the presence of GTP (Frey et al., submitted). The potencies of various GTP analogs in causing dissociation of $[^3H]$etorphine from receptors were measured (figure 14). The order of potencies was GTPγS (0.2 ± 0.05 μM) > GTP (8.5 ± 2.6 μM) > GDP (20.6 ± 6.8 μM). The maximal effects of these guanine nucleotides were approximately the same.

It has been shown that $[^3H]$diprenorphine binds to the mu-opioid receptor on 7315c membranes with an affinity of approximately 1 nM (Puttfarcken et al., 1986). The effects of GTPγS and pertussis toxin treatment on agonist receptor affinity was assessed by measuring the ability of morphine to displace 1 nM $[^3H]$diprenorphine (figure 15). In

Figure 13. [^{3}H]Etorphine Dissociation from the Mu-Opioid Receptor is Enhanced by GTP.

7315c cell membranes were incubated with a saturating concentration of [^{3}H]etorphine as described in Methods. The prebound membranes were further incubated in the absence (○) or presence (●) of 100 μM GTP. Specific [^{3}H]etorphine binding was measured at the indicated time points. In the absence of GTP, 70% of the specific prebound [^{3}H]etorphine remained associated with the mu-opioid receptor after the 30 min incubation. In the presence of GTP, 35% of the prebound [^{3}H]etorphine remained associated; the half-time of dissociation was 9.3 $\pm$ 1.8 min.

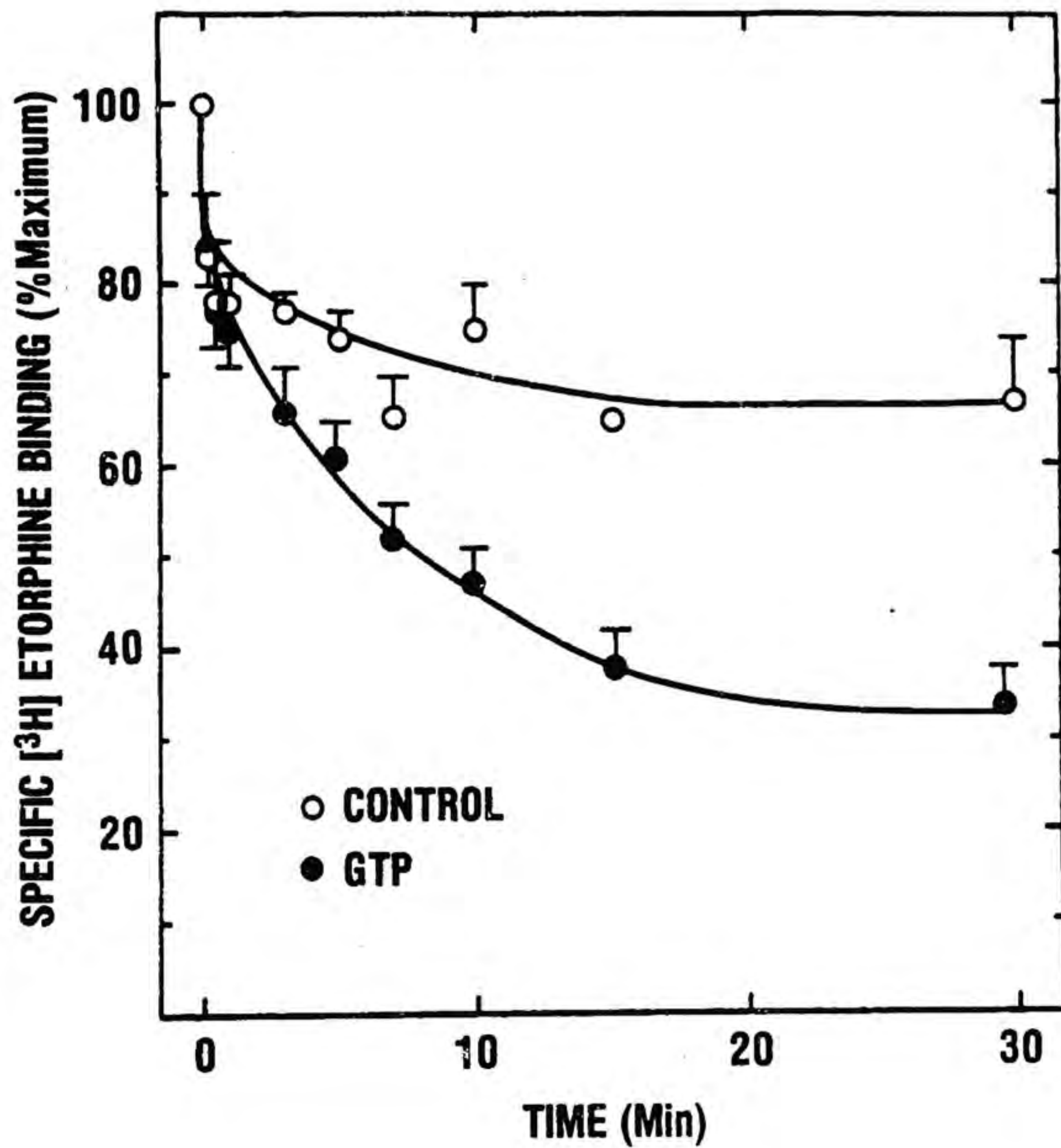
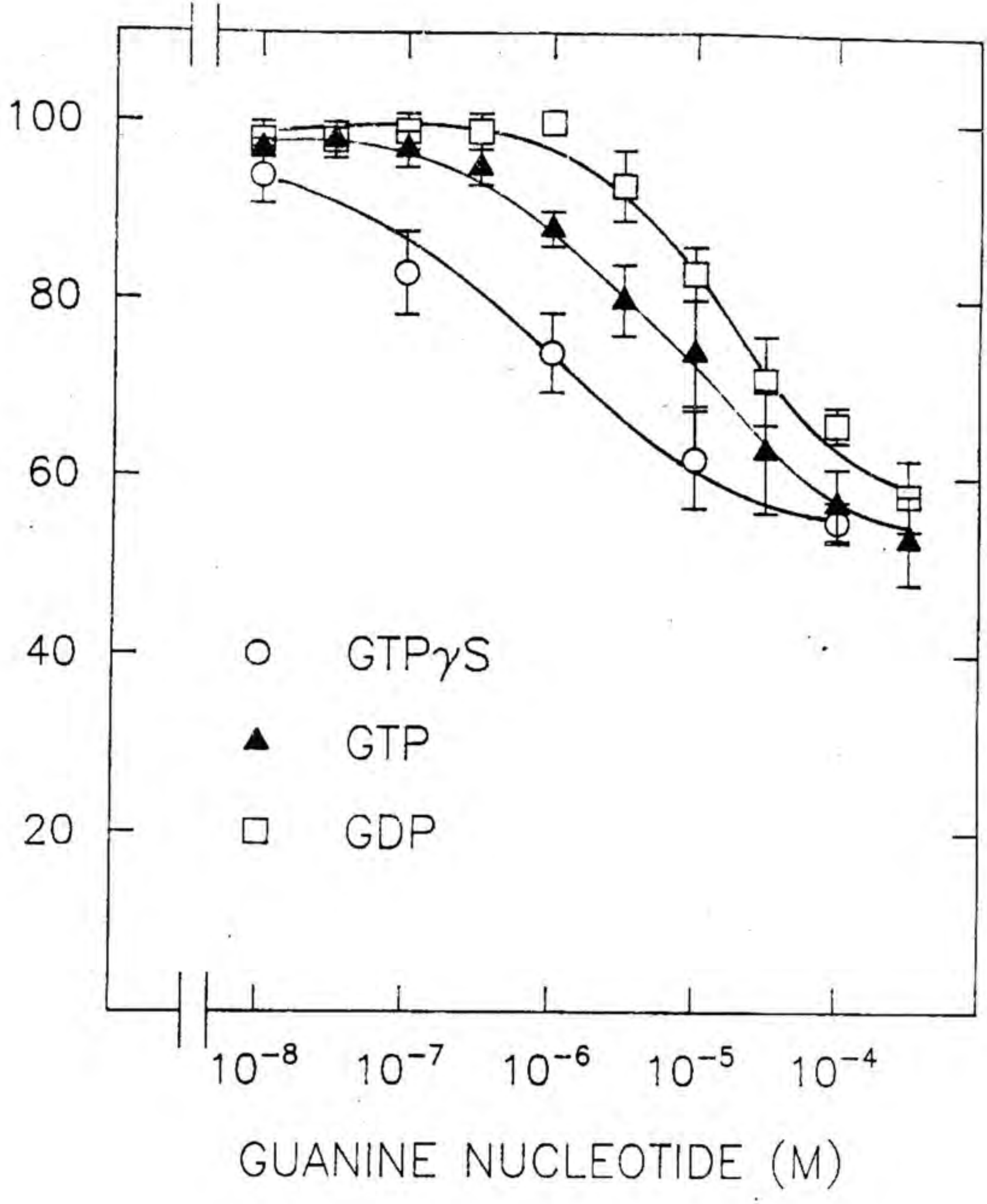

SPECIFIC [3H] ETORPHINE BINDING (%Maximum)
100
80
60
40
20
0
10
20
30
TIME (Min)
CONTROL
GTP

Figure 14. Guanine Nucleotides Enhance [^{3}H]Etorphine Dissociation from Mu-Opioid Receptors in a Concentration-Dependent Manner.

7315c cell membranes were incubated with a saturating concentration of [^{3}H]etorphine as described in Methods. The prebound membranes were further incubated in the presence of the indicated concentrations of guanine nucleotides. The EC_{50}s of the enhanced [^{3}H]etorphine off-rate were 0.2 $\pm$ 0.05 in the presence of GTPγS (○), 8.5 $\pm$ 2.6 μM in the presence of GTP (▲), and 20.6 $\pm$ 6.8 μM in the presence of GDP (□). Data represent the mean $\pm$ SEM from three separate experiments.

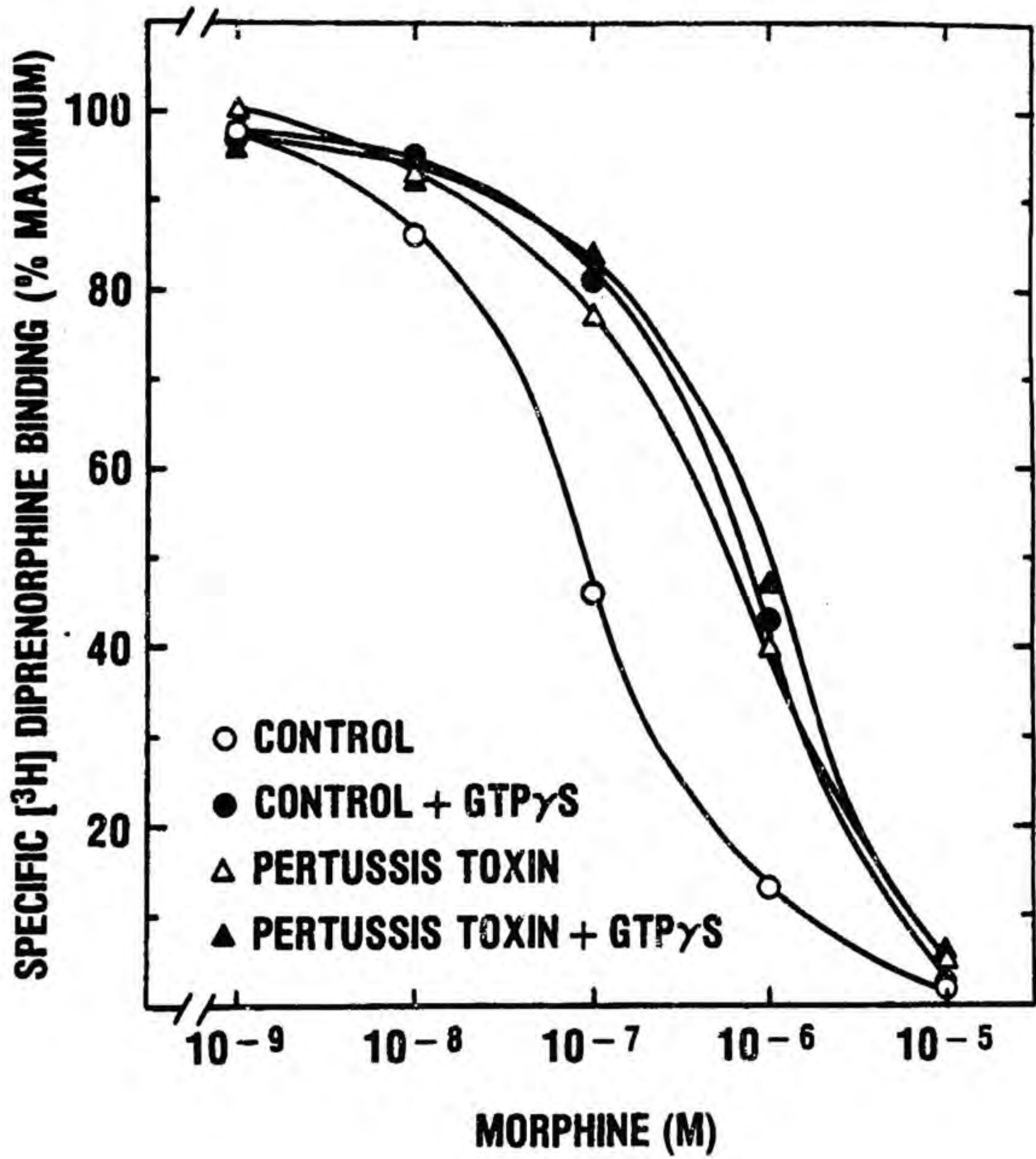

SPECIFIC [3H]ETORPHINE BINDING
(% MAXIMUM)
100
80
60
40
20
GTPγS
GTP
GDP
10−8
10−7
10−6
10−5
10−4
GUANINE NUCLEOTIDE (M)

membranes prepared from untreated cells (figure 15, circles), morphine competed with [^{3}H]diprenorphine for binding to the mu-opioid receptor with a calculated Ki of 17.2 ± 4 nM alone and 141 ± 40 nM in the presence of 10 μM GTPγS (open and filled circles, respectively). Pertussis toxin treatment significantly altered the ability of morphine to compete with [^{3}H]diprenorphine for the mu-opioid receptor and abolished the ability of GTPγS to shift the affinity of the mu-opioid receptor for its agonist (figure 15, triangles). The affinities of morphine for the mu-opioid receptor in pertussis toxin-treated membranes were 110 ± 1 alone or 163 ± 32 nM in the presence of 10 μM GTPγS (open and filled triangles, respectively). Therefore "low" affinity agonist binding occurs in the presence of guanine nucleotides or when the receptor is unable to interact with Gi as seen with pertussis toxin treatment.

The potency of morphine in competing with [^{3}H]diprenorphine for binding to the mu-opioid receptor in the presence of either 0, 1, or 10 μM GTP was compared to the potency of morphine in inhibiting adenylyl cyclase activity in the presence of either 0, 1, or 10 μM GTP (figure 16). Morphine competed with [^{3}H]diprenorphine for mu-opioid receptors in 7315c cell membranes (figure 16, panel A). GTP decreased the affinity of the mu-opioid receptor for morphine in a concentration-dependent manner. A maximal 3-fold effect occurred at a concentration of 10 μM GTP; when the concentration of GTP was increased to 100 μM, no further decrease in affinity for morphine was detected (data not shown). The calculated Ki's of morphine in the presence of 0, 1, or 10 μM GTP were 0.04 ± 0.01, 0.08 ± 0.02, and 0.15 ± 0.05 (n=4), respectively.

Figure 15. GTPγS Decreased the Affinity of Morphine for the Mu-Opioid Receptor. Pertussis Toxin abolished both High Affinity Binding and the Ability of GTPγS to Affect the Affinity of Morphine for the Mu-Opioid Receptor.

7315c cells were incubated for 3 hr in the absence (○,●) or in the presence of 30 ng/ml pertussis toxin (△,▲). Membranes prepared from these cells were incubated with 1 nM [^{3}H]diprenorphine in the absence (open symbols) or presence of 10 μM GTPγS (filled symbols). Nonspecific binding was determined by the addition of 10 μM naloxone. Data are given as a percent specific binding in the absence of any additions. Morphine competed with [^{3}H]diprenorphine for the mu-opioid receptor with a calculated affinity of 17.2 ± 4 nM in the absence (○) and 141 ± 40 nM in the presence of 10 μM GTPγS (●). The affinities of morphine for the mu-opioid receptor in the toxin-treated membranes were 110 ± 1 and 163 ± 32 in the absence (△) and presence of 10 μM GTPγS (▲), respectively.

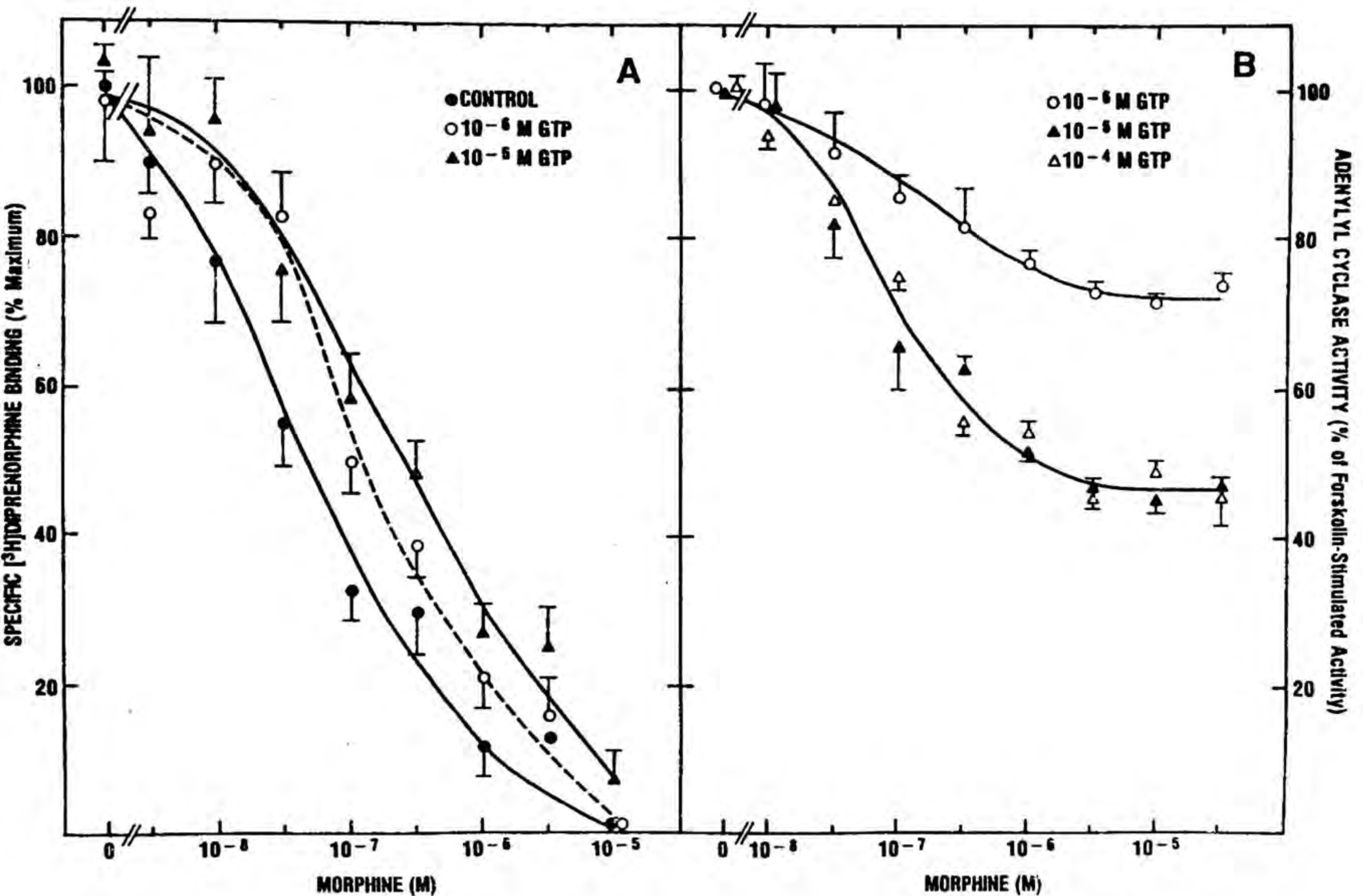

SPECIFIC [3H] DIPRENORPHINE BINDING (% MAXIMUM)
100
80
60
40
20
○ CONTROL
● CONTROL + GTPγS
△ PERTUSSIS TOXIN
▲ PERTUSSIS TOXIN + GTPγS
10 − 9
10 − 8
10 − 7
10 − 6
10 − 5
MORPHINE (M)

The effect of these concentrations of GTP on the potency and efficacy of morphine-inhibiting adenylyl cyclase activity is shown (figure 16, panel B). Opioid inhibition of adenylyl cyclase activity was not observed in the absence of GTP. However, GTP (1-100 μM) had no significant effect, as determined by analysis of variance, on the potency of morphine; the potencies in the presence of 1, 10, 100 μM GTP were 0.11 ± 0.03, 0.08 ± 0.01, and 0.14 ± 0.02 μM, respectively. The potency of morphine in inhibiting adenylyl cyclase activity agreed most closely with the calculated Ki of morphine competing with [^{3}H]diprenorphine for the mu-opioid in the presence of a high concentration of GTP. Therefore, only the "low" affinity agonist binding state of the mu-opioid receptor appears to be involved in receptor-mediated inhibition of adenylyl cyclase activity. These findings explain why etorphine inhibited adenylyl cyclase (in the presence of 10 μM GTP) with a potency that was 10-fold weaker than its binding affinity for the mu-opioid receptor (an assay performed in the absence of GTP, figures 11 and 12).

Figure 16. The Potency of Morphine in Inhibiting Adenylyl Cyclase Activity is Similar to the "Low" Affinity of Morphine in Binding to the Mu-Opioid Receptor.

Panel A: 7315c cell membranes were incubated in the presence of 1 nM [^{3}H]diprenorphine and the indicated concentrations of morphine in the absence (●), or in the presence of 1 μM (○), or 10 μM GTP (▲). Nonspecific binding was determined in the presence of 10 μM naloxone. Data are expressed as percent of maximum specific binding for bound [^{3}H]diprenorphine: 1230, 1242, 1390 dpm for 0, 1, and 10 μM GTP, respectively. Each point represents the mean ± SEM from triplicate samples from an experiment representative of three.

Panel B: 7315c cell membranes were incubated with 0.25 mM ATP, 10 μM forskolin, the indicated concentrations of morphine, and assayed for adenylyl cyclase activity as described in the Methods. In the presence of 1 μM (○), 10 μM (▲) and 100 μM (△) GTP the measured IC_{50}s of morphine-induced inhibition of forskolin-stimulated adenylyl cyclase activity were 110 ± 30, 80 ± 10, and 140 ± 20 nM, respectively. In the presence of 10 μM and 100 μM GTP, the maximal inhibitory effect of morphine was a 43 and 46% inhibition of adenylyl cyclase, respectively. The maximal effect of morphine in the presence of 1 μM GTP was 26% inhibition of adenylyl cyclase.



A
●CONTROL
○10−6 M GTP
▲10−5 M GTP
SPECIFIC [3H]DIPRENORPHINE BINDING (% Maximum)
MORPHINE (M)
B
○10−6 M GTP
▲10−5 M GTP
△10−4 M GTP
ADENYLYL CYCLASE ACTIVITY (% of Forskolin-Stimulated Activity)
MORPHINE (M)
0
10−8
10−7
10−6
10−5
100
80
60
40
20

III. Solubilization and Reconstitution of the Mu-opioid Receptor/G-Protein Complex into Phospholipid Vesicles

Rationale: As a preliminary step in the process of purification of the mu-opioid receptor/G-protein complex from 7315c cell membranes, methods for the detection and characterization of the detergent solubilized proteins were developed. Demonstration of specific mu-opioid receptor binding and the effects of guanine nucleotides on agonist affinity would enable us to monitor the presence of the solubilized receptor and its G-protein. Retention of the active receptor/G-protein complex in detergent solution would make possible the co-purification of these proteins from the 7315c cell membrane.

[^{3}H]Etorphine Binding to Solubilized and Reconstituted Opioid Receptors.

Solubilization of 7315c cell membranes with the detergent CHAPS resulted in low recovery of mu-opioid receptor binding activity that was insensitive to GTP (table 2). Previous studies (Gilman, 1987) have suggested a tighlty associated agonist/receptor/G-protein complex forms in the absence of guanine nucleotides. Therefore, 7315c cell membranes washed free of guanine nucleotides were incubated with [^{3}H]etorphine and then solubilized with CHAPS. 50-70% of the receptors were recovered and were found to be sensitive to guanine nucleotides (Frey et al., 1989). These results suggested that a mu-opioid receptor had been co-solubilized with its G-protein. If 7315c cell membranes were incubated with morphine, solubilized, and then tested for [^{3}H]etorphine binding in the absence or presence of GTP, no specific binding was

Membrane-associated, solubilized, or reconstituted receptors, prepared as described in the Methods, were assayed with 0.8 to 2.0 nM [^{3}H]etorphine in the absence and presence of 100 μM GTP; nonspecific binding was determined by the inclusion of 100 μM naloxone. Membrane-associated or solubilized receptors were incubated at 37 C for 30 min; reconstituted receptors were incubated for 18 h at 4 C. Where indicated, membranes were incubated for 15 min at 37 C with 10 μM morphine prior to and during solubilization (Morphine Pretreatment of Membranes). The data are expressed in terms of mg protein of starting material and represent the mean and S.E.M. from 3 separate experiments. Approximately 8% of the protein in the starting material was recovered after solubilization. It was assumed that 100% of the solubilized protein was recovered when incorporated into the phospholipid vesicles; no actual protein determination was made because bovine serum albumin was a component of the reconstituted vesicles. The specific activity of [^{3}H]etorphine was 69,486 dpm/pmole. There was no specific binding to phospholipid vesicles formed in the absence of material solubilized from membranes.

Table 2

$[^3H]$Etorphine Binding to Membrane-associated, Solubilized, and Reconstituted Receptors from 7315c Membranes

	Specific $[^3H]$Etorphine Binding (fmole/mg protein starting material)		Protein (mg)
Preparation	- GTP	+ GTP	
	No Pretreatment of Membranes		
Membrane-associated	50.8 ± 7.3	32.8 ± 7.5	1.00 ± 0.10
Solubilized	3.0 ± 2.4	2.7 ± 2.2	0.08 ± 0.02
Reconstituted	1.2 ± 0.4	1.4 ± 0.6	0.08 ± 0.02
	Morphine Pretreatment of Membranes		
Solubilized	0	0	0.08 ± 0.02
Reconstituted	20.0 ± 3.0	8.1 ± 0.1	0.08 ± 0.02

detected (table 2). This result indicated that ligand exchange could not occur in detergent solution. Studies of the solubilization and purification of other receptors have shown that following removal of these receptor proteins from the lipid bilayer environments, the receptors must be reinserted into phospholipid vesicles to display appropriate binding activity (Pederson and Ross, 1982; Cerione et al., 1984). Therefore, the next series of experiments were designed to develop a protocol for the co-solubilization and reconstitution of the mu-opioid receptor/G-protein complex.

Specific [^{3}H]etorphine binding to solubilized opioid receptors that were not prebound with agonist was undetectable, even if the solubilized material was reinserted into phospholipid vesicles (table 2). However, if the opioid receptor was prebound with morphine, solubilized, and reinserted into phospholipid vesicles specific agonist binding, as well as GTP reduction in the specific binding, could be measured. Thus receptor occupancy by agonist during the solubilization reconstitution procedures was required in order to measure receptor function. Using this protocol, specific binding of [^{3}H]etorphine was saturable (figure 17) and had a Kd of 0.59 ± 0.22 nM (n=1), a value similar to the receptor affinity for [^{3}H]etrophine measured in intact membranes (figure 11). Specific [^{3}H]etorphine binding that was sensitive to GTP was also detected when receptors were prebound with antagonist (naloxone), solubilized, and reconstituted (table 3). This indicated that an opioid receptor occupancy could maintain the receptor/G-protein complex during the solubilization and reconstitution procedure. No specific binding to the phospholipid vesicles was detected if solubilized membrane extracts were omitted. Approximately

Table 3

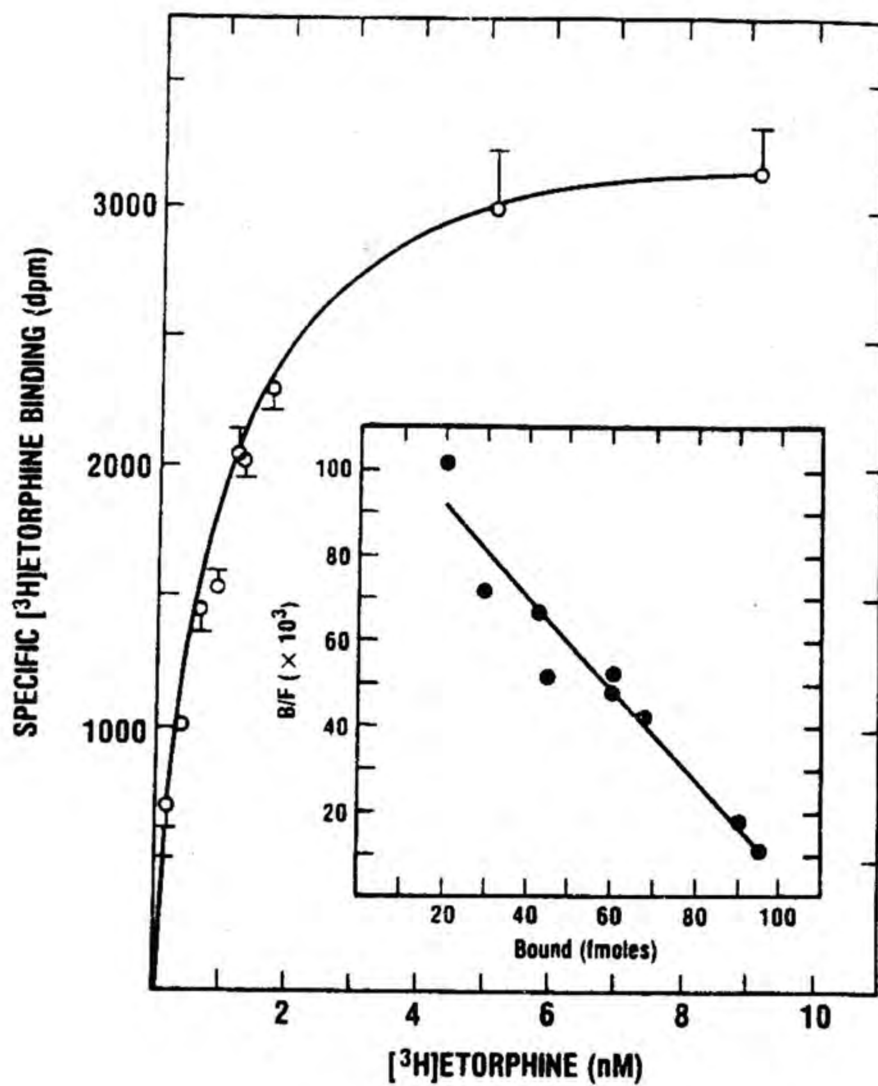
[^{3}H]Etorphine Binding to Solubilized Receptors Reconstituted in Phospholipid Vesicles

	[^{3}H]Etorphine Binding (dpm)		
Preincubation	Total	100 μM GTP	Nonspecific
Morphine (10 μM)	3089 ± 755	733 ± 76	390 ± 66
Naloxone (1 μM)	2233 ± 433	991 ± 353	612 ± 123

Receptors associated with 7315c membranes were preincubated with morphine or naloxone, solubilized, and reinserted into phospholipid vesicles. The vesicles were incubated with [^{3}H]etorphine (1 nM) for 18 h at 4 C in the absence (control) and presence of GTP. Nonspecific binding was determined by inclusion of 100 μM naloxone in the reaction mixtures. Bound [^{3}H]etorphine was separated from free using Sephadex G50 gel filtration chromatography. Specific binding could not be detected without preincubation with either morphine or naloxone. These procedures are detailed in the Methods.

Figure 17. Saturation Binding of [^{3}H]Etorphine to Solubilized and Reconstituted Mu-Opioid Receptor/G-Protein complexes from 7315c Cell Membranes.

Morphine prebound mu-opioid receptor/G-protien complexes were solubilized and reconstituted into phospholipid vesicles as described in the Methods. The phospholipid vesicles were incubated in the presence of the indicated concentrations of [^{3}H]etorphine. Nonspecific binding was determined in the presence of 100 μM naloxone. Specific binding is shown (O). Nonlinear parametric analysis of the saturation curve data (Munson and Rodbard, 1980) resulted in a one-site model with a calculated Kd of 0.59 ± 0.22 nM. Inset: Scatchard analysis of the saturation data is shown. This experiment was conducted in the absence of guanine nucleotides.


SPECIFIC [3H]ETORPHINE BINDING (dpm)
3000
2000
1000
2
4
6
8
10
[3H]ETORPHINE (nM)
B/F (× 10³)
100
80
60
40
20
20
40
60
80
100
Bound (fmoles)

20% (n=3) of opioid receptors from 7315c cell membranes were recovered using the solubilization and reconstitution methods described (table 4).

Evidence of Co-Solubilization of the Mu-Opioid Receptor/G-Protein Complex

The evidence for the co-solubilization and reconstitution of the mu-opioid receptor/ G-protein complex was determined by the effect of guanine nucleotides to decrease mu-opioid agonist binding. In the presence of 100 μM GTP, the specific [^{3}H]etorphine binding was decreased by 85 ± 5% when 7315c cell membranes were preincubated with morphine and 79 ± 11% when preincubated with naloxone (table 3).

To test whether the effect of guanine nucleotides in diminishing agonist binding was an effect on affinity rather than receptor number, a [^{3}H]etorphine saturation binding experiment was performed in the absence or presence of GTPγS (figure 18). Nonlinear parametric analysis of the saturation data (Munson and Rodbard, 1980) resulted in a one-site model (no significant difference from a two site model) with calculated affinities of [^{3}H]etorphine as 0.53 ± 0.25 nM or 3.61 ± 0.2 nM alone or in the presence of 100 μM GTPγS, respectively. GTPγS had no effect on Bmax; 850 ± 125 fmoles mg protein^{-1} and 721 ± 143 fmoles-mg protein^{-1} in the absence or presence of GTPγS, respectively (paired t-test, $p<0.05$).

Guanine nucleotides diminished [^{3}H]etorphine binding. The order of potency (IC_{50}) of the guanine nucleotides was GTPγS (0.54 ± 0.02 μM) > GTP (4.1 ± 1.3 μM) > GDP (14.9 ± 6.8 μM) (figure 19). These potencies are nearly identical to those observed for the membrane associated receptor (figure 14).

Table 4

Saturation Binding of [^{3}H]Etorphine to 7315c Membrane-associated and Reconstituted Receptors

	Specific [^{3}H]Etorphine (10nM) Binding (fmole/mg protein)	Recovery %
Membrane-associated	157 ± 50	100
Reconstituted	850 ± 125	20 ± 2

Membrane-associated and reconstituted receptors, prepared as described in the Methods, were assayed with 10 nM [^{3}H]etorphine, a saturating concentration; nonspecific binding was determined by the inclusion of 10 μM naloxone. Membrane-associated receptors were incubated with the ligand for 30 min at 37 C; reconstituted receptors were incubated for 18 h at 4 C. The average corresponding amounts of protein for membrane-associated and reconstituted receptors were 0.4 mg and 0.04 mg, respectively. The data are the mean and S.E.M. of 3 separate experiments.

Figure 18. [^{3}H]Etorphine Binding to the Reconstituted Mu-Opioid Receptor and the Effect of GTPγS.

Receptors associated with 7315c membranes were exposed to morphine (10 μM), solubilized, and reconstituted, as described in Mehtods. The reconstituted receptors were incubated for 18 hr at 4 C with the indicated concentrations of [^{3}H]etorphine in the absence (open circles) and presence (closed circles) of 100 μM GTPγS. Naloxone (10 μM) was used to define nonspecific binding; GTPγS had no effect on nonspecific binding. Specific binding was 70 and 52% of total binding at 0.1 and 10 nM [^{3}H]etorphine, respectively. Each point represents the mean ± SEM of triplicate samples from an experiment representative of three.

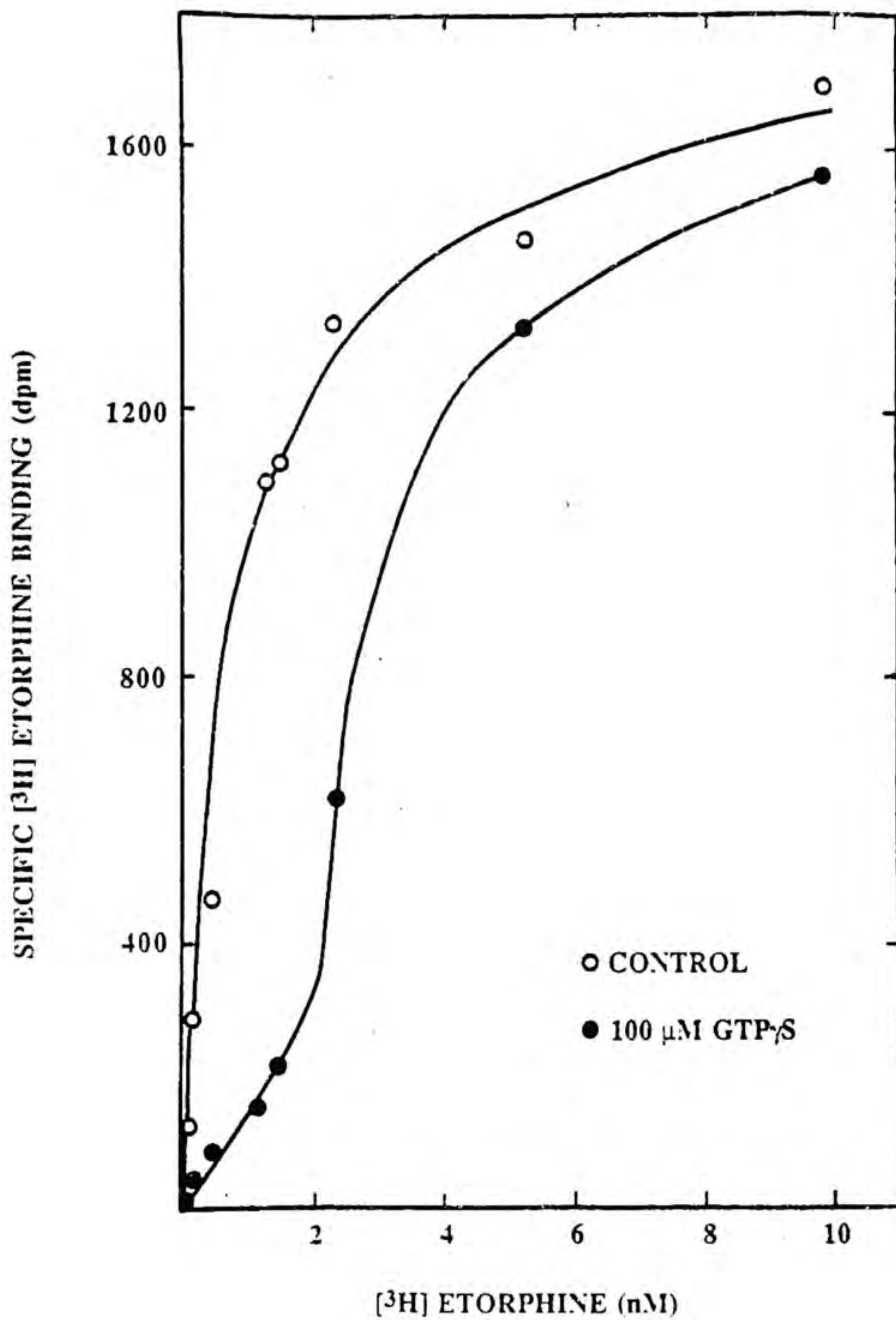
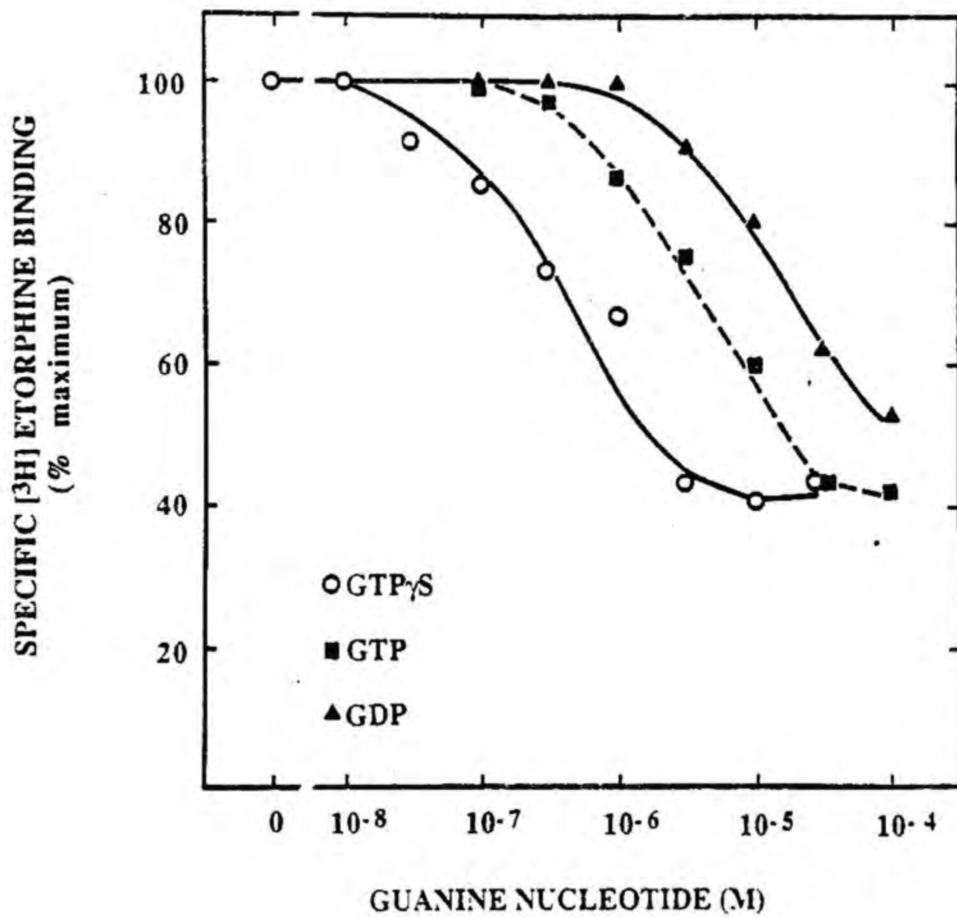

1600
1200
800
400
SPECIFIC [3H] ETORPHINE BINDING (dpm)
CONTROL
100 μM GTPγS
2
4
6
8
10
[3H] ETORPHINE (nM)

Figure 19. Guanine Nucleotides Diminish Specific [^{3}H]Etorphine Binding to Solubilized and Reconstituted Mu-Opioid Receptor/G_i Complexes.

Receptors associated with 7315c membranes were exposed to morphine (10μM), solubilized, and reconstituted, as described in the Methods. The reconstituted receptors were incubated for 18 hr at 4 C with 1 nM [^{3}H]etorphine and the indicated concentrations of GTPγS (○), GTP(■), or GDP (▲). Naloxone (10 μM) was used to define nonspecific binding. Each point represents the mean ± SEM of triplicate samples from an experiment representative of three. The maximal specific binding (binding in the absence of guanine nucleotide) from these three experiments was 1043 ± 353 dpm and was 77 ± 4% of the total binding.



SPECIFIC [3H] ETORPHINE BINDING
(% maximum)
100
80
60
40
20
GTPγS
GTP
GDP
0
10-8
10-7
10-6
10-5
10-4
GUANINE NUCLEOTIDE (M)

IV. Affinity Co-Purification of the Mu-opioid receptor/G-protein Complex.

Rationale: Since opioid agonists are known to inhibit adenylyl cyclase activity (Blume et al., 1979; Cooper et al., 1982; Frey and Kebabian, 1984) as well as activate ion-channel activity (North et al., 1987; Aghajanian and Wang, 1986) through activation of GTP-binding proteins, one might suspect that these differing effector mechanisms are regulated by receptor activation of distinct G-proteins that in turn interact with unique effector proteins. In the present study, identification of the G-protein that interacts in vivo with the mu-opioid receptor will be attempted by co-purifiying these two proteins from 7315c cell membranes. This study takes advantage of the fact that the 7315c tumor cell expresses a homogeneous population of mu-opioid receptors that are functionally coupled to a pertussis toxin sensitive GTP-binding protein. The model of G-protein mediated signal transduction (Gilman, 1987) and our recent studies (section III) indicate that a stable agonist/receptor/Gi complex is formed in the absence of guanine nucleotides. This complex represents a high affinity interaction of the agonist with the receptor and a tight association of this inhibitory receptor with Gi. Thus it is anticipated that this complex will be well suited for the affinity co-purification procedure.

Purification of the Mu-Opioid/Gi Complex from 7315c Tumor Cell Membranes

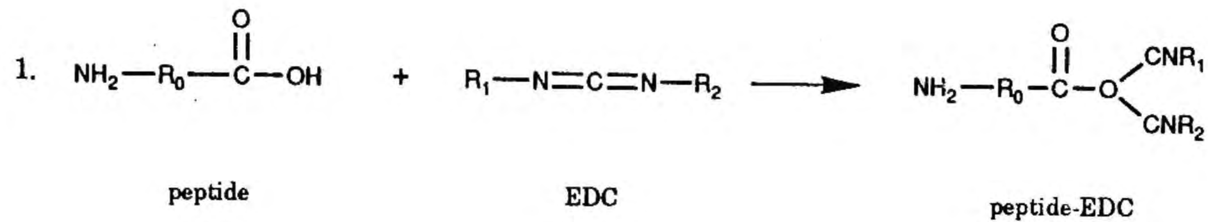
In order to co-purify the mu-opioid receptor with its GTP-binding protein, an agonist affinity column was constructed. Although

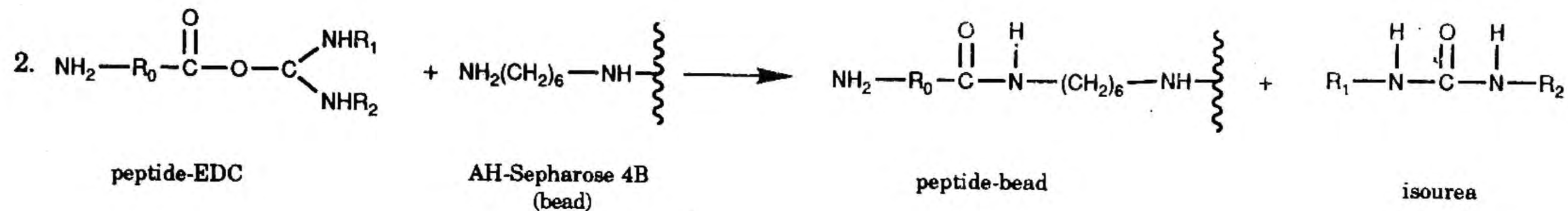
antagonist affinity columns have been used to co-purify inhibitory receptor/G-protein complexes, the preliminary data (section III) suggested that agonist occupation of the mu-opioid receptor in the absence of guanine nucleotides resulted in a tightly associated agonist/receptor/G-protein complex that withstood solubilization with detergent. A peptide agonist, [D-ala^2, D-leu^5]-enkephalin (DADLE) was selected because of the free carboxyl terminal readily available for carbodiimide coupling to AH-Sepharose 4B. Construction of a DADLE-AH-Sepharose 4B column is depicted in figure 20; assessment of the coupling efficiency of this reaction is shown in table 5.

In preliminary studies, it was determined that the mu-opioid receptor must be occupied by an opioid ligand prior to solubulization and must be reconstituted in phospholipids vesicles in order to display mu-opioid receptor binding properties following solubilization with CHAPS (table 2). Therefore, for the co-purification of these proteins, intact membranes were incubated with DADLE-AH Sepharose 4B resin for 1 hr at 20 C prior to solubilization with the detergent, CHAPS. After the affinity resin was extensively washed (as described in Methods), the receptor and G-protein were eluted with 10 μM morphine and 100 μM GTP; [^{3}H]etorphine binding was measured to receptors following reconstitution of the eluant into phospholipid vesicles. This purification scheme is depicted in figure 21. Following this protocol, the mu-opioid receptor was purified 2919 ± 128-fold compared to membrane-associated receptors; 5.6 ± 0.9% of the membrane receptors were recovered (table 6). The DADLE-AH Sepharose 4B specifically purified opioid receptors from the 7315c cell membrane; when the membranes were incubated with the DADLE-Sepharose in the presence of excess naloxone, the purification was

Figure 20. Acid-catalyzed Covalent Coupling Reaction of [D-ala^2, D-leu^5]Enkephalin to AH-Sepharose 4B.

[D-ala^2, D-leu^5]Enkephalin (10 μmoles), EDC (0.1 M), and 1 gram (swollen gel volume, 4 mls) of AH-sepharose 4B (final volume, 12 mls 0.5 M NaCl pH 4.5) were incubated overnight. The pH was adjusted after one hour to 4.5 with dilute NaOH. The prepared gel was washed four times with acetate (0.1 M Potassium Acetate, 0.5 M NaCl, pH 4.0) and bicarbonate (0.1 M Sodium Bicarbonate, 0.5 M NaCl, pH 8.3) buffers. The gel was then washed twice with 1 M NaCl containing 0.01% Thimersol to retard bacterial growth, and the gel was stored at 4 C.





Peptide used: [D-ala^2, D-leu^5] Enkephalin NH$_2$-Tyr-Ala-Gly-Phe- Leu-COOH

Table 5

Coupling Efficiency of [D-ala^2,D-leu^5]Enkephalin to AH-Sepharose 4B.

Exp. #	Starting Solution (dpm x 10^6)	Specific Activity (dpm/nanomole)	[^{3}H]Enkephalin in washed gel (dpm)	% Coupling efficiency	Enkephalin Bound (nmoles bound/ 1 ml of gel)
1	4.93	493	115313	2.3	58.8
2	4.65	465	135180	2.8	72.0
3	3.43	343	65900	1.9	48.5
4	3.10	310	82140	2.6	66.3
Mean ± SEM	4.03 ± 0.45	402.8 ± 44.9	99633 ± 15688	2.4 ± 0.20	61.4 ± 5.1

Preparation of the [D-ala^2,D-leu^5]Enkephalin affinity column was performed as described in Methods. For the determination of the coupling efficiency and nanomoles of [D-ala^2,D-leu^5]Enkephalin bound to the gel, 1 µl of [^{3}H][D-ala^2,D-leu^5]Enkephalin was added to the reaction mixture. An aliquot of the starting material was analyzed for the presence of the tritiated compound. The specific activity was determined by dividing the dpm in the starting material by the moles of unlabeled [D-ala^2,D-leu^5]Enkephalin in the reaction solution. After the acetate and bicarbonate washes, an aliquot of the packed gel was then analyzed for the presence of the tritiated label. The coupling efficiency was determined by dividing the dpm found in the washed packed gel by the dpm of the starting solution. The nanomoles of [D-ala^2,D-leu^5]Enkephalin bound per ml of gel was determined by dividing the dpm in the washed packed gel by the determined specific activity. In a typical purification experiment 7315c membranes were incubated with 1 ml of DADLE-AH Sepharose 4B; the final concentration of DADLE attached to the affinity resin was 10 µM.

Figure 21. Protocol for the affinity purification of the Mu-Opioid receptor/ G-protein complex from 7315c Tumor Cells.

Scheme depicted is the protocol used to affinity purify the mu-opioid receptor/G-protein complex using [D-ala^2,D-leu^5]-enkephalin-AH Sepharose 4B. The buffer used contained 50 mM K^+HEPES pH 7.4, 2 mM EDTA, 100 mM NaCl, 0.1 mM Phenyl Methyl Sulfonyl Fluoride, 1 μM leupeptin, 1 mM dithiothreitol, 20 μM bestatin, and 100 μM 1,10-O-phenanthroline (Buffer C).

7315c cell intact membranes

Affinity Purification

Incubate with 1 ml affinity resin
1 hr at room temp
in a 1.5 x 5 cm column
final volume, 6mls.

↓

Add CHAPS to solution in column
Final concentration, 10mM CHAPS
End-over end mixing
30 min, 4 C

↓

Elute fluid

↓

Wash column with 300 mls of
buffer containing 2 mM CHAPS

↓

Wash column with 50 mls of
buffer containing 1 mM CHAPS

↓

Elute receptor and G-protein
with 5 mls buffer containing 1 mM CHAPS
and 10 μM morphine plus 100 μM GTP

↓

Reduce eluted volume to 1 ml using
ultrafiltration device

↓

Insert 730 μl into phospholipid vesicles.
Use remaining volume to measure protein
(amido black assay)

↓

Dilute phospholipid vesicle mixture
and measure receptor binding

↓

Incubate ≥18 hrs 4 C
Separate free from bound by
Sephadex G50 column chromatography

Intact membrane binding

incubate membranes with
[^{3}H]ligand,
± 10 μM Naloxone
± 100 μM GTP
30 min 37 C
terminate by rapid
filtration

↓

measure protein
(Bradford assay)

Table 6

Saturation Binding to Membrane-Associated and Affinity Purified Mu-Opioid Receptors

Determination of Purification

Membrane Associated (pmoles/mg protein)	Affinity Purified (pmoles/mg protein)	Fold purification
0.202	623	3084
0.157	472	3006
0.300	810	2667
0.220 ± 0.04	635 ± 98	2919 ± 128

The specific binding of 10 nM [^{3}H]etorphine to mu-opioid receptor/G-protein complexes from intact membranes and affinity purified receptors from 7315c tumor cells were determined from data similar to those depicted in figure 5. Specific binding was determined as the difference of the binding of 10 nM [^{3}H]etorphine determined in the absence and presence of 10 μM naloxone. The recovery of the mu-opioid receptor is 5.6 ± 0.9% (n=3). Calculated purity of the mu-opioid receptor/G-protein complex is 9.5%. The values shown above are similar to the data from β-endorphin-AH-Sepharose affinity purification experiments. Protein was determined by the Bradford Assay (membranes) and the Amido black assay (affinity purified receptors).

reduced by 81.1%; from 2307-fold to 645-fold pure (Figure 22). The recovery of [^{35}S]GTPγS binding activity was dependent on recovery of the mu-opioid receptor; the inclusion of naloxone during the purification procedure reduced the recovery of [^{35}S]GTPγS binding activity by 78%.

The eluted mu-opioid receptor/G-protein complexes were found to be soluble (figure 23); after centrifugation at 100,000 x g for 1 hr, only the supernatant contained the mu-opioid receptor/ G-protein complex as assessed by receptor and G-protein binding assays.

The stability of the affinity co-purified receptor/G-protein complexes was tested (figure 24). The eluted proteins could be frozen in liquid nitrgen and thawed without loss of activity as assesed by receptor ligand binding assays at the Kd and saturating concentrations of [^{3}H]etorphine.

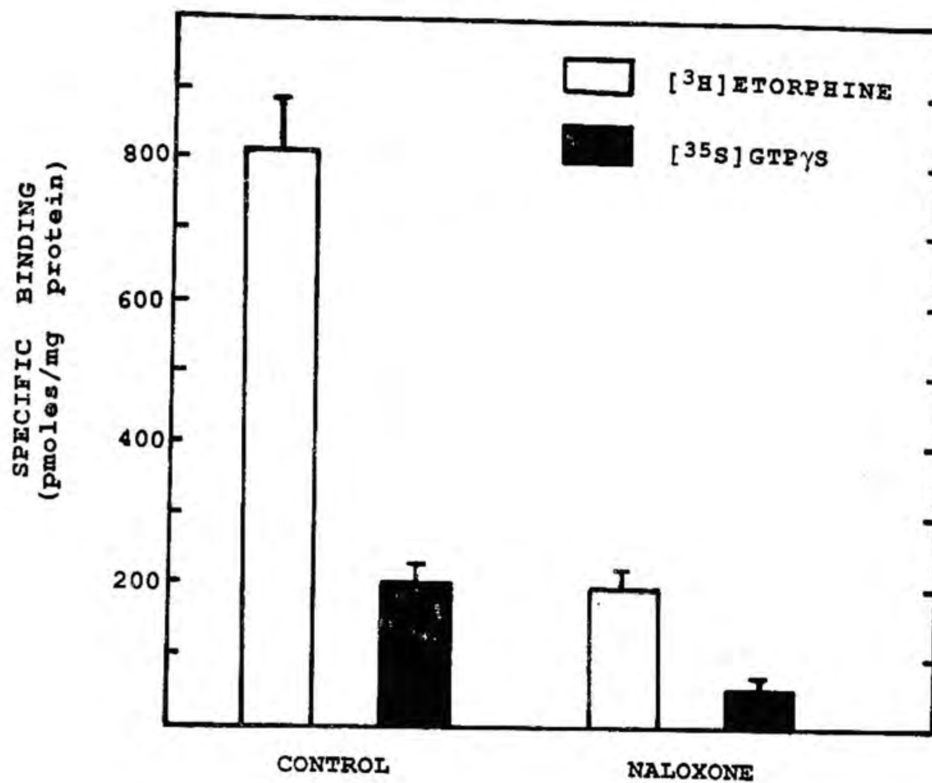
Characterization of the Affinity Purified Mu-Opioid Receptor

The affinity of the purified mu-opioid receptor for [^{3}H]naloxone was determined following reconstitution into phospholipid vesicles. Nonlinear parametric analysis of the saturation curve data from three separate experiments resulted in a one site model with a calculated affinity of 0.90 ± 0.19 nM (figure 25). This affinity is similar to that for the intact membrane receptor from 7315c cells (Puttfarcken et al., 1986) and guinea pig cortex (Werling et al., 1989).

To determine the molecular weight of the affinity purified and membrane-associated mu-opioid receptor, either intact membranes or affinity purified receptors were incubated with 2 nM ^{125}I-β-endorphin in the presence or absence of 10 μM naloxone. The β-endorphin was

Figure 22. DADLE-AH-Sepharose 4B Affinity Chromatography is specific for the Mu-Opioid receptors on 7315c Cell Membranes.

7315c cell membranes were incubated with DADLE-AH-Sepharose 4B in the absence (control) or presence of 10 μM naloxone (naloxone). The mu-opioid receptor/G-protein complex was affinity purified as described in Methods. The mu-opioid receptor/G-protein complexes were eluted with Buffer D containing 10 μM morphine and 100 μM GTP as described in methods. The eluted material was divided; half was reconstituted into phospholipid vesicles and tested for specific 10 nM [^{3}H]etorphine binding. [^{3}H]Etorphine binding was performed in the presence or absence of 10 μM naloxone to determine nonspecific binding; specific binding was 41 and 26% for control and naloxone treated samples. The other half of the eluted samples was dialyzed in Buffer G overnight and tested for [^{35}S]GTPγS binding. Nonspecific binding was determined in the presence of 100 μM GTPγS. Specific binding was 60 or 33% for control or naloxone treated samples. Protein was determined by the Amido Black assay as described in Methods.



SPECIFIC BINDING
(pmoles/mg protein)
800
600
400
200
[^{3}H]ETORPHINE
[^{35}S]GTPγS
CONTROL
NALOXONE

Figure 23. Affinity Purified Mu-Opioid Receptor/G-Protein are found in Detergent Solution.

Mu-opioid receptor/G-protein complex was affinity purified using the DADLE-AH-Sepharose 4B affinity resin as described in Methods. The receptor/G-protein complex was eluted from the affinity column with Buffer D containing 10 μM morphine and 100 μM GTP. The eluted material was divided and half was subjected to ultracentrifugation at 100,000 x g for 1 hr, the other half incubated was on ice. The samples were further divided and control and supernatant samples were each tested for receptor and G-protein binding. Samples for receptor binding were reconstituted into phospholipid vesicles and were incubated with 10 nM [^{3}H]etorphine in the absence and presence of 10 μM naloxone to determine specific binding. Specific binding was 38 and 43% of the total for control and supernatant samples. Samples for G-protein binding were dialyzed overnight in Buffer G as described in Methods. The detergent solutions were then incubated with 1 μM [^{35}S]GTPγS (1843 dpm/pmole) in the absence or presence of 100 μM GTPγS to define specific binding. Specific binding was 57 or 64% of total binding for control or supernatant samples. Detergent samples were saved and protein was measured by the Amido Black assay.

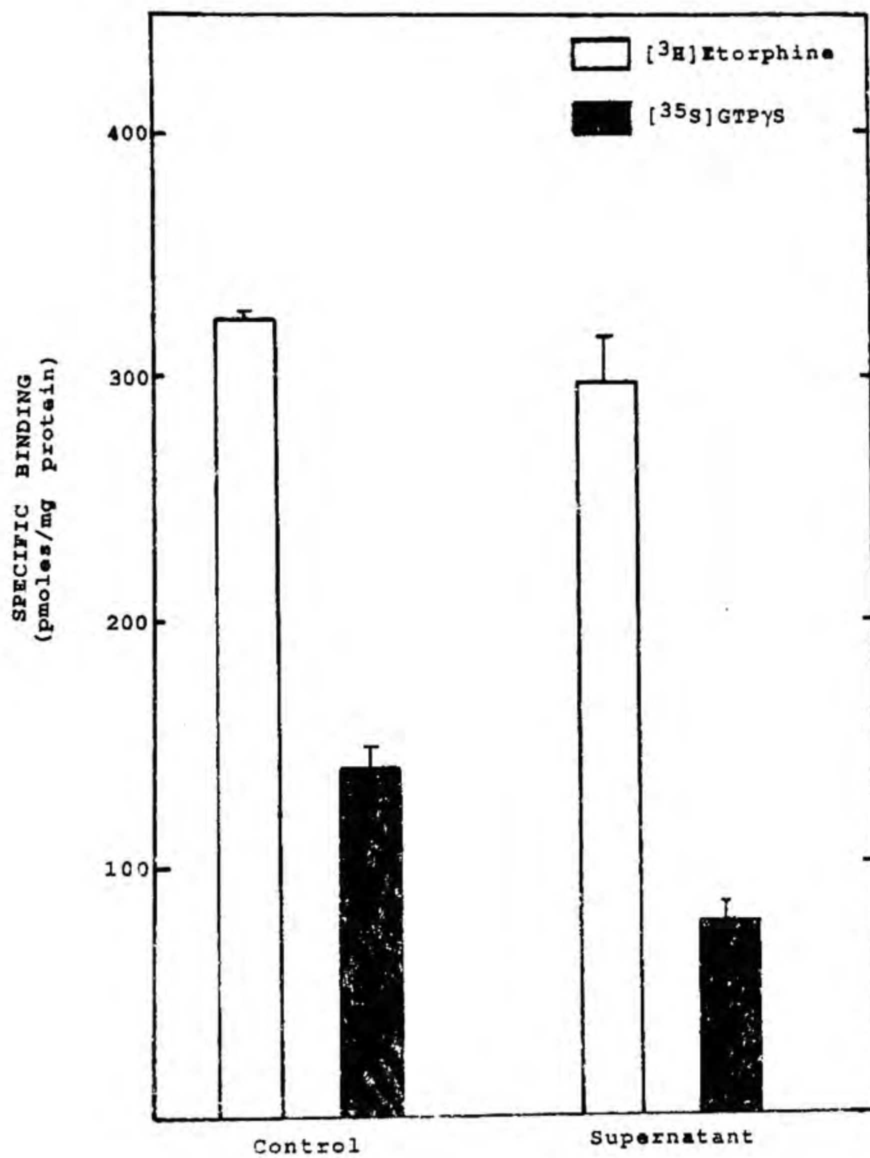

[3H]Etorphine
[35S]GTPγS
400
300
200
100
SPECIFIC BINDING
(pmoles/mg protein)
Control
Supernatant

Figure 24. Affinity Purified Mu-Opioid Receptors Can be Frozen and Thawed Without Loss of Activity.

Mu-opioid receptors were affinity purified using the DADLE-AH-Sepharose 4B affinity resin as described in Methods. Receptors were eluted from the affinity column with buffer containing 10 μM morphine and 100 μM GTP. The eluted material was divided; half was frozen in liquid nitrogen, the other half was incubated on ice. The frozen samples were thawed, and all soluble samples were then tested for opioid receptor binding by reconstituting them into phospholipid vesicles and incubating them with the indicated concentration [^{3}H]etorphine. Membrane-associated receptor binding was performed as described in the Methods. Nonspecific binding determined by incubation in the presence of 10 μM naloxone. Left panel: The binding of a saturating concentration of [^{3}H]etorphine to the reconstituted affinity purified opioid receptors from control and freeze-thaw treated preparations is compared to the binding of membrane-associated opioid receptors from 7315c cells. The specific binding represents 27, 43, and 32% of total binding for membrane, purified, and purified-freeze thaw samples. The contol and freeze-thaw purified receptors are 2073 and 1344 fold-pure, respectively. Right panel: Membrane associated, purified, and purified freeze-thaw opioid receptors were incubated with 0.9 nM [^{3}H]etorphine with the indicated drugs. Naloxone (10 μM) was used to determine nonspecific binding. The specific binding was 74, 68, 65% of the total binding measured for membrane, purified, and purified freeze-thaw opioid receptors.

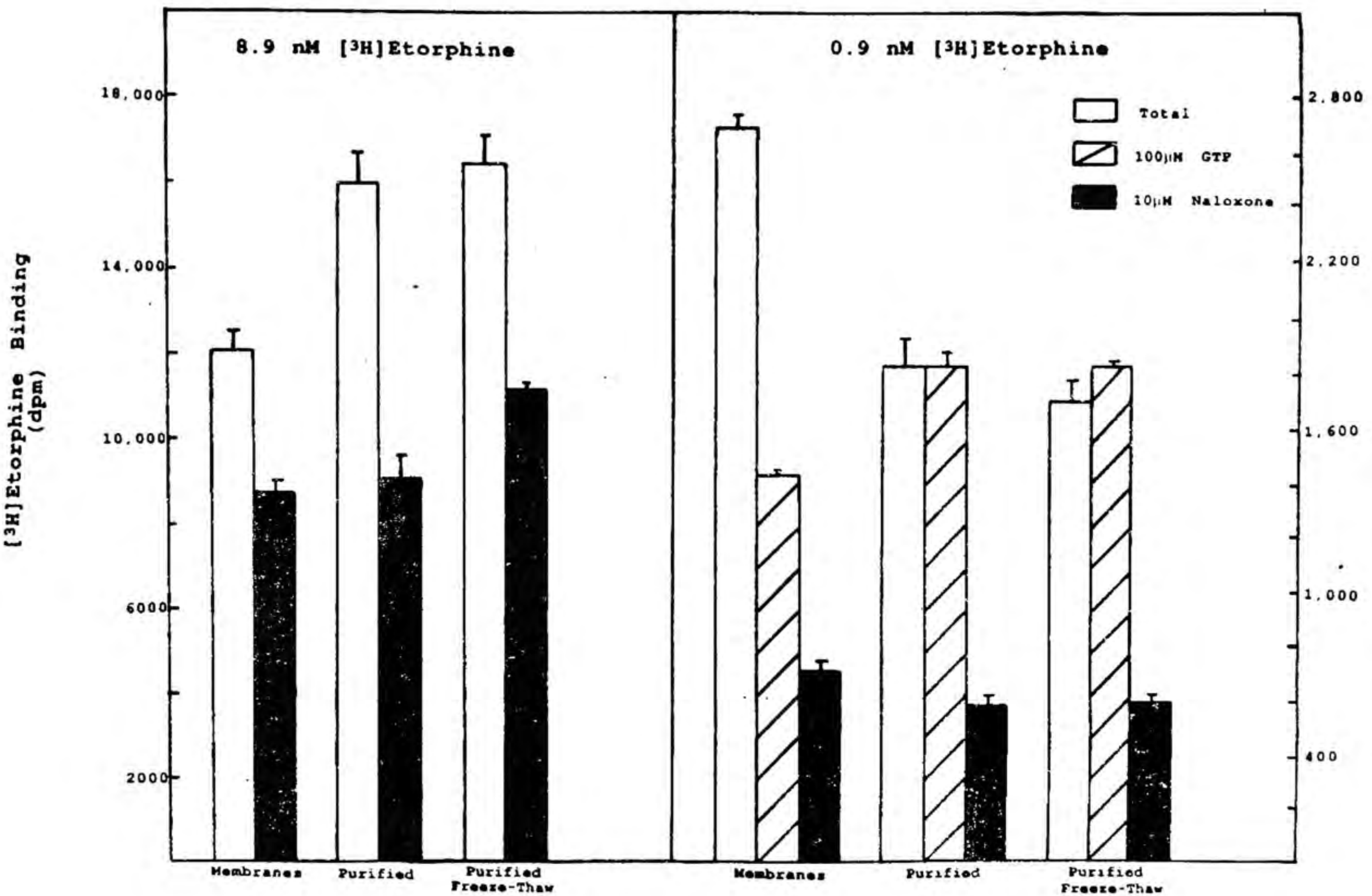
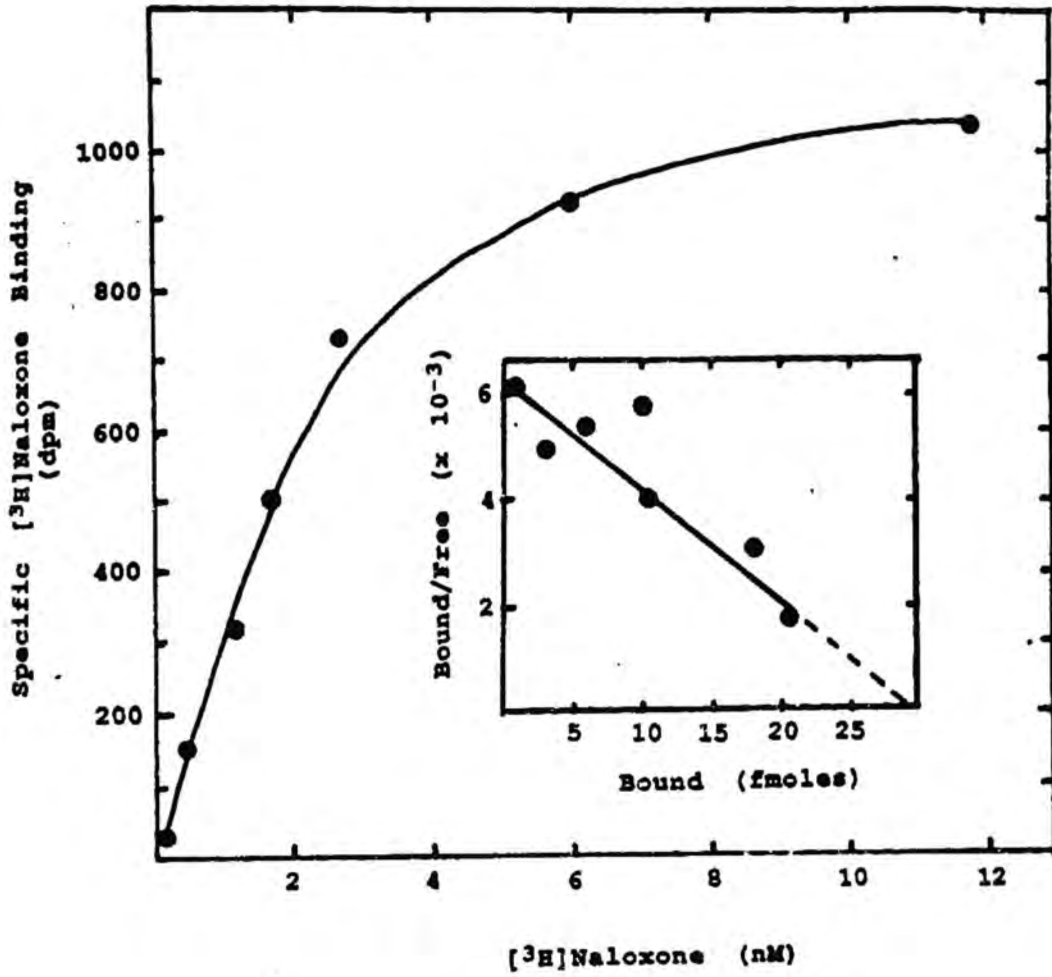

8.9 nM [3H]Etorphine
0.9 nM [3H]Etorphine
Total
100μM GTP
10μM Naloxone
[3H]Etorphine Binding (dpm)
18,000
14,000
10,000
6000
2000
2,800
2,200
1,600
1,000
400
Membranes
Purified
Purified Freeze-Thaw
Membranes
Purified
Purified Freeze-Thaw

Figure 25. [^{3}H]Naloxone Binding to the reconstituted affinity purified Mu-opioid Receptor/G-protein Complex

Mu-opioid receptor/G-protein complexes were affinity purified from 7315c tumor cell membranes using a DADLE-AH-Sepharose 4B column as described in Methods. The purified proteins were reconstituted into phospholipid vesicles and were incubated in the presence of the indicated concentrations of [^{3}H]naloxone. Nonspecific binding was determined in the presence of 10 μM naloxone; specific binding was 52 ± 13 or 72 ± 8% at 10 and 1 nM [^{3}H]naloxone, respectively. The fold purity of receptor/G-protein complexes was 2354 ± 251; and the maximal number of receptor binding sites was 495 ± 26 pmoles/mg protein (n = 3). Nonlinear parametric analysis of the saturation curve data from three separate experiments resulted in a one site model with a calculated affinity of 0.90 ± 0.19 nM. Each point represents the mean ± SEM of triplicate samples from a representive experiment. Inset: Scatchard analysis of the saturation data is shown. This experiment was conducted in the absence of guanine nucleotides.


Specific [³H]Naloxone Binding (dpm)
1000
800
600
400
200
2
4
6
8
10
12
[³H]Naloxone (nM)
Bound/Free (x 10⁻³)
6
4
2
5
10
15
20
25
Bound (fmoles)

crosslinked to the receptor proteins, and the samples were subjected to SDS-PAGE. The autoradiography of the dried gel demonstrated a single protein of molecular weight of approximately 64,000 dalton in both the affinity purified (figure 26, panel B) as well as the membrane proteins (figure 26, panel A). Inclusion of naloxone in both preparations greatly diminished the intensity of the band. This molecular weight is similar to the mu-opioid receptor found in rat brain tissues (Howard et al., 1985).

Evidence for the Co-Purification of the Mu-Opioid Receptor/G-Protein Complex

In our initial experiments, morphine alone was able to elute only a very small amount of the mu-opioid receptor from the affinity resin. However, when 10 μM morphine and 100 μM GTP were added to the elution buffer, a three-fold increase in the amount of receptors was collected in the eluate (figure 27). Furthermore, a potent mu-opioid agonist, FK33824, demonstrated a similar elution pattern in the absence or presence of GTP (figure 27). This experiment provided indirect evidence that a GTP-binding protein was co-purifying with the mu-opioid receptor since the high affinity binding state of a receptor is dependent on the association of the receptor with an unoccupied G-protein (Gilman, 1987). The binding of a guanine nucleotide to the G-protein to dissociate would cause the receptor to bind agonist in the low affinity state (Tota et al., 1987; Haga et al, 1987).

The receptor purified G-protein specifically binds GTPγS; the ratio of $[^{35}S]$GTPγS binding to $[^{3}H]$etorphine binding (maximum G-protein and receptor sites) is shown in figures 22, 23, and table 7. The ratio

Figure 26. Determination of the Molecular Weight of the Affinity Purified Mu-Opioid Receptor from 7315c cells.

Mu-opioid receptors were affinity purified as described in Methods. The purified receptors were eluted from the DADLE-AH-Sepharose 4B affinity resin with Buffer D containing 10 μM morphine and 100 μM GTP and reconstituted into phospholipid vesicles. Membranes from 7315c cells were prepared as described in Methods. 7315c cell membranes (panel A) and reconstituted affinity purified receptors (panel B) were incubated with 2 nM [^{125}I]β-endorphin in the absence (lanes 1 and 3) and presence of 10 μM naloxone (lanes 2 and 4). Free [^{125}I]β-endorphin was removed either by washing the membranes by centrifugation or by subjecting the purified receptors to chromatography on Sephadex G50. The samples were then incubated with 1 mM DSS at 0 C for 15 min. Crosslinking was terminated by the addition of 100 mM Tris buffer. Samples were dried and prepared for SDS-PAGE on a 10% low-bis-acrylamide gel (Goldsmith et al., 1988) (panel B) or a 12% gel (panel A). Migration of molecular weight standards is shown.

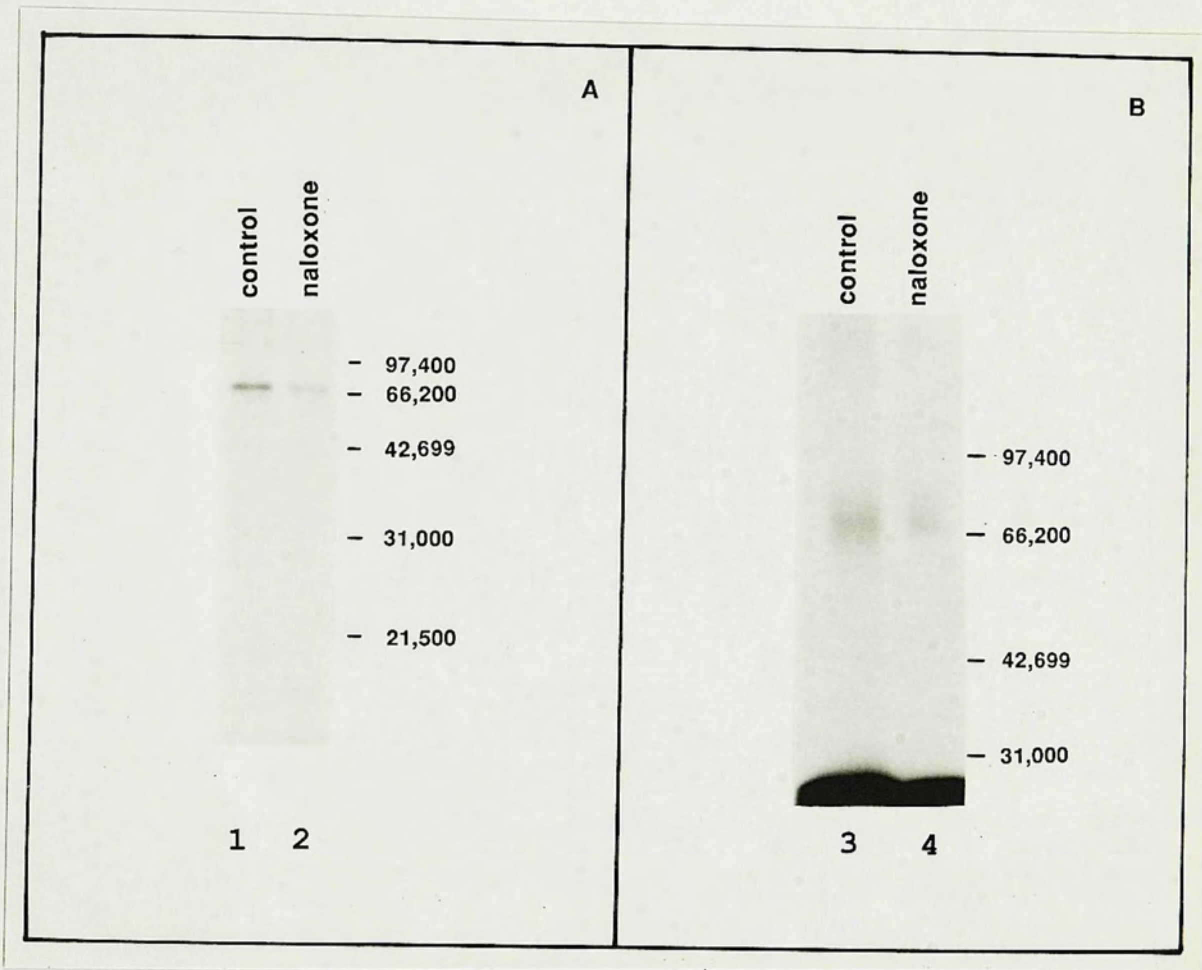

A
control
naloxone
97,400
66,200
42,699
31,000
21,500
1
2
B
control
naloxone
97,400
66,200
42,699
31,000
3
4

Figure 27. Addition of GTP to Elution Buffer Enhances the Elution of the Mu-Opioid Receptor from the DADLE-AH-Sepharose 4B.

Mu-opioid receptor/G-protein complexes were affinity purified from 7315c tumor cell membranes using a DADLE-AH-Sepharose 4B affinity column as described in Methods. The mu-opioid receptors were eluted with Buffer D supplemented with either morphine (10 μM) or FK33824 (1 μM) in the absence or presence of 100 μM GTP. The eluted material was reconstituted into phospholipid vesicles, and tested for specific 1 nM [^{3}H]etorphine binding. Nonspecific binding was determined in the presence of 10 μM naloxone. The specific binding was 34 or 35% of the total binding in the samples eluted with Buffer D supplemented with morphine or FK33824 and 44 or 45% of the total binding samples eluted with Buffer D supplemented with either morphine or FK33824 plus 100 μM GTP.

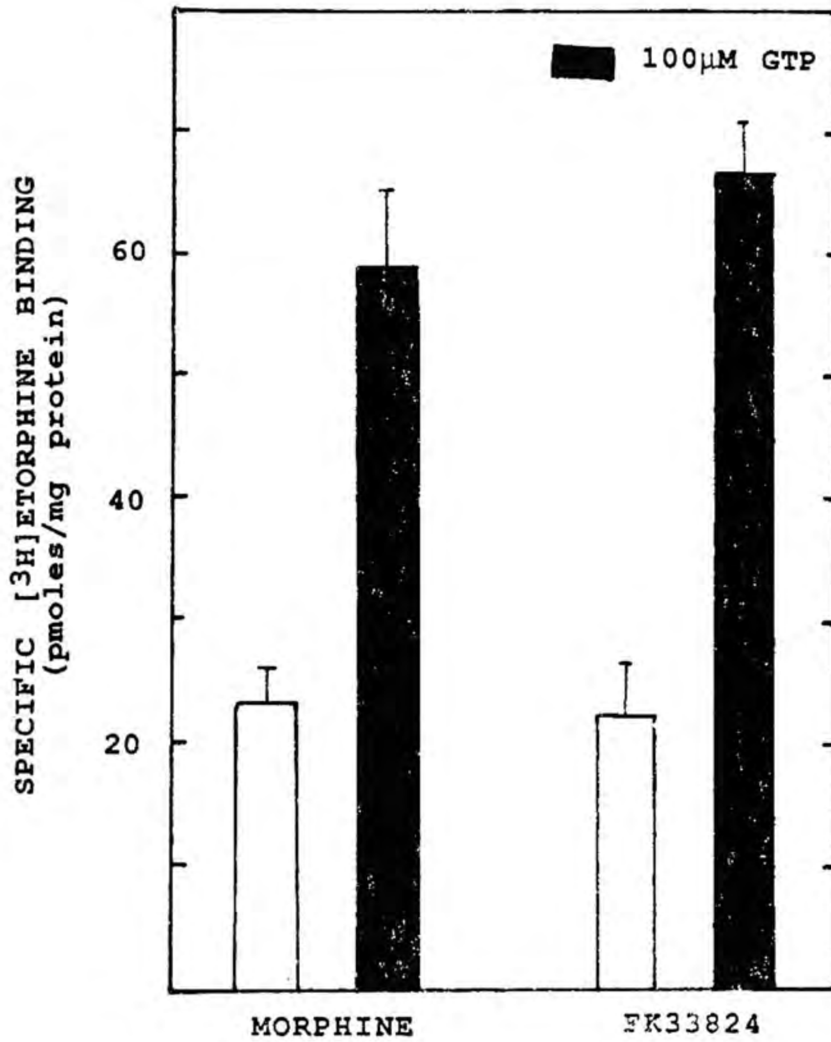

100μM GTP
SPECIFIC [3H]ETORPHINE BINDING
(pmoles/mg protein)
60
40
20
MORPHINE
FK33824

Table 7

Comparison of Mu-Opioid Receptor Binding with Receptor Purified G-Protein Binding

Determination of Co-Purified Receptor to G-Protein Ratio

Exp#	[^{3}H]Etorphine pmoles/mg protein	[^{35}S]GTPγS pmoles/mg protein	Ratio receptor:G-protein
1	473	273	1.0:0.6
2	324	140	1.0:0.4
3	810	191	1.0:0.2
Mean ± SEM	536 ± 144	201 ± 39	1.0: 0.4 ± 0.1

Mu-opioid receptors were affinity co-purified with their GTP-binding proteins as described in the Methods. The fraction eluted with Buffer D supplemented with 10 μM morphine and 100 μM GTP was divided and tested for both receptor and G-protein binding. In the sample to be tested for specific receptor binding activity, the eluted material was reconstituted into phospholipid vesicles and incubated with a saturating concentration (10 nM) of [^{3}H]etrophine in the absence or presence of 10 μM naloxone. The sample to be tested for [^{35}S]GTPγS binding was dialyzed overnight in Buffer G, and incubated in the presence of 1 μM [^{35}S]GTPγS (2538 ± 481 dpm/pmole). Nonspecific binding was determined in the presence of 10 μM GTPγS. Protein concentration was determined for protein concentration in the detergent solution as described in Methods.

of affinity purified mu-opioid receptors to G-proteins is approximately 2.5 receptors to 1 GTP-binding protein. This ratio is maintained when the co-purification is blocked by excess naloxone (figure 22) indicating that the recovery of the G-protein is specifically dependent upon the recovery of the receptor.

The reconstituted affinity purified receptor/G-protein complexes did not demonstrate reduction of specific agonist binding in the presence of GTP (figure 24, right panel). Three explanations of this lack of receptor/G-protein coupling were proposed and were tested.

First, the model of G-protein activation describes active guanine nucleotides (GTP) induction of the dissociation of the heterotrimer into the α and $\beta\gamma$ subunits. Since the heterotrimer is necessary for the G-protein to interact with the receptor (Haga et al., 1986; Tota et al, 1987; Florio and Sternweis, 1989), elution with morphine and GDP (the inactive guanine nucleotide) was utilized to minimize subunit dissociation. Reconstitution and subsequent determination of 1 nM [^{3}H]etorphine binding in the absence or presence of GTP resulted in similar data to the experiments in which receptor/G-protein complexes were eluted using morphine and GTP.

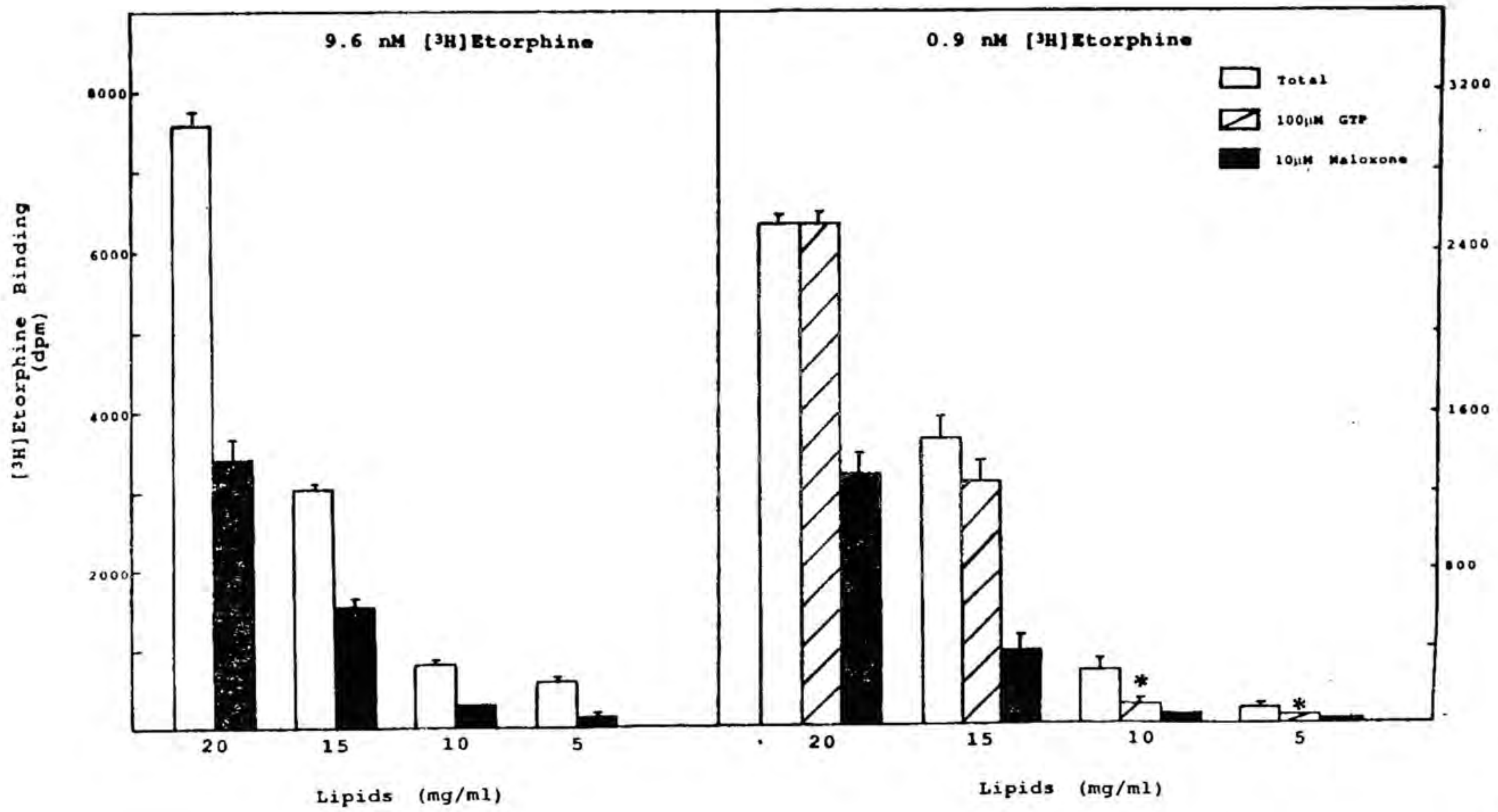
The second tested proposal for the lack of receptor/G-protein interaction is that morphine is not a potent enough agonist to successfully compete with the DADLE-AH Sepharose 4B. This was approached experimentally by eluting the receptor/G-protein with a high affinity agonist FK33824 (the potency in inhibiting adenylyl cyclase activity was 0.7 nM, Frey and Kebabian, 1984) (figure 27). In this experiment it was clear that a significantly greater amount of receptor

eluted when GTP was included with FK 33824 than when FK33824 was used alone.

The third proposed hypothesis was that perhaps the experimental conditions for the reconstitution of the eluted material were preventing the recoupling of the purified receptor and G-proteins. In the plasma membrane a single receptor is thought to interact with multiple G-proteins and following affinity purification less than one G-protein per receptor is recovered. We speculated that the conditions to detect the purified receptor/G-protein interactions would be more sensitive to the phospholipid reconstitution preparation. Therefore, if lower concentrations of lipids were to be used, the reassociation of the receptor with its G-protein would be more likely to occur. The affinity purified mu-opioid receptor/G-protein material was reconstituted with 5, 10, 15, 20 mg/ml sonicated phospholipids (figure 28). The reconstituted vesicle mixtures were then tested for [^{3}H]etorphine binding in the absence or presence of GTP. As it can be clearly seen, the specific binding at a saturating concentration of [^{3}H]etorphine decreased from 735 to 265 pmole mg protein^{-1} (64% reduction) upon lowering the lipid concentration from 20 to 15 mg/ml. A similar result is seen at 1 nM [^{3}H]etorphine binding. Interestingly, the affect of GTP on diminishing agonist binding is detected at the lower concentrations of lipids; a 79% reduction in the specific binding of 0.9 nM [^{3}H]etorphine to purified receptor/G-proteins reconstituted into 10 mg/ml phospholipid vesicles was detected. Although the activity of receptor binding is diminshed when reconstituted into lower concentrations of phospholipids, the affinity co-purified receptor/G-protein complex is active when measured under conditions of lower phospholipids (10 mg/ml). Apparently, the

Figure 28. Interaction of the Affinity Co-Purified Mu-opioid Receptor G-protein Complex is Measured at when Concentrations of Phospholipids are Diminished.

Mu-opioid receptor/G-protein complexes were affinity purified as described in Methods. The receptor/G-protein complex was reconstituted into vesicles formed with the indicated concentrations of sonicated phospholipids. Left panel: The vesicles were incubated with 10 nM [^{3}H]etorphine in the absence or presence of 10 μM naloxone to determine specific binding. Right panel: The vesicles were incubated with 1 nM [^{3}H]etorphine in the absence or presence of 100 μM GTP or 10 μM naloxone. The specific binding to affinity purified receptors reconstituted into 20 mg/ml phospholipid vesicles represent 735 and 220 pmole mg protein^{-1} at 9.6 and 0.9 nM [^{3}H]etrophine, respectively. Specific binding was 55, 49, 65, 77% of total 10 nM [^{3}H]etorphine binding, and was 49, 74, 81, 64% of total 1 nM [^{3}H]etorphine binding at 20, 15, 10, and 5 mg/ml phospholipid, respectively. (*, $p < 0.01$)


9.6 nM [3H]Etorphine
0.9 nM [3H]Etorphine
Total
100μM GTP
10μM Naloxone
[3H]Etorphine Binding
(dpm)
8000
6000
4000
2000
3200
2400
1600
800
20
15
10
5
Lipids (mg/ml)
Lipids (mg/ml)

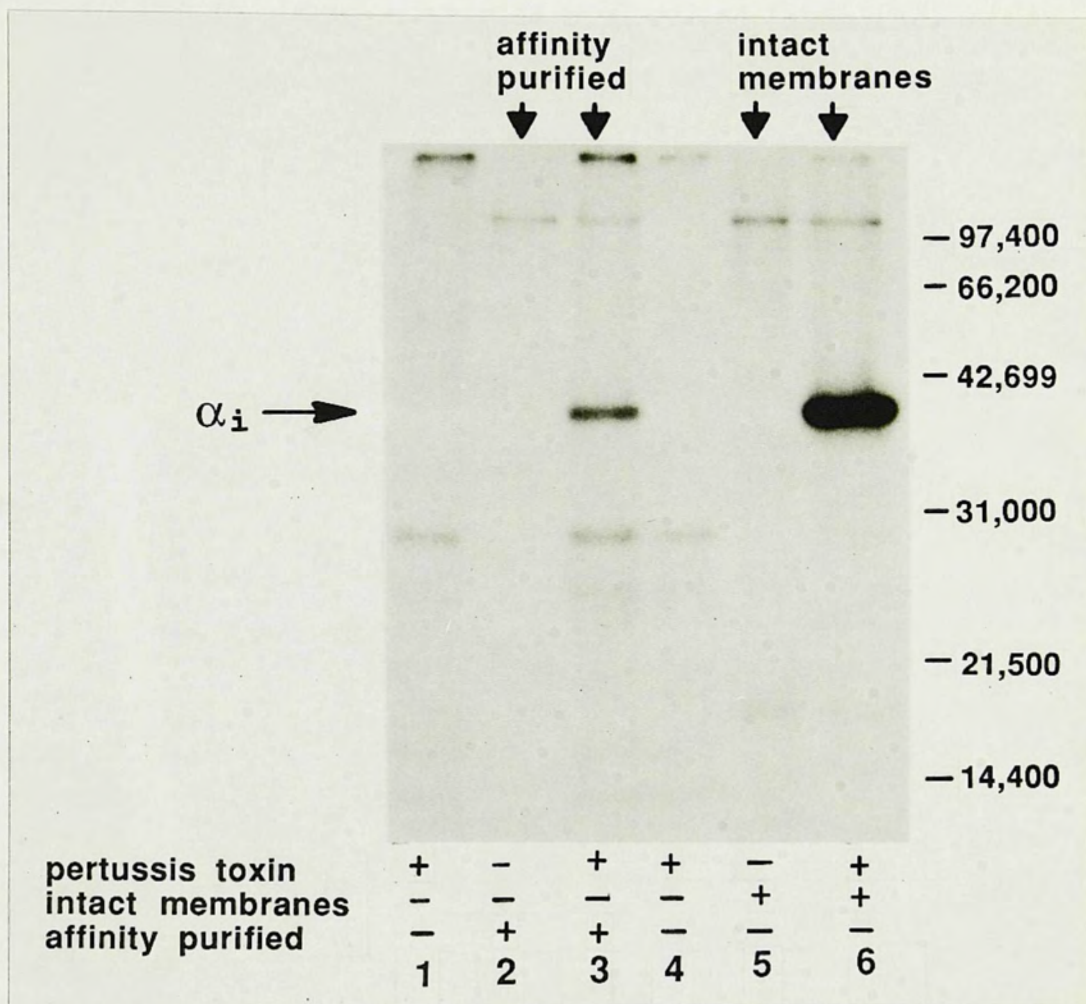
higher density of phospholipid stabilizes the affinity purified mu-opioid receptor. However, the high density of phospholipids also diminishes the interaction bewteen the receptor and its G-protein.

Identification of the Receptor Purified GTP-Binding Protein

Pertussis toxin treatment of the 7315c tumor cell has been shown to abolish mu-opioid agonist-mediated inhibition of adenylyl cyclase (Aub et al., 1986). This effect of pertussis toxin is the result of the ADP-ribosylation of the α subunit of inhibitory G-proteins (Katada and Ui, 1981; Katada and Ui, 1982) leading to uncoupling of the inhibitory GTP-binding protein from the mu-opioid receptor (Cote et al., 1984). Therefore, we looked for the presence of a pertussis toxin substrate by incubating the affinity purified material in the presence of pertussis toxin and [^{32}P]NAD. As shown in figure 29, toxin treatment specifically identified a protein of approximately 41,000 dalton in both membrane (lane 6) and purified G-protein (lane 3). From the determination of the molecular weights of both the mu-opioid receptor and its co-purified GTP-binding protein (assuming the presence of a 35,000 dalton β-subunit and a 10,000 dalton γ-subunit), this complex is calculated to be 9.5% of the eluted protein from the DADLE-AH Sepharose 4B affinity column (table 6; figure. 26; figure 29).

Figure 29. Pertussis Toxin ADP-Ribosylation of the the Mu-Opioid Receptor Purified G-protein for 7315c Tumor cell membranes.

The mu-opioid receptor/G-protein complex was affinity purified using β-endorphin-AH Sepharose 4B as described in Methods. Membranes (lanes 5, 6) and the soluble affinity purified receptor/G-protein complexes (lanes 2,3) were incubated in the presence of 10 mM thymidine, 1 mM ATP, 0.1 mM GTP, 1 mM EDTA, and 25 mM Tris-HCl. In lanes 1-3, 7 µCi and in lanes 4-6, 3.5 µCi [^{32}P]NAD were added to the reaction mixture. Pertussis toxin was included to the reaction mixtures in lanes 1, 3 (2 µg) and in lanes 4, 6 (1 µg). The samples were incubated at 37 C for 30 min. A 30 µl sample (20 µg protein intact membrane and 0.72 µg protein affinity co-purified G-protein) was added to each lane of a 12% SDS-Polyacrylamide gel (3.5% stacking gel). The electrophoresis was conducted at constant voltage, 35 volts, overnight. The dried gel was exposed 16 hours to Kodak X-OMAT AR film. Migration of molecular weight standards are indicated.


affinity purified
intact membranes
— 97,400
— 66,200
— 42,699
α_i
— 31,000
— 21,500
— 14,400
pertussis toxin + − + + − +
intact membranes − − − − + +
affinity purified − + + − − −
1 2 3 4 5 6

Detection of known α-subunits of G-proteins in 7315c Cell Membranes and Affinity Purified Material

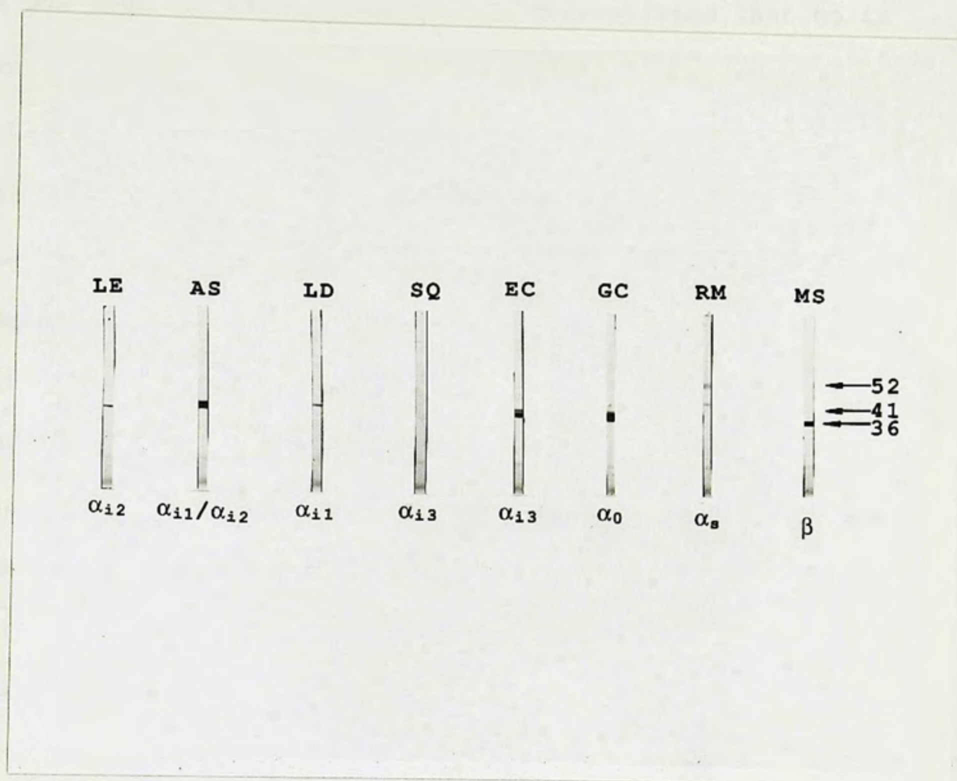
Although the receptor purified pertussis toxin substrate was identified as a 40-41 kDa substrate of pertussis toxin, it remained unclear whether one or several types of pertussis toxin substrates had been co-purified and therefore could interact with mu-opioid receptors in 7315c tumor cell membranes. Antibodies have been raised against specific decapeptide sequences of the three known Gi_{α} proteins, as well as Go_{α} (Goldsmith et al., 1988). These antibodies were used to identify the G-proteins in the 7315c cell membrane and the affinity co-purified preparations.

The G-proteins in the 7315c cell and bovine brain membranes were identified with these antibodies by Western analysis (figure 30). All antisera were affinity purified prior to incubation with the nitrocellulose strips, except MS, a β-subunit specific antisera. The RM antiserum raised against α_s-subunits identifies both the 45 and 52 kDa subunits; these two forms were detected in cholate extracts of bovine brain (figure 30, panel A). In the 7315c cell membrane only the 52 kDa form of the α_s subunit is present (RM, α_s; figure 30, panel B). The LD antisera detected the presence of the 40 kDa α_{i2} subunit in the 7315c cell membrane. The AS antiserum detected both the α_{i1} (41 kDa), and α_{i2} (40 kDa); as shown in the lane marked AS. Antiserum to an internal sequence of α_{i3} (EC) detected only a faint band at approximately 41 kDa; this antisera is less specific since it has been shown to crossreact with the α_{i2} subunit. The SQ antiserum specifically detects the carboxyl terminal of the α_{i3} subunit without crossreactivity. The

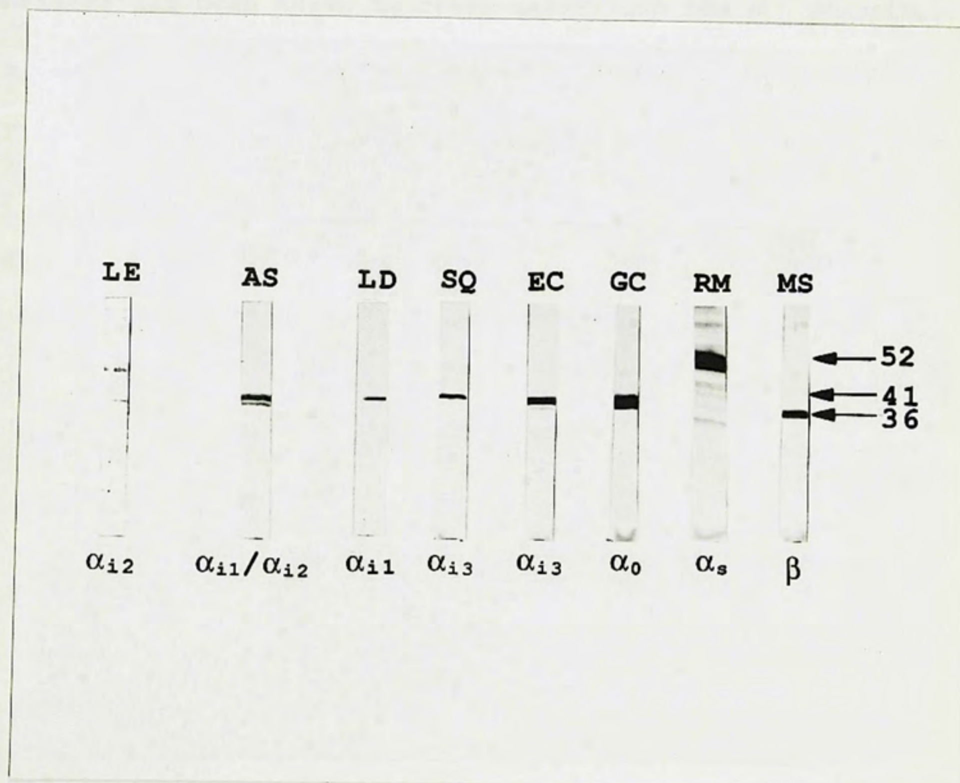
Figure 30. Western Analysis of the α-subunits of the GTP-binding Proteins in 7315c Cell and Bovine Brain Membranes.

Membranes from the 7315c cell were prepared, washed once, and resuspended in Buffer F (8 mg protein/ml) as described in Methods. Cholate extracts of bovine brain membranes were prepared as described (Goldsmith et al., 1988). The 7315c cell membrane suspension and brain detergent extract were diluted (1:1) with SDS-PAGE sample buffer and subjected to gel electrophoresis (Panel A: 150 µg brain extract protein/lane; Panel B: 200 µg 7315c cell membrane protein/lane) using "low" bis-acrylamide 10% gels as described in Methods. After electrophoresis, the proteins were transferred to nitrocellulose filter paper. The paper was cut into strips and incubated with 1-5 µg of the indicated affinity purified antisera: LE (α_{i2}), AS (α_{i1} and α_{i2}), LD (α_{i1}), SQ (an internal sequence of α_{i3}), EC (the carboxyl terminal of α_{i3}), GC (α_o), RM (α_s, both 52 and 45 kdal forms), and crude antiserum MS (β subunit). The strips were washed extensively, and incubated with peroxidase-conjugated IgG. The strips were washed again and developed using buffer containing H_2O_2. Arrows indicate the migration of 52, 41, and 36 kDa proteins.

A



B

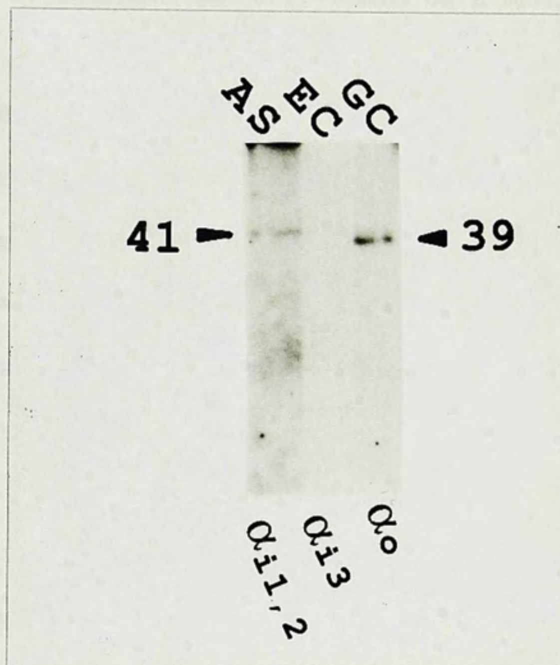


antiserum GC, used to detect α_o (39 kDa), demonstrated that αo is present in the 7315c cell membrane. In addition, the MS antisera detected the presence of $\beta\gamma$ subunits in the 7315c cell membrane.

Since the affinity purified G-protein was shown to be a substrate for pertussis toxin and since all known effects of activation of the mu-opioid receptor were abolished by pertussis toxin treatment, the antisera to the pertussis toxin substrates were used to identify and detect the presence of these α-subunits in the affinity purified preparation. The mu-opioid receptor purified G-protein clearly contains immunoreactive α_o (figure 31, lane α_o). In the same blot the affinity purified G-protein was also immunoreactive with antisera raised to a peptide sequence specific for the carboxyl terminus of both α_{i1} and α_{i2} (AS) and to the antisera raised to an internal sequence of the α_{i3} (EC). The EC antisera has been shown to cross-react with the α_{i2} subunit, therefore further screening using the more specfic antisera SQ would be necessary to unequivocally determine the presence of α_{i3}. The relative intensity of the bands is not quantitative. These data clearly demonstrate that the G-proteins that co-purify with the mu-opioid receptor are Go and Gi_1 and/or Gi_2, implying that these G-proteins normally interact with the receptor in the native 7315c cell membrane.

Figure 31. Western Analysis of the Affinity Co-purified Receptor/G-protein Preparation.

The co-purified receptor/G-protein complex was prepared as described in Methods. The co-purified material was dialyzed overnight in Buffer H and frozen in liquid nitrogen. Ten preparations were thawed, combined, dried under reduced pressure, and resuspended in SDS-PAGE buffer. The sample was subjected to polyacrylamide gel electrophoresis using "low" bis-acrylamide 10% gels as described in Methods. After electrophoresis, the proteins were transferred to nitrocellulose filter paper. The paper was cut into strips and incubated with 1-5 μg of the indicated affinity purified antisera: AS (α_{i1}/α_{i2}), EC (the carboxyl terminal of α_{i3}), and GC (α_o). The strips were washed extensively and incubated with [^{125}I]-Protein A. The paper was developed by autoradiography (5 day exposure). Arrows indicate the migration of the 41 and 39 kDa α-subunits.


AS
EC
GC
41
39
αi1,2
αi3
αo

DISCUSSION

I. Studies of the activated receptor mediated alterations of the guanine nucleotide interaction at Gi.

A fundamental assumption of the current hypothesis of signal transduction by G-proteins is that activation of a membrane receptor by its agonist in some way causes GDP to be replaced by GTP at the G-protein (Gilman, 1987; Birnbaumer et al., 1987). The question then becomes what property of Gi becomes altered that results in the preferential binding of GTP over GDP at the G-protein. In 1978, Cassel and Selinger discovered that stimulation of the β-adrenergic receptor on pigeon erythrocyte membranes enhanced the release of [^{3}H]GDP from the membranes (Cassel and Selinger, 1978). Subsequently, inhibitory receptors were also shown to enhance the release of [^{3}H]GDP from cell membranes (Murayama and Ui, 1984). More recently, carbachol complexed to the muscarinic cholinergic receptor has been shown to reduce the affinity of Gi for GDP when both purified components were reconstituted in phospholipid vesicles (Tota et al., 1987). On the basis of these studies, it has been proposed that the primary function of an agonist-activated receptor is to enhance the release of GDP from its G-protein. The results of the present study are consistent with this proposal, and demonstrate, for the first time, the relevance of guanine nucleotide exchange at Gi to the inhibition of adenylyl cyclase activity.

In the present study, agonist activation of the mu-opioid receptor had no significant effect on the potency of GTPγS in inhibiting adenylyl

cyclase activity. We assume that GTPγS accurately reflects how GTP acts at Gi except that GTPγS remains active at Gi for an extended period of time by virtue of its resistance to hydrolysis. On the other hand, agonist activation of the mu-opioid receptor had a striking effect on the ability of GDP to block the GTPγS-induced inhibition of adenylyl cyclase activity. Since the intracellular concentrations of both GTP and GDP are approximately 100 μM (Chen et al., 1980; Cote et al., 1980), agonist activation of the receptor would insure that GTP, rather than GDP, would bind to and activate Gi. Conversely, in the absence of an agonist, Gi would have an enhanced affinity for GDP and thus maintain the system in an inactive state.

Since an opioid agonist must act through its receptor to affect the interaction of guanine nucleotides with Gi, it was of interest to determine if the mu-opioid receptor itself (unoccupied by an agonist) could exert a negative influence on the interaction of GDP with Gi. Pertussis toxin has been shown to prevent the interaction of receptors and Gi by virtue of the ADP-ribosylation of the cysteine residue near the carboxy-terminus of Gi_{α} (Katada and Ui, 1979; Katada and Ui, 1979; Katada and Ui, 1981; Murayama and Ui, 1983; Kurose et al., 1983; Cote et al., 1984). Apparently, the carboxy-termini of Gs_{α}, Gi_{α}, and Go_{α} are all critical for each G_{α} to interact with its appropriate receptor (Sullivan et al, 1986). In the current study, 7315c cells were incubated with pertussis toxin (30 ng/ml) to uncouple the mu-opioid receptor from Gi. A 3 h treatment was used since these conditions were previously found to abolish the ability of morphine to inhibit adenylyl cyclase activity, and to reduce by more than 70% the radiolabelling of

II. Studies of the mu-opioid receptor interaction with the Inhibitory GTP-binding protein in 7315c cell Membranes.

In the previous section, the influence of the receptor on guanine nucleotide interaction with the G-protein suggested that the receptor may disrupt the interaction of the αβγ heterotrimer to effectively enhance the exchange of GDP for GTP. In this section, the effects of guanine nucleotides on the receptor's affinity for both agonists and antagonists were studied. The effect of guanine nucleotides on opioid agonist binding has been previously demonstrated in brain, and NG108-15 cells (Blume, 1978; Childers and Snyder, 1980; Werling et al., 1988). These data confirm the reported guanine nucleotide effects on agonist binding and clarify the physiologically relevant receptor affinity state.

The high and low affinity binding states of the mu-opioid receptor were defined by determining the ability of the agonist to compete with the [^{3}H]antagonist for the mu-opioid receptor in the presence or absence of guanine nucleotides. The calculated Ki value for the agonist was higher when guanine nucleotides were added to the incubation; antagonist binding was unaffected. Furthermore, guanine nucleotides were tested for their ability to enhance the dissociation of prebound [^{3}H]etorphine from mu-opioid receptors. Guanine nucleotides enhanced agonist dissociation in a concentration-dependent manner. The potencies of the guanine nucleotides in enhancing the agonist dissociation was similar to the rank order of potency of guanine nucleotides in sustaining or blocking (GDP) inhibition of adenylyl cyclase activity; GTPγS > GTP >

Gi_α upon subsequent exposure to [^{32}P]NAD and pertussis toxin (Aub et al., 1987). Pertussis toxin had only a small effect on the IC_{50} of GTPγS in inhibiting adenylyl cyclase. However, the efficacy of GTPγS was substantially diminished. A comparison of the time courses of GTPγS-induced inhibition of adenylyl cyclase in control and pertussis toxin-treated membranes revealed that the difference in the efficacy of GTPγS in the two experimental groups was due to an exaggerated lag in the onset of inhibition of cyclase in the pertussis toxin-treated membranes. The most striking effect of pertussis toxin-treatment was the enhanced apparent potency of GDP in blocking the inhibitory effect of GTPγS; the EC_{50} of GDP was reduced by approximately 10-fold following pertussis toxin-treatment. We suspect that the pertussis toxin-induced tighter binding of GDP to Gi explains the exaggerated lag in the GTPγS-induced inhibition of cyclase; consistent with this proposal was the finding that activation of the receptor in control membranes diminished the lag in the activation of Gi by GTPγS. The enhanced apparent potency of GDP and the exaggerated lag in the onset of cyclase inhibition in the pertussis toxin-treated membranes are consistent with the notion that the unoccupied mu-opioid receptor itself exerts a negative influence on the interaction of GDP with Gi by interacting with the carboxy-terminal of Gi_α (Cote et al., 1982); the binding of an agonist to the receptor amplifies the negative influence of the receptor. This hypothesis can best be tested in a system containing the purified mu-opioid receptor and its GTP-binding protein(s). With this in mind, the co-solubilization and co-purification of the mu-opioid receptor/G-protein complex was undertaken.

GDP. These findings suggested that the low affinity binding state occurred in the presence of guanine nucleotides. The time course experiment revealed that after a 30 min incubation at 37 C in the absence of GTP, 75% of the initial specific binding remained. Based on these observations one could suggest that the receptor would retain a high affinity for the agonist in the absence of GTP, and the affinity for agonist would be shifted to lower affinity once guanine nucleotides interact with the G-protein. Since guanine nucleotide concentrations inside the cell are relatively constant (Chen et al., 1980; Cote et al., 1980) one would suspect that the receptor at any given moment would be found in the low affinity state Therefore, the question then became whether or not the low or high affinity state was physiologically relevant, i.e. which of the two described receptor states is involved in the receptor mediated inhibition of adenylyl cyclase activity.

The ability of morphine to inhibit cyclase activity and to compete with [^{3}H]diprenorphine for the mu-opioid receptor in the absence and presence of varying concentrations of GTP was studied. Although the efficacy of morphine-induced inhibition of adenylyl cyclase at the varying concentrations of GTP differed, the potency of morphine was consistent. In addition, the ability of morphine to compete with [^{3}H]diprenorphine was diminished three-fold when 10 μM GTP was included in the incubation. GTP did not affect the antagonist binding. The potency of morphine to inhibit cyclase activity regardless of the concentration of added GTP agreed most closely with the calculated Ki of morphine to compete with the antagonist in the presence of maximally effective concentrations of GTP. Thus, the low affinity receptor state

correlated best with the potency of the inhibition of adenylyl cyclase activity. This explains the ten-fold higher IC_{50} value for etorphine to induce inhibition of adenylyl cyclase activity as compared to the measured affinity of the receptor for etorphine in the absence of GTP. These data indicate that since the concentration of GDP and GTP are similar (Chen et al., 1980; Cote et al., 1980) and relatively constant in any given moment in the cell, the receptor may be found having low affinity and still remain functionally active.

Since the receptor affinity for agonist is influenced by the guanine nucleotide binding at the G-protein, it was of interest to determine if the receptor, when uncoupled from the G-protein, would exhibit high affinity for the agonist. In order to answer this question experimentally, morphine competition with $[^3H]$diprenorphine binding for the mu-opioid receptor was tested in control and pertussis toxin-treated tissues. As described above, pertussis toxin treatment effectively ADP-ribosylates and uncouples the inhibitory GTP-binding protein from inhibitory receptors. It was anticipated that the effect would be a reduction in the receptor affinity for agonist without changing the affinity for antagonist binding (Kurose et al., 1983; Cote et al., 1984). In an untreated control tissue preparation, the addition of GTPγS to the incubation mixture diminished the ability of morphine to compete with $[^3H]$diprenorphine for binding to the receptor. The calculated Ki for morphine was ten-fold lower in the presence of GTPγS, a result anticipated for this non-hydrolyzable analog of GTP. In the pertussis toxin-treated tissue, antagonist binding was unaffected. Toxin-treatment diminished agonist affinity for the receptor, and

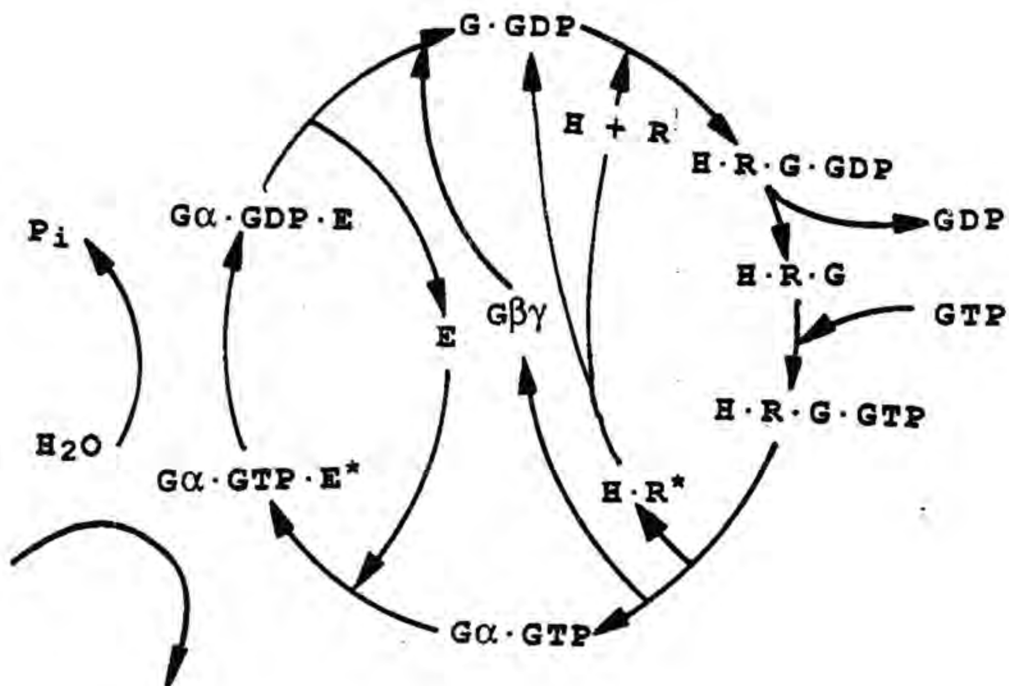
addition of GTPγS did not further diminish agonist binding; the calculated Ki for morphine in this tissue in the absence or presence of guanine nucleotides was similar. Therefore, the mu-opioid receptor exhibits low affinity for the agonist when uncoupled from its G-protein or when guanine nucleotides are bound to the G-protein.

The receptor high affinity state for the agonist appeared in these experiments only when guanine nucleotides were absent from the incubation mixture. The generation of a high affinity state is shown in figure 32. When the agonist interacts with the receptor the agonist/receptor complex selectively diminishes the affinity of the G-protein for GDP. The release of GDP results in a transitory and tightly associated agonist/receptor/G-protein complex. If GTP is present, it will preferentially interact with this complex, and guanine nucleotide-induced reduction of agonist affinity at the level of the receptor as well as activated G-protein interaction with the effector molecule will occur.

This model suggests the generation of a tightly associated transitory agonist/receptor/G-protein complex in high affinity agonist binding occurs. There may be other high agonist affinity receptor states in this cycle. As shown in figure 32, perhaps the agonist/receptor (HR^*) state represents a rapidly generated transitory state of high affinity agonist binding. This complex could conceivably interact with another G-protein (G·GDP), and schematically represents, in part, the ability of the agonist/receptor complex to interact with and activate more than one G-protein as has been previously reported

Figure 32. Agonist-Induced Generation of a Transitory, Tightly Associated Agonist/Receptor/G-protein Complex.

The agonist, H, interacts with its receptor, R. The HR complex has a higher affinity for the GTP-binding protein, G. The G protein is inactive when GDP is bound. The H·R·G·GDP complex enhances the exchange of GDP for GTP at the GTP-binding site (the α-subunit) of the G protein. During the exchange of GDP for GTP, an H·R·G transitory state is formed. This conformation of the interacting proteins alters the guanine nucleotide-binding site such that GTP prefentially binds; this conformation is maintained ("locked-in") until GTP binds. Once GTP binds to the G-protein, the affinity of HR for the G protein is diminished and HR^* complex is free to interact with other available G·GDP units. In the absence of available G·GDP components, the affinity of R for the H is diminished. The active G protein, G·GTP undergoes subunit dissociation; G_α·GTP dissociates from Gβγ. The G_α·GTP activates the enzyme, (E*) which converts ATP to cAMP. The α-subunit hydrolyzes GTP to GDP, the enzyme unit is inactivated, and $G_{\beta\gamma}$ reassociates with G_α forming the heterotrimer G protein with GDP bound (G·GDP) (adapted from Gilman, 1986).



Generation of
Intracellular Signals

Activation of Channel Gating

Adenylyl Cyclase Activity

?

(Pederson and Ross, 1982; Hekman, et al., 1984; Ransnas and Insel, 1988). The rapid turnover of the receptor/G-protein interaction warrants further study. We therefore sought to study this complex away from the other membrane receptor and GTP-binding proteins found in 7315c cell membranes so as to better characterize the mu-opioid receptor G-protein interaction and to unambigously identify the G-protein(s). The transitory agonist/receptor/G-protein complex generated in the absence of guanine nucleotides described above was exploited in our subsequent co-solubilization experiments.

III. Co-Solubilization and Reconstitution of the Mu-Opioid Receptor/G-protein Complex from 7315c Cell Membranes.

The mu-opioid receptor has been solubilized from various brain tissues and studied for its retention of the native receptor protein pharmacologic profile (Ruegg et al., 1980; Bidlack and Abood, 1980; Howells et al., 1982; Simonds et al., 1980; Mollereau et al., 1988). The mu-opioid receptor and its GTP-binding protein have been co-solubilized (Demoliou-Mason and Barnard, 1986; Chow and Zukin, 1983). However, in these reports the studies of the mu-opioid receptor/G-protein interaction were limited. To better understand this receptor activation of GTP-binding proteins and therefore mu-opioid agonist mode of generation of intracellular signal transduction, we sought to co-solubilize the mu-opioid receptor and its G-protein from 7315c tumor cell membranes.

When solubilized in the detergent CHAPS the receptor demonstrated little specific binding, and the detected binding was insensitive to GTP. However, when the receptor prelabeled with [^{3}H]etorphine was solubilized, reasonable specific binding was detected, and GTP was able to enhance the agonist dissociation in a concentration-dependent manner (Frey et al., 1989). This data indicated that the receptor occupancy was a requirement for the retention of the receptor/G-protein complex in detergent solution.

It was also of interest to determine if the solubilized receptor retained competitive equilibrium binding that could be affected by guanine nucleotides. The exchange of [^{3}H]ligand for unlabeled ligand at

the receptor binding site was not possible in detergent solution. This finding indicated that either the tightly associated agonist/receptor/G-protein complex obviates agonist dissociation or that the native receptor protein conformation is lost upon solubilization when a ligand is not bound by the receptor.

The next attempt to measure competitive binding to the solubilized receptor preparation was to reconstitute the receptor/G-protein complex into phospholipid vesicles. As demonstrated in the experiments of binding to the solubilized receptor, specific binding could only be measured if the membrane-associated receptor was first pre-incubated with the agonist, solubilized, and reconstituted into phospholipid vesicles. The affinity of the reconstituted receptor for [^{3}H]etorphine was similar to that of the membrane-associated receptor. As with the membrane-associated receptor (Aub et al., 1987), GTPγS decreased the affinity of an agonist for the reconstituted receptor, strongly suggesting that the reconstituted receptor was complexed with a G-protein. Also, the potencies of GTPγS, GTP, and GDP in diminishing the affinity of [^{3}H]etorphine from membrane-associated and reconstituted receptors were in good agreement. As with other receptors (Smith and Limbird, 1981; Kilpatrick and Caron, 1983; Kelleher et al., 1983; Fitzgerald et al., 1986; Couvineau et al., 1986; Senogles et al., 1987), binding of an agonist to the mu-opioid receptor prior to solubilization was required for the isolation of a functioning receptor-G-protein complex. These results are in contrast with those of others who demonstrated a GTP-sensitive, solubilized mu-opioid receptor in the

absence of receptoe occupancy (Chow and Zukin, 1983; Demoliou-Mason and Barnard, 1986).

In our system the potencies of GTP and GTPγS in diminishing agonist binding to the membrane-associated or reconstituted receptors were 10-fold less than their potencies in regulating adenylyl cyclase. Interestingly, guanine nucleotide potencies in decreasing agonist binding to the solubilized receptor were nearly identical to their potencies in regulating cyclase (Frey et al., 1989). Other investigators have found that a single agonist-bound β-adrenergic receptor can cause GTP to associate with several molecules of Gs in phospholipid vesicles (Pederson and Ross, 1982; Hekman et al., 1984; Ransnas and Insel, 1988) and that the muscarinic, D-2 dopaminergic, and the mu-opioid receptor were reconstituted with nearly ten times the amount of inhibitory G-protein before a reasonable decrease of agonist binding could be detected (Haga, et al., 1987; Ohara et al., 1988; Fujioka et al., 1988; Ueda, et al., 1988). Therefore, to explain the discrepancies in the potencies of GTP and GTPγS described in this study, we speculate that an inhibitory receptor, such as the mu-opioid receptor, causes exchange of GDP for GTP at several Gi's before the agonist dissociates from the receptor, while guanine nucleotide exchange at fewer Gi's is necessary for inhibition of adenylyl cyclase (described above, figure 32). In contrast, the interaction of GTP with the solubilized agonist-receptor/G-protein complex would result in a rapid release of agonist from the receptor due to the unavailability of other G-proteins.

IV. Affinity Co-purification of the Mu-Opioid Receptor/G-protein complex

To date only two types of receptors have been co-purified with their GTP-binding proteins, the D-2 dopaminergic and the muscarinic receptor (Senogles et al., 1987; Tota et al., 1987). In these studies, the preparation of an affinity column involved the covalent attachment of a ligand to a matrix; for the D-2 receptor, an antagonist (spiroperidol) was used. In both studies detergent solubilized extracts of plasma membranes were prepared. These detergent solutions were then passed through the prepared affinity column to capture both the inhibitory receptor and its GTP-binding protein. From the studies discussed in section III above, the solubilized mu-opioid receptor did not exhibit high affinity for an immobile agonist. Furthermore, this experimental design risks co-purifying inhibitory receptors with GTP-binding proteins that would interact with one another only in detergent solution, but not necessarily in the intact membrane. Therefore, we sought to incubate the membranes from 7315c cells with the prepared DADLE-AH Sepharose 4B in the absence of guanine nucleotides to ensure the formation of a stable, tightly associated agonist/receptor/G-protein complex prior to introducing the detergent. The subsequent co-purification of the this complex would thus reflect the *in vivo* receptor G-protein interaction.

A single step affinity chromatography purification procedure of the mu-opioid receptor/G-protein complex from 7315c cell membranes was demonstrated. The co-purified receptor/G-protein complex represents nearly 10% of the agonist plus GTP eluted protein from the affinity

column. The purification of the receptor was approximately 3000-fold; 30% of the membrane-associated mu-opioid receptors were recovered from the starting tissue. The overall yield is comparable to the purification of mu-opioid receptors from brain tissues using other protocols (Maneckjee et al., 1980; Ruegg, et al., 1981; Gioannini et al., 1984; Simonds et al., 1985; Cho et al., 1986). We have characterized the DADLE-AH Sepharose 4B co-purified receptor/G-protein complex. The mu-opioid receptor was shown to specifically bind naloxone, an opioid antagonist, with an affinity similar to the affinity of the intact membrane receptor. The molecular weights of the mu-opioid receptor in the 7315c cell membrane and the affinity purified receptor were similar, both having a molecular weight of approximately 65 kdal. This molecular weight agrees with the results from similar experiments in rat brain (Howard, 1985), and it further supports the evidence of a single protein species of 65 kdal as opposed to the report of several receptor proteins of differing molecular weights (Cho et al., 1986; Ueda et al., 1987). The crosslinking of the membrane-associated or purified receptors with ^{125}I-β-endorphin was shown to be substantially blocked by the addition of excess of naloxone, an opioid antagonist. This finding is consistent with previous reports (Ueda et al., 1987).

The first evidence that a G-protein had been co-purified with the mu-opioid receptor was that recovery of the receptor protein from the affinity resin was three-fold higher when eluted with agonist and GTP than when it was eluted with agonist alone. Additional evidence that the morphine/GTP eluted material contained a G-protein was the finding that pertussis toxin catalyzed the [^{32}P]ADP-ribosylation of a 40 kdal

protein. Finally, immunoreactive α-subunits were shown to be present in the agonist/GTP eluted material and were identified as α_0 and α_{i1} and/or α_{i2}. Given this information, and the fact that the DADLE-AH Sepharose 4B was incubated with the 7315c cell membrane prior to solubilization, we can propose with a reasonable degree of confidence that the pertussis toxin substrates (G-proteins) interacting with the mu-opioid receptor are Go, G_{i1}, and G_{i2}. Therefore, *in vivo* the mu-opioid receptor interacts with more than one type of GTP-binding protein. These data give credence to the recent finding that purified mu-opioid receptors, when reconstituted with either purified Gi or Go, can demonstrate interaction with both types of inhibitory G-proteins (Ueda et al., 1988). In addition, these data are reminiscent of the earlier finding that the D-2 receptor co-purifies with both Gi and Go (Senogles, et al., 1987).

Estimation of the purified mu-opioid receptor to G-protein ratio was derived from the comparison of the maximal number of receptor binding sites and maximal G-protein binding sites in each co-purification sample. We have estimated that the co-purified receptor to G-protein ratio is approximately three to one. However this ratio is only a rough estimate. The GTPγS binding was performed at a concentration of 1 μM, a concentration of GTPγS that probably estimated a sub-maximal concentration for the detection of maximal number of binding sites; at higher concentrations of [^{35}S]GTPγS, nonspecific binding became unmanagable. Furthermore, the two assays were not performed under the exact same procedures; the [^{35}S]GTPγS binding was measured in detergent solution while the receptor binding was measured

in phospholipid vesicles. Despite these shortcomings, we feel that this ratio is within reason.

In the reported studies of the co-purification of the D-2 receptor and the muscarinic receptor G-protein complexes, each receptor system was studied for the functional ability of the receptor to interact with its co-purifiying G-protein. In our purification protocol the receptor/G-protein complex was shown to elute when the eluting buffer was supplemented with GTP. As shown in section II, the presence of GTP lowers the affinity of the receptor for the opioid ligand on the affinity matrix thus allowing the elution of the receptor/G-protein complex. Attempts were made to co-elute the receptor/G-protein using FK38824, a potent mu-opioid agonist, and naloxone. Neither of these two opioid ligands were able to compete with the DADLE-AH Sepharose 4B for the majority of the matrix-bound receptors. In addition, eluting with the inactive guanine nucleotide, GDP, did not preserve the complex during elution and reconstitution procedures. Under the present assay conditions receptor coupling to the G-protein was only detected when the phospholipids were lowered below 20 mg/ml during the reconstitution process. In these preparations, 100 µM GTP was able to reduce specific binding by nearly 80%. However, at the lower phospholipid concentrations, the maximal detectable receptor binding sites was reduced significantly, suggesting that a substantial number of the receptors lacked the ability to bind ligand. On the other hand, one could suggest that the higher phospholipid concentration hinders the ability of the receptor to recouple with the GTP-binding protein. Since the hypothetical ratio is three receptors to one G-protein, any obstacle

to recoupling would result in the inability to detect functional interaction of the receptor with the co-purifying GTP-binding proteins. Furthermore, as discussed in section III and shown in figure 32, the possibility of one agonist/receptor complex interacting with more than one G-protein is likely, and this may be a requirement to experimentally detect functional coupling of the receptor with the G-protein. In other reconstitution studies (Ohara et al., 1988; Ueda et al., 1988; Senogles et al., 1987; Tota et al., 1987), a far greater ratio of G-protein to receptor (usually 10:1) was added to the reconstitution mixture to ensure detection of receptor G-protein interaction by either guanine nucleotide reduction in agonist affinity, agonist induced off-rate of [^{3}H]GDP from the G-protein, or agonist induced GTPase activity. Therefore, either the reconstitution conditions were not conducive for both the receptor binding and receptor/G-protein coupling or addition of batch-purified Go (Go, because it is abundant and can be easily purified from the 7315c tumor cell membrane) may be required to clearly demonstrate functional coupling.

Initially, the model of the receptor/G-protein interaction suggested that one receptor interacted with one type G-protein and this G-protein interacted with one effector (Rodbell, 1980). This model was complicated by the data from reconstitution studies of the purified components of this system. These data suggested that inhibitory receptors could interact with more than one type of G-protein, i.e. Gi and Go (Gilman, 1987). Furthermore, it was suggested that G-proteins could specifically activate a single effector; Gi would simply inhibit adenylyl cyclase activity, Go would alter channel activity, and Gs would

stimulate cyclase. The successful cloning and identification of multiple subtypes of these G-proteins was consistent with the notion that receptor activation of unique subtypes of the G-proteins would translate to agonist-induced specific activation of unique effector molecules. However, as better expression vectors of the cDNAs for these G-proteins have become available, it has recently been shown that the expression of unique subtypes of these G-proteins did not specify unique interaction with unique effectors (Yatani et al., 1988). In fact, multiple subtypes of either stimulatory or inhibitory G-proteins have been shown to interact and affect more than one type of effector (Mattera et al., 1989; Heschler et al., 1987; Von Dongen, 1988). For instance, all forms of Giα (α_{i1}, α_{i2}, α_{i3}) will open K^+ channels and will also mediate the inhibition of adenylyl cylcase activity. Furthermore both long and short forms of Gs_{α} will stimulate adenylyl cyclase and activate Ca^{++} channel gating.

One hypothesis to explain the mu-opioid receptor interaction with multiple G-proteins in the 7315c cell membrane is that mu-opioid agonists inhibit prolactin secretion from this cell via the generation of multiple intracellular signals. For example, Go has been implicated in decreasing the Ca^{++} current from dorsal root ganglion (Yatani et al., 1987b; Heschler et al., 1987) and stimulation of K^+ channels in hippocampal pyramidal neurons (Van Dongen, et al., 1988). Co-isolation of this G-protein would be consistent with the notion of mu-opioid receptor mediated inhibition of prolactin secretion occurs, in part, by decreasing available Ca^{++} or by hyperpolarizing the cell. This receptor activation of Go would represent in part the short term, immediate,

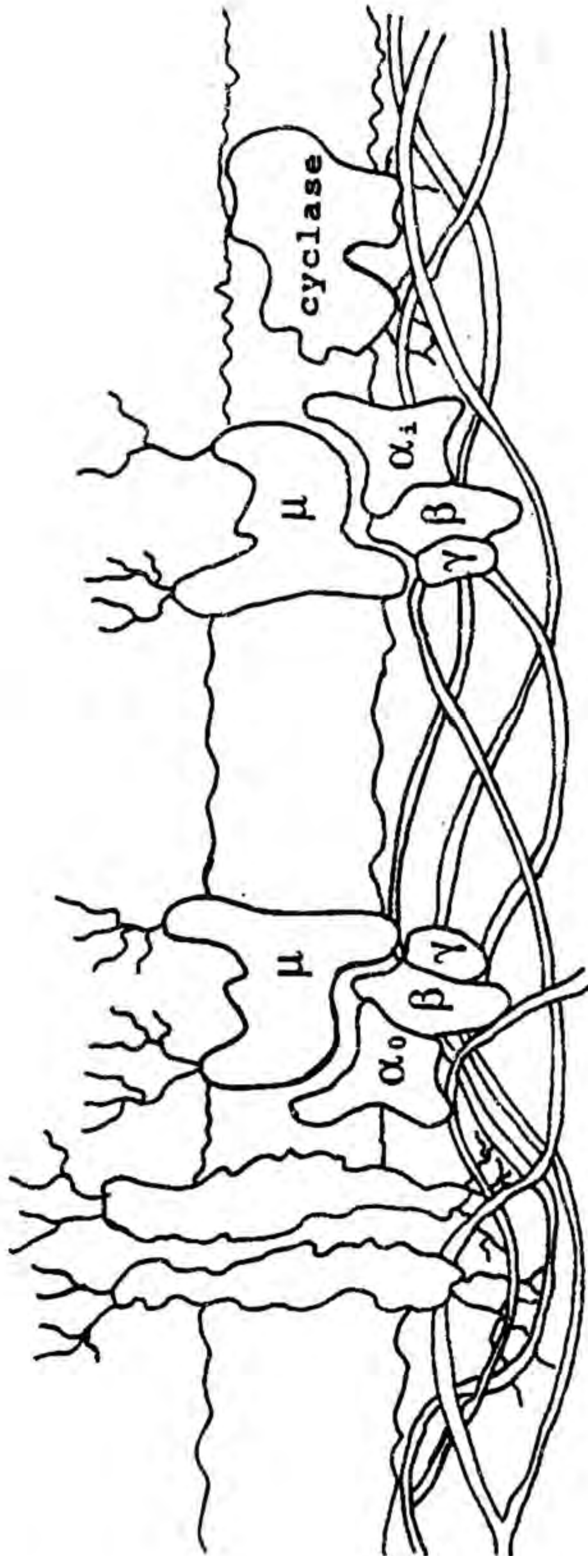
inhibition of prolactin secretion. The receptor mediated activation of either G_{i1} or G_{i2} and subsequent inhibition of adenylyl cyclase activity would inhibit prolactin secretion via the reduction in the cAMP-dependent protein kinase (PKA) mediated phosphorylation cascade. The prolactin gene isolated from a rat genomic library has a cAMP response element located in the 5' flanking sequence upstream from the transcriptional start site (Cooke and Baxter, 1982; Roesler et al., 1988). Thus, decreasing the production of cAMP would result in a reduction in the transcription of the prolactin gene. In addition, cAMP has been implicated in mRNA stability presumably via the activation of PKA. Recently, regulation by cAMP of phosphenolpyruvate carboxykinase (GTP), lactate dehydrogenase, and osteocalcin gene expression occurs via an enhanced transcriptional rate as well as increased stability of the mRNA molecules for each of these gene products (Hod and Hanson, 1988; Jungmann, et al., 1983; Noda et al., 1988). Therefore, it is conceivable that mu-opioid agonist induced inhibition of adenylyl cyclase activity would reduce PKA-mediated enhancement of the transcription of the prolactin gene and/or stability of the prolactin mRNA. Reduction of either transcription or translation of the prolactin gene would represent the long-term reduction of prolactin secretion from the 7315c cell.

A model for the regulation of this receptor-mediated activation of more than one inhibitory GTP-binding protein and their interactions with effectors is shown in figure 33. The cytoskeletal filaments may attach to the receptor, G-protein, and effector molecules to retard their mobility within the lipid bilayer. It is possible that the receptor/G-

protein and effector are co-localized and topographically restricted within the plasma membrane.

Figure 33. Mu-opioid Agonists can Elicit the Generation of Multiple Intracellular Second Messengers Within the 7315c Tumor Cell

Shown is the co-localization of the mu-opioid receptor, G-protein, and effector molecules in the plasma membrane of the 7315c cell. The mu-opioid receptor is shown to interact with both Gi and Go. The cytoskeleton prohibits mobility of the receptor, G-protein, or the effector molecule within the plasma membrane. Agonist interaction with the mu-opioid receptor will activate the co-localized G-protein, and subsequent G-protein interaction with the immobile effector will occur. Mu-opioid receptor activation of Gi or Go will inhibit adenylyl cyclase activity or alter channel gating, respectively.


cyclase
μ
α_i
β
γ
μ
γ
β
α_o

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Appendix 1

Solubilization of the mu-opioid receptor/G-protein complex from 7315c cell membranes has been accomplished (Frey et al., 1989). The following is a description of the procedure used; modifications for the reconstitution studies are found in the Methods section.

Labelling of Membrane-Associated Mu-Opioid Receptors with [^{3}H]Etorphine

Frozen membranes were quickly thawed, diluted 50-fold 25 mM K^+-HEPES, 1 mM $MgSO_4$, and 2 mM EGTA, pH 7.4 (Binding Buffer), and centrifuged at 48,000 x g for 15 min. The membrane pellets were homogenized in buffer either with a Brinkman Polytron (setting 2 for 15 sec) or in a glass cup with 5 strokes of a teflon pestal. The homogenate was incubated with 10 to 12 nM [^{3}H]etorphine in the absence and presence of 100 µM morphine at 37 C for 30 min. Morphine (100 µM) was used to define nonspecific binding. Specific binding was determined by subtracting nonspecific from total binding. Saturation of binding sites was achieved with 10 nM [^{3}H]etorphine within the 30 min incubation (figure 11). The membranes were chilled on ice for 5 min, diluted with about 20 volumes of buffer, and centrifuged for 15 min at 48,000 x g. The resulting pellets were utilized for the solubilization of opioid receptors.

Solubilization of Opioid Receptors.

Pellets of membranes prelabelled with [^{3}H]etorphine were resuspended in buffer supplemented with 10 mM CHAPS by homogenization with 5 strokes of a teflon pestal in a glass cup and incubated for 30

min at 4 C. Oscillation was maintained during the incubation. The membrane suspensions were then centrifuged at 48,000 x g for 30 min. The supernatent was then used in the reconstitution experiments as described.