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Molecular Mechanisms Of Opioid Action

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Abstract

Although several distinct molecular mechanisms for opioid action have been proposed, there is considerable evidence that they may share the common feature of GTP-binding proteins. The proposed research was designed to address the question of whether G proteins mediate opioid action and the nature of the second messenger systems involved. The role of G proteins in three separate systems, inhibition of adenylate cyclase, stimulation of potassium flux and regulation of neuronal calcium, was studied.

The involvement of G proteins can be determined with the use of specific toxins. Pertussis toxin catalyzes the ADP-ribosylation of the GTP-binding subunit of G_i, resulting in an attenuation of its inhibitory action on adenylate cyclase and an uncoupling of G_i from its receptor. It was shown here (and elsewhere) that pertussis toxin specifically labels two proteins in the brain, G_i and G_o.

Opioids have been demonstrated to inhibit adenylate cyclase in the rat brain striatum. It was shown here that this inhibition is mediated by G_i. Furthermore, the opiate receptor is also regulated by G_i in the striatum. This is the first demonstration of a complete second messenger system for opioids in the brain, a complex consisting of opioid receptor + G_i + adenylate cyclase.

The opioid receptor is regulated by G proteins in the cortex as well as in the striatum. In the cortex, opiates inhibit adenylate cyclase, but a possible alternative second messenger system was demonstrated through which opioids may act, i.e., phosphoinositide

turnover. Since both systems (cAMP and phosphoinositide turnover) involve G proteins, the opioid response in the cortex could be mediated through either of these systems.

The role of G proteins in opioid action in other brain areas such as the midbrain is less clear. Although opioid binding is regulated by GTP throughout the brain and G proteins were found in every brain region examined, opioids do not affect cAMP production in the midbrain, nor is opioid binding altered by pertussis toxin treatment. This suggests that the opioid receptor is not regulated by pertussis toxin sensitive G proteins in the midbrain.

The role of G proteins in the mediation of opioid analgesia was studied. During the course of the studies it was determined that pertussis toxin had limited access to neuronal tissues. The toxin could not cross the blood brain barrier, nor could it enter the brain when injected intracerebroventricularly. There was poor penetration of the toxin even when added directly to brain slices. It was not possible, therefore, to demonstrate an effect of G proteins on the physiological endpoint of analgesia.

The proposed research was designed to ask if opioid action in the brain is mediated by G proteins. The results showed definite coupling of opiate receptors and G proteins in the striatal and cortical regions of the brain.

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Introduction

The events by which opioid receptor binding is transduced to opioid action are not fully understood, but several lines of evidence implicate GTP-binding proteins. The most established evidence comes from studies of cultured neuroblastoma cell lines, which are used as a model system for neuronal opioid action, where opioids have been shown to inhibit cAMP production (1,2). The inhibition is mediated through a guanine nucleotide regulatory protein, G_i (or N_i), which couples the opioid receptors in these cells to adenylate cyclase (1,2). While this mechanism of opioid action has only been demonstrated in the striatum, its possible existence throughout the brain is supported by the observation that opioid binding to brain membranes, like that to neuroblastoma cells, is inhibited by guanine nucleotides (3,4).

In addition to evidence linking opioid action and guanine nucleotides, there are other studies suggesting that opioids may activate potassium channels or inhibit calcium channels (5). This is presumably the mechanism by which opioids inhibit firing in these neurons (6). GTP-binding proteins may be involved in these processes also, as they have been implicated in calcium gating (7).

In view of the fact that G proteins are involved in the regulation of adenylate cyclase activity and, possibly, Ca^{++} gating and modulation of receptor binding, the question arose as to whether G proteins mediate opioid action and the nature of the second messenger systems involved. Although several distinct mechanisms may underlie opioid action, they all may share the common feature of mediation by G proteins.

Adenylate cyclase as a second messenger system for opioid action

Opioid inhibition of adenylate cyclase has been firmly established in the cultured neuroblastoma cell lines (1,2). When opioids such as etorphine (an alkaloid) or d-ala-d-leu-enkephalin (DADLE, a synthetic enkephalin) bind to the receptors on these cells they activate G_i, which in turn inhibits adenylate cyclase (1,2). In the neuroblastoma-glioma cell line, G_i is responsible for the GTP-mediated inhibition of opioid agonist binding (2). Opioids have been shown to inhibit adenylate cyclase in the rat brain striatum (8). With the possible exception of the cortex, the striatum is the only brain region shown to be coupled to adenylate cyclase (9,10). Striatal adenylate cyclase exhibits a biphasic response to GTP. At concentrations below 1 μM, GTP will stimulate, whereas at higher concentrations GTP will inhibit adenylate cyclase. Such a response to GTP suggests the presence of both stimulatory and inhibitory guanine nucleotide regulatory proteins in the striatum, since a similar response occurs in systems known to possess both proteins (11). Opioid inhibition of striatal adenylate cyclase is GTP-dependent suggesting the involvement of the inhibitory guanine nucleotide regulatory protein, G_i (8). This inhibition has been shown to involve the opioid receptor since it is stereospecific and naloxone reversible. The extent of inhibition is 20-30%, which is comparable to that seen in neuroblastoma x glioma hybrid cells (30-50%) (1). A variety of opioid alkaloids and peptides inhibit adenylate cyclase (8).

Opioid interaction with ion channels

Potassium channels

Consistent with the theory that opioid action involves an activation of potassium or inhibition of calcium channels, opioids inhibit neuronal firing and transmitter release throughout the brain. In the locus ceruleus these actions can be correlated with an increased potassium conductance (6,12). Intracellular calcium must be elevated prior to potassium channel opening (13). There is evidence to indicate that GTP-binding proteins may regulate calcium gating (7), therefore, these proteins may affect potassium channels.

One possible mechanism for opioid inhibition of neuronal firing and transmitter release might be an increased potassium conductance. An outward potassium flux will hyperpolarize the membrane, rendering it less excitable. Hyperpolarization could also prevent the voltage dependent calcium entry, and, thereby decrease transmitter release.

A series of electrophysiological studies by North et. al. have documented these events in the rat locus ceruleus and guinea pig myenteric plexus (9). The potassium conductance involved appears to be a calcium-dependent potassium current (13). The opioid induced K^+ activation is voltage dependent, so that at potentials less negative than -55 to -60 mV the hyperpolarization is decreased. The fact that the opioid response is reversed at potentials more negative than -105 mV is consistent with the reversal potential for K^+ (12). In addition, K^+ ions affect the potency of opioids in the electrically stimulated mouse vas deferens (14). At high potassium concentrations opioids lose activity (14), a finding which is consistent with a

potassium mediated effect.

Calcium channels and phosphoinositide turnover

Since phosphoinositide (PI) turnover has been implicated in Ca^{++} flux, while PI turnover may be regulated by G proteins, the possibility arises that the opioids may be involved with this second messenger system (15-20). A number of studies point to the involvement of calcium in the action of opioids (21,22). Opioid agonists have been shown to decrease intracellular calcium and calcium antagonists can antagonize analgesia (22). Opioids decrease sensory neuron Ca^{++}-dependent action potentials (21). On the other hand, chronic exposure to opioids can result in an increase in neuronal calcium levels (22). PI turnover is generally associated with an increase in intracellular calcium (15,16). In light of these observations, it might be expected that opioids might decrease PI turnover acutely, but increase it chronically.

The pathway of agonist stimulated PI turnover probably includes a G protein (17-20). An extracellular signal (agonist) binding to its receptor induces the production of inositol triphosphate (IP_3) and diacylgycerol (DAG) by the action of a phospholipase-C-like enzyme on phosphatidylinositol 4,5-bisphosphate (PIP_2), usually resulting in calcium mobilization and activation of protein kinase C (15,16). This pathway has been shown to be a second messenger system for numerous hormones and neurotransmitters (15,16). Pertussis toxin blocks PIP_2 breakdown, concomitant with radiolabelling of a 41,000 dalton protein, a finding suggests the involvement of G_i (17,19,20). GTP analogues added to membranes will directly stimulate PIP_2 breakdown suggesting

the interaction of a GTP-binding protein with the phosphodiesterase/phospholipase which acts on PIP_2 (18). G_i had previously been thought to associate only with adenylate cyclase, but this data implies another role for G_i. Furthermore, somatostatin inhibits hormone release in pituitary cells via inhibition of stimulated adenylate cyclase and a cAMP-independent process which involves calcium; and since pertussis toxin blocks both these processes to a similar extent, both processes may be acting through G_i, the pertussis toxin substrate in these cells (19).

GTP regulation of opioid binding

The fact that opioid receptor binding is modified by guanine nucleotides throughout the brain (3,4,10), implicates GTP-binding proteins in mediating opioid action, since most systems which have GTP-sensitive binding sites are coupled to GTP-binding proteins (2,23,24). In the presence of GTP, opioid agonist but not antagonist binding is decreased (3,4,10). In the neuroblastoma-glioma cell line NG108-15 and in other systems known to be coupled to G proteins, the observed inhibition by GTP results from decreased receptor affinity of opioid agonist (25). In the case of the opioid delta receptor in NG108-15 cells, when agonist binds to the receptor, the conformation of the receptor is altered so that it binds to G_i. This agonist-receptor-G_i complex promotes the dissociation of GDP bound to G_i by GTP after which G_i-GTP dissociates from the receptor. The receptor now assumes a low affinity or uncoupled state.

Proposed research:

The overall objectives of my research are to investigate the role of G proteins in the action of opioids in the higher central nervous system and to determine which second messenger systems and/or ion channels may be involved.

Inhibition of adenylate cyclase

I was interested in determining whether the inhibitory guanine nucleotide protein, G_i, mediates opioid inhibition of adenylate cyclase in brain. With the use of pertussis toxin, (or IAP, islet activating protein), an exotoxin produced by Bordetella pertussis it has been possible to specifically label G_i and modify its regulatory role (2). Pertussis toxin catalyzes the ADP-ribosylation of the GTP-binding subunit of G_i, to result in both an attenuation of its inhibitory action on adenylate cyclase and an uncoupling of G_i from its receptor (26). The GTP-binding subunit of G_i corresponds to a 41 kDa protein, which can be radiolabelled with [α-^{32}P]NAD as the substrate for pertussis toxin.

The strategy was to ask if pertussis toxin treatment of rat brain striatal membranes alters the opioid effect on adenylate cyclase. The predicted result would be an attenuation of opioid inhibited adenylate cyclase, which could then be correlated with radiolabeling of a 41 kDa protein. Such a correlation would strongly suggest the involvement of G_i in opioid interaction.

The effect of opioids on adenylate cyclase in brain areas other than the striatum can be re-examined. Since G proteins comprise about 1% of bovine brain membrane protein and GTP modulation of opioid binding occurs throughout the brain, it is likely that G proteins will be present in most regions of rat brain (3,27). This hypothesis can be tested by labeling different brain regions with pertussis toxin. If G proteins are found throughout the brain, but opioid inhibition of adenylate cyclase is only seen in the striatum, one could infer that opioids are not coupled to adenylate cyclase in other brain regions despite the presence of G proteins in these regions. An alternative possibility would be that the coupling has been disturbed by cellular disruption. The latter possibility can be tested by determining the effect of opioids on cAMP levels in brain slices. A failure to demonstrate any effect would support the conclusion that opioids are not coupled to the adenylate cyclase system in brain regions other than the striatum and cortex.

Effects on ion channels

Potassium channels

In order to investigate the potassium effect at the biochemical level, a study was undertaken to determine if opioids will increase K^+ efflux. If opioids act by increasing K^+conductance, then a small amount of K^+ ions must cross the membrane to change the membrane potential. It has been shown in other systems (e.g., ACh, Glu) that measuring radioactive tracer ion flux is a reasonable alternative to electrophysiological measurements (28,29). One measures the sum of

events in, for example, a slice preparation rather than recording from a single cell, where the signal to noise ratio may be greater. Technical limitations force observation of events in terms of seconds instead of milliseconds. However, the electrophysiological data for the opioid induced K^+ conductance shows an event that occurs in seconds, since there is an after-hyperpolarization which prolongs the response and is also opioid dependent (12).

I chose to study K^+ efflux in hippocampal slices for two major reasons. Firstly, they are easily dissected and their anatomy is well worked out and conserved along the tissue so that separate slices are more homogenous. Secondly, opioid effects have been demonstrated in the hippocampus by electrophysiological techniques (6,30). Since electrophysiological data suggest that opioids increase potassium conductance, it was of interest to determine if this effect could be reproduced biochemically.

The strategy was to look at K^+ flux under basal, acute and chronic opioid conditions to determine if it could be correlated with analgesia and known binding characteristics. The nature of the flux could then be determined, i.e., whether it was related to the action potential or a Ca^{++}-dependent process. Selective channel blockers, such as tetraethylammonium for action potential related conductances and apamin for Ca^{++}-related fluxes, would be used to characterize the response. Since inhibition of Na,K-ATPase will increase K^+ flux oubain would be expected to mimic the opioid effect.

Ca^{++} channels and phosphoinositide turnover

Since opioids may act via a G protein in the brain, and a number of studies indicate the involvement of Ca^{++} in the action of opioids (21,22), the regulation of PI turnover may be a possible second messenger system for opioids. In order to simplify the study of opioid regulation of this system in acute and chronic opioid states, the neuroblastoma-glioma hybrid cell line, NG108-15, provides an ideal model system. Initial studies will thus be done in the cells, followed by testing the effect of opioids on PI turnover in brain slices from various areas of rat brain. The effects of acute and chronic exposure to opioids can be tested, along with naloxone reversibility.

Opioid binding and analgesia

Among the many pharmacologic effects of opioids, analgesia is the one most extensively examined and probably the most relevant to the endogenous opioids. Since the goal of this research is to address the mechanism of action of opioids it is important to try and correlate a physiological endpoint, such as analgesia, with any proposed mechanism. If opioid analgesia is mediated through G_i, then pertussis toxin should uncouple the receptor from G_i, thereby resulting in an alteration of the physiological endpoint in an animal. The experimental approach involves the exposure of rats to pertussis toxin and testing for analgesia by a tail flick assay (31). If G_i or G_o mediate opioid analgesia, then inactivation of these proteins by pertussis toxin should result in a reduced effect of exogenous and endogenous opioids, which would be reflected as a decreased response in the tail flick test.

Along with the effects on analgesia, the effects of pertussis toxin treatment on opioid receptor binding properties can be determined. Membranes from pertussis toxin treated NG108-15 cells exhibit a decreased affinity of agonists for the opioid receptor similar to that seen for GTP in untreated cells (25, 32-34). The decrease in agonist affinity is thought to occur as described for GTP above, except that pertussis toxin is thought to "freeze" G_i in a GDP-bound state (34). The uncoupling effect of pertussis toxin can be correlated with the degree of radiolabeling of the 41 kDa subunit of G_i and is manifest as a decrease in [^{3}H]-agonist binding or as a decreased ability of agonist to displace [^{3}H]-antagonist binding. Such effects of the toxin have been demonstrated for the opioid receptor in NG108-15 cells and the dopamine (D_2) and α_2-adrenergic receptors in brain membranes (2,23,24).

The effects on opioid binding following pertussis toxin treatment will be examined by comparing [^{3}H]-agonist binding in treated and non-treated rat brain membranes. Initially, binding in striatal membranes will be examined since in this brain region opioids inhibit adenylate cyclase which strongly implies the presence of G_i. If pertussis toxin treatment results in uncoupling of the receptor with G_i in these membranes, a correlation between uncoupling, extent of radiolabeling and attenuation of inhibition of adenylate cyclase should be demonstrable. The effects of pertussis toxin on other brain regions, where opioids do not inhibit adenylate cyclase, can then be studied in order to determine whether G proteins modulate receptor binding regardless of whether they are coupled to adenylate cyclase.

Summary

It needs to be emphasized that none of these possible mechanisms of opioid action is likely to account for all opioid effects. The available evidence suggests that different mechanisms of action may exist in different systems, including different regions of the brain. For example, opioid inhibition of adenylate cyclase has been readily observed only in the striatum, a brain region that is not associated with analgesia, or in the neuroblastoma cell lines (1,8). Opioid regulation of potassium channels has been shown in the locus ceruleus or myenteric plexus (12,35). Opioid effects on calcium channels have been shown in the gut and in a dorsal root ganglion-spinal cord co-culture (21,35). However, all these processes may share one common factor, the G proteins. So far, three G proteins have been isolated from the brain, one of which is responsible for stimulation of adenylate cyclase, G_s, one responsible for inhibition of adenylate cyclase, G_i, and another, G_o (27). In addition to the G protein regulation of adenylate cyclase, G proteins have been implicated in regulation of potassium channels in heart (36a,b), and phosphoinositide turnover in many cells (17-20), suggesting similar roles in the brain.

In order to identify any coupler of the opioid receptor it would be ideal to purify and reconstitute the system. Until that is accomplished, one can only assay events and try to correlate them with receptor binding studies. My project is to study the role of G proteins as mediators of opioid action. In order to do so it is necessary to determine: 1) the co-localization of opioid receptors and G proteins; 2) the effects of G proteins on opioid receptor binding; 3) the second

messenger systems through which opioids and G proteins act; 4) the physiological endpoints of the systems. The proposed experiments are designed to address these points, to determine whether G proteins mediate opioid action.

Methods:

Preparation of Membranes:

P2 fractions were prepared according to the method of Law et.al. (8). All steps were carried out at 0-2°C. In a typical preparation, ten rats were sacrificed by decapitation and the striata dissected. Male Sprague-Dawley rats from Charles Rivers were used for all experiments. The striata were prepared by homogenizing in .32 M Sucrose/1.6 mM EGTA/ 10 mM Hepes pH 7.4. Nuclei were pelleted out by centrifugation at 1000 x g for 10 min. The pellets were washed and re-centrifuged at 1000 x g for 10 min. The supernatants were combined and centrifuged at 22,500 x g for 20 min. The supernatant was discarded and the pellet resuspended in membrane buffer (10 mM Hepes/1.6 mM EGTA pH 7.4), then centrifuged at 22,500 x g for 20 min. The final pellet was resuspended in membrane buffer to give a final protein concentration of 10 mg/ml. The preparations were stored as 1 ml aliquots at -60°C until use. Protein concentrations were determined by the Lowry method (37).

Pertussis toxin treatment:

Membranes were treated with pertussis toxin according to a modification of the method of Kurose et. al. (2). Pertussis toxin (obtained from List Biochemicals) was activated with 100 mM dithiothreitol (DTT) for four hours at 25°C. Membranes were incubated with 20.0 μg/ml pertussis toxin in a reaction mixture containing 50 mM Tris HCl pH 8, 20 mM thymidine, 0.5 mM ATP, 20 μM GTP, 5 mM $MgCl_2$, 1 mM EDTA, 5 mM DTT, 20 mM creatine phosphate, 100 U/ml creatine phosphokinase, 1 mM 1,10-o-phenanthroline, and 1 mM NAD. When adenylate

cyclase activity was to be measured after toxin treatment the membranes were centrifuged at 12,000 x g for 10 minutes and resuspended in membrane buffer.

For determinations of the radiolabeling of the G_i protein, 10 μM-1 mM [α^{32}P]NAD (1-2 Ci/mmol) was added in place of the non-radioactive NAD. The membranes were centrifuged at 12,000 x g for 10 minutes, dissolved in 1% sodium dodecyl sulfate (SDS) and subjected to SDS-polyacrylamide gel electrophoresis. The gel was then autoradiographed at -80°C using Kodak X-Omat film. The autoradiogram was used to locate the 41,000-Da subunit of G_i which was then sliced from the gel along with slices from the background. The slices (4 mm x 15 mm) were dissolved in 30% hydrogen peroxide by heating at 60 °C overnight then counted with 5 ml Scintiverse in a liquid scintillation counter.

Adenylate cyclase assay:

Striatal adenylate cyclase activity was determined as described by Law et.al. (8). In a standard assay, the following reagents in their final concentrations are combined to give a total volume of 50 μl: 40 mM Hepes, pH 7.4, 10 mM $MgCl_2$, 40 mM creatine phosphate, 2 mM cAMP, 200 U/ml creatine phosphokinase, 200 mM NaCl, 40 μM GTP, 20 mM theophylline and 2 mM 1,10-o-phenanthroline. Opioid agonists (in a 5 μl volume) were added to the above assay mixture.

The mixture was then incubated with 25 μl of membrane protein for 10 min at 0 °C. Reactions were initiated by adding 20 μl of [α^{32}P]ATP (1-2 μCi per assay tube in 0.1 mM final concentration).

The reactions were carried out at 30°C for 10 min and terminated by the addition of 550 μl of 1N perchloric acid containing 10,000 cpm of [^{3}H]cAMP. The reaction product cAMP was separated from other radioactive contaminants by double columns of Dowex and alumina as outlined by White and Karr (38). Reaction mixtures containing the radiolabeled cAMP were separated from the membranes by centrifugation, and applied to individual Dowex 50-WX4 columns (1 x 3.5 cm) washed with 4 ml 10 mM perchloric acid and eluted onto alumina columns (1 g alumina in a 0.75 cm column) with 7 more ml of perchloric acid. The alumina columns were washed with 5 ml water and the cAMP eluted with 3 ml of imidazole (0.2 M) into counting vials containing 5 ml Scintiverse. The radioactivity was determined by liquid scintillation spectrometry. The percent cAMP recovery could be calculated using an internal standard of [^{3}H]cAMP.

Binding Studies:

The binding characteristics of the brain P2 preparations were assayed according to a modification of the method of Lee et. al. (39). Samples containing 25 mM Hepes, pH 7.4, 0.5-1.0 mg protein and the tritiated ligand in a final volume of 1 ml were incubated at 25 °C in a shaking incubator for 1 hour. In some experiments, 10 mM $MgCl_2$ and 50 μM GppNHp were added to this mixture. Samples were filtered through Whatman GF/B filters under vacuum, washed twice with 5 ml ice cold 5 mM Hepes, pH 7.4 and counted in the presence of 5 ml scintillation fluid in a liquid scintillation counter. All binding assays were carried out in triplicate. Non-specific binding was determined as that remaining in the presence of 1 μM non-labeled ligand.

For the displacement experiments, the binding of 1-2 nM radiolabeled ligand was assayed in the presence of increasing concentrations of non-labeled ligands, and $\pm$ $MgCl_2$ and $\pm$ GTP. The saturation experiments were performed with 10-24 concentrations of the radiolabeled ligand.

Induction of Tolerance:

Male Sprague-Dawley rats were made tolerant to morphine according to the pellet implantation procedure (40,41). Usually three rats were used per group. Placebo or morphine pellets (75 mg) were implanted according to the following protocol: Day 1, 1 pellet; Day 2, 1 pellet; Day 3, 2 pellets; Day 4, sacrifice. When the effects of naloxone to block the morphine effect were to be studied the protocol was modified as follows: Day 1, 2 pellets, either 2 placebo pellets (placebo group), 1 morphine + 1 placebo pellet (morphine group), 1 morphine + 1 naloxone pellet (morphine + naloxone group), or 1 naloxone + 1 placebo (naloxone group); Day 2, 2 pellets for each group; Day 3, 4 pellets per group; Day 4, sacrifice.

Cholate extracts of membranes:

Cholate extracts of membranes were used to solubilize G_i according to the following method (26). Membranes were extracted with 1% sodium cholate in TED buffer (20 mM Tris, pH 8, 1 mM EDTA, 1 mM DTT), usually using 5 mg of membrane per ml of TED-cholate buffer, and stirred on ice one hour. The extracts were layered on 4 ml 1 M sucrose and centrifuged at 143,000 x g for one hour (43,000 rpm in a Beckman SW65 rotor). The clear supernatant removed from the top was then diluted 20-

fold with TED to use for the ADP-ribosylation experiments.

Measurement of cAMP accumulation in slices:

The effect of opioids on intracellular cAMP levels was measured by the method of Schultz and Daly using [^{3}H]-adenine to label the intracellular ATP pools (42). Rats were sacrificed by decapitation immediately prior to the experiment and their brains put in oxygenated Krebs-Ringer solution (124 mM NaCl, 0.76 mM KCl, 1.24 mM KH_2PO_4, 1.3 mM $MgSO_4$, 2 mM $CaCl_2$, 10 mM glucose and 26 mM Tris HCl, pH 7.3) on ice. The areas to be studied, usually the striatum and cortex, were dissected and sliced into 250 x 250 μm cubes with a McIlwain tissue slicer. The slices were centrifuged at low speed (600 x g) and transferred with 10 ml of warmed incubation medium (Dulbecco's modified Eagle's medium H-16; 29.3 mM $NaHCO_3$, 15.3 mM glucose, 15.4 mM NaCl, pH 7.3) to 100 mm cultures dishes for a 10 minute pre-incubation at 37 °C in a 10% CO_2 (in air) incubator. [^{3}H]-adenine (75 μCi per 10 ml) was added along with phosphodiesterase inhibitors isobutylmethylxanthine (IBMX, 0.5 mM) and ZK62711 (0.1 mM) to start the intracellular labeling of the slices. The slices were incubated for 60 minutes. The slices were then transferred to 1.5 ml eppendorf centrifuge tubes and centrifuged at low speed (600 x g). The medium was aspirated and 0.5 ml of incubation medium containing identical concentrations of phosphodiesterase inhibitors and, typically, 10 μM forskolin $\pm$ 1 μM etorphine $\pm$ 200 μM naloxone. The incubation was carried out for 10 minutes at 37 °C and 10% CO_2. The reactions were terminated by the addition of 50 μl 3.3 N perchloric acid. The samples were centrifuged again to separate the slices from the released cAMP. Fifty

microliters of ^{32}P labeled cAMP (10,000 cpm) was added to each sample and the ^{3}H labeled cAMP that was formed during the 10 minute incubation period was separated from other nucleotides by the double column method of White and Karr (38). The pellet remaining from the last spin was resuspended in 1 ml of 1% SDS: 1 N NaOH: H_2O (1:1:1) and incubated overnight at 60 °C, then an aliquot of this was used for protein determination by the Lowry method (37).

Cerebroside sulfate treatment of slices:

Cerebroside sulfate (purified) was dissolved in saline at a concentration of 1-2 mg/ml. Lipid vesicles were prepared by sonication at the maximal output of the sonicator for 10' at 4 °C as described (43). The vesicles were used immediately after preparation and 0.7 mg of vesicles added to freshly prepared slices in incubation medium. Slices were treated for four hours ± cerebroside sulfate at 37°C in a 10% CO_2, 90% O_2 environment. During the last hour of incubation, [^{3}H]- adenine was added to label the intracellular ATP pool.

Pertussis toxin treatment of slices:

When pertussis toxin was to be added to rat brain slices, the slices were prepared as described as above. Pertussis toxin was added to the incubation medium at various concentrations, starting at 100 ng/ml, for 3 or more hours, then either cAMP accumulation was measured as described or membranes were prepared from the slices.

Measuring potassium flux in slices:

Male Sprague-Dawley rats were sacrificed by decapitation and their brains placed in oxygenated Krebs-Ringer's solution on ice. The tissues were oxygenated throughout the procedure in order to keep the tissue viable. The hippocampi were dissected and sliced into 500 μm slices using a McIlwain tissue slicer. The slices were transverse through the hippocampus so that each had the same anatomy. After slicing, the tissues were incubated on ice 30-40 minutes. The slices were then placed in Ringer's containing 1-2 μCi $^{86}Rb^{+}$ for 30 min. at 37°C. After this incubation, they were transferred with sieves through a series of vessels every 30 seconds containing the drug to be tested. The first vessel through which the tissue was transferred served as a "washout" tube for non-specific radioactivity.

The amount of radioactivity in each vessel was determined as well as that present in the tissue at the end of the experiment. The efflux was measured as the percent of radioactivity released from the tissue, calculated as follows: the concentration of $^{86}Rb^{+}$ could be calculated in each tube, then the initial tissue concentration found by adding the final tissue concentration with the sum of each tube. The concentration at each time t was divided by the initial concentration in order to calculate percent remaining (the inverse of that which is released). In order to transfer several tissues at one time into tubes containing different drugs, a sieve holding devise was constructed.

Intravenous injection of pertussis toxin:

Male Sprague Dawley rats (100-200 g) were used for all experiments. Pertussis toxin from lyophilized powder was dissolved in a sterile isotonic solution of 55 mM $NaPO_4$, 100 mM NaCl, pH 7.4, at a concentration of 50 μg/ml. Pertussis toxin (20 μg in 0.4 ml) was injected into a tail vein of each rat. An equivalent amount of saline was injected into the control rats. The rats were observed every day for three days then tested and sacrificed on the fourth day. The animals were weighed before and after the treatment.

Intracerebroventricular injection of pertussis toxin:

Male Sprague Dawley rats (200-300 g) were used for all experiments. Pertussis toxin was prepared in a sterile isotonic solution as for i.v. injections, but 50 μg of toxin were suspended in .25 ml for a final concentration of 0.2 μg/μl. The animals were injected with 5 - 15 μl(1 - 3 μg) of toxin (or saline) via cannulae (previously placed stereotaxically under anesthesia in the fourth ventricle), which was then flushed with 10 μl saline. The animals were sacrificed on the fourth day after the injection.

Measurement of phosphoinositide turnover in NG108-15 cells:

Phosphoinositide hydrolysis was monitered by measuring ^{3}H-labeled inositol phosphate accumulation in the presence of LiCl, which prevents the conversion of inositol-1-phosphate to inositol (44). Phosphoinositides were radiolabeled by incubating NG108-15 cells with growth medium containing 1-3 μCi/ml myo-[2-^{3}H]inositol for 18-20 hours (45). Typically, the cells were plated in 24 well dishes or 6

well plates. Prior to assay, the medium was removed and the monolayers washed with warmed incubation medium. The cells were incubated with incubation medium containing 10 mM LiCl for 10 min. prior to assay. Assays were initiated by the addition of incubation medium containing the appropriate drugs and 10 mM LiCl. The reactions were terminated by aspiration of the medium, the monolayers washed three times with Krebs-Ringers solution and 0.5 ml cold methanol added. The cells were removed by scraping and 0.5 ml methanol added to rinse. Chloroform (1.0 ml) and water (0.9 ml) were added and the tubes centrifuged at 1500 x g for 15 min at 4 °C. An aliquot (100 μl) of the aqueous layer was reserved for determining radioactivity. The remainder of the aqueous layer was loaded onto columns prepared according to Berridge (46).

The inositol phosphates were separated by columns (BioRad econocolumns) containing 1 ml of anion exchange resin (Bio-Rad AG 1X8, 100-200 mesh, formate form). Columns were washed with 10 ml water to remove inositol, 5 ml of 5 mM disodium tetraborate + 60 mM sodium formate to remove glycerophosphoinositol. The inositol phosphates were eluted as follows: 10 ml of 100 mM formic acid + 200 mM ammonium formate for inositol-1-phosphate (IP); 5 ml of 100 mM formic acid + 400 mM ammonium formate for inositol-1,4-diphosphate (IP_2); and 5 ml of 100 mM formic acid + 1 M ammonium formate for inositol -1,4,5-triphosphate (IP_3). This elution pattern was that shown by Berridge to contain these compounds (46). The eluted material was counted in a liquid scintillation counter and normalized according to the radioactivity loaded onto each column.

Some of the cells were treated with 1 μM etorphine for 2 hours prior to assay in order to produce a "chronic" state. These conditions have been shown to desensitize adenylate cyclase in these cells (25).

Measurement of phosphoinositide turnover in brain slices

Phosphoinositide turnover in rat brain slices was measured according to Berridge (44). The cerebral cortex was dissected from rat brains immediately after sacrifice. Cross-chopped slices (width 350 μm) were cut with a McIlwain chopper. The slices were washed thoroughly in warm Krebs-Ringer bicarbonate containing 10 mM glucose (KRB) and then incubated for 30 min with gentle shaking in a 37 °C water bath.

50 μl portions of the gently packed tissue preparations were pipetted into vials containing 0.64 μl myo-[2-^{3}H]inositol in 1 ml of incubation medium. When present, LiCl replaced NaCl in the normal incubation medium to give the appropriate Li concentration. Drug additions were made from stock solutions in KRB buffer. The vials were gassed (95% O_2/10% CO_2) and capped and then incubated at 37°C for 60 minutes in a gently shaking water bath. The incubations were stopped by the addition of 1 ml of chloroform/methanol (1:2, v/v). The slices were homogenized in 0.2 ml 1N HCl, the homogenizer washed with 0.2 ml water, and 0.5 ml chloroform added to separate the phases. The samples were centrifuged at 1500 x g for 15 min at 4°C. The upper (aqueous) phase was removed and the phosphoinositides isolated as described above.

Chapter one: Opioid inhibition of adenylate cyclase

Opioids have been shown to inhibit adenylate cyclase in the rat brain striatum (8). With the possible exception of the cortex, this is the only brain region shown to be coupled to adenylate cyclase (9,10). Opioid inhibition of striatal adenylate cyclase is GTP dependent, suggesting the involvement of the inhibitory guanine nucleotide regulatory protein (8).

I was interested in determining whether the inhibitory guanine nucleotide protein, G_i, mediates this effect of opioids on adenylate cyclase. It is possible to specifically radiolabel and modify the functions of G_i using pertussis toxin, (or IAP, islet activating protein), an exotoxin produced by Bordetella pertussis (2). If G_i mediates opioid inhibition of adenylate cyclase in the striatum, then pertussis toxin treatment would result in uncoupling of G_i from the opioid receptor and adenylate cyclase, and attenuate opioid inhibited adenylate cyclase. This effect could then be correlated with radiolabeling of a 41 kDa protein (the GTP-binding subunit of G_i, the substrate for pertussis toxin). Such a correlation would strongly suggest the involvement of G_i.

Results:

Effect of pertussis toxin on opioid inhibition of adenylate cyclase:

If opioid inhibition of adenylate cyclase is mediated by G_i in the rat brain striatum, the predicted effect of pertussis toxin treatment would be attenuation of the opioid response. In order to

demonstrate such an effect, it was first necessary to establish that the conditions for pertussis toxin treatment did not alter the adenylate cyclase activity. Striatal membranes were treated in the presence or absence of the ADP-ribosylation mixture. The initial mixture consisted of 50 mM Tris, pH 8, 20 mM thymidine, 0.5 mM ATP, 20 μM GTP, 5 mM $MgCl_2$, 1 mM EDTA, 5 mM DTT, and 250 μM dimyristol phosphatidylcholine. It was found that treatment of membranes at 30°C for 60 minutes resulted in 40-60% loss of basal and Mn^{++}-stimulated adenylate cyclase activity (Table 1.1). This was a time dependent loss, but a 60 minute incubation was required for ADP-ribosylation (see fig. 1.1). If the initial ADP-ribosylation mixture was added to the treatment, a further loss (10-20% more) of adenylate cyclase activity occurred (Table 1.1). Since a loss of basal cyclase activity would complicate the interpretation of the effects of toxin addition, reagents were added to stabilize the basal adenylate cyclase activity. The following reaction mixture was found to partially preserve adenylate cyclase activity: 50 mM Tris pH 8, 20 mM thymidine, 0.5 mM ATP, 20 μM GTP, 5 mM $MgCl_2$, 1 mM EDTA, 5 mM DTT, 20 mM creatine phosphate, 100 U/ml creatine phosphokinase, 1 mM 1,10-o-phenanthroline, and 1 mM NAD (Table 1.1 and Methods).

In the presence of 1 μM etorphine, basal adenylate cyclase activity was inhibited 20-30%, as previously reported (fig. 1.1, inset,(8)). This inhibitory effect remains in the presence of the ADP-ribosylation mixture. When 12.5 μg/ml activated pertussis toxin was added, the inhibition dropped to less than 10%, suggesting that the toxin had ADP-ribosylated the G_i protein and that this protein was responsible for

Table 1.1

The loss of adenylate cyclase activity under ADP-ribosylation conditions

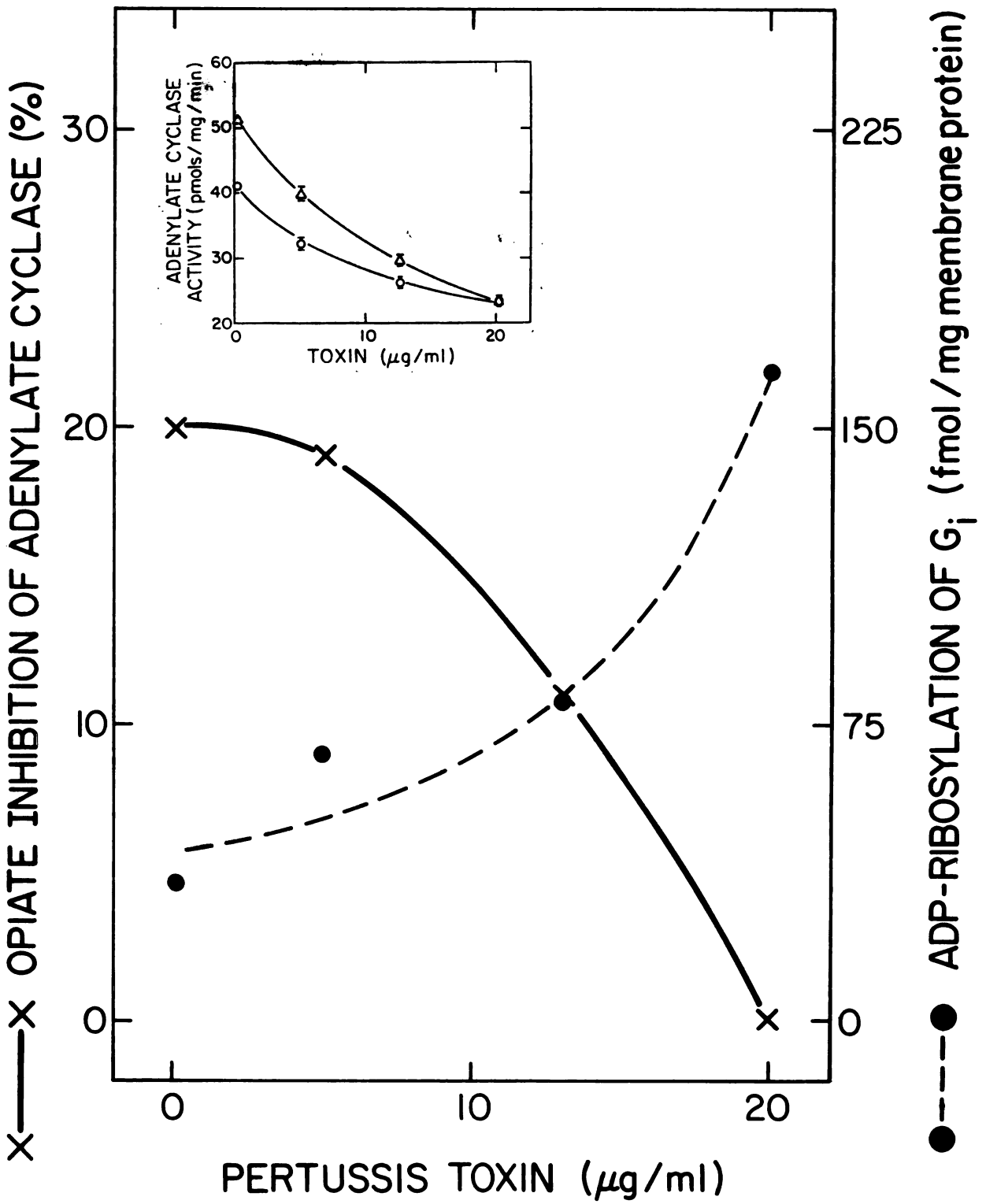
	basal(pmols/mg/min)	Mn^{++}-stimulated
no treatment	113.8 ± 6.4 (100%)	391.7 ± 2.6 (100%)
30°C /1 hr	48.8 ± 1.7 (43%) p<.003	202.8 ± 8.1 (52%) p<.002
" + ADP-R #1	36.0 ± 3.1 (32%) p<.002	n.d.
" + ADP-R #2	59.0 ± 2.6 (52%) p<.008	203.9 ± 7.1 (52%) p<.001

The basal and Mn^{++}-stimulated adenylate cyclase activities of striatal membranes were assayed after the indicated conditions. 5 mM $MnCl_2$ was used to stimulate the catalytic unit of adenylate cyclase. As described in the text, the decrease in cyclase activity after 1 hour was enhanced with the first ADP-ribosylation mixture used (ADP-R #1). With the mixture found to stabilize adenylate cyclase (ADP-R #2) there was still a 48% loss of activity. The data shown are the means of triplicate assay tubes. The means of triplicate assay tubes ± standard deviations are shown. The p values are from a student's t test.

Figure 1.1

Dose-response curves

Striatal P2 membranes were pre-treated at 30°C for 60 minutes with the indicated doses of pertussis toxin with either 1 mM NAD or 50 μM [α-^{32}P]NAD (10 μCi) in the ADP-ribosylation mixture (described in text), then assayed for adenylate cyclase activity or for ADP-ribosylation of G_i, respectively. The number of fmols of ADP-ribose in G_i per mg membrane protein was obtained by counting the slice corresponding to the 41 kDa subunit of G_i and assuming 1 mole of G_i per mole of ADP-ribose. The data shown are the results of a typical experiment. X-X represent percent opiate (opioid) inhibition of adenylate cyclase. ●-● represent ADP-ribosylation of G_i in fmoles per mg membrane protein. In the inset, the adenylate cyclase activity is plotted against the pertussis toxin concentration. This demonstrates the dose-dependent loss of adenylate cyclase activity with toxin treatment. The attenuation of opioid inhibition of adenylate cyclase activity is a separate event, as can be seen here. Δ-Δ represent basal adenylate cyclase activity, o-o represent adenylate cyclase activity in the presence of 1 μM etorphine.


X——X OPIATE INHIBITION OF ADENYLATE CYCLASE (%)
●---● ADP-RIBOSYLATION OF G_i (fmol/mg membrane protein)
PERTUSSIS TOXIN (μg/ml)
0
10
20
30
75
150
225
ADENYLATE CYCLASE ACTIVITY (pmols/mg/min)
TOXIN (μg/ml)

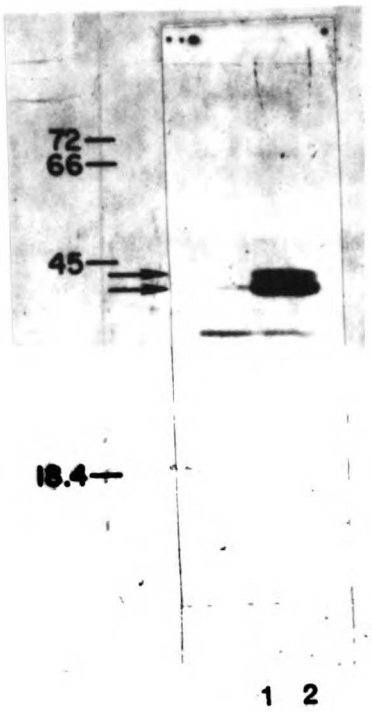
mediating the opioid effect. To demonstrate this further, it was necessary to show a dose-dependent effect of the toxin and correlate this with radiolabeling of the G_i protein. As shown in figure 1.1, pertussis toxin treatment resulted in a dose-dependent attenuation of the opioid inhibition of adenylate cyclase activity, with 20 μg/ml toxin resulting in a complete loss of opioid response. A dose-dependent loss of adenylate cyclase activity was also seen with pertussis toxin, in contrast to the increases usually reported (2). Basal adenylate cyclase activity reflects the balance of several events: those which regulate the production of cAMP, such as inhibition and stimulation by receptors mediated through G_i or G_s, respectively; and those which regulate the destruction of cAMP, via phosphodiesterase activity. The observed decrease suggests that striatal adenylate cyclase activity may be under additional excitatory control (leading to either increased production or decreased destruction of cAMP) which is then inactivated by pertussis toxin treatment, or, conversely, an inhibitory control which is then activated by toxin treatment.

Pertussis toxin ADP-ribosylation of rat brain membranes

Since pertussis toxin has been shown to specifically ADP-ribosylate the 41 kDa GTP-binding subunit of G_i, [α-^{32}P]NAD was used as a substrate for the toxin in order to demonstrate the presence of G_i in the striatum. Pertussis toxin treatment resulted in the specific radiolabeling of two bands in the rat brain striatum (fig. 1.2). Since the α subunit of G_i is located at 41 kDa, it is probably safe to assume that this band on the gel corresponds to α_i (26). G_i has been purified to homogeneity and shown to inhibit adenylate cyclase

Figure 1.2

Autoradiogram of pertussis toxin treated striatal membranes



Striatal membranes were treated at 30°C for 60 minutes with 10 μM [α-^{32}P]NAD (20 μCi) $\pm$ 20 μg/ml pertussis toxin. The molecular weight standards indicated on the bar to the right of the autoradiogram are: 72 kilodaltons, transferrin; 66 kDa, bovine serum albumin; 45 kDa, ovalbumin; and 18.4 kDa, β-lactoglobulin. Lane 1 represents membranes treated in the absence of pertussis toxin, lane 2 represents membranes + pertussis toxin. The arrows indicate the 41 kDa and 39 kDa toxin substrates.

activity, thus this is probably the protein responsible for inhibition in the rat brain striatum (26). The excellent correlation between attenuation of opioid inhibition of adenylate cyclase activity and radiolabeling of the 41 kDa protein suggests that opioid inhibition of adenylate cyclase activity is mediated via G_i. The 39 kDa band specifically radiolabeled probably corresponds to G_o, a protein isolated from brain (27).

In an attempt to quantitate the amount of G_i in the rat brain striatal membrane a saturation curve for NAD was determined. An estimate of 4.3 pmols G_i/mg membrane protein was obtained by assuming one G_i per molecule of ADP-ribose (fig. 1.3). This assumption was based on the 1:1 ratio found with purified rabbit liver G_i (26).

Opioid inhibition of adenylate cyclase has previously been shown in the striatum with one report of inhibition in the cortex (10), whereas opioid receptors are found throughout the brain. A study was undertaken to determine whether other brain regions contained G_i even though they show no opioid effect on adenylate cyclase. In all brain regions examined, G_i was ADP-ribosylated with pertussis toxin to a varying extent: striatum = cortex $\geq$ hippocampus > midbrain = hypothalamus > brainstem (fig 1.4). In agreement with others (8,9), opioid inhibition of adenylate cyclase activity was seen in the striatum with less than 10% inhibition in the cortex and no detectable inhibition in the other brain regions (Table 1.2).

A dose response curve with NAD revealed a decreased amount of G_i as well as a decreased apparent affinity for NAD in the midbrain as

Figure 1.3

Radiolabeling of G_i in striatum versus midbrain

Membranes prepared from either the striatum (●-●) or midbrain (x-x) regions of rat brains were treated at 30°C for 60 min with [α-^{32}P] NAD (7.5 μCi) plus NAD in ADP-ribosylation mixture to give final concentrations ranging from 10μM to 2 mM. The data are plotted as the log [NAD] with 1 - 10μM versus the fmols ADP-ribose in G_i per mg membrane protein (calculated as described in the legend to figure 1.1). The data shown are the average of the results from two separate experiments with less than 10% variance in the points. The maximal incorporation of ADP-ribose in striatal G_i was 4160 fmols/mg membrane protein with an apparent half-maximum at 265 μM NAD, as compared to 2051 fmols/mg protein in the midbrain and a half-maximum at 530 μM NAD.

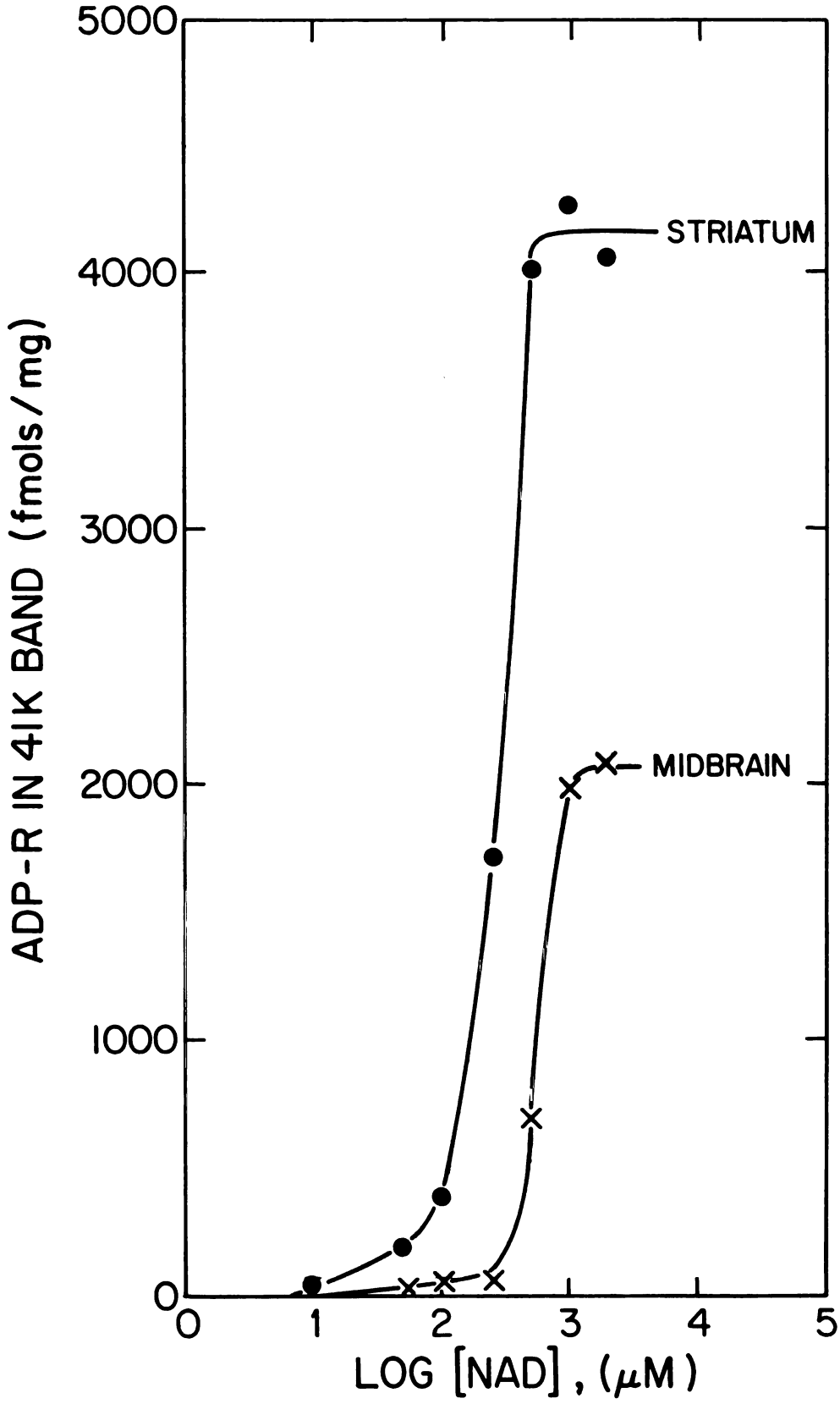
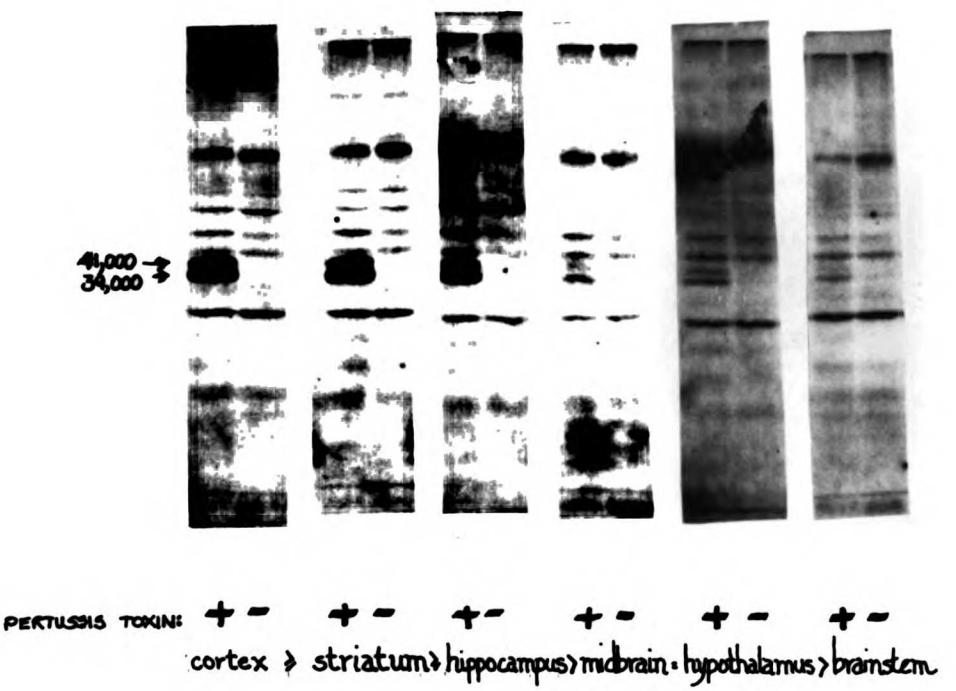

5000
4000
3000
2000
1000
0
0
1
2
3
4
5
ADP-R IN 41K BAND (fmols/mg)
LOG [NAD], (μM)
STRIATUM
MIDBRAIN

Figure 1.4

Radiolabeling of G_i in various brain regions



Membranes prepared from the indicated brain regions were treated at 30°C for 60 minutes with 10 μM [α-^{32}P]NAD (5 μCi).

Table 1.2

Opioid inhibition of adenylate cyclase in various brain regions

	basal (pmols/mg/min)	1 μM etorphine	% inhibition
striatum	184.2 ± 1.5	143.2 ± 5.7	22% p<.001
midbrain	83.9 ± 1.6	82.0 ± 1.1	N.S.
cortex	77.8 ± 1.0	70.3 ± 2.3	10% p<.001
brainstem	54.1 ± 1.0	52.8 ± 3.4	N.S.
hippocampus	55.8 ± 5.8	49.6 ± 2.4	N.S.
hypothalamus	145.4 ± 9.5	143.1 ± 4.6	N.S.

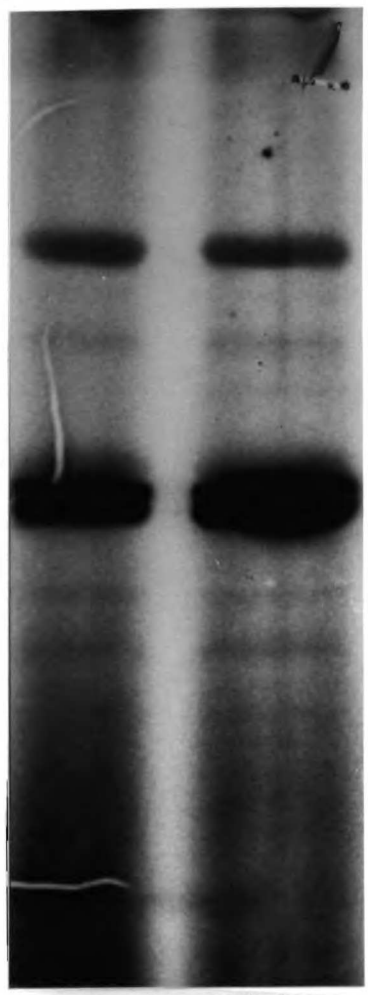
Membranes from the indicated regions were assayed for adenylate cyclase activity with or without 1 μM etorphine as indicated. The results presented are means ± standard deviations from triplicate assay tubes. A student's t-test was used for statistical comparisons.

compared to the striatum (fig. 1.3). In order to determine whether this decrease reflected an increased NADase activity, two further experiments were pursued. Cholate extracts of membranes from these regions were compared for the extent of radiolabeling of G_i. Such extracts presumably contain G_i in the absence of NADase activity. The midbrain region showed a 50% decrease in labeling compared to the striatum (fig 1.5). The possibility remained that some NADase activity was solubilized with G_i, so the membranes from striatum and midbrain were mixed to test this. If increased NADase activity was present in the midbrain, the mixed membranes should have less radiolabeling than the calculated sum of the membranes. This was found to be the case (fig. 1.6). The fact that the dose response curves for NAD reached a plateau suggest that there is a lesser amount of G_i in the midbrain, but the affinity change is probably due to the increased NADase activity. It seems, therefore, that there are two contributing factors to the variable labeling in the brain: an increase in NADase activity and a decrease in the amount of G_i in certain brain regions.

Since G_i is present throughout the brain, the reason opioid inhibition of adenylate cyclase is not seen in other brain areas does not appear to be due to a lack of G_i in these areas. Opioid receptors do not seem to be coupled to adenylate cyclase except in the striatum and cortex. It is possible that the receptors and G_i are in different locations in the neuron, e.g., pre- and post-synaptically. Alternatively, the receptors, G_i proteins, and adenylate cyclase, which are interconnected in the intact cell, are separated during the preparation of membranes. This possibility was explored further

Figure 1.5

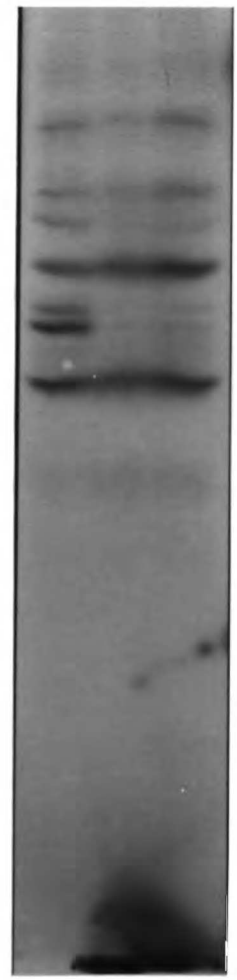
Cholate extracts of midbrain shows decreased radiolabeling compared to striatum



Membranes prepared from striatum or midbrain regions were extracted with 1% sodium cholate as described in methods. These extracts were treated at 30°C for 60 minutes with 2.5 μM [α-^{32}P]NAD (5 μCi), then the excess radioactivity removed by passing the mixture over a Sephadex G-100 column, followed by concentration with an Amicon membrane, after which the samples were subjected to PAGE.

Figure 1.6

Detection of NADase activity in midbrain membranes



Membranes were treated with 100 μM [α-^{32}P]NAD (7.5 μCi). Lane 1 represents 200 μg striatal membranes, lane 2, 200 μg midbrain membranes, lane 3, 100 μg each striatal + midbrain membranes.

(chapter 3). Another possibility, that opioid receptors may couple to an effector other than adenylate cyclase was also investigated (chapter 4,5). Another aim was to determine whether GTP regulation of opioid binding was mediated by G_i or G_o (chapter 2).

Effects of opioids on pertussis toxin labeling:

As it has now been shown that the opioid receptor is coupled to G_i in the striatum, since pertussis toxin treatment alters the opioid inhibition of adenylate cyclase by radiolabeling the G_i protein, it was of interest to see if opioids had any effect on the extent of radiolabeling of G_i. In the α-adrenergic system, and the rod outer segment-transducin-cGMP phosphodiesterase system, the extent of radiolabeling of the coupling protein depends on the ligand/activation state of the receptor (47,48). Transducin, a GTP-binding protein highly homologous to G_i, can be labeled with both cholera toxin and pertussis toxin. Cholera toxin ADP-ribosylation is also found with G_s, and in both cases the presence of a ligand on the receptor increases the amount of labeling (47,48). In the case of transducin, photons are the ligands activating rhodopsin, the receptor; whereas with G_s, the stimulatory ligand is isoproterenol activating the β-adrenergic receptor. On the other hand, with pertussis toxin ADP-ribosylation of transducin, the presence of the ligand decreases the extent of radiolabeling (49). The implications of these results are that the receptor can alter the ability of the coupling protein to serve as a substrate for radiolabeling, most likely by altering the coupling protein's conformation. It is possible to use the toxin as a tool to investigate

the coupling interactions at a structural level. In the case of opioids, it would be of interest to determine whether the extent of radiolabeling changes with opioid tolerance. The most obvious problem with such experiments is that in the brain, as opposed to the rod outer segment, the coupling protein is coupled to more than one receptor so that even if the extent of radiolabeling changes in the population of G_i proteins that interact with the opioid receptor, it may be difficult to detect such a change when looking at the entire population of G_i proteins.

Initial studies with 10 μM etorphine added to the pertussis toxin ADP-ribosylation mixture revealed a 25-40% decrease in radiolabeling of striatal membranes with opioid added (Table 1.3). This finding is consistent with the observation that pertussis toxin radiolabeling of transducin decreases in the light-activated state (49). The effects of chronic opioid treatment were then examined. Rats were implanted with morphine pellets according to the following procedure: three rats were used per group, morphine or placebo; on the first day, the rats received one 75 mg morphine pellet or one placebo pellet, on day 2 the rats received one additional pellet, on day 3 two additional pellets were implanted, and on day 4 the animals were sacrificed. This procedure has been routinely used in this laboratory and demonstrated to produce tolerant animals (40,41). Membranes were prepared from several brain regions, and the extent of radiolabeling by pertussis toxin compared. The initial experiment showed that the radiolabeling was essentially equal in most brain areas, both in the labeling of the 41 kDa (G_i) and 39 kDa (G_o) proteins (Table 1.4). However, the extent of labeling of

Table 1.3

Radiolabeling of G proteins in the presence of etorphine

	fmols/mg	band	% decrease
no drug	16.2	41 kDa	
+ etorphine	9.4	41 kDa	42%
no drug	31.6	39 kDa	
+ etorphine	23.8	39 kDa	25%

Striatal membranes were treated with 20 μg/ml pertussis toxin + 10 μM [α-^{32}P]NAD in the presence or absence of 10 μM etorphine. The gels were sliced at the 41 or 39 kDa bands and counted in a liquid scintillation counter to determine the extent of ADP-ribosylation in fmols/mg protein. Each point is a single determination. Similar results were observed in three separate experiments with <10% variance.

Table 1.4

Radiolabeling of G proteins in morphine-tolerant rats

Group #1

	band	placebo (fmols/mg)	morphine tolerant
striatum	41	297	286
	39	739	788
cortex	41	235	297
	39	387	734
midbrain	41	102	94
	39	175	120
cortex cholate extracts	41 + 39	250,244	566,066

Group #2

		placebo	morphine	naloxone	morphine + naloxone
cortex	41	146	142	116	163
	39	463	446	306	390

Rats were made tolerant to morphine as described in the text, then membranes prepared from the indicated regions were treated with 20 μg/ml pertussis toxin + 50 μM [α-^{32}P]NAD. In addition, cholate extracts were compared from the cortex in order to assess whether the observed 50% change was in the protein or in an NADase activity. The experiments are from two groups of animals, as described in the text. The gels were sliced at the indicated bands and counted in a liquid scintillation counter and the extent of labeling compared. Each point is a separate determination so no statistics is shown.

the 39 kDa protein was two-fold greater in the cortex of the morphine tolerant animals (group #1, Table 1.4). This result was reproducible in these animals, both in membranes and in cholate extracts prepared from these membranes (Table 1.4). To determine if naloxone would prevent this change, rats were implanted with a combination of two pellets per rat as follows: one morphine + one naloxone pellet (75 mg each), one morphine + one placebo, one naloxone + one placebo or two placebo pellets, following the same schedule as outlined above. When membranes from these rats were compared, there was no difference between any of the treatments (group #2, Table 1.4). These results suggest that the initial difference seen was anomalous, since it could not be repeated in another set of animals.

The effects of opioids on the adenylate cyclase activity in these rats was also compared. In morphine tolerant versus placebo animals, no significant difference in the ability of opioids to inhibit cyclase was observed (Table 1.5).

Table 1.5

Adenylate cyclase activity in morphine tolerant rats

	placebo	morphine
no drug	152.8 ± 7.4	100.6 ± 9.9
etorphine	122.8 ± 10.2 (20%) p<.02	79.4 ± 4.0 (21%) p<.02

Adenylate cyclase activity was assayed in striatal membranes prepared from placebo or morphine tolerant rats, as indicated. As can be seen, the addition of 1 μM etorphine resulted in a 20% inhibition of adenylate cyclase acitvity in both cases. The results are means of triplicate assay tubes. The experiment was repeated twice with similar results.

Discussion:

The correlation between attenuation of the opioid effect and radiolabeling of the G_i provides strong evidence for the conclusion that opioids inhibit adenylate cyclase activity in the rat brain striatum via G_i. In addition, under the conditions used for labeling, nearly all of the G_i present in the membranes was ADP-ribosylated (this will be discussed further in another chapter). The earlier studies pointed to an involvement of G_i since the opioid effect was GTP-dependent and Mg^{++}-dependent (8,9).

One obvious question involves the magnitude of the inhibition, i.e., whether a 20-30% effect is sufficient to postulate a mechanism for opioid action. If the opioid inhibition of adenylate cyclase is part of a cascade mechanism, the size of the signal could be amplified via ensuing phosphorylation or dephosphorylation reactions. One of the more striking features of the cAMP system is the fact that changes in the level of cAMP are amplified via cAMP-dependent protein kinase reactions. The stimulation of the protein kinase results in the phosphorylation of many cellular proteins. Thus if the production of cAMP is decreased in the cell the normal rate of activation of cAMP-dependent protein kinase would be decreased resulting in changes in the levels of phosphorylation in the cellular proteins. Another very important amplification system involves the neuronal pathways in the brain. The opioid-sensitive striatal neuron could synapse with 1-1000's of other neurons, thus transmitting the signal to other cells. Therefore, although the magnitude of opioid inhibition of adenylate cyclase activity is not

great, its consequence in terms of neuronal function may be significant.

One of the major objections invoking an inhibition of cAMP production as "the" mechanism of opioid action may be the lack of opioid mediated adenylate cyclase inhibition in other brain areas. The present study has shown, not surprisingly, that this is not due to a lack of G_i in these regions. It is conceivable that in membrane preparations receptors and G_i and cyclase become physically separated and thus uncoupled. In fact, manipulation of the membrane (by lowering the pH) has been shown to increase opioid inhibition of adenylate cyclase (10). Accordingly, a slice preparation was used to determine if cAMP accumulation may be affected by opioids, whereas membranes are not (chapter 4), however, a similar result was found. It seems that opioid receptors are probably not coupled to adenylate cyclase in brain regions other than the striatum and cortex.

The type of opioid receptor mediating inhibition of adenylate cyclase activity is thought to be δ. Since this had been addressed in the initial studies it was not explored further. However, this raises the question of whether one receptor subtype could be coupled to more than one effector system. It is clear that δ receptors mediate inhibition of adenylate cyclase in NG108-15 cells (50), but there are δ receptors throughout the brain in regions that do not appear to be coupled to adenylate cyclase. Electrophysiological studies indicate that δ agonists mediate an activation of a Ca^{++}-activated K^+-channel in the locus ceruleus (12). It is possible to explain the two observations on the basis that an inhibition of adenylate cyclase results in changes in the Ca^{++}- activation of a K^+ channel. This

type of effect has been seen in hippocampal pyramidal neurons where increased concentrations of cAMP result in a reduction of Ca^{++}-dependent K^+ conductances (51). Alternatively, it is conceivable that the δ receptor is coupled to different effectors in various brain regions, or that there are two types of δ receptors, one that is coupled to adenylate cyclase and one that involves K^+ channels.

Chronic exposure to opioids results in tolerance. Presumably, any biochemical system through which opioids act should show some kind of tolerance or desensitization; however, the opioid inhibition of adenylate cyclase activity shows no change in morphine-tolerant animals (Table 1.5). Such a finding might suggest that opioid regulation of adenylate cyclase may not be an important mechanism of opioid action; however, it has been difficult to demonstrate biochemical changes in opioid receptors in the brain with tolerance. Only one binding study has shown a decrease in number of receptors in tolerant animals (52), whereas others have shown an increase (53,54). It is well-established that opioids act via receptors, and the fact that changes in receptor number or affinity are difficult to show suggests that there are either problems with the methods used to measure receptors or that receptor down-regulation/desensitization is not the relevant change responsible for opioid tolerance. It would seem, therefore, that post-receptor events must somehow signal alterations in opioid peptide levels so that the receptors become fully occupied and therefore unresponsive to exogenous opioids. Changes in opioid peptide levels during tolerance have been reported (65).

No reproducible changes were found in the G_i or G_o proteins following chronic opioid treatment (Table 2.3). The problem with these experiments is that the opioid receptors are greatly outnumbered by the amount of G proteins in the membrane (30 fmols/mg vs. 4 pmols/mg, a 150 fold difference), so that changes in the receptor may not be reflected in the entire population of G proteins. The acute effect of etorphine of reducing G_i and G_o labeling may reflect the opioid-induced conformational change in receptor-G protein coupling. The lack of an effect in tolerant animals could be due to the lack of conformational changes in opioid receptors during tolerance.

Chapter 2: The effects of pertussis toxin on opioid receptor binding and analgesia.

In chapter one, it was seen that opioids appear to be coupled to adenylate cyclase in the striatum and cortex only, whereas G proteins were found in all brain regions examined. Inhibition by GTP of opioid agonist binding is also found throughout the brain (10,55). Recently, a number of studies have shown the involvement of G_i as a mediator of Ca^{++} regulation and phosphoinositide turnover (7,17-20). The role of G_i had been thought to be solely to mediate cAMP inhibition. It is, therefore, possible that G proteins are involved in opioid action as mediators in other second messenger systems. In an effort to demonstrate the involvement of G_i or G_o as mediator(s) of the inhibition of opioid binding, use was made of pertussis toxin, a specific probe for examining the role of G_i and G_o proteins.

The effects on opioid binding following pertussis toxin treatment will be examined by comparing [^{3}H]-agonist binding in treated and non-treated membranes. The predicted effect of pertussis toxin would be an uncoupling of the receptor from G_i, manifest as a decrease in agonist affinity, as reported for the opioid receptor in NG108-15 cells and the dopamine (D_2) and α_2-adrenergic receptors in brain membranes (2,23,24). Initially, binding in striatal membranes will be examined. If pertussis toxin treatment results in uncoupling of the receptor with G_i in these membranes, a correlation between uncoupling, extent of radiolabeling and attenuation of inhibition of adenylate cyclase should be demonstrable.

The effect of the toxin on opioid binding will be examined in vitro in brain slices and after administration intracerebroventricularly (i.c.v.), or intravenously (i.v.). One important advantage of in vivo studies with pertussis toxin as opposed to treating membranes is that it permits one to determine whether opioid analgesia is mediated by pertussis-sensitive G proteins. The goal of this research is to address the mechanism of action of opioids. If opioid analgesia is mediated through a G protein then not only should opioid binding be affected but the physiological endpoint as well. Animals treated with pertussis toxin will be tested for their analgesic responses via a tail flick assay (31). If G_i or G_o mediate opioid analgesia, then inactivation of these proteins by pertussis toxin should result in a reduced effect of exogenous and endogenous opioids, which would be reflected as an decreased response in the tail flick test.

Results:

Intravenous injection of pertussis toxin

The first method chosen for introduction of pertussis toxin into rat brains was the intravenous route. As mentioned in the introduction, although a large molecule like pertussis toxin may not be expected to cross the blood brain barrier, there were indications that pertussis toxin had central effects (56). The advantage of the i.v. route over the slice preparation was that the animals could be tested for analgesia following pertussis toxin administration. If G_i or G_o mediate opioid action, then inactivation of these proteins by ADP-ribosylation by pertussis toxin should result in a reduced effect of exogenous (and

endogenous) opioids. One bioassay for analgesia is the tail flick test, where pertussis toxin treatment may raise the basal level as well as the ED_{50} for morphine. The strategy was first to see if i.v. administration was an effective route for introduction of pertussis toxin in the brain (by back ADP-ribosylation as described below) then to see if it has any effect on analgesia and opioid receptor binding.

The initial studies with i.v. administration involved three rats treated with pertussis toxin treated and three as saline controls. The rats were injected via a tail vein with 20 μg of pertussis toxin in 0.4 ml sterile isotonic buffer or with the equivalent volume of saline. After four days the animals were sacrificed and their brains dissected into cortical (cortex + striatum) and subcortical (the rest of the brain minus the cerebellum and brainstem) regions. Four days was chosen as the time for treatment since the toxin had been shown to have a slow onset <u>in vivo</u> , requiring three days after i.p. administration in rats and 4 - 6 days in guinea pigs (57-59). The toxin treated animals lost weight during the four days, whereas the control animals gained weight. Crude (P2) membrane fractions were prepared from each set of animals (n = 3). The membranes were then treated with activated pertussis toxin and [α^{32}P]-NAD to assess the amount of G proteins that were ADP-ribosylated by the <u>in vivo</u> treatment. The cortex regions from the pertussis toxin treated animals showed a 40% reduction in G_i and G_o labeling (Table 2.1).

Since the initial experiment indicated that the toxin had entered the brain, the experiment was repeated with a larger number of animals, this time also testing the effect of i.v. pertussis toxin treatment on

Table 2.1

Effect of i.v. pertussis toxin treatment on G protein labeling

G proteins	control (fmols/mg)	pertussis	% decrease
G_i	197.3	120.3	(40%)
G_o	343.7	205.1	(40%)

Three Sprague Dawley rats were injected with 20 μg pertussis toxin into tail veins. Four days later the animals were sacrificed, the brains pooled, and "cortical" membranes prepared. The membranes were treated with 20 μg/ml (activated) pertussis toxin + 50μM [α-^{32}P]NAD, and subjected to PAGE. The gels were sliced at the indicated bands and counted in a liquid scintillation counter and the extent of labeling compared. The data are from a single determination, so no statistics are available, but similar results were obtained in a second experiment.

opioid analgesia. Eighteen rats were injected with 20 μg (group #1) or 40 μg (group #2) pertussis toxin and an equivalent number were injected with saline. A higher dose of toxin was used to produce more striking results. The 40 μg dose of toxin resulted in the death of three rats during the four day incubation period. The remainder of the rats in both groups appeared ill with puffy faces and bloody noses. As shown in table 2.2, the pertussis toxin treated animals had lost weight when compared to the saline controls (71 g, $p < .005$ in group 2) and also when compared with their pre-treatment weights (37 g, $p < .005$ in group 2). On the fourth day the remaining pertussis treated rats were divided into three groups (of five or six) and tested for analgesia by the tail flick method (31). Basal tail flick responses were determined for all the rats, then morphine (1.25, 2.5, 5 and 10 mg/kg) was injected i.p. and the tail flicks repeated at 30,60, 90 and 150'. The ED_{50} for morphine in the pertussis toxin treated animals was not significantly different from the saline controls since the calculations of the potency ratio and the confidence limits overlapped (table 2.3). The ED_{50} for morphine in the animals treated with 40 μg of pertussis toxin (group #2) was the same as that for the animals treated with 20 μg pertussis toxin (group #1). Compared to the saline ED_{50}'s the direction of change in the toxin treated animals is opposite in the two groups of animals. This supports the calculations which indicate that the difference between toxin treated and saline controls is insignificant.

Pertussis toxin treatment seemed to have an effect on the duration of morphine action (fig. 2.1, 2.2). The pertussis toxin treated animals recovered from morphine analgesia faster at all doses tested (fig 2.1,

Table 2.2

Weight changes following i.v. pertussis toxin treatment

	control	pertussis treated	change
group #1	223.2 ± 14.8	163.4 ± 8.6	60 g ($p<.005$)
group #2 (before)	197.0 ± 18.1	185 ± 23.2	n.s.
(after)	219 ± 19.7	148 ± 8.1	71 g ($p< .005$)

Eighteen rats were injected with 20 μg or 40 μg pertussis toxin or the equivalent volume of saline into tail veins. Four days later the animals were weighed and the change in weight in the saline controls versus the pertussis toxin treated animals noted. Group #1 represents the set of animals given the 20 μg dose of toxin and group #2 the higher dose. The difference in weights was calculated by a student's t-test and shown to be significant in both groups of rats after toxin treatment (numbers in parentheses in the table). In addition, when the animals were weighed before and after treatment (group #2) the toxin treated animals showed a significant weight loss (37 g, $p<.005$).

Table 2.3

The ED50 for morphine in i.v. pertussis toxin treated animals

	saline	pertussis treated
ED_{50} #1	4.0 mg/kg	2.4 mg/kg
f_{ED50} #1	2.23	1.45
PR #1	1.67	
fPR #1	2.42	
ED_{50} #2	1.5 mg/kg	2.4 mg/kg
f_{ED50} #2	7.39	2.83
PR #2	0.625	
fPR #2	>4.0	

The ED_{50} for morphine was calculated according to the method of Litchfield and Wilcoxon (66). The ED_{50} dose is that which gives an analgesic response in 50% of the animals. The f_{ED50} is a factor which is based on the differences between the doses and the number of animals. The potency ratio is calculated as the ratio between ED_{50}'s and the fPR is the factor for the error of the ratio of f_{ED50}'s. If the fPR is greater than the PR the dose deviation is not significant. In both cases the dose deviation was not significant. Another way of expressing the data is to calculate the confidence limits of the ED_{50} as follows: the ED_{50} x the f_{ED50} gives the upper limit and the ED_{50} + the f_{ED50} gives the lower limit. For the first set of animals the saline values were 4.0 (1.79 to 8.92) mg/kg and the pertussis values were 2.4 (1.65 to 3.5) mg/kg. For group #2 the saline values were 1.5 (0.2 to 11.0) mg/kg and the pertussis values were 2.4 (0.84 to 6.8) mg/kg. As can be seen, the range is so great in both cases as to make the dose differences insignificant.

Figure 2.1

The time course of morphine analgesia in control versus pertussis toxin treated rats - #1

Eighteen rats injected with 20 μg of pertussis toxin into tail veins or the equivalent volume of saline were tested on day 4 for morphine analgesia by the tail flick method. The percent of rats analgesic was calculated by comparing the number of rats with a tail flick latency greater than the average basal latency plus 3 standard deviations over the total number of rats. Figure 2.1a represents the time course for the saline treated rats, and figure 2.1b for the pertussis toxin treated animals. The doses of morphine were as follows: ●-● = 1.25 mg/kg; □-□ =2.5 mg/kg; Δ-Δ= 5 mg/kg; o-o = 10 mg/kg.

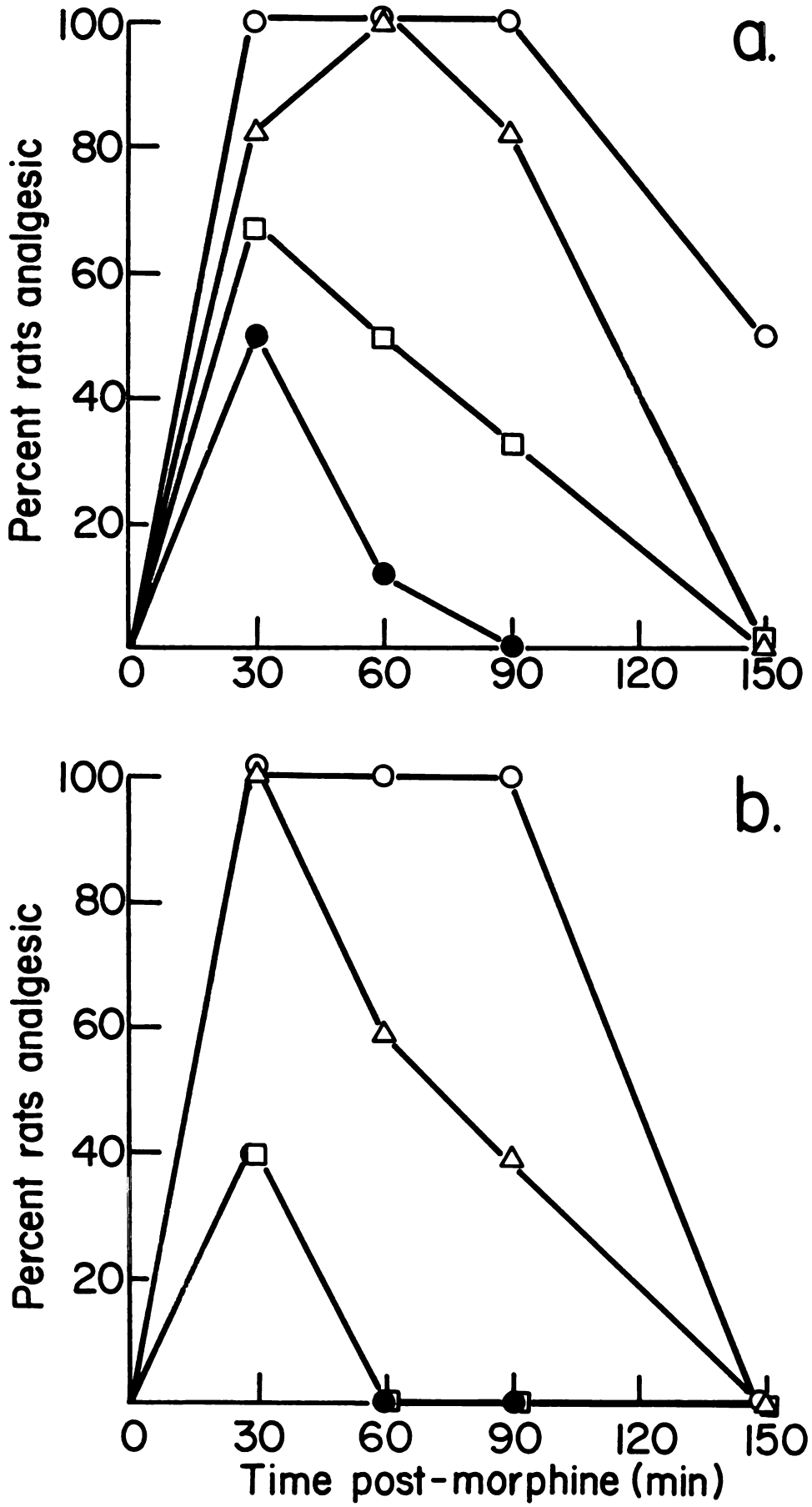
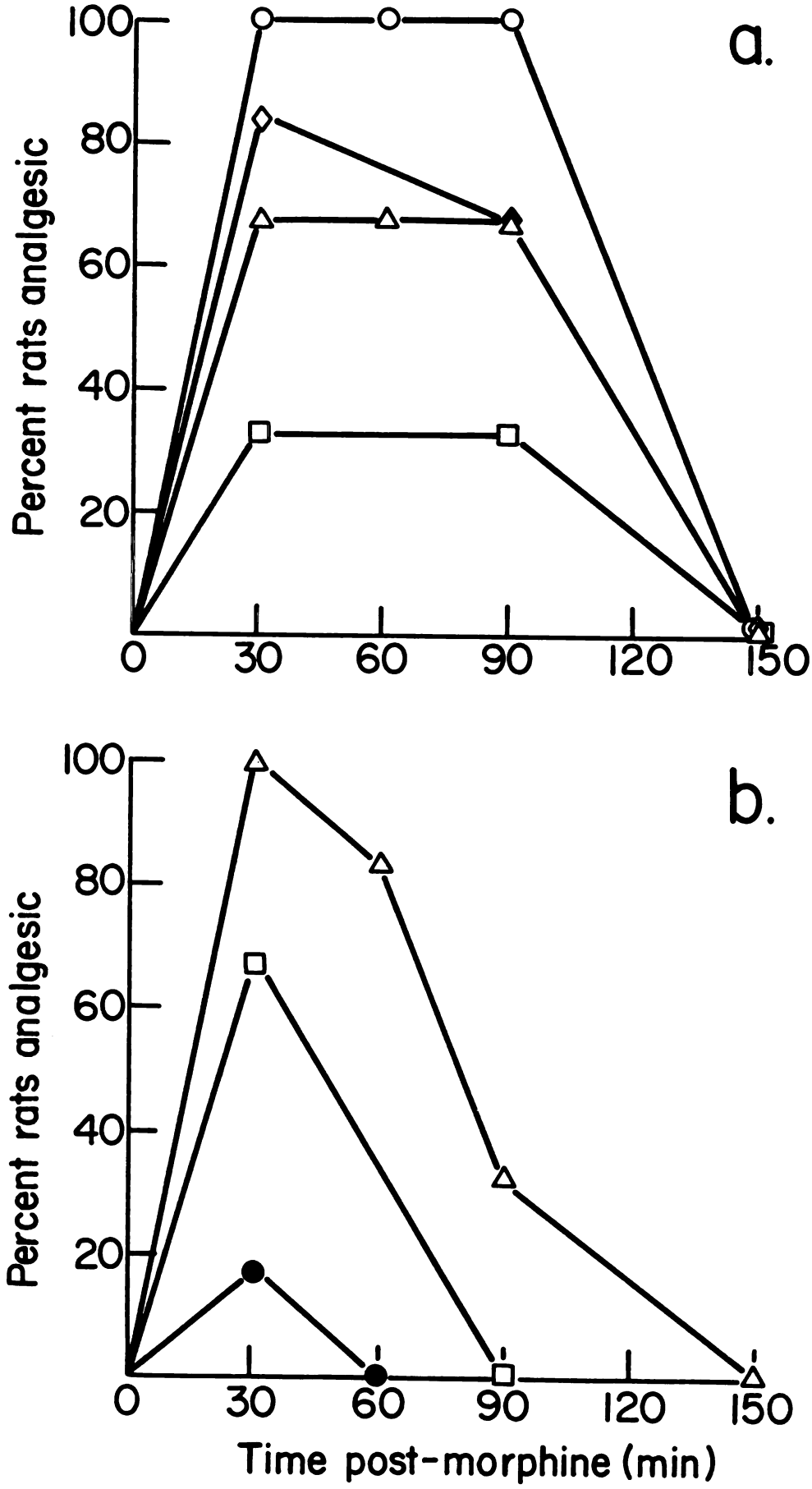

a.
b.
Percent rats analgesic
100
80
60
40
20
0
30
60
90
120
150
Time post-morphine (min)

Figure 2.2

The time course of morphine analgesia in control versus pertussis toxin treated rats - #2

Rats which had been injected with 40 μg of pertussis toxin or the equivalent amount of saline into tail veins were tested on day 4 for morphine analgesia by the tail flick method. The percent of rats analgesic was calculated as in fig 2.1. Fig 2.2a represents the time course for the saline treated rats, n=6 rats per group. Fig 2.2b shows the time course for the pertussis toxin treated rats, n=5 rats per group. The doses of morphine were as follows: ●-● = 1.25 mg/kg; ▢-▢= 2.5 mg/kg; ◇-◇ =5 mg/kg; Δ-Δ= 7.5 mg/kg; o-o = 10 mg/kg.


a.
b.
Percent rats analgesic
100
80
60
40
20
0
30
60
90
120
150
Time post-morphine (min)

2.2). This observation could be due to an effect of the toxin on morphine pharmacokinetics.

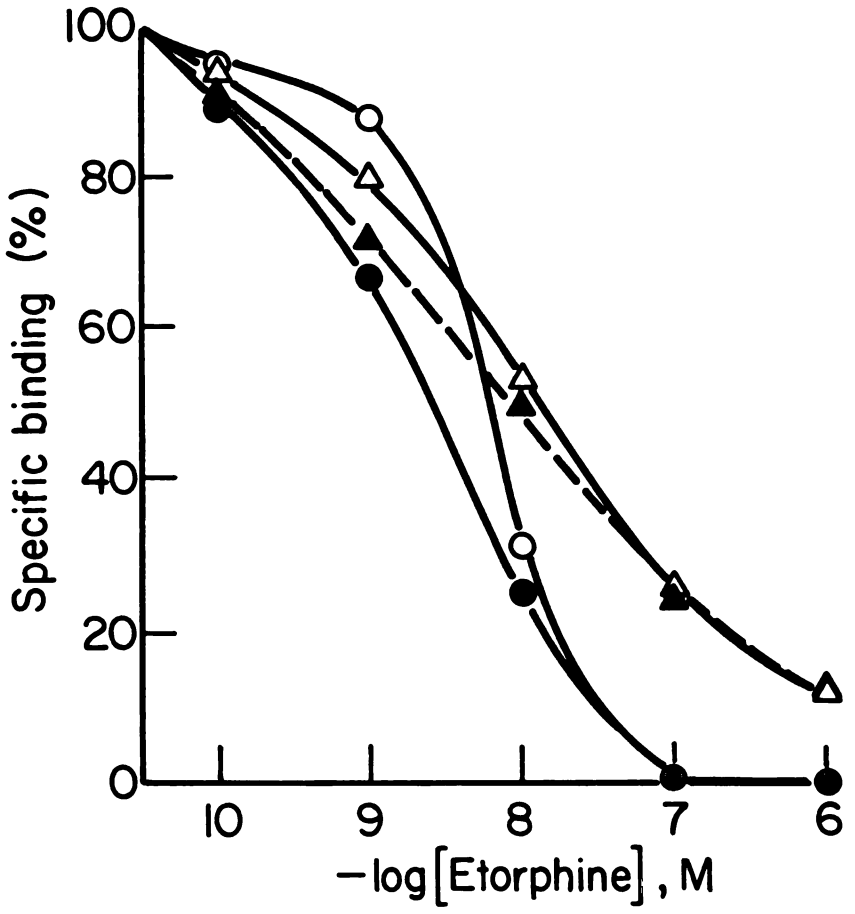
The effect of i.v. pertussis toxin treatment on opioid receptor binding was also tested. The displacement of [^{3}H]diprenorphine (an opioid antagonist) by etorphine (an agonist) shows a shift to lower affinity in the presence of 5'-guanylylimidodiphosphate (GppNHp), a non-hydrolyzable GTP analog (fig 2.3). If i.v. pertussis toxin treatment results in an uncoupling of the G proteins from the receptor, then a shift to the lower affinity should be seen in the opioid binding curves in the pertussis toxin treated animals. However, no consistent shift was seen.

The extent of ADP-ribosylation of G proteins in the pertussis toxin treated animals was reduced by 47% in G_i and 37% in G_o in group #1 (table 2.4). Although this finding suggests that the pertussis toxin had entered the brain, in group #2 there was no significant entry of pertussis toxin into the brain (table 2.4). The ADP-ribosylation of the G proteins was not decreased following pertussis toxin treatment (table 2.4); however, the ADP-ribosylation of G_i in the liver and kidneys was completely abolished in these animals (fig 2.4). It appears that the toxin had entered the rats' bloodstream, acted on the liver and kidneys, but not crossed the blood brain barrier.

It is somewhat surprising that the lower dose <u>in</u> <u>vivo</u> experiments had shown some entry of pertussis toxin into the brain and that the higher dose experiment did not; but the demonstration of a complete effect of i.v. pertussis toxin in the peripheral organs in the last

Figure 2.3

Displacement curves in the presence of Mg and GppNHp



Cortical rat brain membranes were incubated with 1 nM [^{3}H]diprenorphine in the absence or presence of 10 mM Mg^{++} and/or 10 μM GppNHp as indicated by: ▲-▲ = control; ●-● = + Mg^{++}; Δ-Δ = + GppNHp; o-o = + Mg^{++} + GppNHp. The IC_{50} values for the curves were as follows: control, 8.91 nM; Mg^{++}, 2 nM; GppNHp, 14 nM; Mg^{++} + GppNHp, 7.94 nM.

Table 2.4

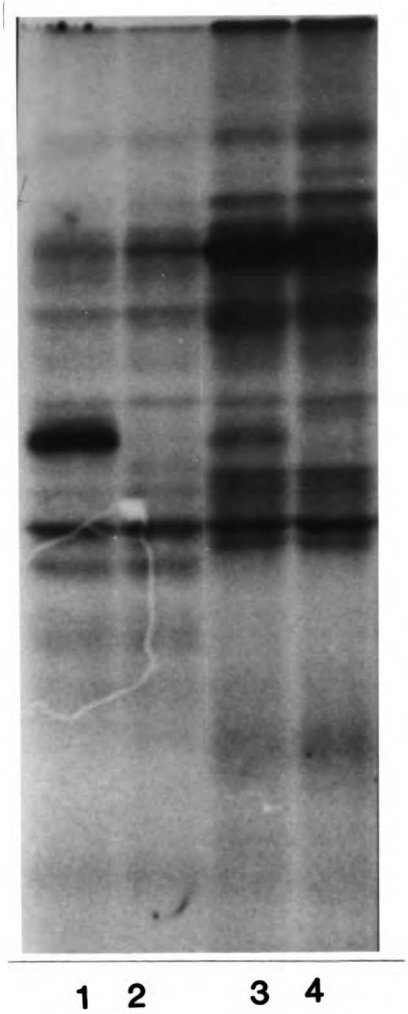
Intravenous pertussis toxin does not necessarily enter the brain

	saline	pertussis	decrease
group #1		(20 μg)	
G_i	32.1 ± 1.0	17.1 ± 1.0 fmols/mg	(47%)
G_o	48.2 ± 2.2	31.5 ± 4.0	(35%)
group #2		(40 μg)	
G_i	27.7 ± 0.9	26.5 ± 7.0 fmols/mg	
G_o	31.1 ± 9.8	37.2 ± 6.6	

"Cortical" (cortex + striatum) brain membranes were prepared from rats which had been injected with 20 (group #1) or 40 μg (group #2) pertussis toxin or the equivalent volume of saline as indicated. The membranes were treated with 20 μg/ml pertussis toxin (activated) + 50 μM [α-^{32}P]NAD and subjected to SDS-PAGE. The gels were sliced at the 41 kDa (G_i) and 39 kDa (G_o) bands and counted in a liquid scintillation counter. The results shown are the average of the 3 groups of pertussis toxin treated rats and the 3 groups of control rats.

Figure 2.4

The effect of i.v. pertussis toxin treatment on ADP-ribosylation of kidneys and liver



Fifteen rats injected with 20 μg pertussis toxin and eighteen rats injected with the equivalent volume of saline into tail veins were sacrificed on day 4 and their brain, kidneys and livers dissected. Membranes were prepared and treated with 20 μg/ml (activated) pertussis toxin + 100 μM [α-^{32}P]NAD and subjected to SDS-PAGE. An autoradiogram of the gel is shown. Lane 1 represents kidney from control rats. Lane 2 represents kidney from pertussis toxin treated animals. Lane 3 shows liver from control rats. Lane 4 shows liver from pertussis toxin treated rats. The amount of protein in each of these lanes is equivalent.

experiment suggests that the amount of toxin that had entered the brain previously was probably not sufficient.

Intracerebroventricular injection of pertussis toxin

Since i.v. administration did not conclusively demonstrate the entry of pertussis toxin into the brain, a more direct approach was tried. A cannula was surgically implanted into the fourth ventricle of the rat brain to allow intracerebroventricular (i.c.v.) injection of pertussis toxin. This method allows the administration of material directly into the cerebrospinal fluid. The toxin still must cross the epidydema to get to the glia and neurons, but a substantial barrier has been removed. The cannulated rats were injected with pertussis toxin 5 days after cannula implantation to allow the animal to recover from the operation. The first doses of pertussis toxin used were 0.2, 2.0 and 20 ng in 2 μl saline i.c.v. then the cannulaes were flushed with 10 μl saline. The animals were observed for four days and sacrificed on the fourth day. Their brains were removed and membranes prepared (minus the cerebellum). To assess the amount of pertussis toxin that had entered the brain in vivo , a back ADP-ribosylation experiment was used. There were no significant changes in the rats at any dose (table 2.5). The amount of radiolabeling in these experiments was reduced overall because the whole brain was used and there is NADase activity in the subcortex (chapter 2).

Since higher doses of pertussis toxin were expected to result in more striking differences, four rats were injected i.c.v. with pertussis toxin or saline as follows: rat #1 received 10 μl saline; rat #2,#3 - 1

Table 2.5

Effect of i.c.v. pertussis toxin on G protein labeling

i.c.v. dose	G_i (fmols/mg)	G_o
0	25.1	92.0
0.2 ng	36.2	82.0
2 ng	41.0	83.6
20 ng	38.5	87.1

Rats were injected with the indicated doses of pertussis toxin through cannulae implanted into the fourth ventricle. Four days later the animals were sacrificed and brain membranes (whole brain minus cerebellum) were prepared. The membranes were treated with 20 μg/ml (activated) pertussis toxin + 100 μM [α-^{32}P]NAD and subjected to SDS-PAGE. The 41 kDa (G_i) and 39 kDa (G_o) bands were cut out and counted in a liquid scintillation counter. The data are from a single experiment. Similar results were obtained in a second experiment.

μg toxin in 5 μl; rat #4 - 2 μg toxin in 10 μl. The cannulae were flushed with 10 μl saline following the toxin injection. Two days later the treated animals had lost weight, while the control had gained weight. The animals were injected with more toxin at this time in the same amount they had previously received. Rat #4 died one night later. The remainder of the rats were sacrificed on the second day following the second injection, and their brains and kidneys removed. When the extent of ADP-ribosylation of G proteins in control versus toxin treated cortical membranes were compared, no significant changes were found (table 2.6). However, a significant decrease in G_i labeling was seen in the kidneys from the pertussis toxin treated animals (table 2.6). Since the toxin was applied directly to the brain (i.c.v.), the effect of toxin on the kidneys indicates that the toxin had exited the cerebrospinal fluid and entered the periphery. However, the toxin had still not entered neuronal tissue evidenced by the lack of effect on the G proteins in the brain. An experiment to see if there was any change in the extent of [^{3}H]DADLE binding in the toxin treated animals again showed no changes (table 2.7). Since it appears that the toxin had not reached neuronal sites, a negative result in the binding experiment would be inconclusive.

The highest dose of toxin injected i.c.v., 4 μg, resulted in death of the animal, so a slightly lower dose, 3 μg was used. Two rats were injected with saline and two rats with 3 μg of pertussis toxin in 15 μl. The cannulae were flushed with 10 μl saline following the toxin injection. Four days later the toxin treated animals had lost weight while the controls had gained weight. The animals were sacrificed and

Table 2.6

The effect of i.c.v. pertussis toxin on brain and kidney G proteins

dose	G_i (cortex)	G_o	G_i (kidneys)
control	294	311	1014
2 μg	237	317	
2 μg	339	256	435 (57%)
control #2	145 ± 15	356.5 ± 58.5	407 ± 50
3 μg	113.5 ± 18.5 (n.s.)	348 ± 57 (n.s.)	240.5 ± 123 (41%) p<.001

Rats were injected i.c.v. with the indicated doses of pertussis toxin. Four days after treatment the animals were sacrificed and membranes prepared from the cortex (+ striatum) and kidneys. The data from the second experiment (#2) are pooled from two separate rats. The membranes were treated with 20 μg/ml (activated) pertussis toxin + 100 μM [α-^{32}P]NAD and subjected to SDS-PAGE. The number of fmols of ADP-ribose per mg of membrane protein in the G_i and G_o bands are indicated. The numbers in parentheses represent the percent decrease in G_i in the kidneys from the pertussis treated animals.

Table 2.7

The effect of i.c.v. pertussis toxin on agonist binding

dose	[^{3}H]DADLE bound (fmols/mg)
control	41.5 ± 14
3 μg	48.5 ± 10 (n.s.)

Rats were injected with the indicated doses of pertussis toxin. Four days after treatment the animals were sacrificed and membranes prepared from the cortex (+ striatum). The membranes were incubated with 2 nM [^{3}H]DADLE at 25 °C for one hour. Non-specific binding was determined with 1 μM DADLE. The data represent the average plus the standard deviations for two separate animals.

their brains and kidneys dissected. Membranes from the cortical regions of the brain (cortex + striata) showed no significant changes in the pertussis toxin treated animals when compared to the controls, but the kidneys from the pertussis treated animals showed a 41% decrease in the G_i protein (table 2.6). Pertussis toxin does not seem to get to the G proteins in the neurons via i.c.v. injection.

Pertussis toxin treatment of slices

The final approach used to ascertain whether pertussis toxin could enter the neurons was a slice technique. Rats were sacrificed immediately before the experiment and their brains rapidly removed and placed in oxygenated Ringer's solution. The regions to be studied, the cortex and striata, were dissected and sliced with a McIlwain tissue slicer. The slices were transferred to incubation medium and kept in a 10% CO_2 (in air) incubator at 37°C. Pertussis toxin was added to the medium at various doses for four or more hours. One problem with this approach is that slices do not remain viable for long periods of time at 37 °C, but pertussis toxin takes hours to act in cells (2). The viability of the opioid receptor or adenylate cyclase systems in the slices was assessed by preparing membranes after the incubation and assaying receptor binding or adenylate cyclase activity, which may also be affected by pertussis toxin treatment. The extent of toxin entry into the slices was assessed by a back ADP-ribosylation experiment.

The initial concentration of pertussis toxin chosen was 100 ng/ml for four hours, the conditions used to treat neuroblastoma-glioma cells (2). The result was an inconsistent incorporation of pertussis toxin

into the slices as assayed by a back ADP-ribosylation experiment. Longer incubation periods were examined (table 2.8). After 8 hours, the toxin had ADP-ribosylated about 50% of the G proteins, but adenylate cyclase activity had dropped to unmeasurable levels. Opioid receptor binding was found to remain constant until 12 hours when it dropped to 40% of control levels (table 2.8). At 16 hours opioid receptor binding, the ability of G proteins to be ADP-ribosylated (after membrane preparation) and adenylate cyclase activity all disappeared (table 2.8). It appears that the toxin is not able to cross the cellular membranes sufficiently in the slices under the incubations attempted.

All the attempts to introduce whole pertussis toxin into the neurons in vivo have failed. The fact that the i.c.v. method failed could be explained by an inability of the toxin to cross epidydymal membranes and/or neuronal membranes. The failure of the i.v. experiments is probably due to poor penetration of the blood brain barrier. The slice preparation is as close to a cellular preparation that can be reasonably attempted for living brain and the toxin should have been able to enter the neurons in this preparation provided it bound to the membrane. It seems that pertussis toxin does not cross neuronal membranes.

Pertussis toxin treatment of membranes

Although the effects of pertussis toxin on opioid receptor binding were negligible in the in vivo experiments (table 2.7,2.8), the results were not conclusive since the toxin showed partial entry. It was decided, therefore, to try a binding assay under conditions where

Table 2.8

The effect of pertussis toxin on striatal slices

time	G_i	G_o	adenylate cyclase	[^{3}H]DADLE binding
4 hr	n.c.	n.c.	-	-
8 hr	53%	32%	n.d.	100%
12 hr	n.c.	n.c.	n.d.	40%
16 hr	n.d.	n.d.	n.d.	n.d.

Striatal slices were prepared as described as described in the text. The slices were incubated with 100 ng/ml pertussis toxin for the indicated lengths of time, after which membranes were prepared. Aliquots of the membranes were treated with 20 μg/ml (activated) pertussis toxin + 100 μM [α-^{32}P]NAD and subjected to SDS-PAGE and the number of fmols of ADP-ribose in the G proteins were determined. Other aliqouts of the membranes were assayed for adenylate cyclase activity or specific binding with 2 nM [^{3}H]DADLE in the presence of 1 μM DADLE.

complete ADP-ribosylation of G proteins had occurred; conditions that would optimize any effect of the toxin on opioid binding. The conditions of labeling that had abolished adenylate cyclase activity should be sufficient to ADP-ribosylate a significant amount of G proteins (c.f. chapter 2). Initial experiments to test this showed that the cyclase conditions of 8 μg (activated) toxin/mg membrane protein was not sufficient (table 2.9). To ADP-ribosylate 60-70% of the G proteins in the membrane required 40 μg toxin/mg membrane protein (table 2.9). These were the conditions chosen for the in vitro pertussis toxin treatment of the membrane.

The effect of pertussis toxin treatment on opioid receptor binding was assessed by the determining changes in the number of agonist binding sites. If pertussis toxin treatment results in a change in the affinity of the receptor for agonist this should be reflected as a loss in the number of agonist binding sites at low agonist concentrations. The effect of pertussis toxin treatment on 2 nM [^{3}H]DADLE binding was examined. Pretreatment of striatal membranes with 40 μg pertussis toxin per mg membrane protein resulted in the loss of 18 - 55% of the [^{3}H]DADLE binding sites (table 2.10), a finding which correlated with the extent of ADP-ribosylation in 4 separate experiments (fig 2.5). The variability of the response was attributable to a loss of pertussis toxin activity when the toxin was frozen (fig 2.6). It appears that under appropriate conditions pertussis toxin does affect opioid binding.

The loss of binding sites at 2 nM [^{3}H]DADLE could reflect either a true loss of sites or a decrease in agonist affinity following pertussis toxin treatment. In order to distinguish between these two

Table 2.9

Pre-treatment of membranes with pertussis toxin

toxin	G_i	G_o
0	22.0	26.3
4 μg/mg	8.9	18.1
8 μg/mg	9.7	29.2
16 μg/mg	9.4	29.8
0	913.8	2596.0
20 μg	424 (54%)	794 (31%)
40 μg	341 (64%)	593 (77%)

Striatal membranes were pre-treated with the indicated amounts of (activated) pertussis toxin for one hour at 30 °C with the standard ADP-ribosylation mixture + 2 mM NAD. The reaction was terminated by centrifugation and the membranes were resuspended in ADP-ribosylation mixture + 20 μg/ml pertussis toxin + 100 μM [α-^{32}P]NAD and subjected to SDS-PAGE. The number of fmols of ADP-ribose per mg of membrane protein are shown. The percent decrease in incorporation is indicated in the parentheses. The data are from single determinations but were reproducible in a second experiment.

Table 2.10

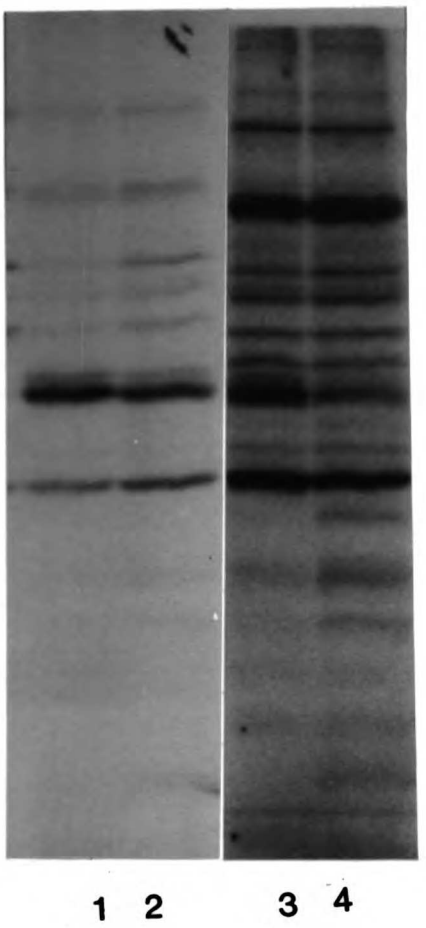
Pre-treatment of striatal membranes with pertussis toxin results in a decrease in opioid agonist binding sites

exp't	control	pertussis toxin	% decrease	
#1	58.5	48.2 fmols/mg	18%	p< .04
#2	92.1	69.6 fmols/mg	24%	p< .03
#3	34.8	20.3 fmols/mg	42%	p< .07
#4	69.7	31.4 fmols/mg	55%	p< .02

Striatal membranes were treated with 40 μg (activated) pertussis toxin per mg membrane protein in the standard ADP-ribosylation mixture containing 2 mM NAD at 30°C for one hour. Control membranes were treated with the mixture without pertussis toxin. After the treatment, the membranes were centrifuged at 22,500 x g for 10 minutes and resuspended in 25 mM Hepes, pH 7.4. The membranes were then assayed for opioid binding with 2 nM [^{3}H]DADLE in 25 mM Hepes, pH 7.4 at 25 °C for one hour. The samples were rapidly filtered and the filters counted in a liquid scintillation counter. Specific binding was determined by the addition of 1 μM DADLE. The specific binding in fmols/mg membrane protein from four separate experiments are shown. Aliquots of each pre-treated membrane were saved for back ADP-ribosylation and protein determination. The data are means of triplicate assay tubes.

Figure 2.5

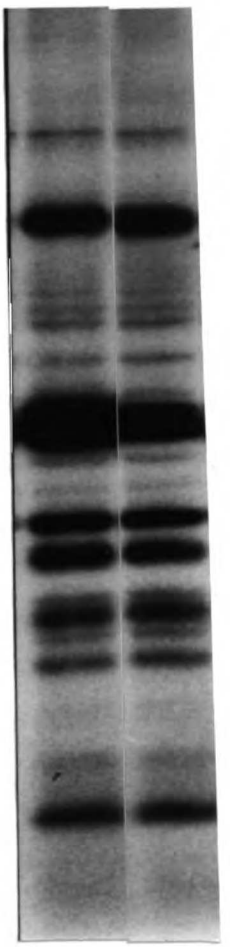
Reduction of G protein labeling following in vitro pre-treatment



Striatal membranes were pre-treated with 40 μg (activated) pertussis toxin per mg membrane protein for one hour at 30 °C with the standard ADP-ribosylation mixture + 2 mM NAD. The reaction was terminated by centrifugation and the membranes were resuspended in ADP-ribosylation mixture + 20 μg/ml pertussis toxin + 100 μM [α-^{32}P]NAD and subjected to SDS-PAGE. The autoradiogram from two separate assays is shown. Lanes 1 and 2 represent - and + pre-treatment from experiment #1 (see Table 2.10). Lanes 3 and 4 are from #3.

Figure 2.6

The effect of freezing on pertussis toxin activity



Striatal membranes were incubated with 20 μg/ml pertussis toxin, either freshly activated (lane 1) or frozen then thawed (lane 2) + 100 μM [α-^{32}P]NAD. The number of fmols of ADP-ribose per mg membranes protein in lane 1 are 3-6 times greater than the frozen (lane 2). Lane 1, G_i = 577 fmols/mg, G_o = 2152; lane 2, G_i = 91, G_o = 640 fmols/mg.

possibilities, the binding at the saturation concentration of [^{3}H]DADLE was examined. If pertussis toxin treatment results in the loss of binding sites then the number of sites should still be reduced at saturating concentrations of agonist. On the other hand, if pertussis toxin treatment results in a change in receptor affinity then the number of sites should be the same at saturating concentrations of agonist exceeding the shift that was induced. The maximum number of binding sites (B_{max}) for [^{3}H]DADLE was reached at 5 nM [^{3}H]DADLE and the K_D was at 2 nM (fig. 2.7). A concentration at which it should be possible to distinguish the two possibilities is 10 nM [^{3}H]DADLE. At this concentration, the number of binding sites was not reduced following pertussis toxin treatment (table 2.11). Pertussis toxin pre-treatment reduced 80-90% of the G proteins in this experiment. It appears, therefore, that pertussis toxin treatment results in a decreased affinity of the opioid receptor for agonist. The most likely explanation for such a change is an uncoupling of the G_i or G_o proteins following ADP-ribosylation by pertussis toxin.

Since the cerebral cortex is an area of the brain with less evidence for adenylate cyclase serving as a second messenger system for opioid action, it was of interest to determine whether pertussis toxin decreased agonist binding as observed in the striatum. As shown in figure 2.8, pertussis toxin treatment of cortical membranes resulted in a 34-71% loss of [^{3}H]DADLE binding at three different concentrations of DADLE. The highest concentration tested, 20 nM, was the B_{max} for DADLE in the cortex (figure 2.9), and the shape of the curve suggests that pertussis toxin may alter the number of sites as opposed

Figure 2.7

Saturation curve for [^{3}H]DADLE binding

Striatal membranes were assayed for opioid binding with [^{3}H]DADLE concentrations ranging from 10 pM to 20 nM in 25 mM Hepes, pH 7.4, at 25 °C for one hour. Non-specific binding was determined as that remaining in the presence of 1 μM DADLE. Specific binding was calculated by subtracting the non-specific binding from the total counts.

Specific binding [3H]- DADL (fmol /mg)
80
70
60
50
40
30
20
10
11
10
9
8
7
-log[3H]-DADL ,M

Table 2.11

Specific binding of [^{3}H]DADLE after pre-treatment of striatal membranes with pertussis toxin

control	pertussis toxin	%
108.3 ± 13.1	121.3 ± 16.0	12% p< .07

Striatal membranes were pre-treated ± 40 μg pertussis toxin/mg membrane protein in the standard ADP-ribosylation mixture containing 2 mM NAD at 30 °C for one hour. After the treatment the membranes were centrifuged at 22,500 x g for 10 minutes and resuspended in 25 mM Hepes pH 7.4. The membranes were then assayed for opioid binding with 10 nM [^{3}H]DADLE in 25 mM Hepes, pH 7.4 at 25 °C for one hour. Specific binding was determined using 1 μM DADLE. The specific binding in fmols/mg membrane protein from triplicate assay tubes is shown. The membranes from the pertussis toxin pre-treatment showed a 12% increase in this experiment. Aliquots of each membrane were saved for back-ADP-ribosylation.

Figure 2.8

Pertussis toxin treatment results in a decrease in opioid agonist binding in cortical membranes

Cortical membranes were treated with 40 μg (activated) pertussis toxin per mg membrane protein in the standard ADP-ribosylation mixture containing 2 mM NAD at 30 °C for one hour. Control membranes were treated with the mixture without pertussis toxin. After the treatment, the membranes were centrifuged at 22,500 x g for 10 min and resuspended in 25 mM Hepes, pH 7.4. The membranes were then assayed for opioid binding at the indicated concentrations of [^{3}H]DADLE in 25 mM Hepes, pH 7.4 at 25°C for one hour. The samples were rapidly filtered and the filters counted in a liquid scintillation counter. Specific binding was determined by the addition of 1 μM DADLE. The specific binding in fmols/mg protein is plotted against the DADLE concentration in nM. The control curve is indicated by o-o, and the pertussis treated by ●-●. An aliquot of each membrane was assayed for ADP-ribosylation, and a 40% decrease in G_o and a 52% decrease in G_i was observed in the pertussis toxin pre-treated membranes. The data are means of triplicate assay tubes. The data were significant at all points with a $p < .007$.

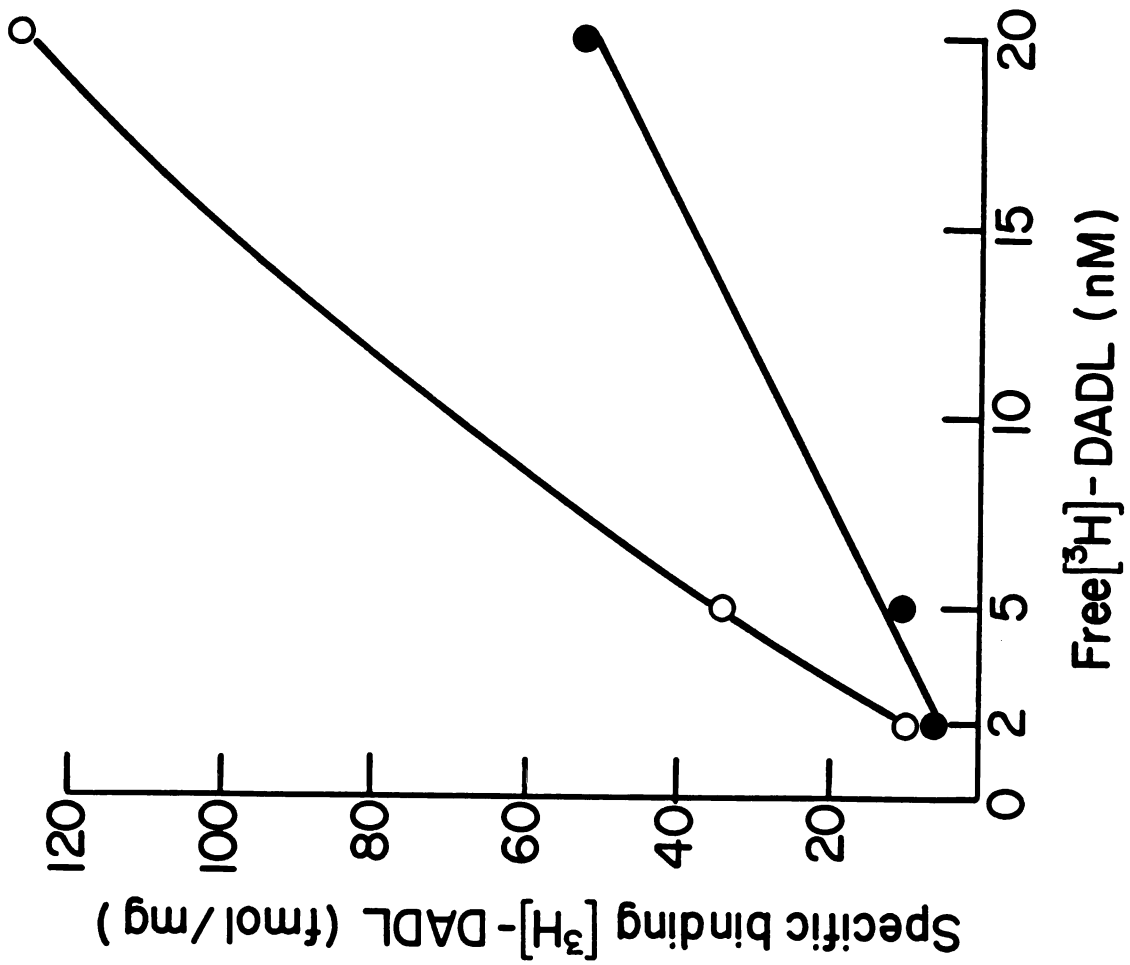
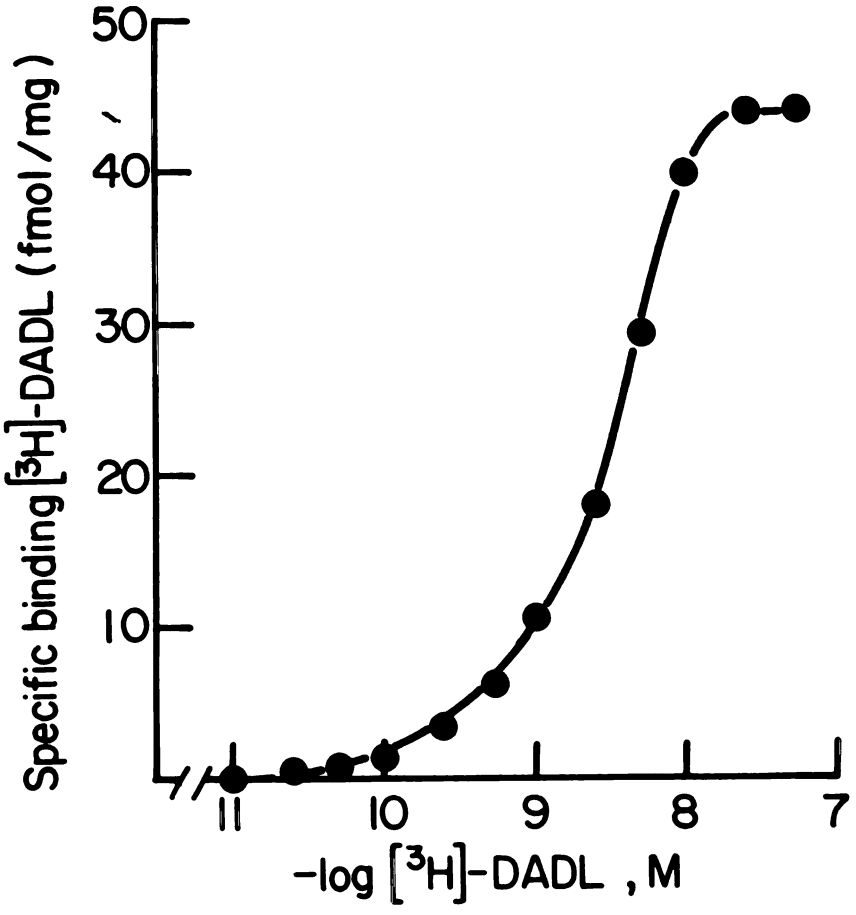

Specific binding [3H]- DADL (fmol/mg)
120
100
80
60
40
20
0
2
5
10
15
20
Free[3H]- DADL (nM)

Figure 2.9

Saturation curve for DADLE binding in the cortex



Cortical membranes were assayed for opioid binding with [^{3}H]DADLE concentrations ranging from 10 pM to 20 nM in 25 mM Hepes, pH 7.4, at 25 °C for one hour. Non-specific binding was determined as that remaining in the presence of 1 μM DADLE. Specific binding was calculated by subtracting the non-specific binding from the total counts.

to the affinity of the receptor for agonist in the cortex.

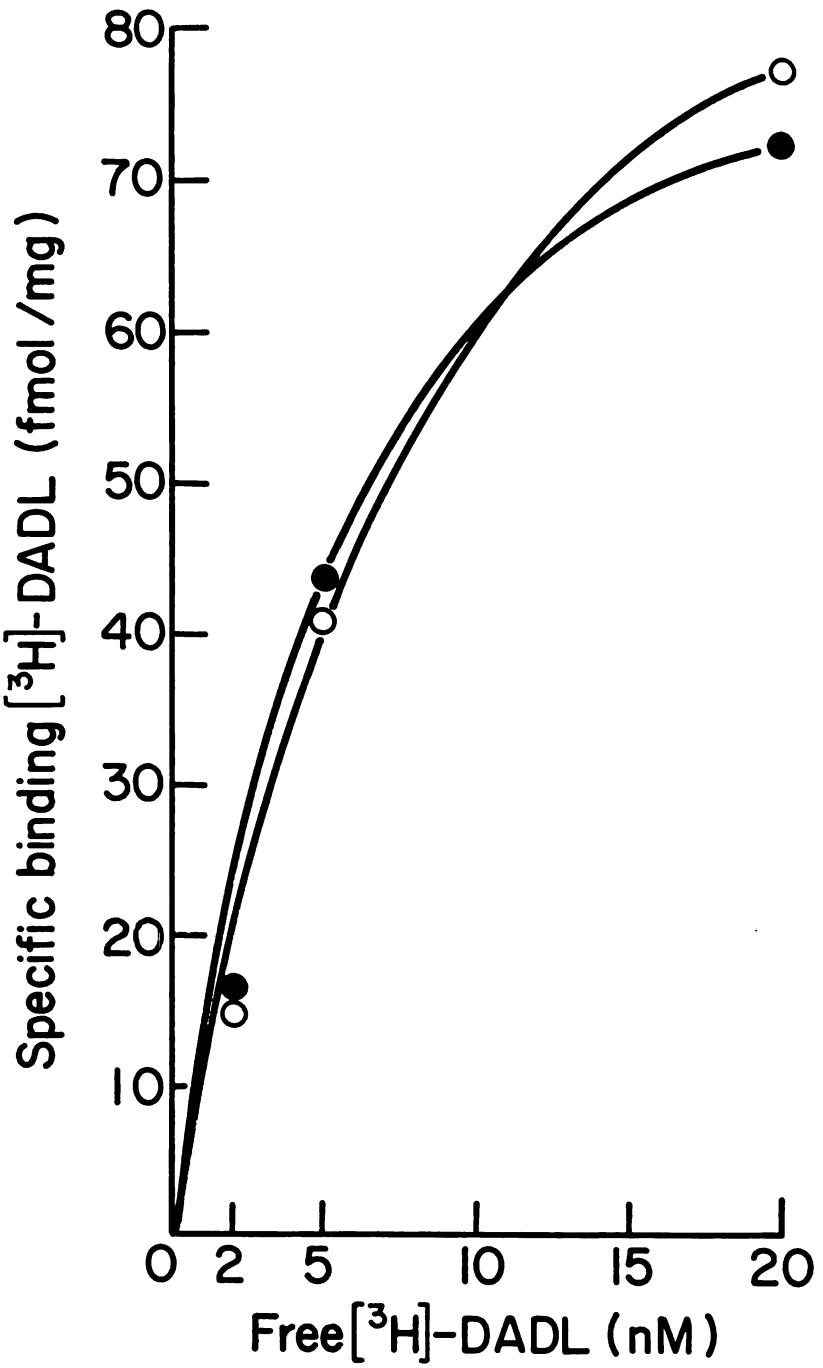
The loss of agonist binding sites was associated with significant ADP-ribosylation of cortical membranes (figure 2.8 legend). In the experiment shown in figure 2.8, 40% of the G_o and 52% of the G_i was labelled during the pretreatment for the binding assay. It can, therefore, be concluded that the pertussis toxin had labeled the G proteins in the cortex and that this was responsible for the appreciable loss of agonist binding.

In the cerebral cortex and the striatum, opioid inhibition of adenylate cyclase and opioid agonist binding is altered by pertussis toxin, implicating G proteins as mediators of opioid action in these areas. In order to address whether regulation of opioid binding by G proteins is limited to brain areas where opioids are coupled to adenylate cyclase, the effect of pertussis toxin on agonist binding in the midbrain was studied. In the midbrain, opioids do not inhibit adenylate cyclase. As shown in figure 2.10, pertussis toxin treatment of membranes prepared from the midbrain region did not alter the number of [^{3}H]DADLE binding sites, although 86% of the G_i and 72% of the G_o in the membranes had been ADP-ribosylated.

Figure 2.10

Effects of pertussis toxin on agonist binding in midbrain membranes

Midbrain membranes were treated with 40 μg pertussis toxin per mg membrane protein in the standard ADP-ribosylation mixture containing 2 mM NAD at 30°C for one hour. Control membranes were treated with the mixture without pertussis toxin. After the treatment, the membranes were treated exactly as described in the legend to figure 2.8. The specific binding in fmols/mg protein is plotted against the DADLE concentration in nM. The control curve is indicated by o-o and the treated by ●-●. The data are means of triplicate assay tubes from two separate experiments. The difference between the points was not significant in a student's t-test. An aliqout of each membrane was assayed for ADP-ribosylation, and a 72% decrease in G_o and a 86% decrease in G_i was observed in the pertussis toxin pre-treated membranes.


80
70
60
50
40
30
20
10
Specific binding [3H]-DADL (fmol/mg)
0
2
5
10
15
20
Free [3H]-DADL (nM)

Discussion:

The studies described in this chapter were designed to determine if pertussis toxin treatment alters two important opioid effects, opioid receptor binding and opioid mediated analgesia. Although it was not possible to demonstrate an effect on analgesia, because pertussis toxin does not seem to cross neuronal membranes; pertussis toxin does alter opioid receptor binding when added directly to membranes. The results of the in vitro binding studies in the striatum suggest that pertussis toxin treatment resulted in a change in the affinity of the opioid receptor for agonists, as opposed to a decrease in the number of binding sites. This was reflected as a decrease in [^{3}H]DADLE binding at low concentrations, e.g. 2 nM, but not at saturation levels, e.g. 10 nM. This result is similar to that seen with opioid, α-adrenergic, and cholinergic receptors in neuroblastoma-glioma NG108-15 cell hybrids, and with α-adrenergic receptors and dopamine receptors in the brain (2,23,24,34). Pertussis toxin seems to result in an uncoupling of the receptor from the G_i or G_o protein, leading to a decreased affinity of the receptor for agonists. This is the first evidence that a G protein mediates the GTP effect on opioid receptor binding in brain.

The studies with pertussis toxin treatment in vivo provide useful data on the entry (rather, the lack of entry) of the toxin into neuronal tissues. This observation has relevance for studies which draw conclusions on the lack of an effect of pertussis toxin without showing that the toxin has entered the tissue. If the back-ADP-ribosylation experiments had not been done on the in vivo studies, then it could have

been concluded that pertussis toxin treatment had no effect on opioid receptor binding or analgesia. Since the experiments showed that pertussis toxin does not enter the neuronal tissue via intravenous or intracerbroventricular injection, or even by incubation with slices, the negative results are not conclusive. Demonstration that pertussis toxin had entered the kidneys (and livers) of the animals injected in vivo strengthens the conclusion that pertussis toxin had not entered the brain despite its presence in the bloodstream following both the i.v. and i.c.v. administration. The presence of pertussis toxin in the livers of the treated animals may have increased the metabolism of morphine in these animals, thereby resulting in their faster recovery from morphine analgesia. The demonstration that the affinity of opioid receptors is altered by pertussis toxin treatment in the striatum supports the notion of a striatal opioid receptor that is linked to adenylate cyclase as shown in chapter two. The present study has demonstrated the existence of a complete second messenger system in the striatum, namely a complex consisting of receptor + G_i + adenylate cyclase which is altered by treatment with pertussis toxin. The question of whether pertussis toxin alters opioid binding in other brain areas was addressed by examining the cortex, a region that showed only a 10% inhibition of adenylate cyclase activity by opioids (chapter 1). A striking decrease in agonist binding following pertussis toxin treatment was observed in the cortex.

In order to determine if opioid receptors in the brain may be coupled to G proteins without the involvement of adenylate cyclase, the effect of pertussis toxin on opioid binding in the midbrain was tested.

Since no decrease in the number of receptors occurred in this region despite the ADP-ribosylation of 80% of the G proteins, it can be concluded that pertussis-sensitive G proteins do not regulate opioid receptors in the midbrain. The number of G proteins is in excess of the number of opioid receptors in the brain, so it could be argued that the remaining G proteins are those necessary for opioid binding. However, the striking effect of pertussis toxin in the striatum and cortex indicates that a sufficient number of G proteins had been altered. The possibility remains that G proteins which are not affected by pertussis toxin mediate opioid action in areas other than the striatum and cortex.

Chapter three: The effect of opioids on cAMP accumulation in rat brain slices

Opioids have been shown to inhibit adenylate cyclase activity in the rat brain striatum via a guanine nucleotide binding protein, G_i, (chapter 2). In other brain areas (except the cortex), opioids do not inhibit adenylate cyclase activity in membranes, despite the presence of G_i (chapter two). It is possible that during membrane preparation, opioid receptors become separated from adenylate cyclase. An example of this may be the neuroblastoma-glioma NG108-15 hybrid cells where opioids cause 70% inhibition of cAMP accumulation in whole cells, but only 40% in membrane preparations (1). A similar phenomenon has been reported with N18TG2 neuroblastoma cells (1). The question posed in this chapter is whether opioid receptors are coupled to adenylate cyclase in tissue slices.

If opioid receptors are coupled to adenylate cyclase in the brain but are separated by cellular disruption, tissue slices of brain should show opioid inhibition of cAMP accumulation. Areas like the striatum and the cortex, where a small inhibition is observed in membranes, would be expected to show an enhanced effect in brain slices. In other areas an effect of opioids on cAMP accumulation may be demonstrable in slices. On the other hand, a failure of opioids to inhibit cAMP accumulation in slices would support the conclusion that opioids are not coupled to the adenylate cyclase system in brain regions other than the striatum and cortex.

The opioid receptor has been shown to be affected by its lipid environment (60). In particular, opioid inhibition of adenylate cyclase

activity can be augmented by pretreatment of rat brain membranes at low pH (10). The addition of cerebroside sulfate or phosphatidylcholine to N18TG2 cells potentiates opioid inhibition of cAMP accumulation (43). If cerebroside sulfate potentiates the opioid effect in brain slices, then it would serve as additional information about opioid action in the brain.

Results:

Striatal cAMP accumulation

The striatum was chosen as the first brain region to be studied for the effects of opioids on cAMP accumulation in slices since this region showed the greatest inhibition in membranes (chapter one). Striatal slices were prepared and cAMP accumulation measured as described in the Methods. Since the basal level of cAMP accumulation was nearly undectable, 10 μM forskolin was added to stimulate the adenylate cyclase enzyme (61). The addition of 1 μM etorphine resulted in a 35-70% inhibition of forskolin-stimulated cAMP accumulation (table 3.1). The etorphine effect was naloxone reversible while naloxone alone had no effect (table 3.1). The extent of etorphine inhibition was greater in the slices than in the membranes, where etorphine inhibited 20-30% of basal adenylate cyclase activity. Although basal cAMP accumulation was small in the slices, 1 μM etorphine resulted in as much as 58% inhibition (table 3.2). The greater inhibition of adenylate cyclase in slices may be attributed to either a more efficient receptor-cyclase coupling or an activation of cAMP phosphodiesterase activity in membrane preparations. The latter possibility is unlikely since

Table 3.1

The effect of etorphine on forskolin stimulated cAMP accumulation in striatal slices

exp't	forskolin	etorphine	etorphine + naloxone
#1	2525 ± 1138	768 ± 643 (70%) p<.02	-
#2	3641 ± 560	1388 ± 161 (62%) p<.003	3387 ± 482 n.s.
#3	2021 ± 377	1310 ± 766 (35%) p<.03	3525 ± 2965 n.s.
#4	2171 ± 319	1292 ± 628 (40%) p<.01	2780 ± 1434 n.s.

Striatal slices were prepared and cAMP accumulation measured as described in the Methods. The data shown are four separate experiments each with triplicate assay tubes plus the standard deviations. The units are cpm/mg protein/minute. The slices were incubated with 10 μM forskolin (column 1), or forskolin + 1 μM etorphine (column 2), or forskolin + 200 μM naloxone (column 3), or forskolin + etorphine + naloxone (column 4). The slices were incubated for 10 minutes at 37 °C in a 90% O_2 10% CO_2 incubator. The numbers in parentheses represent the percent decrease in cAMP accumulation in the presence of etorphine.

Table 3.2

The effect of etorphine on basal cAMP accumulation in striatal slices

basal	etorphine
422 ± 232	178 ± 21 (58%) p< .01

Striatal slices were prepared and cAMP accumulation measured as described in the Methods. The data represent triplicate assay tubes plus the standard deviations from a typical experiment. The units are cpm/mg/min. The slices were incubated for 10 min ± 1 μM etorphine. The numbers in parentheses represent the percent decrease in cAMP accumulation in the presence of etorphine.

phosphodiesterase inhibitors were present throughout the labeling and assay periods.

Since opioid inhibition of adenylate cyclase activity is mediated by G_i, (as demonstrated by attenuation of the effect with pertussis toxin radiolabeling), it was of interest to determine pertussis toxin sensitivity of opioid inhibition of cAMP accumulation; however, as discussed in chapter two, brain slices are impermeable to the toxin. Even when striatal slices were exposed to 500 ng/ml pertussis toxin there was no effect on the extent of etorphine inhibition, presumably because the toxin did not enter the slices (table 3.3, chapter 2).

Cortical cAMP accumulation

Since a greater inhibition of cAMP accumulation was seen in the striatal slices than in the membranes, it was of interest to determine whether the effects of etorphine in the cortex would also be amplified. Etorphine resulted in a 10% decrease in adenylate cyclase activity in cortical membranes (chapter 1). Cortical slices (with the striata dissected away) were prepared and cAMP accumulation measured as described in the Methods. The magnitude of etorphine inhibition in cortical slices varied from 7 to 33% (table 3.4) and was naloxone reversible (table 3.4). When the assay was done in the absence of phosphodiesterase inhibitors, no effect of etorphine was seen (table 3.4, expt #6); therefore, phosphodiesterase activity is not altered by etorphine. It appears that the effect of etorphine on cAMP accumulation is enhanced, but the effect of etorphine is still reduced when compared to striatal slices. The conclusion seems warranted that the coupling of

Table 3.3

The effect of pertussis toxin treatment on cAMP accumulation in striatal slices

pertussis toxin	forskolin	+ etorphine
control #1	847 ± 21	562 ± 130 (34%)
1 ng/ml	1080 ± 54	713 ± 11 (34%) n.s.
100 ng/ml	1440 ± 237	811 ± 41 (44%)
control #2	1251 ± 145	985 ± 154 (21%)
500 ng/ml	1051 ± 120	859 ± 60 (18%) n.s.

Striatal slices were prepared and incubated ± the indicated concentrations of pertussis toxin in a 90% O_2 10% CO_2 incubator at 37°C for 4 hrs. During the last hour [^{3}H]adenine was added (7.5 μCi/ml) to label the intracellular cAMP pools. The radioactive medium was removed and the slices assayed in the presence of 10 μM forskolin ± 1 μM etorphine for 10 minutes. The results shown are from two separate experiments. Each represents the average of triplicate assay tubes plus standard deviations. The units are cpm/mg/min. The numbers in parentheses show the percent decrease in cAMP accumulation in the presence of etorphine.

Table 3.4

The effect of etorphine on forskolin stimulated cAMP accumulation in cortical slices

	forskolin	+ etorphine	+ naloxone
expt #1	1014 ± 289	844 ± 228 (17%) p<.3	-
expt #2	1548 ± 164	1036 ± 219 (33%) p<.1	1643 ± 19 n.s.
expt #3	815 ± 145	687 ± 28 (16%) p<.2	718 ± 42 n.s.
expt #4	778 ± 43	607 ± 52 (15%) p<.2	785 ± 109 n.s.
expt #5	775 ± 99	719 ± 19 (7%) n.s.	-
expt #6	361 ± 24	441 ± 58 n.s.	412 ± 35

Cortical slices were prepared and cAMP accumulation measured as described in the Methods. The data shown are from five different experiments each with triplicate assay tubes plus the standard deviations. The units are cpm/mg protein/minute. The slices were incubated with 10 μM forskolin (column 1), or forskolin + 1 μM etorphine (column 2), or forskolin + etorphine + 200 μM naloxone (column 3). In expt #6 phosphodiesterase inhibitors were absent throughout the labeling and assay periods. The numbers in parentheses represent the percent decrease in cAMP accumulation in the presence of etorphine.

opioid receptors to adenylate cyclase in brain membranes is an accurate reflection of the coupling in the brain, since the effects of etorphine in two different regions of the brain are similar in both slices and membranes.

Midbrain cAMP accumulation

It was of interest to determine whether opioids might inhibit cAMP accumulation in a brain area where no opioid inhibition of adenylate cyclase activity in membranes was observed. The midbrain area was chosen for investigation. As shown in table 3.5, opioids did not inhibit basal or forskolin-stimulated cAMP accumulation.

Cerebroside sulfate treatment

Since the opioid receptor has been shown to be affected by its lipid environment (60), and cerebroside sulfate added to neuroblastoma cells potentiates opioid inhibition of cAMP accumulation (43), the effect of this lipid on opioid inhibition of cAMP accumulation in rat brain slices was examined. Cerebroside sulfate (purified from bovine brain) was added to cortical or striatal slices as described in the Methods. After a 4 hour treatment (sufficient to incorporate the lipid into N18TG2 cells), the slices were assayed for cAMP accumulation as usual. The results are shown in table 3.6. Cerebroside sulfate seemed to attenuate, rather than potentiate the effect of etorphine.

Discussion:

The question posed in this chapter was whether opioid receptors are coupled to adenylate cyclase in tissue slices. The results indicated

Table 3.5

The effect of etorphine on cAMP accumulation in midbrain slices

	control	+ etorphine	+ etorphine + naloxone
basal	442 ± 79	381 ± 45 (n.s.)	311 ± 7
forskolin stimulated	478 ± 83	427 ± 186 (n.s.)	746 ± 87

Midbrain slices were prepared and cAMP accumulation measured as described in the Methods. The results are triplicate assay tubes from a typical experiment. The units are cpm/mg/minute. The slices were incubated in the absence (basal) or presence of 10 μM forskolin. The data were not significantly different in a student's t-test.

Table 3.6

The effect of cerebroside sulfate on etorphine inhibition of cAMP accumulation in rat brain slices

		forskolin	+ etorphine
cortex #1	- CS	1014 ± 289	844 ± 228 (17%) p<.2
	+ CS	566 ± 42	571 ± 27 n.s.
cortex #2	- CS	775 ± 99	719 ± 19 (7%) n.s.
	+ CS	474 ± 70	448 ± 23 n.s.
striatum	- CS	1015 ± 145	571 ± 124 (44%)
	+ CS	642 ± 23	423 ± 56 (34%)

Slices were prepared from the indicated brain regions and treated for four hours ± cerebroside sulfate (CS), then assayed for cAMP accumulation. The data shown are from triplicate assay tubes plus the standard deviations. The units are cpm/mg/minute. The slices were incubated with 10 μM forskolin (column 1) or forskolin + 1 μM etorphine (column 2). The numbers in parentheses represent the percent decrease in cAMP accumulation in the presence of etorphine.

that opioid receptors are coupled to adenylate cyclase in rat brain slices as in membranes. Furthermore, the relative magnitude of the opioid effect in slices prepared from the cortical, midbrain and striatal regions of rat brains parallels that seen in cortical, midbrain and striatal membranes, an observation which suggests that the membrane preparations are an accurate reflection of the opioid inhibited adenylate cyclase system of the rat brain. It appears that the opioid receptor is not separated from the adenylate cyclase enzyme system during membrane preparation; therefore the conclusion of chapter one, that opioids do not inhibit adenylate cyclase in brain areas other than the striatum and cortex, remains intact.

Since cerebroside sulfate had been shown to potentiate opioid inhibition of cAMP accumulation in neuroblastoma cells, it was of interest whether this lipid may affect opioid responses in rat brain slices. Cerebroside sulfate added to rat brain slices decreased both the etorphine effect and the basal level of cAMP accumulation. This suggests that the lipid had blocked the entry of both the opioid and the [^{3}H]adenine or somehow altered the membrane structure such that the receptor-cyclase system became uncoupled. It is not possible to distinguish between these two possibilities at this point, since cerebroside sulfate incorporation has not been shown.

It appears that opioid inhibition of adenylate cyclase is only one mechanism whereby opioids exert their actions in the brain. Insofar as opioid inhibition of the cyclase is demonstrable in slices as well as in membrane preparations, the effect in the latter is not an artifact. The presence of opioid receptors throughout the brain that evidently are not

linked to adenylate cyclase necessitates the continued search for additional second messenger systems.

Chapter four: Opioid interaction with potassium channels

Electrophysiological data suggests that opioids increase potassium conductance (6,12,62). A study was undertaken to determine if opioids could increase potassium flux. If opioids increase potassium conductance, it should be possible to monitor the conductance change by measuring potassium flux using radioactive tracer ions, provided the signal is large enough (28).

This study was performed with the hippocampus because it was an anatomically distinct area where opioid effects had been demonstrated electrophysiologically. If opioids were to produce a receptor mediated effect on potassium flux, it should be naloxone reversible and correlate with binding characteristics and possibly analgesia.

Results:

Potassium efflux was measured as described (28 and Methods) using the release of a radioactive tracer, usually $^{86}Rb^{+}$, from preloaded slices. Initial studies showed that most of the radioactivity from the pre-loaded slices was released within the first five minutes as the tubes were transferred. A significant amount of radioactivity was released in the first tube, which served as a washout tube. Since no significant amount of counts was released from the tissue after five minutes, most experiments involved a five minute time course.

To determine whether morphine altered the efflux rate, tissues were pre-loaded in $^{86}Rb^{+}$ then transferred to vessels containing either morphine or no drug and the efflux followed for five minutes. Initial

studies revealed a rapid (less than thirty seconds) increase in release of tracer in the presence of 10 μM morphine (fig 4.1); however, the change was not very reproducible or dose-dependent as one would expect for a true drug effect. Furthermore, in instances where the effect was seen in several experiments, it was not naloxone reversible. (Table 4.1).

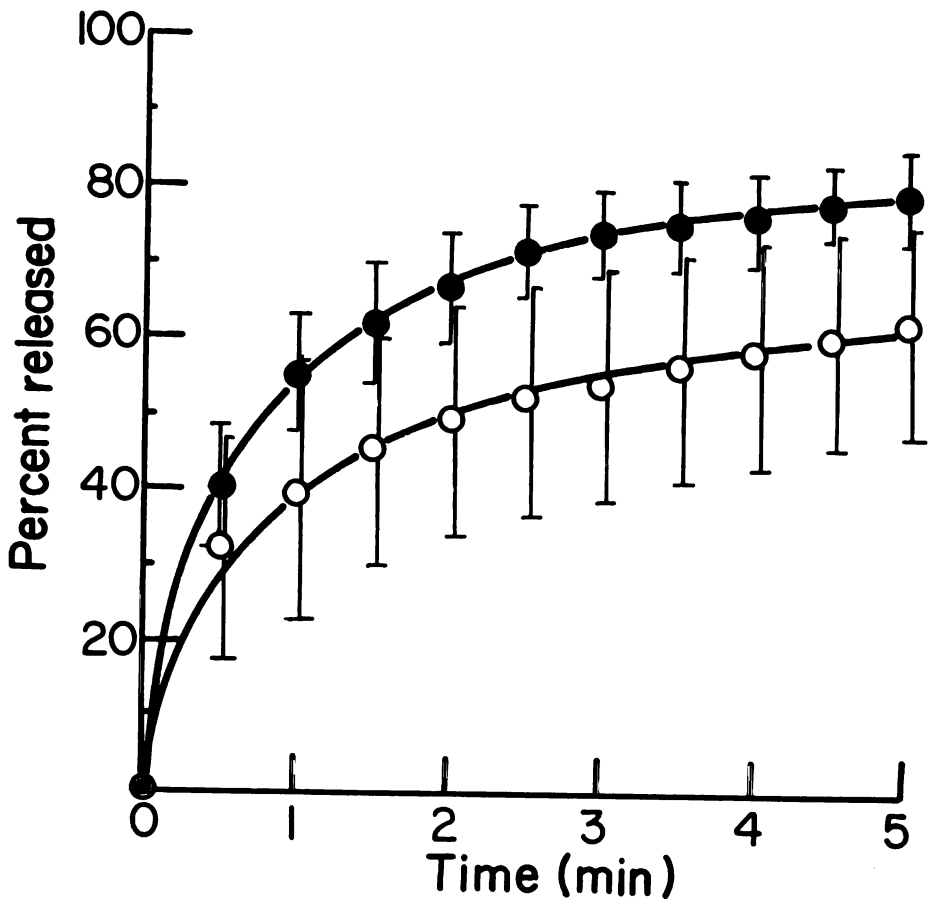
Discussion:

From the study it does not appear that morphine increases potassium efflux via an opioid receptor. During the course of these studies, we learned that the receptor involved in the opening of a potassium channel was in fact blocked by Rb^+ (R.A. North, pers. comm.); however, when the $^{86}Rb^+$ was substituted with $^{42}K^+$, no effect of morphine was observed.

It is possible that the hippocampal slice preparation is not the best tissue for such studies, since opioids act indirectly in the hippocampus (30). Opioids appear to act on inhibitory interneurons within the hippocampus, and 1 of these contacts about 200 pyramidal cells (30). Although the opioid effect on the interneurons is amplified, there are so few interneurons that the signal to noise level is too low to measure biochemically. Because of this difficulty and the technical limitations of using $^{42}K^+$ with its 4 hour half-life, further investigation of potassium efflux in other brain areas was not undertaken. Another difficulty arises from the fact that in a slice preparation one is measuring the sum total of potassium changes in many neuronal pools. In light of these difficulties, this project was dropped.

Figure 4.1

The effect of morphine on potassium efflux in hippocampal slices



The efflux of potassium was measured as the release of $^{86}Rb^{+}$ from hippocampal slices in the presence or absence of 10 μM morphine. The results shown are from three separate experiments.

Table 4.1

The naloxone reversibility of the morphine induced efflux of $^{86}Rb^{+}$

	0	Morphine	Naloxone	Morphine + Naloxone
t=1/2	53 ± 11	58 ± 14	55 ± 14	50 ± 11
t=1	65 ± 13	69 ± 12	66 ± 13	66 ± 10
t=1 1/2	72 ± 13	75 ± 11	72 ± 12	73 ± 10

The time course of efflux was followed for 5 minutes. The results represent the first 90 seconds of release of $^{86}Rb^{+}$ from hippocampal slices. Each column entry is the average of triplicate assays from three separate experiments. There is no statistically significant difference between these tubes.

Chapter 5: Opioids and phosphoinositide turnover

Although G_i is present throughout all regions of the rat brain which were examined, opioid inhibition of adenylate cyclase is observed in the striatum and cortex only (chapter one). Furthermore, the binding of opioids to receptors is modified by treatment of membranes with pertussis toxin, indicating that the opioid receptor is linked with G_i and/or G_o (chapter two). These results necessitate the investigation of second messenger systems other than adenylate cyclase through which opioids may act. The pathway of agonist-stimulated phosphoinositide (PI) turnover has been shown to include a G protein (17-20). This pathway usually results in calcium mobilization (15,16). Electrophysiological and biochemical studies suggest that opioids interact with Ca^{++} and Ca^{++} channels, consistent with the possibility that opioids regulate PI turnover (15,16,21,22). NG108-15 cells have been shown to increase PI turnover in response to bradykinin (63).

In order to simplify the study of opioid reglation of PI turnover in acute and chronic opioid states, neuroblastoma x glioma hybrid NG108-15 cells were used initially. The turnover of phosphoinositides in response to opioids was also studied in brain slices.

Results

Phosphoinositide turnover in NG108-15 cells

The initial studies were performed in neuroblastoma x glioma hybrid NG108-15 cells. The inositol pool was labelled with an eighteen hour

exposure to myo-[2-^{3}H]inositol. The cells were then treated with the appropriate agonists and the phosphoinositides were isolated as described in Methods. Although bradykinin had been reported to increase phosphoinoside hydrolysis (63) it was not observed here. As shown in table 5.1, neither 10 μM bradykinin nor 10 μM etorphine had a significant effect on PI turnover. The slight increase in IP_2 with etorphine was not consistently observed. It was of interest to determine whether higher doses of etorphine or longer incubation periods might increase the opioid effect. At concentrations up to 100 μM, there was no significant effect of etorphine (Table 5.2). Naloxone also had no effect on PI turnover (Table 5.2).

Since opioids have been shown to increase Ca^{++} flux in tolerant animals (22), it was possible opioids may increase PI turnover in chronically treated cells. When NG108-15 cells are treated with 1 μM etorphine for 2 hours, opioids no longer inhibit adenylate cyclase, representing a desensitized or a tolerant state (67). Upon addition of 200 μM naloxone, an increase in cAMP levels is observed, a cellular equivalent of withdrawal (67). However, no effect on PI turnover was observed in either chronic or naloxone withdrawn cells (Table 5.2).

Phosphoinositide turnover in cortical slices

Since there was no change in PI turnover in NG108-15 cells which contain only δ receptors, it was of interest to determine whether opioids had any effect on PI turnover in the rat brain, especially in those areas containing other opioid receptors. The assay conditions chosen were those used to demonstrate the effect of muscarinic agonists on PI

Table 5.1

The effect of etorphine on PI turnover in NG108-15 cells

	IP	IP_2	IP_3
control	3062 ± 148	111 ± 14	192 ± 109
bradykinin	2655 ± 573	110 ± 10	132 ± 17
etorphine	3309 ± 370	157 ± 7 ✓	210 ± 138

NG108-15 cells grown in 6 well plates were incubated with 1 μCi/ml myo-[2-^{3}H]inositol for 18 hours, then the medium was removed and, following a 10 minute pre-incubation with incubation medium, the cells were incubated with 10 mM LiCl (control), or Li + 10 μM bradykinin, or Li + 10 μM etorphine for 10 min. The phosphoinositides were isolated on Berridge columns as described in the Methods. In this experiment, glycerophosphoinositol was not separated from inositol-1-phosphate (IP). IP_2= inositol-1,4-diphosphate and IP_3 =inositol-1,4,5-triphosphate. The units are cpm. The data are means + standard deviations from triplicate assay tubes. The ✓ indicates sigficance at the $p < .05$ level. All other data points were not significant in a student's t-test.

Table 5.2

PI turnover in NG108-15 cells in response to acute and chronic etorphine

	IP	IP_2	IP_3
control	732 ± 249	100 ± 88	105 ± 55
1 μM etorphine	819 ± 121	47 ± 9	131 ± 24
10 μM "	655 ± 74	104 ± 48	155 ± 75
100 μM "	707 ± 60	69 ± 32	145 ± 26
10 μM naloxone	716 ± 69	99 ± 14	103 ± 27
200 μM "	762 ± 112	51 ± 8	135 ± 17
chronic	4015 ± 3641	518 ± 188	1230 ± 114
chronic + naloxone	2042 ± 552	896 ± 211	1200 ± 301

NG108-15 cells grown in 6 well plates were treated with 1 μCi/ml myo-[2-^{3}H]inositol for 18 hours, the medium removed, and following a 10 min preincubation with incubation medium, the cells were treated with the indicated concentrations of drugs + 10 mM LiCl for 20 min. Chronic treatment involved exposure of the cells to 1 μM etorphine for 2 hours. The phosphoinositides were isolated with Berridge columns with IP separated from glycerophosphoinositol as described in the Methods. The units are cpm. The data are from triplicate assay tubes and were not significant at the $p < .05$ level in a student's t-test.

turnover in cerebral cortical slices of rat brain (44). As shown in table 5.3, 10 μM etorphine caused a small (20%) but significant increase in all three products of phosphoinositide hydrolysis (n = 10). It is worth noting that with smaller sample numbers (n = 5) it was difficult to demonstrate a statistically significant increase in PI turnover in response to etorphine (data not shown). Etorphine at 1 μM produced a 10% increase in phosphoinositides (data not shown).

The increase in phosphoinositides induced by 10 μM etorphine was reversible with 20 μM naloxone (Table 5.4). The data shown in Table 5.4 are averaged from three experiments with a total n = 20. The ability of naloxone to reverse the etorphine effect suggests that it is indeed a specific opioid effect. Naloxone alone at 160 μM produced an effect.

Discussion

The data presented in this chapter indicate another second messenger system through which opioids may act. In cerebral cortical slices, 10 μM etorphine increased phosphoinositide turnover from 20 - 80% (Table 5.3,5.4). The increase varied from one experiment to another; however, with sample numbers of n = 10 or more the increase was always statistically significant. This variability could be due to both the small signal and limitations with the experimental method. Etorphine produced a specific opioid effect since it could be reversed by naloxone.

Etorphine produced a small (10%) effect at 1 μM. At 10 μM, the effect of etorphine was reproducibly 20% or greater. It is worth

Table 5.3

The effect of etorphine on PI turnover in cerebral cortical slices

	IP	IP_2	IP_3
control	3746 ± 339	733 ± 212	122 ± 60
etorphine	4605 ± 524	992 ± 334	162 ± 52
statistics n = 9	23% $p < .001$	35% $p < .05$	32% $p < .05$

Cerebral cortical slices from rat brains were prepared as described in the Methods. The slices were incubated with 61 μM (10 μCi/ml) myo-[2-^{3}H]inositol for one hour in 100 mM LiCl - Kreb's-Ringer's bicarbonate buffer in the absence (control) or presence of 10 μM etorphine. Following the incubation the slices were homogenized and the phosphoinositides isolated as described in the Methods. The units are cpm. The data are from nine sample tubes and were significant as indicated in a student's t-test.

Table 5.4

Naloxone reversibility of etorphine effect on PI turnover

	IP	IP_2	IP_3
control	1859 ± 733	354 ± 143	127 ± 81
etorphine	3419 ± 2035 (p< .008) (83%)	481 ± 210 (p< .01) (35%)	224 ± 154 (p< .006) (76%)
etorphine + naloxone	2071 ± 883 (n.s.)	361 ± 169 (n.s.)	146 ± 103 (n.s.)

Cerebral cortical slices from rat brains were prepared as described in the Methods. The slices were incubated with 30 μM (5 μCi/ml) myo-[2-^{3}H]inositol for one hour in 100 mM LiCl- Krebs-Ringer's bicarbonate buffer (control) or plus 10 μM etorphine or plus 10 μM etorphine + 20 μM naloxone. Following the incubation the slices were homogenized and the phosphoinositides isolated as described in the Methods. The units are cpm. The data are the average of three separate experiments with a total n = 20. The % increase in the presence of etorphine is indicated in the parentheses. The etorphine samples were statistically significant in a student's t-test as shown.

noting, however, that with small numbers of samples (n = 5) it was difficult to demonstrate a statistically significant effect of etorphine. This will make further characterization of the opioid increase in PI turnover more difficult. The question arises whether a 20% effect is physiologically relevant. As is the case with opioid inhibition of adenylate cyclase where a 10 - 25% effect is observed (chapter 1), a small effect can be amplified in a second messenger system. PI turnover results in increases in intracellular calcium and activation of protein kinase C (15,16). Small changes in intracellular calcium can regulate ion channels (16). Protein kinase C phosphorylates a number of intracellular proteins which can result in dramatic cellular events, such as increased DNA synthesis (15). Opioid-induced PI turnover could therefore be an important action of opioids in the brain.

Previous studies have demonstrated opioid inhibition of calcium conductances which would result in decreased intracellular calcium (21,22). Other reports have indicated opioid induced increases in intracellular calcium, such as the calcium dependent potassium conductance in locus ceruleus neurons, and the calcium dependent after hyperpolarization produced by opioids in the myenteric plexus and locus ceruleus (12,13,64). The response of a given neuron probably depends on the receptor subtype involved. The demonstration that opioids increase PI turnover is consistent with the data indicating increases in calcium in opioid action.

In summary, the demonstration of opioid induced PI turnover provides a mechanism other than adenylate cyclase through which opioids may act. Since G proteins have been implicated in PI turnover (17-20),

and pertussis toxin alters opioid binding in the cerebral cortex (chapter 2), the action of opioids in the cerebral cortex may be mediated by PI turnover as well as via inhibition of adenylate cyclase.

Conclusions

The proposed research was aimed at determining whether opioid action is mediated by G proteins. Although several distinct processes have been suggested as second messenger systems for opioids, the possibility that they may share a common feature, the G proteins, was examined.

Inhibition of adenylate cyclase

Opioids have been shown to inhibit adenylate cyclase in the rat brain striatum (8,9). With the possible exception of the cortex, the striatum is the only brain region shown to be coupled to adenylate cyclase (8-10). The fact that opioid inhibition of striatal adenylate cyclase is GTP-dependent suggests the involvement of G_i. The specific aims were to determine: whether the opioid of inhibition of adenylate cyclase in the rat brain striatum is mediated by G_i ; and whether the lack of an opioid effect on adenylate cyclase is due to the absence of G_i in these areas.

The role of G_i as a mediator of opioid inhibition of adenylate cyclase was determined with the use of pertussis toxin. Pertussis toxin catalyzes the ADP-ribosylation of the GTP-binding subunit of G_i, resulting in an attenuation of its inhibitory action on adenylate cyclase and an uncoupling of G_i from its receptor. The GTP-binding subunit can be radiolabelled with [α-^{32}P]NAD as the substrate for pertussis toxin. Treatment of striatal membranes with pertussis toxin resulted in a dose-dependent attenuation of the opioid inhibition of adenylate cyclase, which could be correlated with the extent of

ADP-ribosylation of G_i (figure 1.1). This correlation provides strong evidence for the conclusion that opioids inhibit adenylate cyclase in the rat brain striatum via G_i. In addition, opioid inhibition of adenylate cyclase was also demonstrable in the cortex.

With the use of pertussis toxin, it was possible to demonstrate the presence of G_i in all brain regions examined (figure 1.4). Despite the presence of G_i, however, opioid inhibition of adenylate cyclase was seen only in the striatum and cortex (table 1.2). In order to determine if physical separation of the opioid receptor, G_i and adenylate cyclase occur during membrane preparation accounting for a lack of opioid effect in other brain areas, the effect of opioids on cAMP accumulation in rat brain slices was examined. The opioid effect in rat brain slices parallels that in cortical, striatal and midbrain membranes (chapter 3); therefore, it appears that the opioid receptor is not separated from the adenylate cyclase system during membrane preparation.

The conclusions of the studies on opioid inhibition of adenylate cyclase are as follows: 1) opioids inhibit adenylate cyclase in the striatum and cortex only, in both membranes and brain slices; 2) in the striatum, the opioid effect is mediated by G_i; 3) the lack of opioid inhibition of adenylate cyclase is not due to a lack of G_i proteins in other areas.

Regulation of opioid binding and analgesia by G proteins

It was important to try and correlate a physiological endpoint with the proposed mediation of the opioid effect through G proteins. An

appropriate endpoint for opioid action is analgesia. The question of whether G proteins are involved in analgesia was addressed by determining the effect of pertussis toxin on opioid analgesia. Along with analgesia, the effects of pertussis toxin on opioid receptor binding could be studied. The modification of opioid receptor binding by guanine nucleotides throughout the brain (3,4,10) implies G proteins in the action of opioids, since most systems which have GTP-sensitive binding sites are coupled to G proteins (2,23,24).

The effect of pertussis toxin on analgesia was determined by administrating the toxin into rats, i.v. and i.c.v.. The results of these experiments indicated that the toxin did not cross the blood-brain-barrier nor did it enter the brain when injected i.c.v.. By either route, however, the toxin labeled G proteins in the liver and kidneys, a finding which suggests that the toxin is active in vivo and had entered the circulation from its central injection site. Since the toxin had no effect on analgesia, (as measured by tail flick latencies), even after i.c.v. administration it evidently had not entered the brain; therefore, the negative result is inconclusive. The impermeability of intact neural tissue to the toxin was confirmed by demonstrating that the toxin did not act on brain slices. If added directly to membranes in vitro , however, pertussis toxin altered opioid receptor coupling, as evidenced by its effect on adenylate cyclase in the striatum, and its effect on agonist binding (described below). The observation that the toxin did not cross neuronal membranes has relevance for studies which draw conclusions on the lack of an effect of pertussis toxin without demonstration that the toxin has entered the tissue.

The demonstration that the affinity of opioid receptors is altered by pertussis toxin treatment of striatum membranes indicates an uncoupling of the opioid receptor from the G_i or G_o protein. This finding points to the existence of a complete second messenger system in the striatum, namely a complex consisting of receptor + G_i + adenylate cyclase, and it is the first demonstration that opioid receptors are regulated by G proteins in the brain.

The question of whether pertussis toxin alters opioid binding in other brain areas was addressed by examining the cortex, a region that showed only a 10% inhibition of adenylate cyclase activity by opioids. A striking decrease in agonist binding following pertussis toxin treatment was observed in the cortex. Pertussis toxin treatment of midbrain membranes, (an area where opioids do not inhibit adenylate cyclase), revealed no change in opioid agonist binding, despite the ADP-ribosylation of 80% of the G proteins. The results indicate that in areas where opioids do not inhibit adenylate cyclase the opioid receptors are not coupled to pertussis-sensitive G proteins. The possibility remains that G proteins which are not affected by pertussis toxin mediate opioid action in areas other than the striatum and cortex.

Opioids and Ion channels

Potassium channels

Since electrophysiological data in hippocampal slices pointed to an involvement of potassium channels in opioid action, a biochemical study of potassium efflux was undertaken. It was not possible to demonstrate unequivocally a receptor-mediated effect on potassium efflux. The

inconclusiveness of the data may be explained on the basis that since the opioid-sensitive interneurons within the hippocampus are few in number, about 1 in 200 (30), the biochemical signal is understandably small. Consequently, an accurate extimate of the effect was not possible.

Calcium channels and phosphoinositide turnover

The pathway of agonist-stimulated phosphoinositide (PI) turnover has been shown to involve a G protein (17-20). PI turnover usually results in calcium mobilization (15,16). A number of biochemical and electrophysiological studies point to the involvement of calcium and Ca^{++} channels in opioid action, (15,16,21,22), and raise the possibility that opioids regulate PI turnover.

The effects of opioids on PI turnover in cerebral cortical slices were studied, since in this preparation muscarinic receptors have been shown to increase PI turnover (44). It was found that etorphine produces a small but significant increase in PI turnover (table 5.3). This response needs to be further characterized, but it suggests another possible second messenger system through which opioids may act.

Summary

The present findings demonstrate regulation of the opioid receptor by G proteins in the striatum and cortex (chapter 1,2). In the striatum, opioids inhibit adenylate cyclase via G_i (chapter 1). Opioid inhibition of adenylate cyclase is also demonstrable in the cortex, which may also contain an alternative second messenger system

which is opioid-sensitive, i.e., PI turnover (chapter five). Since both systems (cAMP and PI turnover) involve G proteins, the opioid response in the cortex could be mediated through either of these systems.

The role of G proteins in opioid action in other brain areas, such as the midbrain, is not clear. Opioids do not affect cAMP production in the midbrain (chapter 1,3). Opioid binding is not altered by pertussis toxin treatment under conditions where most of the G proteins in the membrane are ADP-ribosylated, a finding which indicates that pertussis toxin-sensitive G proteins do not mediate opioid action in the midbrain. Since opioid binding is regulated by GTP in the midbrain, the possibility remains that other G proteins may be involved in opioid action in this area. Alternatively, the effect of GTP in the midbrain may be directly on the receptor.

The proposed research was aimed at determining whether opioid action in the brain is mediated by G proteins. The results have demonstrated a coupling of opioid receptors and G proteins in the striatal and cortical regions of the brain. Inhibition of cAMP production has been shown to be the second messenger system mediating opioid action in the striatum. In the cortex, opioids may act through an increase in PI turnover as well as an inhibition of adenylate cyclase. It was not possible to demonstrate an involvment of G proteins on the physiological endpoint of analgesia because of the lack of entry of pertussis toxin into neuronal tissues; consequently, this issue remains unresolved. It can be concluded that in at least two different brain regions three of the criteria necessary have been satisfied for demonstrating a coupler of the opioid receptor, namely: 1)

colocalization of opioid receptors and G proteins; 2) an effect of G proteins on opioid receptor binding; 3) and elucidation of the nature of the second messenger systems mediating opioid action via G proteins.

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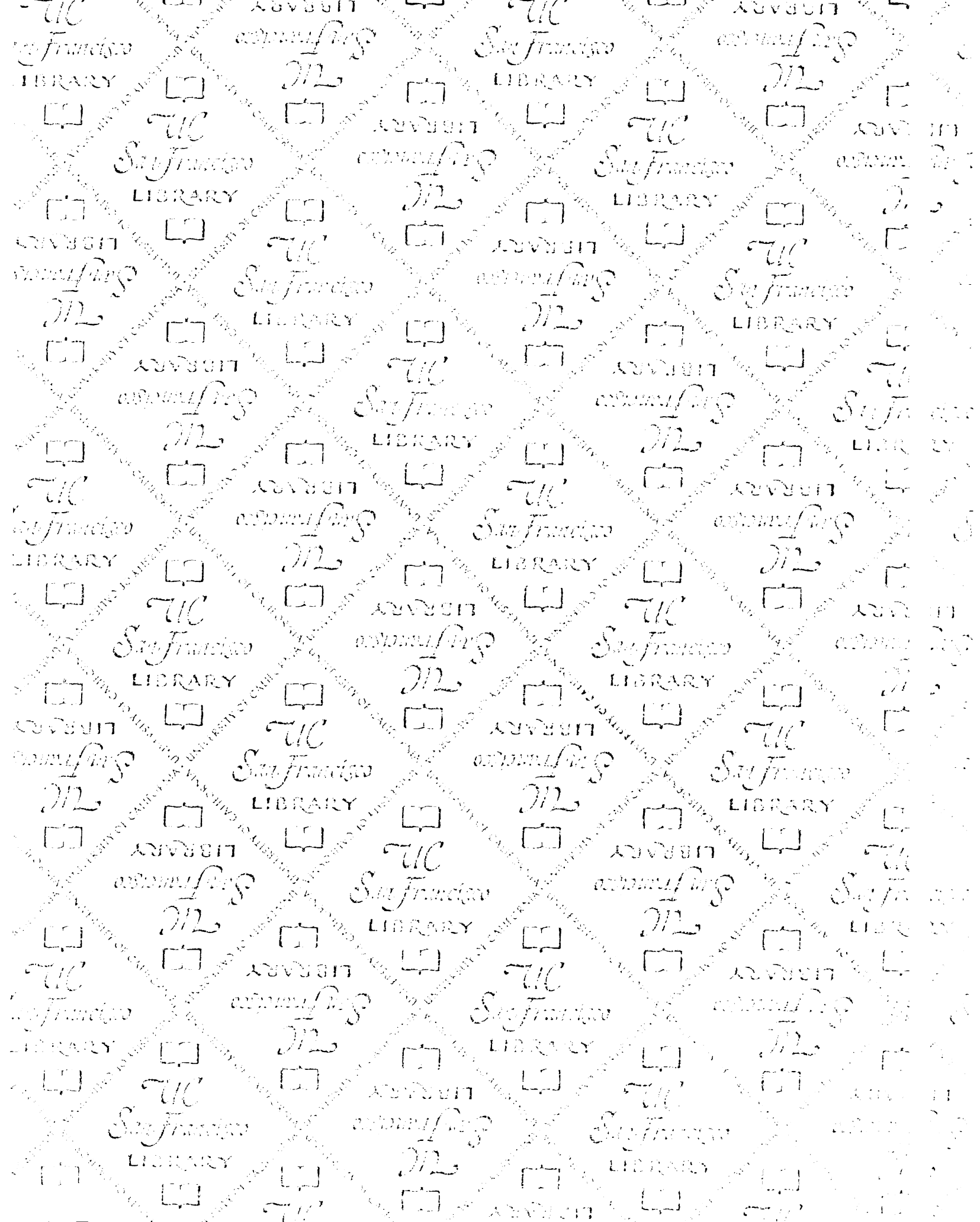
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