REGULATION OF MU AND DELTA OPIOID ACTION IN NORMAL AND MORPHINE-TOLERANT CELLS AND CELL MEMBRANE PREPARATION

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ABSTRACT

Title of Dissertation: Regulation of μ and δ opioid action in normal and morphine-tolerant cells and cell membrane preparations

Pamela Sue Puttfarcken, Doctor of Philosophy, 1988

Dissertation directed by: Brian M. Cox, Professor, Department of Pharmacology

Na^+ and guanyl nucleotides have profound effects on opioid action. Analysis of these effects is confounded by the presence of heterogeneous populations of opioid sites in most tissues investigated. To avoid this complication, the regulatory functions of Na^+ and guanyl nucleotides were studied in cells carrying only δ (NG108-15) or μ (7315c) receptors respectively. Binding studies were performed at 37° C in Krebs-Hepes buffer at pH 7.4. Na^+ and guanyl nucleotides inhibited agonist binding in each cell type, whereas antagonist binding was not affected. Although Na^+ appeared to regulate both μ and δ receptors through intracellular sites, its effects were not identical at each receptor type. Na^+ regulation of opioid inhibition of adenylyl cyclase activity also differed between cell types.

High and low affinity components of binding were characterized by competition of unlabeled μ or δ agonists against antagonist binding in the absence or presence of GTP or GTPγS in both cell types. In contrast, only single agonist affinity states were observed in the presence of GDPβS or in membranes from pertussis toxin treated cells. Data suggest regulation of agonist binding by guanyl nucleotides may be mediated through interconversions among multiple affinity states of the δ and μ opioid receptors in both cell types. These states may represent several forms of the agonist-receptor-guanyl nucleotide protein complex in the transmembrane signalling cycle.

The effects of sustained exposure to morphine on function of μ receptors was studied. Opioid inhibition of adenylyl cyclase activity was lost within 5 hours of morphine incubation but reductions in receptor number were not observed until after 24 and 72 hours of morphine incubation. There appear to be at least two consequences of sustained μ receptor activation: desensitization and down-regulation. Desensitization to agonist is apparently associated with a reduced ability of agonist-occupied receptors to interact with guanyl nucleotide binding proteins. This is followed by receptor down-regulation.

REGULATION OF μ AND δ OPIOID ACTION IN NORMAL AND MORPHINE-TOLERANT CELLS AND CELL MEMBRANE PREPARATIONS

by Pamela Sue Puttfarcken

Dissertation submitted to the Faculty of the Department of Pharmacology
Graduate Program of the Uniformed Services University of the
Health Sciences in partial fulfillment of the
requirements for the degree of
Doctor of Philosophy 1988

Dedication

For Brian and Linda. Thank you for your guidance, support, and friendship.

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I gratefully acknowledge my parents, Klaus and Sue Puttfarcken, who have not only instilled in me the desire to learn, but the strength and ability to pursue my life's ambitions. Without their continuous love and support, I would never have made it this far.

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I wish to express my appreciation to the members of my thesis committee, Dr. Leon Moore, Dr. John Sarvey, Dr. Tom Cote and Dr. Keith Latham, for their continuing interest in my research and their helpful suggestions and comments.

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The abbreviations used are: Bmax, total binding sites; BSA, bovine serum albumin; DADLE, [D-Ala^2-D-Leu^5]enkephalin; cAMP, Adenosine 3'-5'-cyclic monophosphate; DAGO, Tyr-D-Ala^2-Gly-N(Me)Phe-Gly-ol; DIP, diprenorphine; DMEM, Dulbecco's modified essential medium; DPDPE, [D-Pen^5-D-Pen^5]enkephalin; DSLET, [D-Ser^2-Leu^5]enkephalyl-Thr; EBSS, Earle's balanced salt solution; EGTA, ethylene glycol-bis-(β-aminoethyl ether) N-N-tetraacetic acid; EKC, ethylketocyclazocine; ETOR, etorphine; FCS, fetal calf serum; GDP, Guanosine diphosphate; GTP, Guanosine triphosphate; GTPγS, Guanosine-5'-O-(3-thiotriphosphate); HEPES, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid; IC_{50}, concentration of agonist producing 50% maximal effect; ICI174,864, allyl$_2$-Tyr-(a-aminoisobutyric acid)$_2$-Phe-Leu-OH;K_{app} apparent dissociation constant (macroscopic dissociation constant); K_D, equilibrium dissociation constant for labeled ligand; K_e, antagonist equilibrium dissociation constant, K_i, equilibrium dissociation constant for competing ligand; NAL, naloxone; N-MG, N-methyl-D-glucamine; and U50,488H, trans-3,4-dichloro-N-methyl-N-[2-(pyrrolidinyl)-cyclohexyl]benzeneacetamide methansulfonate hydrate.

INTRODUCTION

There was no direct evidence of opioid receptors until the 1970s. At this time, their existence was inferred from structure-activity relationships of narcotic analgesics (Casy, 1973). In the early 1970s the application of radioactive labeled ligands in studies of drug binding to tissue provided the first direct evidence of the presence of high-affinity binding sites presumed to be associated with receptors. However results obtained from these studies were complicated by the fact that the radiolabeled drug bound nonspecifically to many membranes sites aside from the opioid receptor. It was not until Goldstein and coworkers introduced the concept of stereospecific binding, that discrimination between specific and non-specific binding at opioid receptors became feasible (Goldstein et al., 1971). Goldstein and coworkers noted that opioids displayed an extraordinary degree of stereospecificity. Stereospecific binding was defined as the difference between the amount of binding observed in the presence of a high concentration of an inactive (+) enantiomer of a pair of opioid enantiomers and that observed in the presence of the same concentration of active (-) enantiomer. A second factor important in the demonstration of opioid receptors was the introduction of radioligands with high specific activity. Snyder and coworkers (1977) proposed that "success in identifying specific opioid binding depended on the use of opioids with high specific radioactivity ". In this way, the low concentration of radioligand employed would favor binding to specific rather than nonspecific sites on the membrane. Using rat brain homogenates, Pert and Snyder (1973) , Simon et al., (1973) and Terenius (1973), each group working independently, were able to demonstrate high affinity stereospecific binding using [^{3}H]NAL with a specific radioactivity 1000 times greater than that of the radiolabeled opioids employed by Goldstein et al (1971). Relative receptor affinities for a range of opioid drugs obtained from *in vitro* binding studies, correlated well with their relative potencies obtained from *in vivo* experiments (ie. analgesic activity); (Wilson et al., 1975). This suggested that the sites labeled in binding experiments were

probably the receptors through which the drugs worked *in vivo* to produce a pharmacological effect.

Subsequent binding experiments showed other receptor types were subject to regulation by Na^+ and GTP (Tsai and Lefkowitz, 1978; Connolly and Limbird, 1983; Katadi and Ui, 1982; Minnuth and Jacobs, 1986; Simon et al., 1975; Pert et al. 1973; Lefkowitz et al., 1976). A characterization of these regulatory processes and the changes induced by long-term agonist exposure is the subject of this dissertation. The physiological role of the regulatory processes is still not entirely understood.

Sodium Regulation of hormone binding

a. Non-opioid systems

Agonist receptor binding is influenced by several different mono- and divalent cations, as well as nucleotides and circulating hormones. Na^+ in particular appears to regulate agonist-receptor interactions in many different systems. These include the α-adrenergic receptors on human platelet cells (Tsai and Lefkowitz, 1978; Connolly and Limbird, 1983), the α_2-adrenergic receptors of the pancreas (Katadi and Ui, 1982), the muscarinic cholinergic receptor in the heart (Rosenberger et al.,1979; Yamamura et al., 1980), the β-adrenergic receptor of the S49 lymphoma cells (Minnuth and Jacob, 1986), the adipose cells (Burns et al., 1975; Aktories et al., 1979), and the lutropin/ choriogonadotropin receptors on porcine luteal membranes (Buettner and Ascoli, 1984).

b. Opioid systems

The sensitivity of opioid binding to Na^+ was first demonstrated by Simon et al.

(1973) and Pert et al. (1973). In these early studies, Na^+ was shown to increase the binding of a [^{3}H] opioid antagonist and to decrease the binding of [^{3}H] opioid agonist in rat brain homogenates. The differential effect was elicited by Na^+ but not by other ions such as K^+, Cs^+ and Rb^+. This highly specific action suggested that Na^+ was interacting to allosterically regulate the opioid receptor.

Simon and coworkers (1975) reported that the presence of Na^+ led to an increase in the affinity of antagonists and a decrease in the affinity of agonists for the opioid receptor. The total number of binding sites appeared to remain unaffected by Na^+. The apparent reduction in agonist affinity was attributed to Na^+'s ability to increase the rate of agonist dissociation (Pert and Snyder, 1973; Blume, 1978). Simon et al. (1975) proposed that, "opioid receptors existed in two conformational states: a sodium-free state that binds a given agonist and corresponding antagonist with about equal affinity, and a sodium-dependent state in which antagonist binds with a considerably greater affinity than corresponding agonists."

Conversely, Pert and Snyder (1974) suggested the Na^+ induced receptor conformation decreased the number of agonist binding sites and increased the number of antagonist sites. This hypothesis was supported by the observation of increase in [^{3}H]NAL binding sites (ie. antagonist binding sites) and a concurrent decrease in [^{3}H]dihydromorphine sites (ie. agonist binding sites) with the addition of Na^+. Studies by Simantov and coworkers (1976) supported the contention of Pert and Snyder that Na^+ induced a change in the density of opioid binding sites with a minimal change in affinity. These investigators reported an unmasking of "new receptors" when binding a [^{3}H] agonist or [^{3}H]antagonist in the presence of Na^+. Simantov et al.(1976) , in agreement with Pasternak and coworkers (1975) , suggested that this increase in density was due to sodium's ability to accelerate the rate of dissociation of an endogenous agonist-like

inhibitor of opioid binding which was tightly bound to a fraction of the receptors in freshly prepared membranes.

c. Possible reasons for inconsistent interpretations

Resolution of the exact mechanism by which Na^+ affects opioid binding has been complicated by several factors. Until Martin and coworkers (1976) discovered the presence of a heterogeneous population of opioid receptors, investigators had assumed the existence of only one type of opioid receptor. Today there are at least three well characterized opioid receptors that may each be regulated by sodium in a different manner. Another major factor that contributed to inconsistent results was the varied binding conditions used among the early investigators. Several investigators have demonstrated the large influence temperature and ionic concentrations have on opioid receptor binding; these binding conditions varied a great deal among the early studies. Sadee et al. (1982) observed that Na^+, Ca^{2+}, Mg^{2+}, Mn^{2+}, and Cu^{2+} exerted multiple effects on opioid antagonist binding. Na^+ increased on- and off-rates of [^{3}H]DIP binding with a small net change in receptor affinity. Conversely, physiological concentrations of Ca^{2+}, Mg^{2+}, Mn^{2+}, in that order of potency, decreased [^{3}H]DIP receptor dissociation and association rates. Cu^{2+} was the only divalent cation examined that abolished [^{3}H]DIP binding. The investigators suggested that Cu^{2+} was destroying the [^{3}H]DIP binding sites. However these studies did not further examine the Cu^{2+}-induced disappearence of [^{3}H]DIP binding sites. It is possible Cu^{2+}decreased the affinity of the labeled receptor, thus rendering it more difficult to measure binding. Alternatively Cu^{2+} could have induced internalization of the receptor, again leading to the observed decrease in [^{3}H]DIP binding. Although their mechanisms of action have not been elucidated, investigators have agreed that metal ions may exert multiple effects on opioid binding and are likely to regulate the function of opioid receptors *in vivo* (Chapman and Way, 1980).

Recently Paterson and coworkers (1986) compared the effect of four monovalent and three divalent cations on the μ, δ , and κ opioid receptor in guinea pig brain. All four monovalent cations tested, Na^+, K^+, Li^+, and NH_4^+ inhibited agonist binding at the μ, δ and κ receptor. Na^+ was the most potent at each site, although the relative potencies were receptor-dependent. This suggested that the mechanism by which these monovalent cations inhibit binding of the agonist is different for each receptor type. A detailed analysis of the effects of Na^+ on binding site affinities and densities was not reported. Regulation of agonist binding by divalent cations also differed depending upon the receptor examined. Mg^{2+}, Mn^{2+}, and Ca^{2+} potentiated binding of δ agonists. Conversely low concentrations of Mg^{2+} and Mn^{2+} slightly potentiated μ agonist binding but higher concentrations inhibited binding. Furthermore, the investigators reported that the ion effects are different when an [^{3}H] antagonist is bound to the μ, δ or κ receptor.

Guanyl nucleotide regulation of hormone binding and action

a. Hormone receptors stimulating adenylyl cyclase through the G_s protein

Since Rodbell and coworkers (1971) reported that guanyl nucleotides increased the apparent affinity of glucagon for adenylyl cyclase, concurrently decreasing the affinity of [^{125}I]-glucagon for hepatic glucagon receptors, several studies have reported guanyl nucleotide regulation of adenylyl cyclase in parallel with effects on radioligand binding. In 1975 Maguire and coworkers reported that binding of the β-adrenergic antagonist, [^{125}I]iodohydroxybenzylpindolol to β adrenergic receptors in two cultured cell clones was not affected by addition of GTP, GDP or the non hydrolyzable GTP analogue G(pp)NHp, whereas agonist binding was decreased. Similar observations were reported by Lefkowitz and coworkers (1976) who demonstrated a decrease in the agonist binding affinity to the β-

adrenergic receptor of frog erythrocyte membranes with the addition of guanyl nucleotides. This change was not observed for β-adrenergic antagonists, or for agonists in solubilized receptor preparations. It was concluded that occupation of the nucleotide regulatory sites caused an alteration in the β-adrenergic receptor conformation that could only occur when the receptor was coupled to adenylyl cyclase by virtue of agonist binding. The altered conformation resulted in a decreased agonist affinity for the receptor. However addition of GTP appeared to shift agonist dose-response curves for stimulation of adenylyl cyclase to the left. This indicated that GTP caused an increased functional efficacy for agonist in stimulating the enzyme. This latter observation and the fact that the adenylyl cyclase system in a variety of eukaryotic systems was found to be sensitive to guanyl nucleotides, prompted investigators to search for a role for guanyl nucleotides in adenylyl cyclase activation.

The use of the nonhydrolyzable analogues Gpp(NH)p, GTPγS, and Gpp(CH_2)p greatly facilitated this search. In 1975 several investigators studying various β-adrenergic systems observed that these nonhydrolyzable analogues could activate adenylyl cyclase by themselves, although in order for maximal activation of the enzyme, the nucleotide analogue and agonist must act in concert. The activation by the GTP analogues was reported to be quasi-irreversible, and after a short incubation period, neither the GTP analogue nor the hormone was required to maintain activity (Pfeuffer and Helmreich, 1975, Schramm and Rodbell, 1975, Lefkowitz et al., 1976). Conversely , the stimulation of adenylyl cyclase by GTP and hormone was completely reversible and enzyme activity required the presence of both (Salomon et al., 1975; Schramm, 1975).

It was thought that the difference between GTP and its analogues as activators of adenylyl cyclase was due to the analogues' resistance to hydrolysis. It was suggested by several investigators that the adenylyl cyclase system was coupled to a GTPase (Rodbell et

al., 1975; Pfeuffer and Helmreich, 1975). Although studies by Pfeuffer and Helmreich (1975) reported a GTPase to ATPase activity ratio of two in pigeon erythrocyte membranes and Salomon and Rodbell (1975) reported that GTP bound to liver membranes was degraded to GDP and GMP, there was not at the time direct evidence that GTPase activity was directly linked to adenylyl cyclase. However in 1976 Cassel and Selinger demonstrated the existence of a catecholamine-sensitive GTPase in turkey erythrocyte membranes. The investigators proposed that this GTPase was a component of the turkey erythrocyte adenylyl cyclase system: "We suggest that the catecholamine activated state of adenylyl cyclase has an increased ability to hydrolyze GTP. Nevertheless, the hydrolysis is slow enough to allow for an almost continuous presence of GTP at the regulatory site. Hydrolysis of GTP is required ultimately in order to allow the system to return to the basal inactive state."

In order to support this hypothesis, Cassel and Selinger (1977) measured agonist induced adenylyl cyclase activity in cholera toxin treated turkey erythrocytes. Cholera toxin had previously been shown to increase adenylyl cyclase activity in several different cell types (Kimberg et al., 1971; Sharp et al., 1971; Field et al., 1972; Gill, 1975). The investigators reported that cholera toxin concurrently inhibited the GTPase activity and enhanced the adenylyl cyclase activity. Furthermore when GTP was added to the cholera toxin treated membranes, the activation was similar to activation by nonhydrolyzable analogues in untreated membrane preparations. The investigators suggested that the regulation of adenylyl cyclase activity might be achieved not only by controlling the on-rate through the hormone receptor, but also by controlling the off-rate through the GTPase reaction.

These observations were further supported when Cassel and Selinger (1978) were able to demonstrate that the activation of adenylyl cyclase through the β-adrenergic

receptor by a hormone and guanyl nucleotide resulted in release of membrane-bound GDP. The investigators proposed the model, which is still accepted today, to explain hormone stimulation of adenylyl cyclase. The agonist binds to the receptor which causes the release of GDP from the stimulatory GTP binding component. GTP then binds to the stimulatory GTP binding component and subsequently activates adenylyl cyclase. The reaction is terminated by hydrolysis of GTP by the GTPase associated with the stimulatory binding component. This results in a termination of both the activation of adenylyl cyclase and the stimulatory binding component.

In 1978 Cassel and Pfeuffer reported that cholera toxin was responsible for the ADP-ribosylation of membrane components of cholera toxin treated turkey erythrocyte membranes. Extraction and purification of these membranes resulted in the identification of a 42 kDa molecular weight protein. Reconstitution of the 42 kDa protein into a system containing adenylyl cyclase without its GTP binding component, resulted in an activation of the enzyme. The investigators concluded cholera toxin was responsible for ribosylating the GTP-binding component of adenylyl cyclase. Subsequently, Northup et al. (1983) purified the stimulatory binding component G/F (initially named G/F, now named G_s, see footnote on page 11) from rabbit liver membranes. The investigators demonstrated that the purified G_s component reconstitutes guanyl nucleotide-, fluoride-, and hormone-stimulated adenylyl cyclase activity in the G_s deficient cyc^- variant of S49 murine lymphoma cells. The purified G_s was found to consist of three polypeptide subunits with approximate molecular weights of 52, 42, and 35 kDa.

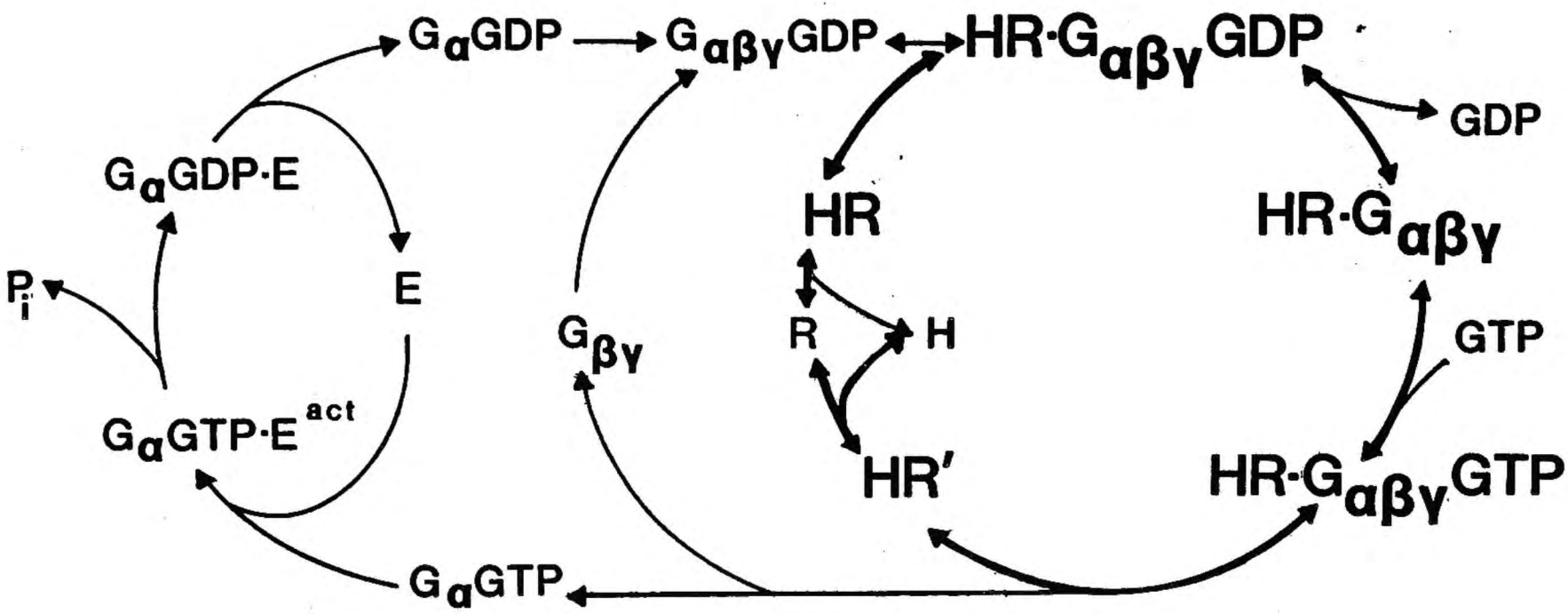
In 1983 Northup et al. separated the subunits of G_s protein and proposed interactions of the subunits (Fig 1). Reconstitution experiments indicated that the 45 kDa α subunit was responsible for activation of adenylyl cyclase whereas the β subunit (35 kDa) increased the deactivation of the α subunit. The investigators proposed that after an agonist

binds to a stimulatory receptor in the presence of GTP, the α,β and γ subunits of G_s dissociate. The α subunit stimulates adenylyl cyclase until hydrolysis of GTP occurs. At this time $G_s\alpha$ and the enzyme dissociate, and the β and γ subunits reassociate with the α subunit to render G_s incapable of further enzyme activation until reactivation by agonist occupied hormone receptors . Two forms of the $G_s\alpha$ subunit (Northup et al., 1980; Sternweis et al., 1981) have been isolated. Both have been expressed in Escherichia coli and are capable of activating hormone-, and GTPγS-, stimulated adenylyl cyclase activity to the same extent . At this date it is difficult to distinguish functional differences between the two isolated $G_{s\alpha}$ subunits (Graziano et al., 1987).

b. Hormone receptors inhibiting adenylyl cyclase through the G_i protein

Since the 1970s investigators have been aware of receptor systems that inhibit adenylyl cyclase activity. These include the α_2-adrenergic , muscarinic, D-2 dopaminergic, and opioid receptors. Initially investigators believed these receptors interacted with G_s to prevent its activation of adenylyl cyclase. However in 1980 Rodbell postulated the existence of a separate inhibitory binding component which they named "N_i " * (Rodbell, M., 1980). The existence of a separate inhibitory guanyl nucleotide binding protein, now named G_i, was confirmed by Cote et al. in 1982. They observed that Gpp(NH)p, the nonhydrolyzable GTP analogue, inhibited adenylyl cyclase in cholera toxin - treated tissue. The investigators assumed that cholera toxin was inactivating the GTPase associated with G_s, hence Gpp(NH)p must be working through a distinct G_i to inhibit adenylyl cyclase.

FIGURE 1. HORMONE/AGONIST-RECEPTOR-G BINDING PROTEIN INTERACTION CYCLE (MODIFIED FROM GILMAN, 1986). H, hormone or agonist; R, opioid receptor; HR, agonist-receptor complex; G, guanyl nucleotide binding protein composed of three subunits α, β, and γ; HR', post-dissociational agonist-receptor complex which may have a different affinity from that of HR; E, enzyme; E^{act}, activated enzyme; P_i, liberated phosphate; GTP, guanosine triphosphate; GDP, guanosine diphosphate.


$G_\alpha GDP$
$G_{\alpha\beta\gamma}GDP$
$HR \cdot G_{\alpha\beta\gamma}GDP$
GDP
$G_\alpha GDP \cdot E$
HR
$HR \cdot G_{\alpha\beta\gamma}$
P_i
E
R
H
GTP
$G_{\beta\gamma}$
$G_\alpha GTP \cdot E^{act}$
HR'
$HR \cdot G_{\alpha\beta\gamma}GTP$
$G_\alpha GTP$

In 1982 Katada and Ui demonstrated that pertussis toxin, when added to intact C6 glioma cells or membranes was able to ADP ribosylate a 41 kDa protein which the investigators hypothesized to be one of the subunits of the guanyl nucleotide regulatory component of the cyclase system. The pertussis toxin sensitive-protein was not identical to the protein ADP-ribosylated by cholera toxin.

Subsequently Northup and coworkers (1983) isolated a guanyl nucleotide binding component which appeared to be the substrate for ADP ribosylation by pertussis toxin in rabbit liver membranes. This component was distinct from G_s, the binding component associated with the stimulation of adenylyl cyclase.

Katada and coworkers (1984) proposed a mechanism to explain the agonist-mediated inhibition of adenylyl cyclase. This model suggested that after the agonist bound to the receptor, there was a dissociation of GDP from G_i and association of GTP with G_i. This caused G_i to dissociate into three subunits, α, β, and γ (Fig.1). The α subunit was predicted to be responsible for the inhibition of the enzyme. However termination of enzyme inhibition occurred following the hydrolysis of GTP by endogenous GTPases. The Gilman group (1984) proposed an alternative mode of inhibition in which the liberated β and γ complex of G_i interacted with $G_s\alpha$ to prevent activation of the enzyme since the

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*The terminology of these guanyl nucleotide proteins has been a matter of dispute. Initially both the stimulatory and inhibitory components were named the nucleotide binding components (N_s and N_i) since early investigators were not certain of their specificity for guanyl nucleotides. After Gilman and coworkers purified both components and reported their specificity in binding guanyl nucleotides, they proposed that they be identified as guanyl nucleotide binding components (G_s and G_i). The forms, G_s and G_i, will be used here. Subsequently other related guanyl nucleotide binding proteins have been identified. The α subunits of the G protein have different though related structures; the β and probably the γ subunits are very similar and can apparently interact with the α subunit of all of the G proteins so far identified.

and γ subunits of G_s and G_i were indistinguishable. An increase in GTPase activity with addition of opioid or other agonists that can inhibit adenylyl cyclase activity has been demonstrated (Koski et al., 1980).

c. Other G proteins and their possible functions: G_t and G_o

Studies examining the pathway for transduction of visual information have revealed the existence of another member of the G protein family, transducin or G_t. Transducin activates a cyclic GMP-specific phosphodiesterase which is ultimately responsible for visual excitation in the retinal rods (Kaupp and Koch,1986).

A fourth guanyl nucleotide binding protein, G_o, originally found in high concentrations in the brain (Rosenthal et al., 1985; Huff, et al., 1985), is the only G protein not associated with a particular function thus far. It has been suggested to interact with muscarinic cholinergic and phorbol ester binding sites (Hoffman et al., 1987). This G protein may also be involved in the functional coupling of δ opioid receptors to calcium channels in the NG108-15 cells (Herscheler et al., 1986; Herscheler et al., 1987). Receptor or GTPγS mediated inhibition of voltage-dependent Ca^{2+} channels in neurons where it is known that cyclic AMP is not involved in channel regulation has been demonstrated (Scott and Dolphin, 1986; Holz et al., 1986). There is evidence suggesting that several neurotransmitter receptors are functionally coupled to ion channels via a guanyl nucleotide binding protein. Using the whole cell patch clamp method to record Ca^{2+} currents in the NG108-15 cells, Herscheler and coworkers (1987) were able to study the effects of opioids, pertussis toxin and G proteins on Ca^{2+} currents. It was reported that DADLE reduced Ca^{2+} conductance in these cells through the δ opioid receptor activation. Pretreatment with pertussis toxin completely abolished the inhibitory effect of DADLE. Intracellular applications of G_i and G_o to the pertussis toxin treated cells restored the ability of DADLE to inhibit the Ca^{2+} channels. The α subunit of G_o was 10 times more potent

than G_i, leading investigators to believe that G_o was involved in the functional coupling of opioid receptors to calcium channels. Norepinephrine, dopamine and GABA agonists are responsible for inhibition of Ca^{2+} channels in the dorsal root ganglia (Dunlap and Fischbach, 1981; Deisz and Lux, 1985; Marchetti et al., 1986). This inhibition is also blocked by pertussis toxin, again suggesting an involvement of a pertussis toxin-sensitive G protein in regulation of neuronal Ca^{2+} channels (Scott and Dolphin, 1986; Holz et al., 1986).

In search of a physiological role for G_o, investigators examined the effects of guanyl nucleotide regulation on the phospholipase C system. As early as 1974 investigators reported the ability of guanyl nucleotides to regulate agonist binding to receptors responsible for activation of phospholipase C, ultimately leading to the mobilization of intracellular Ca^{2+} (Glossman et al., 1974). In 1985 Litosch and coworkers demonstrated that calcium mobilizing receptors were coupled to phospholipase C through a G protein. In an attempt to identify the G protein, investigators measured the ability of an agonist to increase intracellular levels of Ca^{2+} in the presence of cholera toxin or pertussis toxin treated cell membranes (Barrowman et al., 1986; Verghese, et al., 1985; Monreno et al., 1983; Okajima and Ho, 1984). Cholera toxin did not affect the ability of the agonists to increase Ca^{2+} levels, suggesting G_s was not involved in activation of phospholipase C. On the other hand, reports involving pertussis toxin-treated cells yielded inconsistent results. Several investigators reported pertussis toxin was able to inhibit coupling of Ca^{2+} mobilizing receptors to phospholipase C in neutrophils, adipocytes, mast cells and human poly-morphonuclear leukocytes (Barrowman et al., 1986; Verghese et al., 1985; Nakamura and Ui, 1985; Bradford and Rubin, 1985; Monreno et al., 1983; Okajima and Ho, 1984). These results implied that G_i linked the receptor to the phospholipase system in these cells. However, other studies found pertussis toxin to be ineffective at attenuating agonist or guanyl nucleotide stimulation of phospholipase C (Masters et al., 1985; Martin et

al., 1985; Martin et al., 1986; Aub et al., 1986). In contrast to the above results, these findings would imply that the G protein coupled to Ca^{2+} mobilizing receptors was different from the pertussis toxin-sensitive G_i or G_o. Recently, Kikuchi et al. (1986) demonstrated that reconsitution of pertussis toxin-treated HL60 cell membranes with either G_i or G_o resulted in restoration of fMet-Leu-Phe receptor -stimulated formation of inositol phosphate. These results indicated that both rat brain G_i and G_o may be involved in the mechanism of signal transduction. Today the identity of the G-protein responsible for linking Ca^{2+} receptors to phospholipase C activation is still the subject of considerable speculation. Several factors have contributed to the difficulty. Firstly, agonists commonly employed to activate phospholipase C may have effects on adenylyl cyclase activity (presumably working through G_i). In this case investigators can not rule out the possibility that changes in Ca^{2+} mobilization are due to the direct inactivation of G_i, but may be caused by indirect effects on other G proteins and/or effector systems not directly related to the phospholipase system. Secondly pertussis toxin has been used extensively as a tool to elucidate the possible role G_i plays in the phospholipase C system. Recently investigators reported the sequence of amino acids of the α subunits of several G proteins show homology at the site of pertussis toxin action (Itoh et al., 1986; Sullivan et al., 1986; West et al., 1985). This would suggest the demonstration of pertussis toxin sensitivity does not necessarily insure the involvement of G_i or G_o. In summary, the G protein responsible for receptor-mediated activation of phospholipase C has not yet been identified. Results indicate it may be G_i , G_o, or a novel G protein given the designation, G_p.

Several other neurotransmitter receptors have been found to be directly coupled to an ion channels through a G protein. These include the acetylcholine receptor in guinea pig cardiac muscle (Soejima and Noma, 1984; Breitwieser and Szabo, 1985; Pfaffinger et al., 1985); the $GABA_B$ receptor in rat hippocampus (Andrade et al.,1983); the α_2-adrenergic receptor (North et al., 1987) and δ opioid receptor (North et al., 1987) in guinea pig

submucous plexus; and the μ opioid in rat locus coeruleus (North et al., 1987). Although G proteins are implicated in direct regulation of ion channels, at present it is not clear which G proteins are involved.

Guanyl nucleotide regulation of opioid binding

a. Brain membranes

Early studies examining the effects of various guanyl nucleotides on opioid agonist and antagonist binding in rat brain homogenates reported the addition of G(pp)NHp , GTP, or GDP caused a reduction in ligand affinity for the opioid receptor. In this study measurement of ligand dissociation suggested that guanyl nucleotides accelerated the dissociation rates of both agonists and antagonists (Blume, 1978). Subsequent studies have usually found that guanyl nucleotide effects were restricted to changes in the agonist dissociation rate. Blume et al. (1978), Childers and Snyder (1979) reported differentiation by guanyl nucleotides of opioid receptor binding for several agonists and antagonists. The investigators reported a "selective reduction of opioid agonist binding displayed by GTP, Gpp(NH)p, and GMP but not by ATP, ADP or AMP." The decrease was thought to be due to acceleration of the dissociation rate of the agonist . This latter observation coincided with reports of accelerated dissociation of agonist from the β-adrenergic receptor (Williams and Lefkowitz, 1977), the dopamine receptor (Zahniser N.R. and P.B. Molinoff., 1978; Creese and Snyder, 1978), the α-adrenergic receptor (U'Prichard and Snyder, 1976), the serotonin receptor (Peroutka et al., 1979), and the glucagon receptor (Rodbell et al., 1971; Lin et al., 1977) in the presence of guanyl nucleotides. In each case, investigators reported no change in [^{3}H]antagonist binding in the presence of guanyl nucleotides. In 1984 Ishizuka and coworkers investigated the extent to which agonist binding to each class of opioid receptor was regulated by guanyl nucleotides . As previously reported by Childers and Snyder (1978), antagonist affinity at the μ , κ and δ receptor was not affected by addition of GTP. However agonist affinity was decreased at both μ and κ receptors.

Interestingly, the extent of the decrease varied for each receptor type. However, these studies can be criticized for the lack of specificity in the attempted labeling of each type of opioid receptor.

In order to further explain differences in guanyl nucleotide modulation of opioid agonist and antagonist binding, Spain and Coscia (1986) measured rates of [^{3}H]DADLE, the opioid agonist, dissociation in the presence of Gpp(NH)p from δ opioid receptors in bovine hippocampal synaptic plasma membranes after varying periods of incubation with agonist. These studies yielded complex curves, suggesting a multistep process involving the existence of several kinetic intermediates. Three affinity states of the ligand/receptor complex were proposed the 1) ligand-receptor complex (LR), this initial kinetic state displaying a relatively low affinity 2) LR*, a somewhat higher affinity state detected after approximately 7 minutes, and 3) ligand/receptor/G protein complex, displaying a higher affinity and which were detected after 60 minutes of incubation with agonist. Of the three affinity states detected, two were affected by the addition of guanyl nucleotides, LR* and the proposed ligand/receptor/G protein complex. This would suggest that GTP may act at more than one regulatory site. On the other hand, LR, the initial affinity state, was not affected by the addition of guanyl nucleotides. For this reason, the LR complex was thought to be associated with the antagonist binding site. It was proposed that agonists initally bind to LR and then proceed to higher affinity binding states. These higher binding states, LR* and the ligand/receptor/G protein complex, are both regulated by guanyl nucleotides, thus rendering agonist binding sensitive to GTP.

b. NG108-15 cell membranes

Increasing awareness of multiple types of opioid receptors and the possible differential regulation of these types led to the recognition of the necessity to investigate the regulation of opioid binding in a tissue containing a homogeneous population of opioid

receptors. The NG108-15 hybrid cell line were chosen to examine guanyl nucleotide regulation of the δ opioid receptor. Subsequently results from these experiments have been compared to findings obtained in the brain as well as other receptor systems.

In 1979 Blume and coworkers reported an increased dissociation of the opioid-receptor-bound complex in the presence of guanyl nucleotides in the NG108-15 cells . Chang and coworkers (1981) further expanded on this observation by comparing GTP effects on agonist binding to the μ receptor in rat brain and the δ receptor in NG108-15 cells. These investigators also reported an increased dissociation rate of agonist from the μ receptor in rat brain and the δ receptor in the NG108-15 cells and membranes in the presence of GTP. Law et al. (1982) demonstrated the existence of multiple affinity states of the δ receptor in the NG108-15 cells. These multiple states were agonist-specific and were only observed under conditions in which coupling between binding sites and G_i was promoted. The investigators suggested that these multiple affinity states may reflect the interaction of the receptor and other membrane components involved in opioid regulation of adenylyl cyclase. These agonist-specific multiple affinity states have also been observed in acetylcholine receptors of NG108-15 cells (Klein et al.,1982), the β-adrenergic receptor in two clonal lines of cultured cells (Maguire et al., 1975; Lefkowitz et al, 1976), platelet α-adrenergic receptors (Tsai and Lefkowitz, 1979; Hoffman et al., 1982) and the serotonin receptors (Peroutka et al., 1979).

Effects of Chronic Agonist Treatment

Since the late 1960's, the biochemical basis for development of tolerance and physical dependence on narcotics has been the subject of much speculation and experimentation. In 1970 Cochin suggested that the body reacts to narcotic drugs by increasing the rate at which they are destroyed, thus causing smaller proportions of the drug to actually reach the site of action. In 1973 Schulz and Goldstein suggested that processes which are directly

affected by the narcotic analgesics are circumvented or otherwise accommodated through an indirect physiological adaptation. Collier (1980) postulated that tolerance was due to a direct adaptation which affects either the number of receptor sites or the affinity of the narcotic for the receptor. Over the past decade, behavioral and biochemical studies have been employed to elucidate the mechanism of tolerance and a wide range of mechanisms have been invoked. Biochemical studies suggested increases in adenylyl cyclase (Collier H.O.J., 1974); up-regulation of brain calcium channels (Rambumar and El-Fakahany, 1984); and increases in calmodulin levels in specific brain areas (Bonnet et al., 1982) upon chronic morphine treatment.

In order to detect changes in receptor characteristics during tolerance, radioligand binding studies have been used. The use of clonal cell lines have greatly facilitated research concerning the adaptative processes occurring during chronic agonist exposure. Studies employing cell lines have concluded that the number and functional properties of the membrane receptors are generally controlled by the concentration of receptor-active ligands reaching the cell surface. Changes in the receptivity of cells to hormones are most likely important control mechanisms involving amplification or diminishment of hormone induced cellular responses . Extensive studies examining both radioligand and functional studies for the δ opioid receptor in NG108-15 cells (Law et al., 1982, 1984) and the β-adrenergic receptor in frog erythrocytes (Lefkowitz et al., 1980; Su et al., 1980) would indicate at least three time-dependent processes, each involving the coupled state of the receptor and exhibiting unique kinetic properties, occur following chronic agonist exposure. The first is receptor desensitization described as the selective loss of the inducing agent's ability to exert an effect on the system. This change is usually associated with a decrease in the agonist affinity for the receptor. The second step occurs later and involves receptor down-regulation. The third step involves an increase in adenylyl cyclase activity after withdrawal of a chronically administered agonist.

Desensitization and Down-Regulation

a. Nonopioid systems

Over a number of years, many studies have demonstrated that chronic treatments of tissues and cells with many kinds of agonists results in a reduction in the number of receptors through which the agonist acts. This phenomenon is known as "down-regulation." Noble et al. (1978) reported that the acetylcholine receptors of a mouse muscle cell line are down-regulated by prolonged treatment with a cholinomimetic ligand. Cooper and coworkers (1978) reported a similar phenomenon for the α-adrenergic receptor of human platelets after chronic treatment with various α-adrenergic agonists. Su and coworkers (1980) and Lefkowitz and coworkers (1980) reported an agonist-induced multistep desensitization process of the β-adrenergic in 1321N1 astrocytoma cells. These investigators reported exposure of 132N1N1 cells to isoproterenol resulted in a loss of responsiveness of adenylyl cyclase to catecholamines (desensitization) followed by a loss in β-adrenergic receptors (down-regulation). In 1981 Marshall and coworkers reported an *in vitro* insulin-induced loss of insulin receptors on rat adipocytes. The apparent ubiquity of receptor down-regulation induced by sustained agonist exposure suggested that similar events might follow prolonged activation of opioid receptors.

b. Opioid systems

Early studies investigating opioid receptor changes upon chronic agonist treatment yielded conflicting results. In 1973 Klee and Streaty compared [^{3}H]dihydromorphine, a μ agonist, binding in brain homogenates from control and morphine - dependent rats, and concluded that in both cases binding characteristics were indistinguishable. Displacement of [^{3}H]dihydromorphine by NAL also yielded similar results. On the basis of these observations, it was concluded that morphine tolerance was not the result of an alteration in

the number or nature of the specific receptor in rat brain. Similar results have been observed by Lee and coworkers (1976), Pert et al. (1975), Hitzemann et al. (1979) . Cox and Padhya (1977) studied the ability of opioid drugs to inhibit the release of acetylcholine from neurons in the myenteric plexus of ileum preparations from normal and morphine pretreated guinea pigs, in addition to measuring stereospecific binding of [^{3}H]ET in these preparations. The investigators reported pretreatment of guinea-pigs with morphine resulted in a reduction in the ability of lower concentrations of opioid drugs to inhibit electrically stimulated contractions of ileum preparations, although the maximum opioid effect was unchanged. Conversely binding studies indicated tolerance to the opioid effect was not accompanied by a change in the affinity or the number of stereospecific binding sites for [^{3}H]ET. Thus early studies of opioid tolerance failed to provide evidence of receptor changes associated with tolerance. However measurement of functional opioid receptor reserve with irreversible antagonists suggested a reduction in the number of functional receptors in opioid tolerance. Chavkin and Goldstein (1984) found that the fraction of spare receptors was reduced in opioid tolerant guinea pig ileum preparations.

Other studies have demonstrated changes in opioid receptor binding that appeared to be related to prior drug treatment. However, explanations unrelated to the processes underlying tolerance and dependence probably explain these effects. Thus, Pert and Snyder (1974) reported that administration of either opioid agonist or antagonist to rats produced a 50-100% enhancement of [^{3}H]dihydromorphine and [^{3}H]NAL binding to rat brain homogenates. This enhancement was seen after 5 minutes, and antagonists were 10-100 times more potent in eliciting it. It was suggested that the inability of Klee and Streaty (1973) to observe this enhanced binding was due to differences in tissue preparations. Pert and Snyder (1975) agreed with Pasternak and coworkers (1975) in proposing that opioids displace an endogenous morphine-like inhibitor from opioid receptors, thereby causing an apparent increase in the number of binding sites rather than a change in receptor affinity.

Davis and coworkers (1975) reported that chronic morphine, ET or NAL treatment reduced opioid binding to slices by altering the affinity and positive cooperativity of the binding sites. This decreased agonist affinity has also been demonstrated by Way et al. (1969); and Takemori et al. (1975). However washout of the tolerance inducing drug prior to assay of the receptors was not clearly demonstrated in these studies. If residual morphine, ET or NAL was present in their binding preparation, it is not unexpected that a decrease in apparent affinity for agonists was observed.

In 1986 Rogers and El-Fakahany suggested that the use of brain homogenate preparations may impede the demonstration of tolerance-related changes in receptor function. The investigators argued that homogenization disrupted cellular integrity, allowed cytoplasmic contents to come in contact with membrane-bound receptors, and disrupted unstable components of the membrane-bound receptor-effector chain. All of these factors may ultimately affect receptor conformation. The investigators suggested an alternate tissue preparation which involved the use of intact adult rat brain cells, processed in physiologic buffers. Endogeneous and/or exogeneous residual opioids were then removed by extensive washing in sodium-rich buffers. They reported a decrease in [^{3}H]NAL binding in intact cell preparations from tolerant rats, with no change in receptor affinity, thus supporting the view that a reduction in the number of cell surface opioid receptors may occur after chronic agonist treatment. The results obtained in previous opioid binding studies involving tissue homogenization may not have shown this since internalized receptors become accessible to labeled ligand after cell disruption.

Early studies of opioid tolerance clearly demonstrated a loss in the ability of agonist-induced activation to induce a pharmacological response, although it was more difficult to identify changes in receptor binding properties (Cox and Padhya, 1977; Lee et al., 1977; Pert et al., 1975; and Hitzemann et al., 1974). These studies suggested that in the opioid

system desensitization could occur without down-regulation. More recently, studies examining the cellular changes following chronic agonist exposure to the NG108-15 cells, indicate an initial loss in opioid inhibition of adenylyl cyclase activity followed by a significant decrease in the number of δ receptors. These results suggested that both processes occur at the δ receptor upon chronic opioid exposure. Desensitization and down-regulation have not been previously identified in tissues containing a homogeneous population of μ opioid receptors.

Adenylyl cyclase activity upon withdrawal of treatment drug

Aside from desensitization and down-regulation, prolonged exposure of tissues or cells to antagonists may cause an additional phenomenon. A modification in adenylyl cyclase activity was first reported in 1975 upon chronic opioid agonist exposure in the NG108-15 cell (Traber et al., 1975; Sharma et al., 1975). After prolonged exposure to an agonist, investigators observed a 2-fold increase in enzyme activity following withdrawal of the agonist. Several groups have proposed that alterations in adenylyl cyclase during chronic agonist exposure may be a possible biochemical mechanism involved in the development of drug dependence (Sharma et al., 1975; Brandt et al., 1976; Lampert et al., 1976). Recent observations indicate enhancement in adenylyl cyclase activity in a variety of clonal cell lines. It is not certain, however, whether this phenomenon is a critical biochemical correlate of opioid dependence since some studies of chronic narcotic treatment have found no changes in enzyme activity following withdrawal of the treatment drug.

a. Nonopioid systems

Subsequent studies examining the effects of non-narcotic agents on enzyme activity indicate the occurrence of a supersensitive adenylyl cyclase enzyme is not confined to cells containing opioid receptors. Following withdrawal after prolonged agonist exposure, several investigators have reported enhanced cyclase activity in cells containing somatostatin receptors (Reisine et al., 1984; Heisler and Srikant, 1985); muscarinic

cholinergic receptors (Heisler et al., 1985; Traber et al., 1975; Green and Clark, 1981; Meeker and Harden, 1983); α_2-adrenergic receptors (Sabol and Nirenberg, 1979; Northam and Mobley, 1985; Jones et al., 1987); and serotonin receptors (Berrie-Kravis and Dawson, 1985).

b. Opioid systems

NG108-15 hybrid cells have facilitated research concerning the adaptive processes occurring during chronic opioid exposure. Early studies examining supersensitive adenylyl cyclase activity induced by NAL have yielded conflicting results. The first investigators to report enhanced enzyme activity, following removal of the treatment drug, reported an approximate 80% increase in both basal and PGE_1-stimulated adenylyl cyclase (Traber et al., 1975; Sharma et al., 1975). Later studies reported a much smaller (30-40%) enhancement (Griffin et al., 1983). On the other hand, Wuster and coworkers reported no change in the cyclase activity upon addition of NAL to desensitized NG108-15 cells (1983). In order to resolve this apparent inconsistency, Musacchio and Greenspan (1986) examined the NAL precipitated rebound response in NG108-15 cells which had been exposed to morphine, ET and vehicle. These investigators concluded that the extent of enhanced enzyme activity observed following the addition of NAL, depended upon the dissociation rate of the treatment drug. Cells treated with ET, a relatively hydrophobic drug with a slow dissociation rate, did not exhibit as great a rebound response as cells exposed to morphine unless procedures designed to facilitate rapid removal of the retained ET were employed.

Proposed biochemical mechanisms involved in development of tolerance

a. Desensitization

The exact mechanism of desensitization is not known. Elucidating underlying mechanisms involved in this process are further complicated by the existence of two different types of desensitization. Homologous desensitization occurs if, upon sustained

exposure, activators working through a receptor linked to a specific effector system become less effective in causing an effect. However, activators mediating their effects on the same effector system through different receptor types are not affected. Heterologous desensitization occurs when prolonged exposure to a receptor specific - activator of a particular effector system, reduces the efficiency of agonists acting via other receptor types linked to the same effector system. Several biochemical hypothesises have been proposed to account for these phenomena.

Parenti and coworkers (1981) have postulated that chronic opioid treatment causes a significant decrease in low K_m GTPase activity in rat striatal membranes. Therefore, the compensatory mechanism, produced after long term treatment with opioids, could be the result of a prolonged "survival" of GTP bound at the nucleotide regulatory protein, leading to a more efficient activation of adenylyl cyclase after removal of the opioid agonist. Parenti et al. (1981) proposed that the decreased activity of the GTPase may be the initial event leading to opioid tolerance.

In 1983 Wuster and coworkers observed that desensitization (defined as the loss of inhibitory activity of opioids upon cAMP accumulation) in the NG108-15 cell was dose-dependent, non-competitive and highly selective (1983). The investigators noted that the opioid-induced desensitization was not accompanied by a cross-desensitization to other inhibitory hormone receptors, muscarinic and α_2-receptor, found on the NG108-15 cell. Assuming all membrane receptors controlled a common adenylyl cyclase pool (Sabol and Nirenberg, 1979) and all receptors were acting through the same G_i to inhibit adenylyl cyclase, the investigators concluded that opioid-induced desensitization must be caused by an impaired interaction of the receptor with the G_i protein. Further studies supported this hypothesis by reporting similar kinetics of pertussis toxin - induced desensitization to that induced by chronic opioid exposure.

In 1986 Vachon et al. measured the time course of GTPase and adenylyl cyclase desensitization to DADLE and compared it with the change in opioid receptor binding characteristics resulting from chronic DADLE exposure. Results indicated that GTPase and adenylyl cyclase desensitize at different rates. The opioid-GTPase systems undergoes a much more rapid loss of responsiveness as compared to the rate of desensitization measured for adenylyl cyclase activity. The desensitization of GTPase activity and the loss in opioid receptor binding were differentiated into a fast and slow exponential component, whereas the loss in the activity of adenylyl cyclase activity decayed as one slow exponential component. A comparison of the half-lives of these events suggested the rapid phase of desensitization of GTPase activity, was probably due to uncoupling of the receptor from its G protein. On the other hand, the slower phase of GTPase desensitization and the loss of adenylyl cyclase activity were accounted for by down-regulation of the opioid receptor. In order to explain these differences, Vachon et al. (1986) offered two different interpretations. The first hypothesized that the proportion of GTPase activity actually involved in the inhibition of adenylyl cyclase activity was very small and that the δ receptor may be coupled to another effector system in the NG108-15 cells. Subsequent studies showing δ receptor mediated inhibition of Ca^{+2} conductance in NG108-15 cells (Herscheler et al., 1986) provides some direct support for this hypothesis. The second suggested that the interaction between GTPase and cyclase involved an amplification mechanism. In this case, the loss of responsiveness of cyclase to an agonist was a consequence of the desensitization of GTPase activity. These studies suggest that desensitization rates for different consequences of opioid receptor activation may not be identical.

More recently Sibley and coworkers (1986) have proposed a mechanism to explain desensitization of the β-adrenergic receptor following prolonged exposure to isoproterenol

in frog erythrocytes. Studies involving purified β-adrenergic receptor indicate that after prolonged agonist exposure, the receptor becomes phosphorylated by specific β-adrenoceptor kinase (βARK). The phosphorylated receptor is much less effective in interacting with guanyl nucleotide binding proteins, hence there is a decrease in the ability of the agonist to stimulate adenylyl cyclase activity. βARK has been implicated in desensitization of the β-adrenergic receptor, although the receptor also contains phosphorylation sites for other protein kinases. The existence of a cAMP dependent protein kinase has been implicated in heterologous desensitization. Unlike βARK, this kinase will phosphorylate all receptors linked to adenylyl cyclase through G_s. In the case of the β-adrenergic and prostaglandin receptor, sustained exposure of frog erythrocyte cells to either a β-agonist or prostaglandin, resulted in reduced ability of either drug to activate cyclase. In this situation, cAMP dependent protein kinase was responsible for phosphorylation of both receptors (Benovic et al., 1986). As is the case with βARK, receptors phosphorylated by cAMP dependent protein kinase are not as efficient in activating G proteins.

b. Down-regulation

In 1979 Hazum and coworkers demonstrated the existence of clusters of opioid receptors on the cell surface of NG108-15 cells using a fluorescent derivative of enkephalin, Tyr-D-Ala-Gly-Phe-Leu-Lys-rhodamine, to observe the location of opioid receptors on the cell surface. The formation of patches of receptors occurred ten minutes after the addition of the fluorescent ligand and reached their maximum number after a 40 minute incubation period. Careful examination of the patches and the fact that they decreased after thorough washing of the cells, suggested that the receptors remained on the cell surface and did not internalize during the incubation period. Investigators speculated that either patches occurred naturally on the membrane surface or the distribution of cell surface receptors was altered as a result of binding. The addition of 100 mM NaCl greatly

decreased the number of patches observed, which is consistent with binding studies in which [^{3}H]agonist binding to NG108-15 cells is decreased in the presence high concentrations of NaCl.

In 1983 Law et al. proposed an internalization mechanism to explain the down-regulation of the δ opioid receptor observed after chronic treatment in the NG108-15 cells. Evidence for internalization was based on previous cases in which receptor internalization had already been reported (Law et al., 1982; Brown et al., 1983). Down-regulation of the opioid receptor was found to be agonist concentration-dependent, time and temperature-dependent , and blocked by metabolic inhibitors. Law et al. (1984) demonstrated that down-regulation was not due to an increase in the degradation rate or decrease in the synthesis rate of the receptor, since the protein inhibitor cycloheximide did not potentiate agonist-induced receptor-down-regulation and inhibition of lysosome degradation of receptors with chloroquine did not prevent internalization of [^{3}H]DADLE. However, chloroquine treatment resulted in an accumulation of internalized [^{3}H]DADLE. This suggested that subsequent to internalization, the receptor is normally delivered to the lysosomes where it is degraded. Subsequent studies examining agonist-induced down-regulation of the δ opioid receptor (Law et al., 1985), muscarinic cholinergic and α_2-adrenergic receptors (Thomas and Hoffman, 1986) following pertussis toxin treatment, reported decreases in receptor density following prolonged agonist exposure. This would indicate that the G_i does not have to be functionally coupled to the receptor for down-regulation to occur. The fate of internalized opioid receptors is not known. Extensive studies examining the down-regulation of the β-adrenergic receptor indicate phosphorylation by βARK causes the receptors to be relocated into a sequestered region of the membrane (Chang and Costa, 1979). These receptors are then either dephosphorylated in which case they may return to the plasma membrane, or degraded by lysosmal enzymes.

It is possible that similar events occur at opioid receptors, although phosphorylation of opioid receptors has not yet been demonstrated.

c. Adenylyl cyclase supersensitivity

The mechanism involved in the compensatory increase in basal adenylyl cyclase following withdrawal of chronically administered opioids is not known. Early investigators postulated the existence of two or more forms of the adenylyl cyclase enzyme (Constantopoulos,1978; Sharma et al., 1977). More recent studies examining levels of enzyme activity in pertussis toxin treated cells report the necessity of a coupled opioid receptor to observe enhanced cyclase activity following removal of the treatment drug (Thomas and Hoffman, 1986). Thus investigators postulate changes in the enzyme activity may be due to alterations in the guanyl nucleotide binding protein, or in the catalytic unit of adenylyl cyclase. These alterations may include phosphorylation or other covalent modifications (Thomas and Hoffman, 1987).

4. Dependence

More recent studies examining in vitro preparations, have reported the occurrence of tolerance without dependence. Studies involving isolated mouse vas deferens and locus coeruleus slices reported extremely high degrees of opioid tolerance with no signs of dependence (i.e. no withdrawal sign; Schulz et al., 1973, 1980 ; Andrade et al., 1983; Christie et al., 1987). These studies led investigators to suggest that dependence was not necessarily correlated with the development of tolerance in *in vitro* preparations. In contrast, in studies involving intact animals, both tolerance and dependence to opioids are generally observed (Aghajanian, 1978; Cicero and Meyer, 1973; Way et al., 1969; Yano and Takemori, 1977; Cheny and Goldstein; 1971; Martin and Jasinski, 1969) .

Investigators have hypothesized two different adaptative mechanisms. Selective tolerance is commonly observed in in vitro preparations where the nervous system is not intact. In this case, dependence is not commonly observed. In order to observe dependence, the nervous system must be intact and associated with a signal transmission system. These observations led investigators to conclude "tolerance and dependence may be initiated by different biochemical mechanisms located at distinct neuronal levels." Opioid tolerance may be produced by an uncoupling of certain populations of opioid receptor binding sites from their G proteins, consequently resulting in failure to transmit inhibitory signals to various effectors. On the other hand, dependence may occur after a disruption (ie. hypersensitivity of the cyclase enzyme) in any localized mechanism involved in the signal transmission of an opioid-affected pathway (Wuster et al., 1985). The idea that a network of opioid sensitive neurons must remain intact in order to observe both tolerance and dependence is probably not correct, since both phenomena have been observed in systems such as the NG108-15 cells (Sharma et al., 1977) where there is no evidence to indicate the establishment of interacting networks of neurons.

Alternatively dependence may involve the development of homeostatic adjustments which tend to overcome alterations in cellular function induced by the continual presence of treatment drug. Such adjustments may be changes in receptor structure, membrane properties or receptor regulated enzyme systems. In 1985 Griffin et al. proposed a theory to explain enhancement of adenylyl cyclase activity observed after withdrawal of chronic agonist exposure in NG108-15 cells. The investigators hypothesized that the catalytic unit of adenylyl cyclase was under tonic regulation by both G_i and G_s. Upon chronic opioid exposure, the tonic inhibitory regulation of cyclase was lost resulting in the unopposed expression of G_s. Further work is clearly needed to elucidate the mechanisms responsible

for the enhancement of adenylyl cyclase activity after opioid withdrawal in NG108-15 cells.

The NG108-15 cells have greatly facilitated our understanding of the mechanisms involved in the development of tolerance and dependence. These cells were advantageous in that they contain a homogeneous population of δ opioid receptors linked to adenylate cyclase. Thus it has been possible to measure changes in the δ receptor following chronic opioid exposure. However studies examining cellular adaptative changes in tissues containing a homogeneous population of μ opioid receptors would be more clinically relevent, in that most clinically used opioid analgesics as well as many drugs of abuse (ie. morphine, heroin and fentanyl) have preferential affinity for the μ receptor. Thus it is the purpose of this study to examine regulation of μ receptor function, and the effects of chronic μ receptor activation. The availability of the 7315c cells makes this possible.

MATERIALS AND METHODS

A. Maintance of NG108-15 cells and preparation of membranes

NG108-15 cells were placed in 75 cm^2 plastic tissue culture flasks containing Dulbecco's Minimum Essential Medium (DMEM; for composition of DMEM, see Appendix), 0.1μM hypoxanthine, 10μM aminopterin, and 17 μM thymidine, 2 nM glutamine, 0.1M glucose, and 10% fetal calf serum (FCS). The cells were maintained in a 37° humidified atmosphere of 10% CO_2 and 90% air. Cells were removed from the flasks by discarding the growth medium and rinsing the flask with the appropriate buffer. The cell suspension was then decanted into plastic tubes and centrifuged at 450 rpm (40 x g) for 2 minutes. For studies on intact cells, the cells were rinsed twice in the appropriate buffer (in the presence of sodium: 25 mM HEPES, 118 mM NaCl, 4.8 mM KCl, 1.2 mM $MgCl_2$, 2.5 mM $CaCl_2$. In the absence of sodium: 25 mM HEPES, 118 mM N-methyl D-glucamine (N-MG), 4.8 mM KCl, 1.2 mM $MgCl_2$, 2.5 mM $CaCl_2$, pH adjusted 7.4) before resuspension at the required dilution. For studies with cell membranes, the 450 rpm pellet was disrupted by homogenization in the appropriate modified Krebs buffer, using a Teflon-glass homogenizer (four passes at speed setting 70 on T-Line laboratory stirrer; Thomas Scientific, Philadelphia, PA). The suspension was then washed twice by centrifugation at 10,000 x g for 15 minutes.

B. 7315c cells and membranes

1. Propagation of the 7315c tumor

The original source of rats bearing the 7315c tumor was a gift from Dr. Tom Cote. The tumor, weighing approximately 5 grams, was removed and minced in 10 ml of Earle's Balanced Salts without glutamine solution (EBSS or Medium 199; Gibco Laboratories, Grand Island, N.Y., for composition, see Appendix) . Approximately 200 μl of the tumor suspension was then injected i.p. into each recipient female Buffalo rat (NCI, Frederick,

MD.; weighing 150 - 175 g). After 21 days the tumor was removed for further propagation for binding and adenylyl cyclase assays.

2. Preparation of 7315c membranes and cells

Approximately 2 - 5 g of tumor tissue were excised from the peritoneal cavity of a rat carrying the 7315c tumor. The tissue was minced finely with a razor blade in 10 ml of EBSS supplemented with 0.25% BSA (EBSS/0.25% bovine serum albumin (BSA)). The suspension was filtered through gauze into a 50 ml plastic tube and centrifuged at 200 x g for 5 minutes. This procedure was repeated twice resulting in a pellet which consisted of a layer of cells on top of heavier tissue fragments and red blood cells. The layer of cells was removed with a pipet and transferred to another centrifuge tube. Additional cells were obtained by resuspending the heavier tissue fragments in EBSS/0.25% BSA and repeating the above procedure. The second cell suspension was pooled with the first and centrifuged at 200 x g for 5 minutes. The pellet was resuspended in EBSS/0.25% BSA and the suspension layered in EBSS/ 4% BSA. The cell EBSS/ 4% BSA solution was centrifuged at 200 x g for 5 minutes to further remove the red blood cells. The resultant pellet consisted of 7315c cells free of red blood cells or other tissue fragments. The cells were rinsed twice in the appropriate buffer before resuspension at the required concentration. An aliquot of the final cell suspension was diluted in EBSS/0.25% BSA and stained with 0.4% Trypan Blue. Cells were examined under the microscope and the viability of the 7315c cells during various incubation periods was determined. The fact that trypan blue stain has a greater affinity for serum proteins than cellular proteins allows one to differentiate viable (possessing an intact membrane) from nonviable cells. Since nonviable cells stain completely blue and viable cells are only outlined in blue it is possible to count the number of cells using a hemocytometer and obtain an estimate of % viability.

Membranes were prepared from cells by resuspension in 20 volumes of a solution containing 6 mM Tris HCl (pH 7.4), 2 mM EGTA, 1 mM $MgSO_4$, and 250 mM sucrose and disrupted with two to three 5 sec bursts of a Polytron generator (Brinkmann Instruments, Westbury, N.Y.). The homogenate was centrifuged at 120 x g for 10 min, and the supernatant fluid was decanted into a fresh tube and centrifuged at 25,000 x g for 30 min. The resulting pellet was resuspended in 6 mM Tris HCl (pH 7.4), 2 mM EGTA, 1 mM $MgSO_4$, and 10% glycerol. These membranes were stored under liquid nitrogen for later use. Prior to use the membranes were thawed and rinsed three times in the appropriate modified Krebs buffer before resuspension at the required concentration.

For studies examining receptor characteristics upon chronic opioid exposure, the 7315c cells were incubated for the desired time period in 75 cm^2 collagen-coated tissue culture flasks at a concentration of 2 x 10^6 cells/ml Dulbecco's Minimum Essential Medium (DMEM) containing 2 nM glutamine and 10% fetal calf serum. For experiments involving treated cells, the medium was supplemented with 100 μM morphine. For each flask of treated cells, a control flask was incubated for the same time interval without morphine.

75 cm^2 tissue culture flasks were treated with Vitrogen 100, a sterile solution of purified, pepsin-solubilized bovine dermal collagen, for one hour prior to use. After 1 hour the excess Vitrogen was removed from the flask and cells were incubated at a concentration of 10^6 cells/ml DMEM with 100 μM morphine in a humidified atmosphere of 10% CO_2 and 90% air.

C. Opioid Binding Assays

Receptor binding was carried out by incubating membrane suspensions in a 1 ml final reaction volume for experiments examining sodium effects or studies involving membranes which were pertussis toxin-treated or exposed to morphine for extended periods of time.

All other studies involved a 0.5 ml. final reaction volume. Binding assays were carried out in modified Krebs buffer containing physiological concentrations of NaCl, KCl, $MgCl_2$, and $CaCl_2$. In experiments performed in the absence of sodium an equimolar concentration of N-MG (in whole cell preparations) or potassium (in membrane preparations) replaced sodium. Concentrations of all other ions remained the same.

Triplicate samples of membrane suspensions were preincubated at 37° for 5 minutes before addition of radiolabeled ligand. Incubation continued for 20 minutes at which time all radioligands had achieved equilibrium (Fig. 1, 4). The incubation was terminated by the addition of 4 ml ice-cold buffer and rapid filtration through Whatman GF/B glass fiber filter paper using a Brandel Cell Harvester (Gaithersburg, MD). The filters were washed with an additional eight ml buffer and transferred to scintillation vials. Absolute ethanol (0.5 ml) and Beckman EP Ready-Solv were added to vials which were then counted at an efficiency of 30% for 5 minutes.

All saturation experiments employed from 12 to 21 radioligand concentrations between 0.05 and 25 nM. Nonspecific binding was defined as the fraction of radioligand that remained bound in the presence of 1 μM unlabeled levallorphan, diprenorphine or naloxone (all opioid antagonists). This parameter was determined at each concentration of radioligand.

Competition studies employed 28 unlabeled agonist or 21 antagonist concentrations between 0.5 nM and 10 μM. For binding studies examining the effects of guanyl nucleotide regulation of opioid agonist binding, 10 μM GTPγS or 10 μM GDPβS (Boeringer Mannheim, Indianapolis, IN.) were employed (see below). In experiments performed in the presence of GTP (Boeringer Mannheim, Indianapolis, IN.), the nucleotide was added at the start of the reaction to give a concentration of 100 μM (see

below). To compensate for possible degradation, a second addition of an equal amount of GTP was made 10 minutes after the start of the incubation.

All opioid agonists and antagonists employed in this study are listed in Table 1 and 2 respectively. The nonselective agonists [^{3}H]ET and [^{3}H]DADLE were employed in several experiments in order to directly compare binding parameters obtained in both the δ-containing NG108-15 cells and the μ-containing 7315c cell types. The use of the opioid antagonists, diprenorphine (DIP) and naloxone (NAL) were useful in that antagonist binding is insensitive to regulation by sodium and guanyl nucleotides, therefore competition of unlabeled agonists for [^{3}H]antagonist binding provided a useful method to measure agonist affinity in the presence of factors found to decrease agonist affinity (ie. guanyl nucleotides). DAGO, the μ-selective agonist, was used to characterize binding in the 7315c cells; DSLET, the δ-preferring agonist, was used in the NG108-15 cells.

D. Adenylyl Cyclase Assays

Triplicate samples of approximately 100 μg protein/ml control or treated 7315c or NG108-15 membranes were added to assay buffer (80 mM theophylline, 1 mM $MgSO_4$, 8 mM EDTA, 0.01 mM GTP, 0.25 mM ATP and 30 mM NaCl (unless otherwise stated)). Dose response curves for forskolin were performed to obtain an appropriate concentration for the assay (personal communication with Dr. Cote). Concentrations of forskolin higher than 10 μM did not appear to change the percent of opioid-mediated inhibition of cyclase activity and were harder to dissolve into solution. Therefore cyclase activity was assayed in the presence of 10 μM forskolin to increase basal activity of the enzyme, thus improving quantitation of opiate-mediated inhibition of adenylyl cyclase. The standard curve consisted of triplicate samples of increasing concentrations of unlabeled cAMP (in assay buffer). The unknown samples underwent a 10 minute incubation at 30°. At this temperature adenylyl cyclase converts ATP to cAMP. After 10 minutes (7315c cell membranes) or 15 minutes

TABLE 1

OPIOID RECEPTOR SELECTIVITY FOR AGONISTS

AGONIST	OPIOID RECEPTOR RELATIVE SELECTIVITY	APPROXIMATE K_D (nM)		
		μ	δ	κ
Etorphine (ET)	μ = δ = κ	1	1	1*
Tyr-D-Ala-Gly-(Me)Phe-Gly-ol (DAGO)	μ >>> δ = κ	5	> 1000	>1000*
[D-Ala^2-D-Leu^5]enkephalin (DADLE)	δ > μ >>> κ	5	2	>1000*
[D-Ser^5, Leu^5]enkephalin-Thr (DSLET)	δ >> μ >> κ	80	5-10	>1000*
[D-Pen^2-D-Pen^2]enkephalin (DPDPE)	δ >>> μ = κ	1000	1	>1000**
Ethylketocyclazocine (EKC)	κ >> μ >>> δ	20	>1000	1.4*
U50,488H	κ >>> μ = δ	>1000	>1000*	3.5*

---

* K_D values obtained in guinea pig cortical membranes (Werling et al., 1985, 1986 and personal communication). All other values obtained from NG108-15 or 7315c membranes unless otherwise specified.

** K_D values obtained from Cotton et al. (1985).

TABLE 2

RECEPTOR SELECTIVITY FOR ANTAGONISTS

ANTAGONIST	OPIOID RECEPTOR RELATIVE SELECTIVITY	APPROXIMATE K_D or K_i (nM)		
		μ	δ	κ
Naloxone	$\mu >> \delta = \kappa$	1	20	30*
Diprenorphine	$\mu = \delta = \kappa$	1	1	1*
ICI174,864	$\delta >>> \mu = \kappa$	>5000**	100-300*	>5000 ***

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* K_D values obtained in guinea pig cortical membranes (Werling, personal communication). All other values obtained from NG108-15 and 7315c membranes unless otherwise specified.

** K_i values obtained from adenylyl cyclase assays in 7315c cell membranes.

*** K_D values obtained from Corbett et al., 1984

(NG108-15 cell membranes), the reaction was terminated by boiling the assay tubes for 1 minute. Heat denatures the adenylyl cyclase enyzme,whereas cAMP remains stable. Following the termination procedure [^{3}H]cAMP (80,000 cpm/100 µl) and binding protein, obtained from the bovine adrenal gland (Brown et al., 1971), were added to both the standard curve and unknown samples. All tubes were incubated for an hour on ice. During this time, cAMP competes with [^{3}H]cAMP for the sites on the binding protein. The assay is terminated with the addition of a charcoal suspension (5 g Norit SG Carbon, 2 g BSA, 5 ml Antifoam A and 50 ml water) to each tube and a centrifugation step (10,000 x g for 15 minutes). Charcoal adsorbs free cAMP and [^{3}H]cAMP and leaves cAMP and [^{3}H]cAMP bound to the binding protein in the supernatant. A 100 µl aliquot of the supernatant was removed and counted. Picomoles of cAMP formed over a 10 (7315c) membranes) or 15 (NG108-15 membranes) minute incubation period were calculated by extrapolation of the standard curve. The more cAMP produced by the tissue, the lower the bound counts.

Studies confirming µ-mediated inhibition of adenylyl cyclase activity in the 7315c cell membranes, employed the following: DAGO, the µ-selective agonist, DPDPE, the δ-agonist, and U50,488H and EKC, the κ-agonists.

To examine sodium regulation of opioid inhibition of adenylyl cyclase activity, DADLE the nonselective agonist was employed to directly compare agonist-induced inhibition of enzyme activity in both the NG108-15 and 7315c cell membranes. In experiments investigating the effects of chronic morphine exposure on agonist inhibition of cyclase activity in the 7315c cell membranes DADLE was also used. This peptide has been found to act as a full agonist at the µ opioid receptor in the neurons of rat locus coeruleus (Williams and North, 1984) and peripheral µ-opiate receptors (Smith and Rance, 1983).

E. Analysis of Data

All saturation and competition data were analyzed by the computer program LIGAND (Munson and Rodbard, 1980). This program utilizes a nonlinear least squares curve-fitting algorithm and assumes the simultaneous contribution of one or more independent binding sites. With the exception of the sodium experiments, all data were plotted by LIGAND. Curves generated for sodium studies were hand- drawn according to parameters determined by the program. The LIGAND graphics program was not available at this time. Data sets from replicate experiments have been modeled together. The model best fitting the experimental data was selected on the basis of F-test comparisons of the residual variances, and a runs test examining the sequences of positive and negative differences between the actual data points and the estimated binding curve. The estimated reliability of a reported parameter value is indicated as a standard error of the parameter estimate, calculated by the LIGAND program from pooled data from three more independent experiments, each of which contained triplicate samples at each concentration of radioligand (saturation data) or unlabeled competing ligand (competition studies).

All possible models, in which parameters (ie. K_D or B_{max}) obtained from saturation data were either assumed to be independent, or were constrained to be equal, in the presence and absence of sodium or guanyl nucleotides were considered for each radioligand. If the estimates for a parameter were not significantly different ($p > 0.05$) in the presence and absence of either sodium, or guanyl nucleotides, estimates for the model in which this parameter was constrained to be equal under the two experimental conditions are reported.

In analyzing competition studies, curves were modeled for the existence of one or more multiple affinity states. If the estimates for a two site model did not result in a significant improvement in fit over a one site fit, the simpler model is reported. In order to compare the binding parameters of the agonist under varying conditions, computer-generated fits for curves in the presence of guanyl nucleotides were compared to fits in which the affinities were constrained to values obtained in the absence of added nucleotide. Affinity states identified in the presence of different nucleotides (Table 11), were significantly different from one another, with the exception of those labeled in the presence of GTP and GTPγS. In this case, values reported in the presence of GTP were not different from those identified in the presence of GTPγS ($P > 0.05$), although these values were significantly different ($P < 0;05$) from affinity states observed in the presence of GDPβS and control curves. Similarly, the affinity state identified in the presence of GDPβS was significantly different ($P < 0.05$) from those identified in the absence of added nucleotide or in the presence of GTP and GTPγS. To compare the binding parameters in untreated cell membranes to those obtained in the treated 7315c membranes, computer-generated fits for curves in treated membranes were compared to fits in which the affinities were constrained to values obtained in unexposed membranes.

In experiments in which agonists have been used to compete against antagonist our results are consistent with previous studies suggesting the existence of several bound forms of the ligand. The K_i values calculated by LIGAND in this situation are assumed to be effective binding constants in which two or more sequential equilibrium reactions may participate. Since the estimates of K_i values cannot be assigned to a specific reaction, we have chosen to describe such values as apparent dissociation constants, abbreviated K_{app}. Nonspecific binding was estimated as an independent parameter in the computer analysis. In practice, it was found to be comparable to the amount of radioligand that remained bound in the presence of 1 μM unlabeled diprenorphine. For graphical representation of

guanyl nucleotide and chronic studies, the modeled nonspecific binding estimate has been subtracted from the total amount bound and is reported in the legend for each figure. More detailed definitions of the binding parameters obtained from different binding experiments are listed in Table 3.

Table 3
Binding Constants

The following binding constants have been used throughout the text. All constants have been referred to by abbreviation.

Binding constant	Type of experiment employed to determine constant
K_D, equilibrium dissociation constant	1) Saturation analysis 2) Competition studies when homologous equilibrium conditions exist.
K_{app}, apparent dissociation constant	1) Heterologous or homologous competition curves when two or more sequential equilibrium reactions probably occur simultaneously. K_{app} describes a composite binding constant for the overall reactions.
K_i , equilibrium dissociation constant of competing ligand (agonist or antagonist)	1) Heterologous competition studies.
K_e , equilibrium dissociation constant (antagonist)	1) Functional studies of antagonist potency.

Definitions:

Saturation studies: Increasing concentrations of labeled ligand are added to a tissue preparation until the receptor population is saturated. Results are commonly transformed into a Scatchard plots for analysis.

Homologous competition: Receptor populations are labeled with a radiolabled ligand. Increasing concentrations of unlabeled ligand are added to compete for binding with the labeled ligand. In this case the unlabeled ligand is the same as labeled ligand. Binding parameters are for the unlabeled ligand.

Heterologous competiton: Similar to homologous conditions, except that the unlabeled ligand is a different from the labeled ligand. Binding parameters are for the unlabeled ligand.

Functional studies: Studies in which an estimate of the ability of a drug to elicit a physiological response may be obtained. The potency of antagonists in reversing agonist's effects can be obtained.

CHAPTER ONE:
CHARACTERIZATION OF OPIOID BINDING SITES IN NG108-15 AND 7315c CELLS

Rationale

Previous binding (Chang et al., 1978) as well as functional (Sharma et al., 1975) studies have demonstrated the NG108-15 cells contain a homogeneous population of δ opioid binding sites which mediate inhibition of adenylyl cyclase. Functional studies by Frey and Kebabian (1984) have shown that opioids inhibit adenylyl cyclase through a receptor with μ-like characteristics in the 7315c pituitary tumor cells, although binding studies have not been performed to confirm these studies. In order to insure the existence of a homogeneous population of δ receptors in the NG108-15 cells and μ receptors in the 7315c cells under our conditions, both binding and functional studies have been employed in membranes and whole cell preparations from both cell types. In order to obtain results pertinent to the *in vivo* situation it was necessary to conduct binding studies at 37° in the presence of the appropriate concentrations of physiological cations.

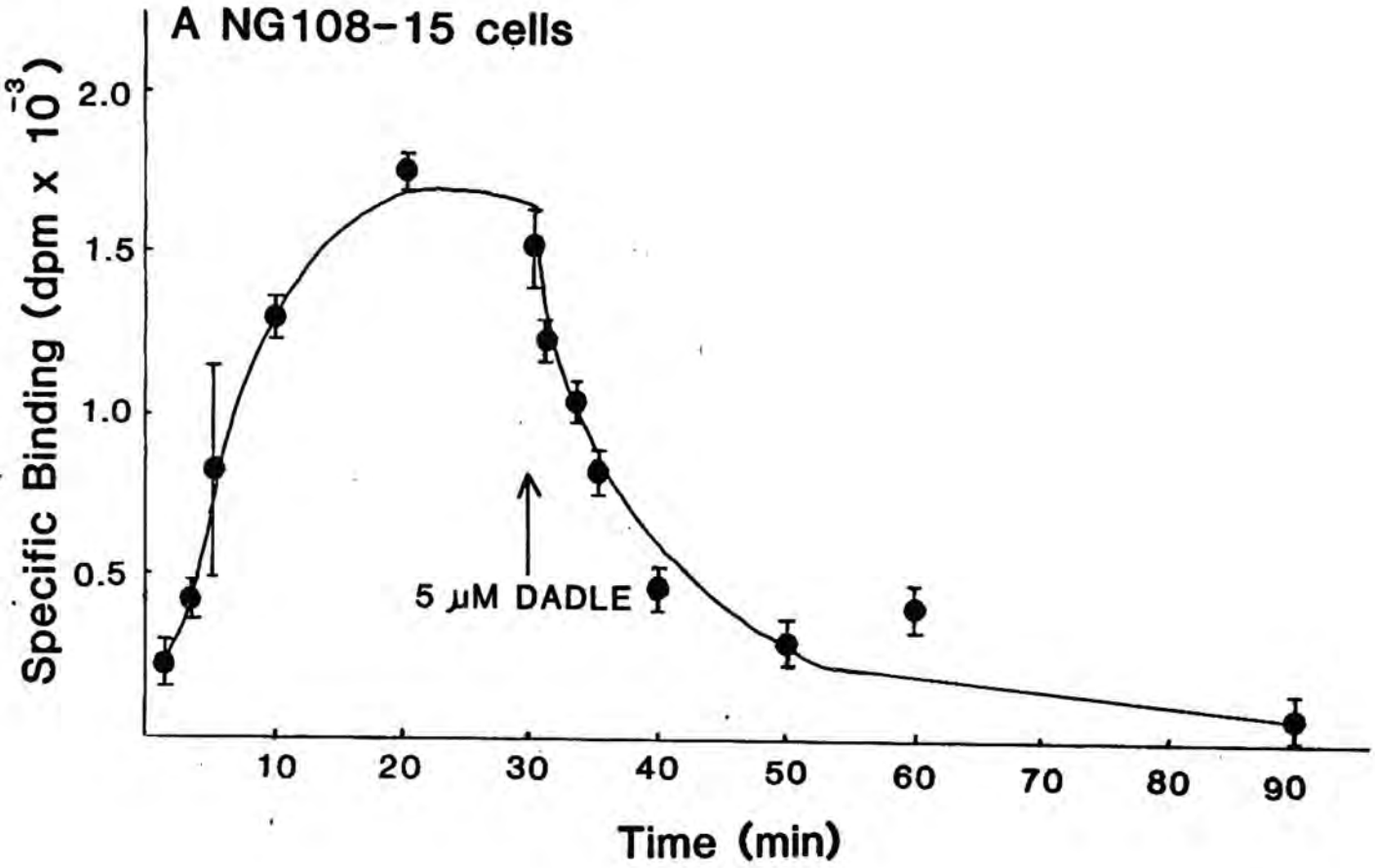
Results

A. Characterization of opioid binding sites in NG108-15 cells

1. Radioligand Binding Studies

In both intact NG108-15 cells (Fig 2), and in membrane suspensions (data not shown), binding of [^{3}H]ET reached equilibrium by 20 min. This time period was chosen as an appropriate time to allow equilibrium to occur between the receptor and labeled ligand in all binding experiments reported herein. To determine if internalization of the ligand/receptor complex occurred during the incubation period, a large excess of unlabeled

FIGURE 2. TIME COURSE OF [^{3}H]ET (2nM) BINDING TO NG108-15 CELLS, AND DISSOCIATION AFTER ADDITION OF 5 μM DADLE. Aliquots of cell suspensions were filtered at indicated times, and specific binding was determined (245 μg of membrane protein/assay tube) in the presence of modified Krebs Hepes buffer containing 118 mM NaCl. Presented data are mean values (± SE) of triplicate measurements from a single experiment, which was replicated (Total N=2) with similar results.


A NG108-15 cells
Specific Binding (dpm x 10^{-3})
2.0
1.5
1.0
0.5
5 μM DADLE
10
20
30
40
50
60
70
80
90
Time (min)

DADLE was added after 30 minutes incubation (Fig 2). Within 60 minutes, DADLE addition produced almost complete dissociation of the labeled ligand (Fig 2), suggesting that most if not all of the agonist/receptor complexes had remained on the cell surface during this time period allowing rapid dissociation of bound ligand. Opioid binding sites on NG108-15 cells have previously been shown to behave as a homogeneous population of sites with properties characteristic of δ receptors (Chang et al., 1978). In the present experiments, in which binding has been measured at 37° in the presence of appropriate cations, this result has been confirmed. Saturation analysis of [^{3}H]ET binding to NG108-15 cell membranes generated a linear Scatchard plot (Fig. 3), results consistent with binding to a single class of binding sites. Computer analysis of [^{3}H]DIP, [^{3}H]NAL, and [^{3}H]DADLE binding to NG108-15 membranes yielded similar results (Table 4). NG108-15 cell membranes and intact cell suspensions, showed high-affinity displaceable binding of [^{3}H]DADLE by DSLET (Fig 4), [^{3}H]DIP by DIP, and DPDPE , [^{3}H]ET by DSLET, ligands with good affinity for δ receptors, but did not show significant high-affinity binding displaceable by DAGO, a μ receptor selective ligand (Fig 4), or EKC, a κ selective ligand (Table 4).

2. Adenylyl Cyclase Studies

Studies demonstrated opioid inhibition of adenylyl cyclase activity in NG108-15 cell membranes. In these studies, DADLE was used as the agonist, since it has a good affinity at the δ opioid receptor. At an intermediate sodium concentration (30 mM), DADLE induced inhibition of adenylyl cyclase, and the maximum inhibition was about 30 to 40% of control enzyme activity (Fig 5). The IC50 for DADLE in NG108-15 cell membranes was about 10 nM. These findings are consistent with the estimated K_D for DADLE at δ receptors obtained from radioligand binding studies and suggest that the action of this peptide is mediated through the δ opioid receptor in the NG108-15 cell membranes.

FIGURE 3. SATURATION ANALYSIS OF [^{3}H]ET BINDING TO NG108-15 CELL MEMBRANES. Scatchard transformation of [^{3}H]ET binding to δ-receptors in NG108-15 membranes suspended in modified Krebs-HEPES buffer containing 118 mM NaCl. Specific binding was measured at increasing radioligand concentrations from 0.05 to 25 nM. Data are mean values of triplicate determinations in a single experiment, which was repeated twice with similar results. Estimates of K_D and B_{max} from the combined experiments are listed in Table 4.

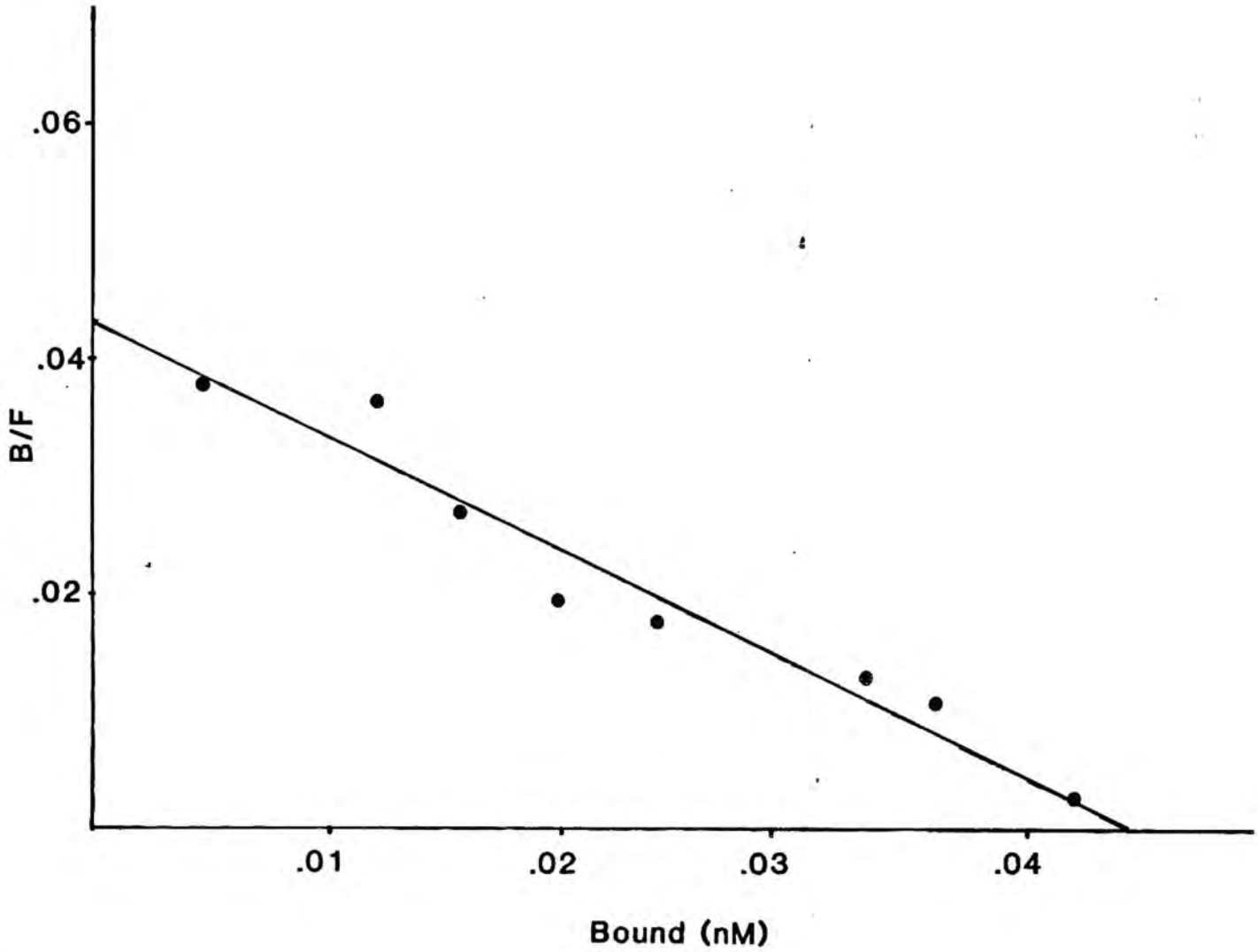

.06
.04
.02
B/F
.01
.02
.03
.04
Bound (nM)

FIGURE 4. COMPETITION FOR [^{3}H]DADLE BINDING BY DAGO AND DSLET TO NG108-15 CELL MEMBRANES. Inhibition by opioids of specific binding of [^{3}H]DADLE, 1 nM, to δ receptors by increasing concentrations (0.05 to 10 μM) of , • DAGO and o, DSLET in the presence of modified Krebs Hepes buffer containing 118 mM NaCl.. Nonspecific binding was estimated in the presence of 1 μM levallorphan. Points are the mean values of triplicate determinations.

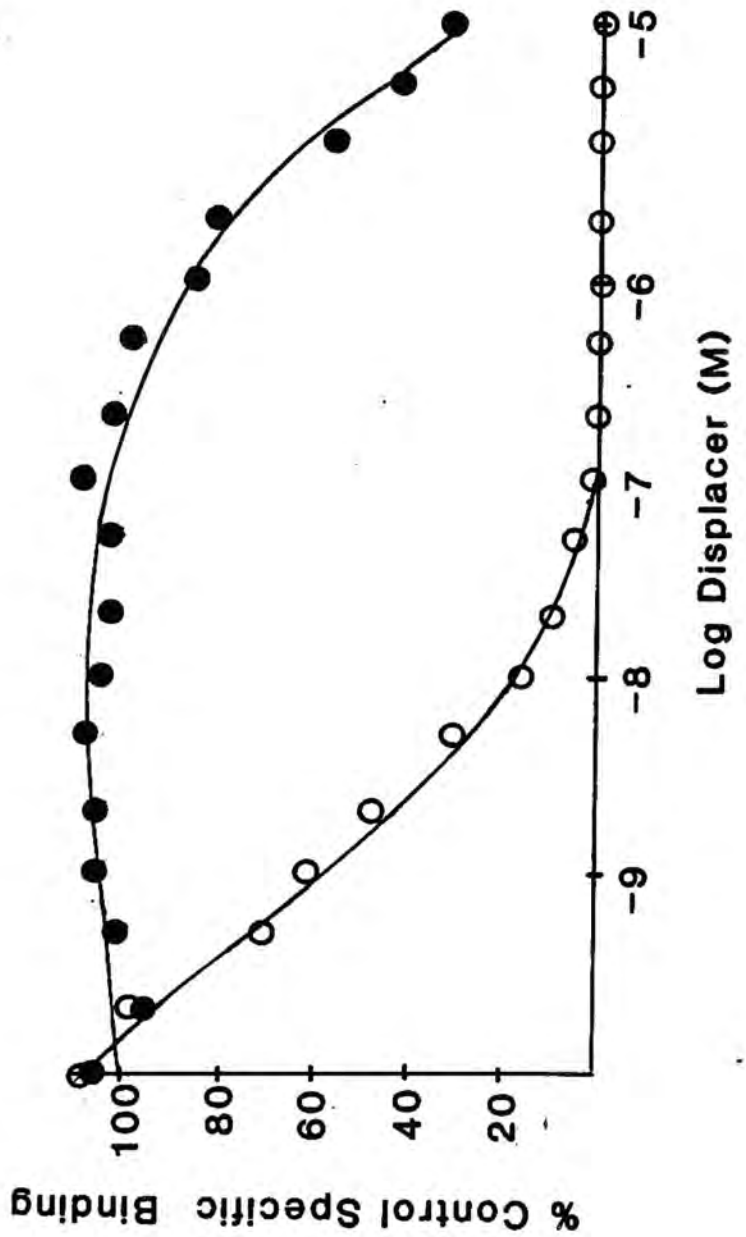
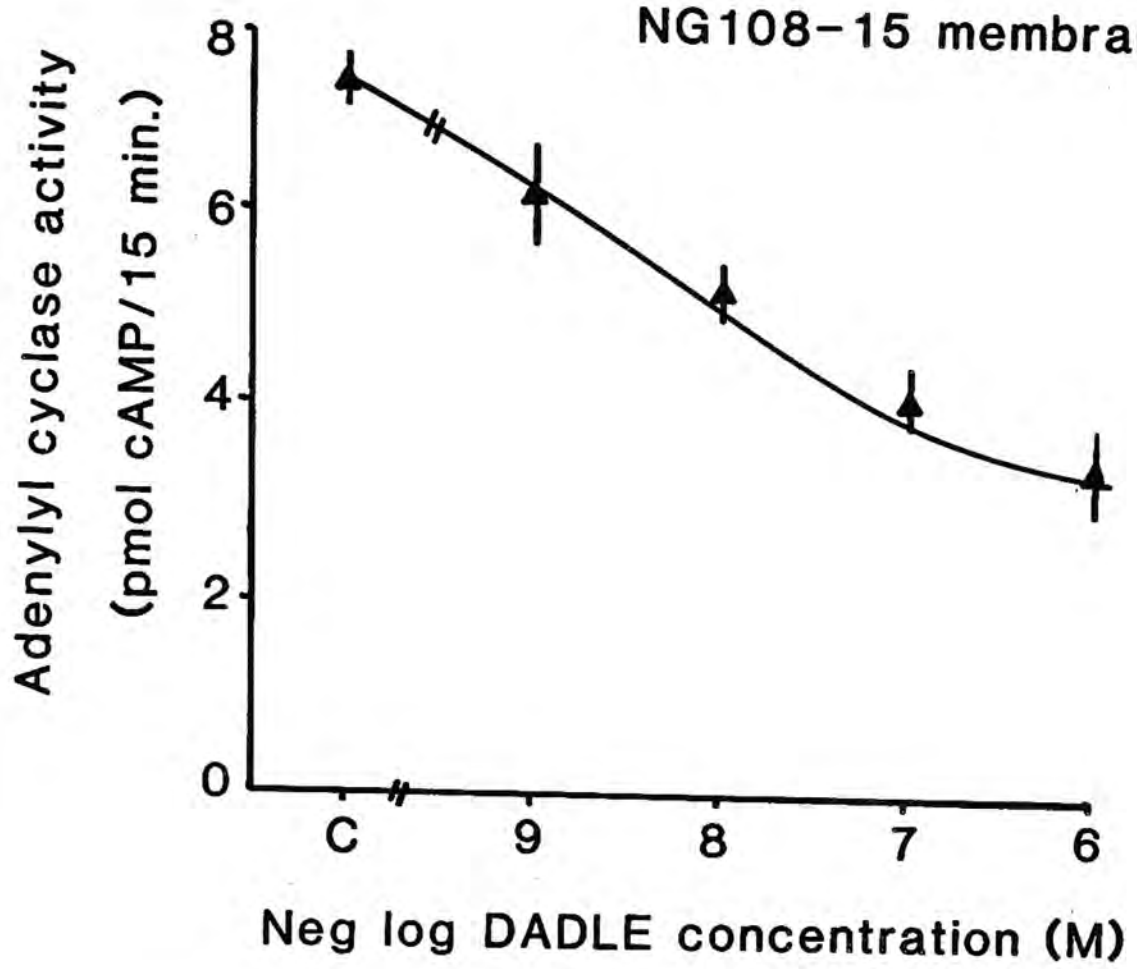

% Control Specific Binding
100
80
60
40
20
-9
-8
-7
-6
-5
Log Displacer (M)

TABLE 4
Summary of Binding Parameters for NG108-15 cell membranes

Membranes were incubated for 20 minutes at 37° C in modified Krebs buffer containing 118 mM NaCl. Results were analyzed by LIGAND, a nonlinear curve fitting algorithm (Munson and Rodbard, 1980). The tabulated values are the estimates of K_i , K_D or K_{app} ± the standard error of the estimate from a combined analysis of all like experiments. In competition experiments, the binding of 1 nM [^{3}H]DIP or 2 nM [^{3}H]ET were was competed for by increasing concentrations of unlabeled ligand. Under conditions where two parameters are stated, a two-site model fitted the data better than a one site fit.

Experiment type	Labeled ligand	Unlabeled ligand	N	K_i, K_{app}, K_D (nM)	B_{max} (fmol/mgprotein)
Saturation	DIP	---	2	1.2 ± 1.0	104 ± 5
Competition	DIP	DIP	3	2.3 ± 1.0	100 ± 20
Saturation	NAL	---	2	5.2 ± 2.5	115 ± 43
Saturation	ET	---	3	0.7 ± 0.1	140 ± 9
Saturation	DADLE	---	2	2.3 ± 0.7	230 ± 27
Competition	ET	DSLET	2	8.7 ± 13	92 ± 2
Competition	DADLE	DSLET	2	5.0 ± 0.23	89 ± 7
Competition	DIP	DSLET	9	3.0 ± 2 428 ± 52	94 ± 9 28 ± 9
Competition	DIP	DPDPE	2	2.3 ± 1 6800 ± 2000	161 ± 21 37 ± 13
Competition	DIP	EKC	2	> 1000	--------
Competition	DIP	DAGO	2	> 1000	--------

FIGURE 5. DADLE-MEDIATED EFFECTS ON ADENYLYL CYCLASE ACTIVITY IN NG108-15 CELL MEMBRANES. Membranes from NG108-15 cells were incubated with increasing concentrations of DADLE (0 to 1 μM) and 30 mM NaCl. Vertical bars indicate the standard errors of the mean estimates of triplicate determinations.


NG108-15 membranes
Adenylyl cyclase activity
(pmol cAMP/15 min.)
8
6
4
2
0
C
9
8
7
6
Neg log DADLE concentration (M)

B. Characterization of opioid binding sites in 7315c cells and membranes

1. Radioligand binding studies

In intact 7315c cell suspensions, binding of [^{3}H]ET reached equilibrium within 15 min (Fig. 6). Addition of a large excess of DAGO after 30 min incubation displaced most (about 75%) of the specifically bound [^{3}H]ET within a further 20 min (Fig 6) indicating that as in the NG108-15 cells, most of the bound ligand was readily displaceable. All other experiments reported here were terminated after 20 min incubation to reduce the extent of possible internalization of bound ligand. Under our incubation conditions, Scatchard plots of [^{3}H]ET binding (Fig. 7), and [^{3}H]NAL (Table 5), in 7315c cells and membranes did not deviate significantly from linearity. The concentration of [^{3}H]ET and [^{3}H]NAL binding sites on 7315c cells was found to be considerably lower than their concentration on NG108-15 cells, and variances in binding estimates were generally higher in experiments with 7315c cells and membranes. The variation in B_{max} values may reflect real differences between different 7315c cell membrane batches since these were derived from different tumors with varying amounts of non-tumor tissue.

The 7315c cell membranes showed high-affinity displaceable binding of [^{3}H]DAGO, [^{3}H]NAL, [^{3}H]DIP, and [^{3}H]ET, ligands with high-affinity for μ receptors (Table 5), but did not display any high-affinity displaceable binding of [^{3}H]DADLE or [^{3}H]EKC (both ligands used in the presence of unlabeled DAGO to block binding to μ receptors). Binding of [3H]ET by 7315c cell membranes was completely inhibited by DAGO, with a calculated K_i for inhibition of 9 nM (Fig. 8). The κ-selective agonist, U50,488H, produced no inhibition at concentrations up to 1 μM (Fig 8). DSLET inhibited [^{3}H]ET binding to 7315c membranes with a K_i of about 90 nM (Fig 8), a value very similar to its estimated K_i for μ receptors in guinea pig brain membranes under similar incubation conditions (Werling et al., 1986) . Concentrations up to 1 μM of DPDPE, the δ-selective agonist, were unable

FIGURE 6. TIME COURSE OF [^{3}H]ET BINDING TO 7315c CELLS. Time course of binding to 2 nM [^{3}H]ET to μ-receptors in 7315c cells, and dissociation after addition of 5 μM DAGO. Aliquots of cell suspensions (34 μg of membrane protein/assay tube) were filtered at indicated times, and specific binding was determined in the presence of modified Krebs Hepes buffer containing 118 mM NaCl.. Presented data are mean values (± SE) of triplicate measurements from a single experiment, which was replicated with similar results. Values for the unlabeled agonist are reported as K_i as determined by LIGAND (Munson and Rodbard, 1980).

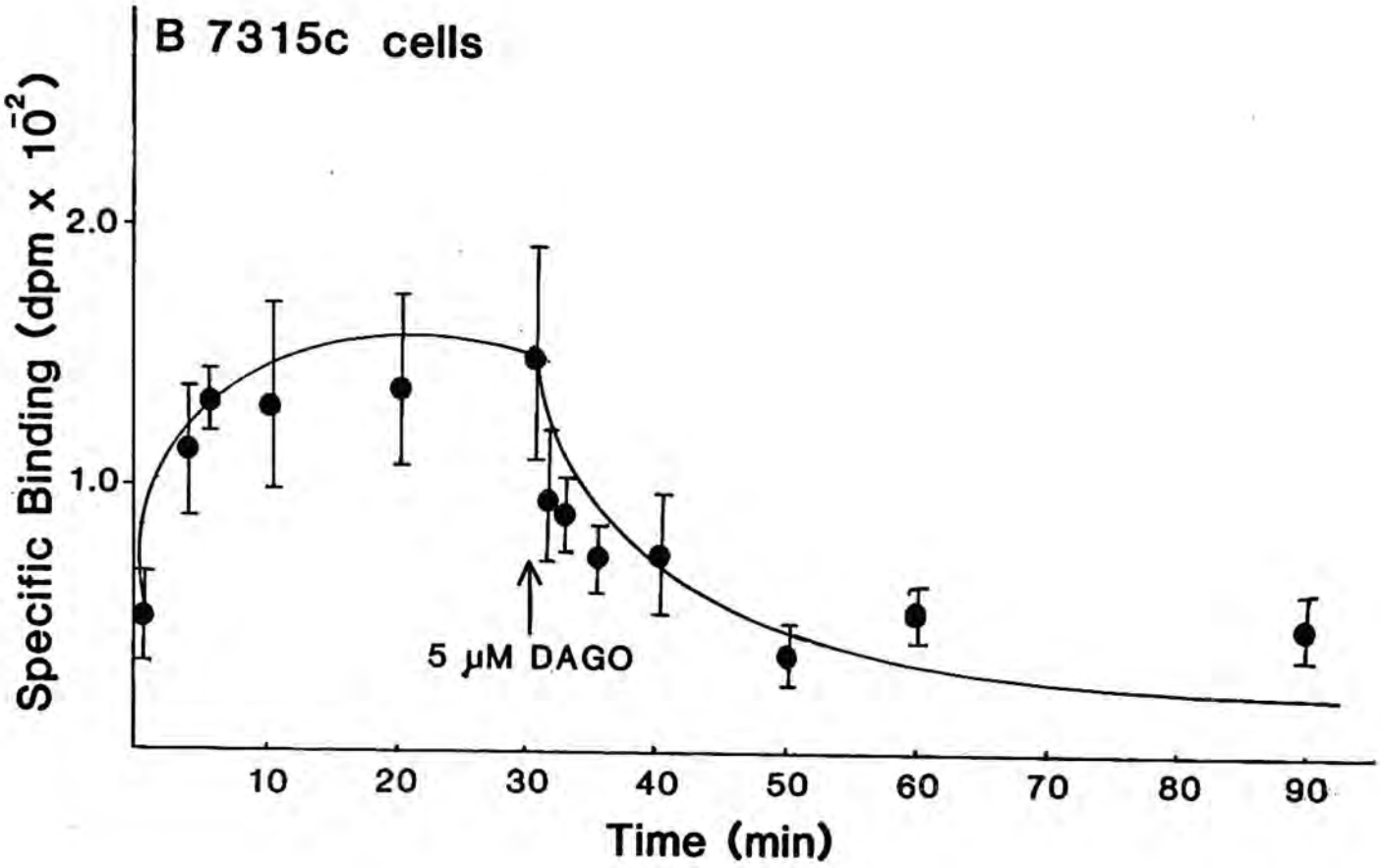

B 7315c cells
Specific Binding (dpm x 10^{-2})
2.0
1.0
5 μM DAGO
10
20
30
40
50
60
70
80
90
Time (min)

FIGURE 7. SATURATION ANALYSIS OF [^{3}H]ET BINDING TO 7315c CELL MEMBRANES. Scatchard transformation of [^{3}H]ET binding to μ-receptors in 7315c cell membranes suspended in modified Krebs HEPES buffer containing 118 mM NaCl. Specific binding was measured at increasing concentrations from 0.05 to 25 nM, in the presence of 118 mM sodium. Data are mean values of triplicate determinations in a single experiment, which was replicated twice with similar results. Estimates of K_D and B_{max} from the combined experiments are listed in Table 5.

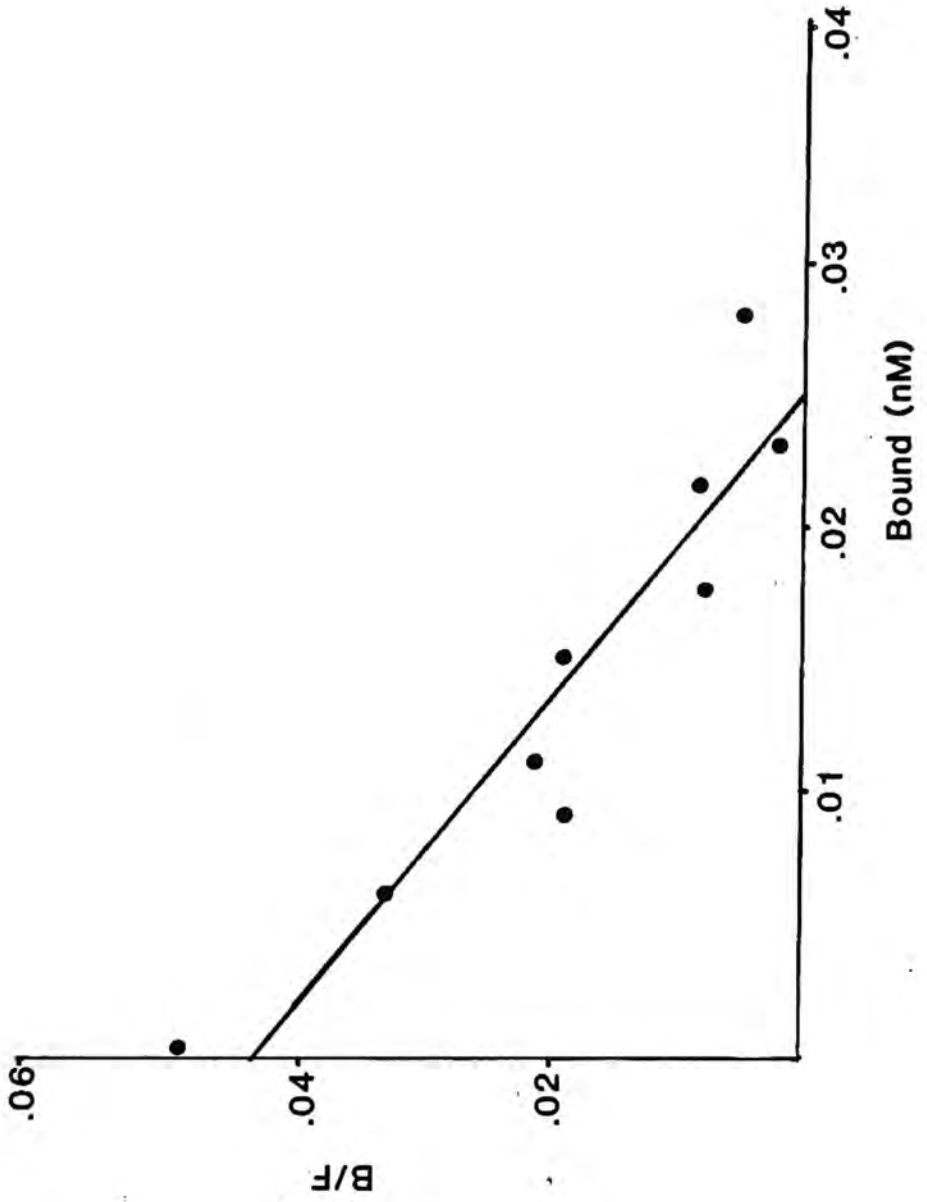
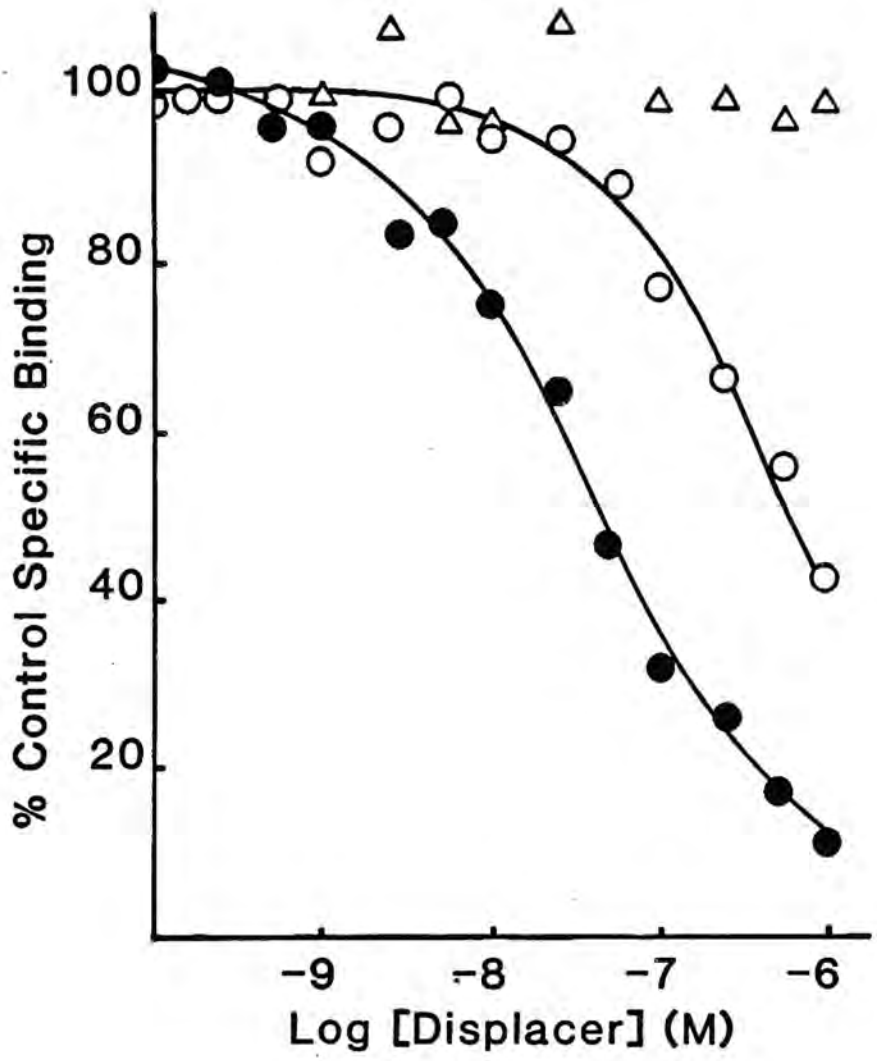


TABLE 5
Summary of Binding Parameters for 7315c Cell Membranes

Membranes were incubated for 20 minutes at 37° C in modified Krebs buffer containing 118 mM NaCl. Results were analyzed by LIGAND, a nonlinear curve fitting algorithm (Munson and Rodbard, 1980). The tabulated values are the estimates of K_D, K_i, or K_{app} ± the standard error of the estimate from a combined analysis of all like experiments. In competition experiments, the binding of 1 nM [^{3}H]DIP or [^{3}H]NAL or 2 nM [^{3}H]ETOR was competed for by increasing concentrations of unlabeled ligand. Under conditions where two parameters are stated, a two-site model fitted the data better than a one site fit.

Experiment type	Labeled ligand	Unlabeled ligand	N	K_D, K_i, or K_{app} (nM)	B_{max} (fmol/mg protein)
Saturation	NAL	---	2	0.9 ± 0.3	21 ± 3.0
Competition	NAL	NAL	3	2.0 ± 0.5	43 ± 4.0
Competition	DIP	DIP	9	0.8 ± 0.1	44 ± 2.0
Saturation	ET	---	3	1.6 ± 0.3	26 ± 2.7
Competition	ET	DAGO	2	9.0 ± 1.5	27 ± 5.0
Competition	DAGO	DAGO	2	5.0 ± 2.0	24 ± 3.4
Competition	NAL	DAGO	9	7.0 ± 2.0 465.0 ± 220	28 ± 3.0 16 ± 20.0
Competition	ET	DSLET	2	90.0 ± 16	--------
Competition	NAL	DPDPE	2	1530 ± 470	--------
Competition	ET	U50488H	2	>1000	--------
Competition	ET	EKC	2	20.0 ± 12	--------

FIGURE 8. COMPETITION FOR [^{3}H]ET BINDING BY DAGO, DSLET, AND U50,488H TO 7315c CELL MEMBRANES. Inhibition by opioids of specific [^{3}H]ET binding, 2 nM, by increasing concentrations (0.05 nM - 1 μM) of •, DAGO; o, DSLET and Δ , U50,488H in the presence of modified Krebs Hepes buffer containing 118 mM NaCl.. Nonspecific binding was estimated in the presence of 1 μM levallorphan. Points are the mean values of triplicate determinations.


100
80
60
40
20
% Control Specific Binding
-9
-8
-7
-6
Log [Displacer] (M)

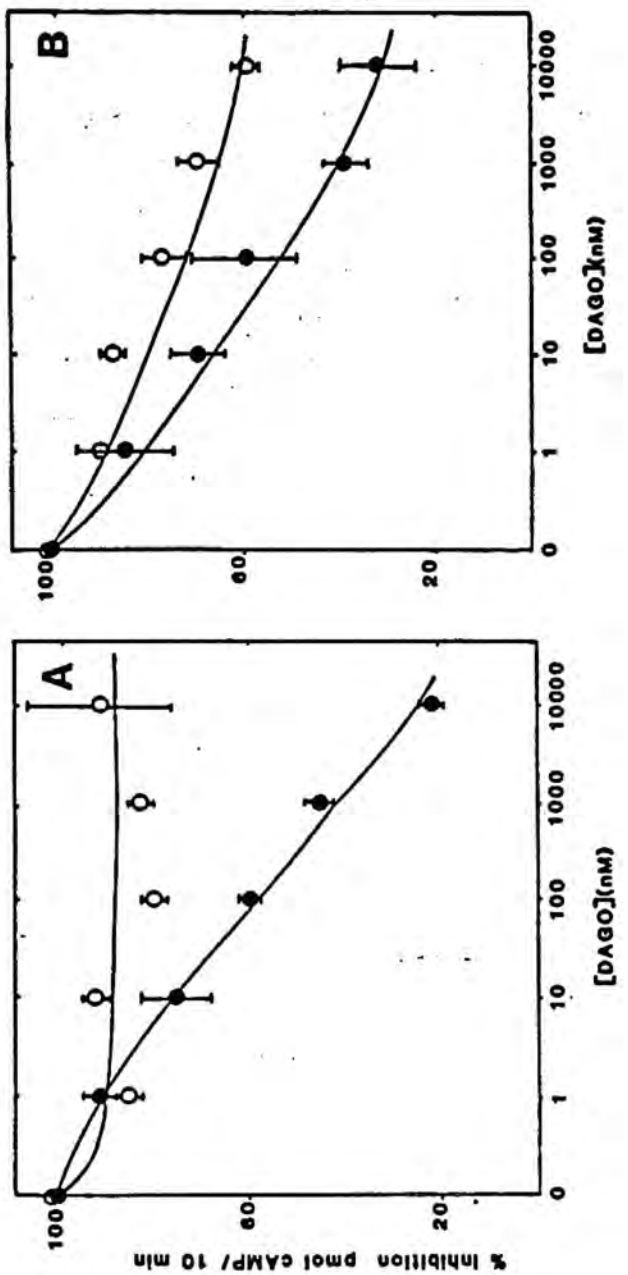
to significantly inhibit binding of [^{3}H]NAL by 7315c membranes (Table 5). These results suggest that the high-affinity binding of [^{3}H]DAGO and [^{3}H]ET, [^{3}H]DIP and [^{3}H]NAL by 7315c membranes was to μ type receptors.

2. Adenylyl Cyclase Studies

In order to confirm earlier reports demonstrating μ-mediated inhibition of adenylyl cyclase activity in the 7315c cells (Frey and Kebabian, 1984), dose response curves were constructed using DAGO, the μ-selective agonist in the presence of 30 mM NaCl (concentration of NaCl found to increase adenylyl cyclase activity, thus making it easier to measure opioid inhibition. For more details, see page 82). DAGO produced a dose-related depression of adenylyl cyclase activity. The concentration of DAGO producing 50% of the maximum inhibitory effect of DAGO was in the range of 10 - 100 nM. At a concentration of 10 μM, DAGO produced a maximum inhibition of adenylyl cyclase activity of about 80%.

To confirm that DAGO was acting through a μ opioid receptor to mediate inhibition of adenylyl cyclase activity, the ability of naloxone, the μ selective-antagonist, and ICI174,864, the δ-selective antagonist, to antagonize DAGO-mediated effects was examined. DAGO concentration-response curves were constructed in the absence of antagonist, and in the presence of 10 nM naloxone (Fig. 9 A) or 300 nM ICI174,864 (Fig. 9 B). Dose-response curves performed in the presence of 10 nM naloxone indicated that this concentration was sufficient to completely reverse the DAGO-mediated inhibition of cyclase activity. On the other hand, a 300 nM concentration of ICI174,864, the δ-selective antagonist, produced only a slight antagonism of the DAGO-mediated depression of cyclase activity.

FIGURE 9. NALOXONE (A) AND ICI174,848 (B) EFFECTS ON DAGO-MEDIATED INHIBITION OF ADENYLYL CYCLASE ACTIVITY IN 7315c CELL MEMBRANES. Membranes were prepared and incubated in the absence and presence of increasing concentrations of DAGO (1nM to 10 μM). Adenylyl cyclase activity was determined as % of control cAMP production in 10 minutes. The enzyme activity was assayed in the presence or absence of 10 nM naloxone or 300 nM ICI174,864. Naloxone antagonized the inhibitory action of DAGO, whereas ICI174,864 only weakly antagonized DAGO-mediated inhibition of enzyme activity. The figures are mean values from replicate experiments (Total N=2). The error bars represent standard error of the mean estimates from six determinations at each concentration of agonist. Closed symbols, DAGO alone; open symbols, DAGO in the presence of 10 nM naloxone (A) or 300 nM ICI174, 864 (B).


A
B
% inhibition pmol cAMP/ 10 min
[DAGO](nM)
[DAGO](nM)
100
60
20
0
1
10
100
1000
10000

Because ICI174,864 slightly antagonized DAGO-mediated depression of adenylyl cyclase activity, the possibility that δ receptors, in addition to μ, inhibit adenylyl cyclase activity in the 7315c cell was evaluated by comparing the effects of the δ selective agonist, DPDPE with those of DAGO (Fig. 10). DPDPE concentration-response curves were constructed in the absence and presence of the antagonists naloxone and ICI174,864. DPDPE was not as potent as DAGO, the μ selective agonist, in reducing cyclase activity. DPDPE at 1-10 μM produced a significant reduction in activity, with a maximum inhibition of 50-70% at 10 μM. This is in contrast to the approximate IC50 of 10 nM for DAGO with a maximum inhibition of 80%. This observation is compatible with the idea that at concentrations in excess of 100 nM the δ-selective agonist, DPDPE, is mediating inhibition of adenylyl cyclase activity through the μ opioid receptor. DPDPE concentration-response curves performed in the presence of 10 nM naloxone indicated that low concentrations of naloxone were able to reverse the inhibition of cyclase activity induced by 10 μM DPDPE (Fig 10 A). DPDPE-mediated inhibition of cyclase activity was only weakly antagonized by ICI174,864 (Fig 10 B), to an extent comparable to its antagonism of the μ-selective agonist DAGO (Fig. 9).

Adenylyl cyclase assays performed in the presence of increasing concentrations of the opioid antagonist naloxone indicated that concentrations of this drug between 1 nM and 100 nM inhibited cyclase activity in the 7315c cell membranes (Table 6) in the absence of opioid agonist. Because of this unexpected effect, we were unable to determine K_e values for reversal of agonist-mediated inhibition in this preparation. Some inhibition of adenylyl cyclase activity was also induced by the δ antagonist ICI174,864 in the absence of added agonist. The apparent direct agonist action of these antagonists was consistently observed, but quantitatively variable. In contrast to naloxone and ICI174,864, enyzme activity measured in the presence of increasing concentrations of another opioid antagonist, diprenorphine, remained unaffected (Table 6). Despite its direct inhibitory activity, it is

FIGURE 10. NALOXONE (A) AND ICI174,848 (B) EFFECTS ON DPDPE-MEDIATED INHIBITION OF ADENYLYL CYCLASE ACTIVITY IN 7315c CELL MEMBRANES. Membranes were prepared and incubated in the absence and presence of increasing concentrations of of DPDPE (1nM to 10 μM). Adenylyl cyclase activity was determined as % of control cAMP production in 10 minutes. The enzyme activity was assayed in the presence or absence of 10 nM naloxone or 300 nM ICI174,864. Naloxone antagonized the inhibitory action of DPDPE, whereas ICI174,864 only weakly antagonized DPDPE-mediated inhibition of enzyme activity. The figures are mean values from two experiments (Total N=2). The error bars represent standard error of the mean estimates from six determinations at each concentration of agonist. Closed symbols, DPDPE alone; open symbols, DPDPE in the presence of 10 nM naloxone (A) or 300 nM ICI174, 864 (B).

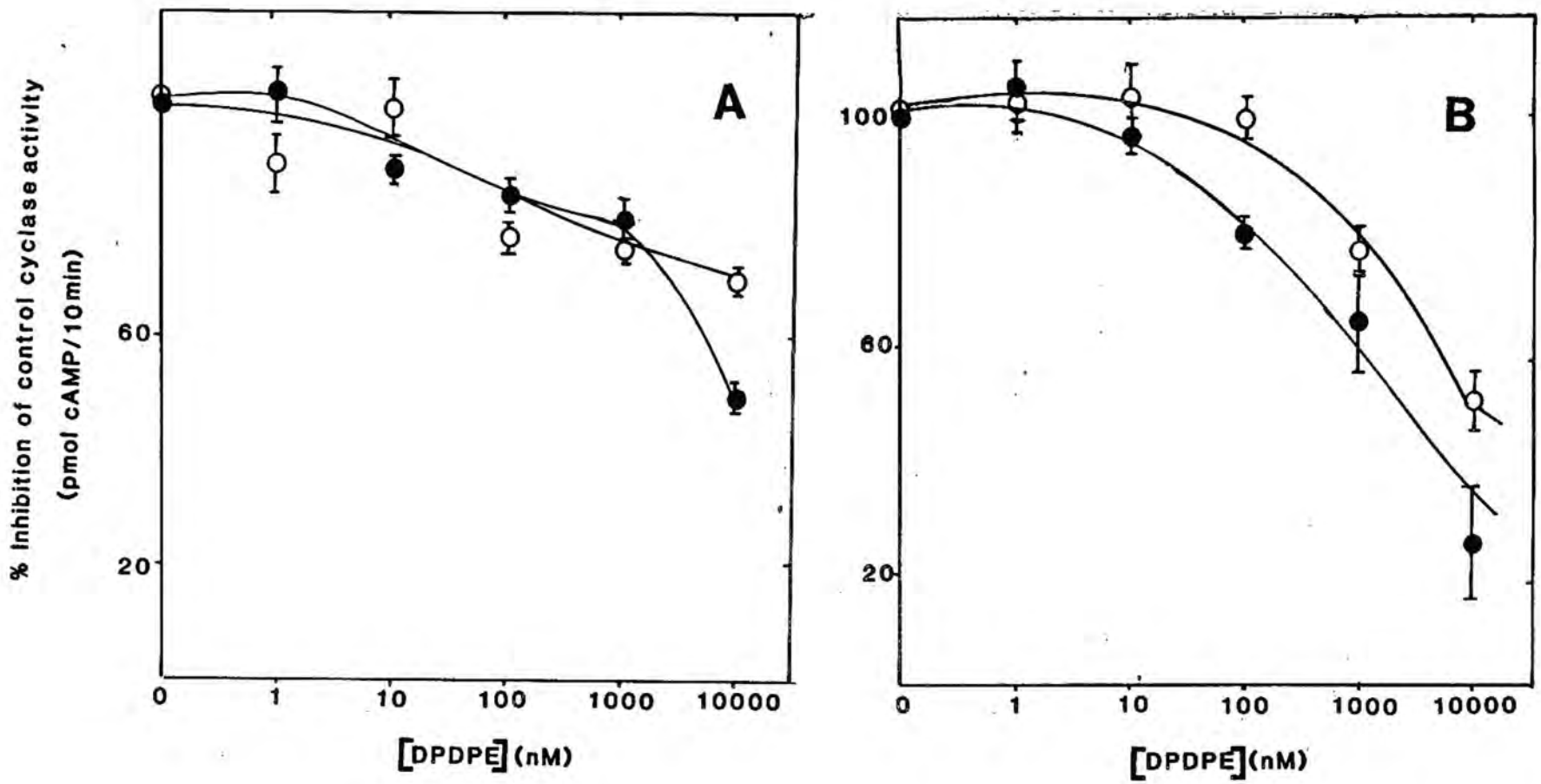

% Inhibition of control cyclase activity
(pmol cAMP/10min)
A
B
100
60
20
0
1
10
100
1000
10000
[DPDPE] (nM)
[DPDPE](nM)

Table 6

Opioid Antagonist Inhibition of Adenylyl Cyclase Activity in the 7315c Cell Membranes

Cell membranes were assayed in the presence of increasing concentrations of naloxone or diprenorphine. For details of the adenylyl cyclase assay see Materials and Methods section of the text. The number of independent replicate experiments is indicated (N). Independent experiments contained triplicate determinations at each antagonist concentration. The tabulated values are the estimates of the % inhibition (± standard error of the mean) produced by the antagonist from a combined analysis of the results from N independent experiments.

Antagonist	N	[Antagonist] (nM)	% Inhibition of Adenylyl Cyclase Activity
Naloxone	4	10	20 ± 20
		100	18 ± 16
		1000	18 ± 12
Diprenorphine	2	10	1 ± 2
		100	1 ± 6
		1000	1 ± 5

clear that naloxone readily antagonizes the actions of submicromolar concentrations of DAGO and micromolar concentrations of DPDPE, a result consistent with the action of both these agonists through μ opioid receptors. It is also evident that ICI174,864 only slightly antagonized the actions of DAGO or DPDPE, supporting the suggestion that the action of both these peptides was mediated through the μ and not the δ opioid receptor. To eliminate the possibility that inhibition of adenylyl cyclase activity was mediated through κ opioid receptors, the ability of the κ-selective agonists U50,488H and EKC to inhibit cyclase activity was evaluated (Table 7). In both cases, concentrations up to 100 nM were unable to significantly depress the enzyme activity. This observation indicated that the κ-receptor activation did not mediate inhibition of adenylyl cyclase activity in 7315c cell membrane preparations.

Discussion

Results from this study support evidence that a homogeneous population of δ and μ opioid-binding sites exist in the NG108-15 and 7315c cells respectively. In both NG108-15 and 7315c membrane preparations and intact cell suspensions, binding reached an apparent equilibrium within 20 minutes. The ability of an excess of unlabeled agonist to compete for previously bound ligand suggests that substantial internalization of receptor-bound ligand had not occurred after 30 minutes of incubation. Since an apparent binding equilibrium was reached in these cells between 15 and 20 minutes, all subsequent incubations were limited to 20 minutes to reduce the extent of possible receptor-ligand internalization. Binding parameters obtained in both intact cells and membrane preparations were very similar. However the percentage of nonspecific binding was found to be smaller in membrane preparations. For this reason, all binding experiments will be performed on membrane suspensions unless otherwise stated.

Table 7

Effect of Kappa Selective Agonists on Adenylyl Cyclase Activity in the 7315c Cell Membranes

Cell membranes were assayed in the presence of increasing concentrations of opioid agonsits. For details of the adenylyl cyclase assay, see Materials and Methods section of the text. The number of independent replicate experiments is indicated in the table (N). Independent experiments contained triplicate determinations at each agonist concentration. The tabulated values are the estimates of the % inhibition (± standard error of the mean) produced by the agonist from a combined analysis of the results from N independent experiments.

Agonist	N	[Agonist] nM	% Inhibition of Adenylyl Cyclase Activity
U50,488H	3	1	2 ± 5
		10	1 ± 2
		100	8 ± 12
EKC	2	1	6 ± 8
		10	2 ± 10
		100	7 ± 5

The 7315c cells and membranes showed high affinity binding of [^{3}H]ET which was readily competed for by the μ–selective agonist, DAGO. The competition of unlabeled NAL for [^{3}H]NAL binding yielded a monophasic curve with an affinity comparable to the antagonist affinity at the μ receptor. DSLET and DPDPE, δ-preferring agonists, competed for [^{3}H]ET binding with affinities similar to their affinities at μ receptors. In contrast the κ agonists, U50,488H and EKC were unable to compete for [^{3}H]ET binding . Scatchard plots of [^{3}H]NAL and [^{3}H]ET binding in the 7315c cell membranes were linear. We reconfirmed initial observations that opioid inhibition of adenylyl cyclase was mediated through the μ receptor (Frey and Kebabian, 1984). DAGO, the μ selective agonist, decreased adenylyl cyclase activity in a dose dependent fashion, whereas DPDPE, the δ selective agonist, and EKC and U50,488H, both κ agonists,were unable to signficantly inhibit cyclase activity. Naloxone (10 nM) antagonized both DAGO- and 10 μM DPDPE-mediated suppression of cyclase. On the other hand, 300 nM ICI174,864, the δ-selective antagonist, produced only a weak antagonism of DAGO and DPDPE-mediated inhibition of adenylyl cyclase activity. These results are consistent with the view that opioid inhibition of adenylyl cyclase in 7315c cell membranes results from activation of μ opioid receptors.

This study also reaffirms δ homogeneity in NG108-15 cell membranes by demonstrating high-affinity competition for binding with [^{3}H]DIP by DPDPE, the delta selective agonist. DPDPE, like DSLET, discriminated high and low affinity states of the δ receptor. The lower affinity state does not represent binding to μ receptors, since previous studies demonstrated high affinity competition binding for [^{3}H]DADLE and [^{3}H]ET by DSLET, the δ-preferring agonist, and the absence of competition by DAGO, the μ-selective agonist . Micromolar concentrations of both EKC, the κ-selective agonist, and DAGO were unable to compete for [^{3}H]DIP binding in NG108-15 cell membranes. Scatchard plots of [^{3}H]DIP, [^{3}H]NAL, [^{3}H]ET, and [^{3}H]DADLE were linear. These results

support previous studies (Chang et al., 1978) indicating the existence of a homogeneous population of δ-opioid receptors in the NG108-15 cells. Functional studies confirm earlier reports of δ–mediated inhibition of adenylyl cyclase activity in these cells (Sharma et al., 1975). DADLE an opioid agonist with a high affinity for the δ receptor, was able to inhibit cyclase activity in a dose dependent manner (Fig. 5).

CHAPTER TWO:
EFFECTS OF SODIUM ON OPIOID BINDING IN NG108-15 AND 7315c CELLS AND CELL MEMBRANES

Rationale

Sodium appears to be a prominent regulator of ligand receptor interactions in several receptor systems. Early work involving sodium regulation of the opioid receptor yielded inconsistent results. A major reason for the discrepancy was that the early investigators were unaware of the heterogeneity of opioid binding sites and the importance sodium played in regulating these sites. Thus it was the purpose of this study to re-examine these regulatory processes using cells which contained a homogeneous population of opioid receptors.

Results

1. [^{3}H]opioid antagonist binding

Binding of the opioid antagonist, [^{3}H]NAL, by membranes from either NG108-15 or 7315c cells was not substantially affected by replacement of sodium by potassium. In both NG108-15 and 7315c membranes, neither K_D nor B_{max} was altered significantly (Table 8). In both cell types, the number of [^{3}H]NAL binding sites was similar to the number of [^{3}H]ET binding sites measured in the presence of sodium.

2. [^{3}H]opioid agonist binding

When sodium in the modified Krebs buffer was replaced by an equimolar concentration of potassium, binding of [^{3}H]DADLE and [^{3}H]ET by NG108-15 cell membranes was increased. Scatchard analysis indicated that the effect was attributable entirely to an increase by about two-fold in apparent B_{max}, with no significant change in

Table 8

Opioid binding by NG108-15 and 7315c cells and cell membranes in the presence and absence of sodium

Cell membranes or intact cell suspensions were incubated for 20 min at 37° with several concentrations of radioligands, in modified Krebs buffer or in a buffer in which sodium was replaced by an equimolar concentration of potassium (for membranes) or N-MG (for intact cells). The number of independent replicate experiments under each condition is indicated in the table (n). Results were analyzed by LIGAND (Munson and Rodbard, 1980). Under each condition, a single-site model fitted the experimental data better than more complex models. Where no significant differences ($p > 0.05$) in K_D or B_{max} estimates between the plus and minus sodium conditions were observed, combined estimates of the parameter value are reported, except in the case of NAL binding in NG108-15 membranes. Tabulated values are the estimates of K_D or B_{max} (± S.E) from a combined analysis of the results from n independent experiments.

Cell type	Radioligand	$[Na^+]$	n	K_D	B_{max}
		mM		nM	fmol/mg protein
Membranes					
NG108-15	DADLE	118	2	2.3 ± 0.7	230 ± 27
		0	2	2.3 ± 0.7	460 ± 64
	ET	118	3	0.67 ± 0.1	140 ± 9
		0	3	0.67 ± 0.1	330 ± 19
	NAL	118	2	5.2 ± 2.5	115 ± 43
		0	2	11.0 ± 5.2	146 ± 56
7315c	ET	118	3	1.6 ± 0.3	26 ± 2.7
		0	3	0.71± 0.15	26 ± 2.7
	NAL	118	2	0.25 ± 0.08	18 ± 2.1
		0	2	0.30 ± 0.09	18 ± 2.1
Intact cells					
NG108-15	ET	118	3	0.5 ± 0.05	195 ± 11
		0	3	0.5 ± 0.05	367 ± 15
7315c	ET	118	3	0.6 ± 0.14	48 ± 5.4
		0	3	0.3 ± 0.09	48 ± 5.4

the affinity (Fig 11 A, Table 8). Replacement of sodium by potassium also increased binding of [^{3}H]ET in 7315c membranes, although in this tissue the effect was attributable to an increase in affinity of the agonist by a factor of about two-fold, with no significant change in the number of binding sites (Fig 11 B, Table 8).

Similar experiments were conducted in intact cell suspensions, with the exception that sodium was replaced with an equimolar concentration of N-methyl D-glucamine (N-MG) (Connolly and Limbird, 1983) instead of potassium. Again, removal of sodium resulted in an increase (88%) in apparent B_{max} with no change in affinity in NG108-15 cells, but a two-fold increase in affinity with no change in B_{max} in 7315c cells (Table 8).

3. Site of sodium regulation of opioid binding

In order to determine the cellular location of sodium regulatory sites at δ receptors in NG108-15 cells, and at μ receptors in 7315c cells, we have employed the sodium selective ionophore, monensin, to increase intracellular sodium concentration without changing the extracellular concentration. In intact NG108-15 cells suspended in sodium containing modified Krebs buffer, monensin reduced the specific binding of [^{3}H]ET by more than 70% (at 50 μM), the concentration of monensin producing half-maximal effect being between 1 and 5 μM (Fig. 12 A). However, in NG108-15 cells in modified Krebs buffer in which sodium was replaced by N-MG, monensin (1 to 50 μM) failed to produce significant inhibition of binding (Fig 12 B). Similar results were obtained in 7315c cell suspensions. Specific [^{3}H]ET binding was reduced about 60% by monensin in the presence of sodium, with half-maximal reduction of binding occurring at a monensin concentration between 1 and 5 μM (Fig. 13 A). In the absence of sodium no consistent dose-related effect of monensin of [^{3}H]ET binding was observed (Fig 13 B). Thus monensin reduced agonist binding in both cell types, provided that sodium was present in the extracellular fluid. In homogenized membrane preparations from NG108-15 and 7315c

FIGURE 11. SATURATION ANALYSIS OF SODIUM EFFECT ON [^{3}H]ET BINDING TO NG108-15 AND 7315c CELLS. Scatchard transformation of [^{3}H]ET binding to δ-receptors in NG108-15 cells (A) or μ-receptors in 7315c cells (B) suspended in Krebs-HEPES buffer. Specific binding was measured at increasing concentrations from 0.05 to 25 nM, in the presence of 118 mM sodium (•) , or after equimolar replacement of sodium by N-methyl D-glucamine (N-MG) (o). Data are mean values of triplicate determinations in a single experiment, which was replicated twice with similar results. Estimates of K_D and B_{max} from the combined experiments are listed in Tables 4 and 5. A, 160 μg of cell protein/assay tube in the presence of sodium, 300 μg of protein/assay tube in the absence of sodium.

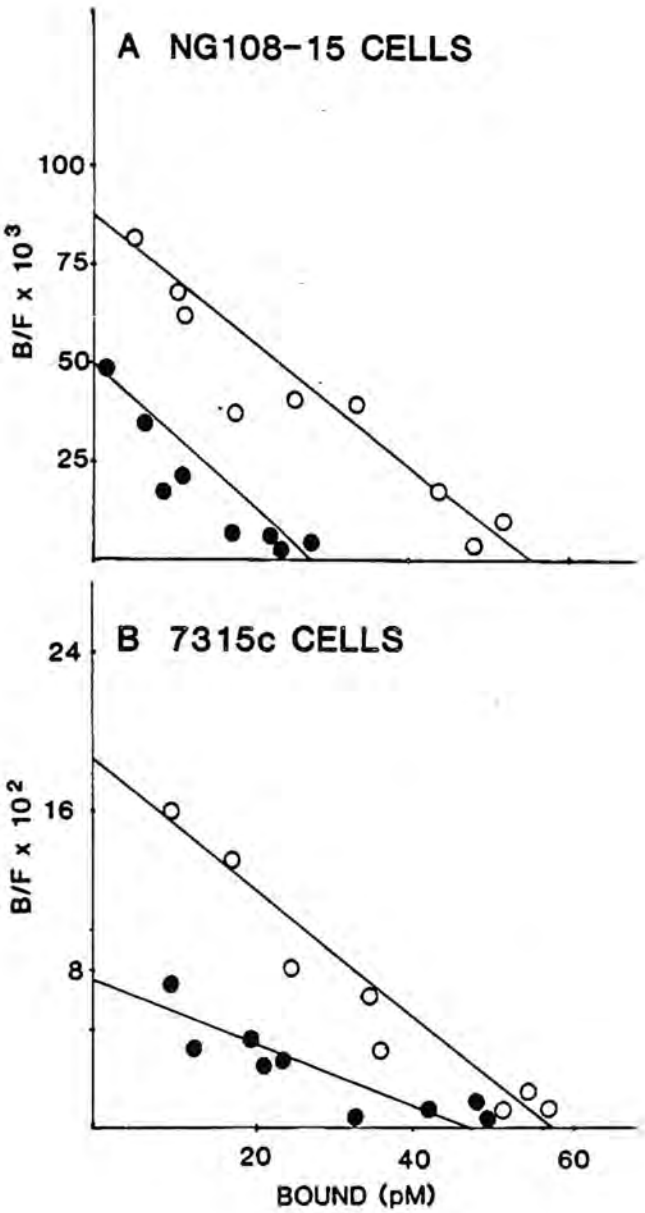

A NG108-15 CELLS
100
75
50
25
B/F x 10³
B 7315c CELLS
24
16
8
B/F x 10²
20
40
60
BOUND (pM)

FIGURE 12. EFFECTS OF MONENSIN ON THE SPECIFIC BINDING OF 2 nM [^{3}H]ET BY δ OPIOID RECEPTORS IN NG108-15 CELLS. Cells were incubated in the absence or presence of increasing concentrations of monensin (1 to 50 μM) in modified Krebs-HEPES buffer containing 118 mM sodium (A), or modified Krebs-HEPES buffer in which sodium was replaced with N-MG (B). Results are the mean ± SE of triplicate determinations. Replicate experiments (Total N=2) yielded similar results.

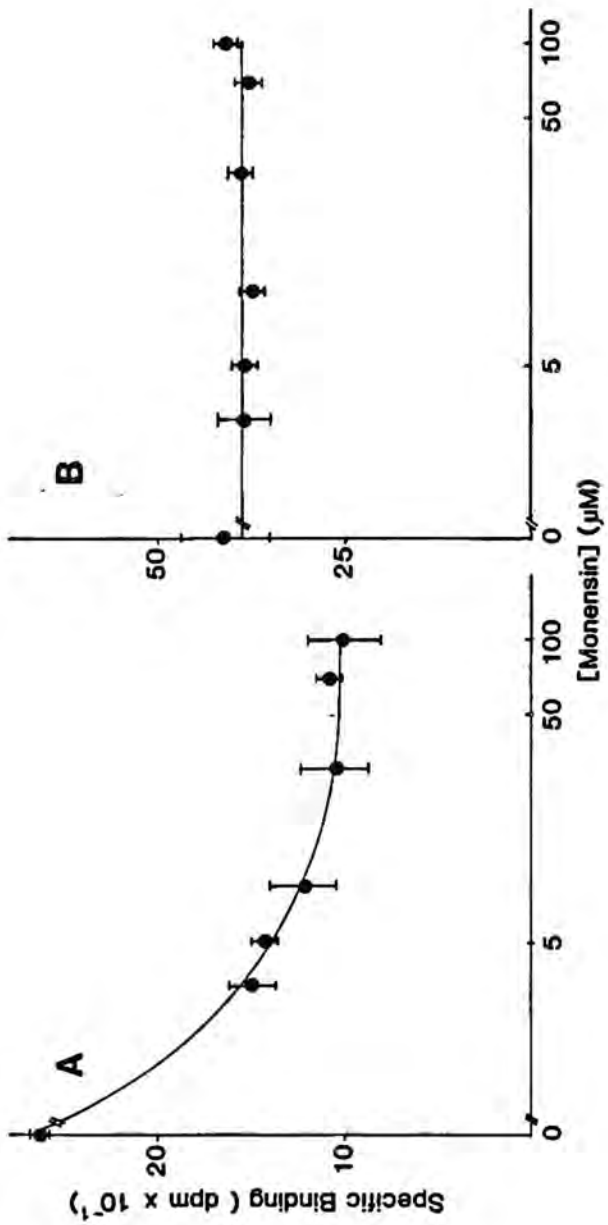
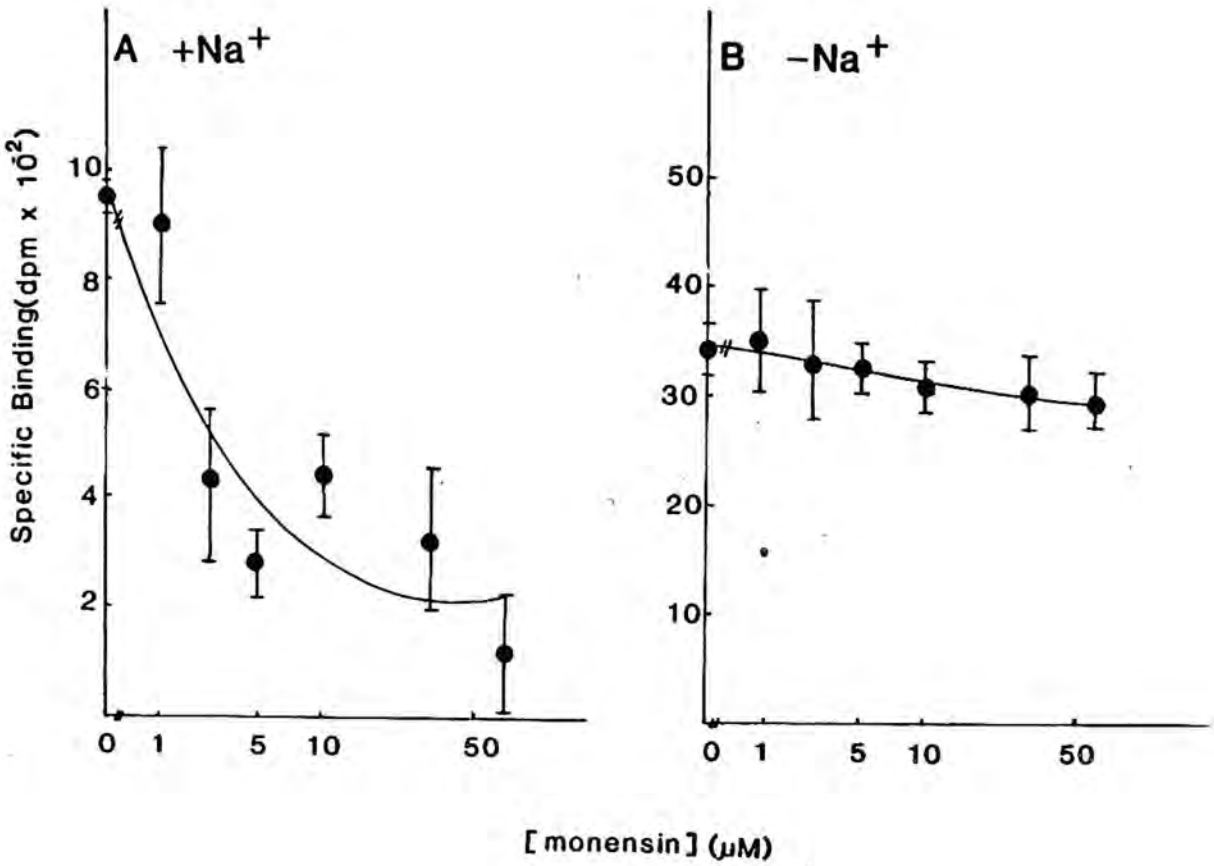

A
B
Specific Binding (dpm x 10^-1)
[Monensin] (µM)
0
5
50
100
10
20
25

FIGURE 13. EFFECTS OF MONENSIN ON THE SPECIFIC BINDING OF 2 nM [^{3}H]ET BY μ OPIOID RECEPTORS IN 7315c CELLS. Cells were incubated in the absence or presence of increasing concentrations of monensin (1 to 50 μM) in modified Krebs-HEPES buffer containing 118 mM sodium (A), or modified Krebs-HEPES buffer in which sodium was replaced with N-MG (B). Results are the mean ± SE of triplicate determinations. Replicate experiments (Total N=2) yielded similar results.


A +Na+
B −Na+
Specific Binding(dpm x 10²)
10
8
6
4
2
50
40
30
20
10
0 1 5 10 50
0 1 5 10 50
[monensin] (μM)

cells, however, monensin produced only a slight inhibition of [^{3}H]ET binding which was independent of monensin concentration between 3 and 100 μM and not altered by the removal of sodium (Table 9).

Saturation analysis of the effects of 30μM monensin on the specific binding of [^{3}H]ET by intact NG108-15 and 7315c cells in the presence of 118 mM sodium demonstrated that monensin reduced the number of specific binding sites for this agonist in NG108-15 cells from 101 to 54 fmol/mg protein (data from two independent experiments modeled together) without any change in affinity (combined estimate of K_D, 0.43 nM). Thus the effect of adding monensin on the number of high-affinity binding sites in these cells is similar to the effect of an increase in sodium concentration. In contrast, addition of monensin to 7315c cells did not change the number of binding sites for [^{3}H]ET (two independent experiments, combined estimate of B_{max}, 19 fmol/mg protein), but reduced affinity (K_D increased from 2.4 to 4.0 nM; $P < 0.01$). This action of monensin in intact 7315c cells also parallels the effects on agonist binding of adding sodium to 7315c membranes suspensions. These results support the conclusion that monensin inhibits opioid binding in intact NG108-15 and 7315c cells by increasing the intracellular concentration of sodium. It therefore is probable that sodium exerts its regulatory effect on opioid binding through an intracellular site of action in each cell type even through the consequences of an increase in intracellular sodium are not identical in the two cell types.

4. Effect of sodium on opioid inhibition of adenylyl cyclase activity

Opioids have been shown to inhibit adenylyl cyclase activity in both NG108-15 and 7315c membranes (Sharma et al., 1975; Frey and Kebabian, 1984). Sodium has also been shown to be essential in the coupling of opioid receptors to adenylyl cyclase in NG108-15 membranes (Blume et al., 1979). In view of the apparent differences in the manner of

Table 9

Effects of monensin on specific binding of [^{3}H]ET by NG108-15 and 7315c membranes

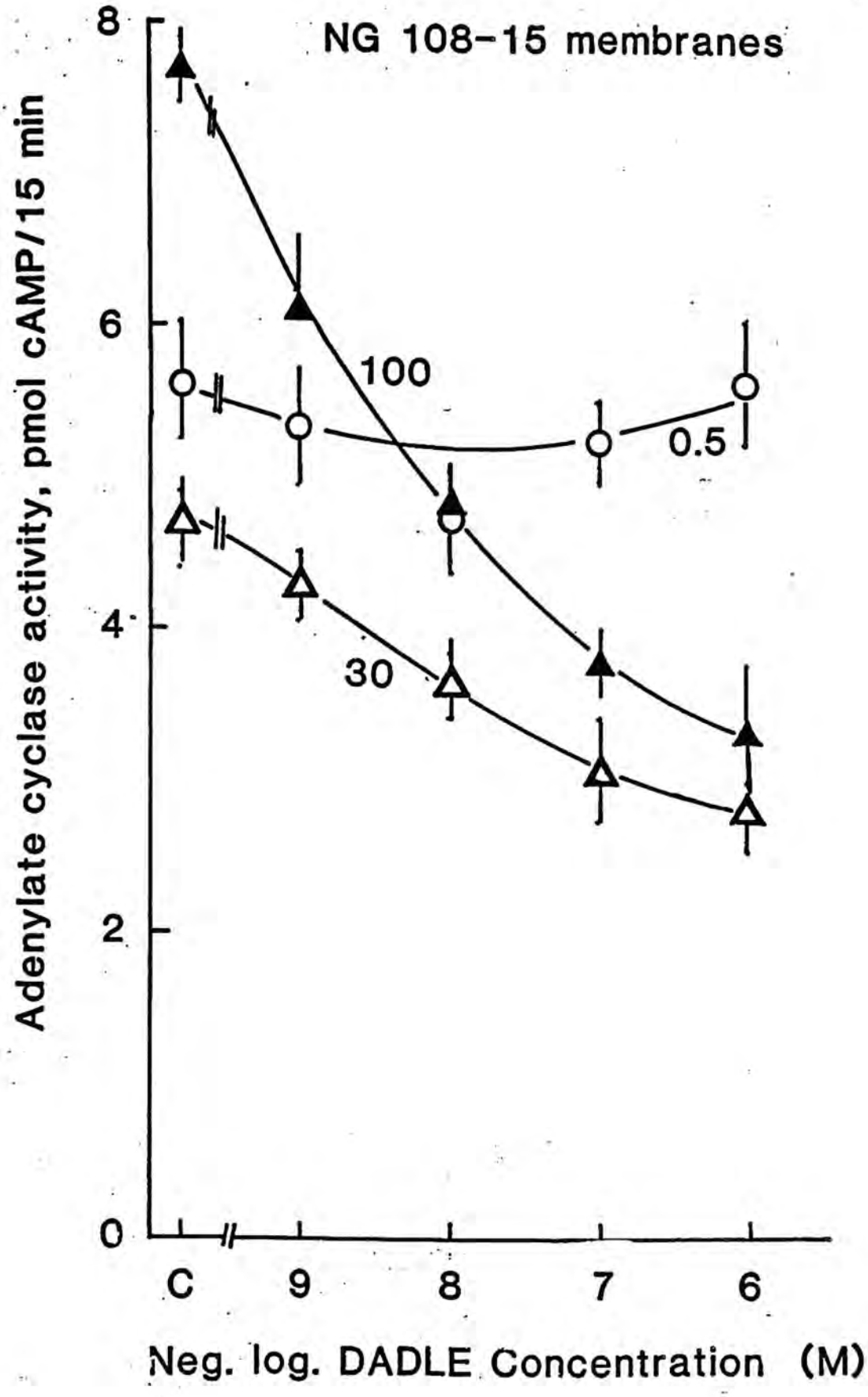
Washed membranes from NG108-15 or 7315c cells were incubated for 20 min with [^{3}H]ET, 2 nM, in modified Krebs buffer containing either 118 mM sodium or 118 mM N-MG, in the presence of varying concentrations of monensin. The table shows the specific binding of [^{3}H]ET in the presence of varying concentrations of monensin, expressed as a percentage (± S.E.M. of triplicate estimates) of the specific binding in the absence of monensin (control binding). The experiment was replicated twice with similar results.

Monensin concentration	Percentage of control binding in absence of sodium			
	NG108-15 membranes		7315c membranes	
	$+Na^+$	$-Na^+$	$+Na^+$	$-Na^+$
μM				
3	97 ± 7	82 ± 4	93 ± 1	92 ± 2
5	88 ± 3	89 ± 9	99 ± 3	100 ± 11
10	86 ± 4	93 ± 9	98 ± 1	99 ± 4
30	77 ± 5	86 ± 12	99 ± 12	100 ± 12
70	81 ± 10	79 ± 7	89 ± 4	91 ± 2
100	88 ± 14	79 ± 8	96 ± 3	98 ± 3

sodium regulation of opioid binding in these two membrane preparations, it was of interest to determine if opioid regulation of adenylyl cyclase activity might be differentially affected by sodium in membranes prepared from the two cell types. In these studies, DADLE was used as the agonist, since it has good affinity at both μ and δ receptors, and the effects of varying the sodium concentration on DADLE regulation of adenylyl cyclase were tested under comparable conditions in each cell type. In the absence of opioid, adenylyl cyclase activity in membrane incubates from both types of cell was enhanced by increasing the sodium concentration from 0.5 to 100 mM. At the lowest sodium concentration, inhibition of adenylyl cyclase in NG108-15 membranes by DADLE (1nM to 1 μM) was negligible. However, at 100 mM sodium, significant inhibition of adenylyl cyclase was observed, with a maximum inhibition of about 50 to 60% of control activity (Fig. 14). At an intermediate sodium concentration (30 mM), DADLE induced inhibition of adenylyl cyclase, but the maximum inhibition was about 30 to 40% of control enzyme activity. The IC50 for DADLE in NG108-15 cell membranes was about 10 nM both at 30 and at 100 mM sodium. The sodium dependence of opioid inhibition of adenylyl cyclase in NG108-15 membranes was also confirmed in experiments with ß-endorphin, where the peptide produced no inhibition of enzyme activity at concentrations up to 10 μM when the sodium concentration was 0.5 mM (personal communication with Dr. Cote). At 100 mM sodium, maximum inhibition of adenylyl cyclase by ß-endorphin was about 50% of control enzyme activity, and the ß-endorphin IC50 was about 100 nM. These results are consistent with previous reports of the sodium dependence of opioid inhibition of adenylyl cyclase in NG108-15 membranes (Blume et al., 1979), and show that the maximum inhibition by opioid, but not the IC50 of the agonist, is dependent on the sodium concentration.

In 7315c membranes in the presence of 0.5 mM sodium, DADLE reduced adenylyl cyclase activity, with a maximum effect of about 50% inhibition at concentrations from 100

FIGURE 14. EFFECTS OF SODIUM ON OPIATE -INDUCED INHIBITION OF ADENYLYL CYCLASE ACTIVITY IN NG108-15 CELL MEMBRANES. Membranes from NG108-15 cells were incubated in the absence or presence of increasing concentrations of DADLE (1 nM to 1 μM), at three sodium concentrations: o, 0.5 mM sodium; Δ, 30 mM sodium and s, 100 mM sodium. Vertical bars indicate the standard errors of the mean estimates of triplicate determinations.

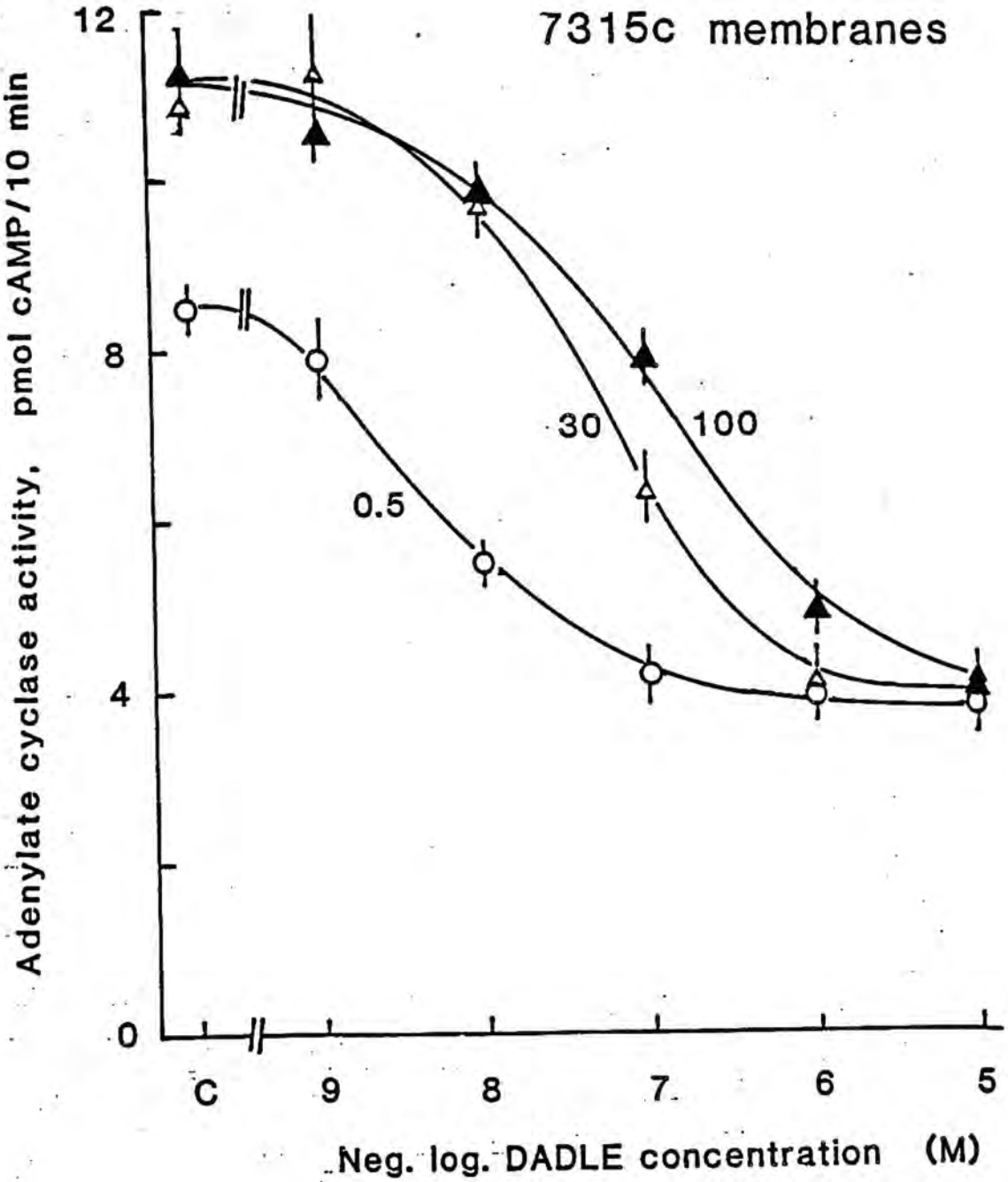

NG 108-15 membranes
Adenylate cyclase activity, pmol cAMP/15 min
8
6
4
2
0
100
0.5
30
C
9
8
7
6
Neg. log. DADLE Concentration (M)

nM to 10 μM. At higher sodium concentrations, enzyme activity increased, as observed in the NG108-15 cell membranes, by about 20 %, and all of the increased activity was inhibitable by DADLE (Fig 15). The IC50 for DADLE also increased as the sodium concentration was raised, from about 4.5 nM at 0.5 mM sodium, through 45 nM at 30 mM sodium, to about 130 nM at 100 mM sodium. In another experiment, the IC50 for morphine inhibition of adenylyl cyclase activity in 7315c membranes increased from 40 nM in 0.5 mM sodium, through 60 nM in 10 mM sodium, 300 nM in 30 mM sodium to 2.5 μM in 100 mM sodium. Thus in 7315c membranes, in contrast to NG108-15 membranes, agonist potency was reduced by an increase in sodium concentration.

DISCUSSION

In 7315c cell membranes, replacement of sodium by potassium resulted in an increase in agonist affinity for the μ receptor by about 2-fold. No change was seen in the number of binding sites following sodium removal. The effects of sodium removal cannot be attributed to a change in ionic strength, since ionic strength was maintained constant in these studies. Similar results were obtained in intact cell suspensions when sodium was removed from the extracellular medium, indicating that the effect of sodium on agonist affinity was not dependent on fragmentation of the cell membrane. Similar results were obtained when either potassium or N-methyl D-glucamine were used as the replacement ion for sodium indicating that the effect of sodium removal is not attributable to the action of a particular replacement ion. Thus, sodium appears to reduce the affinity of agonists at μ-receptors in 7315c cells. The effect of sodium removal on opioid binding in NG108-15 cells was not identical to its effects in 7315c cells. In NG108-15 membranes, an increase by a factor of about 2 in the number of observed specific binding sites was noted following replacement of sodium by potassium, with no change in the affinity of the binding sites.

FIGURE 15. EFFECTS OF SODIUM ON OPIATE -INDUCED INHIBITION OF ADENYLYL CYCLASE ACTIVITY IN 7315c CELL MEMBRANES. Membranes from 7315c cells were incubated in the absence or presence of increasing concentrations of DADLE (1 nM to 1 μM), at three sodium concentrations: o, 0.5 mM sodium; Δ , 30 mM sodium and s, 100 mM sodium. Vertical bars indicate the standard errors of the mean estimates of triplicate determinations.


7315c membranes
12
8
4
0
30
100
0.5
C
9
8
7
6
5
Adenylate cyclase activity, pmol cAMP/10 min
Neg. log. DADLE concentration (M)

This effect occurred both with a peptide agonist, DADLE, and with an alkaloid derivative, ET, and with two different replacement cations, potassium and N-MG. Removal of sodium from the incubation medium also increased the number of [^{3}H]ET binding sites in intact NG108-15 cells. It is therefore unlikely that the effect of sodium removal was associated with a particular ligand or replacement ion, or the integrity of the cell membrane. Thus, in contrast to its effects on μ opioid receptors in 7315c cells, sodium reduces the number of δ binding sites measurable by saturation binding with labeled agonist.

The apparent difference in sodium regulation of opioid binding is probably not a function of different membrane organization or composition in two different types of cells, since similar results have been obtained in parallel experiments using guinea pig cortical membranes where both receptor types are present in the same membrane homogenate (Werling et al., 1986). This demonstration, that the effects of sodium on labeled agonist binding at μ and δ receptors differ, may provide a resolution of the early, apparently conflicting, reports. At that time studies reported the effect of sodium was usually a reduction of opioid agonist affinity (Simantov, et al., 1978; Simon et al., 1975), brought about largely through an increase in the rate of agonist dissociation (Blume, 1978) . However other investigators reported sodium induced changes in receptor density of opioid binding sites (Pasternak et al., 1975; Simantov et al., 1976; Lee et al., 1977).

The ability of sodium to reduce the number of high-affinity δ receptors requires further consideration. It is improbable that binding sites are destroyed by exposure to sodium since the sodium-induced reduction in binding was reversible. Furthermore, the number of binding sites for the antagonist, NAL, was not changed by exposure to sodium. The effect of sodium on δ receptors is probably to reduce the affinity for agonists of about half the sites to very low values, leaving these sites undetectable by radiolabeled agonists

when binding is characterized by Scatchard transformation of saturation data. This argument is supported by the studies of Law et al. (1983). By measuring displacement of radiolabeled antagonist binding at NG108-15 cells δ receptors, they were able to demonstrate that 30-40% of the binding sites had very low-affinity for DADLE when 100 mM sodium was present. In experiments where labeled agonists are employed, these low-affinity sites would not be detectable above the background nonspecific binding. Thus, a large reduction in agonist affinity at a fraction of the binding sites resulted in an apparent decrease in the receptor density. Subsequent experiments in which unlabeled DADLE competed for [^{3}H]diprenorphine binding in the presence and absence of sodium, revealed a low affinity site in the presence of sodium (data not shown). These results reflected as a biphasic curve agree with earlier work by Law et al. (1983) and support the idea that sodium shifted a porportion of δ receptors into a very low affinity state not measurable by direct [^{3}H]agonist binding. Similar experiments performed on the 7315c cell membranes did not expose a low affinity μ site in the presence of sodium, but showed a shift in the affinity of the entire population of labeled μ receptors, analogous to that revealed in Scatchard transformation of saturation data (Fig 11, Table 8).

To establish the cellular location of the site at which sodium induces regulation of agonist binding, the sodium-selective ionophore monensin was employed. Monensin will produce increases in intracellular sodium concentration without changing the extracellular concentration. Monensin has been shown to increase sodium uptake in the NG108-15 cells (Lichtshtein et al., 1979) and has been employed in studies of sodium regulation of α_2-adrenergic receptors (Connolly and Limbird, 1983). Results indicate that in both the NG108-15 and 7315c cells, monensin treatment produced a reduction in opioid agonist binding, provided sodium was present in the extracellular medium. After removal of sodium from the extracellular medium, monensin did not significantly change the opioid

agonist binding. Moreover, monensin had little effect when similar experiments were performed in homogenized membrane preparations. The simplest interpretation of these results is that monensin reduced the agonist binding in NG108-15 and 7315c cells by increasing sodium flux through the cell membrane, allowing the intracellular concentration of this ion in the vicinity of opioid receptors to rise to a level at which agonist binding was affected. This interpretation is supported by the demonstration that monensin behaved in a manner similar to sodium in reducing agonist affinity at the μ receptors in 7315c cells, but reducing the number of receptors in the NG108-15 cells without a change in the affinity of the residual binding sites. Therefore it is concluded that sodium exerts its regulatory effect at an intracellular site in both the NG108-15 and 7315c cells. This finding is supported by experiments designed to examine agonist binding in the presence of increasing concentrations of sodium in guinea pig cortical membranes (Werling et al., 1986). For μ, δ, and κ sites, the concentration of sodium giving half-maximal inhibition of agonist binding was about 10-30 mM, corresponding to the intracellular sodium level. These results suggest small changes in intracellular sodium concentrations, perhaps only in regions of receptors, may be able to regulate agonist affinity and coupling to G proteins.

In order to examine sodium regulation of opioid inhibition of adenylyl cyclase activity, the effect of increasing sodium concentration on the ability of an opioid agonist to inhibit cyclase activity was examined. In NG108-15 cell membranes, opioid inhibition of enzyme activity was dependent on the presence of a sodium concentration in excess of 0.5 mM. Increasing the sodium concentration increased both the activity of the enzyme and the fraction of cyclase activity that was inhibitable by opioid agonist, but did not change the potency of the agonist as measured by the concentration required to inhibit adenylyl cyclase by 50% of the maximum inhibition. These results again suggest that addition of sodium assists in the coupling of the δ receptor to guanyl nucleotide binding proteins and adenylyl

cyclase activity in the NG108-15 cells. In the 7315c cells, significant opioid inhibition of enzyme activity was observed in the presence of a low sodium concentrations (0.5 mM). Increasing the sodium concentration not only increased cyclase activity, but also resulted in an increase in the opioid agonist IC50. This result is consistent with the reduction in agonist affinity observed with increasing sodium concentrations in these cells. Thus, functional studies of opioid effects in the two types of cells support the conclusion from ligand binding studies that sodium regulates agonist binding to μ and δ receptors by different mechanisms.

It should be noted that the difference in sodium regulation between the two receptor types may be associated with the manner of interaction of each receptor with its guanyl nucleotide-binding protein. Opioid receptor-GTP-guanyl nucleotide binding protein complexes may regulate different functions in different cell types, or under different physiological circumstances. In order to investigate the role guanyl nucleotides play in ligand-receptor interactions, it was necessary to compare guanyl nucleotide regulation of agonist binding in both the 7315c and NG108-15 cells.

CHAPTER THREE:
EFFECTS OF GUANYL NUCLEOTIDES ON OPIOID BINDING IN THE NG108-15 AND 7315c CELL MEMBRANES

Rationale

Several reports of guanyl nucleotide regulation of opioid agonist binding have suggested that these nucleotides act to decrease the binding of agonists to their receptors in both brain membranes (Blume, 1978; Childers and Snyder, 1978; Ishizuka et al., 1984) and established cell lines (Blume et al., 1978; Chang et al., 1981). The extent of guanyl nucleotide - induced inhibition for the agonists tested varied according to receptor type (Ishizuka et al., 1984), and depended upon cationic composition of the incubation medium (Childers and Snyder, 1978). In general, the effect of GTP addition to a membrane preparation appeared to be a reduction in agonist affinity as a result of increased agonist dissociation rate (Blume, 1978; Childers and Snyder, 1980). Recent characterization of the proteins which bind these guanyl nucleotides (G proteins) and the model proposed to describe the cycle of interaction of these proteins, nucleotides, and the receptor itself (Fig 1) suggested that the assumption of a single affinity state of a particular opioid receptor in the absence of added guanyl nucleotide and another in the presence may be too simplistic. Assuming that this cycle is continuously operative during the course of a binding incubation, at equilibrium, it is likely that several forms of receptor complex, each with a different affinity, may be identified. Thus it was the purpose of this study to reexamine guanyl nucleotide regulation of the μ and δ opioid receptor. In an attempt to identify multiple affinity states of the receptors, pertussis toxin as well as non-hydrolyzable guanyl nucleotide analogues were utilized.

Results

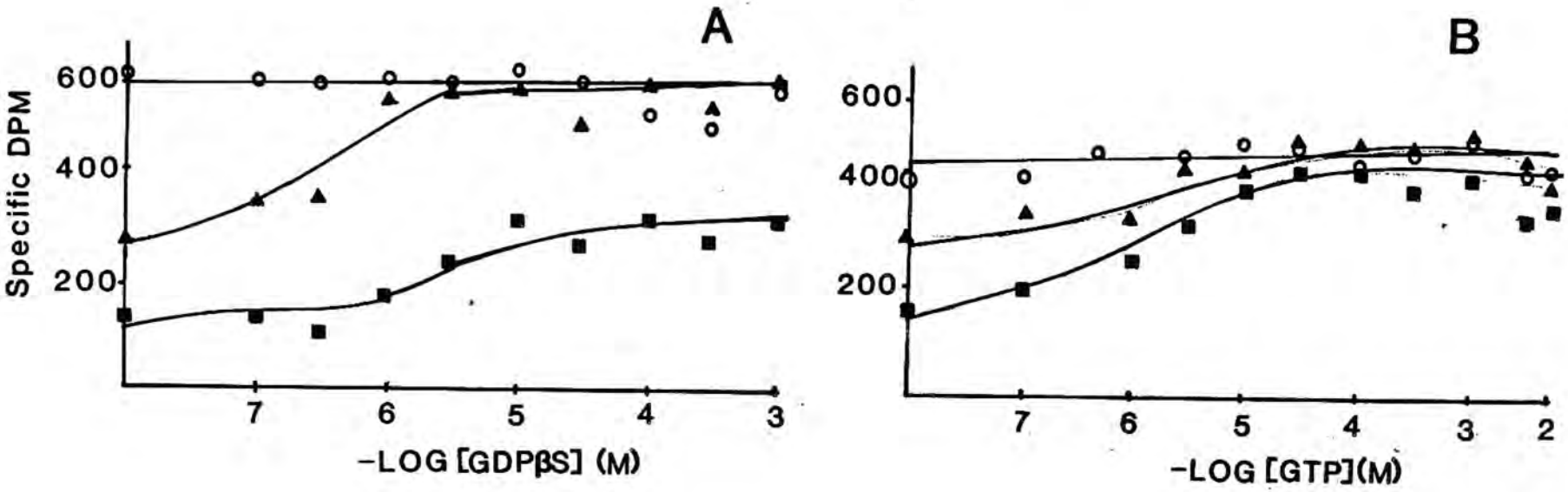
1. Determination of effective guanyl nucleotide concentrations

In order to examine the effects of guanyl nucleotide regulation of opioid agonist binding, GTP, GTPγS and GDPβS nucleotide concentration response curves were constructed to ascertain the maximally effective concentration of each analogue in inhibiting agonist binding at high and low concentrations of agonist (Fig. 16). Concentration curves ranging from 0 to 500 μM were constructed for GTP, and from 0 to 100 μM for GDPβS and GTPγS. For the 7315c membranes, 100 μM GTP and 10 μM GDPβS and GTPγS produced maximal inhibition of 20 nM and 500 nM DAGO competition against [^{3}H]NAL. These concentrations of nucleotides were also maximally effective in inhibiting 10 nM and 500 nM DSLET competition against [^{3}H]DIP binding in the NG108-15 membranes. The binding of the opioid antagonist [^{3}H]NAL, in the 7315c membranes, and [^{3}H]DIP, in the NG108-15 membranes in the absence of agonist, were not reduced by the addition of 100 μM concentrations of GTP, GTPγS or GDPβS .

2. [^{3}H]opioid antagonist binding

Saturation analyses of the specific binding of [^{3}H]NAL to 7315c membranes or of [^{3}H]DIP to NG108-15 membranes were performed in the absence and presence of 100 μM GTP. Scatchard analysis of specific binding resulted in linear plots in both the 7315c and NG108-15 membranes. In the absence of GTP, the [^{3}H]NAL K_D was 0.93 nM ± 0.3 nM. The addition of 100 μM GTP to the 7315c membranes did not significantly alter this affinity (K_D = 1.0 nM ± 0.45 nM). Addition of GTP also had no effect on the estimated number of μ receptor sites. In the absence of GTP the Bmax was approximately 21 ± 3 fmol/mg protein. With the addition of GTP, the B_{max} was 19 ± 4 fmol/mg protein. Similarly in the NG108-15 membranes the addition of 100 μM GTP did not affect [^{3}H]DIP binding. The K_D of [3H]DIP for the δ receptor was 1.2 nM ± 1 nM in the

FIGURE 16. EFFECTS OF INCREASING CONCENTRATIONS OF GDPβS OR GTP ON THE ABILITY OF DAGO TO COMPETE AGAINST [^{3}H]NAL BINDING TO 7315c MEMBRANES. A, (o) competition of [^{3}H]NAL binding by increasing concentrations of GDPβS; (s) competition of [^{3}H]NAL to μ receptors by 20 nM DAGO in the presence of increasing concentrations of GDPβS; (n) competition of [^{3}H]NAL to μ receptors by 500 nM DAGO in the presence of increasing concentrations of GDPβS. B, (o)competition of [^{3}H]NAL binding by increasing concentrations of GTP; (s) competition of [^{3}H]NAL to μ receptors by 20 nM DAGO in the presence of increasing concentrations of GTP; (n) competition of [^{3}H]NAL to μ receptors by 500 nM DAGO in the presence of increasing concentrations of GTP. Data are mean values of triplicate determinations in a single experiment, which was repeated three times with similar results.


A
B
Specific DPM
600
400
200
7
6
5
4
3
2
-LOG [GDPβS] (M)
-LOG [GTP](M)

absence of GTP, with a receptor density of 104 ± 5 fmol/mg/protein. With the addition of GTP the K_D was 1.2 nM ± 5 nM with a B_{max} of 103 ± 2 fmol /mg protein.

Comparable results were obtained when competition studies were performed in the 7315c membranes, using [^{3}H]NAL binding displaced by unlabeled naloxone in the presence and absence of 100 μM GTP (Fig 17, Table 10) . Computer analysis resulted in a one site model with no significant change in the antagonist affinity. These studies also revealed no significant change in the number of binding sites labeled under these conditions. Similar results were obtained in the NG108-15 membranes when [^{3}H]DIP was competed with unlabeled diprenorphine (Fig 18, Table 10). Neither ligand affinity nor binding site density were significantly affected by the addition of 100 μM GTP.

3. Agonist binding

Addition of 100 μM GTP affected both the binding of DSLET, a δ preferring agonist, in the NG108-15 membranes and DAGO, a μ selective agonist, in the 7315c membranes. Competition against [^{3}H]antagonist binding by either DSLET or DAGO in the absence of added nucleotide, resulted in a broad displacement. Analysis suggested two components of agonist binding with high and low affinities. The high-affinity site had a K_{app} of 1-10 nM, the low-affinity K_{app} was 100-500 nM (Figs 19, 20 ; Table 11). In the 7315c membranes, the ratio of the high-affinity site to low-affinity site was approximately 2:1. In the NG108-15 membranes this ratio was approximately 3:1. The lower affinity state was not detectable by direct measurement of labeled agonist binding (Werling et al., 1987). A biphasic curve was also observed when DPDPE, an agonist with greater δ-selectivity than DSLET (Mosberg et al., 1983), competed for [^{3}H]DIP binding in the NG108-15 membranes (Table 1). DPDPE was much less potent in inhibiting binding of [^{3}H]NAL in 7315c cell membranes (Table 5). In both cell types, the number of binding sites labeled

FIGURE 17. COMPETITION FOR [^{3}H]NAL BINDING TO μ OPIOID SITES IN 7315c CELL MEMBRANES BY UNLABELED NAL IN THE PRESENCE (O) OR ABSENCE (•) OF 100 μM GTP. The lines are best fits generated by the LIGAND program. The optimal fit produced from these data was for one binding site under each condition. Nonspecific binding was a modeled parameter equal to 15% of total binding in the presence and 14% in the absence of added nucleotide. For more direct comparison between treatments, this parameter has been subtracted in these plots. Other details of the binding are provided in the Materials and Methods. Data are from a single experiment which was repeated twice with similar results.

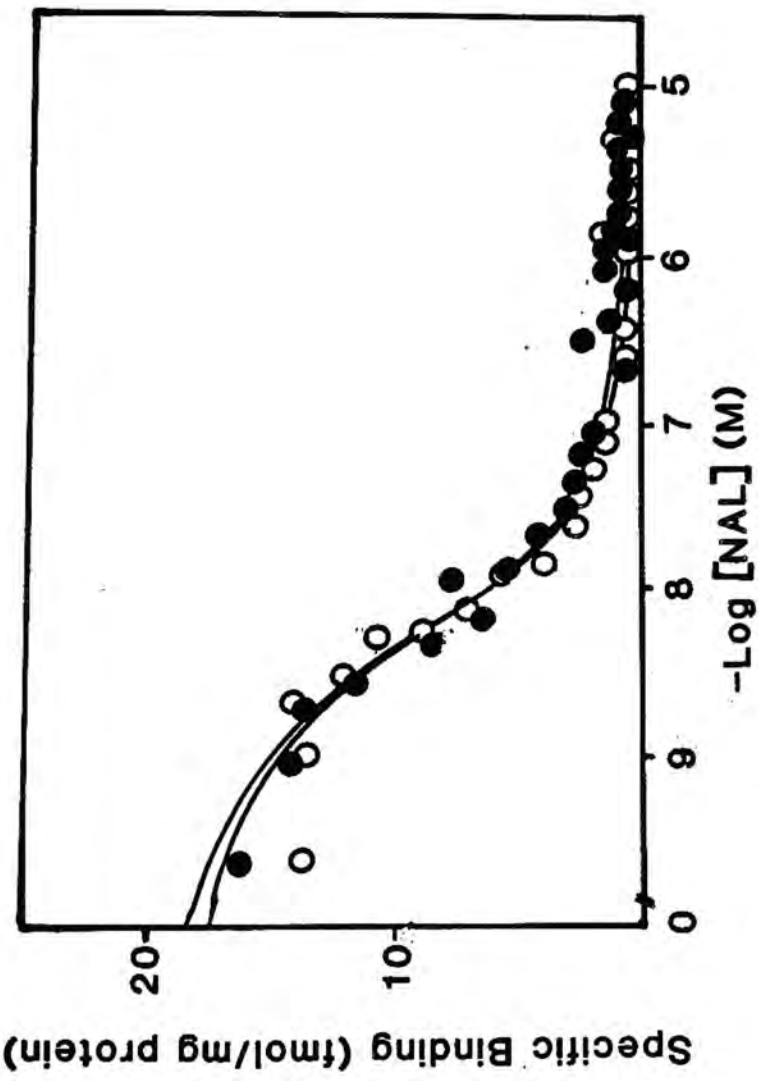

Specific Binding (fmol/mg protein)
20
10
0
9
8
7
6
5
-Log [NAL] (M)

TABLE 10

Antagonist binding by NG108-15 and 7315c cell membranes in the presence and absence of 100 μM GTP

Cell membranes were incubated for 20 minutes at 37° in modified Krebs buffer in the absence or presence of 100 μM GTP. The number of independent replicate experiments under each condition is indicated in the table (N). Results were analyzed by a nonlinear curve-fitting algorithm. The tabulated values are the estimates of K_i or B_{max} (± standard error of the parameter estimate) from a combined analysis of the results from N independent experiments. The concentrations of [^{3}H]NAL was 1 nM and [^{3}H]DIP was 1 nM.

Cell Membrane	Radioligand	Unlabeled Antagonist	[GTP] μM	N	K_i nM	B_{max} fmol/mg protein
7315c	NAL	NAL	none	2	2.0 ± 0.5	43 ± 4
			100	2	1.5 ± 1	59 ± 20
NG108-15	DIP	DIP	none	3	2.3 ± 1	110 ± 20
			100	3	3.6 ± 1	97 ± 7

FIGURE 18. COMPETITION FOR [^{3}H]DIP BINDING TO δ OPIOID SITES IN NG108-15 CELL MEMBRANES BY UNLABELED DIP IN THE PRESENCE (O) OR ABSENCE (•) OF 100 μM GTP. The lines are traced from the best fits generated by the LIGAND program. The optimal fits produced from these data were for one binding site under each condition. Nonspecific binding was a modeled parameter equal to 24% of total binding in the presence and 21% in the absence of added nucleotide. For more direct comparison between treatments, this parameter has been subtracted in these plots. Other details of the binding are provided in the Materials and Methods. Data are from a single experiment which was repeated with similar results (Total N=3).

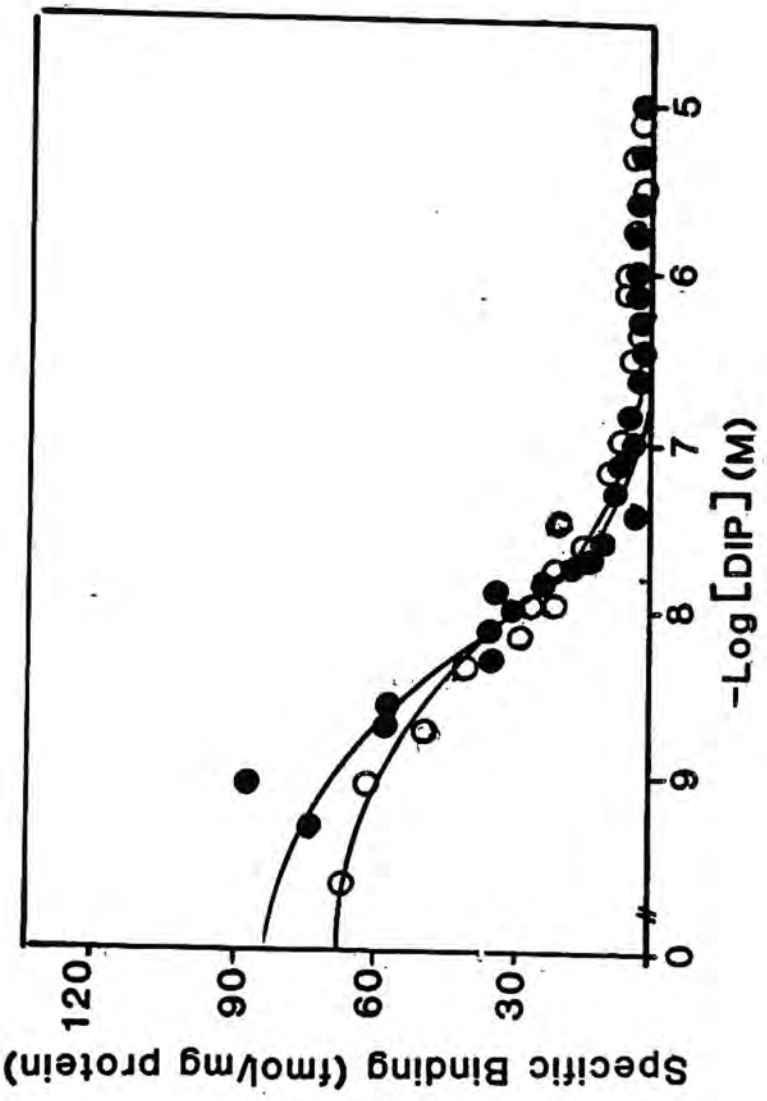

Specific Binding (fmol/mg protein)
120
90
60
30
0
5
6
7
8
9
-Log [DIP] (M)

FIGURE 19. COMPETITION FOR [^{3}H]DIP BINDING TO δ OPIOID SITES IN NG108-15 CELL MEMBRANES BY UNLABELED DSLET IN THE PRESENCE (O) OR ABSENCE (•) OF 10 μM GTPγS. The lines are traced from the best fits generated by the LIGAND program. The optimal fits produced from these data were for two binding sites under each condition. Nonspecific binding was a modeled parameter equal to 36% of total binding in the presence and 37% in the absence of added nucleotide. For more direct comparison between treatments, this parameter has been subtracted in these plots. Other details of the binding are provided in the Materials and Methods. Data are from a single experiment which was repeated with similar results (Total N=3).

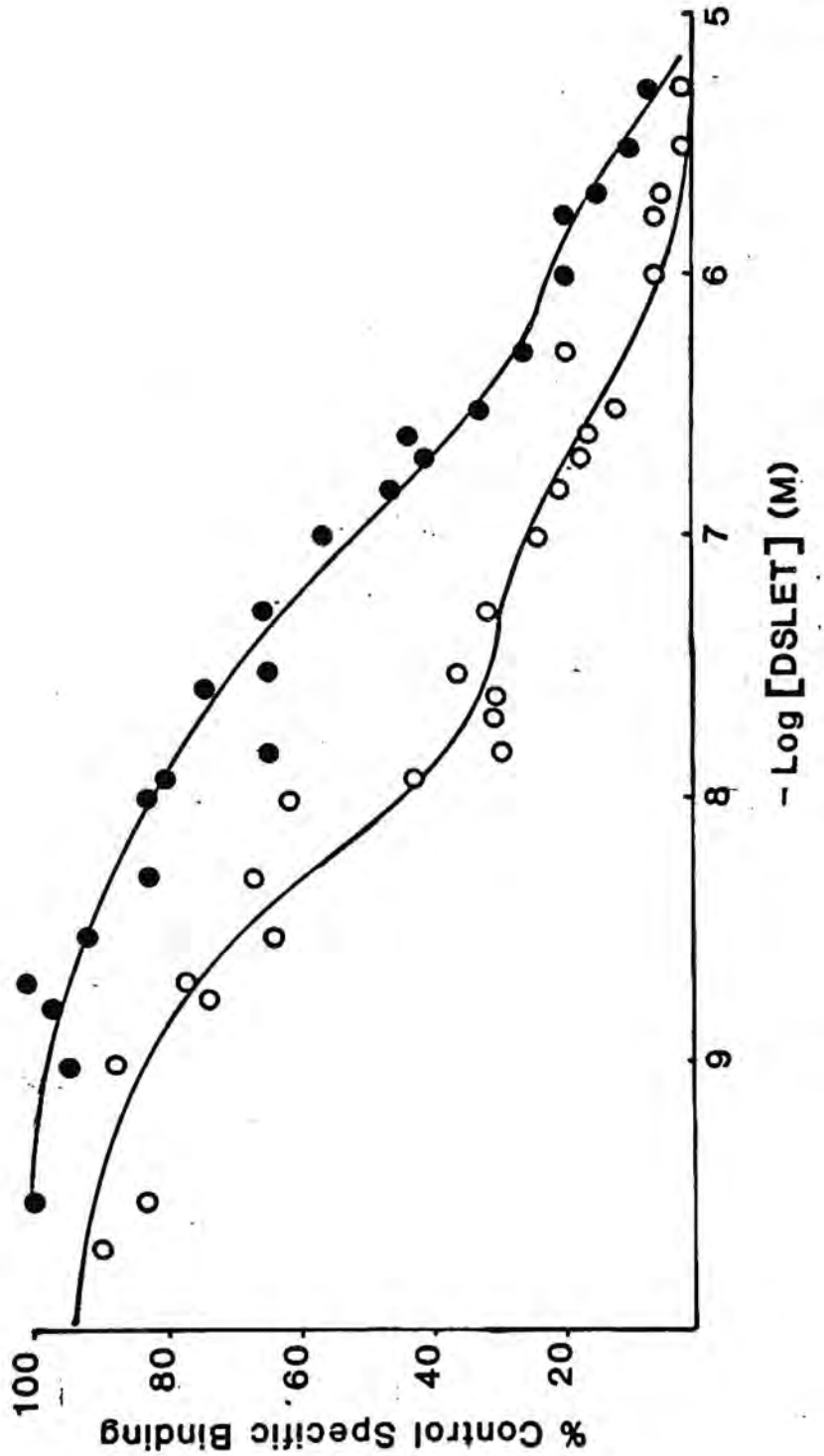
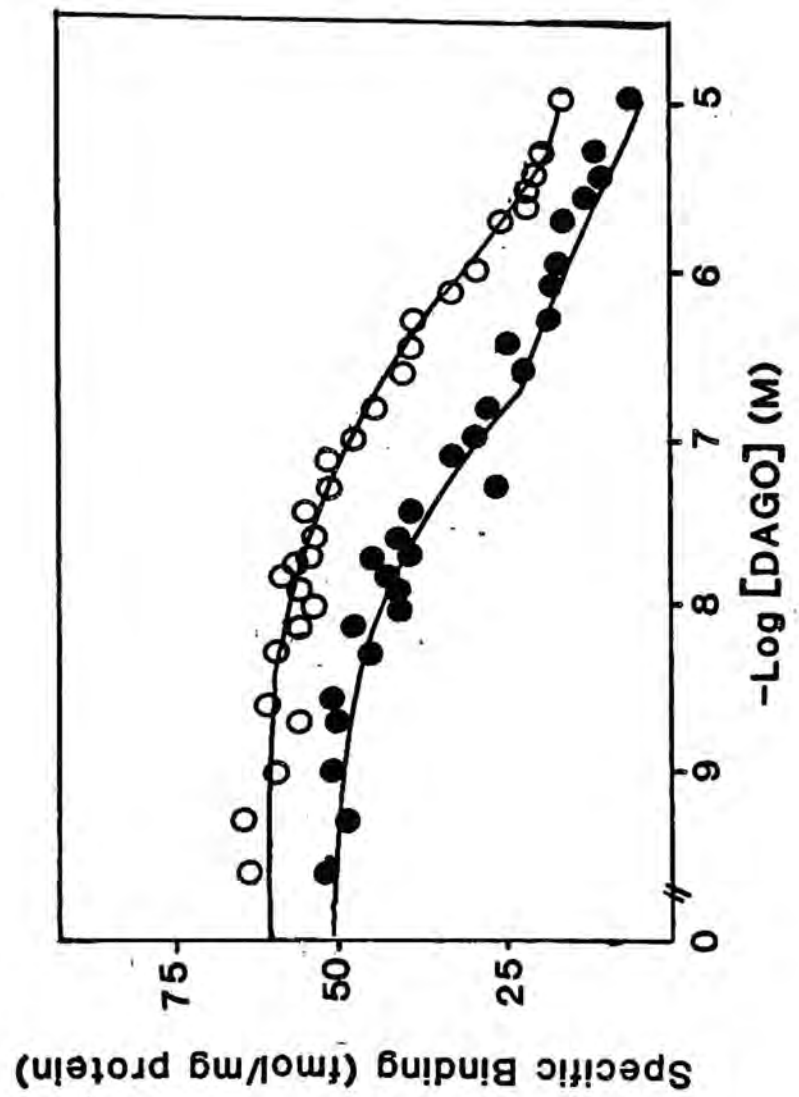

% Control Specific Binding
100
80
60
40
20
5
6
7
8
9
– Log [DSLET] (M)

FIGURE 20. COMPETITION FOR [^{3}H]NAL BINDING TO μ OPIOID SITES IN 7315c CELL MEMBRANES BY UNLABELED DAGO IN THE PRESENCE (O) OR ABSENCE (•) OF 10 μM GTPγS. The lines are traced from the best fits generated by the LIGAND program . The optimal fits produced from these data were for two binding sites under each condition. Nonspecific binding was a modeled parameter equal to 16% of total binding in the presence and 22% in the absence of added nucleotide. For more direct comparison between treatments, this parameter has been subtracted in these plots. Other details of the binding are provided in the Materials and Methods. Data are from a single experiment which was repeated with similar results (Total N=3).


Specific Binding (fmol/mg protein)
75
50
25
0
9
8
7
6
5
-Log [DAGO] (M)

under these conditions was comparable to those measured when a [^{3}H]antagonist was subjected to competition by an unlabeled antagonist (Table 10).

In both the 7315c and NG108-15 membrane preparation, addition of 100 μM GTP caused a rightward shift in the displacement curve, indicating a decrease in agonist affinity at both the high and low-affinity sites (Table 11). Addition of 10 μM GTPγS, the nonhydrolyzable GTP analogue, produced qualitatively similar results. GTPγS caused an approximate 6-10 fold shift in agonist affinity at both the high (K_{app}, 3 ± 2 nM to 18 ± 7 nM) and low-affinity sites (K_{app}, 428 ± 52 nM to 5100 ± 4000 nM) in the NG108-15 membranes (Fig. 19, Table 11). The addition of GTPγS to the 7315c membranes also caused an approximate 5 fold shift in both the high-affinity site (K_{app}, 7 ± 2 nM to 36 ± 15 nM) and the low-affinity site (K_{app}, 465 ± 220 nM to 3600 ± 1200 nM) (Fig. 19, Table 11). In each case, a two-site binding model fit the experimental data significantly better than a one-site model. In both the NG108-15 and 7315c membranes the proportion of binding sites in the low-affinity states was also increased. There was also a tendency for the total number of binding sites to be increased in the presence of GTP and GTPγS.

In contrast to the more complex competition curves observed in the absence of added nucleotide or in the presence of GTP or GTPγS, the addition of 10 μM GDPβS, the nonhydrolyzable GDP analogue, resulted in a steepening of the curve. Computer analysis suggested the existence of one affinity state under these conditions (Table 11). No significant improvement in fitting the data was obtained by employing a two-site or more complex binding model. The addition of 10 μM GDPβS to 7315c membranes decreased the agonist K_{app} to a 50-fold lower value than that observed for the high-affinity site observed in control membranes (K_{app}, 7 to 350 nM) (Table 11). In the NG108-15 membranes, 10 μM GDPβS decreased the agonist K_{app} approximately 10-fold compared

TABLE 11

Opioid binding by NG108-15 and 7315c cell membranes in the presence and absence of guanine nucleotides

Cell membranes were incubated for 20 minutes at 37° in modified Krebs buffer containing 118 mM NaCl in the absence or presence of 100 μM GTP or 10 μM guanine nucleotide analogues. The number of independent replicate experiments under each condition is indicated in the table (N). Results were analyzed by a nonlinear curve-fitting algorithm. The tabulated values are the estimates of K_i or B_{max} (± standard error of the parameter estimate) from a combined analysis of the results from N independent experiments. The concentration of [^{3}H]NAL was 1 nM and [^{3}H]DIP was 1 nM. For more details regarding modeling procedures, see Analysis of Data in Materials and Methods.

Cell Membrane	Radioligand	unlabeled agonist	Guanine Nucleotide	N	K_{app} nM	B_{max} fmol/mg protein
7315c	NAL	DAGO	none	8	7 ± 2 465 ± 220	28 ± 3 16 ± 20
			GDPβS	3	348 ± 110	32 ± 2
			GTP	3	18 ± 7 5200 ± 4000	12 ± 7 51 ± 6
			GTPγS	3	36 ± 15 3600 ± 1200	41 ± 4 60 ± 4
NG108-15	DIP	DSLET	none	8	3 ± 2 428 ± 52	94 ± 9 28 ± 9
			GDPβS	3	20 ± 3	100 ± 3
			GTP	3	7 ± 5 4600 ± 2900	100 ± 20 52 ± 14
			GTPγS	3	18 ± 4 5100 ± 4000	52 ± 3 57 ± 10

to that observed in control membranes (K_{app}, 3 to 20 nM) and the low-affinity state under control conditions was no longer apparent (Fig. 21). Addition of GDPβS did not significantly affect the total number of binding sites in the 7315c or NG108-15 membrane preparations (Table 11). The binding curves generated in the presence of GDPβS were not satisfactorily fitted a two-site model by employing the parameter estimates obtained from binding measured in the presence of GTP, or GTPγS.

4. Pertussis toxin-treated cell membranes

a. Treatment

Cultures of 7315c and NG108-15 cells were incubated at an approximate concentration of 2 x 10^6 cells/ml DMEM/BSA in the presence of 30 ng/ml pertussis toxin . The effects of guanyl nucleotide regulation of agonist binding were examined after the indicated incubation time to determine if functional uncoupling of the guanyl nucleotide binding protein from opioid receptor had occurred. After 3 hours, GTP was no longer able to inhibit agonist binding at the μ opioid receptor in 7315c cells. Preliminary experiments indicated that GTP regulated agonist binding in NG108-15 cells exposed to pertussis toxin for up to 24 hours, but by 36 hours, GTP regulation of agonist binding was no longer apparent.

b. Agonist binding

In order to determine the agonist K_{app} for the receptor uncoupled from its guanyl nucleotide binding protein, competition studies were performed on membranes from pertussis toxin-treated cells. [^{3}H]NAL displaced by DAGO in membranes from pertussis toxin-treated cells yielded a monophasic displacement curve (Table 12). Computer analysis confirmed the existence of a single agonist affinity state with approximately 25-fold lower affinity than the high-affinity state observed in control 7315c membranes (untreated, 7 nM ± 2 nM; pertussis toxin treated, 88 ± 24 nM). Similar results were obtained in membranes

TABLE 12

Opioid binding by pertussis toxin-treated NG108-15 and 7315c cell membranes in the presence and absence of GTP

Cells were treated with 30 ng/ml pertussis toxin for 36 hours. Cell membranes were incubated in modified Krebs Hepes buffer containing 118 mM NaCl for 20 minutes at 37^{o} in the absence or presence of 100 μM GTP. The number of independent replicate experiments under each condition is indicated in the table (N). Results were analyzed by a nonlinear curve-fitting algorithm . The tabulated values are the estimates of K_i or B_{max} (± standard error of the parameter estimate) from a combined analysis of the results from N independent experiments. The concentration of [^{3}H]NAL was 1 nM and [^{3}H]DIP was 1 nM.

Cell Membrane	Radioligand	unlabeled agonist	[GTP] μM	N	K_{app} nM	B_{max} fmol/mg protein
7315c	NAL	DAGO	none	2	255 ± 100	22 ± 4
			100	2	140 ± 45	18 ± 2
NG108-15	DIP	DSLET	none	2	158 ± 90	56 ± 3
			100	2	118 ± 30	60 ± 3

FIGURE 21: COMPETITION FOR [^{3}H]DIP BINDING TO δ OPIOID SITES IN NG108-15 CELL MEMBRANES BY UNLABELED DSLET IN THE PRESENCE (O) OR ABSENCE (•) OF 10 μM GDPβS. The lines are traced from the best fits generated by the LIGAND program. The optimal fits produced from these data were for two binding sites in the absence and one binding site in the presence of GDPβS. Nonspecific binding was a modeled parameter equal to 36% of total binding in the presence and 29% in the absence of added nucleotide. For more direct comparison of the binding between treatments, this parameter has been subtracted in these plots. Other details of the binding are provided in the Materials and Methods. Data are from a single experiment which was repeated with similar results (Total N=3).

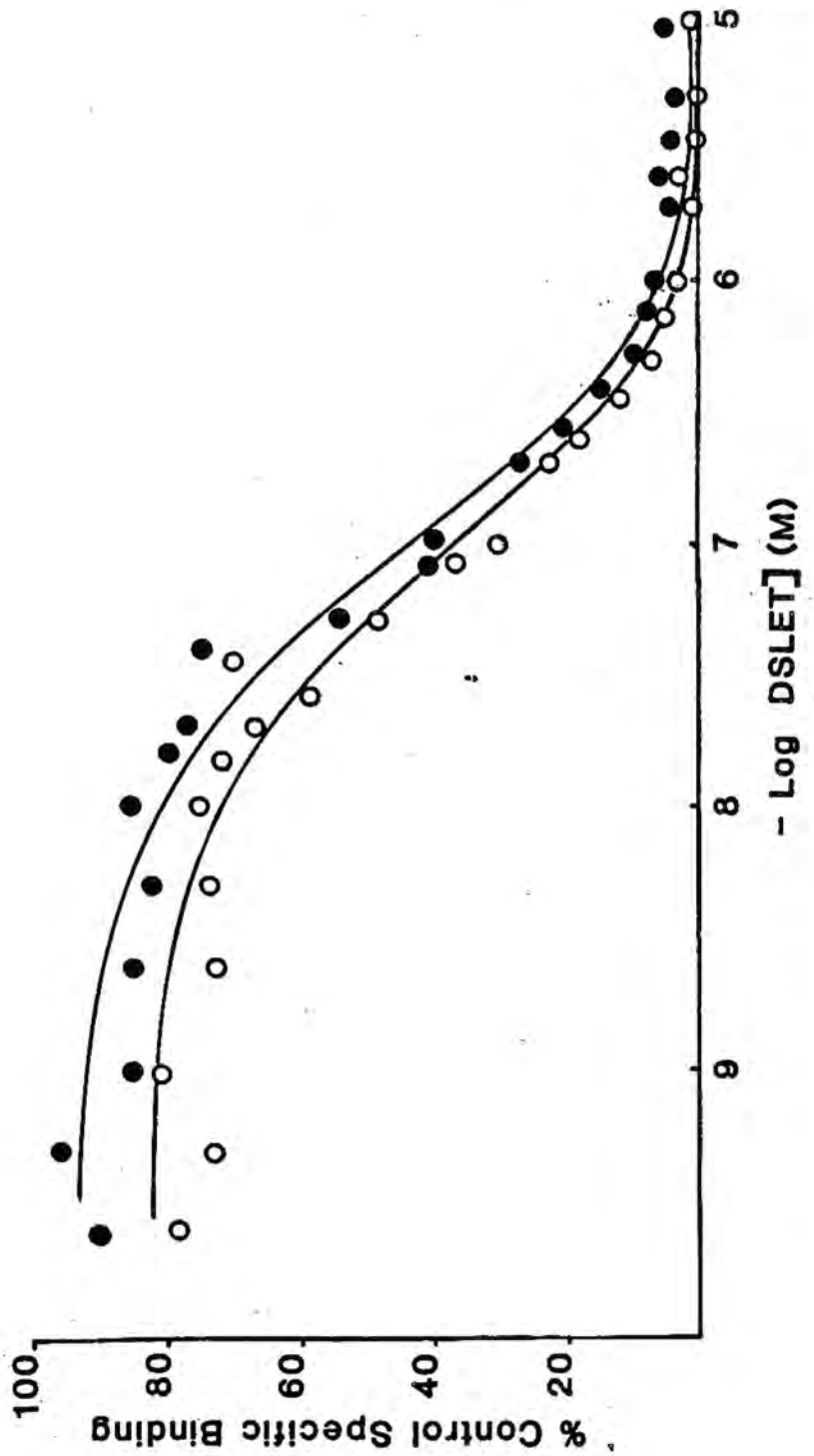
Specific Binding (fmol/mg protein)
300
200
100
0
5
6
7
8
9
-Log [DSLET] (M)

from pertussis toxin-treated NG108-15 cells. [^{3}H]DIP displaced by DSLET resulted in a monophasic curve with a substantially lower agonist affinity than the high-affinity state observed in control NG108-15 membranes (Fig 22) (untreated, 3 ± 2 nM; pertussis toxin treated, 100 ± 37 nM). Pertussis toxin treatment of both the 7315c and NG108-15 cells appeared to decrease the total number of binding sites labeled by the [^{3}H]antagonist. [^{3}H]NAL binding competed by DAGO in control 7315c cell membranes resulted in a calculated total site density of 44 fmol/mg protein. However in pertussis toxin-treated 7315c cell membranes , the receptor density was decreased to 35 fmol/mg protein. In control NG108-15 cell membranes, DSLET competition for [^{3}H]DIP binding yielded a total site density of 122 fmol/mg protein. In pertussis toxin treated NG108-15 cell membranes the receptor density was 47 fmol/mg protein. Addition of 100 μM GTP did not induce any additional change in affinity of the agonist or the receptor density in the pertussis toxin-treated membranes from either 7315c or NG108-15 cell membranes (Table 12).

Discussion

Findings from this study demonstrate that the regulation of opioid agonist binding by guanyl nucleotides may be through multiple affinity states of the δ and μ opioid receptors in NG108-15 and 7315c cell membranes. These multiple affinity states do not represent contamination by other opioid receptor types, as these cell lines contain only homogeneous populations of μ or δ (Puttfarcken et al., 1986; Chang et al., 1981) receptors. Initial experiments examined guanyl nucleotide regulation of opioid antagonist binding. Binding curves were constructed in which unlabeled antagonist competed for [^{3}H]antagonist binding in the presence and absence of GTP or GTPγS. The addition of either GTP or its nonhydrolyzable analogue did not significantly affect the antagonist K_i or

FIGURE 22. COMPETITION FOR [^{3}H]DIP BINDING TO δ OPIOID SITES IN PERTUSSIS TOXIN-TREATED NG108-15 CELL MEMBRANES BY UNLABELED DSLET IN THE PRESENCE (O) OR ABSENCE OF 100 μM GTP (•). The lines are traced from the best fits generated by the LIGAND program . The optimal fits produced from these data was for one binding site under each condition. Nonspecific binding was a modeled parameter equal to 40% of total binding in the presence and 32% in the absence of added nucleotide. For more direct comparison of the binding between treatments, this parameter has been subtracted in these plots. Other details of the binding are provided in the Materials and Methods. Data are from a single experiment which was repeated with similar results (Total N=2).


% Control Specific Binding
100
80
60
40
20
5
6
7
8
9
- Log DSLET] (M)

B_{max}. Having confirmed the insensitivity of opioid antagonist binding to guanyl nucleotides, as originally reported by Childers and Snyder (1978), [^{3}H]NAL was used to label μ sites and [^{3}H]DIP to label δ sites, and examined the pattern of competition by the μ selective agonist DAGO at μ receptor, or the δ preferring agonist DSLET at the δ receptor. Employing a wide concentration range spanning five orders of magnitude, at least two affinity states of each receptor in control membranes were identified. Under conditions in which coupling between binding sites and the guanyl nucleotide binding protein were promoted, we confirmed the existence of multiple agonist-induced affinity states of the δ opioid receptor in the NG108-15 cells, as originally reported by Law et al. (1983) and also report the existence of multiple affinity states of the μ opioid receptor in the 7315c cell membranes.

These agonist-specific multiple affinity states probably represent several forms of the agonist-receptor-guanyl nucleotide protein complex in the cycle responsible for transmembrane signalling (Fig 1). The fact that GTP is required to demonstrate the inhibitory effect of an opioid agonist on adenylyl cyclase activity, in both the 7315c cell (Frey and Kebabian, 1984) and the NG108-15 cell (Blume et al., 1979), suggests the presence of an inhibitory guanyl nucleotide binding protein coupled to the opioid receptor in each cell type. Gilman (1986) suggests that several forms of the G protein-bound receptor exist (Fig. 1). The interaction of the agonist with the receptor - G protein complex is ultimately responsible for exerting regulatory control of a coupled effector system. In the case of the NG108-15 and 7315c cells the effector system is an adenylyl cyclase which is subject to inhibitory regulation by opioids. The fact that the antagonist-receptor complex is not affected by addition of GTP suggests that antagonist binding does not result in association of the antagonist-receptor complex with a G protein. An assumption inherent in the analysis presented here is that equilibria exist between free ligand, receptor complexes, and each of the G-protein-guanyl nucleotide associated forms, the relative proportions of

each form being determined by the agonist/antagonist properties of the ligand and the properties of the guanyl nucleotides present. The analysis is complicated by the probable presence of more than one type of G protein. It is probable that opioids regulate adenylyl cyclase in NG108-15 cells through agonist-receptor interactions with the cyclase-inhibitory G protein, G_i , but there is known to be a high concentration of at least one other G protein, G_o, in the membranes of these cells (Milligan, et al., 1986). Similarly, there is probably more than one G protein in the membranes of the 7315c cells (Aub et al., 1986). The extent to which agonist-occupied opioid receptors can interact with a G protein other than G_i is not known. There are indications that opioids regulate cellular functions other than adenylyl cyclase through G protein coupled systems in which G proteins different from G_i might participate (Herscheler et al., 1987). Also, several affinity states of each G-protein opioid receptor complex might exist. If these all have different affinities for agonists, it is unlikely that each form will be discriminable by equilibrium binding techniques. Despite these potential complexities a limited number of discrete agonist-affinity forms of each receptor type were observed under the experimental conditions employed in these studies. In general, the agonist affinity states that were discriminated in these experiments appeared consistent with those proposed by Gilman (1986) to be involved in the transmembrane signalling pathways (Fig 1).

Pertussis toxin has been demonstrated to induce functional uncoupling of a subset of G proteins by promoting ADP ribosylation of the alpha subunit of G_i (Katada and Ui, 1982). In both the 7315c and NG108-15 membranes prepared from pertussis toxin-treated cells, the competition against [^{3}H]antagonist binding by an agonist resulted in a monophasic curve. In the 7315c membranes from pertussis toxin-treated cells, the agonist affinity was approximately 35-fold lower than the high-affinity state observed in under control membranes. In membranes from treated NG108-15 cells, the affinity of the agonist for the δ receptor was approximately 50-fold lower than the high-affinity state

present in membranes from control cells. These findings are reminiscent of those reported in a cholinergic receptor system (Kurose and Ui, 1983; Aguilar et al., 1986) indicating that *in vitro* pertussis toxin treatment of cells reduces the affinity of agonists for muscarinic receptors. The addition of GTP to membranes from pertussis toxin-treated cells did not further shift the agonist affinity at the μ or δ receptor. This loss of guanyl nucleotide regulation following pertussis toxin treatment was also reported for the muscarinic cholinergic receptor (Aguilar et al., 1986), and suggests that the agonist-receptor complex labeled in membranes prepared from pertussis toxin-treated 7315c and NG108-15 cells represent the H-R complex uncoupled from G protein as described by Gilman (1986). Our data suggest that this uncoupled agonist-receptor complex has an affinity for agonists comparable to the low affinity state observed in membranes from control cells in the absence of added GTP. Thus, one component of the lower affinity state apparent in the absence of added nucleotide might be H-R.

Since pertussis toxin treatment functionally uncouples the guanyl nucleotide binding protein from the receptor, the affinity state measured under these conditions (H-R) is different from the affinity state detected in the presence of GDPβS , presumed to be H-R-G-GDP, although some H-R complex might be present in membranes incubated with GDPβS. The addition of the nonhydrolyzable GDP analogue, GDPβS, resulted in a steepening of the agonist competition curve for the μ and δ receptor. Under these conditions only one affinity site was identified. These observations support earlier findings (Blume, 1978) that GDP caused a shift in the opioid agonist affinity for the μ receptor in brain membranes. The agonist affinity observed in both cell types was lower than that observed for the high-affinity state in control membranes. The addition of GDPβS did not affect the receptor density in either membrane preparation. In guinea pig cortical membranes the addition of GDPβS also resulted in an intermediate affinity state between the high and low affinity states observed in the absence of added nucleotide at both the μ

and δ opioid receptors (Werling et al., 1987). Observations in the NG108-15 and 7315c membranes suggest that the form described by Gilman, the H-R-G-GDP complex, represents a state with an affinity intermediate between the high and low affinity states. The H-R-G-GDP affinity state does not appear to have as low an affinity for agonist as that of the states generated in the presence of GTPγS, since it was not possible to fit data generated in the presence of GDPβS with binding parameters estimated in the presence of GTP or GTPγS. The difference between the effects of GDPβS and GTPγS or GTP is not due to the use of a submaximal concentration of GDPβS since maximally effective concentrations of each nucleotide were used.

In the absence of added guanyl nucleotides, we observed the existence of a high- and low-affinity state of the δ receptor in the NG108-15 cell membranes and of the μ receptor in the 7315c cell membranes. The apparent agonist affinities at δ receptors in NG108-15 cell membranes were similar to those observed for the δ opioid receptors in guinea pig cortical membranes (Werling et al., 1987). At the μ receptor in 7315c cells the state with highest apparent affinity of DAGO appeared to bind the agonist more tightly than the comparable affinity state at μ receptors in guinea pig cortical membranes. In both guinea pig cortical membranes and 7315c cells, the lower affinity state exhibited an apparent affinity of about 400 - 900 nM for DAGO in the absence of added nucleotide. Since there is likely to be GDP present in our membrane preparations it is possible that H-R-G-GDP also contributes to the lower affinity forms apparent in the absence of added nucleotide. However agonists are presumed to facilitate dissociation of GDP from the H-R-G-GDP complex (Gilman, 1986). In the absence of added nucleotide, the high-affinity agonist binding apparent in the 7315c, NG108-15, and guinea pig cortical membranes might therefore well be the H-R-G complex.

With the addition of GTP, there was a decrease in the agonist affinity at both the high- and low-affinity sites observed in the NG108-15 and 7315c cell membranes. To insure the results obtained in the presence of GTP were not due to a product of its hydrolysis, experiments were performed in the presence of the nonhydrolyzable GTP analogue, GTPγS. Similar results were obtained. The decrease in apparent affinity induced by GTP and GTPγS was also observed for the μ and δ opioid receptors in guinea pig cortical membranes (Werling et al., 1987). In all cases GTP and GTPγS decreased the low - affinity K_{app} into the 2.5 - 5 μM range for both DAGO and DSLET. These data suggest that the very low-affinity site, labeled in the presence of GTP and GTPγS, and not after any other treatment, is the H-R-G-GTP complex described by Gilman (1986). Alternatively, the H-R complex liberated after GTP dissociation may exist transiently in a very low-affinity state (H-R') prior to reversion to the intermediate affinity state found in the absence of guanyl nucleotide interactions. In either case the presence of this very low-affinity state seems likely to be associated with opioid agonist-mediated activation of G protein. The fact that two affinity states have been identified in the presence of GTP and GTPγS, suggests the existence of the H-R-G-GTP and the H-R' complex. However, other explanations are also possible. Slight modification of the Gilman model may ultimately be neccessary.

Earlier functional studies have reported the requirement of GTP for opioid agonist inhibition of adenylyl cyclase in the NG108-15 (Blume et al, 1979) and 7315c cells (Frey and Kebabian, 1984). It is possible that the formation of the low-affinity state of the agonist-guanyl nucleotide binding protein-receptor complex we have identified in both the NG108-15 and 7315c membranes is an essential concomitant of inhibition of adenylyl cyclase. These results suggest that G protein(s) are critical in the coupling of both μ and δ opioid receptors with their effector systems.

CHAPTER FOUR:
EFFECTS OF CHRONIC MORPHINE EXPOSURE IN 7315c CELL MEMBRANES

Rationale

Extensive studies involving chronic treatment of the δ opioid receptor in the NG108-15 cells have been reported (Law et al., 1982, 1984). These investigators reported at least two time-dependent cellular processes which take place after chronic exposure. The first was desensitization, in which a decrease in the ability of the agonist to elicit an effect was observed. The second process was down-regulation, this involved a decrease in the receptor density. Although the NG108-15 cells provided a useful model to study development of tolerance, it appears more clinically relevant to study chronic exposure of the μ opioid receptor in the 7315c pituitary cells since addictive narcotics are known to work through this receptor. Investigators have examined the chronic effect of opioids on the μ receptor in mammalian brain, but chronic opioid effects in a homogeneous populations of μ receptors have not been studied.

Results

Preliminary studies indicated that the 7315c cells must be attached to a growth surface in order for significant levels of adenylyl cyclase activity to be observed for more than a few hours after incubation in untreated culture dishes in a 90% air 10% CO_2 humidified atmosphere. When 7315c cells were plated onto collagen coated plates, opioid inhibition of adenylyl cyclase could be observed in cells maintained for as long as 18 days in culture.

To insure that residual morphine did not remain in the preparation after chronic treatment, we examined the retention of [^{3}H]morphine through the regular washing

procedure. Cells were incubated with [^{3}H]morphine at 100 μM for 48 hours and washed as usual. All of the [^{3}H]morphine was removed from the 7315c cells by the routine wash procedure (Table 13).

1. Opioid inhibition of adenylyl cyclase activity

In order to examine the adaptative processes induced by tolerance in 7315c cell membranes, cells were treated for varying periods of time with 100 μM morphine or control medium and the ability of the opioid agonist, DADLE, to inhibit cyclase activity was examined. In untreated cells maintained in culture without added opiate drug for varying time periods, DADLE significantly depressed adenylyl cyclase activity in a dose-dependent manner (Fig 23). The agonist concentration producing 50% inhibition of cyclase activity was within the range of 10-100 nM at each incubation time. At a concentration of 10 μM, DADLE produced a maximum inhibition of adenylyl cyclase activity of approximately 80% at all incubation times. In other experiments, DAGO-mediated inhibition of adenylyl cyclase has been observed after maintenance of 7315c cells for periods as long as 18 days in culture. Although the cells remained viable at 37°, cell counts and protein determinations indicated that cells were not dividing or increasing in tissue mass during incubation. Protein concentrations in membrane preparations remained similar throughout incubations for at least 72 hours (1hr, 250 ± 50 and 72 hr , 261 ± 17 μg/250 μl membrane suspension). In cells that were treated with 100 μM morphine for up to 72 hours, membrane protein concentrations also remained stable, and did not differ significantly from control values (1 hr, 201 ± 31 and 72 hr , 296 ± 36 μg/250 μl membrane suspension). After 1 and 3 hour time periods in 100 μM morphine, DADLE decreased adenylyl cyclase activity in a dose dependent fashion, and the maximum inhibition of adenylyl cyclase activity was reduced from 80% in untreated cells to 60-70% at 3 hours (Fig 23). The DADLE IC50 values after these periods of incubation with morphine were similar to those observed in untreated cells. After approximately 5 hours of 100 μM

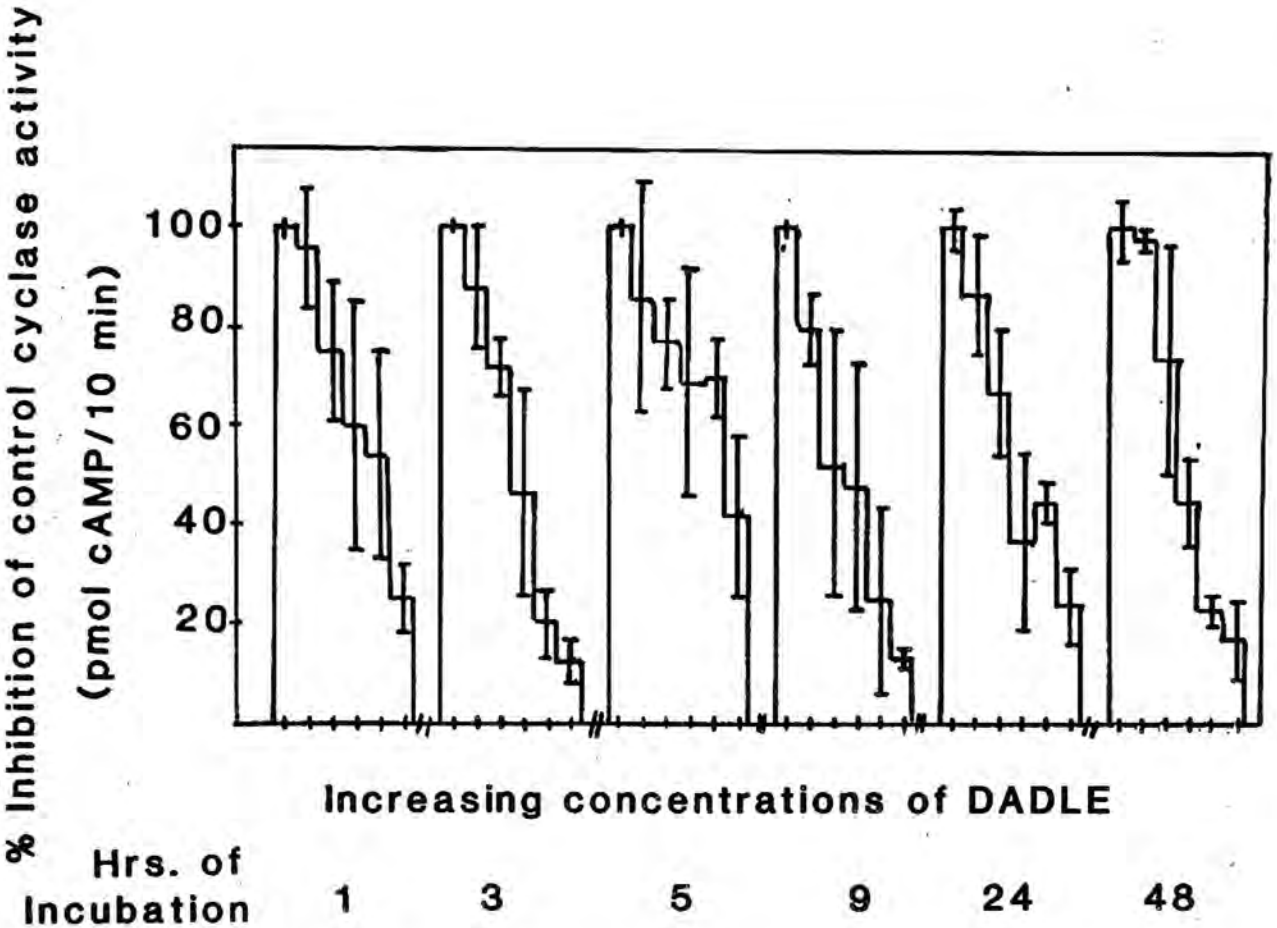
Table 13

Amount of [^{3}H]morphine remaining bound to 7315c cells after 48 hour exposure

7315c cells were treated with 100 μM [^{3}H]morphine for 48 hours. Afterward, cells were removed from flasks and resuspended in drug-free DMEM. The cell suspension was centrifuged (200 x g at room temperature for 5 minutes) and aliquots of supernatent were counted after each wash. The values represent averages of triplicate determinations (± standard errors of the mean).

Treatment	DPM remaining bound
Before wash	28911 ± 400
After first wash	132 ± 200
After second wash	52 ± 8
After third wash	3 ± 5

FIGURE 23. DADLE INHIBITS ADENYLYL CYCLASE ACTIVITY IN UNTREATED 7315C CELL MEMBRANES AFTER INCUBATION OF DISPERSED PRIMARY CULTURES OF INTACT 7315c CELLS AT 37° FOR THE INDICATED TIMES. After the specified incubation period, membranes were prepared and adenylyl cyclase activity assayed in the absence and presence concentrations of DADLE ranging from 1 nM to 10 μM. At each incubation time, the left most bar indicates the control adenylyl cyclase activity in the absence of added drug, set at 100%. Each step then indicates the effect of a ten-fold increase in DADLE concentrations, from 1 nM to 10 μM. Mean control adenylyl cyclase values in the absence of added DADLE did not vary as incubation time was increased (1 hr, 15 pmol cAMP/mg protein/10 min; 3 hr, 18.5 pmol cAMP/mg protein/10 min; 5 hr 17 pmol cAMP/mg protein/10 min; 9 hr, 15 pmol cAMP/mg protein/10 min; 24 hr, 16.5 pmol cAMP/mg protein/10 min; 48 hr, 21 pmol cAMP/mg protein/10 min). The histograms report mean values from four replicate experiments. The error bars represent the standard error of the mean.


% Inhibition of control cyclase activity
(pmol cAMP/10 min)
100
80
60
40
20
Increasing concentrations of DADLE
Hrs. of
Incubation
1
3
5
9
24
48

morphine treatment, DADLE inhibition of adenylyl cyclase activity was much reduced. By 9 hours, the adenylyl cyclase activity in the presence of 10 μM DADLE was comparable to the activity measured in the absence of peptide (Fig. 24).

2. Adenylyl cyclase activity after morphine withdrawal

In order to examine the effects of morphine withdrawal on enzyme activity, the ability of naloxone to reverse the loss of opioid regulation of adenylyl cyclase was examined in 72 hour treated cells. In membranes from NG108-15 cells exposed to 100 μM morphine, nanomolar concentrations of the opioid antagonists naloxone and diprenorphine increased activity of the enzyme above those observed in untreated membranes in the absence of added opioid agonist (Fig 25). In contrast, increasing concentrations of naloxone and diprenorphine did not affect baseline cyclase activity in 7315c cells exposed to morphine for 72 hours (Fig 26).

5. Binding studies

a. Antagonist binding

In order to detect time-dependent changes in receptor density in chronically exposed 7315c cell membranes, [^{3}H]antagonist competition studies were performed on membranes from cells which had been exposed to 100 μM morphine for varying periods of time. When [^{3}H]DIP binding was competed by unlabeled DIP in control cell membranes, computer analysis resulted in a one site model with an antagonist K_i of 0.77 ± 0.1 nM and receptor density of 44 ± 2 fmol/mg protein (Table 14).

Competition studies in membranes from cells which had been treated with 100 μM morphine for 5 hours yielded similar results (Fig 27 A). The antagonist binding parameters, K_i and B_{max}, for membranes which had been treated for 5 hours did not significantly change when compared to the equivalent values obtained in untreated 7315c membranes (Table 14). [^{3}H]DIP binding competed by unlabeled DIP in membranes from

FIGURE 24. DADLE-MEDIATED INHIBITION OF ADENYLYL CYCLASE IN TREATED 7315c CELL MEMBRANES. 7315c cells were exposed to 100 μM morphine at 37° for the indicated times. Membranes were prepared and adenylyl cyclase activity was assayed in the absence and presence of concentrations of DADLE ranging from 1 nM to 10 μM as described in the legend to Fig 23. Mean control adenylyl cyclase values in the absence of added DADLE, did not vary as incubation time was increased (1 hr, 18 pmol cAMP/mg protein/10 min.; 3 hr, 21 pmol cAMP/mg protein/10 min.; 5 hr, 17.5 pmol cAMP/mg protein/10 min; 9 hr, 16.8 pmol cAMP/mg protein/10 min.; 24 hr, 20.4 pmol cAMP/mg protein/10 min.; 48 hr, 16 pmol cAMP/mg protein/10 min). The histograms report mean values from four replicate experiments. The error bars represent the standard error of the mean.

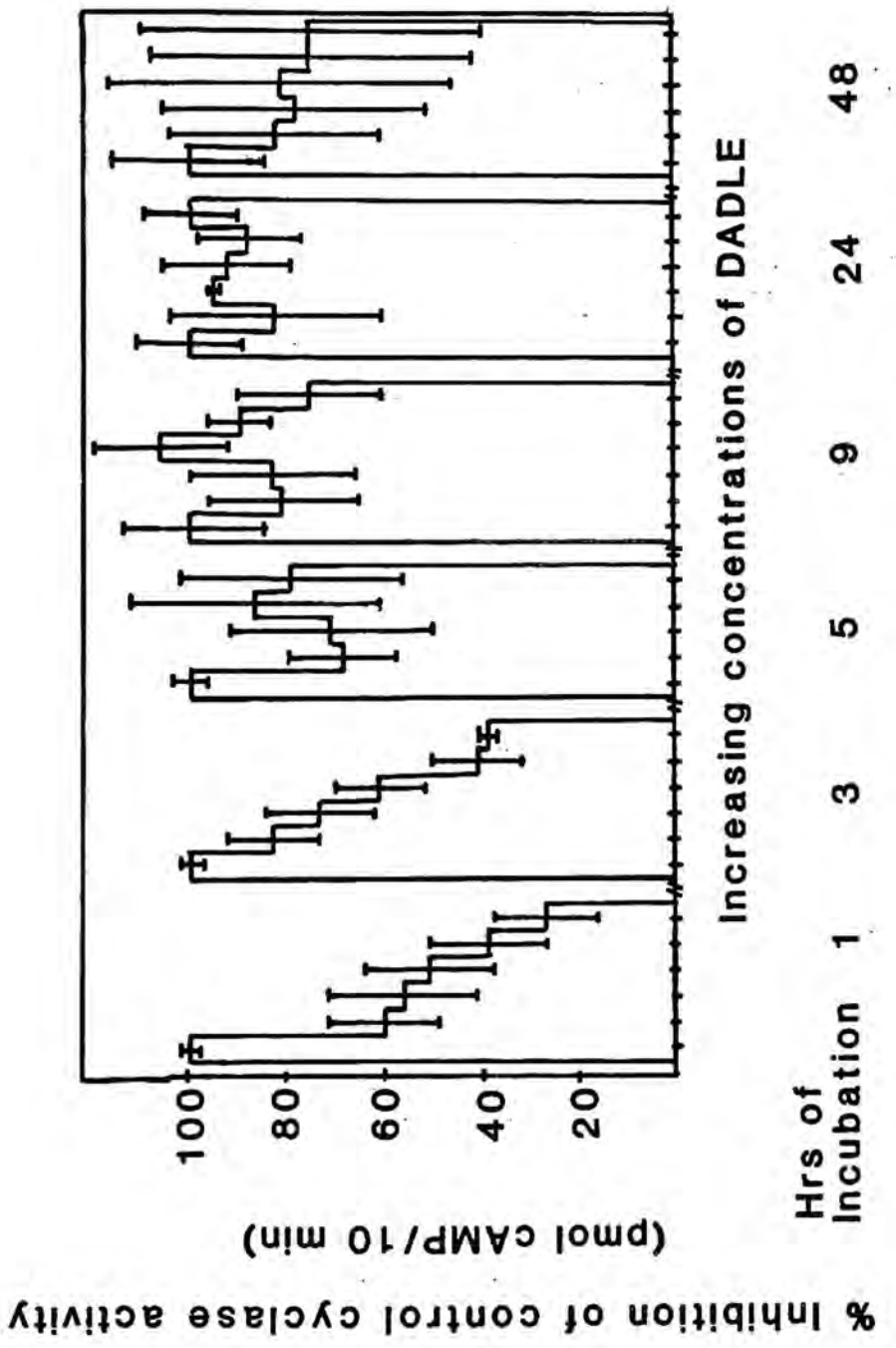

% Inhibition of control cyclase activity
(pmol cAMP/10 min)
100
80
60
40
20
Hrs of Incubation
1
3
5
9
24
48
Increasing concentrations of DADLE

FIGURE 25. NALOXONE AND DIPRENORPHINE EFFECTS ON ADENYLYL CYCLASE ACTIVITY IN MEMBRANES FROM NG108-15 CELLS EXPOSED TO 100 μM MORPHINE FOR 72 HOURS. Membranes were prepared from cells exposed to 100 μM morphine for 72 hours, and adenylyl cyclase activity expressed as % of control activity. The enzyme activity was assayed in the absence and presence of increasing concentrations of DIP (A) or NAL (B) ranging from 1 nM to 10 μM. At each incubation time, the left most bar indicated the control enzyme activity in the absence of added drug, set at 100%. Each step then indicates the effect of a ten-fold increase in DIP (A) or NAL (B) concentration. Data are from a single experiment which was repeated twice with similar results (Total N=3).

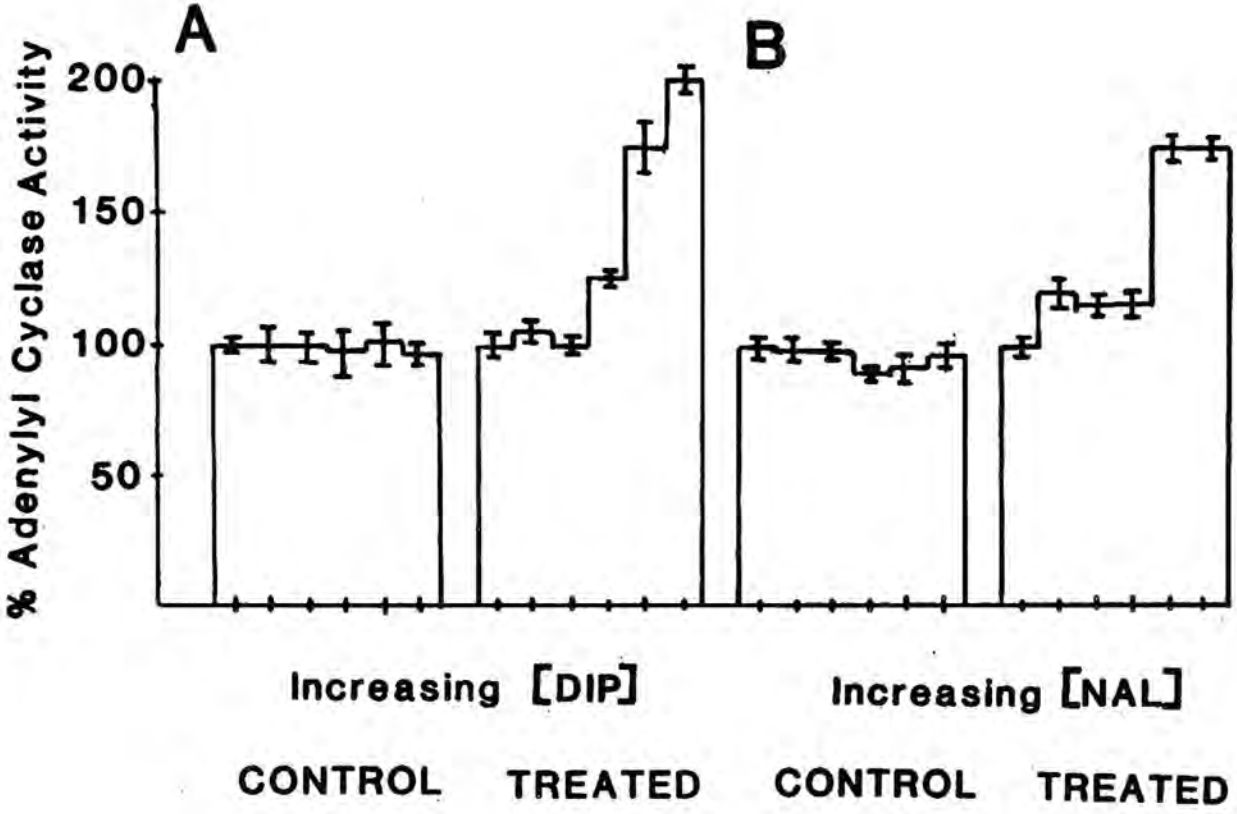

A
B
% Adenylyl Cyclase Activity
200
150
100
50
Increasing [DIP]
Increasing [NAL]
CONTROL
TREATED
CONTROL
TREATED

FIGURE 26. NALOXONE AND DIPRENORPHINE EFFECTS ON ADENYLYL CYCLASE ACTIVITY IN MEMBRANES FROM 7315c CELLS EXPOSED TO 100 μM MORPHINE FOR 72 HOURS. Membranes were prepared from cells exposed to 100 μM morphine for 72 hours, and adenylyl cyclase activity expressed as % of control activity. The enzyme activity was assayed in the absence and presence of increasing concentrations of DIP (A) or NAL (B) ranging from 1 nM to 10 μM. At each incubation time, the left most bar indicated the control enzyme activity in the absence of added drug, set at 100%. Each step then indicates the effect of a ten-fold increase in DIP (A) or NAL (B) concentration. Data are from a single experiment which was repeated twice with similar results (Total N=3).

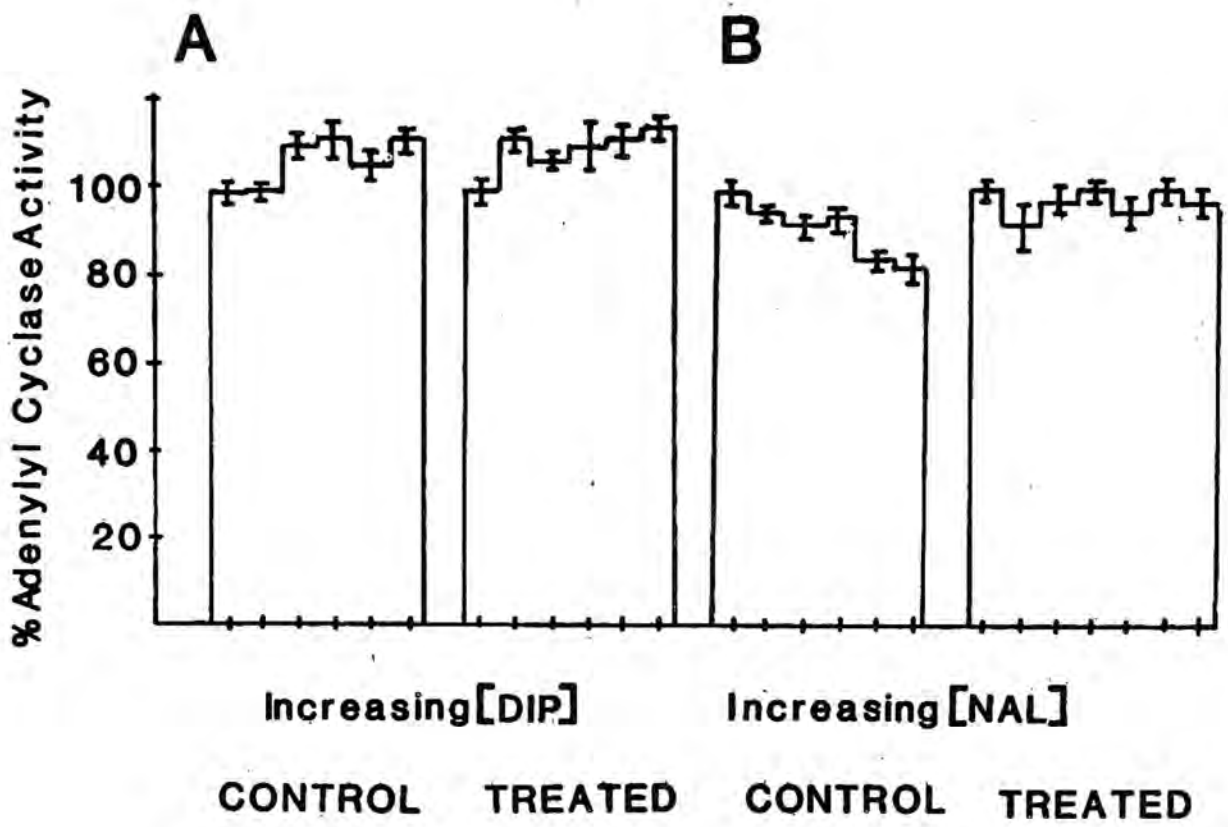

A
B
% Adenylyl Cyclase Activity
100
80
60
40
20
Increasing [DIP]
Increasing [NAL]
CONTROL
TREATED
CONTROL
TREATED

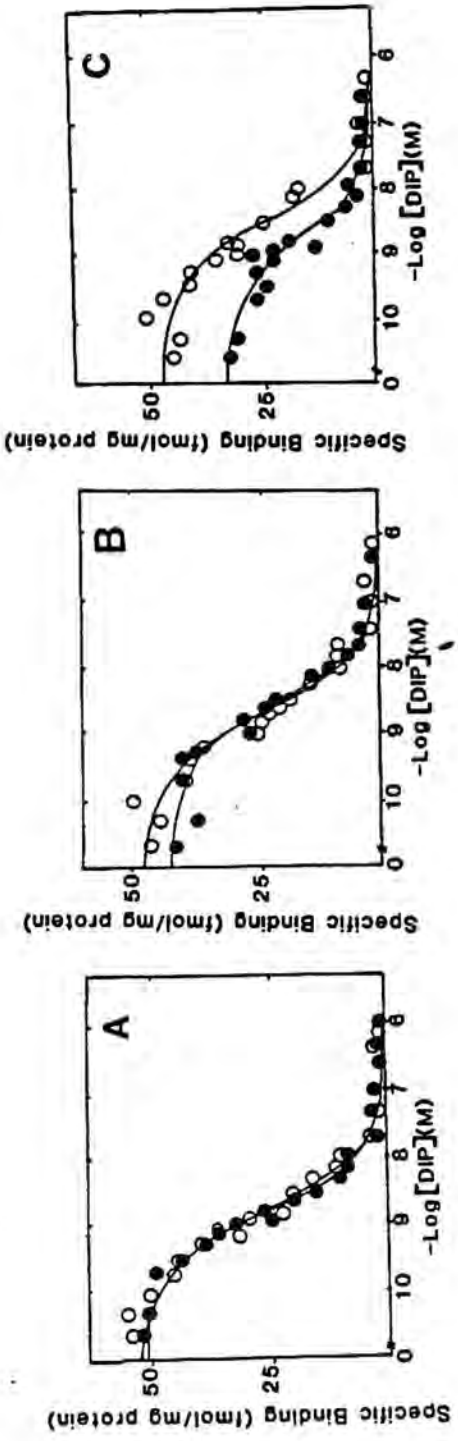
Table 14

Diprenorphine Binding in Membranes from Control and Treated 7315c Cells

7315c cells were incubated at 37 ^{o} in the absence or presence of 100 μM morphine for the indicated time. The number of independent replicate experiments under each condition is indicated in the table (N). Parameters for untreated 7315c cells were obtained from cells incubated for 5, 24, 72 hours in the absence of 100 μM morphine. Results were analyzed by a nonlinear curve-fitting algorithm (Munson and Rodbard, 1980). The tabulated values are the estimates of K_i and B_{max} (± standard error of the parameter estimates) for DIP from a combined analysis from N experiments. The concentration of [^{3}H]DIP was 1 nM.

hours of 100 μM morphine treatment	N	K_i nM	B_{max} fmol/mg protein
untreated	9	0.77 ± 0.1	44 ± 1.8
5	3	0.75 ± 0.3	43 ± 7
24	3	0.73 ± 0.1	35 ± 1.5
72	3	0.84 ± 0.26	14 ± 1.3

FIGURE 27. COMPETITION FOR [^{3}H]DIP (1 nM) BINDING BY UNLABELED DIP IN CONTROL (o) AND TREATED (•) 7315C CELL MEMBRANES EXPOSED FOR 5 HOURS (A), 24 HOURS (B) AND 72 HOURS (C) TO 100 μM MORPHINE. The lines are traced from the best fits generated by the LIGAND program. The optimal fits produced from these data were for one binding site under each condition. Nonspecific binding was a modeled parameter equal to 19% in the untreated cells, 21% in cells treated for 5 hours, 24% in cells treated for 24 hours and 18% in cells treated for 72 hours with 100 μM morphine. For more direct comparison between treatments, this parameter has been subtracted in these plots. Other details of the binding are provided in the Materials and Methods. Data are from a single experiment which was repeated with similar results (Total N=3).


A
B
C
Specific Binding (fmol/mg protein)
50
25
0
6
7
8
9
10
-Log [DIP](M)

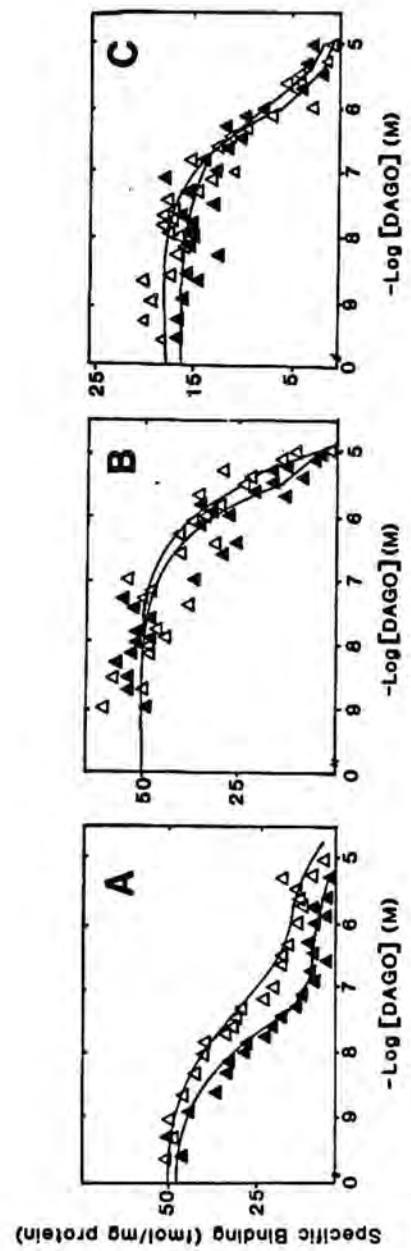
cells treated with 100 μM morphine for 24 hours resulted in a monophasic curve, very similar to that obtained in untreated cell membranes (Fig. 27 B). The antagonist K_i did not change significantly after 24 hour 100 μM morphine treatment from that observed in untreated cells or cells exposed to 100 μM morphine for 5 hours, while receptor density was slightly reduced. Although graphical representation of these data would suggest only a slight reduction in B_{max}, computer analysis indicated the receptor density labeled following 24 hours of morphine exposure was significantly lower than that observed in membranes prepared from both untreated cells and those exposed to morphine for 5 hours. Computer analysis of [^{3}H]antagonist competition studies in membranes from cells treated with 100 μM morphine for 72 hours again resulted in a preferred one site fit, with no significant change in the antagonist K_i (Fig 27 C, Table 14). However, receptor density measured in membranes from cells which had been exposed to morphine for 72 hours, was decreased to 32% of that observed in untreated cells or those exposed to 100 μM morphine for 5 hours.

b. Guanyl nucleotide regulation of agonist binding

To detect changes in the agonist-receptor-G protein interactions during the development of morphine tolerance, the ability of GTPγS, the nonhydrolyzable GTP analogue, to regulate agonist binding in 7315c cell membranes was examined. The possibility that changes in agonist/receptor coupling may be dependent on the duration of agonist exposure was investigated by measuring the agonist K_{app} in membranes from cells which had been treated with morphine for varying amounts of time in the absence and presence of GTPγS. The agonist Kapp was determined by competition against [^{3}H]antagonist binding .

Initially competition studies in the presence and absence of GTPγS were performed in membranes from untreated cells (Fig. 28, Table 15). Competition against [^{3}H]DIP binding

FIGURE 28. COMPETITION FOR [^{3}H]DIP (1 nM) BINDING BY UNLABELED DAGO IN CONTROL (A) AND TREATED 7315C CELL MEMBRANES EXPOSED FOR 5 HOURS (B) AND 72 HOURS (C) TO 100 μM MORPHINE. Studies were performed in the absence (s) and presence (Δ) of 10 μM GTPγS. The lines are traced from the best fits generated by the LIGAND program. The optimal fits produced from data in control membranes (A) were for two binding sites in both the presence and absence of 10 μM GTPγS. The best fit produced from 5 (B) and 72 (C) hour treated 7315c cell membranes resulted in one binding site in the both the absence and presence of 10 μM GTPγS. Nonspecific binding was a modeled parameter equal to 19% in the absence and 22% in the presence of GTPγS in untreated membranes, 24% in the absence and 17% in the presence of GTPγS in treated membranes. For more direct comparison between treatments, this parameter has been subtracted in these plots. Other details of binding are provided in the Materials and Methods. Data are from a single experiment which was repeated with similar results (Total N=3).


A
B
C
Specific Binding (fmol/mg protein)
-Log [DAGO] (M)
-Log [DAGO] (M)
-Log [DAGO] (M)
50
25
50
25
25
15
5
0
0
0
5
5
5

by DAGO in the absence of added nucleotide resulted in a complex displacement curve (Fig. 28 A). Computer analysis indicated two components of agonist binding with high and low affinities. The high-affinity site had a K_{app} of 1-10 nM and the low-affinity K_{app} of 100-500 nM. Addition of 10 μM GTPγS caused a rightward shift in the competition curve, indicating a decrease in agonist affinity at both the high and low-affinity state. The addition of GTPγS caused a rightward shift in both the high and low-affinity sites (Table 15) and an increase in the proportion of binding sites in the low-affinity state, with a concomitant increase in the total number of measured binding sites (from 47 ± 17 fmol/mg protein to 93 ± 21 fmol/mg protein). These observations are similar to those obtained when competition studies against [^{3}H]NAL binding by DAGO in the absence and presence of GTPγS were performed (Fig. 20).

The competition of [^{3}H]DIP binding by DAGO in membranes from cells that had been treated with morphine for 5 hours resulted in a monophasic curve (Fig. 28 B). Analysis suggested a one-site model fit the experimental data significantly better than a two-site model (Table 15). The K_{app} for DAGO was reduced to 404 ± 200 nM, whereas the receptor density (44 ± 9 fmol/mg protein) was comparable to that estimated when [^{3}H]DIP was subjected to competition by DAGO in untreated membranes (Table 15) and when [^{3}H]DIP was subjected to competition by DIP in membranes obtained from cells incubated in the absence of morphine for 5 hours. The addition of 10 μM GTPγS, produced no significant additional change in the agonist K_{app} or B_{max} in membranes subject to 5 hours of morphine treatment ($P > 0.05$).

[^{3}H]DIP binding competed by DAGO in membranes from cells that had been treated with morphine for 72 hours also yielded a monophasic competition curve (Fig 28 C). Computer analysis confirmed the existence of a single agonist affinity state with a K_{app} for DAGO of 134 ± 44 nM. These studies confirmed that the number of binding sites

Table 15

Opioid Binding by membranes from treated 7315c cells in the presence and absence of GTPγS

Cell membranes were incubated for 20 minutes at 37° in modified Krebs buffer in the absence or presence of 10 μM GTPγS, the nonhydrolzable GTP analogue. The number of independent replicate experiments under each condition is indicated in the table (N). Results were analyzed by a nonlinear curve-fitting algorithm (Munson and Rodbard). The tabulated values are the estimates of K_D and B_{max} (± standard error of the parameter estimate) for DAGO from a combined analysis of the results from N independent experiments. Competition studies against [^{3}H]NAL in untreated cells were obtained from a previous paper (Puttfarcken et al., 1987). Similar results were obtained using [^{3}H]DIP (N=1). The concentration of [^{3}H]DIP was 1 nM.

hours of 100 μM morphine treatment	Radioligand	N	[GTPγS] μM	DAGO K_{app} nM	DAGO B_{max} fmol/mg protein
0	NAL	3	none	7 ± 2 465 ± 220	28 ± 3 16 ± 20
		3	10	36 ± 15 3600 ± 1200	41 ± 4 60 ± 13
0	DIP	1	none	10 ± 2 400 ± 20	27 ± 5 20 ± 12
		1	10	40 ± 5 4000 ± 1000	35 ± 13 58 ± 8
5	DIP	3	none	404 ± 200	44 ± 9
		3	10	238 ± 100	41 ± 4
72	DIP	3	none	134 ± 44	11 ± 1
		3	10	110 ± 33	9 ± 1

competed for by DAGO after 72 hours of morphine treatment was reduced significantly ($P < 0.01$) relative to control values and those obtained from experiments utilizing membranes obtained from cells incubated for 72 hours in the absence of morphine. GTPγS regulation of agonist affinity was also not apparent after 72 hours of incubation with morphine (Table 15). Thus both high-affinity and very low-affinity states of the receptor detectable in the absence and presence of guanyl nucleotides, respectively, were no longer apparent after exposure to morphine for as short as 5 hours. Reduction in agonist affinity, and loss of guanyl nucleotide regulation of agonist affinity occurred before there was a significant reduction in the number of binding sites.

Discussion

Results from this study demonstrate the occurrence of two time dependent processes: desensitization, characterized as the loss in the ability of the opioid agonist to inhibit adenylyl cyclase activity, and down-regulation, defined as the decrease in the number of binding sites, upon chronic morphine treatment of the μ receptor in the 7315c cell (Table 16). In correlation with these findings our binding data show that after desensitization has occurred, there is a decrease in the opioid agonist affinity, and agonist binding is no longer under GTP control. There was no change in the receptor density after 5 hours of morphine treatment when the agonist response was significantly attenuated. However, after 72 hours of 100 μM morphine exposure, there was a significant decease in the receptor density. Again, no GTP effect on agonist binding was observed. The adaptive processes described for the μ opioid receptor appear very similar to those previously reported for the δ opioid receptor in the NG108-15 cell (Law et al., 1982; 1984) and the β-adrenergic receptor (Su et al., 1980; Lefkowitz et al., 1980) upon chronic agonist treatment. Although the

Table 16

Functional and/or Binding Changes After 100 μM Morphine Treatment of 7315c Cells

Treatment (Hrs)	Change
5	loss in the ability of the opioid agonist to inhibit adenylyl cyclase activity (desensitization) decrease in the agonist affinity loss of GTPγS regulation of opioid agonist binding
24	desensitization small decrease (20%) in receptor density (down-regulation) (agonist affinity and GTPγS regulation not measured)
72	desensitization approximately a 60% down-regulation decrease in agonist affinity loss of GTPγS regulation of opioid agonist binding

dispersed 7315c cells did not divide in culture, the cells survived for at least 18 days. Opioid inhibition of adenylyl cyclase was observed in membranes from cells cultured for that period of time. The membrane protein content of each incubate did not change during 72 hours of incubation. Thus changes in receptor number reported here presumably reflect changes in receptor synthesis, internalization, or degradation and are not related to or influenced by cell growth or replication.

Morphine was chosen as the appropriate drug for chronic treatment. Morphine, a relatively μ- selective agonist, (Miller et al.1986) has been used widely to study the development of opioid tolerance and dependence. We chose conditions analogous to the studies of Law and coworkers (1982, 1984) describing δ opioid receptor desensitization and down-regulation for the NG108-15 cells upon chronic agonist exposure. Maximal inhibition of PGE_1-stimulated adenylyl cyclase activity in both control and morphine-treated NG108-15 cells (Law et al., 1982; Sharma et al., 1975) was observed with 100 μM morphine. This concentration should give almost complete occupation of all functional receptors, including the very low agonist affinity states of the μ receptor apparent in the presence of GTP (Puttfarcken et al., 1986), throughout the chronic exposure period. We have shown that despite the high concentration, morphine can be completely removed from cell membranes prior to assay of enzyme or receptor properties. The opioid agonist DADLE was chosen to examine opioid inhibition of adenylyl cyclase activity in cells which had been treated with 100 μM morphine for varying time periods. This peptide has been found to act as a full agonist at the μ opioid receptor in the neurons of the rat locus coeruleus (Williams and North; 1984) and in peripheral μ-opiate receptors (Smith and Rance, 1983), and was chosen to correlate with earlier studies examining agonist-mediated inhibition of cyclase activity in 7315c cells (Puttfarcken et al., 1986).

We observed loss of DADLE inhibition of adenylyl cyclase after 5 hours of 100 μM morphine treatment, and after 9 hours of exposure, concentrations of DADLE as high as 10 μM failed to inhibit cyclase activity. Forskolin-stimulated enzyme activity remained at levels comparable to those observed in untreated cells even after incubation with morphine for 48 hours. The onset of desensitization in the 7315c cells appeared to occur later than reported for the δ opioid receptor in the NG108-15 cells after 10 nM ETphine exposure (Puttfarcken et al, 1986) or the β-adrenoceptor in S49 lymphoma cells after 10 μM isoproterenol exposure (Sibley et al., 1986). In these systems significant desensitization was apparent after 3 hours of agonist exposure, at a time when the agonist effect was only slightly reduced in the 7315c cells.

The mechanisms underlying agonist-induced loss of agonist potency or effect have been investigated in many receptor systems (Law et al., 1982; Stadel et al., 1983; Sibley et al., 1986; Sabol and Nirenberg, 1979; Parenti et al., 1983), and several biochemical events appear to be associated with this phenomenon. Measurement of the number of opioid binding sites detectable in 7315c cell membranes with the labeled antagonist [^{3}H]DIP after exposure of cells to morphine for 5 hours, a time when desensitization was clearly apparent, shows that the number of receptors was not changed. However, their ability to interact with agonist, and the regulation of agonist affinity, may have been impaired. Binding experiments were performed on membranes from cells exposed to 100 μM morphine for 5 hours (onset of desensitization) in the presence and absence of 10 μM GTPγS. Since the binding affinity of opioid antagonists at μ receptors in 7315c cells was unaffected by guanyl nucleotides (Werling et al., 1987), we used [^{3}H]DIP to label μ sites and examined the pattern of competition by DAGO. Employing an agonist concentration range spanning five orders of magnitude, we were able to identify only one agonist affinity state in membranes from 7315c cells treated with morphine for 5 hours. Computer analysis yielded a preferred one-site fit with a K_{app} for DAGO of approximately 200 nM. This is in

contrast to the identification of at least two agonist affinity states of the μ receptor in membranes prepared from untreated 7315c cells or in cortical membranes from untreated guinea pigs (Werling et al., 1987). The DAGO affinity for μ receptors in membranes exposed to 100 μM morphine for 5 hours was 30 fold-lower than the high-affinity state identified in untreated membranes. The existence of reduced agonist affinity states of G protein-coupled receptors following short term agonist exposure has been reported for the δ opioid receptor (Law et al., 1983), and the β-adrenergic receptor (Su et al., 1980; Lefkowitz et al., 1980). The state of the 7315c cell μ receptor, with a K_{app} of 200-400 nM after 5 hours of morphine exposure, is apparently comparable to that identified in pertussis toxin-treated 7315c cell membranes with a K_{app} of 255 ± 100 nM. These results are consistent with previous work (Costa et al., 1983; Wuster et al., 1983) in which investigators reported a similarity in opioid agonist binding characteristics at δ receptors in NG108-15 cells which had been treated with pertussis toxin and those which had been chronically treated with the opioid agonist, DADLE. The addition of GTPγS, the nonhydrolyzable GTP analogue, to membranes from morphine-treated cells did not significantly change the agonist K_{app} or B_{max}. The loss in guanyl nucleotide regulation of agonist binding is thought to be associated with the onset of the desensitization process in several receptor systems (Law et al., 1982; 1984; Su et al., 1980; Lefkowitz et al., 1980) and is reminiscent of the agonist insensitivity to GTP regulation observed in pertussis-toxin treated 7315c membranes . The results suggest that an impaired interaction of the μ opioid receptor with the guanyl nucleotide binding protein might be responsible for the desensitization observed in the 7315c cells upon chronic morphine treatment.

In addition to desensitization, a second cellular adaptative process, down regulation, was observed in 7315c cell membranes following continuous opioid agonist exposure. Competition studies employing the opioid antagonist unlabeled DIP against [^{3}H]DIP demonstrated a gradual decline in the total number of μ opioid receptors as the duration of

100 μM morphine treatment increased. The mechanism of down-regulation is not known, although investigators have suggested an internalization of the ligand-receptor complex into sequestered regions of the membrane which are inaccessible to hydrophilic ligands (Brown et al., 1982; Roth and Coscia, 1984). Unlike the fairly rapid onset of desensitization, down-regulation was first apparent after approximately 24 hours exposure of 7315c cells to morphine. Since a relatively high-speed centrifugation step was used to prepare the membranes from the treated cells, both surface and internalized opioid receptors were probably labeled in our studies. After 72 hours of 100 μM morphine treatment, there was a 60-70% reduction in the number of labeled μ opioid binding sites . Thus it is likely that receptor degradation followed internalization. It is possible that desensitized receptors uncoupled from G proteins are more readily internalized and degraded than G protein coupled receptors. It may be relevant that a reduction in μ receptor density was also observed following sustained pertussis toxin treatment in 7315c cells (Chang et al, 1978). Earlier studies have reported a 50-70% decrease in the number of δ opioid receptors following pertussis toxin treatment in the NG108-15 cells (Chang et al., 1978; Minuth and Jakobs, 1986) .

Using binding and functional studies, we have demonstrated the occurrence of two different cellular adaptation processes which occur upon chronic morphine exposure of the μ opioid receptor in the 7315c cell (Table 13). These processes are similar to those described for other receptor systems coupled to guanyl nucleotide binding proteins after chronic agonist exposure. Functional studies suggest desensitization is characterized by a loss in the ability of the opioid agonist to inhibit adenylyl cyclase activity. Binding studies indicate that during desensitization there is a decrease in the opioid agonist binding affinity along with a loss in guanyl nucleotide regulation of agonist binding. The second process, receptor down-regulation, occurs only after 24 hours of morphine exposure.

Specific mechanisms involved in μ opioid receptor desensitization and down-regulation remain to be elucidated. Evidence presented here suggests that desensitization of the μ opioid receptor in the 7315c cells is associated with the uncoupling of the ligand receptor complex from the guanyl nucleotide binding protein. Desensitization is followed by a loss of opioid receptors by an as yet undefined mechanism.

CHAPTER FIVE:
SUMMARY AND CONCLUSIONS

Major objectives of this project were comparisons of the regulation of opioid agonist binding to μ and δ opioid receptors by sodium and guanyl nucleotides. The mechanisms underlying the loss of opioid potency (tolerance) that follows chronic exposure to an opioid agonist have also been examined. It was hypothesized that opioid tolerance would be associated with changes in opioid receptor function.

Sodium and guanyl nucleotides both appear to be prominent regulators of ligand receptor interactions in several receptor systems. Early work involving sodium regulation of the opioid receptor yielded inconsistent results. A major reason for the discrepancy was that the early investigators were unaware of the heterogeneity of opioid binding sites and the importance both sodium and guanyl nucleotides played in regulating these sites. Thus it was the purpose of this study to reexamine these regulatory processes using a homogeneous population of opioid receptors. In order to obtain results pertinent to the in vivo situation it was necessary to conduct binding studies in the presence of the appropriate concentrations of physiological cations. The NG108-15 and 7315c cells were chosen as they contain a pure population of δ and μ opioid receptors respectively. Using these cell types it was possible to study the effects of sodium and guanyl nucleotides without having to consider possible interactions with other opioid receptor types.

The effects of varying the sodium concentration on opioid binding at δ and μ opioid receptors in NG108-15 and 7315c cells have been examined. The binding of [^{3}H]ET to μ receptors on 7315c cells was increased by replacing the sodium in the incubation medium with potassium (membrane preparation) or N-MG (intact cell preparation). This effect was shown to be attributable to an increase in affinity, with no change in receptor density, both

in whole cell and membrane suspensions. On the other hand, replacement of sodium with potassium or N-MG in NG108-15 membrane or intact cell suspensions also resulted in an increase in ^{3}H]ET binding, but in these cells the effect was associated with an increase in the number of binding sites measurable under these experimental conditions. The effects of sodium on opioid inhibition of adenylyl cyclase in membrane preparations from both NG108-15 and 7315c cells also differed. Sodium reduced apparent agonist affinity in the 7315c cell membranes. In NG108-15 membranes, sodium was essential for the demonstration of opioid inhibition of cyclase activity. Increasing sodium concentration from 0.5 mM to 100 mM resulted in an increase in the fraction of total enzyme activity inhibited by opioid, but the opioid IC50 did not change. An increase in intracellular sodium concentration without a change in extracellular concentration was affected by incubation of 7315c and NG108-15 cells with the sodium-selective ionophore, monensin. When sodium was present in the extracellular medium, monensin reduced [^{3}H]ET binding by 50% or more at both receptor types. In the absence of sodium, monensin treatment produced only a small inhibition of binding and monensin had little effect on opioid binding to homogenized cell membranes in the presence or absence of sodium. These results suggest that sodium acts at an intracellular site to regulate opioid agonist binding at both μ and δ receptors, but the mode of regulation is not identical at each site. Since a reduction in intracellular sodium concentration by removal of extracellular sodium increased agonist binding, and an increase in intracellular sodium following monensin treatment reduced agonist binding, it is probable that the intracellular sodium concentration is a critical regulator of opioid agonist binding in intact cells.

Although sodium is most likely regulating agonist receptor interactions through an intracellular site, it is more difficult to determine the exact target of sodium's action within the intracellular compartment. In general it is thought sodium regulation of receptor binding does not require the α subunit of either G_i or G_s. This belief is supported by

studies examining sodium regulation of various receptor types, including platelet α_2-adrenoceptors, δ opioid receptors and β-adrenergic receptors. Investigators reported sodium continued to regulate agonist binding to membranes even after treatment with N-ethylmaleimide, an agent known to inactivate the α subunit of G_i (Aktories et al., 1984), pertussis toxin (Wuster et al., 1984) or heat activation (Limbird et al., 1982, 1984). In contrast, under all these conditions, the regulation of the receptor by guanyl nucleotides was lost. Furthermore sodium regulation of agonist binding was also observed in the G_s-deficient cyc⁻ S49 lymphoma cells (Minuth and Jakobs, 1986). Therefore it is highly unlikely that sodium regulates binding to various types of hormone receptors through the α subunit of a guanyl nucleotide protein. If G protein is the target for sodium action, it is more probable that sodium interacts with the β and/or γ subunits common to all guanyl nucleotide binding proteins and therefore present in the S49 cyc⁻ membranes. However, sodium may also exert its action directly on the receptors themselves or on an as yet unidentified additional component of the receptor-effector system. To examine these possibilities, investigators have attempted to study the effect of sodium on solubilized opioid receptor preparations (Chow and Zukin, 1981; Ruegg et al., 1983). Studies have demonstrated a sodium-selective inhibition of μ binding, although regulation by guanyl nucleotides was also observed. The latter observation suggested the solubilized preparation also contained a G protein, thus making it impossible to determine sodium's effect on the receptor itself. In order to answer this unresolved question, studies must be performed with purified receptors.

Multiple affinity states of opioid receptors of the μ and δ types have been identified in membranes prepared from cells which bear only one type of opioid receptor (μ receptor in the 7315c cells, δ receptor in the NG108-15 cells). States of μ and δ receptors which have agonist affinities too low to be identified by radiolabeled agonist have been measured indirectly by agonist competition for sites labeled by radioactive antagonist. Using

analogues of guanyl nucleotides, this study has examined the competition of the μ and δ agonists DAGO and DSLET against [^{3}H]DIP or [^{3}H]NAL binding to opioid receptors and identified several agonist affinity states. In the absence of added nucleotide, competition of DSLET for [^{3}H]DIP binding to δ receptors and DAGO for [^{3}H]NAL binding to μ receptors revealed the presence of two binding sites with differing apparent affinities. Addition of GDPβS to the NG108-15 or 7315c cell membranes produced a steep monophasic curve which was best fit by a one site model. In contrast, in the presence of added GTP or GTPγS, two affinity states were again apparent for DSLET competition at the δ receptor and DAGO competition at the μ receptor. Pertussis toxin treatment resulted in a monophasic agonist competition curve which was best fitted by a single site model in both the NG108-15 and 7315c cell membranes. Addition of 100 μM GTP did not affect the agonist K_{app} or B_{max} after pertussis toxin treatment, suggesting that sites labeled under these conditions are not functionally associated with a G-protein. In general, the effects of guanyl nucleotides were qualitatively similar at δ and μ opioid receptors. The multiple apparent affinity states of each receptor probably reflect the preferential occurrence of different forms of agonist-receptor-G protein-guanyl nucleotide complex depending on the agonist or antagonist properties of the ligand and the guanyl nucleotides present.

The effects of prolonged morphine exposure on the μ opioid receptor in 7315c pituitary tumor cell membranes have been examined. Although extensive studies involving chronic treatment of the δ opioid receptor in the NG108-15 cells have been reported (Law et al., 1982, 1984), it appeared more clinically relevant to study chronic exposure of the μ opioid receptor in the 7315c pituitary cells since addictive narcotics are known to work through this receptor. Investigators have examined the chronic effect of opioids on the μ receptor in mammalian neuronal membranes, but no one has studied these effects in a homogeneous populations of μ receptors.

Studies measuring the ability of [D-Ala^2-D-Leu^5]enkephalin (DADLE), an opioid agonist, to inhibit adenylyl cyclase in cells that had been exposed to 100 μM morphine for varying periods of time, indicated that the agonist lost the ability to inhibit enzyme activity after 5 hours morphine exposure. Measurements of [^{3}H]antagonist binding in membranes from cells exposed to morphine, demonstrated a decreased receptor density after 24 hours of 100 μM morphine exposure with no change in the antagonist affinity. Computer analysis indicated a 20% decrease in the number of μ receptors labeled after 24 hours of morphine exposure and a 60% decrease after 72 hours of exposure. Computer analysis of agonist competition against [^{3}H]antagonist binding confirmed the existence of one binding site with an affinity intermediate between the high and low apparent affinity states observed in membranes from untreated cells. Addition of 10 μM GTPγS did not affect the agonist affinity or receptor density in membranes from morphine-treated cells, suggesting that the receptors were uncoupled from G proteins, as observed in 7315c cell membranes that have been treated with pertussis toxin. Thus chronic morphine treatment induced a rapid loss of opioid μ receptor mediated inhibition of adenylyl cyclase (desensitization), and a more slowly developing reduction in receptor number. The desensitization was accompanied by a loss of guanyl nucleotide regulation of agonist affinity. These findings are comparable to results reported for the δ opioid receptor and the beta adrenergic receptor upon prolonged agonist exposure. Although direct measurements of opioid receptor binding in brain and other tissues from animals made tolerant to morphine have generally not revealed any significant differences in either receptor affinity or number (Klee and Streaty, 1974; Cox and Pakhya, 1977; Hollt et al., 1975; Dum et al., 1979). However, these studies used nonselective radioligands which could bind to all opioid receptor types (ie. μ, δ, κ) present in the tissue preparation. It is not certain that one could detect changes in one type of receptor if other types of opioid receptors were not affected by chronic opioid exposure. If changes in the distribution of receptors between plasma and intracellular membrane compartments (ie. down-regulation) were taking place during prolonged opioid exposure,

they could not be detected in studies using tissue homogenates or treatment drugs which were highly lipophilic. In fact in studies where investigators used [^{3}H]NAL to measure receptor changes in intact brain cell preparations pretreated with vehicle or morphine, a 25% decrease in binding sites was reported after morphine exposure (Rogers and El-Fakahany, 1986).

In regard to the relationship of tolerance and dependence, the unitary mechanism once postulated to describe the underlying mechanism of these two phenomena may have to be modified. Today several studies have suggested that it is possible to dissociate tolerance and dependence *in vitro* . This seems to be the case in the 7315c cells, where it is possible to detect changes associated with tolerance yet no signs of dependence. If this is the case, it may be speculated that tolerance and dependence are initiated by different biochemical mechanisms and are localized at distinct neuronal levels.

In summary, this project was novel in several ways. First, the study of sodium and guanyl nucleotide regulation of opioid receptors in the presence of physiological concentrations of the appropriate cations provided physiological relevance to the *in vitro* preparations. The 7315c pituitary cells were helpful in studying receptor characteristics of the μ receptor, as well as gaining an understanding as to how endogenous or exogenous substances elicit their effects via the μ receptor. Chronic treatment of the μ receptor provided insight into the mechanism of tolerance/dependence induction by μ selective opioid agonists.

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