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Title: Interaction of the Muscarinic Acetylcholine Receptor

with Effector Proteins

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The purified porcine atrial muscarinic acetylcholine receptor was
reconstituted into a lipid environment with proteins with which it was
believed to interact in vivo. One such protein was the inhibitory
guanine nucleotide binding protein (G_i). This signal transducing
protein was purified from porcine atria and reconstituted with the
mAcChR into a defined lipid system consisting of phosphatidylcholine,
phosphatidylserine and cholesterol (1:1:0.1 w/w). The proteins were
shown to be interactive when mAcChR, reconstituted with G_i, displayed
a high-affinity guanine-nucleotide sensitive carbachol binding site.
5'-Guanylyl imidodiphosphate (0.1 mM) converted this high affinity
site (K_d equal to 1 μM) to low affinity (K_d equal to about 100 μM)
but had no effect on the binding of the muscarinic antagonist
L-quinuclidinylbenzilate. The agonist carbachol was able to increase
the GTPase of mAcChR coupled G_i by 11 fold. Carbachol also reduced
the affinity of G_i for GDP by 50 fold and increased the observed rate
constant for GDP dissociation by 38 fold. Thus, the increase in

steady-state GTPase activity observed for carbachol is largely, if not exclusively, due to the increase in GDP dissociation from G_i--probably the rate-limiting step in the steady-state mechanism. The carbachol-stimulated GTPase and high-affinity agonist binding were sensitive to ADP-ribosylation of reconstituted G_i by pertussis toxin.

A protein complex consisting of the bovine brain stimulatory guanine nucleotide binding protein (G_s) and calmodulin-sensitive adenylyl cyclase was added to the reconstituted mAcChR and G_i. Conditions were chosen so that each of the G proteins could be selectively activated by guanosine 5'-0-(3-thiotriphosphate) (GTPγS) in the presence of GDP. The inhibitory effects of G_i were examined with non-activated, G_s activated, forskolin activated, and calcium-calmodulin activated adenylyl cyclase. Addition of carbachol to the mAcChR-G_i complex further activated G_i by increasing the rate of GDP release and allowed more GTPγS to bind. GTPγS bound G_i then mediated inhibition of adenylyl cyclase. Adenylyl cyclase activated by calcium plus calmodulin was the most sensitive to inhibition by carbachol.

Studies using the resolved subunits of G_i showed that while the $\beta\gamma$ subunits could inhibit all forms of adenylyl cyclase, only the calmodulin stimulated enzyme was inhibited by the α subunit.

Interaction of the Muscarinic Acetylcholine

Receptor with Effector Proteins

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CHAPTER I

Introduction

The muscarinic acetylcholine receptor (mAcChR) was one of the

first neurohormone receptors to be studied. Initial investigation of

the naturally occurring alkaloid muscarine played an important role

in developing modern neurohormonal theory. The mushroom Amanita

muscaria, the source of muscarine, has been known since ancient times

as a deadly poison. The action of muscarine was correlated with a

response to vagal stimulation of the heart, and it was proposed that

the vagus nerve released a muscarine like substance (Dixon, 1907).

Vagal stimulation results in a decrease in the rate of contraction in

the heart. Several other muscarinic ligands have been known for

centuries. Pilocarpine, isolated in 1875 from the leaflets of the

South American shrub of the genus Pilocarpus, acts as an agonist for

the mAcChR. An agonist is a chemical which binds to and activates a

receptor. Chewing of the leaf was known to cause salivation, which is

a muscarinic response of the salivary glands. Atropa belladonna,

which contains the anti-muscarinic drugs atropine and scopolamine,

have been used as medicine (or poison) since the Middle Ages. As

early as 1867, atropine was demonstrated to block the effects of vagal

stimulation in cardiac tissue (Koelle, 1975). Dale (1914) used these

naturally occurring compounds to investigate the pharmacology of

acetylcholine. Acetylcholine had recently been identified as the physiological agent that acted like muscarine (Ewins, 1914). The acetylcholine effects were separated into two categories. The first was muscarinic and was antagonized by atropine, and the second was nicotinic and was blocked by curare and by large doses of nicotine. Acetylcholine was confirmed as a neurotransmitter by Loewi (1921) and was the first molecule demonstrated to be a neurotransmitter compound.

The activation of the mAcChR results in a decrease in the rate and force of contraction in the heart, constricts airways, increases motility and secretion in the gastrointestinal tract, and increases secretion from salivary and sweat glands. Muscarinic receptors are also present in the central nervous system and are involved in memory, learning, and control of movement (Nathanson, 1987).

While the nicotinic acetylcholine receptor is in itself an ion channel and will thus directly effect the movement of ions across the cell membrane of target cells, the mAcChR does not contain an ion channel. Activation by muscarinic agonists leads to interaction of the receptor with other proteins, such as guanine nucleotide binding proteins, which in turn regulate effector proteins, either ion channels or enzymes which regulate second messenger levels. The biochemical responses regulated by muscarinic receptors include decreased levels of cAMP, increased levels of cGMP, and an increased turnover of some inositol phospholipids. Muscarinic activation is also known to regulate the conductance of ion channels, such as potassium, chloride, and calcium (Giles and Noble, 1976; Oron et al., 1985; Biegon and Pappano, 1980; Nathanson, 1987).

Biochemical Responses Mediated by the mAcChR

Inhibition of Adenylyl Cyclase

Activation of the mAcChR in many cell lines leads to a decrease in cAMP levels. This muscarinic effect has frequently been shown to require GTP (Watanabe et al., 1978; Jakobs et al., 1979) and is sensitive to Pertussis toxin (Kurose et al., 1983; Hazeki and Ui, 1981; Schlegal et al., 1985). The mAcChR does not directly interact with adenylyl cyclase, but is coupled via Pertussis toxin sensitive guanine nucleotide binding (G) proteins. While adenylyl cyclase is coupled to stimulatory receptors (e.g. β-adrenergic receptors) via the stimulatory guanine nucleotide binding protein (G_S), it is also coupled to inhibitory receptors via the inhibitory guanine nucleotide protein (G_i), which is ADP-ribosylated by the toxin from Bordetella pertussis (Gilman and Casey, 1987; Gilman, 1988, for recent reviews). G_o (the "other" guanine nucleotide binding protein) can also interact with adenylyl cyclase (Katada et al., 1986a, 1987) and is sensitive to Pertussis toxin. Muscarinic acetylcholine receptors from the brain interact with both brain G_i and G_o (Florio and Sternwies, 1985; Kazuko et al., 1986).

In 1321N1 astrocytoma cells (Huges et al., 1984) a muscarinic induced decrease in cAMP was observed. This was not sensitive to Pertussis-toxin and was believed to be the result of activation of a calmodulin stimulated phosphodiesterase (Tanner et al., 1986). Thus there must be another mechanism whereby muscarinic activation regulates cAMP levels, presumably by altering the amount of free

calcium in the cell available for calmodulin.

The force of cardiac contraction is related to cAMP levels. Activation of the β-adrenergic receptor leads to an accumulation of cAMP which activates cAMP dependent protein kinases. Subsequent phosphorylation of calcium channels (Hosey et al., 1986) will open the channels and allow extracellular calcium to enter and bind to troponin C, thus allowing a muscle contraction cycle to begin (Katz, 1977).

In addition to the hormone-regulated adenylyl cyclase, there also exists a calmodulin-regulated adenylyl cyclase which is stimulated by calcium and calmodulin. This calmodulin-sensitive adenylyl cyclase has been documented in only a few cell types including kidney (Sulimovici et al., 1983), adrenal medulla (Valverde et al., 1979), pancreatic islet cells (Le Donne and Coffee, 1979), and brain (Brostorm et al., 1975). Calmodulin-sensitive adenylyl cyclase has been purified from bovine brain. Purification may or may not include a 5'guanylylimidodiphosphate (GppNHp) pretreatment of membranes. Pretreatment of membranes results in a higher yield and higher specific activity of adenylyl cyclase but renders the enzyme insensitive to activation by GppNHp and thus is undesirable for studies involving regulation by G_s. The non GppNHp treated adenylyl cyclase was stimulated by guanosine triphosphates (which activate G_s), but was more sensitive to stimulation by calmodulin (Yeager et al., 1985a; Rosenberg et al., 1987). The purified, untreated calmodulin-sensitive adenylyl cyclase has been reconstituted with pure β-adrenergic receptor. Cyclase activity was enhanced by β-adrenergic agonists, but to a lesser degree than by calmodulin (Rosenberg et al., 1987).

Phosphatidylinositol Turnover
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Many hormones also regulate cellular events by stimulating the

hydrolysis of phosphoinositides. The hormone receptor activates a

phospholipase C which then cleaves phosphatidylinositol

4,5-bis-phosphate (PIP_2) to produce two second messengers, inositol

1,4,5-trisphosphate (IP_3) and diacylglycerol. Phospholipase A_2 may

then act on diacylglycerol and ultimately produce arachidonate, a

precursor of prostaglandins (Berridge, 1984).

Receptors, such as the mAcChR, are believed to couple to

phospholipase C through a guanine nucleotide binding protein. This

proposed G_p has so far eluded detection. Coupling of the mAcChR to

phospholipase C has been demonstrated to be insensitive to

pertussis toxin in chick heart cells (Helper and Harden, 1984) and in

1321N1 astrocytoma cells (Masters et al., 1985). Thus, G_i and G_o are

ruled out as possible candidates for G_p in these systems. However,

other systems have shown sensitivity to pertussis toxin (Ohta et al.,

1985). Phospholipase A_2 also shows sensitivity to pertussis toxin and

may be another enzyme regulated by G protein coupled receptors

(Nakahima et al., 1987).

Inositol 1,4,5-trisphosphate acts as a second messenger and

releases calcium from internal reserves. The mechanism of how IP_3

releases calcium has not yet been established (Berridge, 1984). A

mAcChR induced mobilization of calcium has been observed in

neuroblastoma cells (Oshako and Deguchi, 1984), pancreatic acinar

cells (Clandler and Williams, 1978), chicken fibroblasts (Oettling et

al., 1985), and astrocytoma cells (Masters et al., 1984). The

increase in calcium may be the means by which secretory cells are stimulated by muscarinic activation. A rise in intracellular calcium may also activate calmodulin regulated phosphodiesterases, thus providing an alternative mechanism for lowering the levels of cAMP (Nathanson, 1987; Schimerlik, 1988).

Diacylglycerol serves as a second messenger by activating protein kinase C. The mechanism of activation is to increase the affinity of calcium for protein kinase C, which is calcium dependent. Thus, diacylglycerol acts synergistically with IP_3, which raises the level of available calcium. Although the targets of protein kinase C are largely unknown, it is known that tumor promoting phorbol esters can activate the kinase and transform the target cell (Berridge, 1984). Protein kinase C may be involved in a feedback regulation of mAcChR coupling to phospholipase C (Orellana, 1987) and potassium channels (Dascal et al., 1985), possibly by phosphorylating and inactivating the G protein involved.

Stimulation of cGMP synthesis

By an as yet unidentified mechanism, muscarinic stimulation causes an increase in the levels of the second messenger cGMP. The muscarinic cGMP response in NE1-115 neuroblastoma cells can be lowered by disrupting metabolism of arachidonate or by inhibiting the phospholipase A_2 catalyzed release of arachidonate. These results suggest that a metabolite of arachidonate may regulate guanylyl cyclase (McKinney and Richelson, 1986; Nathanson, 1987). It has been proposed that guanylyl cyclase is activated by a metabolite of

arachidonate, and that arachidonate is liberated by a calcium

dependent phospholipase A_2. Thus receptors that mobilize

intracellular calcium may activate guanylyl cyclase through some

metabolite of arachidonate and by releasing calcium (Berridge, 1984).

The role of cGMP as a second messenger is still somewhat unclear.

In heart tissue cGMP activates a cGMP dependent phosphodiesterase,

thus reducing cAMP dependent calcium entry into the cell (Hartzell and

Fischeister, 1986). Therefore, cGMP can function as an antagonist to

cAMP. A cGMP-dependent protein kinase in smooth muscle cells has also

been linked with a muscarinic induced relaxation of vascular smooth

muscles (Nathanson, 1987).

Ion Channels

The mAcChR regulated decrease in the rate of cardiac contraction

is due to an increased permeability of the cardiac sarcolemma to K^+,

resulting in a hyperpolarizing effect (Katz, 1977). The ion channel

responsible for the increased permeability is the inward rectifying

potassium channel. The lengthy time lag following muscarinic

activation and the temperature dependence of the phenomena suggested

that the mAcChR does not contain, or is not directly coupled to the

ion channel. Regulation by cyclic nucleotides was also ruled out

(Nathanson, 1987). The muscarinic response is sensitive to Pertussis

toxin, implying that either G_i and/or G_o are involved in this signal

transduction (Martin et al., 1985). Recent work has suggested that

the transducing protein is G_i, the mechanism by which G_i regulates the

channel is currently under debate (see below, under G proteins).

Muscarinic activation may also result in depolarization in some tissues. In these tissues the muscarinic activation decreases an M-current. M-currents are K^+ channels which are activated by depolarization. Attenuation of M-currents results in an excitatory postsynaptic potential. Spinal chord neurons (Nowak and MacDonald, 1983) and hippocampal neurons (Cole and Nicoll, 1984) are examples of tissue which shows this type of regulation.

Muscarinic Receptor Subtypes and Structure

The large diversity of physiological and biochemical responses that occur as a consequence of muscarinic activation can be explained, in part, by the existence of multiple muscarinic receptor subtypes. The existence of receptor subtypes was suggested by the heterogeneous response of mAcChRs to the antagonist pirenzepine (Hammer et al., 1980). Following isolation of homogeneous mAcChR proteins (Peterson et al., 1984; Haga and Haga, 1985) it has been possible to obtain molecular clones of the mAcChR from porcine atria (PM2 receptors, Kubo et al., 1986b; Peralta et al., 1987) and porcine cerebellum (PM1, Kubo et al., 1986). Those clones revealed that the two muscarinic subtypes were polypeptide products from two different genes, but share the same overall structure of seven hydrophobic transmembrane regions. The structural motif is similar to that observed for other G protein coupled receptors, such as the β-adrenergic receptor and visual rhodopsins (Dohlman et al., 1987).

While screening a human genomic library to isolate HM1 (human mAcChR type 1) and HM2 (human mAcChR type 2), two additional human

subtypes were isolated and characterized. These were designated HM3 and HM4. The seven transmembrane regions of the four receptors appeared to be homologous, the region of largest amino acid sequence diversity was a large hydrophilic stretch between transmembrane segment 5 and 6. This region was used as a probe for Northern analysis to localize the receptor subtypes to specific tissues (Peralta et al., 1988).

The HM1 subtype showed a 98.9% sequence homology to porcine M1, and showed a closer homology to HM4 than to HM2 or HM3. The finding that HM1 has high affinity for the M1 selective antagonist pirenzipine, and low affinity for the cardioselective antagonist AFDX-116, were consistent with HM1 being located in the cerebral cortex, but not the heart (Peralta et al., 1988).

Conversely, HM2 has a low affinity for pirenzipine and a high affinity for AFDX-116. HM2 shared a high identity with HM3, and a 97.4% identity with PM2 from the myocardium. The only subtype detected in the heart was HM2, although HM2 was also detected in the brain (Peralta et al., 1988). The PM2 receptor appears to be more tightly coupled to regulation of adenylyl cyclase than to regulation of phospholipase C (Ashkenazi et al., 1987; Peralta et al., 1988).

The HM3 receptor shares the highest homology with HM2, but has a high affinity for pirenzepine and a low affinity for AFDX-116. Despite the difference in ligand selectivity, the HM3 receptor appears to be similar to HM2 in that it is more tightly coupled to adenylyl cyclase than to phosphoinosatide turnover. HM3 is abundant in the brain.

The HM4 receptor also has a high affinity for pirenzepine and a

low affinity for AFDX-116. While HM4 has been located in the brain, it is the only receptor subtype expressed in pancreas cells. This observation, together with the distinctively large molecular weight of this subtype (590 amino acids vs 460 in HM1) has led to the suggestion that HM4 is a glandular type receptor. A high molecular weight mAcChR has been observed in pancreas. Muscarinic mediated phosphatidylinositol turnover and calcium mobilization seem to be linked to excretion in glandular type cells, so HM4 may be coupled to phosphatidylinositol turnover. Despite the difference in molecular weight, HM4 has the highest overall sequence identity with HM1 (Peralta et al., 1988).

Since HM4 shares a high sequence identity with HM1, it is interesting to note that HM1 has also been observed to couple to phosphatidylinositol turnover (Lai et al., 1988). So, there appear to be two receptor subtypes that couple to phosphatidylinositol turnover, and two subtypes that couple to adenylyl cyclase. It has been noted that individual subtypes do not exclusively, but preferentially couple to a particular biochemical response (Peralta et al., 1988).

Ligand Binding

Most muscarinic antagonists bind to the mAcChR with a single class of binding sites (Hulme et al., 1978). Labelled antagonists such as [^{3}H] L-quinuclidinyl benzilate ([^{3}H] L-QNB) and [^{3}H] N-methyl scopalomine have been used to identify and characterize the mAcChR. Kinetic analysis of [^{3}H] L-QNB binding to the porcine atrial mAcChR has shown that the binding of QNB is a two step process involving a

rapid binding step followed by a slow isomerization of the receptor-ligand complex to a more stable form (Schimerlik and Searles, 1980).

Displacement of labelled antagonist by unlabelled agonists has demonstrated that agonists bind to the receptor with at least two classes of binding sites. The binding sites were designated as high and low affinity with the possible existence of a super high affinity site. Selective alkylation of the receptor revealed that the heterogeneity of agonist binding was not due to negative cooperativity, but to two separate classes of binding sites (Birdsall et al., 1978).

High affinity sites are converted to low affinity sites by the addition of guanine nucleotides in the heart (Berrie et al., 1979) and in the cerebellum and brainstem (Korn et al., 1983). It has been postulated that the high affinity binding sites represent mAcChR coupled to a G protein. The high affinity hormone-receptor-G protein complex binds GTP and 1) dissociates the G protein from the receptor, returning the receptor to the low affinity state and 2) activates the G protein (Gilman, 1988). It is apparently the high affinity form of the mAcChR that is coupled to adenylyl cyclase (Brown and Brown, 1984; Martin et al., 1985) indicating that the G protein is involved in signal transduction.

Muscarinic agonists display some heterogeneity with regard to receptor response. Certain agonists show a preference in coupling to adenylyl cyclase rather than inositol lipid metabolism. Despite a higher affinity for the receptor, the partial agonist oxotremorine was much more effective in inhibiting cAMP formation than stimulating phosphoinositide breakdown (Brown and Brown, 1984).

G Proteins

Most of our knowledge of muscarinic signal transduction pertains
to the coupling of the mAcChR to G proteins. These regulatory
proteins are probably the major intermediate by which the receptor
communicates with biochemical effector systems in target cells.

The G proteins are a family of membrane associated proteins which
transduce information from a variety of hormone receptors to effector
enzymes or ion channels (recent reviews can be found in Gilman, 1987;
and Casey and Gilman, 1988).

The G proteins characterized to date are heterotrimers of α, β,
and γ subunits. The α subunits bind guanine nucleotides with high
affinity, are the substrates for ADP-ribosylation by bacterial toxins,
and are unique for the different classes of G proteins. Since the α
subunits provide the major structural diversity for the G proteins, it
is not unexpected that they play an important role in the function of
most G proteins. The activation of a G protein occurs when the α
subunit binds one guanosine triphosphate and the protein then
dissociates into α and $\beta\gamma$ subunits. The subunits then perform the
regulatory activity associated with the particular G protein. An
alpha subunit containing a bound GTP is considered the active form of
this subunit.

The stimulatory guanine nucleotide binding protein (G_s) has an
alpha subunit of about 45K which is ADP-ribosylated by cholera toxin.
Guanosine triphosphate bound $G_{s\alpha}$ stimulates adenylyl cyclase.
Treatment with Cholera toxin results in persistent activation of the
protein, presumably by preventing the hydrolysis of GTP. Hydrolysis

of GTP to GDP by the α subunit results in deactivation. There are at least 4 types of $G_{s\alpha}$ subunits, but their significance is not yet known (Casey and Gilman, 1988).

The inhibitory guanine nucleotide binding protein (G_i) has an α subunit of about 40-41K and is ADP-ribosylated by Pertussis toxin. Treatment with Pertussis toxin uncouples G_i from interacting with hormone receptors. Since the normal role of the protein is to mediate inhibition of adenylyl cyclase, treatment with Pertussis toxin also results in a persistent activation of adenylyl cyclase. The physiological mechanism of $G_{i\alpha}$ function is somewhat uncertain. It may bind to and weakly inhibit adenylyl cyclase, or compete with $G_{s\alpha}$ for a binding site on the enzyme (Cerione et al., 1986; Katada et al., 1984b, 1986a, 1987). As discussed below, the $\beta\gamma$ subunits may also play an important role for G_i. There are also at least three subtypes of $G_{i\alpha}$, and their functional differences have not yet been determined (Casey and Gilman, 1988). The G protein (G_k) which is believed to interact with the atrial potassium channel may be the same as G_i (Codina et al., 1987).

The α subunit of G_o is a 40K peptide which is also a substrate for ADP-ribosylation by Pertussis toxin. An explicit role for $G_{o\alpha}$ has not yet been determined, thus its designation as the "other" G protein. However, $G_{o\alpha}$ has been recently shown to mediate the neuropeptide Y induced inhibition of dorsal root ganglion calcium channels (Ewald et al., 1988).

The α subunit of transducin is 40K and can be ADP-ribosylated by either pertussis toxin or cholera toxin. There are two forms of this peptide, one for the rod outer segment and one for cones (Lerea et

al., 1986). Transducin communicates signals from visual opsins to cGMP phosphodiesterases. Both of the transducin α subunits activate the phosphodiesterase.

The β and γ subunits remain closely associated and, in general, are considered interchangeable among the G protein classes. There are two similar forms of the β subunit, one 35K and one 36K. The two forms of the β subunit are usually seen together except for transducin which only has a 36K β subunit associated with it. The γ subunits are about 8-10K and there may be multiple forms. It is clear that the γ subunit of transducin is distinct from those of the other G proteins (Hildebrandt et al., 1985).

The $\beta\gamma$ subunits serve several roles. As a consequence of being more hydrophobic, they help to anchor the α subunit to the membrane (Sternweis, 1986). They also seem to be required for G protein coupling to receptors (Florio and Sternweis, 1985). The most established functional role for the $\beta\gamma$ subunits is to deactivate G_s. In this sense both G_i and G_o could serve as inhibitory proteins for adenylyl cyclase by providing an excess of $\beta\gamma$ subunits, thus removing free $G_{s\alpha}$ by mass action equilibrium (Gilman, 1987).

There is some evidence that the $\beta\gamma$ subunits may directly inhibit adenylyl cyclase (Katada et al., 1986a). There have also been observations that G_i and G_o interact with calmodulin. The $\beta\gamma$ subunits and $G_{i\alpha}$ inhibited calmodulin activated phosphodiesterase activity, and it appeared that G_i and G_o functioned as inhibitors by binding to calmodulin (Asano et al., 1986), thus reducing the amount available for the phosphodiesterase activation. It was later observed (Katada et al., 1987) that $\beta\gamma$ subunits could bind to calmodulin and inhibit

the calmodulin-sensitive adenylyl cyclase, presumably by reducing the calmodulin available for adenylyl cyclase.

A highly debated role for G proteins is the regulation of the muscarinic regulated atrial inward rectifying potassium channel. Some investigators have observed that the α subunit of a G_i-like protein from erythrocytes activated the channel (Codina et al., 1987). Others claim the channel is regulated by $\beta\gamma$ subunits (Logothetis et al., 1987), or perhaps both α and $\beta\gamma$ (Clapham and Neer, 1988).

There has been some confusion with regard to the term G_p. It may pertain to the as yet unidentified G protein that regulates phospholipase C (Schimerlik, 1988). Alternatively, G_p pertains to a $GTP_\gamma S$-binding protein with an apparent molecular weight of 21,000. This protein has been isolated from placenta; it is not a substrate for ADP-ribosylation by cholera toxin or pertussis toxin, and does not associate with $\beta\gamma$ subunits (Evans et al., 1986). No function has been assigned to this protein. It is possible that the two G_p's define the same protein.

As stated above, G proteins are activated by binding GTP which results in a separation of subunits. Activation is terminated when GTP is hydrolyzed to GDP and the subunits reassociate. The GDP bound form of G_i is believed to associate with a hormone receptor, the receptor then being transformed to a high affinity agonist form. The formation of a hormone-receptor-G protein complex renders the G protein in an "open" conformation by increasing the rate of GDP release and perhaps increasing the rate of GTP binding. Association of GTP to this complex uncouples the receptor from the G protein.

Reconstitution

The successful solubilization of mAcChR using the detergent mixture of digitonin and cholate (Cremo et al., 1981) and subsequent purification of the receptor to homogeneity from atria (Peterson et al., 1984) and brain (Haga and Haga, 1985) have opened the possibility for reconstitution of the pure receptor with regulatory and effector proteins.

Reconstitution procedures are required to remove the receptor from a detergent environment before interaction of the receptor with effector proteins can be studied. An example of the disruption of the interaction between the mAcChR and G_i caused by digitonin can be seen when the receptor is solubilized. The crude extract showed an apparently homogeneous class of agonist binding sites (Herron et al., 1982) indicating uncoupling from G_i, even though GTP$_\gamma$S binding studies indicated that G_i was present in the extract and could be purified from the extract (Chapter II, this thesis).

Reconstitution of partial purified brain mAcChR with pure brain G_i and G_o demonstrated that both proteins could interact with the receptor and induce a guanine nucleotide sensitive high affinity site (Florio and Steinweiss, 1985). The $\beta\gamma$ subunits alone did not have an effect on the receptor but enhanced the effects of the α subunit.

The ability of pure brain mAcChR to interact with pure G_i and G_o was confirmed by Haga et al. (1986). The receptor was also able to increase the V_{max} of the intrinsic GTPase of G_i by 20-50%, and increase the apparent association constant of GTP$_\gamma$S (Kurose et al., 1986).

Objectives

The intent of this thesis was to explore the mechanism of signal transduction from the muscarinic receptor to effector proteins. It was necessary to place the mAcChR into a lipid environment where its interactions with other protein components could be examined. Emphasis was placed on examining the interaction of the atrial mAcChR with atrial G_i.

Chapter II describes the purification of atrial G_i. Atrial G_i was isolated as a by product from the purification of atrial mAcChR. The availability of this protein for study will be important because it is certain to be compatible with atrial mAcChR. It could also provide some clues about the regulation of the atrial potassium channel. The putative G_k that has been tested with the atrial potassium channel is from erythrocytes. Data obtained with this protein indicated that the α subunit was the active component, not the $\beta\gamma$ subunits (Codina et al., 1987). Other groups using brain G_i claim that the α subunit was ineffective, while the $\beta\gamma$ subunits were active (Logothetis et al., 1987). This quandary provides additional motivation for isolating the atrial form of G_i.

A fortuitous advantage of the atrial G_i purification was the limited number of G proteins isolated. The only major contaminant was a GTPγS binding protein of 23K which was not a substrate for ADP-ribosylation and did not associate with $\beta\gamma$ subunits. Speculations are made as to whether this protein is similar to the placental G protein (Evans et al., 1986), or a proteolytic degradation of other G proteins.

Chapter III describes the reconstitution of atrial mAcChR and G_i into a lipid environment in order to examine the mechanism of activation of G_i by the agonist-bound receptor. One consequence of activation was an increased GTPase activity of G_i. The rate limiting step of the GTPase reaction under non-stimulated conditions was shown to be the rate of GDP release. It will be shown that the agonist bound muscarinic receptor functions as an efficient catalyst and increases the rate of GDP release, thus the receptor-G_i had a lower affinity of GDP for G_i. However, the affinity for $GTP\gamma S$ remained unaltered, implying only the affinity for the diphosphate was effected. Most of these measurements would not have been possible in a heterogeneous system. The effects of the mAcChR on GDP release have not been previously directly demonstrated.

In Chapter IV the reconstituted system was extended to include G_s, calmodulin, and the calmodulin-sensitive adenylyl cyclase, thus reconstituting a hormone mediated inhibition of adenylyl cyclase. In addition to establishing the minimum components required for reconstitution, these experiments also demonstrate a new role for $G_{i\alpha}$, inhibiting only the calmodulin stimulated adenylyl cyclase. Thus cells which contain calmodulin-sensitive adenylyl cyclase and muscarinic receptors may have an as yet unexplored mechanism of regulation.

The hormone mediated stimulation of adenylyl cyclase has also been performed in a reconstituted system using purified components (Cerione et al., 1985; May et al., 1985; Rosenberg et al., 1987). Thus it is now possible to perform experiments studying the regulation of reconstituted adenylyl cyclase with multiple receptors and G proteins

present. The existence of multiple muscarinic biochemical responses and the identification of multiple muscarinic subtypes and multiple G proteins that could potentially interact with them have posed the questions of which receptors interact with which G proteins. An important advantage in these reconstitution experiments is that specific mAcChR subtypes and G protein subtypes may be examined.

Chapter II

Purification of the Inhibitory Guanine Nucleotide-binding

Protein (G_i) from Porcine Atria

Contributions of Co-authors

This chapter describes the purification and characterization of

atrial G_i. I originally made the observation that the procedure used

for solubilizing mAcChR also solubilized a large amount of G proteins.

The bulk of these G proteins could be separated from the mAcChR during

the first subsequent purification step, the wheat germ agglutinin

column. Professor Schimerlik then began to develop purification

procedures based on the purification of G_i from brain. One of the

major problems of this purification was the presence of a 23K Da

contaminating G protein. Professor Schimerlik, Gary Peterson and I

contributed to solving this problem.

Gary Peterson also contributed to the majority of the

electrophoresis work and offered interpretation and suggestions

regarding various stages of purification. He also pursued isolating

and identifying the 23K Da peptide, G protein $\beta\gamma$ subunits, and other G

proteins that might have been lost during the purification.

My contribution was to adapt for use in this lab the $[^{35}S]GTP_\gamma S$

binding assay, the assay for GTPase activity, and the methodology for

the ADP-ribosylation of G_i. These methodologies were essential for

the isolation and identification of G_i. I also contributed to the

adaptation of published G_i purification procedures to our system. One

problem was how and when to switch detergents from digitonin-cholate to cholate.

I was also responsible for characterizing the interaction of the purified G_i with guanine nucleotides. After the initial purification, I was responsible for the handling and storage of the protein.

Abstract

The inhibitory guanine nucleotide-binding protein (G_i) was purified from porcine atria as a by-product of muscarinic acetylcholine receptor purification. By using guanosine 5'-O-(3-thio-triphosphate) (GTPγS) to monitor G protein purification, a G protein was isolated which consisted of an α subunit of 41 kDa, a β subunit of 35/36 kDa and a low molecular weight γ subunit. The α subunit was a substrate for pertussis toxin. On the basis of its molecular weight, its ability to act as a substrate for pertussis toxin, and its interaction with guanine nucleotides, this protein was assumed to be G_i. Little or no G_o was isolated, but a low molecular weight GTPγS binding protein (approximately 23 kDa) co-purified with G_i through most of the purification and was finally separated by sucrose gradient centrifugation. It is not certain whether this low molecular weight protein was a proteolytic fragment of G_i or another G protein. Reconstituted G_i had a high affinity for GTPγS (K_d = 456 p$\underline{M}$) and demonstrated GTPase activity (K_m = 8 n$\underline{M}$, k_{cat} = 0.19 min^{-1}).

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Abbreviations

mAcChR, muscarinic acetylcholine receptor; PC, soybean

L-α-phosphatidylcholine; PS, bovine brain L-α-phosphatidyl-L-serine;

CHAPS, (3-[3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate; G_s,

the stimulatory guanine nucleotide binding protein; G_i, the inhibitory

guanine nucleotide binding protein; GppNHp,

5'guanylylimidodiphosphate; $GTP_\gamma S$, guanosine

5'-0-(3-Thio-triphosphate); DTT, dithiotreitol; HEPES,

4-(2-hydroxyethyl)-1-piperazineethanesulfonate; EGTA,

ethyleneglycol-bis-(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid;

EDTA, ethylene diamine tetraacetic acid; PMSF, phenylmethylsulfonyl

fluoride; TED, 20 m$\underline{M}$ Tris HCl, 1 m$\underline{M}$ EDTA, 1 m$\underline{M}$ DTT, and 0.1 m$\underline{M}$ PMSF,

pH 8.0; HEMD, 10 m$\underline{M}$ Na HEPES, 0.1 $\underline{M}$ NaCl, 1 m$\underline{M}$ EGTA, 5 m$\underline{M}$ $MgCl_2$, 1 m$\underline{M}$

DTT and 0.1 m$\underline{M}$ PMSF, pH 7.4.

Introduction

The guanine nucleotide-binding proteins are a family of membrane associated proteins consisting of trimers of heterologous subunits ($\alpha\beta\gamma$). Two members of this family were originally characterized with regard to the regulation of adenylyl cyclase. The stimulatory (G_s) and inhibitory (G_i) guanine nucleotide-binding proteins appear to be involved in signal transduction from either stimulatory or inhibitory receptors respectively, to adenylyl cyclase (see Rodbell, 1980; Stryer and Bourne, 1986; for reviews).

The G_i has been purified from several tissue sources including brain (Neer et al., 1984; Sternweis and Robshaw, 1984; Katada et al., 1986b; Milligan and Klee, 1985), erythrocytes (Codina et al., 1984) and liver (Bokoch et al., 1984). The distinguishing α subunit of G_i, molecular weight of 40 or 41 kDa was shown to be a substrate for ADP-ribosylation by pertussis toxin. Several preparations of G_i have also included a pertussis toxin substrate of 39 kDa (Katada et al., 1984; Sternweis and Robishaw, 1984; Neer et al., 1984), classified as the α subunit of another G protein, G_o. All purifications report the existence of a 35 kDa and/or 36 kDa β subunit. The β doublet seen in many preparations has been determined to be two distinct proteins (Evans et al., 1987; Fong et al., 1987). Although sometimes difficult to distinguish by SDS electrophoresis because of low molecular weight and poor silver staining qualities, a γ subunit was also believed to be associated with G proteins (Hilderbrandt et al., 1984).

The mAcChR is one of the inhibitory types of receptors coupled through G_i to adenylyl cyclase. There has been direct evidence that

the brain mAcChR can interact with both bovine brain G_i and G_o (Haga et al., 1986). A guanine nucleotide binding protein other than G_i or G_o is thought to couple the mAcChR to phosphatidylinositol turnover (Nathanson et al., 1987). In addition, the atrial mAcChR is coupled through a G protein to an inward rectifying potassium channel (Breitweiser and Szabo, 1985; Pfaffinger et al., 1985). This G protein is thought to be G_i; however, there is considerable disagreement as to whether the α or $\beta\gamma$ subunit interacts directly with the potassium channel (Codina et al., 1987; Logothetis et al., 1987; Yatina et al., 1987). Thus it is uncertain how many different G proteins the atrial mAcChR recognizes and if there are any G proteins unique to the atria.

To date, no G proteins have been isolated from atrial tissue, perhaps because this tissue has a lower abundance of GTP binding proteins and is more difficult to work with than other sources, such as brain. In the present study, atrial G_i was purified as a by-product in the purification of atrial mAcChR. The availability of a purified atrial G_i should help to clarify the role of this protein in atria.

Materials and Methods

$[^{35}S]GTP_\gamma S$ (1000 Ci/mmol), $[\gamma-^{32}P]GTP$ (20-30 Ci/mmol), and
[adenylate-^{32}P] NAD (31 Ci/mmol) were purchased from New England
Nuclear. Cholesterol, P.C. (type III-s), P.S. (bovine brain), CHAPS,
digitonin, GTP, NAD and activated charcoal were purchased from Sigma.
Cholic acid was purchased from Sigma and purified by the method of
Ross and Shatz (1978). GDP and GTP$_\gamma$S were purchased from Boehringer
Mannheim. The purity of GTP and GTP$_\gamma$S was determined by thin layer
chromatography using a solvent of 1-propanol:NH$_4$OH:H$_2$O (6:3:1). When
necessary, these nucleotides were purified by applying them to DEAE
Biogel A and eluting with a 0-0.5 $\underline{M}$ LiCl gradient. Amido Black 10B
was from BioRad. Pertussis toxin was from List Biological
Laboratories and was stored in 50 m$\underline{M}$ sodium phosphate, 0.25 $\underline{M}$ NaCl, pH
7.0, at 100 µg/ml. A mixture of brain G_i/G_o was a generous gift from
Dr. Lutz Birnbaumer.

Membrane Preparation and Detergent Extraction. Porcine atrial
plasma membranes were enriched for mAcChR and extracted with 0.4%
digitonin and 0.08% sodium cholate as described (Peterson and
Schimerlik, 1984) except for the addition of 1 µg/ml soybean trypsin
inhibitor and 1 m$\underline{M}$ benzamidine (protease inhibitor) to all buffers.
The solubilized protein (extract) was applied to a wheat germ
agglutinin (WGA) column as described for the purification of the
mAcChR (Peterson et al., 1984) except that the extract was
supplemented with 5 m$\underline{M}$ MgCl$_2$ to increase the yield of mAcChR from the
WGA column by improving glycoprotein binding to the immobilized
lectin. The material that did not bind to the WGA column was

collected and referred to as WGA void (600-700 ml/preparation).

The WGA void was supplemented with 0.9% Na cholate and 1 m$\underline{M}$ DTT, and

the pH was adjusted to 8.0 with 1 $\underline{M}$ tris base. This material was

either used immediately for G_i purification or supplemented with 75 m$\underline{M}$

sucrose and stored at -80 C.

The WGA voids (1.8 to 2.1 liters) from three mAcChR preparations

were applied to a 4.9 x 42 cm DEAE Sephacel column which was

equilibrated in TED/0.9% cholate/2 µg/ml STI. The column was washed

at 2 ml/min with 350 ml of equilibration buffer containing 20 m$\underline{M}$ NaCl

and eluted with a 20-400 mM NaCl gradient in 2.2 l of equilibration

buffer. Twenty ml fractions were collected at 2 ml/min. Figure II-1A

shows the profile of [^{35}S]GTP$_\gamma$S binding and absorbance at 280 nM for

the DEAE Sephacel column. Fractions 46-65 containing GTP$_\gamma$S binding

activity were pooled and concentrated to 16.5 ml by pressure

filtration through an Amicon YM-10 membrane.

The concentrated DEAE pool was then loaded on a 4.9 x 51 cm

Ultragel AcA 34 (LKB) exclusion column which was equilibrated with

TED/0.9% cholate/0.1 $\underline{M}$ NaCl/2 µg/ml STI. Equilibration buffer was

used to elute the column at 1 ml/min collecting 10 ml/tube (Fig.

II-1B).

Fractions 75 to 98 containing the peak GTP$_\gamma$S binding activity from

the Ultragel column were pooled and diluted three fold into TED/0.1 $\underline{M}$

NaCl/2 µg/ml STI to yield 540 ml of solution in TED buffer containing

0.1 M NaCl and 0.3% sodium cholate. This was loaded at 0.4 ml/min

onto a 3 x 25 cm octyl-sepharose column equilibrated in the same

buffer and then washed with 100 ml of equilibration buffer followed by

400 ml of TED/0.3% cholate/0.5 $\underline{M}$ NaCl/2 µg/ml STI. The column was

eluted with a 500 ml NaCl/sodium cholate gradient starting

with 0.25 $\underline{M}$ NaCl, 0.3% sodium cholate and ending with 50 $\underline{mM}$ NaCl, 1.2%

sodium cholate in TED/2 µg/ml STI at a flow rate of 0.5 ml/min. 6.8

ml fractions were collected.

Fractions 50 to 60 of the octyl-sepharose column, which contained

the peak of the GTPγS binding activity Fig. II-2, were concentrated on

a 1.4 x 2.5 cm hydroxylapatite column prepared and equilibrated in

TED/0.9% cholate/0.1 $\underline{M}$ NaCl and eluted with equilibration buffer plus

0.2 $\underline{M}$ potassium phosphate, pH 8.0. The column was loaded and eluted

at 0.3 ml/min and 0.75 ml fractions were collected.

The final purification procedure was a 5-20% linear sucrose

gradient. Multiple 35 ml gradients in TED/0.9% cholate were used and

2-3 ml of material concentrated by hydroxylapetite (approximately 3 µ$\underline{M}$

in [^{35}S]GTPγS sites) were applied to each gradient and were

centrifuged in a SW28 rotor at 27,000 rpm for 72 hours. Gradients

were fractionated from the bottom into 1 ml fractions. Protein which

bound [^{35}S]GTPγS and contained the 41 kDa subunit as determined by SDS

polyacrylamide gel electrophoresis (Fractions 15-21, Fig. II-3), was

pooled and concentrated by applying to 1/10 volume of DEAE Bio-Gel A

which had been equilibrated in TED/0.9% cholate. The G$_i$ was eluted

with TED/0.9% cholate/0.3 $\underline{M}$ NaCl/75 $\underline{mM}$ sucrose and dialyzed against

TED/0.9% cholate/75 $\underline{mM}$ sucrose. The purified G$_i$ was either used

directly or supplemented with 5 $\underline{mM}$ MgCl$_2$ and stored at -80 C. G$_i$ was

also concentrated by using a Centricon 10 microconcentrator (Amicon).

[^{35}S]GTPγS binding. The binding of [^{35}S]GTPγS was performed

essentially as described by Northup et al. (1982). The samples in

cholate were diluted at least 10-fold into (HEMD) buffer supplemented

with 25 m$\underline{M}$ MgCl$_2$, 0.1% lubrol PX, and 1 $\mu\underline{M}$ [^{35}S]GTP$_\gamma$S (3-8 ci/mmol),

incubated for one hour at 32°C and filtered over nitrocellulose paper.

Assays of reconstituted G$_i$ were performed in HEMD buffer without

lubrol for one hour at 32°C.

GTPase activity was determined by measuring ^{32}P released as

described (Appendix II).

Protein Determination. Protein was estimated either by the Folin

phenol method (Peterson, 1983) or by staining with Amido Black as

described by Schaffner and Weissman (1973).

SDS-Polyacrylamide gel electrophoresis. Fractions obtained during

purification of the G proteins were analyzed on 8-18% linear

acrylamide gradient gels (0.7 cm x 8 cm x 8 cm) in SDS according to

Laemmli (1970). For high resolution of 41 kDa and 39 kDa α subunit

bands, the samples were alkylated with N-ethylmaleimide (Sternweis and

Robishaw) and run on long (0.7 cm x 8 cm x 15 cm) 12% acrylamide gels.

The gels were either stained with Coomassie blue G-250 or silver (Wray

et al., 1981) as indicated in the figure legends.

ADP ribosylation. Reconstituted G$_i$ was diluted 10 fold into HEMD

buffer supplemented with 5 m$\underline{M}$ thymidine and 3 $\mu\underline{M}$ [adenylate-^{32}P]NAD.

Pertussis toxin was activated for 15 minutes at 32°C with 10 m$\underline{M}$ DTT

and 50 $\mu\underline{M}$ ATP. The activated toxin was diluted 20 fold into the

ADP-ribosylation mixture to give a final concentration of 4.5 μg/ml.

The mixture was incubated for one hour at 32°C and the reaction

stopped by adding 1/4 volume of 5x Laemmli sample buffer (Laemmli,

1970).

Determination of bound GDP. The amount of GDP bound to G$_i$ was

determined by the method of Ferguson et al. (1986). The inhibitory

guanine nucleotide binding protein (1.5 µM in $GTP_\gamma S$ sites) was
denatured by boiling for 2-3 minutes in TED/0.8% cholate buffer.
After cooling, the denatured protein was diluted 10 fold into
HEMD/0.1% lubrol buffer which contained a final concentration of 10 nM
active G_i (based on $GTP_\gamma S$ binding). [^{35}S]$GTP_\gamma S$ was then added to a
concentration of 10 nM (1100 Ci/mmol). This mixture was incubated at
32°C for 1 hour and then filtered as described above. The degree of
inhibition from the denatured protein was compared to the inhibition
obtained from adding known amounts of GDP to active G_i.

Reconstitution. Atrial G_i was reconstituted into a lipid mixture
of 1:1:0.1 PC, PS, and cholesterol as described (Appendix I). In
brief, the procedure involved adding G_i to the lipid mixture which was
sonicated in the presence of 7 mM CHAPS. The mixture was diluted 25-
fold, polyethylene glycol was added to a final concentration of 10%
w/v, and the precipitate collected by centrifugation at 250,000 x g.

Results and Discussion

Table II-1 summarizes the purification of atrial G_i from membranes enriched in mAcChR. Identification and purification of G_i was facilitated by using $[^{35}S]GTP_{\gamma}S$ binding activity and, at latter steps of the purification, SDS gel electrophoresis. The use of membranes enriched in mAcChR for purification of G proteins not only conserves resources and effort, but is also a logical strategy, used previously by Sternweis and Robishaw (1984), as an enrichment in mAcChR should result in an enrichment of associated G proteins. The double extraction procedure that was optimized for mAcChR (Peterson and Schimerlik, 1984) was also successful in solubilizing $GTP_{\gamma}S$ binding proteins. The first digitonin-cholate extract (E1) solubilized 20% of the $GTP_{\gamma}S$ binding sites resulting in a slight decrease in specific activity (0.079 nmol/mg), while the second digitonin-cholate extract (E2) contained 56.3% of the $GTP_{\gamma}S$ binding sites and a 2.6-fold increase in specific activity to 0.23 nmol $GTP_{\gamma}S$ bound/mg protein. The first extract was discarded and the second extract was used for purification of mAcChR and G_i.

The $GTP_{\gamma}S$ binding proteins were separated from the mAcChR on the WGA column. The WGA column bound the receptor while the majority of the $GTP_{\gamma}S$ binding proteins do not bind and were collected in the fraction termed "WGA void". The mAcChR was displaced from the column by eluting with 0.2 M N-acetyl-glucoseamine (Peterson et al., 1984). There was some $GTP_{\gamma}S$ binding activity eluting with the receptor (about 5%), which probably represented GTP binding proteins coupled to the mAcChR as well as other GTP binding proteins which associated with

glycoproteins. There has been no indication that the G proteins themselves are glycosylated. The α and β subunits of G_s and G_i behave as standard proteins on SDS-PAGE (Codina et al., 1984), and the apparent subunit molecular weights on SDS-PAGE agree closely with their predicted molecular weights (Fong et al., 1987; Sullivan et al., 1986).

The elution profile of the WGA void chromatographed over a DEAE-Sephacel column (See Fig. II-1A) consistently showed two peaks of GTPγS binding. The reasons for this heterogeneity are not known. The two peaks may represent the separation of two different G proteins, the separation of G proteins from proteolytic products, or the separation of monomeric subunit from heterotrimer. The two peaks did interact differently with octyl-sepharose. Most of the GTPγS binding activity of the higher salt DEAE column peak did not bind to octyl sepharose when treated separately (data not shown). In any case, these two peaks were combined and concentrated as described in methods. The DEAE-Sephacel column was not very effective for purification but it was effective in concentrating the material and exchanging digitonin/cholate for cholate. Digitonin was necessary for the stability of the mAcChR but not for the GTPγS binding protein. Most G protein purifications have been performed in cholate, and switching to cholate facilitated a simpler and less expensive purification more analogous to published procedures.

After concentration, the DEAE pool was applied to an Ultragel ACA 34 column. The GTPγS binding proteins eluted after the majority of the protein (Fig. II-1B) and resulted in a three fold enrichment in GTPγS protein (Table II-1). Fractions that contained the peak of

GTP$_\gamma$S activity were pooled, diluted to a cholate concentration of 0.3% and applied to an octyl-sepharose column, and eluted with a reverse gradient of NaCl and cholate as described in methods. The resulting GTP$_\gamma$S binding profile (Fig. II-2) showed a single peak which eluted after a large protein peak giving a 4.5-fold enrichment in GTP$_\gamma$S binding protein. The SDS-PAGE profile of the octyl-sepharose column (Fig. II-2) showed the peak of GTP$_\gamma$S binding to represent nearly purified G protein (α, β and γ subunits) while the low detergent-high salt end of the elution profile contained mostly excess $\beta\gamma$ subunits. Fractions containing GTP$_\gamma$S binding were concentrated on an HT column as described in methods. It was almost always necessary to further purify the G protein by sucrose gradient centrification. The sucrose gradient showed two peaks of GTP$_\gamma$S binding activity (Fig. II-3), where the faster sedimenting peak contained the typical $\alpha\beta\gamma$ heterotrimer expected for G proteins. Fractions from the faster sedimenting peak were pooled and concentrated as described in methods. An SDS gel of the final purified and concentrated atrial G protein is shown in Fig. II-4 lane A.

To determine the identity of the isolated atrial α subunit, a sample was alkylated with N-ethylmaleimide (NEM) (Sternweis and Robishaw, 1984) and run on a 12% acrylamide gel alongside a mixture of NEM alkylated brain G_i/G_o (Fig. II-4 lanes B and C). The atrial α subunit comigrated with brain G_i (41 kDa) with little or no detectable α_o subunit. As shown in Fig. II-4 lane E, the 41 kDa atrial α subunit was ADP-ribosylated by Pertussis toxin and ^{32}P-NAD. On the basis of its ability to tightly bind GTP$_\gamma$S, its $\alpha\beta\gamma$ heterotrimer structure, its comigration with brain G_i, and its ability to act as a substrate for

ADP-ribosylation catalyzed by pertussis toxin, the atrial G protein was provisionally termed G_i. The β subunit in Fig. II-4 was a doublet of 35 kDa and 38 kDa. Other G proteins have been observed to contain a β subunit doublet (Sternweis and Robishaw, 1984; Evans et al., 1987) and these have been demonstrated to be two distinct proteins (Fong et al., 1987).

The slower sedimenting $GTP_\gamma S$ peak from the sucrose gradient contained a conspicuous band of about 23 kDa. The recovery of this protein relative to G_i was variable. It was not present in all preparations, but in some preparations it was the only band which could account for the observed $GTP_\gamma S$ binding activity. At present it is not clear whether this polypeptide is a stable proteolytic fragment of $G_{i\alpha}$ similar to that generated by Winslow et al. (1986) or a low M_r G protein similar to the placental 23 kDa G protein reported by Evans et al. (1986). The 23 kDa protein isolated from atria was similar to the placental G protein in that it did not associate with the $\beta\gamma$ subunits on the sucrose gradient (Figure II-3) and was not significantly ADP-ribosylated by Pertussis toxin (data not shown). However, these properties could be a result of proteolysis. The amino acid residue which is ADP ribosylated is located near the C terminal of G_i.

The lack of G_s in our preparation was notable since a comparison of bovine brain and bovine heart cholate extracts revealed that heart extracts contained almost five times more G_s per mole of $GTP_\gamma S$ binding sites then brain extracts (Sternweis and Robishaw, 1984). However, octyl-sepharose chromatography has been demonstrated to completely separate G_s from G_i (Katada et al., 1986b). In the present study only

one GTPγS binding peak was eluted from this column, but represented

less than 50% of the applied GTPγS binding sites. A small (<5%)

amount of the GTPγS binding sites were recovered in the load and

subsequent washes. Some additional GTPγS binding was eluted after the

gradient with 2% cholate (data not shown). Since brain G_s was eluted

from heptylamine-sepharose at higher cholate concentrations than brain

G_i/G_o (Sternweis and Robishaw, 1984), it was conceivable that any G_s

present was not eluted from octyl-sepharose with a 0.3-1.2% cholate

gradient. These observations are consistent with an estimation of G_s

and G_i hydrophobicity based on their ability to bind detergent, G_s

binding three times as much detergent as G_i (Codina et al., 1984).

The lack (or nearly so) of G_o in atrial preparations was particularly

interesting since octyl-sepharose was not successful in separating G_i

from G_o in previous purification approaches using brain (Millagan and

Klee, 1985) and thus could not explain its absence. It is not known

if G_o composes a smaller fraction of GTPγS binding proteins in atrial

tissue, or if G_o (as well as G_s) were separated from G_i at some

earlier purification step, such as the first detergent extract.

Purified G proteins from liver and brain have been determined to

contain tightly bound GDP (Ferguson et al., 1986). Atrial G_i has also

been determined to contain a tightly bound guanine nucleotide. A

direct spectrophotometric analysis of atrial G_i for the determination

of bound GDP was not practical because of the large protein

requirements. Bound GDP was assayed by denaturing a small amount of

sample and measuring its inhibition of [^{35}S]GTPγS binding to active

G_i. As determined from a standard curve of added GDP, atrial G_i was

found to contain 2/3 to 1 GDP/GTPγS binding site (data not shown).

When the denatured G_i was first treated with charcoal and the charcoal removed by centrifugation, there was no inhibition of GTP$_\gamma$S binding by the supernatant. It was assumed that this compound absorbed to the charcoal was GDP.

Using reconstituted G_i, [^{35}S]GTP$_\gamma$S was found to bind to a single class of high affinity (K_d = 456 pm) binding sites within the range of nucleotide tested (Fig. II-5). Reconstituted G_i also possessed a high affinity GTPase. Figure II-6 shows a Lineweaver Burke plot of the GTPase activity between 10 and 110 nM GTP. The K_m (8 nM) was somewhat lower than the K_m of 40 nM reported for detergent free erythrocyte G_i (Sunyer et al., 1984) but closer to the K_m of 17 nM reported for reconstituted G_i brain (Kurose et al., 1986). Although atrial G_i bound GTP rather tightly, it has a low turnover (0.19 min^{-1}), similar to the value obtained for reconstituted brain G_i (0.37 min^{-1}) (Kurose et al., 1986) but an order of magnitude higher then the turnover of 0.013 for erythrocyte G_i (Sunyer et al., 1984). It has been noted that lipid also increases the GTPase activity of the 40 kDa α subunit G protein (Neer et al., 1984).

In conclusion, G_i was purified from porcine atrial membranes as a by product of mAcChR purification. The overall yield of G_i was low (1% from cholate extract) when compared to brain purifications which ranged from 5-20% (Sternweis and Robishaw, 1984; Milligan and Klee, 1984; Katada et al., 1986b). This may indicate that atrial tissue is not as good a source for G_i as brain tissue. Bovine heart extracts show 1/3 the total GTP$_\gamma$S binding sites compared to bovine brain extracts and a high proportion of GTP$_\gamma$S sites in heart extracts is due to G_s (Sternweis and Robishaw, 1984). Thus heart membranes have less

total G proteins and a smaller proportion of these proteins are G_i.

Another source for low yields might have been proteolysis, as

suggested by the presence of the lower molecular weight $GTP_\gamma S$ binding

protein. Due to longer processing time, preparation of G_i from atria

has more potential for proteolytic problems than preparations from

brain. For example, to produce 3.2 g of detergent extracted protein,

1.3 kg of brain tissue from six bovine brains were processed (Milligan

and Klee, 1985). In our preparations, 20 Kg of atria from several

hundred animals must be processed to produce 2.4 g of detergent

extracted protein. The time required for the collection and

preparation of this amount of heart tissue could exacerbate problems

due to proteolysis. Despite these limitations, G_i was purified in

quantities sufficient for further investigation.

The purified $GTP_\gamma S$ binding protein was determined to be G_i on the

basis of its electrophoretic mobility compared with brain G_i and on

its ability to act as a substrate for ADP-ribosylation catalyzed by

pertussis toxin. Reconstituted atrial G_i also hydrolyzes GTP in a

manner similar to brain G_i (Kurose et al., 1986). In addition to G_i a

23 kDa GTP binding protein was isolated. Experiments are currently in

progress to determine if this is a breakdown product of G_i or another

G protein. The recovery of G_o was low, but it was not determined

whether G_o is lost during purification or is less abundant in atria.

Isolation of atrial G_i should now allow direct investigations into

the mechanism of cholinergic modulation of cardiac activity. Future

work should help to clarify the role of G_i and its individual subunits

in the coupling of the atrial mAcChR to effector proteins such as

phospholipase C, adenylyl cyclase, and the inward rectifying potassium

channel. It will also be possible to examine particular aspects of the interaction between atrial mAcChR and G_i, such as hormone induced changes in interaction of G_i with guanine nucleotides and guanine nucleotide induced changes in hormone binding to the mAcChR. Finally, using mAcChR and G_i which were both isolated from atria will remove any possible artifacts or interpretive reservations that may arise as a result of using proteins derived from different tissue sources. Studies of the interaction of atrial G_i and atrial mAcChR have already been described (Chapter III) and are ongoing in this laboratory.

Table II-1

Purification of G_i from Porcine Atria

Purification Step	Protein (mg)	$[^{35}S]GTP\gamma S$ binding sites (nmol)	Site recovery (%)	Specific activity (nmol/mg)	Relative purification (fold)
Membrane	10,958[a]	950	100.0	0.087	1.0
Extract 1	2,352[a]	186	19.6	0.079	0.9
Extract 2	2,327[a]	535	56.3	0.230	2.6
WGA void	996[b]	404	42.5	0.406	4.7
DEAE-Sephacel	335[b]	177	18.6	0.528	6.1
Ultragel AcA34	70.2[b]	113	14.0	1.61	18.5
Octyl-sepharose	5.39[b]	41.4	4.36	7.68	88.3
Sucrose gradient	0.70[b]	5.26	0.55	7.51	86.3

[a]Determined by Folin method (Peterson (1983)

[b]Determined by staining with Amido Black (Schaffner and Weisman, 1973)

Figure II-1A Chromatography of $GTP_\gamma S$-binding proteins through

DEAE-Sephacel. See methods for explanation.

II-1B Chromatography of $GTP_\gamma S$-binding proteins through
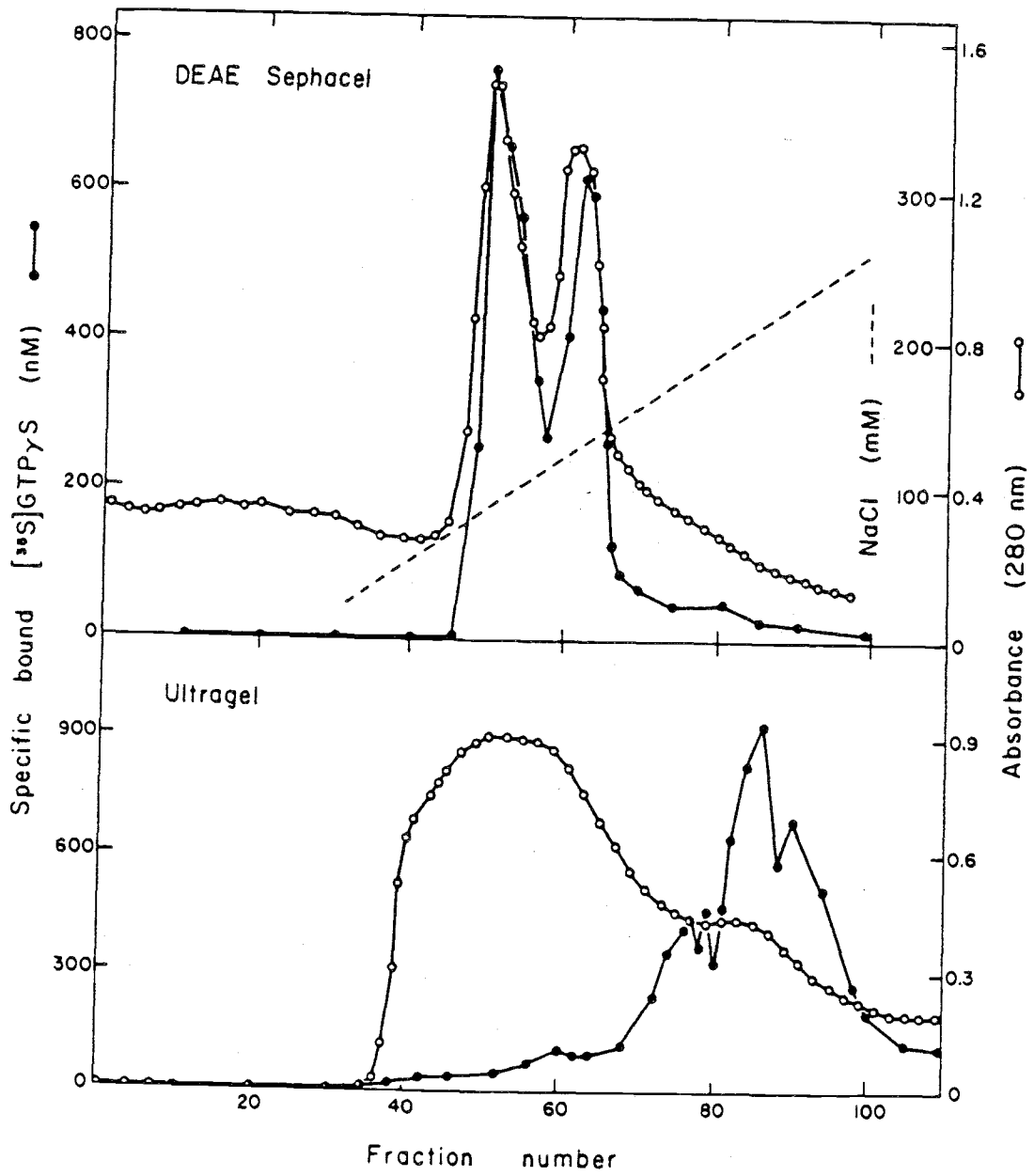
Ultragel AcA 34. See methods for explanation.

Figure II-1

Figure II-2   Chromatography of GTP$_\gamma$S-binding proteins through

Octyl-Sepharose. See methods for explanation. Aliquots

(20 µl) of indicated fractions were analyzed by

SDS-polyacrylamide (8-18%) electrophoresis and

visualized by Coomassie staining (upper panel).

Figure II-2

Figure II-3   Chromatography of GTP$_\gamma$S-binding proteins through sucrose

gradient. See methods for explanations. Aliquots (20

µl) of indicated fractions were analyzed by

SDS-polyacrylamide (8-18%) electrophoresis and

visualized by Coomassie staining (upper panel).

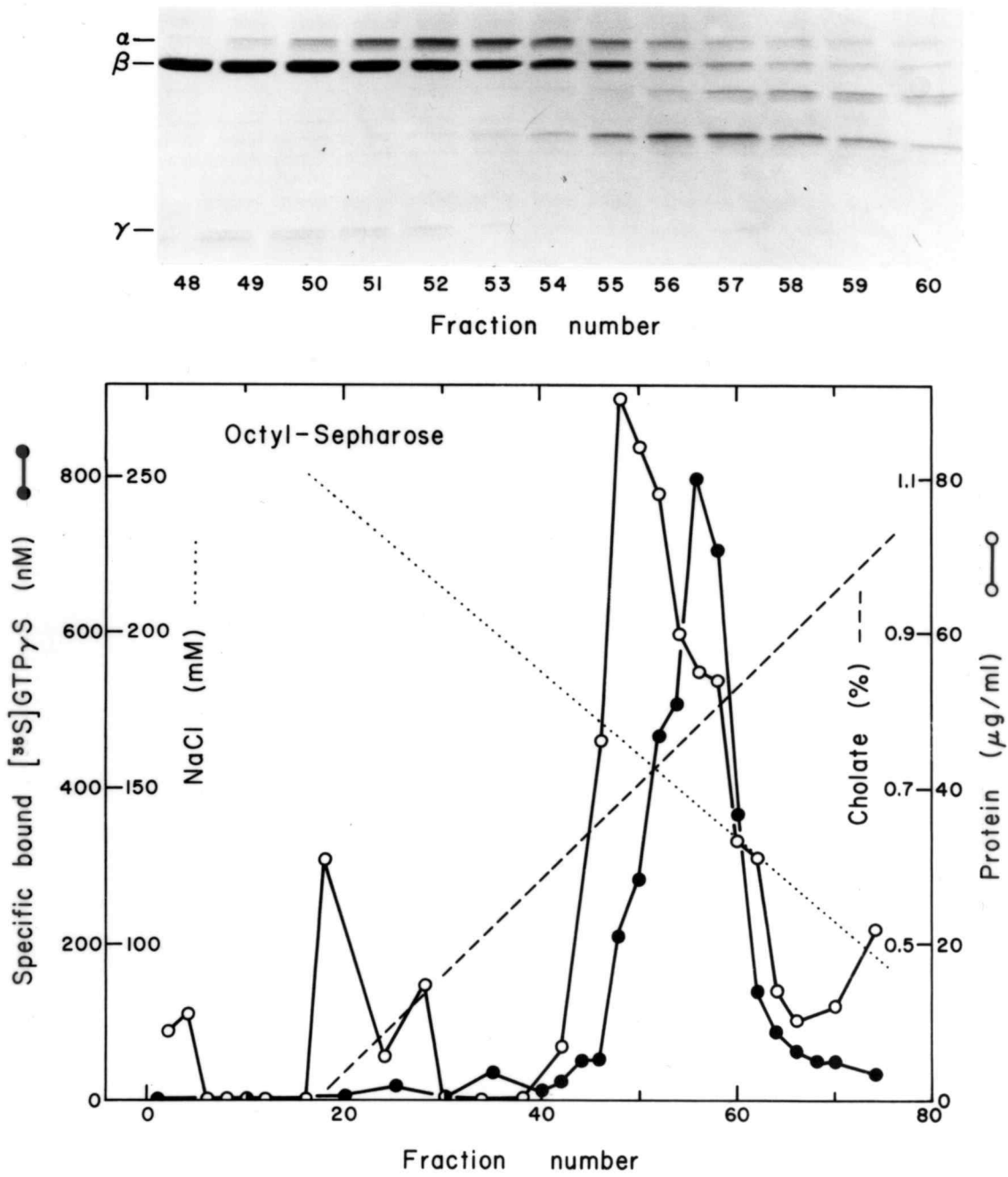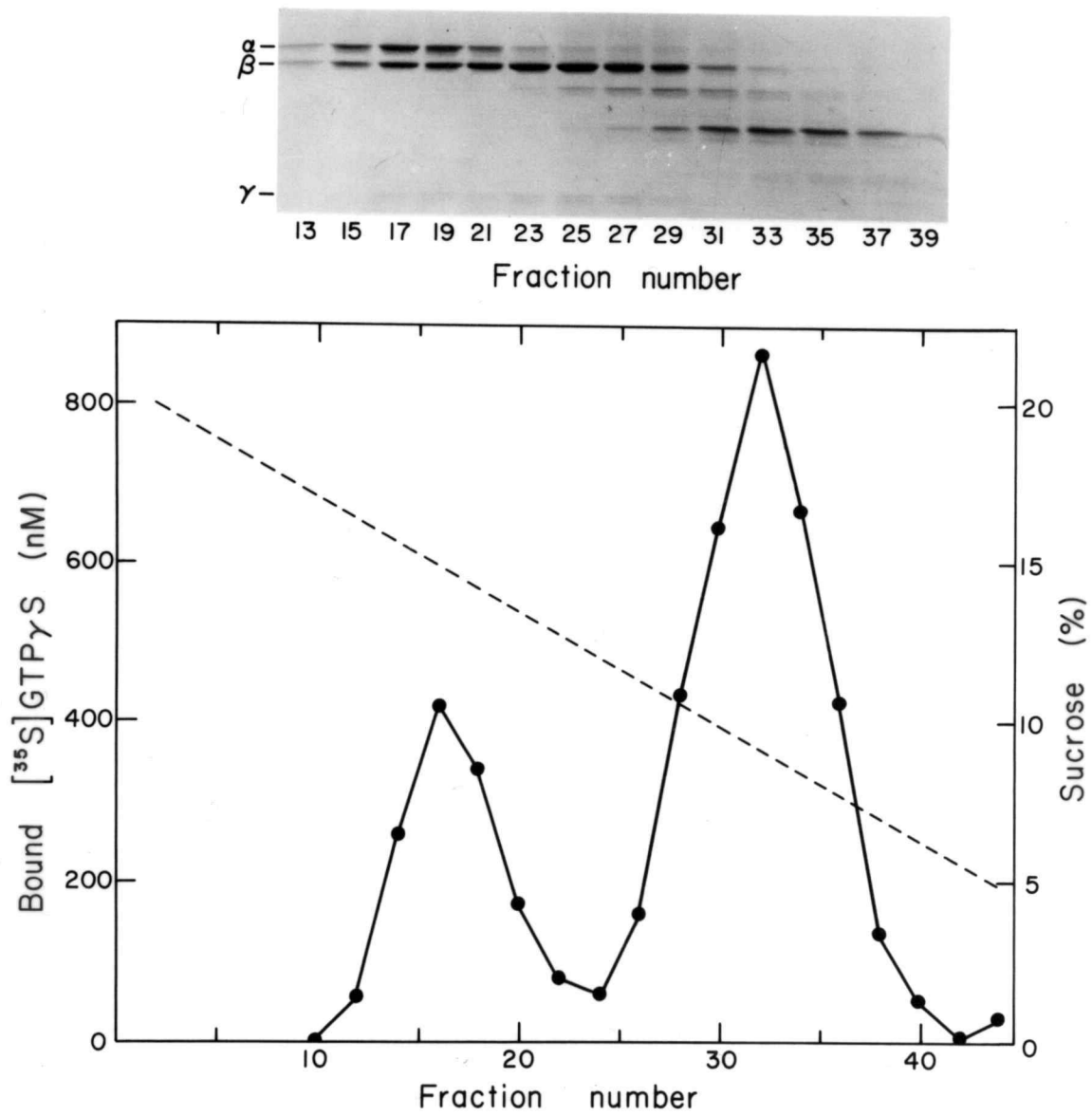Figure II-3

Figure II-4   Sodium dodecyl sulfate polyacrylamide gel

electrophoresis of atrial G_i. Lane A, Sucrose gradient

purified atrial G_i analyzed using a 8-18% linear

acrylamide gel. G_i (400 ng) was electrophoresed along

with molecular weight markers (phosphorylase a, M_r

97,114; bovine serum albumen, M_r 66,296; aldolase, M_F

39,210; Chymotropsinogen A, M_r 25,666; soybean trypsin

inhibitor, M_r 20,095; and lysozyme, M_r 14,314) and

visualized with silver staining. Lane B and C, atrial

G_i and brain G_i/G_o respectively were run on 12%

acrylamide gel as described in methods to resolve α_i

from α_o. The gels were visualized by silver staining.

Lane D, Reconstituted G_i was ribosylated as described in

methods and electrophoresed on a 12% acrylamide gel.

The gel was visualized by silver staining and exposed to

x-ray film for 3 days (Lane E). The additional lower

molecular weight bands represent pertussis toxin while

the higher molecular weight bands are from contaminating

α keratins seen as a consequence of overstaining the gel

to visualize G_i.
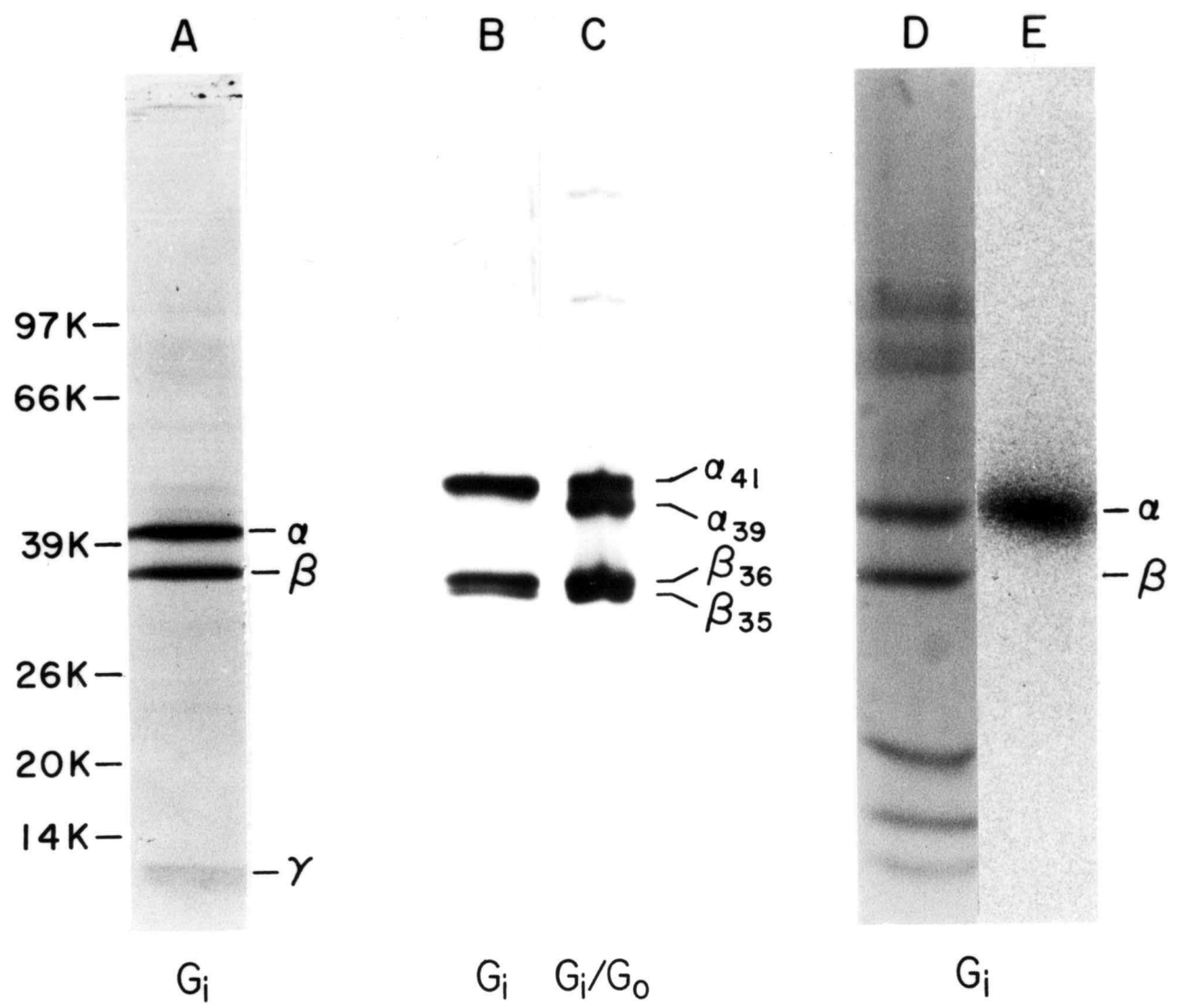
Figure II-4

Figure II-5   Binding of [^{35}S]GTP$_\gamma$S to reconstituted G$_i$.

Reconstituted G$_i$ was incubated with 0.1 to 11 n$\underline{M}$

[^{35}S]GTP$_\gamma$S (SA = 120 ci/mmol). Duplicate 100 µl aliquots

were filtered as described under Methods. Data were

analyzed in the form of a Scatchard plot (insert).

Least squares analysis gave a dissociation constant of

456 ± 41 p$\underline{M}$ and a total site concentration of 487 ± 52

p$\underline{M}$.
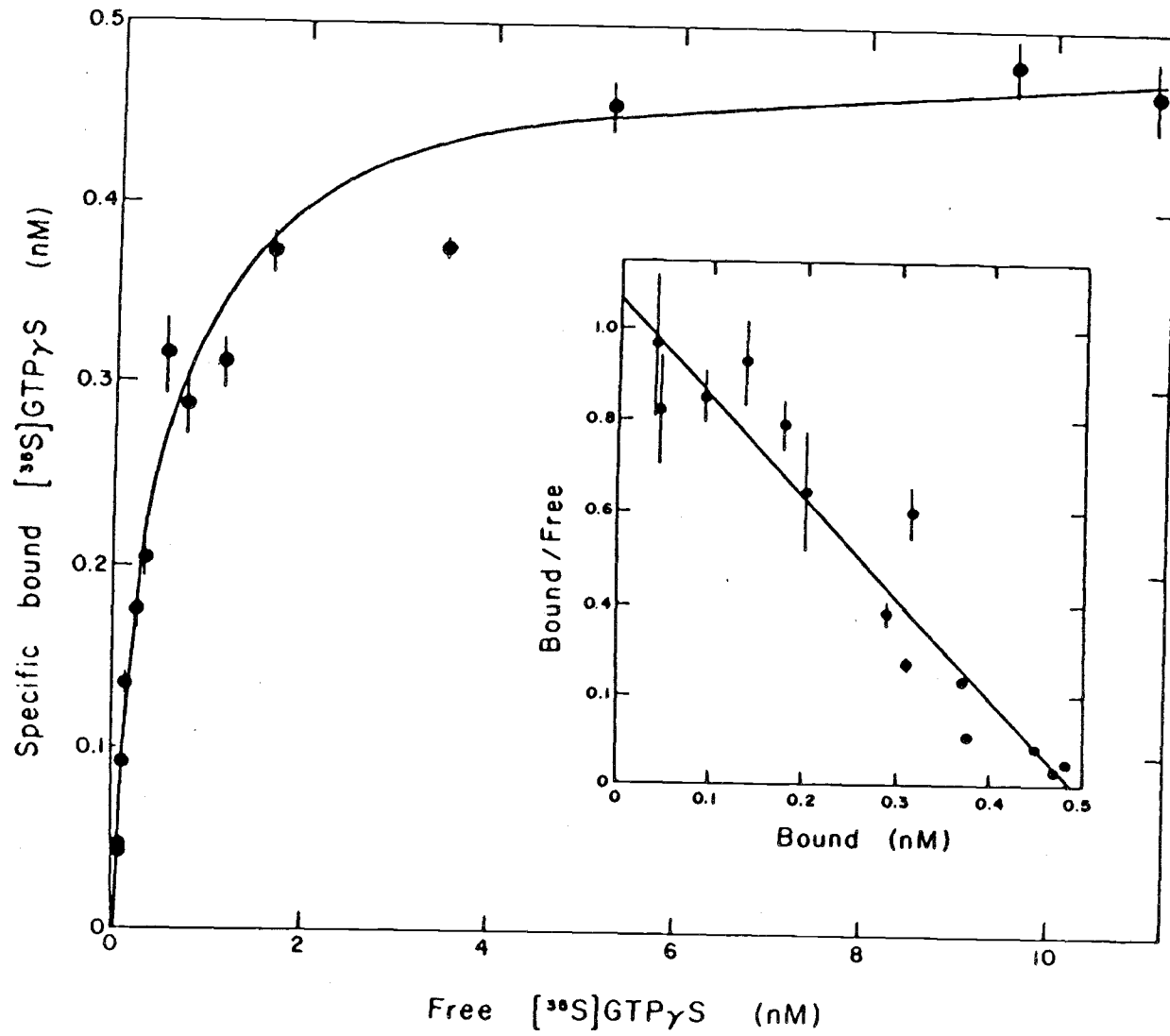
Figure II-5

Figure II-6   Dependence of GTPase on GTP concentration. G_i was

diluted to a concentration of 1.76 n$\underline{M}$ and incubated for

15 minutes at 32°C in HEMD buffer with the indicated

concentration of [^{32}P]GTP. Least squares analysis of

the Lineweaver-Burke plot gave a turnover of 0.19 ± 0.01

min^{-1} and a K_m of 7.98 ± 0.74 n$\underline{M}$.
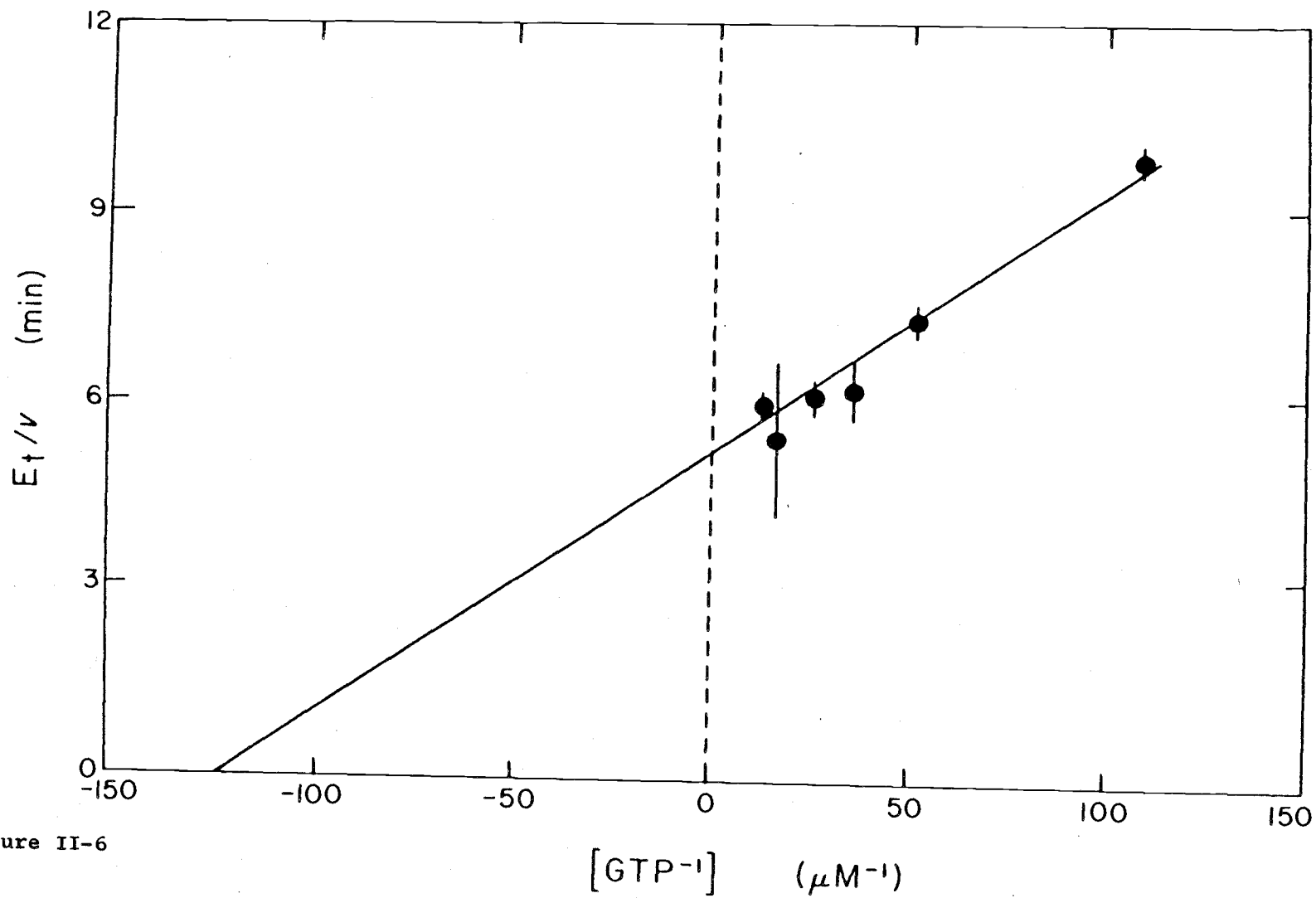
Figure II-6

Acknowledgments

Chapter III

Reconstitution of the Purified Porcine Atrial Muscarinic

Acetylcholine Receptor with Purified Atrial Inhibitory

Guanine Nucleotide Binding Protein

Contribution of Co-Authors

Kimberly Kahler assisted with some of the $[^{35}S]GTP\gamma S$ binding experiments and was involved with some of the data analysis for these experiments. The remaining experiments were performed and analyzed by myself under the guidance of Professor Michael Schimerlik. G. Scott Herron assisted in the development of the reconstitution procedure.

Abstract

Purified porcine atrial muscarinic receptor (mAcChR) was reconstituted with purified porcine atrial inhibitory guanine nucleotide binding protein (G_i) in a lipid mixture consisting of phosphatidylcholine: phosphatidylserine and cholesterol (1:1:0.1 w/w). 5'-Guanylylimidodiphosphate (0.1 m$\underline{M}$) had no effect on the binding of the muscarinic antagonist L-quinuclidinyl benzilate but converted high affinity carbachol binding sites (K_d equal to 1 $\mu\underline{M}$) in the reconstituted preparation to the low affinity state (K_d equal to about 100 $\mu\underline{M}$). Steady-state kinetic measurements of GTPase activity showed that the turnover number was increased from 0.19 min^{-1} in the presence of the muscarinic antagonist L-hyoscyamine to 2.11 min^{-1} for the agonist carbachol. The affinity of G_i for GDP was reduced by about fifty-fold upon interaction with the carbachol-mAcChR complex, and the observed rate constant for GDP dissociation was increased by thirty-eight fold from 0.12 min^{-1} to 4.5 min^{-1}. Thus the increase in steady-state GTPase activity observed for muscarinic agonists is largely, if not exclusively, due to the increase in GDP dissociation from G_i--probably the rate limiting step in the steady-state mechanism. Carbachol stimulated GTPase was sensitive to ADP-ribosylation of the reconstituted G_i by pertussis toxin, but the high affinity agonist binding was uncoupled only when the reconstituted preparation was treated with pertussis toxin in the presence of GTP and the agonist acetylcholine. These results suggest that association with the mAcChR protects G_i from ADP-ribosylation by pertussis toxin.

Abbreviations

mAcChR, muscarinic acetylcholine receptor; Buffer A, 10 $\underline{mM}$ HEPES, 0.1 $\underline{M}$ NaCl, 1 $\underline{mM}$ EGTA, 5 $\underline{mM}$ $MgCl_2$, 1 $\underline{mM}$ DTT, and 0.1 $\underline{mM}$ PMSF, pH 7.4; Buffer B, 10 $\underline{mM}$ HEPES, 50 $\underline{mM}$ NaCl, 1 $\underline{mM}$ EGTA, 5 $\underline{mM}$ $MgCl_2$, 1 $\underline{mM}$ DTT, and 0.1 $\underline{mM}$ PMSF, pH 7.4; Buffer C, 25 $\underline{mM}$ Imidazole, 0.1 $\underline{M}$ NaCl, 1 $\underline{mM}$ EDTA, 5 $\underline{mM}$ $MgCl_2$, 1 $\underline{mM}$ DTT, and 0.1 $\underline{mM}$ PMSF, pH 7.4; PC, soybean L-α-phosphatidylcholine; PS, bovine brain L-α-phosphatidyl-L-serine; CHAPS, (3-[3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate; L-QNB, the L isomer of quinuclidinyl benzilate; G_s, the stimulatory guanine nucleotide binding protein; G_i, the inhibitory guanine nucleotide binding protein; G_T, transducin; GppNHp, 5'guanylylimidodiphosphate; GTP$_\gamma$S, guanosine 5'-0-(3-Thio-triphosphate); DTT, dithiotreitol; IAP, islet-activating protein (pertussis toxin); HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonate; BHT, butylated hydroxytoluene; EGTA, ethyleneglycol-bis-(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid; EDTA, ethylene diamine tetraacetic acid; PMSF, phenylmethylsulfonyl fluoride.

Introduction

The mAcChR[1] belongs to the class of neurotransmitter receptors in which signal transduction is mediated by guanine nucleotide binding regulatory proteins. Receptors that stimulate adenylyl cyclase have been shown to interact through G_s, while receptors that inhibit the enzyme, such as the mAcChR, appear to be coupled through G_i (Stryer and Bourne, 1986). In atrial tissue, G_i activated by mAcChRs has also been shown to function as a G_K (Pfaffinger et al., 1985; Breitweisser and Szabo, 1985; Yatani et al., 1987; Logothetis et al., 1987; Codina et al., 1987) to activate inward rectifying potassium channels. An analogous system can be found in vertebrate photoreceptors where light-activated rhodopsin stimulates cGMP phosphodiesterase via G_T (Fung, 1983; Stryer and Bourne, 1986).

The guanine nucleotide binding regulatory proteins are heterotrimers with differing α subunits (α_i = 41 kDa, α_s = 45 kDa, α_o = 39 kDa) and similar β (35 kDa) and γ (5-10 kDa) subunits (Northup et al., 1983; Bokoch et al., 1984; Codina et al., 1984; Stryer and Bourne, 1986). It is thought that light-activated rhodopsin or agonist-bound receptors activate the G protein by catalyzing the exchange of bound GDP for GTP (Cassel and Selinger, 1978; Brandt and Ross, 1986). The binding of GTP will then uncouple the G protein from the receptor (Rodbell, 1980) or rhodopsin (Fung and Stryer, 1980) as well as cause the dissociation of the heterotrimers into α plus $\beta\gamma$ subunits (Hildebrandt et al., 1984; Katada et al., 1984a; Katada et al., 1984b). The activation of the G protein results in an enhancement of G protein-mediated GTPase activity. The β-adrenergic

receptor-agonist complex binding to G_s is thought to induce a conformational change in G_s that increases the rate of GDP release and GTP association (Cassel and Selinger, 1978; Brandt and Ross, 1986).

The interactions of purified brain G_i and mAcChRs resolved from guanine nucleotide binding proteins (Florio and Sternweiss, 1985) as well as the interactions of purified brain mAcChR and purified G_i (Haga et al., 1985; Kurose et al., 1986; Haga et al., 1986) have been demonstrated in reconstituted systems. These studies have shown that the mAcChR agonist complex was capable of stimulating the GTPase activity of G_i and that association with G_i results in high affinity agonist binding to the mAcChR. ADP ribosylation of G_i prior to reconstitution (Haga et al., 1986) prevented the purified brain components from interacting in the reconstituted system. ADP ribosylation of G_i also uncouples the mAcChR from inhibition of adenylyl cyclase in heart tissue (Martin et al., 1985).

The purpose of this study is to characterize the interaction of purified porcine atrial mAcChR and G_i in a reconstituted system. Since the heart and brain mAcChRs are known to be different gene products (Kubo et al., 1986a; Kubo et al., 1986b; Peralta et al., 1987b) and the sequence homology of brain and heart G_i are as yet unknown, these studies should provide additional information regarding the similarity and possible differences in mAcChR-effector interactions in different tissues.

Materials and Methods

[^{35}S]GTP$_\gamma$S (1000 Ci/mmol), [γ-^{32}P]GTP (20-30 Ci/mmol), and [α-^{32}P]GTP (800 Ci/mmol) were purchased from New England Nuclear. [^{3}H]L-QNB (46 Ci/mmol) was purchased from Amersham. Cholesterol, P.C. (type III-s), P.S. (bovine brain), CHAPS, digitonin, carbachol, acetylcholine, L-hyoscyamine, GTP, GppNHp, acetylcholine esterase (type V-S), succinic thiokinase, pyruvate kinase (type VII), and activated charcoal were purchased from Sigma. GDP and GTP$_\gamma$S were purchased from Boehringer Mannheim. The purity of GTP and GTP$_\gamma$S was determined by thin layer chromatography using a solvent of 1-propanol:NH$_4$OH:H$_2$O (6:3:1). When necessary, these nucleotides were purified by applying them to DEAE Biogel A and eluting with a 0-0.5 $\underline{M}$ LiCl gradient. Pertussis toxin was from List Biological Laboratories and was stored in 50 m$\underline{M}$ sodium phosphate, 0.25 $\underline{M}$ NaCl, pH 7.0, at 100 µg/ml. mAcChR (10-15 nmol [^{3}H]L-QNB/mg) was purified from porcine atria (Peterson et al., 1984). G$_i$ (1.4-8 nmol [^{35}S]GTP$_\gamma$S/mg protein) was purified as a byproduct of the mAcChR purification procedure by sequential chromatography on DEAE Sephacel, ultragel ACA-34, and octyl sepharose, followed by sucrose gradient centrification in cholate buffers (details will be published elsewhere).

Reconstitution of mAcChR and mAcChR/G$_i$

PC:PS (1:1), plus 5% w/w cholesterol, stored in toluene:ethanol (1:1), plus 0.02% BHT, were rotovapped to dryness and resuspended in 25 m$\underline{M}$ imidazole, 0.1 M NaCl, 1 m$\underline{M}$ EDTA, 8.4 m$\underline{M}$ CHAPS, pH 7.4, to a

final concentration of 1.2 mg total lipid per ml. The suspension was sonicated to clarity at 0°C under argon. DTT (1 mM) and MgCl$_2$ (5 mM) were then added. The lipid-detergent solution (330 µl) was transferred to a 10 ml polycarbonate centrifuge tube. mAcChR alone (10 pmol in 10 to 20 µl of 25 mM imidazole, 1 mM EDTA, 0.08% digitonin, and 0.016% sodium cholate, pH 7.4) or mAcChR plus G$_i$ (50 pmol in 30-40 µl of 20 mM Tris, 1 mM EDTA, 5 mM MgCl$_2$, 75 mM sucrose, and 0.8% sodium cholate, pH 8.0) were added to the lipid detergent mixture along with acetylcholine to 50 µM. The final lipid concentration was 1 mg/ml and the final CHAPS concentration was 7 mM in a volume of 0.4 ml. This mixture was incubated on ice for five minutes and then diluted slowly with ice cold buffer C to a volume of 8 ml. Lipid was then precipitated by adding 2 ml of 50% PEG 8000 containing 5 mM MgCl$_2$ and 0.1 M NaCl to yield a final PEG concentration of 10%. This solution was incubated on ice for one hour followed by centrifugation at 4°C in a Beckman Ti 75 rotor at 250,000 x g for 75 minutes. The resulting pellet was resuspended in 0.25 ml of buffer A or buffer C. Typical recoveries after reconstitution were 30-40% for both mAcChR and G$_i$.

Preparation of [α-^{32}P]GDP

[α-^{32}P]GDP was prepared from the treatment of [α-^{32}P]GTP with succinic thiokinase. One volume of [α-^{32}P]GTP (usually 160 pmoles in 12.5 µl of 10 mM Tricine, pH 7.6) was added to one volume of solution containing 0.2 M Tris acetate, pH 8.0, 100 µM Coenzyme A, 20 mM sodium succinate, 10 mM MgCl$_2$, and 0.4 units/ml of succinic thiokinase. This

reaction was allowed to proceed for 30 minutes at room temperature after which EDTA was added to 7.5 $m\underline{M}$ and the sample was diluted into 0.5 ml of H_2O and heated at 100°C for two minutes. The sample was then applied to a 4x1 cm DEAE Bio-Gel A column which had been equilibrated with 5 $m\underline{M}$ ammonium bicarbonate, pH 7.5. A 5 to 500 $m\underline{M}$ linear gradient of ammonium bicarbonate was used to elute the column at 0.1 ml/min. Fractions which contained radioactivity and eluted at the same position as unlabelled GDP were pooled, concentrated under vacuum, applied to 15x0.75 cm column of Bio-Gel P-2 pre-equilibrated in H_2O, and eluted with H_2O at 0.1 ml/min. The fractions which contained radioactivity were lyophilized at least twice to remove any residual ammonium bicarbonate. Purity of the $[\alpha-^{32}P]GDP$ was monitored by thin layer chromatography on Merk Silica Gel 60 F254 plates using a solvent of 6:3:1 1-propanol:NH_4OH:H_2O, and exposing the TLC plate to X-ray film. At least 90% of the radioactivity migrated with GDP. This material was stored at -20°C in H_2O until used.

Ligand Binding

The mAcChR was quantitated in terms of $[^3H]$L-QNB binding sites using the DEAE filter disk assay (Peterson and Schimerlik, 1984). All binding assays for reconstituted material were performed in buffer B using 125 µl of sample. Non-specific binding was determined in the presence of 10 $\mu\underline{M}$ L-hyoscyamine or 0.1 $\underline{M}$ carbachol. Total mAcChR concentration was determined using 20 $n\underline{M}$ $[^3H]$L-QNB plus or minus 10 $\mu\underline{M}$ L-hyoscyamine or lipids without mAcChR for nonspecific binding.

$[^{35}S]GTP\gamma S$ binding was performed as described by Northup et al.

(1982). Detergent soluble G_i was incubated for 60 minutes at 32°C in buffer A, supplemented with 25 mM $MgCl_2$, 0.1% lubrol PX, and 1 μM [^{35}S]GTP$_\gamma$S (3-8 Ci/mmol). Non specific binding was determined in the absence of protein and was less than 0.5% of the total radioactivity. Reconstituted G_i was assayed in buffer A. In some instances the reaction mix was diluted into wash buffer (10 mM Hepes, 0.1 M NaCl, and 25 mM $MgCl_2$) plus 100 μM GTP to further reduce nonspecific binding.

The determination of bound [α-^{32}P]GDP was done in a similar manner except that the reaction mixture (50 μl) was diluted into ice cold wash buffer containing 0.5 mM GDP and immediately filtered and washed with 2 ml of buffer. The time from dilution to the end of the final wash was about 7 seconds.

For Scatchard plots and titration curves, duplicate aliquots of the reaction mixture were removed to determine the total radioligand present.

In experiments to determine the binding of nonlabelled GTP, a GTP regenerating system was included so that GDP, produced by the GTPase activity of G_i, would not accumulate. The regenerating system consisted of buffer B plus 5 mM KCl, 1 mM phosphoenolpyruvate, and 70 ng/ml of pyruvate kinase.

GTPase

GTPase activity was determined as described in Sunyer et al. (1984) with the following alterations. Vesicles were diluted with buffer A to a final concentration between 1 and 4 nM [^{35}S]GTP$_\gamma$S sites.

Muscarinic ligands were added as described in the text and allowed to equilibrate for five minutes at 32°C. The GTPase reaction was initiated by adding $[\alpha-^{32}P]GTP$ and incubated at 32°C for the desired amount of time. The reaction (50 μl) was quenched by the addition of 0.25 ml of an ice cold suspension of activated charcoal (5% w/v) in 20 mM sodium phosphate (pH 2.3). This mixture was placed on ice for five minutes, then centrifuged at 15,000 x g for 15 minutes at 4°C. The ^{32}P in 0.1 ml of the clear supernatant was measured by Cerenkov counting. Nonenzymatic hydrolysis was subtracted from all determinations. The time course of the GTPase activity was linear under the conditions in which the GTP concentration dependence was performed.

ADP-Ribosylations

Pertussis toxin was activated by incubating with 10 mM DTT and 50 μM ATP at 32°C for 15 minutes. Reconstituted G_i in buffer A was ADP-ribosylated by diluting into an assay mixture to give final concentrations of 5 μM thymidine, 2 mM NAD, and about 0.4 μg/ml activated pertussis toxin. This resulted in a two-fold dilution of vesicles. The reaction was allowed to proceed for 30 minutes at 32°C and was then diluted at least 20 fold into buffer A or B before subsequent experiments were executed.

Data Analysis

Titration curves were fit to equation 1, using Marquardt's algorithm as described by Duggleby (1984).

$$\bar{Y} = \frac{[L]}{K} \left(\frac{F_1}{1 + [I]/K_1 + [L]/K} + \frac{F_2}{1 + [I]/K_2 + [L]/K} \right) \qquad (1)$$

In equation (1) $\bar{Y}$ equaled the fractional saturation of the protein by radioligand and [L] and [I] are the free radioligand and inhibitor concentrations, respectively. F_1 and F_2 are the fractions of binding sites having high affinity and low affinity for inhibitor with dissociation constants K_1 and K_2, respectively, and K is the overall dissociation constant for the radioligand. Data were normalized according to equation (2) where $\bar{Y}_O$ was the fractional saturation in the absence of inhibitor.

$$\% \text{ specific bound} = \left(\frac{\bar{Y}}{\bar{Y}_O} \right) \times 100 \qquad (2)$$

Since K appeared to vary slightly depending on the preparation, its value was calculated for each experiment using the law of mass action and the concentration of bound protein, free protein, and [L] determined in the absence of inhibitor.

Steady state GTPase kinetics were analyzed using Marquardt's algorithm (Duggleby, 1984) by a two component fit to the observed steady-state velocity.

$$v = \frac{(F_1)(V_{m1})[S]}{K_{m1} + [S]} + \frac{(F_2)(V_{m2})[S]}{K_{m2} + [S]} \tag{3}$$

In equation 3, v is the observed steady-state velocity at GTP concentration S; F_1 and F_2 equal the fractions of enzyme having a maximum velocity of V_{m1} and V_{m2} and a Michaelis constant of K_{m1} and K_{m2}, respectively. Turnover numbers for V_{m1} and V_{m2} were obtained by dividing each maximal velocity by the concentration of [^{35}S]GTP$_\gamma$S binding sites.

Kinetic analysis of [α-^{32}P]GDP dissociation was done using nonweighted least squares fitting to either equation (4) or (5)

$$y(t) = A_1 e^{-t/\tau_1} \tag{4}$$

$$y(t) = A_1 e^{-t/\tau_1} + A_2 e^{-t/\tau_2} \tag{5}$$

where y(t) equaled the specifically bound ligand at time t, A_i's are the amplitudes and τ_i^{-1}'s the reciprocal relaxation times for the exponential decay processes. For equation 5, the slow phase was analyzed after the decay of the fast kinetic phase. The fast phase was then evaluated after by subtraction of the slower component.

Results

Purified porcine atrial muscarinic mAcChR reconstituted with

purified porcine atrial G_i (SDS gel shown in Fig. III-1) was able to

bind [^{3}H]L-QNB in a concentration dependent manner showing a single

class of binding sites within the concentration range examined (Fig.

III-2). The binding of L-QNB to the mAcChR was not effected by the

presence of 0.1 mM GppNHp in agreement with findings on reconstituted

porcine brain muscarinic receptor and G_i (Haga et al., 1986). Figure

III-3A shows the carbachol displacement of [^{3}H]L-QNB bound to mAcChR

reconstituted without G_i. The titration curve was analysed assuming

one class of carbachol binding sites and the resulting dissociation

constant was not significantly different from the low affinity

carbachol binding site for membrane-bound and detergent-solublized

atrial mAcChR (Schimerlik et al., 1986). However, when mAcChR was

reconstituted with G_i (Fig. III-3B) there appeared a class of high

affinity carbachol binding sites which were sensitive to 0.1 mM

GppNHp. Treatment of the reconstituted mAcChR and G_i with 0.1 mM

GppNHp caused an apparent uncoupling of the two proteins since the

mAcChR bound carbachol as if it were reconstituted without G_i. The

average K_d value (n=4) for the high affinity binding site was 1.1 ±

0.5 μM and 44 ± 11% of the [^{3}H]L-QNB binding sites were sensitive to

guanine nucleotides.

Coupling of the mAcChR to G_i was also demonstrated by the ability

of carbachol to increase the GTPase activity associated with G_i (Fig.

III-4). The apparent dissociation constant for carbachol-stimulated

GTPase activity (2.1 μM) was in good agreement with the GppNHp

sensitive high affinity carbachol binding site seen in the titration curves (Fig. III-3B). The carbachol induced increase in GTPase was blocked by 10 $\mu\underline{M}$ hyoscyamine. The GTPase activity in the presence of L-hyoscyamine was slightly lower than in the absence of ligands (76 ± 11%, n=4). The presence of acetylcholine esterase during the GTPase reaction did not alter the inhibition due to the antagonist, thus contamination by acetylcholine from the reconstitution was unlikely.

The dependence of the conversion of low affinity muscarinic agonist binding to the high affinity state on G_i concentration is shown in Fig. III-5. The amount of 0.1 $m\underline{M}$ GppNHp sensitive high affinity carbachol binding was near maximal when G_i and mAcChR were reconstituted in a 5:1 molar ratio.

In order to more completely examine the coupling of the mAcChR to G_i in terms of the muscarinic agonist-stimulated GTPase, GTP concentration dependence of the GTPase activity of G_i was determined in the presence of saturating concentrations of either carbachol or L-hyoscyamine. As seen in Figure III-6, treatment of the reconstituted system with carbachol resulted in an apparent four fold increase of V_{max} and a 2.6 fold increase in the K_m for GTP. The apparent turnover numbers in this experiment were 0.19 min^{-1} and 0.76 min^{-1} for the L-hyoscyamine and carbachol treated preparations, respectively.

In order to more fully elucidate the properties of the G_i coupled to the mAcChR, a series of ligand binding and kinetic studies were initiated. The first probe used was the nonhydrolyzable GTP analogue GTPγS. Because of the possibility of interference from bound GDP (Ferguson et al., 1986; Chapter II, this thesis) unpublished results) we did not directly examine the association rate constant of GTPγS.

The equilibrium dissociation constant for $[^{35}S]GTP_{\gamma}S$ was determined in the presence of carbachol or L-hyoscyamine (Fig. III-7). The binding studies showed that the ligand bound to homogeneous class of sites (K_d equal to about 400 $p\underline{M}$) and the dissociation constant was not strongly affected by muscarinic ligands within the range of concentrations examined.

The effects of carbachol on GTP binding was investigated by measuring the inhibition of the initial rate of $GTP_{\gamma}S$ binding. The binding of $[^{35}S]GTP_{\gamma}S$ for the first 60-90 seconds of the time course was linear. The inverse of this initial rate was plotted as a function of GTP. A straight line was drawn through both the agonist and antagonist data sets and had a common intersection point on the X axis. Therefore, both the agonist and antagonist treated samples showed an inhibition constant of about 10 $n\underline{M}$ for GTP binding to G_i (Fig. III-8). As with $GTP_{\gamma}S$, the affinity of this triphosphate was unchanged by carbachol.

The dissociation constant of GDP was, however, strongly affected by muscarinic ligands (Fig. III-9). When incubated with L-hyoscyamine, the displacement of $[^{35}S]GTP_{\gamma}S$ by GDP could be fit by assuming one class of binding sites for GDP (K_d = 7 $n\underline{M}$). Incubation with carbachol produced a shallower titration curve which was fit by assuming two classes of binding sites for GDP. Thus the presence of carbachol had produced a population of G_i with a low affinity for GDP (32%, K_d = 499 $n\underline{M}$) with no significant change in the remaining population (K_d = 12.7 $n\underline{M}$).

In order to determine to what extent the change in GDP affinity caused by carbachol was due to a change in the dissociation rate of

GDP, the release of $[\alpha-^{32}P]$GDP was measured in the presence of a muscarinic agonist and antagonist (Fig. III-10). The results indicated a single phase of GDP release from antagonist treated material with τ^{-1} of 0.1 min^{-1}. In the presence of carbachol there was a rapid phase of GDP release ($\delta A_1/\delta At$ = 27%, τ_1^{-1} = 4.5 min^{-1}) followed by a slower phase ($\delta A_2/\delta At$ = 63%, τ_2^{-1} = 0.13 min^{-1}) which was similar to the rate of GDP release in the presence of L-hyoscyamine. Although the fast phase was poorly defined, a value could be estimated that was 33 to 44 times faster than the slow phase of GDP release. The change in K_d for GDP could therefore be explained by a change in the dissociation rate for GDP.

Since 30% of the $[^{35}S]GTP_\gamma S$ sites were affected by muscarinic agonists, it was necessary to re-evaluate the carbachol stimulated GTPase data assuming that 30% of the G_i was responsive to carbachol and the remaining protein hydrolysed GTP with a rate similar to that found in the presence of L-hyoscyamine. The concentration dependence of the steady-state velocity of GTP hydrolysis in the presence of carbachol was then analyzed as the sum of two simultaneous reactions (see Fig. III-6, Equation 3) where 30% of the total enzyme appeared to interact with the mAcChR. This gave a K_m for GTP equal to (31 ± 4) nM and a turnover number of 2.1 ± 0.1 min^{-1} for the carbachol stimulated GTPase. In three experiments where the ratio of G_i/mAcChR was between 5:1 and 4:1 the average value of the carbachol stimulated turnover was 2.17 ± .07 min^{-1}. This value was independent of slight variations in the ratio of G_i and mAcChR recovered after reconstitution, and the value of 30% obtained from the two GDP binding experiments was used without corrections in other experiments where the ratio of the two

proteins were not exactly the same. Therefore carbachol promoted an actual 11-fold increase in turnover number rather than the observed 4 fold stimulation.

The effectiveness of guanine nucleotides in altering the binding of [^{3}H]L-QNB in the presence of 100 μM carbachol could be related to their affinity for G_i in the presence of agonist (Fig. III-11). The apparent K_d for GDP was similar to the thermodynamic dissociation constant obtained from competition binding with [^{35}S]GTPγS and the apparent dissociation constant for GTP was similar to the K_m value obtained from the GTP concentration dependence of the carbachol-stimulated GTPase activity. The order of potency was GTPγS > GTP > GDP where GTP was about 17 fold more potent than GDP in eliciting a change in carbachol binding.

The effect of ADP-ribosylation on muscarinic agonist-stimulated GTPase was then determined for the reconstituted preparation. A solution containing 0.13 pmol of [^{3}H]L-QNB sites and 0.61 pmol of [^{35}S]GTPγS sites in 8 μl was ADP-ribosylated as described in methods in a total reaction volume of 20 μl. The IAP-treated mixture was diluted 20-fold into buffer A and assayed for GTPase activity with 500 nM [γ^{32}P]GTP and either 2 mM carbachol or 10 μM L-hyoscyamine (assays were performed in triplicate). When NAD was omitted, 67.2 ± 0.2 nM [^{32}P] was released in one hour in the presence of carbachol while 25.6 ± 3.4 nM was released in the presence of L-hyoscyamine. When 2 mM NAD was included the amount of [^{32}P] released was 26.0 ± 3.2 and 21.6 ± 4.4 nM for carbachol and L-hyoscyamine treated samples, respectively. Thus pertussis toxin plus NAD attenuated most, if not all, of the agonist stimulated GTPase. Neither the non-stimulated GTPase nor the

amount of $[^{35}S]GTP_\gamma S$ sites were affected by ADP-ribosylation (data not shown). A similar treatment of reconstituted material had no effect on the ability of carbachol to displace $[^3H]L-QNB$ or on the ability of 0.1 m$\underline{M}$ GppNHp to alter carbachol binding (Fig. III-12A and III-12B). The ADP-ribosylation experiment was then repeated in the presence of acetylcholine and GTP. Prior to titration curve experiments acetylcholine was removed from the system by treatment with acetylcholine esterase and the majority of the GTP should have been hydrolysed to GDP by the activated G_i. Any residual GTP and GDP were diluted below a concentration (less than 2 n$\underline{M}$) in which they were effective in uncoupling the proteins. Under these conditions the binding of carbachol to the mAcChR was decreased and there was no further effect of 0.1 m$\underline{M}$ GppNHp (compare Fig. III-12C and III-12D).

Discussion

The data presented above demonstrated that porcine atrial mAcChR effectively coupled to porcine atrial G_i in the reconstituted system. The criteria for coupling were (1) the ability of muscarinic ligands to alter the interaction of guanine nucleotides with G_i, (2) the ten fold stimulation of steady-state GTP hydrolysis by the muscarinic agonist carbachol and (3) the appearance of a guanine nucleotide-sensitive high affinity carbachol binding by the mAcChR. Treatment of the coupled system with 0.1 $\underline{mM}$ GppNHp resulted in an apparent uncoupling of the two proteins. After treatment with the nucleotide the receptor interacted with carbachol in a manner similar to mAcChR reconstituted without G_i. mAcChR reconstituted alone had a homogenous low affinity binding as seen in Fig. III-3. It should be noted that there was some variability between receptor preparations and that mAcChR reconstituted alone or mAcChR and G_i treated with 0.1 $\underline{mM}$ GppNHp did not always show a homogenous low affinity carbachol binding site. This heterogeneity with respect to carbachol binding has been previously observed both in detergent soluble preparations (Peterson and Schimerlik, 1984) and for the reconstituted brain mAcChR (Haga et al., 1986), and has not yet been adequately explained. Although, a fraction of the mAcChR high affinity sites occassionally remained in the high affinity state in the presence of saturating GppNHp, guanine nucleotide sensitive high affinity agonist binding was observed for all reconstituted preparations. The average dissociation constant for carbachol binding to the GppNHp sensitive high affinity site was 1.1 ± 0.5 $\underline{\mu M}$ and 44 ± 11% of the [^{3}H]L-QNB sites were

sensitive to guanine nucleotides. This maximum value of mAcChR that can be coupled to G_i agrees with that found for the brain mAcChR reconstituted with brain G_i ($48 \pm 11\%$, Haga et al., 1986); however, in the atrial system only a five-fold excess of G_i over receptor was necessary to see the maximum effect as opposed to a 20-fold excess for the brain system. The reasons for this difference are not known, but may be due to the different structures of the brain and heart mAcChRs (Kubo et al., 1986a; Kubo et al., 1986b; Peralta et al., 1987) or to the differences in reconstitution procedures. The K_d of carbachol for the coupled receptor (1.1 $\mu\underline{M}$) agreed quite well with the K_{app} for the carbachol stimulated GTPase activity ($K_{app} = 2.1 \pm 0.3$ $\mu\underline{M}$). This was further evidence that the high affinity carbachol binding site was interacting with G_i.

The mAcChR could also be uncoupled from G_i by treatment with other guanine nucleotides. The order of potency for this effect was GTP$_\gamma$S > GTP > GDP (Fig. III-11). The ability of GDP to uncouple the mAcChR from G_i has also been noted by others (Haga et al., 1986). A K_{app} was determined for each of these nucleotides and the K_{app} for GDP (557 n$\underline{M}$) agreed with the dissociation constant of GDP from receptor activated G_i (500 n$\underline{M}$). A K_{app} for GTP was determined to be 33 n$\underline{M}$. It was somewhat difficult to rigorously interpret this value, since even though there was a GTP regenerating system included, GTP would be converted to GDP on G_i. However the value obtained was similar to the K_m of GTP for the carbachol-activated G_i (31 n$\underline{M}$). Determination of the K_{app} for GTP$_\gamma$S was also not straight forward. Because of the low K_d (about 0.4 n$\underline{M}$ from Scatchard analysis) certain assumptions had to be made in terms of free ligand. Since both carbachol and

L-hyoscyamine treated mAcChR-G_i had about the same high affinity binding for $GTP_\gamma S$, all of the G_i present was assumed to participate equally in binding $GTP_\gamma S$ for the calculation of free nucleotide. This gave a value of 2.7 ± 3.4 n$\underline{M}$ for an estimation of K_{app} which was almost an order of magnitude greater than the estimated K_d. However the value for K_{app} is not well defined and may reflect the inaccuracies arising from the use of an inappropriate G_i concentration. In any case, it was clear that $GTP_\gamma S$ was more effective than GTP in uncoupling the receptor. Also both GTP and GDP uncouple the receptor by binding to the activated agonist mAcChR-G_i complex.

Coupling between mAcChR and G_i was also sensitive to ADP-ribosylation by pertussis toxin. As seen for brain system (Haga et al., 1985), ADP-ribosylation interferes with the carbachol stimulated GTPase. However, ADP-ribosylation using similar conditions did not show any effect on carbachol binding. The mAcChR still demonstrated high affinity guanine nucleotide-sensitive carbachol binding (Fig. III-12A,B). Addition of GTP and acetylcholine during ADP-ribosylation resulted in a loss of this guanine nucleotide-sensitive carbachol binding site. Kurose et al. (1986) also report a similar uncoupling when G_i was ADP-ribosylated before reconstitution. In the reconstituted system the ADP-ribosylation must be performed in the presence of acetylcholine and GTP in order to see an effect on carbachol binding, but these conditions were not necessary for seeing an attenuation of carbachol-stimulated GTPase. A possible explanation was that the addition of GTP during the GTPase assay is sufficient to increase ADP-ribosylation efficiency either by

further activating the toxin or stabilizing G_i. Such a phenomena has been previously noted (Mattera et al., 1986).

Based on IC50 values, ATP binds to pertussis toxin about 140 times tighter than GTP (Mattera et al., 1986). Therefore, it seems unlikely that 500 n$\underline{M}$ GTP would have an effect in the presence of 25 $\mu\underline{M}$ ATP. It is also unlikely that the effect was due to additional stabilization of G_i. Without any added guanine nucleotides during ADP-ribosylation there was still some G_i coupled to the mAcChR. If G_i was denatured during the half hour preincubation with IAP, it would not explain the ability of GppNHp to convert high affinity agonist sites to lower affinity (Fig. III-12B). An alternative explanation is that the mAcChR was precoupled to G_i and this protected the associated G_i from ADP ribosylation. Free G_i was still ADP-ribosylated. The GTPase assay of the ADP-ribosylated sample showed no increase of activity when carbachol was added because all the free G_i was ADP-ribosylated and each precoupled G_i was only able to complete one GTPase cycle before it was also ADP-ribosylated.

The regulation of G_i by agonist occupied mAcChR was shown by a stimulation of GTPase activity. As seen in Figure III-6 there was an apparent four fold stimulation of the GTPase activity of carbachol treated G_i-mAcChR compared to L-hyoscyamine treated, and a 2.6 fold increase in K_m. The K_m (9.8 n$\underline{M}$) and V_{max} (0.19 min^{-1}) obtained for L-hyoscyamine treated reconstituted mAcChR and G_i were similar to the values determined with brain mAcChR and brain G_i (Kurose et al., 1986), determined in the absence of ligand. However, the apparent stimulation observed in the presence of carbachol was greater in the present study. Using the assumption that 30% of the G_i was stimulated

by the mAcChR the GTPase kinetics were re-evaluated to give a turnover of 2.1 min^{-1} for G_i coupled to receptor. This value was similar to the turnover obtained for the activated catacholamine stimulated GTPase associated with G_s after similar corrections were performed (Brandt and Ross, 1986). L-hyosycamine-treated mAcChR and G_i showed a 24% lower GTPase activity compared to the absence of ligands (n=4). Addition of acetylcholine esterase (PMSF was omitted for this experiment) did not alter these results. Thus contamination by acetylcholine seems unlikely. The reason for these observations are not yet known.

.It has been suggested that activation of G_s by its associated receptors results in an "open" G protein in which there is an accelerated turnover of guanine nucleotides (Stryer and Bourne, 1986) and a recent study suggested that the agonist stimulation of G_s involves both an increase in the rate of GTP binding and dissociation of GDP (Brandt and Ross, 1986). In other hormone-G protein systems, agonists were seen to regulate the binding of $GTP_\gamma S$ (Brandt and Ross, 1986) and an agonist-induced effect on the apparent association rate of $GTP_\gamma S$ has been noted for the reconstituted brain mAcChR and brain G_i (Kurose et al., 1986). In the present study differences in $GTP_\gamma S$ binding between carbachol and L-hyosycamine treatments were observed (data not shown); however, the effects of bound GDP can not be ruled out. At 100 n$\underline{M}$ $GTP_\gamma S$ in the presence of L-hyosycamine, the rate of $GTP_\gamma S$ binding was similar to the rate of GDP release and was preceeded by a burst phase. In the presence of carbachol the predominant difference was a change in the amplitude of the burst phase which was assumed to represent the fraction of G_i which had no GDP bound or

bound GDP with low affinity. When the equilibrium binding of GTPγS was examined there was no significant difference between incubation with carbachol or L-hyoscyamine (Fig. III-7). Although there was no change in GTPγS binding, there was a 3.2 fold increase in the K_m of GTP when the GTPase reaction was performed in the presence of carbachol. It is not yet clear if this is due to a difference in the affinity of G_i for GTP, or a change in some other intermediate step of the mechanism.

The GTPase activity of G_i precluded or at least complicated measuring the K_d of GTP by displacement of GTPγS in an equilibrium titration curve as was done for GDP. An alternative was to measure the initial rate of [^{35}S]GTPγS binding in the presence of simultaneously added GTP. It was hoped this would minimize the effects of GTP hydrolysis. The faster GTPγS binding observed in the presence of carbachol was due to the apparent burst phase GTPγS binding caused by the agonist induced increase in GDP dissociation. The initial linear portion of the binding curve was used. A replot of the inverse of initial rate as a function of GTP concentration gave a straight line for such data set (Fig. III-8). The extrapolated dissociation constant for GTP (~10 n$\underline{M}$) was unchanged by the addition of muscarinic agonist and was similar to K_m values (9.8 n$\underline{M}$ for L-hyoscyamine treated, 31.0 n$\underline{M}$ for carbachol treated) estimated for GTPase and was similar to the K_{app} of GTP for uncoupling mAcChR from G_i (33 n$\underline{M}$). This is further evidence that the affinity of GTP for the mAcChR-G_i complex was unchanged by muscarinic agonists.

The data presented in Figure III-9 demonstrated that agonist stimulation of mAcChR coupled to G_i resulted in a decrease in affinity

of GDP for G_i. The change in affinity for GDP (50-fold) could be explained by the change in GDP dissociation rate (38-fold) if the binding of GDP was a simple bimolecular reaction. Such an assumption would predict an association rate between 1.5×10^5 and 2.0×10^5 M^{-1} sec^{-1}. This value is within the range of values determined for the association rate of GDP binding to G_o (Higashijima et al., 1987c).

The average value for the slow phase of GDP release was about 0.12 min^{-1} and the turnover of the GTPase reaction in the presence of L-hyoscyamine was 0.19 min^{-1}. The rate limiting step for the non-stimulated GTPase was therefore the release of GDP. Any stimulation of V_{max} must alter this step, and stimulation caused by agonist bound mAcChR increases V_{max} to about 2.1 min^{-1}. Therefore stimulation by the mAcChR causes an increase in GDP release; however, GDP release may be only partially rate-limiting in the steady-state mechanism. (The GDP dissociation rate in the presence of carbachol was 4.5 min^{-1}.)

In summary, the atrial mAcChR is able to functionally interact with atrial G_i. The evidence indicates that the proteins are precoupled after reconstitution. Treatment with agonist caused an activation of the GTPase associated with G_i by, at least in part, increasing the dissociation rate of GDP. Guanine nucleotides were able to uncouple the mAcChR from G_i. After uncoupling, the low affinity form of the receptor for agonists may then combine with another (GDP bound) G_i. Both rhodopsin (Fung and Styrer, 1980) and the adrenergic receptor (Brandt and Ross, 1986) seem to act catalytically with respect to G protein activation. The findings in this study indicated that if the G_i/mAcChR ratio is about 5:1 and 30%

of the G_i is activated then about 1.5 G_i are activated per mAcChR.

Since only 40% of the mAcChR can couple, then 3-4 G_i are activated per

receptor.

Figure III-1   SDS polyacrylamide gel of purified porcine atrial

mAcChR and purified porcine atrial G_i used for

reconstitution studies.   Samples and molecular weight

markers (phosphorylase a, M_r 97,114; bovine serum

albumen, M_r 66,296; aldolase, M_r 39,210;

chymotrypsinogen A, M_r 25,666; soybean trypsin

inhibitor, M_r 20,095; and lysozyme, M_r 14,314) were

electrophoresed on an 8-18% linear acrylamide gradient

gel using the discontinuous buffer system of Laemmli

(1970) and visualized by silver staining (Wray et al.,

1981).
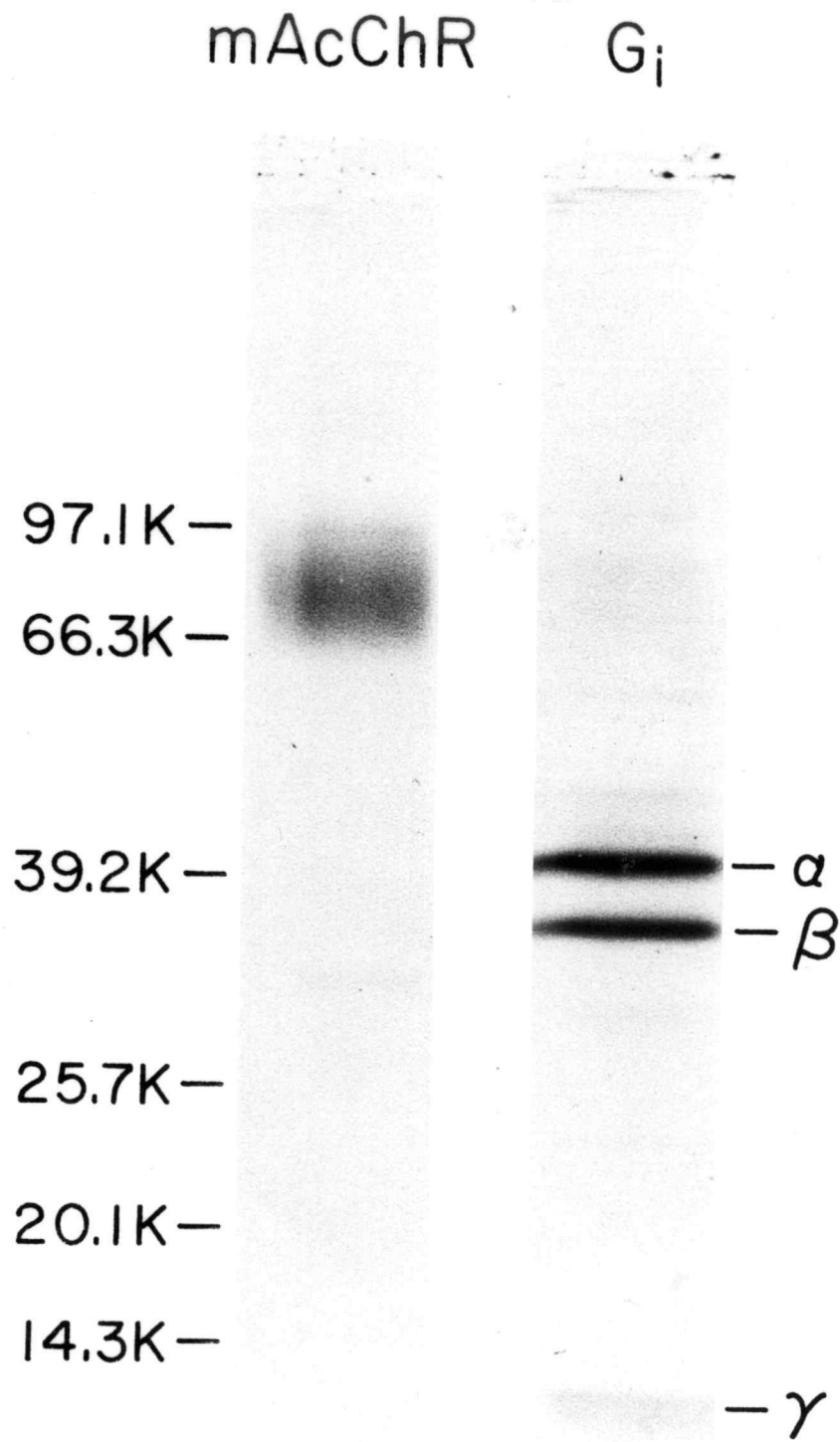
Figure III-1

Figure III-2   Binding of [^{3}H]L-QNB to reconstituted G$_i$ and mAcChR.

The reconstituted preparation containing approximately

150 p$\underline{M}$ [^{3}H]L-QNB sites and 975 p$\underline{M}$ [^{35}S]GTP$_\gamma$S sites was

equilibrated in buffer B with 0.05 to 5.00 n$\underline{M}$ [^{3}H]L-QNB

for two hours at 32°C in the presence (○) or absence

(●) of 100 μ$\underline{M}$ GppNHp. The data points represent the

mean ± SD of triplicate determinations. After

correction for nonspecifically bound label in the

presence of 10 μ$\underline{M}$ L-hyoscyamine, data were analysed in

the form of a Scatchard plot (insert). Least squares

analysis gave a dissociation constant of (297 ± 26) p$\underline{M}$

and a total site concentration of (154 ± 16) p$\underline{M}$ for the

control and a dissociation constant of 292 ± 12 p$\underline{M}$ and

a site concentration of (146 ± 8) p$\underline{M}$ for GppNHp treated

vesicles.
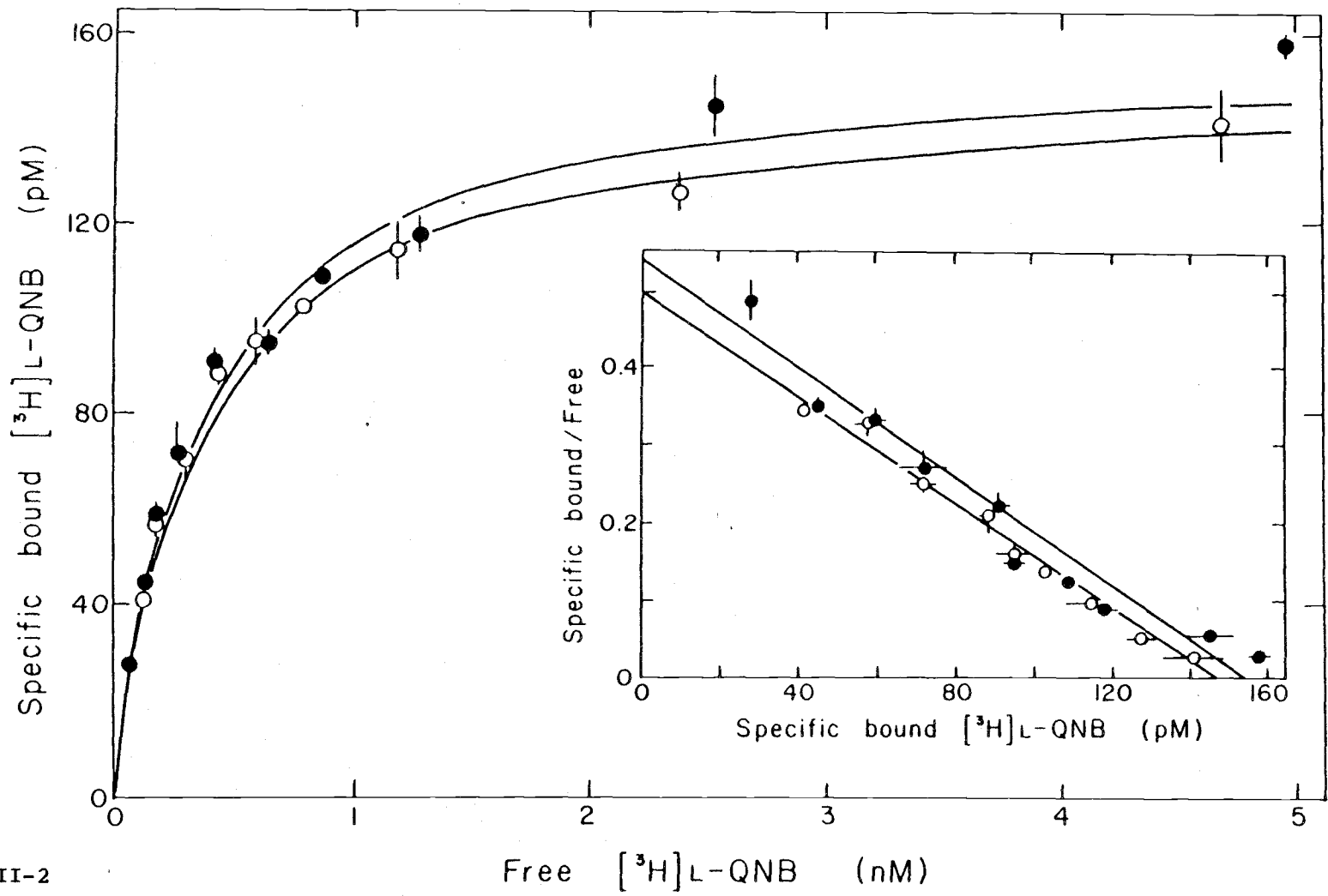
Figure III-2

Figure III-3   Carbachol titration of specifically bound [³H]L-QNB.

A.   Reconstituted mAcChR alone was diluted in buffer B

to a concentration of 141 p$\underline{M}$ in [³H]L-QNB sites.

Samples were added to tubes containing the indicated

final concentration of carbachol and after 30 minutes

at 32°C, 550 p$\underline{M}$ [³H]L-QNB was added. After 2 hours

specific binding was determined as described in

methods. Data points represent the average ± SD of

triplicate determinations. The data were fit to

equation (1) holding F_1 at 1.0 to give a K_d for

carbachol of (122 ± 11) $\mu\underline{M}$ using a K_d of 305 p$\underline{M}$ for

L-QNB.   B.   Reconstituted mAcChR and G_i were diluted

into buffer B in the presence (●) or absence (○) of

0.1 m$\underline{M}$ GppNHp and titrated with carbachol as described

above. The titration in the absence of GppNHp

contained 73 p$\underline{M}$ [³H]L-QNB sites and 350 p$\underline{M}$ [³⁵S]GTP$_\gamma$S

sites. The data were fit to equation (1) using a K_d

for L-QNB of 460 p$\underline{M}$. Analysis gave F_1 equal to 0.53 ±

.04, K_1 equal to (1.0 ± 0.3) $\mu\underline{M}$, F_2 equal to 0.47 ±

0.04 and K_2 equal to (66.5 ± 11.7) $\mu\underline{M}$. In the presence

of GppNHp the curve was fit to equation (1) holding F1

at 1.0 using 93 p$\underline{M}$ [³H]L-QNB total binding sites and a

K_d for L-QNB equal to 431 p$\underline{M}$. The K_d for carbachol was

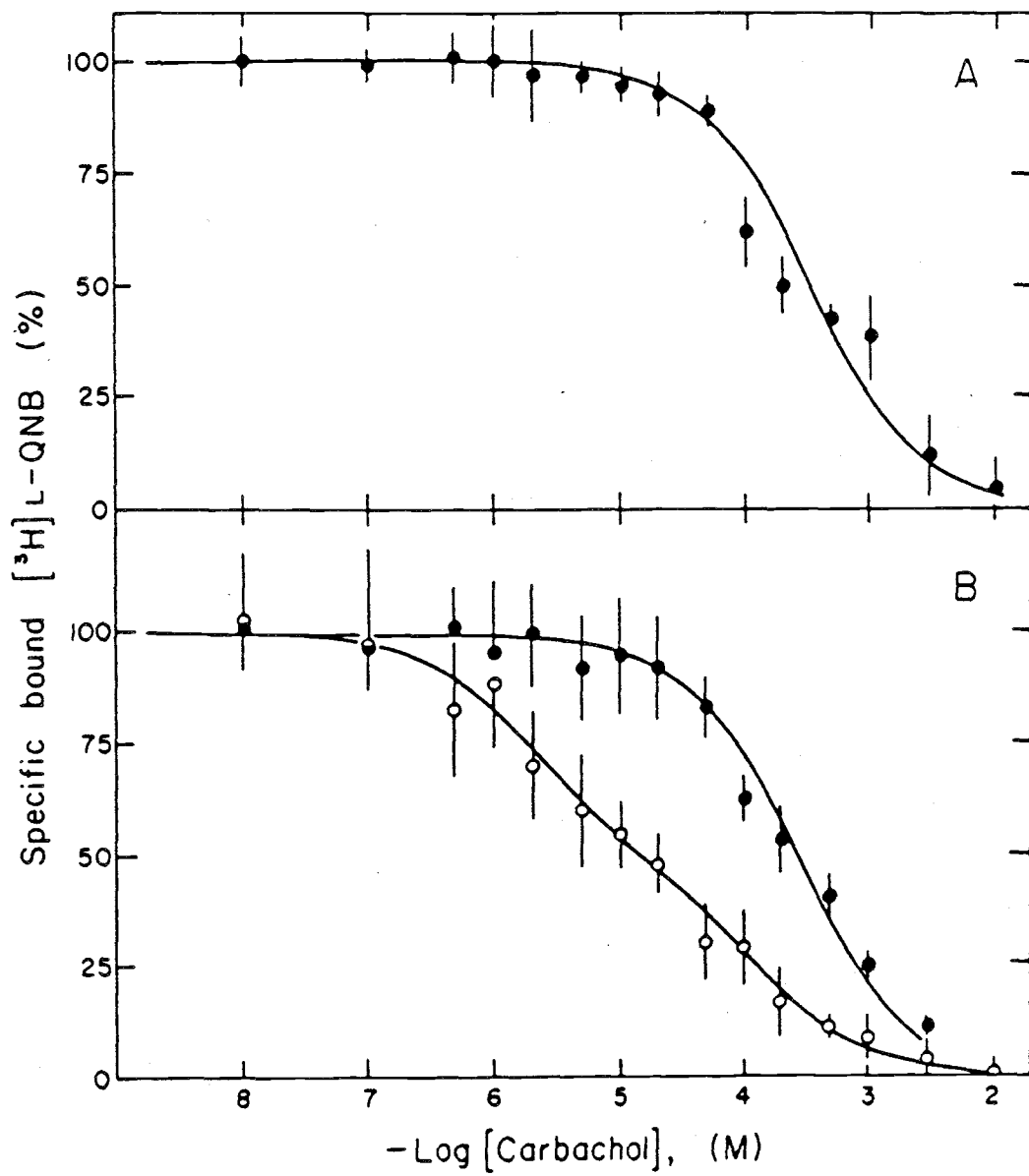calculated to equal (121.6 ± 10.3) $\mu\underline{M}$.

Figure III-3

Figure III-4   Carbachol dependence of mAcChR stimulated GTPase.

Samples were pre-incubated in buffer A for 5 minutes at

32°C with the indicated final carbachol concentrations.

The GTPase reaction was initiated by adding $[\gamma-^{32}P]GTP$

to a concentration of 500 n$\underline{M}$ and incubating for an

additional hour. Samples (50 µl containing 4.8 n$\underline{M}$

$[^{35}S]GTP_\gamma S$ sites and 0.82 n$\underline{M}$ $[^3H]$L-QNB sites) were

assayed for ^{32}P released as described in methods. The

data points represent the average ± SD of triplicate

determinations and were fit to the law of mass action

to give an apparent dissociation constant of (2.1 ±
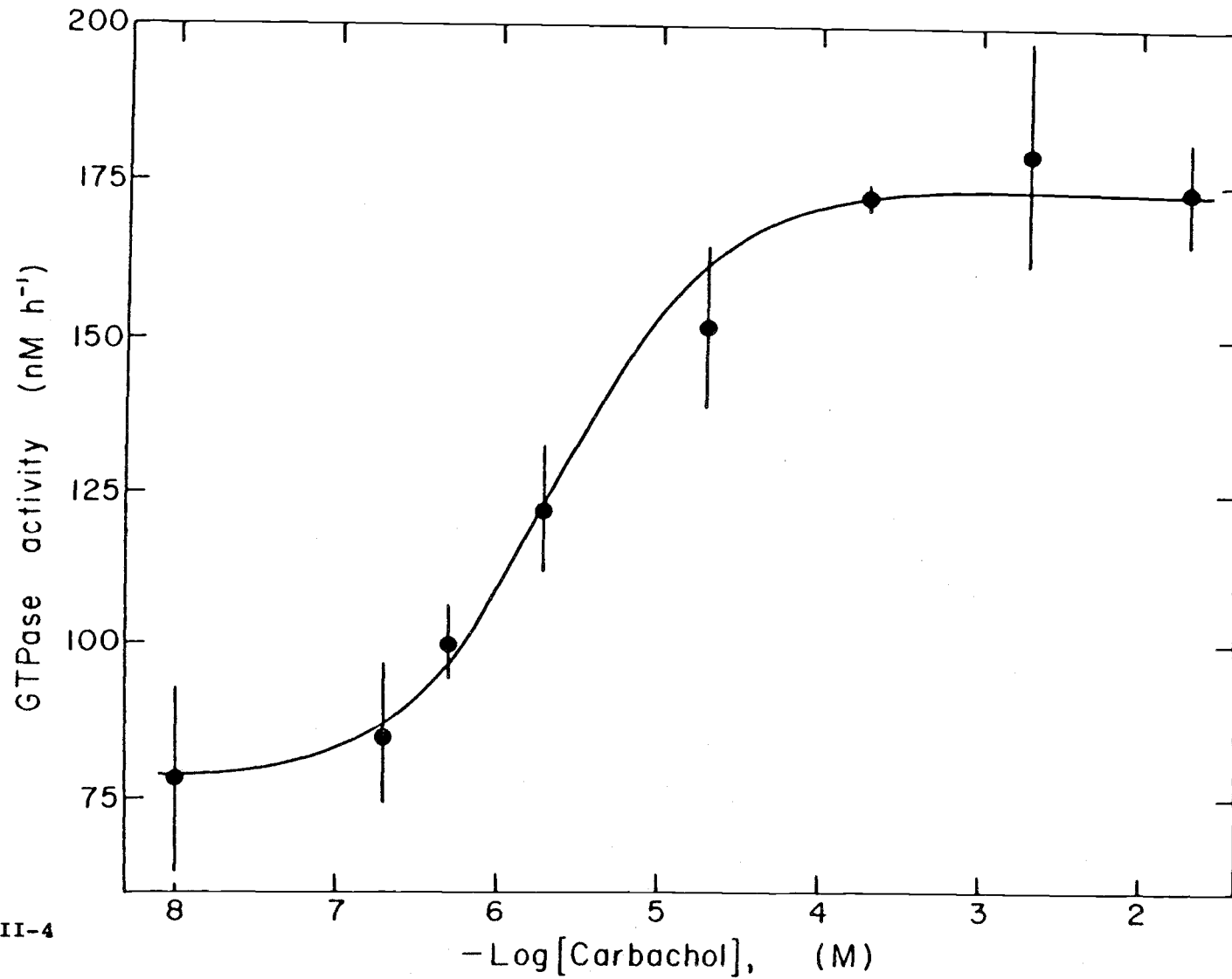
0.3) µ$\underline{M}$ for carbachol.

Figure III-4

Figure III-5   Dependence of high agonist affinity nucleotide

sensitive binding sites on G_i concentration present

during reconstitution. mAcChR (10 pmol) was

reconstituted with the indicated amounts of G_i as

described in methods. The recovered mAcChR was then

adjusted to 500 pM [^{3}H]L-QNB binding sites in buffer B

and incubated for 30 min with 10 μM carbachol either

with or without 0.1 mM GppNHp. [^{3}H]L-QNB was then

added to 500 pM, allowed to incubate for 2 hours, and

filtered as described in methods. Assays were

performed in triplicate. The fraction of conversion

was defined as $\left(1-(\text{sp. bd. cpm in the absence of}\right.$

GppNHp/sp. bd. cpm with GppNHp present)$\left.\right)$ and plotted

as percent of maximum. The line drawn through the data

points has no theoretical significance.
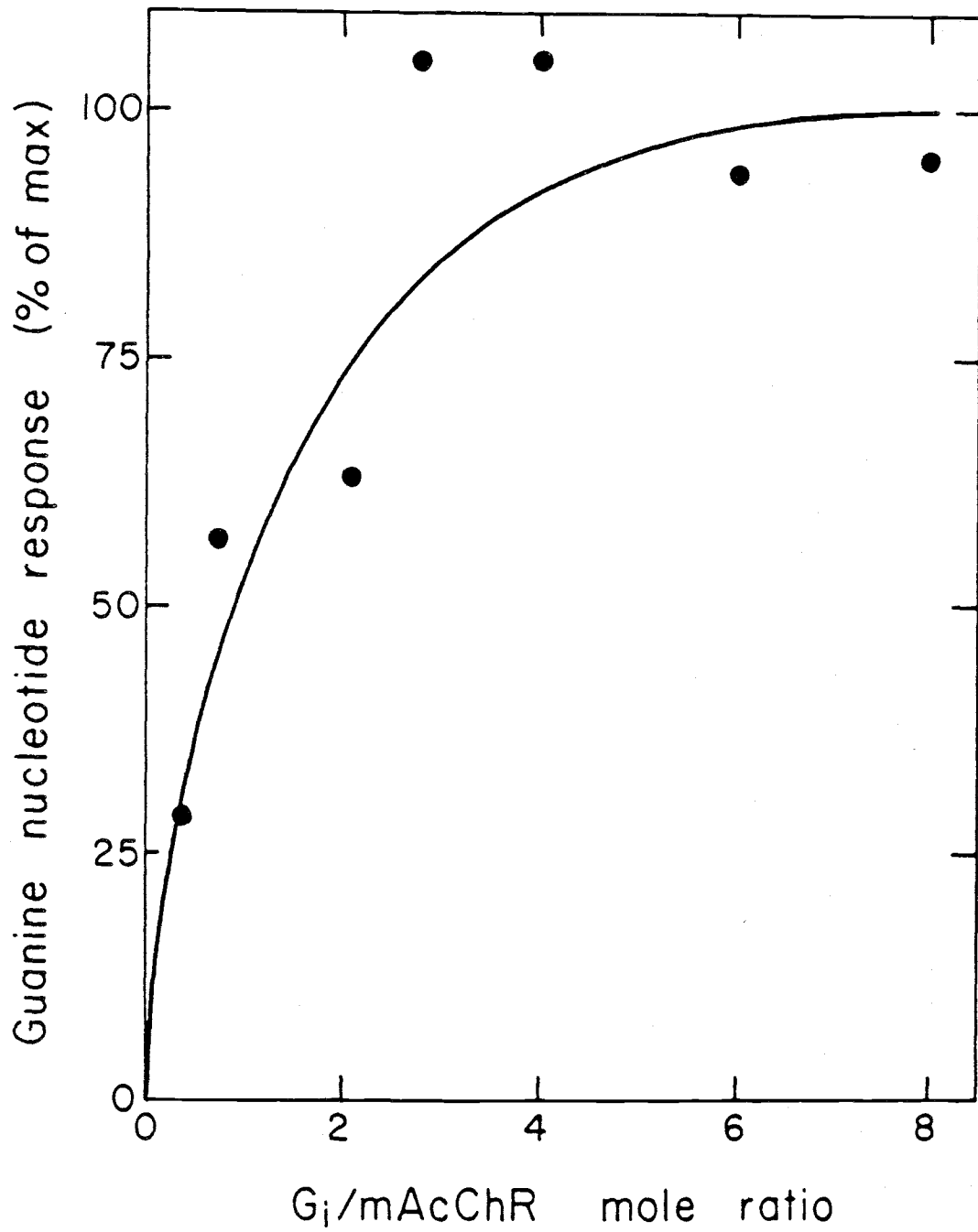
Figure III-5

Figure III-6   Dependence of GTPase Activity of G_i Reconstituted with

mAcChR on GTP concentration. Reconstituted material

(0.8 n$\underline{M}$ [^{35}S]GTP$_\gamma$S binding sites and 0.2 n$\underline{M}$ [^{3}H]L-QNB

binding sites) in buffer A was incubated with either 2

m$\underline{M}$ carbachol ($\bullet$); or 10 μ$\underline{M}$ L-hyoscyamine (O) for five

minutes at 32°C and the GTPase reaction was started by

adding [γ-^{32}P]GTP and allowed to continue for 15

minutes. Values represent the mean ± standard

deviation of triplicate determinations. Data were

analysed by a direct fit to the Michaelis-Menton

equation to give a K_m of (9.79 ± 1.42) n$\underline{M}$ and a V_{max}

of(154 ± 8) p$\underline{M}$ min^{-1} for L-hyoscyamine treated vesicles

and a K_m of (25.5 ± 1.3) n$\underline{M}$ and a V_{max} of (612 ± 11) p$\underline{M}$

min^{-1} for carbachol treated vesicles. Alternatively,

data were analysed according to equation (3) as

described in results assuming 30% interactive G_i. The

theoretical curve from this analysis was virtually
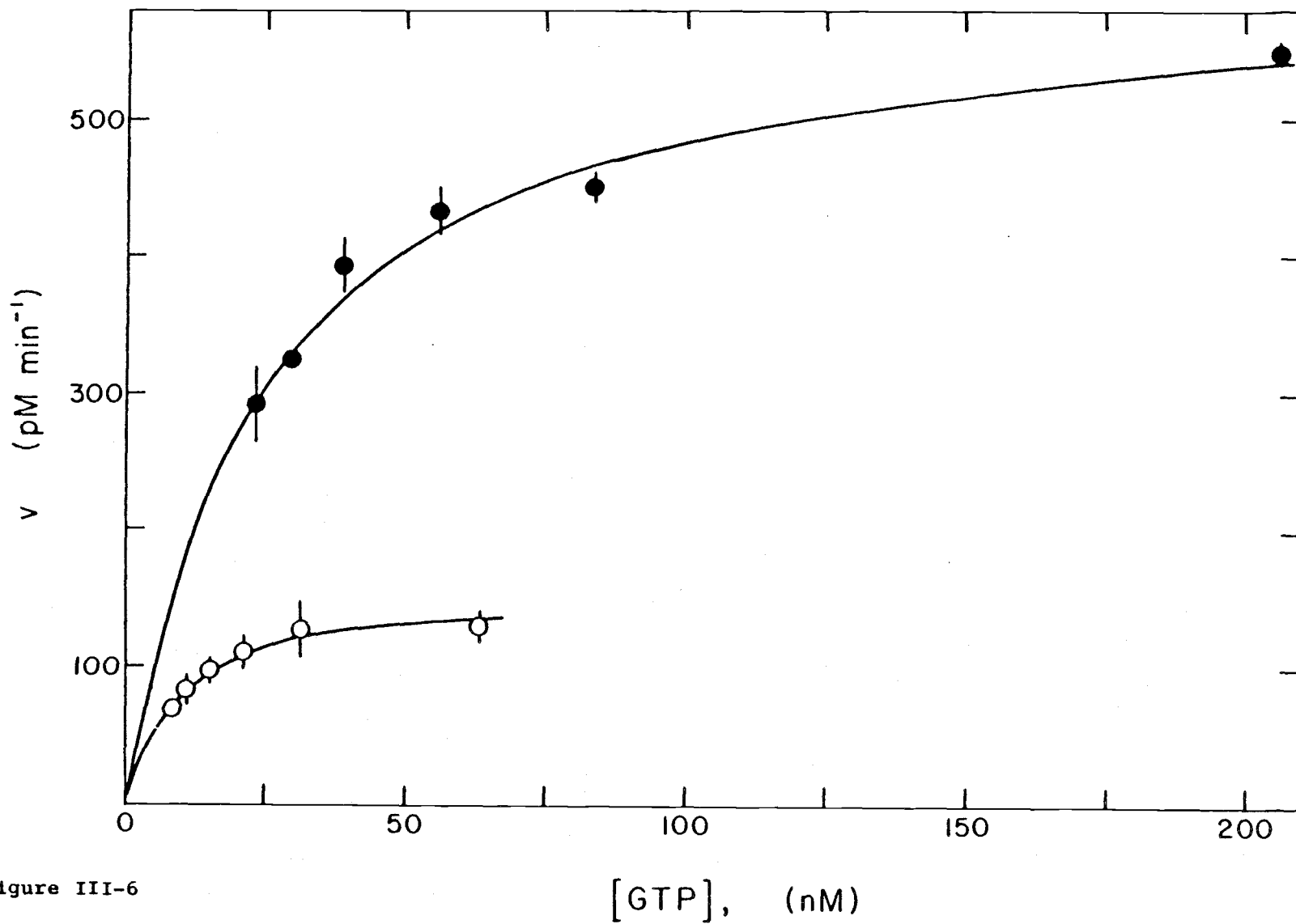
superimposable over the curve drawn in the Figure.

Figure III-6

Figure III-7   Binding of [^{35}S]GTP$_\gamma$S to reconstituted G$_i$ plus mAcChR.

The reconstituted preparation containing approximately

122 p$\underline{M}$ [^{35}S]GTP$_\gamma$S sites and 35 p$\underline{M}$ [^{3}H]L-QNB binding

sites was equilibrated in buffer A with [^{35}S]GTP$_\gamma$S for

2 hours at 32°C in the presence of either 2 m$\underline{M}$

carbachol (●) or 10 μ$\underline{M}$ L-hyoscyamine (O). Duplicate

100 μl aliquots were filtered as described under

Methods. Data were analysed in the form of a Scatchard

plot (insert). Least squares analysis gave a

dissociation constant of (332 ± 32) p$\underline{M}$, and a total

site concentration of (128 ± 15) p$\underline{M}$ for carbachol

treated vesicles. L-hyoscyamine treated vesicles

showed a dissociation constant of (454 ± 70) p$\underline{M}$ and a
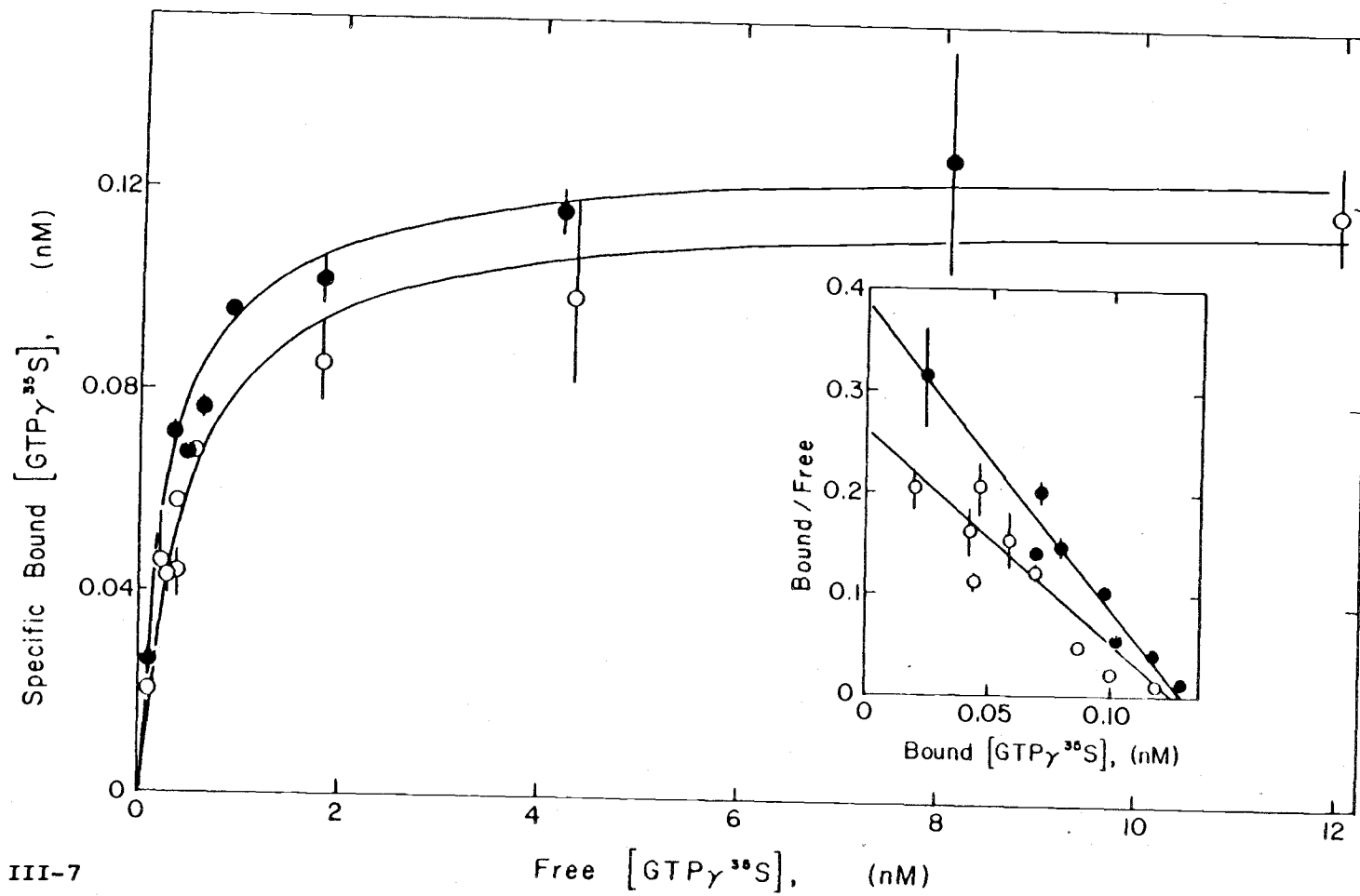
total site concentration of (117 ± 21) p$\underline{M}$.

Figure III-7

Figure III-8   The inhibition of the initial rate of [^{35}S]GTP$_\gamma$S

binding to G_i by GTP. A liposome preparation

containing 0.40 n$\underline{M}$ [^{35}S]GTP$_\gamma$S binding sites and 70.0 p$\underline{M}$

[^{3}H]L-QNB sites was incubated with either 2 m$\underline{M}$

carbachol (●) or 10 µM L-hyoscyamine (O) for 5

minutes at 32°C. The reaction was initiated by adding

[^{35}S]GTP$_\gamma$S together with GTP to give 10 n$\underline{M}$ [^{35}S]GTP$_\gamma$S

and the indicated concentrations of GTP. Samples were

removed and filtered at 15-30 second intervals for 5

minutes. Using data from the first 60-90 seconds of

the reaction, the inverse of the initial rate of

[^{35}S]GTP$_\gamma$S binding was plotted as a function of GTP

concentration. The data was then fit by a linear least

squares method.

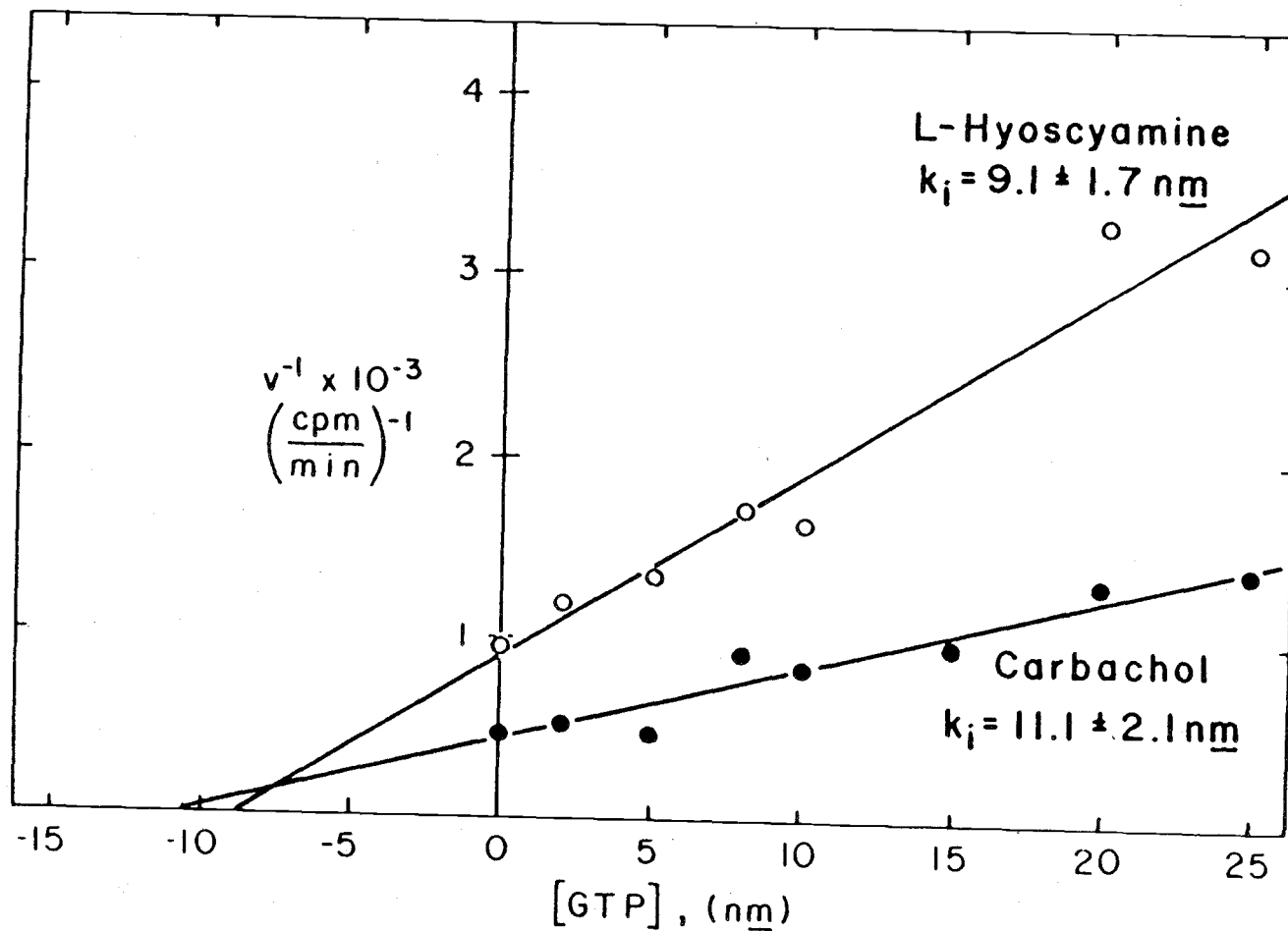# Inhibition of Initial Rate of GTPγS Binding by GTP

L-Hyoscyamine
$k_i = 9.1 \pm 1.7$ n$\underline{m}$

Carbachol
$k_i = 11.1 \pm 2.1$ n$\underline{m}$

$v^{-1} \times 10^{-3}$
$\left(\dfrac{cpm}{min}\right)^{-1}$

[GTP], (n$\underline{m}$)

Figure III-8

Figure III-9   GDP titration of specifically bound [^{35}S]GTP$_\gamma$S. The

reconstituted preparation containing approximately 170

p$\underline{M}$ [^{3}H]L-QNB binding sites and 940 p$\underline{M}$ [^{35}S]GTP$_\gamma$S sites

in buffer A was supplemented with either 2 m$\underline{M}$ carbachol

($\bullet$) or 10 $\mu\underline{M}$ L-hyoscyamine (O). Samples were then

added to tubes containing the indicated final

concentrations of GDP and incubated for 15 minutes at

32°C. [^{35}S]GTP$_\gamma$S was then added to a final

concentration of 1.6 n$\underline{M}$ and the incubation continued

for an additional two hours at which time samples were

filtered as described in Methods. Data for carbachol

treated vesicles were analysed using equation (1) and a

K_d of 280 p$\underline{M}$ for [^{35}S]GTP$_\gamma$S and 188 p$\underline{M}$ for the total

number of GTP$_\gamma$S sites. A fit to equation (1) gave F_1 =

0.68 ± 0.04, K_1 = (12.7 ± 2.1) n$\underline{M}$, F_2 = 0.32 ± 0.04,

and K_2 = (499 ± 86) n$\underline{M}$. The analysis for L-hyoscyamine

treated vesicles assumed one class of binding site with

a K_d of 277 p$\underline{M}$ for [^{35}S]GTP$_\gamma$S and 164 p$\underline{M}$ in GTP$_\gamma$S

binding sites. The dissociation constant for GDP was

calculated to equal (7.0 ± 0.3) n$\underline{M}$ from the fit to
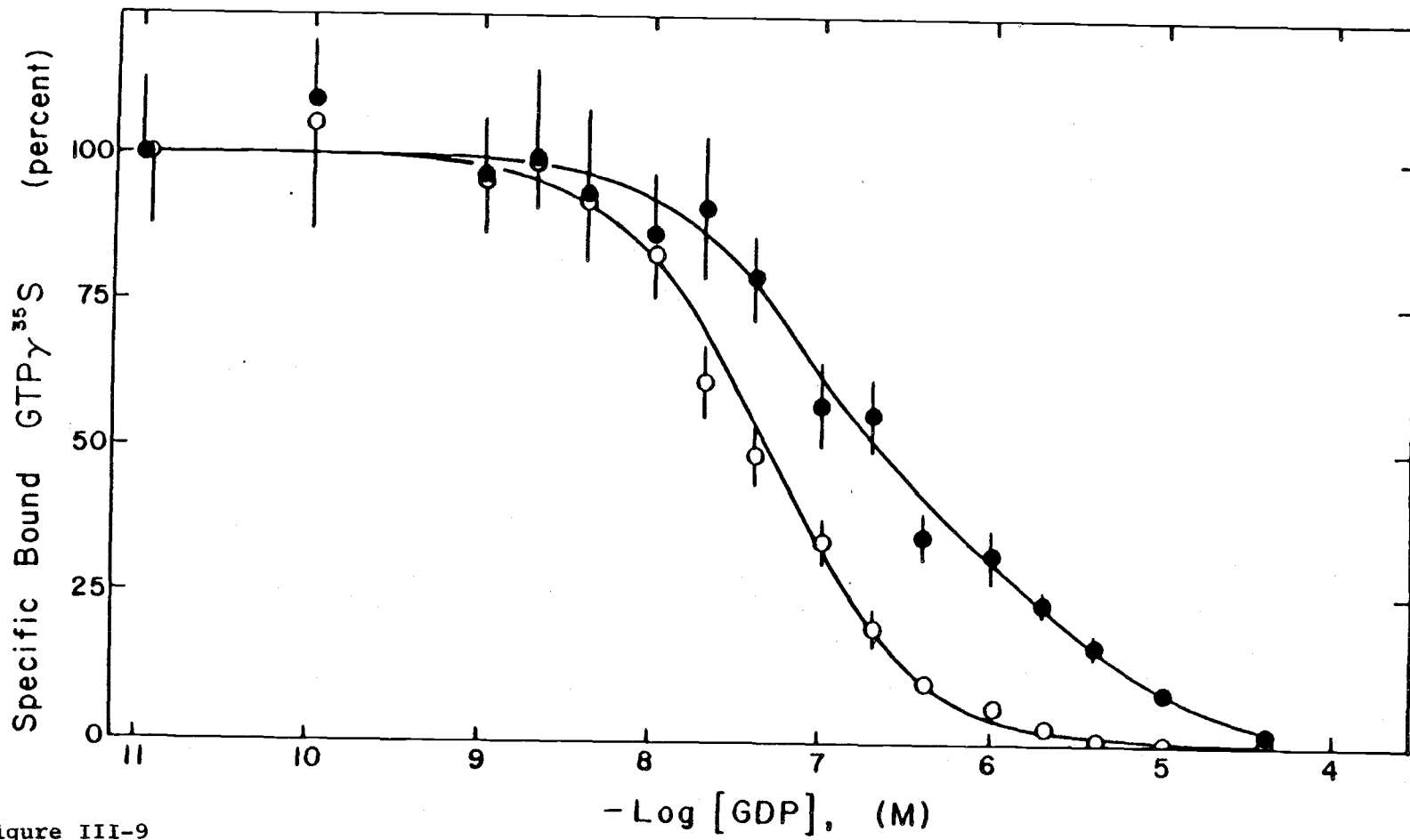
equation (1) fixing F_1 at 1.0.

Figure III-9

Figure III-10   Time course for $[\alpha-^{32}P]GDP$ dissociation. Reconstituted

mAcChR (360 pM in $[^{3}H]L$-QNB binding sites) and G_i (2 nM

in $[^{35}S]GTP_\gamma S$ sites) were incubated in buffer A at 32°C

in the presence of 100 nM $[\alpha-^{32}P]GDP$ for 30 minutes.

The dissociation reaction was initiated by adding

unlabelled GDP to a final concentration of 100 µM plus

either 2 mM carbachol ($\bullet$) or 10 µM L-hyoscyamine (O).

Aliquots were removed at the time indicated and

filtered as described in methods. L-hyoscyamine data

were analysed by a least squares fit to a single

exponential (equation 4) which gave an ordinate

intercept of 94 ± 3% bound and a rate constant of

(0.100 ± 0.004) min^{-1}. The time course for

dissociation in the presence of carbachol contained two

kinetic phases and was analysed according to equation

5. The slow phase ($\delta A_2/\delta A_t$ = 63 ± 4%; τ_2^{-1} = (0.136 ±

0.001) min^{-1}) was analysed by a least squares fit using

data obtained after one minute. The fast phase was

then evaluated after subtraction of the slow phase.

($\delta A_1/\delta A_t$ = 27 ±8%; τ_1^{-1} = 4.5 ± 0.8 min^{-1}).
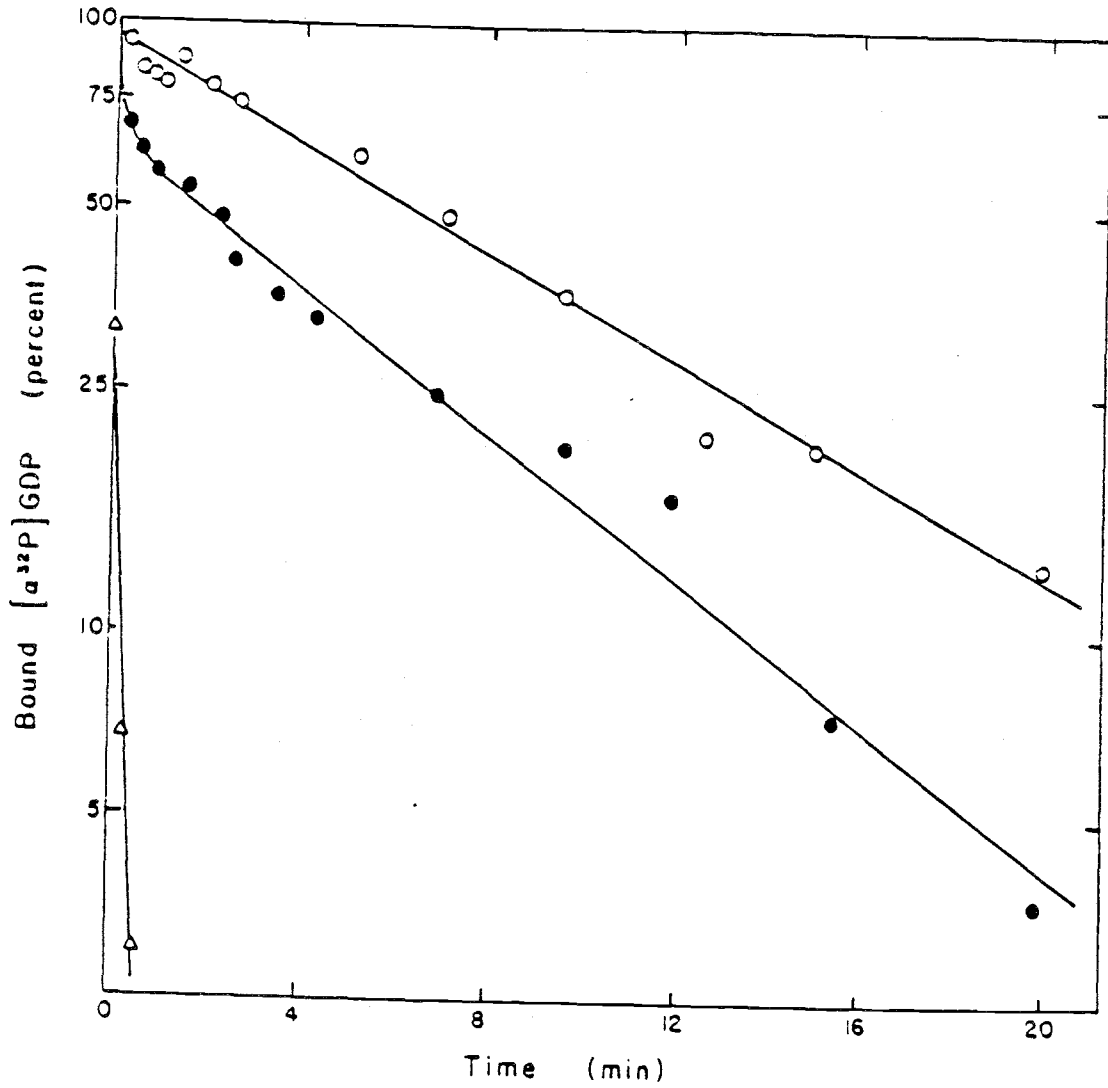
Figure III-10

Figure III-11   Guanine nucleotide titration of carbachol affinity

change. Respective guanine nucleotides (●, GTP$_\gamma$S; △,

GTP; 0 GDP) were added to the reconstituted preparation

in buffer B containing 0.25 n$\underline{M}$ [^{3}H]L-QNB binding sites

and 3 n$\underline{M}$ [^{35}S]GTP$_\gamma$S sites plus 0.1 m$\underline{M}$ carbachol to give

the final concentrations of nucleotides shown. Samples

which contained GTP also contained the GTP regenerating

system described in methods. After one half hour

incubation at 32°C [^{3}H]L-QNB was added to 585 p$\underline{M}$.

After incubation for 2 hours, samples were filtered as

described in methods. Data points are the average ± SD

of triplicate determinations and represent the

percentage of [^{3}H]L-QNB binding in the presence of 0.1

m$\underline{M}$ carbachol and 0.1 m$\underline{M}$ GppNHp compared to [^{3}H]L-QNB

binding in absence of GppNHp. Data were fit to the law

of mass action to give K_{app} of (2.7 ± 3.4) n$\underline{M}$ for

GTP$_\gamma$S, (33.2 ± 5.3) n$\underline{M}$ for GTP, and (557.4 ± 207.3) n$\underline{M}$
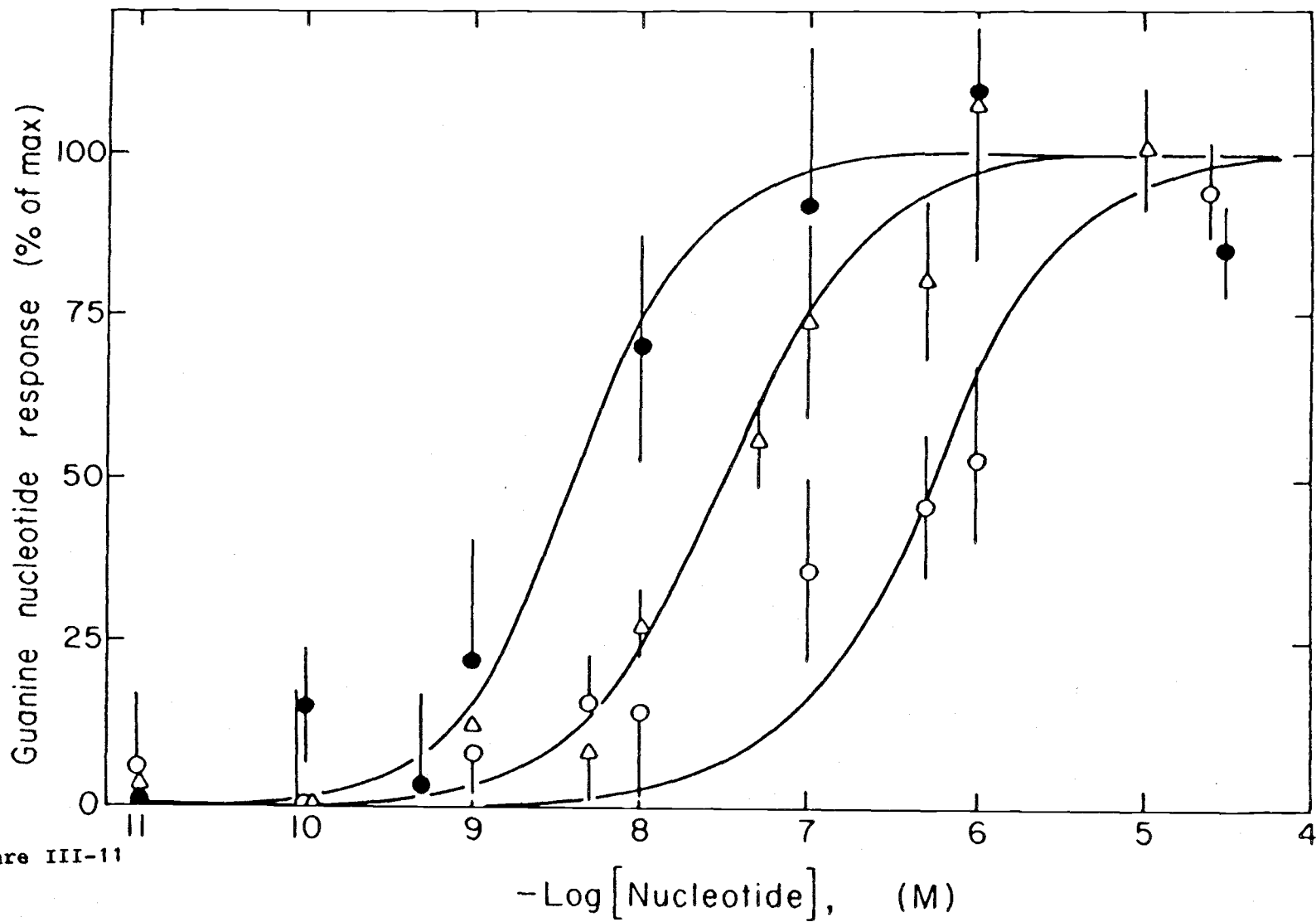
for GDP.

Figure III-11

Figure III-12   Effect of ADP-ribosylation on binding of carbachol to

mAcChR. mAcChR and G_i were ADP-ribosylated as

described in Methods with (B,D) or without (A,C) NAD.

C and D were supplemented with 500 n$\underline{M}$ GTP and 5 $\mu\underline{M}$

acetylcholine and after a 30 minute incubation were

allowed to incubate 5 additional minutes at 32°C with

0.76 U ml^{-1} of acetylcholine esterase. Reaction

mixtures were then diluted into buffer B to give an

[^{3}H]L-QNB site concentration of 72 p$\underline{M}$ and a GTP$_\gamma$S site

concentration of 334 p$\underline{M}$ for A and B and 78 p$\underline{M}$ [^{3}H]L-QNB

sites and 400 p$\underline{M}$ GTP$_\gamma$S sites for C and D. Carbachol

titration of specifically bound [^{3}H]L-QNB was performed

in the presence (●) or absence (○) of 100 $\mu\underline{M}$ GppNHp.

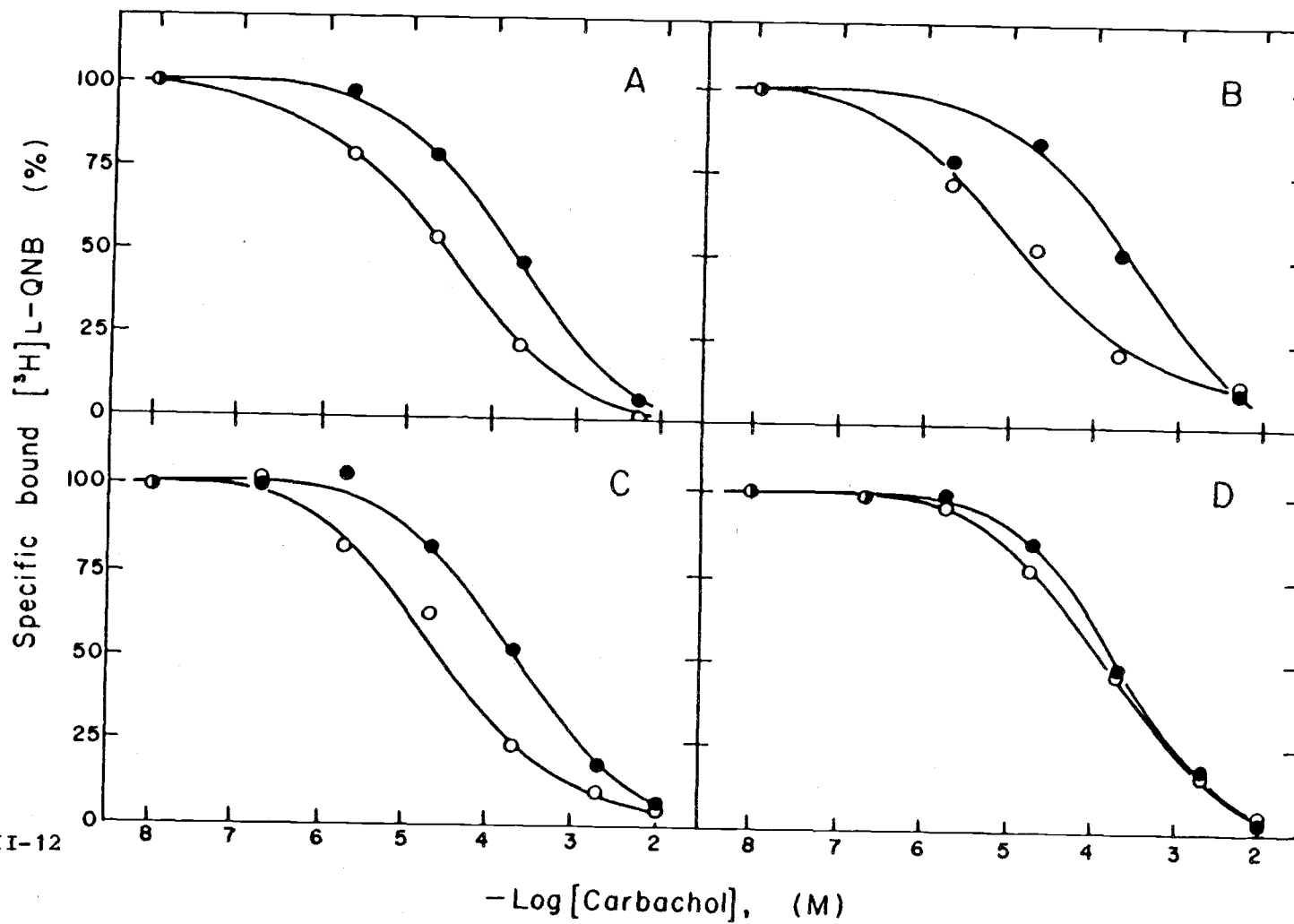Lines through the data were drawn by eye and have no

theoretical basis.

Figure III-12

Acknowledgments

Chapter IV

Reconstitution of Acetylcholine Mediated

Inhibition of Adenylyl Cyclase

Contributions of Co-authors

Zhengui Xia, working under the direction of Professor Daniel R. Storm, purified and assayed the adenylyl cyclase used in this study. The remainder of the experiments and procedures were performed by myself under the direction of Professor Schimerlik. Professor Storm also offered some suggestions on how to manipulate the adenylyl cyclase and suggested detergents which would interfere the least with adenylyl cyclase-G_s interactions.

Abstract

Inhibition of bovine brain calmodulin-sensitive adenylyl cyclase,
initiated by the muscarinic agonist carbachol, was examined in a
system consisting of the reconstituted purified porcine atrial
muscarinic acetylcholine receptor and inhibitory guanine nucleotide
binding protein (G_i) in addition to the stimulatory guanine nucleotide
binding protein (G_s) adenylyl cyclase complex. Experimental
conditions were chosen such that, in the absence of carbachol, G_i was
complexed mainly to GDP while adenylyl cyclase was selectively
preactivated with guanosine 5'-O-(3-thiotriphosphate) (GTPγS).
Adenylyl cyclase was also activated by forskolin and calcium plus
calmodulin. Addition of carbachol to form the receptor-carbachol
complex increased the dissociation rate of GDP from G_i (Tota, M.R.,
Kahler, K.R., and Schimerlik, M.I. (1987) Biochemistry 26, 8175-8182)
such that GTPγS could bind and initiate G_i mediated inhibition of the
enzyme. Adenylyl cyclase activated by calcium plus calmodulin was
more sensitive to inhibition by carbachol than either nonstimulated
enzyme or enzyme activated by GTPγS or forskolin.

Studies using the resolved subunits of G_i showed that while the $\beta\gamma$
subunits could inhibit all forms of the adenylyl cyclase, only the
calmodulin stimulated enzyme was inhibited by the α subunit. Possible
explanations are given for these results.

Abbreviations

mAcChR, muscarinic acetylcholine receptor; PC, soybean L-α-phosphatidylcholine; PS, bovine brain L-α-phosphatidyl-L-serine; CHAPS, (3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate; G_s, the stimulatory guanine nucleotide binding protein; G_i, the inhibitory guanine nucleotide binding protein; G_o, a similar guanine nucleotide binding protein of unknown function; GppNHp, 5'guanylylimidiophosphate; $GTP\gamma S$, guanosine 5'-O-(3-Thio-triphosphate); DTT, dithiothreitol; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonate; EGTA, ethyleneglycol-bis-(β-aminoethyl ether)N,N,N',N'-tetraacetic acid; EDTA, ethylenediaminetetraacetic acid; PMSF, phenylmethylsulfonyl fluoride.

Introduction

Activation of mAcChR's[1] in several tissues including heart (Murad et al., 1962) and brain (Olianas et al., 1983) results in a decrease in the rate of cAMP accumulation. In atrial tissue, as in several other cell types (reviewed in Nathanson, 1987), the acetylcholine induced attenuation of cAMP levels is mediated by pertussis toxin sensitive guanine nucleotide binding proteins G_i and/or G_o. This mechanism is not absolute, however, since it has been shown in at least one system (1321N1 astrocytoma cells; Hughes et al., 1984; Hughes and Harden, 1986) that mAcChR's regulate cAMP levels by activating a calmodulin-sensitive phosphodiesterase in a Pertussis toxin insensitive manner.

Recent work (Ashkenazi et al., 1987; Peralta et al., 1988a) has indicated that the PM2 muscarinic subtype, found in the atria, interacts preferentially with guanine nucleotide binding proteins that regulate adenylyl cyclase. Of the four mAcChR human subtypes whose coupling to physiological effector systems have been characterized, the HM2 and HM3 mAcChR appear to couple more tightly to adenylyl cyclase inhibition than to stimulation of inositol phospholipid metabolism (Peralta et al., 1988). Furthermore, Northern analysis has indicated that both of these subtypes are found in the brain, suggesting that they play a role in the regulation of adenylyl cyclase activity in that tissue.

Regulation of adenylyl cyclase by G proteins requires that the G proteins are first activated by their respective receptors. The binding of hormone to the receptor will stimulate the turnover of a

tightly bound GDP on the α subunit of the G protein and allow a GTP to bind. When GTP is bound to the G protein the heterotrimer apparently dissociates into α and βγ subunits. The individual subunits are then thought to be involved in the regulation of adenylyl cyclase activity. The GTP bound α subunit of G_s acts to stimulate adenylyl cyclase activity. Stimulation is terminated when GTP hydrolyzes to GDP and the G_s subunits reassociate (Gilman, 1987).

Recently, several laboratories have examined the effects of isolated G protein subunits on adenylyl cyclase activity using reconstituted (Smigel, 1986; Cerione et al., 1986; Cerione et al., 1987; Hekeman et al., 1987) or detergent solublized preparations (Katada et al., 1986a; Katada et al., 1987). The results from these studies were somewhat conflicting with regard to the function of the G_i subunits. One proposed mechanism is that inhibition of adenylyl cyclase by the βγ subunits of pertussis toxin-sensitive G proteins is predominantly due to mass action equilibria favoring reassociation of the G_s heterotrimer (Katada et al., 1984b; Cerione et al., 1986). However, alternative mechanisms have also been proposed (Katada et al., 1986a, 1987) in which (1) $G_{i\beta\gamma}$ could inhibit adenylyl cyclase by either binding to the protein directly or, in the case of calmodulin sensitive adenylyl cyclase, competing for the calcium calmodulin complex or (2) $G_{i\alpha}$·GTPγS could compete directly with $G_{s\alpha}$ for a binding site on the enzyme. $G_{i\alpha}$·GTPγS also directly inhibited adenylyl cyclase in Cyc⁻ S49 cells, but the effect was small (Katada et al., 1984a).

Adenylyl cyclase, G_s, and stimulatory hormone receptors have been successfully coreconstituted into liposomes (Cerione et al., 1984; May

et al., 1985; Rosenberg et al., 1987). These studies define the minimum requirement for hormone induced activation of adenylyl cyclase. It has been more difficult to prepare a reconstituted system in which the adenylyl cyclase was under the control of inhibitory receptor proteins. Light activated rhodopsin had little effect when reconstituted with adenylyl cyclase, G_s, and either G_i or transducin because, under the experimental conditions chosen, both regulatory G proteins were activated (Cerione et al., 1985).

Reconstitution of the atrial mAcChR and G_i has been reported (Tota et al., 1987). The purpose of this study was to demonstrate that the purified atrial mAcChR and atrial G_i are able to interact with calmodulin sensitive adenylyl cyclase·G_s complex from bovine brain in a reconstituted system. Conditions were chosen such that adenylyl cyclase could be activated through G_s and/or calmodulin while remaining sensitive to inhibition by mAcChR activated G_i. It was also possible to test the effects of the resolved subunits of G_i on the activity of the unstimulated adenylyl cyclase or adenylyl cyclase activated by G_s or calmodulin.

Methods

Porcine atrial mAcChR was purified as described by Peterson et al.
(1984). Atrial G_i was purified as a by product of mAcChR purification
(Tota et al., 1987). Bovine brain calmodulin-sensitive adenylyl
cyclase was prepared as described by Yeager et al. (1985a) and had a
specific activity of 10 nmol/min/mg protein when assayed in the
presence of 10 mM $MnCl_2$. The preparation was not pretreated with
guanine nucleotides and thus co-purified with G_s and maintained
sensitivity to GTP analogs. mAcChR and G_i were reconstituted as
described (Tota et al., 1987). Briefly, both proteins were added to a
mixture of 7 mM CHAPS and 1 mg/ml lipid (1:1:0.1 PC:PS:cholesterol),
diluted 25 fold, precipitated by adding 10% PEG, and collected by
centrifugation. All experiments used between 30 and 50 pmol of G_i and
between 20 to 30 pmol of muscarinic receptor. The concentration of
recovered proteins is indicated in the figure legends.

Adenylyl Cyclase Assays. One volume of adenylyl cyclase·G_s
complex (64 µg/ml in 0.1% Tween 20) was added to 9 volumes of either
control lipids or lipids with reconstituted G_i and mAcChR. GDP was
added to 167 µM and the mixture was incubated on ice for 10 minutes.
Aliquots were then diluted 3.3 fold to a final volume of 50 µl and a
final concentration of 10 mM Na HEPES pH 7.4, 100 mM NaCl, 5 mM $MgCl_2$,
240 nM GTPγS, 50 µM GDP, 50 µM cAMP, 5 mM theophylline, 0.2 mg/ml BSA,
1 mM DTT, 0.1 mM PMSF, and 500 µM ATP with about 3×10^6 cpm of $[\alpha^{32}P]$
ATP (120 cpm/pmol) per assay. In experiments which examined
calmodulin stimulated adenylyl cyclase, 50 nM calmodulin and 100 µM
$CaCl_2$ were added. In experiments where calcium·calmodulin was omitted

100 μM EGTA was added. The examination of G_s stimulated adenylyl cyclase was performed by preincubating the Tween soluble adenylyl cyclase·G_s complex with 8 μM GTP$_\gamma$S for 30 minutes at 32°C before addition to lipids and GDP. No additional GTP$_\gamma$S was added in these experiments and the final GTP$_\gamma$S concentration was 240 nM, as in the controls. All assays contained a residual Tween 20 concentration of 0.003% v/v and 2.5% v/v ethanol (stock solutions of theophylline were prepared in ethanol).

The subunits of G_i were separated as described by Katada et al. (1986b). G_i was incubated with 10 μM GTP$_\gamma$S for 2 hours at 32°C (50 μg G_i in 250 μl of 10 mM Na·HEPES pH 7.4 mM MgCl$_2$, 5 mM EGTA, 1 mM DTT, 0.1 mM PMSF, and 0.1% Lubrol PX). The activated protein was diluted into 5 ml of 20 mM Tris·HCl pH 8.0, 10 mM MgCl$_2$, 1 mM EDTA, 0.1 M NaCl, 0.25% cholate, 0.1 mM PMSF, and 1 mM DTT and then applied to 10 ml of heptyl agarose. The column was washed with 20 ml of equilibration buffer and then eluted with a 60 ml linear gradient starting with 0.25% cholate, 0.25 M NaCl and ending with 1.2% cholate, 0.025 M NaCl. All buffers contained Tris, MgCl$_2$, DTT, and PMSF as in the equilibration buffer. All chromatography was performed at 4°C. $G_{i\alpha}$ was eluted in the wash and $G_{\beta\gamma}$ was eluted at about 0.8% cholate. Heptyl agarose was prepared by reacting Affigel 10 (Biorad) with an excess of heptylamine.

Liposomes were prepared with 30 pmol of α subunit or 1.0 μg of $\beta\gamma$ subunit. The recovery of the α subunit was similar to that of the G_i heterotrimer (about 30%) and the final concentration in the adenylyl cyclase assay was 7.8 nM. The recovery of the $\beta\gamma$ subunit was monitored by gel electrophoresis (12% acrylamide gel; Laemmli, (1970))

using silver staining (Wray et al., 1981). The recovery was about 40% which gave a final protein concentration of 0.63 μg/ml (about 13 nM) in the adenylyl cyclase assay.

The adenylyl cyclase reactions were started by adding ATP, [α^{32}P] ATP, cAMP, theophylline, BSA, and HEPES buffer as a 5X stock solution and the reaction was allowed to proceed for one hour at 32°C. [α^{32}P] cAMP was then isolated as described by Salomon (1979) using [^{3}H] cAMP to quantitate recoveries.

Binding assays. [^{3}H]L-QNB and [^{35}S] GTP$_\gamma$S assays were performed for the reconstituted proteins as described by Tota et al. (1987). In order to examine L-QNB and GTP$_\gamma$S binding under the conditions of the adenylyl cyclase assays, slight modifications were made. When [^{3}H]L-QNB binding was performed in a 50 μl volume with high ionic strength, aliquots were removed and diluted 2.5 fold with 10 mM sodium phosphate, 1 mM EDTA pH 7.4 buffer immediately before applying to DE81 paper. [^{35}S]GTP$_\gamma$S binding was assayed by substituting 240 nM [^{35}S]GTP$_\gamma$S for unlabeled nucleotide and filtering 15 μl aliquots.

Materials. Bovine brain calmodulin was a generous gift from Sonia R. Anderson. The source of all other materials used have been described previously (Tota et al., 1987).

Results

Adenylyl cyclase activity was stimulated by either forskolin, G_s, calcium-calmodulin, or a combination of G_s and calcium-calmodulin. Assays were performed on adenylyl cyclase added to either control lipids, or lipids containing reconstituted mAcChR and G_i with either the muscarinic antagonist L-hyoscyamine or the agonist carbachol. Stimulation by G_s was achieved by preactivating the Tween soluble adenylyl cyclase·G_s complex with GTPγS before adding to lipids and GDP. The results (Fig. IV-1) indicate that adenylyl cyclase was activated by forskolin, G_s, or calcium·calmodulin. The largest single stimulation was by forskolin (4.2-fold), followed by calcium·calmodulin (4.1-fold). Stimulation by G_s was more modest (1.9-fold). Activating G_s by 193 μM instead of 8 μM GTPγS gave no additional stimulation. Increasing calmodulin from 50 to 250 nM gave only a 3% further increase in activity while a 5 fold increase in calcium reduced activity by 40% (data not shown). A combination of calcium·calmodulin and G_s stimulation gave more than an additive response.

The purpose of performing these assays in the presence of high concentrations of GDP was to inhibit binding of GTPγS to G_s and G_i. This concentration of GDP, 50 μM, completely blocked GTPγS binding to G_s. Based on the known affinities of GDP and GTPγS (Chapter III), a small fraction ($\sim$16%) of G_i was expected to bind GTPγS at the concentrations of GDP and GTPγS used. Activation of G_i by agonist bound mAcChR should decrease the affinity of G_i for GDP but not affect the GTPγS binding affinity (Chapter III). Thus when an agonist bound

receptor is present, there would be an increase in GTPγS binding to G_i (Fig. IV-2). GTPγS-activated G_i would then function to inhibit adenylyl cyclase activity, while G_s would not be activated.

As seen in Figure 1, the addition of adenylyl cyclase to liposomes containing mAcChR, G_i, and L-hyoscyamine resulted in a decrease in adenylyl cyclase activity when compared to adenylyl cyclase added to control lipids. (Inhibition ranged from 14 to 36%, depending on the mode of stimulation, Fig. IV-1.) Replacing L-hyoscyamine with carbachol resulted in a further decrease in adenylyl cyclase activity resulting from mAcChR mediated activation of G_i. Stimulation of adenylyl cyclase by calcium·calmodulin was most sensitive to inhibition by G_i and mAcChR. G_s and forskolin-stimulated adenylyl cyclase were less sensitive to G_i while nonstimulated adenylyl cyclase also showed some mAcChR mediated inhibition. The inhibition of adenylyl cyclase activity in the presence of carbachol as compared to L-hyoscyamine was 30±1% for calmodulin stimulated, 16±3% for calmodulin and GTPγS stimulated, 9±3% for GTPγS stimulated, 5±4% for forskolin stimulated, and 17±4% for nonstimulated adenylyl cyclase.

Liposomes containing calmodulin activated adenylyl cyclase, G_i, and mAcChR were titrated with carbachol and the inhibition of adenylyl cyclase activity was measured (Fig. IV-3). The apparent dissociation constant for carbachol measured in this manner (K_{app}=9.5 ± 0.6 µM) agreed with the binding constant of the high affinity site for carbachol for the receptor as measured by the displacement of [^{3}H]L-QNB (Fig. IV-4, K_1=19 ± 10 µM).

In order to investigate the mechanism of receptor induced inhibition of adenylyl cyclase by G_i in more detail, adenylyl cyclase

was added to liposomes containing GTP$_\gamma$S plus either $G_{i\alpha}$ or $G_{i\beta\gamma}$. The

results in Fig. IV-5 suggest that while the $\beta\gamma$ subunit could inhibit

G_s stimulated, calmodulin stimulated, or basal adenylyl cyclase

activity, $G_{i\alpha}$ could only inhibit calmodulin stimulated adenylyl

cyclase. The forskolin stimulated activity was also inhibited by $\beta\gamma$

subunits and not GTP$_\gamma$S$\cdot$G$_{i\alpha}$ (data not shown).

Discussion

The adenylyl cyclase used for this study was stimulated by G_s, calcium·calmodulin, and forskolin. That calmodulin and forskolin were more effective than G_s, is in agreement with previous studies (Yaeger et al., 1985a).

In order to study the inhibition of the adenylyl cyclase·G_s complex by G_i it was necessary to choose conditions which would permit G_s and G_i to be activated independently of each other and still allow G_i to be sensitive to changes in nucleotide binding caused by the mAcChR. The experimental conditions were chosen (50 µM GDP and 240 nM GTPγS) such that GTPγS would not bind to G_s unless it was preincubated with it, and the activation of G_i by the mAcChR would give a maximal effect on adenylyl cyclase activity. Under these conditions a small amount of GTPγS could still bind to G_i, and thus some inhibition of adenylyl cyclase activity was found in the absence of muscarinic agonists. Therefore, when adenylyl cyclase was added to liposomes containing G_i, mAcChR, and the muscarinic antagonist L-hyoscyamine, there was an inhibition of adenylyl cyclase activity when compared to adenylyl cyclase added to control liposomes (Fig. IV-1). GTPγS-activated G_i was expected to inhibit adenylyl cyclase activity (Katada et al., 1986a; Katada et al., 1987). When the assay was performed in the presence of the muscarinic agonist carbachol instead of an antagonist, there was a further decrease in adenylyl cyclase activity resulting from a larger fraction of G_i activated by GTPγS (Figs. IV-1 and IV-2). The increase in GTPγS-activated G_i presumably resulted from a decrease in affinity of G_i for GDP caused by agonist

bound receptor (Chapter III), permitting more GTP$_\gamma$S to bind.

It has been previously demonstrated that soluble adenylyl cyclase could reconstitute into preformed liposomes (Yeager et al., 1985b). Since an interaction between adenylyl cyclase and reconstituted mAcChR and G_i was observed, it is reasonable to assume that in this case the adenylyl cyclase was reconstituting into liposomes containing mAcChR and G_i. This preparation of adenylyl cyclase has been functionally reconstituted with the β-adrenergic receptor and the β-adrenergic agonist isoproterenol was able to stimulate the calmodulin-sensitive adenylyl cyclase two fold (Rosenberg et al., 1987).

The calcium·calmodulin stimulated adenylyl cyclase was clearly more sensitive to inhibition by G_i than either the forskolin or GTP$_\gamma$S activated enzyme. As was observed by others (Katada et al., 1987), the effects of G_i on forskolin and G_s stimulated activity were much less potent. It seems that this form of adenylyl cyclase is more sensitive to regulation by calcium than by stimulatory hormones. However, since G_s and calcium·calmodulin have a more than additive effect on adenylyl cyclase activity (2.7 fold for GTP$_\gamma$S activated, 3.3 fold for calmodulin, and 10 fold for both stimulation together), the mechanism of regulation appears to be complex.

The mAcChR mediated inhibition of adenylyl cyclase observed in this reconstituted system (about 30%) was comparable to inhibition caused by muscarinic agonists in native membrane preparations. Developing chick atrial membranes have been reported to show a carbachol-induced inhibition of 26-30% (Halvorsen and Nathanson, 1984). Acetylcholine inhibited basal adenylyl cyclase activity by 30-40% in rat striation synaptic plasma membranes (Olianas et al.,

1983). GTP-isoproterenol activated adenylyl cyclase was inhibited 17% by methacholine in canine myocardial membranes, or 26% in canine myocardial homogenates (Watanabe et al., 1976). The degree of hormone mediated inhibition was about the same when the calmodulin sensitive adenylyl cyclase was known to be involved. In the presence of calmodulin and GTP, the adenosine receptor agonist (1)-N^6-phenylisopropyladenosine was able to inhibit adenylyl cyclase activity by 26% in rat cerebral cortex membranes (Perez-Reyes and Cooper, 1987).

Titration of mAcChR with carbachol indicated that the mAcChR population having high affinity for agonists was involved in inhibition of adenylyl cyclase (Figs. IV-3 and IV-4). This was expected since this is the receptor form that interacts with the G_i.

The actual affinity of ligands for the receptor was somewhat lower than expected for the reconstituted system (Chapter III). The reason for this effect was believed to be the residual Tween 20 in the adenylyl cyclase assay. When adenylyl cyclase (and Tween 20) were added to the liposomes, the receptor L-QNB complex was unstable. Most of the receptor L-QNB complex was not recovered after 90 minutes at 32°C, whereas without Tween 20 nearly 100% was recovered. Although these conditions were not optimal for the receptor, the mAcChR was stable enough to stimulate G_i. As seen in Fig. IV-2, the presence of adenylyl cyclase (and Tween 20) has little effect on the ability of the receptor to promote GTPγS binding to G_i.

Since the effect of GTPγS binding to G_i is to promote subunit dissociation (reviewed in Gilman, 1987), the effects of resolved subunits on adenylyl cyclase activity were examined and the results

are shown in Figure IV-5. The $\beta\gamma$ subunit was able to inhibit calmodulin, G_s, and forskolin stimulated adenylyl cyclase in addition to inhibiting nonstimulated adenylyl cyclase. These data argue that the $\beta\gamma$ subunits must inhibit the enzyme by means other than reducing the concentration of activated $G_{s\alpha}$. Katada et al. (1986a, 1987) have reported that the $\beta\gamma$ subunits inhibited adenylyl cyclase by direct binding and also inhibited the calmodulin sensitive form by competing with the enzyme for calmodulin. In the present study it was not possible to distinguish between these two possible mechanisms, but since $\beta\gamma$ seemed equally effective at inhibiting basal and calmodulin stimulated adenylyl cyclase, it would favor a mechanism where $\beta\gamma$ acted primarily by directly inhibiting the enzyme.

The α subunit was also able to inhibit calmodulin stimulated adenylyl cyclase but not G_s stimulated adenylyl cyclase. $G_{i\alpha}$ has not been previously tested directly on the calmodulin sensitive adenylyl cyclase, but in studies using other adenylyl cyclase preparations $G_{i\alpha}$ was able to inhibit only the G_s stimulated adenylyl cyclase (Katada et al., 1986a). There are several possibilities to explain these discrepancies. That $G_{i\alpha}$ did not compete for $G_{s\alpha}$ in these experiments could be explained by there not being a high enough concentration of $G_{i\alpha}$ to displace $G_{s\alpha}$, or that the dissociation rate of $G_{s\alpha}$ was too slow in this reconstituted system. Alternatively, $G_{i\alpha}$ may not directly compete with $G_{s\alpha}$ for binding to the calmodulin sensitive adenylyl cyclase.

$G_{i\alpha}$ was not expected to inhibit calmodulin activated adenylyl cyclase. Perhaps when the adenylyl cyclase was activated by calmodulin it was able to release tightly bound $G_s \cdot GDP$, allowing

$G_{i\alpha}$•GTP$_\gamma$S to bind. $G_{i\alpha}$•GTP$_\gamma$S bound to the calmodulin sensitive adenylyl cyclase then resulted in a direct inhibition of adenylyl cyclase activity.

Another mechanism that can not be ruled out from these data is that $G_{i\alpha}$•GTP$_\gamma$S could compete with calmodulin for binding to adenylyl cyclase. This would explain why the α subunit could only inhibit the calmodulin activated adenylyl cyclase. This mechanism may be supported by a recent report that mastoparan can activate G proteins, possibly by mimicking a mastoparan like structure found in many receptors (Higashijima et al., 1988). Mastoparan forms a tight complex with calmodulin (Malencik and Anderson, 1983). It is possible that both $G_{i\alpha}$•GTP$_\gamma$S and calmodulin recognize similar targets and $G_{i\alpha}$•GTP$_\gamma$S could compete with calmodulin for binding to the calmodulin activated adenylyl cyclase.

In conclusion, this study demonstrated that the only components necessary for muscarinic receptor mediated inhibition of calmodulin-sensitive adenylyl cyclase were the adenylyl cyclase, the G protein(s) and the receptor. A methodology was also developed to allow the examination of all these components together.

The coupling of the atrial muscarinic receptor with brain calmodulin-sensitive adenylyl cyclase may be physiologically relevant since HM2 receptors are also located in the brain. The mechanism for coupling of PM2 or HM2 receptors to calmodulin sensitive adenylyl cyclase may also serve as a model for other receptor subtypes (i.e. HM3), and other inhibitory receptors that couple to calmodulin sensitive adenylyl cyclase (Perez-Reyes and Cooper, 1987). The data presented here may also suggest another mechanism for inhibition by

$G_{i\alpha}$ other than competing for a $G_{s\alpha}$ binding site on the adenylyl cyclase. The alternative role for $G_{i\alpha}$ could be to inhibit the calmodulin activated adenylyl cyclase, possibly by competing with calmodulin for a binding site on adenylyl cyclase.

Figure IV-1. G_i mediated inhibition of adenylyl cyclase activity in

the presence of various adenylyl cyclase stimulating

agents. Adenylyl cyclase was assayed in the presence of

A, EGTA (control); B, EGTA and GTP$_\gamma$S activated G_s;

C, CaCl$_2$ and 50 nM calmodulin; D, EGTA and forskolin; and

E, CaCl$_2$, calmodulin, and GTP$_\gamma$S-activated G_s. Adenylyl

cyclase was added to liposomes containing mAcChR, G_i,

and 2 mM carbachol,▓ ; to liposomes containing mAcChR,

G_i, and 1 µM L-hyoscyamine,☐ ; or to liposomes

containing no protein or ligands,▨ . The final

concentration of adenylyl cyclase in the assay was 1.9

µg/ml. The final concentration of mAcChR and G_i were

6.9 nM 10.8 nM, respectively. Assays were performed as

described in methods. Measurements were the average of

triplicate determinations and the standard deviations
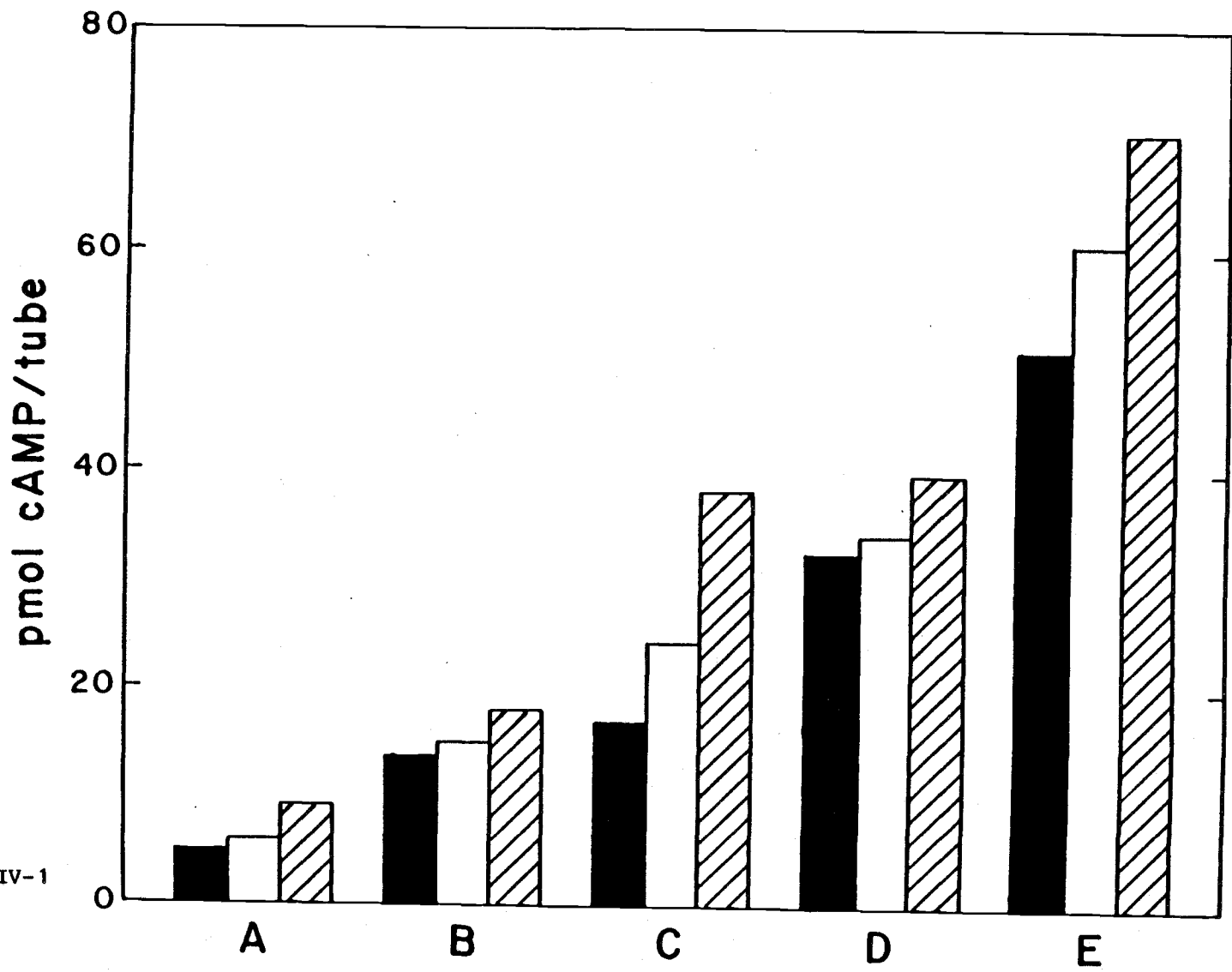
were less than 4% of the average values.

Figure IV-1

Figure IV-2.   GTP$_\gamma$S binding to liposomes containing mAcChR and G$_i$.

Assay conditions for (A) were identical to adenylyl

cyclase assay conditions described in Figure IV-1 except

that no [α^{32}P]ATP was added and [^{35}S]GTP$_\gamma$S was used.

Samples contained either Carbachol ▧ , or L-hyoscyamine

▨ . (B) No adenylyl cyclase or Tween 20 was added. (C)

No GDP was added. The error bars indicate the standard

deviation of triplicate determinations. The receptor

concentration was estimated to be 5.4 nM.

Figure IV-2
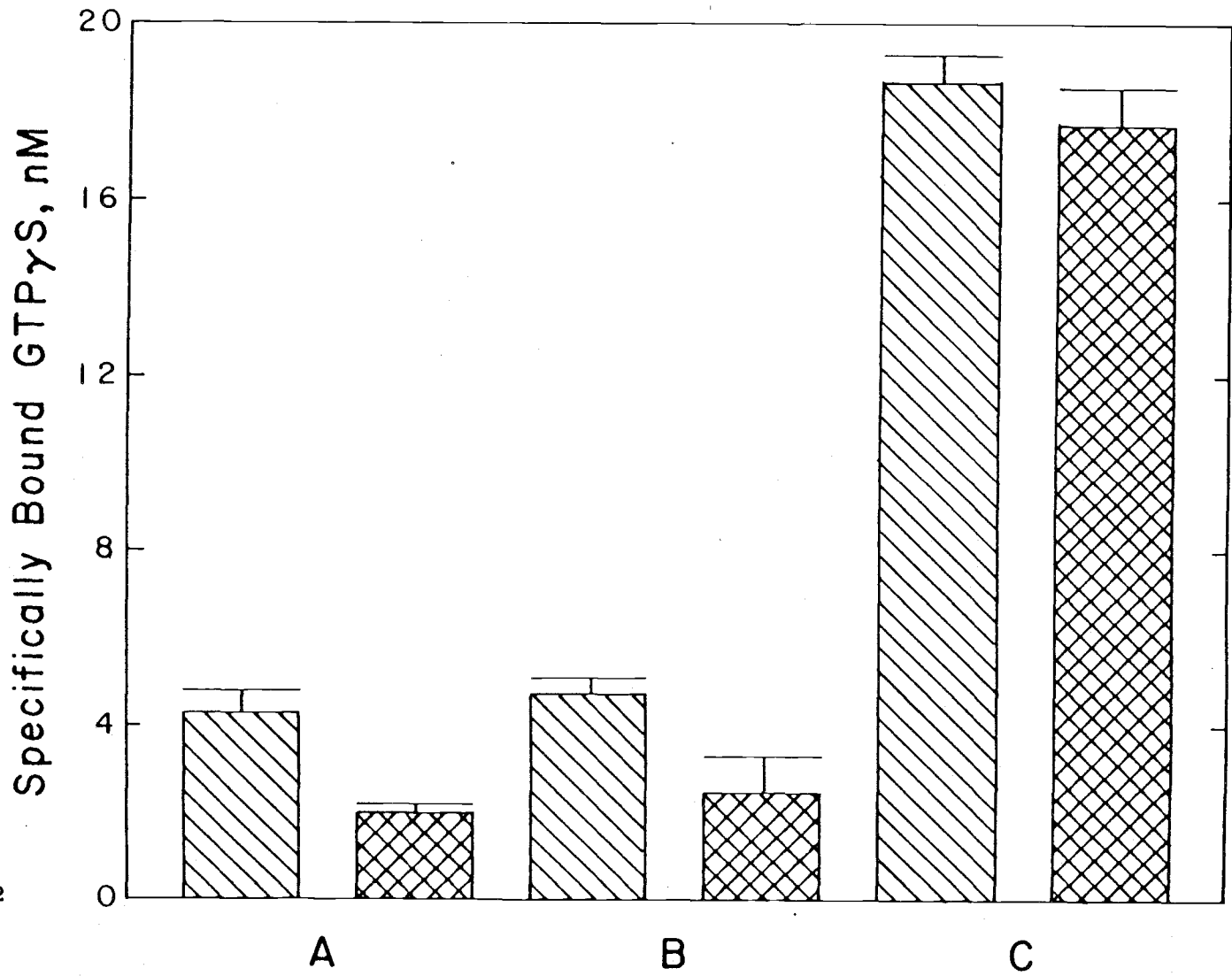
Figure IV-3. Dependence of mAcChR mediated adenylyl cyclase

inhibition on carbachol concentration. Calcium

calmodulin activated adenylyl cyclase was measured as

described in Figure IV-1 with the indicated concentration

of carbachol. The receptor concentration was estimated

to be 6.5 nM and the G_i concentration was 11.3 nM.

Data points were the average of duplicate determinations

and the data was fit assuming one class of carbachol

binding sites giving a K_d of 9.5 ± 0.6 μM. The curve

through the data points is the theoretical curve

calculated using the above K_d for carbachol. The

activity in the absence of carbachol was 44.0 ± 2.3

pmol/tube, and at the maximum carbachol concentration,
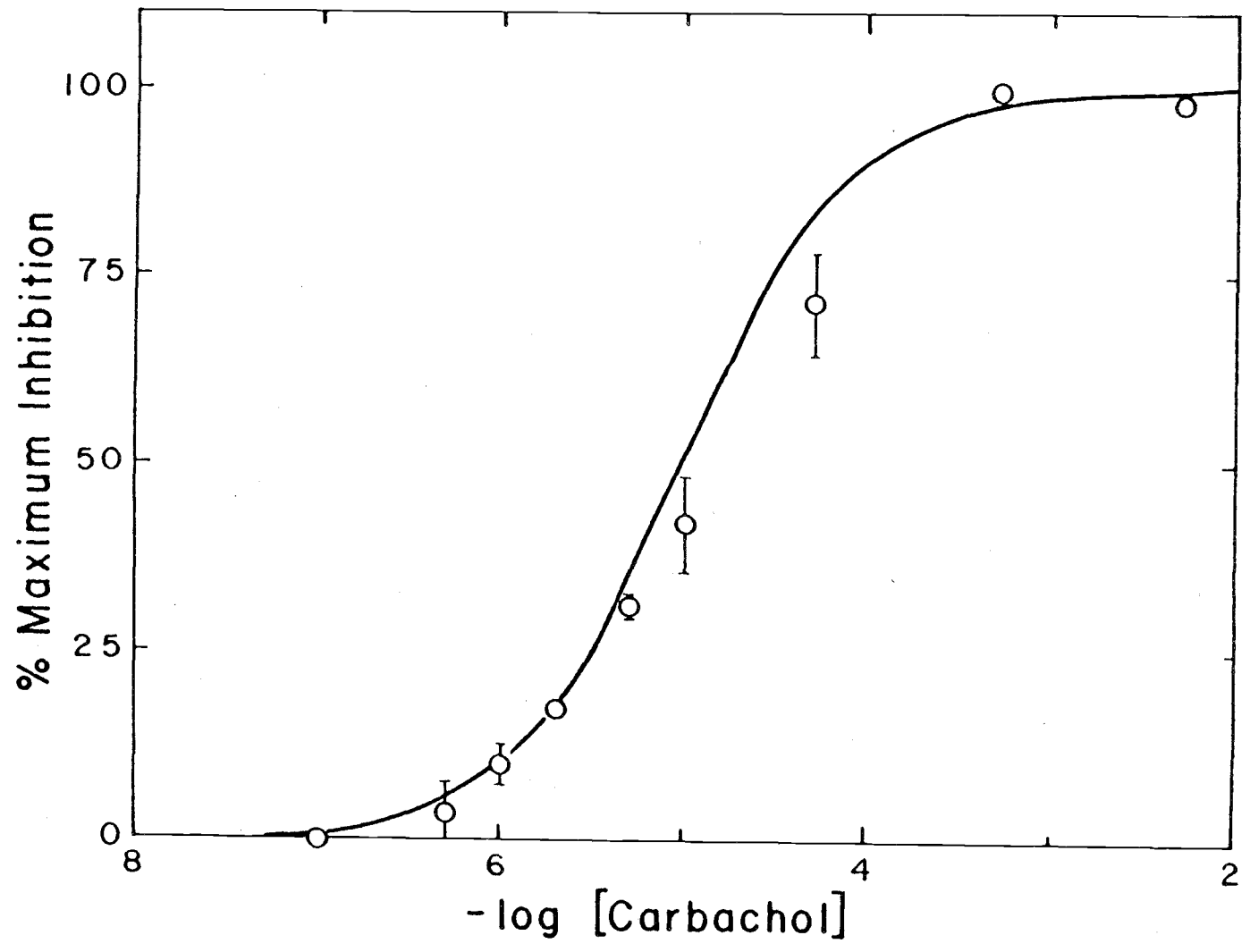
the activity was 28.7 ± 0.1 pmol/tube.

Figure IV-3

Figure IV-4. Carbachol titration of [³H]L-QNB binding sites.

[³H]L-QNB binding was performed in the presence of

varying carbachol concentrations under the same

conditions as Figure IV-1 except that labeled ATP was

replaced by unlabeled ATP and the reaction was allowed

to proceed for 20 minutes instead of one hour. Also,

guanine nucleotides were omitted for this assay. The

samples were diluted and immediatly applied to DE81

paper and the assay completed as described in methods.

The data were fit assuming 4.7 nM L-QNB sites, and a K_d

for L-QNB of 12.1 nM. The K_d for L-QNB was determined

by Scatchard analysis under similar conditions (data not

shown). The fitted parameters were F1 = 0.22 ± 0.03, K1

= 19.0 ± 10.5 µM, F2 = 0.78 ± 0.03, K2 = 4.1 ± 0.7 mM.
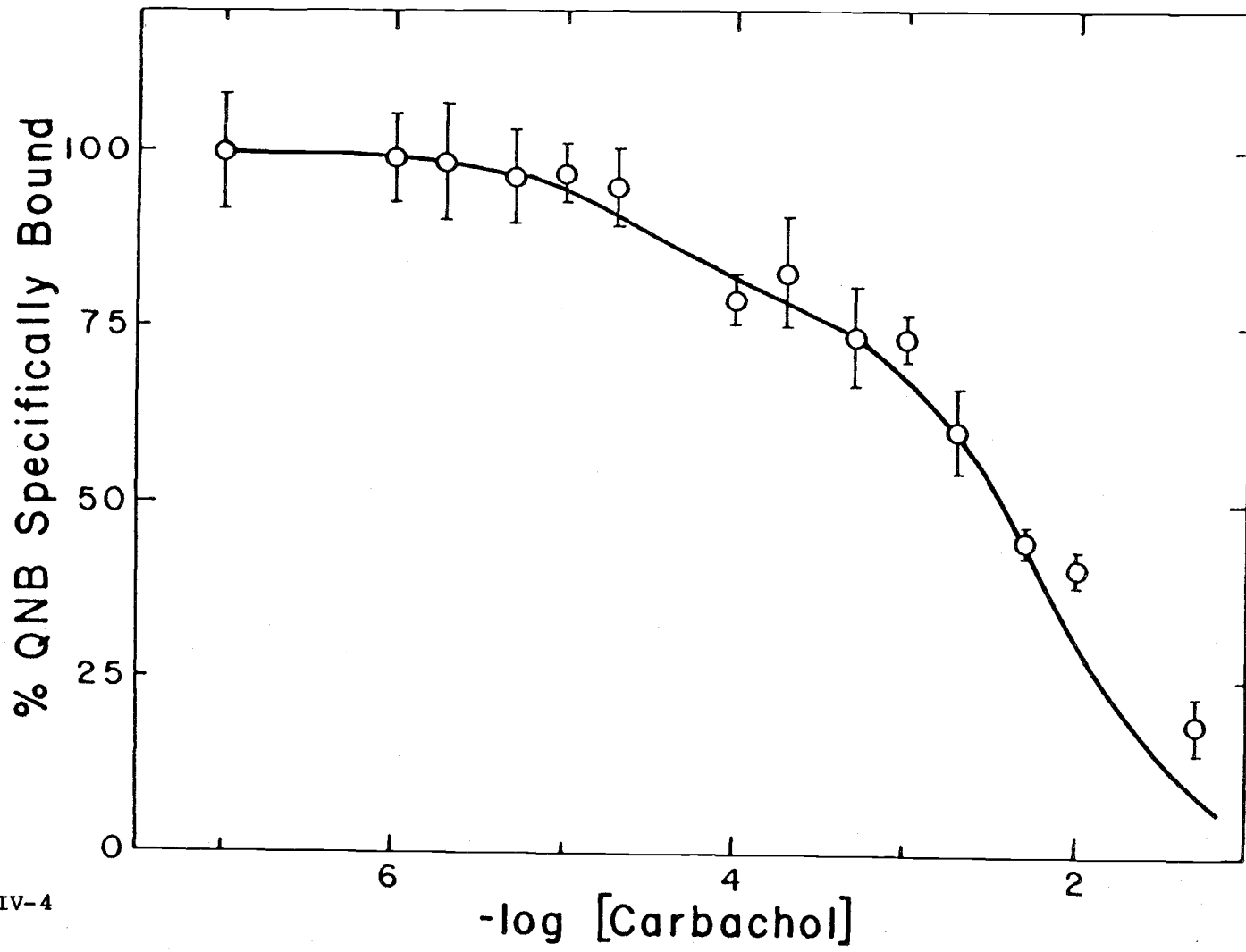
Figure IV-4

Figure IV-5. Effects of G_i subunits on adenylyl cyclase activity.

Liposomes were reconstituted as described in methods

with GTPγS $G_{i\alpha}$ subunit,▨ ; $\beta\gamma$ subunits,▩ ; or with no

subunits,▧ . The adenylyl cyclase·G_s complex was

added and adenylyl cyclase was stimulated by (A), G_s;

(B), calmodulin; or (C), no stimulation. Measurements

were the average of triplicate determinations and the

standard deviations were less than 4%.
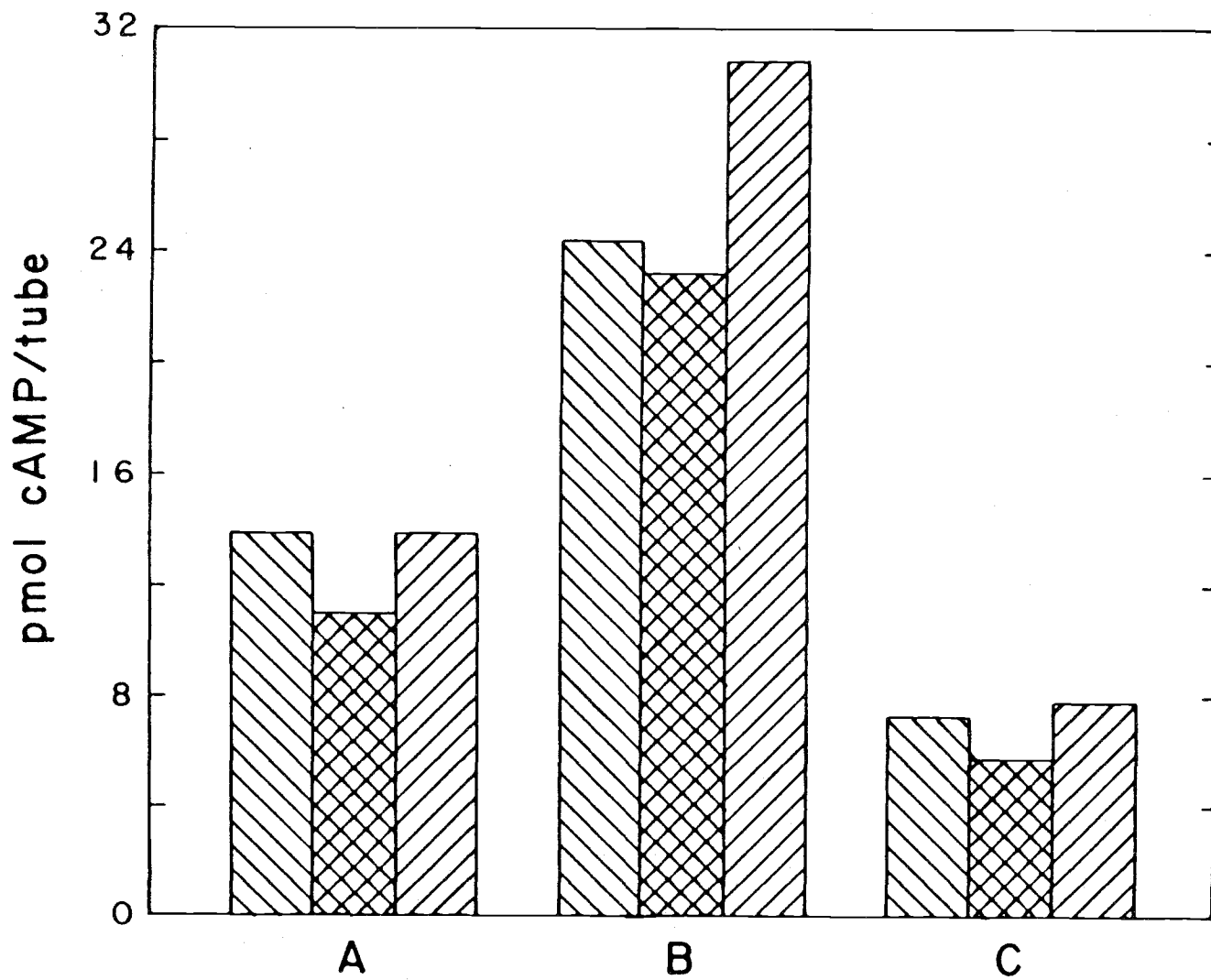
Figure IV-5

Acknowledgments

Chapter V

Discussion

The initial objective of this thesis was to study the interaction of the atrial mAcChR with atrial G_i. The successful co-reconstitution of mAcChR and G_i, together with the availability of the bovine brain calmodulin-sensitive adenylyl cyclase, prompted the further investigation of the interaction of mAcChR and G_i with this effector enzyme.

The reconstituted mAcChR and G_i were judged to be functionally interactive on the basis of the appearance of high affinity-guanine nucleotide sensitive-agonist binding sites when the mAcChR was reconstituted with G_i. Functional coupling could also be observed from the perspective of the G protein when the intrinsic GTPase activity of G_i was stimulated by a muscarinic agonist. An increased GTPase activity of G_i could generally be associated with activation of the G protein. Studies were then undertaken to explore the mechanism by which the mAcChR activated this GTPase. From these experiments, the increase in GTPase activity could be explained almost entirely by an increase in the rate of GDP release from G_i, the rate limiting step in the basal GTPase reaction of G_i. Equilibrium binding studies showed that the dissociation constant for GTP$_\gamma$S was not altered by muscarinic agonists. The inhibition of the initial rate of GTP$_\gamma$S binding by GTP showed that muscarinic agonists had no effect on the dissociation constant of GTP.

Although it is assumed that receptors activate G proteins by

forcing them into an "open" state with respect to guanine nucleotide binding (Cassel and Selinger, 1978), there seems to be some question as to whether the open state results solely from the increased rate of GDP release, or if there is also an increase in the rate of GTP binding. The β-adrenergic receptor was shown to activate G_s by increasing the rate of GDP release and by increasing the rate of GTPγS association (Brandt and Ross, 1986). However, more recent work has seemed to emphasize that much of the observed increase in GTPγS binding can be explained by an increase in GDP release, followed by rapid binding of GTPγS to the unliganded G protein (May and Ross, 1988). The data presented in Chapter III supports a similar conclusion. Therefore, reports of the brain mAcChR stimulating GTPγS to brain G_i (Kurose et al., 1986) should be viewed with some hesitation as the effects on GDP release were not taken into consideration. The mAcChR acts as an efficient catalyst and increases the slowest step in the GTPase reaction of G_i, the rate of GDP release. GTP will then rapidly bind to the alpha subunit and the protein will be ready to begin the next step in the regulation of target proteins. Carbachol increased the rate of GDP release by 38 fold, but only increased k_{cat} by 11 fold. The rate of GDP release may not be limiting in this case, but could still be a partial rate limiting step for the hormone activated G_i.

The rate of GDP release from G_i and the k_{cat} for activated G_i are controlling factors by which the mAcChR regulates effector proteins. These values should then be related to the time it takes the mAcChR to initiate its response. The direct determination of guanine nucleotide binding parameters and GTPase parameters for receptor coupled G

proteins could be difficult in heterogeneous systems, such as isolated cellular membranes. Interference from a large excess of G proteins and other enzymes that hydrolyze GTP would make data interpretation difficult. It is here that a defined reconstituted system would be useful, and the values generated from this system can begin to be related to specific biochemical responses of mAcChR activation, such as the activation of ion channels.

There is widely believed to be a delay time of 100-300 msec for the hormone bound mAcChR to activate the inward rectifying potassium channel in cardiac tissue (Hills-Smith and Purves, 1978; Hartzel, 1980; Nargeot et al., 1982). A rate constant for activation of 230 min^{-1} was determined by Nargeot et al. (1982). Recent studies on the atrial inward rectifying potassium channel have indirectly measured guanine nucleotide binding properties of G_k in vivo by studying the rate of potassium channel activation with various nonhydrolyzable GTP analogues (Breitweiser and Szabo, 1988). The investigators concluded that the rate of channel activation in the absence of muscarinic agonist was limited by the release of GDP from G_k, a rate of 0.3 min^{-1}. This value agrees with the rate of GDP release from detergent soluble G_o (Higashijima et al., 1987b), with the k_{cat} of nonstimulated reconstituted brain G_i (Kurose et al., 1986), and with the k_{cat} and rate of GDP release determined in this study for nonstimulated G_i. In the presence of acetylcholine, the k_{cat} for GTP turnover was estimated to be 135 min^{-1}. While this k_{cat} is consistent with the time required for potassium channel activation, it is significantly faster than the k_{cat} determined for mAcChR activated G_i as determined in Chapter III (k_{cat} = 2 min^{-1}). The problem of tissue compatibility

between the mAcChR and G_i can be ruled out in this study since both proteins used in this study were from the atria. Breitweiser and Szabo (1988) noted that detergent solubilized G_o also had a k_{cat} of about 2 min^{-1} (Higashijima et al., 1987a) and speculated that this low k_{cat} could be due to deleterious effects of detergent. While the presence of phospholipids was shown to effect the GTPase activity of G_i, this effect was only about 2 fold (Neer et al., 1984). In this study detergent was removed and the k_{cat} was still about 2 min^{-1}. Reconstituted hormone activated G_s also had a k_{cat} of 1.7 min^{-1} (Brandt and Ross, 1986). It is possible that the receptor-G protein interaction could be very sensitive to the lipid environment or any residual detergents and function correctly only in native membranes. Poor or improper receptor-G protein coupling might not reveal the maximum k_{cat} possible for G_i.

An alternative explanation is that there is a missing component in the transducing system. The missing component would perhaps assist the mAcChR in increasing the GTPase activity of G_i. There is already one protein identified that might perform such a function. The GTPase activating protein (GAP) was required to demonstrate the full GTPase activity associated with the ras protein (Trahey and McCormik, 1987). This ras protein is a novel G protein regulator whose cellular activity is related to its GTPase activity. The target for the ras protein is unknown, but certain mutations are known to transform mammalian cells. The role of GAP is unclear, but it is speculated to be the elusive target of the ras protein (Sigal, 1988). If GAP represents the existence of another class of proteins involved in signal transduction, then it could explain the paradox observed

between the *in vivo* k_{cat} and the k_{cat} observed for reconstituted G_i.

The reconstituted system described here provides a quantitative description of the signal transduction mechanism and may provide evidence that a key component is still lacking. The reconstituted mAcChR and G_i have proved useful in several other respects. These liposomes have been used to study the effects of phosphorylation by the cAMP dependent protein kinase on the mAcChR. Reconstituted mAcChR was phosphorylated at a fifteen fold higher level than the detergent solubilized protein and, unlike the detergent solubilized mAcChR, was still able to bind L-QNB after phosphorylation. In the presence of G_i, the reconstituted receptor was able to show a carbachol-stimulated increase in phosphorylation (Rosenbaum et al., 1987). These results may provide clues as to how chronic exposure of the receptor to agonists results in a down regulation of receptor number (Galper and Smith, 1980). Phosphorylation of the mAcChR by cAMP and calmodulin dependent protein kinases has been suggested as a means to regulate receptor number (Burgoyne et al., 1983). Recently, the involvement of these kinases was disputed, but correlation of the phosphorylation of high affinity GTP-sensitive agonist-binding sites with mAcChR desensitization was emphasized (Kwatra et al., 1987). In any case, the data presented by Rosenbaum et al. (1987) suggested that the G protein-coupled agonist-bound receptor was more susceptible to phosphorylation than was antagonist bound, unliganded, or uncoupled mAcChR.

The reconstituted mAcChR and G_i were also used to examine the hormonal regulation of calmodulin-sensitive adenylyl cyclase. These experiments were the first to show a hormone-mediated inhibition of

adenylyl cyclase using purified components in a reconstituted system. This establishes a minimum requirement for the components that need to participate in cyclase regulation and provides a good foundation for future work with this system. An unexpected result was the ability of the alpha subunit of G_i to inhibit the calmodulin-sensitive adenylyl cyclase only in the presence of calmodulin. $G_{i\alpha}$ may perform this function by competing for the binding of calmodulin to adenylyl cyclase. In any case, an as yet unexplored role for $G_{i\alpha}$ has been uncovered. Although the calmodulin-sensitive adenylyl cyclase has not yet been observed in the atria, the ability of the atrial G_i/mAcChR to regulate the brain calmodulin-sensitive adenylyl cyclase has interesting implications for other tissues. Both of the mAcChR subtypes that are believed to couple to adenylyl cyclase (M2 and M3) have been identified in the brain (Peralta et al., 1988). It is not unlikely that certain mAcChR subtypes may naturally attenuate the calmodulin-activated adenylyl cyclase activity. The mAcChR may also be involved in a complicated feedback loop as certain subtypes also function to release calcium, possibly resulting in stimulation and inhibition of cyclase activity. These situations could be resolved when individual cell types are characterized with respect to mAcChR subtypes, muscarinic biochemical responses, adenylyl cyclase subtypes, and prevalent G proteins.

Much of the future work on mAcChR's will undoubtedly entail trying to match mAcChR subtypes, with G proteins, and with effector proteins. It should be possible to purify all subtypes and reconstitute them with various G proteins. Effector enzymes, such as adenylyl cyclase, could also be included and the overall interactions of the mAcChR, G

protein, and effector enzyme coupling could be monitored. Although interactions between these components could be examined individually, a multiple component system could be used to monitor the interactions of one or more mAcChR subtypes with one or more G proteins using regulation of the effector enzyme as an assay for coupling. Thus, heterogeneous in vivo systems may be studied in a direct manner.

In conclusion, the work presented here has contributed to the understanding of the signal transduction mechanism used by the mAcChR and should provide the basis for future work involving the signal transduction mechanism, mAcChR regulation, and the role of individual mAcChR subtypes.

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APPENDICES

Appendix I

Reconstitution of the Muscarinic Acetylcholine

Receptor and G_i into Liposomes

Preparation of the lipid stock solution. All lipids were

purchased from Sigma Chemical Company. Soybean

L-α-phosphatidylcholine (Sigma P-6263) was supplied as a chloroform

solution at 100 mg/ml. Bovine brain L-α-phosphatidyl-L-serine was

supplied as a chloroform:methanol (95:5) solution (Sigma P-8518) or as

a solid (Sigma P-7769). Cholesterol (Sigma C-8253) was supplied as a

solid, but dissolved in chloroform at 10 mg/ml immediately before use.

Phosphatidylcholine (25 mg), phosphatidylserine (25 mg), and

cholesterol (2.5 mg) were rotovaped to dryness in a 50 ml roundbottom

flask. The solid was resuspended in 2.5 ml of toluene:ethanol (1:1)

with 0.02% butylated hydroxytoluene. The solution was stored under

argon at -80°C in a 3 ml reacti-vial. If solid phosphatidylserine was

used, it was added to the toluene:ethanol solution of

phosphatidylcholine and cholesterol at a concentration of 10 mg/ml.

The final ratio of lipids was phosphatidylcholine:phosphatidylserine:

cholesterol 1:1:0.1 (w/w).

Reconstitution. A typical reconstitution used 30 pmol of mAcChR

and 30 pmol of G_i. The lipid was removed from the stock vial with a

Hamilton syringe. For two reconstitutions, 50 µl of lipid (21 mg/ml;

1.05 mg) was removed and placed in a glass centrifuge tube. The

solution was rotovaped to dryness and 787 µl of 25 mM imidazole pH

7.4, 100 mM NaCl, 1 mM EDTA, and 0.02% sodium azide was added,

followed by 47 µl of 0.15 M CHAPS in 25 mM imidazole pH 7.4, 1 mM

EDTA. The solution was supplemented with 1 mM DTT, capped under

argon, and sonicated to clarity. After sonication, 8.3 µl of 0.5 M

MgCl$_2$ was added to give a final concentration of 5 mM. Next, 333 µl

of the CHAPS-lipid solution was added to one µl of a 20 mM

acetylcholine solution in a polycarbonate centrifuge bottle (Beckman

355651). G$_i$ (30 pmol; about 20 µl of 1.5 µM GTP$_\gamma$S sites in 20 mM Tris

HCl pH 8.0, 1 mM EDTA, 0.8% sodium cholate, and about 10% sucrose) and

mAcChR (30 pmol; about 20 µl of 1.5 L-QNB sites in 25 mM imidazole pH

7.4, 250 mM NaCl, 1 mM EDTA, 0.08% digitonin, 0.016% sodium cholate

and 0.02% sodium azide) were added to the CHAPS-lipid solution. The

volume was brought to 400 µl with dilution buffer (25 mM imidazole pH

7.4, 100 mM NaCl, 1 mM EDTA, 5 mM MgCl$_2$, 1 mM DTT, 0.1 mM PMSF, and

0.02% sodium azide). The final lipid concentration was about one

mg/ml, and the final CHAPS concentration was 7.0 mM. This solution

was kept on ice for 5 minutes and then diluted to 8 ml by adding 7.6

ml of ice cold dilution buffer, one ml at a time. Liposomes

precipitation was begun by adding 2 ml of 50% polyethylene glycol

(molecular weight 8000, Sigma P-2139) containing 100 mM NaCl, 5 mM

MgCl$_2$, and 0.02% sodium azide. The biphasic solution was gently

rocked until homogeneous. After a one hour incubation on ice, the

liposomes were centrifuged for 75 minutes at 61000 rpm in a Ti75 rotor

(250000 Xg). The pellet was resuspended in 0.25 ml of dilution buffer

by repeated pipetting with a sterile transfer pipette. Typical

recoveries for [^{3}H] L-QNB sites and [^{36}S]GTP$_\gamma$S sites were about

25-35%. The binding sites were stable for about one week on ice. If

a muscarinic ligand binding study was to be performed, the lipids were

resuspended in 10 m$\underline{M}$ HEPES pH 7.4, 100 m$\underline{M}$ NaCl, 1 m$\underline{M}$ EDTA, 5 m$\underline{M}$ MgCl$_2$,

1 m$\underline{M}$ DTT, and 0.1 m$\underline{M}$ PMSF instead of the imidazole buffer. Imidazole

binds to the mAcChR ligand binding site and therefore interferes with

ligand binding studies (Peterson and Schimerlik, 1984).

Appendix II

Enzyme Assays and Ligand Binding Assays

[^{3}H] L-QNB Assays. The quantitation of mAcChR was achieved by assaying the specific binding of [^{3}H] L-QNB using a DEAE filter disk assay essentially as described by Peterson and Schimerlik (1984). Slight modifications were made to accommodate reconstituted mAcChR. Saturation binding was routinely achieved by incubating 10 μl of reconstituted mAcChR with 140 μl of 10 mM Na HEPES, 50 mM NaCl, 1 mM EGTA, 5 mM MgCl$_2$, 1 mM DDT, 0.1 mM PMSF, and 20 nM [^{3}H] L-QNB (20-40 ci/mmol, New England Nuclear NET-656 or Amersham TRK.604). The solution was incubated for 90-120 minutes at 32°C and then 125 μl was applied to a 2.5 cm DE81 ion exchange paper which was perforated with a pin and suspended in air. After soaking for about 30 seconds the paper was placed in a beaker containing about 500 ml of 10 mM sodium phosphate pH 7.4, 1 mM EDTA, and 0.05% (v/v) triton X-100. The paper was washed in this buffer for 10 minutes, the buffer drained and replaced with fresh, and washed for an additional 10 minutes. After this washing, the filter paper was blotted dry on a paper towel, placed in a scintillation vial, and dried for 5 minutes at 80°C. The vial was filled with 3.5 ml of scintillation fluid and counted on a Beckman LS 6800 on user 1 (channels 0-400) for tritium with an efficiency of 0.443. The scintillation cocktail was 23.8% v/v triton X-100, 0.3% w/v Permablend III (United Technologies Packard, 91% PPO {2,5 diphenyloxazole} 9% bis-MSB {1,4 Bis(2-methylstyryl)benzene} in toluene).

[^{35}S]GTP$_\gamma$S Assays. This method of quantitating G$_i$ was adapted from Northup et al. (1982). Labelled GTP$_\gamma$S was purchased from New England Nuclear (NEN-030H) and diluted with unlabeled GTP$_\gamma$S from Boehringer Mannheim. It was sometimes necessary to purify the Boehringer Mannheim product in order to remove contaminating GDP. This procedure was described in Chapter III.

Detergent solubilized G$_i$ was assayed by incubating 5 μl of sample with 45 μl of 10 mM Na HEPES pH 7.4, 0.1 M NaCl, 1 mM EGTA, 30 mM MgCl$_2$, 0.1% deionized lubrol PX, 1 mM DDT, 0.1 mM PMSF, and 1.1 μM [^{35}S]GTP$_\gamma$S (3-8 ci/mmol). The final nucleotide concentration was one μM. The assay was allowed to incubate for one hour at 32°C, at which time 2 ml of ice cold 10 mM Na HEPES pH 7.4, 100 mM NaCl, and 25 mM MgCl$_2$ was added and the entire solution was filtered under vacuum through a BA85 nitrocellulose paper that was prewashed with 2 ml of the above cold buffer. The filter was finsed 4 more times with 2 ml of the same ice cold buffer. The nitrocellulose filters were added to a scintillation vial, dried for 5 minutes at 80°C, cooled, and dissolved with one ml of ethylene glycol monomethyl ether. Scintillation fluid was then added (3.5 ml) and the mixture was vortexed until homogeneous and counted on a standard ^{14}C channel with a counting efficiency of 0.70.

Reconstituted G$_i$ was assayed in a similar manner except the buffer used was 10 mM Na HEPES pH 7.4, 100 mM NaCl, 1 mM EGTA, 5 mM MGCl$_2$, 1 mM DDT, and 0.1 mM PMSF (the same buffer as the GTPase assay). The final [^{35}S]GTP$_\gamma$S concentration was 100 nM (100 ci/mmol).

Determination of the background value was done by using either non G$_i$ containing lipids or buffer alone and was less than 0.5% of the

total radioactivity. The background could be lowered by adding 100 μM cold GTP to the first 2 ml of wash buffer used to dilute the reaction.

GTPase Measurements. The assay for GTPase used gamma ^{32}P labelled GTP as a substrate. [^{32}P] phosphate was released upon hydrolysis and unreacted GTP was removed from solution by the addition of activated charcoal followed by centrifugation. The supernatent was then counted for [^{32}P]. The procedures used here were modeled after Sunyer et al (1984). For measuring a GTP concentration dependence, the following protocol was used: Reconstituted G_i (with or without mAcChR) was diluted to a concentration of 1 nM ([^{35}S]GTP$_\gamma$S sites) with 10 mM sodium HEPES pH 7.4, 100 mM NaCl, 1 mM EGTA, 1 mM MgCl$_2$, 1 mM DDT, and 0.1 mM PMSF. Any muscarinic ligands were added at this point and the solution was incubated at 32°C for 5 minutes. The final volume was about 175 μl. The GTPase reaction was initiated by adding the labelled nucleotide (about 5 Ci/mmol of [$\gamma^{32}P$]GTP, obtained from a dilution of New England Nuclear NEG-004 with the appropriate volume of unlabeled GTP of about the same concentration). GTP was added to give a final concentration between 10 and 75 nM for L-hyoscyamine treated liposomes, or between 25 and 200 nM for agonist treated liposomes. After 15 minutes at 32°C, 50 μl aliquotes were removed and added to 250 μl of an ice cold suspension of 5% w/v activated charcoal in 20 mM phosphate, pH 2.3, made by combining appropriate amounts of sodium phosphate and phosphoric acid. (The activated charcoal (Sigma C-5260) was previously washed 10 times in distilled H$_2$O to remove fines before being suspended in the sodium phosphate solution.) The mixture of charcoal and enzyme reaction solution was placed on ice for 5 minutes and then centrifuged at 15,000 g for 15 minutes at 4°C in an Eppendorf

centrifuge. The $[^{32}P]$ in 0.1 ml of the clear supernatent was measured by Cerenkov counting (user channel 3, open counting window). A sample of the original reaction mix was also measured by Cerenkov counting to accurately determine the concentration of total GTP added.

Routine assays were also performed using a G_i concentration between 1 and 4 n$\underline{M}$, and a GTP$\gamma^{32}P$ concentration of 500 n$\underline{M}$. The time could also be extended to either 30 minutes or 1 hour. Assays performed in this manner gave a larger signal.

Adenylyl Cyclase Assay. Cyclic AMP production was monitored by the method of Salomon (1979). $[^{32}P]$ ATP (New England Nuclear NEG-003X) was used as the substrate and the reaction product was isolated by chromatography over a Dowex AG 50W-X4 column followed by chromatography over a neutral alumina WN-3. Details of how to prepare these columns and several other reagents can be found in Salomon (1979). Prepackaged Dowex columns were purchased from Biorad (Biorad 731-6225). Before the assay is begun, these columns are prepared by (1) washing the alumina column (0.5 g) with 2 x 4 ml of 0.1 $\underline{M}$ imidazole pH 7.4, and (2) by washing the Dowex column with 2 x 8 ml of H_2O.

Routine adenylyl assay solutions were 50 µl in volume and contained 10 m$\underline{M}$ Na HEPES pH 7.4, 100 m$\underline{M}$ NaCl, 5 m$\underline{M}$ MgCl$_2$, 5 m$\underline{M}$ theophylline, 0.2 mg/ml BSA, 1 m$\underline{M}$ DDT, 0.1 m$\underline{M}$ PMSF, 50 µ$\underline{M}$ cAMP, 500 µ$\underline{M}$ ATP, and 3×10^6 cpm $[\alpha^{32}P]$ ATP (120 cpm/pmol). EGTA, calcium, calmodulin, and guanine nucleotides were added according to the needs of the particular experiment (see Chapter IV). Assays of reconstituted adenylyl cyclase also contained 8 m$\underline{M}$ Imidazole pH 7.4 and 300 µ$\underline{M}$ EDTA, these components were carried over from the

reconstitution buffer. Each assay contained 1.9 µg/ml of adenylyl cyclase. The reaction was initiated by the addition of 10 µl of a cocktail containing a 5x solution of the Na HEPES, BSA, Theophylline, cAMP, and ATP to 40 µl containing the remaining components. Theophylline was originally prepared as a 100 mM solution in 50% ethanol. The enzyme assay was incubated at 32°C for one hour and then quenched by the addition of 100 µl of stopping solution (2% sodium dodecyl sulfate, 45 mM ATP, 1.3 mM cAMP adjusted to pH 7.5 with Tris base). [^{3}H] cAMP standard was then added (New England Nuclear NET-275; 75 µl of an H_2O solution containing about 2500 cpm. A stock solution was routinely prepared by diluting one µl of the New England Nuclear product into 20 ml of H_2O). The quenched reaction mix was then diluted to one ml with H_2O, was applied to the 2 ml Dowex column, and allowed to drain to a waste container. The column was washed with 2x 1.5 ml of H_2O, then placed over the alumina column and eluted with 5 ml of H_2O. After the alumina column had dripped dry, it was eluted with 4 ml of 0.1 M imidazole pH 7.4 into 14 ml of scintillation fluid. The scintillation vials were shaken until homogeneous and counted on user 6. User 6 was a duel label counting program set to count tritium between channels 0-200 and ^{32}P between channels 400-1000. In this manner both ^{3}H and ^{32}P cAMP could be counted and the recovery of the [^{3}H] cAMP standard was used to monitor the loss of [^{32}P] cAMP during the purification procedure. [^{3}H] cAMP recovery was usually between 50 and 80%.