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The Opioid Receptors of the Rat Periaqueductal Gray

by

Joseph P. Fedynyshyn

DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

Pharmacology

in the

GRADUATE DIVISION

of the

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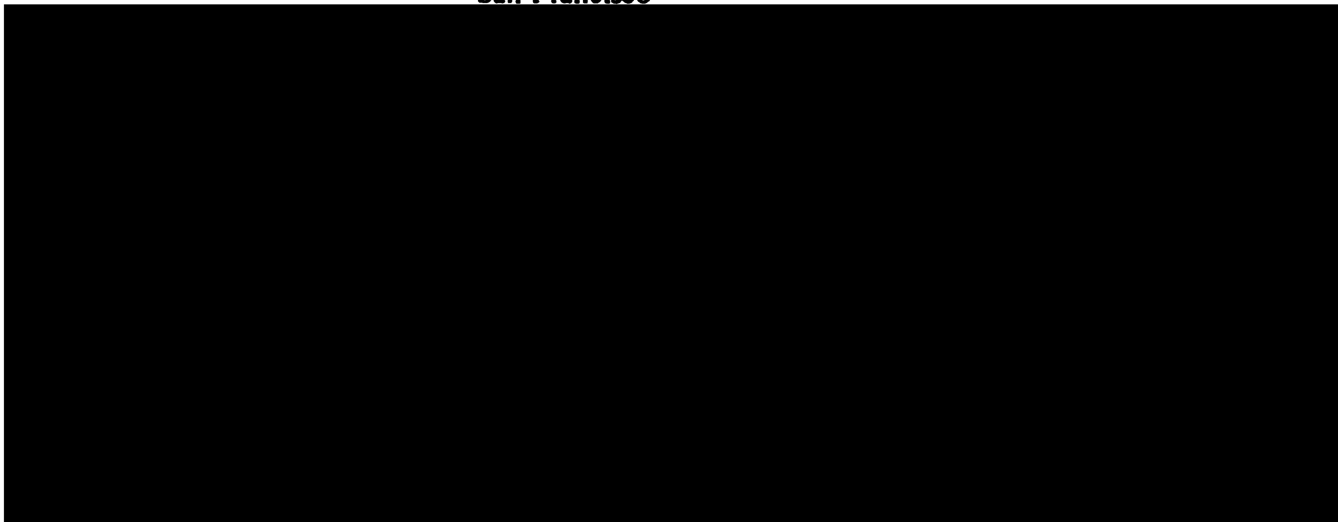
San Francisco



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This thesis is dedicated to my parents who have unselfishly and unfalteringly provided me with support throughout the long years of my education.

Preface

I wish to thank my research advisor Dr. Nancy M. Lee for both her guidance and support throughout the course of my graduate studies.

I also wish to thank Dr. Horace H. Loh and Dr. Anthony J. Trevor for serving on my dissertation committee, and Dr. Loh, Dr. Ping Y. Law, Dr. Sabita Roy, Dr. Y.X. Zhu, and Nancy Tang for additional guidance and/or technical assistance.

The Opioid Receptors of the Rat Periaqueductal Gray
Joseph P. Fedynyshyn

Abstract

The periaqueductal gray (PAG) region of the midbrain has been implicated in both stimulation produced and opioid induced strong analgesia. In this thesis project both the opioid binding characteristics of the rat PAG and the signal transduction mechanisms of the opioid receptors assoicated with this binding were examined with in vitro radioligand binding, GTPase, adenylyl cyclase, and inositol phosphate assays.

In particular the nonselective ligand ^{3}H-ethylketocyclazocine (EKC), the μ and δ selective ligand ^{3}H-[D-Ala^2, D-Leu^5] enkephalin (DADLE), the μ selective ligand ^{3}H-[D-Ala^2, N-Methyl Phe^4, Glyol^5] enkephalin (DAGO), and the δ selective ligand ^{3}H-[D-Pen^2, D-Pen^5] enkephalin (DPDPE) were separately used as tracer ligands to label opioid binding sites in rat PAG enriched P_2 membrane in competition with unlabeled DADLE, DAGO, DPDPE, or the κ selective ligand trans-3,4-dichloro-N-[2-(1-pyrrolidinyl)cyclohexyl] benzeneacetamide, methane sulfonate, hydrate (U50,488H). Only μ selective high affinity opioid binding was observed. No high affinity δ or κ selective binding was detected. The radioligand competition binding data are consistent with the presence of a single population of μ selective high affinity sites to which δ and κ selective ligands also bind with low affinity.

^{3}H-DAGO was also used as a tracer ligand to selectively label μ selective high affinity opioid binding sites in PAG enriched P_2 membrane in competition with unlabeled β-endorphin, dynorphin A (1-17), BAM-18, methionine enkephalin, dynorphin A (1-8), and leucine enkephalin. Of these endogenous opioid peptides , all of which are present in the PAG, only those with previously reported high affinity μ type opioid binding activity competed with ^{3}H-DAGO for binding sites in rat PAG enriched P_2 membrane with affinities similar to that of unlabeled DAGO. The observed ability of these endogenous peptides to compete with ^{3}H-DAGO binding also

generally paralleled the previously reported ability of these peptides to induce strong supraspinal analgesia.

The nonhydrolyzable GTP analog guanyl-5'-yl-β-γ-imidodiphosphate (GppNHp) inhibited specific hgih affinity ^{3}H-DAGO binding in rat PAG enriched P_2 membrane in a dose dependent manner. DAGO stimulated total GTPase activity in rat PAG enriched P_2 membrane in a saturable, dose dependent, μ agonist selective, stereoselective, and naloxone reversible manner. This DAGO stimulation of total GTPase activity was also dependent on Na^+ and Mg^{2+} and was abolished by pretreatment of the membrane with pertussis toxin. The data are consistent with the coupling of μ type opioid receptors to guanine nucleotide binding proteins (G proteins) in the rat PAG.

DAGO inhibited basal adenylyl cyclase activity in rat PAG enriched P_2 membrane in the presence of Na^+ in a dose dependent, μ agonist selective, and naloxone reversible manner. This DAGO inhibition of adenylyl cyclase activity could also be abolished by pretreatment of the membrane with pertussis toxin. While DAGO did no appear to significantly affect phospholipase C activity in intact PAG cell tissue suspensions such an effect could not be conclusively ruled out. The data are consistent with the G protein mediated coupling of μ type opioid receptors to at least the inhibition of adenylyl cyclase activity in the rat PAG. It is possible that the μ type opioid receptors of the rat PAG are also coupled to additional G protein mediated effector systems.

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Introduction

The opioid alkaloid morphine and its many partial and fully synthetic derivatives produce a wide variety of pharmacological effects both in the central nervous system and in the periphery. Consequently morphine and its many congeners are useful in the clinical treatment of a number of medical problems including the treatment of acute pulmonary edema, the control of diarrhea, the suppression of cough, the relief of anxiety, particularly when they are used as adjuncts in preoperative anasthesia, and the management of pain. Of these therapeutic uses the management of pain is the most important. The main indication for the use of morphine and related opioids is the relief of severe acute pain or the relief of severe chronic pain associated with malignant cancer.

In contrast to aspirin and other nonsteriodal anti-inflammatory weak analgesics which act in the periphery, opioid analgesics are strong analgesics which act within the central nervous system. Pain is a complex sensation with both perception and reaction elements, and as such involves a major central nervous system component. The actual anatomical and physiological systems through which pain is processed and through which strong analgesia is manifested is an active area of research. A. I. Basbaum and H. I. Fields propose that both supraspinal and spinal components are involved in strong analgesia and endogenous pain control. In particular Basbaum and Field describe a descending, three-tiered endogenous pain control system which includes the periaqueductal gray (PAG) region of the midbrain, the nucleus raphe magnus (NRM) and adjacent nuclei of the rostral ventral medulla (RVM), and the dorsal horn of the spinal cord (Basbaum and Fields 1984). Opioid induced strong analgesia involves activation of this endogenous pain control system.

The PAG region of the midbrain has been particularly implicated in endogenous pain control and opioid induced analgesia. Focal electrical stimulation within the PAG results in a profound analgesia which is unaccompanied by general motor or behavioral depression (Mayer, Wolfe et al. 1971). Intracerebral microinjection of analgesic opioids into the PAG results in a similar analgesia (Yaksh,

Yeung et al. 1976), and intracerebroventricular injection of opioids into the third ventricle and the vicinity of the PAG is now commonly used to activate the supraspinal components of antinociception. It appears that this opioid induced analgesia in the PAG is mediated by an opioid receptor in that it is stereoselective, dose dependent, and reversed by the opioid antagonist naloxone (Yaksh and Rudy 1978). It also appears that opioid agonism within the midbrain is important if not essential for the mediation of the strong analgesia observed after systemic administration of opioids. The analgesia produced by typical systemic doses of morphine can be reversibly attenuated by intracerebroventricular microinjection of naloxone into the third ventricle in a dose dependent manner (Yeung and Rudy 1980).

A second well known property of morphine and other opioids is their addictive properties, their ability to induce both tolerance and physical dependence. Upon repeated administration of an opioid there is a gradual loss of pharmacological responsiveness to the drug and ultimately a decrease in the clinical effectiveness of a set dosage. In order to maintain a constant level of clinical effect drug dosage must be continually increased. The development of tolerance to opioids is particularly marked in opioid induced analgesia. In addition upon chronic opioid administration a failure to continue administration of the drug or a related congener results in a characteristc withdrawl or abstinance syndrome which reflects an exaggerated rebound from several of the acute pharmacological effects of the drug. This ability of opioids to induce tolerance and physical dependence places significant limitations upon the clinical usefulness of opioid drugs.

The actual pharmacological and biochemical mechanisms through which opioids induce strong analgesia are still unknown. A knowledge of these mechanisms may allow for the logical design of more effective strong analgesics and perhaps even provide an understanding of the mechanisms underlying opioid induced tolerance and physical dependence. It is the purpose of this thesis project to examine the opioid receptors of the PAG region of the rat midbrain, shown in Fig. 1, with the aim of identifying the pharmacological mechanisms mediating opioid induced analgesia. In

particular this study includes: 1) an examination of the opioid binding characteristics of the PAG with an emphasis on identifying the type of opioid binding sites and presumably receptors present in PAG tissue, 2) an examination of the coupling mechanisms associated with these opioid receptors, and 3) an examination of potential signal transduction and effector mechanisms to which these opioid receptors are coupled.

Fig. 1.
The location of the PAG in the midbrain of the rat.

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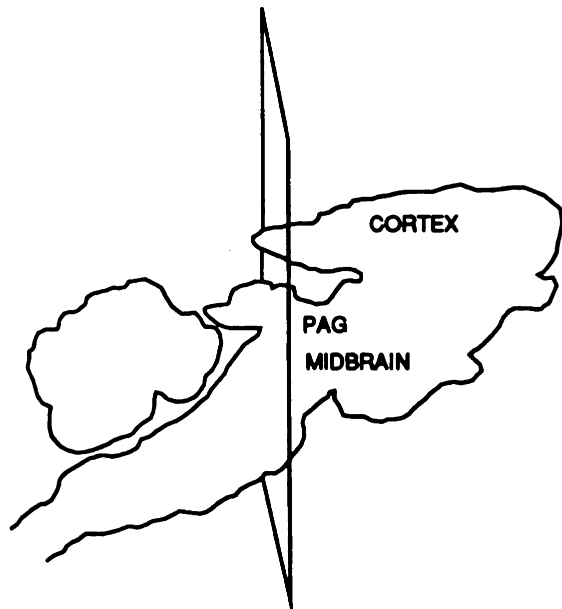
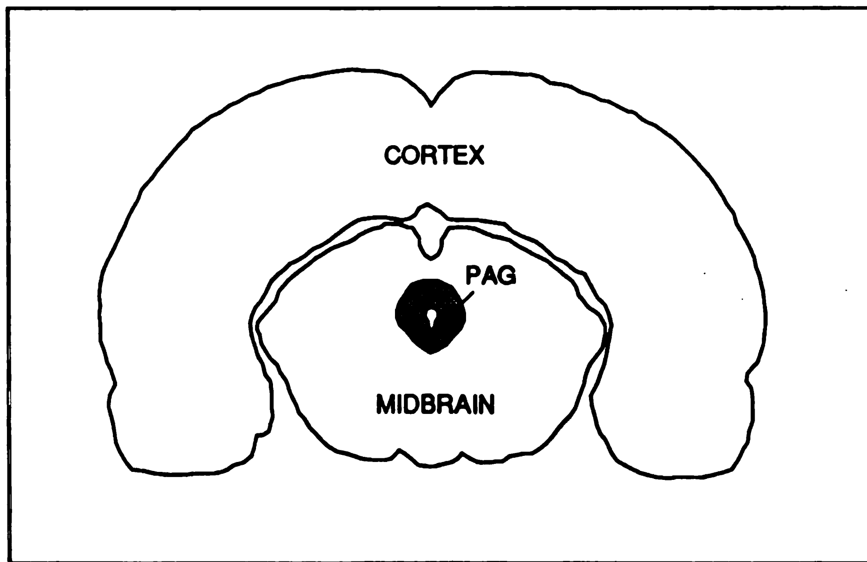


Midline Sagital Section



Transverse Section

Methods

Membrane Preparation

Male Sprague-Dawley rats weighing 180-200 g were used in all experiments. Rats were killed by decapitation, and the brains minus cerebellum were immediately dissected over ice. The PAG region of the midbrain of each rat was initially isolated by three transverse cuts along the main cerebral axis; the first cut caudal to the corpus collosum, the second cut between the superior and inferior colliculi, and the third cut caudal to the inferior colliculi. PAG tissue was further concentrated by removing the white matter surrounding the visible gray matter in the central region of the two inner transverse sections produced by the three previous transverse cuts. The individually isolated PAG regions were pooled, washed in either ice-cold 25 mM Hepes-0.32 M Sucrose buffer (pH 7.7) when prepared for binding assays or in 10 mM Hepes-0.32 M Sucrose-1.6 mM EGTA buffer (pH 7.4) when prepared for GTPase and adenylate cyclase assays, and homogenized in a Teflon-glass homogenizer. The homogenate was centrifuged at 1000 x g for 10 min.. The supernatant was saved and the pellet resuspended in buffer and again centrifuged at 1000 x g for 10 min.. This pellet was discarded and the pooled supernatant from both 1000 x g spins was centrifuged at 22,500 x g for 20 min.. The resulting pellet was resuspended in buffer and again centrifuged at 22,500 x g for 20 min.. The final P_2 pellet was resuspended in either 25 mM Hepes-0.32 M Sucrose buffer (pH 7.7) when prepared for binding assays or in 10 mM Hepes-1.6 mM EGTA buffer (pH 7.4) when prepared for GTPase and adenylate cyclase assays, and frozen in aliquots for future use. Typically the PAG enriched P_2 membrane was resuspended to and stored in an average protein concentration of either 11.5 mg per ml when prepared for binding assays or in 4.8 mg per ml when prepared for GTPase and adenylate cyclase assays as determined by Lowry assay (Lowry, Rosebrough et al. 1951). Thawed PAG enriched P_2 membrane was used once and never refrozen.

Pertussis Toxin Pretreatment of Membrane

PAG enriched P_2 membrane was pretreated with Pertussis toxin utilizing a modified version of a technique described by Kurose et. al. (Kurose, Katada et al. 1983). Pertussis toxin was first activated with 100 mM dithiothreitol for 4 hrs. at room temperature. Membrane was incubated with various concentrations of pertussis toxin in a reaction mixture containing 50 mM Tris buffer (pH 8), 20 mM thymidine 0.5 mM ATP, 20 mM GTP, 5 mM $MgCl_2$, 1 mM EDTA, 5 mM dithiothreitol, 20 mM creatine phosphate, 10 units of creatine phosphokinase, 1 mM 1,10-0-phenanthroline, and 1 mM NAD at room temperature for 1 hr.. The ADP-ribosylation reaction was terminated with the addition of 250 µl of ice-cold 25 mM Hepes buffer (pH 7.4), and the samples were centrifuged at 850 x g for 15 min.. The resulting pellet was resuspended to an appropriate protein concentration in either deionized water for immediate use in GTPase assays or in 10 mM Hepes-1.6 mM EGTA buffer (pH 7.4) for immediate use in adenylyl cyclase assays.

Radioligand Binding Assay

Borosilicate glass tubes were utilized in all binding experiments. The incubation mixture of each radioligand binding assay consisted of 25 mM Hepes buffer (pH 7.7), 10 µM unlabeled opioid, 1 nM or 2 nM tritiated tracer ligand, 50 µl of PAG enriched P_2 membrane, and various concentrations of guanyl-5'-yl-β,γ-imidodiphosphate (GppNHp) when appropriate to a total volume of 1 ml. The incubation mixture of each saturation binding assay consisted of 25 mM Hepes buffer (pH 7.7), either no or 10 µM DAGO, various concentrations of ^{3}H-DAGO, and 50 µl of PAG enriched P_2 membrane also to a total volume of 1 ml. Incubation was carried out for 1 hr. at a constant pH of 7.7 and at a constant temperature of 25°C. In radioligand binding assays utilizing endogenous opioid peptide ligands incubations were carried out for 2.5 hr. at a constant pH of 7.7 and a constant temperature of 0°C. Samples were filtered under vacuum through glass fiber filters (Whatman GF/B) after the incubation period. Filters were washed twice with 5 ml of ice-cold 5 mM Hepes buffer (pH 7.7) and placed in counting vials. Scintillation

cocktail was added to each vial, and the vials were counted 48 hrs. later. All assays were carried out in triplicate unless otherwise indicated. Specific binding was determined as the difference between total bound and that binding remaining in the filter from samples containing both membrane and 10 μM excess unlabeled form of the tracer ligand. One way analysis of variance was utilized in statistical testing.

GTPase Assay

GTPase activity in PAG enriched P_2 membrane was assayed via the release of $^{32}P_i$ from γ^{32}P-GTP utilizing a modified version of the technique described by Koski and Klee (Koski and Klee 1981). The reaction mixture of each GTPase assay consisted of 50,000 cpm of γ^{32}P-GTP, 0.5 μM GTP, 1 mM adenosine-5'-β,γ-imidotriphosphate (AppNHp), 1 mM oubain, 10 mM creatine phosphate, 5 units of creatine phosphokinase, 100 mM NaCl, 5 mM $MgCl_2$, 2 mM dithiothrietol, 0.1 mM EDTA, 12.5 mM Tris-HCl buffer (pH 7.4), and various concentrations of opioid agonists and antagonists prepared on ice. The reaction was initiated in each sample with the addition of 5 μg of the protein contained in PAG enriched P_2 membrane to bring the total reaction volume to 100 μl. Samples were immediately incubated at 37°C for 15 min.. The reaction was terminated with the addition of 900 μl of ice-cold 20 mM H_2PO_2 (pH 2.3) containing 5% weight per volume of activated charcoal, and the samples were centrifuged at 10,000 x g for 15 min.. 200 μl of each supernatant was collected and placed in a counting vial, scintillation cocktail was added, and the vials were counted. All assays were carried out in at least triplicate. One way analysis of variance and unpaired Student's t Tests were utilized in statistical analysis.

Adenylyl Cyclase Assay

Adenylyl cyclase activity in PAG enriched P_2 membrane was assayed via the formation of ^{32}P-cAMP utilizing a modified version of the technique described by White and Karr (White and Karr 1978). The reaction mixture of each adenylyl cyclase assay consisted of 10 mM $MgCl_2$, 40 mM creatine phosphate, 2 mM cAMP, 10 units of

creatine phosphokinase, 40 mM GTP, 20 mM theophyllin, 2 mM 1,10-O-phenanthroline, 100 mM NaCl where appropriate, 40 mM Hepes buffer (pH 7.4), and various concentrations of opioid agopnists and antagonists to a total volume of 50 µl. The reaction mixture was incubated with 25 µl of PAG enriched P_2 membrane for 10 min. at 0°C. Each sample contained 43.5 or 62.5 µg of protein. The reaction was initiated with the addition of 20 µl of a α^{32}P-ATP solution. The final concentration of ATP per assay was 0.1 mM, and each assay contained 1 µCi of α^{32}P-ATP. The reaction was terminated with the addition of 150 µl of 1 M $HClO_4$.

10,000 cpm of ^{3}H-cAMP was added to each sample as an internal standard for cAMP recovery. Reaction product cAMP was separated from other labeled compounds with double columns of Dowex and alumina as described by White and Karr. Collected cAMP was placed in individual counting vials, scintillation cocktail was added, and the vials were counted. All assays were carried out in triplicate. One way analysis of variance and unpaired Student's t tests were utilized in statistical analysis.

Inositol Phosphate Assays

Method 1

Inositol phosphate formation in PAG cells was assayed via the formation of ^{3}H-inositol phosphates from ^{3}H-phosphatidylinositol 4,5-biphosphate utilizing a modified version of the technique desribed by Berridge et. al. (Berridge, Downes et al. 1982). Male Sprague-Dawley rats were killed by decapitation, and the brain minus cerebellum was immediately dissected over ice. The PAG region of the midbrain of each rat was initially isolated by three transverse cuts along the main cerebral axis; the first cut caudal to the corpus collosum, the second cut between the superior and inferior colliculi, and the third cut caudal to the inferior colliculi. PAG tissue was further concentrated by removing the white matter surrounding the visible gray matter in the central region of the two inner transverse sections produced by the three previous transverse cuts. The PAG tissue was finely cross-chopped and placed in a closed vial with carbogenated Krebs-Ringer bicarbonate solution. The

individually isolated intact PAG cell tissue suspensions were pooled and incubated at 37°C for 30 min. with gentle shaking.

200 μl of intact PAG cell tissue suspension was added to 1 ml of a reaction mixture also in a closed vial and incubated at 37°C for an additional 1 hr. with gentle shaking. Each reaction mixture consisted of 0.5 μM myo- [2 ^{3}H] inositol and various concentrations of opioid ligands in carbogenated Krebs-Ringer bicarbonate solution. The reaction was terminated with the addition of 1 ml of a 1:2 (volume:volume) mixture of chloroform and methanol. Each reaction mixture was individually homogenized with 200 μl of 0.5 M HCl and 200 μl of water in a Teflon-glass homogenizer, and an additional 500 μl of chloroform was added. Each sample were centrifuged at 1500 x g for 10 min., and the upper aqueous phases was transferred to an anion exchange column for separation and collection of ^{3}H-inositol phosphates.

Each anion exchange column consisted of 1 ml of AG1-X8, 200-400 mesh of anion exchange resin in formate form. Inositol was eluted from the columns with 10 ml of water, and glycerolphosphoinositols were eluted from the columns with 5 mM sodium tetraborate and 60 mM sodium formate. Inositol phosphate (IP) was eluted from the columns with 5 ml of 100 mM formic acid and 200 mM amonium formate, inositol biphosphate (IP_2) was eluted from the columns with 5 ml of 100 mM formic acid and 400 mM amonium formate, and inositol triphosphate (IP_3) was eluted from the columns with 5 ml of 100 mM formic acid and 1 M amonium formate. All eluted inositol phosphates were collected in counting vials, 10 ml of scintillation cocktail was added, and the vials were counted. All assays were carried out in triplicate.

Krebs-Ringer bicarbonate solution consisted of either 110 mM NaCl or 110 mM LiCl, 12 mM KH_2PO_4, 1 mM $MgSO_4$, 25 mM $NaHCO_3$, 10 mM glucose, and 1.8 mM $CaCl_2$ (pH 7.4).

Method 2

(1,4,5)IP_3 formation in PAG cells was directly assayed with a commercially avilable D-myo-inositol 1,4,5-triphosphatre quantitative assay system utilizing high specific activity tritiated

tracer and a specific IP_3 binding protein. Assayed IP_3 was extracted from PAG intact cell tissue suspensions utilizing a modified version of the technique described by Sharpes and McCarl (Sharpes and McCarl 1982). Intact PAG cell tissue suspension was prepared in carbogenated Krebs-Ringer bicarbonate solution as detailed in Method 1.

200 µl of intact PAG cell tissue suspension was added to 500 µl of a reaction mixture in a closed polypropylene microcentrifuge tube and incubated at 37°C for 1 hr. with gentle shaking. Each reaction mixture consisted of various concentrations of opioid ligands in carbogenated Krebs-Ringer bicarbonate solution. The reaction was terminated with the addition of 500 µl of ice-cold 10% (volume:volume) $HClO_4$. Samples were incubated at 0°C for 10 min. and centrifuged at 2000 rpm on a bench top centrifuge for 5 minutes. The supernatant was transferred to a polypropylene microcentrifuge tube containing 100 µl of 100 mM ETDA (pH 7.0), and samples were neutralized with the addition of 300 µl of a 1:1 (volume:volume) mixture of 1,1,2-trichlorotrifluoroethane and tri-n-octylamine. Samples were vortexed and centrifuged at 2000 rpm for 1 min. 300 µl of the upper phase was transferred to polypropylene tubes for assay of 1,4,5-IP_3.

Each IP_3 assay consisted of assay buffer, either PAG intact cell tissue extract or various known concentrations of (1,4,5) IP_3 ranging from 0.19 pmol to 25 pmol, 6000 cpm of ^3H-(1,4,5) IP_3, and IP_3 binding protein. Assays were incubated at 0°C for 15 min. and centrifuged at 1000 x g for 10 min at 4°C. The supernatants were disgarded and each pellet was individually resuspended in 200 µl of water in a separate counting vial. 3 ml of scintillation cocktail was added, and the vials were counted. The concentration of IP_3 in the PAG intact cell tissue extracts was determined on the basis of its ability to compete with the specific binding of ^3H-(1,4,5) IP_3 to IP_3 binding protein relative to the known IP_3 concentration standards. Specific binding was determined as the difference between total bound and the binding remaining in the pellet from assays containing 4 nM excess unlabeled (1,4,5) IP_3. All assays were carried out in triplicate.

Chapter 1: Exogenous Opioid Ligand Binding

The heterogeneity of opioid receptors is now widely accepted. μ, κ, and δ opioid receptors have been postulated on the basis of pharmacological data obtained in the chronic spinal dog (Martin, Eades et al. 1976) and *in vitro* guinea pig ileum and mouse vas deferens preparations (Lord, Waterfield et al. 1977). High affinity μ, κ, and δ binding sites presumably associated with these receptors have subsequently been identified in brain membrane (Chang and Cuatrecasas 1979; Chang, Hazum et al. 1981; Kosterlitz, Paterson et al. 1981), and respective μ, κ, and δ selective ligands have all been implicated in the induction of strong analgesia (Tyers 1980; Ward and Takemori 1983). High affinity opioid binding sites have also been identified in the PAG region of the midbrain with *in vivo* autoradiographic localization techniques (Atweh and Kuhar 1977). However in this initial study the high affinity opioid binding sites were defined with ^{3}H-diprenorphine, a ligand which is relatively nonselective for the proposed μ. κ, and δ receptor types. A more recent *in vitro* autoradiographic study utilizing more selective tritiated opioid ligands and slide mounted brain sections has identified high affinity μ but not δ or κ binding sites in the PAG (Mansour, Lewis et al. 1986). In contrast a second study utilizing a similar autoradiographic technique has reported both high affinity μ and κ binding sites in this brain region (Tempel and Zukin 1987). Consequently it remains unclear as to which type or types of opioid binding sites and presumably receptors are actually present in the PAG and account for the reported high affinity opioid binding.

To determine the opioid binding site types actually present in the PAG the opioid binding activity of rat PAG enriched P_2 membrane was directly assessed with *in vitro* radioligand competition binding assays. In particular a strategy of reversibly displacing a tritiated tracer ligand with unlabeled ligands which have been previously shown to be relatively selective for μ, δ, and κ opioid binding sites was employed.

Results

2 nM ^{3}H-EKC was first used as a tracer ligand to nonselectively label all opioid binding sites in PAG enriched P_2 membrane. Since previous studies have established that this nominal κ ligand also binds to μ and δ opioid binding sites with high affinity (Chang, Hazum et al. 1980; Snyder and Goodman 1980). 2 nM ^{3}H-EKC should nonspecifically label all three types of opioid binding sites which may be present in PAG membrane. This ^{3}H-EKC tracer ligand was placed in competition with the relatively selective μ ligand [D-Ala^2, N-Methyl Phe^4, Glyol^5] enkephalin (DAGO) (Kosterlitz and Paterson 1980), δ ligand [D-Pen^2, D-Pen^5] enkephalin (DPDPE) (Mosberg, Hurst et al. 1983), and κ ligand U50,488H (Vonvoightlander, Lahti et al. 1983). Although these ligands are not specific for their respective opioid receptor type they are among the most selective μ, δ, and κ ligands currently available. The results are shown in Fig. 2. The shallowness and biphasic nature of the ^{3}H-EKC and DAGO competition curve suggests that ^{3}H-EKC and DAGO share more than one population of common binding sites. DAGO competed with ^{3}H-EKC for at least one population of binding sites with high affinity. In contrast both DPDPE and U50,488H each competed with ^{3}H-EKC for an apparent single population of binding sites with low affinity.

2 nM ^{3}H-DAGO was next used as a tracer ligand to selectively label only high affinity DAGO binding sites in PAG enriched P_2 membrane in competition with DAGO, DPDPE, and U50,488H. The results are shown in Fig. 3. As expected DAGO competed with ^{3}H-DAGO with high affinity for a single population of binding sites. However both DPDPE and U50,488H competed with ^{3}H-DAGO with low affinity in a manner very similar, if not identical, to their competition with ^{3}H-EKC.

2 nM ^{3}H-[D-Ala^2, D-Leu^5] enkephalin (DADLE), a partially selective δ ligand, was also used as a tracer ligand in competition with DADLE, DAGO, and DPDPE. Since DADLE has significant affinity for both δ and μ opioid binding sites (Chang 1984), 2 nM ^{3}H-DADLE should label both high affinity δ and μ binding sites which may be present in PAG membrane. The results are shown in Fig. 4. DAGO competed with ^{3}H-DADLE in a manner similar to its competition with

Fig. 2.
Competition binding curves of DAGO, DPDPE, and U50,488H with 2 nM ^{3}H-EKC. Binding was measured under equilibrieum conditions at 25° C. Nonspecific ^{3}H-EKC binding was defined with 10 μM unlabeled EKC. Each data point is the mean of triplicate determinations.

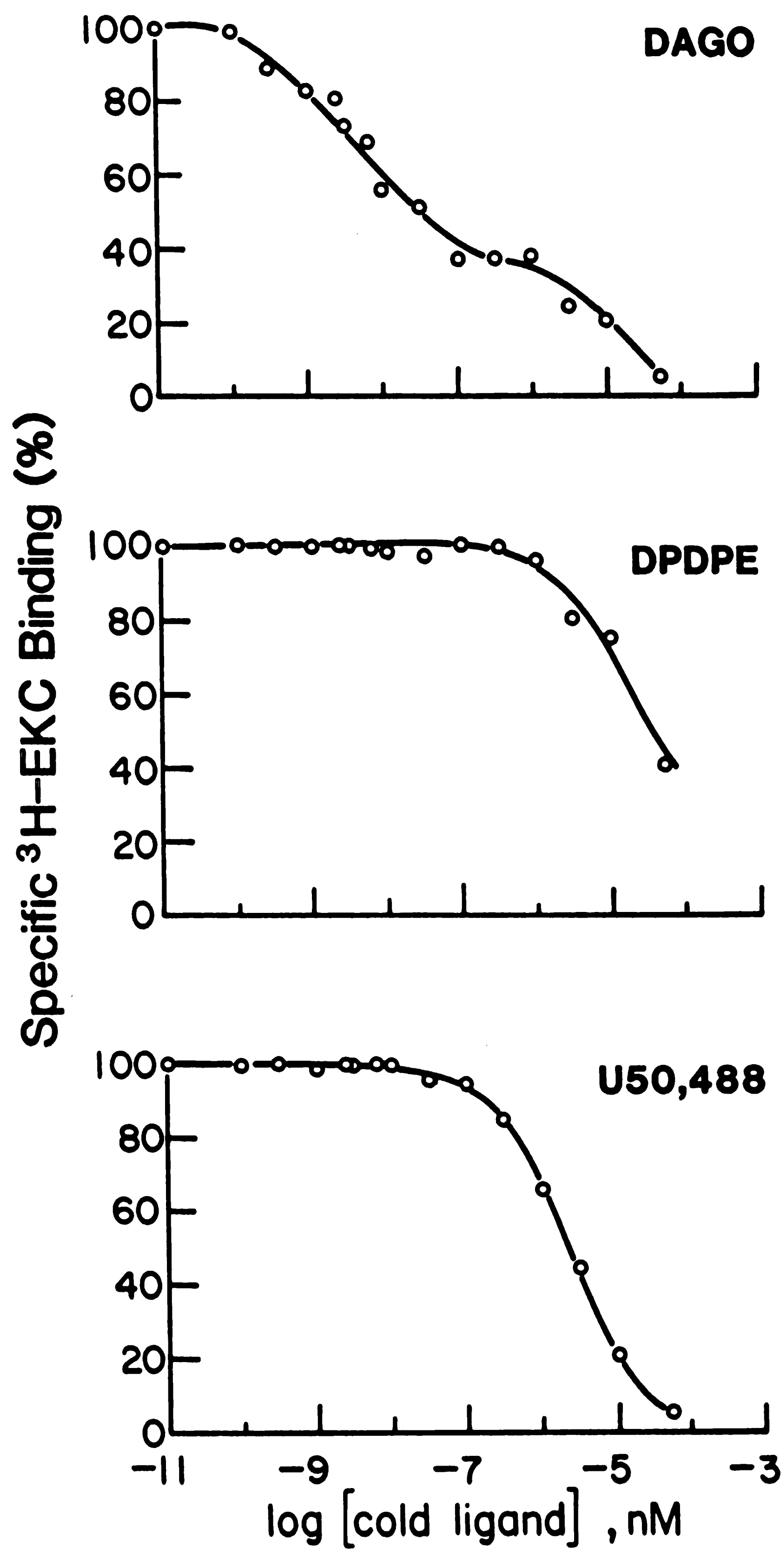

DAGO
DPDPE
U50,488
Specific ^{3}H-EKC Binding (%)
100
80
60
40
20
0
-11
-9
-7
-5
-3
log [cold ligand] , nM

Fig. 3.
Competition binding curves of DAGO, DPDPE, and U50,488H with 2 nM ^{3}H-DAGO. Binding was measured under equilibrium conditions at 25°C. Nonspecific ^{3}H-DAGO binding was defined with 10 μM unlabeled DAGO. Each data point is the mean of triplicate determinations.

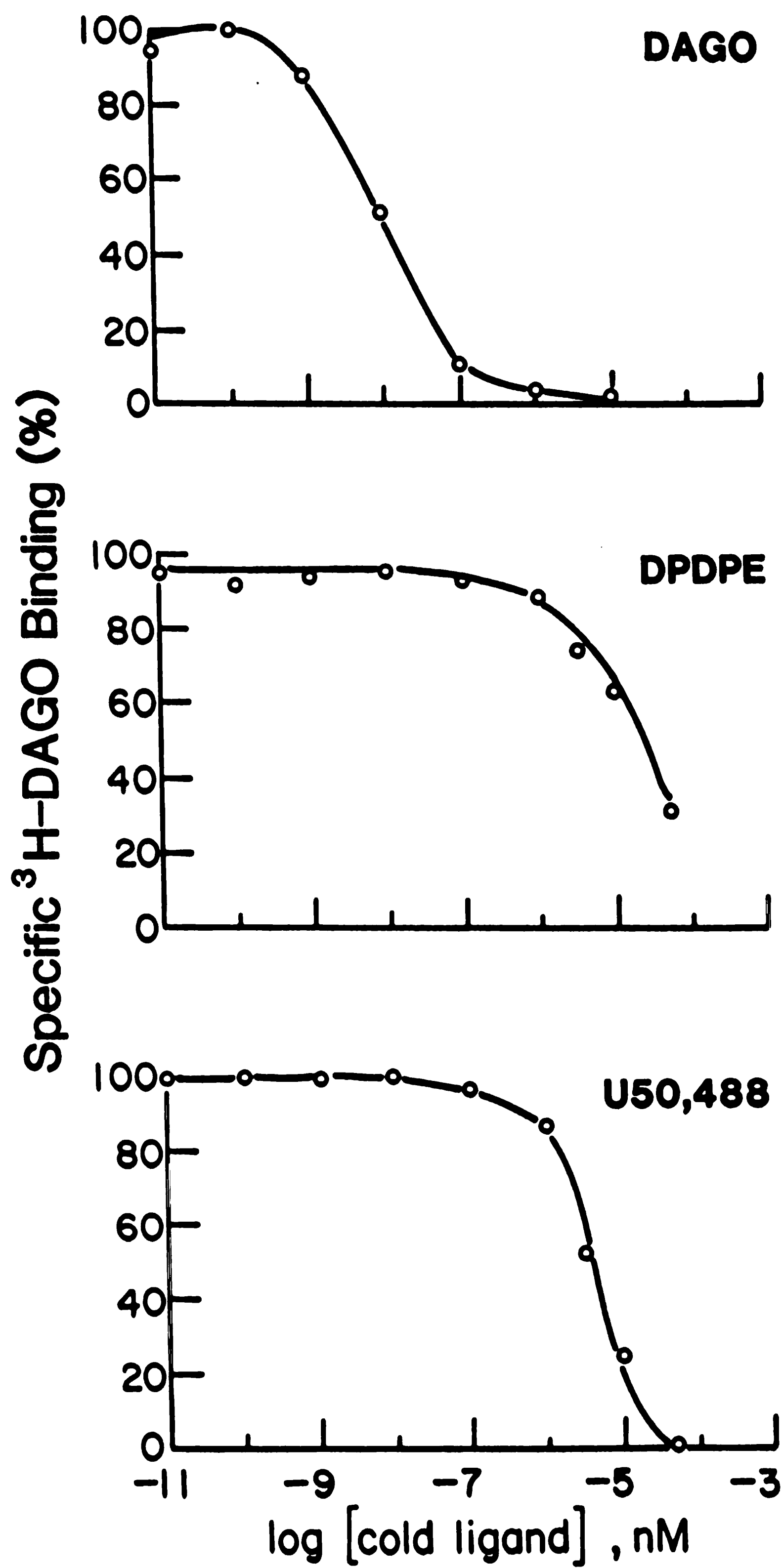
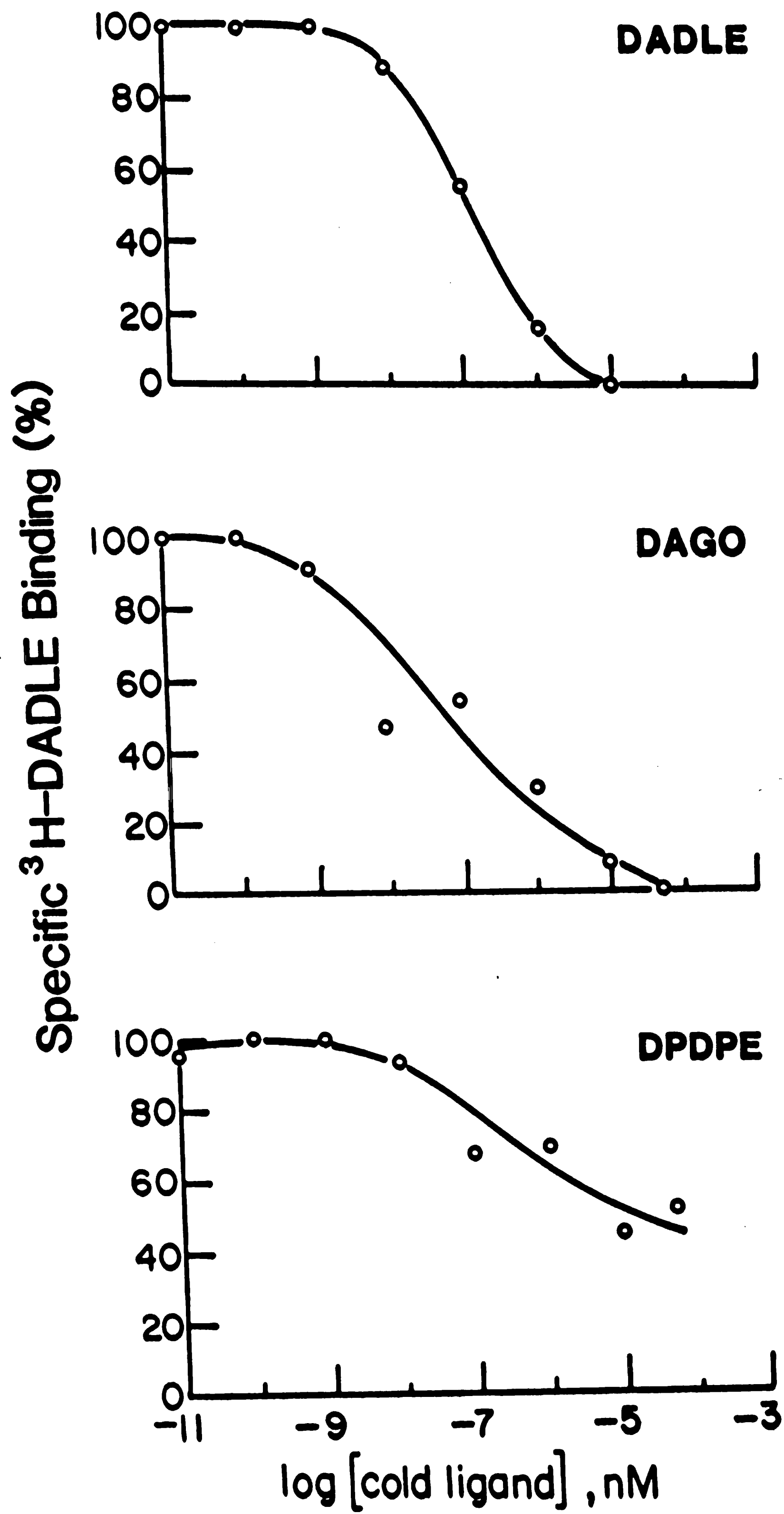

DAGO
DPDPE
U50,488
Specific ^{3}H–DAGO Binding (%)
100
80
60
40
20
0
−11
−9
−7
−5
−3
log [cold ligand] , nM

Fig. 4.
Competition binding curves of DADLE, DAGO, and DPDPE with 2 nM ^{3}H-DADLE. Binding was measured under equilibrium conditions at 25°C. Nonspecific ^{3}H-DADLE binding was defined with 10 μM unlabeled DADLE. Each data point is the mean of triplicate determinations.


DADLE
100
80
60
40
20
0
DAGO
100
80
60
40
20
0
DPDPE
100
80
60
40
20
0
-11
-9
-7
-5
-3
Specific ^{3}H-DADLE Binding (%)
log [cold ligand] , nM

^{3}H-EKC shown in Fig. 1 with a broad curve suggestive of both high and low affinity binding sites. DPDPE however competed with ^{3}H-DADLE for a single population of binding sites with low affinity. There is no component to the ^{3}H-DADLE binding with which DPDPE competed with high affinity.

Finally 2 nM ^{3}H-DPDPE was used as a tracer ligand in competition with DPDPE and DAGO. Specific binding of 2 nM ^{3}H-DPDPE to PAG enriched P_2 membrane was much less than that seen with identical concentrations of ^{3}H-EKC, ^{3}H-DAGO, and ^{3}H-DADLE. The results are shown in Table 1. 1 nM DAGO competed completely with ^{3}H-DPDPE for the little specific binding of this tracer ligand which was detected.

Specific binding of ^{3}H-DAGO in PAG enriched P_2 membrane is saturable. The results are shown in Fig. 5.

Discussion

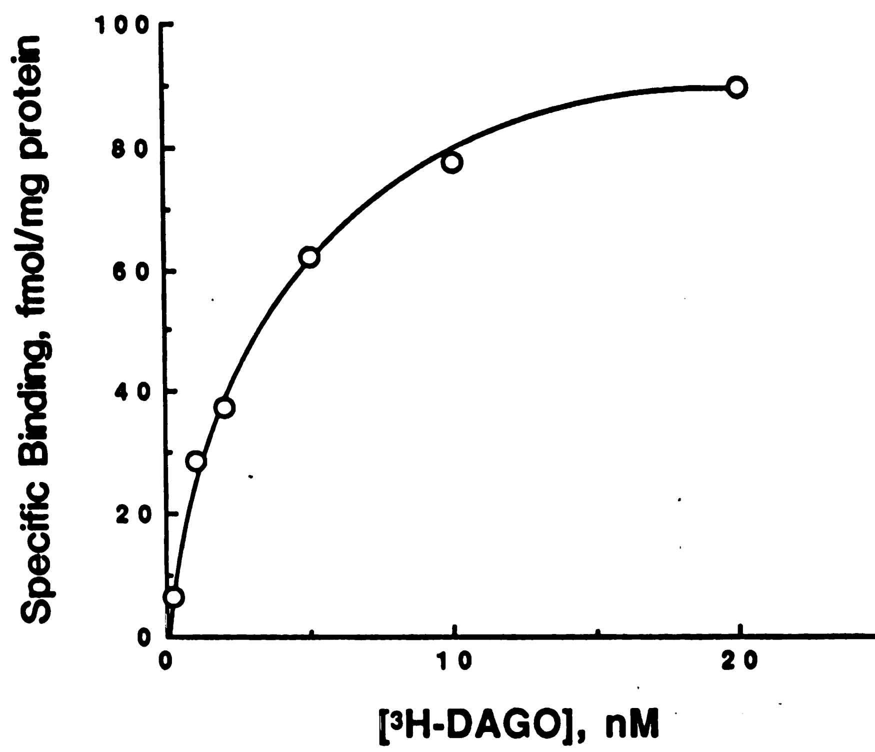
It is apparent from the radioligand competition binding data that only the μ selelctive ligand DAGO reversibly displaces 2 nM ^{3}H-EKC from PAG enriched P_2 membrane with high affinity; the δ ligand DPDPE and the κ ligand U50,488H displace both 2 nM ^{3}H-EKC and 2 nM ^{3}H-DAGO from PAG enriched P_2 membrane with very similar low affinities. These results are most parsimoniously explained by the presence of a single population of primarily μ selective high affinity opioid binding sites in the PAG which are labeled by both ^{3}H-EKC and ^{3}H-DAGO snd to which δ and κ ligands are still capable of binding but with much lower affinity relative to more μ selective ligands. Neither high affinity δ nor high affinity κ binding was detected in PAG membrane.

The absence of high affinity δ binding sites in PAG enriched P_2 membrane is further supported by both the ^{3}H-DADLE and ^{3}H-DPDPE binding data. DAGO competed with the 2 nM ^{3}H-DADLE more completely and with higher affinity than DPDPE. Similarly a lower 1 nM concentration of DAGO competed with the 2 nM ^{3}H-DPDPE for all of the small amount of detectable binding of this tracer ligand. DADLE also competed with ^{3}H-DADLE in PAG P_2 membrane with lower affinity than DAGO competed with its tritiated counterpart, ^{3}H

Table 1. Specific Binding Activity of Exogenous Tracer Ligands

tracer ligand	specific binding to PAG enriched P_2 membrane
2 nM ^{3}H-EKC	44.6 fmol/mg protein
2 nM ^{3}H-DAGO	17.8 fmol/mg protein
2 nM ^{3}H-DADLE	11.8 fmol/mg protein
2 nM ^{3}H-DPDPE	3.0 fmol/mg protein

Fig. 5.
Saturation binding curve of ^{3}H-DAGO. Binding was measured under equilibrium conditions at 25°C. Nonspecific ^{3}H-DAGO binding was defined with 10 μM unlabeled DAGO. Each data point is the mean of triplicate determinations.



Specific Binding, fmol/mg protein
100
80
60
40
20
0
0
10
20
[3H-DAGO], nM

DAGO. DADLE has a higher afinity for δ binding sites than does DAGO, but DAGO has a higher afinity for μ binding sites than does DADLE. These results are consistent with both ^{3}H-DADLE and ^{3}H-DPDPE primarily labeling only μ selective high afinity binding sites in PAG membrane.

It is reasonable to assume that the μ selective high affinity opioid binding sites detected in the PAG enriched P_2 membrane are associated with μ opioid receptors. Specific high affinity ^{3}H-DAGO binding in this tissue is saturable in a manner consistent with binding to physiologically relevant receptors. The nature of the low affinity DAGO displaceable ^{3}H-EKC binding and the low affinity DAGO displaceable ^{3}H-DADLE binding is less clear but could represent a second population of μ binding sites. This is consistent with evidence from several laboratories that at least two distinct μ opioid binding sites exist (Loew, Keys et al. 1986; Nishimura, Recht et al. 1983; Wolozin and Pasternack 1981).

The existence of a single population of high affinity μ opioid receptors in the PAG is consistent with reported physiological data and the idea that supraspinal analgesia produced by both endogenous (Chaillet, Coulaud et al. 1983; Chaillet, Coulaud et al. 1984) and exogenous (Fang, Fields et al. 1986; Wood, Rackham et al. 1981) opioids is mediated by the μ type opioid receptor. While both μ and δ selective opioid agonists induce strong analgesia after intracerebroventricular injection it appears that a single population of μ receptors is sufficient for mediating this effect. This conclusion is principally based on similar apparent pA_2 values for naloxone antagonism of the analgesia produced by both μ and δ selective opioid drugs. It has also been reported that the observed increase in body temperature (Widdowson, Griffiths et al. 1983) and the observed increase in hind-limb muscle tone (Widdowson, Griffiths et al. 1986) after microinjection of opioids into the PAG are both mediated by the μ opioid receptor. The in vitro binding data is in agreement with in vivo pharmacological data.

The radioligand competition binding data while very suggestive of an absence of high affinity δ and κ opioid receptors in the PAG can not conclusively rule out the presence of these receptor types in this

brain region. It is still possible that a small number of δ and κ opioid receptors exist in the PAG below the detection limits of the radioligand binding assay, and this small number of receptors may be physiologically important. It is also possible that δ and κ opioid receptors in the PAG are more sensitive to their environment relative to their μ receptor counterpart, and as such may not be functional and detectable under the in vitro binding conditions of the radioligand competition binding assays. However if, as the data indicates, only a single population of μ opioid receptors is present or significantly predominates in the PAG, PAG tissue represents a system in which the function of the μ opioid receptor may be characterized free from the confounding presence of other opioid receptor types.

Chapter 2: Endogenous Opioid Ligand Binding

The opioid alkaloid morphine and its many partially and fully synthetic derivatives such as EKC and U50,488H as well as the opioid peptides DAGO, DADLE, and DPDPE are exogenous compounds which are not native to central nervous system or peripheral tissue. These exogenous drugs elicit their pharmacological effects by mimicing the action of a variety of endogenous opioid peptides which are synthesized and released by neural and endocrine cells as neurotransmitters, neuromodulators, and neurohormones.

A number of endogenous opioid peptides have been isolated, sequenced, and characterized, and three distinct endogenous opioid peptide gene families have been identified and subsequently cloned. The pre-pro-opiomelanocortin gene codes for the production of β-endorphin (Mains, Eipper et al. 1977; Nakanishi, Inoue et al. 1979), the pre-pro-enkephalin A gene codes for the production of the enkephalin and BAM family of opioid peptides (Noda, Furutani et al. 1982), and the pre-pro-enkephalin B gene codes for the production of the dynorphin and neo-endorphin family of opioid peptides (Kakidani, Furutani et al. 1982).

Several endogenous opioid peptides from each of these gene families have been implicated in the mechanisms underlying strong analgesia. β-endorphin and BAM-18 themselves induce strong analgesia when injected intracerebroventricularly (Loh, Tseng et al. 1976; Stevens, Leslie et al. 1986). Methionine enkephalin and leucine enkephalin themselves may also induce strong analgesia when injected intracerebroventricularly. However these endogenous opioid peptides only induce a transcient analgesia and only if injected in very high doses (Belluzzi, Grant et al. 1976). In contrast dynorphin A, which is not itself a strong analgesic, and leucine enkephalin when injected intracerebroventricularly modulate the strong analgesia induced by morphine and other opioids (Tulunay, Jen et al. 1981; Vaught and Takemori 1979; Vaught and Takemori 1979). All of these endogenous opioid peptides are present in the PAG as demonstrated by immunohistochemical and

immunocytochemical localization techniques (Bloom, Battenberg et al. 1978; Moss, Glazer et al. 1983; Watson, Khachaaturian et al. 1982).

To determine if and with what relative affinity these endogenous opioid peptides bind to the high affinity μ selective opioid binding sites identified in the PAG the opioid binding activity of rat PAG enriched P_2 membrane was once again assessed with in vitro radioligand competition binding assays. In particular a strategy of reversibly displacing ^{3}H-DAGO with unlabeled endogenous opioid peptides at 0°C, a temperature which is less conducive to the potential degradation of the endogenous ligands, was employed.

Results

2 nM ^{3}H-DAGO was used as a tracer ligand to selectively label high affinity μ selective binding sites in PAG enriched P_2 membrane. This ^{3}H-DAGO tracer ligand was placed in competition with DAGO, β-endorphin, dynorphin A (1-13), a synthetic derivative of the endogenous opioid peptide dynorphin A (1-17) which has all the pharmacological properties of the larger naturally occuring peptide (Goldstein, Fischli et al. 1981), BAM-18, methionine enkephalin, dynorphin A (1-8), a major degradative product of dynorphin A (1-17) (Weber, Evans et al. 1982), and leucine enkephalin.

The shallowness of the ^{3}H-DAGO and DAGO competition curve at 0°C suggests that ^{3}H-DAGO labels more than one population of binding sites at this low temperature. DAGO competed with ^{3}H-DAGO for at least two populations of common binding sites with both intermediate and low affinity. The results are shown in Fig. 6.

The shallowness and biphasic nature of the ^{3}H-DAGO and β-endorphin or dynorphin A (1-13) competition curves suggest that ^{3}H-DAGO and β-endorphin and dynorphin A (1-13) also share more than one population of common binding sites. β-endorphin and dynorphin A (1-13) each competed with ^{3}H-DAGO for at least one population of binding sites with intermediate affinity and at least one additional, clearly defined, population of binding sites with much lower affinity. The results are shown in Fig. 7.

Fig. 6.
Competition binding curve of DAGO with 2 nM ^{3}H-DAGO. Binding was measured under equilibrium conditions at 0°C. Nonspecific ^{3}H-DAGO binding was defined with 10 μM unlabeled DAGO. Each data point is the mean of triplicate determinations.

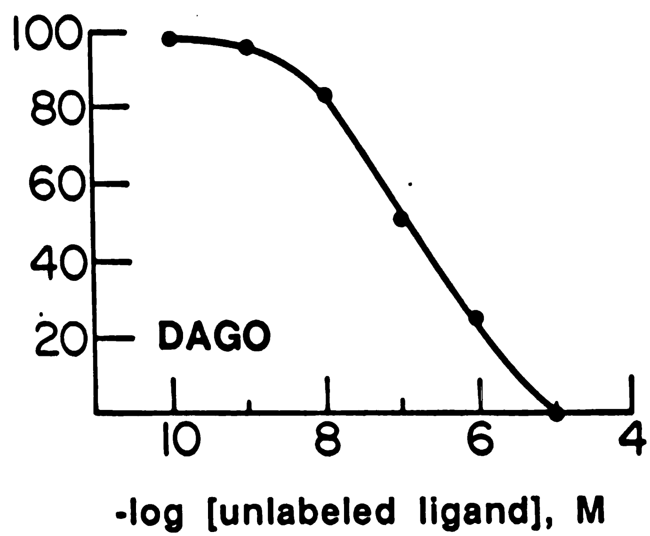
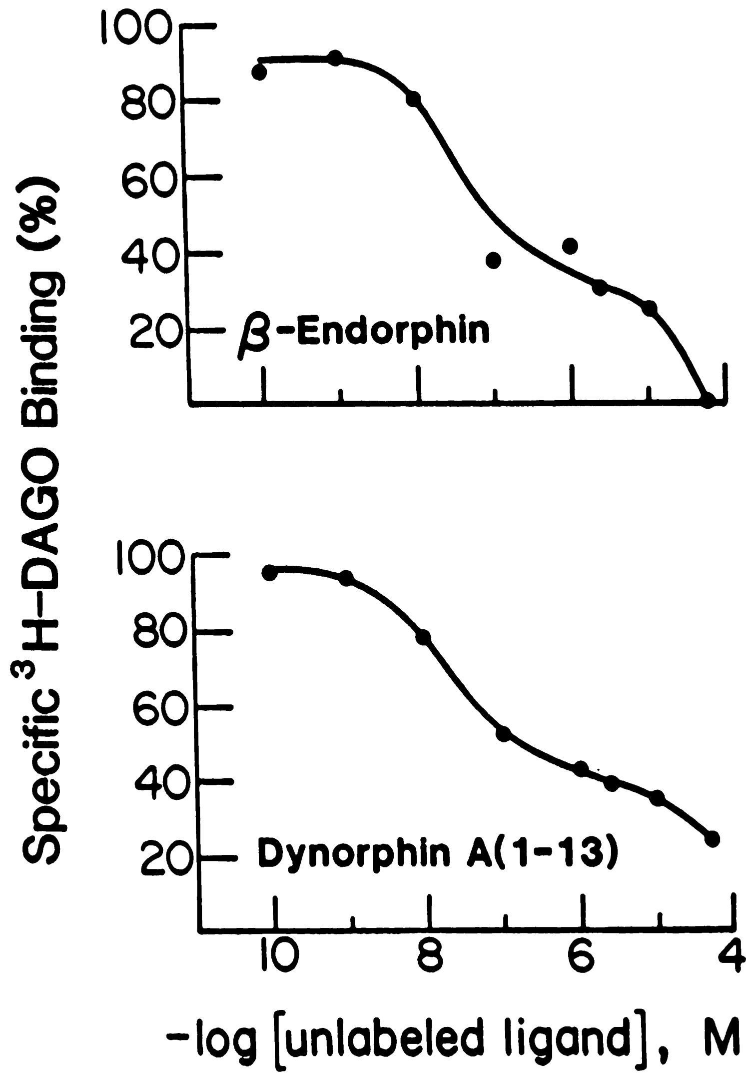

Specific ^{3}H-DAGO Binding (%)
100
80
60
40
20
DAGO
10
8
6
4
-log [unlabeled ligand], M

Fig. 7.
Competition binding curves of β-endorphin and dynorphin A (1-13) with 2 nM ^{3}H-DAGO. Binding was measured under equilibrium conditions at 0°C. Nonspecific ^{3}H-DAGO binding was defined with 10 μM unlabeled DAGO. Each data point is the mean of triplicate determinations.


Specific ^{3}H-DAGO Binding (%)
100
80
60
40
20
β-Endorphin
100
80
60
40
20
Dynorphin A(1-13)
10
8
6
4
-log [unlabeled ligand], M

In contrast BAM-18 competed with ^{3}H-DAGO for an apparent single population of binding sites with intermediate affinity. The results are shown in Fig. 8.

Methionine enkephalin, dynorphin A (1-8), and leucine enkephalin also competed with ^{3}H-DAGO for an apparent single population of binding sites but with low affinity. The results are shown in Fig. 9.

Discussion

It is apparent from a comparison of the radioligand competition binding data shown in chapters 1 and 2 that the specific DAGO binding in PAG enriched P_2 membrane observed at 0°C is different from that observed at 24°C. At the lower temperature used in the endogenous opioid peptide competition binding assays the ^{3}H-DAGO and DAGO competition curve is shifted to the right indicating that at this lower temperature DAGO reversibly displaces ^{3}H-DAGO from μ selective opioid binding sites with decreased affinity. This result is not unexpected. While the effects of incubation temperature on *in vitro* opioid binding in brain tissue are complex, in general the affinity of opioid agonist binding decreases with decreasing temperature (Creese, Pasternack et al. 1975; Law and Loh 1978). At the lower temperature of 0°C 2 nM ^{3}H-DAGO also labels more than one population of μ selective opioid binding sites. This result is most apparent in the ^{3}H-DAGO and β-endorphin or dynorphin (1-13) competition curves, and is again consisitent with evidence from several laboratories that at least two distinct populations of μ selective opioid binding sites exist. At low temperature two populations of μ selective opioid binding sites can be detected in the PAG.

As expected only those endogenous opioiod peptides with reported high affinity μ type opioid binding activity competed with ^{3}H-DAGO for binding sites in PAG enriched P_2 membrane with affinities similar to that of DAGO. Both β-endorphin (Lord, Waterfield et al. 1977) and BAM-18 (Hurlbut, Evans et al. 1987) each competed with ^{3}H-DAGO with intermediate affinity. Dynorphin A (1-13) which was initially described as a κ selective opioid ligand (Chavkin, James

Fig. 8.
Competition binding curve of BAM-18 with 2 nM ^{3}H-DAGO. Binding was measured under equilibrium conditions at 0°C. Nonspecific ^{3}H-DAGO binding was defined with 10 μM unlabeled DAGO. Each data point is the mean of triplicate determinations.

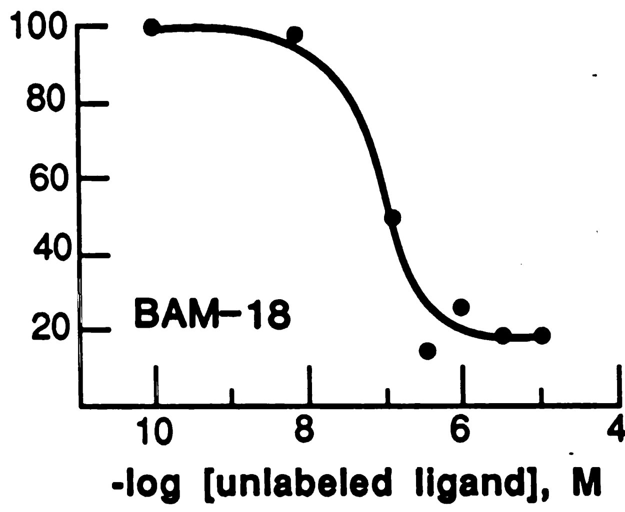
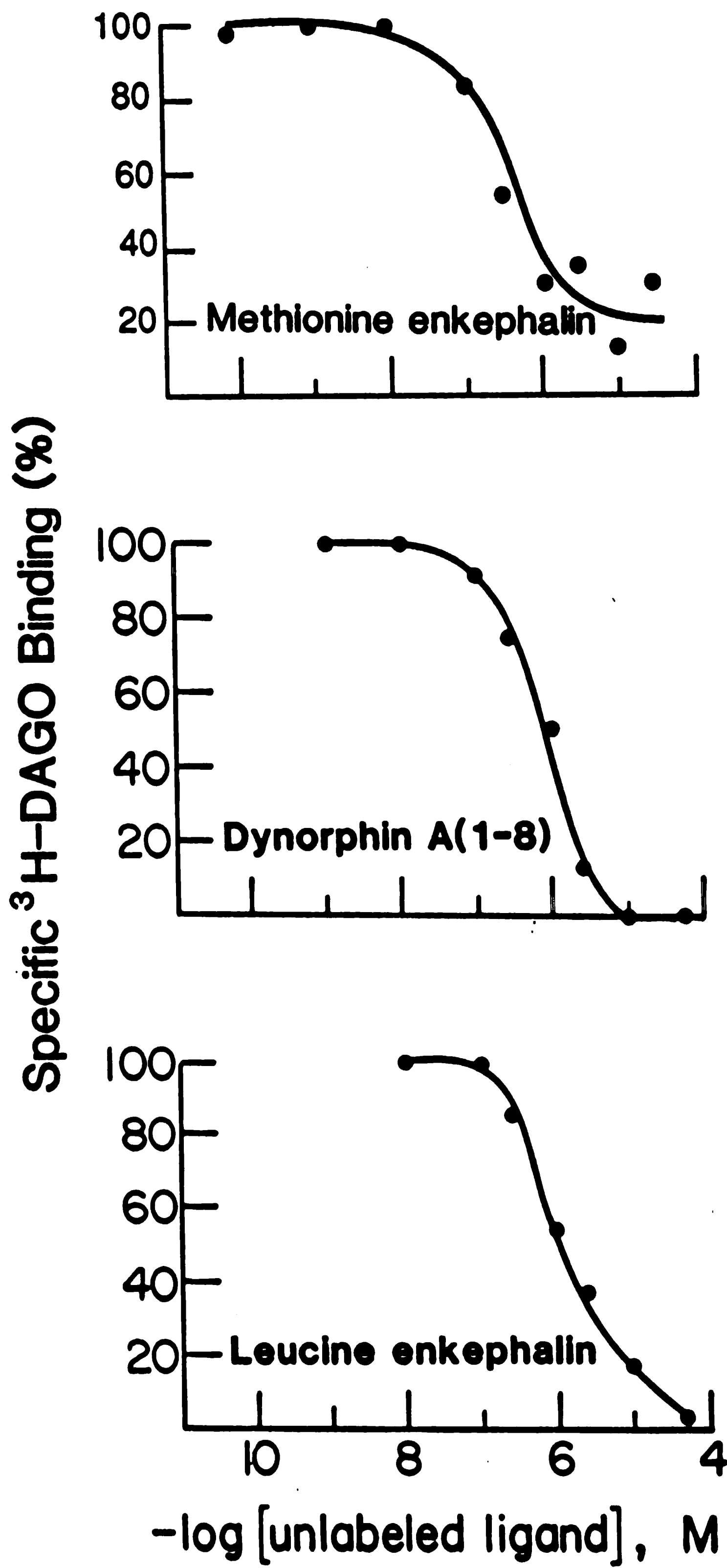

Specific ^{3}H-DAGO Binding (%)
100
80
60
40
20
BAM-18
10
8
6
4
-log [unlabeled ligand], M

Fig. 9.
Competition binding curves of methionine enkephalin, dynorphin A (1-8), and leucine enkephalin with 2 nM ^{3}H-DAGO. Binding was measured under equilibrium conditions at 0°C. Nonspecific ^{3}H-DAGO binding was defined with 10 μM unlabeled DAGO. Each data point is the mean of triplicate determinations.



Specific ^{3}H-DAGO Binding (%)
100
80
60
40
20
Methionine enkephalin
Dynorphin A(1-8)
Leucine enkephalin
10
8
6
4
-log [unlabeled ligand], M

et al. 1982) but which has subsequently been shown to have μ type opioid binding activity as well (Garzon, Sanchez-Blazquez et al. 1984; James and Goldstein 1984) also competed with ^{3}H-DAGO with intermediate affinity. Methionine enlephalin and leucine enkephalin both of which have reported δ type opioid binding selectivity (Chang and Cuatrecasas 1979; Corbett, Paterson et al. 1982; Lord, Waterfield et al. 1977) and dynorphin A (1-8) which has reported κ type opioid binding selectivity (Corbett, Paterson et al. 1982) each competed with ^{3}H-DAGO binding with only low affinity.

The ability of these endogenous opioid peptides to compete with ^{3}H-DAGO for opioid binding sites in PAG enriched P_2 membrane generally parallels the ability of these endogenous opioid peptides to induce strong analgesia. Relative to morphine the rank order of potency of the endogenous peptides to induce strong analgesia after intracerebroventricular injection can be ranked as β-endorphin > morphine > BAM-18 >> methionine enkephalin > leucine enkephalin (Stevens, Leslie et al. 1986; Vaught, Rothman et al. 1982). The most potent analgesic peptides, β-endorphin and BAM-18, also showed the highest affinity for competing with ^{3}H-DAGO binding while the least analgesic peptides, the enkephalins, showed the lowest affinity for competing with ^{3}H-DAGO binding. The in vitro endogenous opioid binding data is in general agreement with in vivo pharmacological data , and is consistent with the idea that supraspinal strong analgesia is mediated by the μ type opioid receptor.

In contrast the ability of dynorphin A (1-13) to compete with ^{3}H-DAGO for opioid binding sites in PAG enriched P_2 membrane does not correlate with strong analgesic activity. However while dynorphin A (1-13) is not itself analgesic after intracerebroventricular injection it can antagonize the analgesia induced by both β-endorphin and morphine in opioid naive animals (Tulunay, Jen et al. 1981). Together the in vitro binding data and the in vivo pharmacological data suggest that dynorphin A (1-17) may act as an endogenous antagonist of μ receptors in the PAG.

The ability of leucine enkephalin to compete with ^{3}H-DAGO for binding sites in PAG enriched P_2 membrane with only low affinity

also does not explain the ability of this peptide to potentiate morphine analgesia after intracerebroventricular injection (Vaught and Takemori 1979; Vaught and Takemori 1979). It is possible that additional non-opioid binding sites and receptors for leucine enkephalin and perhaps the other endogenous opioid peptides are also present in the PAG, and these non-opioid peptide receptors may be associated with their own pharmacological effects. There is no a priori reason that endogenous opioid peptides act exclusively through classically defined opioid receptors. It is also possible that after intracerebroventricular injection leucine enkephalin acts upon opioid receptors in brain regions adjacent to the PAG in order to potentiate morphine induced strong analgesia.

Overall the endogenous opioid ligand binding data demonstrates that those endogenous opioid peptides which are found in the PAG can bind with varying affinities to the μ selective opioid binding sites also identified in this brain region. Furthermore the data suggests that these μ selective opioid binding sites are involved with the strong analgesia associated with endogenous opioid peptides in the PAG.

Chapter 3: Receptor-Effector Coupling

The data obtained from the radioligand competition binding assays indicate the apparent exclusive presence of high affinity μ selective opioid binding sites in PAG tissue. However high affinity binding of an agonist ligand in biological tissue is itself not indicative of a pharmacological receptor. Such high affinity binding must also be coupled to a biochemical event or events and ultimately to a physiological response. Are the high affinity μ selective opioid binding sites identified in PAG enriched P_2 membrane associated with true pharmacological receptors, and if so in what manner and to what biochemical events and signal transduction mechanisms are they coupled?

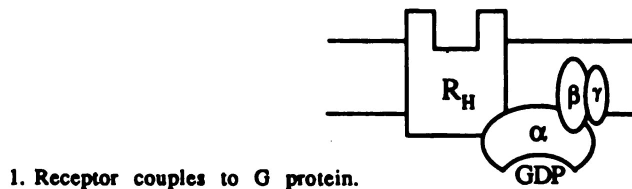
Membrane bound cell surface receptors may be coupled to a biochemical response and signal transduction event either directly or indirectly. For instance agonist binding at a cell surface receptor may be directly coupled to the openning of an ion channel in the cell membrane. Nicotinic (Noda, Takahashi et al. 1983), glycine (Grenningloh, Rienitz et al. 1987), and γ-aminobutyric acid ($GABA_A$) (Schofield, Darlison et al. 1987) receptors are themselves ion channels or part of large ion channel complexes. More commonly agonist binding at a cell surface receptor is indirectly coupled to a signal transduction event via an intermediary guanine nucleotide binding protein (G protein). Muscarinic (Florio and Sternweis 1985), both α and β adrenergic (Cerione, Codina et al. 1984; Cerione, Regan et al. 1986), dopaminergic (Clement-Lormier, Parrish et al. 1975; Creese, Usdin et al. 1979; Zahniser and Molinoft 1978), and serotonergic and $GABA_B$ (Andrade, Malenka et al. 1986) receptors are all coupled to biochemical and physiological responses via G proteins, and a number of functionally and physically distinct G proteins have been identified or postulated in a variety of tissues including the brain. Transducin (Baehr, Morila et al. 1982) is involved in the light activated regulation of cyclic GMP phosphodiesterase, G_s (Sternweis, Northup et al. 1981) and G_i (Katada, Bokoch et al. 1984) are involved in the regulation of adenylate cyclase activity, and G_K (Yatani, Codina et al. 1987) is involved in the regulation of K^+ channels. The function

of G_o (Sternweis and Robishaw 1984) which accounts for as much as 1% of all brain protein remains unknown.

As implied in their name G proteins bind guanine nucleotides with high affinity, and it is this property which plays an important role in their coupling of agonist binding to a signal transduction mechanism (Gilman 1987). In general cell surface receptors couple to the inactive, heterotrimeric form of a G protein to which GDP is bound. When coupled to the G protein the recptor has its highest affinity for agonist ligands. Extracellular binding of an agonist to the receptor results in the displacement of the bound GDP with GTP, and the receptor dissociates from the now active G protein. Dissociated G protein in turn activates an intracellular enzyme system or alters ion channel function. The uncoupled active GTP bound form of the G protein also contains an intrinsic GTPase activity which hydrolyzes the bound GTP to GDP, and thereby cycles the G protein back to its inactive, receptor coupled form. A more detailed schematic of G protein function is shown in Fig. 10. As a consequence of this functional scheme: 1) nonhydrolyzable GTP analogs will effectively and irreversibly uncouple cell surface receptors from G proteins and reduce their affinity for agonist, and 2) agonist binding will stimulate GTPase activity by stimulating the exhange of GTP for GDP bound to the G protein.

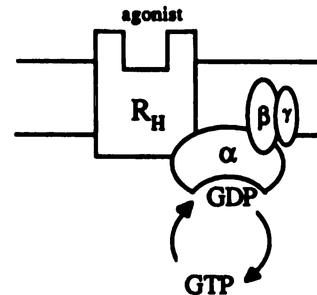
In the NG-108,15 neuroblastoma-glioma cell line, a cell line which expresses only a single opioid receptor type (Chang and Cuatrecasas 1979), agonism at the δ opioid receptor is coupled to the inhibition of adenylyl cyclase activity most likely via G_i (Kurose, Katada et al. 1983). To determine if agonist binding at brain μ selective binding sites in PAG enriched P_2 membrane is similarly coupled to a G protein or proteins both the effects of a nonhydrolyzable GTP analog upon high affinity DAGO binding and the effects of opioid ligands on GTPase activity were respectively assessed in this tissue with radioligand binding and GTPase assays.

Fig. 10.
The functional coupling of cell surface receptor to G protein.

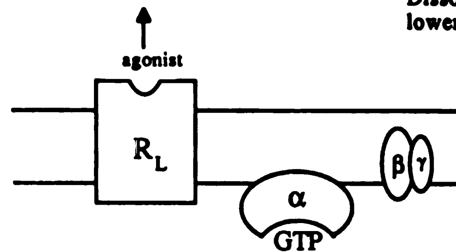


1. Receptor couples to G protein. Coupled receptor has higher affinity for agonist ligand.

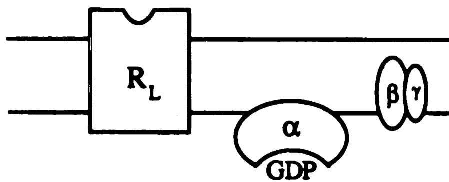
2. Binding of agonist ligand to receptor stimulates the exchange of GTP for GDP on the G protein heterotrimer.



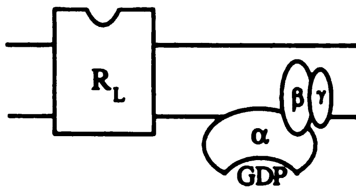
3. Binding of GTP leads to the dissociation of the receptor from the G protein and the dissociation of the G protein subunits. Dissociated α subunit acts upon effector system. Dissociated receptor has lower affinity for agonist.



4. Intrinsic GTPase activity on the α subunit hydolyzes bound GTP to GDP.



5. G protein subunits reassociate to form heterotrimer.



Results

A 2 nM concentration of the μ selective opioid agonist ligand ^{3}H-DAGO was used to label high affinity μ selective binding sites in PAG enriched P_2 membrane. Increasing concentrations of the nonhydrolyzable GTP analog guanyl-5'-yl-β-γ-imidodiphosphate (GppNHp) decreased the total specific binding of 2 nM ^{3}H-DAGO in a dose dependent manner. The results are shown in Fig. 11.

Increasing concentrations of DAGO stimulated total measurable GTPase activity of PAG enriched P_2 membrane in a dose dependent and saturable manner. The results are shown in Fig. 12 and Fig. 13. Stimulation of total GTPase activity is maximal at a 10 μM concentration of DAGO. This effect is selective for opioid agonists with a reported μ profile. Neither the δ selective agonist ligand DPDPE nor the κ selective agonist ligand U50,488H increased total GTPase activity to a significant extent. The results are shown in Fig. 14. In subsequent experiments higher concentrations of morphiceptin, an opioid ligand with greater selectivity but lower affinity for μ opioid binding sites than DAGO (Chang, Killian et al. 1981), did significantly and maximally increase total GTPase activity in PAG enriched P_2 membrane. The results are shown in Fig. 15.

DAGO stimulation of total GTPase activity in PAG enriched P_2 membrane was also antagonized by the opioid antagonist naloxone in a stereoselective manner. Only a 10 μM concentration of the (-) enantomer of naloxone and not of the (+) enantiomer of this ligand antagonized DAGO stimulation of total GTPase activity. A 10 μM concentration of naloxone(-) itself did not significantly affect total GTPase activity. The results are shown in Fig. 16.

Both Na^+ and Mg^{2+} are necessary for DAGO stimulation of total GTPase activity in PAG enriched P_2 membrane. In the absence of these cations total GTPase activity was reduced and a 1 μM concentration of DAGO had no significant effect on the lower total GTPase activity. The results are shown in Fig. 17.

Pretreatment of PAG enriched P_2 membrane with increasing concentrations of activated pertussis toxin abolished DAGO stimulation of total GTPase activity. After pretreatment of

Fig. 11.
Dose response curve of GppNHp inhibition of 2 nM ^{3}H-DAGO binding expressed as percent decrease in total specific binding. Binding was measured under equilibrium conditions at 25°C. Nonspecific ^{3}H-DAGO binding was defined with 10 μM unlabeled DAGO. Each data point is the mean of triplicate determinations. $F=10.0674032$, $p<0.001$.

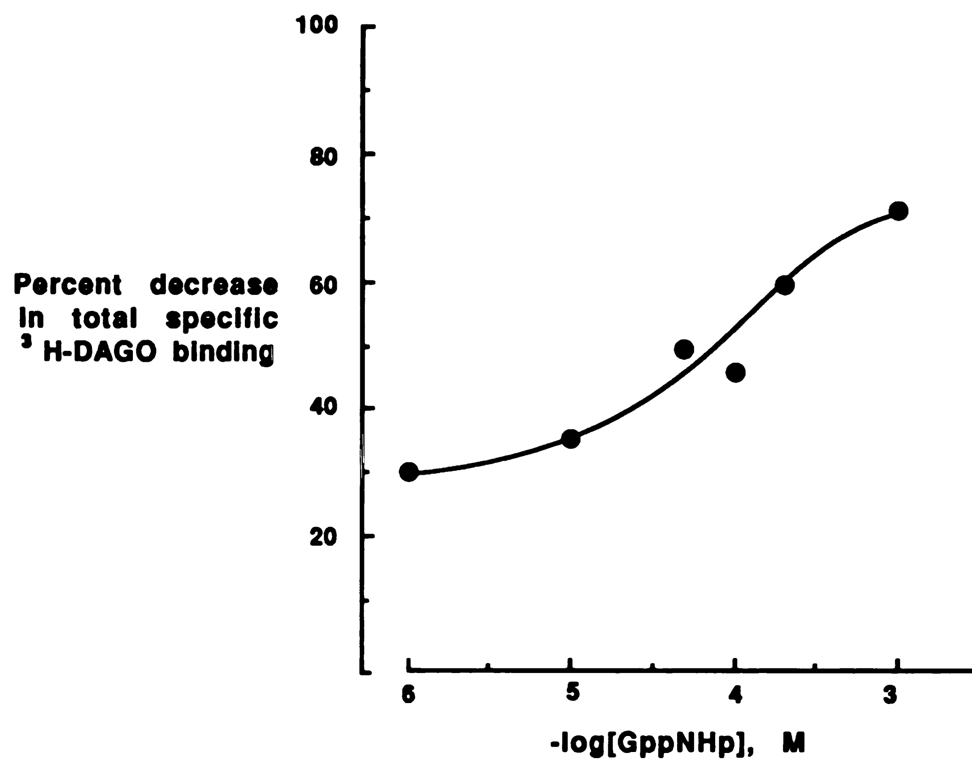

100
80
60
40
20
Percent decrease
in total specific
3 H-DAGO binding
6
5
4
3
-log[GppNHp], M

Fig. 12.
Effects of DAGO on total GTPase activity. The data are expressed as percent increase in activity relative to the basal total GTPase activity of non-opioid treated controls. Each column represents the mean of triplicate determinations.
Basal activity=38.03±2.32 pmol $^{32}P_i$/min/mg protein.

LI LI

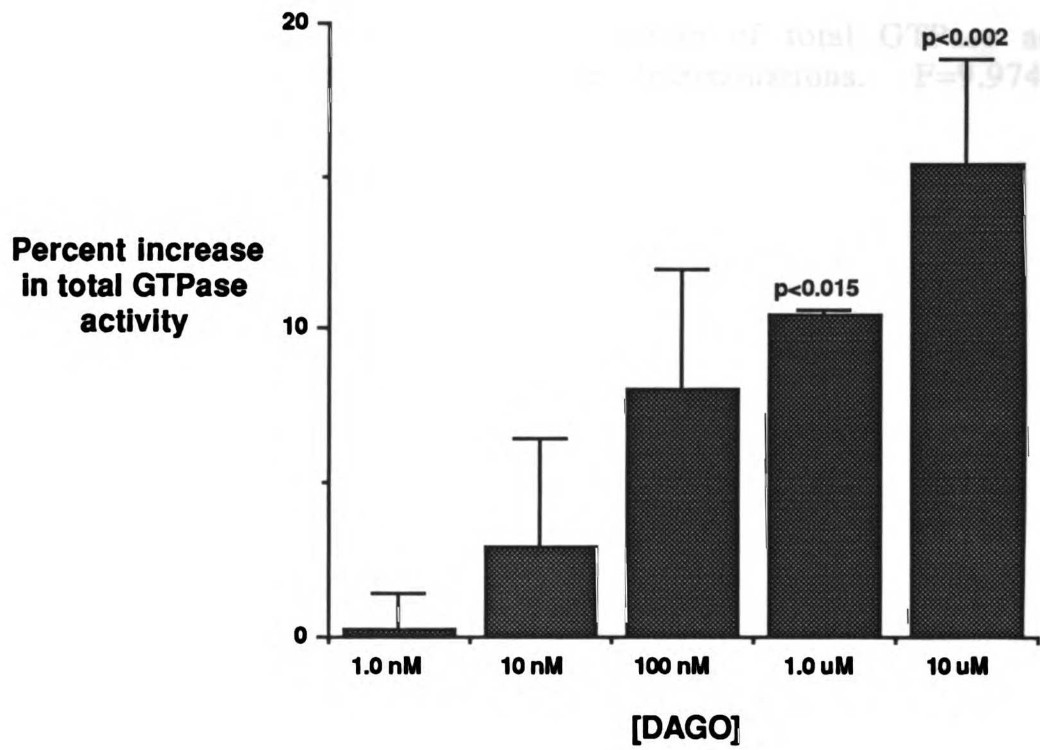

20
10
0
Percent increase
in total GTPase
activity
p<0.015
p<0.002
1.0 nM
10 nM
100 nM
1.0 uM
10 uM
[DAGO]

Fig. 13.
Dose response curve of DAGO stimulation of total GTPase activity. Each point is the mean of triplicate determinations. $F=9.97451311$, $p<0.002$.

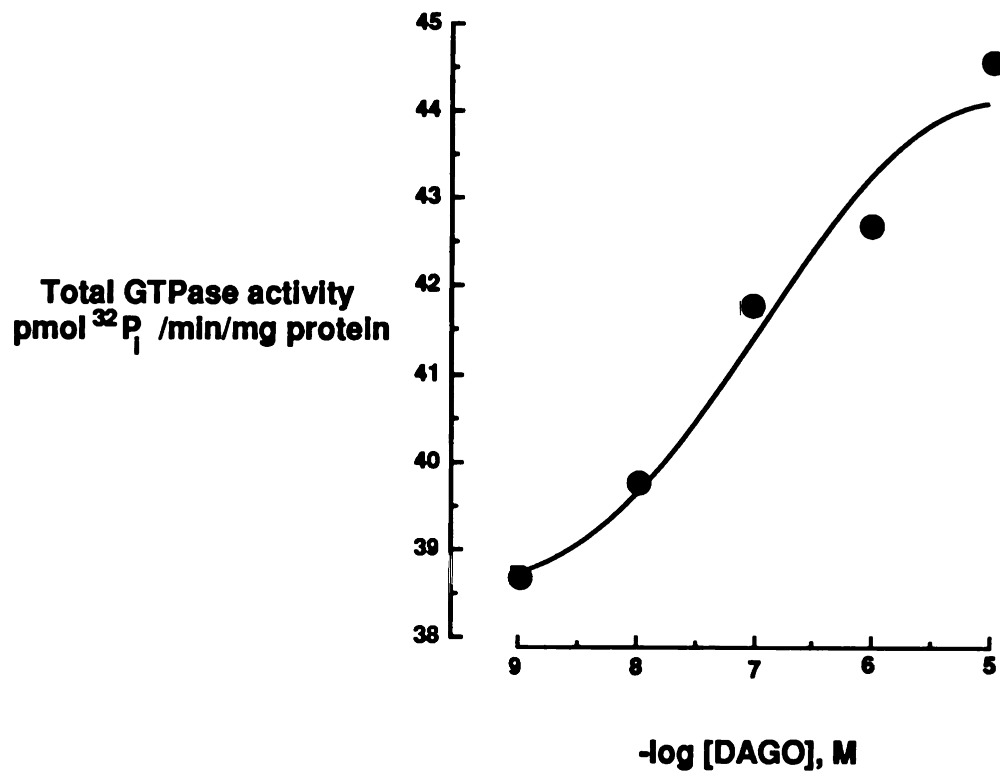
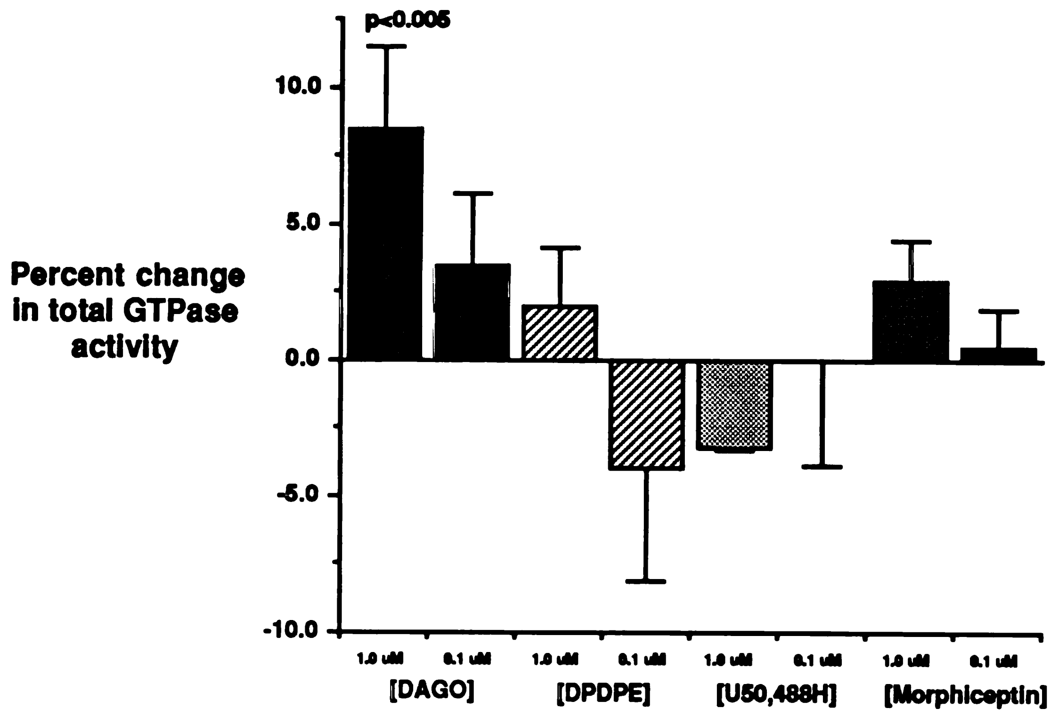

Total GTPase activity
pmol $^{32}P_i$ /min/mg protein
45
44
43
42
41
40
39
38
9
8
7
6
5
-log [DAGO], M

Fig. 14.
Effects of DAGO, DPDPE, U50,488H, and morphiceptin on total GTPase activity. The data are expressed as percent change in activity relative to the basal total GTPase avtivity of non-opioid treated controls. Each column represents the mean of triplicate determinations.
Basal activity=75.80±2.45 pmol $^{32}P_i$/min/mg protein.



p<0.005
10.0
5.0
0.0
-5.0
-10.0
Percent change in total GTPase activity
1.0 uM
0.1 uM
1.0 uM
0.1 uM
1.0 uM
0.1 uM
1.0 uM
0.1 uM
[DAGO]
[DPDPE]
[U50,488H]
[Morphiceptin]

Fig. 15.
Effects of morphiceptin on total GTPase activity. The data are expressed as percent change in activity relative to the basal total GTPase activity of non0opioid treated controls. Each column represents the mean of triplicate determinations.
Basal activity=20.91±0.60 pmol $^{32}P_i$/min/mg protein.

LI LI

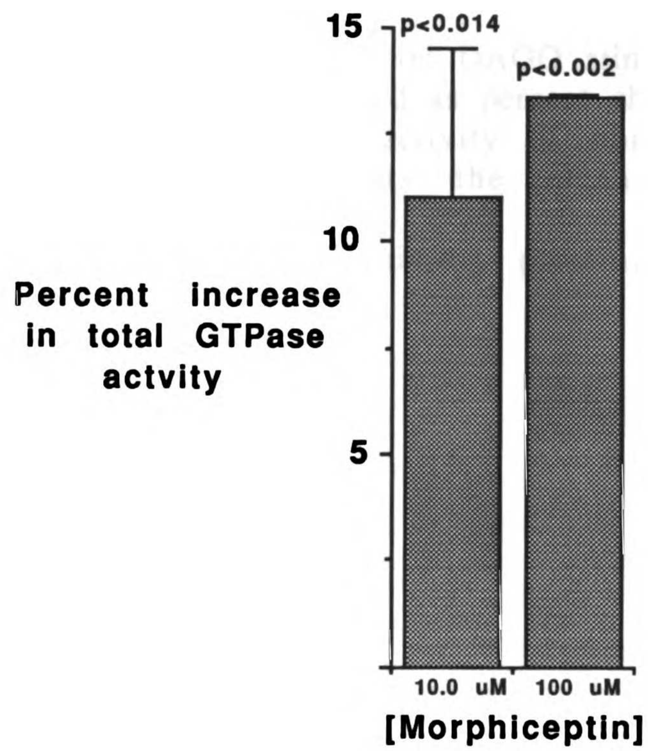

15
p<0.014
p<0.002
10
5
Percent increase in total GTPase actvity
10.0 uM
100 uM
[Morphiceptin]

Fig. 16.
Effects of naloxone(+) and naloxone(-) on DAGO stimulation of total GTPase activity. The data are expressed as percent change in activity relative to the basal total GTPase activity of non-opioid treated controls. Each column represents the mean of triplicate determinations.
Basal activity=26.89±1.77 pmol $^{32}P_i$/min/mg protein.

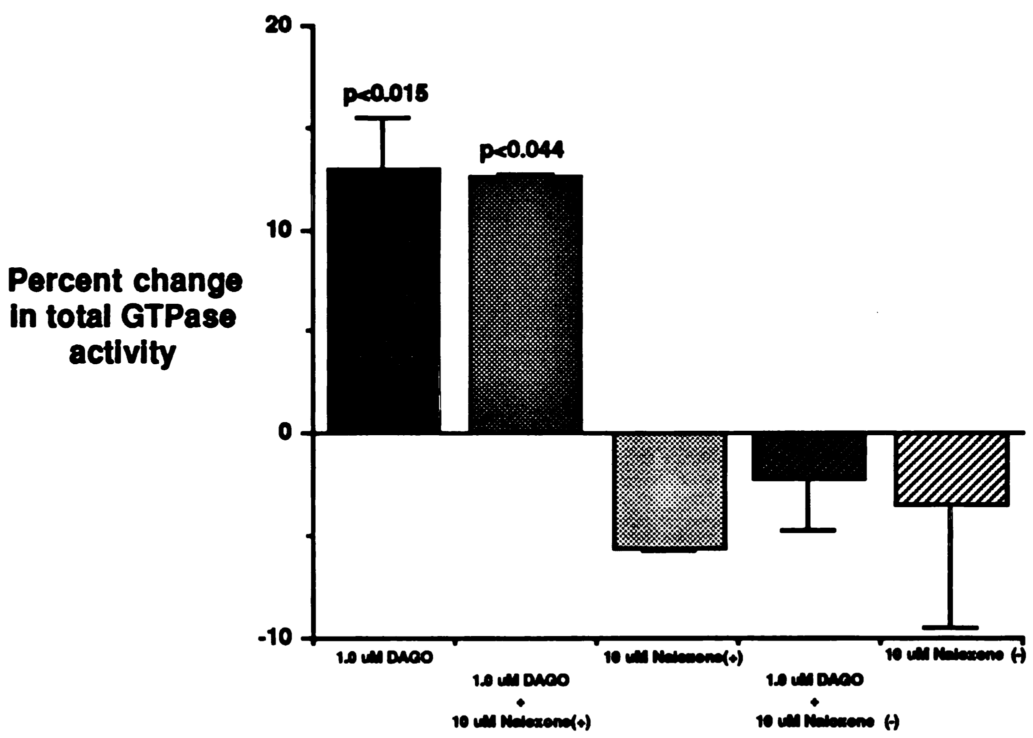
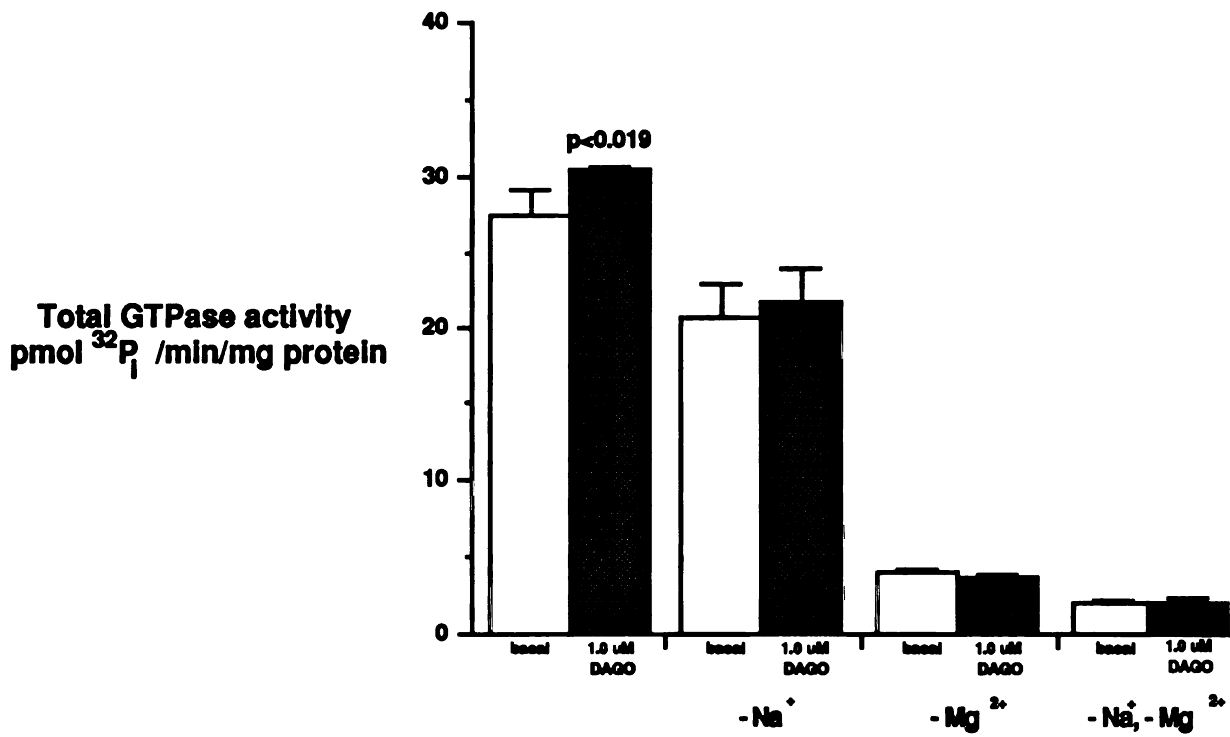

20
10
0
-10
p<0.015
p<0.044
Percent change
in total GTPase
activity
1.0 uM DAGO
1.0 uM DAGO
+
10 uM Naloxone(+)
10 uM Naloxone(+)
1.0 uM DAGO
+
10 uM Naloxone (-)
10 uM Naloxone (-)

Fig. 17.
Effects of Na^{+} and Mg^{2+} on DAGO stimulation of total GTPase activity. Each column represents the mean of triplicate determinations.


Total GTPase activity
pmol $^{32}P_i$ /min/mg protein
40
30
20
10
0
p<0.019
basal
1.0 uM DAGO
basal
1.0 uM DAGO
basal
1.0 uM DAGO
basal
1.0 uM DAGO
- Na^+
- Mg^{2+}
- Na^+, - Mg^{2+}

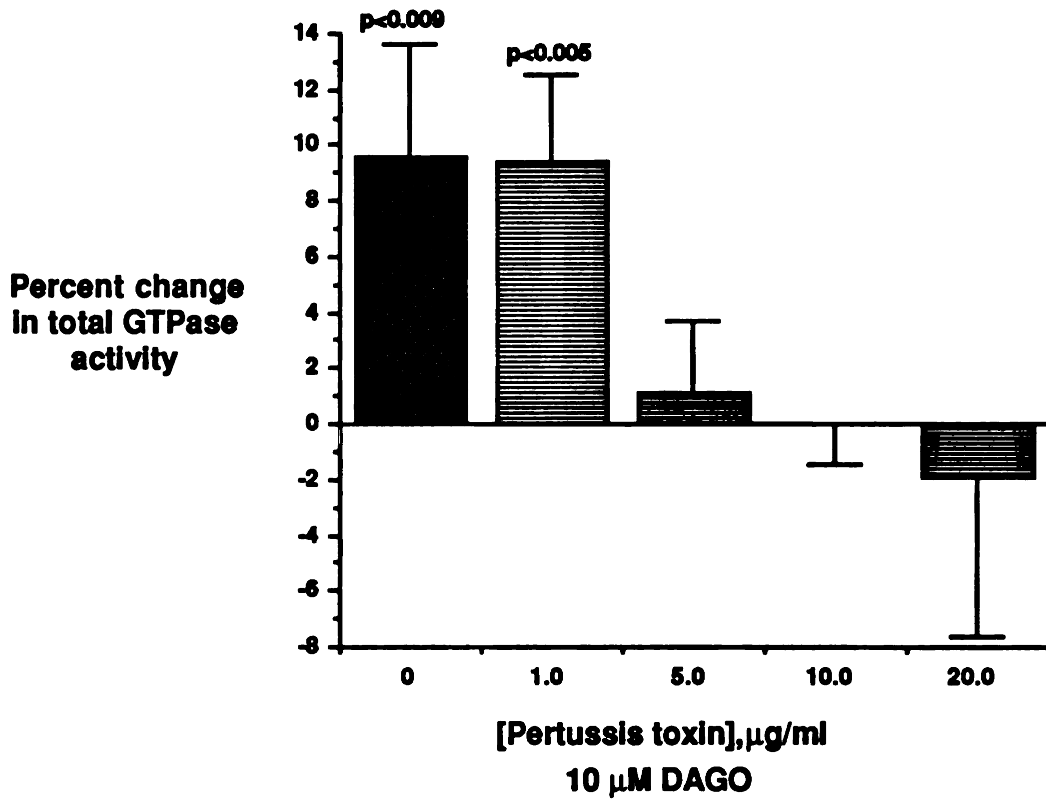
membrane with a 5 μg/ml or greater concentration of activated pertussis toxin a 10 μM concentration of DAGO had no significant effect on total GTPase activity relative to similarly treated pertussis toxin minus controls. The results are shown in Fig. 18.

Discussion

In PAG enriched P_2 membrane the nonhydrolyzable GTP analog GppNHp reduces total specific binding of 2 nM ^{3}H-DAGO in a dose dependent manner. Such a decrease in total specific binding can be due to either a decrease in affinity of binding sites for the ligand or to a decrease in the actual number of binding sites. In a radioligand binding assay it is technically difficult to differentiate between these two possibilities since it is difficult to distinguish the loss of high affinity binding , i.e. the conversion of high affinity binding sites to low affinity binding sites, from the actual loss of high affinity binding sites. The first of these alternatives is consistent with the coupling of high affinity μ selective binding sites to G proteins. The second alternative is both unlikely and not easily explained. In order for GppNHp to decrease the actual number of opioid binding sites in P_2 membrane GppNHp must act as a noncompetitive or irreversible antagonist at the opioid binding sites. To date there is no evidence that GTP, GppNHp, or any GTP analog directly antagonizes opioid binding. This observed GppNHp mediated reduction of ^{3}H-DAGO binding in PAG enriched P_2 membrane is consistent with several previous studies reporting similar guanine nucleotide regulation of opioid binding in rat whole brain membrane (Blume 1978; Chang, Hazum et al. 1981; Childers and Snyder 1980).

In addition the μ selective opioid agonist DAGO stimulates total GTPase activity in PAG enriched P_2 membrane. Such an increase in total GTPase activity is consistent with an agonist associated stimulation of the intrinsic GTPase activity of G proteins coupled to μ opioid receptors in this tissue. This DAGO stimulation of total GTPase activity appears to be mediated by a true μ type opioid receptor in that it is saturable, dose dependent, stereoselective, naloxone reversible, and μ opioid agonist selective. δ and κ selective opioid agonists do not significantly affect total GTPase activity. These

Fig. 18.
Effects of DAGO on total GTPase activity following Pertussis toxin pretreatment of membrane. The data are expressed as percent change in activity relative to the basal total GTPase activity of non-opioid treated controls. Each column represents the mean of triplicate determinations.
Basal activity after 0, 1.0, 5.0, 10.0, and 20.0 μg/ml Pertussis toxin pretreatment=29.62±0.89, 32.47±0.40, 36.11±2.63, 38.30±1.32, and 33.79±0.93 pmol $^{32}P_i$/min/mg protein resepectively.


Percent change in total GTPase activity
p<0.009
p<0.005
14
12
10
8
6
4
2
0
-2
-4
-6
-8
0
1.0
5.0
10.0
20.0
[Pertussis toxin],µg/ml
10 µM DAGO

findings are consistent with another recent study reporting μ opioid agonist stimulation of low-K_m GTPase activity in rat whole brain membrane (Clark and Medzihradsky 1987).

Statistically significant stimulation of GTPase activity in PAG enriched P_2 membrane requires relatively high μM concentrations of DAGO and not the nM concentrations of this agonist ligand shown to saturate specific binding sites in this tissue as shown in Fig. 3. This discrepancy is not unexpected. The GTPase assays and the radioligand binding assays were carried out under different experimental conditions and in different buffer systems. Radioligand binding assays are generally carried out in non-physiological buffer systems which are formulated to optimize ligand affinity. The buffer system utilized in the GTPase assays contained Na^+ and GTP, both of which reduce opioid agonist binding (Childers and Snyder 1978; Pert and Snyder 1974; Simon, Hiller et al. 1975), and more closely approximated physiological conditions.

A second property of G proteins is that the cation Mg^{2+} is essential to their function. In particular it appears that the presence of Mg^{2+} is required for the agonist mediated exchange of GTP for GDP on the G protein and the subsequent dissociation of the active form from the cell surface receptor (Brandt and Ross 1986). It has also been demonstrated that the cation Na^+ is required in the coupling of opioid receptors to G proteins and adenylate cyclase activity in the NG-108,15 neuroblastoma-glioma cell line (Blume 1978; Blume, Lichtchstein et al. 1979). In the present study we have shown that in the absence of Na^+ and Mg^{2+} DAGO does not stimulate total GTPase activity in PAG enriched P_2 membrane.

A third important and useful property of G proteins is their known predisposition to act as substrates for ADP-ribosylation by specific bacterial endotoxins. One such endotoxin is pertussis toxin which is known to catalyze the ADP-ribosylation of the inactive forms of transducin (Manning, Fraser et al. 1984), G_i and G_o (Sternweis and Robishaw 1984), and perhaps other G proteins. Such ADP-ribosylation renders a G protein incapable of coupling to cell surface receptors. Consequently extracellular agonist binding to these receptors no longer exerts an effect on G protein function.

After pretreatment of PAG enriched P_2 membrane with a sufficient concentration of activated pertussis toxin DAGO does not stimulate total GTPase activity in this tissue. This result suggests the specific involvement of G proteins in the observed DAGO stimulation of total GTPase activity in PAG membrane.

While the maximal effect of DAGO on total GTPase activity in PAG enriched P_2 membrane is small, in the range of 10% to 15%, it is statistically significant and reproducible. Such a 15% change in total GTPase activity may reflect physiologically significant activation of G proteins. Also even though the basal total GTPase activity may vary from tissue preparation to tissue preparation in different experiments, the percent stimulation of this activity by DAGO remains consistent.

Together the binding and GTPase assay data presented in this study provide strong evidence that high affinity μ selective binding sites in the PAG region of the midbrain are indeed associated with pharmacological receptors, and these μ opioid receptors are coupled to guanine nucleotide binding proteins in a manner similar to a variety of other neurotransmitter cell surface receptors. This coupling of μ type opioid receptors to G proteins in the PAG may then also explain the low affinity μ selective binding observed in the radioligand competition binding assays. It is possible, and reasonable to assume, that the low affinity μ selective binding sites detected in PAG enriched P_2 membrane may simply be those binding sites associated with uncoupled μ opioid receptors.

Chapter 4: Effector Systems - Inhibition of Adenylyl Cyclase

The data obtained from the GTP assays clearly indicate that high affinity μ selective opioid agonist binding in PAG tissue is coupled to the stimulation of G protein mediated hydolysis of GTP to GDP. This is then but the first of a potentially long chain of biochemical events which ultimately lead to a measurable physiological response. To which G protein mediated effector system or systems are the μ type opioid receptors of the PAG coupled?

G proteins act as intermediaries in a number of different signal transduction mechanisms. G proteins either have been shown to be involved in or have been implicated in the regulation of adenylyl cyclase activity (Gilman 1984), phospholipase C activity (Cockcroft and Gomperts 1985; Fain, Wallace et al. 1988; Wallace and Fain 1985), K^+ channel activity (Codina, Yatani et al. 1987; Logothetis, Kurachi et al. 1987; Yatani, Codina et al. 1987), and Ca^{2+} channel activity (Hescheler, Rosenthal et al. 1987; Holz IV, Rane et al. 1986). Of these transmembrane signal transduction mechanisms the G protein regulation of adenylyl cyclase activity is best characterized and understood.

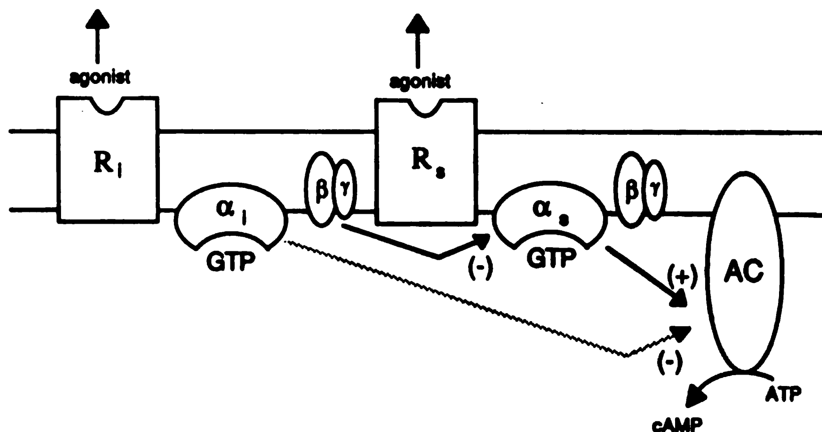
Adenylyl cyclase is the catalytic enzyme responsible for the production of the second messenger cyclic AMP (cAMP). As a second messenger cAMP activates both cytosolic and membrane bound cAMP dependent protein kinases including protein kinase A (Rubins 1979). Once activated these protein kinases can phosphorylate and consequently either activate or deactivate or modify the activity of a number of additional cytosolic and membrane bound enzymes, ion channels, and other functional proteins (Cohen 1982). Adenylyl cyclase is subject to both stimulation and inhibition by activated G proteins. A more detailed schematic of adenylyl cyclase function and the cAMP second messenger system is shown in Fig. 19. As a consequence of this functional scheme agonist binding to an appropriate cell surface receptor will either stimulate or inhibit the production of intracellular cAMP.

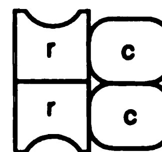
As mentioned in the introduction of the preceeding chapter in the NG-108,15 neuroblastoma-glioma cell line agonism at δ type

Fig. 19.
The G protein mediated coupling of cell surface receptors to adenylyl cyclase and the cAMP second messenger system.

LI
LI

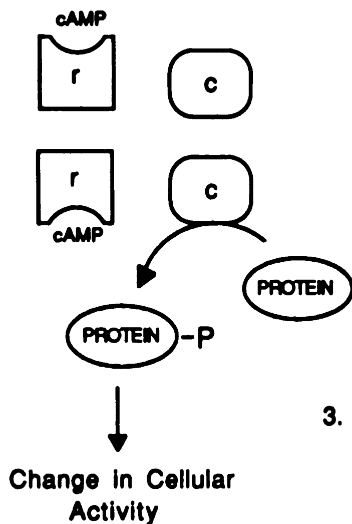
1. Specific receptors activate stimulatory and inhibitory G protein heterotrimers as shown in Fig. 10.
Dissociated α_s subunit directly stimulates adenylyl cyclase activity and promotes the formation of cAMP.
Dissociated α_i subunit directly, but weakly, inhibits adenylyl cyclase. The primary inhibitory action of G_i is the attenuation of the stimulatory action of G_s.
Dissociated $\beta\gamma$ subunits from activated G_i are functionally identical to those from activated G_s. Dissociated $\beta\gamma$ subunit from activated G_i indirectly inhibits adenylyl cyclase by reassociating with α_s subunit to form inactive G_s heterotrimer as shown in Fig. 10.

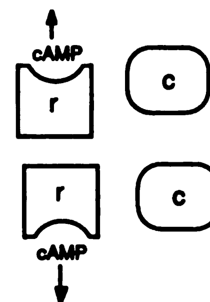




2. Binding of cAMP to regulatory subunits of inactive cAMP dependent protein kinase tetramer leads to the dissociation of cAMP dependent protein kinase subunits. Binding of cAMP is a cooperative process.

4. cAMP dissociates from regulatory subunits of cAMP dependent protein kinase, and kinase subunits reassociate to form inactive tetramer. Phosphodiesterases degrade free cAMP.





3. Dissociated catalytic subunit of cAMP dependent protein kinase phosphorylates protein substrates.
Functions of the phosphorylated proteins are altered, and changes in cellular activity occur.

opioid receptors is coupled to the inhibition of adenylyl cyclase activity. To determine if agonist binding at μ type opioid receptors is similarly coupled to the inhibition of adenylyl cyclase activity the effects of opioid ligands on cAMP formation in PAG enriched P_2 membrane were assessed with adenylyl cyclase assays.

Results

In preliminary experiments a 1 μM concentration of the potent but nonselective opioid agonist etorphine (Magnan, Paterson et al. 1982) inhibited adenylyl cyclase activity in both PAG enriched P_2 membrane and striatal P_2 membrane in a naloxone reversible manner. 1 μM etorphine inhibited both untreated basal adenylyl cyclase activity and the adenylyl cyclase activity stimulated by 5 μM forskolin. The results are shown in Table 2.

In subsequent experiments a 10 μM concentration of the μ selective opioid agonist DAGO also inhibited both basal and forskolin stimulated adenylyl activity in PAG enriched P_2 membrane. DAGO inhibition of forskolin stimulated adenylyl cyclase activity was not consistently dose dependent. The results are shown in Fig. 20. The DAGO inhibition of forskolin stimulated adenylyl cyclase activity was not naloxone reversible. The opioid antagonist naloxone itself significantly inhibited forskolin stimulated adenylyl cyclase activity, even when present in low concentration. The results are shown in Table 3.

DAGO inhibition of basal adenylyl cyclase activity was also not consistently dose dependent. However the DAGO inhibition of basal adenylyl cyclase activity was naloxone reversible. Naloxone itself did not significantly affect adenylyl cyclase activity. The results are shown in Fig. 21.

In the presence of 100 mM NaCl DAGO did inhibit basal adenylyl cyclase activity in a more dose dependent manner with borderline statistical significance. In the presence of 100 mM NaCl DAGO inhibition of adenylyl cyclase activity was naloxone reversible. The results are shown in Fig. 22 and Fig. 23. This effect is also selective for opioid agonists with a reported μ profile. Neither DPDPE

Table 2. Etorphine Inhibition of Basal and Forskolin Stimulated Adenylyl Cyclase Activity

	PAG enriched P_2 membrane		Striatal P_2 membrane	
	pmol ^{32}P-cAMP/ min / mg protein	% decrease	pmol ^{32}P-cAMP/ min / mg protein	% decrease
Basal activity	24.45 ± 2.04		29.71 0.45	
+1.0 μM Etorphine	22.58 ± 1.70	7.65 ± 6.95	23.92 ± 0.52	19.49 ± 1.75**
+1.0 μM Etorphine, 100 μM Naloxone	24.41 ± 1.48	0.02 ± 6.05	27.79 ± 1.33	6.46 ± 4.48
5.0 μM Forskolin stimulated activity	69.77 ± 3.40		367.87 ± 37.46	
+1.0 μM Etorphine	57.78 ± 2.22	17.19 ± 3.18*	316.04 ± 11.56	14.09 ± 3.14
+1.0 μM Etorphine, 100 μM Naloxone	66.81 ±.3.40	4.24 ± 4.87	350.43 ± 15.25	0.37 ± 4.37

* $p<0.01$
** $p<0.001$

Fig. 20.
Effects of DAGO on basal and forskolin stimulated adenylyl cyclase activity. The data are expressed as percent change in activity relative to the basal adenylyl cyclase activity of non-opioid treated controls. Each column represents the mean of triplicate determinations.

Basal activity=49.67±1.17 pmol ^{32}P-cAMP/min/mg protein.

Non-opioid treated, forskolin stimulated activity= 62.96±4.20 pmol ^{32}P-cAMP/min/mg protein.

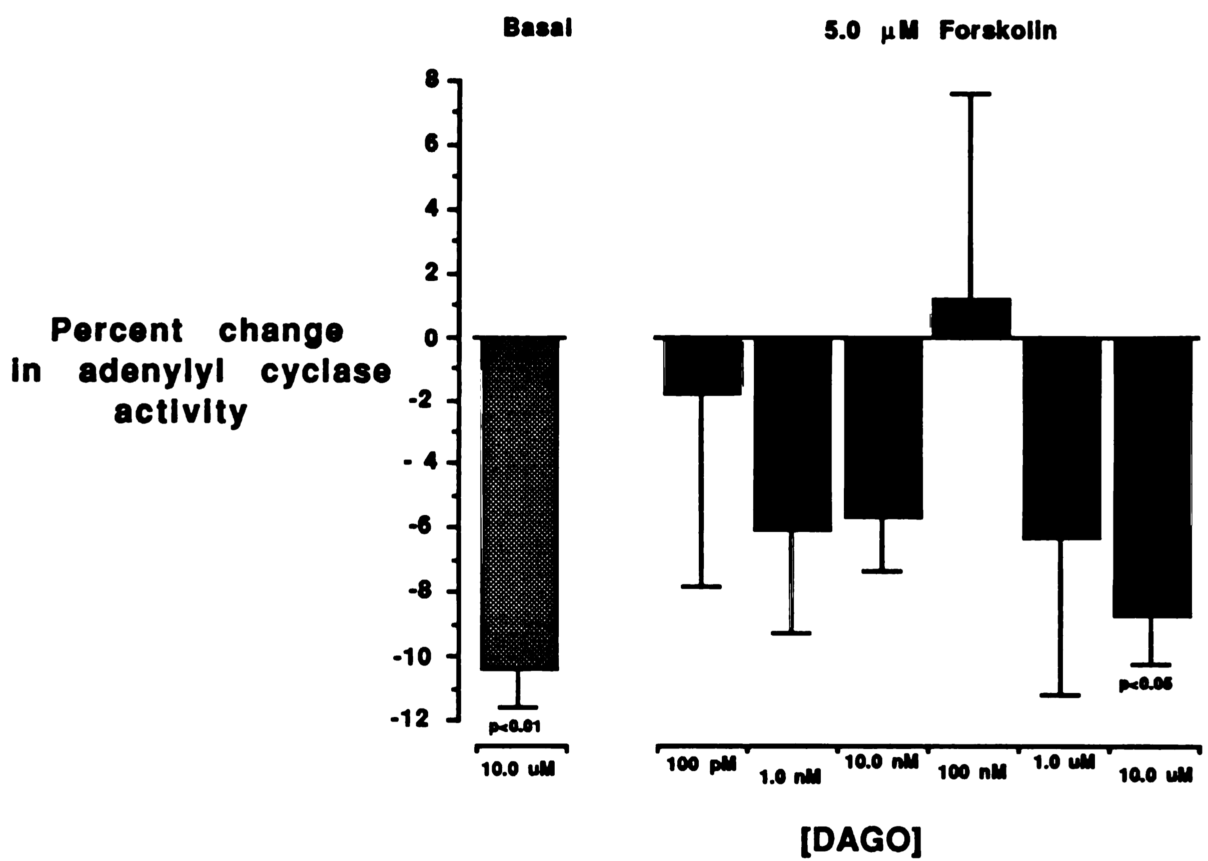

Basal
5.0 µM Forskolin
Percent change in adenylyl cyclase activity
8
6
4
2
0
-2
-4
-6
-8
-10
-12
p<0.01
p<0.05
10.0 uM
100 pM
1.0 nM
10.0 nM
100 nM
1.0 uM
10.0 uM
[DAGO]

Table 3. DAGO and Naloxone Inhibition of Forskolin Stimulated Adenylyl Cyclase Activity

	pmol ^{32}P-cAMP/ min / mg protein	% decrease
5.0 μM Forskolin stimulated activity	69.10 ± 0.78	
+10.0 μM DAGO	55.89 ± 0.72	19.11 ± 1.04*
+10.0 μM DAGO, 100 μM Naloxone	59.96 ± 1.33	13.22 ± 1.92*
+100 μM Naloxone	52.68 ± 3.03	23.76 4.38*
+10.0 μM Naloxone	49.94 ± 4.58	27.73 ± 6.63*
+ 1.0 μM Naloxone	53.41 ± 2.06	22.71 ± 2.98*
+ 0.1 μM Naloxone	44.03 ± 1.03	36.28 ± 1.49*

* $p<0.001$

Fig. 21.
Effects of DAGO on basal adenylyl cyclase activity. The data are expressed as percent change in activity relative to the basal adenylyl cyclase activity of non-opioid treated controls. Each column represents the mean of triplicate determinations.
Basal activity=46.89±2.36 pmol ^{32}P-cAMP/min/mg protein.

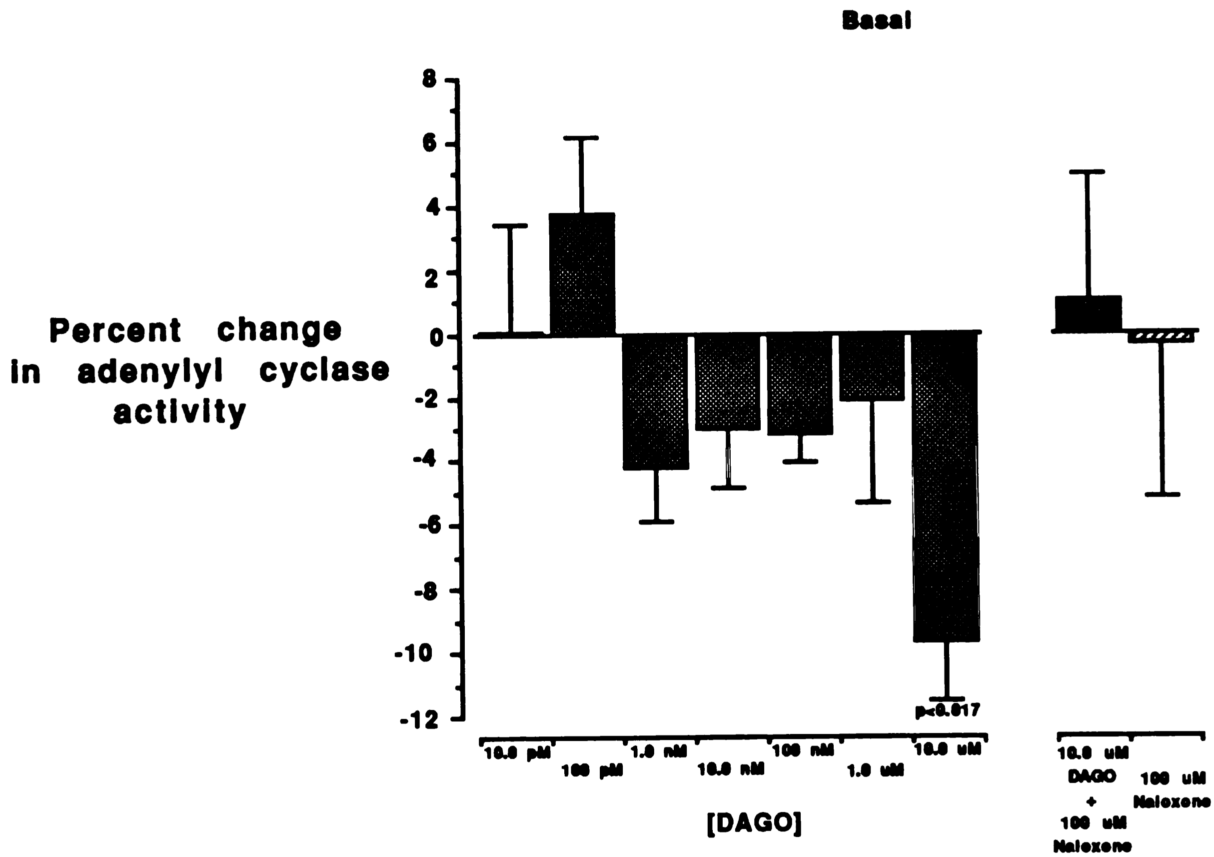

Basal
Percent change in adenylyl cyclase activity
8
6
4
2
0
-2
-4
-6
-8
-10
-12
p<0.017
10.0 pM
100 pM
1.0 nM
10.0 nM
100 nM
1.0 uM
10.0 uM
[DAGO]
10.0 uM DAGO + 100 uM Naloxone
100 uM Naloxone

Fig. 22.
Effects of DAGO on basal adenylyl cyclase activity in the presence of Na^+. The data are expressed as percent change in activity relative to the basal adenylyl cyclase activity of non-opioid treated controls. Each column represents the mean of triplicate determinations.
Basal activity=45.04±3.07 pmol ^{32}P-cAMP/min/mg protein.

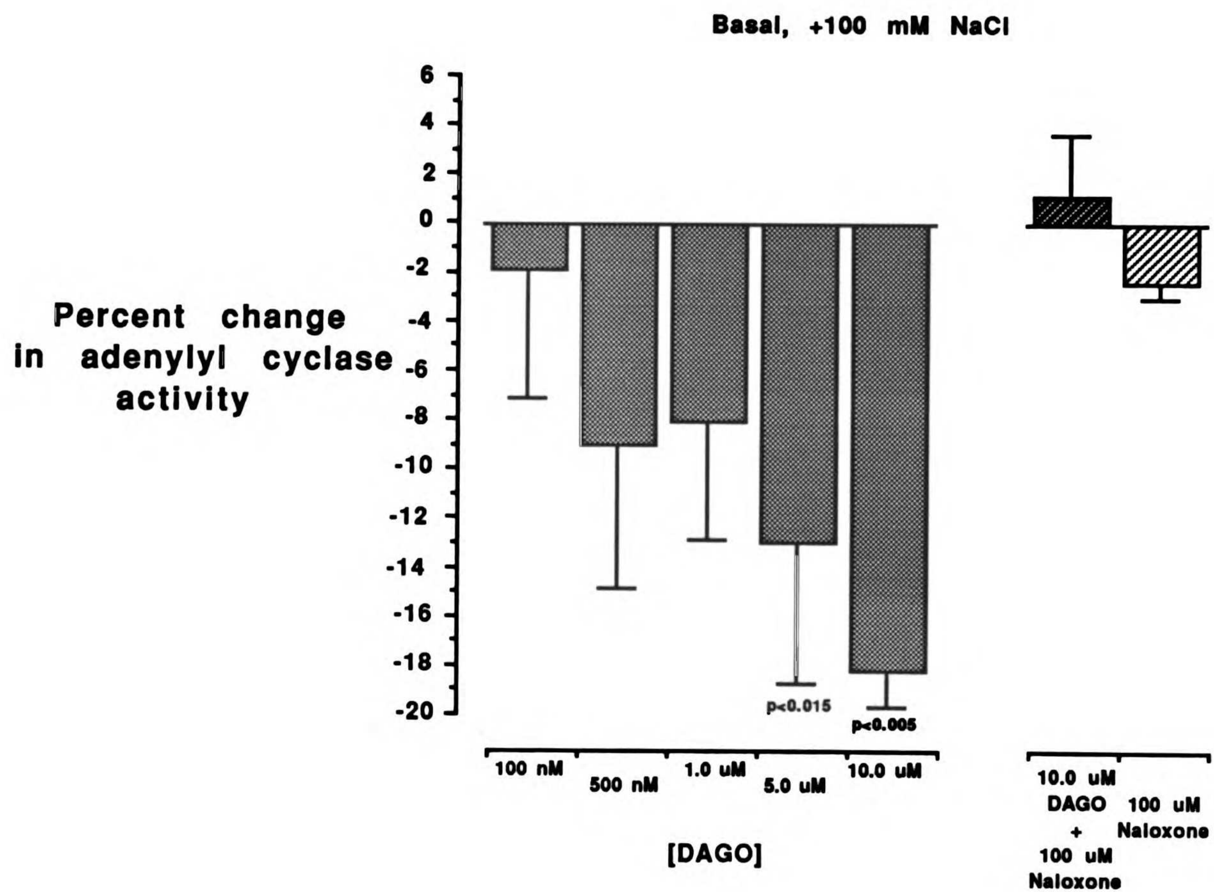
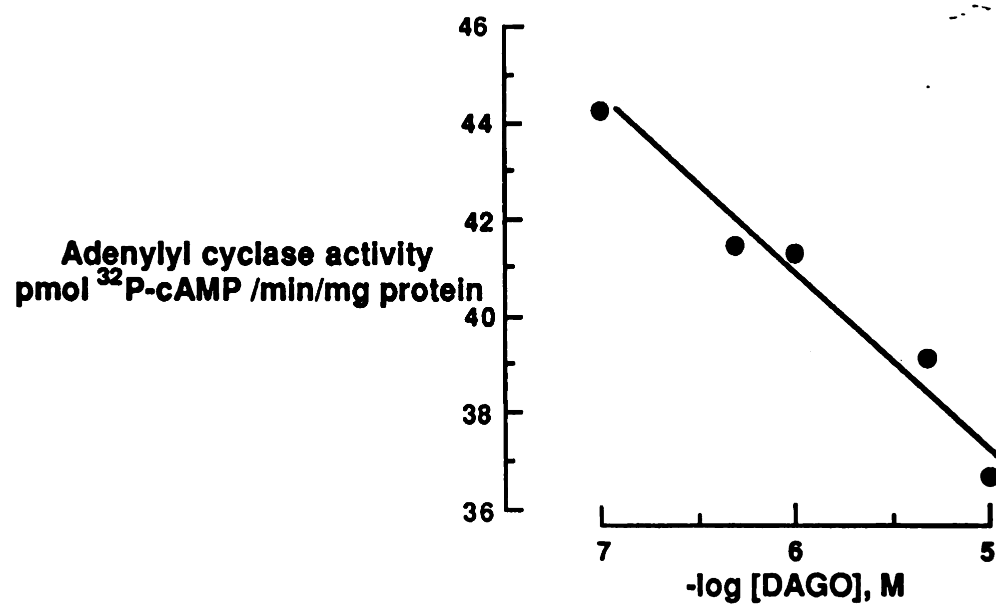

Basal, +100 mM NaCl
Percent change in adenylyl cyclase activity
6
4
2
0
-2
-4
-6
-8
-10
-12
-14
-16
-18
-20
p<0.015
p<0.005
100 nM
500 nM
1.0 uM
5.0 uM
10.0 uM
[DAGO]
10.0 uM DAGO + 100 uM Naloxone
100 uM Naloxone

Fig. 23.
Dose response curve of DAGO inhibition of basal adenylyl cyclase activity in the presence of Na^+. Each point is the mean of triplicate determinations. $F=3.50449532$, $p<0.055$.


Adenylyl cyclase activity
pmol ^{32}P-cAMP /min/mg protein
46
44
42
40
38
36
7
6
5
-log [DAGO], M

nor U50,488H affected basal adenylyl cyclase actitivity to a significant extent. The results are shown in Fig. 24.

Pretreatment of PAG enriched P_2 membrane with activated pertussis toxin abolished DAGO inhibition of adenylyl cyclase activity. This effect was not consistently dose dependent. The results are shown in Fig. 25.

Discussion

The nonselective opioid agonist etorphine inhibits adenylyl cyclase activity in PAG enriched P_2 membrane in a manner comparable of its inhibition of adenylyl cyclase activity in striatal P_2 membrane. Opioid agonists have previously been shown to inhibit adenylyl cyclase activity in striatal tissue most likely through agonist activity at δ type opioid receptors (Cooper, Londos et al. 1982; Law, Wu et al. 1981) as in the NG-108,15 neuroblastoma-glioma cell line. While this etorephine inhibition of adenylyl cyclase is not entirely statistically significant it is suggestive of the coupling of opioid receptors to adenylyl cyclase in the PAG.

The plant derived diterpine forskolin is commonly used in the study of adenylyl cyclase. Forskolin is believed to directly stimulate the catalytic component of adenylyl cyclase (Seamon and Daly 1981). Since adenylyl cyclase has not yet been purified the basis for this forskolin stimulation is not yet understood. Forskolin is a particularly useful tool in the study of the inhibition of adenylyl cyclase activity. It seems that forskolin stimulation of adenylyl cyclase activity within an assay system provides a higher degree of enzyme activity in which inhibitory signal may be more readily detectable. Etorphine inhibition of adenylyl cyclase activity in PAG enriched P_2 membrane is most apparent and is statistically significant in the presence of forskolin.

The μ selective opioid agonist DAGO also inhibits adenylyl cyclase activity in PAG enriched P_2 membrane in what initially may seem to be a confusing manner. Both basal and forskolin stimulated adenylyl cyclase activity are significantly inhibited, but in the presence of forskolin DAGO inhibition of adenylyl cyclase activity is neither dose dependent nor naloxone reversible. Surprisingly

Fig. 24.
Effects of DAGO, DPDPE, and U50,488H on basal adenylyl cyclase activity in the presence of Na^+. The data are expressed as percent change in activity relative to the basal adenylyl cyclase activity of non-opioid treated controls. Each column represents the mean of triplicate determinations.
Basal activity=58.05±2.47 pmol ^{32}P-cAMP/min/mg protein.

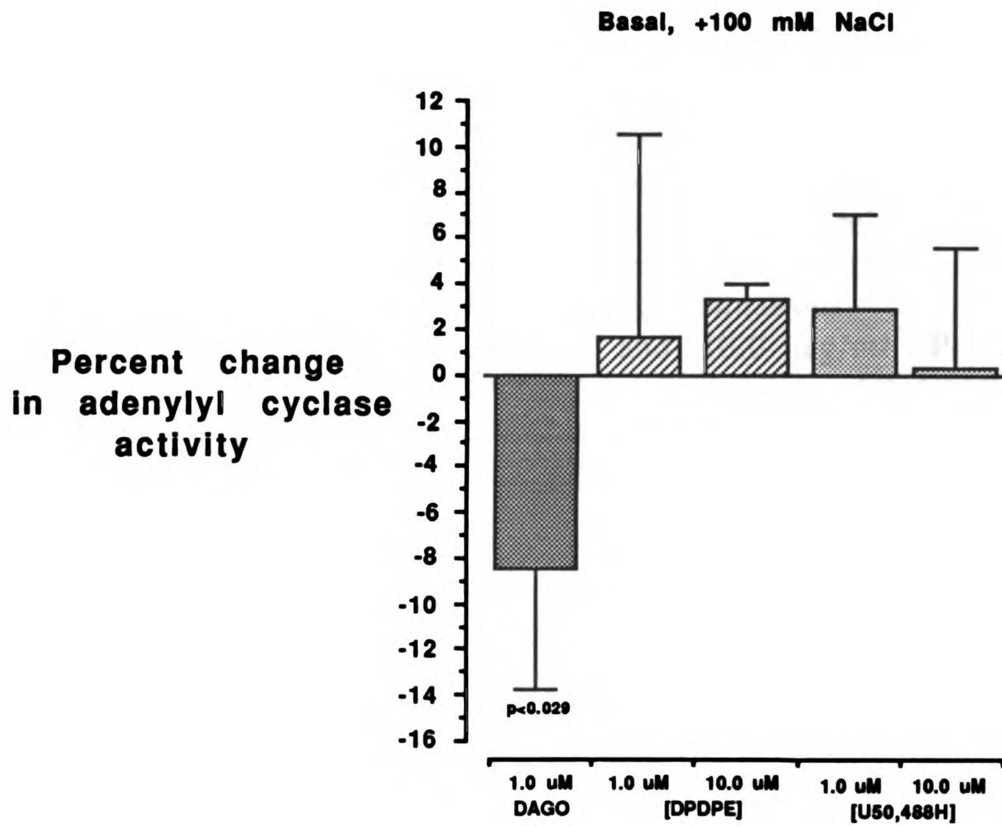
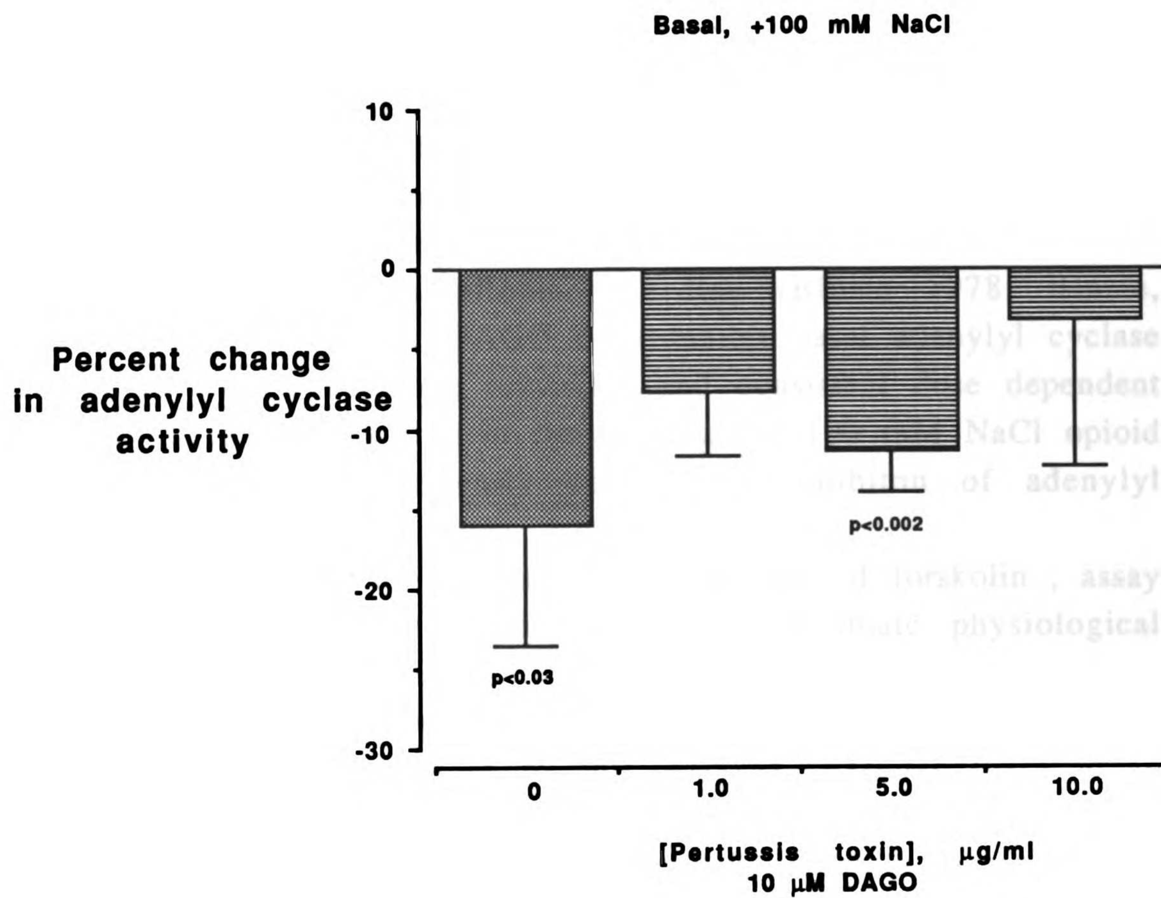

Basal, +100 mM NaCl
Percent change in adenylyl cyclase activity
12
10
8
6
4
2
0
-2
-4
-6
-8
-10
-12
-14
-16
p<0.029
1.0 uM DAGO
1.0 uM
10.0 uM
[DPDPE]
1.0 uM
10.0 uM
[U50,488H]

Fig. 25.
Effects of DAGO on basal adenylyl cyclase activity in the presence of Na^+ following Pertussis toxin pretreatment of membrane. The data are expressed as percent change in activity relative to the basal adenylyl cyclase activity of non-opioid treated controls. Each column represents the mean of triplicate determinations.
Basal activity after 0, 1.0, 5.0, and 10.0 μg/ml Pertussis toxin pretreatment=57.71±0.89, 65.07±0.65, 60.49±0.74, and 56.99±2.44 pmol ^{32}P-cAMP/min/mg protein respectively.



Basal, +100 mM NaCl
Percent change in adenylyl cyclase activity
10
0
-10
-20
-30
p<0.03
p<0.002
0
1.0
5.0
10.0
[Pertussis toxin], µg/ml
10 µM DAGO

naloxone itself significantly inhibits forskolin stimulated adenylyl cyclase activity, also in a non-dose dependent manner.

In contrast DAGO inhibition of basal adenylyl cyclase activity is naloxone reversible, and in the absence of forskolin naloxone itself does not significantly affect adenylyl cyclase activity. It is possible that naloxone somehow interferes with the forskolin stimulation of adenylyl cyclase in a manner unrelated to its antagonism of opioid receptors. Such interference of forskolin stimulation of adenylyl cyclase would appear as inhibition of this enzyme.

Although high doses of DAGO significantly inhibit basal adenylyl cyclase activity in PAG enriched P_2 membrane, such DAGO inhibiton is not consistently dose dependent. However in the presence of Na^+, a cation which has been shown to be required in the coupling of opioid receptors to G proteins and adenylyl cyclase in the NG-108,15 neuroblastoma-glioma cell line (Blume 1978; Blume, Lichtchstein et al. 1979), DAGO does inhibit basal adenylyl cyclase activity in both a naloxone reversible and consistent dose dependent manner. It is possible that in the presence of 100 mM NaCl opioid receptors are more efficiently coupled to inhibiton of adenylyl cyclase in the PAG.

In the presence of Na^+ and in the absence of forskolin , assay conditions which probably more closely approximate physiological conditions, DAGO inhibits adenylyl cyclase in PAG enriched P_2 membrane in a manner which is consistent with G protein mediated coupling of opioid receptors to this enzyme. Under these assay conditions DAGO inhibition of adenylyl cyclase is not only apparently dose dependent and clearly naloxone reversible, but is also μ opioid agonist selective. δ and κ opioid agonists do not significantly affect adenylyl cyclase activity. In addition after pretreatment of PAG enriched P_2 membrane with a sufficient concentration of activated pertussis toxin DAGO does not significantly inhibit adenylyl cyclase activity. While this effect of pertussis toxin pretreament is not entirely consistent it suggests the involvement of G proteins in the observed DAGO inhibition of adenylyl cyclase activity in PAG membrane.

Similar to the DAGO stimulation of GTPase activity in PAG enriched P_2 membrane the maximal effect of DAGO on adenylyl cyclase activity is small, in the range of 15% to 20%, but it is statistically significant, reproducible, and consistent from tissue preparation to tissue preparation regardless of the amount of basal activity measured.

Overall the adenylyl cyclase assay data presented in this study provide clear evidence that μ opioid receptors can couple to adenylyl cyclase to inhibit the production of the second messenger cAMP in the PAG. This finding is consistent with a report that μ opioid receptors in rat 7315c pituitary tumor cells are also coupled to the inhibtion of adenylyl cyclase via G proteins (Frey and Kebabian 1984).

Chapter 5: Effector Systems - Stimulation of Phospholipase C

As mentioned in the introduction of Chapter 4 G proteins can couple agonist binding at cell surface receptors to phospholipase C activity. Phospholipase C is the catalytic enzyme responsible for the direct production of two second messengers and the indirect activation of a third second messenger system. Phospholipase C catalyzes the conversion of the phospholipid phosphatidylinositol 4,5-biphosphate to 1,2 diacylglycerol (DAG) and inositol 1,4,5-triphosphate (1,4,5-IP_3) (Berridge 1987). As second messengers DAG stimulates protein kinase C activity (Bell 1986; Nishizuka 1986), and 1,4,5-IP_3 stimulates the release of Ca^{2+} from intracellular stores (Berridge and Irvine 1984). Further metabolites of 1,4,5-IP_3 such as 1,3,4,5-IP_4, IP_5, and IP_6 may also have some biological activity, but the physiological significance of this activity is unclear (Downes 1988). Free intracellular Ca^{2+} is itself a second messenger which modulates the activity of additional protein kinases and other enzyme systems through the activity of the calcium binding protein calmodulin (Means and Dedman 1980). A more detailed schematic of phospholipase C function and the second messenger systems with which it is associated is shown in Fig. 26. As a consequence of this functional scheme agonist binding to an appropriate cell surface receptor will stimulate the production of intracellular 1,4,5-IP_3.

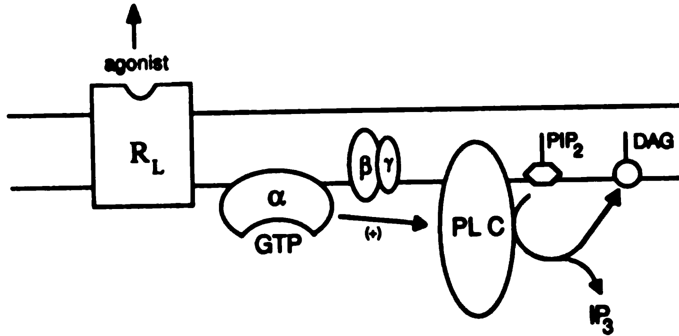
To determine if agonist binding at the μ type opioid receptor is coupled to the stimulation of phospholipase C activity the effects of opioid agonists on 1,4,5-IP_3 formation in intact PAG cells were assessed with two different inositol phosphate assays.

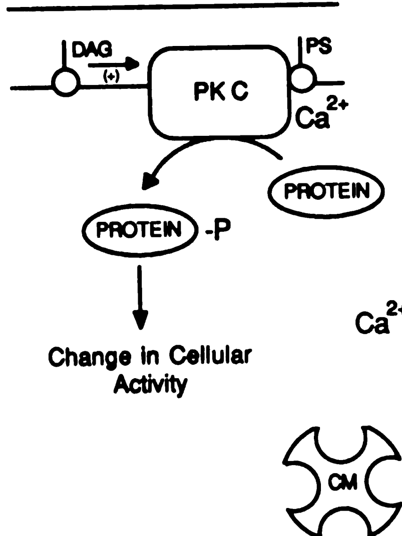
Results

10 μM DAGO, a concentration of this opioid agonist which produced maximal effects in both GTPase assays and adenylyl cyclase assays, did not significantly affect the levels of tritiated inositol phosphates in intact PAG cell tissue suspensions incubated with myo-[2 ^{3}H]inositol. Since inositol is metabolized to phosphatiylinositol 4,5-biphosphate in intact cells such tritiated inositol is incorporated into intact cell membrane as phophatidyl

Fig. 26.
The G protein mediated coupling of cell surface receptors to phospholipase C and the DAG, IP_3, and Ca^{2+} second messenger systems.

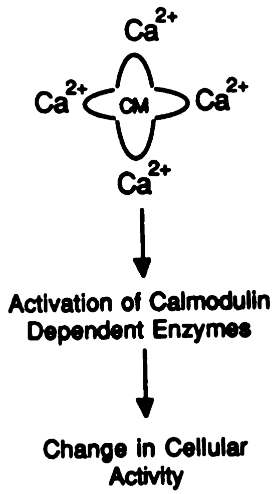
1. Specific receptors activate G protein heterotrimer as shown in Fig. 10. Dissociated α subunit stimulates phospholipase C activity and promotes the formation of 1,2 diacylglycerol (DAG) and inositol 1,4,5-triphosphate (IP_3) from phosphatidylinositol 4,5-biphosphate (PIP_2).





2. DAG stimulates protein kinase C activity in conjunction with phosphatidyl serine and Ca^{2+}. Protein kinase C phosphorylates protein substrates. Functions of phosphorylated proteins are altered, and changes in cellular activity occur. IP_3 stimulates the release of Ca^{2+} from intracellular storage sites. This effect may be mediated by a specific receptor. DAG is sequentially metabolized to CDP-diacylglycerol. IP_3 is sequentially degraded to IP_2, IP, and inositol. CDP-diacylglycerol and inositol are metabolized to PIP_2.

3. Binding of free intracellular Ca^{2+} to calmodulin leads to the activation of this protein and to the subsequent activation of calmodulin dependent enzymes. Binding of Ca^{2+} is a cooperative process. Activation of calmodulin dependent enzymes produce additional changes in cellular activity.



4. Ca^{2+} dissociates from calmodulin, and free intracellular Ca^{2+} is resequestered into intracellular storage sites.

[2 ^{3}H]inositol 4,5-biphosphate, and any subsequent phospholipase C activity results in the measurable generation of tritiated 1,4,5-IP_3 and its principle inositol phosphate catabolites inositol 1,4-biphosphate (1,4-IP_2) and inositol 1-phosphate (1-IP) or inositol 4-phosphate (4-IP). 10 μM DAGO both alone and in the presence of 100 μM naloxone did not significantly alter the levels of tritiated 1,4,5-IP_3, tritiated 1,4-IP_2, and tritiated IP. The results are shown in Table 4.

10 μM DAGO both alone and in the presence of 100 μM naloxone also did not significantly alter the levels of tritiated 1,4,5-IP_3, tritiated 1,4-IP_2, and tritiated IP when LiCl replaced NaCl in the Krebs-Ringer bicarbonate solution of the intact PAG cell tissue suspension. The substitution of Li^+ for Na^+ in the Krebs-Ringer bicarbonate solution did however alter the basal levels of the three different inositol phosphates assayed. The levels of tritiated 1,4,5-IP_3 and tritiated 1,4-IP_2 were increased while the level of tritiated IP was decreased. The results are shown in Table 5.

The levels of 1,4,5- IP_3 in intact PAG cell tissue suspension extracts both in the presence and absence of 10 μM DAGO were below the detection limit of a specific 1,4,5-IP_3 quantitative assay system and could not be determined.

Discussion

The μ selective opioid agoinist DAGO does not appear to stimulate the production of 1,4,5-IP_3 or its principle catabolites. It therefore appears that μ type opioid receptors are not coupled to phospholipase C activity in this brain region. However the method used in measuring the formation of tritiated 1,4,5-IP_3 following incubation of intact cells with D-myo-[2 ^{3}H]inositol is crude, and the use of intact cells rather than the P_2 membrane used in the GTPase assays and adenylyl cyclase assays introduces some potential complications. Degradation, both physiological and non-physiological, of tritiated inositol phosphates may occur following their formation and during the extraction procedure in which the PAG cells are homogenized and the water soluble inositol phosphates recovered. Loss of tritiated inositol phosphates may also occur during their

Table 4. Effects of DAGO on Phospholipase C Activity in Krebs-Ringer Bicarbonate Solution

	^{3}H-IP	^{3}H-IP$_2$ cpm/mg tissue	^{3}H-IP$_3$
Basal activity	40.51±2.17	9.66±1.65	4.73±0.57
+10 μM DAGO	40.50±2.51	8.25±0.80	4.15±0.74
+10 μM DAGO, 100 μM Naloxone	36.37±1.41	10.31±1.57	3.68±0.47

Table 5. Effects of DAGO on Phospholipase C Activity in Li^+ Krebs-Ringer Bicarbonate Solution

	^3H-IP	^3H-IP_2 cpm/mg tissue	^3H-IP_3
Basal activity	22.30±2.52	16.45±2.35	11.93±2.55
+10 μM DAGO	21.91±0.18	16.99±1.93	10.14±2.34
+10 μM DAGO, 100 μM Naloxone	20.12±2.90	18.73±1.29	9.03±1.47

separation in and collection from the anion exchange columns. It is possible that small but significant DAGO induced changes in the level of 1,4,5-IP_3 may be obscurred by a reduction in the tritiated 1,4,5-IP_3 signal.

In order to prevent potential physiological enzymatic degradation of tritiated inositol phosphates following their formation the NaCl in the Krebs-Ringer bicarbonate solution of the intact PAG cell tissue suspension was replaced with LiCl. Li^+ is commonly used in inositol phosphate assays to prevent the loss of tritiated inositol phosphate signal. Li^+ is known to inhibit the catabolism of inositol phosphates. In particular Li^+ is believed to inhibit the enzyme inositol 1- phosphatase which catalyzes the breakdown of 1-IP to free inositol (Berridge, Downes et al. 1982). As expected in the presence of Li^+ the levels of tritiated 1,4,5-IP_3 and tritiated 1,4-IP_2 are increased. However the level of tritiated IP is paradoxically decreased. Nevertheless even in the presence of Li^+ a high concentration of DAGO does not stimulate the production of 1,4,5-IP_3 or its principle catabolites.

In order to circumvent the potential loss of 1,4,5-IP_3 during separation in and collection from anion exchange columns a second type of specific inositol triphosphate assay was utilized. Instead of anion exchange mediated separation of tritiated 1,4,5-IP_3 from other compounds present in the aqueous environment of PAG cell extracts, the level of 1,4,5-IP_3 in PAG cell extracts was directly assayed on the basis of ability to compete with standard tritiated 1,4,5-IP_3 binding to a specific 1,4,5-IP_3 binding protein. Unfortunately the levels of 1,4,5-IP_3 in PAG cell extracts from both untreated and DAGO treated cells were below the 0.19 pmol detection limit of the sensitive quantitative assay. The discrepancy between the measurable levels of tritiated 1,4,5-IP_3 and the undetectable levels of 1,4,5-IP_3 in the two different ionsitol phosphate assays may be due to differences between the extraction procedures of the two assays. Technical requirements of the quantitative 1,4,5-IP_3 assay system prevented the use of identical extraction procedures.

Overall the inositol phosphate assay data presented in this study can not adequately address the question of whether μ type

opioid receptors in the PAG are coupled to phospholipase C activity, and such coupling can not be ruled out. Although relatively high concentrations of DAGO apparently did not stimulate the formation of 1,4,5-IP_3 in intact PAG cells, it is possible that small, subtle, but physiologically significant changes in the level of 1,4,5-IP_3 may not be detectable in the inositol phosphate assays used. The negative data are of limited value especially in the absence of appropriate positive controls. The PAG is a small region of the midbrain which has not been closely characterized either pharmacologically or biochemically, and the choice of appropriate positive controls for the G protein mediated stimulation of phospholipase C activity in intact PAG cell tissue suspensions remains empirical. Due to technical considerations the empirical determination of such proper positive controls is impractical. The determination of whether μ type opioid receptors are coupled to phospholipase C activity in the PAG must await refinements in the assay of brain tissue 1,4,5-IP_3 and the defining of appropriate positive controls for this brain region.

Conclusion

The binding data presented in this study strongly suggest that the opioid binding sites which can be detected in rat PAG enriched P_2 membrane are exclusively or a least predominantly μ selective and are functionally coupled to G proteins in a manner consistent with their being true pharmacological receptors. These μ type opioid receptors also appear to be involved with the physiological induction of strong analgesia in the PAG region of the rat midbrain in that the affinity of endogenous opioid peptides for these receptors generally parallels the ability of these endogenous peptides to induce strong analgesia following icv injection.

The biochemical data presented in this study confirm that the opioid binding sites identified in rat PAG enriched P_2 membrane are opioid receptors of the μ type and can functionally couple to G protein mediated signal transduction mechanisms. μ type opioid receptors in rat PAG enriched P_2 membrane stimulate GTPase activity and inhibit adenylyl cyclase activity in a manner characteristic of the G protein mediated coupling of cell surface receptors to an effector system.

It is therefore reasonable to conclude that 1) the opioid receptors associated with the rat PAG are predominantly of the μ type, 2) these opioid receptors are functionally coupled to G proteins, and 3) these opioid receptors are then at least coupled to the inhibition of adenylyl cyclase activity and the regulation of the cAMP second messenger system.

There is however no a priori reason for the μ opioid receptors of the rat PAG to be limited to coupling with a single effector system such as adenylyl cyclase. The functional coupling of μ opioid receptors to G proteins in the rat PAG provides a mechanism through which these receptors may also couple to additional G protein mediated effector systems in this brain region. Unfortunately at present it is not possible to adequately address the question of whether the μ opioid receptors of the rat PAG are also coupled to such known G protein mediated effector systems as phospholipase C and K^+ channels and Ca^{2+} channels.

Data from experiments in this study which test the possible coupling of opioid agonism to phospholipase C activity in intact PAG cells are inconclusive but suggestive that such coupling does not occur.

Experiments which test the possible direct coupling of opioid receptors to ion channels in PAG cell membrane are beyond the scope of this thesis project. There is ample electrophysiological evidence that μ type opioid receptors are coupled to K^+ channels elsewhere in the rat brain (North 1986). Intracellular recording techniques have revealed that μ selective opioid agonism can modulate K^+ flux across locus coeruleus cell membranes. However intracellular recording techniques can not differentiate between direct and indirect action on ion channels, and therefore it is not known whether this opioid effect is the direct consequence of G protein mediated coupling of μ type opioid receptors to K^+ channels or is the indirect consequence of opioid modulation of other second messenger systems such as cAMP. K^+ channel activity can be modulated by cAMP dependent protein kinases. In order to demonstrate direct G protein mediated coupling of μ opioid receptors to K^+ channels or perhaps Ca^{2+} channels free from the confounding influence of other second messenger effects on other cellular activities patch clamp techniques must be utilized. Patch clamping of excised cell membrane allows for the measurement and segregation of membrane bound receptor and ion channel activity away from cytoplasmic or cytoplasmic dependent enzyme activity. Unfortunately the PAG region of the rat midbrain is a very heterogenous community of neuronal and non-neuronal cell types and as such is not suitable for the practical application of patch clamp techniques.

Nevertheless the finding that μ type opioid receptors can couple to G protein mediated signal transduction mechanisms and effector systems in the PAG is significant. Such coupling provides the means for μ opioid agonists to modulate a wide variety of enzyme systems and cellular functions in those PAG neurons which express μ opioid receptors on their surfaces. How these opioid induced cellular changes are translated into the induction of strong analgesia awaits a

more complete understanding of the relationship between cellular functions and cellular activity and the neuronal circuitry associated with the supraspinal management of pain.

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Appendix: Receptor-Antibody Interactions

Are the μ type opioid receptors identified in the PAG region of the rat midbrain similar or identical to and thereby typical of the μ selective opioid binding sites and receptors identified throughout the CNS? This is an important but difficult question to address. Since little is now known of the biochemistry and pharmacology of the μ type opioid receptors found in brain regions other than the PAG, a direct comparison of the μ type opioid receptors of the PAG with μ type opioid receptors of other brain regions is not yet possible. However it is possible to indirectly address this question.

There has been limited success in the purification of opioid binding sites from brain membrane, but a fully functional μ type opioid receptor has yet to be reconstituted in a lipid environment and characterized. One such opioid binding protein has been purified from whole rat brain (Cho, Hasagawa et al. 1986) and consequently cloned (Schofield, McFarland et al.). Initially this opioid binding protein was described as displaying μ selective opioid binding activity, but subsequent radioligand binding studies utilizing more up to date and selective opioid peptide ligands do no support this earlier observation. F_{ab} fragments of an IgM monoclonal antibody 3B4F11 raised against this purified opioid binding protein specifically inhibit opioid binding in whole rat brain membrane (Roy, Zhu et al. 1988). F_{ab} fragments of this monoclonal antibody were used because of potential problems involving the access of the very large intact pentameric IgM molecules to membrane bound proteins. Do the F_{ab} fragments of this monoclonal antibody also recognize and interact with the μ type opioid receptors in PAG membrane? Such an interaction would at least suggest significant similarity or shared identity between the μ opioid receptors of the PAG and a purified opioid binding site and presumably receptor derived from whole brain tissue.

To determine if F_{ab} fragments of the monoclonal antibody 3B4F11 interact with the μ type opioid receptors of the PAG the effects of these F_{ab} fragments on high affinity DAGO binding and

DAGO stimulated GTPase activity in PAG enriched P_2 membrane were respectively assessed with radioligand binding and GTPase assays.

Methods

PAG enriched P_2 membrane was pre-incubated with F_{ab} fragments of monoclonal antibody 3B4F11 for 1 hr. at room temperature and immediately used in radioligand binding assays or GTPase assays as previously described.

Results

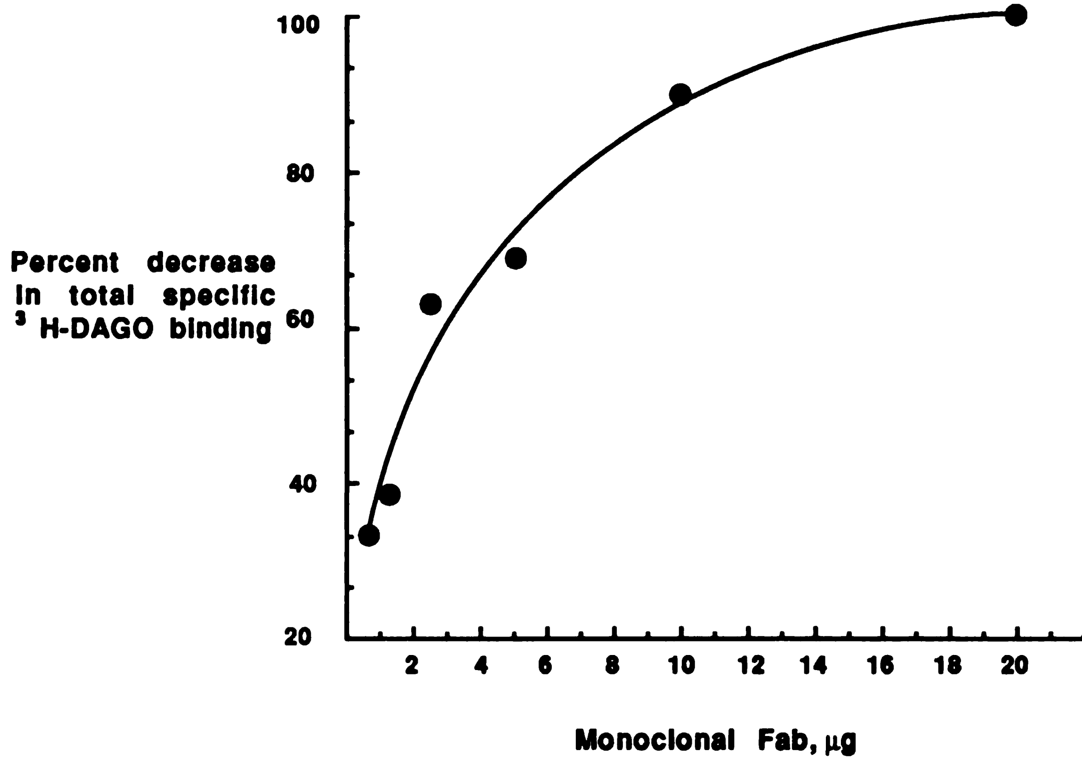
A 1 nM concentration of the μ selective opioid ligand ^{3}H-DAGO was used to label high affinity μ selective binding sites in PAG enriched P_2 membrane which had been pre-incubated with F_{ab} fragments of monoclonal antibody 3B4F11. Pre-incubation with increasing concentrations of F_{ab} fragments of the monoclonal antibody decreased the total specific binding of 1 nM ^{3}H-DAGO in a dose dependent manner. The results are shown in Fig. A1 and Fig. A2.

DAGO stimulation of total GTPase activity was greatly enhanced in PAG enriched P_2 membrane which had been similarly pre-incubated with 3 μg of F_{ab} fragments. Pre-incubation of PAG enriched P_2 membrane with 3 μg of F_{ab} fragments alone also stimulated total GTPase activity in a naloxone reversible manner. This stimulation of total GTPase activity was specific to the monoclonal antibody 3B4F11. Similar pre-incubation of PAG enriched P_2 membrane with 3 μg of F_{ab} fragments derived from a control IgM monoclonal antibody which had not affected opioid binding in whole rat brain membrane in previous testing did not significantly affect total GTPase activity. The results are shown in Fig. A3. The stimulation of total GTPase activity is also due to an interaction of the F_{ab} fragments with a membrane bound protein and is not associated with a property of the F_{ab} fragments themselves. In the absence of membrane 3 μg of F_{ab} fragments displayed no intrinsic GTPase activity.

The F_{ab} fragments of monoclonal antibody 3B4F11 interact with the μ opioid receptors of the PAG in a complex manner. Both

Fig. A1.

Monoclonal antibody 3B4F11 F_{ab} fragment inhibition of 1 nM ^{3}H-DAGO binding expressed as percent decrease in total specific binding. Binding was measured under equilibrium conditions at 25°C. Nonspecific ^{3}H-DAGO binding was defined with 10 μM unlabeled DAGO.



Percent decrease in total specific ³H-DAGO binding
100
80
60
40
20
2
4
6
8
10
12
14
16
18
20
Monoclonal Fab, μg

Fig. A2.
Dose response curve of monoclonal antibody 3B4F11 F_{ab} fragment inhibition of 1 nM ^{3}H-DAGO binding expressed as percent decrease in total specific binding. Binding was measured under equilibrium conditions at 25°C. Nonspecific ^{3}H-DAGO binding was defined with 10 μM unlabeled DAGO.

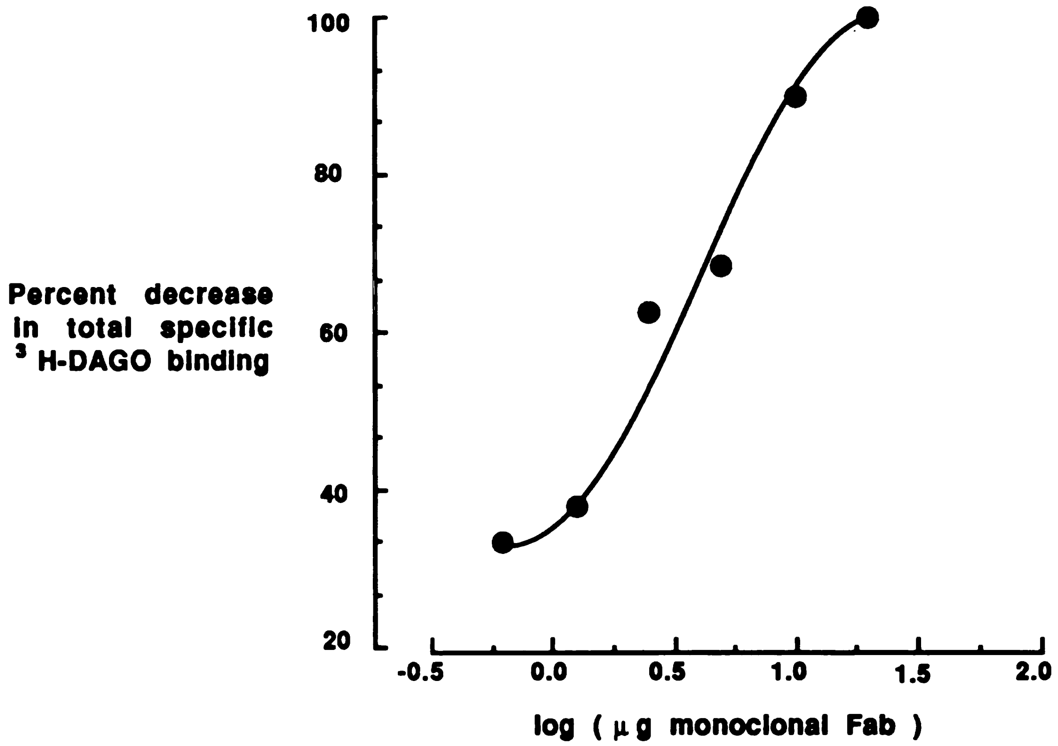
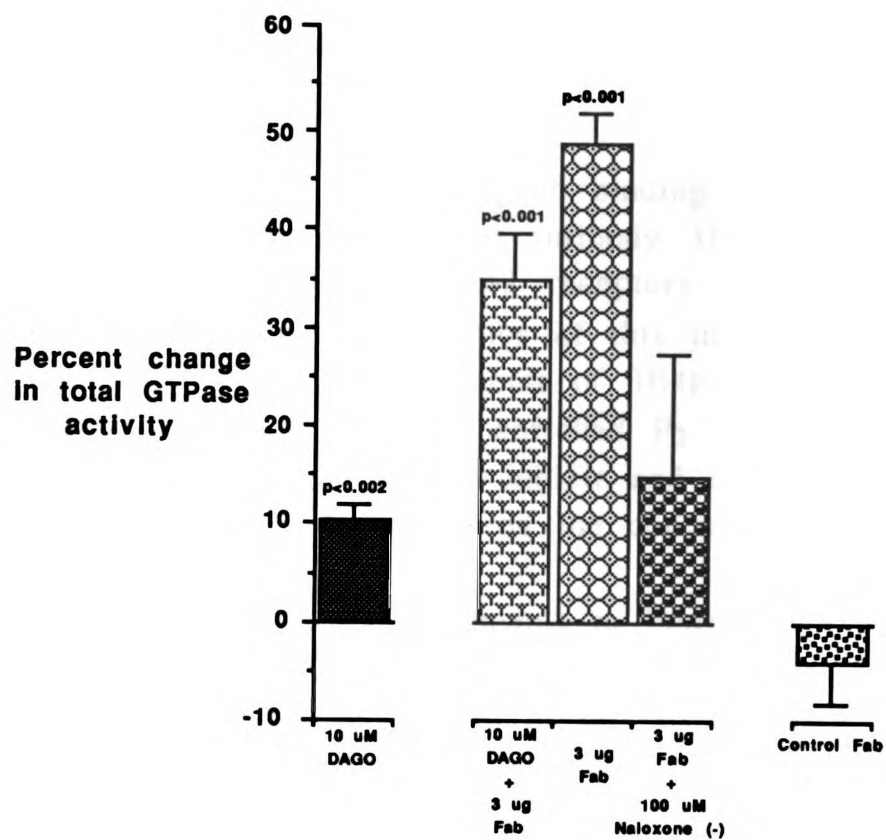

Percent decrease
in total specific
³ H-DAGO binding
100
80
60
40
20
-0.5
0.0
0.5
1.0
1.5
2.0
log (μ g monoclonal Fab)

Fig. A3.
Effects of DAGO and monoclonal antibody 3B4F11 F_{ab} fragments on total GTPase activity. The data are expressed as percent change in activity relative to basal total GTPase activity of non-opioid and non-antibody treated controls. Each column represents the mean of triplicate determinations.
Basal activity=8.91±0.20 pmol $^{32}P_i$/min/mg protein.



Percent change in total GTPase activity
60
50
40
30
20
10
0
-10
p<0.002
p<0.001
p<0.001
10 uM DAGO
10 uM DAGO + 3 ug Fab
3 ug Fab
3 ug Fab + 100 uM Naloxone (-)
Control Fab

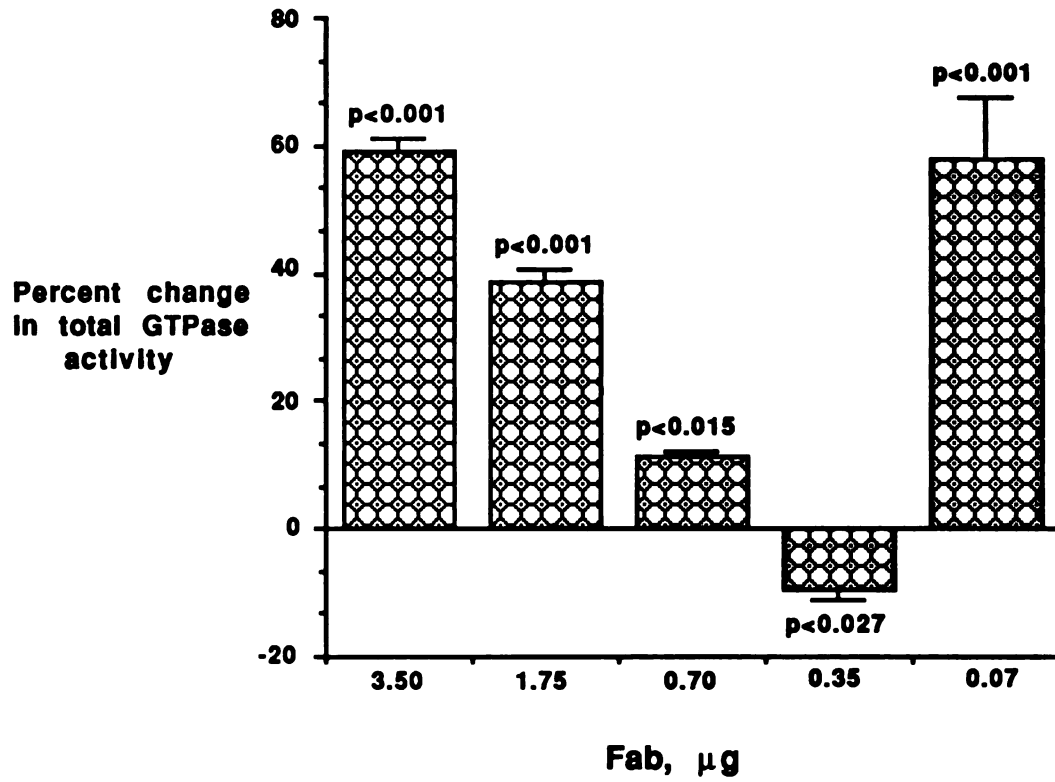
high and low doses of F_{ab} fragments significantly stimulated total GTPase activity in PAG enriched P_2 membrane. In contrast intermediate doses of F_{ab} fragments significantly inhibited total GTPase activity in this tissue. The results are shown in Fig. A4. Subsequent assays utilizing a different preparation of F_{ab} fragments of monoclonal antibody 3B4F11 generated generally similar results. Although the stimulation of total GTPase activity in PAG enriched P_2 membrane was less and the dose response was somewhat shifted in these subsequent assays, a complex, biphasic pattern of dose response was again observed. The results are shown in Fig. A5.

Discussion

It is clear from the radioligand binding and GTPase data that F_{ab} fragments of the monoclonal antibody 3B4F11 recognize, bind, and interact with μ type opioid receptors in PAG enriched P_2 membrane. However the dynamics of this interaction appear to be quite complex. While F_{ab} fragments of 3B4F11 inhibit high affinity opioid agonist binding in PAG enriched P_2 membrane these same fragments can both significantly inhibit and stimulate total GTPase activity in this tissue depending on the concentration in which they are present.

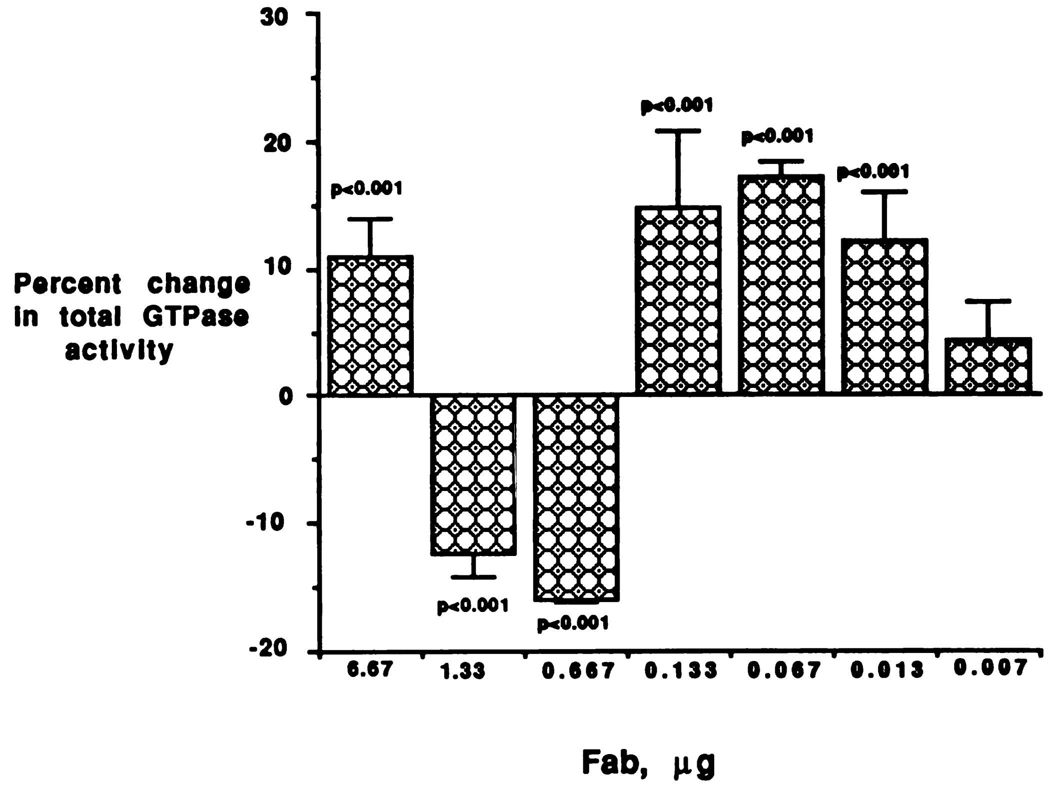
The ability of the F_{ab} fragments to inhibit high affinity opioid agonist binding in PAG enriched P_2 membrane is not unexpected if the μ type opioid receptors found in this tissue share some identity and antigenicity with the purified opioid binding protein, and presumably receptor, against which the monoclonal antibody 3B4F11 was raised. It is easy to envision that the binding of the relatively large F_{ab} fragments to antigenic sites on opioid receptors sterically hinders the access of opioid ligands to their binding site or sites on the receptor molecules. It is not easy to envision how the binding of the same F_{ab} fragments to opioid receptors results in either inhibition or stimulation of total GTPase activity in PAG enriched P_2 membrane even in the absence of the appropriate opioid agonist. It can be argued that both these effects on total GTPase activity and the inhibition of opioid agonist binding are due to the interaction of the F_{ab} fragments of monoclonal antibody 3B4F11 not with opioid

Fig. A4.
Effects of monoclonal antibody 3B4F11 F_{ab} fragments on total GTPase activity. The data are expressed as percent change in activity relative to basal total GTPase activity of non-antibody treated controls. Each column represents the mean of triplicate determinations.
Basal activity=19.13±1.18 pmol $^{32}P_i$/min/mg protein.



80
60
40
20
0
-20
Percent change in total GTPase activity
p<0.001
p<0.001
p<0.015
p<0.027
p<0.001
3.50
1.75
0.70
0.35
0.07
Fab, μg

Fig. A5.
Effects of monoclonal antibody 3B4F11 F_{ab} fragments on total GTPase activity. The data are expressed as percent change in activity relative to basal total GTPase activity of non-antibody treated controls. Each column represents the mean of triplicate determinations.
Basal actvity=20.91±0.75 pmol $^{32}P_i$/min/mg protein.


30
20
10
0
-10
-20
Percent change in total GTPase activity
p<0.001
p<0.001
p<0.001
p<0.001
p<0.001
p<0.001
6.67
1.33
0.667
0.133
0.067
0.013
0.007
Fab, μg

receptors but with G proteins in PAG enriched P_2 membrane. Perhaps the binding of F_{ab} fragments to G proteins uncouples opioid receptors from these proteins, and it is this uncoupling which results in the observed decrease in opioid agonist binding as discussed in chapter 3. However the specificity of monoclonal antibody-antigen interactions, the ability of monoclonal antibody 3B4F11 to inhibit antagonist binding in rat whole brain membrane, and the observed naloxone reversibility of the F_{ab} fragment mediated stimulation of total GTPase activity in PAG enriched P_2 membrane suggest a more direct involvement of the μ type opioid receptor. It is possible that the binding of F_{ab} fragments of monoclonal antibody 3B4F11 to opioid receptor hinders the access of opioid ligands to their binding sites on the receptor molecule and also induces those changes in the receptor molecule which are involved in the activation of the G protein to which the receptor is coupled. The biphasic nature of the F_{ab} fragment effect on total GTPase activity might then involve the general nature of antibody-antigen interaction. At optimal concentrations of antibody and antigen aggregation may occur. As a consequence GTP may no longer have access to its binding sites on those G proteins coupled to opioid receptors, and further GTPase activity is inhibited. At other than optimal concentrations of antibody and antigen, i.e. the ratio of F_{ab} fragments of antibody to antigen is low or high, GTPase activity is still possible and is stimulated.

Regardless of the actual molecular dynamics of the F_{ab} fragment interaction with μ type opioid receptors in PAG enriched P_2 membrane, monoclonal antibody 3B4F11, a monoclonal antibody raised against opioid binding sites purified from whole brain, clearly recognizes the μ type opioid receptors of the PAG. Overall the data from these antibody studies suggest that the μ type opioid receptors of the PAG are not unique among opioid receptors and may be similar to or typical of those μ type opioid receptors found throughout the brain.

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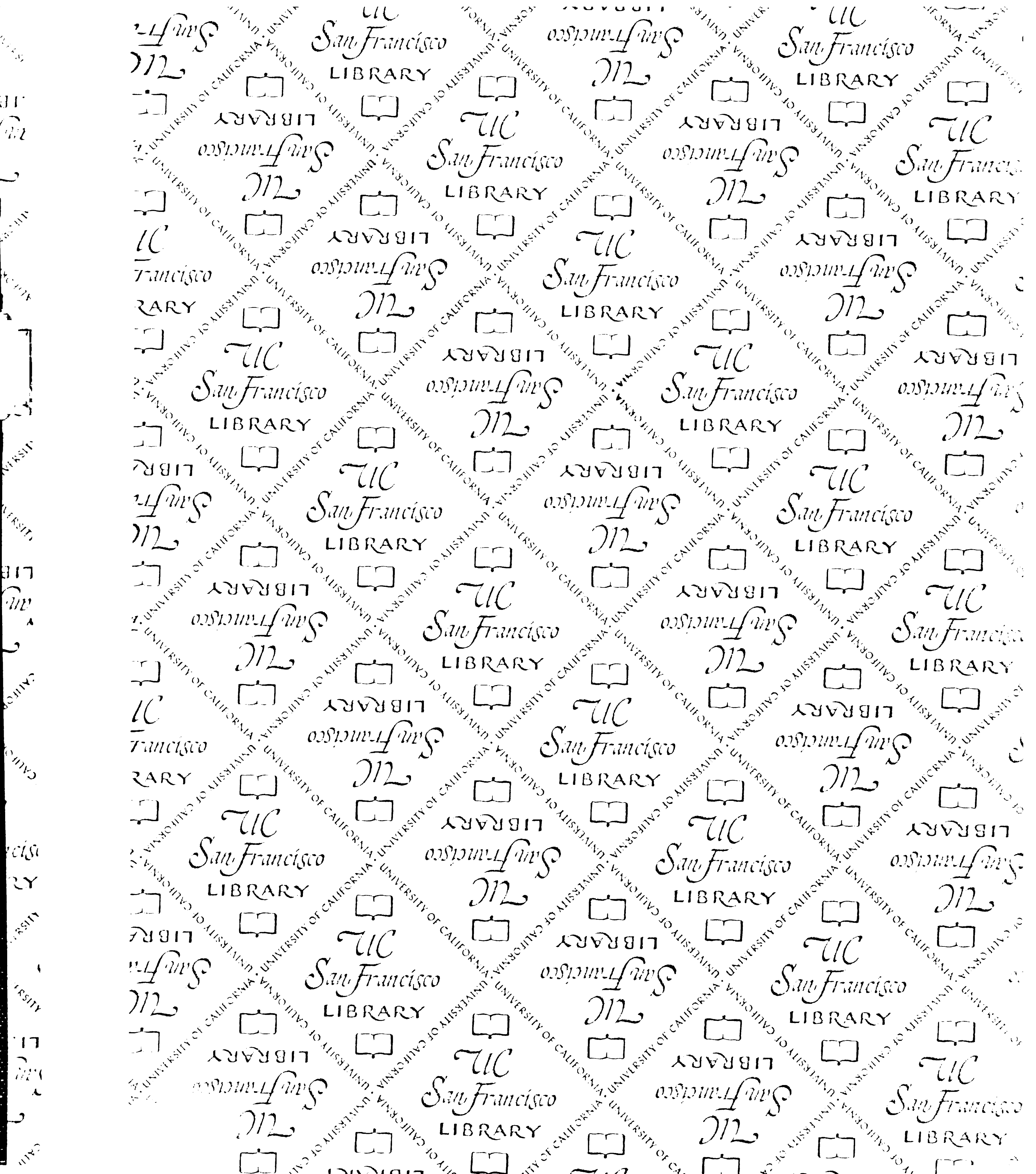
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