The goal of this work was to elucidate the mechanism of ligand binding to the muscarinic receptor. Chinese hamster ovary cell membranes containing the recombinant porcine m2 muscarinic receptor were used for continuous supply of a homogeneous receptor population. Antagonist binding to these membranes followed a hyperbolic isotherm. In contrast, agonist binding curves were shallow and fit to a model assuming three-independent classes of binding sites. Evidence is presented that the class with the highest agonist affinity is a complex of the receptor with G-proteins. One of the two lower affinity classes may be a receptor aggregate, but this hypothesis awaits further proof.

Agonist binding to all three classes of sites was controlled by a residue with a $pK_A$ of 6.6. Mutagenesis studies suggested that this amino acid was aspartate 103 in the third transmembrane region. Antagonists showed a low $pK_A$ of 4.4 that was shifted to 5.4 after detergent treatment of the membranes. Evidence is presented that this $pK_A$ was due to protonation of an allosteric residue involved in a conformational change of the receptor. Aspartate 120 is a strong candidate for this residue.

Finally, a model for superhigh affinity agonist binding is proposed based on detailed kinetic studies of binding of the agonist oxotremorine M. Kinetic experiments
showed a two-exponential association of oxotremorine M with the superhigh affinity class of sites. Both phases were independent of ligand concentration. The number of phases in dissociation experiments was dependent on the presence of a competing ligand and on the fractional saturation of the receptor. The proposed model, that is in agreement with all of these findings, involves the formation of asymmetric receptor dimers. It may be applicable to other ligands and possibly other G-protein coupled receptors.
Ligand Binding to the Muscarinic Receptor
— Equilibrium and Kinetic Studies

by

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A THESIS
submitted to
Oregon State University

in partial fulfillment of
the requirements for the
degree of
Doctor of Philosophy

Completed November 11, 1993
Commencement June 1994
APPROVED:

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Date thesis is presented November 11, 1993

Typed by Barbara Hanson for Birgit T. Hirschberg
This thesis is dedicated to Scott D. Rullman for the love and companionship that made the last years a special time.
First and most of all I would like to thank Dr. Michael I. Schimerlik for his support and guidance throughout my years in his laboratory. Mike has been an excellent advisor, and I will always think of him as an outstanding scientist. Our many late-night discussions were much appreciated.

Further, I would like to thank Dr. Gary L. Peterson for preparing the membranes I used in most of my binding studies. In addition, Gary taught me many experimental techniques and was always available for advice.

Dr. David J. Broderick patiently taught me tissue culture techniques and provided some of the mutants I used in the pH dependence studies. The other mutants were generously supplied by Walter K. Vogel. Dylan A. Bulseco has helped me tremendously with all tasks involving computers. His enthusiasm for science has been a source of inspiration for me. My ligand binding experiments on cell membranes were complemented by whole cell experiments performed by Valerie A. Mosser, who also maintained and supplied the tissue culture cells expressing the wild-type muscarinic receptor.

I am thankful to Drs. Michael H. Nesson and Charles H. Robert for many helpful and encouraging discussions.

Thanks to Drs. P. Shing Ho, Sonia R. Anderson, W. Curtis Johnson and Peter K. Freeman for serving on my committee and to Barbara Hanson for typing my thesis.

Finally, I would like to thank all of my friends—without whom I would not have been able to do any of this work—and my parents.
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LIGAND BINDING TO THE MUSCARINIC RECEPTOR
— EQUILIBRIUM AND KINETIC STUDIES

CHAPTER I
INTRODUCTION

Historic review

As is the case with many other hormone receptors, the first indication of muscarinic effects on the nervous system was obtained from the study of long-known poisonous plants. Some plants that are now known to contain muscarinic ligands are the solanaccae Atropa bella donna (deadly nightshade), Datura stramonium (Jimson weed) and Hyoscyamus niger (henbane), the shrub Pilocarpus and the mushroom Amanita muscaria. Although Amanita muscaria was mainly used for its psychoactive qualities, the action of these plants on the heart was well-known. Von Bezold and Bloebaum found that atropine, the muscarinic agent present in the Solanaceae mentioned, blocks action of the vagus nerve (see Brown, 1989, for review).

In 1869 Schmiedeberg and Koppe made extracts of Amanita muscaria and showed that they could use it to arrest a frog heart. They isolated the active alkaloid from their extracts and named it 'muscarine'. After administration of atropine, muscarine was without effect. Although at the time there was no notion of neurotransmitters, Schmiedeberg and Koppe (1869) suggested a connection between the actions of muscarine and electrical stimulation of the vagus nerve.

In 1906 Hunt and Taveau synthesized acetylcholine and showed that it lowered blood pressure—an effect that was blocked by atropine. In 1914 Dale found that at high concentrations acetylcholine increased blood pressure. This effect was similar to that of
nicotine, which is why these two effects of acetylcholine and acetylcholine analogues were termed 'muscarinic actions' and 'nicotinic actions'.

Dale and Dudley (1929) were the first to discover acetylcholine in an animal tissue in 1929 namely in bovine and equine spleen. The first evidence for chemical mediation of nerve signals came from an experiment done by Loewi in 1921. He showed that fluid that was collected from a frog heart after electrical stimulation of the vagus nerve could weaken the heartbeat of a second frog heart. In contrast fluid from an unstimulated heart was without effect. He named the active substance 'vagusstoff' and five years later identified it as acetylcholine. This made acetylcholine the first recognized neurotransmitter.

Smooth muscle tissue was used in the first successful receptor binding studies performed by Paton and Rang in 1965. Radiolabeled atropine was used in this study. With the availability of a number of radiolabeled muscarinic ligands, muscarinic receptors were soon demonstrated in the heart, central nervous system and secretory glands of many vertebrate species in addition to some neuronal cell lines (see Brown, 1989, for review). These studies also showed that the receptor was located in the membrane fraction of these tissues/cells.

In the 70s and 80s muscarinic receptors were solubilized from brain and heart tissue using a number of different detergents. Finally, in 1984 Peterson et al. were able to purify active receptor from porcine atria. At about the same time Haga and Haga (1985) obtained purified muscarinic receptor from porcine brain.

**Receptor subtypes**

Soon after purification of the muscarinic receptor was achieved, Kubo et al. (1986a,b) cloned the porcine cerebral muscarinic receptor and the cardiac muscarinic receptor (later termed the m1 and m2 receptor) from cDNA libraries. The deduced
sequence of the m1 receptor was compared to the β2 adrenergic receptor (Bonner et al., 1987), and a region of sequence homology was used to screen rat brain cDNA libraries. Using this technique the muscarinic receptor subtypes m3 and m4 were identified. At the same time another group succeeded at cloning all four muscarinic subtypes from genomic libraries (Peralta et al., 1987). A year later a fifth receptor subtype was identified (Bonner et al., 1988). The five subtypes are differentially distributed throughout the body with only m2 being present in atrial tissue. Working with atrial tissue therefore circumvents the problem of receptor subtype heterogeneity that has to be addressed with other tissues. Most muscarinic ligands show little subtype specificity. Although all muscarinic receptors share a fair amount of sequence homology, the m1, m3 and m5 receptors couple preferentially to stimulation of hormone sensitive phospholipases, and the m2 and m4 receptors mainly inhibit adenylate cyclase.

Receptor structure

Hydropathy analysis of the amino acid sequence of muscarinic receptors suggests that they share a great deal of structural homology with rhodopsin as well as with other receptors such as the adrenergic receptors (Hulme et al., 1990). All of these receptors contain seven transmembrane regions connected through alternating extracellular and intracellular loops as shown in Figure 1.1. Along with structural homology, this family of receptors shares the ability to interact with guanine nucleotide binding proteins. The N-terminal end of the muscarinic receptor is located on the extracellular side of the plasma membrane and contains a number of potential glycosylation sites. The intracellular C-terminus contains a palmitoylated cysteine residue, and the seven transmembrane regions are thought to form a helical wheel-type structure with the ligand binding site in the center (for review see Hulme et al., 1990).
The transmembrane regions are relatively well conserved among G-protein coupled receptors and especially among muscarinic receptor subtypes. The second and third membrane-spanning domains contain 3 highly conserved aspartate residues. At least one of these aspartates is thought to participate in charge-charge interactions with the positively charged nitrogen moiety in most muscarinic agonists. Transmembrane regions 4, 5, 6 and 7 each contain a proline residue that is conserved and that is likely to introduce a bend in the helix (Lefkowitz et al., 1988). Extracellular loops 1 and 2 each contain a cysteine residue, and the two residues have been shown to form a disulfide bond (Kurtenbach et al., 1989). Another residue that is conserved and that may play an important role in ligand binding and/or receptor function is a tyrosine residue in membrane-spanning domain 7. Site-directed mutagenesis studies are underway to examine the significance of this conservation. Another important feature of muscarinic receptors is the rather large intracellular loop that connects transmembrane regions 5 and 6. At least part of the G-protein coupling specificity of different muscarinic receptor subtypes resides within this third intracellular loop. Subtypes 1, 3 and 5 on one hand and subtypes 2 and 4 on the other hand show the most homology in this region parallel with their preference for different effector systems. Experiments with chimeric receptors have shown that the membrane-proximal portions of the third intracellular loop are most important in determining effector specificity (Wess et al., 1990).

G-proteins

As mentioned above muscarinic receptors assert at least part of their effects through coupling to guanine nucleotide binding proteins. The first evidence for this interaction came from ligand binding studies in the presence of guanine nucleotides. GTP and its analogues decrease binding of muscarinic agonist, whereas antagonist binding is not affected (Berrie et al., 1979). Other evidence is the time course of effector activation,
which is much slower for muscarinic receptors than, for example, for nicotinic receptors. Nicotinic receptors mediate the aforementioned nicotinic actions of acetylcholine and contain an ion channel. They assert their effects through directly controlling the flow of ions across the cell membrane, which is a very rapid effect.

The mechanism of action of G-protein coupled receptors is complex and is illustrated in Figure 1.2. It is thought that upon binding of an agonist the liganded receptor binds to and activates a G-protein. Activation of the G-protein results in an exchange of GTP for GDP at the protein's nucleotide binding site. The nucleotide exchange is due to a higher dissociation rate of GDP in the presence of the receptor-agonist complex (Tota et al., 1987). The GTP-bound G-protein then interacts with effector proteins such as adenylate cyclase, phospholipase C or ion channels.

Guanine nucleotide binding proteins are heterotrimers comprised of a 39-45 kDa α-subunit, a 35-36 kDa β-subunit and a 5-10 kDa γ-subunit (see Stryer & Bourne, 1986, for review). The β- and γ-subunits stay associated, whereas the α-subunit dissociates and reassociates during the activation cycle of the protein. The α-subunit carries the guanine nucleotide binding site. In the inactive state of the protein it is thought to have GDP bound and be associated with the βγ-subunit (Northup et al., 1983). 0.2 to 0.9 moles of GDP per mol G-protein were found in highly purified preparations of G-proteins (Ferguson et al., 1986). As mentioned above, binding of the receptor-agonist complex to the heterotrimeric G-protein results in a decrease in affinity for GDP, allowing GTP to bind in its place. GTP binding is thought to cause the G-protein to dissociate from the receptor as well as dissociate into its subunits. The GTP-bound α-subunit is then free to interact with effector proteins such as adenylate cyclase until the protein's endogenous GTPase activity hydrolyzes the bound GTP to GDP and inorganic phosphate. Upon GTP hydrolysis the α-subunit reassociates with the βγ-subunit. Although it has been assumed that the βγ-subunit only acts as a membrane anchor for the α-subunit and facilitates interactions with the receptor, it has also been suggested that the βγ-subunit itself can
interact with effectors (Logothetis et al., 1987; Jelsema & Axelrod, 1987). Complicating the picture is the fact that there are innumerable, different G-proteins that vary in their specificity both for the receptor and for different effectors. Cloning and sequencing have identified more than 30 different α-subunits, 4 different β-subunits and 5 different γ-subunits (for review see Simon et al., 1991). Until recently it was thought that only the α-subunits confer specificity to G-proteins. Based on sequence similarities of the α-subunits, G-proteins were grouped into four classes: Gs, Gi, Gq and G12. Gi, the inhibitory G-protein, and Gs, the stimulatory G-protein, are named for their effects on adenylate cyclase. Aside from sequencing, the four classes can be distinguished by use of certain bacterial toxins. Cholera toxin catalyzes the ADP-ribosylation of the Gs class, and Pertussis toxin, the agent that causes whooping cough, ADP-ribosylates the Gi class. In both cases NAD functions as the ADP-ribose donor. Evidence is now emerging that the β- and γ-subunits are also important in determining the heterotrimer's specificity. Specific combinations of β- and γ-subunits seem to be required for coupling to different receptors (Kleuss et al., 1991 and 1993).

Second messenger systems

Muscarinic ligands have been demonstrated to affect cellular levels of cAMP, phospholipid metabolites, calcium and cGMP as well as a number of ion conductances (for review see Brown, 1989). Levels of cAMP are regulated through an effect of the muscarinic receptor on the enzyme adenylate cyclase. Activation of the muscarinic receptor primarily leads to an inhibition of adenylate cyclase, although much higher concentrations of muscarinic agonists result in a stimulatory phase (W.K. Vogel, unpublished results). Whether the inhibitory and the stimulatory effect are mediated through the same adenylate cyclase subtype is unclear at the moment. The inhibition of adenylate cyclase is blocked by pertussis toxin, a toxin from Bordetella pertussis that
catalyzes ADP-ribosylation of G\textsubscript{i} and G\textsubscript{o} (Gilman, 1987). This effect of pertussis toxin together with the requirement for GTP for inhibition of the enzyme (for review see Stryer & Bourne, 1986) suggests that G-proteins are involved in the coupling of muscarinic receptors to adenylate cyclase.

Two mechanisms have been suggested for the interactions between muscarinic receptors, G-proteins and adenylate cyclase. Either the \( \alpha \)-subunit of G\textsubscript{i} directly interacts with and inhibits adenylate cyclase (Gilman, 1984a; Katada et al., 1984) or the muscarinic receptor causes the \( \alpha \)-subunit of G\textsubscript{i} to dissociate, resulting in an increase in cellular concentrations of the \( \beta\gamma \)-subunit, which then combines with the \( \alpha \)-subunit of the stimulatory G-protein, G\textsubscript{s} (Birnbaumer, 1987). The latter mechanism suggest an indirect effect of G\textsubscript{i}, inactivating the stimulatory G-protein and thus preventing stimulation of adenylate cyclase.

Another mechanism by which muscarinic receptors can decrease cAMP levels is through activation of phosphodiesterases that catalyze the break-down of cAMP. This pathway has been demonstrated in thyroid slices (van Sande et al., 1977) and astrocytoma cells (Gross & Clark, 1977). The phosphodiesterase involved is stimulated by calcium-calmodulin. As will be discussed later, muscarinic agonists increase cytoplasmic calcium levels, which could account for the observed phosphodiesterase activation.

Similar to effects on adenylate cyclase, the regulation of phospholipid metabolism by muscarinic ligands has been well demonstrated. Muscarinic agonists activate phospholipases C, A\textsubscript{2} and D. The most studied of these has been the activation of an inositol phospholipid-specific phospholipase C which in turn hydrolyzes phosphatidyl inositol-4,5-bisphosphate. The hydrolysis gives rise to the two second messengers diacylglycerol and inositol-1,4,5-trisphosphate. Inositol-1,4,5-trisphosphate releases calcium from the endoplasmic reticulum and thus increases the cytoplasmic calcium concentration. Changes in cytoplasmic calcium concentration have profound effects on many proteins, including the mentioned calcium-calmodulin stimulated
phosphodiesterase, protein kinase C and calcium-activated potassium channels. The other second messenger produced, diacylglycerol, is together with calcium involved in activating protein kinase C. As with adenylate cyclase the effect of muscarinic ligands on phospholipase C involves guanine nucleotide binding proteins (Litosch et al., 1985). In Chinese hamster ovary cells (CHO cells) overexpressing the m2 receptor, this effect is sensitive to pertussis toxin (D.A. Bulseco, unpublished results).

Muscarinic effects on cyclic GMP levels have been far less studied, but increases in cGMP concentration following muscarinic activation have been demonstrated in many tissues (Goldberg & Haddox, 1977). Since this phenomenon is only seen in whole cells or tissue, it is likely to be an indirect effect, although the second messengers involved have not been identified.

Finally, the muscarinic receptor is involved in regulation of a number of ion channels. The system that is best understood is the inward rectifying potassium channel in the heart. This channel is opened during muscarinic activation leading to a hyperpolarization of the cell membrane up to several millivolts. This hyperpolarization is the basis for the actions of the vagus nerve on the heart. Patch-clamp studies have shown that no diffusible second messengers are involved in coupling of the receptor to the inward rectifying potassium channel. The involvement of a pertussis toxin sensitive G-protein has been demonstrated (Breitweiser & Szabo, 1985). This G-protein was named Gk, but might be identical with G13 (Gilman, 1987b). Whether the G-protein α- or βγ-subunit is active in this pathway has been an issue of debate and has not been decided unequivocally.

In the heart muscarinic ligands also affect calcium channels (Loffelholz & Pappano, 1985; Trautwein et al., 1975). The observed decrease in calcium current is the result of the protein kinase C activation that follows muscarinic stimulation of phosphatidyl inositol hydrolysis (Nelson, M.T., personal conversation).
Other ion conductances (e.g. sodium and chloride currents) have also been shown to be influenced by muscarinic stimulation. Much less is known about these responses, and they may be indirect effects caused by changes in the levels of second messengers already mentioned.

**Ligand binding**

Ligands can generally be divided into two classes: agonists and antagonists. Agonists cause the same response as the physiological ligand (i.e. acetylcholine in the case of the muscarinic receptor). Antagonists block the response of the physiological ligand. A large number of compounds are known to bind to the muscarinic receptor, most of which show no or little specificity for the different receptor subtypes. Although muscarinic ligands belong to very different chemical classes (e.g. cyclopentene and tetrahydrofurane derivatives), they share two important features: a fully or partially charged ammonium group and a nearby group of high electron density. The latter corresponds to the ester bond in acetylcholine. Muscarinic agonists are generally fairly small, hydrophilic molecules, whereas antagonists tend to be rather hydrophobic and often contain bulky aromatic groups (see Ringdahl, 1989, for review). The structures of some muscarinic ligands are shown in Figure 1.3.

A number of radioactively labeled muscarinic ligands are commercially available and are used in binding studies on tissue slices, whole cells and membrane preparations. After binding of the labeled ligand comes to equilibrium, the bound and the unbound ligand have to be separated. In membrane preparations, separation can be achieved by filtration, centrifugation and dialysis. Filtration has the advantage that the bound label can be quantitated directly rather than be calculated from the difference between total and free ligand, which tends to reduce the error. On the other hand filtration can only be used accurately, if the dissociation rate is slow enough that the bound label is not lost during
the washing steps. Independent of what separation method is used, the amount of nonspecific binding of the radioligand has to be determined. Nonspecific binding is defined as low-affinity binding to an infinite number of sites, whereas specific binding is saturable and of higher affinity. Nonspecific binding is determined as the amount of binding of the radioligand left after the specific binding sites are saturated with an unlabeled ligand (see Järvi and Barfai, 1988, for review).

Antagonists display hyperbolic binding isotherms as predicted for simple mass-action binding to a single class of sites. Different antagonists displace each other completely over a concentration range of about two orders of magnitude. Binding of agonists displays more complex behavior. As is seen with other G-protein coupled receptors, muscarinic agonists can displace antagonists completely, but do so over a concentration range of 5-6 orders of magnitude. Agonists show shallow binding curves that sometimes contain marked inflections. Such a binding behavior can have a number of interpretations. The receptor may exist in different states or contain more than one binding site. In this case antagonist binds to all states/sites with the same affinity, whereas agonists have different affinities. Negative cooperativity only seen with agonists is another possible explanation for shallow binding curves, although it is inconsistent with the presence of inflections in some binding curves. Finally, shallow binding isotherms can be caused by other molecules that bind to the receptor and modulate its affinity for agonists. This is generally referred to as a ternary complex model. In the case where the modulating factor is present at concentrations lower than that of the receptor and binds to the receptor with high affinity, it becomes identical to a multiple state model. All of the models mentioned for agonist binding can be found in the literature (Burgen, 1987; DeLean et al., 1980). At present the model assuming multiple states of the receptor is most widely accepted. In most membrane preparations three states are required to adequately fit the binding data, whereas two states are sufficient in whole cells. Different
binding models and their implications are discussed in more detail in the following chapter.
Figure 1.1  Structure and amino acid sequence of the porcine m2 muscarinic receptor.
Figure 1.1
Figure 1.2 Activation cycle of G-proteins.

$G_\alpha$ and $G_\beta\gamma$ stand for the $\alpha$- and $\beta\gamma$-subunits of G-proteins, R for a hormone or neurotransmitter receptor and A for a hormone or neurotransmitter.
Figure 1.2
Figure 1.3  Chemical structures of muscarinic ligands.
**AGONISTS:**

- Acetylcholine: $\text{CH}_3\text{-C-O-CH}_2\text{-CH}_2\text{-N-(CH}_3)_3$
- Carbachol: $\text{NH}_2\text{-C-O-CH}_2\text{-CH}_2\text{-N-(CH}_3)_3$
- Oxotremorine M: $\text{N-CH}_2\text{-C=CH}_2\text{-N-(CH}_3)_3$

**ANTAGONISTS:**

- L-quinuclidinyl benzilate (L-QNB)
- L-hyoscyamine
- N-methyl scopolamine (NMS)

Figure 1.3
CHAPTER II
EQUILIBRIUM BINDING STUDIES

INTRODUCTION

It has long been known that neurotransmitter receptors that couple to guanine-nucleotide binding proteins (G-proteins) bind ligands in a characteristic manner. At least in membranes and broken cell preparations antagonist binding displays a simple mass-action relationship, as expected for non-cooperative binding to a homogeneous class of binding sites. Different antagonists recognize the same number of sites and are able to displace each other fully over a concentration range of two orders of magnitude. In contrast, a concentration range of up to 6 orders of magnitude is needed to fully displace an antagonist by an agonist. Due to this large concentration range agonist binding is generally studied in competition experiments using a radiolabeled antagonist as a tracer. The complex binding behavior of agonists was initially interpreted as binding to two noninterconvertible classes of independent sites with different affinities for agonists and the same affinity for antagonists. Guanine nucleotides leave antagonist binding unchanged, but affect the binding of agonists (Birdsall & Hulme, 1976). This effect has been interpreted as a shift of receptors from the high affinity to the low affinity state (Waelbroeck et al., 1982) without a change in the dissociation constant of either state. It was suggested that the high affinity state of the receptor is the result of the formation of a receptor G-protein complex (Kurose & Ui, 1983), whereas binding to the receptor itself is of low affinity. This fairly simple picture of receptor ligand interaction was invalidated when Birdsall et al. (1980) showed convincingly that for their preparations two classes of sites were insufficient in fitting the data, and at least three classes of sites were needed to obtain a satisfactory fit. The three classes were termed superhigh, high and low affinity classes of sites. Whereas two populations can be explained in terms of free receptors and
receptor G-protein complexes, the physical identity of a third population is obscure. Receptor dimerization, complex formation with a second G-protein and posttranslational modifications are a few of the possibilities. It is also unclear what the significance of different affinity states is in terms of the receptor's ability to elicit cellular responses.

Alternatively, the shallow nature of agonist binding curves has been attributed to the formation of ternary complexes involving the receptor, the agonist and a G-protein (DeLean et al., 1980). In this model an equilibrium exists between the receptor G-protein complex and its free components, and agonists support complex formation by binding with higher affinity to the complex than to the free receptor. A number of variations of the ternary complex model have been proposed, some of which include additional steps like a receptor isomerization (Samama et al., 1993) or G-protein subunit dissociation (Onaran et al., 1992). One of the weaknesses of any ternary complex model is that they fail to explain why purified receptors have been shown in some cases to have more than one affinity state (Peterson et al., 1984).

Both models, the ternary complex model and the noninterconverting classes of sites model, persist in the muscarinic receptor literature and most data sets can be fit equally well to both models. However, the parameters obtained from either fit are completely different, and parameters from one fit generally carry no meaning in the other binding scheme.

One would assume that the binding pattern of agonists is somehow linked to the mechanism by which G-protein coupled receptors elicit their cellular responses, and understanding this pattern seems crucial for the interpretation of any ligand induced phenomenon. Also, differences in binding patterns between whole cells and broken cell preparations have been observed, making the validity of in vitro binding assays questionable. For these reasons the following study was aimed at gaining a better understanding of agonist binding behavior. Four muscarinic agonists were chosen, including the physiological agonist acetylcholine and the partial agonist pilocarpine.
Their binding to the pm2 mAChR was studied in whole cells, broken cell preparations and membrane preparations. The effect of G-proteins and receptor density on muscarinic agonist binding was examined. Implications of the results for the choice of a binding model and for the differences between in vivo and in vitro studies are discussed. All of the in vivo binding experiments were carried out by Valerie A. Mosser. Experiments using purified muscarinic receptors were done by Dr. Gary L. Peterson.
MATERIALS AND METHODS

Abbreviations

BCM, benzilylcholine mustard; CHO cells, Chinese hamster ovary cells; DTT, dithiothreitol; EDTA, ethylene diamine tetraacetic acid; EGTA, ethyleneglycol-bis-(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid; DHFR, dihydrofolate reductase; G-protein, guanine nucleotide binding protein; GDP, guanosine diphosphate; GppNHp, guanylylimido-diphosphate; GTP, guanosine triphosphate; GTPγS, guanosine 5'-O-(3-thio-triphosphate); Hepes, 4-(2-hydroxyethyl)-1-piperazine ethane sulfonate; NMS, N-methyl scopolamine; Oxo M, oxotremorine M; PEI, polyethylene imine; pm2, porcine m2 muscarinic receptor subtype; PMSF, phenylmethylsulfonyl fluoride; PTX, pertussis toxin; L-QNB, L-quinuclidinyl benzilate; Tris, Tris(hydroxy-methyl)amino methane; buffer A, 10 mM Hepes, 5 mM MgCl2, 1 mM EDTA, 1 mM EGTA, pH 7.4; buffer B, ice-cold 50 mM sodium phosphate, 1 mM EDTA, pH 7.4; buffer C, 20 mM Tris HCl, 1 mM EDTA, 0.1 mM PMSF, 1 mM DTT, 0.9% cholate, 73 mM sucrose, 5 mM MgCl2, pH 8.0.

Materials

All radioligands were purchased from DuPont, New England Nuclear. Specific activities were 41.9 Ci/mmol for [3H] L-QNB, 80.4 Ci/mmol for [3H] NMS and 87.5 Ci/mmol for [3H] oxotremorine M. Unlabeled oxotremorine M, pilocarpine and acetylcholine were purchased from Research Biochemicals Inc., 2-hyoscyamine from Sigma and carbachol from Aldrich Chemical Company. All guanine nucleotides were purchased from Boehringer Mannheim. Pertussis toxin was obtained from List Biological Laboratories. Glass fiber filters were either from Schleicher & Schuell (#32) or from Whatman (GF/B). Benzilylcholine mustard was synthesized according to Gill
and Rang (1966). The pko-neo vector was a generous gift from Dr. G.F. Merrill and was constructed by Dr. Doug Hanahan.

**Receptor preparation**

The receptor used in this study is exclusively the recombinant m2 subtype of the porcine muscarinic acetylcholine receptor. The gene for this receptor was cloned into the pSVE vector containing the gene for dihydrofolate reductase, which was then used to transform DHFR- Chinese hamster ovary (CHO) cells (Peralta et al., 1987a). Transformed cells were selected using the DHFR inhibitor methotrexate. The obtained stably transfected cell line overexpressed the m2 receptor at levels of 1-2 million receptors per cell and contained no endogenous muscarinic receptors. It expressed the stimulatory G-protein, Gs, all three subtypes of the inhibitory G-protein, Gi, and members of the Gz family but no Go (Dr. Janet Robishaw, unpublished results). The CHO cell line CHOipm2 was generated by transfection of CHO cells with 10 µg of a 20:1 (w/w) ratio of pSV2pm2 to pko.neo and selected at 100 µg/ml G418. pSV2pm2, provided by M.I. Schimerlik, was obtained by cloning the pm2 receptor gene into the Hind III/BgLI site in place of the DHFR gene in pSV2DHFR (Subramani et al., 1981).

When whole cells were used in binding assays, they were grown to near confluency on 2 mm culture dishes. They were then treated with agonists for 15 minutes before the radiolabeled tracer was added and allowed to equilibrate. After equilibration the media containing the free ligand was aspirated and the cells were washed, solubilized in triton and counted for bound radioligand. Nonspecific binding was assessed in the presence of an excess of an unlabeled antagonist.

Alternatively, the cells were harvested, homogenized on ice with ten strokes in a Potter-Elvehjem homogenizer with a Teflon pestle and used in binding experiments or used to purify membranes. Membrane purification involved differential centrifugation.
and a sucrose gradient and was performed as described for porcine atrial membranes (Peterson & Schimerlik, 1984).

**Binding assays**

Agonist binding data for broken cell preparations and membranes were obtained using a competition assay. Membranes or broken cells were diluted to a L-QNB site concentration of 100-200 pM into buffer A and incubated with different concentrations of the unlabeled agonist typically ranging from 10 pM to 1 mM. In the case of the physiological agonist acetylcholine, 10 µM eserine sulfate were added to inhibit any acetylcholine esterase present in the preparation. If binding was studied in the presence of guanine nucleotides, the nucleotides were added to the preparation before incubating with the agonist. After an incubation period of 20 minutes, the radioactive tracer, either [3H]-L-QNB or [3H]-NMS, was added and allowed to equilibrate for 2 hours. [3H]-L-QNB was used at a concentration of 150 pM, [3H]-NMS at 1.5 nM. Nonspecific binding was assessed in the presence of 100 µM β-hyoscyamine, and the total binding site concentration was determined with saturating concentrations of the radiolabeled antagonist. After equilibration samples were filtered through #32 glass fiber filters either on a filter manifold or a Brandell cell harvester and washed with 4x3 ml of buffer B. In case of the positively charged tracer [3H] NMS the glass fiber filters were soaked in 0.1% PEI prior to use in order to reduce adsorption of the ligand to the filters. After filtration 3.5 ml of a triton-toluene based scintillation cocktail was added and samples were counted for 5 minutes in a Beckman liquid scintillation spectrometer.

**PTX treatment**

Cells were treated with 100 ng/ml PTX for 14 hours prior to harvesting.
Mustard treatment

BCM at 100 µM was cyclized for 30 minutes at room temperature in 10 mM sodium phosphate, 1 mM EDTA, pH 7.4. Cells on tissue culture plates were rinsed and incubated with 1 µM of the cyclized BCM at 37°C for 20-30 minutes. The cells were rinsed twice before use in ligand binding experiments.

Reconstitution

0.5 ml of the membrane preparation (~1 µM receptor sites) were incubated with 0.1 mM acetylcholine on ice for 30 minutes, after which 1 ml of purified G-proteins in buffer D were added. The final cholate concentration was 0.6%. The suspension was dialyzed overnight at 4°C against 0.5 L of buffer A, causing the fairly hydrophobic G-proteins to insert into the membranes. The dialysate was diluted 8-fold into buffer A and centrifuged at 40000 rpm for 1 hour. The resulting pellet was resuspended in 7 ml of buffer A, recentrifuged, resuspended in 0.5 ml of buffer A and frozen at -80°C.

Data analysis

All data were corrected for nonspecific binding by subtracting the amount of radioligand bound in the presence of 100 µM L-hyoscyamine. Agonist binding data were generally analyzed in terms of a noninteracting three-site model. The equation describing this model is

\[
\bar{Y} = \frac{[L]}{K} \left( \frac{F_1}{1 + \frac{[L]}{K_1} + \frac{[I]}{K_1}} + \frac{F_2}{1 + \frac{[L]}{K_2} + \frac{[I]}{K_2}} + \frac{F_3}{1 + \frac{[L]}{K_3} + \frac{[I]}{K_3}} \right)
\]
where $\bar{Y}$ is the fractional saturation of the receptor with the radioactive tracer. $[L]$ is the free tracer concentration and $[I]$ the free agonist concentration assumed to equal the total agonist concentration. $K$ is the dissociation constant of the tracer. $F_1$, $F_2$ and $F_3$ are the fractions of total binding sites that show the dissociation constants $K_1$, $K_2$ and $K_3$ for the agonist (so that $F_1 + F_2 + F_3 = 1$). Agonist binding data were fit to this model using Marquardt's algorithm (Duggleby, 1984). The fractional saturation was normalized to 1 in the absence of agonist.

To evaluate the ternary complex model the equations describing the model were simulated using "Scopfit" (Simulation Resources Inc., Berrien Springs, MI). The concentrations of the receptor and the two competing ligands were determined experimentally, and the G-protein concentration, the dissociation constants and the coupling factors were fitted in "Scopfit" by an iterative method.

Guanine nucleotide effects on Oxo M binding were fitted to a hyperbola described by the equation $A = A_o - \frac{B[N]}{[N]+K}$ where $A$ is the amount of $^{3}$H Oxo M bound at the guanine nucleotide concentration $N$. $A_o$ is the amount of $^{3}$H Oxo M bound in the absence of guanine nucleotides and $K$ is the dissociation constant of the G-protein for the guanine nucleotide. $B$ describes the maximum amount of Oxo M that dissociates as a result of nucleotide treatment. Again, Marquardt's algorithm was used to fit the data.
RESULTS

Figure 2.1 shows a typical binding curve for binding of the agonist carbachol to membranes of CHO cells overexpressing the m2 receptor. The binding curve was obtained by titrating the bound, radiolabeled antagonist [³H] NMS with unlabeled carbachol ranging in concentration from 10 pM to 1 mM. The graph shows the fractional saturation of the receptor with [³H] NMS as a function of carbachol concentration. As mentioned in the introduction the binding curve is shallow and extends over a concentration range of 6 orders of magnitude. It was fit assuming three classes of agonist binding sites as described under 'Materials and Methods'. Two classes of sites were insufficient in fitting the data since fitting to two classes resulted in systematic errors, whereas fitting to three classes gave residuals randomly scattered around zero. In this experiment carbachol bound with the three dissociation constants $K_1 = (6.3 \pm 6.8)$ nM, $K_2 = (2.6 \pm 1.5)$ µM and $K_3 = (54 \pm 31)$ µM to fractions of $F_1 = 0.20 \pm 0.05$, $F_2 = 0.52 \pm 0.10$ and $F_3 = 0.28 \pm 0.11$ of L-QNB binding sites. The experiment shown is representative of at least ten experiments, and the shape of the binding curves and the fitted parameters were reproducible. The dissociation constants obtained varied somewhat from one experiment to the next, and up to 5-fold differences were observed. This variation may be due to differences in the membrane preparation used, but more likely stems from difficulties in fitting the data.

To examine the effect of guanine nucleotide binding proteins on the agonist binding properties of the receptor, membranes containing the receptor were reconstituted with different amounts of G-proteins. Reconstitution was performed as described under 'Materials and Methods' with the exception that 0.8 mL, 1 mL, 1.5 mL and 2 mL of G-proteins in buffer C were used resulting in cholate concentrations of 0.55%, 0.6%, 0.68% and 0.89% before dialysis. Binding curves using the agonist carbachol were performed on the reconstituted membranes and are shown in Figure 2.2. The parameters obtained
from the fitting routine are summarized in Table 2.1. Classes of sites that were present at 15% or less of total ligand binding sites often gave dissociation constants that were not well determined due to problems in fitting the data. However, dissociation constants were reproducible in a large number of experiments. As can be seen in Figure 2.2, varying the G-protein to receptor ratio had a profound effect on the shape of agonist binding curves. This effect is reflected in an increase in $F_1$, the fraction of superhigh affinity binding sites, in Table 2.1. Thus, the results from this experiment suggested that the ratio of G-proteins to receptor controlled the fraction of receptors showing superhigh affinity agonist binding without affecting any of the dissociation constants. It should be pointed out that it was possible to increase $F_1$ to 72% whereas in the past a maximum of 50% had been observed (Dr. M. Tota, unpublished results).

Another way of looking at the role of G-proteins is to examine the effect of guanine nucleotides on agonist binding. Binding experiments using the agonist Oxo M were performed in the presence of different concentrations of the nonhydrolyzable GTP analogue GppNHp. Figure 2.3 shows the binding curves in the presence of zero and 84 nM GppNHp. In the presence of GppNHp the binding curve was shifted to the right. This shift could be attributed to a decrease in the fraction of superhigh affinity binding, $F_1$, as can be seen from the fitted parameters summarized in Table 2.2. No effect of GppNHp on the dissociation constants of the agonist was seen. Figure 2.4 shows a plot of the percentage of L-QNB sites showing superhigh affinity for Oxo M as a function of GppNHp concentration. Nonlinear least squares fitting of the data to an inverse hyperbolic function yielded an apparent dissociation constant for GppNHp of $(1.64 \pm 0.48)\, \text{nM}$. Even at very high concentrations of the guanine nucleotides $(11 \pm 2)\%$ of L-QNB sites showed superhigh affinity for Oxo M. In this set of experiments the decrease in $F_1$ seemed to be correlated with an increase in $F_3$, the low affinity class of binding sites. The results shown suggested that superhigh affinity Oxo M binding can be used to quantitate the effects of guanine nucleotides on agonist binding and to obtain
apparent dissociation constants for guanine nucleotide binding to G-proteins in the presence of liganded receptor. Reconstituted membranes were incubated with 1 nM [3H] Oxo M and varying concentrations of the four guanine nucleotides GDP, GTP, GTP\textsubscript{S} and GppNHp for 1 hour. 1 nM [3H] Oxo M binding decreased as a function of nucleotide concentration for each of the guanine nucleotides tested and could be fit to a hyperbola. This procedure yielded the following apparent dissociation constants: K\textsubscript{D}(GDP) = (447 ± 121) nM, K\textsubscript{D}(GTP) = (10.8 ± 1.7) nM, K\textsubscript{D}(GTP\textsubscript{S}) = (0.55 ± 0.18) nM and K\textsubscript{D}(GppNHp) = (1.6 ± 0.9) nM. It can be seen from Table 2.1 that, if the G-protein to receptor ratio was low enough, the superhigh affinity binding site became unobservable. This was the case for some CHO cell clones that expressed the muscarinic receptor at very high levels. If homogenized cells from these clones were used in agonist binding experiments, no effect of guanine nucleotides was observed. Table 2.3 shows the results from binding experiment with three different agonists in the presence and absence of 10 μM of the nonhydrolyzable GTP analogue GTP\textsubscript{S}. The nomenclature K\textsubscript{2}, K\textsubscript{3}, F\textsubscript{2} and F\textsubscript{3} was used for the sake of consistency with lower expression clones that in addition showed F\textsubscript{1} and K\textsubscript{1}. The radioactive tracer used in these experiments was [3H] N-methyl scopolamine ([3H] NMS). In the homogenized cell preparation used NMS bound to the same number of sites as L-QNB, and no differences were observed between agonist titration curves with [3H] NMS or [3H] L-QNB as tracer. In addition to the previously mentioned agonists carbachol and Oxo M the table shows binding parameters for pilocarpine. Pilocarpine is a partial agonist (Tota & Schimerlik, 1990), and at low receptor expression levels recognized only two classes of sites (data follows), whereas three classes of sites were needed to fit carbachol and Oxo M binding data obtained under the same conditions. Table 2.3 shows that the higher affinity site recognized by pilocarpine was sensitive to guanine nucleotides and disappeared in the presence of 10 μM GTP\textsubscript{S}. The dissociation constant of the lower affinity site was unaffected in site of the observed variability of this parameter.
As mentioned in the introduction, pertussis toxin catalyzes the ADP-ribosylation of the G_{i} family of G-proteins. Table 2.4 shows carbachol and Oxo M binding data for a homogenized cell preparation that had previously been treated with pertussis toxin as described under 'Materials and Methods'. Like guanine nucleotides, pertussis toxin treatment only affected the superhigh affinity class of agonist binding sites. This class of sites was not observed after PTX-treatment, although the number of antagonist binding sites was unaffected. Unfortunately, the data did not allow a conclusion about the partitioning of the former F_{1} sites into the two lower affinity classes of sites. In whole cells only two affinities were observed with the agonists carbachol, Oxo M and acetylcholine and a single class of sites with the partial agonist pilocarpine (V.A. Mosser, unpublished results). None of these sites were affected by PTX treatment.

In whole cells, binding curves were right-shifted by approximately an order of magnitude compared to homogenized cells and membrane preparations. This rightward shift was lost when the cells, while still attached to culture plates, were permeabilized with 0.05% digitonin (Mosser & Hirschberg, unpublished results). Permeabilization was monitored with trypan blue, which is excluded by intact cells but enters permeabilized cells, making them appear blue under a light microscope. Cells that die during permeabilization lift off from the culture plates and are lost during the wash steps. The following binding experiment was thus not complicated by the presence of dead cells. In contrast to permeabilization of whole cells, freezing and thawing of cell homogenates and use of culture media instead of buffer A was without effect on agonist binding curves.

To try to discern between different ligand binding models it was of interest to systematically compare binding of different agonists. For this purpose four membrane preparations were chosen, and agonist titrations with carbachol, Oxo M, acetylcholine and pilocarpine were performed in parallel. The tracer used was [\textsuperscript{3}H] NMS. The results are shown in Table 2.5 for the two preparations of lower receptor density and in Table 2.6 for the higher receptor density preparations. The specific activities were 39 and 14
30 pmoles receptor per mg protein for the low receptor density membranes and 377 and 150 pmoles per mg protein for the membranes with higher receptor density. It should be pointed out that the fraction of pilocarpine binding sites listed as F2 is the site that is sensitive to guanine nucleotides and thus corresponds to F1 for the other agonists. Again, the guanine nucleotide sensitive classes of sites were either reduced in the case of pilocarpine or unobservable in membranes with high receptor density. In contrast, the agonist dissociation constants seemed unaffected by the 10-fold difference in specific activity between preparations 1 and 2 and preparations 3 and 4. For the ligands carbachol, Oxo M and acetylcholine the fraction of high affinity sites, F2, was close to 50% in all of the membrane preparations.

When whole cells were treated with BCM as described under 'Materials and Methods', about 70% of surface sites, defined as sites accessible to [3H] NMS binding, were lost due to irreversible alkylation. The remaining sites showed a single apparent affinity for all four agonists previously mentioned when binding was measured in whole cells (V.A. Mosser, unpublished results). In contrast, when the treated cells were homogenized and the homogenate was used in binding experiments, three classes of sites were necessary to fit the data. The binding of Oxo M to homogenized BCM-treated cells is shown in Figure 2.5. It is virtually identical to binding curves obtained with untreated homogenized cells. Table 2.7 shows the fitted parameters for carbachol and Oxo M binding to homogenized BCM-treated cells. They are not significantly different from the previously reported parameters for untreated homogenized cells. This is in agreement with results from membrane preparations that had been treated with either BCM or acetyethylcholine mustard on the membrane-level instead of the whole cell-level (C. Hopf, unpublished results).

The effect of low receptor numbers per cell can be more directly examined in cell lines that do not overexpress the receptor. The Chinese hamster ovary cell line CHO_Lpm2 expresses the same subtype of the muscarinic receptor (porcine m2) as the previously
discussed cell lines, but at approximately two orders of magnitude lower levels. Agonist binding to whole cells of this cell line could be fit assuming a single class of binding sites (V.A. Mosser, unpublished results). Alternatively, the cells were homogenized and binding of oxotremorine M was examined in the homogenate. The obtained binding data are shown in Figure 2.6 and required three classes of sites for adequate fitting. Nonlinear least squares fitting yielded the three dissociation constants $K_1 = (13 \pm 3)$ nM, $K_2 = (0.40 \pm 0.17)$ μM and $K_3 = (25 \pm 4)$ μM corresponding to fractions of sites $F_1 = 0.52 \pm 0.05$, $F_2 = 0.28 \pm 0.04$ and $F_3 = 0.20 \pm 0.06$. While the dissociation constants did not seem to be affected by the low level of receptor, this binding curve showed the largest fraction of super high affinity agonist binding sites observed in nonreconstituted membranes.
DISCUSSION

The data presented generally fit well to a model assuming three noninteracting classes of sites producing no systematic errors. The following discussion will examine the results within the frame of this model and try to assign physical meaning to the classes of sites. Other binding models and their implication will also be discussed and compared to the three-site model.

Results from reconstitution experiments, guanine nucleotide treatment and pertussis toxin treatment all pointed to a link between guanine nucleotide binding proteins and the superhigh affinity class of agonist binding sites. Increasing the G-protein to receptor ratio by reconstituting membranes with an excess of purified G-protein resulted in a large increase in the fraction of superhigh affinity binding sites. The total number of binding sites was unaffected, and the increase in F₁ could be correlated with a decrease in F₃. None of the dissociation constants were affected. The opposite effect was observed when the preparation was incubated with guanine nucleotides. With increasing guanine nucleotide concentration the fraction of superhigh affinity binding sites were shifted to low affinity. Again there was no effect on any of the dissociation constants.

The results from both experiments agreed with results from rat heart membranes (Burgen, 1987) and were consistent with the F₁ site being a receptor G-protein complex. This complex had to be able to form from the low affinity fraction of sites in the presence of unliganded G-proteins. Guanine nucleotides dissociated the complex causing the receptor to return to its low affinity state. To be consistent with the notion of independent, noninterconvertible classes of sites, the amount of receptor G-protein complex formed had to be limited by the availability of G-proteins, so that the receptors that are able to couple to G-proteins constitutes one class of binding sites and receptors without available G-proteins represent a second, independent class. The same conclusion is reached, if formation of the ternary complex involving the ligand, the receptor and a G-
protein is assumed to be a two-step process. Depending on the order of the ligand-binding and the G-protein coupling step two such mechanisms are possible and both will be discussed briefly.

(1) \[ R + L + G \rightleftharpoons RL + G \rightleftharpoons RGL \]

where \( R \) is the pm2 mAChR, \( L \) the ligand and \( G \) the free G-protein.

Equilibrium constants for each step are described as

\[
K_1 = \frac{[R][L]}{[RL]} \quad \text{and} \quad K_2 = \frac{[RL][G]}{[RGL]}
\]

two limiting cases have to be considered:

a) \( G_{Tot} \gg R_{Tot} \)

with \([RGL] = \frac{R_{Tot}G_{Tot}[L]}{K_2 + G_{Tot}} \frac{K_1K_2}{K_2 + G_{Tot}} + [L] \)

and b) \( R_{Tot} \gg G_{Tot} \)

with \([RGL] = \frac{G_{Tot}[L]}{K_1K_2} \frac{R_{Tot}}{R_{Tot} + [L]} \left(1 + \frac{K_2}{R_{Tot}} \right) \)

Only the second case predicts an effect of G-protein concentration on maximum formation of the ternary complex but not on the apparent dissociation constant for \( L \).

However, in order for this mechanism to be in agreement with the reconstitution experiment (Table 2.1, Figure 2.2), only 2% of detected GTP\(_7\)S binding sites would be attributed to G-proteins capable of coupling to the pm2 receptor. This conflicts with
results obtained with the same G-protein preparation that showed 30% of the GTP\textsubscript{S} sites being responsive to the muscarinic agonist carbachol (Tota, 1987).

\begin{equation}
R + G + L \rightleftharpoons RG + L \rightleftharpoons RGL
\end{equation}

with \( K_1 = \frac{[R][G]}{[RG]} \) and \( K_2 = \frac{[RG][L]}{[RGL]} \)

\begin{align*}
a) \quad & G_{Tot} \gg R_{Tot} \quad \text{with} \quad [RGL] = \frac{R_{Tot} [L]}{K_1 K_2 + K_2 + [L]} \\
b) \quad & R_{Tot} \gg G_{Tot} \quad \text{with} \quad [RGL] = \frac{G_{Tot} [L]}{R_{Tot} K_1 K_2 + K_2 + [L]}
\end{align*}

This mechanism predicts no effect of G-protein concentration on the apparent dissociation constant, if \( R_{Tot} \gg G_{Tot} \) or \( G_{Tot} \gg K_1, R_{Tot} \). The latter case shows only one affinity since all of the receptor can be coupled to G-proteins. In order to see two affinities \( R_{Tot} \) has to be greater than \( G_{Tot} \), which is equivalent to the previous statement that RG formation is limited by the availability of G-proteins.

The two mechanisms shown can be added to form a square, which is referred to as the ternary complex model (Jacobs and Cuatrecacas, 1976) and will be discussed below. If one assumes that \( F_1 \) is the fraction of receptor responsible for the physiological response, the ligand could be activating this fraction by changing the affinity of the complex for GDP (Tota et al., 1987). A negative allosteric effect of agonists on GDP binding agrees with the finding that GDP caused dissociation of superhigh affinity \(^3\text{H}\) Oxo M binding. However, GTP and its analogues have to cause Oxo M dissociation by a different mechanism, since no effect of agonists on GTP\textsubscript{S} affinity was observed (Tota et al., 1987). Numerous such mechanisms are possible, but the most straight-forward seems to be a negative allosteric effect of GTP and its analogues on G-protein subunit
interactions. That only a fraction of the total receptor population is active in causing a physiological response is not unreasonable considering that the receptor was expressed at unphysiologically high levels. However, in porcine atria the fraction that showed nucleotide-sensitive superhigh affinity agonist binding was still only 30% of total receptor (unpublished results). On the other hand, if one assumes that one or both of the lower affinity states are the active species, one has to again conclude that the ligand-effect lies downstream from G-protein coupling. Since G-proteins are involved in eliciting a physiological response, the active receptor species has to be capable of interacting with G-proteins. The lack of effect of guanine nucleotides and pertussis toxin on the two lower affinity states showed that G-proteins do not change the ligand affinity of these states. On the basis of microscopic reversibility it follows that ligand binding to these states does not alter G-protein coupling, thus the effect has to lie downstream.

The IC50s for guanine nucleotide inhibition of 1 nM [3H]Oxo binding were in good agreement with thermodynamic dissociation constants obtained for GTPγ35S binding and GDP displacement of GTPγ35S in the presence of muscarinic agonists (Tota et al., 1987). Why a fraction of superhigh affinity Oxo M binding sites seemed insensitive to GppNHp is unclear.

Since the three-site model assumes that the fractions of sites with different binding affinity are ligand-independent, it predicts that they should be the same for different ligands. To test this hypothesis the experiment using four different agonists and four membrane preparations was initiated. In light of the difficulties in fitting the data, the fractions observed with the full agonists carbachol, Oxo M and acetylcholine were in agreement (Tables 2.5 and 2.6). In contrast the partial agonist pilocarpine clearly recognized different fractions. Fitting pilocarpine binding data from both whole cells and membrane preparations generally requires one class of sites less than fitting data from the other three agonists. The fraction that bound pilocarpine with higher affinity equaled the superhigh affinity fraction observed with the other agonists, if one assumes that fractions
of 10% are not always observable when fitting to three classes of sites. Together with the guanine nucleotide sensitivity this data suggests that the site that was termed F2 for pilocarpine is the same as F1 for carbachol, Oxo M and acetylcholine. Further, pilocarpine binds with the same affinity to the other two classes of sites. Whether this difference is directly related to pilocarpine's action as a partial agonist is unclear at the moment. The factors relating the dissociation constants $K_1$, $K_2$ and $K_3$ were similar for carbachol, Oxo M and acetylcholine, and the following order was observed $\frac{K_3}{K_1} > \frac{K_2}{K_1} > \frac{K_3}{K_2}$ for pilocarpine $> \frac{K_3}{K_2}$. It will be of interest to test whether any of these factors are correlated to agonist efficacies in physiological assays. Whereas the fractions of sites were dependent on the receptor density of a given membrane preparation as previously discussed, no effect on any of the dissociation constants was observed. For all of the membrane preparations F2 seen with the full agonists tested was close to 50%. Whether this finding is significant is unclear.

The same set of data was alternatively fit to a ternary complex model as described by Jacobs and Cuatrecacas (1976). The model is described by the following set of equations

\[
\begin{align*}
R+L & \rightleftharpoons RL & (K_1) \\
R+G & \rightleftharpoons RG & (K_2) \\
RL+G & \rightleftharpoons RLG & (\alpha K_2) \\
RG+L & \rightleftharpoons RLG & (\alpha K_1)
\end{align*}
\]

where the thermodynamic dissociation constants are shown in brackets. In order to fit data to this model, the model was simulated allowing some of the binding parameters to vary, as described under 'Materials and Methods'. The fitted curve generally agreed well with the data and often looked identical to the curve obtained from fitting to the three-site model. For binding data obtained with the same ligand, the ligand-dependent parameters
(K₁ and α) were in good agreement, and preparation-dependent parameters (the G-protein concentration and K₂) were similar for titration curves using the same preparation. However, several observations lead to the conclusion that the ternary complex model was inadequate in explaining the presented results. It was found that only a narrow range of receptor to G-protein ratios produced simulated binding curves shallow enough to fit the data. For example, if the previously discussed set of experiments was fit to the ternary complex model, the obtained receptor to G-protein ratio only varied by a factor of two between the preparations of low and high receptor density, although the receptor expression levels varied by a factor of 10. Also, if one assumes that the receptor G-protein complex has higher affinity for agonists than the free receptor, the ternary complex model only allows for inflections in the top half of the binding curve, and only if the total G-protein concentration is less than the total receptor concentration. This clearly disagrees with the results in Figure 2.2, which show an inflection at 0.3 fractional saturation for a preparation with a 28-fold excess of GTP₅S sites over QNB sites.

The limitations of the ternary complex are discussed in detail by Wells and his colleagues (Lee et al., 1986 and Wong et al., 1986). The most obvious disagreement between the presented results and the ternary complex is the fact that in the absence of G-protein coupling (e.g. after guanine nucleotide or pertussis toxin treatment) two classes of sites were required to fit the data. In the ternary complex model deviations from simple hyperbolic binding isotherms are entirely due to G-protein effects, thus without G-protein coupling one class of sites should be adequate to fit binding data.

The idea that two classes of agonist sites are intrinsic to the receptor population is supported by data obtained with purified pm2 receptor (Peterson et al., 1984). The recombinant m2 receptor was purified from CHO cell membranes and stored in 0.08% digitonin and 0.016% sodium cholate. At low temperatures (equal to or less than 25°C) this preparation showed two classes of sites with respect to carbachol binding. The dissociation constants were similar to the two lower affinity dissociation constants in
membrane preparations. For purified receptor preparations the shape of agonist binding
curves is dependent on temperature and on detergent concentration (G.L. Peterson,
unpublished results). Low temperature and low detergent concentration favored the
appearance of the higher affinity class of sites, whereas only the low affinity class was
seen at temperatures higher than 25°C. This finding suggested that receptor aggregation
or isomerization is the basis for the two agonist affinities rather than differential post-
translational modifications.

Results discussed in Chapter IV also pointed to the possibility of receptor
aggregation. In order to produce two ligand affinities, aggregate formation and
dissociation or receptor isomerization would have to be slow on the time scale of the
ligand binding experiment. If these reactions occurred rapidly, only one intermediate
affinity would be measured. Since aggregate formation is most likely when the receptor
concentration is high, the ligand binding experiments using homogenates from BCM-
treated cells and CHO_{Lpm2} cells were initiated. Similar to untreated preparations, BCM-
treated cell homogenates showed approximately 50% of binding sites having intermediate
affinity for carbachol and Oxo M. No significant effect on the relative amounts of F_2 and
F_3 were observed. However, it should be kept in mind that the concentration of receptor
protein was not reduced in BCM-treated cells. Only the number of free binding sites was
reduced which may not effect receptor aggregation. In contrast, the receptor
concentration was reduced by approximately 100-fold in CHO_{Lpm2} cells. Oxo M binding
to CHO_{Lpm2} cell homogenates showed only 28% of binding sites having intermediate
affinity. This finding agrees with the idea of receptor aggregation, although it certainly
does not prove it. In Chapter IV it was concluded that reconstitution reduces receptor
aggregation. Generally, reconstituted membrane preparations showed lower values for F_2
than nonreconstituted preparations. If the hypothesis of monomers and receptor
aggregates being responsible for the two lower affinity states is extended to the whole cell
studies described, it has to be concluded that homogenization affects the aggregation state
of the receptor. This could happen either through a change in membrane organization (e.g. receptor patching) or a change in surface area going from whole cells to membrane vesicles/sheets.

In conclusion, the presented data fit best to a model assuming three ligand-independent classes of binding sites with the highest affinity class being a precoupled receptor G-protein complex. The physical identity of the two lower affinity classes cannot be assigned unequivocally, but the simultaneous existence of receptor monomers and dimers or higher aggregates agrees with the present observations. Although the presented discussion leaves many unanswered questions, it is hoped that it will be helpful in designing future experiments and instructive in evaluating different models.
Figure 2.1  Carbachol binding to CHO cell membranes containing the recombinant pm2 muscarinic receptor.

Membranes from CHO cells overexpressing the receptor were diluted 500-fold into buffer A resulting in a final L-QNB site concentration of 192 pM. Carbachol binding was determined as described under 'Materials and Methods' using 1.5 nM [³H] NMS as the tracer. The normalized fractional saturation of the receptor with [³H] NMS is plotted versus the competing concentration of carbachol. The line shows the best fit of the data to the equation describing the three-site model. Fitted parameters were $K = (0.56 \pm 0.05)$ nM, $K_1 = (6.3 \pm 6.8)$ nM, $K_2 = (2.6 \pm 1.5)$ nM, $K_3 = (54 \pm 31)$ pM, $F_1 = 0.20 \pm 0.05$, $F_2 = 0.52 \pm 0.10$ and $F_3 = 0.28 \pm 0.11$. 
Figure 2.1
Figure 2.2  Effect of the G-protein to receptor ratio on carbachol binding.

Reconstitution of CHO cell membranes with purified G-proteins was performed as described under 'Materials and Methods' using 0.8, 1, 1.5 and 2 ml of G-proteins in buffer C. Panels A, B, C and D show carbachol displacement of 150 pM [3H] L-QNB for the four reconstituted preparations in order of increasing G-protein to receptor ratio. The receptor concentration in the assay was 212 pM, 153 pM, 71 pM and 47 pM, respectively. The fitted curves are shown as the solid lines and the parameters are listed in Table 2.1.
Figure 2.2
Figure 2.3  Effect of guanine nucleotides on Oxo M binding.

CHO cell membranes reconstituted with purified G-proteins were diluted into buffer A to give a final receptor concentration of 130 pM. Oxo M binding was analyzed in competition with 150 pM [³H] L-QNB as described under 'Materials and Methods'. Binding curves in the absence of guanine nucleotides and in the presence of 84 nM GppNHp are shown. The fitted parameters are listed in Table 2.2.
Figure 2.3
Figure 2.4 Superhigh affinity agonist binding as a function of guanine nucleotide concentration.

F₁ from Table 2.2 was converted into percent and plotted versus GppNHp concentration. The line represents a nonlinear least squares fit of the data to a hyperbolic function. The fitted parameters are listed in the text.
Figure 2.4

Superhigh affinity OXO M binding (%) vs. [GppNHp] (nM)
Figure 2.5  Oxo M binding to homogenized, BCM-treated cells.

CHO cells overexpressing the muscarinic receptor were treated with BCM as described under 'Materials and Methods'. Following mustard treatment the cells were homogenized and diluted to a receptor concentration of 230 pM in L-QNB sites. Oxo M binding was analyzed in competition with 1.5 nM [³H] NMS. Binding parameters are listed in Table 2.7.
Figure 2.5

Fractional saturation with $[\text{H}^3\text{NMS}]$ vs [Oxo M] (µM)

- X-axis: [Oxo M] (µM)
- Y-axis: Fractional saturation with $[\text{H}^3\text{NMS}]$
Figure 2.6  Oxo M binding to homogenized CHO_LEpm2 cells.

CHO_LEpm2 cells expressing the m2 muscarinic receptor at roughly 10000 receptors per cell were homogenized and diluted into buffer A to a final receptor concentration of 59 pM. Oxo M binding was examined in competition with 1.5 nM [3H] NMS. The line represents a fit of the data to the equation describing the three-site model. The obtained parameters were $K = (1.22 \pm 0.02)$ nM, $K_1 = (13 \pm 3)$ nM, $K_2 = (0.40 \pm 0.17)$ μM, $K_3 = (25 \pm 4)$ μM, $F_1 = 0.52 \pm 0.05$, $F_2 = 0.28 \pm 0.04$ and $F_3 = 0.20 \pm 0.06$. 
Figure 2.6

Fractional saturation with \([H^3]NMS\) vs. \([\text{Oxo M}] (\mu\text{M})\)
Table 2.1  Effect of G-protein to receptor ratio on carbachol binding.

Listed are the parameters obtained from fitting the carbachol binding curves shown in Figure 2.2 to the equation describing the three-site model.
<table>
<thead>
<tr>
<th>[QNB sites] (nM)</th>
<th>[GTP(_\gamma)S sites] (nM)</th>
<th>GTP(_\gamma)S sites L-QNB sites</th>
<th>F1</th>
<th>F2</th>
<th>K1</th>
<th>K2</th>
<th>K3</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.12</td>
<td>1.34</td>
<td>11</td>
<td>---</td>
<td>0.15±0.03</td>
<td>---</td>
<td>0.23±0.20</td>
<td>66±6</td>
</tr>
<tr>
<td>0.11</td>
<td>1.51</td>
<td>14</td>
<td>0.10±0.05</td>
<td>0.29±0.07</td>
<td>2.34±3.29</td>
<td>0.91±0.74</td>
<td>35±7</td>
</tr>
<tr>
<td>0.07</td>
<td>1.46</td>
<td>20</td>
<td>0.58±0.02</td>
<td>0.13±0.03</td>
<td>2.36±0.50</td>
<td>1.00±0.84</td>
<td>39±7</td>
</tr>
<tr>
<td>0.08</td>
<td>2.33</td>
<td>28</td>
<td>0.72±0.04</td>
<td>0.08±0.09</td>
<td>1.84±0.72</td>
<td>0.81±2.60</td>
<td>14±9</td>
</tr>
</tbody>
</table>

Table 2.1
Table 2.2  Effect of guanine nucleotides on Oxo M binding.

Binding of Oxo M was analyzed in the presence of different concentrations of GppNHp. The L-QNB site concentration in the assay was 130-140 pM and 150 pM [3H] L-QNB was used as tracer. The table lists the binding parameters obtained from nonlinear least squares fitting.
<table>
<thead>
<tr>
<th>[GppNHp] (nM)</th>
<th>$K_1$ (nM)</th>
<th>$K_2$ (μM)</th>
<th>$K_3$ (μM)</th>
<th>$F_1$</th>
<th>$F_2$</th>
<th>$F_3$</th>
</tr>
</thead>
<tbody>
<tr>
<td>---</td>
<td>0.52±0.07</td>
<td>0.069±0.025</td>
<td>3.80±0.60</td>
<td>0.43±0.02</td>
<td>0.23±0.02</td>
<td>0.34±0.03</td>
</tr>
<tr>
<td>0.42</td>
<td>0.80±0.14</td>
<td>0.184±0.047</td>
<td>6.30±2.14</td>
<td>0.35±0.02</td>
<td>0.43±0.03</td>
<td>0.22±0.04</td>
</tr>
<tr>
<td>0.84</td>
<td>1.78±0.38</td>
<td>0.197±0.049</td>
<td>7.94±2.59</td>
<td>0.32±0.02</td>
<td>0.46±0.03</td>
<td>0.22±0.04</td>
</tr>
<tr>
<td>1.68</td>
<td>0.77±0.15</td>
<td>0.155±0.029</td>
<td>7.96±1.84</td>
<td>0.28±0.01</td>
<td>0.46±0.02</td>
<td>0.26±0.02</td>
</tr>
<tr>
<td>2.52</td>
<td>0.55±0.10</td>
<td>0.204±0.052</td>
<td>6.04±1.82</td>
<td>0.30±0.01</td>
<td>0.42±0.04</td>
<td>0.28±0.04</td>
</tr>
<tr>
<td>4.20</td>
<td>0.44±0.12</td>
<td>0.077±0.013</td>
<td>5.15±0.68</td>
<td>0.20±0.01</td>
<td>0.44±0.02</td>
<td>0.36±0.02</td>
</tr>
<tr>
<td>5.88</td>
<td>3.55±1.65</td>
<td>0.216±0.064</td>
<td>6.20±1.43</td>
<td>0.16±0.03</td>
<td>0.48±0.04</td>
<td>0.36±0.05</td>
</tr>
<tr>
<td>8.40</td>
<td>0.93±0.43</td>
<td>0.086±0.019</td>
<td>4.42±0.64</td>
<td>0.14±0.02</td>
<td>0.45±0.02</td>
<td>0.41±0.03</td>
</tr>
<tr>
<td>84</td>
<td>1.72±1.21</td>
<td>0.140±0.073</td>
<td>5.21±0.97</td>
<td>0.14±0.03</td>
<td>0.33±0.04</td>
<td>0.53±0.05</td>
</tr>
</tbody>
</table>

Table 2.2
Table 2.3  Effect of guanine nucleotides on agonist binding to the lower affinity sites. Membrane preparation with a very high specific activity of 377 pmoles receptor per mg protein were diluted into buffer A to a final receptor concentration of 190-300 pM. Agonist binding was analyzed in competition with 1.5 nM \(^{3}H\) NMS in the presence and absence of 10 \(\mu\)M GTP\(_{\gamma}S\) as described under 'Materials and Methods'. The table shows the binding parameters obtained from fitting the data to the three-site model.
<table>
<thead>
<tr>
<th>Agonist</th>
<th>Guanine nucleotide</th>
<th>$K_2$ (µM)</th>
<th>$K_3$ (µM)</th>
<th>$F_2$</th>
<th>$F_3$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carbachol</td>
<td>---</td>
<td>1.82±1.08</td>
<td>15±10</td>
<td>0.63±0.24</td>
<td>0.37±0.24</td>
</tr>
<tr>
<td></td>
<td>10µM GTPγS</td>
<td>0.33±0.44</td>
<td>2.67±3.31</td>
<td>0.57±0.29</td>
<td>0.43±0.29</td>
</tr>
<tr>
<td>Oxo M</td>
<td>---</td>
<td>0.25±0.19</td>
<td>2.88±1.30</td>
<td>0.60±0.15</td>
<td>0.40±0.15</td>
</tr>
<tr>
<td></td>
<td>10µM GTPγS</td>
<td>0.093±0.044</td>
<td>0.99±0.57</td>
<td>0.73±0.11</td>
<td>0.27±0.11</td>
</tr>
<tr>
<td>Pilocarpine</td>
<td>---</td>
<td>0.14±0.15</td>
<td>26±4</td>
<td>0.19±0.05</td>
<td>0.81±0.05</td>
</tr>
<tr>
<td></td>
<td>10µM GTPγS</td>
<td>---</td>
<td>13±1</td>
<td>---</td>
<td>1.00</td>
</tr>
</tbody>
</table>

Table 2.3
Table 2.4  Effect of pertussis toxin treatment on agonist binding.

Whole cells were treated with pertussis toxin for 14 hours, homogenized and diluted to 110 pM L-QNB sites into buffer A. Agonist binding was analyzed in competition with 1.5 nM [3H] NMS as described under 'Materials and Methods'. Parameters describing the binding of carbachol and Oxo M are shown.
<table>
<thead>
<tr>
<th>Agonist</th>
<th>PTX-treatment</th>
<th>K₁</th>
<th>K₂</th>
<th>K₃</th>
<th>F₁</th>
<th>F₂</th>
<th>F₃</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(nM)</td>
<td>(µM)</td>
<td>(µM)</td>
<td>(µM)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Carbachol</td>
<td>-</td>
<td>1.52±1.30</td>
<td>0.75±8.17</td>
<td>5.3±4.3</td>
<td>0.31±0.06</td>
<td>0.07±0.55</td>
<td>0.62±0.55</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td></td>
<td>0.22±0.12</td>
<td>17.7±5.0</td>
<td>---</td>
<td>0.41±0.05</td>
<td>0.59±0.05</td>
</tr>
<tr>
<td>Oxo M</td>
<td>-</td>
<td>2.76±1.56</td>
<td>0.39±0.10</td>
<td>10.7±2.9</td>
<td>0.20±0.03</td>
<td>0.57±0.03</td>
<td>0.23±0.04</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td></td>
<td>0.28±0.07</td>
<td>9.8±3.3</td>
<td>---</td>
<td>0.77±0.04</td>
<td>0.23±0.04</td>
</tr>
</tbody>
</table>

Table 2.4
Table 2.5  Binding of four muscarinic agonists to membrane preparations of low receptor density.

The tables summarize the binding parameters obtained for binding of carbachol, Oxo M, acetylcholine and pilocarpine to two membrane preparations with specific activities of 39 and 14 pmoles receptor per mg protein, respectively. The preparations were diluted into buffer A to a final receptor concentration of 100-240 pM and 1.5 nM [³H] NMS was used as tracer.
<table>
<thead>
<tr>
<th>Membrane preparation</th>
<th>Agonist</th>
<th>$K_1$</th>
<th>$K_2$</th>
<th>$K_3$</th>
<th>$F_1$</th>
<th>$F_2$</th>
<th>$F_3$</th>
</tr>
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<tr>
<td></td>
<td></td>
<td>(nM)</td>
<td>(μM)</td>
<td>(μM)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>Carbachol</td>
<td>6.3±6.8</td>
<td>2.6±1.5</td>
<td>54±31</td>
<td>0.20±0.05</td>
<td>0.52±0.10</td>
<td>0.28±0.11</td>
</tr>
<tr>
<td></td>
<td>Oxo M</td>
<td>4.8±5.8</td>
<td>0.77±0.27</td>
<td>17±12</td>
<td>0.17±0.05</td>
<td>0.70±0.06</td>
<td>0.13±0.08</td>
</tr>
<tr>
<td></td>
<td>Pilocarpine</td>
<td>---</td>
<td>0.23±0.31</td>
<td>11±7</td>
<td>---</td>
<td>0.29±0.11</td>
<td>0.71±0.11</td>
</tr>
<tr>
<td>2</td>
<td>Carbachol</td>
<td>29±16</td>
<td>6.1±3.2</td>
<td>177±254</td>
<td>0.38±0.05</td>
<td>0.52±0.08</td>
<td>0.10±0.09</td>
</tr>
<tr>
<td></td>
<td>Oxo M</td>
<td>1.9±1.6</td>
<td>0.48±0.23</td>
<td>9.5±6.4</td>
<td>0.26±0.05</td>
<td>0.57±0.08</td>
<td>0.17±0.09</td>
</tr>
<tr>
<td></td>
<td>Acetylcholine</td>
<td>0.65±0.21</td>
<td>0.32±0.08</td>
<td>6.4±2.6</td>
<td>0.36±0.02</td>
<td>0.52±0.03</td>
<td>0.12±0.04</td>
</tr>
<tr>
<td></td>
<td>Pilocarpine</td>
<td>---</td>
<td>0.36±0.23</td>
<td>31±6</td>
<td>---</td>
<td>0.30±0.05</td>
<td>0.70±0.05</td>
</tr>
</tbody>
</table>

Table 2.5
Table 2.6  Binding of four muscarinic agonists to membrane preparations of high receptor density.

Binding parameters for binding of carbachol, Oxo M, acetylcholine and pilocarpine to membranes with specific activities of 377 and 150 pmoles receptor per mg protein are summarized. Binding parameters were obtained from displacement studies using 100-240 pM L-QNB binding sites and 1.5 nM $[^3H]$ NMS as a tracer.
<table>
<thead>
<tr>
<th>Membrane preparation</th>
<th>Agonist</th>
<th>K2</th>
<th>K3</th>
<th>F2</th>
<th>F3</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>Carbachol</td>
<td>0.81±0.38</td>
<td>17±7</td>
<td>0.57±0.10</td>
<td>0.43±0.10</td>
</tr>
<tr>
<td></td>
<td>Oxo M</td>
<td>0.25±0.13</td>
<td>2.9±1.3</td>
<td>0.60±0.15</td>
<td>0.40±0.15</td>
</tr>
<tr>
<td></td>
<td>Acetylcholine</td>
<td>0.10±0.05</td>
<td>2.1±0.6</td>
<td>0.48±0.08</td>
<td>0.52±0.08</td>
</tr>
<tr>
<td></td>
<td>Pilocarpine</td>
<td>0.16±0.19</td>
<td>15±3</td>
<td>0.11±0.04</td>
<td>0.89±0.04</td>
</tr>
<tr>
<td>4</td>
<td>Carbachol</td>
<td>1.40±0.91</td>
<td>38±14</td>
<td>0.45±0.11</td>
<td>0.55±0.11</td>
</tr>
<tr>
<td></td>
<td>Oxo M</td>
<td>0.54±0.30</td>
<td>5.6±5.1</td>
<td>0.76±0.18</td>
<td>0.24±0.18</td>
</tr>
<tr>
<td></td>
<td>Acetylcholine</td>
<td>0.64±0.22</td>
<td>11±5</td>
<td>0.72±0.10</td>
<td>0.28±0.10</td>
</tr>
<tr>
<td></td>
<td>Pilocarpine</td>
<td>0.30±0.54</td>
<td>19±4</td>
<td>0.10±0.06</td>
<td>0.90±0.06</td>
</tr>
</tbody>
</table>

Table 2.6
Table 2.7  Agonist binding to homogenized, BCM-treated cells.

Fitted parameters are shown for the binding of carbachol and oxotremorine M to homogenized cells treated with BCM prior to homogenization. The data for oxotremorine M binding are shown in Figure 2.5. Receptor concentrations in the assay were 200 pM for carbachol binding and 230 pM for Oxo M binding.
<table>
<thead>
<tr>
<th>Agonist</th>
<th>K₁ (nM)</th>
<th>K₂ (μM)</th>
<th>K₃ (μM)</th>
<th>F₁ (μM)</th>
<th>F₂ (μM)</th>
<th>F₃ (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carbachol</td>
<td>42±14</td>
<td>9.68±4.62</td>
<td>132±57</td>
<td>0.27±0.02</td>
<td>0.42±0.09</td>
<td>0.31±0.09</td>
</tr>
<tr>
<td>Oxo M</td>
<td>27±6</td>
<td>3.55±0.74</td>
<td>61±17</td>
<td>0.36±0.02</td>
<td>0.48±0.03</td>
<td>0.16±0.04</td>
</tr>
</tbody>
</table>

Table 2.7
CHAPTER III
INFLUENCE OF pH ON BINDING OF MUSCARINIC LIGANDS

INTRODUCTION

Several lines of evidence point to an ionic interaction of muscarinic ligands with a negatively charged residue on the receptor. Most, if not all, muscarinic ligands are either quaternary ammonium or sulfonium ions or tertiary amines expected to be protonated at physiological pH (Ringdahl, 1989). The positive charge resides at roughly the same distance from the end of the molecule on what has been termed the headgroup of the ligand. In the case of tertiary amine ligands it has been shown that protonation is needed for agonist activity (Burgen, 1965; Hanin et al., 1966). Asselin et al. (1983) showed that protonation was required for binding of the agonists pilocarpine and oxotremorine. The two ligands showed pKs of 7.05 and 8.60, respectively. In contrast binding of the antagonist scopolamine was also reduced at high pH, but the uncharged, deprotonated ligand retained significant affinity for the receptor. Scopolamine possessed a pK of 7.6 and bound with a 6-fold higher dissociation constant in the deprotonated form (Asselin et al., 1983). This observed difference between muscarinic agonists and antagonists agreed with results from other studies suggesting that antagonist binding largely involves hydrophobic interactions whereas agonist binding is mainly facilitated by ionic interactions. Waelbrook et al. (1985) reached this conclusion through studies of the enthalpy and entropy changes involved in ligand binding to muscarinic receptors in rat heart membranes. Almost a decade earlier Aronstam et al. (1977) had suggested that binding of the antagonist L-quinuclidinyl benzilate to membranes from rat brain was mostly driven by hydrophobic interactions.

Ionic interactions with the positive charge on muscarinic ligands requires a complementary negatively charged residue on the muscarinic receptor. Protonation of
this residue should reduce, if not abolish, ligand binding. Thus it should be possible to determine the $pK_A$ of this acidic amino acid by measuring ligand binding as a function of pH. Indeed, Ehlert et al. (1980) showed that binding of the muscarinic agonist (cis)-dioxolane was inhibited significantly below pH 5.5. The same author later determined the $pK_A$ governing N-methylscopolamine binding in rat heart and corpus striatum to be 5.5 (Ehlert & Delen, 1990). Another group studied the pH dependence of binding of a number of muscarinic antagonists in rat heart and found three titratable groups on the receptor (Birdsall et al., 1989). Binding of antagonists specific for the m2 receptor was governed by $pK_A$s in the range of 6.5-6.8. In contrast, antagonists without subtype specificity gave $pK_A$ values ranging from 5.5 to 6.1. The value of 5.9 for N-methylscopolamine agreed reasonably well with the $pK_A$ determined by Ehlert et al. Finally, three $pK_A$ values were obtained in binding studies with the m2-specific antagonist methoctramine. Methoctramine belongs to a group of polymethylene tetramines (Cassinelli et al., 1986). This group is structurally quite different from classical muscarinic antagonists, but binds with high affinity to the m2 subtype. In addition to two $pK_A$s with values of 5.4 and 6.8 methoctramine binding decreased as the pH increased above 7.0. It was concluded that an ionizable group with a $pK_A$ of 7.5 is involved in a conformational change.

The identity of the residues with the mentioned $pK_A$s has not been determined. Candidates for these residues should be conserved throughout the five muscarinic receptor subtypes and possibly in other monoamine receptors such as the adrenergic receptors. This requirement points to four conserved aspartic acid residues in transmembrane regions 2 and 3, namely asp 69, asp 97, asp 103 and asp 120 in the m2 sequence. Mutagenesis studies and affinity labelling with the irreversible antagonist propylbenzylcholine mustard should yield more information about the involvement of these residues. In studies with purified brain muscarinic receptors the chemically reactive aziridinium compound [$^3$H] propylbenzylcholine mustard mainly alkylated asp 103 (m2
sequence), although asp 97 was labeled to a lesser degree (Curtis et al., 1989). That the ligand binding site resides within the transmembrane regions agrees with proteolysis studies (Peterson, unpublished results) and comparisons with the structurally homologous rhodopsins. Also, studies with the β-adrenergic receptor using the fluorescent antagonist carazolol indicated that the ligand binding site is buried in the hydrophobic pore formed by the membrane-spanning domains (Tota and Strader, 1990).

Valuable information has been revealed by site-directed mutagenesis. Mutation of the aspartate corresponding to asp 103 to asparagine in the m1 muscarinic receptor and the β-adrenergic receptor greatly decreased ligand affinity (Fraser et al., 1989; Strader et al., 1988). Mutation of the aspartate corresponding to asp 69 to asparagine in the α2-adrenergic receptor resulted in guanine nucleotide-insensitive agonist binding and a loss of physiological response (Wang et al., 1991). Site-directed mutagenesis studies in this laboratory using the porcine m2 muscarinic receptor agree with this finding. The mutation D69N resulted in loss of superhigh affinity, guanine nucleotide-sensitive agonist binding (W.K. Vogel and Dr. G.L. Peterson, unpublished results) as well as a loss of any detectable physiological response (W.K. Vogel, unpublished results). In α2-adrenergic receptors the aspartate corresponding to asp 120 seemed to be involved in receptor G-protein coupling. Mutation of this residue to asparagine required 100-fold higher agonist concentrations to elicit the full physiological response and showed no superhigh, guanine nucleotide-sensitive agonist binding (Wang et al., 1991). The corresponding mutation in the pm2 muscarinic receptor was either not expressed or resulted in complete loss of ligand binding (Dr. D.J. Broderick, unpublished results). Finally, the mutation D97N did not adversely affect agonist or antagonist binding (W.K. Vogel and Dr. G.L. Peterson, unpublished results). Other conserved amino acid residues are pointed out in the Introduction. However, these residues do not contain groups ionizable in the right pH range to account for the observed pKₐs.
The following study was designed to answer the question whether agonist and antagonists interact with the same residue(s) on the muscarinic receptor, and to try to identify the residue(s) giving rise to the observed pK$_A$ (s). To address the first question the effect of pH on the binding of two agonists and two antagonists to wild-type pm2 receptors was examined. Following, the involvement of specific amino acid residues in ligand binding was tested by studying the pH dependence of ligand binding to a number of site-directed mutants to the muscarinic receptor.
MATERIALS AND METHODS

Abbreviations

CHO cells, Chinese hamster ovary cells; EDTA, ethylene diamine tetraacetic acid; EGTA, ethylene glycol-bis-(β-amino ethyl ether)-N,N',N'-tetraacetic acid; G-protein, guanine nucleotide binding protein; Hepes, 4-(2-hydroxy-ethyl)-1-piperazine ethane sulfonate; NMS, N-methylscopolamine; Oxo M, oxotremorine M; pm2, porcine m2 muscarinic receptor; L-QNB, L-quinuclidinyl benzilate; Tris, Tris (hydroxymethyl amino methane); buffer A, 10 mM Hepes, 5 mM MgCl2, 1 mM EDTA, 1 mM EGTA, pH 7.4; buffer B, 10 mM sodium acetate, 10 mM 3-(N-Morpholino) propane sulfonate, 10 mM Hepes, 5 mM MgCl2, 1 mM EDTA, 1 mM EGTA; buffer D, 20 mM Tris (hydroxymethyl) amino methane, 1 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol, 0.9% cholate, 75 mM sucrose, 5 mM MgCl2, pH 8.0. The mutant receptors were named using the one letter code for amino acids, where D69N stands for the mutation of aspartate residue 69 to asparagine. D97-103N has aspartate residues 97 and 103 changed to asparagine residues.

Materials

All radioligands were purchased from DuPont-New England Nuclear. Specific activities were 41.9 Ci/mmol for [3H] L-QNB, 80.4 Ci/mmol for [3H] NMS and 87.5 Ci/mmol for [3H] Oxo M. Unlabeled Oxo M was obtained from Research Biochemicals Inc., and carbachol was purchased from Aldrich Chemical Company.

All buffers were purchased from Sigma except Hapes which was from Research Organics, Inc. All other reagents were also from Sigma with the exception of MgCl2 from Mallinckrodt, Inc. and sucrose from United States Biochemical Corp.
Membrane preparation

CHO cells expressing the recombinant pm2 muscarinic receptor were used as a source and membranes were prepared as described for porcine atrial membranes (Peterson & Schimerlik, 1984). Prepared membranes were frozen at -80°C in buffer A.

Site-directed mutants were supplied by Walter K. Vogel (D69N, D97N, D69-97N, D97-103N) and Dr. David J. Broderick (D103E, D103C). The mutants were produced according to the method of Kunkel et al. (1987) and stably expressed in CHO cells. In the case of the mutants D69N, D97N, D69-97N and D97-103N membranes were prepared and stored at -80°C. Cells containing the mutant receptors D103E and D103C were homogenized on ice with ten strokes using a Potter-Elvehjem homogenizer with teflon pestle, and the homogenates were used in binding experiments.

In some cases membranes containing the wild-type pm2 muscarinic receptor were reconstituted with purified G-proteins in buffer D as described in Chapter II.

Ligand binding

Ligand binding experiments were performed as described in Chapter II except that membranes/homogenized cells were diluted into buffer B instead of buffer A. Buffer B contained 10 mM sodium acetate, 10 mM 3-(N-Morpholino) propane sulfonic acid (MOPS), 10 mM 4-(2-hydroxyethyl)-1-piperazine ethane sulfonate (Hepes), 5 mM MgCl₂, 1 mM EDTA and 1 mM EGTA and was adjusted to the pH specified. This buffer system was chosen to minimize differences in ionic strength over the range pH 4-9.
Data analysis

Ligand binding data were analyzed as described in Chapter II, and the obtained dissociation constant were converted to their negative logarithm and plotted versus the pH. The dependence of the $p(K_D)_{App}$ on the pH was fit assuming that protonation of one residue on the receptor results in complete loss of ligand binding. This model can be described as follows:

\[
\begin{align*}
&\text{H}^+ \\
&+ \\
&A^+ + R^- \xleftrightarrow[K_D]{K_A} RA \\
&\downarrow \\
&RH
\end{align*}
\]

where R is the receptor, A the ligand and $K_D$ and $K_A$ are the dissociation constants governing ligand binding and protonation, respectively.

The fractional saturation of the receptor with ligand, $\overline{Y}$, is given by

\[
\overline{Y} = \frac{[L]}{K_D} \left( 1 + \frac{[L]}{K_D} + \frac{[H]}{K_A} \right)
\]

Thus the apparent dissociation constant of the ligand is

\[
(K_D)_{App} = K_D \left( 1 + \frac{[H]}{K_A} \right) \quad \text{(Equation 1)}
\]

Results from pH dependence studies are fit to this equation using a nonlinear least squares fitting routine described by Duggleby (1984).
RESULTS

First, the pH dependence of binding of two antagonists was tested. Figure 3.1 shows the binding of the quaternary amine NMS at pH 7.0 and 4.5 to CHO cell membranes reconstituted with purified G-proteins. At both pH values NMS binding was saturable and was corrected for nonspecific binding. The amount of bound radioligand was normalized as percent saturation to account for different receptor concentrations in the two experiments. At pH 4.5 the binding curve was right-shifted by a little over one order of magnitude. Figure 3.2 shows Scatchard plots of the two binding curves. Over a concentration range of two orders of magnitude the Scatchard plots gave straight lines indicating that for both pH values binding was noncooperative and to a homogeneous population of sites. The dissociation constants obtained from direct binding experiments with the radioligand [3H] NMS are plotted versus the pH in Figure 3.3. Thus each point in this figure represents a separate experiment. All binding experiments were performed in the same buffer system as described under 'Materials and Methods' to minimize variations in the ionic strength of the buffer. Specific binding was stable at pH 6-8.5. At low pH (<5.0) approximately 40% of specific binding was lost irreversibly, but since complete binding curves were obtained and analyzed for each pH value and binding was allowed to equilibrate, this loss does not cause errors in the pH dependence. At pH values below 5.5 the affinity of the receptor for NMS is decreased, and the data was fit assuming that protonation of one specific residue on the receptor results in complete loss of ligand binding. The equation describing this model is derived under 'Materials and Methods'. The pKₐ that was obtained for the residue involved in NMS binding was 5.45 ± 0.14. This result was in good agreement with the values of 5.5 and 5.9 measured by Ehlert & Delen (1990) and Birdsall et al. (1989).

The other antagonist examined was the tertiary amine L-quinuclidinyl benzilate (L-QNB). Direct binding of the radiolabeled drug [3H] L-QNB to membranes reconstituted
Figure 3.4 shows the pH dependence of binding of two agonists was examined. As discussed in Chapter II, agonist binding takes place over a concentration range of 5-6 orders of magnitude. In order to measure complete binding curves, the radiolabeled antagonist [³H] L-QNB was used as a tracer and displaced by increasing concentrations of the agonist. This method and the fitting of the data to three independent classes of binding sites is discussed in detail in Chapter II. Membranes reconstituted with purified G-proteins are used in the experiments illustrated in Figure 3.7. The two curves show the displacement of the tracer by increasing concentrations of the quaternary amine carbachol at pH 7.5 and 5.5. While the shape of the curves is similar, the binding isotherm is right-shifted at lower pH. The same experiment was repeated at a number of pH values, and the set of experiments was evaluated with respect to the fractions of receptor in the different classes of agonist binding sites. No trends were observed, and suitable fractions were chosen and fixed in the following separate analysis of the binding curves. The dissociation constants obtained for the superhigh, high and low affinity binding sites are plotted versus the pH in Figure 3.8. Panel D shows the pH dependence of carbachol binding to the low affinity class of sites obtained from a second set of experiments. Only
two classes of binding sites were needed to fit this set of experiments, and the
dissociation constants of the high affinity class were too poorly determined to give a
meaningful pH dependence. Although the apparent $K_D$ values obtained from the second
set of experiments are somewhat smaller, the pH dependence is very similar. Nonlinear
least squares fitting to Equation 1 gave $pK_A$ values of $6.52 \pm 0.20$ for the lowest affinity
class of sites in the first set and $6.71 \pm 0.16$ for the second set. The superhigh and high
affinity classes show a decrease in binding at a pH greater than 7.5. It is possible that this
decrease was an artefact in the data fitting caused by conversion of binding sites to the
low affinity state. This phenomenon was previously observed by Asselin et al. (1983) for
rat heart membranes. For this reason dissociation constants at pH 8.0 and 8.5 were left
out during the analysis of superhigh and high affinity carbachol binding, although the
data points are included in panels A and B. The $pK_A$s obtained for the superhigh and
high affinity classes of sites were $6.84 \pm 0.06$ and $6.61 \pm 0.08$ respectively. Binding of
the second agonist, Oxo M, is analyzed in very much the same way and is illustrated in
Figures 3.9 and 3.10. Like carbachol Oxo M contains a quaternary ammonium group.
Binding to reconstituted membranes at pH 7.5 and 5.5 is shown in Figure 3.9, and binding
over the whole pH range examined is illustrated in Figure 3.10 for each class of binding
sites separately. $pK_A$s were $6.53 \pm 0.12$, $6.67 \pm 0.08$ and $6.84 \pm 0.04$ for superhigh, high
and low affinity binding, respectively. Since Oxo M could be obtained radioactively
labeled, it was possible to directly measure binding to the superhigh affinity class of sites
as shown in Figure 3.11 for pH 7.5 and 5.5. Figure 3.12 shows Scatchard plots of the
data, indicating that binding of the radioligand was indeed saturable and obeyed the law
of mass action. The pH dependence obtained from the direct binding experiments is
shown in panel D of Figure 3.10 for comparison with data obtained from displacement
studies. The observed $pK_A$ was $6.40 \pm 0.22$. Since the two agonists and the two
antagonists gave essentially the same results, as will be discussed later, in the following
studies only one antagonist (NMS) and one agonist (carbachol) were used.
To control for possible effects of the reconstitution procedure, NMS and carbachol binding was examined in nonreconstituted (native) membranes. Carbachol binding to these membranes determined by displacement of L-QNB showed only high and low affinity sites, and the pH dependence of binding to these sites was identical to the one obtained with reconstituted membranes. In contrast NMS binding at low pH was much more stable in native membranes than in reconstituted preparations. Figure 3.13 shows the pH dependence of NMS binding to native membranes. At pH 4.0 the affinity for the ligand was decreased by less than one order of magnitude, whereas \[^{3}H\] NMS binding to reconstituted membranes was barely detectable at pH 4.0 (Figure 3.3). The pK\(_A\) determined for NMS binding to native membranes was 4.50 ± 0.08—one pH unit less than what was found for reconstituted membranes. One would not expect G-proteins to greatly affect antagonist binding, thus it seemed more likely that the shift in pK\(_A\) was due to the reconstitution procedure itself rather than an action of the G-protein. Indeed, when membranes were subjected to a reconstitution procedure substituting buffer D for G-proteins in the same buffer, the treated membranes showed the same pH dependence of NMS binding as the reconstituted membranes. A plot of the negative logarithm of the dissociation constants is shown in Figure 3.14. The pK\(_A\) obtained (5.44 ± 0.13) was indistinguishable from the pK\(_A\) seen with membranes reconstituted in the presence of G-proteins (5.45 ± 0.14). For the following pH dependence studies only nonreconstituted membranes were used.

The second part of this study attempted to identify the amino acid residues on the pm2 receptor that give rise to the observed pK\(_A\) values. Likely candidates are pointed out in the introduction. These residues, namely aspartates 103, 69, 97 and 120, were changed to asparagines by site-directed mutagenesis. The mutants D103N and D120N were either not expressed or showed no detectable ligand binding. This points to an important role of these two residues, although it is possible that the mutation interfered with stable expression independent of ligand binding, e.g. by making the receptor more susceptible to
proteolysis. Fortunately, information about the aspartate residue 103 could be obtained from the double-mutant D97-103N, that was expressed and showed saturable ligand binding.

Another double-mutant, D69-97N, was the first mutant used in pH dependence studies. Figure 3.15 shows direct binding of the radiolabeled antagonist [³H]NMS to native membranes of CHO cells expressing this mutant receptor. Binding was saturable and gave rise to hyperbolic isotherms at all pH values studied. A plot of the negative logarithm of the obtained dissociation constants versus the pH is shown in Figure 3.16. Nonlinear least squares fitting to Equation 1 yielded a pKₐ of 4.26 ± 0.05. Values for the two single mutants D69N and D97N were 4.54 ± 0.10 and 4.43 ± 0.03, respectively. Data are not shown here. The double-mutant D69-97N shows mostly low affinity binding of the agonist carbachol. Carbachol displacement of the tracer [³H]L-QNB at pH 7.5 and 5.5 is shown in Figure 3.17. The experiment was repeated at a number of pH values, and the dissociation constants obtained for the low affinity class of binding sites are plotted versus the pH in Figure 3.18. The curve yielded a pKₐ of 6.70 ± 0.18 for low affinity carbachol binding to the double-mutant. Since this value was identical with results obtained for the wild-type receptor, the single mutants were not examined separately. Since the membranes containing the double-mutant were not reconstituted, this also confirms that agonist binding is not affected by the reconstitution procedure outside of a change of the fractions with different affinities.

To examine the role of aspartate 103 ligand binding to the double-mutant D97-103N was studied. Incubation of the mutant with 10 nM [³H]Oxo M, equivalent to 10 times the wild-type dissociation constant, failed to yield any detectable radioligand binding. Also 1 mM carbachol was ineffective in displacing L-QNB from the mutant receptor at L-QNB concentrations close to its dissociation constant. Thus, the double-mutant D97-103N does not seem to have significant affinity for agonists. The affinity for antagonist is also reduced—by about 50-fold for N-methylscopolamine, but is still measurable.
Figure 3.19 shows the pH dependence of NMS binding to the double-mutant D97-103N. Nonlinear least squares fitting of the data to Equation 1 yielded a pKₐ of 5.64 ± 0.17. The membranes containing the mutant receptor were not reconstituted, although the high pKₐ is in the range of values obtained with reconstituted preparations. The aspartate to glutamate and aspartate to cysteine mutants D103E and D103C also showed no displacement of 15 nM [³H]L-QNB with Oxo M or acetylcholine at concentrations up to 1 mM. Antagonist affinity was reduced similarly to the double-mutant D97-103N. However, since these mutants were expressed at lower levels, it was not possible to obtain meaningful pH dependences.
DISCUSSION

Tables 3.1 and 3.2 summarize the pK_A values obtained with wild-type and mutant pm2 muscarinic receptors. The data were grouped into antagonist pK_As in Table 3.1 and agonist pK_As presented in Table 3.2. The two antagonists examined, NMS and L-QNB showed the same pH dependence of binding to CHO cells reconstituted with purified G-proteins. Within the error, the pK_A values were identical and in good agreement with the value published for NMS binding. Without undergoing a reconstitution procedure native membranes were less sensitive to low pH, and NMS binding to native membranes was governed by a pK_A one pH unit less than that seen with reconstituted membranes. This shift in pK_A was independent of whether G-proteins were added during the reconstitution and was most likely due to the detergent treatment of the membranes. During the reconstitution process membranes were incubated with 0.6% cholate. This concentration of detergent could alter the pH dependence of ligand binding by changing the conformation of the receptor or by changing the lipid environment immediately adjacent to the receptor. In the first case a conformational change of the receptor could either change that residue(s) with which the ligands interact, or change the pK_A of a given residue. The alternative explanation of changing the lipid environment is supported by indications of a role of phospholipids in binding of muscarinic antagonists (Aronstam et al., 1977). It was found that acidic phospholipids enhance the binding of L-QNB, and that L-QNB binding in turn was decreased by pretreatment of the membranes with phospholipases. The authors suggested that phospholipids associated with the muscarinic receptor may provide accessory hydrophobic binding areas—a concept proposed by Ariëns and Simonis (1967).

Membranes from CHO cells expressing the mutant receptors were not subjected to detergent treatment. NMS binding to the mutant receptors D69N and D97N and to the double-mutant D69-97N showed the same pH dependence as binding to wild-type
receptors in a nonreconstituted system. Thus, at least in native membranes, NMS binding seems to involve neither aspartate 69 nor aspartate 97. This finding is supported by the high affinity of these mutants for the antagonist.

In contrast, the double-mutant D97-103N shows reduced affinity for both NMS and L-QNB. In addition, the $pK_A$ for NMS binding to this mutant was shifted by roughly one pH unit. It was very similar to the $pK_A$ values obtained for antagonist binding to reconstituted membranes containing the wild-type receptor. This might indicate that the double-mutant D97-103N is locked into a similar conformation as the detergent-treated wild-type receptor or has the same phospholipid environment. The latter case seems unlikely since both mutations are located within the central pore of the receptor.

Aspartate 103 cannot be the residue giving rise to the $pK_A$ of 5.4, since this $pK_A$ was seen in D97-103N. The $pK_A$ of 4.4 also cannot be associated with aspartate 103. If aspartate 103 had a $pK_A$ value of 4.4 and the mutation D97-103N forced ligand binding to a less preferred residue with a $pK_A$ of 5.6, reconstitution of CHO cell membranes with G-proteins should also lower ligand affinity, which was not observed.

In summary $pK_A$s governing antagonist binding fall into two groups: a group around 4.4 for native membranes and a group around 5.4 for reconstituted membranes. Protonation of the aspartates 69, 97 and 103 did not seem to affect antagonist binding.

As seen in Table 3.2 only one $pK_A$ around 6.6 was observed in agonist binding studies. This $pK_A$ was seen with both agonists tested, carbachol and Oxo M, and described binding to all three classes of sites. This value was significantly higher than any of the $pK_A$s observed with antagonists and was also unaffected by reconstitution of the membranes. This indicated that a different residue was involved in charge-charge interactions with antagonists than with agonists. The superhigh and high affinity classes of sites showed a decrease in agonist binding at a pH greater than 7.0. This was not due to a change in the ligand, since both agonists tested were quaternary amines. However, it might be an effect on the fractions of binding sites showing superhigh and high affinity
binding rather than on the dissociation constants. The decrease was more pronounced for
carbachol than for Oxo M. Whether the fractions or the dissociation constants were
affected, the change in binding had to be due to the deprotonation of a residue at an
allosteric site.

Mutating aspartate residues 69 and 97 to asparagines had no effect on the pH
dependence of carbachol binding, indicating that neither residue is involved in ionic
interactions with the agonist's headgroup. The demonstrated necessity of aspartate 103
for agonist binding indicates that it may be the residue with the pKₐ of 6.6. Since agonist
binding is completely abolished in any mutant involving aspartate 103, this hypothesis
cannot be tested directly.

To end this discussion with a little speculation, the presented results are in
agreement with ionic interactions with aspartate 103 being the major force in agonist
binding and antagonist binding to the same site being controlled mostly by hydrophobic
forces. In this model the two pKₐs observed in antagonist binding studies are due to the
protonation of an allosteric site resulting in loss of ligand binding. The allosteric residue
may very well be aspartate 120. Since aspartate 120 is located at the interface of the cell
membrane and the cytoplasm, it is expected to have a lower pKₐ value than residues
buried in the hydrophobic core of the protein. A conformational change caused either by
detergent treatment or by the mutation D97-103N then results in a shift of the pKₐ of the
allosteric residue. Small conformational changes can conceivably cause rather large pKₐ
shifts of residues located at the membrane interfaces.
Figure 3.1  [³H] NMS binding to reconstituted membranes at pH 4.5 and 7.0.

The binding experiment was performed as described in Chapter IV. CHO cell membranes reconstituted with G-proteins were diluted into buffer B adjusted to the given pH. The final binding site concentration was approximately 1.6 nM. Membranes were incubated with concentrations of [³H] NMS between 40 pM and 100 nM for 1 hour at 25°C. Bound and free radioligand were separated by filtration through Whatman GF/B filters pretreated with 0.1% polyethylene imine. Bound ligand was normalized to 100% at saturation, and the curve through the data was calculated using the fitted parameters (K_D)_{App} = 51 nM at pH 4.5 and (K_D)_{App} = 2.2 nM at pH 7.0 from Figure 3.2.
Figure 3.1 

Fractional saturation with $[\text{H}^3\text{NMS}]$ (%) vs $[[\text{H}^3\text{NMS}]]$ (nM) at pH 7.0 and pH 4.5.
Figure 3.2 Scatchard analysis of [$^3$H] NMS binding to reconstituted membranes.

Data from Figure 3.2 were plotted as bound ligand/free ligand versus bound ligand as described by Scatchard (1949). This transformation was used to assure that binding took place to a homogeneous population of sites. It also allows fitting the data by linear regression and yielded the apparent dissociation constants $(K_D)_{\text{App}} = (51 \pm 6) \text{nM at pH 4.5}$ and $(K_D)_{\text{App}} = (2.2 \pm 0.3) \text{nM at pH 7.0}$. 
Figure 3.2 Bound \([\text{H}^3\text{NMS}](\text{nM})\)

**pH 4.5**

**pH 7.0**

Figure 3.2
Figure 3.3  pH dependence of the dissociation constant of NMS in reconstituted membranes.

Data points in this plot represent separate binding experiments, two of which are shown in Figure 3.1. The dissociation constants obtained from these experiments were converted to the negative logarithm and plotted versus the pH. The line represents a fit of the data to Equation 1 with $pK_A = 5.45 \pm 0.14$ and $K_D = (2.0 \pm 0.3)$ nM.
Figure 3.3
Figure 3.4  Binding of [{sup[3]H}] L-QNB to reconstituted membranes at pH 4.5 and 5.0.

The binding experiment was performed as described in Chapter IV, except that reconstituted membranes were diluted into buffer B adjusted to pH 5.0 and 4.5, respectively. Final binding site concentrations were 1.8 nM at pH 5.0 and 1.6 nM at pH 4.5. Bound ligand was normalized to 100% at saturation, and the curve through the data was calculated using the fitted parameters from Figure 3.5 $K_D^{App} = 86.4$ nM at pH 4.5 and $K_D^{App} = 1.8$ nM at pH 5.0.
Figure 3.5  Scatchard analysis of \([^{3}H]\) L-QNB binding to reconstituted membranes.

Data from Figure 3.4 were plotted according to Scatchard (1949). The lines represent a fit of the data by linear regression yielding \((K_{D})_{\text{App}} = (6.4 \pm 0.4)\) nM at pH 4.5 and \((K_{D})_{\text{App}} = (1.8 \pm 0.)\) nM at pH 5.0.
Figure 3.5
Figure 3.6  pH dependence of the dissociation constant of L-QNB in reconstituted membranes.

Each data point represents a separate experiment. Data points at pH 4.5 and 5.0 were obtained from the direct binding experiments shown in Figure 3.4. All other data points were obtained from the analysis of displacement studies using [3H] L-QNB as a tracer. Displacement studies and their analysis are described in detail in Chapter II. All data points were fit to Equation 1 as shown by the solid line. Fitted data were pK_A = 5.39 \pm 0.17 and K_D = (98 \pm 8) \text{ pM}.
Figure 3.6
Figure 3.7  Carbachol binding to reconstituted membranes at pH 5.5 and 7.5.

The binding curves were obtained by a displacement study using 150 pM [³H] L-QNB as a tracer as described in Chapter II. Binding site concentrations in the assay were approximately 100 pM. Plotted is the fractional saturation of the receptor with [³H] L-QNB normalized to 1 in the absence of carbachol. The lines represent a nonlinear least squares fit of the data to a model assuming three independent classes of binding sites (pH 5.5: K = 191 pM, F₁ = 0.33, F₂ = 0.4, K₁ = 37 nM, K₂ = 2.6 μM, K₃ = 530 μM; pH 7.5: K = 124 pM, F₁ = 0.33, F₂ = 0.4, K₁ = 5.3 nM, K₂ = 0.17 μM, K₃ = 49 μM).
Figure 3.7
Figure 3.8  pH dependence of the dissociation constants of carbachol in reconstituted membranes.

Dissociation constants obtained from displacement studies including the ones shown in Figure 3.7 were converted to the negative logarithm and plotted versus the pH. Panels A, B and C show the pH dependence for the superhigh, high and low affinity classes of sites found in one set of experiments. The data obtained for the low affinity class of sites in a second set of experiments is illustrated in panel D. All data were fit to Equation 1. Fitted parameters were panel A: $pK_A = 6.84 \pm 0.06$ and $K_D$ fixed at $5 \text{nM}$; panel B: $pK_A = 6.61 \pm 0.08$, $K_D$ fixed at $0.15 \mu\text{M}$; panel C: $pK_A = 6.52 \pm 0.20$, $K_D = (46 \pm 16) \mu\text{M}$; panel D: $pK_A = 6.71 \pm 0.16$, $K_D = (20 \pm 6) \mu\text{M}$. Data points at pH 8.0 and 8.5 in panels B and C were omitted during the fitting as discussed in the text.
Figure 3.8
Figure 3.9  Oxo M binding to reconstituted membranes at pH 5.5 and 7.5.

CHO cell membrane reconstituted with G-proteins were diluted into buffer B at pH 5.5 and 7.5 to give final binding site concentrations of 70 pM.

150 pM [3H] L-QNB were displaced by increasing concentrations of Oxo M. Plotted is the fractional saturation of the receptor with [3H] L-QNB normalized to 1 in the absence of Oxo M. The data were fit assuming three independent classes of sites (pH 5.5: $K = 161 \text{ pM}$, $F_1 = 0.4$, $F_2 = 0.4$, $K_1 = 4.6 \text{ nM}$, $K_2 = 3.6 \text{ \mu M}$, $K_3$ undetermined; pH 7.5: $K = 81 \text{ pM}$, $F_1 = 0.4$, $F_2 = 0.4$, $K_1 = 0.5 \text{ nM}$, $K_2 = 0.2 \text{ \mu M}$, $K_3 = 9.5 \text{ \mu M}$).
Figure 3.9

The graph shows the fractional saturation with [H\(^3\)]\text{L}-QNB plotted against [Oxo M] (µM) for pH 5.5 and pH 7.5.
Figure 3.10  pH dependence of the dissociation constants of Oxo M in reconstituted membranes.

Dissociation constants obtained from a set of displacement studies as illustrated in Figure 3.9 are shown as a function of pH. Panels A, B and C represent the superhigh, high and low affinity classes of sites, respectively. Panel D shows the pH dependence of [³H] Oxo M binding to the superhigh affinity class of sites as determined by direct binding experiments illustrated in Figure 3.11. All data were fit to Equation 1 (panel A:  $pK_A = 6.53 \pm 0.12$, $K_D = 0.42 \pm 0.09$ nM; panel B:  $pK_A = 6.67 \pm 0.06$, $K_D = 0.19 \mu$M fixed; panel C:  $pK_A = 6.84 \pm 0.04$, $K_D$ fixed at 9 $\mu$M; panel D:  $pK_A = 6.40 \pm 0.22$, $K_D = 0.81 \pm 0.11$ nM).
Figure 3.10
Figure 3.11 Binding of [$^3$H] Oxo M to the superhigh affinity class of sites in reconstituted membranes at pH 5.5 and 7.5.

Binding experiments were performed as described in Chapter IV, except that reconstituted membranes were diluted into buffer B at pH 5.5 and 7.5 to give final binding site concentrations of approximately 2 nM. Membranes were incubated with [$^3$H] Oxo M concentrations between 40 pM and 10 nM for 1 hour at 25°C. Bound ligand was normalized to 100% at saturation, and the curve through the data was calculated using the fitted parameters from Figure 3.12 ((K_D)_{App} = 165 pM at pH 5.5 and (K_D)_{App} = 0.69 nM at pH 7.5).
Figure 3.11

Fractional saturation with $[\text{H}^3]\text{Oxo M}$ (%) vs. $[\text{H}^3]\text{Oxo M}$ (nM)

- pH 7.5
- pH 5.5

Horizontal axis: $[\text{H}^3]\text{Oxo M}$ (nM)
Vertical axis: Fractional saturation with $[\text{H}^3]\text{Oxo M}$ (%)
Figure 3.12  Scatchard analysis of [³H] Oxo M binding to the superhigh affinity class of sites in reconstituted membranes.

Binding data from Figure 3.11 were plotted according to Scatchard (1949). The good agreement of the data with the fitted line obtained by linear regression shows that at the concentrations used binding was only detected to the superhigh affinity class of binding sites. Fitted parameters were

\((K_D)_{\text{App}} = (1.6 \pm 0.2) \ \text{nM at pH 5.5 and } (K_D)_{\text{App}} = (0.69 \pm 0.07) \ \text{nM at pH 7.5.}\)
Figure 3.12
Figure 3.13  pH dependence of NMS binding to native membranes.

Membranes not subjected to a reconstitution procedure were diluted into buffer B at different pH values to yield final receptor concentrations around 1 nM. Direct binding of [3H] NMS was measured as described in Chapter IV, and the negative logarithm of the obtained dissociation constants was plotted versus the pH. Data were fit to Equation 1 yielding $pK_A = 4.50 \pm 0.08$ and $K_D = (6.3 \pm 0.6)$ nM.
Figure 3.13
Figure 3.14  pH dependence of NMS binding to membranes reconstituted in the absence of G-proteins.

CHO cell membranes were subjected to a reconstitution procedure as described in Chapter II except that G-proteins in buffer D were replaced with buffer D alone. The membranes were then diluted into buffer B at various pH values to give receptor concentrations around 1 nM and used in binding experiments with NMS. The obtained dissociation constants are shown as a function of pH together with a fit of the data to Equation 1 with pK_A = 5.44 ± 0.13 and K_D = (1.4 ± 0.2) nM.
Figure 3.14
Figure 3.15 Binding of [³H] NMS to the double-mutant D69-97N at pH 4.5 and 7.5.

CHO cell membranes containing the mutant receptor D69-97N were diluted into buffer B at pH 4.5 and 7.5 to a final receptor concentration of 2 nM. Direct binding of [³H] NMS was measured as described in Chapter IV. Data were transformed according to Scatchard (1949) and fit by linear regression yielding $(K_D)_{App} = (5.13 \pm 0.35)\text{ nM}$ at pH 4.5 and $(K_D)_{App} = (3.66 \pm 0.19)\text{ nM}$ at pH 7.5.
Figure 3.15 

Fractional saturation with [\(\text{H}^3\text{NMS}\)] (nM)
Figure 3.16  pH dependence of NMS binding to D69-97N.

Dissociation constants obtained from the binding experiments illustrated in Figure 3.15 were converted to the negative logarithm and plotted versus the pH. Data were fit to Equation 1 yielding $pK_A = 4.26 \pm 0.05$ and $K_D = (3.5 \pm 0.4) \text{nM}$. 
Figure 3.16
Figure 3.17 Carbachol binding to the double-mutant D69-97N of pH 5.5 and 7.5.

Binding of carbachol to the mutant receptor D69-97N was examined in displacement studies using the tracer $[^3H]$ L-QNB. Binding site concentrations were 90 pM and 70 pM in buffer B at pH 7.5 and 5.5, respectively. The data were analyzed assuming two independent classes of binding sites as described in Chapter II (pH 5.5: $K = 55$ pM, $F_1 = 0$, $F_2 = 1$, $K_2 = (98 \pm 7) \mu$M; pH 7.5: $K = 29$ pM, $F_1 = 0.19 \pm 0.04$, $F_2 = 0.81 \pm 0.04$, $K_1 = (0.90 \pm 0.61) \mu$M, $K_2 = (29 \pm 2) \mu$M.)
Figure 3.17
Figure 3.18  pH dependence of carbachol binding to D69-97N.

Dissociation constants for the low affinity class of binding sites obtained from displacement studies are plotted as a function of pH. The line represents a nonlinear least squares fit of the data to Equation 1 with $pK_A = 6.70 \pm 0.18$ and $K_D = (17 \pm 2) \mu M$. 
Figure 3.18 pH

[Graph showing the relationship between pH and p(K_D)_{App}]

The graph illustrates the change in p(K_D)_{App} with pH. The data points at pH 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, and 8.5 are marked with error bars indicating variability.
Figure 3.19  pH dependence of NMS binding to the double-mutant D97-103N.

CHO cell membranes containing the double-mutant D97-103N were diluted into buffer B at various pH values to yield final receptor concentrations of 40 nM. [3H] NMS binding was measured as described in Chapter IV. Fitting of $p(K_D)_{App}$ versus pH to Equation 1 yielded $pK_A = 5.64 \pm 0.17$ and $K_D = (64 \pm 11) \text{nM}$. 
Figure 3.19
Table 3.1  Summary of pK$_A$s governing antagonist binding.

The pK$_A$s listed for the specified type of receptor and type of ligand in the presence or absence of reconstitution with purified G-proteins was obtained by performing direct binding experiments over a pH range from 4.0 to 8.5 and fitting the obtained dissociation constants to Equation 1. The receptor concentration in the binding assay was approximately 1 nM.
<table>
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<th>Reconstitution</th>
<th>Ligand</th>
<th>pKₐ ±SD</th>
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<td>Wild-type</td>
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<td>L-QNB</td>
<td>5.39±0.17</td>
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<tr>
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<td>NMS</td>
<td>5.44±0.13</td>
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<tr>
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<td>NMS</td>
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<td>NMS</td>
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<tr>
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<td>NMS</td>
<td>4.54±0.10</td>
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<td>D97N</td>
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<tr>
<td>D97-103N</td>
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<td>NMS</td>
<td>5.64±0.17</td>
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</table>

Table 3.1
Table 3.2  Summary of pKₐs governing agonist binding.

The table summarizes the pKₐs obtained with the agonists carbachol and Oxo M. Displacement studies, illustrated in Figures 3.7 and 3.9, were performed to obtain dissociation constants at different pH values, which were then fit to Equation 1 to yield the listed pKₐ. The third row shows the result of the direct binding experiments with [³H] Oxo M shown in Figure 3.11.
<table>
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<th>Receptor</th>
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<th>Ligand</th>
<th>Class of sites</th>
<th>pKₐ ±SD</th>
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<td></td>
<td></td>
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<td>F₃</td>
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<td>Oxo M</td>
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<td>6.53±0.12</td>
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<td></td>
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<td></td>
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<td>F₃</td>
<td>6.84±0.04</td>
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<tr>
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<td>Carbachol</td>
<td>F₃</td>
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</table>

Table 3.2
CHAPTER IV

KINETICS OF OXOTREMORINE M BINDING TO THE SUPERHIGH AFFINITY CLASS OF SITES

INTRODUCTION

Agonist binding to muscarinic receptors has mostly been examined in competition binding studies using radiolabeled antagonists as tracers. Data obtained in this way have to be fit employing nonlinear least squares fitting routines. The equation fit necessarily contains the investigators bias about the mechanism of binding. For example the same data set can be fit assuming three independent classes of receptor binding sites or a ternary complex model involving a second protein that interacts with the muscarinic receptor. In some cases the data will fit to both models equally well, but the parameters that are obtained from the two fits are different and have completely different physical meaning. For the three-site model, three parameters specific for the ligand and two parameters specific for the membrane preparation are obtained, i.e. the three dissociation constants and the fraction of sites in each class. The ternary complex model contains two ligand-specific parameters (the dissociation constant and the factor by which the modulating protein influences ligand binding) and two preparation-dependent parameters (the concentration and the affinity of the modulating protein). Obviously, the uncertainty about the right binding model poses a major problem in interpreting any competition binding data. But even if one—as currently the majority of researchers do—fits binding data to the three-site model, data analysis is not unambiguous. Most binding curves do not show marked inflections. This is in agreement with dissociation constants separated by less than two orders of magnitude, but it allows for more than one set of parameters to fit the data. Changes in the fractions of sites in each class can be compensated by changes in dissociation constants to give essentially the same fitted curve.
Considering these problems, it is advantageous to directly measure agonist binding where possible, and to try to obtain information on the mechanism of binding. The discovery of the potent muscarinic agonist oxotremorine M has made this methodology available, since radiolabeled oxotremorine M is now commercially available and binds with high affinity to a saturable class of sites. The fraction of muscarinic antagonist sites that bind [3H] oxotremorine M with high affinity varies in different membrane preparations and corresponds to the highest affinity class of sites in the three-site model. As shown in Chapter II, this is the class of sites that is sensitive to guanine nucleotides. Therefore aside from ligand binding per se, [3H] oxotremorine M binding might also reveal information about receptor G-protein interactions.

Several researchers have undertaken binding studies using [3H] oxotremorine M. The dissociation constants obtained for the highest affinity site are generally in good agreement and range from 300 pM in rat heart at 4°C (Harden et al., 1983) to 6.5 nM at 25°C for rat heart and cortex (Gillard et al., 1987). Association and dissociation kinetics were also measured (Harden et al., 1983 and Gillard et al., 1987), but these measurements were mostly used to support equilibrium binding data and were not designed to look in detail at the binding mechanism. The association kinetics were measured at only one ligand concentration, which means that the reported rate relies on the assumption that binding occurs in a single reversible step. Since the obtained kinetic data agreed with equilibrium binding measurements, there was no reason to look for a more complicated binding mechanism. Although in rat heart Gillard et al. found biphasic association and dissociation rates, they could be explained by two receptor populations with the same affinity for [3H] oxotremorine M but different kinetics. Schimerlik (unpublished observations) obtained similar results using the antagonist [3H] L-quinuclidinyl benzilate and membranes from Chinese hamster ovary cells overexpressing the m2 muscarinic receptor. On the other hand when porcine atria was used the results were more complex (Schimerlik & Searles, 1980). Measuring [3H] L-quinuclidinyl benzilate dissociation two
exponentials were observed, but the amplitudes of the two phases were dependent on the concentration of the competing ligand. At very high concentrations of the competing ligand only a single phase was observed. At low concentrations of competing ligand dissociation was also monophasic but showed a 16-fold greater relaxation time. In between two phases were observed with the same slow and fast relaxation times, and the amplitudes of the two phases were a function of the competing ligand concentration. At the time it was concluded that the competing ligand either binds to an allosteric site on the receptor or alters the properties of the membrane preparation.

Similarly complex kinetic behavior has been observed with other G-protein coupled receptors. Results obtained with glucagon and histamine receptors may serve as an example for the kinds of complex phenomena that occur and might also be related to the experiments discussed in this chapter. Bharucha and Tager (1990) studied interactions of glucagon with the canine hepatic glucagon receptor and in addition to fast and slow association and dissociation phases found a component of irreversibly bound ligand. This component was only present at higher temperatures. From these studies it was concluded that two non-interacting populations of sites were present giving rise to the slow and fast association and dissociation phases and that a fraction of each population can be converted to nondissociable sites in a subsequent step. However, not all of the observations agree with this fairly simple model. The formation of irreversibly bound ligand is found to be a first-order reaction rather than a zero-order reaction, and the fraction of ligand that becomes irreversibly bound is a function of ligand concentration. In addition, the biological significance of the formation of nondissociable receptor-ligand-complexes is unclear, although it has been demonstrated for a number of receptors.

Sinkins et al. (1983) studied histamine binding to membranes from guinea pig cerebral cortex. It is unclear whether their preparation contains H2 or H3 histamine receptors or a mixture of both, but receptor heterogeneity is clearly insufficient to explain all of the observations. Two classes of sites are needed to fit the equilibrium binding
data, and association and dissociation kinetics are biexponential. However, the amplitudes of the two phases in the association and dissociation measurements are not the same and also do not correspond to the fractions found in equilibrium binding studies. Further, the fractions corresponding to the two dissociation constants are different for different ligands—both agonists and antagonists. Sinkins et al. end the summary of their paper saying that the difficulty in interpreting equilibrium binding data "may reflect the failure to assess binding in the correct mechanistic context".

Similar problems with equilibrium binding data in this laboratory are the reason why detailed kinetic studies were initiated using the tritium-labeled agonist oxotremorine M. Association of oxotremorine M was measured as a function of ligand concentration, and the effect of different experimental protocols on oxotremorine M dissociation was examined. A presentation of the results is followed by a discussion of different binding models in an attempt to better understand the mechanistics of oxotremorine M binding. Hopefully, the discussion presented here will in some form be applicable to other muscarinic ligands as well as to other G-protein coupled receptors.
MATERIALS AND METHODS

Abbreviations

CHO cells, Chinese hamster ovary cells; DEAE, diethylaminoethyl; DTT, dithiothreitol; EDTA, ethylene diamine tetraacetic acid; EGTA, ethyleneglycol-bis-(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid; G-protein, guanine nucleotide binding protein; G\textsubscript{i}, the inhibitory G-protein; G\textsubscript{o}, the 'other' G-protein; G\textsubscript{s}, the stimulatory G-protein; GDP, guanosine diphosphate; GTP, guanosine triphosphate; GTP\textsubscript{y}S, guanosine 5'-O-(3-thio-triphosphate); GppNHp, guanylylimidodiphosphate; Hepes, 4-(2-hydroxyethyl)-1-piperazine ethane sulfonate; MFD, Dulbecco's Modified Eagle Medium with F-12 Nutrient Mixture (Gibco/Bethesda Research Laboratories); NMS N-methylscopolamine; Oxo M, oxotremorine M; PMSF, phenylmethylsulfonylfluoride; L-QNB, L-quinuclidinylbenzilate; SDS, sodium dodecylsulfate; SH\textsubscript{a}, superhigh affinity; Tris, Tris(hydroxymethyl)aminomethane; buffer A, 10 mM Hapes, 5 mM MgCl\textsubscript{2}, 1 mM EDTA, 1 mM EGTA, pH 7.4; buffer B, ice cold phosphate buffered saline, 0.1 mM PMSF, 1 mM benzamidine, 1 μg/ml soybean trypsin inhibitor, pH 7.0; buffer C, 25 mM imidazole, 250 mM sucrose, 50 mM EDTA, 1 μg/ml pepstatin A, 17 μg/ml PMSF, 1 μg/ml benzamidine, 10 μg/ml bacitracine, 5 μg/ml leupeptin, 2 μg/ml aprotinin, 10 μg/ml soybean trypsin inhibitor, pH 7.4; buffer D, 20 mM Tris HCl, 1 mM EDTA, 0.1 mM PMSF, 1 mM DTT, 0.9% cholate, 73 mM sucrose, 5 mM MgCl\textsubscript{2}, pH 8.0; buffer E, ice cold 50 mM sodium phosphate, 1 mM EDTA, pH 7.4.

Materials

[\textsuperscript{3}H] Oxo M was purchased from DuPont-New England Nuclear at a specific activity of 87.5 Ci/mmol. Carbachol was from Aldrich Chemical Company and 9-
hyoscyamine from Sigma. Oxo M, NMS and L-QNB were purchased from Research Biochemicals Inc. Guanine nucleotides were purchased from Boehringer Mannheim.

Membrane preparation

Three kinds of membrane preparations derived from Chinese hamster ovary cells expressing the porcine m2 muscarinic receptor were used in the following studies. Except for one set of experiments using the CHOLEI,m2 cell line described in Chapter II, the CHO cells used were expressing the muscarinic receptor at levels of 10 to 300 pmol binding sites per mg protein. They were grown to near confluency in 100 mm tissue culture dishes and MFD culture media supplemented with 10% calf serum. For harvesting the media was replaced by buffer B, and the cells were manually scraped off the dishes. They were then pelleted for 5 minutes in a table top centrifuge and resuspended in twice the packed cell volume of buffer C. The resuspended cells were homogenized on ice with ten strokes in a Potter-Elvehjem homogenizer with teflon pestle. The homogenate was either used directly or to prepare membranes as described by Peterson & Schimerlik (1984). No difference was detected in binding studies using either homogenized cells or membrane preparations. The homogenates, stable at 4°C for several days, were kept on ice, whereas membrane preparations were frozen at -80°C. The Oxo M binding properties of membrane preparations seemed to be unaltered by freezing and thawing and were stable for several months at -80°C. For some experiments membrane preparations were reconstituted with purified G-proteins in an attempt to increase the G-protein to receptor ratio and thus the concentration of superhigh affinity oxotremorine M binding sites. 0.5 ml of the membrane preparation (~1 μM receptor sites) were incubated with 0.1 mM acetylcholine on ice for 30 minutes, after which 1 ml of purified G-proteins in buffer D were added. The final cholate concentration in the mixture was 0.6%. The suspension was dialyzed overnight at 4°C against 0.5 L of buffer.
A, causing the fairly hydrophobic G-proteins to insert into the membranes. The dialysate was diluted 8-fold into buffer A and centrifuged at 40000 rpm for 1 hour. The resulting pellet was resuspended in 7 ml of buffer A, recentrifuged, resuspended in 0.5 mL of buffer A and frozen at -80°C. Freezing and thawing of this preparation affected the concentration of superhigh oxotremorine M binding sites without changing the ligands dissociation constant or kinetic behavior. As will be shown in this chapter results obtained with reconstituted preparations differ from the ones obtained with nonreconstituted membranes.

**G-protein purification**

G-proteins were purified from porcine atria by a method developed by Tota et al. (1987). Briefly, porcine atrial membranes were extracted with a digitonin/cholate mixture. Solubilized G-proteins were then purified employing chromatography on wheat germ agglutinin, DEAE Sephacel, gel filtration and octyl-sepharose columns and a sucrose gradient centrifugation. SDS polyacrylamide gel electrophoresis of the purified protein showed only three bands corresponding to the α, β and γ subunits of the heterotrimeric G-protein. The preparation contained a mixture of $G_{11}$ and $G_{13}$ with no detectable $G_s$ or $G_o$. It was stored at -80°C in buffer D.

**Receptor binding assays**

To measure binding of radiolabeled ligands to the receptor membrane preparations were incubated with the radioligand until equilibrium was reached, i.e. 1 hour at 25°C. At this point unbound ligand can be separated from receptor and bound ligand by a number of methods. In the experiments discussed below filtration through Whatman GF/B or Schleicher & Schuell #32 glassfiber filters was used. Filtration was performed either on a
filter manifold from Hoefer Scientific Instruments or on a Brandell cell harvester. The filters were washed with 4x3 ml of buffer E. Nonspecific binding was determined by repeating the binding experiment in the presence of a 1000 to 10000-fold excess of the antagonist 2-hyoscyamine. Nonspecific binding to the glassfiber filters can be quite high for positively charged ligands such as [3H] Oxo M and [3H] NMS. Where higher concentrations of these ligands (≥1 nM) were used, the filters were pretreated with 0.1% (v/v) polyethylene imine to reduce the background. Using filtration to separate bound from free ligand has the disadvantage that binding of fast-dissociating ligands may be underdetermined if some of the ligand dissociates during the wash steps. Therefore, a centrifugation method that circumvents this problem was also used, and the results from both procedures were compared. To use the centrifugation method, membranes were diluted 15-fold into buffer A to a total volume of 230 µL and incubated with the radioligand for 1 hour at 25°C. 100 µl were removed and the total radioligand concentration was determined by scintillation counting. The remaining 130 µl were centrifuged for 30 minutes in a Beckman airfuge at 25°C in Beckman polyallomer centrifuge tubes. To determine the concentration of unbound radioligand 100 µl of the supernatant were removed and counted. The disadvantage of this method is that the concentration of bound ligand has to be calculated by subtracting two large numbers with correspondingly large errors. Because of this error source, the following binding data were obtained using the filtration procedure after results from both procedures had been compared to assure that all of the binding was detected in the filtration assay.

After filtration 3.5 ml of a triton-toluene based scintillation cocktail were added to the filter discs and equilibrated for at least one hour at room temperature. The radioligand was quantitated by scintillation counting in a Beckman LS6800.
Equilibrium binding experiments

Receptor concentrations were generally around 100 pM. 10 nM[^3H] L-QNB was used to determine the total concentration of receptor binding sites, and 10 nM oxotremorine M was used to estimate the concentration of superhigh affinity agonist sites. Dissociation constants were obtained either from Scatchard analysis of direct binding data (Scatchard, 1949) or from competition binding curves. The latter are discussed in detail in Chapter II. All equilibrium binding experiments were performed at 25°C in buffer A. Direct binding experiments were incubated for 1 hour, competition binding curves for 2 1/2 hours. Nonspecific binding was assessed in the presence of 100 μM 1-hyoscyamine.

Kinetic studies

In association experiments membranes were diluted to approximately 100 pM SHA Oxo M sites into buffer A. At time zero the labeled ligand was added, and after specified time periods aliquots (1 mL unless noted otherwise) were filtered through Whatman GF/B or Schleicher & Schuell #32 glassfiber filters on a filter manifold. Filters were pretreated with 0.1% polyethylene imine. Nonspecific binding was determined in a separate experiment using 100 μM 1-hyoscyamine. The concentration of bound ligand at equilibrium was determined at least in duplicate. (C eq - C t), where C eq and C t are the concentrations of specifically bound ligand at equilibrium and time t, was fit to either a single- or a bi-exponential decay, given by A = A o exp (-t,τ) (equ. 1) and A = A fast exp (-t,τ fast) + A slow exp (-t,τ slow) (equ. 2), using a nonlinear least squares fitting routine (Duggleby, 1984). In dissociation experiments membranes were diluted to about 100 pM SHA Oxo M sites into buffer A and equilibrated with the labeled ligand for one hour at 25°C. The concentration of specifically bound ligand was determined, and at time zero dissociation was initiated either by diluting the sample 10-fold or by adding an excess of
an unlabeled ligand. Again, samples were removed at specified times and filtered. Plots of \((C_t - C_{eq})\) versus time were fit to a single- or bi-exponential decay given by \(A = A_0 + A_1 \exp(-t,T)\) (equ. 3) and \(A = A_0 + A_{fast} \exp(-t,T_{fast}) + A_{slow} \exp(-t,T_{slow})\) (equ. 4). Unless otherwise noted all kinetic experiments were performed at 25°C.
RESULTS

Figure 4.1 shows a binding curve for \(^3\text{H}\) Oxo M binding to a preparation of CHO cell membranes reconstituted with purified guanine nucleotide binding proteins as described under 'Materials and Methods'. The resulting G-protein to receptor ratio was 20:1. \(^3\text{H}\) oxotremorine binding to this preparation was saturable with a dissociation constant of \((1.2 \pm 0.1)\) nM. The inset shows a Scatchard plot of the same data. The linear Scatchard plot indicated that the receptor population observed was homogeneous and that binding to this population was not cooperative. The total number of \(^3\text{H}\) Oxo M binding sites as obtained from least squares fitting analysis corresponded to \((49 \pm 2)\)% L-QNB binding sites. This was in good agreement with the competition binding experiment shown in Figure 4.2, which showed \((53 \pm 3)\)% of L-QNB sites having superhigh affinity for Oxo M with a dissociation constant of \((0.57 \pm 0.42)\) nM.

Figure 4.3 shows the time course for association of 0.1 nM \(^3\text{H}\) Oxo M with the same reconstituted membrane preparation. As can be seen from the logarithmic transformation in the inset association followed a single exponential and reached equilibrium in 15 minutes. Nonlinear least squares fitting of the \((C_{eqv}-C_i)\) data to a single exponential decay yielded a reciprocal relaxation time of \((0.19 \pm 0.02)\) min\(^{-1}\). At equilibrium only 13% of the radioligand was bound which justified treating the association as a pseudo-first order reaction. If the mechanism by which binding occurs is unknown, the reciprocal relaxation time should be determined as a function of ligand concentration in order to determine the true, chemical rate constant(s). Also, determining the dependence of the association rate can provide information about the binding mechanism of the ligand. This dependence is shown in Figure 4.4, where the reciprocal relaxation time is plotted versus the Oxo M concentration. Each point in this diagram represents a separate experiment. Errors were between 5% and 10%, and error bars were omitted for the sake of clarity. The graph showed a negligible dependence of the
apparent association rate on [³H] Oxo M concentration, and linear regression yielded a slope of \((0.013 \pm 0.007) \text{ min}^{-1}\text{nM}^{-1}\) and an intercept of \((0.20 \pm 0.03) \text{ min}^{-1}\). Implications of this finding will be discussed in the following section. Finally, the rate of [³H] Oxo M dissociation from the superhigh affinity class of sites was measured as shown in Figure 4.5. In this experiment dissociation was initiated by the addition of 1 µM unlabeled Oxo M after equilibration of the reconstituted membrane preparation with 1 nM [³H] Oxo M—a concentration equal to the dissociation constant for the ligand. Dissociation followed a single exponential decay with a reciprocal relaxation time of \((0.26 \pm 0.01) \text{ min}^{-1}\). Levels of bound [³H] Oxo M remaining after about 30 minutes can be accounted for by nonspecific binding. The data shown are representative of four experiments with reciprocal relaxation times ranging from 0.22 min⁻¹ to 0.28 min⁻¹.

Because of the possibility that the introduction of G-proteins foreign to the CHO cell membranes affects the nature of receptor G-protein interactions and thus the superhigh affinity class of agonist binding sites, the experiments described above were repeated with membrane preparations that had not undergone reconstitution. Homogenized CHO cell preparations or membranes were used in the following experiments without observing any differences. Scatchard plots of the direct binding of [³H] Oxo M to nonreconstituted preparations appeared linear over a range of [³H] Oxo M concentrations from 500 pM to 5 nM and were generally in agreement with data obtained from competition curves. Dissociation constants varied some from one preparation to another. The average value of 10 determinations was \((3.12 \pm 1.58) \text{ nM}\) with an average of \((3.62 \pm 3.00) \text{ nM}\) for Scatchard analysis and \((3.00 \pm 1.35) \text{ nM}\) for competition experiments. The association of 1 nM [³H] Oxo M with a nonreconstituted membrane preparation is shown in Figure 4.6. Shortly after addition of the labeled ligand binding was quite rapid and reached equilibrium after about 20 minutes. This time course could not be adequately fit to a single exponential, but was fit well by a sum of two exponentials and yielded apparent association rates of \((3.10 \pm 0.70) \text{ min}^{-1}\) and \((0.22 \pm
0.01) min\(^{-1}\) for the fast and slow phase respectively. All the binding expected from equilibrium measurements could be accounted for in the two phases with 40% appearing in the fast phase. The inset of Figure 4.6 shows a more careful determination of the fast association rate. With an association rate of (3.7 \(\pm\) 1.5) min\(^{-1}\) and 47% fast phase it agreed well with the previous determination. As with the reconstituted preparations the reciprocal relaxation time was measured as a function of \[^3\text{H}\] Oxo M concentration as plotted in Figure 4.7 for both the fast and slow phase. Each data point represents a separate experiment. Since two exponentials were fit errors are somewhat larger than with the reconstituted preparation but are below 20%. Both association rates showed essentially no dependence on ligand concentration. As shown in Chapter II preincubating membranes with guanine nucleotides such as the nonhydrolyzable GTP analogue GTP\(_\gamma\)S reduces the superhigh affinity binding of \[^3\text{H}\] Oxo M at equilibrium. The widely accepted notion is that GTP\(_\gamma\)S shifts superhigh affinity sites to lower affinity by uncoupling G-proteins from the receptor. Antagonist binding was not affected. Preincubating with guanine nucleotides can be used to examine effects of the concentration of unbound G-proteins. Preincubating membranes with 100 nM GTP\(_\gamma\)S for 35 minutes at 25°C reduced equilibrium superhigh Oxo M binding by 65%, but failed to affect the rates or the relative amplitudes of \[^3\text{H}\] Oxo M association. Since association experiments yielded different results for reconstituted versus untreated membrane preparations, dissociation should also be measured in both systems. Dissociation from untreated membranes is illustrated in Figure 4.8. The receptor concentration in the assay was 14 nM. The membranes were incubated with 1 nM \[^3\text{H}\] Oxo M for 1 hour at 25°C after which dissociation was initiated by addition of 1 \(\mu\)M unlabeled Oxo M. The experiment shown is representative of 10 experiments. A single exponential was clearly inadequate in fitting the data. Assuming a sum of two exponentials significantly improved the fit yielding reciprocal relaxation times of (2.7 \(\pm\) 0.4) min\(^{-1}\) for the fast phase and (0.11 \(\pm\) 0.01) min\(^{-1}\) for the slow phase with the amplitude of the fast phase being (53
± 3)% of the total dissociating ligand. Again, dissociation was only measured from the superhigh affinity class of binding sites, since 1 nM [3H] Oxo M was used in the preincubation. At this ligand concentration binding to the two lower affinity sites was insignificant. Figure 4.9 shows a different kind of dissociation experiment where dissociation was initiated by diluting the labeled ligand to a concentration below its dissociation constant. In the experiment shown the ligand was diluted 10-fold to a final total concentration of 0.1 nM after it had been equilibrated with the membranes and the concentration of bound ligand had been determined. Dissociation was adequately fit by a single exponential decay with a reciprocal relaxation time of (0.24 ± 0.08) min⁻¹. Equilibrium was reached after about 15 minutes, although only (39 ± 8)% of the ligand dissociated. This behavior was reproducible, and relaxation times measured in four separate experiments ranged from 0.06 min⁻¹ to 0.24 min⁻¹. Because of the differences between different kinds of membrane preparations as well as between different experimental protocols, dissociation from the superhigh affinity site was examined in detail. Only one exponential was observed in reconstituted preparations and when dissociation was initiated by dilution. Competition experiments using nonreconstituted preparations showed an additional fast phase, and it was of interest to find factors controlling the amplitude of this phase. Effects on the relaxation times can also provide information about the processes governing the two phases. Tables 4.1-4.4 show a summary of the conditions tested and the resulting reciprocal relaxation times and amplitudes. As mentioned above, addition of guanine nucleotides reduces superhigh affinity Oxo M binding and thus is a third path by which dissociation of [3H] Oxo M can be initiated. This path should include binding of the nucleotide either to the free G-protein or to the receptor G-protein complex and uncoupling of the complex. Dissociation should either take place from the receptor G-protein complex after nucleotidide has bound or from the free receptor after uncoupling of the complex. Interestingly, dissociation initiated by this method was very fast and seemed to be
complete in 10 seconds making it impossible to measure its relaxation time without special equipment. Relatively high concentrations of guanine nucleotides (equal to or greater than 100 nM for GTP$_s$) were needed to initiate dissociation, and at 10 μM GTP$_s$ only half of the ligand dissociated. If on the other hand guanine nucleotides were added together with the radiolabeled Oxo M and allowed to equilibrate, adding an excess of unlabeled Oxo M yielded the same two relaxation times seen without guanine nucleotides. This suggested that uncoupling of G-proteins from the muscarinic receptor was either not involved or not rate-limiting unless [³H] Oxo M dissociation was initiated by addition of guanine nucleotides. Supporting this conclusion were experiments directed at elucidating the mechanism by which reconstitution changed the kinetic behavior of the preparation. In the first of these experiments membranes were subjected to a reconstitution procedure without G-proteins, i.e. the membranes were incubated with acetylcholine and the G-protein storage buffer containing 0.9% cholate and then dialyzed. The result is shown in Figure 4.10. This preparation showed the same dissociation time course as preparations reconstituted with G-proteins—that is a single exponential dissociation rate with a reciprocal relaxation time of (0.27 ± 0.03) min$^{-1}$. In the next set of experiments the reconstitution procedure was examined in more detail. Three aliquots of membranes were either dialyzed only, incubated with acetylcholine and then dialyzed or treated with cholate and dialyzed. The first two samples showed the biexponential ligand dissociation typical for nonreconstituted membranes. In contrast the third sample could be adequately fit to a single exponential ($\tau_r = (0.23 ± 0.03) \text{ min}^{-1}$). Fitting to two exponentials ($\tau_{\text{fast}}^{-1} = (3.7 ± 2.7) \text{ min}^{-1}$ and $\tau_{\text{slow}}^{-1} = (0.17 ± 0.02) \text{ min}^{-1}$) yielded an amplitude of the fast dissociation phase of (25 ± 5)% compared to (59 ± 3)% in the absence of detergent treatment. It was concluded that detergent treatment rather than an effect of the G-proteins was the factor that influenced the kinetics of Oxo M binding.

Receptor dimers have been suggested in the literature (e.g. Potter et al., 1990), and it is conceivable that detergents like cholate assert their effects through disrupting
receptor aggregates. Alternatively, detergents might affect the receptor directly or through changes in the membrane environment. Aggregates are most likely to form, when the receptor density is high as is the case in cells overexpressing the muscarinic receptor. Thus it was of interest to compare cell lines with different receptor numbers. In this study cell line CHO\textsubscript{Lepm2} was used which contained the same gene for the m2 receptor as the overexpression system discussed above, but does not overexpress the protein. The receptor numbers per cell (10\textsuperscript{4}) were two orders of magnitude lower than in the overexpressing cell line (10\textsuperscript{6}). As illustrated in Figure 4.11 Oxo M dissociation from this preparation was described by a single exponential decay similar to results obtained with reconstituted membranes. The experiment was performed under the same conditions as with the other preparations using 1 \textmu M unlabeled Oxo M to initiate dissociation. The reciprocal relaxation time was three- to four-fold larger at (0.87 ± 0.07) min\textsuperscript{-1} but was reproducible.

If formation and dissociation of aggregates or conformational changes within aggregates takes place, varying the incubation time with the labeled ligand may yield information about the time course of these events. Instead of an incubation time of one hour, which was used in previous experiments and allowed the labeled ligand to equilibrate, incubation periods of 1, 5 and 7 minutes were tested as shown in Table 4.1. After these time periods [\textsuperscript{3}H] Oxo M binding reached 39, 82 and 95\% of completion. Neither the reciprocal relaxation times nor the relative amplitudes of the two phases were affected suggesting that slow isomerization or aggregation steps following ligand binding were not rate-limiting for dissociation. Since the fast phase was only seen when a competing ligand was added, experiments were undertaken to test the dependence of this phenomenon on the nature and the concentration of the competing ligand. In addition to an excess of unlabeled Oxo M, the agonist carbachol and the muscarinic antagonists \textsuperscript{L}-hyoscyamine and L-quinuclidinylbenzylate (L-QNB) caused the same effect as shown in Table 4.2. It was thus not an Oxo M-specific effect and also not limited to muscarinic
agonists. The amplitude of the fast dissociation phase should in some manner depend on
the concentration of the competing ligand, since it was zero if no second ligand was
added and, depending on the preparation, increased to 40-70% at 1 μM Oxo M. As
shown in Table 1.2 increasing the concentration of unlabeled drug 100-fold failed to
further increase the fraction dissociating with the fast relaxation time. Decreasing this
concentration to 10 nM Oxo M also was without effect. At even lower concentrations
only a fraction of the labeled ligand would be displaced making the signal to noise ratio
very unfavorable. For this reason the membrane equilibrated with the radioligand were
diluted into a larger volume of buffer A containing the unlabeled ligand. In these
experiments the measured dissociation was caused by dilution and competition, but since
dilution gave rise to only one slow dissociation phase, the effect of the second ligand
could be determined. The data is summarized in Table 4.3. Three observations could be
made: the reciprocal relaxation times were independent of the concentration of
competing ligand, the transition between monophasic and biphasic dissociation curves
took place between 0.5 nM and 1 nM Oxo M (or between 1 nM and 2 nM in a second set
of experiments not shown), and the transition was rather steep. These results suggested
that the sites that the unlabeled Oxo M interacts with were indeed superhigh affinity sites
and that the interaction is cooperative.

If the competing ligand has to bind to free sites on the receptor in order to cause the
fast dissociation of the first ligand, saturating all of the superhigh affinity sites should
prevent this effect. Indeed, if along with the [3H] Oxo M enough L-QNB was added to
saturate the remaining sites, only a single exponential off-rate was observed as illustrated
in Figure 4.12. The same effect was achieved, if high concentrations of [3H] Oxo M were
used in the preincubation. At 10 nM [3H] Oxo M only the slow dissociation phase was
seen. This dependence is illustrated in Figure 4.13, where the relative amplitude of the
fast dissociation phase is plotted versus the percentage of unoccupied sites after
preincubation, and the data are summarized in Table 4.4.
A dissociation experiment with porcine heart membranes suggested that they behave like the nonreconstituted membranes of overexpressing CHO cells. Porcine heart membranes were diluted to a SHa Oxo M site concentration of 120 pM and incubated with 1 nM [3H] Oxo M for 1 hr at 25°C. Addition of 1 μM unlabeled Oxo M yielded two dissociation phases with $\tau_{\text{fast}} = (1.4 \pm 0.4) \text{ min}^{-1}$, $\tau_{\text{slow}} = (0.05 \pm 0.02) \text{ min}^{-1}$ and $\frac{A_{\text{fast}}}{A_{\text{fast}} + A_{\text{slow}}} = 0.59 \pm 0.08$.

Finally, the temperature dependence of ligand dissociation was looked at briefly. Membranes were incubated with 1 nM [3H] Oxo M at 4°C for 3.5 hours after which dissociation was initiated by addition of 1 μM unlabeled Oxo M. The temperature was kept at 4°C throughout the experiment. The resulting dissociation curve was biphasic, and both reciprocal relaxation times were decreased by a factor of 2-3 to $(1.68 \pm 0.35) \text{ min}^{-1}$ and $(0.043 \pm 0.016) \text{ min}^{-1}$ respectively. This relatively small temperature dependence was in agreement with the notion that agonist binding is mostly controlled by enthalpic terms (Waelbroeck et al., 1985).
DISCUSSION

The results presented here clearly show that the mechanism of Oxo M binding to the superhigh affinity binding site is complex and is not a single step process as Harden et al. (1983) claim. Two parallel single step mechanisms, as proposed by Gillard et al. (1987), are also insufficient in explaining the data. However, it is easily seen how one would arrive at their conclusions, if the apparent association rate is not measured over a range of ligand concentration.

For a reversible single step binding mechanism of the form \( R + L \xrightleftharpoons[k_2]{k_1} RL \) dissociation is described by \( RL = (RL_0)e^{-k_2t} \) and association takes the form \( RL = RL_{eq} (1 - e^{-(k_1[L]+k_2)t}) \). Thus, a plot of the reciprocal relaxation time versus the ligand concentration should yield a straight line with a slope equal to the association rate constant \( k_1 \) and the intercept equal to the dissociation rate constant \( k_2 \). In this mechanism, the equilibrium dissociation constant is described by the ratio of dissociation to association rate constant \((k_2/k_1)\). In membrane preparations reconstituted with purified G-proteins Oxo M, association and dissociation followed a single exponential. However, the reciprocal relaxation time showed no or very little dependence on the Oxo M concentration. In a plot of reciprocal relaxation time versus ligand concentration, the intercept equaled the observed dissociation rate, \( k_2 \), but the slope was very small and together with the intercept would predict a dissociation constant of 31 nM. This clearly disagreed with equilibrium binding data showing a dissociation constant around 1 nM. Thus, a more complex reaction mechanism had to be evoked. Two-step mechanisms of the form \( R + L \xrightleftharpoons{\kappa_2}{\kappa_1} RL \xrightarrow{\kappa_2}{\kappa_1} RL' \) or \( R \xrightarrow{\kappa_2}{\kappa_1} R' + L \xrightarrow{\kappa_2}{\kappa_1} RL \) generally possess two relaxation times unless special assumptions are made. A common assumption that reduces the number of relaxation times to one is that the ligand binding step is in rapid equilibrium. This kind of a mechanism predicts a hyperbolic ligand dependence of the apparent association rate contradictory to what was observed here. Alternatively, one can choose rate constants
such that a formally biphasic reaction appears monophasic, because the amplitude of one of the phases becomes negligible. The following derivation proves, that it is possible to choose rate constants, for which a simple two step binding mechanism shows a single ligand-independent association rate equal to the dissociation rate.

\[
\begin{align*}
& \frac{r}{k_1} \overset{R}{\underset{k_1}{\overset{k_2}{\underset{k_2}{\bar{R}}}}} \\
& \quad \frac{d[r]}{dt} = k_1[R] - k_1[r] \\
& \quad \frac{d[R]}{dt} = k_1[r] + k_2[RL] - (k_1 + k_2[L])[R] \\
& \quad \frac{d[RL]}{dt} = k_2[L][R] - k_2[RL]
\end{align*}
\]

Assume: \( R_{\text{Tot}} = [r] + [R] + [RL] \)
\((L) >> [R_{\text{Tot}}]\) (pseudo-first order assumption)
\( k_1, k_2 >> k_1, k_2 \) and \( \lambda_1 >> \lambda_2 \)

where \( \lambda_1 \) and \( \lambda_2 \) are the reciprocal relaxation times of the fast and slow phase. The set of differential equations above are solved using a Laplace-Carson transformation (Roberts, 1977) giving:

\[
[RL] = \left( \frac{k_1 + k_2[L]}{\lambda_1 \lambda_2} \right) RL_o + \frac{k_2[L] R_o + k_1 k_2[L] R_{\text{Tot}}}{\lambda_1} e^{-\lambda_1 t} \\
+ \left( \frac{k_1 + k_2[L] - \lambda_2}{\lambda_1 (\lambda_1 - \lambda_2)} \right) RL_o + \frac{k_2[L] R_o + k_1 k_2[L] R_{\text{Tot}}}{\lambda_1 (\lambda_1 - \lambda_2)} e^{-\lambda_1 t} \\
+ \left( \frac{k_1 + k_2[L] - \lambda_2}{\lambda_2 (\lambda_2 - \lambda_1)} \right) e^{-\lambda_2 t}
\]

where the subscript "0" indicates concentrations at time zero.
\[ \lambda_1 = k_1 + k_2[L] \]

\[ \lambda_2 = \frac{k_1k_2 + k_1k_2[L]}{k_1 + k_2[L]} \]

**Association:**

For ligand association \( R_o = R_{Lo} = 0 \) can be assumed, and the above equation simplifies to

\[ [RL] = \frac{k_1k_2[L]R_{Lo}}{\lambda_1\lambda_2} \left(1 - e^{-\lambda_2t}\right) \]

**Dissociation:**

For ligand dissociation the assumption \([L] = 0\) is made, and the same equation yields

\[ [RL] = \frac{[RL_o]}{\lambda_1} e^{-\lambda_2t} \]

The reciprocal relaxation time for ligand association and dissociation equals \( \lambda_2 \).

At \([L] = 0\) \( \lambda_2 = k_{-2} \)

at high ligand concentration \( \lambda_2 = k_1 \)

Thus if \( k_1 = k_{-2} \), the association rate is ligand-independent and equals the dissociation rate.

In addition to predicting the right kinetic behavior this mechanism also predicts a linear Scatchard plot and thus agrees with all of the findings for the reconstituted system. G-proteins are needed for superhigh affinity agonist binding, and there are two alternatives to include G-proteins in the above mechanism: both \( r \) and \( R \) equal receptor
G-protein complexes, and the first receptor isomerization step involves coupling of the receptor to a G-protein. The second possibility predicts a dependence of the equilibrium dissociation constant on the concentration of G-proteins. However, changing the G-protein concentration by reconstituting with different amounts of the protein may not result in a significant change in the effective concentration in each lipid vesicle, and thus this dependence may be obscured.

Finding a binding mechanism that is consistent with all the observations made in the nonreconstituted system, is a lot more challenging. If only the association experiments and the dissociation involving a competing ligand are considered, two receptor populations each binding ligands according to the above two-step mechanism could explain the data. However, neither the dilution experiments nor the dependence of the fast phase on unoccupied receptor sites is consistent with this notion. The dependence of the amplitude of the fast phase on the fractional saturation of the receptor demands an interaction of occupied and free superhigh affinity sites. Such an interaction necessitates the formation—at least transiently—of a receptor dimer or higher aggregate. Aggregate formation or dissociation necessarily involve steps that are not first-order or pseudo-first-order reactions, making it necessary to simulate such mechanisms since they cannot be solved analytically. A simulation program called "Scopfit" (Simulation Resources Inc., Berrien Springs, MI) was used to analyze a large number of binding mechanisms. Mechanisms involving up to 18 rate constants were investigated, before a model was found that could account for all the experimental observations. While the author is confident that no simpler models will fit all of the data, there may be other binding mechanisms of the same or greater complexity that work equally well. For this reason the schematic of the binding mechanism illustrated below is followed by a discussion aimed at pointing out the features of this model, that would have to be included in any alternative mechanism.
In this schematic, r and R denote different receptor conformations, and the dimer is asymmetric in that the two ligand binding sites have different affinity for the ligand. Binding initially takes place to two separate receptor populations, the monomer r and the dimer rr. This allows the amplitudes of the two association phases to be independent of the ligand concentration. Binding to each population requires an isomerization step before the actual ligand binding step. As pointed out during the discussion of the reconstituted system, this is necessary in order to make the two relaxation times ligand-independent and equal to the observed dissociation rates. Two ligated monomers can interact to form the fully ligated dimer RLRL. The two binding sites on this asymmetric dimer have different affinity and different binding kinetics. Ligand dissociation from the
fully ligated dimer can proceed by two different pathways. Each pathway involves the intermediate formation of a receptor dimer with one ligand molecule bound. One of these species, RLR, has the ligand bound in the higher affinity site, whereas the ligand is bound to the lower affinity site in RRL. It is assumed, that equilibrium constants for the individual species are such, that at ligand concentrations close to the overall dissociation constant the four species r, rr, RL and RLR predominate. At higher ligand concentration preferentially RL is formed, since it is assumed that the formation of RL from RLRL is favorable. All liganded species can exchange ligands in a competition experiment. In contrast, dilution of the ligand results in dissociation mainly from RL, since the ligand is bound with higher affinity in RLR. If one assumes that the ligand dissociates slowly from RL and rapidly from RLR, this results in mainly slow dissociation in dilution experiments and a slow and a fast phase in competition experiments. At high ligand concentrations, where RL is favored over RLR, dissociation is also slow. As discussed for the proposed binding mechanism for the reconstituted preparation, G-proteins are either included in all receptor species or are bound in the isomerization steps from r to R and from rr to RR. As predicted from the presence of different ligand affinities, the Scatchard plot for this model is somewhat nonlinear and indicates negative cooperativity. However, at ligand concentrations close to half saturation (within 5-fold from the apparent dissociation constant) the deviation from linearity is fairly small, and the predicted Scatchard plot is in agreement with the experimental data.

At this level of complexity, the proposed model can not reasonably be used for data fitting. While some rate constants have to be equal to the measured reciprocal relaxation times, others can only be determined relative to each other. Also, when choosing rate constants, the constraint on equilibrium constants imposed by the presence of a closed loop has to be considered \( \frac{k_9}{k_{10}} \times \frac{k_{13}}{k_{14}} = \frac{k_7}{k_8} \times \frac{k_{11}}{k_{12}} \). Figures 4.14 through 4.18 are designed to show that it is possible to choose rate constants for which the proposed model agrees with all of the experimental observations. The lines in these figures do not
represent a fit to the data shown as squares, but are the result of simulations of the model under appropriate conditions. It should be pointed out that the curves obtained from the simulations may not formally be described by one or two exponentials, but are well fit by these approximations. Figures 4.14 and 4.15 show association time courses at ligand concentrations of 1 nM and 10 nM, respectively. Since the model agrees with measured association rates at both ligand concentrations, it follows that the model predicts the right ligand-dependence of the association rate. Figures 4.16 and 4.17 show dissociation time courses for reactions initiated either by competition with 1000-fold excess of a second ligand or by a 10-fold dilution. Figure 1.16 shows an experimental and a simulated Scatchard plot.

The binding mechanism that is described for the reconstituted membranes and that also seems to fit systems with low receptor concentrations such as the described CHOLEpm2 cells, follows from this more complicated model, if dimer formation is not allowed.

To end this discussion it may be pointed out that the proposed model or variations thereof may account for some seemingly obscure results obtained with other G-protein coupled receptors as mentioned in the introduction. Formation of higher affinity binding sites may look like formation of nondissociable ligand as was seen by Bharucha and Tager (1990) with glucagon receptors. The different relative amplitudes of the two phases in association and dissociation experiments observed by Sinkin et al. (1993) with histamine receptors might also be explained. In the association reaction, the amplitudes are mostly controlled by the relative amounts of r and rr, whereas the equilibrium constants relating RLR and RL largely determine the amplitudes in the dissociation reaction. On this admittedly very superficial level it seems that the suggested binding mechanism may be adapted to explain the ligand binding behavior of different G-protein coupled receptors.
Figure 4.1  Binding curves for [$^3$H] Oxo M to a preparation of CHO cell membranes reconstituted with purified G-proteins.

The final concentrations in the assay were 624 pM L-QNB sites, 300 pM SHa Oxo M sites and 12.5 nM [$^{35}$S] GTP$_\gamma$S binding sites. The concentrations of [$^3$H] Oxo M ranged from 20 pM to 10 nM, and the obtained values for bound [$^3$H] Oxo M were corrected for nonspecific binding. Nonspecific binding was determined in the presence of 100 µM α-hyoscyamine. All samples were incubated at 25°C for 30 minutes. The inset shows a Scatchard plot of the binding data, which was fit by linear regression to give a dissociation constant of (1.2 ± 0.1) nM.
Figure 4.1
Figure 4.2  Oxo M displacement of specifically bound \(^3\)H L-QNB.

A preparation of CHO cell membranes reconstituted with G-proteins was diluted into buffer A to a L-QNB site concentration of 125 pM. The dilution was preincubated at 25°C for 20 minutes with different concentrations of Oxo M ranging from 10 pM to 1 mM. 1.6 nM \(^3\)H QNB were added and equilibrated for 90 minutes at 25°C. Samples were then filtered through Whatman GF/B glass fiber filters soaked in 0.1% polyethylene imine, equilibrated with nonaqueous scintillation coctail and quantitated by liquid scintillation counting. Nonspecific binding of \(^3\)H L-QNB was determined in the presence of 100 μM L-hyoscyamine. Fitting of the data to a model assuming three independent classes of sites yielded \(F_1 = 0.53 \pm 0.03\), \(F_2 = 0.44 \pm 0.05\), \(K_1 = (0.57 \pm 0.42)\) nM, \(K_2 = (0.66 \pm 0.42)\) μM and \(K_3 = (10 \pm 30)\) μM.
Figure 4.2
Figure 4.3  Association of 0.1 nM [3H] Oxo M with a reconstituted membrane preparation.

CHO cell membranes reconstituted with purified G-proteins were diluted into buffer A to give a L-QNB site concentration of 412 pM and a guanine nucleotide binding site concentration of 11.6 nm. At time 0 0.1 nM [3H] oxotremorine M was added, and at specified times 1 ml aliquots were filtered through Whatman GF/B glass fiber filters soaked in 0.1% polyethylene imine. Bound radioligand was quantitated by liquid scintillation counting. The experiment was performed at 25°C. The inset shows a semilogarithmic plot \((C_{equ} - C_t)\) versus time. \((C_{equ} - C_t)\) was fit to equ. 1 yielding \(\tau^{-1} = (0.19 \pm 0.02)\) min\(^{-1}\) at a total SHa Oxo M binding site concentration of \((18 \pm 1)\) pM.
Figure 4.3

[Graph showing the relationship between time (min) and bound Oxo M (cpm).]
Figure 4.4 Ligand dependence of the reciprocal relaxation time of $[^3\text{H}]$ Oxo M in reconstituted membranes.

Each data point represents a separate experiment, and experiments were performed as described under 'Materials and Methods'. Total L-QNB site concentrations were approximately 400 pM, SHa Oxo M sites were 20 pM and the temperature was 25°C. Linear regression yielded a slope of $(0.013 \pm 0.007) \text{ min}^{-1} \text{ nM}^{-1}$ and an intercept of $(0.20 \pm 0.03) \text{ min}^{-1}$. 
Figure 4.4
Figure 4.5  Dissociation of $[^3H]$ Oxo M from the highest affinity site in a reconstituted membrane preparation.

CHO cell membranes reconstituted with G-proteins were diluted into buffer A to give a L-QNB site concentration of 2.7 nM, a SHa Oxo M site concentration of 0.18 nM and a guanine nucleotide binding site concentration of 77 nM. The membranes were incubated with 1 nM $[^3H]$ Oxo M for 1 hour at 25°C. Two 1 ml samples were removed and filtered through glass fiber filters. At time zero 1 µM Oxo M (unlabeled) was added, and at specified times 1 ml samples were removed and filtered. Bound ligand was quantitated by liquid scintillation counting. The inset shows a semilogarithmic plot of the data. Non linear least squares fitting of the data to equ. 3 yielded $\tau^{-1} = (0.26 \pm 0.01)$ min$^{-1}$. 
Figure 4.5
Figure 4.6 Association of 1 nM [3H] Oxo M with nonreconstituted membranes.

A nonreconstituted membrane preparation was diluted into buffer A to give a L-QNB site concentration of 1.4 nM, 200 pM SHa Oxo M sites and a guanine nucleotide binding site concentration of 15 nM. The experiment was performed at 25°C as described for the reconstituted preparation except that 1 nM of the radioligand was used. Fitting \( \frac{A_{fast}}{A_{fast} + A_{slow}} = \) 0.40 ± 0.04. The inset shows an experiment using similar conditions but shorter incubation periods and yielded \( A_{fast} = (3.7 ± 1.5) \text{ min}^{-1} \) at a SHa Oxo M site concentration of 7 pM.

\[ \tau_{slow} = (0.22 ± 0.01) \text{ min}^{-1} \quad \text{and} \quad \tau_{fast} = (3.10 ± 0.70) \text{ min}^{-1} \]
Figure 4.6
Figure 4.7  Ligand dependence of the fast and slow reciprocal relaxation times of $[\text{H}]$ Oxo M in nonreconstituted membranes.

Each data point represents a separate experiment performed at 25°C with final SHa Oxo M site concentrations of 200 pM. The data was fit by linear regression to give a slope of $(0.072 \pm 0.066)$ min$^{-1}$ nM$^{-1}$ and an intercept of $(2.81 \pm 0.38)$ for the fast phase and a slope of $(-0.001 \pm 0.008)$ min$^{-1}$ nM$^{-1}$ and an intercept of $(0.29 \pm 0.046)$ for the slow phase.
Figure 4.8 Dissociation of $[^3H]$ Oxo M from nonreconstituted membranes initiated by addition of unlabeled Oxo M.

Nonreconstituted membranes were diluted into buffer A to a L-QNB site concentration of 14 nM, a SHa Oxo M site concentration of 0.5 nM and a guanine nucleotide binding site concentration of 154 nM. The sample was incubated with 1 nM $[^3H]$ Oxo M for 1 hour at 25°C. Two 0.5 ml samples were filtered. At time zero 1 µM unlabeled Oxo M was added, and at specified times 0.5 ml aliquots were removed and filtered. Bound radioligand was quantitated by liquid scintillation counting and fit to equ. 4 using nonlinear least squares fitting yielding $\tau_{\text{fast}} = (2.7 \pm 0.4) \text{ min}^{-1}$, $\tau_{\text{slow}} = (0.11 \pm 0.01) \text{ min}^{-1}$ and $\frac{A_{\text{fast}}}{A_{\text{fast}} + A_{\text{slow}}} = 0.53 \pm 0.03$. 
Figure 4.8
Figure 4.9  Dissociation of $[^3H]$Oxo M from nonreconstituted membranes initiated by dilution.

Nonreconstituted membranes were diluted into buffer A to give 85 nM L-QNB sites, 0.3 nM SHa Oxo M sites and 13 nM GTP$_7$S sites. After a 1 hour incubation with 1 nM $[^3H]$Oxo M at 25°C, two 100 µl samples were removed and filtered. At time 0 the mixture was diluted 10-fold into buffer A, and at specified times 1 ml aliquots were removed and filtered. Data were fit to equ. 3. $\tau^{-1}$ was $(0.24 \pm 0.08)$ min$^{-1}$ and $(39 \pm 8)$% of the ligand dissociated.
Figure 4.9
Figure 4.10  [³H] Oxo M dissociation from membranes "reconstituted" without G-proteins.

0.5 ml of membranes were incubated with 0.1 mM acetylcholine on ice for 30 minutes after which 1 ml of buffer D (G-protein storage buffer) was added. The mixture was dialyzed and centrifuged as described under "Materials and Methods". The membranes were resuspended in the original volume, diluted 100-fold to give 160 pM SHₐ Oxo M sites and 580 pM L-QNB sites and equilibrated with 1 nM [³H] Oxo M. Dissociation was initiated by addition of 1 µM unlabeled Oxo M and 0.5 ml aliquots were filtered. Nonlinear least squares fitting to equ. 3 yielded $\tau^{-1} = (0.27 \pm 0.03)$ min⁻¹.
Figure 4.11 Dissociation of [³H] Oxo M from homogenized CHO_{LEpm2} cells.

CHO_{LEpm2} cells were harvested and homogenized as described under "Materials and Methods". Homogenized cells were diluted 15-fold to give a L-QNB site concentration of 247 pM and a SHa Oxo M site concentration of 52 pM. The sample was equilibrated with 2 nM [³H] Oxo M for 1 hour at 25°C. At time 0 dissociation was initiated by addition of 1 μM unlabeled Oxo M, and at specified times 0.5 ml aliquots were removed and filtered. Data were fit to equ. 3 yielding $\tau^{-1} = (0.87 \pm 0.07) \text{ min}^{-1}$. 
Figure 4.11
Figure 4.12 Dissociation of $[^{3}\text{H}]$ Oxo M in the presence of saturating concentrations of L-QNB.

Membranes were diluted into buffer A to a final L-QNB site concentration of 50 pM and a final SHa Oxo M site concentration of 6 pM. The dilution was incubated with 1 nM $[^{3}\text{H}]$ Oxo M and 0.2 nM QNB for 90 minutes at 25°C. At time 0 dissociation was initiated by addition of 1 μM unlabeled Oxo M, and at specified times 5 ml aliquots were removed and filtered. Fitting the data to equ. 3 yielded $\tau^{-1} = (0.06 \pm 0.01) \text{ min}^{-1}$. 
Figure 4.12
Figure 4.13 Dependence of the amplitude of the fast dissociation phase on the percentage of unoccupied sites.

Membranes were diluted into buffer A to a L-QNB concentration of 13 nM and a SHα Oxo M site concentration of 0.15 nM. Membranes were then incubated with concentrations of $[^3H]$ Oxo M ranging from 0.1 nM to 10 nM, and the percentage of unoccupied sites was determined. At time zero 1 μM unlabeled Oxo M was added to initiate dissociation. The dissociation curves were fit by nonlinear least squares fitting, and the relative amplitude of the fast phase was plotted versus the percentage of unoccupied sites.
Figure 4.14  Simulated and experimental association rate at 1 nM ligand.

The experimental data were obtained at a SHa Oxo M site concentration of 100 pM as described under 'Materials and Methods' and are shown as squares. The line is the result of simulating the proposed binding model under the following conditions: $k_1 = 0.2 \text{ min}^{-1}, k_2 = 100 \text{ min}^{-1}, k_3 = 100 \text{ min}^{-1} \text{ nM}^{-1}, k_4 = 0.2 \text{ min}^{-1}, k_5 = 0.005 \text{ min}^{-1} \text{ nM}^{-1}, k_6 = 1.125 \text{ min}^{-1}, k_7 = 100000 \text{ min}^{-1}, k_8 = 10 \text{ min}^{-1} \text{ nM}^{-1}, k_9 = 0.1 \text{ min}^{-1}, k_{10} = 100 \text{ min}^{-1} \text{ nM}^{-1}, k_{11} = 4 \text{ min}^{-1}, k_{12} = 4000 \text{ min}^{-1} \text{ nM}^{-1}, k_{13} = 1000 \text{ min}^{-1}, k_{14} = 0.1 \text{ min}^{-1} \text{ nM}^{-1}, k_{15} = 400 \text{ min}^{-1}, k_{16} = 4 \text{ min}^{-1}, r_o = 0.06 \text{ nM}, r_{ro} = 0.02 \text{ nM}, L_o = 1 \text{ nM}, R_o = R_{lo} = LR_{Ro} = RRR_{Ro} = 0$ where the subscript "O" denotes initial concentrations at time zero. The software used was "Scopfit".
Figure 4.14

Spec. bound ligand (%) vs. Time (minutes)

- Simulated
- Experimental
Figure 4.15  Simulated and experimental association rate at 10 nM ligand.

Experimental data shown as squares were obtained as described under 'Materials and Methods' at a final SHa Oxo M site concentration of 0.1 nM. The line resulted from simulating the proposed binding model. The parameters used in the simulation were the same as for Figure 4.14 except for \( L_o = 10 \text{ nM}, r_o = 0.02 \text{ nM} \) and \( r_{r_o} = 0.04 \text{ nM} \).
Figure 4.15
Figure 4.16  Simulated and experimental dissociation rate involving competition with a second ligand.

Experimental data (shown as squares) were obtained from a dissociation experiment involving equilibration of 0.1 nM SHa Oxo M sites with 1 nM [3H] Oxo M and addition of 1 μM unlabeled Oxo M. The experiment was simulated by first simulating the association of 1 nM ligand and using the obtained equilibrium concentrations as starting conditions. A second ligand x was introduced into the model with xo (concentration of x at time 0) being 1 μM. Rate constants were the same as for Figure 4.14.
Figure 4.16

Spec. bound ligand (%) vs. Time (minutes)

- simulated
- experimental
Figure 4.17 Simulated and experimental ligand dissociation after dilution.

The squares represent a dissociation experiment involving equilibration of 0.1 mM $\text{SH}_a$ Oxo M sites with 1 nM [³H] Oxo M followed by a 10-fold dilution. The experiment was simulated using initial concentrations 10-fold lower than the equilibrium concentrations obtained from simulating the association of 1 nM ligand. Rate constants were the same as for Figure 4.14.
Figure 4.17 Time (minutes)

Spec. bound ligand (%) vs Time (minutes)

- simulated
- experimental
Figure 4.18  Simulated and experimental Scatchard plot.

Experimental data are shown as squares and were obtained as described for Figure 4.1 at a SHa Oxo M site concentration of 0.58 nM. The association of different ligand concentrations was simulated and the resulting equilibrium concentrations were used to produce the curve. Rate constants were the same as for Figure 4.14 except for $k_3 = 15 \text{ min}^{-1} \text{nM}^{-1}$, $k_{12} = 600 \text{ min}^{-1} \text{nM}^{-1}$ and $k_{10} = 13 \text{ min}^{-1} \text{nM}^{-1}$. These changes reflect the somewhat higher dissociation constant seen with the experimental data, but do not influence the kinetic behavior predicted by the proposed model. $r_o$ was 0.3 nM and $r_{ro}$ 0.14 nM.
Table 4.1 [\(^3\)H] Oxo M dissociation in the presence of guanine nucleotides and after short incubation periods.

The table summarizes the results of a number of dissociation experiments. The first column shows the conditions during ligand binding, i.e. before dissociation was initiated. SHa Oxo M binding site concentration was approximately 0.1 nM in all experiments. The second column gives the nature and concentration of the competing ligand that was added to initiate dissociation of [\(^3\)H] Oxo M. The remaining three columns show the results of nonlinear least squares fitting of the data to equ. 4.
<table>
<thead>
<tr>
<th>Preincubation conditions</th>
<th>Competing ligand</th>
<th>$\tau^{-1}$ (fast) (min$^{-1}$) ±SD</th>
<th>$\tau^{-1}$ (slow) (min$^{-1}$) ±SD</th>
<th>$A_{(fast)}$ (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 nM [H$^3$]Oxo M for 1 hr</td>
<td>1 μM Oxo M</td>
<td>2.76±0.37</td>
<td>0.11±0.01</td>
<td>53±3</td>
</tr>
<tr>
<td>1 nM [H$^3$]Oxo M + 100 μM GppNHp for 1 hr</td>
<td>1 μM Oxo M</td>
<td>3.60±0.87</td>
<td>0.18±0.03</td>
<td>64±6</td>
</tr>
<tr>
<td>1 nM [H$^3$]Oxo M + 10 μM GDP for 1 hr</td>
<td>1 μM Oxo M</td>
<td>4.82±1.38</td>
<td>0.10±0.01</td>
<td>59±5</td>
</tr>
<tr>
<td>1 nM [H$^3$]Oxo M for 1 min</td>
<td>1 μM Oxo M</td>
<td>4.49±0.57</td>
<td>0.53±0.08</td>
<td>74±4</td>
</tr>
<tr>
<td>1 nM [H$^3$]Oxo M for 5 min</td>
<td>1 μM Oxo M</td>
<td>6.03±0.98</td>
<td>0.48±0.04</td>
<td>65±3</td>
</tr>
<tr>
<td>1 nM [H$^3$]Oxo M for 7 min</td>
<td>1 μM Oxo M</td>
<td>2.58±0.19</td>
<td>0.33±0.04</td>
<td>71±3</td>
</tr>
</tbody>
</table>

Table 4.1
Table 4.2  Effect of the nature and concentration of the competing ligand on $[\text{H}]$ Oxo M dissociation.

Approximately 0.1 nM SHa Oxo M sites were incubated with 1 nM $[\text{H}]$ Oxo M for 1 hour at 25°C. At this point a second ligand, specified in the second column of this table, was added to initiate dissociation of $[\text{H}]$ Oxo M. Columns 3-5 show the results of nonlinear least squares fitting of the data to equ. 4.
<table>
<thead>
<tr>
<th>Preincubation conditions</th>
<th>Competing ligand</th>
<th>$\tau^{-1}_{(\text{fast})}$ (min$^{-1}$) ±SD</th>
<th>$\tau^{-1}_{(\text{slow})}$ (min$^{-1}$) ±SD</th>
<th>$A_{(\text{fast})}$ (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1nM $[\text{H}^3]\text{Oxo M}$ for 1 hr</td>
<td>100μM Carbachol</td>
<td>4.39±0.74</td>
<td>0.12±0.01</td>
<td>68±4</td>
</tr>
<tr>
<td></td>
<td>10μM Hyoscyamine</td>
<td>2.65±0.59</td>
<td>0.09±0.01</td>
<td>56±4</td>
</tr>
<tr>
<td></td>
<td>2μM QNB</td>
<td>4.21±1.53</td>
<td>0.09±0.01</td>
<td>59±5</td>
</tr>
<tr>
<td>1nM $[\text{H}^3]\text{Oxo M}$ for 1 hr</td>
<td>100μM Oxo M</td>
<td>3.13±0.60</td>
<td>0.10±0.01</td>
<td>52±5</td>
</tr>
<tr>
<td></td>
<td>100nM Oxo M</td>
<td>3.55±0.45</td>
<td>0.11±0.01</td>
<td>61±3</td>
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<tr>
<td></td>
<td>10nM Oxo M</td>
<td>3.25±1.17</td>
<td>0.18±0.03</td>
<td>47±7</td>
</tr>
<tr>
<td></td>
<td>1nM Oxo M</td>
<td>3.16±2.49</td>
<td>0.22±0.19</td>
<td>67±22</td>
</tr>
</tbody>
</table>

Table 4.2
Table 4.3  Effect of the concentration of the competing ligand on \[^{3}H\] Oxo M dissociation.

Approximately 1 nM SHa Oxo M sites were incubated with 1 nM \[^{3}H\] Oxo M for 1 hour at 25°C. At time zero dissociation of \[^{3}H\] Oxo M was initiated by diluting the sample 10-fold into buffer A containing the concentration of unlabeled Oxo M specified in the first column. Data were fit to equ. 4, and the results are shown in columns 2-4.
<table>
<thead>
<tr>
<th>[Oxo M] (nM)</th>
<th>$\tau^{-1}_{\text{fast}}$ (min$^{-1}$) ±SD</th>
<th>$\tau^{-1}_{\text{slow}}$ (min$^{-1}$) ±SD</th>
<th>$A_{\text{fast}}/(A_{\text{fast}}+A_{\text{slow}})$ (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1</td>
<td>---</td>
<td>0.34±0.01</td>
<td>0</td>
</tr>
<tr>
<td>0.5</td>
<td>---</td>
<td>0.47±0.01</td>
<td>0</td>
</tr>
<tr>
<td>1</td>
<td>2.53±0.34</td>
<td>0.14±0.01</td>
<td>36±2</td>
</tr>
<tr>
<td>2</td>
<td>4.10±0.70</td>
<td>0.13±0.01</td>
<td>43±2</td>
</tr>
<tr>
<td>5</td>
<td>2.89±0.22</td>
<td>0.12±0.01</td>
<td>43±1</td>
</tr>
<tr>
<td>10</td>
<td>2.21±0.12</td>
<td>0.11±0.01</td>
<td>33±1</td>
</tr>
<tr>
<td>100</td>
<td>4.15±0.48</td>
<td>0.14±0.01</td>
<td>34±1</td>
</tr>
</tbody>
</table>

Table 4.3
Table 4.4  Effect of the fractional saturation of the receptor on the dissociation of $[^3\text{H}]$ Oxo M.

Approximately 0.1 nM SHa Oxo M sites were incubated for 1 hour at 25°C with the concentration of $[^3\text{H}]$ Oxo M or $[^3\text{H}]$ Oxo M and L- QNB listed in the first column. 1 µM unlabeled Oxo M was used to initiate dissociation. Data obtained were fit to equ. 4, and the results are shown in columns 3-5.
<table>
<thead>
<tr>
<th>Preincubation conditions</th>
<th>Competing ligand</th>
<th>$\tau^{-1}_{\text{fast}}$ (min$^{-1}$) ±SD</th>
<th>$\tau^{-1}_{\text{slow}}$ (min$^{-1}$) ±SD</th>
<th>$A_{\text{fast}}$ (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1nM [H$^3$]Oxo M + 0.2nM QNB</td>
<td>1μM Oxo M</td>
<td>---</td>
<td>0.06±0.01</td>
<td>0</td>
</tr>
<tr>
<td>0.1nM [H$^3$]Oxo M</td>
<td>1μM Oxo M</td>
<td>3.62±1.02</td>
<td>0.15±0.01</td>
<td>46±5</td>
</tr>
<tr>
<td>0.5nM [H$^3$]Oxo M</td>
<td>1μM Oxo M</td>
<td>1.14±0.31</td>
<td>0.13±0.02</td>
<td>44±7</td>
</tr>
<tr>
<td>1nM [H$^3$]Oxo M</td>
<td>1μM Oxo M</td>
<td>2.55±0.96</td>
<td>0.13±0.02</td>
<td>39±7</td>
</tr>
<tr>
<td>2nM [H$^3$]Oxo M</td>
<td>1μM Oxo M</td>
<td>0.88±0.24</td>
<td>0.19±0.05</td>
<td>23±3</td>
</tr>
<tr>
<td>5nM [H$^3$]Oxo M</td>
<td>1μM Oxo M</td>
<td>1.16±0.33</td>
<td>0.17±0.01</td>
<td>25±4</td>
</tr>
<tr>
<td>10nM [H$^3$]Oxo M</td>
<td>1μM Oxo M</td>
<td>---</td>
<td>0.12±0.03</td>
<td>0</td>
</tr>
</tbody>
</table>

Table 4.4
CHAPTER V

CONCLUSIONS

The studies presented here were designed to gain a better understanding of ligand binding to the muscarinic receptor. The receptor subtype used was the recombinant porcine m2 muscarinic receptor expressed in Chinese hamster ovary cells. The m2 subtype is the only muscarinic receptor subtype expressed in atrial tissue. Therefore, results obtained in this study should be directly comparable to findings with porcine atrial membranes. Indeed, no differences between equilibrium ligand binding and oxotremorine M binding kinetics were observed between porcine atrial membranes and membranes containing the recombinant receptor.

Although agonist binding was studied extensively, many questions remain unanswered. Agonist binding data presented fit reasonably well to a model assuming three independent classes of sites. This finding has to be reconciled with the traditional view of how muscarinic receptors function. One important question is which classes of sites are active in eliciting a physiological response. Whole cells show only two classes of binding sites. Since both classes are insensitive to pertussis toxin treatment, it is assumed that they correspond to the high and low agonist affinity classes seen in membranes. The agonist affinities are approximately an order of magnitude lower in whole cells, but increase to the values observed with membrane preparations, if the cells are permeabilized with 0.05% digitonin. The simplest explanation for this finding is that the cytoplasm contains an inhibitor of agonist binding that is diluted upon permeabilization. Several cations inhibit ligand binding to the muscarinic receptor. It will be interesting to see if any of them are present at high enough concentrations in the cytoplasm to cause this effect. Alternatively, the lower affinity observed in whole cells may be due to an aspect of membrane organization, e.g. the membrane potential or a specific organization of membrane proteins in arrays.
In addition to the two sites seen in whole cells, membranes also show a class of
binding sites with superhigh affinity for agonists. This class is sensitive to guanine
nucleotides and pertussis toxin as shown in Chapter II. Together with the reconstitution
experiments presented this is evidence for the involvement of G-proteins in superhigh
affinity agonist binding. All results presented here are in agreement with the superhigh
affinity class of sites being a receptor G-protein complex. The amount of superhigh
affinity binding seems to be limited by the availability of G-proteins. It is likely that this
site is not observed in whole cells because of the concentration of guanine nucleotides in
the cytoplasm uncoupling the complex. Although complex formation with G-proteins is
assumed to be a step in the pathway by which muscarinic receptors elicit a physiological
response, EC50s from physiological assays disagree with the superhigh affinity class
being the active species of the receptor. However, one has to keep in mind that ligand
binding experiments in whole cells are not equilibrium measurements, but represent a
steady state. In a steady state measurement the EC50 is the result of all the steps involved
and may be lower than the affinity of one or some of the intermediates.

The difference between the high and low affinity classes of sites is unclear. In
whole cells the appearance of the low affinity state for agonists was dependent on
receptor density, and at very low receptor per cell numbers only the high agonist affinity
was seen. This dependence points to receptor aggregation as a possible explanation. At
the same low receptor density that gives only one agonist affinity in whole cells,
membranes show three agonist affinities. The reason for this difference may be
differences in the surface area in whole cells and membrane vesicles/sheets. Studies are
underway to test this hypothesis. If receptors are organized in patches rather than
distributed randomly and the membrane surface area is smaller in membrane preparations
than in whole cells, it is conceivable that aggregation is different in whole cells and
membrane preparations. The one site seen in whole cells with low receptor density could
be a receptor monomer or an aggregate. Monomers would seem to be favored at low
receptor density. On the other hand, there may be areas of the plasma membrane where receptors are inserted preferentially. In these areas receptor might form arrays. Once these arrays are occupied, additional receptors may be distributed over other areas of the plasma membrane.

Additional support for receptor aggregation comes from kinetic studies of oxotremorine M binding to the superhigh affinity class of sites. Although these studies only involve one class of sites, receptor-receptor interactions have to be postulated to explain all of the results presented in Chapter IV. If receptors can interact in the presence of G-proteins to give rise to complex superhigh affinity agonist binding kinetics, they may also interact in the absence of G-proteins resulting in a different agonist affinity. Thus, the model proposed to explain superhigh affinity oxotremorine M binding may be expanded to include the two lower affinity classes of sites. This hypothesis could be tested by cross-linking studies. Cross-linking experiments with glutaraldehyde in the presence of high agonist concentrations failed to show enough receptor dimers and trimers to account for the observed high and low affinity fractions of sites (Dr. G. Peterson, unpublished results). However, in addition to monomers and some dimers and trimers, cross-linking resulted in the formation of high molecular weight structures unable to penetrate SDS polyacrylamide gels. Receptor aggregates might be hidden in these structures. Analogy with the model for superhigh affinity agonist binding suggests an alternative explanation. High agonist concentrations may promote receptor dissociation, and cross-linking studies at lower agonist concentrations may be more suitable to demonstrate receptor aggregation.

Detergent treatment abolished receptor-receptor interactions involved in superhigh affinity agonist binding. Interestingly, detergent-treatment seemed to also decrease the high affinity fraction of binding sites in membranes and high detergent concentrations favor low affinity agonist binding to purified pm2 muscarinic receptors (Dr. G. Peterson, unpublished results). Detergents may change receptor interactions by promoting a
conformational change of the receptor. This possibility is supported by results obtained from the pH dependence studies. Upon detergent-treatment membranes showed a shift in the pKₐ governing antagonist binding. No shift in the pKₐ involved in agonist binding was seen. The classes of sites having different agonist affinity show the same pKₐ. Thus, a conformational change affecting the fractions of high and low agonist affinity sites is not expected to yield a shift in pKₐ for agonists.

pH dependence studies were not successful at identifying the receptor residue interacting with the positive charge on muscarinic ligands. It was shown that agonist and antagonist binding was governed by different pKₐs. Neither pKₐ belongs to aspartates 69 and 97. Aspartate 103 is likely to be responsible for the pKₐ seen with agonists. The antagonist pKₐ may be due to a conformational change involving aspartate 120. Thus, the role of aspartate 120 should be studied in more detail. Maybe the more conservative mutation D120E rather than D120N will result in expressed protein for ligand binding studies.

In summary, the presented study yielded new information about ligand binding; the two major findings being the model describing agonist binding to the superhigh affinity site and the differences between agonist and antagonist binding suggested by the pH dependence studies. In addition, the study points to possible future experiments, some of which were pointed out in this chapter.
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