Modulation of the M₂ Muscarinic Cholinergic Receptor by Cholesterol

by

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A thesis submitted in conformity with the requirements for the degree of Doctor of Philosophy Graduate Department of Pharmaceutical Sciences University of Toronto

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ABSTRACT

M2 muscarinic receptor extracted from Sf9 cells in cholate-NaCl differs from that extracted from porcine sarcolemmal membranes. Whereas the latter has been shown to exhibit non-competitive effects in the binding of N-methylscopolamine (NMS) and quinuclidinylbenzilate (QNB), which can be explained in terms of cooperativity within a receptor that is at least tetravalent, binding to the former is essentially competitive. Levels of cholesterol in Sf9 membranes were only 5% of those in sarcolemmal membranes and were increased to about 100% by means of cholesterol-methyl-β-cyclodextrin. M2 receptors extracted from CHL-treated Sf9 membranes resembled those from heart; that is, cholesterol induced a pronounced heterogeneity detected in the binding of both radioligands, including a shortfall in the apparent capacity for [3H]NMS, and there were marked discrepancies in the apparent affinity of NMS as estimated directly and via the inhibition of [3H]QNB. The data can be described quantitatively in terms of cooperative effects among six or more interacting sites, apparently within an oligomer. Cholesterol also was found to increase the affinity of the receptor for NMS and QNB, and the effect was examined for its possible relationship to the known interconversion of cardiac muscarinic receptors between an agonist-specific (R*) and an antagonist-specific (R) state. Cholesterol and N-ethylmaleimide (NEM) were compared for their effect on the affinity of NMS, QNB and four muscarinic agonists, and the data were assessed in terms of an explicit mechanistic model for a receptor that interconverts spontaneously between two states (i.e., R Ú R*). The data can be described equally well
by an effect of cholesterol on either the distribution of receptors between R and R* or the affinity of all ligands for both states, with an accompanying effect of NEM on either the affinity or the distribution between states, respectively. Since NEM is known from other data to favor R* over R, cholesterol appears to increase affinity \textit{per se}. Cholesterol therefore is a determinant of affinity and cooperativity in the binding of orthosteric ligands to the M$_2$ receptor. Both effects are observed in solution and therefore appear to arise from a direct interaction between the lipid and the receptor.
I never think of the future – it comes soon enough.

*Albert Einstein*
ACKNOWLEDGEMENTS

I thank my father and mother, Wilfredo and Adelina Colozo for their unwavering support in all of my endeavors and I thank them for always trying to instill in me the value of hardwork and education.

I am grateful for all the help of those whom I have worked with during my time here, Chi Sum, Paul Park, Amy Ma, Luca Pisterzi, Dar’ya Redka, Rabindra Shivnaraine, David Greiss, Judy Chou and John Dong.

I thank my advisory committee members, Dr. Christine Bear, Dr. Hubert Van Tol and Dr. Christopher Yip for their guidance and helpful insights throughout the completion of this work.

I am grateful to my supervisor James W. Wells for always making time and to whom I am very much indebted.

This work was supported by the Canadian Institutes of Health Research (CIHR), the Heart and Stroke Foundation of Ontario (HSFO), Ontario Graduate Scholarships (OGS) and an Ontario Graduate Scholarship in Science and Technology (OGSST).
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PR_j \iff [P]/K_{Pj} \iff R_j \iff [A]/K_{Aj} \iff AR_j
\]

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<th>Full Form</th>
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<tbody>
<tr>
<td>AICc</td>
<td>second-order Akaike’s information criterion</td>
</tr>
<tr>
<td>AFM</td>
<td>atomic force microscopy</td>
</tr>
<tr>
<td>BRET</td>
<td>bioluminescence resonance energy transfer</td>
</tr>
<tr>
<td>DAMGO</td>
<td>[D-Ala²,N-MePhe⁴,Gly-ol⁵]enkephalin</td>
</tr>
<tr>
<td>BS³</td>
<td>bis[sulfosuccinimidyl]suberate</td>
</tr>
<tr>
<td>CHL</td>
<td>cholesterol</td>
</tr>
<tr>
<td>CHO</td>
<td>Chinese hamster ovary</td>
</tr>
<tr>
<td>EM</td>
<td>Electron microscope</td>
</tr>
<tr>
<td>FRET</td>
<td>fluorescence (Förster) resonance energy transfer</td>
</tr>
<tr>
<td>GMP-PNP</td>
<td>guanylylimidodiphosphate</td>
</tr>
<tr>
<td>GPCR</td>
<td>G protein-coupled receptor</td>
</tr>
<tr>
<td>GTPγS</td>
<td>guanosine 5’-O-[3-(γ-thio)triphosphate]</td>
</tr>
<tr>
<td>HEN</td>
<td>HEPES, EDTA and NaCl</td>
</tr>
<tr>
<td>MβCD</td>
<td>methyl-β-cyclodextrin</td>
</tr>
<tr>
<td>p-MPPF</td>
<td>(4-2’-methoxy)-phenyl-1-[2’-(N-2”-pyridinyl-p-fluorobenzamido]ethyl-piperazine</td>
</tr>
<tr>
<td>NEM</td>
<td>N-ethylmaleimide</td>
</tr>
<tr>
<td>NMS</td>
<td>N-methylscopolamine</td>
</tr>
<tr>
<td>Oxo-M</td>
<td>oxotremorine-M</td>
</tr>
<tr>
<td>PC</td>
<td>phosphatidylcholine</td>
</tr>
<tr>
<td>PMSF</td>
<td>phenylmethylsulfonylfluoride</td>
</tr>
<tr>
<td>PS</td>
<td>phosphatidylserine</td>
</tr>
<tr>
<td>QNB</td>
<td>(−)-quinuclidinylbenzilate</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis</td>
</tr>
<tr>
<td>Sf9</td>
<td><em>Spodoptera frugiperda</em></td>
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CHAPTER 1

GENERAL INTRODUCTION
1.1 The Receptor Concept

The origins of the idea of the receptor can be traced back to the works of Paul Ehrlich and John Newport Langley between the period of 1878 and 1908 [1]. Although the nature of their research was quite different, the ideas that emerged from their studies eventually led to the modern concept of the receptor. In the case of Paul Ehrlich, his early work was concerned with the drug resistance of trypanosomes, for which he developed a ‘side chain theory’ of immunity in order to give a theoretical platform to the chemotherapy of such micro-organisms [2]. As for John Langley, his research interest was mainly focused on the effects of alkaloids on muscle and nerve. In his early work as a student at Cambridge, Langley was involved in examining the opposing actions of atropine and pilocarpine on the secretion of saliva by the sub-maxillary gland [3].

It was in 1905 when Langley first spoke of the existence of what he referred to as specific ‘receptive substances’ in cells [4]. He had shown that nicotine causes tonic contraction in certain muscles of the fowl, frog and toad, that this contraction is prevented by a sufficient quantity of curari and that the action of both ‘poisons’ in the fowl is unaltered by degeneration of the nerve endings [5].

Paul Ehrlich initially rejected Langley’s notion of the receptor in favor of his own ‘receptive side chain’ hypothesis. In Ehrlich’s view, the protoplasm of a cell is akin to a giant molecule which contains a central structure which possesses chemical side chains that are responsible for the specific activity of that given cell type [1]. According to Ehrlich, a particular ‘receptive side chain’ has an atomic group which has specific combining properties for a particular toxin. Such groups are said to act as antibodies or antitoxins by combining with foreign agents in the bloodstream, thus preventing the toxin from attaching to cells. The relative ease by which drugs can be removed from tissues with the use of solvents led Ehrlich to conclude that, unlike toxins, drugs are not bound firmly to cells. He believed that the action of drugs did not apply to his proposed ‘receptive side chains’ because, unlike toxins which are bound to the protoplasmic molecule of the cell by chemical union, the former in his view lacked the necessary
atomic groups to do so. By 1907, however, the growing body of evidence from Langley’s work had convinced Ehrlich that the concept of the receptor as articulated by his contemporary also applied to chemical substances in general. Thus the ‘receptive substance’ described initially by John Newport Langley was designated a ‘chemoreceptor’ by Paul Ehrlich [6].

The receptor concept was not met with universal acceptance. During the period between 1895 and 1930, there were several alternative hypotheses for drug action [4,7]. A few were based on homeopathy while others were based on physicochemical considerations. Examples of the latter posed by far the stronger challenges to the receptor concept. One of the more prominent ones was the physical theory, a notion championed by Walther Straub who was an internationally renowned pharmacologist at the time. The physical theory emphasized that the surface tension of the cell membrane greatly influenced the concentration gradient between the inside and outside of the cell, and thus controlled the effect of the drug on the receptive organ. With experiments on the isolated heart of the snail, Walther Straub observed a decrease or a complete cessation of the heart rate upon addition of muscarine [4,8]. He concluded that this effect depended upon the absorption of the alkaloid into the muscle cell, rather than the direct effect of the poison once inside the cell itself. Straub was convinced that the decisive factor that controlled toxicity was the difference in the poison concentration between the inside and the outside of the cell, which he referred to as the ‘concentration potential’. He reasoned that, as the poison entered the cell through the membrane, the concentration gradient worked to hinder the latter from excreting the chemical waste products of the former thus damaging the cell and eventually bringing its functions to a standstill. Although it is now clear that these alternatives were misguided, the ensuing years following Langley and Ehrlich’s proposal would see much debate and skepticism over their concept of a receptor as the basis for hormone and drug action. It would take several more decades before it was shown that receptors really do exist and are not merely an abstract concept.

Although much of the early work that emerged from the notion of receptors was qualitative in nature, some investigators began to develop the idea in more quantitative terms. For example in 1909, Archibald Vivian Hill, who was a student of John Langley,
made an early attempt to provide a quantitative basis for the receptor concept [9]. In his mathematical analysis of the size and time course of the contraction curves for nicotine and of the relaxation curves at different concentrations of curare, Hill supported Langley’s view in his conviction that the curves reflected a gradual combination of the drug with some constituent of the muscle. This was in contrast to a gradual diffusion of the drug into or out of the muscle preparation, as physical theories of drug action would have suggested. He concluded that the action of the drug on the muscle could be understood as a reversible chemical combination of the nicotine molecule with some constituent of the muscle.

The idea of the receptor was further carried forward by Alfred Joseph Clark, another student of Langley. His major contribution to the development of the concept was linked with his research on acetylcholine, which he regarded as suitable for the study of drug actions on cells as it produced reversible and graded effects over a wide range of concentrations and its effects differed with different tissues. Clark worked on isolated ventricular strips from the frog heart [10] and on the rectus abdominis muscle of the frog [11], where he observed a decrease and an increase in the force of contraction upon addition of acetylcholine, respectively. The resultant data resembled a sigmoid-shaped curve when the action of acetylcholine was plotted against the logarithm of its concentration.

The main observation that Clark gathered from his data was that the amount of acetylcholine required to elicit an effect in fact was very small. He calculated that the minimal amount of acetylcholine that would be sufficient to produce a demonstrable response was roughly 2,000 molecules per cell. Such an amount was not enough to form a continuous layer over the heart cells or to cover a large area inside them, and it therefore was contrary to the ‘poison-potent’ hypothesis touted by Walther Straub. Moreover, Clark observed no direct relation between the amount of acetylcholine entering the heart muscle and the action produced. He therefore suggested that the simplest way to explain the concentration-action relationship, which was in the form of a rectangular hyperbola, was to assume that a reversible, monomolecular reaction occurred
between the drug and some receptor in the cell or on the cell surface. His conclusions were in agreement with those of Archibald Hill.

In addition to his own work, Clark’s approach to the receptor concept also led him to collect and collate data from a large number of diverse pharmacological studies which he analyzed and re-analyzed quantitatively [12]. He recognized that the Law of Mass Action which previously was used by Hill and Langmuir to describe the adsorption of a gas onto a metal surface also can be applied to the interaction between drugs and cells, because he noticed that, for many drugs, the relationship between drug concentration and biological effect followed a simple hyperbolic function. Clark ultimately concluded that the hyperbolic nature of drug action reflected an equilibrium between a drug present in excess that reacts with a finite number of cell receptors to form an easily dissociable complex, and that the response that is elicited is directly proportional to the number of receptors occupied. This linear relationship between occupancy and response has been referred to as the receptor-occupancy theory, and it allowed for thermodynamic properties of the drug-receptor interaction to be inferred from measurements of a dose-dependent response [13]. Clark’s scheme demonstrated the essential value of a mechanistic model in that it allowed access to properties of a system that were not directly measurable at the time. The accuracy of his inferences, however, would depend on how closely this simple model represents the system. Nevertheless, his proposal would become the commonly accepted scheme for interpreting drug dose-response relationships up until the early 1950s.

An important contribution to the development of the receptor idea was made by John Henry Gaddum in 1937 when he introduced the idea of competitive antagonism [14]. He suggested that antagonistic drugs acted by competing with the agonist for the same receptors by blocking them inertly. He demonstrated this by deriving the very simple equations for the binding of two mutually exclusive compounds at the same population of sites. Since the binding of drugs to their receptors could not yet be measured directly at that time, Heinz Otto Schild further elaborated on this notion of competitive antagonism. Schild circumvented the lack of knowledge regarding the relationship between agonist binding and response by keeping the level of response constant; that is, he assumed only
that occupancy of a specified fraction of receptors by agonist would always produce the same response regardless of whether other receptors were occupied by antagonist. His treatment of competitive antagonism made it possible to measure the equilibrium constant for the binding of an antagonist [15–18], as it does not include the agonist as an explicit variable and therefore avoids the uncertainty associated with an empirical quantity such as the inhibitory potency of an antagonist.

Although some of the early data by Clark and others were consistent with the postulate that the fraction of receptors that is occupied by agonist correlated directly with the fractional response that is elicited, certain data did conflict with this straightforward relationship. For example, there were data for which the slope of the dose-response relationship was steeper or sometimes shallower than what is predicted by the Hill-Langmuir binding isotherm. There also were numerous examples where the application of supramaximal concentrations of stimulatory agents did not elicit a maximal level of response, which suggested that even at full receptor occupancy certain agonists do not elicit maximum physiological effects [12]. Moreover, there also was the problem that structurally similar drugs could differ considerably in their biological effects. In the 1930’s and 1940’s, researchers were still puzzled by the variety of excitatory and inhibitory responses of different tissues to adrenaline and similar substances.

At that time, there was the notion that the mediator substance of sympathetic nerves was what had been called sympathins. This notion was echoed by Walter B. Cannon and Arturo Rosenblueth, two prominent physiologists who suggested that sympathins combined with either excitatory or inhibitory receptive substances in tissues to form either excitatory sympathin E or inhibitory sympathin I [19]. These complexes were thought to be released into the bloodstream which led to either the stimulation or the inhibition of other tissues. In 1948, however, Raymond P. Ahlquist proposed an alternative view by suggesting the existence of two types of adrenoceptors which he named alpha and beta. Ahlquist compared the potency of action of six sympathomimetic amines in experiments on dogs, cats, rats and rabbits in terms of several physiological functions [20]. The differences in effect of the six sympathomimetics could not be reconciled with Cannon and Rosenblueth’s view of the receptor. According to Ahlquist,
epinephrine had all the chemical, physical and physiological properties necessary to be the only adrenergic mediator. His proposal of the existence of two fundamental types of adrenotropic receptors was directly opposed to the concept of two adrenergic mediators (sympathin E and sympathin I), and this distinction subsequently developed into the modern concept of nicotinic and muscarinic acetylcholine receptors.

Despite some of the inconsistencies associated with a quantitative approach to the receptor concept, the idea as a whole was steadily gaining acceptance over those based on physical theories of drug action. In the 1950s, the receptor concept took another step forward when Everhardus Jacobus Ariens and Robert P. Stephenson revisited Clark’s theory in their attempts to account for partial agonism. Ariens observed dualistic behavior from the action of phenylethylamines in elevating blood pressure in decapitated cats; that is, they elicited both agonistic and antagonistic effects [21]. Ariens therefore introduced the concept of ‘intrinsic activity’ as a new factor that determined the maximum effect of a drug. According to Ariens, the biological effect of a drug had to be described not only as a function of its dose and its affinity for the receptor but also as a function of the degree of effect that it is able to produce upon combining with the receptor. Although Arien’s introduction of ‘intrinsic activity’ did not alter the fundamental principles articulated by Alfred Clark, in that the maximal effect of a given drug still required full occupancy of the entire receptor population, it did address some of the anomalous observations wherein the apparent saturation of all receptor sites by some agonists did not elicit a maximal response.

Robert Stephenson addressed the phenomenon of partial agonism with his proposed concept of ‘efficacy’. It was a major conceptual advance in the understanding of the quantitative relationship between receptor occupancy and receptor-elicited effects. According to Stephenson, efficacy is a characteristic of the drug that describes its ability to activate receptors and is distinct from its affinity for those receptors. In 1956, he postulated three principles which he believed governed receptor-mediated effects and could explain the anomalous observations that some agonist-response curves often were steeper than the dose-response relationships predicted by simple law of mass action [22]. They are as follows. First, a maximum effect can be produced by an agonist when
occupying only a small proportion of the receptors. Second, the response is not linearly proportional to the number of receptors occupied. Third, different drugs may have varying capacities to initiate a response and consequently occupy different proportions of the receptors when producing equal responses. The last point is what Stephenson referred to as the efficacy of the drug. Critical to this idea is the notion that a maximal tissue response does not necessarily correspond to full receptor occupancy but could occur when only a small proportion of the receptors are occupied. Stephenson’s approach introduced the concept of spare receptors and, in contrast to Arien’s description of partial agonism, it moved away from Clark’s initial postulate that agonist concentration-effect curves tracked the corresponding curves for occupancy of the receptor.

The existence of ‘spare receptors’ was confirmed by the work of Robert F. Furchgott with the use of haloalkylamine antagonists such as dibenamine [23]. Dibenamine is known to block histamine and catecholamine receptors irreversibly. When used sparingly, it yielded data on the blockade of histamine-induced contractions that resembled the pattern expected for reversible, competitive antagonism: that is, a parallel shift to the right of the agonist log concentration-response curves with no change in the slope or the maximal response. At higher concentrations of dibenamine, a progressive inactivation of the receptors was accompanied by a rightward shift in the agonist log dose-response curves and a concomitant reduction in the maximal response. He emphasized that a non-proportionality between occupancy and response was commonly observed [24,25]. A similar pattern was described by Nickerson when he demonstrated that occupancy of only 1% of the histamine receptor population in guinea pig ileum was sufficient to give rise to maximal contractile effects [26]. His observations also suggested the existence of a large reserve for histamine receptors in this tissue. As more dose-response studies were carried out in different tissues, it slowly became clear that the system was rather more complicated than articulated initially by Clark in his prototypic scheme.

It was shortly after Stephenson had introduced his concept of efficacy that the development of several novel technologies would facilitate the merger between
biochemistry and pharmacology [27]. The 1960s and 1970s saw major advances in receptor research with the emergence of radioligand binding and affinity labeling techniques, detergent solubilization, purification via affinity chromatography and lipid reconstitution.

It can be argued that methods based on radioligand binding were the technical advance that opened the door to the molecular era of receptor research; through liquid scintillation counting of compounds radiolabeled to high specific activity, it became possible to measure directly the binding of drugs to receptors rather than to infer those properties from activities measured at effectors downstream [27]. Sir William Drummond Macdonald Paton and Humphrey P. Rang performed the first studies on atropine uptake by the smooth muscle of guinea-pig ileum, where they demonstrated the existence of saturable binding to sites with the characteristics of muscarinic receptors [28]. Shortly thereafter, Gill and Rang also prepared and radiolabeled benzilylcholine mustard, which is an irreversible atropine-like compound, in an attempt to purify the receptor protein [29]. It was not very long after these pioneering studies that the nicotinic acetylcholine receptor would be successfully labeled with \( \alpha \)-bungarotoxin [30] and the \( \beta \)-adrenoeceptor with alprenolol [31]. Such methods eventually would be employed by others and would lead to new insights into the dynamic regulation of receptor number and behavior as well as insights into their molecular coupling properties.

In 1971, Earl Sutherland was awarded the Nobel Prize for his work on the mechanism of action of hormones. In 1958, he isolated a previously unknown compound called cyclic adenine monophosphate (cAMP), and by the 1970s he had shown that it acted as a secondary messenger mediating the actions of dozens of receptors [32]. In particular, his discovery implied that epinephrine induced the formation of cAMP in liver cells, where the nucleotide converted the inactive phosphorylase to its active form which ultimately led to the formation of glucose. Moreover, he also discovered the enzyme that was responsible for the conversion of ATP to cAMP, which he then named adenylyl cyclase.
In 1992, Edwin Krebs shared the Nobel Prize with Edmond Fischer for their work on reversible phosphorylation as a mechanism of protein activation which played important roles in regulating various cellular processes \[33,34\]. Their work showed that an enzyme called protein kinase takes phosphate from adenosine triphosphate (ATP) and adds it to inactive phosphorylase resulting in activation. Moreover, they showed that another enzyme called protein phosphatase reverses this process and deactivates phosphorylase by removing the phosphate. Their work had an immense impact in many areas of biology, including signaling via G protein-coupled receptors, and it became evident over the years that reversible protein phosphorylation is a general mechanism that cells employ to regulate activity.

In 1994, Martin Rodbell shared the Nobel Prize with Alfred Gilman for their discovery of GTP-binding proteins (G proteins) and for their work in uncovering the role of those proteins in signal transduction. In particular, Rodbell studied the properties of the glucagon-sensitive adenylyl cyclase system in liver membranes, where he noted that ATP could reverse the binding action of glucagon to a yet undefined receptor of the cell \[35\]. He later realized that his adenylyl cyclase assay was contaminated with GTP. When he used synthetic, GTP-free, AMP-PNP as the substrate instead of “dirty” ATP, he discovered that the hormone could not act unless GTP also was added. This observation led him to propose, in 1971, the existence of a guanine nucleotide regulatory protein (G protein) that serves as a transducer between hormone receptors and adenylyl cyclase. Gilman and his colleagues eventually would demonstrate the existence of this protein, which they successfully purified and named Gs.

Taken together, the works of Sutherland, Krebs, Fisher, Rodbell and Gilman identified the essential components for the initial series of molecular events that are required to communicate a signal from the outside of the cell to the inside. The ability to tag receptors and other proteins directly, a technique that became available in the 1970s and 1980s, allowed for the purification of each of these signaling components. One of the earliest purifications of a receptor was achieved using affinity chromatography. The $\beta_2$-adrenoceptor \[36–38\] and the $\alpha_{2A}$- [39] and $\alpha_{1B}$-adrenoceptors [40] were purified essentially to homogeneity using this procedure. It later would be demonstrated that the
receptor can be successfully reconstituted with purified $G_s$ and the catalytic moiety of adenylyl cyclase to give a fully functioning system in which catecholamines could stimulate the enzyme \[41,42\]. Such experiments revealed the minimum number of components that are necessary for the agonist-sensitive regulation of adenylyl cyclase.

The advent of receptor cloning and advances in micro-sequencing techniques by the mid-1980s hastened the identification and characterization of a multitude of receptors as well as the other components of the G protein-mediated signaling pathway. The earliest example came in 1983 when the gene and cDNA encoding bovine rhodopsin was successfully cloned \[43\]. Shortly thereafter, clones of other receptors including the M$_1$ and M$_2$ muscarinic receptors \[44,45\], $\beta_2$-adrenoceptor \[46\], $\alpha_2$-adrenoceptor \[47\] and dopamine D$_1$ receptor \[48\] were obtained, as well as those of the stimulatory and inhibitory G proteins and mammalian adenylyl cyclase. Since then, more than 800 different G protein-coupled receptor genes have been identified, and the G proteins to which they couple to have been found to derive from at least 35 different genes \[49\]. The system which Alfred Clark had attempted to describe mechanistically in the early 1930s, when he related drug or hormone concentration to tissue response, turns out to be not quite as simple as it first seemed.

1.2 The G protein-Coupled Receptor Superfamily

The receptor responsible for mediating the opposing actions of pilocarpine and atropine, which John Langley first observed as a student at Cambridge, would come to be known as a member of the superfamily of G protein-coupled receptors (GPCRs). It is the largest group of cell surface proteins that are involved in signaling across biological membranes. It has been estimated that there are over 1000 sequences, which constitute roughly 3–4 % of all human genes, and of which 500 are odorant or taste receptors and 450 are receptors for endogenous ligands \[50\]\[51\]. They are involved in many physiological processes and also are linked to a multitude of diseases such as retinitis pigmentosa \[52\], hyperfunctioning thyroid adenomas \[53\], and acromegaly \[54\] to name a few. Over the
years, they have become an attractive avenue for therapeutic intervention in that approximately 50% of all drugs on the market today are targeted toward G protein-coupled receptors [55]. All members of this family of membrane proteins are likely to operate through a similar molecular mechanism, and there accordingly is a vast potential for drug discovery in understanding the manner by which they transduce signals across biological membranes

G protein-coupled receptors are commonly classified into three major families based on the homology of their amino acid sequences [56,57]. Family I is composed of the rhodopsin-like receptors, which happens to be the largest of the three and is further divided into three other subclasses. Family II is made up of the secretin/glucagon receptor group, which is a small collection of peptide receptors. Family III includes the metabotropic glutamate receptors, the calcium sensor, the γ-aminobutyric acid receptor, and a family of ~100 pheromone receptors.

Recently, G protein-coupled receptors have been further classified into five main families on the basis of their phylogeny [58]. This classification system has been named GRAFS. It is an acronym for Glutamate, Rhodopsin, Adhesion, Frizzled and Secretin, and it corresponds to the names of the five main groups. Receptors that make up the families of Rhodopsin, Secretin and Glutamate in GRAFS are generally the same as those receptors of Families I, II and III, respectively. In GRAFS, the newly classified Adhesion family consists of receptors with GPCR-like transmembrane-spanning regions fused at the N-terminus to one or several functional domains containing adhesion-like motifs [59,60]. Such domains include EGF-like repeats, mucin-like regions and conserved cysteine-rich motifs. The other newly classified family of G protein-coupled receptors is that of Frizzled, and it includes the frizzled and the taste-2 (TAS2) receptors.

G protein-coupled receptors are known to undergo post-translational modifications such as palmitoylation and phosphorylation, which have been shown to play a role in G protein-coupling selectivity and also in the process of desensitization [57,61]. Rhodopsin
was the first G protein-coupled receptor for which autopalmitoylation was demonstrated in vitro [62]; that is, palmitoylation occurs at two highly conserved cysteine residues in the carboxyl-terminus of the protein, 14 amino acids away from the putative membrane/cytosol border. Similarly, other G protein-coupled receptors also have been found to contain one or more highly conserved cysteine residues within their C-terminus located at a position analogous to that found in rhodopsin. Functional analyses of mutant receptors lacking these conserved C-terminal cysteines have failed to reveal a common functional role for receptor palmitoylation. It appears to depend upon the specific receptor examined, as it has been shown to have several different effects on receptor phosphorylation, sequestration, desensitization, cell-surface trafficking, and the efficiency of G protein-coupling [61].

Phosphorylation by a number of kinases has been shown to initiate a cascade of events that lead to receptor desensitization [63,64]. It has been suggested that the sites of phosphorylation are in the vicinity of those of palmitoylation, and a model for the concerted regulation of these two post-translational modifications has been proposed [61]. In that context, palmitoylation has been suggested to act as a molecular switch, regulating the accessibility of phosphorylation sites involved in the desensitization of the receptor, rather than to exert direct control over the interaction between the receptor and the G protein.

Despite the remarkable structural diversity of their activating ligands and the variety of their amino acid sequences, G protein-coupled receptors share a common architecture; that is, their tertiary structure consists of seven transmembrane \( \alpha \)-helices which are linked by alternating intracellular and extracellular loops [57]. The ligand-binding site of those receptors that belong to Family I is located within a crevice formed by the cluster of transmembrane domains; the interaction with heterotrimeric G proteins occurs at the intracellular surface and involves the C-terminal domain of the receptor [65,66].

The high-resolution crystal structures of several G protein-coupled receptors from Family I are now available. They include rhodopsin [67], opsin [68,69], the \( \beta_1 \)- [70] and \( \beta_2 \)-adrenergic receptors [71,72] and the \( A_{2A} \) adenosine receptor [73]. The general
organization of the seven-helical bundle was initially identified by cryo-electron microscopy, and this general topology was confirmed by the first X-ray structure of rhodopsin [67]. The crystal was resolved at 2.8 \( \text{D} \), and it is of the dark state (inactive) of rhodopsin. It can be seen that the helical bundle at the intracellular side of the receptor is tightly packed. That is in contrast to the extracellular segments, which diverge from one another, thus creating the main ligand-binding pocket. The crevice that forms the binding pocket is completely covered by a \( \beta \)-sheet structure that comprises major parts of the second extracellular loop, forming what appears to be a lid above retinal.

For several years, the crystal structure of rhodopsin was the only one which was available, and therefore it has been used as the structural template for many other G protein-coupled receptors [74,75]. Recently, the crystal structure of a \( \beta_2 \)-adrenergic receptor-T4 lysozyme fusion protein was resolved at 2.4 \( \text{D} \) [71]. The receptor presumably was in its inactive state, as it was crystallized while bound to the partial inverse agonist carazolol. When superimposed onto the structure of rhodopsin, it was found that, despite an overall similarity of the transmembrane segments, the \( \beta_2 \)-adrenergic receptor appears to have a relatively more open structure. In contrast to the buried \( \beta \)-sheet structure of the second extracellular loop in rhodopsin, the corresponding region of the \( \beta_2 \)-adrenergic receptor is more exposed to the solvent and contains an extra helical segment. Moreover, the N-terminus of the \( \beta_2 \)-adrenergic receptor does not make extensive contact with the second extracellular loop, which also is in contrast to that of rhodopsin. It has been suggested that this lack of interaction between the N-terminus and the second extracellular loop of the \( \beta_2 \)-adrenergic receptor facilitates access of a diffusible ligand to the binding site [71].

It appears that carazolol and retinal occupy similar spaces in their respective receptors. The main difference is that retinal extends deeper into the binding pocket of rhodopsin and contacts residues on helices V and VI. There is a highly conserved tryptophan residue in helix VI which has been proposed to act as a ‘toggle switch’ for receptor activation, and the interaction between this residue and cis-retinal likely accounts for the absence of basal activity in rhodopsin. Carazolol does not interact directly with this proposed switch, and it has been suggested that it lowers the basal
activity of the β2-adrenergic receptor by interacting with two phenylalanine residues that surround that highly conserved tryptophan in helix VI [71].

Even when the β2-adrenergic receptor is occupied by the inverse agonist carazolol, the relatively high constitutive activity is suppressed by only 50%. It appears that the carazolol-bound β2-adrenergic receptor is not the functional counterpart of the dark-adapted state of rhodopsin, which is known to have no detectable basal activity. The difference in the arrangement of the cytoplasmic ends of the transmembrane segments between these two receptors has provided structural insights into basal receptor activity. For example, it appears that a network of hydrogen bonds and charge interactions referred to as the ‘ionic lock,’ which helps to maintain rhodopsin in an inactive conformation, serves a similar role in the β2-adrenergic receptor [72]. It previously has been shown that mutations of these amino acids in the β2-adrenergic receptor or of those in other adrenergic receptors lead to constitutive activity [74,76].

It appears that constitutive activity is a common property of numerous wild-type G protein-coupled receptors and many of them are members of Family 1a, which includes the biogenic amine receptors, cannabinoid and opioid receptors [77]. It has been suggested that the comparatively more open structure of the β2-adrenergic receptor likely is more reflective of the general architecture of other G protein-coupled receptors, particularly those of Family I, than is that of the known structure of rhodopsin.

Unlike rhodopsin, generating high-resolution crystals of other G protein-coupled receptors has proven difficult owing to their low natural abundance, inherent structural flexibility, and instability in detergent solutions [72]. Successful crystallization of the β2-adrenergic receptor skirted these issues by expression of the protein in Sf9 insect cells, by the use of a monoclonal antibody and by incubation of the receptor with an inverse agonist of picomolar affinity. Protein expression in Sf9 cells allowed for the production of large quantities of the receptor, which was purified to homogeneity using antibody and ligand-affinity chromatography. The production of a monoclonal antibody that bound to the relatively unstructured region of the third intracellular loop of the receptor provided sufficient conformational stability. The instability in detergent solution was avoided by
incubation of purified $\beta_2$-adrenergic receptor with an inverse agonist of picomolar affinity, which helped to maintain the receptor in its less labile inactive state.

It is likely that the strategies employed in the structural analysis of the $\beta_2$-adrenergic receptor are applicable to other G protein-coupled receptors, especially to those of Family I. One therefore can expect that the crystal structures of other G protein-coupled receptors will become available with increasing frequency, contributing in turn to a better understanding of structure and mechanism of action within this large family of membrane proteins.

1.3 Mechanism of Signaling

There appear to be three principal components involved in the initial stages of transmembrane signaling: namely, the receptor, the heterotrimeric G protein to which it couples and the effector enzyme which it regulates. The key to understanding the mechanism of signaling via G protein-coupled receptors lies in understanding the nature of the interaction between the receptor and its cognate G protein. Not long after the discovery of the latter in 1971 \[35\], mechanistic models were conceived to rationalize GPCR-mediated effects. Radioligand studies, which directly measure the binding of a ligand to a receptor, have shown that there is a dispersion of affinities in the binding of agonists \[78\]. At thermodynamic equilibrium, there are three possible explanations for the observed dispersion: a heterogenous mixture of mutually independent and non-interconverting sites \(i.e.,\) multi-site model; \(e.g.,\) Reference \[79\]), interaction of the receptor with a limiting quantity of a third component such as a G protein \(i.e.,\) ternary complex model; \[80\]), and cooperativity among interacting sites \[81\].

In 1972, a general approach was put forward to describe the reversible binding reactions between multiple species of ligand and multiple species of binding molecule, which in this case would be the receptor, in a system at thermodynamic equilibrium \[82\]. This general model is referred to here as the ‘multi-site’ model, and it assumes that each species of receptor behaves as if it were mutually independent in the binding of ligand;
that is, there is no interconversion between different classes of sites and no interaction between one site and another. In the absence of structural or mechanistic information on GPCR-mediated systems, the multi-site model was widely adopted for the analysis of data that revealed multi-phasic behavior in the binding of agonists (i.e., \( n_H < 1 \)). The dispersion of affinities seen in the binding of agonists, therefore, has been attributed to multiple classes of receptor [79]. Although the model can describe the observed heterogeneity, it does so at the price of mechanistic consistency [83]. For example, the effect of guanyl nucleotides suggests that the receptor can interconvert between at least two states differing in their affinity for agonists; that is, the presence of the nucleotide appears to convert the receptor from a state of high affinity for the agonist to a state of low affinity [80,84,85]. This interconversion is manifested as a steepening of the binding curve such that the value of \( n_H \) approaches 1 and an overall shift of the curve to the right. The effect has been generally referred to as the ‘GTP-shift’ . Moreover, it has been shown that the fraction of receptors in one or another class can be different for different agonists even when characterized under the same experimental conditions [78,83,86–89]. If the apparent heterogeneity indeed derives from different classes of receptor, such as different receptor subtypes or the same subtype with different post-translational modifications, then all agonists for a particular receptor are expected to exhibit the same proportion of high- and low-affinity sites. The apparent interconversion of the receptor from high to low affinity in the presence of guanyl nucleotides and the inconsistencies in the proportion of high- and low-affinity sites for different agonists highlight the failure of the multi-site model to provide a mechanistically consistent account of the data [83].

Although the multi-site model is not tenable as a mechanistic basis for G protein-mediated signaling, it does provide a quantitative measure of the dispersion of affinities revealed in the binding of agonists. Thus, the ratio of affinities for the states of high and low affinity (i.e., \( K_L/K_H \)) correlates with pharmacological properties such as efficacy and intrinsic activity [e.g., 86,90]. Similarly, the fraction of receptors ostensibly in the state of high affinity for agonists and the magnitude of the shift effected by guanyl nucleotides also correlate with efficacy [86,91]. The binding patterns thus appear to be a manifestation of the mechanistic events that culminate in a response, and it is widely
accepted that sites of low and high affinity for agonists represent coexisting populations of uncoupled and G protein-coupled receptors.

The limitations of models based on classical saturation functions such as the multi-site model prompted De Lean et al. (1980) to propose that the low Hill coefficients arise from an interaction among components of a ternary complex composed of agonist (A), receptor (R), and its cognate G protein (G) [80]. Since that time, the ternary complex model has become the most widely accepted view in rationalizing the mechanism of signal transduction across biological membranes. Its basic premise in terms of G protein-mediated signaling is the notion of a spontaneous equilibrium between free receptor (R) and free G protein (G) on the one hand and a receptor-G protein complex on the other (RG) (i.e., R + G Ú RG) [85]. The freely mobile components of the ternary complex are assumed to assemble through random collisions within the membrane and, once formed, the complex is presumed transient. Thus, the equilibrium dissociation constants for the binding of A to R and to RG, and for the binding of G to R and to AR, can be represented by $K_A$, $K_{AG}$, $K_G$, and $K_{GA}$, respectively [85]. Four quantities control the binding of agonists in the ternary model: (a) the ratio of total G protein to total receptor within the membrane ($[G]_t/[R]_t$); (b) the relative affinity of the agonist (A) for the receptor alone (R) and for the complex (RG) between receptor and G protein ($K_A/K_{AG}$); (c) the relative affinity of the radiolabeled antagonist (P) for the two forms of the receptor ($K_P/K_{PG}$); and (d) the total concentration of receptor within the membrane relative to its affinity for the G protein ($[R]_t/K_G$). Moreover, it is implicit in the model that agonists are without effect on $[G]_t/[R]_t$.

The equilibrium between the uncoupled (R) and coupled (RG) states of the receptor is regulated by agonists and guanyl nucleotides in accord with the principle of microscopic reversibility; that is, compounds with higher affinity for free R or G must promote uncoupling, and those with higher affinity for the complex must promote coupling [85]. The model predicts that a drug may bind with higher affinity either to the G-coupled form ($K_A/K_{AG} > 1$) or to the uncoupled form ($K_A/K_{AG} < 1$) of the receptor. The fraction of receptors coupled to G protein thus will be either increased ($K_G/K_{GA} > 1$) or decreased ($K_G/K_{GA} < 1$) upon addition of the drug. Data in the literature suggest that
agonists have higher affinity for the complex and therefore will promote the association of the receptor with the G protein \[92,93\]. In contrast, guanyl nucleotides have higher affinity for free G protein and therefore will promote uncoupling of the complex (RG), leaving the receptor in the form for which agonists have lower affinity. Antagonists either are indifferent to presence of the G protein \((K_A = K_{AG})\) or, in the case of inverse agonists, there are data to suggest that they mimic the effect of guanyl nucleotides: that is, they have higher affinity for the uncoupled form of the receptor and thus promote uncoupling \[94–98\].

Qualitatively, the ternary model appears to describe the binding patterns of agonists and to account for those effects that are inconsistent with the assumptions of the multi-site model. Moreover, the model also is in apparent agreement with several biochemical observations. For example, there was an apparent increase in the size of the \(\alpha\)-adrenergic \[99\], \(\beta\)-adrenergic \[100\] and D2-dopamine \[101\] receptors upon treatment of membranes with agonists, which corresponds to the association of the receptor with its cognate G protein. In the absence of agonist, or after treatment with either antagonist or guanyl nucleotide, only free receptor and free G protein were detected. It also has been shown that co-immunoprecipitation of the RG complex for muscarinic receptors from heart requires pre-treatment of the membranes with agonists \[102,103\]. Moreover, the amount of G protein that co-precipitated with the receptor exhibited a dose-dependence on the concentration of the agonist, and the level of co-immunoprecipitation was higher with a full agonist than with a partial agonist. Such evidence lends support to the notion of a ligand-regulated equilibrium between free receptor and free G protein on the one hand and the receptor-G protein complex on the other.

In order for the ternary complex model to account quantitatively for the dispersion of affinities observed in the binding of agonists, three conditions have to be satisfied; that is, the agonist must differ in its affinity for R and RG, the number of G proteins must be limiting, and the total concentration of the receptor must be bracketed by its affinity for the G protein in the absence and presence of ligand \[85\]. If these conditions are not fulfilled, then the ternary complex model predicts that agonist binding curves will have the form of a rectangular hyperbola (\(i.e., n_H = 1\)).
It is clear that the agonist must differ in its affinity for R and RG for it to be able to differentiate between the uncoupled and coupled forms of the receptor. Otherwise, agonist binding curves would be monophasic. Indeed, there is evidence to show that agonists do differ in their affinity for receptor alone and for receptor coupled to a G protein. It has been shown that muscarinic receptor purified from porcine brain exhibits predominantly low affinity in the binding of acetylcholine both in the absence of nucleotide and in the presence of GMP-PNP [104]. When the receptor was reconstituted with either Gi or Go, however, the receptor exhibited predominantly high affinity in the binding of acetylcholine; upon treatment with GMP-PNP, the majority of sites of high affinity were converted to sites of low affinity. Moreover, in those same preparations, the antagonist atropine did not exhibit any difference in its affinity for purified receptor alone or for purified receptor reconstituted with either Gi or Go. Similar patterns have been observed in other studies of purified M₂ muscarinic receptor [93], and also in studies of purified µ-opioid receptor, [105–107], purified D₂ dopamine receptor [108], and purified A₁ adenosine receptor [109].

In principle, three situations can exist with respect to the relative amounts of G protein and receptor: [G]ᵢ greater than [R]ₑ, [G]ᵢ equal to [R]ₑ, and [G]ᵢ less than [R]ₑ [85]. The nature of the dispersion of affinities in the binding of agonists that is predicted by the ternary model derives from the fixed ratio of [G]ᵢ to [R]ₑ and the mutual depletion of both. The Hill coefficient is 1 when [G]ᵢ is 0 and tends to 1 as [G]ᵢ exceeds [R]ₑ sufficiently that relatively little change can occur in the free concentration of G. In the case when [G]ᵢ is greater than [R]ₑ, it has been shown that a 1.4-fold excess of the former over the latter places a lower limit of 0.85 on the Hill coefficient, and a two-fold excess results in a lower limit of 0.91. Experimental error in binding assays generally would render the latter value virtually indistinguishable from 1, which highlights the fact that a comparatively small excess of G protein over receptor would be sufficient to yield curves that appear monophasic. It follows that the number of G proteins must be severely limiting in order for the system to give rise to the manifestly biphasic curves observed in the binding of agonists.
The number of G proteins is limiting in situations when $[G]_t$ equals $[R]_t$ or when $[G]_t$ is less than $[R]_t$ [85]. When $[G]_t$ equals $[R]_t$, the binding profiles for agonists can appear biphasic with appropriate values of $[R]/K_G$ and $K_A/K_{AG}$. In that case, it has been shown that no agonist irrespective of its relative affinities for R and RG can exhibit a Hill coefficient less than a minimum determined by the ratio of $[R]_t$ to $K_G$. Similarly, no change in $[R]_t/K_G$ can reduce the Hill coefficient of an agonist below a minimum determined by $K_A/K_{AG}$. A Hill coefficient of 0.67 is the smallest that can be obtained when the number of G proteins equals the number of receptors. This theoretical minimum reflects the limitation on the apparent heterogeneity possible with the ternary model, and it shows that there is a very narrow range of values for either $[R]_t/K_G$ or $K_A/K_{AG}$ which will give rise to binding profiles that are manifestly biphasic.

Similar restrictions on the values of $[R]_t/K_G$ and $K_A/K_{AG}$ are observed when $[G]_t$ is less than $[R]_t$ [85]. In this case, however, the origin of a multiphasic curve is self-evident. When there are fewer G proteins than there are receptors, some receptors will be free of G proteins even in the presence of an agonist that has a very high affinity for RG simply because there are not enough G proteins. Intuitively, one expects that there will be two components to the binding profile; that is, the agonist will exhibit one affinity for the free receptor and a different affinity for the G protein-coupled receptor. Interestingly, however, there actually are at least three components. One component arises from the excess of receptors over G proteins. It reflects the interaction of the agonist with free receptor and thus exhibits a Hill coefficient near or equal to 1. The second and third components reflect the effect of the agonist within the ternary system. The Hill coefficient for these two components is less than 1, and the value is controlled by $K_A/K_{AG}$ and $[R]_t/K_G$ in a manner similar to the case when $[G]_t$ equals $[R]_t$.

Whenever $[G]_t$ approximates $[R]_t$, the total concentration of the latter must be bracketed by the affinity of the receptor for the G protein in the absence and presence of the ligand in order for the ternary model to predict multi-phasic curves for the binding of agonists (i.e., $K_{GA} < [R]_t < K_G$ or $K_{GA} > [R]_t > K_G$) [85]. This requirement relates to the ability of the ligand to cause maximal perturbation of the system; that is, in the coupling or uncoupling of the receptor and G protein. This will occur when two conditions are
met: first, $K_G$ must be such that the system in the absence of a ligand exists predominantly in the state not favored by that ligand; second, $K_{GA}$ must be such that a sufficient concentration of the ligand can preclude measurable quantities of uncoupled R and G ($K_A/K_{AG} > 1$) or the RG complex ($K_A/K_{AG} < 1$). For example, when $K_{GA} < [R]_t < K_G$, the system is such that, in the absence of ligand, the receptor exists predominantly in the uncoupled form. Upon addition of an agonist that has a high affinity for RG, the system is perturbed maximally in that the receptor becomes predominantly coupled to the G protein. However, when $[R]_t$ falls outside this range (i.e., $[R]_t > K_G$ or $[R]_t < K_{GA}$), maximal perturbation of the system will not be achieved. When $[R]_t > K_G$, the receptor exists predominantly in the coupled form even in the absence of ligand, and the effect of an agonist is to shift the equilibrium further in that direction, that is, towards the coupled state. When $[R]_t < K_{GA}$, the receptor exists predominantly in the uncoupled form in the absence of ligand and remains largely uncoupled even in the presence of agonist.

Several lines of evidence are at odds both qualitatively and quantitatively with various precepts of the ternary complex model. For example, there is evidence to suggest that the receptor and the G protein associate to form a stable complex rather than one which is transient as presumed in the ternary model. It previously has been shown through immunoblotting that the M$_2$ muscarinic receptor is accompanied by a mixture of G proteins including G$_{i1}$, G$_{i2}$, G$_{i3}$, G$_o$, G$_{q/11}$ and G$_s$ when affinity-purified from the sarcolemmal membrane of porcine atria [110,111]. G proteins which include G$_{i1/2}$ and G$_o$ also have been found to accompany the A$_1$ adenosine receptor when the latter is affinity-purified from membranes of bovine cerebral cortex [112]. Similarly, $\mu$-opioid receptor solubilized from rat brain can be co-immunoprecipitated with the $\alpha$-subunits of G$_o$, G$_{i1}$ and G$_{i3}$ [113,114], and the vasopressin receptor was found to co-sediment with its cognate G protein following solubilization from plasma membranes of rat liver [115].

It has been shown using fluorescence resonance energy transfer (FRET) that in the absence of agonists in intact living cells, the $\alpha_{2A}$-adrenergic receptor, M$_4$ muscarinic receptor, A$_1$ adenosine receptor and the D$_2$ dopamine receptor are each in a complex with G$_o$ in their resting state [116]. Similarly, the conformational rearrangements at the interface between the $\alpha_{2A}$-adrenergic receptor and Ga$_{i1}\beta_1\gamma_2$ heterotrimer were monitored
using bioluminescence resonance energy transfer (BRET), and it was demonstrated that at least a fraction of the receptor exists in pre-associated complexes with the G protein even in the absence of receptor activation [117]. Moreover, changes in the BRET signal suggested that there is a conformational change between the N-terminus and the helical domain of the γ- and α-subunits of the G protein, respectively, rather than the full dissociation of the latter upon activation of the receptor with an agonist. Similar results have been obtained in other studies in which resonance energy transfer techniques were applied to G proteins of the Gi family [118,119].

The ability of the receptor-G protein complex to survive the procedures of solubilization and purification argues against the notion that the two are loosely bound in a transient association. Also, the observation that they can be found in a complex in the absence of agonist-promoted receptor activation argues against a ligand-regulated equilibrium between receptor and G protein. Moreover, the observation that the latter may not necessarily dissociate fully from the former upon activation of the receptor by an agonist is further evidence that the complex may not be transient. Taken together, these data are at variance with the notion of a reversible equilibrium between two, membrane bound proteins that are presumed to associate and dissociate in random fashion such that all members of one pool potentially can interact with all members of the other.

There is evidence to suggest that the level of G proteins often exceeds that of the receptor in systems where apparent heterogeneity is detected in the binding of agonists [120 and references therein]. For example, in preparations of purified plasma membranes from human platelets and in the postmortem brains of humans, it was found that there is a 20–100-fold excess and over a 1000-fold excess of G proteins over α2-adrenergic and ρ-opioid receptors, respectively [121,122]. When studies were carried out to quantify the components involved in signaling through the β-adrenergic receptor, it was found that the ratio of receptor:G protein:adenylyl cyclase in S49 murine lymphoma cells was approximately 1:100:3 [123]. It has been shown that very large quantities of the Gi and Go subtypes of G protein can be purified from membranes of bovine brain, accounting for about 1.5% of the total membrane protein [124]. At such high levels, those G proteins are likely to be in great excess relative to their cognate receptors. The multi-phasic
binding of agonists in those systems therefore argues against the ternary model, as only a
twofold excess is required to render the model unable to predict manifestly bi-phasic
curves. In the case of the M2 muscarinic receptor, which couples to G proteins of the
Gt/Go family, saturation of the former with the latter was ensured by treatment of the
system with propylbenzilylcholine mustard, which resulted in the irreversible blockade of
about 80% of the receptors. Even in this extreme case, multi-phasic curves were obtained
for the binding of agonists [125].

Quantitative application of the ternary complex model to describe the binding of
agonists to β-adrenergic receptors [80], D2 dopaminergic receptors [126], A1 adenosine
receptors [127], α2 adrenergic receptors [128], and M2 muscarinic receptors [91,129–131]
commonly has implied that guanyl nucleotides such as GMP-PNP cause an irreversible
loss of G proteins from the system. Such an irreversible loss cannot be accounted for by
the model, and it is incompatible with the basic notion of a thermodynamic equilibrium.
In the case of the M2 muscarinic and β-adrenergic receptors, the ratio of total G protein to
total receptor ([G]t/[R]t) inferred from the model was reported to differ with different
agonists [80,82], which implied that the agonist itself determines the total number of G
proteins that is accessible to the receptor [83]. In principle, [G]t/[R]t is expected to be
independent of the agonist, and the observed variability is inconsistent with the ternary
model. The nucleotide is expected to alter \( K_G \), that is, the affinity of the receptor for the
G protein. It thereby ought to perturb the equilibrium between coupled and uncoupled
receptor, which in turn ought to yield a shift in the inhibitory potencies of agonists [85].
In the case of the M2 muscarinic receptor, those shifts are not as great as those predicted
by the model [85]. Moreover, the model also is unable to describe the high-affinity sites
recognized by agonists at saturating concentrations of GMP-PNP.

Just as heterogeneity is observed in the binding of agonists to G protein-coupled
receptors, heterogeneity also is observed in the binding of guanyl nucleotides to receptor-
coupled G proteins. It has been shown that muscarinic agonists can modulate the
interconversion of G proteins from higher to lower affinity for GDP in a manner that is
analogous to the effect of nucleotides on the binding of agonists to the M2 receptor
[132,133]. The system therefore appears symmetrical in that the effect of agonists on the
binding of GDP mirrors the effect of guanyl nucleotides on the binding of agonists. If this apparent symmetry is to be described by the ternary model, then its parametric values ought to be the same regardless of whether the system is viewed through radioligands to the receptor or to the G protein. This is not the case with the M₂ muscarinic receptor in that estimates of \( K_G \) obtained from assays with [³H]NMS imply that there is no appreciable coupling of R and G in the absence of ligands to either protein; in contrast, those obtained from assays with [³⁵S]GTP\(\gamma\)S imply that R and G are more than 95% coupled [125]. There also is a discrepancy between the concentration of receptor as inferred from the values of [G] \(_t\) and [R]/[G] \(_t\) in assays with [³⁵S]GTP\(\gamma\)S and that which is measured directly with [³H]NMS. Similarly, different guanyl nucleotides reveal different numbers of G proteins in assays with [³⁵S]GTP\(\gamma\)S. Finally, there is a discrepancy in the affinity of guanyl nucleotides for the uncoupled G protein in that the value estimated with [³⁵S]GTP\(\gamma\)S differs from those values estimated with [³H]NMS.

Although there are biochemical and biophysical data to support both sides of the argument with regard to whether or not the receptor-G protein complex is transient or whether or not the number of G proteins is limiting, the ternary complex model fails to provide a mechanistically consistent description of the data when it is applied in a quantitative manner to G protein-mediated systems. Moreover, this inconsistency cannot be resolved by expanding the model to include two pools of receptor: one that lacks G proteins and another that contains a heterogeneous population of G proteins [85,125].

The problem with using the ternary complex model and its variants to describe signaling via G protein-coupled receptors is that it has become common practice to analyze ligand-binding data and the accompanying effects of guanyl nucleotides in terms of the multi-site model but then to rationalize those effects in terms of the ternary complex model [80,83,134]. The two schemes are not equivalent, and the estimates of high and low affinity sites from the former (\( K_{A1}, K_{A2} \)) are not necessarily equivalent to the affinities of the agonist for the coupled and uncoupled forms of the receptor in the latter (\( K_{AG}, K_A \)) [85]. The estimates of affinity from the multi-site model fall in between those values from the ternary model (\( i.e., K_{AG} < K_{A1} < K_{A2} < K_A \)). Therefore, if the ‘GTP-shift’, which correlates well with efficacy, indeed derives from the uncoupling of
the RG complex as a result of the effect of guanyl nucleotides, then the resulting curve would be markedly to the right of that obtained experimentally [85]. It therefore argues against the validity of the ternary complex model as a mechanistic basis for signaling.

As described above, a dispersion of affinities for agonists could arise in at least three ways if the system is at thermodynamic equilibrium. Given the inability of the multi-site and ternary complex models to provide a mechanistically consistent account of the data, one is left with the possibility that the apparent heterogeneity may arise from cooperativity in the binding of successive equivalents of the ligand to interacting sites within a receptor-oligomer.

Although now much in evidence, oligomers of G protein-coupled receptors went almost unrecognized for many years, and a growing body of evidence also suggests that the major components of signaling, namely, the receptor, the G protein, and the effector, can occur in precisely packaged and stable complexes [135,136]. Such systems are therefore in contrast to the fundamental premise underlying the ternary complex model and related schemes [e.g., 137,138].

Non-competitive effects in ligand binding were detected early on for the β-adrenergic receptor [139], the histamine receptor [87,140–143] and the muscarinic receptor [110,144–146]. It has been reported that the rate of dissociation of [3H]alprenolol from β-adrenergic receptors was faster when the process was initiated by addition of the unlabeled analogue than when it was initiated by dilution of the reaction mixture [138]. Hill coefficients greater than 1 have been reported for the inhibitory effect of histaminic ligands on the specific binding of [3H]histamine to membranes of guinea pig cortex [87,140,143]. Hill coefficients which exceed 1 also have been reported for the specific binding of [3H]QNB to cardiac membranes [144,146]. Similarly, apparently noncompetitive behavior also has been described in the binding of antagonists to muscarinic receptors in membranes from rat adenohypophysis [145].

Moreover, purified M2 receptors from porcine atria revealed a difference in capacity for the antagonists [3H]NMS and [3H]QNB; that is, the former labeled only about one-
half the sites labeled by the latter, yet binding at saturating concentrations of \([{}^3\text{H}]\text{QNB}\) was inhibited fully at comparatively low concentrations of unlabeled NMS \([110]\). Essentially the same pattern has been found with M$_2$ receptor extracted from sarcolemmal membranes of porcine atria and that purified from membranes of Sf$_9$ cells \([147,148]\). Data from the former were inconsistent with the multi-site model but could be accounted for in terms of cooperativity among at least four interacting sites, presumably within a tetramer or larger oligomer \([147]\). In the case of the latter, M$_2$ receptors were purified as monomers but were reconstituted into phospholipid vesicles as tetramers where they gave rise to essentially the same pattern in the binding of muscarinic antagonists as that found with M$_2$ receptor from porcine atria \([148]\).

It can be seen that cooperativity in M$_2$ muscarinic receptors is manifested in the membrane, in solution, and in a preparation where the receptor was first purified as monomers followed by reconstitution in phospholipid vesicles as tetramers. In those examples, the multi-site model is unable to provide a consistent account of the data regardless of the number of sites that are added to the model; it therefore is inadequate at any level of complexity. In contrast, models based on cooperativity can provide a good approximation of those same data and, although their success stems in part from their intrinsic complexity and their comparatively large number of parameters, they do so in a mechanistically consistent manner.

The observed differential capacity for \([{}^3\text{H}]\text{QNB}\) and \([{}^3\text{H}]\text{NMS}\) can be attributed to pronounced negative cooperativity in the binding of the latter. Cooperativity previously has been investigated as the mechanistic basis for effects that cannot be accounted for by the ternary complex model with cardiac muscarinic receptors in membranes from Syrian golden hamsters \([81]\). The dispersion of affinities observed in the binding of agonists, which the ternary complex model attributes to uncoupled and G protein-coupled receptors, can be rationalized in terms of cooperative interactions between sites within the oligomer; that is, binding of one equivalent of an agonist to a site on the vacant oligomer reduces the affinity of successive equivalents of ligand for the remaining sites. The binding of an agonist typically is inferred from its effect on the binding of a radiolabeled antagonist, and in such cases the binding pattern of the agonist is determined in part by the
magnitude of cooperative interactions between the agonist and the radioligand. The effects of GMP-PNP on the binding of agonists can be attributed to changes in the degree of cooperativity at various levels of receptor occupancy, with corresponding changes in the affinity of the agonist at each level [81,148].

In addition to the non-competitive effects exhibited by G protein-coupled receptors, they also have been found to exist in multiple interconverting states. It has been shown previously in early studies that the sulfhydryl reagent N-ethylmaleimide (NEM) appeared to convert the receptor from a state of low affinity to a state of high affinity for agonists [149,150]. An explicit scheme for such an arrangement is referred to here as the two-state model, which presupposes that the receptor interconverts spontaneously between an inactive state (R) and an active (R*) state (Scheme 3) [e.g., 151]. The preference of a ligand for one state over the other shifts the equilibrium accordingly; thus, ligands with higher affinity for the R* state are agonists, and ligands that are indifferent or prefer the R state are antagonists. Those antagonists with a preference for the R state typically are referred to as inverse agonists [152]. This notion of a ligand-regulated equilibrium between at least two states is consistent with the general pattern revealed in binding studies; that is, guanyl nucleotides are found to reduce the apparent affinity of G protein-coupled receptors for agonists. Conversely, agonists reduce the apparent affinity of receptor-linked G proteins for GDP.

The non-competitive effects revealed in the binding of antagonists and the apparent symmetry that is present in G protein-mediated systems—as manifested in the effect of agonists on the binding of GDP and the reciprocal effect of guanyl nucleotides on the binding of agonists—can be accommodated within a framework in which oligomers of receptor and oligomers of G protein exist in a stable heteromeric complex that interconverts spontaneously between at least two states. In this context, G proteins and guanyl nucleotides can be regarded as allosteric effectors that shift the equilibrium of the receptor-oligomer between R and R*. In the absence of an explicit mechanism for such effects, the shift can be modeled empirically as a change in the corresponding equilibrium constant (i.e., $K_R = [R]/[R^*]$). The equilibrium between R and R* also will be regulated by agonists and antagonists to the extent that the ligand differs in its affinity for the two
states at each level of occupancy. It therefore appears that an oligomer of receptor that can interconvert spontaneously between two states that differ in their cooperative properties can account for the several phenomena that seem likely to share a common mechanistic basis: namely, the occurrence of constitutive activity, the differential effects of agonists and inverse agonists, the multiple states of affinity, and the effects of guanyl nucleotides [81].

1.4 Oligomers of G protein-coupled receptors

The oligomers of G protein-coupled receptors implied by the non-competitive effects revealed in radioligand binding studies have now been demonstrated through various biochemical and biophysical techniques [153]. Early biochemical evidence involved the detection of immuno-reactive bands on western blots, which often showed the presence of molecular masses equivalent to two, three and sometimes larger multiples of the mass expected of a receptor monomer [e.g., 154]. For example, detection of multimeric forms of the M2 receptor purified from porcine atrial tissue has shown that the protein can migrate as a trimer and a tetramer during electrophoresis on SDS-polyacrylamide gels [110]. In recent years, the most common biochemical approach used to show the existence of oligomers has been the co-immunoprecipitation of differently tagged receptors from extracts of cells expressing both proteins. Some of the aggregates identified in this manner include homo-oligomers of the M2 and M3 muscarinic receptors [155,156], the β2-adrenergic receptor [157], the D3 dopamine receptor [158], the δ-opioid receptor [159], the somatostatin SST2A receptor [160], the mGluR5 receptor [161] and the α-factor receptor [162].

Co-immunoprecipitation also has been used to demonstrate the existence of hetero-oligomers of closely and distantly related G protein-coupled receptors. Examples of the former include the R1 and R2 subunits of the GABA receptor [163–165], the δ-opioid and κ-opioid receptors [166], the δ-opioid and μ-opioid receptors [167,168], and the SST3 and SST2a somatostatin receptors [160]. Examples of the latter include the A1 adenosine
and D₁ dopamine receptors [169], the AT₁ angiotensin and B₂ bradykinin receptors [170], and the δ-opioid and β₂-adrenergic receptors [171].

Bioluminescence and fluorescence resonance energy transfer is the non-radiative transfer of energy between the electromagnetic dipoles of a donor and an acceptor [172]. BRET and FRET recently have become valuable tools for demonstrating the existence of receptor oligomers in living cells. Both require that an overlap exists between the emission and excitation spectra of the donor and acceptor molecules, respectively. The donor and acceptor molecules also must be in close proximity to one another, generally less than 100 Å apart.

One of the earliest demonstrations that G protein-coupled receptors could form dimers or larger oligomers in living cells came from the appearance of BRET between fusion proteins in which GFP and Renilla luciferase were linked to the carboxyl-terminus of the human β₂-adrenergic receptor [173]. Oligomers of other G protein-coupled receptors that also have since been identified in this manner include the A₁ and A₂A adenosine receptors, AT₁ angiotensin receptor, δ-, κ- and μ-opioid receptors, and the TRHR1 and TRHR2 receptors to name a few [174].

Similarly, the many different spectral mutants of the jellyfish green fluorescent protein now available have made the application of FRET an attractive approach in the study of GPCR oligomerization, both in solution and in living cells [174,175]. For example, the α-mating factor receptor Ste2 has been fused to CFP and YFP, and energy transfer was shown to occur in whole cells of yeast and in purified membrane fractions [176]. With FRET, the prolonged fluorescence characteristics of lanthanide compounds, such as europium and allophycocyanin, can be exploited by allowing the short-lived autofluorescence of the cell to decay in order to achieve a greater signal-to-noise ratio. Antibodies to G protein-coupled receptors have been labeled with these compounds and incubated with cells expressing receptors tagged with different immunological epitopes. Using a technique called time-resolved FRET, in which a europium-labeled anti-FLAG antibody was used as the donor fluorophore and an allophycocyanin-labeled anti-Myc antibody was used as the acceptor fluorophore, oligomers of the δ-opioid receptor were
detected on the surface of HEK-293 cells [177]. Oligomers of the $\alpha_{1A}$-adrenergic receptor also have been identified in this manner [178].

Another application of FRET stems from an intrinsic property of a fluorophore called photobleaching, which is characterized by the fading of the fluorescent signal upon continuous exposure to excitation light. It is useful for the detection of oligomers because the close proximity of an acceptor fluorophore offers an alternate route for the deactivation of the energetically excited state of the donor fluorophore thus resulting in the slower photobleaching of the latter. Receptor oligomers identified using this approach include the somatostatin SSTR5 receptor [179], the neuropeptide Y receptor [180], the thyrotropin receptor [181] and hetero-oligomers of the somatostatin SSTR5 and D$_2$ dopamine receptors [182].

High-resolution structures of membrane proteins have been obtained recently using atomic force microscopy and from the three-dimensional reconstructions of single-particle images obtained using cryoelectron microscopy [183]. Both biophysical approaches are capable of monitoring the oligomeric status of G protein-coupled receptors, and atomic force microscopy, in particular, can do so under near-native conditions. Atomic force microscopy is a biophysical technique wherein a very sharp tip is dragged across a sample, and the change in the vertical position reflects the topography of the surface. Therefore, by collecting the height data across the sample area, it is possible to construct a three-dimensional map of the surface features. With this approach it has been shown that rhodopsin can occur in densely packed paracrystalline arrays of what appears to be rows of dimers in native mouse disc membranes [184–186]. With the use of a cell-free expression system which allows for the production of membrane proteins that can directly associate with detergent micelles upon their translation, large quantities of the V$_2$ vasopressin and the corticotropin receptors were produced for studies using cryoelectron microscopy [187]. Single particle analyses of electron micrographs of both those receptors revealed homogenously distributed particles similar to that of rhodopsin, which exhibited topologies that are indicative of the formation of homodimers [187].
A functional role for oligomers is implied by the distinct properties that can emerge from the co-expression of two isoforms. For example, membranes from COS-7 cells transfected with the genes for two complementary chimeras of the $\alpha_2$-adrenergic and M$_3$ muscarinic receptors were found to bind adrenergic or muscarinic antagonists only when the recombinant proteins were coexpressed [188]. Similarly the K102A and K199A mutants of the angiotensin AT$_{1A}$ receptor bound peptidic agonists and the antagonist [3H]DuP753 only when coexpressed, and the maximal effect occurred when the two genes were transfected in equal amounts. In that case, however, recovery of binding was not accompanied by recovery of a receptor-mediated response [189]. A constitutively desensitized mutant of the $\beta_2$-adrenergic receptor has been shown to regain the native stimulatory effect on adenylate cyclase when coexpressed with the wild-type receptor [190]. It therefore appears that oligomers have distinct implications for binding on one hand and for efficacy or intrinsic activity on the other. Although it is now widely accepted that G protein-coupled receptors occur as oligomers, the basic functional oligomeric-unit remains unclear.

1.5 Cholesterol

The discovery of cholesterol is credited to the French chemist Michel Chevreul, who found it as a component of human gallstones in 1815 [191]. Its empirical formula (C$_{27}$H$_4$O$_6$) was established by Friedrich Reinitzer in 1888, and its chemical structure was elucidated by Heinrich Wieland and Adolf Windaus. For their work, Wieland and Windaus were awarded the Nobel Prize in Chemistry in 1927 and 1928, respectively. Following the determination of its structure, cholesterol became the focus of many investigations into its biological synthesis, a complicated pathway that involves more than 30 different enzymes and numerous cofactors [191]. The work of Konrad Bloch, in particular, contributed much to our understanding, as he established the biosynthetic origin of the twenty-seven carbons of cholesterol. His efforts garnered him the Nobel Prize in Physiology and Medicine in 1964.
Cholesterol is the major sterol that is present in membranes of mammalian cells [192]. It has been estimated that more than 90% of cellular cholesterol is located at the plasma membrane, where the level as measured in terms of the molar ratio of cholesterol to phospholipids can vary for different biological membranes and may be as high as 1:1 [192,193]. For example, estimates at the lower end have been reported for rat and mouse lymphocytes [194], synaptic plasma membranes of rat forebrain [195], and plasma membranes of CHO cells [196] to be 0.25, 0.56, and 0.45, respectively. At the higher end, estimates of 0.83, 0.90 and 1.32 have been reported for the membranes of liver cells [197], erythrocytes [198,199] and myelin [198,200], respectively.

It previously has been suggested that the structure of cholesterol is the product of evolutionary selection pressures where improvement of function was the primary driving force [201]. For example, yeast is a sterol auxotroph when grown under anaerobic conditions, whereas insects have lost the ability to make sterols altogether and therefore require exogenous cholesterol to survive [201,202]. In both cases, lanosterol has been shown to be a poor substitute for cholesterol in terms of membrane function. The former is the first cyclization product of squalene and the earliest intermediate in the sterol pathway. This cyclization requires oxygen, and it has been suggested that primitive organisms, which evolved early in evolution when oxygen was believed to be scarce if not absent, do not contain or require sterols [202]. The reasons why cholesterol became the ‘sterol of choice’ for mammalian membrane function are not clear, but it has been suggested that a sterol that fits and interacts most effectively with the phospholipid partner will alter the physical state of the membrane much more than a molecule that is poorly accommodated [201]. Thus, in both artificial and natural membranes, it has been shown that lanosterol, the earliest intermediate, fits least whereas cholesterol, the end product, fits best; the fit of partially de-methylated intermediates lies somewhere in between [201].

Naturally occurring membranes are not homogenous, which makes it difficult to assess the individual contribution of each component to the overall physical state of the bilayer. Model membranes—that is, those with a defined synthetic lipid composition—therefore are attractive experimental systems, and they have been employed widely in
recent years in studies on the nature of the physical effects of cholesterol and the possible consequences for the function of membrane proteins. It previously has been shown that the presence of cholesterol in lipid membranes can increase lipid acyl chain order as well as induce the formation of segregated domains [203–206].

When present in lipid bilayers, cholesterol is perpendicularly oriented relative to the plane of the membrane. Early studies have suggested that it interacts differently with different lipids, exhibiting a general preference in the order of sphingomyelin > phosphatidylserine > phosphatidylcholine > phosphatidylethanolamine [207]. It has been shown that the affinity of cholesterol for different lipids varies significantly with the polar headgroup and backbone structure of the latter and also with the degree of saturation of their acyl chains. The strength of the cholesterol-lipid interaction is generally found to decrease as the extent of lipid unsaturation increases [208].

Over the years, several models have been proposed in an attempt to provide a general framework that describes cholesterol-lipid interactions [209]. They are based on the profile for the chemical activity of cholesterol in the membrane: that is, its chemical potential as a function of its mole fraction. For example, the aforementioned effect of acyl-chain saturation on the strength of cholesterol-lipid interactions is encompassed within the framework of the ‘Condensed Complex’ model, which predicts that cholesterol can form condensed complexes with those lipids with which it can mix favorably. Such lipids include those of phosphatidylcholine with long saturated chains and those of sphingomyelins. Recently, data from mixtures of phospholipid and cholesterol that contain fluorescent analogues of the latter have indicated that cholesterol can form superlattice structures in lipid bilayers. Such data have led to the proposal of the ‘Superlattice’ model, which suggests that the difference in cross-sectional area between cholesterol and other lipid molecules can result in a long-range repulsive force among molecules of the former and thereby produce superlattice distributions. The model also suggests that these long-range repulsive forces play the dominant role in cholesterol-lipid interactions. Data acquired from studies on the maximum solubility of cholesterol in lipid bilayers have led to the proposal of the ‘Umbrella’ model, which attributes the
preferential association of cholesterol with certain membrane molecules to the mismatch between the small polar headgroup of cholesterol and its large nonpolar body.

Among naturally occurring lipids, cholesterol interacts preferentially with phosphatidylcholine and sphingomyelin. The latter also is known to have a rigidifying effect on biomembranes [193,210,211], and together they constitute more than one half of the phospholipids found in cell membranes wherein both are known to function primarily as structural components. It has been suggested that the interaction between cholesterol and sphingomyelin may amplify the ordering effects of both compounds, and it also may promote lipid phase separation into more and less ordered membrane domains. Both effects previously have been shown to have an indirect influence on the proper functioning of several membrane proteins, including G protein-coupled receptors.

The influence of the lipid environment on the operation of the nicotinic acetylcholine receptor (AChR) has been studied for more than twenty years. Early studies demonstrated that delipidation and detergent-solubilization of the protein can affect its affinity for agonists. Moreover, the ion-gating activity of the receptor has been shown to be sensitive to its lipid environment in that reconstitution without neutral phospholipids results in a pronounced decrease in the ability of the receptor to allow cation translocation in response to the binding of cholinergic agonists. Similarly, delipidation of the β-adrenergic receptor by solubilization in deoxycholate abolished virtually all binding of the antagonist [125I]iodocyanopindolol [212]. Re-addition of soybean lipids restored specific radioligand binding, and the reconstituted receptor demonstrated agonist and antagonist binding affinities which were not very different from those of the native β-adrenergic receptor. Photoactivation of rhodopsin involves a conformational change from the photointermediate metarhodopsin I to the fully active metarhodopsin II, which is accompanied by volume expansion of the latter. The equilibrium between these two states has been shown to be sensitive to the surrounding membrane environment in that a reduction of the free volume of the bilayer that is caused by cholesterol inhibits the formation of metarhodopsin II [213]. Another G protein-coupled receptor whose function is known to depend upon the physical properties of the membrane is the cholecystokinin receptor (CCKβR). It has been shown that the ligand-binding properties
of the receptor depend upon the fluidity of the membrane, in that the function of the protein did not exhibit any specific requirement for the structure of the sterol but required only that the steroids restore the rigidity of the membrane to a level similar to that achieved by cholesterol [214].

The lateral organization of biological membranes also can have functional consequences for membrane proteins. Recent studies have suggested that cholesterol plays a role in maintaining the protein complement of cholesterol-rich regions of the plasma membrane such as caveolae and lipid rafts [215]. The former are 50–70 nm invaginations in the plasma membrane which are associated with a family of proteins called caveolins. The latter are somewhat more difficult to identify morphologically, and there is much debate surrounding their composition. It is widely believed that lipid rafts correspond to those areas of the plasma membrane that exhibit resistance to solubilization at 4 °C by non-ionic detergents such as Triton X-100, and glycosyl phosphatidylinositol-anchored proteins often have been used as markers. That notion has been questioned, however, in the light of evidence that such detergent-resistant membranes may be created by the detergent [216–218].

Both forms of membrane domain are enriched in cholesterol and sphingolipids, and both have been suggested to serve as platforms for numerous signaling pathways. In recent years, a number of receptor tyrosine kinases, G protein-coupled receptors, G proteins, kinases and phosphatases have been located in lipid rafts and/or caveolae [219]. Sequestration of certain components of a signaling pathway likely will have mechanistic implications, especially in the case of G protein-coupled receptors.

It has been shown that there is an upper limit on the concentration of cholesterol that can be accommodated within a given lipid bilayer; any amount in excess of that limit has been found to precipitate as crystals of pure cholesterol monohydrate [220]. In model membranes of phosphatidylcholine and phosphatidylserine, the solubility limits for cholesterol have been determined to be 66 and 51 mol%, respectively. Estimates of the cholesterol content in plasma membranes of animal cells typically are in the range of 25–50 mol% [221]. The significance of the level of cholesterol in terms of its effect on the
physical properties and lateral organization of the membrane has been well characterized, and the consequences for the functioning of membrane proteins are evident. In contrast, relatively less is known about the number of functionally relevant molecules of cholesterol that interact directly with any given membrane protein.

In terms of affecting the activity of membrane proteins, lipids generally have been classified as either annular or non-annular [222]. The former constitute those lipids found in the bulk, which interact relatively non-specifically with the protein and act as a solvent by forming an annular shell. The latter constitute those lipids that interact with the protein with much greater specificity and may regulate its activity in a manner similar to that of a traditional co-factor. Non-annular lipids are often bound between transmembrane $\alpha$-helices, either within a protein or at protein-protein interfaces in multi-subunit complexes [222].

Early evidence for the presence of non-annular sites came from studies on the effects of cholesterol on the Ca$^{2+}$-ATPase of sarcoplasmic reticulum [223]. It was shown that the activity of the enzyme did not necessarily relate to the physical properties of the membrane but depended rather on the occupancy of non-annular sites by cholesterol and other hydrophobic molecules. Similar effects also have been observed for several G protein-coupled receptors. For example, although cholesterol previously has been shown to have an indirect effect on the function of rhodopsin, it also can directly influence the activation of the receptor as indicated by the cholesterol-sensitive transfer of resonance energy between tryptophanyl residues of the protein and the fluorescent sterol cholestatrienol [224]. The oxytocin and galanin receptors were shifted from high to low affinity for agonists when cholesterol was removed from the plasma membranes of uterine myometrium and Chinese hamster ovary cells, respectively. High-affinity binding was re-established upon the restoration of cholesterol to the depleted membranes. Similarly, the $\mu$-opioid receptor was driven toward a high-affinity state for the agonist [3H]DAMGO either by the removal of ergosterol from the plasma membrane of yeast or by the addition of cholesterol. The oxytocin, galanin, and $\mu$-opioid receptors appear to be regulated directly, as indicated by the selectivity of ergosterol and analogues of
cholesterol in mimicking the effects of cholesterol on membrane fluidity but not those on the affinity of agonists.

Thus, G protein-coupled receptors are a family of transmembrane proteins whose activity is subject to regulation by the lipid environment either directly, indirectly or both. Although the exact manner by which signals are transduced across biological membranes is unclear, there is evidence to suggest that cooperativity within an oligomer may underlie the signaling process. Central to an understanding of signaling are questions regarding the oligomeric status of the receptor as well as the complement of cognate G proteins and the nature of their association with the receptor. The influence of the surrounding lipids and the role that cholesterol may play within the context of these central themes, however, is not clear. For example, signaling may be sensitive to an effect of cholesterol on processes that promote receptor oligomerization or to a direct effect of cholesterol on already formed oligomeric complexes of the receptor. Similar questions arise regarding the role that cholesterol may play in the nature of the interaction between receptor and G protein. Such questions will have important implications for the mechanistic basis of signaling.
1.6 An Outline of the Thesis

The nucleotide-sensitive dispersion of affinities that is revealed almost universally in the binding of agonists correlates with pharmacological properties such as efficacy and intrinsic activity. Despite the body of work that has been accumulated over the past 30 years, however, the mechanistic basis of signal transduction via G protein-coupled receptors remains elusive. Although classical schemes such as Clark’s occupancy theory and the later introduction of efficacy by Stephenson and Furchgott were sufficient at the time of their inception, when the interaction of an agonist with its receptor could not be measured directly, they do not account for a pattern wherein the dose-dependent binding of a ligand to its receptor deviates from a rectangular hyperbola. Other mechanistic schemes such as the multi-site and ternary complex models have been examined, and both can account superficially for the heterogeneity detected in the binding of agonists, but neither yields a mechanistically consistent description of the data when applied in a quantitative manner. In contrast, cooperativity has been shown to provide a mechanistic basis for effects that otherwise cannot be accounted for by previous schemes. It therefore follows that models based on cooperativity are at least tenable, and they in turn imply the existence of oligomers such as those which now appear to be ubiquitous.

It previously has been shown that purified M<sub>2</sub> receptor [110] as well as M<sub>2</sub> receptor extracted from sarcolemmal membranes of porcine atria and solubilized in cholate-NaCl [147] exhibited non-competitive effects in the binding of muscarinic antagonists. Such apparent cooperativity implies the existence of oligomers, and multimeric forms of M<sub>2</sub> receptor purified from porcine atrial tissue have been found on immunoblots to migrate with the mobility expected of trimers and tetramers [110]. Moreover, further evidence for the existence of oligomers of the M<sub>2</sub> receptor has been obtained from the co-immunoprecipitation of differently epitope-tagged receptors that have been co-expressed in insect S<sub>9</sub> cells [225,226]. In contrast to M<sub>2</sub> receptor from porcine atria, however, that extracted from membranes of S<sub>9</sub> cells in cholate-NaCl failed to exhibit non-competitive effects in the binding of muscarinic antagonists despite their occurrence as oligomers [227].
G protein-coupled receptors are membrane proteins and are likely subject to modulation by the surrounding lipid environment. It has been shown that there is a marked difference in the level of cholesterol between the sarcolemmal membranes of porcine atria and those membranes of Sf9 cells [227]. This difference may account for the difference in the binding patterns of muscarinic antagonists to M2 receptors between the two preparations. When native Sf9 membranes were supplemented with cholesterol, the apparently cooperative effects that were observed previously with M2 receptor from porcine atria were regained. This suggests that cholesterol is a determinant of cooperativity in the M2 muscarinic receptor.

The multiphasic curves revealed by agonists cannot be described by prevailing schemes (e.g., multi-site, ternary complex), but they can be described in terms of cooperativity [81]. Data of the kind normally acquired are mechanistically ambiguous, but the cooperative potential of the system is clear from the binding of antagonists, and the realization of that potential requires cholesterol.

CHAPTER 2: Cholesterol as a determinant of cooperativity in the M2 muscarinic cholinergic receptor

The underlying mechanism which gives rise to the heterogeneity detected in the binding of agonists may also be present in the binding of antagonists, although the heterogeneity is more subtle in the case of the latter and therefore has not been examined in as much detail. In this work, the non-competitive effects observed in the binding of muscarinic antagonists to the M2 receptor were shown to depend upon the level of cholesterol that is present in the membranes of Sf9 cells. In native Sf9 membranes, which contain low levels of cholesterol, binding was wholly competitive; that is, there was no difference in the capacity of [3H]NMS and [3H]QNB, and there were no discrepancies in the affinity of NMS as estimated directly in assays at graded concentrations of the radioligand and as inferred from the inhibitory effect of the unlabeled analogue on the binding of near-saturating and sub-saturating concentrations of [3H]QNB. In cholesterol-treated Sf9
membranes, however, $[^3\text{H}]$NMS labeled only a little over half the sites labeled by $[^3\text{H}]$QNB, yet comparatively low concentrations of unlabeled NMS inhibited the binding of $[^3\text{H}]$QNB to all of the sites. Such effects were shown to be inconsistent with the notion of a heterogeneous population of mutually independent and non-interconverting sites. A mechanistically consistent description of the data was obtained in terms of cooperativity among at least six interacting sites, presumably within an oligomer of the M$_2$ receptor.

**CHAPTER 3:** *Effect of cholesterol on the binding of antagonists to the muscarinic cholinergic receptor extracted from porcine atria*

It previously has been shown that M$_2$ muscarinic receptor from sarcolemmal membranes of porcine atria extracted in cholate-NaCl exhibited non-competitive effects in the binding of muscarinic antagonists [147]. Whereas those effects were incompatible with the notion of a competition for a heterogeneous population of non-interconverting and non-interconverting sites, they could be described in terms of cooperativity among at least four interacting sites. As described in Chapter 2, cholesterol is a determinant of cooperativity in the M$_2$ muscarinic receptor. In the work described here, sarcolemmal membranes from porcine atria were depleted of cholesterol, using methyl-$\beta$-cyclodextrin, to a level that was comparable to that found in native Sf9 membranes. M$_2$ receptor extracted from cholesterol-depleted membranes of porcine atria in cholate-NaCl exhibited no difference in capacity for $[^3\text{H}]$NMS and $[^3\text{H}]$QNB, and any discrepancies found in the affinity of NMS were much less pronounced than those observed with receptor extracted from native atrial membranes. Although there was a small non-competitive component in the system, it appears that removal of cholesterol from native mammalian tissue such as porcine atria transforms the ligand-binding properties of the M$_2$ muscarinic receptor from being manifestly cooperative in nature to one that is largely competitive.
CHAPTER 4: Mechanistic basis for the effect of cholesterol on the binding of agonists and antagonists to the M₂ muscarinic cholinergic receptor

It has been shown in Chapters 2 and 3 that cholesterol is a determinant of cooperativity in the M₂ muscarinic receptor. Receptors from native Sf9 membranes, which reveal competitive binding, can be made to resemble those from porcine atria, which reveal non-competitive binding, and vice versa, through the addition of cholesterol to Sf9 membranes or the removal of cholesterol from atrial membranes. The interconversion of cardiac muscarinic receptors between an agonist-specific state (R*) and an antagonist-specific state (R) previously has been shown to be an intrinsic property of the protein [228]. Cholesterol appears to favor a state in which binding is cooperative over one in which binding is competitive. It also increased the apparent affinity of antagonists, as expected if it were to favor the inactive state (R) over the active state (R*) in the equilibrium that underlies phenomena such as constitutive activity and, presumably, the effects of guanyl nucleotides. That possibility therefore was examined in the work presented here, in which cholesterol and N-ethylmaleimide (NEM) were compared for their effect on the binding of two antagonists and four agonists. An empirical description of the data suggested that NEM drives the equilibrium towards R* and cholesterol causes an increase in affinity per se. Although those results argued against an effect of cholesterol on the interconversion between R and R*, the latter process was not explicit in the model used to calculate apparent affinities. When the model is expanded to include such a process, however, the data can be described equally well by an effect of cholesterol on either the interconversion or affinity per se, with an accompanying effect of NEM on either affinity or the interconversion, respectively. This ambiguity illustrates the limitations of a model that fails to describe a proposed mechanism in an explicit manner. Since NEM is known from other data to favor R* over R, cholesterol appears to increase the affinity of all ligands for the receptor.
CHAPTER 2

CHOLESTEROL AS A DETERMINANT OF COOPERATIVITY IN THE

M₂ MUSCARINIC CHOLINERGIC RECEPTOR


Biochemical Pharmacology, 74, 236–255.
2.1 Abstract

M₂ muscarinic receptor extracted from Sf9 cells in cholate–NaCl differs from that extracted from porcine sarcolemma. The latter has been shown to exhibit an anomalous pattern in which the capacity for N-[³H]methylscopolamine (NMS) is only 50% of that for [³H]quinuclidinylbenzilate (QNB), yet unlabeled NMS exhibits high affinity for all of the sites labeled by [³H]QNB. The effects can be explained in terms of cooperativity within a receptor that is at least tetravalent [Park, P. S.-H. et al. (2002) Biochemistry 41, 5588–5604]. In contrast, M₂ receptor extracted from Sf9 membranes exhibited no shortfall in the capacity for [³H]NMS at either 30 °C or 4 °C, although there was a time-dependent inactivation during incubation with [³H]NMS at 30 °C; also, any discrepancies in the affinity of NMS were comparatively small. The level of cholesterol in Sf9 membranes was only 4% of that in sarcolemmal membranes, and it was increased to about 100% by means of cholesterol-methyl-β-cyclodextrin. M₂ receptors extracted from treated Sf9 membranes were stable at 30 °C and 4 °C and resembled those from heart. Cholesterol induced a marked heterogeneity detected in the binding of both radioligands, including a shortfall in the apparent capacity for [³H]NMS, and there were significant discrepancies in the apparent affinity of NMS as estimated directly and via the inhibition of [³H]QNB. The data can be described quantitatively in terms of cooperative effects among six or more interacting sites. Cholesterol therefore appears to promote cooperativity in the binding of antagonists to the M₂ muscarinic receptor.
2.2 Introduction

G protein-coupled receptors occur at least partly and perhaps exclusively as dimers or larger aggregates. They appear to be assembled at the time of biosynthesis and transported as such to the plasma membrane, where they may remain intact throughout the signaling process [229–231]. Although now much in evidence, oligomers of GPCRs went almost unrecognized for many years, and their functional role remains largely unexplored. It has been suggested, however, that they host a complex interplay of cooperative effects whereby signaling is regulated by agonists and guanyl nucleotides [81,110,132].

Most GPCRs embody an intriguing heterogeneity that emerges in binding assays as a GTP-sensitive dispersion of affinities for agonists [230,232]. The effect of guanyl nucleotides is mediated by the G protein and cooperative in nature (e.g., Ref. [233]). It was recognized early on that cooperativity also might account for the heterogeneity detected by agonists (e.g., Refs. [78,80,234], but tests for such effects in the muscarinic receptor proved negative [78]. Furthermore, most biochemical data suggested that GPCRs exist wholly or largely as monomers, at least in solution (e.g., Refs. [235–238]). There also was widespread acceptance of the view that heterogeneity derives from a transient complex between a monomeric receptor and the G protein [80,235], although such schemes can be problematic when applied in a quantitative manner [85,125].

If cooperativity underlies the heterogeneity revealed by agonists, it seems likely to occur in other contexts. It was inferred from the early observation that the net dissociation of [3H]alprenolol from β-adrenergic receptors was faster when initiated by an unlabeled ligand than by dilution [139,239], although rebinding of the radioligand could not be ruled out as a determinant of the rate following dilution [239]. Negative cooperativity has been invoked more recently to account for kinetic effects in the binding of bradykinin to the B2 bradykinin receptor [240], but it could not be detected in kinetic studies on the D2 dopamine receptor [241].
In studies at equilibrium, Hill coefficients greater than 1 have prompted suggestions of positive cooperativity in the binding of \[^3\text{H}\]QNB to muscarinic cholinergic receptors [144,146] and of some antagonists to receptors labeled by \[^3\text{H}\]histamine [142]. Positive cooperativity also is apparent in the observation that one ligand can promote the binding of another in some systems. With M\(_2\) muscarinic receptors and G proteins purified as a complex from porcine atria, the binding of \[^3\text{H}\]AF-DX 384 was bell-shaped with respect to the concentration of the agonist oxotremorine-M [110]. With heteromers of the \(\delta\)- and \(\kappa\)-opiate receptors, the apparent affinity of the \(\delta\)-selective agonist [D-Pen\(^2\),D-Pen\(^5\)]enkephalin was at least 50-fold higher in the presence of the \(\kappa\)-selective agonist U69593 and vice versa [166]. Similarly, \(\beta_1\)-selective ligands increased the affinity of \(\beta_2\)-selective ligands and vice versa at heteromers of the \(\beta_1\)- and \(\beta_2\)-adrenergic receptors [242]. Negative cooperativity has been inferred from noncompetitive inhibitory effects at muscarinic [145] and histamine receptors [87,141]. With heteromers of the CCR5 and CCR2b chemokine receptors, the CCR5-specific ligand MIP-1\(\beta\) was found to inhibit the binding of \[^{125}\text{I}\]MCP-1 to CCR2b and vice versa [243].

A pattern of latent sites and attendant noncompetitive effects has pointed to cooperativity in the binding of antagonists to cardiac muscarinic receptors extracted in cholate–NaCl or Lubrol-PX [110,147] and to D\(_2\) dopamine receptors in membranes from CHO and S/9 cells [241,244]. In the case of the muscarinic receptor, those effects can be rationalized quantitatively in terms of cooperativity among at least four interacting sites [147]. That in turn implies a tetramer or larger oligomer, and differently tagged mutants of the M\(_2\) muscarinic receptor have been coimmunoprecipitated from extracts of baculoviral-infected S/9 cells [155,226]. In preliminary studies, however, the M\(_2\) receptor extracted from S/9 membranes failed to exhibit the cooperative effects found in myocardial extracts under the same conditions.

Manifestations of cooperativity appear to depend upon environmental factors. The cardiac muscarinic receptor exhibits latent sites and noncompetitive binding upon extraction in cholate–NaCl but not in digitonin–cholate [147]. Similar effects at the D\(_2\) dopaminergic receptor are lost or much diminished in the presence of sodium ions [244]. It has been suggested that cooperativity in the muscarinic receptor may be regulated by
cholesterol [147], which is known to modulate the properties of several GPCRs and other membrane proteins [214]. Levels of cholesterol in the plasma membrane of Sf9 cells are comparatively low [245]. In the present report, M2 muscarinic receptors extracted from cholesterol-supplemented Sf9 membranes are shown to exhibit the cooperative effects found with the receptor from heart.

2.3 Materials and Methods

*Ligands, detergents, and other materials*

\(N\)\(^{-}[\text{3H}]\text{Methylscopolamine was purchased as the chloride salt from PerkinElmer Life Sciences (lots 3406081 and 3474009, 83.5 Ci/mmol; 3436143, 3499213, and 3538031, 81.0 Ci/mmol) and as the bromide salt from Amersham Biosciences (batches B-32 84.0 Ci/mmol; B-35, B-36, 81.0 Ci/mmol). Mass spectra provided by the manufacturer indicated that the samples from Amersham Biosciences were devoid of contaminating scopolamine. The purity of material from PerkinElmer Life Sciences has been confirmed previously [147]. (−)\([\text{3H}]\text{Quinuclidinylbenzilate was purchased from PerkinElmer (lots 3363717, 37.0 Ci/mmol; 3467373, 39.0 Ci/mmol; 3467877, 3499844, 42.0 Ci/mmol) and Amersham (batch B-49, 49 Ci/mmol). Unlabeled NMS hydrobromide was purchased from Sigma-Aldrich, and scopolamine hydrobromide was from RBI-Sigma.}

Sodium cholate was purchased from Sigma-Aldrich, and digitonin was from Wako Bioproducts. Solubilized receptor was concentrated as required by means of Centricon-10 and Centriprep-30 filters (Amicon) purchased from Millipore. HEPES and Tris were obtained as the free bases from Boehringer Mannheim and Sigma-Aldrich (Trizma), respectively. EDTA and EGTA were obtained as the free acids from British Drug Houses or Bioshop Canada. Bacitracin, all protease inhibitors, and Sephadex G-50 were from Sigma-Aldrich. Other chemicals were obtained from the sources shown below. Protein was estimated by means of bicinechonic acid using the BCA Protein Assay Kit from Pierce. Bovine serum albumin was taken as the standard.
M2 muscarinic receptor from Sf9 cells

FLAG-tagged M2 muscarinic receptor was expressed in Sf9 cells as described previously [155]. The cells were cultured at 27 °C in Ex-Cell 400 insect media (JRH Biosciences) containing 2% fetal bovine serum, 1% Fungizone, and 0.1% gentamycin (Life Technologies, Gibco-BRL). Confluent cells growing at a density of about 2 × 10^6 cell/mL were infected with baculovirus at a multiplicity of infection of 5. Between 48 and 72 h after infection, the cells were collected by centrifugation at 1000 × g. The pellet then was suspended in Buffer A (20 mM KH₂PO₄, 20 mM NaCl, 1 mM EDTA, 0.1 mM PMSF, 200 µg/mL bacitracin, 2 µg/mL leupeptin, 20 µg/mL peptatin A, 156 µg/mL benzamidine, and NaOH to pH 7.40) with three bursts of a Brinkman Polytron (setting 6, 10 s), and the resulting homogenate was centrifuged for 45 min at 4 °C and 100,000 × g. The membranes were washed once by resuspension and centrifugation in the same manner, and the pellet was stored at –75 °C.

Incorporation of cholesterol into Sf9 membranes

Cholesterol was incorporated by means of an inclusion complex prepared as described previously [246]. Cholesterol and methyl-β-cyclodextrin (Sigma-Aldrich) were added to Buffer B (20 mM KH₂PO₄, 20 mM NaCl, 1 mM EDTA, 0.1 mM PMSF, and NaOH to pH 7.6), and the mixture was sonicated periodically in a bath sonicator (Haake SWB20) to disrupt aggregates of cholesterol. The process was carried out at 80 °C, yielding a clear solution at a concentration of 1.5 g/L with respect to cholesterol and 50 g/L with respect to methyl-β-cyclodextrin. An increase in the ratio of cholesterol to methyl-β-cyclodextrin resulted in undissolved particles. The inclusion complex was allowed to cool to room temperature prior to use.

To supplement Sf9 membranes prepared as described above, the frozen pellets were thawed on ice and suspended in Buffer B. A portion of the homogenate was
supplemented with cholesterol-methyl-β-cyclodextrin such that the final concentration of cholesterol was 0.5 mM; the balance was supplemented with Buffer B alone. The final concentration of total protein was 1.5 g/L. Each suspension then was shaken on a horizontal shaker for 30 min at room temperature and centrifuged for 45 min at 4 °C and 100,000 × g. The pelleted membranes from the cholesterol-treated sample were washed once by resuspension in Buffer B followed by centrifugation for 45 min at 4 °C and 100,000 × g. Pellets were stored at -75 °C until required for solubilization. To supplement Sf9 cells during growth, the cholesterol-methyl-β-cyclodextrin complex was added to the culture at the time of infection with the baculovirus. The concentration of the complex was 0.09 mM with respect to cholesterol.

The amount of cholesterol in native and cholesterol-treated Sf9 membranes and in sarcolemmal membranes from porcine atria was quantified using a cholesterol assay kit from Boehringer Mannheim (R-Biopharm). Sarcolemmal membranes were prepared as described previously [110].

*Extraction of M2 muscarinic receptor into detergent*

Solubilization in cholate plus NaCl was carried out essentially as described previously [147]. Native and cholesterol-treated Sf9 membranes were thawed on ice and suspended in Buffer C (40 mM NaH$_2$PO$_4$, 1 M NaCl, 1 mM EGTA, 0.16% sodium cholate, 0.1 mM PMSF, and NaOH to pH 7.40; 2 mg of protein/mL). The mixture was shaken for 1 h at room temperature and then diluted 1:1 with a solution of 40 mM NaH$_2$PO$_4$, 1 mM EGTA, and 0.1 mM PMSF adjusted to pH 7.40 with NaOH. The diluted suspension was centrifuged for 45 min at 4 °C and 100,000 × g, and the supernatant fraction was stored on ice until required. The final concentrations of cholate and NaCl were 0.08% and 0.5 M, respectively.

*Chemical cross-linking, electrophoresis, and immunological assays*
M₂ receptor was extracted from native and cholesterol-treated membranes and reacted for 30 min at room temperature with BS³ (Pierce Biotechnology, Inc.) at a concentration of 2 mM. Controls were prepared in the same manner except that BS³ was omitted. The reaction was terminated by the addition of Tris-HCl (pH 7.5) to a final concentration of 20 mM.

Electrophoresis was carried out in a Mini-Protean II cell (Bio-Rad) essentially as described previously [225]. Aliquots of the extract from Sf9 membranes were mixed with buffer (62.5 mM Tris, 10% glycerol, 2% SDS, 0.1 M dithiothreitol, 0.001% bromophenol blue, and HCl to pH 6.80) and heated for 5 min at 65 °C prior to loading on precast polyacrylamide gels (10%) (Bio-Rad, Ready Gel Tris-HCl). This procedure has been shown not to induce aggregation [225]. The resolved material was transferred to nitrocellulose membranes (Bio-Rad, 0.45 µm) in a Mini Trans-Blot Transfer Cell (Bio-Rad) and incubated with an antibody to the M₂ receptor (MA3-044, Affinity Bioreagents) at a dilution of 1:1,000. The bands were visualized by chemiluminescence using reagents and film purchased from Amersham Biosciences (ECL™, Hyperfilm MP). The images were digitized at a resolution of 300 dots per inch, and the intensities of the bands were estimated from the densitometric trace using ImageJ [247]. Molecular mass was estimated from the mobility of standards purchased from Amersham.

**Binding assays**

The radioligand and any unlabeled ligand were dissolved in Buffer D (20 mM HEPES, 20 mM NaCl, 1 mM EDTA, 5 mM MgSO₄, 0.1 mM PMSF, and NaOH to pH 7.40) supplemented with cholate plus NaCl at a concentration of 0.1% and 20 mM, respectively. An aliquot of the ligand-containing solution (48 µL) was added to the solubilized receptor (5 µL), and the reaction mixture was incubated at 30 °C or 4 °C. Except where stated otherwise, the time of incubation for [³H]NMS was as follows: 45 min at 30 °C and 6 h at 4 °C (receptor from native membranes), 3 h at 30 °C and 6 h at 4 °C.
°C (receptor from cholesterol-treated membranes). The time of incubation for all samples containing [³H]QNB was 2 h at 30 °C and 12 h at 4 °C.

To terminate the reaction and separate the bound radioligand, an aliquot of the sample (50 µL) was applied to a column of Sephadex G-50 fine (0.8 × 6.5 cm). The column was pre-equilibrated and eluted with Buffer E (20 mM HEPES, 20 mM NaCl, 1 mM EDTA, 5 mM MgSO₄, and NaOH to pH 7.40) supplemented with cholate plus NaCl at a concentration of 0.017% and 20 mM, respectively. All of the eluant up to and including the void volume was collected (1.60 mL) and assayed for radioactivity.

Binding assays generally were carried out within one week of solubilization. Nonspecific binding was taken throughout as binding in the presence of 1 mM unlabeled NMS and increased linearly with the concentration of either radioligand. To compare the binding of [³H]NMS and [³H]QNB, the two radioligands were assayed in parallel; similarly, the inhibitory effect of unlabeled NMS on the binding of [³H]QNB was measured in parallel at two concentrations of the latter.

Analysis of data

All data were analyzed with total binding taken as the dependent variable (B_{obsd}) and with the total concentrations of all ligands taken as the independent variables. Any subsequent manipulations were for the purpose of presentation only and did not alter the relationship between the data and the fitted curve. Levels of specific binding (B_{sp}), estimates of total receptor ([R]₀), and estimates of maximal specific binding (B_{max}) are presented as the concentration in the binding assay (pM); similarly, the concentrations of ligands denote the total molar concentration in the binding assay. Assays at graded concentrations of either radioligand were performed with and without unlabeled NMS (1 mM), and both sets of data shared a single estimate of nonspecific binding in the analyses described below.
For data acquired at graded concentrations of [³H]QNB or [³H]NMS, the Hill equation was formulated as shown in Eq. 1. The variable \([P]_t\) represents the total concentration of the radioligand. The quantity \(B_{sp}\) represents specific binding at the corresponding value of \([P]_t\), and the parameter \(B_{max}\) represents maximal specific binding. The parameter \(K\) is the concentration of unbound radioligand at half-maximal occupancy, and \(n_H\) is the corresponding Hill coefficient. The parameter NS represents the fraction of unbound radioligand that appears as nonspecific binding. Equation 1 was solved numerically in the manner described previously [248].

\[
B_{obsd} = B_{max} \frac{([P]_t - B_{sp})^{n_H}}{K^{n_H} + ([P]_t - B_{sp})^{n_H}} + NS([P]_t - B_{sp}) \tag{1}
\]

For data acquired at graded concentrations of unlabeled NMS, the Hill equation was formulated as

\[
B_{obsd} = (B_{[A]=0} - B_{[A]\to\infty})K^{n_H} / (K^{n_H} + [A]^{n_H}) + B_{[A]\to\infty}.\]

The variable \([A]_t\) is the total concentration of unlabeled ligand, and the parameters \(B_{[A]=0}\) and \(B_{[A]\to\infty}\) represent the asymptotic levels of binding when \([A]=0\) and as \([A]\to\infty\).

Mechanistic analyses were performed in terms of intrinsic heterogeneity (Scheme 1) and cooperativity (Scheme 2). Estimates of total binding were fitted according to the equation \(B_{obsd} = B_{sp} + NS([P]_t - B_{sp})\), in which \(P\) represents the radioligand and other quantities are as described above for Eq. 1. The value of \(B_{sp}\) was computed according to Scheme 1 or Scheme 2.

Scheme 1 represents an intrinsically heterogeneous system that comprises \(n\) classes of noninterconverting and mutually independent sites (\(R_j, j = 1, 2, ..., n\)). Those of type \(j\) constitute the fraction \(F_j\) of all sites (i.e., \(F_j = [R_j]_t / [R]_t\), where \([R]_t = [R_j] + [AR_j] + [PR_j]\), and \([R]_t = \sum_{j=1}^{n} [R_j]_t\)). The ligands \(P\) and \(A\) compete for \(R_j\) with the equilibrium dissociation constants \(K_{Pj}\) and \(K_{Aj}\), respectively. Total specific binding of a radiolabeled probe was calculated according to Eq. 2, and the required values of \([PR_j]\) were obtained as described below.
Scheme 2 represents a cooperative system in which a multivalent receptor (R) can bind up to \(n\) equivalents of either A or P. The receptor presumably is oligomeric, and it is assumed that the quaternary structure remains formally intact under the conditions of the binding assays; that is, there is no exchange of individual subunits within the system. The model therefore can accommodate processes in which dissociated monomers regroup without exchanging partners. Asymmetry cannot be detected with the present data, and all sites of the vacant oligomer (R) were assumed to bind A or P with the microscopic dissociation constant \(K_A\) or \(K_P\), respectively (e.g., \(K_p=[R][P]/[RP]\)). Similarly, the microscopic dissociation constant was taken as the same for all vacant sites on partially liganded R (e.g., \([P][PPOA]/[PPOA]=[P][POOA]/[POPA]\), where ‘O’ represents a vacant site on tetravalent R). The parameters \(f_{i_0}\) and \(f_{0_j}\) represent the cooperativity factors for binding of the \(i\)th equivalent of A (\(i=2\)) or the \(j\)th equivalent of P (\(j=2\)) to form A\(_i\)R or RP\(_j\), respectively (e.g., \([RP_{j-1}][P]/[RP_j]=\prod_{k=2}^{j} f_{0_k} K_p\)). For the present purposes, this type of interaction is referred to as homotropic cooperativity. The parameters \(f_{ij}\) represent cooperativity factors in the formation of mixed complexes (i.e., A\(_i\)RP\(_j\)), which is referred to as heterotropic cooperativity. Either form of cooperativity may be positive (\(f<1\)) or negative (\(f>1\)). The value of \(f_{00}\), \(f_{10}\), and \(f_{01}\) is defined as 1 in each case. Total specific binding of the probe was calculated according to Eq. 3.

\[
B_{sp} = \sum_{j=1}^{n} \sum_{i=0}^{n-j} \binom{n}{j} \binom{n-j}{i} [A,RP_j]
\] (3)

The values of \([PR_j]\) in Eq 2 and \([A,RP_j]\) in Eq. 3 were calculated from the expansions in terms of the total concentration of R and the free concentrations of A and P. The latter were computed numerically from the corresponding implicit equations for \([A]\_i\) and \([P]_j\). Further details concerning the formulation of Schemes 1 and 2 have been described elsewhere [81,110,147,248,249].
Analyses in terms of Schemes 1 and 2 involved data combined from several experiments. Both schemes were applied in a mechanistically consistent manner, in that parametric values were shared among different sets of data in strict accord with the premises of the model. In such analyses, ligand P represents $[^3\text{H}]$QNB, and ligand A represents both isotopic forms of NMS. Thus, the parameters $K_{P_j}$ (Scheme 1) and $K_P$ (Scheme 2) denote the affinity of $[^3\text{H}]$QNB, and $K_{A_j}$ (Scheme 1) and $K_A$ (Scheme 2) denote the affinity of labeled and unlabeled NMS. Other parameters of Scheme 2 relate to P and A as described above. Scheme 1 also was applied in an empirical manner, in that different sets of data were permitted separate values of parameters that ought to be invariant (i.e., Table 2-2, Figs. 2-5 and 2-6). In such analyses, ligand P represents the radiolabeled probe—either $[^3\text{H}]$QNB or $[^3\text{H}]$NMS as indicated, and ligand A represents unlabeled NMS.

Separation on Sephadex G-50 yields low levels of non-specific binding [147], which represented about 0.06% of the unbound radioligand in the case of $[^3\text{H}]$QNB and extracts from cholesterol-treated membranes (i.e., NS = 0.0006). At the higher concentration of $[^3\text{H}]$QNB used to examine the inhibitory effect of NMS (i.e., 28–35 nM), the level of binding at saturating concentrations of the unlabeled ligand was only about 4% of that in its absence. Since both NS and the asymptotic value of $B_{\text{obsd}}$ were near zero, the values of NS for such data were not well defined. The optimization of NS therefore tended to destabilize that of other parameters, particularly in the case of Scheme 2 at larger values of $n$. Those values of NS therefore were determined from empirical analyses in terms of Scheme 1 and fixed accordingly in all subsequent analyses in terms of Scheme 1 or Scheme 2.

The results of analyses involving multiple sets of data from replicated experiments have been presented in each case with reference to a single fitted curve. To obtain the values plotted on the y-axis, estimates of $B_{\text{obsd}}$ were adjusted according to the expression $B'_{\text{obsd}} = B_{\text{obsd}} \left\{ f(\bar{x}, \bar{a}) / f(x_i, a) \right\}$ [132]. The function $f$ represents the fitted model, and the vectors $x_i$ and $a$ represent the independent variables at point $i$ and the parameters, respectively. Individual values of $B'_{\text{obsd}}$ at the same $x_i$ were averaged to obtain the mean.
and standard error plotted in the figure. Specific binding ($B_{\text{sp}}$) was calculated as $B'_{\text{obsd}}$ less the fitted estimate of nonspecific binding at the same concentration of unbound radioligand.

Statistical procedures

All parameters were estimated by nonlinear regression [250]. Affinities and cooperativity factors were optimized throughout on a logarithmic scale (i.e., log $K_{Pj}$, log $K_P$, log $f_{ij}$, etc.). Some values of $f_{i0}$, $f_{0j}$, and $f_{ij}$ ($i, j \geq 2$) in Scheme 2 were undefined at higher values of $n$, and the number of parameters therefore was reduced to avoid false minima. In some cases, an unknown cooperativity factor was fixed arbitrarily at a value that had no adverse effect on the sum of squares; in others, two or more cooperativity factors shared a single parametric value. Details regarding the assignment of shared parameters are described where appropriate. Values of [R], were assigned separately to data from separate experiments.

Weighting of the data, tests for significance, and other statistical procedures were performed as described elsewhere [81,132,248]. Fits involving nested models, such as Scheme 1 at different values of $n$, were compared by means of the $F$-statistic [248]. The fit of Scheme 2 at different values of $n$ was compared by means of the second-order Akaike’s information criterion, which can be used to assess the benefit of additional parameters in nested and non-nested models [251]. Weighted residuals were of comparable magnitude within each set of data. In simultaneous analyses, individual sets of data generally made comparable contributions to the total weighted sum of squares; thus, the fits were not dominated by the data from one experiment or group of experiments. Mean values calculated from two or more individual estimates of a parameter or other quantity are presented together with the standard error. For parametric values derived from a single analysis of one or more sets of data, the errors were estimated from the diagonal elements of the covariance matrix. Such values reflect the range within which the weighted sum of squares is essentially the same.
2.4 Results

*Effect of cholesterol on electrophoretic mobility, apparent capacity and stability*

Levels of cholesterol in membranes from Sf9 cells were only about 4% of those in sarcolemmal membranes from porcine atria (Fig. 2-1). The level in Sf9 membranes increased to 108% of that in sarcolemmal membranes upon treatment of the former with the inclusion complex at a concentration of 0.5 mM with respect to cholesterol. This method was used to prepare the treated Sf9 membranes used in subsequent experiments. Cholesterol-methyl-β-cyclodextrin also was added to growing Sf9 cells, but the complex proved toxic at the concentration used to supplement harvested membranes. When the complex was added at the lower concentration of 0.09 mM with respect to cholesterol, the cells survived to yield a level of cholesterol that was about 21% of that attained in Sf9 membranes treated after harvest.

Densitometric scans of western blots prepared with an antibody to the M2 receptor indicated that cholesterol was without significant effect on the distribution of immunoreactive material among different bands (Fig. 2-2). In each case, material migrating with the mobility expected of a monomer accounted for a minor fraction of the total density (native membranes, 18 ± 6%, N = 5; cholesterol-treated membranes, 15 ± 4%, N = 7). About 20% of the density resembled smaller oligomers (n = 2–4) (native, 22 ± 5%, N = 5; treated, 19 ± 2%, N = 7), and the largest fraction corresponded to aggregates with a relative molecular mass in excess of 250,000 (native, 40 ± 8%, N = 5; treated, 47 ± 8%, N = 7). The balance corresponded to fragments smaller than a monomer (native, 20 ± 7%, N = 5; treated, 19 ± 5%, N = 7). After treatment of the extract with the cross-linking reagent BS3, at least 80% of the immunoreactive material from either preparation migrated with a relative molecular mass greater than 250,000.

In extracts of native Sf9 membranes at 30 °C, the apparent capacity for [3H]NMS after incubation for 45 min was 81% of that for [3H]QNB (Table 2-1). The muscarinic receptor from porcine atria has been shown to exhibit a relative capacity of 56% under
the same conditions, and the shortfall was attributed to cooperativity in the binding of \(^{3}\text{H}\)NMS [147]. In the present case, however, the shortfall resulted from a time-dependent loss of sites during incubation with \(^{3}\text{H}\)NMS. An increase in the time of incubation from 15 min to 180 min was accompanied by a decrease in \(B_{\text{max}}\), from almost 100% to less than 70% of that for \(^{3}\text{H}\)QNB, and a 3–4-fold increase in \(K\) (Fig. 2-3B). Most of the change in \(K\) occurred between 45 min and 3 h. The binding of \(^{3}\text{H}\)QNB appeared to attain equilibrium within 2 h and remained unchanged for at least 1 h thereafter (Fig. 2-3A).

Receptor from native Sf9 membranes was stable at 4 °C, and there was no appreciable difference in the capacity for \(^{3}\text{H}\)NMS and \(^{3}\text{H}\)QNB (Table 2-1, Fig. 2-3D). The binding patterns obtained for \(^{3}\text{H}\)NMS were virtually superimposable after incubation for 6 h, 12 h (Fig. 2-3D), and 18 h, as were those for \(^{3}\text{H}\)QNB after incubation for 12 h and 15 h (Fig. 2-3C). The system therefore appeared to attain equilibrium with respect to both radioligands. In the case of \(^{3}\text{H}\)NMS, data from samples incubated for different times between 6 h and 18 h were combined in subsequent analyses.

With receptor extracted from cholesterol-treated membranes, the apparent capacity for \(^{3}\text{H}\)NMS was 67% of that for \(^{3}\text{H}\)QNB in assays at 30 °C (Table 2-1). As described below, \(^{3}\text{H}\)NMS bound rather slowly to some of the sites but appeared to attain equilibrium within 3 h. There was no time-dependent inactivation (Fig. 2-4A), in contrast to the decrease in \(B_{\text{max}}\) observed at 30 °C with receptor from native membranes (Fig. 2-3B). The binding of \(^{3}\text{H}\)QNB was the same after incubation for 2 h, 4 h, or 6 h (data not shown). Cholesterol therefore appears to stabilize the receptor and to introduce a heterogeneity in which at least 30% of the sites are of anomalously weak affinity for \(^{3}\text{H}\)NMS.

The binding of \(^{3}\text{H}\)NMS to receptor from cholesterol-treated membranes at 30 °C is illustrated in Figure 2-4A, where the measurements were taken after incubation for 15 min, 45 min, 3 h, and 6 h. The pooled data were described empirically in terms of Scheme 1 and required two classes of sites differing in affinity for the radioligand. Since
the curves are superimposable at lower concentrations of \[^3\text{H}]\text{NMS} \,(i.e., \,< \,10 \,\text{nM}),\) binding to the sites of higher affinity \((K_{P1})\) appeared to attain equilibrium within 15 min and to remain unchanged thereafter. The sites ostensibly of weaker affinity \((K_{P2})\) were not discernible after 15 min, implying a value of \(K_{P2}\) that exceeds the highest concentration of \[^3\text{H}]\text{NMS}.\) Upon longer incubation, binding at higher concentrations of the radioligand increased in a manner consistent with a decrease in the value of \(K_{P2}.\) The binding profiles obtained after 3 h and 6 h were indistinguishable \((P = 0.62).\)

The data shown in Figure 2-4A are drawn from two of several experiments in which the receptor was incubated for different times from 15 min to 6 h. A similar analysis was performed on all of the data \((\text{Eq.} \, 2, \, n = 2),\) and the mean values of \(K_{P1}\) and \(K_{P2}\) for \[^3\text{H}]\text{NMS} are presented in Figure 2-4B. Whereas \(K_{P1}\) was constant at about 6 nM, the value of \(K_{P2}\) decreased from about 2 µM after 15 min to a minimum of about 0.1 µM by 180 min. The system therefore appeared to attain equilibrium at both classes of sites, but the process was slower at those corresponding to \(K_{P2}.\)

In assays at 4 °C, the apparent capacity for \[^3\text{H}]\text{NMS} in extracts of cholesterol-treated membranes was 63% of that for \[^3\text{H}]\text{QNB (Table 2-1). The binding of \[^3\text{H}]\text{NMS was the same after incubation for either 6 h or 12 h; similarly, the binding of \[^3\text{H}]\text{QNB was the same after incubation for 12 h or 15 h (data not shown). The system therefore appeared to attain equilibrium with respect to both radioligands. Cholesterol led to a marked heterogeneity in the binding of \[^3\text{H}]\text{QNB at 4 °C, as indicated by a decrease in the Hill coefficient from 0.90 to 0.55 (Table 2-1). At either temperature, treatment with cholesterol led to a shortfall in the apparent capacity for \[^3\text{H}]\text{NMS.\)\)

\textit{Nature of the effects introduced by cholesterol and assessment of the data in terms of Scheme 1}

Treatment with cholesterol introduced a heterogeneity detected in the binding of both \[^3\text{H}]\text{NMS and \[^3\text{H}]\text{QNB. To examine the nature of those effects, the binding of \[^3\text{H}]\text{NMS was compared with the inhibitory effect of unlabeled NMS on the binding of \[^3\text{H}]\text{QNB.\)\)\)
The data were assessed in the context of Scheme 1 to obtain the results illustrated in Figures 2-5 and 2-6 for receptor from native and cholesterol-treated membranes, respectively. The lines illustrate the best fit of the model to the pooled data obtained with each preparation at each temperature, and the corresponding parametric values are listed in Table 2-2.

Each analysis was empirical, in that the affinity of NMS was estimated separately for the radiolabeled form \( (K_P) \) and for the unlabeled analogue \( (K_A) \) at each concentration of \( [^3\text{H}]\text{QNB} \). If Scheme 1 describes the system, the measured affinity for a particular class of sites is expected to be the same regardless of the nature of the assay: that is, regardless of whether the variable is radiolabeled or unlabeled NMS or whether the latter is examined at a lower or higher concentration of \( [^3\text{H}]\text{QNB} \). Any difference constitutes a discrepancy between the model and the data. The affinity of \( [^3\text{H}]\text{QNB} \) \( (K_P) \) was taken as the same throughout, as was the value of \( F_2 \) when two classes of sites were required. The latter restriction is consistent with the assumption of non-interconverting sites, which is implicit in Scheme 1. These constraints are consistent with the data (Figs. 2-5 and 2-6), and they permit a direct comparison of the affinities estimated for NMS.

The total concentration of receptor generally was estimated as a single parameter for all data acquired in parallel, either with \( [^3\text{H}]\text{NMS} \) and \( [^3\text{H}]\text{QNB} \) or with unlabeled NMS at two concentrations of \( [^3\text{H}]\text{QNB} \). The only exception was for data obtained at 30 °C with receptor from native membranes, when \([R]\) was estimated separately for \( [^3\text{H}]\text{NMS} \) and \( [^3\text{H}]\text{QNB} \) in the same experiment. The effect of this arrangement is to attribute the shortfall in the capacity for \( [^3\text{H}]\text{NMS} \) (Fig. 2-5A) to the time-dependent loss of sites described above (Fig. 2-3B). Prolonged incubation leads to a concomitant decrease in potency, as reflected in the value of \( K \) estimated from the Hill equation (Fig. 2-3B), but \( [^3\text{H}]\text{NMS} \) was incubated for only 45 min in the experiments selected for these analyses. Any change in \( K \) therefore was negligible, and the value of \( K_P \) is assumed to approximate the equilibrium dissociation constant for the purposes of Scheme 1.

With receptor extracted from native membranes, two classes of sites are required to describe the pooled data obtained at 30 °C \( (i.e., \, n = 2) \) \( (P < 0.00001) \) (Figs. 2-5A and 2-
One class is sufficient for the data obtained at 4 °C (Figs. 2-5C and 2-5D). The heterogeneity at 30 °C derives primarily from the binding of $[^3\text{H}]$QNB ($n_H = 0.83$, Table 2-1), and the fitted estimates of affinity ($K_{Pj}$) are listed in the legend to Table 2-2. The receptor was essentially homogeneous with respect to NMS, and the fit was essentially the same with one estimate of affinity rather than two for the radioligand (i.e., $K_{P1} = K_{P2}$) and for the unlabeled analogue at either concentration of $[^3\text{H}]$QNB (i.e., $K_{A1} = K_{A2}$ ($P = 0.03$). With receptor from cholesterol-treated membranes, two classes of sites are required for the data obtained at either temperature ($P < 0.00001$) (Fig. 2-6).

All conditions yield anomalous differences among the values of $K_{Pj}$ and $K_{Aj}$ estimated for labeled and unlabeled NMS (Table 2-2). The differences are comparatively small with receptor from native membranes at either temperature (i.e., < 4-fold), but there is a 1.3–1.4-fold increase in the sum of squares when the number of parameters is reduced to enforce mechanistic consistency ($P < 0.00001$); that is, when the affinity of NMS is represented by one parameter rather than two at 4 °C (i.e., $K_{P1} = K_{A1}$) or by one parameter rather than three at 30 °C. Such discrepancies are not eliminated with three classes of sites rather than two (30 °C) or with two classes rather than one (4 °C).

The discrepancies are larger and more striking with receptor extracted from cholesterol-treated membranes. In assays at 30 °C, about 26% of the sites were of unusually low affinity for $[^3\text{H}]$NMS ($\log K_{P2} > -6.1$) and for the unlabeled analogue at the lower concentration of $[^3\text{H}]$QNB ($\log K_{A2} = -6.64$) (Table 2-2). Moreover, the value of $K_{A2}$ inferred for unlabeled NMS at the higher concentration of $[^3\text{H}]$QNB ($\log K_{A2} = -7.79$) is 14-fold smaller than that inferred at the lower concentration ($\log K_{A2} = -6.64$); it is at least 50-fold smaller than the minimum value of $K_{P2}$ that is consistent with the binding of $[^3\text{H}]$NMS ($\log K_{P2} > -6.1$). Affinities estimated in terms of Scheme 1 therefore exhibit an anomalous dependence upon both the isotopic form of the ligand and the concentration of $[^3\text{H}]$QNB used in assays with unlabeled NMS. The high value of $K_{P2}$ for $[^3\text{H}]$NMS is the counterpart, in terms of Scheme 1, of the apparent shortfall in capacity as estimated in terms of the Hill equation.
Similar inconsistencies emerged at 4 °C, where about 42% of the sites were of unusually low affinity for labeled and unlabeled NMS (Table 2-2). The value of $K_{A2}$ estimated for unlabeled NMS at the higher concentration of $[^3H]$QNB is 6-fold smaller than that estimated at the lower concentration and 48-fold smaller than the value of $K_{P2}$ estimated for $[^3H]$NMS (Table 2-2). There also is a 5-fold difference in the two values of $K_{A1}$.

Such differences lead to large effects on the sum of squares when Scheme 1 is applied in a mechanistically consistent manner. With the data obtained at 30 °C, there is a 2-fold increase when the affinity of NMS for each class of sites is represented by one parameter rather than three (i.e., $K_{Pj} = K_{Aj(LOW)} = K_{Aj(HIGH)}$, where ‘low’ and ‘high’ refer to the concentration of $[^3H]$QNB) ($P < 0.00001$). With the data obtained at 4 °C, there is a 1.5-fold increase ($P < 0.00001$). In both cases, the constraint leads to marked deviations between the fitted curves and the data.

The inability of Scheme 1 to provide a consistent account of the combined data cannot be overcome by increasing the value of $n$ (Fig. 2-7). At either temperature, an increase from two classes of sites to three has little effect on the sum of squares when the affinity of NMS for each class is assumed to be the same for the radioligand and for the unlabeled analogue at each concentration of $[^3H]$QNB ($\leq 6.4\%, P \geq 0.013$). It also does not eliminate the increase in the sum of squares that occurs with one estimate of the affinity rather than three (> 1.5-fold, $P < 0.00001$). An increase from three classes of sites to four classes or more is essentially without effect (< 1.5%, $P > 0.5$). The sum of squares obtained from mechanistically consistent analyses at larger values of $n$ greatly exceeds that obtained from an empirical analysis, as indicated by the dashed lines in Figure 2-7. The data therefore cannot be described by Scheme 1 at any level of complexity (cf. Refs. [87,147]).

The implications of these anomalies are illustrated by the simulated data shown as dotted lines in Figure 2-6. The simulations in the left-hand panels depict the binding profile of $[^3H]$NMS as calculated from the values of $K_{Aj}$ inferred for unlabeled NMS at the higher concentration of $[^3H]$QNB (Table 2-2). Those in the right-hand panels depict
the inhibitory profile that is predicted for unlabeled NMS at the higher concentration of 
$[^3]$H]QNB on the basis of the values of $K_{\text{Aj}}$ inferred from the data at the lower 
concentration. The difference between the simulated and fitted curves accounts in each 
case for the substantial increase in the sum of squares when the parameters are assigned 
to enforce mechanistic consistency in terms of Scheme 1.

At both temperatures, the values of $K_{Pj}$ obtained empirically for $[^3]$H]NMS agree 
more closely with the corresponding values of $K_{\text{Aj}}$ estimated at the lower concentration of 
$[^3]$H]QNB than with those estimated at the higher concentration (Table 2-2). That 
agreement is reflected in the effect on the sum of squares when $K_{Pj}$ is set equal to $K_{\text{Aj}}$ at 
each class of sites. The increase is comparatively small when $K_{\text{Aj}}$ was defined at 1–2 nM 
$[^3]$H]QNB ($P \geq 0.007$) but much larger at 28–35 nM ($P < 0.00001$). It therefore appears 
that the non-competitive contribution implied by a discrepancy in the values of $K_{Pj}$ and 
$K_{\text{Aj}}$ for NMS is greater at higher concentrations of $[^3]$H]QNB.

Cholesterol-induced effects assessed in terms of Scheme 2

The inability of Scheme 1 to describe the binding of NMS and QNB to receptor from 
cholesterol-treated membranes suggests that the interaction was not strictly competitive. 
That in turn implies cooperativity, and the data represented in Figure 2-6 therefore were 
reanalyzed in terms of Scheme 2. All parameters were assigned to enforce mechanistic 
consistency, and the number of interacting sites ($n$) was incremented by 1 or 2 in 
successive analyses until reasonable agreement was obtained. The benchmark for 
agreement was taken as the sum of squares from an unconstrained, empirical analysis in 
terms of Scheme 1 (i.e., Fig. 2-6). This follows from the nature of Schemes 1 and 2, both 
of which are described by rational functions [248]. An empirical fit in terms of Scheme 1 
therefore seems likely to approximate a mechanistically consistent fit in terms of Scheme 
2 if the latter indeed describes the system.

The sums of squares obtained with Scheme 2 at all values of $n$ are compared in 
Figure 2-7. Essentially the same pattern emerges at both temperatures. When $n$ is two or
three, neither Scheme 1 nor Scheme 2 can account simultaneously for the data in the left- and right-hand panels of Figure 2-6. In contrast to Scheme 1, however, Scheme 2 yields a progressively better fit as the number of interacting sites is increased from three to four or more. When $n$ is six, the sum of squares approximates that obtained empirically with Scheme 1 (Fig. 2-7). Further increases in complexity lead to comparatively small improvements in the fit. The AICc scores suggest that optimal agreement at either temperature is achieved with six interacting sites (Fig. 2-8), and the fitted curves from those analyses are illustrated in Figure 2-9.

The results summarized in Figures 2-7 to 2–9 indicate that Scheme 2 can describe the data without compromises that violate the basic tenets of the model. The affinity of NMS for the vacant receptor ($K_A$) and the homotropic cooperativity factor for each successive equivalent after the first ($f_{i0}$) are defined independently by three sets of data: that for [$^3$H]NMS in panel A or C of Figure 2-9 and the two sets in panel B or D, respectively. Similarly, the heterotropic cooperativity factors for the interaction between NMS and [$^3$H]QNB ($f_{ij}$) are defined independently by the two sets of data in panel B or D. One value of each parameter is sufficient, and the model therefore can account for the data in a mechanistically consistent manner. Further details regarding the analysis in terms of Scheme 2 are described in the Appendix.

2.5 Discussion

*Noncompetitive effects induced by cholesterol*

Cooperativity in the binding of agonists may play a central mechanistic role in signaling via muscarinic and other GPCRs [81,87,110,132,146]. Evidence for cooperativity includes a pattern of latent sites and noncompetitive inhibition revealed by antagonists at the muscarinic receptor extracted from porcine atria in cholate–NaCl. The apparent capacity for [$^3$H]QNB is about twice that for [$^3$H]NMS [147], yet comparatively low concentrations of unlabeled NMS inhibit the binding of [$^3$H]QNB at all of the sites. NMS therefore appears to be inhibitory at sites to which it does not bind, a noncompetitive
effect that can be described quantitatively in terms of cooperative interactions within a receptor that is at least tetravalent [147]. Essentially the same pattern has been found with cardiac muscarinic receptor purified in digitonin–cholate under conditions that yield a complex of receptor and G protein [110].

A different pattern has emerged with $M_2$ receptor extracted from native Sf9 membranes, which bound antagonists in an essentially competitive manner that could be approximated by Scheme 1. There were small discrepancies in the affinity of NMS, but the differences were markedly less pronounced than those in myocardial extracts. Also, the apparent capacity for $[^3\text{H}]$NMS and $[^3\text{H}]$QNB was the same, at least in assays at 4 °C. There was a shortfall in the capacity for $[^3\text{H}]$NMS at 30 °C, but it differed in origin from that in myocardial extracts. With receptor from Sf9 cells, the value of $B_{\text{max}}$ decreased over time with a concomitant decrease in apparent affinity upon more prolonged incubation (Eq. 1). The capacity for $[^3\text{H}]$QNB was unchanged. A similar pattern has been described for affinity-purified muscarinic receptor from porcine atria [228], and the changes appear to arise from a thermally unstable state of the unliganded receptor. QNB exchanges slowly and therefore is highly protective; NMS exchanges rapidly, and the transient occurrence of receptor in a labile, unliganded state leads to inactivation. There was little or no shortfall in the capacity for $[^3\text{H}]$NMS after incubation for only 15 min, and instability was avoided altogether in assays at 4 °C.

Sf9 membranes are deficient in cholesterol, which therefore was increased to the level found in atrial membranes. Upon extraction of the receptor in cholate–NaCl, 26–42% of the sites were of anomalously weak affinity for $[^3\text{H}]$NMS in terms of Scheme 1 (i.e., log $K_{P_2} > -6.1$); moreover, the affinity of NMS inferred from its inhibitory effect at near-saturating concentrations of $[^3\text{H}]$QNB exceeded that estimated via $[^3\text{H}]$NMS by 50-fold or more (i.e., $K_{A_2} < K_{P_2}$). Cholesterol therefore imparted the heterogeneity and attendant noncompetitive effects that have been found in extracts from heart but not in extracts from native Sf9 membranes. In addition, cholesterol prevented the time-dependent loss of sites that otherwise occurred at 30 °C in the presence of $[^3\text{H}]$NMS. Cholesterol also may account for the stability of the cardiac muscarinic receptor extracted in cholate–NaCl [147].

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With receptor from cholesterol-treated membranes, the sites of weaker affinity for $[^3\text{H}]\text{NMS}$ lead to the apparent shortfall in capacity that emerges from the Hill equation. Analyses in terms of Scheme 1 presuppose that the system is at thermodynamic equilibrium, a condition that presumably is met once binding has become independent of time. Equilibration at 30 °C was slower at the sites of weaker affinity than at those of higher affinity but appeared to be complete after about 3 h. Since the concentration of $[^3\text{H}]\text{NMS}$ required to occupy the former was comparatively high (i.e., $> 100 \text{ nM}$), it seems likely that their appearance is rate-limited by a conformational change within the receptor.

A shortfall in capacity and related noncompetitive effects also can emerge if $[^3\text{H}]\text{NMS}$ is not cleared of the synthetic precursor scopolamine [249], but the radiolabeled products used in the present investigation were free of such contamination; moreover, anomalous differences in the affinity inferred for unlabeled NMS at two concentrations of $[^3\text{H}]\text{QNB}$ indicate that the discrepancies are not unique to the radiolabeled form. It has been shown previously that the specific binding of $[^3\text{H}]\text{NMS}$ is not reduced through dissociation of the radioligand from the receptor during separation of the complex on Sephadex G-50 [147].

An explicit model for cooperativity in the receptor from cholesterol-treated membranes

The inconsistencies that emerge from Scheme 1 imply cooperativity, and all of the data at either temperature can be described quantitatively in terms of Scheme 2. With six or more interacting sites, the model returns a fit that is equivalent, according to the global sum of squares, to that obtained from an unconstrained application of Scheme 1. Although the required number of sites is comparatively large, the ability to describe the data confirms that the model is tenable. That agreement contrasts with the failure of Scheme 1, which is inadequate at any level of complexity.

In terms of Scheme 2, the sites of anomalously weak affinity for $[^3\text{H}]\text{QNB}$ or $[^3\text{H}]\text{NMS}$ arise from negative homotropic cooperativity between successive equivalents.
of the radioligand. The magnitude of the effect is such that the sites affected by the cooperative transition are unoccupied at concentrations of the radioligand that otherwise would be saturating. At 30 °C, [3H]QNB is restricted to five out of six sites at a radioligand concentration of about 100 nM; similarly, [3H]NMS is restricted to three out of six sites. The apparent capacity for [3H]NMS is therefore about 60% of that for [3H]QNB. At 4 °C, [3H]QNB appears to label all of the sites, albeit over a wide range of concentration, whereas [3H]NMS labels only four sites out of six. At either temperature, the noncompetitive interaction between [3H]QNB and unlabeled NMS emerges as heterotropic cooperativity between the two ligands at various levels of occupancy.

The failure to achieve saturation by [3H]NMS is highlighted by the comparison with [3H]QNB. The latent sites for [3H]QNB at 30 °C are less conspicuous, but they have been observed previously with muscarinic receptors extracted from porcine atria. In that case, the capacity for [3H]QNB increased upon the addition of digitonin to extracts prepared in cholate–NaCl [147]. The heterogeneity evident in the binding of [3H]QNB at 4 °C is similar in nature to the latent sites for [3H]NMS, although not of sufficient magnitude to preclude saturation under the conditions of the assays. Such effects in the binding of both ligands raise the possibility that negative homotropic cooperativity is common among antagonists to muscarinic and other GPCRs. If so, there may be a general tendency for radioligands to underestimate the true capacity in such systems.

In an oligomeric receptor, sites of anomalously low affinity could derive from negative cooperativity, as in Scheme 2, or from asymmetry [249]. Both phenomena are described by an Adair equation when there is only one independent variable [248], and the two schemes are indistinguishable in assays at graded concentrations of a radioligand. Although the receptors are exclusively of the M2 subtype, an asymmetric system might emerge as a consequence of oligomerization. Asymmetry is a special case of Scheme 1 [249], however, and that model cannot reconcile the binding of [3H]NMS with the inhibitory effects of its unlabeled analogue. It follows that the binding properties arise at least in part from cooperativity within a system that may be either symmetric or asymmetric in the absence of ligand. If the system is cooperative, any asymmetric arrangement has a symmetric equivalent; therefore, cooperativity has been taken
arbitrarily as the cause of both the noncompetitive effect of NMS and the heterogeneity evident in the binding of either radioligand.

Cholesterol appears to promote cooperative effects that otherwise are absent or attenuated. In extracts of native Sf9 membranes, M2 receptors may exist primarily as monomers or as oligomers in which the sites are largely independent (i.e., \( f_{ij} \approx 1 \) in Scheme 2). The small but significant discrepancies in the affinity estimated for NMS in terms of Scheme 1 may derive from weak or residual cooperativity associated with low levels of cholesterol. Such effects are not inevitable, however, and some preparations show no evidence of noncompetitive behavior in the binding of antagonists. Data from similar experiments on digitonin-solubilized M2 receptor from porcine sarcolemma were found to be consistent with Scheme 1 [147], as are data from similar experiments on monomers of the M2 receptor purified from native Sf9 membranes [225]. Attempts to remove endogenous cholesterol by means of methyl-\( \beta \)-cyclodextrin abolished the specific binding of \([3H]QNB\) to receptor extracted in cholate–NaCl.

The possibility that cholesterol acts to promote oligomerization of the receptor or to reduce the fragmentation of a native oligomer is difficult to examine directly. Attempts to monitor the oligomeric status by means of immunologically distinct tags were unsuccessful owing primarily to the low efficiency of immunoprecipitation in cholate–NaCl [155]. Treatment of the membranes with cholesterol was without appreciable effect on the densitometric pattern from western blots, where the positions of the immunoreactive bands were suggestive of monomers and homooligomers of various sizes. The predominant band corresponded to a hexamer. The cross-linking reagent BS3 caused virtually all of the receptor from both native and treated membranes to migrate as aggregates equivalent in size to a hexamer or larger oligomer (\( M_r > 300,000 \)). These results favor the suggestion that cholesterol is without effect on the oligomeric status of the M2 receptor but acts rather to enhance cooperativity within the complex. The composition of the aggregates identified on the gels has not been determined, however, and their relationship to the oligomeric status of the receptor in the extract is speculative.
Oligomeric size

The optimal number of interacting sites estimated for the M₂ receptor from cholesterol-treated membranes is six at either temperature. In contrast, four interacting sites have been sufficient to account for the binding of antagonists and agonists to solubilized and membrane-bound receptor from heart [81,110,147]. The reason for this difference in the value of \( n \) is unclear. Oligomers from cholesterol-treated Sf9 membranes indeed may be larger than those from heart, although that seems unlikely if attendant properties such as cooperativity serve a functional role. They also may be more heterogeneous. Scheme 2 presupposes that the system is homogeneous, and heterogeneity would tend to increase the number of parameters required for the model to describe the data.

A heterogeneous system might emerge as a consequence of solubilization or viral-driven overexpression, which could lead either to a mixture of different multimeric forms or to differences in functionality among oligomers of the same size. Some proteins have been found to aggregate in hypotonic buffers [252,253]. In the case of the M₂ receptor, oligomers do not appear to be induced during or after extraction, nor can their cooperative properties be attributed to factors other than cholesterol. There is no coimmunoprecipitation when membranes from singly infected Sf9 cells expressing differently tagged receptors are mixed prior to the addition of detergent [155,226]. Also, receptors from untreated and cholesterol-treated membranes were extracted and assayed in the same buffers and exhibited the same electrophoretic mobility, either before or after cross-linking with BS³, yet only receptor from treated membranes exhibited marked noncompetitive behavior. If heterogeneity contributes to the value of \( n \) in Scheme 2, it seems likely to emerge during expression, from the non-uniform incorporation of cholesterol, or from the disruption of oligomers during or after extraction.

Lower values of \( n \) may suffice in the case of Scheme 2 if the model is examined with insufficient data. In the present investigation, the inhibitory effect of unlabeled NMS was measured at two concentrations of [³H]QNB. Sarcolemmal extracts were assayed at only one concentration [147], and the data therefore were less restrictive. Four interacting
sites are sufficient to reconcile the binding of labeled and unlabeled NMS to receptor from cholesterol-treated Sf9 membranes if the inhibitory effect at the lower concentration of [3H]QNB is omitted from the analyses.

There is little direct evidence regarding the oligomeric state of cardiac muscarinic receptors, although bands corresponding to tetramers have been identified on western blots of the purified receptor from porcine atria [110]. With the M2 receptor from Sf9 membranes, oligomers containing three differently tagged forms have been purified from triply infected Sf9 cells solubilized in digitonin–cholate; moreover, the prevalence of such tri-labeled complexes suggested that an appreciable fraction of the material was tetrameric or larger [226]. Also, the optimal value of $n$ estimated for the receptor from cholesterol-treated Sf9 membranes is consistent with the relative molecular mass of the major band on western blots, which approximates that of a hexamer (Fig. 2-2). Such agreement may be fortuitous, as the composition of the band is unknown, but the conditions of electrophoresis were selected to avoid inducing aggregation [225].

The notion of a tetramer or larger oligomer differs from the widespread view that GPCRs are dimeric (e.g., Refs. [229,231]). Other exceptions include the β2-adrenergic and C5a receptors, both of which have been found to occur as larger oligomers after chemical cross-linking and disulfide trapping, respectively [254,255]. In the case of rhodopsin, atomic force microscopy of native disc membranes from mice has revealed extensive paracrystalline arrays that appear to be rows of dimers [256]. Extended arrays of rhodopsin also can be inferred from the high degree of amplification observed in the activation of transducin [257–259]. A cooperative assembly of bacterial chemoreceptors is implied by Hill coefficients as high as 10 for the activation of a receptor-regulated kinase by chemoattractants [260,261]. In very large arrays, cooperativity may be localized within a subdomain in a manner similar to that proposed for the binding of oxygen to hemocyanins [262].

**Role of cholesterol**
Plasma membranes of Sf9 cells have been shown previously to contain very little cholesterol [245]. The level in membranes from untreated cells in the present investigation was 27-fold lower than that in porcine sarcolemma; similarly, a 20-fold difference has been reported between Sf9 membranes and the plasma membrane of guinea-pig myometrium [246,263]. Levels in porcine sarcolemma are somewhat higher, however, than those found in various cell lines of mammalian origin. Whereas sarcolemmal membranes contained 0.16 mg of cholesterol per mg of total protein (Table 2-1), the levels were 3–16-fold lower in Chinese hamster ovary cells (0.05 mg CHL/mg protein, [264]), human embryonic kidney cells (0.03 mg CHL/mg protein, [265]), and African green monkey kidney cells (0.01 mg CHL/mg protein, [266]). It follows that the amount of cholesterol in treated Sf9 membranes and porcine sarcolemma may exceed the minimum required for cooperative interactions.

Cholesterol-related effects on the affinity and stability of the M2 receptor recall those on other GPCRs. The oxytocin and galanin receptors were shifted from high to low affinity for agonists when cholesterol was removed from the plasma membranes of uterine myometrium and Chinese hamster ovary cells, respectively [246,264]. High-affinity binding was re-established upon the restoration of cholesterol to the depleted membranes. Similarly, the µ-opioid receptor was driven toward a high-affinity state for the agonist [3H]DAMGO either by the removal of ergosterol from the plasma membrane of yeast or by the addition of cholesterol [267]. The state of high affinity stabilized by cholesterol was shown to be independent of the interaction between receptor and G protein.

In the present investigation, the antagonist [3H]QNB bound sixfold more tightly to the M2 receptor from cholesterol-treated Sf9 membranes than to that from native membranes, at least at 30 °C. There also was an increase in the affinity of NMS, although the effect is complicated by the emergence of cooperativity. A similar effect was found with the 5-HT1A receptor from cholesterol-depleted bovine hippocampal membranes, which exhibited increased binding of the antagonist [3H]p-MPPF after the membranes were replenished with cholesterol [268]. The stabilizing effect of cholesterol
on the M₂ receptor resembles that on the oxytocin receptor, which was protected against proteolytic degradation and against inactivation by heat and extremes of pH [269].

Recent studies have suggested that cholesterol plays a role in maintaining the protein complement of cholesterol-rich regions of the plasma membrane such as caveolae and lipid rafts [215]. It has been proposed that those microdomains serve as platforms for assembling the components of signaling cascades [270], with possible consequences for the functionality of receptors and related proteins. Thus, localization of the oxytocin receptor within caveolae was found to alter its regulatory effects on cell proliferation [271,272]. Similarly, translocation of the agonist-activated M₂ muscarinic receptor into sarcolemmal caveolae was important for its signaling via nitric oxide synthase [273,274]. Sequestration of GPCRs within caveolae and lipid rafts also tends to increase their local concentrations. That in turn raises the possibility that localization within cholesterol-enriched regions of the plasma membrane promotes oligomerization. In the present investigation, however, there was no difference in the cross-linking pattern obtained with receptors from native and cholesterol-treated membranes following treatment with BS³. Cholesterol therefore appears to modulate cooperativity within an existing oligomer of the M₂ receptor rather than to promote oligomerization per se.

Cholesterol can modulate the function of GPCRs via a direct interaction with the protein or by altering the biophysical properties of the membrane [214]. Rhodopsin has been reported to undergo both modes of regulation [224,275]. A direct effect is suggested by the cholesterol-sensitive transfer of resonance energy between tryptophanyl residues of the receptor and the fluorescent sterol cholestatrienol [224]. The oxytocin, galanin, and µ-opioid receptors also appear to be regulated directly, as indicated by the selectivity of ergosterol and analogues of cholesterol in mimicking the effects of cholesterol on membrane fluidity but not those on the affinity of agonists [246,264,267]. Molecular modeling has suggested that the interaction of cholesterol with the oxytocin receptor involves residues on the surface of transmembrane segments 5 and 6 [276]. In the present investigation, the effects of cholesterol were monitored in mixed micellar solutions obtained by solubilization of the lipid bilayer. It therefore seems likely that the
noncompetitive behavior found in extracts of cholesterol-treated membranes derives from cholesterol acting directly on the receptor rather than indirectly via the membrane.

Cholesterol favors a state of the M₂ receptor in which binding is cooperative, at least in the case of antagonists; otherwise, the sites function in a largely independent manner. Such an arrangement recalls the suggestion that a multimer comprising multiple equivalents of both receptor and G protein interconverts spontaneously between two states that differ in their cooperative properties [81]. By perturbing the distribution of such a heterooligomer between the two states, agonists affect cooperativity in the binding of GDP to interacting G proteins; conversely, guanyl nucleotides affect cooperativity in the binding of agonists to interacting receptors. Access of the receptor to one or both of those states, and the cooperative effects themselves, may depend upon cholesterol.

2.6 Appendix

Nature of the cooperative effects inferred from Scheme 2

The results illustrated in Figures 2-7 to 2-9 indicate that Scheme 2 can reconcile the shortfall in the apparent capacity for [³H]NMS with the inhibitory effect of unlabeled NMS on the binding of [³H]QNB. At least four interacting sites are required, and six afford the most efficient use of parameters (Fig. 2-8). The model therefore is tenable as a mechanistic description of the data, in contrast to the notion of intrinsic heterogeneity as embodied in Scheme 1. Somewhat paradoxically, the data require a level of complexity at which a number of parameters are correlated or otherwise undefined; nonetheless, the results of the analyses yield some insight into how cooperativity can account for such effects.

With samples equilibrated at 30 °C, there was little net homotropic cooperativity in the binding of [³H]QNB to four out of the six sites. The individual estimates of $f_{02}, f_{03}$, and $f_{04}$ are correlated, but the mean value of log $f_{0j}$ for those interactions is near 1. Binding of the fifth equivalent of the radioligand is largely prevented by negative
homotropic cooperativity (log $f_{05} > 2$), and that in turn precludes binding of the sixth. The cooperativity factor for the sixth equivalent is undefined (i.e., $f_{06}$). It follows that negative homotropic cooperativity appears to prevent saturation of the receptor by $[^3H]$QNB at the concentrations used in the assays, as illustrated by the fitted curve in Figure 2-9A. Restraints that prevent this effect lead to a significant increase in the sum of squares at any value of $n$ greater than three ($P < 0.00001$).

The sites of anomalously weak affinity for $[^3H]$NMS at 30 °C arise from pronounced, negative homotropic cooperativity at the fourth equivalent of the radioligand (log $f_{40} > 2$). Values of $f_{00}$ for the second and third equivalents are near or equal to 1 ($f_{20} = -0.28 \pm 0.20$, $f_{30} = 0.13 \pm 0.28$). These effects lead to a plateau at concentrations of $[^3H]$NMS near 0.1 µm, which causes an apparent shortfall in capacity when the data are analyzed in terms of the Hill equation. Binding is seen to increase further at the highest concentration of $[^3H]$NMS, but two sites are involved and the cooperativity factors are not defined (i.e., $f_{50}$ and $f_{60}$). The non-competitive effect of unlabeled NMS on the binding of $[^3H]$QNB derives from a mix of positive and negative cooperativity between the two ligands at the different levels of occupancy (i.e., $f_{ij} < 1$ or $f_{ij} > 1$). The individual values of $f_{ij}$ tend not to be defined, owing in part to multiple correlations.

A similar pattern emerges from the data obtained at 4 °C. The heterogeneity evident in the binding of $[^3H]$QNB arises from negative homotropic cooperativity between the fifth and sixth equivalents of the radioligand (log $f_{05} > 1.5$), and a parallel effect at the same level of occupancy accounts for the low apparent capacity for $[^3H]$NMS (log $f_{50} > 1.5$). The inhibition of $[^3H]$QNB by unlabeled NMS arises from both positive and negative heterotropic cooperativity; in most cases, however, the individual values of $f_{ij}$ are undefined.

The degree to which the binding properties can be rationalized in terms of specific parametric values is limited at either temperature by the number of parameters required for a satisfactory fit. There are only three cooperativity factors when the receptor is divalent (i.e., $f_{20}, f_{02},$ and $f_{11}$); all are well defined, but the model cannot describe the data. The total number of homotropic factors increases by 2 for each increment in $n$ ($f_{00}$ and $f_{0j}$),
and the number of heterotrophic factors increases by $n-1$. They become increasingly correlated and undefined as the receptor is assumed to be trivalent, tetravalent or larger. A comparatively high value of $n$ is required for the model to describe the data, as illustrated in Figure 2-7; at that point, however, the data are not sufficient to define a unique set of parametric values.

The need for such complexity is related to the mixed nature of the inhibitory effect of NMS on the binding of [$^3$H]QNB. Whereas the initial phases of the inhibition are primarily noncompetitive, the process becomes increasingly competitive as it approaches saturation of the receptor by the unlabeled ligand (i.e., $A_n R$ in Scheme 2). This latter effect accounts for the shape of the fitted curves in Figure 2-9B, where approximately 25% of the labeled sites appear to be of low affinity for NMS (i.e., $K = 10 \mu$M). The relative size of this component is reduced to 18% when $n$ is increased from six to eight, since the opportunity for noncompetitive effects is greater. Similarly, it is reduced further to approximately 11% of the labeled sites when $n$ is increased from eight to ten. The model becomes increasingly noncompetitive in nature at higher values of $n$, and it thereby becomes better able to account for the inhibitory effect of unlabeled NMS at concentrations greater than 1 $\mu$M. Since comparatively few data points are involved, the overall decrease in the sum of squares is comparatively small.

2.7 Acknowledgements

The managers and staff of Quality Meat Packers Limited are gratefully acknowledged for their generous donation of porcine atria. This investigation was supported by the Canadian Institutes of Health Research (MOP43990) and the Heart and Stroke Foundation of Ontario (T4914, T5650).
Table 2-1 – Empirical characterization of specific binding

<table>
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</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>log $K$</td>
<td>$n_H$</td>
<td>$B_{max}$ (pM)</td>
</tr>
<tr>
<td>Native (6,6)</td>
<td>30</td>
<td>0.75</td>
<td>−7.73 ± 0.04</td>
<td>0.99 ± 0.07</td>
<td>241–472</td>
</tr>
<tr>
<td>Native (6,4)</td>
<td>4</td>
<td>6–18</td>
<td>−8.04 ± 0.05</td>
<td>0.96 ± 0.03</td>
<td>608–824</td>
</tr>
<tr>
<td>CHL-treated (3,3)</td>
<td>30</td>
<td>3</td>
<td>−8.03 ± 0.01</td>
<td>0.91 ± 0.05</td>
<td>157–224</td>
</tr>
<tr>
<td>CHL-treated (3,3)</td>
<td>4</td>
<td>6</td>
<td>−7.75 ± 0.25</td>
<td>0.96 ± 0.06</td>
<td>139–191</td>
</tr>
</tbody>
</table>

M$_2$ receptor was extracted from native Sf9 membranes and from membranes supplemented with cholesterol (CHL). Binding was measured at graded concentrations of $[^3]$HNMS and $[^3]$QNB taken in parallel. The time of incubation with $[^3]$HNMS is shown in the table, with some experiments including measurements after equilibration for two different times (i.e., 6 h and 12 h, 6 h and 18 h). The time of incubation with $[^3]$QNB was 2 h at 30 °C and 12 h at 4 °C. The number of sets of data is shown in parentheses ($[^3]$HNMS, $[^3]$QNB). Each set was analyzed independently in terms of the Hill equation, except as noted below, and the individual estimates of log $K$ and $n_H$ were averaged to obtain the means (± S.E.M.) listed in the table. The relative capacity for $[^3]$HNMS and $[^3]$QNB was calculated from parallel estimates of $B_{max}$ (i.e., $B_{max}$ [$[^3]$HNMS]/$B_{max}$ [$[^3]$QNB]), and the individual values were averaged to obtain the mean (± S.E.M.) listed in the table. $^a$ Owing to the low value of $n_H$ for $[^3]$QNB, the values of $K$ and $B_{max}$ were estimated by means of Eq. 2 ($n = 2$) as described previously [249]. The mean values of the fitted parameters are as follows: $F_2 = 0.52 ± 0.09$, log $K_{P1} = -9.92 ± 0.15$, log $K_{P2} = -7.96 ± 0.05$. 

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$[^{249}]$
Table 2-2 – Affinities of NMS for M$_2$ receptor extracted from native and cholesterol-treated Sf9 membranes, estimated empirically in terms of scheme 1

<table>
<thead>
<tr>
<th>Preparation</th>
<th>Temp. (°C)</th>
<th>Ligands</th>
<th>$[^3]$H]QNB (nM)</th>
<th>Time of incubation (h)</th>
<th>Log $K_{L1}$</th>
<th>Log $K_{L2}$</th>
<th>L</th>
</tr>
</thead>
<tbody>
<tr>
<td>Native membranes $^a, e$</td>
<td>30</td>
<td>$[^3]$H]NMS (6)</td>
<td>−8.03 ± 0.03</td>
<td>3</td>
<td>−8.03 ± 0.03</td>
<td>&gt; −6.1 $^d$</td>
<td>P</td>
</tr>
<tr>
<td></td>
<td></td>
<td>NMS/$[^3]$H]QNB (3)</td>
<td>1–2</td>
<td>2</td>
<td>−7.96 ± 0.06</td>
<td>−6.64 ± 0.18</td>
<td>A</td>
</tr>
<tr>
<td></td>
<td></td>
<td>NMS/$[^3]$H]QNB (3)</td>
<td>30–35</td>
<td>2</td>
<td>−8.24 ± 0.05</td>
<td>−7.79 ± 0.16</td>
<td>A</td>
</tr>
<tr>
<td>Native membranes</td>
<td>4</td>
<td>$[^3]$H]NMS (6)</td>
<td>−8.02 ± 0.03</td>
<td>6–18</td>
<td>−8.36 ± 0.05</td>
<td>c</td>
<td>P</td>
</tr>
<tr>
<td></td>
<td></td>
<td>NMS/$[^3]$H]QNB (3)</td>
<td>27–29</td>
<td>12</td>
<td>−5.63 ± 0.45</td>
<td>c</td>
<td>A</td>
</tr>
<tr>
<td>CHL-treated membranes $^e$</td>
<td>30</td>
<td>$[^3]$H]NMS (3)</td>
<td>−7.74 ± 0.04</td>
<td>6</td>
<td>−7.47 ± 0.41</td>
<td>−6.53 ± 0.12</td>
<td>A</td>
</tr>
<tr>
<td></td>
<td></td>
<td>NMS/$[^3]$H]QNB (2)</td>
<td>1–2</td>
<td>12</td>
<td>−8.17 ± 0.10</td>
<td>−7.31 ± 0.13</td>
<td>A</td>
</tr>
<tr>
<td>CHL-treated membranes $^e$</td>
<td>4</td>
<td>$[^3]$H]NMS (3)</td>
<td>−7.81 ± 0.03</td>
<td>0.75</td>
<td>−7.81 ± 0.03</td>
<td>b</td>
<td>P</td>
</tr>
<tr>
<td></td>
<td></td>
<td>NMS/$[^3]$H]QNB (3)</td>
<td>1–2</td>
<td>2</td>
<td>−7.57 ± 0.07</td>
<td>b</td>
<td>A</td>
</tr>
<tr>
<td></td>
<td></td>
<td>NMS/$[^3]$H]QNB (3)</td>
<td>32–38</td>
<td>2</td>
<td>−7.25 ± 0.05</td>
<td>b</td>
<td>A</td>
</tr>
<tr>
<td></td>
<td></td>
<td>P</td>
<td></td>
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<td></td>
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<td>A</td>
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</table>
LEGEND TO TABLE 2-2

The data illustrated in the paired left- and right-hand panels of Figures 2-5 and 2-6 were pooled (i.e., panels A and B, panels C and D) and analyzed according to Eq. 2 \((n = 1 \text{ or } 2)\) to obtain the parametric values listed in the table. NMS was present either as the radioligand \((L = P, \text{ left-hand panel})\) or as the unlabeled analogue \((L = A, \text{ right-hand panel})\). QNB was present only as the radioligand. The number of experiments is shown in parentheses. Two classes of sites were required, except where indicated otherwise, and a single value of \(F_2\) was common to all of the data in both panels. Affinities were estimated as follows: for \([3H]\)QNB, single values of \(K_{Pj}\) were common to all of the relevant data in both panels; for NMS, values of \(K_{Pj}\) were assigned to data obtained with the radioligand (left-hand panel), and separate values of \(K_{Aj}\) were assigned to data obtained with the unlabeled analogue at each concentration of \([3H]\)QNB (right-hand panel). Values of \([R]_i\) were assigned as described in the text. The fitted value of \(F_2\) and the values of \(K_{Pj}\) for \([3H]\)QNB are as follows: receptor from native membranes at 30 °C, \(F_2 = 0.90 \pm 0.04\), \(log K_{P1} = -10.07 \pm 0.33\), \(log K_{P2} = -8.67 \pm 0.05\); at 4 °C, \(log K_{P1} = -8.76 \pm 0.02 \,(n = 1)\); receptor from treated membranes at 30 °C, \(F_2 = 0.26 \pm 0.01\), \(log K_{P1} = -9.43 \pm 0.05\), \(log K_{P2} = -10.34 \pm 0.15\); at 4 °C, \(F_2 = 0.42 \pm 0.02\), \(log K_{P1} = -8.20 \pm 0.09\), \(log K_{P2} = -10.01 \pm 0.09\).  

Data acquired with \([3H]\)QNB and \([3H]\)NMS in the same experiment were assigned separate values of \([R]_i\), as described in the text. The sites appeared homogeneous to NMS and heterogeneous to QNB, as described in the text.  

There is no appreciable decrease in the sum of squares with two classes of sites rather than one \((P = 0.080)\).  

\(K_{P2}\) for \([3H]\)NMS is defined only by a lower bound, which was mapped to identify the value listed in the table. Smaller values of \(K_{P2}\) cause a significant increase in the weighted sum of squares \((P < 0.05)\); higher values are without effect on either the sum of squares or the values of other parameters, which were estimated with the value of \(log K_{P2}\) fixed at \(-5\).  

There is a small decrease in the sum of squares with three classes of sites rather than two \((< 11 \%, \, 0.014 \geq P \geq 0.0058)\), but the parametric values are not well defined.
Fig. 2-1 – Incorporation of cholesterol into the membranes of Sf9 cells. Membranes were supplemented with cholesterol via an inclusion complex with methyl-β-cyclodextrin, either during growth of the cells (0.09 mM with respect to cholesterol) or after harvest and homogenization (0.5 mM with respect to cholesterol). The levels of incorporation were as follows (mg of cholesterol per mg of protein): native Sf9 membranes, 0.0058 ± 0.0032 (N = 6); treated Sf9 membranes, 0.17 ± 0.01 (N = 17); membranes from Sf9 cells treated during growth, 0.034 ± 0.003 (N = 2). The level in sarcolemmal membranes prepared from porcine atria was 0.16 ± 0.01 mg cholesterol/mg protein (N = 2).
Fig. 2-2 – Electrophoretic mobility of M₂ receptor extracted from native and cholesterol-treated Sf9 membranes. Membranes from baculoviral-infected Sf9 cells were solubilized in cholate–NaCl, and the extracts were incubated in the absence (Lanes 1 and 4) and presence of BS³ (Lanes 2 and 5). The gels were loaded with about 80 µg of total protein containing 0.42 ng of receptor, as determined from the binding of [³H]QNB at a concentration of 100 nM. Uninfected Sf9 cells were solubilized in cholate–NaCl, and an aliquot containing about 80 µg of protein served as a negative control (Lane 3). The results presented in the figure are from one of five gels. In each case, samples from native and cholesterol-treated membranes were run in parallel. Two of the gels contained two lanes of cross-linked, cholesterol-treated receptor rather than one. The relative molecular masses of the major bands are as follows (Mᵣ ± s.e.m.): a, 50,900 ± 2,700; b, 105,600 ± 7,300; c, 154,300 ± 11,700; d, 320,000 ± 25,400. The open arrow indicates the position expected for a monomer of the M₂ muscarinic receptor, based on the molecular weight calculated from the amino acid composition (i.e., 51,517 Da).
Fig. 2-3

A

B

C

D

Specific binding (pM)

30 °C

4 °C

log [³H]Quinuclidinylbenzilate

Specific binding (pM)

log [³H]Antagonist
Fig. 2-3 – Stability of binding to M₂ receptor extracted from native Sf9 membranes. Receptor and ligand were incubated at 30 °C (A, B) or 4 °C (C, D). Total binding of [³H]NMS or [³H]QNB was measured in the absence (upper curves) and presence of unlabeled NMS (1 mM) (baseline). When shown together (B, D), [³H]NMS and [³H]QNB were assayed in parallel. The lines depict the best fits of the Hill equation to the pooled data represented in each panel. Data acquired under the same conditions in two or more experiments shared single values of \( K \) and \( n_H \). (A) The receptor was incubated with [³H]QNB for either 2 h (■ and □) or 3 h (● and ○). Open and closed symbols denote data from different experiments (\( N = 2 \)). The parametric values are as follows: 2 h, \( \log K = -8.77 \pm 0.04, n_H = 0.86 \pm 0.04, B_{max} = 719 \) and 665 pM; 3 h, \( \log K = -8.72 \pm 0.03, n_H = 0.87 \pm 0.03, B_{max} = 751 \) and 707 pM. There is no appreciable increase in the sum of squares with single rather than separate values of \( K, n_H, \) and \( B_{max} \) for the data obtained after 2 h and 3 h (\( P = 0.080 \)). (B) The receptor was incubated with [³H]QNB for 2 h (■) and with [³H]NMS for 15 min (○), 45 min (△), or 180 min (◇). Data from two experiments were included in the analysis; for clarity, only one set for [³H]QNB and at each time for [³H]NMS is shown in the figure. The parametric values obtained for [³H]QNB are as follows: \( \log K = -8.83 \pm 0.05, n_H = 0.91 \pm 0.06, B_{max} = 305 \) and 633 pM. The values for [³H]NMS are as follows, where RC denotes the capacity relative to that for [³H]QNB: 15 min, \( \log K = -8.16 \pm 0.04, n_H = 1.27 \pm 0.08, \) RC = 0.91 ± 0.13; 45 min, \( \log K = -7.94 \pm 0.07, n_H = 1.02 \pm 0.06, \) RC = 0.86 ± 0.08; 180 min, \( \log K = -7.57 \pm 0.09, n_H = 1.03 \pm 0.08, \) RC = 0.69 ± 0.01. (C) The receptor was incubated with [³H]QNB for either 12 h (●, ■ and ▲) or 15 h (○, □ and △). Different symbols denote data from different experiments (\( N = 3 \)). The parametric values are as follows: 12 h, \( \log K = -8.60 \pm 0.03, n_H = 1.15 \pm 0.05, B_{max} = 543–752 \) pM; 15 h, \( \log K = -8.67 \pm 0.03, n_H = 1.19 \pm 0.06, B_{max} = 542–703 \) pM. There is no appreciable increase in the sum of squares with single rather than separate values of \( K, n_H, \) and \( B_{max} \) for the data obtained after 12 h and 15 h (\( P = 0.19 \)). (D) The receptor was incubated with [³H]QNB for 12 h (■) and with [³H]NMS for either 6 h (●) or 12 h (○). The parametric values for [³H]QNB are as follows: \( \log K = -8.71 \pm 0.05, n_H = 0.85 \pm 0.06, B_{max} = 709 \) pM. The values for [³H]NMS are: 6 h, \( \log K = -7.92 \pm 0.05, n_H = 1.02 \pm 0.06, \) RC = 0.97; 12 h, \( \log K = -7.95 \pm 0.04, n_H = 1.04 \pm 0.04, \) RC = 0.95. There is no appreciable increase in the sum of squares with a single value of \( B_{max} \) for all of the data and single rather than separate values of \( K \) and \( n_H \) for [³H]NMS after incubation for 6 h and 12 h (\( P = 0.83 \)).
Fig. 2-4

A

Specific binding (pM)


30 °C

B

log K

Time (min)
Fig. 2-4 – Equilibration of \(^{3}H\)methylscopolamine with \(M_2\) receptor extracted from cholesterol-treated \(Sf9\) membranes. (A) Total binding of \(^{3}H\)NMS was measured at 30 °C in the absence (upper curves) and presence of unlabeled NMS (baseline). The data are from two experiments in which the receptor was incubated with \(^{3}H\)NMS for 15 min (●) and 45 min (■) or for 45 min (○), 180 min (—), and 360 min (--). The lines represent the best fit of Eq. 2 \((n = 2)\) to all of the data taken together. Single values of \(K_{Pj}\) were common to both sets of data obtained after incubation for 45 min, and a single value of \(F_2\) was common to all of the data. A single value of \([R]\) was assigned to data from the same experiment. The parametric values are as follows: \(F_2 = 0.66 \pm 0.03\); 15 min, \(\log K_{P1} = -8.38 \pm 0.04\); 45 min, \(\log K_{P1} = -8.38 \pm 0.04\), \(\log K_{P2} = -5.98 \pm 0.10\); 180 min, \(\log K_{P1} = -8.18 \pm 0.08\), \(\log K_{P2} = -6.97 \pm 0.12\); 360 min, \(\log K_{P1} = -8.09 \pm 0.09\), \(\log K_{P2} = -6.94 \pm 0.13\). The value of \(\log K_{P2}\) after 15 min was defined only by a lower bound of \(-5.20 (P = 0.05)\) and was fixed arbitrarily at \(-4\). There was no appreciable increase in the sum of squares with common rather than separate values of \(\log K_{Pj}\) for the data obtained after incubation for 3 h and 6 h \((P = 0.62)\). (B) The receptor was incubated with \(^{3}H\)NMS in six experiments, each of which involved two or three sets of data differing in the time of incubation. The data were analyzed in terms of Scheme 1 (Eq. 2, \(n = 2\)). Separate values of \(K_{Pj}\) were assigned to each set of data; single values of \(F_2\) and \([R]\) were common to all data acquired in the same experiment. The fitted values of \(\log K_{P1} (\square)\) and \(\log K_{P2} (\bigcirc)\) from individual sets of data were averaged to obtain the means (± s.e.m.) plotted on the ordinate, and the corresponding time of incubation is shown on the abscissa. The values of \(\log K_{Pj}\) are follows: 15 min \((N = 3)\), \(\log K_{P1} = -8.42 \pm 0.03\), \(\log K_{P2} = -5.60 \pm 0.06\); 30 min \((N = 3)\), \(\log K_{P1} = -8.45 \pm 0.07\), \(\log K_{P2} = -6.07 \pm 0.17\); 45 min \((N = 5)\), \(\log K_{P1} = -8.36 \pm 0.07\), \(\log K_{P2} = -6.36 \pm 0.23\); 120 min \((N = 1)\), \(\log K_{P1} = -8.39\), \(\log K_{P2} = -6.83\); 180 min \((N = 2)\), \(\log K_{P1} = -8.22 \pm 0.06\), \(\log K_{P2} = -6.84 \pm 0.22\); 360 min \((N = 2)\), \(\log K_{P1} = -8.24 \pm 0.02\), \(\log K_{P2} = -6.60 \pm 0.03\). The mean value of \(F_2\) is 0.52 ± 0.04 \((N = 6)\).
Fig. 2-5 – Binding to M<sub>2</sub> receptor extracted from native Sf9 membranes, analyzed empirically in terms of Scheme 1. Incubations were carried out at 30 °C (A, B) and 4 °C (C, D). (A, C) Total binding of [³H]QNB (closed symbols, ×) or [³H]NMS (open symbols, +) was measured in the absence (upper curves) and presence of unlabeled NMS (baseline). The time of incubation with [³H]QNB was 2 h at 30 °C (A) and 12 h at 4 °C (C); that with [³H]NMS was 45 min at 30 °C (A) and 6 h, 12 h, or 18 h at 4 °C (C). Each experiment included assays with both radioligands taken in parallel, and different symbols denote data from different experiments (e.g., ●, ○; ■, □; ×, +; etc.) (N = 6). (B, D) Total binding was measured at near-saturating (B, □, 32–38 nM, N = 3; D, □, 27–29 nM, N = 3) and subsaturating (B, ○, 1–2 nM, N = 3) concentrations of [³H]QNB and graded concentrations of unlabeled NMS. The time of incubation was 2 h at 30 °C and 12 h at 4 °C. The lines indicate the best fit of Scheme 1 to the pooled data represented in panels A and B (n = 2) and, in a separate analysis, to the pooled data represented in panels C and D (n = 1). The parametric values and further details regarding the analyses are given in Table 2-2. Values plotted at the lower end of the x-axis in panels B and D indicate binding in the absence of NMS. The mean values of the Hill coefficient obtained for unlabeled NMS are as follows: B, 0.82 ± 0.06 at 32–38 nM [³H]QNB, 1.07 ± 0.05 at 1–2 nM [³H]QNB; D, 1.17 ± 0.14 at 27–29 nM [³H]QNB.
Fig. 2-6 – Binding to M<sub>2</sub> receptor extracted from cholesterol-treated Sf9 membranes, analyzed empirically in terms of Scheme 1. Incubations were carried out at 30 °C (A, B) and 4 °C (C, D). (A, C) Total binding of [3H]QNB (closed symbols) or [3H]NMS (open symbols), was measured in the absence (upper curves) or presence of unlabeled NMS (baseline). The time of incubation with [3H]QNB was 2 h at 30 °C (A) and 12 h at 4 °C (C); that with [3H]NMS was 3 h at 30 °C (A) and 6 h at 4 °C (C). The results from 3 independent experiments are shown in each panel, and different symbols denote data from different experiments (i.e., ●, ○; ■, □; ◆, ◇). (B, D) Total binding was measured at near-saturating (B, □, 30–35 nM, N = 10; D, □, 28–30 nM, N = 2) and subsaturating (B, ○, 1–2 nM, N = 7; D, ○, 1–2 nM, N = 2) concentrations of [3H]QNB and graded concentrations of unlabeled NMS. The time of incubation was 2 h at 30 °C (B) and 12 h at 4 °C (D). The solid lines represent the best fits of Scheme 1 (n = 2) to the pooled data represented in panels A and B and, in a separate analysis, to the pooled data represented in panels C and D. The parametric values and further details regarding the analyses are given in Table 2-2. The dotted lines were simulated as described in the text and illustrate discrepancies in the affinity of NMS as estimated under different conditions.
Fig. 2-7 – Comparison of Schemes 1 and 2 for goodness of fit to data from cholesterol-treated Sf9 membranes. The data represented in the corresponding left- and right-hand panels of Figure 2-6 were pooled and analyzed in terms of Schemes 1 and 2 to obtain the global sum of squares at different values of $n$ (A, binding at 30 °C; B, binding at 4 °C) (hatched bars, Scheme 1; grey bars, Scheme 2). The value from each analysis is plotted as a percentage of the value obtained when $n$ was taken as 1, which is the same for both models. All parameters were assigned throughout in a mechanistically consistent manner, in contrast to the empirical assignments depicted in Figure 2-6; thus, all relevant data shared single values $K_{pj}$, $K_{j}$, and $F_j$ (Scheme 1) and of $K_p$, $f_{0j}$, $K_A$, $f_{0b}$, and $f_{ij}$ (Scheme 2). In each case, single values of $[R]_i$ were assigned to data from assays performed in parallel within the same experiment. Further details are described in the text and in the legend to Figure 2-9. The dashed line in each panel indicates the relative sum of squares from an unconstrained, empirical analysis in terms of Scheme 1 (A, $n = 2$; B, $n = 3$). The fitted curves from Scheme 2 with $n = 6$ are shown in Figure 2-9.
Fig. 2-8 – Optimal number of interacting sites for the fit of Scheme 2 to data from cholesterol-treated membranes. The value of AICc was calculated from the weighted sum of squares and the degrees of freedom at each value of $n$ (A, binding at 30 °C; B, binding at 4 °C). When a cooperativity factor was fixed arbitrarily at a specific value, or when two or more cooperativity factors were subsumed in a single parametric value, the restriction was disregarded when calculating the degrees of freedom. The probability that one of two models is correct was calculated from the difference in AICc, and the complementary estimates of P (%) are shown joined by a horizontal line at the tops of the corresponding bars.
Fig. 2-9 – Binding to M<sub>2</sub> receptor extracted from cholesterol-treated Sf9 membranes, analyzed in terms of Scheme 2. Incubations were carried out at 30 °C (A, B) or 4 °C (C, D). The data are the same as those shown in Figure 2-6. (A, C) Total binding of [³H]QNB (closed symbols) or [³H]NMS (open symbols) was measured in the absence (upper curves) or presence of unlabeled NMS (baseline). (B, D) Total binding was measured at near-saturating (□) and sub-saturating (○) concentrations of [³H]QNB and graded concentrations of unlabeled NMS. The lines represent the best fit of Scheme 2 (n = 6) to the pooled data obtained at 30 °C (panels A and B) and, in a separate analysis, to those obtained at 4 °C (panels B and C). All parameters were assigned to enforce mechanistic consistency. Single values of K<sub>p</sub> and f<sub>0j</sub> ([³H]QNB), K<sub>A</sub> and f<sub>0i</sub> ([³H]NMS and NMS) and f<sub>ij</sub> ([³H]QNB and NMS) were common to all relevant data in both panels. The fitted values of log K<sub>p</sub> and log K<sub>A</sub>, respectively, are as follows: 30 °C, –9.51 ± 0.36 and –7.85 ± 0.06; 4 °C, –9.18 ± 0.39 and –7.73 ± 0.09. Single values of [R]<sub>t</sub> were assigned to data acquired in parallel: that is, to data obtained with [³H]QNB and [³H]NMS (A, C) and to data obtained with unlabeled NMS at both concentrations of [³H]QNB (B, D) in the same experiment. Further details are described in the text and in the legend to Figure 2-7.
CHAPTER 3

EFFECT OF CHOLESTEROL ON THE BINDING OF ANTAGONISTS TO THE MUSCARINIC
CHOLINERGIC RECEPTOR EXTRACTED FROM PORCINE ATRIA
3.1 Abstract

Muscarinic receptors were extracted from native and cholesterol-depleted sarcolemmal membranes in cholate–NaCl and compared for the binding of quinuclidinylbenzilate (QNB) and N-methylscopolamine (NMS). Methyl-β-cyclodextrin was used to remove cholesterol, which was reduced 13-fold to levels comparable to those in Sf9 cells. Both preparations exhibited the same or similar capacity for [3H]QNB and [3H]NMS, and two classes of receptor were required to describe the data. With receptor from native membranes, 22% of the sites exhibited an anomalously weak affinity for [3H]NMS, while the balance were of higher affinity. Similar estimates of the affinity were inferred from the inhibitory effect of unlabeled NMS on the binding of [3H]QNB, and the interactions therefore appear to be competitive. Receptor from cholesterol-depleted membranes revealed a similar pattern, including sites of anomalously weak affinity for [3H]NMS, but the affinities estimated from assays at graded concentrations of [3H]NMS differed significantly from those inferred from the inhibitory effect of the unlabeled analogue on [3H]QNB, suggesting that the receptor is capable of cooperative interactions. Depletion of cholesterol also was accompanied by a threefold decrease in the affinity of [3H]QNB. These results indicate that removal of cholesterol from cholesterol-rich sarcolemmal membranes does not necessarily have an effect opposite to that of adding cholesterol to cholesterol-poor Sf9 membranes. In the present investigation, however, the receptor from native myocardial membranes failed to exhibit the non-competitive effects described previously. It therefore appears that cholesterol is not the sole determinant of the latter.
3.2 Introduction

In recent years, much evidence has been gathered for the existence of oligomers of G protein-coupled receptors in native [110,147] and heterologous [225,227] systems using various biochemical and biophysical techniques [277]. Although such oligomers now appear to be ubiquitous, the prevailing view has been that the receptors are mutually independent and that signaling involves a ligand-regulated interconversion between free receptor (R) and free G protein or subunits thereof on the one hand and a 1:1 heteromeric RG complex on the other [80,137]. For many years, the dispersion of affinities revealed in the binding of agonists generally has been rationalized, at least qualitatively, in terms of such an arrangement. The multiple states of affinity therefore are assumed to be induced by the G protein, but this notion has proven difficult to sustain on quantitative grounds [85,110,125].

In an alternative view, the dispersion of affinities revealed in the binding of agonists derives from cooperativity or perhaps asymmetry among the constituent components of an oligomer. Owing to the relationship between the multiple affinities of agonists and efficacy, such a view implies that interactions within the oligomer lie in the mechanistic pathway of signaling. The determinants of cooperativity and the cooperative potential of oligomers are therefore questions that relate to our understanding of signaling via G protein-coupled receptors.

It previously has been shown that the ligand-binding properties of the muscarinic receptor extracted from the sarcolemmal membranes of porcine atria differed in different detergents; that is, the the binding of antagonists was competitive in digitonin-cholate and non-competitive in cholate-NaCl or Lubrol-PX [145]. Digitonin is a plant glycoalkaloid saponin which is known to form water-insoluble, 1:1 complexes with cholesterol [278], and it has been suggested that the difference in ligand-binding characteristics can be attributed to the ability of digitonin to sequester cholesterol from the receptor [147]. Also, it has been shown that M2 receptor extracted from native S9 membranes in cholate–NaCl bound muscarinic antagonists in a wholly competitive manner. Such effects in cholate–NaCl were in contrast to the non-competitive behavior observed
previously in extracts of porcine atria, but the membranes of Sf9 cells contain comparatively low levels of cholesterol. Treatment of Sf9 membranes with exogenous cholesterol regained the pattern of non-competitive effects in the binding of muscarinic antagonists that was observed with atrial extracts. Thus, the apparent capacity for [3H]QNB exceeded that for [3H]NMS, while the specific binding of [3H]QNB was inhibited fully at comparatively low concentrations of unlabelled NMS.

In the present investigation, sarcolemmal membranes were depleted of cholesterol in a reversal of the process employed with Sf9 membranes. Although the capacity was essentially the same for [3H]QNB and [3H]NMS, the sites appeared heterogeneous to both ligands, and the inhibition of [3H]QNB by unlabelled NMS was not strictly competitive. Since the receptor from native membranes bound NMS and QNB in an essentially competitive but otherwise similar manner, cholesterol had comparatively little effect on the binding properties of the cardiac muscarinic receptor in the present investigation.

3.3 Materials and Methods

Ligands, detergents, and other materials

N-[3H]Methylscopolamine was purchased as the chloride salt from NEN Life Science Products (lot 3589315, 71.6 Ci/mmol) and as the bromide salt from Amersham Pharmacia Biotech (batch 38, 80 Ci/mmol). Mass spectra provided by the manufacturer indicated that the samples from Amersham Biosciences were devoid of contaminating scopolamine. The purity of material from PerkinElmer Life Sciences has been confirmed previously [147]. (-)[3H]Quinuclidinylbenzilate was purchased from Amersham Biosciences (batch B-50, 48 Ci/mmol). Unlabeled NMS hydrobromide was purchased from Sigma-Aldrich, and scopolamine hydrobromide was from RBI-Sigma.
Sodium cholate was purchased from Sigma-Aldrich. Solubilized receptor was concentrated as required by means of Centricon-10 and Centriprep-30 filters (Amicon) purchased from Millipore. HEPES and Tris were obtained as the free bases from Boehringer Mannheim and Sigma-Aldrich (Trizma), respectively. EDTA and EGTA were obtained as the free acids from British Drug Houses or Bioshop Canada. Bacitracin, all protease inhibitors, and Sephadex G-50 were from Sigma-Aldrich. Other chemicals were obtained from the sources shown below. Protein was estimated by means of bicinchoninic acid using the BCA Protein Assay Kit from Pierce. Bovine serum albumin was taken as the standard.

Membrane preparation and solubilization of $M_2$ muscarinic receptor

Sarcolemmal membranes were collected from a sucrose gradient and spun down in buffer A (20 mM imidazole, 1 mM Na$^{2+}$-EDTA, 0.1 mM PMSF, 0.02% (w/v) sodium azide, 1 mM benamidine, 2 µg/mL pepstatin A, 0.2 µg/mL leupeptin, 200 µg/mL bacitracin, pH 7.60 with HCl) essentially as described previously [110]. The gradient typically yielded 81–287 mg of protein from about 350–375 g of left plus right atria. The sarcolemmal membranes were washed twice to remove imidazole and endogenous ligands. The washing buffer was the same as that to be used in subsequent steps (i.e., buffer B for solubilization in cholate–NaCl, 20 mM NaH$_2$PO$_4$, 20 mM NaCl, 1 mM EGTA, 0.1 mM PMSF, pH 7.40 with NaOH). The product from the sucrose gradient was suspended in the appropriate buffer (120 mL) with one burst of a Brinkmann Polytron (setting 5, 5 s), and the suspension then was centrifuged for 40 min at 4 °C and 100,000 × g. This step was repeated, and the pellets from the second spin were stored at −75 °C.

Cholesterol was removed from the sarcolemmal membranes of porcine atria by mean of methyl-β-cyclodextrin [246]. Briefly, methyl-β-cyclodextrin (Sigma-Aldrich) was added to buffer C (20 mM KH$_2$PO$_4$, 20 mM NaCl, 1 mM EDTA, 0.1 mM PMSF, and NaOH to pH 7.60), and the mixture was vortexed and sonicated periodically in a bath
sonicator (Haake SWB20) until a clear solution was obtained at a stock concentration of 300 g/L.

To remove cholesterol from sarcolemmal membranes of porcine atria, prepared as described above, the frozen pellets were thawed on ice and suspended in buffer C. A portion of the homogenate was supplemented with methyl-β-cyclodextrin such that the final concentration of methyl-β-cyclodextrin was 100 g/L with respect to the total volume of the suspension; the balance was supplemented with buffer C alone. The final concentration of total protein was 1.5 g/L. Each suspension then was shaken on a horizontal shaker for 3 h at room temperature and centrifuged for 45 min at 4 ºC and 100,000 × g. Pellets were stored at –75 ºC until required for solubilization.

The amount of cholesterol in native and cholesterol-depleted sarcolemmal membranes from porcine atria was quantified using a cholesterol assay kit from BioVision.

Solubilization in sodium cholate plus NaCl was carried out as described previously [147] in a procedure modified from that described in an earlier report [279]. Washed sarcolemmal membranes were thawed on ice and suspended in buffer D (40 mM NaH₂PO₄, 1 M NaCl, 1 mM EGTA, 0.16% sodium cholate, 0.1 mM PMSF, NaOH to pH 7.40, 2 mg of protein/mL), and the mixture was shaken for 1 h at room temperature. The suspension then was diluted 1:1 with a buffer (40 mM NaH₂PO₄, 1 mM EGTA, NaOH to pH 7.40) and centrifuged for 40 min at 4 ºC and 100,000 × g. The supernatant fraction was stored on ice until required for the binding assays. The final concentrations of cholate and NaCl were 0.08% and 0.5 M, respectively.

**Binding assays**

The radioligand and any unlabeled ligand were dissolved in buffer E (20 mM HEPES, 20 mM NaCl, 1 mM EDTA, 5 mM MgSO₄, 0.1 mM PMSF, and NaOH to pH 7.40) supplemented with cholate plus NaCl at concentrations of 0.1% and 20 mM, respectively.
An aliquot of the ligand-containing solution (48 µL) was added to the solubilized receptor (5 µL), and the reaction mixture was incubated at 30 °C. The time of incubation for [3H]NMS was 45 min, and the time of incubation for all samples containing [3H]QNB was 2 h.

To terminate the reaction and separate the bound radioligand, an aliquot of the sample (50 µL) was applied to a column of Sephadex G-50 Fine (0.8 cm × 6.5 cm). The column was pre-equilibrated and eluted with buffer F (20 mM HEPES, 20 mM NaCl, 1 mM EDTA, 5 mM MgSO₄, and NaOH to pH 7.40) supplemented with cholate plus NaCl at a concentration of 0.017% and 20 mM, respectively. All of the eluant up to and including the void volume was collected (1.40 mL) and assayed for radioactivity.

Binding assays generally were carried out within one week of solubilization. Nonspecific binding was taken throughout as binding in the presence of 1 mM unlabeled NMS and increased linearly with the concentration of either radioligand. To compare the binding of [3H]NMS and [3H]QNB, the two radioligands were assayed in parallel; similarly, the inhibitory effect of unlabeled NMS on the binding of [3H]QNB was measured in parallel at two concentrations of the latter.

**Analysis of data**

All data were analyzed with total binding taken as the dependent variable (B_{obsd}) and with the total concentrations of all ligands taken as the independent variables. Any subsequent manipulations were for the purpose of presentation only and did not alter the relationship between the data and the fitted curve. Levels of specific binding (B_{sp}), estimates of total receptor ([R]_t), and estimates of maximal specific binding (B_{max}) are presented as the concentration in the binding assay (pM); similarly, the concentrations of ligands denote the total molar concentration in the binding assay. Assays at graded concentrations of either radioligand were performed with and without unlabeled NMS (1 mM), and both sets of data shared a single estimate of nonspecific binding in the analyses described below.
For data acquired at graded concentrations of $[^3\text{H}]$QNB or $[^3\text{H}]$NMS, the Hill equation was formulated as shown in Eq. 1. The variable $[P]_t$ represents the total concentration of the radioligand. The quantity $B_{sp}$ represents specific binding at the corresponding value of $[P]_t$, and the parameter $B_{\text{max}}$ represents maximal specific binding. The parameter $K$ is the concentration of unbound radioligand at half-maximal occupancy, and $n_H$ is the corresponding Hill coefficient. The parameter NS represents the fraction of unbound radioligand that appears as nonspecific binding. Equation 1 was solved numerically in the manner described previously [248].

$$B_{\text{obsd}} = B_{\text{max}} \frac{([P]_t - B_{sp})^{n_H}}{K^{n_H} + ([P]_t - B_{sp})^{n_H}} + \text{NS}([P]_t - B_{sp})$$  \hspace{1cm} (1)

For data acquired at graded concentrations of unlabeled NMS, the Hill equation was formulated as $B_{\text{obsd}} = (B_{[A]_t=0} - B_{[A]_t\to\infty})K^{n_H}/(K^{n_H} + [A]_t^{n_H}) + B_{[A]_t\to\infty}$. The variable $[A]_t$ is the total concentration of unlabeled ligand, and the parameters $B_{[A]_t=0}$ and $B_{[A]_t\to\infty}$ represent the asymptotic levels of binding when $[A] = 0$ and as $[A] \to \infty$.

Mechanistic analyses were performed in terms of intrinsic heterogeneity (Scheme 1). Estimates of total binding were fitted according to the equation $B_{\text{obsd}} = B_{sp} + \text{NS}([P]_t - B_{sp})$, in which P represents the radioligand and other quantities are as described above for Eq. 1. The value of $B_{sp}$ was computed according to Scheme 1.

Scheme 1 represents an intrinsically heterogeneous system that comprises $n$ classes of noninterconverting and mutually independent sites ($R_j$, $j = 1, 2, ..., n$). Those of type $j$ constitute the fraction $F_j$ of all sites (i.e., $F_j = [R_j]/[R]_t$, where $[R_j]_t = [R_j] + [AR_j] + [PR_j]$, and $[R]_t = \sum_{j=1}^{n}[R_j]_t$). The ligands P and A compete for $R_j$ with the equilibrium dissociation constants $K_{Pj}$ and $K_{Aj}$, respectively. Total specific binding of a radiolabeled probe was calculated according to Eq. 2, and the required values of $[PR_j]$ were obtained as described below.

$$B_{sp} = \sum_{j=1}^{n}[PR_j]$$  \hspace{1cm} (2)
The values of \([PR_j]\) in Eq. 2 were calculated from the expansions in terms of the total concentration of R and the free concentrations of A and P. The latter were computed numerically from the corresponding implicit equations for \([A]_t\) and \([P]_t\) as described previously [248].

Analyses in terms of Scheme 1 involved data combined from several experiments. Scheme 1 was applied empirically and in a mechanistically consistent manner. In the case of the latter, parametric values were shared among different sets of data in strict accord with the premises of the model. In such analyses, ligand P represents \([^3\text{H}]\text{QNB}\), and ligand A represents both isotopic forms of NMS. Thus, the parameters \(K_{Pj}\) (Scheme 1) denote the affinity of \([^3\text{H}]\text{QNB}\), and \(K_{Aj}\) (Scheme 1) denote the affinity of labeled and unlabeled NMS. When Scheme 1 was applied in an empirical manner, different sets of data were permitted separate values of parameters that ought to be invariant (i.e., Table 3-2, Fig. 3-2). In such analyses, ligand P represents the radiolabeled probe—either \([^3\text{H}]\text{QNB}\) or \([^3\text{H}]\text{NMS}\) as indicated, and ligand A represents unlabeled NMS.

The results of analyses involving multiple sets of data from replicated experiments have been presented in each case with reference to a single fitted curve. To obtain the values plotted on the y-axis, estimates of \(B_{\text{obsd}}\) were adjusted according to the expression

\[
B'_{\text{obsd}} = B_{\text{obsd}} \left\{ f(\bar{x}_i, \bar{a}) / f(x_i, a) \right\} [132].
\]

The function \(f\) represents the fitted model, and the vectors \(x_i\) and \(a\) represent the independent variables at point \(i\) and the parameters, respectively. Individual values of \(B'_{\text{obsd}}\) at the same \(x_i\) were averaged to obtain the mean and standard error plotted in the figure. Specific binding \((B_{\text{sp}})\) was calculated as \(B'_{\text{obsd}}\) less the fitted estimate of nonspecific binding at the same concentration of unbound radioligand.

Statistical procedures
All parameters were estimated by nonlinear regression [250]. Affinities were optimized throughout on a logarithmic scale (i.e., log $K_P$). Values of $[R]_i$ were assigned separately to data from separate experiments.

Weighting of the data, tests for significance, and other statistical procedures were performed as described elsewhere [81,132,248]. Fits involving nested models, such as Scheme 1 at different values of $n$, were compared by means of the $F$-statistic [248]. Weighted residuals were of comparable magnitude within each set of data. In simultaneous analyses, individual sets of data generally made comparable contributions to the total weighted sum of squares; thus, the fits were not dominated by the data from one experiment or group of experiments. Mean values calculated from two or more individual estimates of a parameter or other quantity are presented together with the standard error. For parametric values derived from a single analysis of one or more sets of data, the errors were estimated from the diagonal elements of the covariance matrix. Such values reflect the range within which the weighted sum of squares is essentially the same.

3.4 Results

*Effect of cholesterol on apparent capacity and affinity*

Sarcolemmal membranes from porcine atria were depleted of cholesterol via treatment with methyl-β-cyclodextrin at a final concentration of 66.7 mg/mL with respect to the total amount of protein. The level of cholesterol in treated membranes was 13-fold less than that in native sarcolemmal membranes (Fig. 3-1).

In extracts of cholesterol-depleted membranes, the apparent capacity for $[^3H]$NMS after incubation for 45 min at 30 °C was 94% of that for $[^3H]$QNB in terms of the Hill equation; in extracts of native sarcolemmal membranes, the apparent capacity for $[^3H]$NMS was 80% of that for $[^3H]$QNB (Table 3-1). The difference in capacity reported here for native sarcolemmal membranes is less than that found previously with the
muscarinic receptor from porcine atria. In the earlier study, \(^{3}\text{H}\)NMS labeled only 56% of the sites labeled by \(^{3}\text{H}\)QNB, and the shortfall was attributed to cooperativity in the binding of \(^{3}\text{H}\)NMS [147].

There was a 2.5-fold decrease in the overall potency of \(^{3}\text{H}\)QNB when cholesterol was removed from the sarcolemmal membranes of porcine atria (native, \(\log EC_{50} = -9.33 \pm 0.06\); CHL-depleted, \(\log EC_{50} = -8.92 \pm 0.09\)), whereas the potency of \(^{3}\text{H}\)NMS was essentially unchanged (native, \(\log EC_{50} = -8.03 \pm 0.03\); CHL-depleted, \(\log EC_{50} = -8.02 \pm 0.11\)) (Table 3-1).

Assessment of the data in terms of Scheme 1

The data were assessed empirically in terms of Scheme 1, in that the affinity of NMS was estimated separately for the radiolabeled form (\(K_{P}\)) and for the unlabeled analogue (\(K_A\)) at each concentration of \(^{3}\text{H}\)QNB. If the model describes the system, the measured affinity for a particular class of sites is expected to be the same regardless of the nature of the assay: that is, regardless of whether the variable is radiolabeled or unlabeled NMS or whether the latter is examined at a lower or higher concentration of \(^{3}\text{H}\)QNB. Any difference constitutes a discrepancy between the model and the data. The affinity of \(^{3}\text{H}\)QNB (\(K_P\)) was taken as the same throughout, as was the value of \(F_2\) when two classes of sites were required. The latter restriction is consistent with the assumption of noninterconverting sites, which is implicit in Scheme 1. These constraints are consistent with the data, as indicated by the lack of effect on the sum of squares (\(P = 0.96\)), and they permit a direct comparison of the affinities estimated for NMS.

With muscarinic receptor from native sarcolemmal membranes, at least two classes of sites were required to describe the pooled data in panels A and B of Figure 3-2 (\(P < 0.00001\)), and the parametric values are listed in Table 3-2. The total concentration of receptor was estimated as a single parameter for all data acquired in parallel, either with \(^{3}\text{H}\)NMS and \(^{3}\text{H}\)QNB or with unlabeled NMS at two concentrations of \(^{3}\text{H}\)QNB. There was little or no increase in the weighted sum of squares with one estimate rather
than two for the affinity of unlabeled NMS obtained at sub-saturating and near-saturating concentrations of $[^3H]QNB$ ($P = 0.018$, Table 3-2). Similarly, there was little effect on the sum of squares when mechanistic consistency was enforced by reducing the number of parameters for the affinity of NMS from three to one at each class of sites ($P = 0.0055$, Table 3-2). Binding therefore was essentially competitive, although the small differences among the values of $K_{Pj}$ and $K_{A_j}$ for NMS may indicate weak cooperative effects.

With muscarinic receptor from cholesterol-depleted membranes, at least two classes of sites were required to describe the pooled data in panels C and D of Figure 3-2 ($P = 0.00002$), and the parametric values are listed in Table 3-2. The total concentration of receptor was estimated as a single parameter for all data acquired in parallel, either with $[^3H]NMS$ and $[^3H]QNB$ or with unlabeled NMS at two concentrations of $[^3H]QNB$. There was no appreciable increase in the weighted sum of squares with one estimate rather than two for the affinity of unlabeled NMS obtained at sub-saturating and near-saturating concentrations of $[^3H]QNB$ ($P = 0.051$, Table 3-2). There was a significant increase in the sum of squares when mechanistic consistency was enforced by reducing the number of parameters for the affinity of NMS from three to one (14%, $P < 0.00001$). Although the differences in affinity are comparatively small ($\leq 4.5$-fold), the level of significance suggests that cooperative interactions were somewhat stronger than those observed with receptor from native membranes.

3.5 Discussion

It previously has been shown that $[^3H]NMS$ labels only about 50% of the sites labeled by $[^3H]QNB$ in preparations of muscarinic receptor extracted from sarcolemmal membranes in cholate–NaCl [147]. In terms of Scheme 1, the sites inaccessible to $[^3H]NMS$ implied a value of $K_{P2}$ that exceeded the highest concentration of radioligand used in the assays; moreover, that value was at least 70-fold greater than the value of $K_{A2}$ inferred from the inhibition of $[^3H]QNB$. Such a discrepancy implies that NMS inhibits at sites to which it cannot bind, a non-competitive effect that could be described quantitatively in terms of
cooperativity among at least four interacting sites, presumably within a tetramer. A similar pattern has been observed more recently with M\textsubscript{2} receptor purified from Sf\textsubscript{9} cells and reconstituted into phospholipid membranes, where the receptor was shown to exist exclusively as a tetramer [148].

In the present investigation, only 22% of the sites labeled by [\textsuperscript{3}H]NMS in sarcolemmal extracts prepared in cholate–NaCl were of anomalously weak affinity for the radioligand; moreover, the value of \(K_{P2}\) was 4–5-fold smaller than that observed previously (cf. \(\log K_{P2} = -6.11\), Table 3-2; \(\log K_{P2} = -5.45\), Ref. [147]). Owing to these differences in the binding of [\textsuperscript{3}H]NMS, the estimates of affinity derived for the radioligand in the present investigation are consistent with those derived from the inhibitory effect of unlabeled NMS on [\textsuperscript{3}H]QNB, and binding to the receptor extracted from native membranes was essentially competitive. The reason for the absence of non-competitive effects is unclear, although it may relate in part to differences in the level of cholesterol. Whereas cholesterol previously was over 20-fold more abundant in sarcolemmal membranes than in Sf\textsubscript{9} membranes, the difference in the present investigation was only 13-fold. The distribution of cholesterol within the cell is unknown, and the overall difference may mask larger differences in regions important to the functioning of the receptor.

Muscarinic receptor extracted from cholesterol-depleted membranes resembled that extracted from native membranes, despite a level of cholesterol that was comparable to the level in Sf\textsubscript{9} membranes. About 25% of the sites were of anomalously weak affinity for [\textsuperscript{3}H]NMS, and the values of both \(K_{P1}\) and \(K_{P2}\) approximated the corresponding values of \(K_{A1}\) and \(K_{A2}\) inferred from the inhibitory effect of unlabeled NMS on the binding of [\textsuperscript{3}H]QNB. The interactions therefore appeared to be largely competitive, although the differences between \(K_{Pj}\) and \(K_{Aj}\) were sufficiently large to suggest that there was a non-competitive component (\(P < 0.00001\)).

The present results indicate that depletion of cholesterol was essentially without effect on the mechanism whereby antagonists bind to M\textsubscript{2} receptor extracted from cholesterol-rich porcine sarcolemmal membranes, in contrast to the changes observed
when cholesterol was added to native Sf9 membranes [227]. Binding was competitive or nearly so throughout, although the removal of cholesterol may have unmasked weak non-competitive effects that otherwise were latent. Since the cholesterol-depleted preparation from heart resembled the preparation from Sf9 membranes, the lack of an effect derived from the absence of non-competitive behavior in the preparation from native sarcolemmal membranes. These considerations suggest that the manifestation of cooperative effects can differ among different myocardial preparations for reasons that remain unclear. They also indicate that cooperativity is not determined exclusively by the average level of cholesterol within the membrane.

Although cholesterol was without appreciable effect on the mechanism of binding, the overall affinity of [3H]QNB was about fourfold weaker in the cholesterol-depleted preparation, as determined from the values of $K_{P1}$ and $K_{P2}$ estimated in terms of Scheme 1. That difference is consistent with a sixfold increase in affinity that was obtained when cholesterol was added to Sf9 membranes [227]. It therefore appears that the effects of cholesterol on the mechanism of binding and on affinity are independent; that is, the state of the receptor in which binding is cooperative with respect to NMS and QNB is not necessarily the state of lower affinity for those ligands.

The propensity of [3H]QNB to bind with higher affinity to M2 receptor from cholesterol-enriched preparations recalls the existence of G protein-coupled receptors in multiple states that differ in their ligand-binding preferences for agonists and antagonists. An explicit scheme for such an arrangement presupposes that the receptor interconverts between an inactive state (R) and an active state (R*) (Scheme 3). The preference of a ligand for one state over the other shifts the equilibrium accordingly; thus, ligands with higher affinity for the R* state are agonists, and ligands that are indifferent or prefer the R state are antagonists. This context has served as the basis for a further examination of the effect of cholesterol on the affinity of muscarinic ligands, as described in Chapter 4.

3.6 Acknowledgements
The managers and staff of Quality Meat Packers Limited are gratefully acknowledged for their generous donation of porcine atria. This investigation was supported by the Canadian Institutes of Health Research (MOP43990) and the Heart and Stroke Foundation of Ontario (T4914, T5650).
Table 3-1 – Empirical characterization of specific binding

<table>
<thead>
<tr>
<th>Preparation</th>
<th>Temp. (°C)</th>
<th>log $K$</th>
<th>$n_H$</th>
<th>$B_{max}$ (pM)</th>
<th>log $K$</th>
<th>$n_H$</th>
<th>$B_{max}$ (pM)</th>
<th>Relative capacity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Native (4,4)</td>
<td>30</td>
<td>−8.03 ± 0.03</td>
<td>0.99 ± 0.07</td>
<td>162–269</td>
<td>−9.33 ± 0.06</td>
<td>0.91 ± 0.02</td>
<td>236–299</td>
<td>0.80 ± 0.05</td>
</tr>
<tr>
<td>CHL-depleted (5,5)</td>
<td>30</td>
<td>−8.02 ± 0.11</td>
<td>0.87 ± 0.07</td>
<td>27–348</td>
<td>−8.92 ± 0.09</td>
<td>0.92 ± 0.05</td>
<td>34–311</td>
<td>0.94 ± 0.06</td>
</tr>
</tbody>
</table>

$M_2$ receptor was extracted from native and cholesterol-depleted sarcolemmal membranes of porcine atria. Binding was measured at graded concentrations of $[^3]$H]NMS and $[^3]$H]QNB taken in parallel. The time of incubation was 0.75 h for assays containing $[^3]$H]NMS alone and 2 h. for assays containing $[^3]$H]QNB. The number of sets of data is shown in parentheses ([$^3$]H]NMS, $[^3]$H]QNB). Each set was analyzed independently in terms of the Hill equation, and the individual estimates of log $K$ and $n_H$ were averaged to obtain the means ($±$ S.E.M.) listed in the table. The relative capacity for $[^3]$H]NMS and $[^3]$H]QNB was calculated from parallel estimates of $B_{max}$ ($i.e., B_{max,[3H]NMS}/B_{max,[3H]QNB}$), and the individual values were averaged to obtain the mean ($±$ S.E.M.) listed in the table.
Table 3-2 – Affinities of NMS for M2 receptor extracted from native and cholesterol-depleted sarcolemmal membranes of porcine atria, estimated empirically in terms of Scheme 1

<table>
<thead>
<tr>
<th>Preparation</th>
<th>Protocol</th>
<th>Temp. (°C)</th>
<th>Ligands</th>
<th>$[^3\text{H}]\text{QNB}$ (nM)</th>
<th>Time of incubation (h)</th>
<th>Affinity</th>
<th>NMS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Native membranes</td>
<td></td>
<td>30</td>
<td>$[^3\text{H}]\text{NMS}$ (4)</td>
<td>0.75</td>
<td>−8.07 ± 0.03</td>
<td>−6.11 ± 0.44</td>
<td>P</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>NMS/$[^3\text{H}]\text{QNB}$ (3)</td>
<td>0.2–0.4</td>
<td>2</td>
<td>−8.16 ± 0.08</td>
<td>−6.10 ± 0.56</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>NMS/$[^3\text{H}]\text{QNB}$ (5)</td>
<td>20–37</td>
<td>2</td>
<td>−7.92 ± 1.53</td>
<td>−7.06 ± 5.68</td>
</tr>
<tr>
<td>CHL-depleted membranes</td>
<td></td>
<td>30</td>
<td>$[^3\text{H}]\text{NMS}$ (5)</td>
<td>0.75</td>
<td>−8.20 ± 0.04</td>
<td>−6.47 ± 0.29</td>
<td>P</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>NMS/$[^3\text{H}]\text{QNB}$ (4)</td>
<td>1–2</td>
<td>2</td>
<td>−7.55 ± 0.12</td>
<td>−6.60 ± 0.73</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>NMS/$[^3\text{H}]\text{QNB}$ (10)</td>
<td>18–24</td>
<td>2</td>
<td>−6.60 ± 0.73</td>
<td>A</td>
</tr>
</tbody>
</table>

The data illustrated in the paired left- and right-hand panels of Figure 3-2 were pooled (i.e., panels A and B, panels C and D) and analyzed according to Scheme 1 ($n = 1$ or 2) to obtain the parametric values listed in the table. NMS was present either as the radioligand (L = P, left-hand panel) or as the unlabeled analogue (L = A, right-hand panel). QNB was present only as the radioligand. The number of experiments is shown in parentheses. Two classes of sites were required throughout, and a single value of $F_2$ was common to all of the data in both panels. Affinities were estimated as follows: for $[^3\text{H}]\text{QNB}$, single values of $K_{Pj}$ were common to all of the relevant data in both panels; for NMS, values of $K_{Pj}$ were assigned to data obtained with the radioligand (left-hand panel), and separate or common values of $K_{A}$ were assigned to data obtained with the unlabeled analogue at the two concentrations of $[^3\text{H}]\text{QNB}$ (right-hand panel). Values of $[R]_t$ were assigned as described in the text. The fitted value of $F_2$ and the values of $K_{Pj}$ for $[^3\text{H}]\text{QNB}$ are as follows: receptor from native porcine atria ($n = 2$), $F_2 = 0.22 ± 0.03$, log $K_{P1} = −9.54 ± 0.03$, log $K_{P2} = −8.65 ± 0.14$; receptor from CHL-depleted porcine atria ($n = 2$), $F_2 = 0.25 ± 0.04$, log $K_{P1} = −9.04 ± 0.04$, log $K_{P2} = −7.66 ± 0.23$. There is no appreciable increase in the weighted sum of squares with one estimate rather than two for the affinity of unlabeled NMS at each class of sites at both concentrations of $[^3\text{H}]\text{QNB}$ ($P = 0.05$).
Fig. 3-1 – Levels of cholesterol in membranes from porcine sarcolemma, Sf9 cells and CHO cells. Membranes of Sf9 cells were supplemented with cholesterol via an inclusion complex with methyl-β-cyclodextrin after harvest and homogenization (0.5 mM with respect to cholesterol). Sarcolemmal membranes of porcine atria were depleted of cholesterol via incubation with methyl-β-cyclodextrin alone (66.7 mg of MβCD/mg of protein). The levels of cholesterol were as follows (mg of cholesterol per mg of protein): native Sf9 membranes, 0.07 ± 0.01 (N = 7); treated Sf9 membranes, 2.58 ± 0.33 (N = 5); native porcine membranes, 0.94 ± 0.20 (N = 3); depleted porcine membranes, 0.07 ± 0.02 (N = 3). The level in membranes prepared from Chinese hamster ovary cells was 0.17 ± 0.01 (N = 3).
Fig. 3-2 – Binding to M2 receptor extracted from native (panels A and B) and cholesterol-depleted (panels C and D) sarcolemmal membranes of porcine atria, analyzed empirically in terms of Scheme 1. Incubations were carried out at 30 °C. (A and C). Total binding of [3H]QNB (closed symbols) or [3H]NMS (open symbols), was measured in the absence (upper curves) or presence of unlabeled NMS (baseline). The time of incubation was 2 h and 45 min for samples containing [3H]QNB and [3H]NMS, respectively. The results from four (panel A) and five (panel C) independent experiments are shown in each panel, and different symbols denote data from different experiments. (B and D) Total binding was measured at near-saturating (B, □, 20–37 nM, N = 5; D, □, 18–24 nM, N = 10) and subsaturating (B, ○, 0.2–0.4 nM, N = 3; D, ○, 1–2 nM, N = 4) concentrations of [3H]QNB and graded concentrations of unlabeled NMS. The time of incubation was 2 h. The lines represent the best fits of Scheme 1 to the pooled data represented in panels A and B (n = 2) and, in a separate analysis, to the pooled data represented in panels C and D (n = 1). The parametric values and further details regarding the analyses are given in Table 3-2.
CHAPTER 4

MECHANISTIC BASIS FOR THE EFFECT OF CHOLESTEROL ON THE AFFINITY OF AGONISTS
AND ANTAGONISTS FOR THE M2 MUSCARINIC CHOLINERGIC RECEPTOR
4.1 Abstract

*N*-Ethylmaleimide (NEM) has been used together with cholesterol to examine the effect of the latter on the binding of ligands to the M$_2$ muscarinic cholinergic receptor. Upon treatment with NEM, receptors extracted from native and cholesterol-treated *Sf*9 membranes in cholate-NaCl underwent an increase in their apparent affinity for agonists and a decrease in that for antagonists. Treatment of *Sf*9 membranes with cholesterol-methyl-β-cyclodextrin increased the affinity of the solubilized receptor for agonists and antagonists, irrespective of NEM. The data were in good agreement with a model in which the receptor interconverts spontaneously between an agonist-specific state akin to the active state (R*) and an antagonist-specific, inactive state (R). Two equivalent fits emerged from the analyses. In the first, NEM shifted the equilibrium between R and R* toward the latter without affecting affinity *per se*, and cholesterol increased the affinity of all ligands for both states without affecting the position of the equilibrium. In the second, cholesterol shifted the equilibrium without affecting affinity *per se*, and NEM increased the affinity of agonists while decreasing that of antagonists. Previous studies on the purified M$_2$ receptor have shown that NEM acts exclusively to favor R* over R [Sum C.S. *et al.* (2002) *J. Biol. Chem.* 277, 36188–36203]; the effect of cholesterol therefore can be attributed to an increase in the affinity of each ligand for both states. Although the alternative solution can be rejected in this case, its existence is not self-evident and illustrates the insight to be gained from mechanistically explicit models.
4.2 Introduction

Muscarinic and other G protein-coupled receptors interconvert spontaneously between at least two states: an active state favored by agonists (R*) and an inactive state favored by inverse agonists (R) \([137,280–282]\). The distribution of receptors between those states can be modulated via the G protein, and the allostERIC interaction between agonists and guanyl nucleotides regulates signaling across the membrane \([97,283,284]\). Other factors that can or may affect the distribution include sodium and magnesium \([283,285–289]\), mutations that lead to constitutive activity \([280,290]\), sulfhydryl-specific reagents \([228]\), and extraction in detergent \([212,228,291,292]\).

The structural basis for the difference between R and R* remains unclear. It also is not clear how that interconversion relates to the GTP-sensitive dispersion of affinities revealed in the binding of agonists, although both phenomena are linked to efficacy \([91,293,294]\), nor is it understood how the heterogeneity discerned by agonists emerges from a single class or subtype of receptor. If the system is at thermodynamic equilibrium, a population of identical and mutually independent sites is expected to appear homogeneous \((e.g., n_H = 1)\) irrespective of the number of conformational states accessible to a single molecule \([85,248]\). It has been suggested that heterogeneity is induced by G proteins interacting transiently with the receptor to yield a ligand-sensitive distribution of coupled and uncoupled states \(i.e., R + G \overset{\text{RG}}{\underset{\text{R}}{\rightleftharpoons}}\) \([80,137,294]\). Such schemes become problematic when applied quantitatively \([85,125]\), however, and it may be that the RG complex is less transient than assumed in the models \([110,111,119,295,296]\). To reconcile these observations, it has been suggested that the interconverting species is an oligomer in which the observed heterogeneity derives from cooperativity or asymmetry with respect to the binding of the agonist \([81,110,148]\).

The multiple states of GPCRs have exhibited some variability in their sensitivity to the sulfhydryl reagent N-ethylmaleimide. In the case of muscarinic receptors in membranes from rat brain, the reagent was shown early on to cause an apparent interconversion from a state of low affinity for agonists to a state of higher affinity \([94,149]\); in other studies, however, it was found to mimic and thereby to pre-empt the
interconversion from high affinity to low affinity brought about by GTP [297]. The latter effect also has been found repeatedly with the muscarinic receptor in membranes from rat heart (e.g., Ref. [298]).

Such apparent inconsistencies may be a consequence of different experimental conditions and their effect on the interaction between the receptor and the G protein, which would be expected to affect the binding patterns revealed by agonists. In the case of the cardiac muscarinic receptor, the effect of N-ethylmaleimide was clarified in an examination of the purified receptor devoid of G protein [228]. Under those conditions, alkylation favored a thermally unstable state of high affinity for agonists and low affinity for antagonists over a comparatively stable state of low affinity for agonists and high affinity for antagonists. N-Ethylmaleimide and agonists therefore appear to drive the receptor toward the same state, at least in the absence of G protein.

Sulfhydryl-specific reagents have been useful probes of the relationship between structure and function [299–303]. Such reagents have been tagged with environmentally sensitive fluorescent or spin labels and used to detect the conformational change between the inactive and active states [299–301]. Results from such studies have led to the suggestion that activation involves the rotation or tilting of the sixth transmembrane helix [280,299,301–303].

Evidence for the existence of multiple interconverting states also has emerged from constitutive activity as seen, for example, in mutants of the β2 adrenergic receptor [137,151,304] and the M5 muscarinic receptor [305]. In the latter case, the ligand-regulated activity of 13 constitutively active mutants differing only at position 465 in the sixth helix could be accounted for by shifts in a single equilibrium between an active and an inactive state [280]. Similarly, the notion of spontaneously interconverting states is consistent with the constitutive activity and related properties of M1–M4 muscarinic receptors overexpressed in Chinese hamster ovary cells [306] and of M5 muscarinic receptors coexpressed with comparatively large amounts of Gq [305].
The ligand-binding properties of G protein-coupled receptors also can be affected by modifications to their lipid environment, as indicated by the changes that occur when the receptor is extracted in detergent. Upon solubilization in digitonin–cholate, muscarinic receptors extracted from \textit{Sf9} cells, porcine cerebral cortex, and porcine atria have been shown to undergo a decrease in their apparent affinity for antagonists; high-affinity binding was restored upon their reconstitution into brain membranes or into liposomes prepared from cholesteryl hemisuccinate, phosphatidylcholine, and phosphatidylinositol [291,292]. Similarly, delipidation of the \(\beta\)-adrenergic receptor from erythrocyte membranes through solubilization in deoxycholate abolished virtually all binding of the antagonist [\(^{125}\text{I}\)]iodocyanopindolol; upon reconstitution with soybean lipids, the receptor bound agonists and antagonists with affinities similar to those of the receptor in native membranes [212]. Such effects suggest that the binding properties are sensitive to lipids acting either directly on the receptor or via their contribution to properties of the membrane.

Cholesterol is essential to many eukaryotic organisms, in which 65–90\% of cellular free cholesterol can reside in the plasma membrane at an average concentration of 200–300 \(\mu\text{g}/\text{mg}\) protein [214]. It has been shown to modulate the properties of many membrane proteins, including G protein-coupled receptors [214]. In the case of the M\(_2\) muscarinic receptor, the antagonist \([^3\text{H}]\text{quinuclidinylbenzilate}\) was found to bind sixfold more tightly to receptor extracted from cholesterol-treated \(\text{Sf9}\) membranes than to that from native membranes [227]. Moreover, the former preparation was more resistant to thermal instability than the latter. The effect of cholesterol therefore differs from that of \(N\)-ethylmaleimide, which was found to favor a thermally unstable state of high affinity for agonists and low affinity for antagonists [228]. This comparison raises the possibility that cholesterol acts directly on the muscarinic and perhaps other G protein-coupled receptors to modulate the equilibrium between the active and inactive states, shifting the distribution toward the latter.

In the present study, \(N\)-ethylmaleimide has been examined for its effect on the affinity of agonists and antagonists for the M\(_2\) muscarinic receptor extracted from native and cholesterol-treated \(\text{Sf9}\) membranes. The data are quantitatively consistent with a
model in which the receptor interconverts spontaneously between a state of high affinity for antagonists and low affinity for agonists and a state of low affinity for antagonists and high affinity for agonists. Whereas \textit{N}-ethylemaleimide shifts the equilibrium toward the state favored by agonists, cholesterol increases the affinity of all ligands for both states.

4.3 Materials and Methods

\textit{Ligands, detergents, and other materials}

\textit{(-)}-\textsuperscript{3}H\textit{Quinuclidinylbenzilate} (QNB) was purchased from Amersham Biosciences (batch B-49, 49 Ci/mmol; batch B-50, 48 Ci/mmol). Oxotremorine-M, carbamylcholine (carbachol) chloride, arecoline hydrobromide, pilocarpine hydrochloride, and \textit{N}-methylscopolamine hydrobromide were purchased from Sigma-Aldrich.

Sodium cholate was purchased from Sigma-Aldrich. Solubilized receptor was concentrated as required by means of Centricon-10 and Centriprep-30 filters (Amicon) purchased from Millipore. HEPES and Tris were obtained as the free bases from Boehringer Mannheim and Sigma-Aldrich (Trizma), respectively. EDTA and EGTA were obtained as the free acids from British Drug Houses or Bioshop Canada. \textit{N}-Ethylemaleimide (ultra grade), bacitracin, all protease inhibitors, and Sephadex G-50 were from Sigma-Aldrich. Protein was estimated by means of bicinchoninic acid using the BCA Protein Assay Kit from Pierce. Bovine serum albumin was taken as the standard.

\textit{Preparation of M\textsubscript{2} muscarinic receptor, addition of cholesterol, and treatment with \textit{N}-ethylemaleimide}

FLAG-tagged \textit{M\textsubscript{2}} muscarinic receptor was expressed in baculoviral-infected \textit{Sf9} cells, and the harvested membranes were stored at \textdegree 75 \textdegree C. Native membranes were supplemented with exogenous cholesterol by means of an inclusion complex with methyl-\textit{\beta}-cyclodextrin. Sarcolemmal membranes were prepared from porcine atria.
Further details concerning these and related procedures have been described previously [110,147,155,227,246].

Cholesterol was assayed using the Cholesterol/Cholesteryl Ester Quantitation Kit from BioVision Research Products. The level in untreated Sf9 membranes was about 7% of that in sarcolemmal membranes and increased to about 270% upon treatment of the membranes with the cholesterol-methyl-β-cyclodextrin inclusion complex. The amounts in absolute terms were as follows (mg of cholesterol per mg of protein): native Sf9 membranes, 0.07 ± 0.01 (N = 7); treated Sf9 membranes, 2.58 ± 0.33 (N = 5); porcine sarcolemmal membranes, 0.94 ± 0.20 (N = 3).

Receptor was solubilized from native and cholesterol-treated Sf9 membranes as described previously [147,227]. The extraction was performed in sodium cholate plus NaCl at final concentrations of 0.08% and 0.5 M, respectively. Solubilised material was divided into aliquots of 1–2 mL and stored at −75 °C until required.

For reaction with N-ethylmaleimide, aliquots of solubilized extract were thawed and supplemented with the reagent at a final concentration of 10 mM. Unless otherwise indicated, the reaction was carried out on ice for 2 h and 24 h with receptor from native and cholesterol-treated Sf9 membranes, respectively. Following the period of alkylation, aliquots containing the the treated receptor were introduced directly into the binding assays. A fresh solution of N-ethylmaleimide was prepared for each experiment, and controls lacking the reagent were prepared in an otherwise identical manner. Further details regarding the treatment with N-ethylmaleimide have been described previously [228].

**Binding assays**

All assays were performed on receptor extracted from Sf9 membranes. The radioligand and any unlabeled ligand were dissolved in Buffer A (20 mM HEPES, 20 mM NaCl, 1 mM EDTA, 5 mM MgSO₄, 0.1 mM PMSF, and NaOH to pH 7.40) supplemented with
cholate plus NaCl at a concentration of 0.1% and 20 mM, respectively. An aliquot of the ligand-containing solution (48 µL) was added to the solubilized receptor (5 µL), and the reaction mixture was incubated for 2 h at 30 °C. M2 receptor extracted from native and cholesterol-treated Sf9 membranes has been shown to equilibrate with [3H]quinuclidinylbenzilate and accompanying ligands under those conditions [227].

To terminate the reaction and separate the bound radioligand, an aliquot of the sample (50 µL) was applied to a column of Sephadex G-50 Fine (0.8 cm × 6.5 cm). The column was pre-equilibrated and eluted with Buffer B (20 mM HEPES, 20 mM NaCl, 1 mM EDTA, 5 mM MgSO4, and NaOH to pH 7.40) supplemented with cholate plus NaCl at a concentration of 0.017% and 20 mM, respectively. All of the eluant up to and including the void volume was collected (1.60 mL) and assayed for radioactivity.

Binding assays generally were carried out within one week of solubilization. Nonspecific binding was taken throughout as binding in the presence of 1 mM unlabeled N-methylscopolamine and increased linearly with the concentration of [3H]quinuclidinylbenzilate. The inhibitory effect of one or more of oxotremorine-M, carbachol, arecoline, and pilocarpine on the binding of [3H]quinuclidinylbenzilate was assayed in parallel with that of N-methylscopolamine, which was included throughout as a control.

Analysis of data

All data were analyzed with total binding taken as the dependent variable \(B_{\text{obsd}}\) and with the total concentrations of all ligands taken as the independent variables. Any subsequent manipulations were for the purpose of presentation only and did not alter the relationship between the data and the fitted curve. Levels of specific binding \(B_{\text{sp}}\), estimates of total receptor \([R]_t\), and estimates of maximal specific binding \(B_{\text{max}}\) are presented as the concentration in the binding assay (pM); similarly, the concentrations of ligands denote the total molar concentration in the binding assay.
For data acquired at graded concentrations of [3H]quinuclidinylbenzilate, the Hill equation was formulated as shown in Eq. (1).

\[
B_{\text{obsd}} = \frac{B_{\text{max}} (\left[ P \right]_t - B_{\text{sp}})_{n_H}}{K^n_H + (\left[ P \right]_t - B_{\text{sp}})_{n_H}} + \text{NS}(\left[ P \right]_t - B_{\text{sp}})
\]  

(1)

The variable [P]t represents the total concentration of the radioligand. The quantity B_sp represents specific binding at the corresponding value of [P]t, and the parameter B_max represents maximal specific binding. The parameter K is the concentration of unbound radioligand at half-maximal occupancy, and n_H is the corresponding Hill coefficient. The parameter NS represents the fraction of unbound radioligand that appears as nonspecific binding. Eq. (1) was solved numerically in the manner described previously [248]. For data on the binding of [3H]quinuclidinylbenzilate at graded concentrations of an unlabeled ligand, the Hill equation was formulated as shown in Eq. (2).

\[
B_{\text{obsd}} = (B_{[A]=0} - B_{[A] \rightarrow \infty}) \frac{K^n_H}{K^n_H + [A]_t} + B_{[A] \rightarrow \infty}
\]  

(2)

The variable [A]t is the total concentration of unlabeled ligand, and K is the value of [A]t at which the specific binding of the radioligand is reduced by 50%. The parameters B[A]=0 and B[A]→∞ represent the asymptotic levels of binding when [A]=0 and [A]→∞.

Mechanistic analyses were performed in terms of Schemes 1 and 3, in which an unlabeled ligand (A) and [3H]quinuclidinylbenzilate (P) compete for a population of mutually independent sites. In Scheme 1, the receptor exists in a single state to which ligands A and P bind with equilibrium dissociation constants KA and KP, respectively (e.g., KA = [A][R]/[AR], KP = [P][R]/[PR]). In Scheme 3, the receptor can exist in two spontaneously interconverting states designated R and R*. The ligands A and P bind to R with equilibrium dissociation constants KA and KP, respectively, and the relative affinity of the ligand for the two states is designated aA and aP (i.e., aA = K*A/KA, K*A = [A][R*]/[AR*]; aP = K*P/KP, K*P = [P][R*]/[PR*]). The parameter KR represents the
relative concentrations of R and R* at equilibrium in the absence of ligand (i.e., \([R]/[R^*] = KR\)).

Schemes 1 and 3 were fitted to the data by means of the equation \(B_{obsd} = B_{sp} + NS([P]_t - B_{sp})\), in which P represents the radioligand and other quantities are as described above for Eq. (1). The value of \(B_{sp}\) was computed according to Eq. (3) or Eq. (4), respectively.

\[
B_{sp} = [PR] \quad (3)
\]

\[
B_{sp} = [PR] + [PR^*] \quad (4)
\]

The values of \([PR]\) and of \([PR]+[PR^*]\) were calculated from the expansions in terms of the total concentration of R and the free concentrations of A and P. The latter were computed numerically from the corresponding implicit equations for \([A]_t\) and \([P]_t\). Further details concerning the formulation of Schemes 1 and 3 have been described elsewhere [228,248].

Separation on Sephadex G-50 yields low levels of nonspecific binding [147,227], which represented about 0.06% of unbound \([^3H]\)quinuclidinylbenzilate with all preparations used in the present investigation. At the concentrations of \([^3H]\)quinuclidinylbenzilate used to examine the inhibitory effect of other ligands (i.e., 15–27 nM), nonspecific binding never exceeded 8% of binding in the absence of the unlabeled ligand. Nonspecific binding in such experiments was taken as the fitted asymptote at saturating concentrations of the unlabeled ligand, which was poorly defined under some conditions with some ligands. A single value of NS therefore was common to data acquired in parallel, which included values at graded concentrations of \(N\)-methylscopolamine in addition to those obtained with one or more other ligands as described above.

The results of analyses involving multiple sets of data from replicated experiments have been presented in each case with reference to a single fitted curve. To obtain the values plotted on the y-axis, estimates of \(B_{obsd}\) were adjusted according to the expression
\[ B_{\text{obsd}}' = B_{\text{obsd}} \left\{ f(\bar{x}_i, \bar{a} / f(x_i, a)) \right\} \] \[81\]. The function \( f \) represents the fitted model, and the vectors \( x_i \) and \( a \) represent the independent variables at point \( i \) and the fitted parameters for the set of data under consideration; \( \bar{x}_i \) and \( \bar{a} \) are the corresponding vectors in which values that differ from experiment to experiment have been replaced by the means for all experiments associated with the fitted curve. Individual values of \( B_{\text{obsd}}' \) at the same \( x_i \) were averaged to obtain the mean and standard error plotted in the figure. Specific binding (\( B_{\text{sp}} \)) was calculated as \( B_{\text{obsd}}' \) less the fitted estimate of nonspecific binding at the same concentration of unbound radioligand.

**Statistical procedures**

All parameters were estimated by nonlinear regression. Affinities and equilibrium constants were optimized throughout on a logarithmic scale \([i.e., \log K_L, \log \alpha_L (L \equiv A \text{ or } P), \log K_R]\). In analyses of data from two or more experiments, the data from each experiment were assigned separate values of \([R]\) and NS. Other parameters were shared as described in the legends to the tables and figures.

Fits of nested models were compared by means of the \( F \)-statistic. Weighted residuals were of comparable magnitude within each set of data; in simultaneous analyses, individual sets of data generally made comparable contributions to the total weighted sum of squares. Mean values calculated from two or more individual estimates of a parameter or other quantity are presented together with the standard error. For parametric values derived from a single analysis of one or more sets of data, the errors were estimated from the diagonal elements of the covariance matrix.

Further details regarding the optimization of parameters, weighting of the data, tests for significance, and other statistical procedures have been described previously \([81,248]\).
4.4 Results

Reaction of the M2 receptor with N-ethylmaleimide

N-Ethylmaleimide reduced the apparent affinity of $[^3]H$quinuclidinylbenzilate and increased that of oxotremorine-M for M2 receptor extracted from native and cholesterol-treated Sf9 membranes. The time-dependence of the effect is illustrated in Fig. 4-1. With the receptor from native membranes, the value of $K$ for $[^3]H$quinuclidinylbenzilate (Eq. 1) increased to a maximum within the first hour of alkylation and then remained unchanged for up to 6 h (Fig. 4-1A). The effect was mirrored in the value of $K$ for oxotremorine-M (Eq. 2), which decreased to a minimum in a parallel manner (Fig. 4-1B). Essentially the same pattern was found with receptor from cholesterol-treated membranes, where a higher or lower value of $K$ was attained within 6 h and remained unchanged for at least 24 h.

Attainment of a plateau in the value of $K$ suggests that alkylation of the receptor is complete. In accord with this interpretation, the affinity of $[^3]H$quinuclidinylbenzilate for receptor extracted from native membranes was the same after incubation for 30 min with N-ethylmaleimide irrespective of whether or not an additional amount of the reagent was added to the reaction mixture after 15 min. It has been shown previously that the affinity of the alkylated receptor for $[^3]H$quinuclidinylbenzilate is the same regardless of whether or not unreacted N-ethylmaleimide is removed immediately prior to the binding assay [228].

Preparations of the M2 receptor extracted from native Sf9 membranes are known to undergo a time-dependent loss of sites during incubation at 30 °C, an effect that is avoided if the membranes are supplemented with cholesterol or if the preparation is maintained at 4 °C [227]. In previous studies, the reaction with N-ethylmaleimide was conducted in an ice bath for a period 24 h [228]. The receptor remains functionally viable under those conditions, and the same procedure was used for receptor from cholesterol-treated membranes in the present investigation. In the case of receptor from
native membranes, the time of alkylation was reduced to 2 h in order to avoid any possibility of thermal inactivation.

Effects of \( N \)-ethylmaleimide and cholesterol, assessed in terms of the Hill equation and Scheme 1

Treatment with cholesterol and \( N \)-ethylmaleimide led to four preparations of M2 receptor, each of which was characterized for the binding of \([^3H]\)quinuclidinylbenzilate and five unlabeled ligands to obtain the data illustrated in Figure 4-2. Receptor extracted from native Sf9 membranes appeared homogeneous with respect to all six ligands, as indicated by Hill coefficients near or indistinguishable from 1 (Table 4-1). The value of \( n_H \) underwent little or no change upon pretreatment of the membranes with cholesterol. Treatment with \( N \)-ethylmaleimide tended to decrease the value of \( n_H \), at least with receptor from native membranes, although the effect was comparatively small in most cases. The data for all ligands in all preparations were described to at least a first approximation when the value of \( n_H \) was taken as 1, and this constraint was implicit in subsequent analyses in terms of Schemes 1 and 3. The possible consequences of heterogeneity were considered in attempts to fit an expanded version of Scheme 3 that comprised multiple classes of receptor (i.e., Scheme 3 in Ref. 228), but the parameters tended to be highly correlated and poorly defined.

Cholesterol and \( N \)-ethylmaleimide affected the affinity of each ligand in a characteristic manner that can be quantified empirically in terms of Scheme 1, in which the receptor is assumed to exist in a single state. The data were analyzed in concert to obtain estimates of the 24 parameters that denote the affinity of each ligand for each preparation of receptor. The values are listed in Table 4-2, and the fitted curves are compared with the data in Figure 4-2. With receptor from native or cholesterol-treated membranes, treatment with \( N \)-ethylmaleimide increased the affinity of agonists and decreased that of antagonists. With native or \( N \)-ethylmaleimide-treated receptor, agonists and antagonists bound with higher affinity to receptor from cholesterol-treated
membranes than to that from native membranes. These effects suggest a pattern in which cholesterol increased affinity for all ligands whereas N-ethylmaleimide favored an agonist-specific state over an antagonist-specific state. Such an arrangement is not explicit in Scheme 1, and the data therefore were examined in terms of the two-state receptor depicted in Scheme 3.

Effects of N-ethylmaleimide and cholesterol, assessed in terms of Scheme 3

With six ligands and four preparations of receptor, an unconstrained application of Scheme 3 requires 48 parameters to describe the affinity for R ($K_L$) and the relative affinity for R and R* ($\alpha_L$) of each ligand in each preparation. Different preparations also may differ in the distribution of vacant receptors between R and R* ($K_R$), resulting in a total of 52 such parameters overall. A series of analyses therefore was performed in which the number of parameters was reduced to the minimum that was consistent with the data. The baseline for consistency was taken as a sum of squares equal or comparable to that obtained with Scheme 1, applied as described above (Fig. 4-2, Table 4-2).

Neither cholesterol nor N-ethylmaleimide appeared to affect the relative affinities for R and R*. In a test of all ligands taken together, there was no appreciable increase in the weighted sum of squares with a single value of $\alpha_L$ for each ligand rather than with separate values for each of the four preparations of receptor ($P > 0.1$). With that constraint, two distinct but equivalent scenarios emerged with respect to the effects of cholesterol and N-ethylmaleimide on $K_L$ and $K_R$. The parametric values are listed in Table 4-3, and in each case the fitted curves are virtually superimposable with those obtained with Scheme 1 and illustrated in Fig. 4-2.

In the first scenario, N-ethylmaleimide is without effect on affinity (i.e., $K_L$) but shifts the equilibrium between R and R* toward the latter (i.e., a decrease in $K_R$), while cholesterol is without effect on the equilibrium between R and R* but causes a decrease in the dissociation constant of each ligand for R (Table 4-3A). Since the values of $\alpha_L$ are the same in all preparations, cholesterol also causes a decrease in the dissociation...
constants for R* (i.e., $\alpha L K_L$). Thus, the data are well described with only two values of $K_R$: one for the native receptor and one for receptor treated with N-ethylmaleimide; similarly, the data require only two values of $K_L$ for each ligand: one for the receptor from native membranes and one for that from membranes supplemented with cholesterol. These constraints can be applied without an appreciable increase in the sum of squares over that from the wholly unconstrained analysis or that from the analysis with six values of $\alpha_L$ ($P \geq 0.08$). There is an increase of at least twofold upon the further assumption that N-ethylmaleimide is without effect on $K_R$ or that cholesterol is without effect on $K_L$ for any ligand ($P < 0.00001$).

In the second scenario, cholesterol is without effect on affinity (i.e., $K_L$) but shifts the equilibrium between R and R* (i.e., $K_R$), while N-ethylmaleimide increases the affinity of agonists and decreases that of antagonists without affecting the equilibrium between the two states (Table 4-3B). There accordingly are two values of $K_R$: one for receptor from native membranes and one for that from membranes supplemented with cholesterol; similarly, there are two values of $K_L$ for each ligand: one for the native receptor and one for receptor treated with N-ethylmaleimide. These constraints have little effect on the sum of squares ($P \geq 0.025$), whereas there is an increase of 1.6-fold or more upon the further assumption that cholesterol is without effect on $K_R$ or that N-ethylmaleimide is without effect on $K_L$ ($P < 0.00001$).

In either application of Scheme 3, the constraints permitted by the data reduced the total number of parameters corresponding to $K_L$, $\alpha_L$, and $K_R$ from 52 to 20. The minimum sum of squares was virtually the same irrespective of whether $K_R$ was deemed sensitive to N-ethylmaleimide (WSSQ = 127,200, dof = 1,517) or cholesterol (WSSQ = 127,000, dof = 1517), and the value was comparable to that obtained with Scheme 1 with 24 parameters corresponding to $K_P$ and $K_A$ (WSSQ = 125,500, dof = 1,513). Scheme 3 therefore provides a fit equivalent to that of Scheme 1 but requires four fewer parameters.

The two analyses summarized in Table 4-3 were performed with the values of log $K_R$ fixed at 3 and −1 with respect to treatment with N-ethylmaleimide or the addition of cholesterol. In the first case, the native receptor is almost wholly in the R state (log $K_R =
3), and the alkylated receptor is predominantly in the \( R^* \) state (\( \log K_R = -1 \)); in the second, the receptor from native membranes is wholly in the \( R \) state (\( \log K_R = 3 \)), and that from cholesterol-treated membranes is predominantly in the \( R^* \) state (\( \log K_R = -1 \)).

The need for such assignments was dictated by uncertainty over the absolute values of \( K_R \), which were highly correlated in each analysis. To determine the domains of acceptable values, the weighted sum of squares was mapped as described below with respect to the values of \( \log K_R \) for unreacted (\( \log K_R(-) \)) and alkylated (\( \log K_R(+) \)) receptors in the first case and for receptor from native (\( \log K_R(-) \)) and cholesterol-treated (\( \log K_R(+) \)) membranes in the second. The values of \( \log K_R \) selected for the analyses summarized in Table 4-3 lie within the domain defined by those combinations that yield the minimum in the sum of squares, and the fitted values of other parameters (i.e., \( \log K_L \) and \( \log \alpha_L \)) are the same for all combinations of \( \log K_R(-) \) and \( \log K_R(+) \) within that domain.

**Effect of N-ethylmaleimide on \( K_R \) in Scheme 3**

The dependence of the sum of squares on the value of \( \log K_R(-) \) is illustrated in Figs. 4-3A and 4-3B for analyses in which \( \log K_R(+) \) was fixed at \(-2 \) and \( 2 \), respectively. Both maps show a peak when \( K_R \) is assumed to be unaffected by \( N \)-ethylmaleimide: that is, when \( \log K_R(-) \) equals \( \log K_R(+) \). When \( \log K_R(-) \) exceeds \( \log K_R(+) \), the sum of squares decreases to a minimum of 127,200 when \( \log K_R(+) \) is \(-2 \) (Fig. 4-3A) and 129,300 when \( \log K_R(+) \) is \( 2 \) (Fig. 4-3B). When \( \log K_R(-) \) is less than \( \log K_R(+) \), the sum of squares decreases to a minimum that is markedly higher than that obtained when \( \log K_R(-) \) exceeds \( \log K_R(+) \).

The results for all combinations of \( \log K_R(-) \) and \( \log K_R(+) \) between \(-4 \) and \( 4 \) are illustrated in Fig. 4-4. Best fits of the model are obtained when \( \log K_R(-) \) is \( 2 \) or more and \( \log K_R(+) \) is \( 0 \) or less, as defined by the depression at the left of the figure (WSSQ = 127,200). At those values of \( K_R \), the receptors exist almost wholly in the \( R \) state before treatment with \( N \)-ethylmaleimide ([\( R \] > 100[\( R^* \)]) and predominantly in the \( R^* \) state thereafter ([\( R \] ≤ [\( R^* \)]). A marginally higher plateau emerges when \( \log K_R(+) \) exceeds \( 0 \) while remaining less than \( \log K_R(-) \) by 2 units or more (WSSQ = 129,200). Reasonable
agreement therefore requires only that N-ethylmaleimide effect a 100-fold increase in the fraction of receptors in the R* state, regardless of the magnitude of that fraction before or after alkylation.

Another plateau, located at the right of Fig. 4-4 (WSSQ = 156,300), corresponds to the opposite arrangement in which native receptors are predominately in the R* state and interconvert to the R state upon treatment with N-ethylmaleimide (i.e., \( \log K_{R(-)} \leq -1 \) and \( \log K_{R(+)} \geq 2 \)). The sum of squares exceeds that at the minimum by 23\% (\( P < 0.00001 \)), and the data therefore permit a clear distinction between the two possible effects of alkylation. Such a domain constitutes a false minimum that could be misidentified as the best fit in the absence of a map such as that presented in the figure.

Effect of cholesterol on \( K_R \) in Scheme 3

The dependence of the sum of squares on the value of \( \log K_{R(-)} \) is illustrated in Figs. 4-3C and 4-3D for analyses in which \( \log K_{R(+)} \) was fixed at \(-2\) and \(2\), respectively. The pattern is similar to that described above for the effect of N-ethylmaleimide, in that both maps show a peak when \( K_R \) is assumed to be unaffected by the addition of cholesterol (i.e., \( \log K_{R(-)} = \log K_{R(+)} \), WSSQ = 211,500). Unlike N-ethylmaleimide, however, cholesterol is found not to discriminate in its effect on \( K_R \); that is, the sum of squares is essentially the same regardless of whether the addition of cholesterol is assumed to favor R over R* (\( \log K_{R(-)} < \log K_{R(+)} \), WSSQ = 127,500) or R* over R (\( \log K_{R(-)} > \log K_{R(+)} \), WSSQ = 127,000).

The results for all combinations of \( \log K_{R(-)} \) and \( \log K_{R(+)} \) are illustrated in Fig. 4-5, where the two possible effects of cholesterol are seen to be nearly symmetrical. If cholesterol favors R, best fits are obtained at values of \( K_R \) within the domain defined by the depression at the right [i.e., \( \log K_{R(-)} \leq -1 \), and \( \log K_{R(+)} > \log (100K_{R(-)}) \)]. If cholesterol favors R*, best fits lie within the domain defined by the depression at the left [i.e., \( \log K_{R(-)} \geq 2 \), and \( \log K_{R(+)} < \log (K_{R(-)}/100) \)]. The fitted values of \( K_L \) and \( \alpha_L \) listed in Table 4-3B are for the situation in which cholesterol favors R* over R. Those listed in
Table 4-4 are for the alternative possibility in which cholesterol favors R over R*, and they are the same for all combinations of log $K_{R(-)}$ and log $K_{R(+)}/$R within the domain corresponding to the minimum. Whereas $N$-ethylmaleimide increases the affinity of agonists and decreases that of antagonists in both cases, the values of $K_L$ are smaller for all ligands when cholesterol is assumed to shift the equilibrium toward R (cf. Tables 4-3B and 4).

4.5 Discussion

Cholesterol is at least tenfold more abundant in sarcolemmal membranes from porcine atria than in membranes from $S/f$ cells, with consequences for the binding properties of the $M_2$ muscarinic cholinergic receptor [227]. Receptor extracted from sarcolemmal membranes in cholate–NaCl has been shown to exhibit a pattern of non-competitive effects wherein $N$-[3H]methylscopolamine labels only about one half of the sites labeled by [3H]quinuclidinylbenzilate, yet binding at near-saturating concentrations of the latter is inhibited fully at comparatively low concentrations of unlabeled $N$-methylscopolamine. In contrast, receptor extracted from native $S/f$ membranes exhibits the same capacity for $N$-[3H]methylscopolamine and [3H]quinuclidinylbenzilate, and the inhibition of the latter by unlabeled $N$-methylscopolamine is essentially competitive. Upon the enrichment of $S/f$ membranes with cholesterol, the solubilized receptor exhibits the difference in capacity and attendant non-competitive effects characteristic of that extracted from porcine atria. A quantitative analysis of those effects suggested that cholesterol is required for the manifestation of cooperativity in the binding of antagonists to at least four interacting sites, presumably within a tetramer or larger oligomer [227].

The propensity of a ligand to favor the active (R*) or the inactive form (R) of a GPCR derives from the difference in its affinity for the two states. Whereas agonists bind with higher affinity to R*, antagonists either are indifferent ($i.e.$, classical antagonists) or bind with higher affinity to R ($i.e.$, inverse agonists). A shift in the equilibrium toward R* therefore is expected to increase the apparent affinity of agonists
and to decrease that of inverse agonists; similarly, a shift toward R is expected to
decrease the affinity of agonists and to increase that of inverse agonists. Purified M₂
receptor devoid of G protein has been shown to interconvert between agonist- and
antagonist-specific states that resemble R and R*, suggesting that the process is intrinsic
to the receptor [228].

The effect of cholesterol on cooperativity in the binding of antagonists was
accompanied by a sixfold increase in the apparent affinity of [³H]quinuclidinylbenzilate
and a somewhat smaller, twofold increase in that of N-[³H]methylscopolamine [227].
Cholesterol also prevented a thermal inactivation that otherwise occurs with G protein-
coupled receptors in their active state, particularly in the absence of an orthosteric ligand
[e.g., 137,227,228,300]. Such effects suggest that cholesterol may act on the equilibrium
between R and R*, shifting the distribution of sites toward the former. To examine that
possibility, M₂ receptors extracted from native and cholesterol-treated Sf9 membranes
were compared with respect to their affinity for [³H]quinuclidinylbenzilate, N-
methylscopolamine and four muscarinic agonists. The effect of cholesterol was compared
with that of the thiol reagent N-ethylmaleimide, which is known to drive the equilibrium
toward R* and therefore provides a point of reference (Ref. [228], and references cited
therein).

An empirical analysis of the data in terms of Scheme 1 indicated that N-
ethylmaleimide increases the apparent affinity of agonists and decreases that of
agonists irrespective of cholesterol, whereas cholesterol increases the affinity of all
ligands irrespective of N-ethylmaleimide (Table 4-2). The effect of N-ethylmaleimide
agrees qualitatively with the notion that alkylation causes a net interconversion of
receptors from the inactive to the active state. Such an arrangement is consistent the
results obtained with Scheme 3 (Table 4-3A), which yields a fit equivalent to that of
Scheme 1 and constitutes an explicit description of the supposed equilibrium between the
two states. Thus, N-ethylmaleimide is seen to increase the value of $K_R$, thereby favoring
R* over R and hence the binding of agonists over that of antagonists, whereas cholesterol
increases the affinity per se of all ligands for both states (i.e., $K_L$ and $\alpha L K_L$).
In the case of Scheme 3, however, the analyses identified another region of parameter space in which the fit is virtually identical to that described above. In this second scenario, cholesterol is seen to affect the value of $K_R$, which can be either decreased (Table 4-3A) or increased (Table 4-4), while $N$-ethylmaleimide acts to increase the affinity of agonists and to decrease that of antagonists. This interpretation is not self-evident from the results in Table 4-2, and its emergence illustrates the limitations of an empirical description such as that provided by Scheme 1. The present data therefore are ambiguous when taken alone, but alkylation by $N$-ethylmaleimide is known from independent studies to cause a net interconversion of sites for $R$ to $R^*$ without affecting either $K_L$ or $a_L$ [e.g., 228]. It follows that the effect of cholesterol is to increase the affinity of agonists and antagonists for both states.

The conditions of the present experiments were selected to avoid behavior that cannot be accommodated by Scheme 3. Under some of the conditions described here, the M2 receptor exhibits a heterogeneity that is detected as a shortfall in its apparent capacity for $N$-[^3]H]methylscopolamine [147,148,227]. Assays with $N$-[^3]H]methylscopolamine also reveal a time-dependent loss of sites when the radioligand is incubated at 30 °C with receptor purified from porcine atria [228] or extracted from native S/9 membranes [227]. Such effects were avoided through the use of[^3]H]quinuclidinylbenzilate, which labeled all sites with the same or similar affinity (Fig. 4-2F) and has been shown to protect the receptor from thermal inactivation [227,228]. The latter effect can be attributed to the slow kinetics of binding encountered with quinuclidinylbenzilate and the reduced likelihood of finding the receptor in a labile, unliganded state. Scheme 3 therefore provided at least a first approximation of the system and served to distinguish the effect of cholesterol from the known effect of $N$-ethylmaleimide.

Lipids can affect the activity of membrane proteins through direct interactions or by influencing the biophysical properties of the surrounding membrane. The latter effects depend upon the composition of lipids within an annulus that forms around the protein; the former arise from the binding of so-called non-annular lipids at specific sites [307]. Both effects have been implicated in the influence of cholesterol on the ligand-binding
properties of membrane proteins, including G protein-coupled receptors, and related events [214]. The influence of cholesterol on aspects of G protein-mediated signaling has been linked to its concentration in liquid-ordered domains known as lipid rafts, which also are enriched in G protein-coupled receptors, G proteins, and effectors [308]. The colocalization of different constituents within such domains has implications for the assembly of specific complexes before and during signaling [308]. In the case of the β2-adrenergic receptor, localization has been associated with reduced mobility and hence reduced signaling through Gs [309,310].

Rhodopsin has been shown to undergo both direct and indirect regulation by cholesterol (Ref. [214], and references cited therein). A direct effect is suggested by the cholesterol-sensitive transfer of resonance energy between tryptophanyl residues of rhodopsin and the fluorescent sterol cholestatrienol, and it has been estimated that one molecule of cholesterol interacts with one molecule of rhodopsin. An indirect effect is suggested by the sensitivity of the conformational equilibrium between metarhodopsin I and metarhodopsin II to the level of cholesterol in the membrane; that is, increasing the amount of cholesterol shifts the equilibrium towards that of metarhodopsin I, which is the inactive photointermediate. This effect was attributed to a reduction in the free volume of the bilayer, since the formation of metarhodopsin II has been shown to be accompanied by volume expansion of the protein. Cholesterol also has been found to protect rhodopsin from thermal inactivation, and it therefore has been suggested to serve a functional role in stabilizing the protein during trafficking to the plasma membrane. A similar protective effect has been observed with the M2 muscarinic and other G protein-coupled receptors [227,269].

In the present investigation, the effects of cholesterol were monitored in mixed micellar solutions obtained by solubilizing the membranes in cholate–NaCl. Sodium cholate does not form a precipitable complex with cholesterol [311], in contrast to digitonin, and any influence of the membrane on the receptor presumably was lost upon solubilization. It therefore appears that the increased affinity of muscarinic ligands in extracts of cholesterol-treated membranes derives from a direct interaction between cholesterol and the receptor itself.
Non-annular lipids affect the activity of membrane-embedded proteins in the manner of cofactors, and it has been suggested that they are located between transmembrane α-helices or at protein-protein interfaces [307]. In several cases, they have been shown to co-purify with the protein and have been resolved in high-resolution structures of proteins or protein complexes such as cytochrome c oxidase, the potassium channel, bacteriorhodopsin, the β2-adrenergic receptor, and the A2A adenosine receptor [307 and references cited therein].

Early evidence for the existence of non-annular sites came from studies on the effects of cholesterol on the Ca\(^{2+}\)-ATPase of sarcoplasmic reticulum [223]. The sterol increased enzymatic activity without affecting the thickness of the lipid bilayer and therefore appeared to act directly on the protein [312]. Cholesterol also is required for the functioning of the nicotinic acetylcholine receptor, and five to ten non-annular binding sites have been suggested to reside in the interstices of the five receptor subunits [313]. There was no correlation between the effect of cholesterol on the action of the ion channel and changes in membrane fluidity, again pointing to a direct interaction with the protein [314,315].

Molecular modeling studies of the oxytocin receptor have suggested a site of action for cholesterol on the surface of transmembrane segments 5 and 6 [276]. A recent crystal structure of the β2-adrenergic receptor bearing T4 lysozyme in place of the third intracellular loop has revealed a parallel dimer; at the interface between the two protomers, six molecules of cholesterol and two covalently-bound molecules of palmitic acid form a two-fold symmetric sheet [71]. The functional significance of that cholesterol remains unclear, however, owing in part to uncertainty over the physiological significance of the dimer itself. A later structure of the same construct shows the receptor as an anti-parallel dimer. No cholesterol appears at the interface, but each monomer of the receptor binds two molecules of cholesterol within a pocket formed by transmembrane helices 1–4 [316].

The general increase in affinity brought about by cholesterol in the present investigation recalls a similar effect described previously for other G protein-coupled
receptors (Ref. 227, and references cited therein). Those earlier studies were carried out on receptors in membrane fragments, and a contribution from annular lipids therefore cannot be ruled out; nonetheless, it seems likely that the effect derived at least in part from structured cholesterol of the sort identified in recent crystallographic studies. Thus, cholesterol has been shown to increase the affinity of antagonists for membrane-bound oxytocin, µ-opioid, and 5-HT1A receptors. Similarly, agonists bound with weaker affinity to the oxytocin, galanin, and cholecystokinin receptors when cholesterol was removed from the plasma membranes of uterine myometrium and Chinese hamster ovary cells, respectively; high affinity-binding was re-established when cholesterol was restored to the depleted membranes. The agonist [3H]DAMGO bound with higher affinity to the µ-opioid receptor in the plasma membrane of yeast upon the removal of ergosterol or the addition of cholesterol. In the case of the µ-opioid and oxytocin receptors, the increase in affinity for agonists was shown to be independent of the G protein.

Whereas cholesterol acts to enhance stability and increase affinity through a direct interaction with the receptor, at least under the conditions described here, there is no effect on correlates of efficacy. The increase in affinity in terms of Scheme 3 was similar in magnitude for agonists and antagonists (Table 4-3A). Also, the effect on agonists was independent of endogenous G proteins: membranes from Sf9 cells expressing the M2 receptor exhibit no agonist-stimulated binding of [35S]GTPγS [317,318], and they are immunonegative to antibodies that recognize αi1, αi2, αi3, and αo [317]. Lack of signaling through the M2 receptor contrasts with the activity of the M1, M3, and M5 receptors, which have been shown to increase the levels of inositol-1,4,5-trisphosphate and intracellular calcium when expressed in Sf9 cells [319,320]. Finally, the contrast with N-ethylmaleimide indicates that the nature of the effect achieved by cholesterol differs from that of an agent known to perturb the distribution of receptors between the active and inactive states. If the direct interaction of cholesterol with the receptor is a determinant of activity, perhaps through the facilitation of cooperative effects [227], it involves properties that have not been measured or affected in the present investigation.
4.6 Acknowledgements

We thank Rabindra V. Shivnaraine for helpful discussions during the preparation of the manuscript. This investigation was supported by the Canadian Institutes of Health Research (MOP43990) and the Heart and Stroke Foundation of Ontario (T5650, T6280). A. T. C. was the recipient of an Ontario Graduate Scholarship.
### Table 4-1 – Empirical characterization of specific binding in terms of the Hill equation

<table>
<thead>
<tr>
<th>Ligand</th>
<th>No N-ethylmaleimide</th>
<th>Treated with N-ethylmaleimide</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Native membranes</td>
<td>CHL-treated membranes</td>
</tr>
<tr>
<td>Oxo-M (3,3,3,3)</td>
<td>$-3.75 \pm 0.04 (1.34 \pm 0.14)$</td>
<td>$-2.96 \pm 0.04 (0.74 \pm 0.04)$</td>
</tr>
<tr>
<td>Carbachol (3,3,3,3)</td>
<td>$-2.37 \pm 0.07 (1.07 \pm 0.17)$</td>
<td>$-2.36 \pm 0.03 (0.84 \pm 0.05)$</td>
</tr>
<tr>
<td>Arecoline (3,3,3,3)</td>
<td>$-2.87 \pm 0.12 (0.98 \pm 0.23)$</td>
<td>$-2.89 \pm 0.24 (0.99 \pm 0.49)$</td>
</tr>
<tr>
<td>Pilocarpine (3,3,4,4)</td>
<td>$-2.59 \pm 0.10 (1.23 \pm 0.30)$</td>
<td>$-2.63 \pm 0.05 (1.83 \pm 0.31)$</td>
</tr>
<tr>
<td>NMS (10,10,8,8)</td>
<td>$-6.01 \pm 0.04 (1.09 \pm 0.09)$</td>
<td>$-5.89 \pm 0.03 (0.77 \pm 0.03)$</td>
</tr>
<tr>
<td>$[^3]$H]QNB (5,5,3,3)</td>
<td>$-8.54 \pm 0.05 (0.90 \pm 0.05)$</td>
<td>$-9.51 \pm 0.03 (1.05 \pm 0.06)$</td>
</tr>
</tbody>
</table>

M₂ receptor was extracted from native and CHL-treated S9 membranes and reacted with NEM as required. Binding of $[^3]$H]QNB was measured at graded concentrations of the radioligand or at graded concentrations of an unlabeled ligand (oxotremorine-M, carbachol, arecoline, pilocarpine, N-methylscopolamine) and a single concentration of the radioligand (15–27 nM). The number of independent experiments for each ligand is shown in parentheses, with a value for each of the four preparations. The data were analyzed in terms of Eq. (1) or (2) to obtain fitted estimates of log $K$ and $n_H$ for each experiment, and the individual values were averaged to obtain the means ($\pm$ S.E.M.) listed in the table. The data are illustrated in Fig. 4-2.
Table 4-2 – Affinities of agonists and antagonists, estimated in terms of Scheme 1

<table>
<thead>
<tr>
<th>Ligand</th>
<th>log ( K_L )</th>
<th>( \text{No } N\text{-ethylmaleimide} )</th>
<th>( \text{Treated with } N\text{-ethylmaleimide} )</th>
<th>( \text{Native membranes} )</th>
<th>( \text{CHL-treated membranes} )</th>
<th>( \text{Native membranes} )</th>
<th>( \text{CHL-treated membranes} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oxotremorine-M</td>
<td></td>
<td>(-4.80 \pm 0.03 ) (3)</td>
<td>(-5.36 \pm 0.10 ) (3)</td>
<td>(-6.47 \pm 0.19 ) (3)</td>
<td>(-4.96 \pm 0.04 ) (3)</td>
<td>(-4.66 \pm 0.10 ) (3)</td>
<td>(-5.60 \pm 0.15 ) (3)</td>
</tr>
<tr>
<td>Carbachol</td>
<td></td>
<td>(-3.37 \pm 0.04 ) (3)</td>
<td>(-4.67 \pm 0.09 ) (3)</td>
<td>(-4.66 \pm 0.10 ) (3)</td>
<td>(-4.39 \pm 0.04 ) (3)</td>
<td>(-5.82 \pm 0.18 ) (3)</td>
<td>(-5.60 \pm 0.15 ) (3)</td>
</tr>
<tr>
<td>Arecoline</td>
<td></td>
<td>(-3.74 \pm 0.04 ) (3)</td>
<td>(-4.58 \pm 0.12 ) (4)</td>
<td>(-4.58 \pm 0.12 ) (4)</td>
<td>(-4.82 \pm 0.10 ) (3)</td>
<td>(-5.37 \pm 0.19 ) (4)</td>
<td>(-5.82 \pm 0.18 ) (3)</td>
</tr>
<tr>
<td>Pilocarpine</td>
<td></td>
<td>(-3.54 \pm 0.04 ) (3)</td>
<td>(-6.72 \pm 0.09 ) (8)</td>
<td>(-6.72 \pm 0.09 ) (8)</td>
<td>(-4.54 \pm 0.07 ) (3)</td>
<td>(-7.53 \pm 0.12 ) (8)</td>
<td>(-5.37 \pm 0.19 ) (4)</td>
</tr>
<tr>
<td>( N\text{-Methylscopolamine} )</td>
<td></td>
<td>(-7.05 \pm 0.02 ) (10)</td>
<td></td>
<td>(-7.53 \pm 0.12 ) (8)</td>
<td>(-7.81 \pm 0.04 ) (10)</td>
<td></td>
<td>(-7.53 \pm 0.12 ) (8)</td>
</tr>
<tr>
<td>([\text{H}]\text{Quinuclidinylbenzilate} )</td>
<td>(-8.60 \pm 0.02 ) (5)</td>
<td>(-8.34 \pm 0.09 ) (3)</td>
<td>(-9.29 \pm 0.06 ) (3)</td>
<td>(-9.57 \pm 0.03 ) (5)</td>
<td>(-8.40 \pm 0.10 ) (3)</td>
<td>(-9.29 \pm 0.06 ) (3)</td>
<td></td>
</tr>
</tbody>
</table>

The data illustrated in all panels of Fig. 4-2 were pooled and analyzed in concert according to Scheme 1 to obtain the value of \( \log K_L \) (\( L \equiv P \) or \( A \)) for each ligand in each preparation of receptor. The number of independent experiments is shown in parentheses. Data obtained in all experiments on the same preparation of receptor were assigned a single value of \( \log K_P \) for \([\text{H}]\text{QNB} \) (\( L \equiv P \)) and a single value of \( K_A \) for each unlabeled ligand (\( L \equiv A \)), and the fitted values are listed in the table. Most experiments involved the characterization of at least two ligands, and assays were performed on aliquots from the same batch of receptor. All data from the same experiment therefore were assigned single values of \([R]_t\) and NS.
### Table 4-3 – Affinities of agonists and antagonists, estimated in terms of Scheme 3

<table>
<thead>
<tr>
<th>Ligand</th>
<th>( \log \alpha_L ) (±CHL, ±NEM)</th>
<th>( \log K_L ) (±NEM)</th>
<th>( \Delta \log K_L )</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>A.</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>K_R</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Affected</td>
<td>by NEM; ( K_L ) affected by CHL</td>
<td></td>
<td></td>
</tr>
<tr>
<td>( \log K_R ) (−NEM) = 3.0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>( \log K_R ) (+NEM) = −1.0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Carbachol</strong></td>
<td>−0.61 ± 0.08</td>
<td>−4.79 ± 0.03</td>
<td>−4.99 ± 0.04</td>
</tr>
<tr>
<td><strong>Arecoline</strong></td>
<td>−1.25 ± 0.08</td>
<td>−3.37 ± 0.04</td>
<td>−4.40 ± 0.04</td>
</tr>
<tr>
<td><strong>Pilocarpine</strong></td>
<td>−0.91 ± 0.07</td>
<td>−3.73 ± 0.04</td>
<td>−4.84 ± 0.09</td>
</tr>
<tr>
<td><strong>NMS</strong></td>
<td>0.46 ± 0.08</td>
<td>−7.05 ± 0.02</td>
<td>−7.82 ± 0.04</td>
</tr>
<tr>
<td>( [^3]H )QNB</td>
<td>0.40 ± 0.07</td>
<td>−8.61 ± 0.02</td>
<td>−9.58 ± 0.03</td>
</tr>
<tr>
<td><strong>B.</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>K_R</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Affected</td>
<td>by CHL; ( K_L ) affected by NEM</td>
<td></td>
<td></td>
</tr>
<tr>
<td>( \log K_R ) (−CHL) = 3.0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>( \log K_R ) (+CHL) = −1.0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Carbachol</strong></td>
<td>−0.97 ± 0.06</td>
<td>−3.39 ± 0.04</td>
<td>−4.68 ± 0.07</td>
</tr>
<tr>
<td><strong>Arecoline</strong></td>
<td>−1.06 ± 0.10</td>
<td>−3.75 ± 0.04</td>
<td>−4.71 ± 0.07</td>
</tr>
<tr>
<td><strong>Pilocarpine</strong></td>
<td>−0.94 ± 0.08</td>
<td>−3.55 ± 0.04</td>
<td>−4.57 ± 0.09</td>
</tr>
<tr>
<td><strong>NMS</strong></td>
<td>−0.70 ± 0.04</td>
<td>−7.07 ± 0.02</td>
<td>−6.77 ± 0.06</td>
</tr>
<tr>
<td>( [^3]H )QNB</td>
<td>−0.92 ± 0.04</td>
<td>−8.62 ± 0.02</td>
<td>−8.38 ± 0.06</td>
</tr>
</tbody>
</table>

The data illustrated in all panels of Fig. 4-2 were pooled and analyzed in concert according to Scheme 3. A total of 102 sets of data were obtained with the six ligands, and the number of independent experiments per ligand is shown in Table 4-2. Two analyses were performed, and the fitted curves in each case are virtually superimposable with those illustrated in Fig. 4-2. In the first analysis (A), the value of \( \log K_R \) was set at 3.0 and −1.0 for all data acquired with unreacted receptor and NEM-treated receptor, respectively; single values of \( \log K_L \) were assigned to the data obtained with the same ligand in all experiments performed on receptor from native membranes on the one hand and from CHL-treated membranes on the other, irrespective of NEM. In the second analysis (B), the value of \( \log K_R \) was set at 3.0 and −1.0 for all data acquired with receptor from native and cholesterol-treated membranes, respectively; single values of \( \log K_L \) were assigned to the data obtained with the same ligand in all experiments performed on unreacted receptor on the one hand and on NEM-treated receptor, irrespective of cholesterol. In both analyses, single values of \( \log \alpha_L \) were common to all of the data relevant to each ligand, irrespective of NEM and cholesterol. The radioligand was present throughout, and the values of \( \log K_L \) and \( \log \alpha_L \) shown for \( [^3]H \)QNB were common to all of the data. Single values of \( [R]_L \) and NS were assigned to all data acquired in the same experiment. The weighted sum of squares was 127,200 in (A) and 127,000 in (B).
Table 4-4 – Affinities of agonists and antagonists, estimated in terms of Scheme 3

<table>
<thead>
<tr>
<th>Ligand</th>
<th>( \log \alpha_L ) (±CHL, ±NEM)</th>
<th>( \log K_R ) (±CHL)</th>
<th>( \log K_L ) (±CHL)</th>
<th>( \Delta \log K_L )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oxo-M</td>
<td>0.25 ± 0.05</td>
<td>-5.02 ± 0.04</td>
<td>-5.66 ± 0.08</td>
<td>-0.64</td>
</tr>
<tr>
<td>Carbachol</td>
<td>1.09 ± 0.06</td>
<td>-4.45 ± 0.04</td>
<td>-5.71 ± 0.08</td>
<td>-1.26</td>
</tr>
<tr>
<td>Arecoline</td>
<td>1.18 ± 0.10</td>
<td>-4.90 ± 0.09</td>
<td>-5.82 ± 0.11</td>
<td>-0.92</td>
</tr>
<tr>
<td>Pilocarpine</td>
<td>1.05 ± 0.08</td>
<td>-4.58 ± 0.07</td>
<td>-5.55 ± 0.11</td>
<td>-0.97</td>
</tr>
<tr>
<td>NMS</td>
<td>0.83 ± 0.04</td>
<td>-7.87 ± 0.04</td>
<td>-7.53 ± 0.06</td>
<td>0.34</td>
</tr>
<tr>
<td>[^3\text{H}\text{QNB}])</td>
<td>1.03 ± 0.04</td>
<td>-9.63 ± 0.03</td>
<td>-9.34 ± 0.05</td>
<td>0.29</td>
</tr>
</tbody>
</table>

The data illustrated in all panels of Fig. 4-2 were pooled and analyzed according to Scheme 3. The parameters were assigned as described for the analysis represented in Table 4-3B, and the two calculations differed only in the values of \( \log K_R \) for the receptor from native and cholesterol-treated membranes. The weighted sum of squares was 127,500.
Fig. 4-1 – Effect of N-ethylmaleimide on the apparent affinity of oxotremorine-M and [³H]quinuclidinylbenzilate for M₂ receptor extracted from native and cholesterol-treated Sf9 membranes. Extracts from native (●) and cholesterol-treated (○) membranes were reacted with NEM (10 mM) for the times shown on the abscissa. Total binding to untreated (t = 0) and NEM-treated samples then was measured at graded concentrations of [³H]QNB (A) or at a near-saturating concentration of [³H]QNB (21–27 nM) and graded concentrations of oxotremorine-M (B). The data were analyzed in terms of Eq. (1) or Eq. (2) to obtain the individual values of $K$ and $n_H$ for each experiment, and the mean values of log $K$ from 1–5 experiments at each time are plotted in the figure (A, $EC_{50}$; B, $IC_{50}$). The data obtained with each ligand and each preparation of receptor were analyzed in concert to test for the significance of any time-dependent effect of NEM. In each case, there is no appreciable increase in the sum of squares with single values of log $K$ and $n_H$ for all of the data rather than with a separate value of each parameter for the 1–5 sets of data obtained after each time of incubation ($P ≥ 0.27$).
Fig. 4-2 – Effect of cholesterol and N-ethylmaleimide on the binding of agonists and antagonists to M₂ receptor extracted from Sf9 membranes. Receptor from native (□, ■) and cholesterol-treated membranes (○, ●) was characterized without further treatment (□, ○) and after reaction with NEM (■, ●). (A–E) Total binding was measured at a single concentration of [³H]QNB (15–27 nM) and graded concentrations of the unlabeled ligand identified on the abscissa. Points shown at the lower end of the abscissa indicate binding in the absence of unlabeled ligand. (F) Total binding was measured at graded concentrations of [³H]QNB either alone (upper curves) or in the presence of 1 mM unlabeled NMS (baseline). The lines indicate the best fit of Scheme 1 to the data represented in all panels taken together, and the parametric values are listed in Table 4-2. Further details regarding the analysis are given in in the legend to Table 4-2.
Fig. 4-3 – Fit of Scheme 3 mapped with respect to $K_R$ as affected by N-ethylmaleimide and cholesterol. The data represented in all panels of Fig. 4-2 were pooled and analyzed according to Scheme 3 to obtain the weighted sum of squares plotted on the ordinate. Parameters were assigned as described in the legend to Table 4-3, with the value of log $K_R$ taken as follows for the two scenarios with respect to NEM and cholesterol. (A, B) The value log $K_R$ was fixed at $-2$ (A) or $+2$ (B) for NEM-treated receptor, and the corresponding value for unreacted receptor was fixed as shown on the abscissa (cf. Table 4-3A). (C, D) The value of log $K_R$ was fixed at $-2$ (C) or $+2$ (D) for receptor from cholesterol-treated membranes, and the corresponding value for receptor from native membranes was fixed as shown on the abscissa (cf. Table 4-3B).
Fig. 4-4 – Domain of acceptable parametric values for an effect of $N$-ethylmaleimide on $K_R$ in Scheme 3. Analyses such as those represented in Figs. 4-3A and 4-3B were conducted with the values of log $K_R$ taken as shown for data acquired with unreacted receptor (−NEM) and with receptor treated with $N$-ethylmaleimide (+NEM). The weighted sum of squares from each analysis is plotted on the $z$-axis. The parametric values listed in Table 4-3A are from the analysis in which log $K_R$ was fixed at 3 (−NEM) and −1 (+NEM).
Fig. 4-5 – Domain of acceptable parametric values for an effect of cholesterol on $K_R$ in Scheme 3. Analyses such as those represented in Figs. 4-3C and 4-3D were conducted with the values of log $K_R$ taken as shown for data acquired with receptor from native (−CHL) and cholesterol-treated membranes (+CHL). The weighted sum of squares from each analysis is plotted on the z-axis. The parametric values listed in Table 4-3B are from the analysis in which log $K_R$ was fixed at 3 (−CHL) and −1 (+CHL); those listed in Table 4-4 are from the analysis in which log $K_R$ was fixed at −3 (−CHL) and 1 (+CHL).
CHAPTER 5

GENERAL DISCUSSION
5.1 Insights from the Thesis

The mechanistic basis of GPCR-mediated signaling is encoded in the nucleotide-modulated dispersion of affinities revealed in the binding of agonists. Quantitative measures of such dispersions previously have been shown to correlate with efficacy or intrinsic activity \([86,90,91,321]\), but the underlying cause remains unclear. The binding patterns obtained for agonists at most G protein-coupled receptors are inherently ambiguous in that data typically are acquired at a single concentration of the radioligand, and there is insufficient information to distinguish among mechanistic schemes that predict a dispersion of affinities for a system at thermodynamic equilibrium.

The dispersion could arise in at least three ways, as follows: first, two or more classes of distinct, non-interconverting, and mutually independent sites may differ in their affinity for the ligand (multi-site model); second, intrinsically identical sites may exist in two or more interconverting states arising from transient association with other, stoichiometrically limiting proteins within the membrane (ternary complex model); and third, successive equivalents of the ligand may bind to interacting sites in a cooperative manner (cooperativity).

It previously has been shown that schemes based on the first two scenarios cannot describe radioligand-binding data from G protein-mediated systems in a mechanistically consistent manner and therefore are not tenable as a mechanistic basis for signaling \([83,85,86,125]\). When formulated mathematically, those schemes are described by rational functions only if one can disregard the equations of state for all interacting species except the receptor; that is, binding to the receptor should not deplete the free concentration of one or more ligands \([248]\). At odds with this restriction is the requirement, in schemes based on the ternary complex model, that the G protein must be limiting for the model to give rise to a dispersion of affinities in the binding of agonists \([85]\). Moreover, there is an important distinction between the binding patterns that arise from the multi-site and ternary complex models. For a mixture of independent and dissimilar sites, points of inflection measured on the abscissa of a semilogarithmic plot can be related directly to the equilibrium dissociation constants of the ligand for the
different classes of sites. With the ternary model, however, increases in the concentration of agonist are accompanied by changes in the concentration of uncoupled G protein; accordingly, the apparent affinity of an agonist varies with its concentration [85]. A consequence of such changes is that at no point is there a simple relationship between the concentration of the drug and its equilibrium dissociation constant for uncoupled and G-protein coupled receptor.

Although the multi-site model is not tenable as a mechanistic basis for signaling, it does not suffer from the complications found with the ternary complex model which arise from the mutual depletion of receptor and G protein. It therefore can be applied empirically when the aim is simply to draw a curve through a series of experimentally acquired points [248]. In so doing, it provides a good first approximation of the data, and the fit may resemble that of the elusive true model. The multi-site model and models based on cooperativity share the same functional form in that they are both rational functions. They accordingly are indistinguishable under certain conditions. For example, a sum of hyperbolic terms can describe both the multi-site model and negative cooperativity when there is only one independent variable. Moreover, the latter is indistinguishable from the special case of the former in which the capacity of each subclass is some multiple of a basic unit. The data are ambiguous, but the choice is limited when the number of interacting sites equals 2; the possibilities increase as the oligomer becomes more complex. For instance, a tetramer would be indistinguishable from 1, 2, 3 or 4 classes of mutually independent sites, depending upon the degree to which each equivalent of ligand affected the affinity of the next. The ambiguity can be avoided by increasing the number of independent variables [87]. In binding studies, a convenient and informative procedure is to characterize the inhibitory behavior of an unlabeled ligand at two or more concentrations of the radiolabeled probe; the data then can be analyzed in concert to distinguish between competing mechanistic proposals that otherwise are equivalent.

G protein-coupled receptors are membrane proteins that transduce signals across biological membranes, and it therefore seems likely that their local environment influences the manner by which they operate. Cholesterol is well known to be an integral
part of cellular membranes, particularly those of eukaryotes where it previously has been shown to exert its influence on the functioning of many membrane proteins both directly and indirectly [307]. Its physicochemical effects have been well characterized for many membrane systems. In recent years, it has been identified as a key component of membrane microdomains such as caveolae and lipid rafts, both of which have been suggested to serve as platforms for various signaling systems [215, 322, 323]. In the present investigation, cholesterol has been identified as a determinant of cooperativity in that the M₂ muscarinic cholinergic receptor was found to exhibit non-competitive effects in the binding of muscarinic antagonists in the presence of the sterol.

Cooperativity implies the existence of oligomers, and questions regarding the role of those structures in signal transduction relate in part to the size of the basic functional unit and to its structural integrity during signaling. The ability of cholesterol to elicit non-competitive effects from the M₂ receptor raises the question of whether cholesterol does so by facilitating the formation of oligomers or by modulating the cooperative properties within already formed complexes.

5.2 Cooperativity

In the present investigation, M₂ muscarinic receptor from native membranes of Sf9 cells bound muscarinic antagonists in an essentially competitive manner that could be approximated by Scheme 1. The apparent difference in capacity for [³H]NMS and [³H]QNB at 30 °C can be attributed to thermal instability and was avoided when the assays were performed at 4 °C. When the data are analyzed simultaneously and mechanistic consistency is enforced in terms of Scheme 1, there are no discrepancies in the affinity of NMS as estimated from data obtained at graded concentrations of [³H]NMS and from those obtained at graded concentrations of unlabeled NMS at near-saturating and sub-saturating concentrations of [³H]QNB. The competitive behavior that is exhibited by M₂ receptor from native Sf9 membranes is in contrast to that previously observed with M₂ receptor extracted from sarcolemmal membranes of porcine atria [147].
The difference in the ligand-binding properties of the M\(_2\) receptor from the two preparations can be attributed to the level of cholesterol that is present in the membrane prior to extraction and solubilization of the protein in cholate-NaCl.

When native Sf9 membranes were supplemented with cholesterol, receptors from those preparations resembled receptors from porcine atria; that is, \([^3\text{H}]\text{NMS}\) labeled only a little more than one half of the sites labeled by \([^3\text{H}]\text{QNB}\), yet comparatively low concentrations of unlabeled NMS inhibited the binding of \([^3\text{H}]\text{QNB}\) at all of the sites. NMS therefore appears to be inhibitory at sites to which it does not bind, a non-competitive effect that emerges as discrepancies in the affinity of NMS in terms of Scheme 1. Moreover, unlike receptors from native Sf9 membranes, those from cholesterol-treated membranes are thermally stable and the non-competitive effects occur at both 30 °C and 4 °C. The inability of Scheme 1 to provide a consistent account of the combined data from either temperature cannot be overcome by increasing the number of independent sites, which indicates that the model is inadequate at any level of complexity. With six or more interacting sites in terms of Scheme 2, the model returns a fit that is equivalent, according to the global sum of squares, to that obtained from an unconstrained application of Scheme 1 which therefore suggests that models based on cooperativity are tenable.

When sarcolemmal membranes from porcine atria were depleted of cholesterol, the receptors resembled those in extracts from native Sf9 membranes in that \([^3\text{H}]\text{NMS}\) labeled essentially the same number of sites as did \([^3\text{H}]\text{QNB}\). Nevertheless, there was a relatively small fraction of sites (25%, Table 3-2) at which the affinity of \([^3\text{H}]\text{NMS}\) was anomalously weak; also, the affinities estimated from assays at graded concentrations of \([^3\text{H}]\text{NMS}\) differed significantly from those inferred from the inhibitory effect of the unlabeled analogue on \([^3\text{H}]\text{QNB}\), suggesting that the receptor remains capable of cooperative interactions. The discrepancies are smaller, however, than those found with receptor extracted from cholesterol-treated Sf9 membranes or from native sarcolemmal membranes of porcine atria [147]. The non-competitive component of the overall interaction therefore appears to be small relative to the competitive contribution. Small discrepancies in the affinity of NMS also were observed with receptors from native Sf9
membranes, which also exhibited no shortfall in capacity when the binding assays were performed at 4 °C.

It previously has been shown that the ligand-binding properties of the M₂ receptor differ in different detergents [147]. For example, when M₂ receptor from porcine atria is extracted in digitonin-cholate, binding of muscarinic antagonists is competitive. In contrast, when the receptor is extracted in cholate-NaCl, it exhibits non-competitive effects in the binding of ligands. Digitonin is a plant glycoalkaloid saponin which is known to form water-insoluble, 1:1 complexes with cholesterol [278]. It has been shown that although treatment of membranes with digitonin does not physically deplete the membrane of cholesterol, it does effectively reduce cholesterol-receptor interactions [324,325]. This suggests that receptors solubilized in digitonin-cholate are largely devoid of cholesterol relative to those solubilized in cholate-NaCl, which may account for the differences in their ligand-binding properties. In the present investigation, receptor from native and cholesterol-treated Sf9 membranes, and receptor from native and cholesterol-depleted sarcolemmal membranes of porcine atria all were extracted in cholate-NaCl. The manifestation of cooperativity therefore appears to depend upon the presence of cholesterol in the membranes of both preparations prior to the extraction and solubilization of the receptor into detergent.

Oligomers of the M₂ receptor do not appear to be induced during or after extraction in cholate-NaCl, nor can their cooperative properties be attributed to factors other than cholesterol. Receptors from native and cholesterol-treated Sf9 membranes were extracted and assayed in the same buffers and exhibited the same electrophoretic mobility, either before or after cross-linking, yet only receptor from treated membranes exhibited marked non-competitive behavior. Moreover, the effects of cholesterol were monitored in mixed micellar solutions obtained by solubilization of the lipid bilayer. It therefore seems likely that the non-competitive behavior found in extracts of cholesterol-treated Sf9 membranes derives from cholesterol acting directly on the receptor rather than indirectly via the membrane. The manifestation of cooperativity therefore appears to arise from a specific effect of the sterol on already formed oligomeric complexes.
G protein-coupled receptors are known to exist in multiple states. It has been shown that the binding of agonists to the cardiac muscarinic receptor can be described in terms of an oligomer that interconverts spontaneously, and in a guanyl nucleotide-sensitive manner, between two states that differ in their cooperative properties [81]. In the present investigation, the manner by which cholesterol modulates the manifestation of cooperativity in oligomers of the M2 receptor has been examined in a context similar to that adopted for guanyl nucleotides; that is, cholesterol may perturb the known conformational equilibrium of the receptor between R and R* by driving the system towards R, which generally is accepted to be the inactive state of the receptor. The effect of cholesterol was compared with that of NEM, a sulfhydryl reagent that is known to drive the receptor towards the active state (R*) and therefore served to calibrate the system. The effects of cholesterol and NEM on conformational equilibria were monitored through the ligand-binding preferences of the M2 receptor for agonists and antagonists.

In terms of Scheme 1, agonists bound more tightly to receptors treated with NEM than to untreated receptors; antagonist exhibited the opposite preference in that they bound more tightly to unalkylated receptors than to those treated with NEM. In contrast, both agonists and antagonists bound more tightly to receptors from cholesterol-treated Sf9 membranes than to receptors from native membranes. The opposing effects of NEM on the affinity of agonists and antagonists suggest that NEM drives the conformational equilibrium towards R* whereas cholesterol simply appears to increase the affinity of all the ligands and seems to have no effect on the conformational equilibrium.

The seemingly clear effect of cholesterol on the affinity of muscarinic ligands and that of NEM on conformational equilibria revealed by Scheme 1 is potentially deceptive, however, inasmuch as the model does not describe the proposed mechanism in an explicit manner. When the Scheme 1 is expanded to Scheme 3, which allows for the spontaneous interconversion of the receptor between two states, the data were found to be ambiguous, notwithstanding the pattern that emerged from the analysis according to Scheme 1. In terms of Scheme 3, the data can be described equally well by an effect of cholesterol on either the interconversion or affinity per se, with an accompanying effect of NEM on
either affinity or the interconversion. Since NEM is known from other data to favor $R^*$ over $R$ [137,151,228,280,304], cholesterol appears to increase the affinity of all the ligands for the receptor.

It has been demonstrated in the present investigation that $M_2$ receptors from $S_f9$ cells, which behave competitively, can be made to resemble those from porcine atria, which behave non-competitively, by treatment of $S_f9$ membranes with cholesterol. The manner by which cholesterol gives rise to the observed non-competitive effects was examined in the context of an equilibrium between two states akin to the inactive and active states, which it was thought might differ in the degree to which they exhibit cooperative effects. There was no effect on the position of the specific equilibrium known to be affected by NEM; rather, cholesterol acted to increase the affinity of all ligands irrespective of their identity as agonists and antagonists. It follows that cholesterol and NEM do not appear to regulate the same equilibrium in an opposing manner, and the state favored by cholesterol does not appear to be the same as the inactive state favored by inverse agonists. Cholesterol is necessary as a determinant of cooperativity in the $M_2$ muscarinic receptor, however, and it remains to be seen whether or not its presence is a general requirement for non-competitive effects in oligomers of other G protein-coupled receptors.

5.3 Functional Role of Cholesterol

In the present investigation, methyl-$\beta$-cyclodextrin was used to adjust the amount of cholesterol in membranes of $S_f9$ cells and porcine atria, supplementing the former and depleting the latter. $\alpha$-, $\beta$-, and $\gamma$-cyclodextrins are cyclic oligomers of glucose consisting of six, seven, and eight D-glucopyranose units, respectively [326]. The glucose units are linked via $\alpha$-glycosidic bonds such that the external surface of the molecule is hydrophilic and the internal cavity is hydrophobic. Hydrophobic molecules of appropriate size can be accommodated within the internal cavity through the formation of inclusion complexes, thus making cyclodextrins excellent carriers of lipid molecules. It has been shown that the methylated form of $\beta$-cyclodextrins is most suited for studies of cholesterol. It is least
susceptible to cholesterol precipitation upon dilution, and it appears to be the most effective in loading cholesterol into cells over a short period of time. It also is most effective in enriching and depleting cells with cholesterol at much lower concentrations than other modified forms of \( \beta \)-cyclodextrins [327].

It is the ratio of the amount of cholesterol to that of cyclodextrin that determines whether the inclusion complex acts as a cholesterol donor or a cholesterol acceptor [328]. Moreover, the efficiency of cholesterol transfer from the inclusion complex to biological membranes has been shown to depend on the cyclodextrin-to-cholesterol molar ratio, the cyclodextrin-cholesterol concentration, and the duration of exposure of biological membranes to the inclusion complex. In the present study, membranes of \( Sf9 \) cells were successfully enriched with cholesterol by incubation with the inclusion complex at a cholesterol concentration of 0.5 mM for 30 min. It has been shown that methyl-\( \beta \)-cyclodextrin becomes saturated with cholesterol at molar ratios of approximately 20-to-1 [327]. The concentrations employed in the present work were sufficient to enrich the membranes of \( Sf9 \) cells with cholesterol to levels that are comparable to or greater than those in sarcolemmal membranes of porcine atria. Similar concentrations of cholesterol within the inclusion complex and a similar duration of exposure also were employed in the enrichment of plasma membranes of guinea pig myometrium and \( Sf9 \) cells in studies with the oxytocin receptor [246,263]. In those studies, the levels of cholesterol were increased two- and sixteen-fold, respectively. It has been shown that the enrichment procedure varies for different systems, and exposure to inclusion complexes which contain saturating amounts of cholesterol generally leads to levels of cholesterol that exceed those in controls [328].

\( \beta \)-Cyclodextrins also are efficient in depleting cholesterol from biological membranes, and it has been estimated that 80-90% of total cellular cholesterol can be removed when cells are exposed to high concentrations of methyl-\( \beta \)-cyclodextrin (5–10 mM) for a prolonged period of time (>2 h) [329,330]. The removal of cholesterol from the membrane has been suggested to occur by diffusion. A molecule of cyclodextrin is thought to move into the immediate vicinity of the plasma membrane, where molecules of cholesterol can diffuse directly into its hydrophobic pocket. This arrangement makes
cyclodextrins much more efficient than other lipid carriers such as high density lipoproteins, in that there is no need for cholesterol to desorb completely through the aqueous phase before being picked up by the acceptor molecule. It has been shown that the activation energy for the transfer of cholesterol to cyclodextrin (7 kcal/mol) is much lower than that for transfer to a phospholipid-absorbing particle (20 kcal/mol) [331]. Similar to the procedures for cholesterol enrichment, the precise concentration and incubation time that is required for sufficient cholesterol depletion varies for different systems [328]. In the present study, sarcolemmal membranes of porcine atria were treated with methyl-β-cyclodextrin at a final concentration of 75 mM for 6 h. The same concentration was used to deplete the plasma membranes of guinea-pig myometrium in studies with the oxytocin receptor. Although this concentration is much higher than those generally employed in other studies [328], the present conditions were effective in lowering the amount of cholesterol to levels that are comparable to those of native membranes of Sf9 cells, apparently with no adverse effects on binding. The observation that receptors from Sf9 membranes can be made to resemble those from heart suggests that the effects of cholesterol are reversible.

Prolonged exposure of cells to cholesterol-methyl-β-cyclodextrin can lead to toxicity [327], and the growth of Sf9 cells in the present investigation was compromised at levels of the inclusion complex greater than about 0.09 mM with respect to cholesterol. Sf9 membranes were therefore treated with the inclusion complex after harvest of the cells but prior to solubilization in detergent. The ligand-binding properties of solubilized M2 receptors from porcine atria have been shown to depend in part on the detergent. The differential capacity for [3H]NMS and [3H]QNB and the related non-competitive effects observed in cholate-NaCl and Lubrol-PX were not found in digitonin-cholate, where the two ligands appear to compete directly for all of the sites [147]. Moreover, the effects of the detergents appear to be reversible in that digitonin can recover those sites that are latent upon extraction of the receptor in cholate-NaCl. Digitonin is a plant glycoalkaloid saponin which forms water-insoluble complexes with cholesterol [278] and has been shown to be less effective than sodium cholate in extracting lipids from biological membranes [332,333]. It follows that molecules of cholesterol may be removed from
non-annular sites within the supposed oligomer of the M₂ receptor upon extraction in digitonin-cholate. The difference in the cooperative properties of the receptor between the solubilized preparations may therefore depend upon the amount of cholesterol that remains associated with the protein. In the resolved crystal structure of the β₂-adrenergic receptor, six molecules of cholesterol and two molecules of palmitic acid have been found to form a two-fold symmetric sheet between two receptor molecules [71]. Molecular modeling has suggested that the interaction of cholesterol with the oxytocin receptor involves residues on the surface of transmembrane segments 5 and 6 [276].

Solubilization of the M₂ receptor presumably eliminates any indirect effect of the membrane environment on the functioning of the protein, and it therefore seems likely that the non-competitive behavior found in extracts of cholesterol-treated S99 membranes derives from cholesterol acting directly on the receptor. Moreover, receptor from porcine atria can be made to exhibit largely competitive behavior in the binding of antagonists simply by depleting sarcolemmal membranes of cholesterol prior to solubilization in cholate-NaCl. Although there is no longer a difference in capacity between [³H]NMS and [³H]QNB in those preparations, there are small but significant discrepancies in the affinity estimated for the radiolabeled and unlabeled forms of NMS. The discrepancy is much smaller, however, than that in extracts of receptor from native sarcolemmal membranes from which cholesterol was not removed [147].

The ability of cholesterol to crystallize along with the β₂-adrenergic receptor indicates that the two are tightly coupled, and the minor non-competitive component that is detected in preparations of cholesterol-depleted porcine atria suggests that residual amounts of cholesterol may remain bound to the receptor even after treatment with very high concentrations of methyl-β-cyclodextrin. Small but significant discrepancies also were detected with M₂ receptor from native S99 membranes when the binding assays were performed at 4 °C in order to avoid thermal instability and a decrease in the capacity for [³H]NMS. Such discrepancies also may derive from weak or residual cooperativity associated with low levels of cholesterol. Such residual effects suggest that the level of cholesterol in the membranes of mammalian cells greatly exceeds what is required for the proper functioning of some receptors.
Cholesterol has been shown to occupy non-annular sites within membrane proteins or within multi-subunit complexes of membrane proteins [222,307]. Its direct influence at those sites is distinct from the more general effects that arise from its influence on the membrane environment. This is the case for the \( \text{CA}^{2+}-\text{ATPase} \) [223], rhodopsin [224], the nicotinic acetylcholine receptor [314,315], the oxytocin receptor [246], the \( \mu \)-opioid receptor [267], and the galanin receptor [264] to name a few. In the case of the latter three, the functional role of cholesterol was related to its effect in promoting the high-affinity state for the binding of agonists, which was independent of the interaction between those receptors and their cognate G proteins. The present results demonstrate that cholesterol is required for the manifestation of cooperativity in binding of antagonists to the M2 receptor. There also is a general increase in the affinity of muscarinic ligands, both agonists and antagonists, that appears to be independent of the known interconversion of the receptor between an inactive and an active state. The relationship between the increase in ligand-affinity and the manifestation of cooperativity is not clear.

Activated G protein-coupled receptors are comparatively unstable. For example, constitutively active mutants of the \( \beta_2 \)-adrenergic receptor [137,334] and the \( \alpha_{2A} \)-adrenergic receptor [335,336] have been reported to undergo spontaneous inactivation more rapidly than the corresponding wild-type receptors. With the M2 muscarinic receptor, it has been shown that either purification or solubilization of the protein from the membrane leaves the receptor in a labile form akin to the active state [228]. In the present investigation, M2 receptors from native Sf9 membranes have been found to be thermally unstable at 30 °C. Such instability is not observed with receptor from cholesterol-treated Sf9 membranes at the same temperature, which indicates that cholesterol offers some protection. The stabilizing effect of cholesterol on the M2 receptor resembles that on the oxytocin receptor, which was protected against proteolytic degradation and against inactivation by heat and extremes of pH [269]. NEM previously has been shown to drive the conformational equilibrium of the M2 receptor further towards the active state and to react more readily when G protein-coupled receptors are in their agonist-specific state [228]. Thus, the instability exhibited by solubilized M2 receptors from native Sf9 membranes suggests that those receptors are in the active state,
and the protective effect of cholesterol against thermal inactivation raises the possibility that receptors from cholesterol-treated membranes are in the inactive state. It previously has been shown, however, that sufficient concentrations of agonists or antagonists can protect the M_2 receptor against thermal inactivation [228]. This is consistent with the present observation that treatment with cholesterol, and hence its protective effect, does not arise from a shift in the equilibrium distribution of receptors from the active to the inactive state.

Ligands can serve as pharmacological chaperones by assisting in the maturation and transport of G protein-coupled receptors to the cellular surface [337]. In the case of the M_2 receptor, it has been shown that solubilization in digitonin-cholate leads to active monomers and inactive oligomers [225]. Moreover, QNB has been found to preserve the functionality of oligomers, in that treatment of Sf9 cells with QNB after harvest led to a gain of function in that oligomers were retained by the affinity-column and purified in an active state [225]. QNB therefore appears to increase the number of oligomers that were functional in the membrane or remain so upon solubilization; accordingly, it may promote oligomerization in the membrane, slow or prevent the fragmentation of pre-existing oligomers, or exert a protective effect on their activity.

In the present investigation, exogenous cholesterol also was added to Sf9 cells after harvest in a manner similar to that of QNB. Solubilization has been shown to alter the affinities of various ligands for muscarinic receptors expressed in Sf9 cells [292]. Similarly, detergents have been shown to modulate the binding properties of muscarinic receptors from mammalian sources, and the measured affinity for some ligands can vary 20-fold from one detergent to another (Ref. 147 and references cited therein). It also has been suggested that oligomers of the M_2 receptor from Sf9 cells may be unusually sensitive to dissociation or inactivation owing to the absence of an additional element that imparts stability in other systems [225]. Cholesterol may therefore act in a manner similar to that of the chaperone-like effect of QNB; that is, it may promote oligomerization in the membrane, slow or prevent the fragmentation of pre-existing oligomers, or exert a protective effect on their activity in the sense of providing the necessary conditions for the manifestation of cooperativity.
The present data indicate that cholesterol acts to increase the affinity of all the ligands, which suggests that it reverses the general lowering effect of solubilization on the affinity of the M₂ receptor. Moreover, cholesterol appears to have no effect on the oligomeric status of the M₂ receptor, as there was no difference in the cross-linking pattern obtained with receptors from native and cholesterol-treated S/9 membranes. It therefore seems unlikely that cholesterol acts to promote oligomerization of the receptor or to reduce the fragmentation of a native oligomer. Detergent-related effects on binding point to differences in cooperative interactions among linked sites, presumably within an oligomer [147]. M₂ receptors were extracted in cholate-NaCl, which preserves the specific association between cholesterol and the protein and the higher affinity for ligands; resistance to thermal inactivation and the manifestation of cooperativity in the M₂ muscarinic receptor can therefore be attributed to cholesterol in the present work.
CHAPTER 6

FUTURE DIRECTIONS
6.1 Specificity in the Effects of Cholesterol on Cooperativity and Affinity

In the work presented here, cholesterol was identified as a determinant of cooperativity in the M₂ muscarinic receptor. Cholesterol also was shown to protect the receptor from thermal instability and to increase the affinity of the receptor for both agonists and antagonists.

The minimum level of cholesterol that is required for the manifestation of non-competitive effects in the binding of muscarinic antagonists remains unclear. Native Sf9 membranes were not completely devoid of cholesterol, yet binding was competitive. Moreover, specific binding was lost altogether when native Sf9 membranes were treated with methyl-β-cyclodextrin alone, which presumably reduced further the amount of cholesterol from levels that were low at the outset. These observations suggest that native Sf9 membranes contain a level of cholesterol that is necessary for ligand-binding but insufficient for the manifestation of cooperativity. That in turn suggests that cholesterol can affect the receptor in different ways, perhaps via different sites. It therefore would be useful to supplement native membranes with graded amounts of cholesterol in order to determine the level at which binding becomes non-competitive. Cooperativity can be monitored as the magnitude of the apparent difference in capacity for [³H]NMS and [³H]QNB.

A related issue here is the increase effected by cholesterol in the affinity of muscarinic agonists and antagonists, which implies a cholesterol-regulated interconversion between states distinct from the active and inactive states revealed in phenomena such as constitutive activity and the effects of NEM. It remains unclear whether the induction of cooperativity and the increase in affinity are separate and independent effects of cholesterol or two manifestations of the same interaction between the lipid and the receptor. It has been suggested that the effects of guanyl nucleotides on the binding of agonists—which are a correlate of efficacy—derive from differences in cooperativity within a tetramer that interconverts between an inactive and an active state [81]. If so, the effects of cholesterol on cooperativity and affinity may arise from different interactions. A comparison of the relationship between the level of cholesterol
and the appearance of cooperativity on the one hand and the increase in affinity on the other may shed light on this question.

Detergent-solubilized receptor has been used throughout the present investigation, which suggests that the effects of cholesterol derive from a direct interaction between the lipid and the receptor. It therefore would be of interest to probe the specificity of those effects by examining analogues of cholesterol such as ergosterol, stigmasterol and 5-cholesten-3-one.

Studies on the dose-dependence of sterol-related effects will require a greater degree of control than has been exercised to date over the levels of cholesterol in native Sf9 membranes. A portion of the cholesterol in Sf9 cells is acquired from fetal bovine serum present in the culture medium. Recent studies in our laboratory have shown that the cells can be adapted to grow and to express the M2 receptor in serum-free media devoid of or supplemented with cholesterol as required. Such cultures can be expected to yield levels of cholesterol that are more consistent, particularly at the lowest levels. There also may be differences between the properties of receptor extracted from cells grown in the presence of cholesterol and those of receptor extracted from membranes supplemented with cholesterol after the cells have been harvested. Other concerns include the thermal instability that has been found with receptor extracted from membranes containing low levels of cholesterol; experiments with such preparations are likely to be more straightforward if carried out at 4 °C.

6.2 Oligomeric Status of the Receptor

Electrophoretic mobility and the modeling of non-competitive effects both suggest that the M2 receptor extracted from Sf9 membranes in cholate–NaCl is obtained as a hexamer. That is in contrast to previous observations that the receptor is a tetramer when extracted from sarcolemmal membranes of porcine atria [110,147] and when purified from Sf9 cells and reconstituted in phospholipid vesicles [148]. This discrepancy ought to be
reconciled, as it seems likely that there is only one oligomeric state of functional relevance.

The solubilized extract in cholate-NaCl may contain a mixture of oligomeric states such as monomers, dimers, and tetramers, the electrophoretic mobility notwithstanding. In such a case, an artifactually large number of interacting sites might be required for a model such as Scheme 2 to describe the data. To investigate this possibility, the data from cholesterol-treated $S/9$ membranes can be re-analyzed in terms of models that include a single oligomeric species of the receptor plus a population of monomers (e.g., 148). The AIC score can then be re-calculated for the various models, and it can be determined whether or not a hexavalent form remains the optimal size consistent with the data. Models that incorporate more than one oligomeric species also could be examined. In the absence of additional data, however, it seems likely that such calculations would be compromised by the emergence of correlated and undefined parameters.

The major band detected upon gel electrophoresis of solubilized receptor extracted from both native and cholesterol-treated $S/9$ membranes migrated as a hexamer. Upon cross-linking of both preparations, the samples migrated as a broad band of which the leading edge corresponded to a hexamer. The samples were from unprocessed extracts, however, and other proteins may have associated with the receptor to yield a fortuitous agreement between electrophoretic mobility on the one hand and the results of mechanistic analyses on the other. To probe for the presence of such proteins, samples of the receptor could be extracted from the gels and subjected to a proteomic screen by means of mass spectrometry. Positive results could be confirmed by means of western blotting with appropriate antibodies. To avoid detecting unassociated components that co-migrated with the cross-linked receptor, the latter could be purified on an antagonist-affinity column (e.g., 110) or, in the case of the hexahistidyl-tagged receptor now available in our laboratory, on a chelating column.

There are limits to what can be achieved by applying quantitative models and biochemical assays to comparatively uncontrolled preparations such as the extracts used in the present investigation. The oligomeric status of the $M_2$ receptor and its attendant
ligand-binding properties both can be examined under more defined conditions by reconstituting the purified receptor into phospholipid vesicles prepared with and without cholesterol or other sterols. It previously has been shown that the M₂ receptor can be purified as monomers, which then can be reconstituted exclusively as tetramers within cholesterol-containing phospholipid vesicles [148]. Because the receptor is highly purified, its oligomeric status can be identified unambiguously from the mobility of cross-linked material during electrophoresis. Whereas monomers were found to bind muscarinic antagonists in a competitive manner, the reconstituted tetramers exhibited the shortfall in capacity for [³H]NMS and related non-competitive effects characteristic of receptors extracted from porcine sarcolemmal membranes or cholesterol-treated Sf9 membranes.

The reconstituted preparation is a promising system for further studies on the relationship between cholesterol, oligomeric status and functional properties such as cooperativity and affinity. The present results suggest that purified monomers reconstituted in the absence of cholesterol will form tetramers to which antagonists bind in a competitive manner. Stepwise increases in the level of cholesterol will permit the investigation of such questions as the relationship between changes in affinity on the one hand and the emergence of non-competitive effects on the other. Subsequent work may take advantage of the observation that reconstituted tetramers survive solubilization of the vesicles [148] and more recent evidence that they can be stabilized, if necessary, by cross-linking without affecting the binding properties. The extent to which cholesterol-related effects are retained after solubilization will help to distinguish direct interactions from effects determined by the properties of the membrane.

Finally, reconstitution with and without cholesterol may yield insight into the relationship between sterols and oligomeric status. The present data suggest that the existence of oligomers is independent of cholesterol, but even the native preparations examined to date contained residual levels. If reconstitution of the receptor in the absence of cholesterol were to yield monomers, dimers or a mixture of oligomeric states, it would point to a role for cholesterol in maintaining both the oligomer per se and the properties that derive therefrom.
APPENDIX

ASSESSING THE OLIGOMERIC STATUS OF THE M₂ MUSCARINIC RECEPTOR

USING ATOMIC FORCE MICROSCOPY
The atomic force microscope (AFM) was invented in 1986 by Gerd Binnig, Calvin Quate and Christoph Gerber. It is an instrument that gathers information with the use of an atomically sharp probe attached to the end of a flexible cantilever, which makes physical contact with the specimen as it is scanned across the surface. The tip of the probe is often made of silicon or silicon nitride and has a radius of curvature of the order of nanometres. An early example of the capability of this emerging technology when applied to the imaging of biological systems achieved lateral and vertical resolutions of about 0.5 nm and 0.1 nm, respectively [338]. When the tip is brought close to the surface of the sample, forces between the tip and the sample lead to a deflection of the cantilever. The magnitude of the deflection is determined by the underlying topographical features of the sample surface, and it typically is measured using a laser spot reflected from the top of the cantilever onto an array of photodiodes. In contrast to the more traditional approaches of examining biological structure, such as electron microscopy and X-ray crystallography, atomic force microscopy can be applied directly to biological specimens in near-native conditions. In the case of membrane proteins, they can be examined while embedded in the lipid bilayer and immersed in a physiological salt solution [339]. Atomic force microscopy therefore can be used to assess the size of membrane proteins and to resolve their sub-substructural features. It also can be used to monitor changes in the overall lateral organization of those proteins within native and artificial membranes.

Various membrane proteins have now been examined using atomic force microscopy, and they include rhodopsin [184], bacteriorhodopsin [340], halorhodopsin [341], porin OmpF [342], staphylococcal α-hemolysin [343], aquaporins [344], rotors from various F0 ATP synthases [345], and bacterial light-harvesting complexes [346]. Most of these high-resolution topographs were initially achieved for those proteins that were crystallized in two dimensions, which prompted early suggestions that a crystalline assembly was a pre-requisite for observing membrane proteins at sub-nanometer resolution by the AFM. Later images of the G protein-coupled receptor rhodopsin, which was obtained from the rod outer segments of native mouse disc membranes, demonstrated that a comparable spatial resolution also could be achieved from non-crystalline
assemblies. In that work, it was shown that rhodopsin occurs in extensive arrays of what appears to be rows of dimers \[184\]. Moreover, atomic force microscopy has revealed that those single-layered discs were 7–8 nm thick and circular in shape, from which it was estimated that, on average, rhodopsin molecules protruded from the surface by a little more than 1 nm.

The M\(_2\) muscarinic receptor has been shown to bind antagonists in a competitive manner behave when extracted from membranes containing low levels of cholesterol and non-competitively when extracted from membranes containing significantly higher levels of the sterol. The latter behavior implies the existence of oligomers, which have been detected independently by the co-immunoprecipitation of differently tagged M\(_2\) receptors expressed in S/9 cells \[155,225,226\] and by bioluminescence resonance energy transfer \[347\]. Moreover, receptors from untreated and cholesterol-treated S/9 membranes exhibited the same electrophoretic mobility, either before or after cross-linking with BS\(^3\), even though only the receptor from treated membranes exhibited marked non-competitive behavior. It follows that receptors from native S/9 membranes also occur as oligomers but require the presence of sufficient cholesterol for the non-competitive effects to be manifested. In the present work, atomic force microscopy has been employed in an attempt to assess the oligomeric status in various preparations of solubilized and purified extracts of the M\(_2\) receptor and also to identify factors that may affect its state of oligomerization.  

**MATERIALS AND METHODS**

*Development of substrate*

A general procedure for the reconstitution of purified M\(_2\) receptor into liposomes involves a mixture of phosphatidylcholine (PC), phosphatidylserine (PS), and cholesterol (CHL) (all from Sigma-Aldrich) at a molar ratio of 4.5:4.5:1.0, respectively. The lipid mixtures were dissolved in chloroform and dried uniformly under argon gas to a thin film which was stored at –20 °C prior to use. Unless otherwise stated, the dried film was
rehydrated with 1.8 mL of HEN buffer (20 mM HEPES, 0.83 mM EDTA, 160 mM NaCl, pH = 8.0) at a final total lipid concentration of 1 mM, and the mixture was sonicated until a clear, homogenous solution was obtained. This lipid mixture was initially used to create a pre-formed film on the surface of the mica onto which various preparations of the M₂ receptor could be plated (e.g., solubilized, purified, and reconstituted receptor).

For clarity, lipid vesicles alone refer to those samples where the dried lipid film is rehydrated with HEN buffer and are not mixed with preparations of the receptor. Reconstituted lipid vesicles alone refer to those samples where the dried lipid film is rehydrated with HEN buffer which are not mixed with preparations of the receptor and are passaged down a column of Sephadex G-50. Reconstituted receptor refers to those samples where the dried lipid film is rehydrated with HEN buffer which are mixed with preparations of the receptor followed by passaging down the column of Sephadex G-50.

Preparations of the M₂ muscarinic receptor

M₂ muscarinic receptor was detergent-solubilized and affinity-purified from sarcolemmal membranes of porcine atria as described previously [110]. The concentration of digitonin in solubilized and purified samples of the receptor was 0.86% and 0.1%, respectively. Reconstitution of the purified receptor into phospholipid vesicles also was carried out essentially as described previously [148]. Unless otherwise stated, the dried film of lipid (phosphatidyl choline:phosphatidyl serine:cholesterol = 4.5:4.5:1.0) was suspended in 1.8 mL of HEN buffer containing 0.18% deoxycholate (Sigma-Aldrich) and 0.04% sodium cholate (Sigma-Aldrich). The purified receptor was incubated with carbachol (10 mM) for 15 min at 4 °C, mixed with the lipid suspension (100 µL) and applied to a column of Sephadex G-50 (0.8 × 5.0 cm) pre-equilibrated with HEN buffer.

AFM imaging
Solution tapping mode atomic force microscopy images were acquired at ambient temperature on a Digital Instruments IIIa Multimode scanning probe microscope (SPM, Digital Instruments/Veeco) equipped with an “E” scanner. Scanning was performed using 100–200 µm long oxide-sharpened silicon nitride V-shaped cantilevers with a nominal spring constant of 0.06–0.58 N/m, as indicated by the manufacturer. The AFM cantilevers were irradiated with UV light prior to use to remove any adventitious organic contaminants. A contact/tapping mode fluid cell was sealed against a freshly cleaved muscovite mica substrate with a silicone O-ring. The fluid cell was fitted with inlet and outlet tubing to allow exchange of solution in the cell during imaging. Prior to injection of the sample, HEN buffer supplemented with calcium chloride (final [Ca\(^{2+}\)] = 10 mM) was flowed into the cell and over the surface of the mica, which generally served as the negative control or as the reference image for all subsequent experiments. Unless otherwise stated, samples also were supplemented with calcium chloride (final [Ca\(^{2+}\)] = 10.7 mM) prior to their injection into the fluid cell. The injection was followed by a 30 min waiting period to allow the sample to adsorb onto the surface of the mica, which was then washed with a solution EDTA (25 mM) to chelate the calcium ions and also to remove any loosely bound material.

RESULTS

The initial substrate, which is a lipid mixture of PC, PS and CHL suspended in HEN buffer, formed a lipid bilayer with an average thickness of 6.0 ± 0.3 nm on the surface of the mica (Fig. A-1A, Fig. A1-B, \(N = 2\)). Unpurified M\(_2\) receptor solubilized in digitonin-cholate (0.86%/0.17%) was reconstituted in this lipid mixture, and the resultant receptor-containing vesicles were collected and laid over the initial lipid bilayer that was already present on the mica. The addition of receptor-containing vesicles resulted in the disruption of the pre-formed lipid bilayer, which may have been due to the presence of significant amounts of digitonin in the solubilized extract (Fig. A1-C).
To probe for possible disruptive effects of digitonin, reconstituted lipid vesicles lacking both receptor and detergent were prepared by passage of the mixture of lipids down a column of Sephadex G-50. The images were examined under the AFM, and preliminary results indicated that the reconstituted vesicles were capable of forming a bilayer on the mica surface without the prior application of an initial lipid film. That bilayer had a thickness of about 6.1 ± 0.4 nm (Fig. A1-D, \(N = 2\)), which was comparable to the thickness of the initial substrate formed from a lipid mixture that had not been processed on Sephadex G-50. When reconstituted lipid vesicles containing receptor solubilized in digitonin-cholate (0.86%/0.17%) were laid directly onto mica, however, no bilayer structures can be identified (Fig. A1-C). It follows that a constituent of the receptor preparation, which seems likely to be the detergent, hinders the formation of the lipid bilayer.

The possible disruptive effect of digitonin was examined further with samples of the purified M₂ receptor, in which the concentration of digitonin (0.1%) is about 8-fold less than that in unprocessed extracts (0.86%). When reconstituted lipid vesicles lacking receptor were layered on mica and then overlaid with a solution of purified M₂ receptor from porcine atria, the preformed lipid bilayer remained intact (Fig. A1-E). To determine if the retention of the initial bilayer was due to the much lower digitonin content in the purified receptor sample, receptor purified from porcine atria was reconstituted in lipid vesicles for examination under the AFM. When that sample was imaged, however, no discernible bilayer structures were present. Instead, the surface of the mica contained numerous non-specific globular structures (Fig. A1-F). Such structures may be vesicles that have failed to fuse with one another and adopt a planar, bilayer arrangement on the surface of the mica. This lack of membrane fusion could be the result of lipid vesicles that were too small.

Preliminary studies by Riyad Raghu, a summer student in our laboratory, have shown that repetitive freezing and thawing of lipid vesicles increased their mean diameter from 30 ± 11 nm to 260 ± 60 nm (\(N = 80\)), and the enlarged vesicles yielded films with a thickness of about 5 nm. M₂ receptor purified from porcine atria therefore was reconstituted in lipid vesicles, and the sample was subjected to five freeze-thaw cycles in
a dry ice-acetone bath. Prior to imaging with the AFM, the samples were first examined under an electron microscope to assess whether or not the procedure had produced larger vesicles. There appears to be no significant difference in size between those lipid vesicles that underwent five freeze-thaw cycles (Fig. A-2A, mean diameter > 100 nm) and those that did not (Fig. A-2B, mean diameter > 100 nm).

Although the freeze-thaw procedure failed to increase vesicular size, the vesicles from these two preparations were larger than those obtained by Riyad Raghu prior to his freeze-thaw procedure. It appears that the formation of larger vesicles in the present investigation was due to the reconstitution procedure rather than to the freeze-thaw cycles, as lipid vesicles mixed with purified M₂ receptor that had not undergone the reconstitution procedure, but were subjected to the cycles of freezing and thawing failed to yield vesicles with a mean diameter larger than 100 nm (Fig. A-2C). This also is evident in samples of lipid vesicles alone, which had not undergone reconstitution but were subjected to five cycles of freezing and thawing, and which gave liposomes that were relatively smaller in size (Fig. A2-D).

When examined with the AFM, those vesicles that were relatively small in size under the EM appeared to give better lipid bilayers than did larger vesicles. For example, lipids that were not subjected to the reconstitution procedure but were subjected to the freeze-thaw cycles gave relatively small vesicles under the EM (Fig. A-2D). An aliquot of this sample readily formed an extensive lipid bilayer on the mica (Fig. A-2E). The existence of that bilayer can be discerned by the appearance of several holes on its surface, through which the underlying mica can be seen. An extensive bilayer also appeared when the injection of an aliquot of this same sample was followed by an injection of purified receptor (Fig. A-2F). Again, the presence of several holes confirms the presence of the lipid bilayer, as the holes reveal the surface of the mica substrate underneath.

In the case of the image in Figure A-2F, it is difficult to ascribe any of the elevated features found on the surface of the lipid bilayer to the purified M₂ receptor. Various constituents could give rise to such features when topography alone is being monitored. For example, they may derive from the receptor, aggregates of lipid or a mixture of both.
Given that a rise in topography as monitored by the AFM can derive from almost anything on the surface of the mica, it will be difficult to identify which of the elevated features are, in fact, receptor despite the purity of the preparation. It is difficult to ascertain whether or not the purified receptor successfully embedded within the pre-formed lipid bilayer already on the mica or if the vesicles remained on top following injection and subsequently were flushed away. The latter scenario seems likely given the lack of propensity of receptor-containing reconstituted vesicles to fuse with one another during the freeze-thaw cycles and from their inability to form a bilayer when injected directly on the mica surface (Fig. A-1F). An identifiable recurring pattern on the surface of the lipid bilayer may be required in order to distinguish between the presence of the receptor and that of non-specific lipid aggregates. Thus, a sample of purified M2 receptor in a two-dimensional crystalline array is what likely is necessary for visualization and identification of the protein under the AFM.

Lipid vesicles that had undergone reconstitution with purified receptor and the cycles of freezing and thawing were larger (Fig. A-2A). Although larger, these vesicles did not give as extensive a bilayer as those that were not subjected to the reconstitution procedure. In contrast to the numerous and predominantly globular structures observed previously with a similar sample (Fig. A-1F), this batch was able to give more flattened but isolated bilayer structures on the surface of the mica, perhaps owing to their larger size (Fig. A-2G). The difference between the extent of the bilayer structures formed by lipids that did and did not undergo reconstitution can be seen in Figs. A2-G and A-2F, respectively. The former preparation was injected into the AFM subsequently followed by the injection of the latter. It can be seen that the lipids from the former preparation occur largely as isolated structures (Fig. A2-G), whereas subsequent injection of the latter preparation resulted in the disappearance of these isolated lipid islands, which were replaced by a massive lipid bilayer on the surface of the mica (Fig. A2-H).
DISCUSSION

In the present work, formation of a lipid bilayer on the surface of the mica was achieved from samples of either unreconstituted or reconstituted lipid vesicles that did not contain receptor. Two preparations of the receptor were reconstituted in lipid vesicles; that is, one from a crude solubilized-extract and another from a purified receptor-preparation. The latter preparation contains a much lower concentration of digitonin than does that of the former. When a sample of digitonin-solubilized receptor that was reconstituted in lipid vesicles was overlaid over an existing lipid bilayer on the surface of the mica, the lipid bilayer was disrupted (Fig. A-1C). In contrast, when a sample of purified receptor that was reconstituted in lipid vesicles was overlaid over an existing lipid bilayer, the underlying bilayer structure was preserved. The disruption may be due to the significant amounts of digitonin that is present in the solubilized extract.

Although a sample of reconstituted, purified receptor does not cause bilayer disruption, it does result in the appearance of large aggregates on the surface of the bilayer which suggests that the receptor-containing lipid vesicles have failed to fuse with the pre-formed lipid bilayer that is already present on the mica (Fig. A-1E). Therefore, the step of laying an initial lipid bilayer on the mica was skipped, and the purified receptor-containing lipid vesicles were laid directly onto the mica surface. It, however, did not produce a flat lipid bilayer on the surface of the mica which suggested that the receptor-containing vesicles have failed to fuse (Fig. A-1F).

It initially was presumed that the lack of membrane fusion may be due to the small size of the purified receptor-containing vesicles. Those samples were therefore subjected to freeze-thaw cycles in an attempt to facilitate fusion and thus create larger vesicles that would form a bilayer when laid onto the surface of the mica. However, such attempts at creating larger vesicles did not appear to work. What is clear from the present study is that those vesicles that do not contain receptor were able to form an identifiable lipid bilayer on the mica irrespective of the reconstitution procedure while those vesicles that do contain receptor did not. It therefore appears that a constituent belonging to the receptor preparation which may be the receptor itself is hindering bilayer formation. It
also may again be due to the digitonin which is still present in the purified receptor preparation, albeit in a much lower concentration than that from the solubilized receptor-extract.

There are likely many factors that affect membrane fusion. It previously has been suggested that molecular shape plays an important consideration in membrane modeling; that is, the physical dimensions of a membrane component can influence its phase preference upon hydration [348]. By taking into account the interaction free energies, molecular geometry, and entropy of lipid systems, theoreticians have developed a dimensionless packing parameter, $S$, that is useful in determining the size and shape of lipid aggregates upon hydration. For instance, it had been predicted from the values of $S$ and shown experimentally that single-chain lipids with the shape of an inverted cone or a wedge form micelles; in contrast, double-chain lipids such as phosphatidylcholines, which have large head groups, fluid chains and a shape resembling that of a cylinder, form bilayers. Cholesterol and some double-chain lipids with small head groups, which have a shape resembling that of a truncated cone, have been shown to adopt hexagonal phases upon hydration [349]. In terms of these criteria, it has been shown that cylindrical and wedge-shaped molecules were essential for spontaneous vesiculation [350] and bilayer stabilization [351,352], and that molecules having complementary molecular shapes also are able to form bilayer structures.

It is possible that either digitonin or the receptor or both alter the nature of the packing of phospholipids within vesicles, thus affecting the overall shape and size of the latter. This therefore could have consequences for their propensity to fuse and to adopt a planar, bilayer arrangement when adsorbed onto the surface of the mica. A method that can monitor the order of molecular packing and the fluidity of membranes is that of fluorescence anisotropy, which is directly proportional to the former and inversely proportional to the latter. With this technique, digitonin previously has been shown to cause biphasic changes in membrane fluidity; that is, at lower concentrations, digitonin significantly increased the fluidity of membranes, but at higher concentrations, the anisotropy of fluorescence polarization started to increase until it finally formed micelles that were more rigid than the natural membranes [353]. In the present investigation, the
amount of digitonin in samples of purified receptor may have been high enough to cause a rigidifying effect on lipid vesicles. Therefore, although the level of digitonin in samples of purified receptor may not have been sufficient to cause bilayer disruption per se, as it did with samples of solubilized receptor-extracts (Fig. A1-C), it also may not be sufficiently low to increase membrane fluidity and allow for vesicle fusion (Fig. A1-F). Moreover, digitonin also has been shown to form a tight complex with cholesterol [354], and this sequestering property of the detergent also could have unwanted effects on bilayer formation and vesicle fusion.

The M2 receptor itself also may influence the ability of the vesicles to fuse together and adopt a planar arrangement on the surface of the mica. It previously has been suggested that the presence of hydrophobic mismatch—that is, a mismatch in length between the hydrophobic part of membrane-spanning proteins and the thickness of the hydrophobic segment of the lipid bilayer—has consequences not only on protein conformation and activity but also on membrane structure and organization [355].

For instance, in studies of gramicidin embedded within bilayers of phosphatidylcholine of differing thickness, it has been shown that there was a significant increase in the average lipid chain order for those phospholipids with relatively shorter backbones [356,357]. It also has been shown that such stretching or disordering of the acyl chains is a means by which lipids adapt to the presence of a hydrophobic mismatch [357], where the lipids directly adjacent to the protein are perturbed the most and those further in the bulk are perturbed the least [358]. From the work of Riyad Raghu and in the present investigation, an estimate of about 5–6 nm has been obtained for the thickness of the lipid bilayer that is plated on the surface of the mica. In comparison, an estimate of about 7–8 nm was obtained for the thickness of single-layered mouse disc membranes from the surface of which rhodopsin protruded by another 1 nm. If the M2 receptor is assumed to have dimensions similar to those of rhodopsin, there potentially could be a 4 nm discrepancy in thickness between the receptor and the bilayer that is plated on the mica. Thus, the potential for hydrophobic mismatch is highly likely, which therefore would result in changes in lipid chain order.
Hydrophobic mismatch also can affect the melting transition temperature of lipid bilayers. It has been shown that peptides or proteins with relatively long hydrophobic segments stabilize the thicker gel phase whereas those that are relatively short stabilize the fluid phase. For example, bacteriorhodopsin has been shown to induce an increase in the phase transition temperature in bilayers of PC with short acyl chain lengths, but a decrease in those bilayers with relatively longer acyl chains [359]. Similar to G protein-coupled receptors, bacteriorhodopsin also contains seven transmembrane helices, and thus the effect of the former on the phase transition temperature of lipid bilayers is likely similar to that of the latter. In the present work, the preparation of the lipid suspensions, the reconstitution of the lipid vesicles and the imaging of the lipid bilayers with the AFM were all done at room temperature. Since the effect of temperature on the phase transitions of the lipids was not examined, the effect of the M2 receptor on this process is unknown.

Although biological membranes have an overall bilayer organization, they contain large amounts of lipids that preferably adopt non-lamellar structures such as that of the inverted HII phase [360]. Moreover, the presence of membrane proteins, which could create a hydrophobic mismatch with their surrounding lipid environment, can facilitate the formation of these non-lamellar phases. For example, even though phosphatidylcholine is a typical bilayer-forming lipid, it has been shown that the presence of high concentrations of gramicidin A can induce the formation of a non-lamellar, HII phase [361]. In the present work, although the M2 receptor may not promote the formation of a non-lamellar phase per se, it does appear to support the adoption of non-lamellar structures, in the sense that the presence of the receptor preparation hinders the formation of planar bilayer structures on the surface of the mica.

Again through hydrophobic mismatch, the presence of membrane proteins also can induce preferential protein-lipid interactions, which therefore can influence the formation of lipid microdomains [362,363]. For example, in mixtures of di-C12:0-PC and di-C18:0-PC, bacteriorhodopsin has been shown to have a preference for the former when both lipids are in the gel state, but a preference for the latter when both are in the fluid state [364]. In the present investigation, the M2 receptor was reconstituted in two types of
phospholipid, each of which came from natural sources and therefore contained a mixture of lipid species of different acyl chain lengths. Moreover, cholesterol also was included in the reconstituted sample, creating the potential for microdomain formation based not only on lipid type but also on lipid size.

The presence of membrane proteins therefore can affect the properties of the lipid bilayer in a manner analogous to the known effects of the latter on the function of the former. The influence of membrane proteins on physico-chemical factors such as lipid chain order, phase transition temperature, and the formation of non-lamellar phases and lipid microdomains likely will have consequences for vesicle fusion and overall membrane organization. Most of the work discussed above on the effects of hydrophobic mismatch was conducted in model membrane systems composed of a single lipid component. In the present investigation, however, the M₂ receptor was reconstituted in vesicles formed from heterogeneous preparations of phosphatidylcholine and phosphatidylserine. The effect of the receptor on its lipid environment is therefore likely to be complex; nonetheless, reconstituted vesicles that contained purified M₂ receptor (Figs. A-2A and A-2B) were generally larger than those which were not subjected to the reconstitution procedure but were mixed with purified receptor (Fig. A-2C) and those which were neither subjected to reconstitution nor mixed with purified receptor (Fig. A-2D). These results show that the receptor is not incorporated into the liposomes simply by mixing a solution of purified receptor with a lipid suspension. Thus, the increase in size reflects the incorporation of the receptor within the vesicles upon passage of the mixture down a column of Sephadex G-50, a process that presumably is accompanied by a degree of re-organization of the surrounding lipid molecules to accommodate the presence of the protein. As noted above, such adjustments in the molecular packing of the lipid molecules will affect the physico-chemical properties of the resultant vesicles, perhaps with consequences for membrane fusion and bilayer organization.

The intent behind the use of lipids obtained from natural sources in the development of a substrate amenable for studies with the AFM was to adopt an established procedure for the reconstitution of purified M₂ receptor was reconstituted in lipid vesicles for use in biochemical and pharmacological assays. In that way, any structural information
gathered from atomic force microscopy could be assessed and related directly to data obtained from the other techniques.

There did not appear to be any major problems in terms of obtaining a bilayer on the surface of the mica either with a suspension of PC:PS:CHL or with reconstituted vesicles alone. The difficulties arise when the receptor was introduced into the preparation, either in purified form or as an unprocessed extract. Direct injection of the purified receptor into the AFM appeared to preserve the underlying lipid bilayer that already was present on the surface of the mica (Fig. A-1E). This was in contrast to the result obtained upon injection of the extract, which caused a major disruption of the pre-existing lipid bilayer on the mica surface (Fig. A-1C). Thus, the purified receptor preparation was preferred not only because it preserved the underlying lipid bilayer but also because, in a purified sample, the M2 receptor would essentially be the predominant protein in the preparation, which would make interpretation of the AFM data much simpler.

The heterogeneity of the lipids, in terms of the lengths of their acyl chains, did not pose a problem with regard to the formation of a bilayer as long as the sample did not contain receptor. Thus, the failure to form a lipid bilayer cannot be attributed solely to the heterogeneity of the lipids; rather, it appears to result from the digitonin or the receptor protein itself, as both are likely to affect the physico-chemical properties of the surrounding lipids. The disruptive effect of digitonin appears to have been dampened by employing purified receptor preparations which contain lower levels of digitonin rather than solubilized extracts. The reasons for the inability of reconstituted vesicles containing purified receptor to form bilayers on the mica are not clear. Perhaps the failure to form bilayers stems from the hydrophobic mismatch between the lipids and the protein, which likely gives rise to altered membrane and vesicular properties. One way to simplify the system would be to use pure synthetic lipids in the reconstitution procedure and thereby to avoid the heterogeneity inherent in natural products. The receptor also could be reconstituted in one lipid at a time to probe for specificity in the ability to form a bilayer.
FIGURE A-1
Fig. A-1 – Development of substrate and Incorporation of the M2 receptor. (A) Mica. (B) A lipid mixture consisting of phosphatidylcholine (PC), phosphatidylserine (PS) and cholesterol (CHL) at a molar ratio of 4.5:4.5:1.0, respectively, and rehydrated with HEN buffer (HEPES, 20 mM; Na$^{2-}$-EDTA, 0.83 mM; NaCl, 160 mM) at a total lipid concentration of 1 mM. The lipid mixture was supplemented with CaCl2 to a final concentration of 10.7 mM. (C) FLAG-tagged M2 receptor solubilised in digitonin–cholate (0.86%/0.17%) and reconstituted with PC:PS:CHL (HEN buffer plus 10.7 mM CaCl2) at a total lipid concentration of 1 mM. (D) Reconstituted PC:PS:CHL alone (no receptor, HEN buffer, 10.7 mM CaCl2, 1 mM total lipid). (E) A sample of purified M2 receptor (digitonin–cholate, 0.10%/0.02%) reconstituted in PC:PS:CHL (HEN buffer, 10.7 mM CaCl2, 1 mM total lipid)) was overlaid on top of the preformed lipid bilayer in Figure A-1D. (F) Purified M2 receptor (digitonin–cholate, 0.10%/0.02%) reconstituted in PC:PS:CHL (HEN buffer, 10.7 mM CaCl2, 1 mM total lipid) was laid directly onto the mica surface (i.e., without a preformed lipid bilayer).
Fig. A-2 — Effect of repetitive freezing and thawing on vesicular size assessed by electron microscopy and atomic force microscopy. Images obtained with the electron microscope (Panels A–D) were taken at 50,000× magnification, and those obtained with the atomic force microscope (Panels E–H) were taken from an area having a scan size of 5 μm × 5 μm. (A) Purified M2 receptor (digitonin–cholate, 0.10%/0.02%) reconstituted in phosphatidylcholine (PC), phosphatidylserine (PS), and cholesterol (CHL) (PC:PS:CHL at a molar ratio of 4.5:4.5:1.0) was subjected to five freeze-thaw cycles in a dry ice-acetone bath. The black horizontal bar demarcates a width of 100 nm. (B and G) Purified M2 receptor reconstituted in PC:PS:CHL (4.5:4.5:1.0) which had not undergone repetitive freezing and thawing. (C and F) Purified M2 receptor was mixed with PC:PS:CHL (4.5:4.5:1.0) and subjected to five freeze-thaw cycles. (D and E) PC:PS:CHL (4.5:4.5:1.0) alone, which had not undergone the reconstitution procedure but was subjected to five freeze-thaw cycles. (H) The image was obtained by injecting an aliquot of the sample from Figure A-2G and subsequently injecting an aliquot of the sample from Figure A-2F.
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