AN ABSTRACT OF THE THESIS OF

Christine Cremo for the degree of Doctor of Philosophy in Biochemistry and Biophysics presented on July 15, 1983.

Title: Characterization and Photoaffinity Labeling of the Muscarinic Acetylcholine Receptor

Abstract approved: Redacted for privacy

Michael I. Schimerlik

The muscarinic acetylcholine receptor, identified by tritiated L-quinuclidinyl benzilate (L-{^{3}H}QNB) binding, was solubilized from porcine atrial membranes using a 5:1 (w/w) ratio of digitonin and cholate. Specific binding activities of the solubilized receptor solutions usually exceeded 1.0 nmol L-{^{3}H}QNB sites per gram of protein, representing 75-98% total site recovery and a two-to threefold enrichment over untreated atrial membranes. Two rapid assays for measuring the binding activities of detergent extracts were devised and compared with equilibrium dialysis. All three methods gave similar results. The equilibrium dissociation constant of the solubilized receptor for L-{^{3}H}QNB as determined by the three methods varied from 230 to 450 pM depending on the method and temperature.

The interaction of alkyl guanidines and decahydro-

histrionicotoxin with the membrane-bound and solubilized muscarinic acetylcholine receptor (mAcChR) from porcine atria was described. Alkyl guanidines with alkyl chain lengths from one to ten carbons displaced $\{^3H\}$L-quinuclidinyl benzilate ($\{^3H\}$L-QNB) competitively from a single class of sites for the membrane-bound mAcChR. From a plot of $-\ln K_i$ versus alkyl carbon chain number, a value of $-(473 \pm 30)$ cal/mol was estimated as the energetic contribution per methylene group to the total binding energy. The binding of alkyl guanidines to the digitonin/cholate solubilized mAcChR was complex in nature resulting in titration curves that did not obey the law of mass action for simple competitive inhibition at higher alkyl carbon numbers and a sigmoidal plot of $-\ln K_i$ versus carbon number. Decahydrohistrionicotoxin bound in a competitive manner versus $\{^3H\}$L-QNB to both the membrane-bound ($K_i = (6.9 \pm 1.4) \times 10^{-6}$ M) and the solubilized ($K_i = (1.5 \pm 0.3) \times 10^{-5}$ M) preparations.

The synthesis and properties of a radiolabeled muscarinic antagonist photoaffinity probe, $\{^3H\}$ p-azidoatropine methyl iodide were reported. The molecule bound specifically in phosphate buffer in the dark to the membrane-bound, digitonin-cholate solubilized and wheat germ affinity purified mAcChR with an equilibrium dissociation constant of approximately 75 nM.

Ultraviolet irradiation of the {^{3}H} probe and partially purified mAcChR fractions, followed by analysis of covalently incorporated radiolabel by sodium dodecyl sulfate gel electrophoresis showed specific labeling (protectable by L-QNB) of one or more polypeptides at 70-79,000 relative molecular weight.

CHARACTERIZATION AND PHOTOAFFINITY LABELING OF THE MUSCARINIC ACETYLCHOLINE RECEPTOR

by

Christine Cremo

A THESIS

submitted to

Oregon State University

in partial fulfillment of
the requirements for
the degree of

Doctor of Philosophy

Completed July 15, 1983

Commencement June 1984

APPROVED:

Redacted for privacy

Professor of Biochemistry/Biophysics in charge of major

Redacted for privacy

Head of Department of Biochemistry/Biophysics

Redacted for privacy

Dean of Graduate School

Date thesis is presented July 15, 1983

Typed by Jackie Morse for Christine Cremo

Acknowledgements

I thank Professor Mike Schimerlik for his help with every aspect of my scientific training. During the course of my research I benefitted from discussions with Gary Peterson, Tambouie Deffo, Sonia Anderson, Dean Malencik and Boyd Haley. I thank Professor Anderson for use of her fluorescence spectrophotometer and Professor I. Isenberg for use of his darkroom. Chi Juinn Pan must be especially thanked for her work of endlessly supplying membrane protein for my work. Steve Miller wrote computer programs that proved very useful in my data analysis. Steve Miller, Wendy-Lou Manley, Tom Tibbitts and Merri Strayer all provided excellent technical assistance. Thank you, Jackie Morse for your practically flawless typing job. Finally, I want to thank Mike Williams for recording the melting point of p-azidoatropine methyl iodide, and Caroline Cremo for the gift of a coffee cup which I used throughout the duration of my work at OSU.

Table of Contents

LIST OF FIGURES

LIST OF FIGURES
(Continued)

LIST OF TABLES

CHARACTERICATION AND PHOTOAFFINITY LABELING OF THE MUSCARINIC ACETYLCHOLINE RECEPTOR

An Overview

Ion channels in membranes play an important role in the transmission of electrical and chemical signals in biological systems. The muscarinic acetylcholine receptor (mAcChR) in the heart is thought to mediate important changes in ion fluxes and concentrations across membranes of special classes of heart cells that are responsible for regulating the heartbeat. It is not known how the interaction of *in vivo* chemical signals with the mAcChR evoke the well studied physiological responses by way of modulations in membrane ion conductances.

Much of what is known about recognition of extracellular signals and subsequent transduction into electrophysiological responses pertains specifically to the fast responses of the post synaptic potentials of the skeletal neuromuscular junction or motor nerve terminals (Katz, 1966). The acetycholine receptor mediating these responses is classified pharmacologically as nicotinic, on the basis of its interation with certain drugs. The nicotinic acetylcholine receptor (nAcChR) is the first neurotransmitter receptor to be purified; significant

progress has been made toward elucidating a complete mechanism of receptor activation and describing how the receptor controls the flow of ions across the post-synaptic membrane (Conti-Tronconi and Raftery, 1982).

The mAcChR in cardiac cells mediates a slow inhibitory (hyperpolarizing) postsynaptic potential, defined as a response which begins with a lag period of tens of milliseconds after transmitter release. In the heart, these slow responses can last several seconds (Hartzell, 1981). These different characteristic response times of the respective postsynaptic membranes mediated by mAcChR vs. nAcChR indicate fundamental differences that are clues to their mechanisms of action.

In general, the ionic basis of the cardiac action potential differs significantly from the more thoroughly characterized ionic mechanisms responsible for the action potential of the motor end plate. These differences probably reflect specializations in the ion channels and the mechanisms by which they are modulated.

mAcChRs are distributed throughout peripheral tissues that receive parasympathetic ennervation (intestines, urinary tract, vascular smooth muscle, exocrine glands and intraocular) and the central system. Despite this widespread distribution and importance to neural function, biochemical characterization of the mAcChR lags far behind that for the nAcChR. To date, the

mAcChR has not been purified to homogeneity from any tissue. It is possible that cardiac mAcChRs differ significantly from those isolated from other organs.

The remainder of this introduction is divided into two parts. The first is a general short discussion of the physiology of the mAcChR in the heart. The above introductory statements will be extended, in the second part, to a discussion of the kinetics of the slow muscarinic responses in the heart and how they are possibly mediated. The introductions of Chapters 2, 3 and 4 will summarize some recent advances in the structural and functional characterization of mAcChRs from various tissues.

The three manuscripts presented in this thesis describe some structural, kinetic and pharmacological properties of the mAcChR isolated from the atria of the pig heart. The manuscripts are presented in the order that the experiments were performed. Discussions of solubilization and pharmacological characterization of the mAcChR are found in the introductions to Chapters 2, 3 and 4. Because the technique of photoaffinity labeling is central to the structural characterization described in Chapter 4, Chapter 4 will include a review of this technique.

In general, the purpose of the research described in this thesis is to contribute ideas and information useful

to the complete characterization of the mechanism of action of the mAcChR. This in turn will supplement knowledge, at a fundamental biochemical level, of the mechanisms of ion channels in membranes and how they are controlled by receptor proteins. This research, being pharmacologically oriented, may also contribute to advances in clinical treatments of cardiac disfunctions.

Physiology of the mAcChR in the Heart

The sinoatrial node acts as the normal pacemaker in the adult heart (Katz, 1977). The rhythmic heartbeat reflects a cyclic change in the membrane potentials of pacemaker cells (known as automaticity) in the sinoatrial node (West, 1972; Katz, 1977). Pacemaker cells initiate a wave of electrical depolarization that is responsible for initiating systole in all regions heart (West, 1972; Katz, 1977). Cardiac muscle normally behaves as a functional synctium where a rapid spread of electrical excitation travels to all regions of the heart after stimulation of one region (Sperelakis, 1979). Nerves do not participate in the propogation of the wave of excitation through the heart. The sinoatrial node frequency and conduction in the sinoatrial node, atrioventricular node and atrial tissue is modulated in a complex manner by the antagonistic effects of the parasympathetic and sympathetic efferent (from the central to peripheral nervous system) fibers of the autonomic (visceral or involuntary) nervous system (Higgins et al., 1973; Levy, 1971; Vanhoutte and Levy, 1980; Muscholl, 1980).

Acetylcholine (AcCh) released by the vagal nerves, inhibits, and norepenephrine, liberated by the sympathetic nerves, facilitates the pacemaker. The postsynaptic receptor for AcCh in the heart is distinguished pharmacologically as muscarinic (Dale, 1914; Purves, 1976; Magazanik, 1976). It is in general agreement that AcCh interaction with the muscarinic acetylcholine receptor in the heart results in a decrease in the rate (negative chronotropic) and amplitude (negative inotropic) of the action potential in the sinoatrial node, atrioventricular node, and atrial tissue (Higgins et al., 1973). The results of electrophysiological studies indicate that the effect of AcCh to slow the heart rate and decrease force of contraction is a result of an increase in the permeability of the sarcolemma to potassium ions (Trautwein et al., 1956; Giles and Noble, 1976; Burgen and Terroux, 1953; Trautwein and Dudel, 1958; Hartzell et al., 1977; Ten Eick et al., 1976) and a reduction in the amplitude of the slow inward calcium current (Giles and Noble, 1976; Ikemoto and Goto, 1977; Nathan and Beeler, 1975; Nawrath, 1976; Hino and Ochi, 1980) respectively.

In contrast to nerve tissue, potassium conductance decreases during depolarization in the myocardium (Katz, 1977). This voltage-independent K^+ current was first demonstrated by Noble and Tsein (1969). Stimulation of the vagus nerve causes AcCh release, which then binds to

its postsynaptic receptor (mAcChR). The electrical consequences of vagal stimulation increases outward potassium current which slows the heartrate can be explained in terms of resting potential changes immediately prior to depolarization. A partially depolarized state prior to normal electrical stimulation produces a more slowly rising action potential, and thus a slower heartbeat. Thus, the effect of vagal interaction which is mediated by the mAcChR is inhibitory, decreasing the likelihood that the pacemaker cell will reach threshold and initiate an action potential.

A decrease in the slow inward calcium current results in a lowered calcium concentration in the cardiac muscle cell. Contractile protein function in smooth muscle in general (Bolton, 1979) and in cardiac muscle (Katz, 1977; Nawrath, 1977) depends primarily upon ionized calcium concentrations within the sarcoplasm.

In smooth and cardiac muscle cells that generate and propagate action potentials, the changes in calcium current resulting from the action potential itself and Ca^{2+} release from the sarcoplasmic reticulum are the most important mechanisms effecting changes in calcium concentrations important to contractile protein function (Bolton, 1979; Kehoe and Marty, 1980; Reuter, 1983). AcCh interaction with the mAcChR leads to changes in membrane electrical function which relate to the frequency and magnitude of impulse generation.

Kinetics and Mediation of the Muscarinic Response

Changes in potentials that are elicited by muscarinic agents in the heart are grouped together under the heading of slow postsynaptic potentials (Kehoe and Marty, 1980). Slow postsynaptic potentials in general are defined as responses with a lag period (latency) of tens of milliseconds (Hartzell, 1981) and may be either excitory or inhibitory. The inhibitory response (slowing of the heartbeat) observed by stimulation of the vagus nerve, releasing AcCh that binds to receptors classified as muscarinic (Mayer, 1980; Dale, 1914) has two features: a minimum latency near 100 ms (Purves, 1978; Kehoe and Marty, 1980; Hartzell, 1981) and a duration of stimulus 0.5 second or longer. This is in contrast to the skeletal neuromuscular junction (Neher and Stevens, 1977; Heidmann and Changeux, 1978) where the effects of AcCh are observed in 0.2 msec and synaptic potentials last only 30-100 msecs. The utility of the slow synaptic response may be recognized in the heart, where transmitter action must extend over several cycles of the heartbeat (Purves, 1976). If the inhibitory inputs of vagal stimulation on the pacemaker for example were as fast as at the neuromuscular junction, only those inputs arriving immediately before discharge would have any effect. Most impulses would be ineffective. Slow postsynaptic potentials can increase the active time

of the synapse, thus increasing temporal interactions of many responses (Hartzell, 1981). The long response times (latency) after release of neurotransmitter have no obvious functional significance (Purves, 1976), and are not well explained by slow presynaptic events, like slow diffusion of transmitter to receptor due to a peculiar morphology (Kehoe and Marty, 1980; Hartzell, 1981; Purves, 1976), but is probably a postsynaptic phenomenon (Hartzell, 1981). For example in cardiac muscle, receptors are not localized to regions which appear to be inaccessible to transmitter (Hartzell, 1980).

Electrophysiological experimentation has provided clues to the mechanism of slow synaptic responses on the basis of kinetic measurements. A variety of mechanisms have been proposed to explain slow synaptic events mediated by receptors for neurotransmitters. This section is a review of the events mediated by mAcChRs in the heart from and electrophysiological and biochemical point of view.

Muscarinic agonists activate a specific K^+ conductance in heart muscle (Giles and Noble, 1976; Noble and Tsein, 1969; review, Higgins, et al., 1973; Vassalle, 1979). Kehoe and Marty (1980) have reviewed recent electrophysiological studies which point to a channel rather than a carrier permeation mechanism with very long channel open times.

The mechanism underlying the rapid potential changes observed at the neuromuscular junction are relatively well characterized (Neher and Stevens, 1977). For example, a rapid potential change results when AcCh interacts with the nAcChR, which in turn directly activates a permeation process (selective for Na^+ and K^+ that was otherwise inactive (Conti-Tronconi and Raftery, 1982). These results are well explained by the two-state model for channel activation,

$$A + R \underset{\text{fast}}{\rightleftharpoons} AR \underset{\text{rate-limiting}}{\rightleftharpoons} (AR)^*$$

where A=agonist, R=receptor, AR=closed channel, (AR)*= open-channel. The major differences between these fast channels and the slow K^+-dependent channels are the mean channel open times and the latency of response. The slow decay (related to channel open times) depends upon the on and off rates of transmitter-receptor interactions. If agonists dissociate slowly enough, the slow decay could be explained (Birdsall and Hulme, 1976). The latency of the slow response however, is not well explained by kinetics of simple transmitter-induced conformational channels of a channel. Therefore, models involving cooperative protein-protein coupling within the membrane (Cuatracasas, 1974) and intermediate

compounds such as Ca^{2+} and cyclic mucleotides (Greengard, 1976), which insert several steps between agonist binding and channel opening times, have been proposed. Presently, the evidence for Ca^{2+} involvement in the regulation of the K^+ channel is not conclusive and evidence for a role for cyclic nucleotides is either lacking or negative (Kehoe and Marty, 1980).

The decrease in pacemaker activity in the heart due to release of AcCh is not only due to an increase in permeability of the myocardial cell membrane for K^+ ions but also a reduction is the slow inward voltage dependent Ca^{2+} current (Vassalle, 1979; Reuter, 1979). There are two inward membrane currents responsible for the action potential in the heart, one specific for sodium, one for calcium (I_{si}, slow inward). I_{si} is important for the plateau phase of the action potential and can be modulated by neurotransmitters (Reuter, 1979). Adrenaline and noradrenaline acting through β-adrenergic receptors increase I_{si} and muscarinic agonists, acting through muscarinic receptors, decrease I_{si}. This decrease in calcium entering the cell corresponds to the negative inotropic (decrease in contraction force) response to vagal stimulation in the heart.

The slow Ca^+ current probably involves quantitatively different mechanisms than K^+-dependent slow current.

Neurotransmitters seem to modify a conductance that can be activated by membrane potential changes not related to neurotransmitters (Kehoe and Marty, 1980). Therefore, the amplitude of a voltage-dependent membrane permeability (I_{si}) is determined by neurotransmitter action.

A principal direction of research on the β-adrenergic and muscarinic receptor systems at this time is to answer the question of how I_{si} and other functions are modulated. The answer will be closely related to the structure of the receptors and channels and any other proteins or messengers involved in the regulatory mechanisms.

Kinetic electrophysiological results suggest that transmitters don't affect the voltage-dependent kinetics of I_{si} but greatly increase the maximal conductance (Reuter, 1983). This may mean that at a given voltage, muscarinic agents decrease the average number of open channels (Hartzell, 1982; Reuter, 1979), as opposed to transitions of individual channels from one state to another.

A second messenger model has often been invoked to explain transmitter-induced changes in voltage dependent conductances (Greengard, 1976) such as I_{si} in post-synaptic cells. The question as to second messenger involvement has not been resolved for the muscarinic receptor mediation, much the result of the lack of

structural characterization. The very long time courses of muscarinic responses are consistent with a second messenger mechanism (Purves, 1976; Hartzell, 1981; Kehoe and Marty, 1980; Birdsall and Hulme, 1976).

Greengard (1976) has outlined some of the experimental criteria that might indicate mediation by cyclic nucleotides of postsynaptic potentials. There is increasing evidence that cyclic quanosine monophosphate (c-GMP) is involved as a second messenger in mediating the muscarinic response, although this is still an open question. This mediation may be a result of a reduction in availability of Ca^{2+} channels or of membrane proteins associated with Ca^{2+} channels through phosphorylation by c-GMP-dependent protein kinases. A similar system involving a receptor associated adenylate cyclase (Strosberg et al., 1980) and c-AMP-dependent protein kinases may be involved in the β-adrenergic effect on I_{ca} in the heart (Reuter, 1977; Kehoe and Marty, 1980; Tsein, 1977). Nathanson et al. (1978) have reported a mAcChR mediated inhibition of adenylate cyclase in cultured neuronal cells. The question of reciprocal modulation of adenylate cyclase by β-adrenergic and muscarinic agents is still not answered (Hartzell and Titus, 1982).

George et al. (1970) showed significant increases

in c-GMP levels (w/o changes in c-AMP) in AcCh perfused rat hearts which paralled the negative inotropic effect (associated with I_{si}) but not the negative chronotropic effect (associated with K^+ dependent channels). Lee et al. (1972) showed that this effect could be blocked by muscarinic antagonists but not nicotinic antagonists. Using an indirect measure of contractile force, the negative inotropic action of 8-bromo-c-GMP in rat auricles (Nawrath, 1976) was shown to be due to effects on I_{si} (Nawrath, 1977). Therefore, c-GMP has been shown to mimic the conductance changes ellicited by AcCh. However, Linden and Brooker (1979) could not mimic these physiological effects with c-GMP in rat atria. Therefore, the role of c-GMP is still subject to debate. Only similarities in conductance changes and not absolute identity of ionic events has been proven to date.

The next step in characterizing and proving a direct role of c-GMP-protein kinase systems in changing post-synaptic potentials affected by muscarinic agents is to identify target proteins whose phosphorylation is essential to the ultimate effect on the heartbeat. Rinaldi et al. (1981) have demonstrated a β-adrenergic effect on a c-AMP-dependent specific membrane protein phosphorylation which paralells a stimulation of Ca^{2+} flux in sarcolemmal vesicles. A c-GMP dependent protein kinase sensitive

to c-GMP induced by AcCh has been identified in rat heart (Lincoln, 1981). No endogenous substrates in the heart for a c-GMP-dependent protein kinase have been identified.

Several other second messenger systems could possibly play a role in Ca^{2+}-dependent slow postsynaptic potentials mediated by mAcChRs. Hokin and Hokin (1953) were the first to describe the now well-studied effect of stimulation of phosphatidylinositol (PI) and phosphatidic acid turnover by muscarinic agents. Michell (1975) has postulated that increased hydrolysis of PI to diacylglycerol and inositolphosphates may be a general mechanism whereby certain neurotransmitters and hormones activate Ca^{2+} gates. Although no specific mechanism to explain this behavior has been proposed, phosphatidic acid has been shown to act as a Ca^{2+} ionophore (Tyson, 1976). Although various receptors capable of regulating calcium flux are also capable of stimulating phosphatidylinositol turnover (Cohen et al., 1983) no direct relationship between PI turnover and Ca^{2+} conductance changes have been demonstrated. Although the nature of a plasma membrane phospholipase activity possibly activated by neurotransmitters is not well established, the cytoplasmic phospholipase is activated by Ca^{2+} itself (Hawthorne, 1982). Thus, it is possible that Ca^{2+} and not neurotransmitters stimulate the phospholipid breakdown. In

general the dependence upon Ca^{2+} of the PI effect (Fisher and Agranof 1980; Griffin et al., 1979) is crucial to the calcium gating theory proposed by Michell (1975) and the confused state of this matter does not allow for confirmation of the theory at this time (Hawthorne, 1982).

Another possible mechanism for the regulation of the postsynaptic sensitivity mediated by mAcChRs is "down-regulation," or regulation by a decrease in the number of receptors by prolonged agonist exposure (Nathanson, 1982). Galper and Smith (1980) have shown that down regulation by agonists is correlated with a decrease in the negative chronotropic response in cultured embryonic chick heart cells.

There is evidence that cardiac mAcChRs can be regulated by guanine nucleotides. Results from this laboratory (Schimerlik and Searles, 1980) and other laboratories (Cavey et al., 1977; Fields et al., 1978; Galper and Smith, 1978; Galper et al., 1977) indicate that binding of agonists to cardiac mAcChRs does not follow the law of mass action for a single class of sites. In the heart, guanine nucleotides significantly decrease the affinity of agonists without affecting antagonist affinity (Berrie, et al., 1979; Rosenberger, 1979; Wei and Sulakhe, 1980; Waelbroeck, 1982). It is

not known whether mAcChR mediated postsynaptic conductances changes are associated with guanine nucleotide effects. It has been proposed that the nucleotide binding protein associated with the β-adrenergic receptor coupled adenylate cyclase (Strosberg, 1980) may be also associated with the mAcChR (Sokolovsky and Bartfai, 1981).

SOLUBILIZATION OF THE ATRIAL MUSCARINIC ACETYLCHOLINE RECEPTOR: A NEW DETERGENT SYSTEM AND RAPID ASSAYS

Acknowledgement of Co-Authorship

I was a co-author of this publication, which resulted from a research effort initiated and directed by Dr. M.I. Schimerlik. This paper was published under the authorship: Christine Cremo, G. Scott Herron, and M.I. Schimerlik. In accordance with graduate school requirements I will designate my contribution to this publication.

At the time I began research towards my Ph.D. under the supervision of Dr. M.I. Schimerlik, the membrane-bound muscarinic acetylcholine receptor from pig hearts had been recently characterized with respect to mechanism of ligand binding (Schimerlik and Searles, 1980). The primary focus at that time was two-fold; first to solubilize and purify the membrane-bound receptor away from other membrane-bound proteins in some way and second, to develop a quick, relatively easy assay to measure the concentration of the soluble receptor with respect to $\{^3H\}$L-QNB binding sites. The only method we had at the time to determine this was to separate quickly, reversibly bound from free $\{^3H\}$L-QNB using a P10 gel filtration column. I participated along with G. Scott Herron, W.L. Manley, Steve Miller, and Tom

Tibbets in screening detergent solubilization procedures with this method, which ultimately led to the published method. Similarily, I worked to develop rapid assay procedures suitable for use with the digitonin/cholate detergent system which was finally found to solubilize the receptor. Both Scott Herron and I wrote the manuscript which was revised by M.I. Schimerlik.

Introduction

Molecular characterization of the mAcChR[1] has been hampered by the fact that a rich source of this receptor is not available (Rang, 1975). In addition, solubilized mAcChRs that retain ligand binding activity have been difficult to prepare (Birdsall and Hulme, 1974). Relatively low yields of muscarinic ligand binding sites have been obtained in soluble form from brain tissue using saturated digitonin (Aronstam et al., 1978; Beld and Ariens, 1974; Garrisen et al., 1978; Hurko, 1978; Rue and Liefländer, 1979; Repke and Matthies, 1980), Lubrol PX (Haga, 1980) and high salt concentrations (Alberts and Bartfai, 1976; Carson et al., 1977). Extraction of atrial membranes with the mixed detergent system to be described permitted a near quantitative {^{3}H}L-QNB site recovery. This paper describes the first solubilization of mAcChRs from cardiac tissue by any method. Rapid assays employing PEG-6000 to preciptate the receptor-QNB complex and DEAE filter discs were developed and used to quantitate specific binding activities in detergent extracts.

---

[1]Abbreviations used: mAcChR, muscarinic acetylcholine receptor; PEG-6000, polyethylene glycol-6000; DEAE, diethylaminoethyl; PMSF, phenylmethanesulfonyl fluoride; {^{3}H}L-QNB, L isomer of tritium labeled quinuclidinyl benzilate; EDTA, ethylenediaminetetraacetic acid.

Methods

A. Materials

{^{3}H}L-QNB (40.2 Ci/mMol), purchased from New England Nuclear, cochromatographed with a non-labeled standard (the generous gift of Dr. W.E. Scott, Hoffman-La Roche Inc.) on Merck silica gel 60 plates in chloroform:methanol:water:acetic acid (65:25:5:5; R_f=0.58 and acetone:methanol:diethanolamine (10:10:0.3; R_f=0.38) solvent systems. Over 90% of the radioactivity was found in the QNB spot. EDTA, bovine serum albumin, polyethylene glycol 6000, digitonin (Lot No. 19C-0329), cholic acid, aprotinin, pepstatin, γ-globulin, phenylmethanesulfonyl fluoride, bacitracin, sodium azide, and atropine were purchased from Sigma Chemical Co. 2.4 and 2.5 cm DEAE (DE81) and GF/B filter discs were purchased from Whatman. Buffer A included 50 mM sodium phosphate pH 7.4, 1 mM EDTA, 0.1 mM PMSF, 10 μg/ml bacitracin and 0.02% sodium azide. Buffer B is defined as Buffer A with 0.4% (w/v) digitonin and 0.08% (w/v) cholate included. Buffer C is defined as Buffer A with 4% (w/v) digitonin and 0.8% (w/v) cholate.

B. Preparation and solubilization of membranes

Atrial membrane preparation. Atrial microsomes were prepared from fresh or frozen pig hearts as described by Schimerlik and Searles (1980). Specific activities generally ranged between 300 and 500 pMol QNB

sites per gram protein.

Solubilization procedure. Frozen atrial membranes were thawed immediately before use and diluted with warm (22°C) Buffer A and Buffer C to final protein and detergent concentrations of 3.0-3.5 mg/ml and 0.4% (w/v) digitonin, 0.08% (w/v) cholate, respectively. After 5 minutes of occasional swirling at room temperature, the mixture was centrifuged at 100,000 x g for 60 min at 4°C. The clear amber supernatant was removed with a pipette and stored at 0°C.

C. {^{3}H}L-QNB binding assays

{^{3}H}L-QNB binding to membrane preparations. mAcChR concentration was quantitated in terms of {^{3}H}L-QNB sites as described by Yamamura and Snyder (1974). Protein concentration was measured by the method of Lowry et al. (1951), as modified by Peterson (1977), with crystalline bovine serum albumin as a standard.

{^{3}H}L-QNB binding to solubilized receptors. PEG precipitation assay. 0.1 to 0.8 ml of detergent extract was mixed with Buffer B to a final volume of 1.0 ml in the presence of 10 nM {^{3}H}L-QNB. Nonspecific binding controls included 100 μM atropine. Equilibrium was reached after incubation for 60 min at room temperature unless otherwise stated. The mixture was placed on ice for 10 min and then precipitated by adding 0.1 ml of cold 0.6% (w/v) γ-globulin and 0.9 ml of cold 36%

(w/v) PEG and vortexing for 10 sec. After 25 min on ice, duplicate 0.9 ml aliquots were pipetted onto 2.4 cm glass fiber filters (GF/B) mounted on an Amicon vacuum manifold and immediately washed 3 times with 5 ml of ice cold 16% PEG. The dry discs were then placed into 5 ml of Triton-toluene scintillation fluid and allowed to stand at least 1 hr before counting.

DEAE filter disc assay. The DEAE filter binding assay was modified from that originally developed by Schmidt and Raftery (1973) and later used by Hurko (1978) in studies on soluble brain mAcChRs. Detergent extracts were incubated as described in the PEG precipitation assay except that the buffer consisted of 10 mM sodium phosphate pH 7.4, 1mM EDTA, 0.4% (w/v) digitonin, 0.08% cholate, 0.02% sodium azide, 10 μg/ml bacitracin, 0.1 mM PMSF. Aliquots were pipetted onto DEAE filter discs that were impaled and suspended on pins or placed flat on a sheet of aluminum foil. Maximum volumes pipetted were 0.1 ml for 2.4 cm and 0.15 ml for 2.5 cm filters. After 1-10 min, filters were washed at room temperature by stirring in 200-500 ml of 10 mM sodium phosphate pH 7.4, 1mM EDTA buffer for 20 min. Filters were removed from the wash, blotted on paper toweling and placed into 5 ml Triton-toluene scintillation fluid and counted after one hour.

Equilibrium dialysis. To quantitate the solubilized

receptor in terms of QNB binding sites, aliquots of detergent extract in 1 cm dialysis tubing were dialyzed separately against 25 ml of Buffer B containing 10 nM {^{3}H}L-QNB for 30 hr at 4°C. For equilibrium constant determinations equal aliquots of extract were dialyzed against varying QNB concentrations in the dialysate. Non-specific binding controls included 100 μM atropine. 100 μl aliquots were removed from the bags and dialysate and counted in 5 ml of Triton-toluene scintillation fluid. Net binding was measured by the difference in radioactivity between equal volumes of protein solution and dialysate.

D. Stability studies

Frozen membranes that had been prepared in the presence of 10 μg/ml bacitracin and 0.1 mM PMSF were diluted 100 fold in 10 mM sodium phosphate pH 7.4, 1 mM EDTA buffer and centrifuged at 30,000 x g for 1 hr at 4°C. Pellets were resuspended and extracted as described above except that no protease inhibitors were present in the buffers. At time = 0, {^{3}H}L-QNB and/or various protease inhibitors were added to aliquots of the extract and the mixture was either placed on ice or allowed to stand at room temperature. Protease inhibitors were added to the following final concentrations: pepstatin, 1 μg/ml; aprotinin, 20 Killikrein Inhibitor Units/ml; bacitracin, 10 μg/ml; and PMSF, 0.1 mM.

{^{3}H}L-QNB binding was measured as described for the DEAE assay except that the {^{3}H}L-QNB concentration was 15 nM.

Results and Discussion

Solubilization. Opalescent suspensions of aqueous digitonin clarify in the presence of cholate, thus eliminating the need for heat and subsequent precipitation to prepare accurate detergent buffers. Buffer B did not precipitate at 4°C for up to six months. The mixed detergent ratio of 5:1 digitonin to cholate represents the minimum proportion of cholate which completely solubilized a 1% (w/v) suspension of aqueous digitonin.

The concentration effect of this 5:1 detergent mixture on the extraction of mAcChRs was determined over the range of 0.1%-1.4% (w/v) digitonin (data not shown). A maximum in the specific activity (nMole of {^{3}H}L-QNB sites per gram of protein) of the extract was found at digitonin concentrations between 0.3-0.4% (w/v). The higher detergent concentrations did not significantly increase the total activity solubilized (or inactivate the solubilized mAcChR); however, lower specific activities were found due to an increase in total protein extracted from the microsomes.

In contrast to previous methods which used saturated digitonin solutions to solubilize brain tissue at 0°C for 1 hr, extraction with the digitonin-cholate detergent system was complete in 5 min at 19-22°C. Membrane protein concentration during extraction was varied between 0.5 and 6.2 mg/ml (data not shown).

Maximum {^{3}H}L-QNB specific binding activity and site recovery was obtained with protein concentrations between 2.7 and 3.2 mg/ml. Centrifugation of the detergent treated membranes between 15,000 and 150,000 x g for 1 hr made no difference in the recovery of total {^{3}H}L-QNB sites from the supernatant. However, at the higher angular velocities, specific binding activities were increased by approximately 10% if the supernatant was carefully decanted to just above a viscous, yellow fluid which layered over the unsolubilized membranes.

Under solubilization conditions described above, specific binding activities of 1.0 nMol {^{3}H}L-QNB sites/ g protein and protein concentrations of approximately 1.0 mg/ml were routinely obtained in the 100,000 x g supernatants. These values ranged between 0.7-2.0 nMol {^{3}H}L-QNB sites per gram protein and 0.7-1.2 mg/ml, respectively, depending on the membrane preparation used for solubilization. In a typical experiment, about 87% of the total QNB sites and 28% of the total protein in the membrane fractions were extracted using the mixed detergent system. Recoveries of QNB sites ranged between 75% and 98% which corresponded to a 2-3 fold purification over untreated membranes.

Using saturated digitonin solutions, solubilized muscarinic ligand binding site recoveries from various brain preparations between 5 and 50% have been reported.

Haga (1980) reported 26% QNB site recovery from rat brain using 0.32% Lubrol PX. We found 43% recovery of QNB sites from atrial membranes and binding activities of approximately 450 pMol {^{3}H}L-QNB sites/ g protein using saturated digitonin solutions according to the solubilization procedure of Hurko (1978).

Assay results. PEG and other high molecular weight non-ionic polymers have been used to fractionate macro-molecules by selective exclusion from the solvent system (Fried and Chun, 1971). Methods for separating protein-ligand complexes from free ligand using PEG and a coprecipitant such as γ-globulin have been developed for radioimmunoassays (Desbuquois and Aurbach, 1971) and peptide hormone receptor complexes in detergent solutions (Cuatrecases, 1972). A modification of this procedure was used here to precipitate {^{3}H}L-QNB bound mAcChRs in a digitonin-cholate buffer. The PEG assay requires approximately one third (25 vs 90 min) the precipitation time required by the ammonium sulfate precipitation procedure of Hurko (1978). In addition, the presence of the cholate ion may interfere with the salting-out process, although this possibility has not been tested. The interference of other ions is unlikely in PEG pre-cipitation since the relative decrease in solubility of proteins in polymer solutions is independent of salt concentration and pH (Fried and Chun, 1971).

The results shown in Fig. 1 demonstrate that $\{^3H\}$L-QNB binding to detergent extracts, as measured by the PEG assay, is linear between 35 and 260 μg protein filtered. For the PEG assay, binding curves are linear up to 900 μg of protein filtered. At saturating levels of radioligand, both DEAE filter disc and equilibrium dialysis assays also yielded linear plots of specifically bound $\{^3H\}$L-QNB versus protein nearly identical to the representative binding curve shown in Fig. 1. Using the same extract, specific activities obtained by these three methods are in good agreement, as can be seen in Table I. Therefore, it is reasonable to assume that the PEG assay as described above quantitatively precipitates the radioligand-receptor complex from the mixed digitonin-cholate detergent buffer.

The results of optimization experiments in which the concentrations of PEG and γ-globulin were independently varied are presented in Fig. 3. Quantitative precipitation of QNB sites occurred only at PEG and γ-globulin concentrations equal to or higher than 16% (w/v) and 0.03% (w/v), respectively. QNB site precipitation does not vary between 0.13% (w/v) and 0.03% (w/v) digitonin (5:1 digitonin-cholate) present at the precipitation step. At 0°C, at least 25 min was required for complete precipitation.

The results of optimization experiments in which

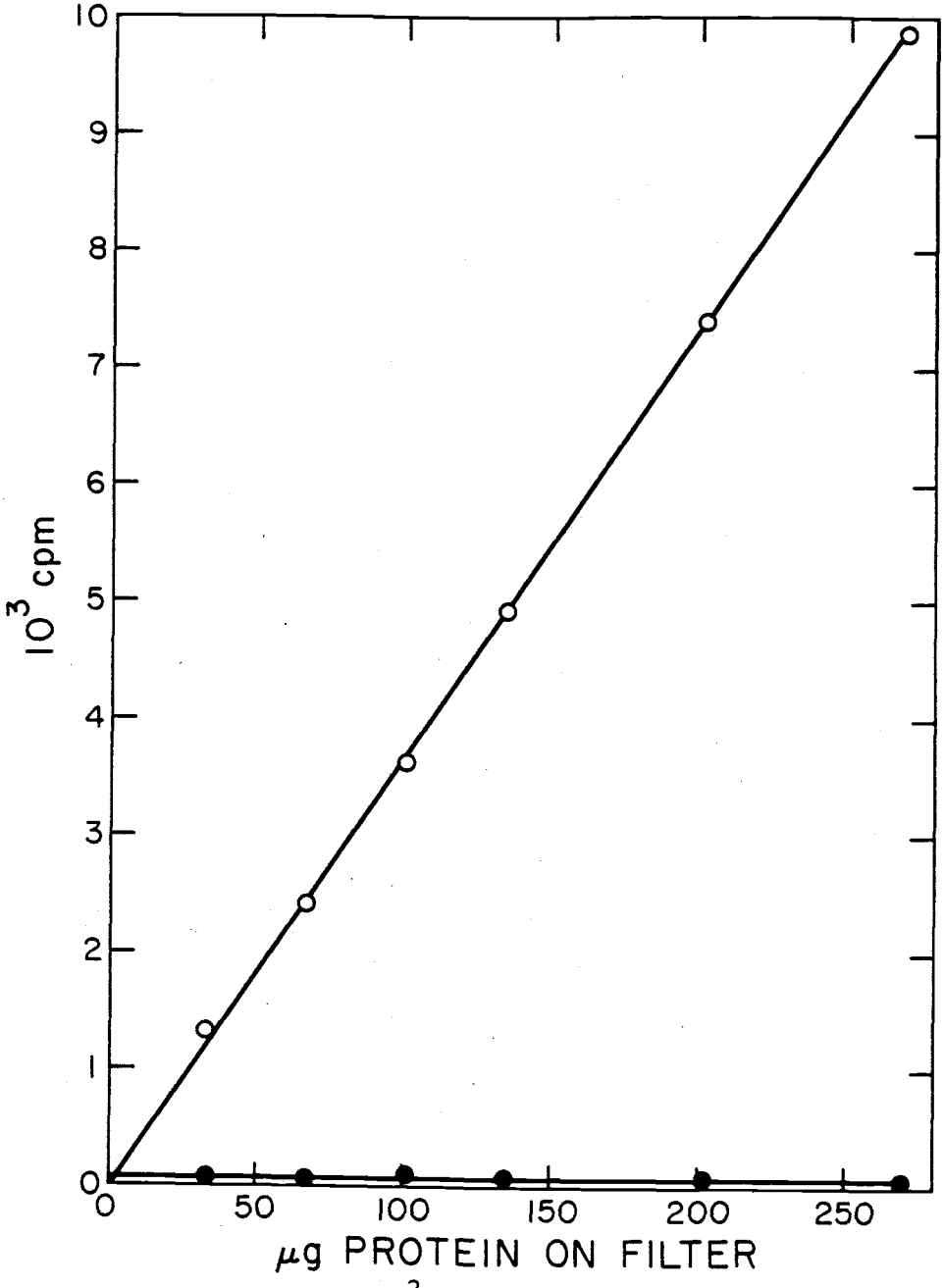


Fig. 1 Binding of {^{3}H}L-QNB to detergent extracts of atrial membranes detected by the PEG precipitation assay as described in Materials and Methods. Each data point is the mean of duplicate filtrations. A specific binding activity of (938 ± 12) pMol {^{3}H}L-QNB sites/g protein was calculated using the weighted least squares method described by Bevington, (1964). o——o, net cpm bound, (i.e. total minus background cpm). ●——●, cpm bound in the presence of 10^{-4}M atropine.

Table I. Comparison of Assay Methods for $\{^3H\}$L-QNB Binding to Solubilized Receptor

Method	Specific binding pMol $\{^3H\}$L-QNB/g protein)[a]
PEG	938 ± 36
DEAE	973 ± 58
Equilibrium dialysis	918 ± 46

[a]These data represent binding assays of a single detergent extract. Non-specific binding for all three methods was less than 3% of the total binding. Uncertainties in each determination were calculated as described in Figure 1.

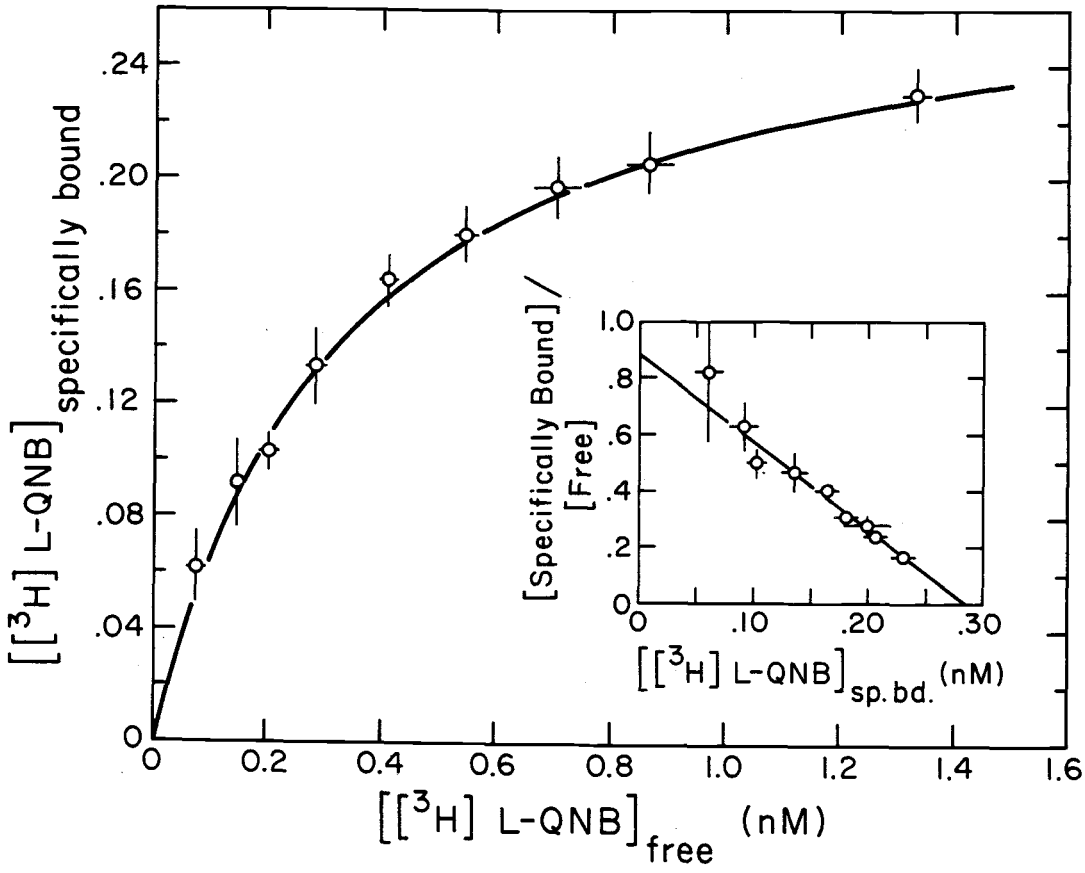


Fig. 2 {^{3}H}L-QNB binding to solubilized muscarinic receptor. A digitonin-cholate extract of atrial membranes, 270 pM in {^{3}H}L-QNB sites, was equilibrated for 24 hr at 4°C with varying concentrations of {^{3}H}L-QNB. Non-specific binding controls included 100 μM atropine. {^{3}H}L-QNB bound mAcChR concentration was measured by the DEAE filter binding assay. After correction for nonspecifically bound {^{3}H}L-QNB data were plotted in the form of a Scatchard plot (insert). Data points were the average of triplicate pipettings at each {^{3}H}L-QNB concentration. Linear weighted least-squares analysis gave a dissociation constant, calculated from the reciprocal of the slope, equal to 323 $\pm$ 26 pM and a total concentration of {^{3}H}L-QNB sites from the abscissa, equal to 284 $\pm$ 26 pM. The curve drawn through the data points was calculated from the law of mass action by using the calculated dissociation constant.

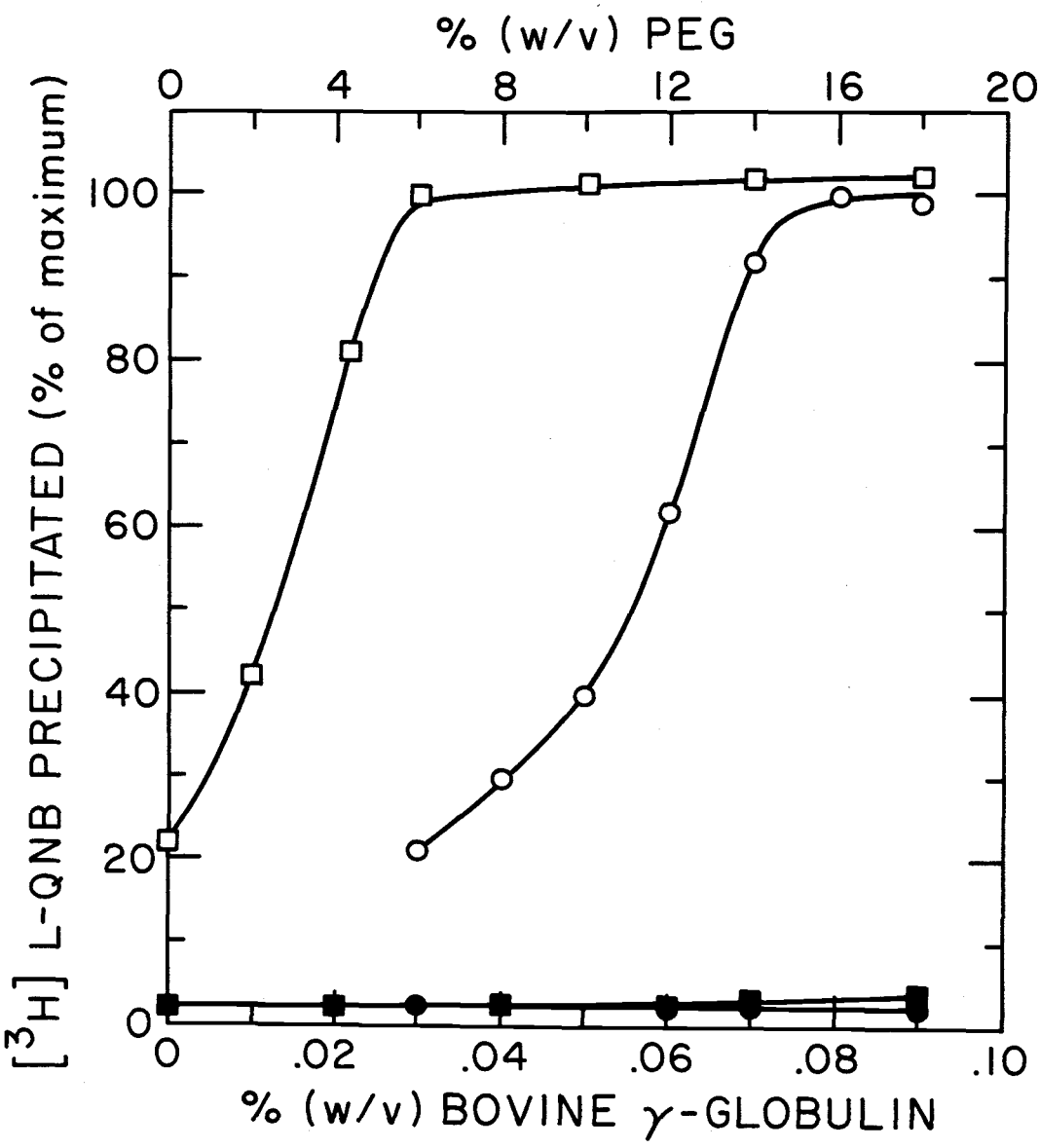


Fig. 3 Effects of PEG and γ-globulin on the solubility of {^{3}H}L-QNB-receptor complex in detergent (Buffer B). Percent precipitation values were calculated by dividing the cpm at each point by cpm obtained under conditions as described in Materials and Methods. □—■, varying γ-globulin concentration at 16% PEG, 500 μg filtered per point; ○—●, varying PEG concentration at 0.03% γ-globulin, 271 μg filtered. Open symbols, total {^{3}H}L-QNB binding; closed symbols, 10^{-4}M atropine controls.

the concentrations of PEG and γ-globulin were independently varied are presented in Fig. 3. Quantitative precipitation of QNB sites occurred only at PEG and γ-globulin concentrations equal to or higher than 16% (w/v) and 0.03% (w/v), respectively. QNB site precipitation does not vary between 0.13% (w/v) and 0.03% (w/v) digitonin (5:1 digitonin-cholate) present at the precipitation step. At 0°C, at least 25 min was required for complete precipitation.

The data in Table I indicate that the modified DEAE filter disc binding assay also quantitatively measured the {^{3}H}L-QNB binding sites solubilized in the digitonin-cholate detergent system. At saturating ligand concentrations, the specific binding of {^{3}H}L-QNB to the solubilized mAcChR was linear over the range of 5 to 150 μg protein applied to the filters. The small fluid capacity (0.1 to .15 ml) of the filters and the low protein concentrations (1 mg/ml) of our extracts precluded evaluation of binding above 0.15 mg under the conditions of the assay. Nonspecific binding was less than 3% of the total {^{3}H}L-QNB binding.

Solubilized porcine atrial mAcChR interactions with {^{3}H}L-QNB. The insert of Fig. 2 shows a Scatchard plot (Scatchard, 1949) of {^{3}H}L-QNB binding to receptors at 4°C as assayed by the DEAE filter method. From the slope of the regression line, an overall dissociation

constant of $(3.23 \pm 0.26) \times 10^{-10}$ M was calculated. The weighted means of various K_d values obtained using the PEG, DEAE and equilibrium dialysis methods are shown in Table II. Schimerlik and Searles (1980) report a dissociation constant of $(4.1 \pm 0.7) \times 10^{-11}$ M for $\{^3H\}$L-QNB binding to the membrane bound mAcChR from pig atria.[2] These results indicate that the solubilized mAcChR has a different affinity for $\{^3H\}$L-QNB than that found in the membrane bound state. This is not surprising, considering that work in many laboratories (Raftery et al., 1975; Heidmann and Changeaux, 1978) has shown that the state of the nicotinic acetylcholine receptor is strongly dependent on its lipid/detergent environment. Aronstam et al. (1977) have shown that the phospholipid environment effects QNB binding to the membrane bound mAcChR in brain. For digitonin solubilized brain mAcChRs, Aronstam et al. (1978) and Hurko (1978) reported dissociation constants of $(1.9 \pm 1.0) \times 10^{-10}$ M for D,L-QNB at $4^{o}C$ and 3.7×10^{-10} M for L-QNB. The dissociation constant for L-QNB determined in our laboratory from a single experiment using saturated digitonin solutions prepared according to the method of Hurko (1978) equaled $(8.6 \pm 1.2) \times 10^{-10}$ M.

---

[2]Schimerlik and Searles (1980) report a K_d of $(1.22 \pm 0.12) \times 10^{-10}$ M using a mixture of the D and L isomers of QNB. $(4.1 \pm 0.7) \times 10^{-11}$ M is the calculated value of the K_d for the L isomer, assuming the D isomer does not bind to the receptor.

Table II. Equilibrium Dissociation Constants

Method	T (°C)	K_d (pM)[a]	n[b]
Equilibrium Dialysis	4	232 ± 9	2
PEG	4	233 ± 10	2
DEAE	4	323 ± 26	1
PEG	22	459 ± 74	2
DEAE	22	254 ± 10	3

[a]Except for the K_d determination at 4°C by DEAE, each value is the weighted mean of separate experiments as represented by the data in Figure 2. Uncertainties are the standard error of the weighted mean.

[b]Number of experiments.

Stability of Solubilized mAcChR. At 0°C or at room temperature, the solubilized mAcChR irreversibly loses {^{3}H}L-QNB binding activity at a faster rate than the receptor-QNB complex (see Fig. 4). The decrease in {^{3}H}L-QNB binding with time is biphasic for free receptors at room temperature. In the initial fast phase, 50% of the sites are destroyed in approximately 9 hr, whereas the second slow phase has a half-life of approximately 80 hrs. At room temperature, the solubilized QNB-receptor complex loses approximately 5% of its initial {^{3}H}L-QNB binding activity in 70 hr. At 0°C, the free receptor loses approximately 25% and QNB bound receptor 5% of the initial QNB binding activity in one week. The loss in binding activity of the free receptor with time at 0°C or room temperature was not altered by the protease inhibitors pepstatin, aprotinin, bacitracin or PMSF (data not shown). Although the observed inactivation may be due to proteolysis, these data indicate that the respective proteases inhibited by these agents did not inactivate the receptor. Another possible cause for the slow decrease in activity may be that the mAcChR changes slowly to an inactive conformation when removed from its native membrane environment. Aguilar et al. (1980) showed that atropine protected against the inhibitory effect of Triton X-100 on {^{3}H}L-QNB binding to mAcChRs in rat cerebral cortex. Perhaps L-QNB

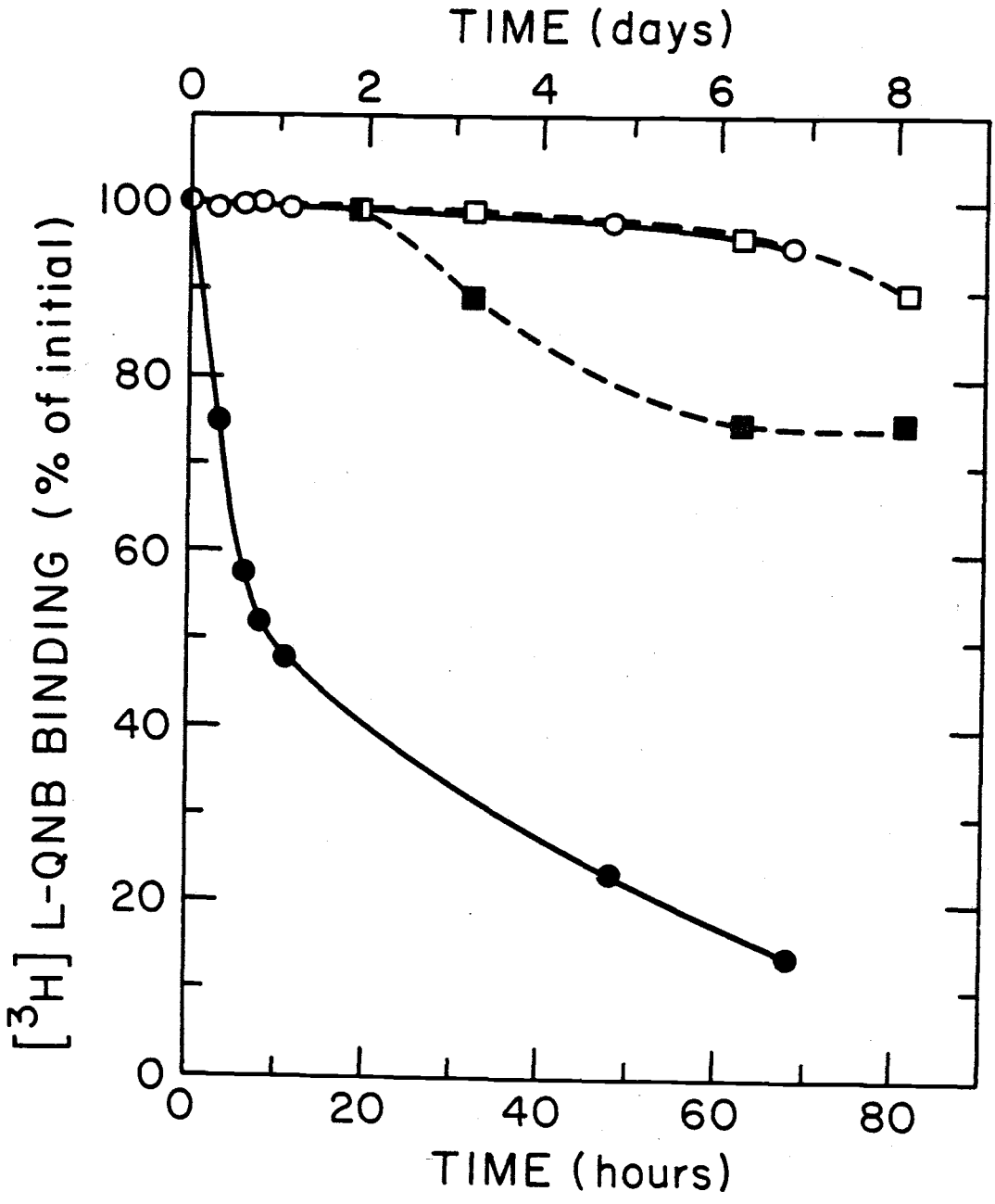


Fig. 4 Stability of the solubilized mAcChR. Lower axis, room temperature (19-22°C): ● —— ●, no QNB present; o —— o, 15nM {^{3}H}L-QNB present. Upper axis, 0°C: ■ -- ■ , no QNB present; □ -- □ , 15 nM QNB present. {^{3}H}L-QNB binding was measured by the DEAE method. 85 μg protein were pipetted onto 2.4 cm discs. All time points are averages of duplicate pipettings and represent the cpm at time = t divided by cpm at time t = 1 hr.

binding induces a conformational change in the solubilized mAcChR that either stabilizes the active conformation or protects the protein from proteolysis. The effects of other agents such as exogenous lipids on mAcChR stability is worthy of future study.

Aronstam et al. (1978) and Carson et al. (1977) reported stabilities of digitonin solubilized brain receptors at 4 and 5°C, respectively, similar to those shown for 0°C in Fig. 4. Hurko (1978), however, reported a 20% loss in QNB binding activity in 24 hr at 4°C.

In summary, the mixed detergent system described here solubilizes mAcChRs in high yields from porcine atrial membranes. This is the first report of mAcChR solubilization by any method from cardiac tissue. Detergent extracts have {^{3}H}L-QNB specific binding activities 2-3 fold higher than unextracted membranes and are shown to be very stable at 0°C. Excellent agreement was obtained between the results of the two rapid assays with respect to specific binding activities (Table I). Dissociation constants measured by the PEG assay at 4°C agreed well with equilibrium dialysis while DEAE assay results were slightly higher (Table II). At 22°C, the PEG assay gave a value for the dissociation constant of {^{3}H}L-QNB almost 2 fold higher than the DEAE assay. The reasons for this difference merit further study.

While digitonin has been used extensively to solubilize membrane associated proteins (Hoppel and Cooper, 1968; Hubbard, 1954), little is known about its mode of action. Suggestions as to how it may disrupt cell membranes have included digitonide formation with membrane cholesterol (Hoppel and Cooper, 1968; Cooper and Lehninger, 1956). However, recent evidence indicates that cholesterol need not be present for digitonin to cause significant lysis of phospholipid vesicles (Rosenqrist et al., 1980).

Commercially available digitonin is a mixture of several plant glycosides (The Merck Index, 1976; Strain, 1943).[3] When two of the major digitalis saponins, digitonin and gitonin, were isolated and tested separately for their ability to solubilize brain tissue (Repke and Matthies, 1980), the recoveries of soluble mAcChRs were low compared to extraction using a 3:2 digitonin/gitonin mixture. Since pure gitonin is essentially insoluble in the aqueous solution, whereas pure digitonin is soluble (Repke and Matthies, 1980), previous methods for preparing "saturated" digitonin solutions, which involve heating to clarification and subsequent precipitation may effectively change the ratio of digitonin to gitonin or other saponins.

---

[3]The commercial product contains 70-80% digitonin, 10-20% gitonin and tigonin, and 5-15% minor saponins.

Aronstam et al. (1978) have reported that extraction of QNB sites from brain tissue varied significantly depending upon the Sigma digitonin lot number used. Similarly, preparations of both retinal rhodopsin (Hubbard, 1954; Adams, 1969) and submitochondrial particles (Hoppel and Cooper, 1968; Cooper and Lehninger, 1956; Schnaitman et al., 1967) vary depending on the source and the method by which digitonin solutions were prepared.

The presence of cholate was required for optimum extraction of active mAcChRs from atrial membranes. The combination of digitonin and cholate has not, to our knowledge, been used previously to prepare soluble mAcChRs. It is tempting to suggest that preferential extraction of specific components which stabilize the membrane bound mAcChR may involve cholate ions and/or the correct ratio of digitalis saponins. Alternatively, low concentrations of cholate may be important to mAcChR stability only after solubilization. Finally, together with any or all of the above effects, cholate may simply augment the extraction efficiency of certain membrane proteins by digitonin.

HISTRIONICOTOXIN AND ALKYL GUANIDINE INTERACTIONS WITH THE SOLUBILIZED AND MEMBRANE-BOUND MUSCARINIC ACETYLCHOLINE RECEPTOR FROM PORCINE ATRIA

Introduction

Vagal interactions with the atria of the heart are mediated by the neurotransmitter acetylcholine. Acetylcholine binding to the mAcChR results in an increase in the permeability of the sarcolemma to potassium ions (Purves, 1978; Trautwein et al., 1956), decreasing both the rate and amplitude of the action potential. It is not currently known whether the mAcChR contains both ligand binding and potassium ion translocating systems or if the latter function is somehow mediated via a second messenger response (Kehoe and Marty, 1980).

Ligand interactions with cardiac mAcChRs have been studied in this laboratory using the membrane-bound (Schimerlik and Searles, 1980) and detergent-solubilized (Herron et al., 1982) preparations from porcine atria and in other laboratories using either rat heart (Cavey et al., 1977; Fields et al., 1978) or tissue culture preparations (Galper and Smith, 1980; Halvorsen and Nathanson, 1981). For all muscarinic systems studied to date, hydrophobic muscarinic antagonists have dissociation constants several orders of magnitude lower than the more polar agonists.

In studies of alkyl guanidine binding to the potential dependent sodium channel in eel electroplax membranes (Reed and Trzos, 1979), the authors determined the dependence of alkyl guanidine affinity, measured by competition with tetrodotoxin, on hydrocarbon chain length and the energetic contribution to binding from hydrophobic interactions. Morello et al. (1980) have shown that nonyl guanidine reversibly blocked both inward and outward sodium currents if applied to the interior of squid giant axons. When alkyl guanidines with chain lengths from one to eight were used as a probe of the ligand binding site topology of the nAcChR[4] (Miller, 1979), it was found that these compounds did not displace nicotinic agonists and antagonists. However, alkyl guanidines with chain lengths from one to eight carbons competitively displaced H_{10}-HTX, a toxin that binds to the site responsible for ion translocation (Elliot and Raftery, 1979; Albuquerque et al., 1973).

The purpose of this paper is to characterize the nature of H_{10}-HTX and alkyl guanidine interactions with the solubilized and membrane-bound porcine atrial mAcChR

---

[4]Abbreviations: ANS, 8-anilino-1-naphthalene-sulfonic acid; H_{10}-HTX, (2pR, 6S, 7pS, 8aS)-7-(butane)-8-hydroxy-2-(cis-4-pentene)-1-azaspiro {5.5} undecane or perhydro-histrionicotoxin; H_{12}-HTX, (2pR, 6S, 7pS, 8aS)-7-(butane)-8-hydroxy-2-(pentane)-1-azaspiro {5.5} undecane or perhydro-histrionicotoxin; H_2-HTX, (2pR, 6S, 7pS, 8aS)-7-(cis-1-buten-3ynyl)-8-hydroxy-2-(cis-2, 3-pentene)-1-azaspiro {5.5} undecane or iso-dihydro-histrionicotoxin.

using the highly specific muscarinic antagonist $\{^3H\}$L-QNB as a probe. Since the binding sites for both alkyl guanidines and H_{10}-HTX are distinct from that of nicotinic agonists and antagonists, the competitive displacement of $\{^3H\}$L-QNB by these compounds in the muscarinic system may indicate that they interact in a different manner with mAcChR compared to the nAcChR. From the dependence of the affinity of alkyl guanidines on hydrocarbon chain length, the hydrophobic contribution of binding to the antagonist binding site of the solubilized and membrane-bound mAcChR can be determined. These results provide information about the binding site environment and alterations that may have occurred upon solubilization.

Experimental Procedures

Materials

{^{3}H}L-QNB (40.2 and 26.8 Ci/mmole), purchased from New England Nuclear, cochromatographed with a non-radiolabeled standard (the generous gift of Dr. W.E. Scott, Hoffman-La Roche, Inc.) on Merck silica gel 60 plates in chloroform:methanol:water:acetic acid (65:25:5:5); R_f=0.58) and acetone:Methanol:diethanolamine (10:10:0.3; R_f=0.38) solvent systems. Ninety-five percent of the radioactivity was found in the QNB spot. Bovine serum albumin, sodium azide, digitonin (Lot No. 19C-0329), cholic acid and atropine sulfate were purchased from Sigma Chemical Company; 2.4 cm DEAE (DE 81) and GF/B filters were purchased from Whatman; ANS and N-phenyl-1-naphthylamine were purchased from Eastman and Molecular Probes, Inc., respectively. Alkyl guanidines were synthesized according to Reed and Trzos (1979). H_{10}-HTX was a generous gift from Professor M.A. Raftery.

Preparation and solubilization of membranes

mAcChR-enriched membranes from frozen pig atria were prepared as described by Peterson and Schimerlik (1982). The mAcChR was solubilized at a protein concentration of 5 mg/ml and a final detergent concentration of 0.4% (w/v) digitonin, 0.08% (w/v) cholic acid according to Cremo *et al*. (1981). Specific activities of the solubilized membranes varied between 1 and 5 nMol of

L-QNB sites per gram of protein, and recoveries of total L-QNB sites from membranes were between 85% and 98%. Protein concentration was measured by the method of Lowry et al. (1951) as modified by Peterson (1977), with crystalline bovine serum albumin as a standard.

{^{3}H}L-QNB binding assays and equilibrium titrations

Membrane-bound mAcChR concentration was quantitated at 21°C in terms of {^{3}H}L-QNB sites as described by Yamamura and Snyder (1974). The solubilized receptor was quantitated in terms of {^{3}H}L-QNB sites using a DEAE filter disc assay developed by Schmidt and Raftery (1973) for the nAcChR and modified in this laboratory for use with the digitonin/cholate mixed detergent system (Cremo et al., 1981).

Fluorescence studies

Fluorescence titrations were performed at 21°C using a Perkin-Elmer MPF-2A spectrofluorimeter. Excitation wavelengths for ANS and N-phenyl-1-naphthylamine were 360 and 330 nm and emissions were observed at 460 and 400 nm. The final concentrations of ANS and N-phenyl-1-naphthylamine were 10 and 3.3 μM. The concentration of {^{3}H}L-QNB sites for the solubilized receptor was 1 nM and the protein concentration equaled 0.32 mg/ml. The membrane-bound receptor was titrated at a concentration of {^{3}H}L-QNB sites equal to 0.05 nM and a protein concentration of 0.12 mg/ml.

Data evaluation

Equilibrium titration curves measuring $\{^3H\}$L-QNB displacement were analyzed by using a weighted least-squares fit to either Equation 1 or 2.

$$\{RQ\} = \frac{\{R_o\}\ \{Q\}}{K(1 + \{I_o\}/K_i) + \{Q\}} \tag{1}$$

$$\frac{\{I_o\}}{\{R_o\}\ (1 - \frac{\overline{RQ}}{\overline{RQ}_o})} - 1 = \bar{J} = \frac{\{Q\}}{\{RQ\}}\ \frac{K_i}{K} \tag{2}$$

Equation 1 was adapted from the weighted least-squares fitting programs of Cleland (1967) modified for use in ligand binding studies. The total concentration of $\{^3H\}$L-QNB sites ($\{R_o\}$) and the dissociation constants for $\{^3H\}$L-QNB (K) and the inhibitor (K_i) were determined in a single experiment by measuring the concentration of specifically bound $\{^3H\}$L-QNB (RQ) as a function of free $\{^3H\}$L-QNB (Q) at differing fixed levels of total inhibitor concentration (I_o). Significant binding of inhibitor did not occur until the total inhibitor concentration was in large excess over the concentration of L-QNB sites; thus the free inhibitor concentration essentially equaled the total inhibitor concentration.

In Equation 2, the value of the dissociation constant (K_i) for a given competitive inhibitor was calculated from the slope of the plot of $\bar{J}$ function (Best-

Belpomme and Dessen, 1973) versus {Q}/{RQ} where {Q} and {RQ} are defined as in Equation 1 and $\overline{RQ}$ and $\overline{RQ}_o$ equal the fractional saturation L-QNB sites in the presence of total inhibitor concentration $\{I_o\}$ and in the absence of inhibitor, respectively. Thus, from the slope of the plot of $\bar{j}$ versus {Q}/{RQ} and the value of K, the dissociation constant for L-QNB, the K_i values could be computed for the various inhibitors.

Fluorescence data were analyzed by using Equation 3, where ΔF equaled the change in fluorescence

$$\Delta F = \frac{\Delta F_{max} \{I_o\}}{K_{app} + \{I_o\}} \tag{3}$$

observed at total ligand concentration $\{I_o\}$ and ΔF_{max}, the change in fluorescence observed at saturation with ligand. K_{app} equaled the apparent dissociation constant for the ligand. Titrations were analyzed using a least-squares fit to Equation 4, the reciprocal of Equation 3, where

$$\frac{1}{\Delta F} = \frac{K_{app}}{\Delta F_{max}} \frac{1}{\{I_o\}} + \frac{1}{\Delta F_{max}} \tag{4}$$

ΔF_{max} and K_{app} were determined from the slope and Y-intercept of a plot of $1/\Delta F$ versus $1/\{I_o\}$ (Figure 9).

Results

Figure 5 shows an octyl guanidine titration of specifically bound {^{3}H}L-QNB to the membrane-bound mAcChR. The intersecting pattern demonstrates that this ligand binds competitively versus {^{3}H}L-QNB. The equilibrium dissociation constant for octyl guanidine obtained from the fit of the data to Equation 1 equaled $(4.5 \pm 0.9) \times 10^{-6}$ M and the dissociation constant for {^{3}H}L-QNB equaled $(44 \pm 11) \times 10^{-12}$ M. The latter value agreed with the value previously determined by Scatchard analysis (1949) in this laboratory $(41 \pm 7) \times 10^{-12}$ M (Schimerlik and Searles, 1980, corrected for equal amounts of D and L-QNB). Additional experiments where the dissociation constant for octyl guanidine was re-determined from titration curves of specifically bound {^{3}H}L-QNB *versus* inhibitor concentration using Equation 2 agreed quite well with the value determined from double reciprocal plots. Therefore, the data were fitted to Equation 2 for all other inhibitors tested. A summary of the dissociation constants (K_i) determined for all competitive inhibitors of {^{3}H}L-QNB binding to the membrane-bound mAcChR is shown in Table III.

The results in Table III were replotted as $-\ln K_i$ *versus* chain length of alkyl guanidine in Figure 6. For the membrane-bound mAcChR, the dissociation constants decreased with increasing chain length up to

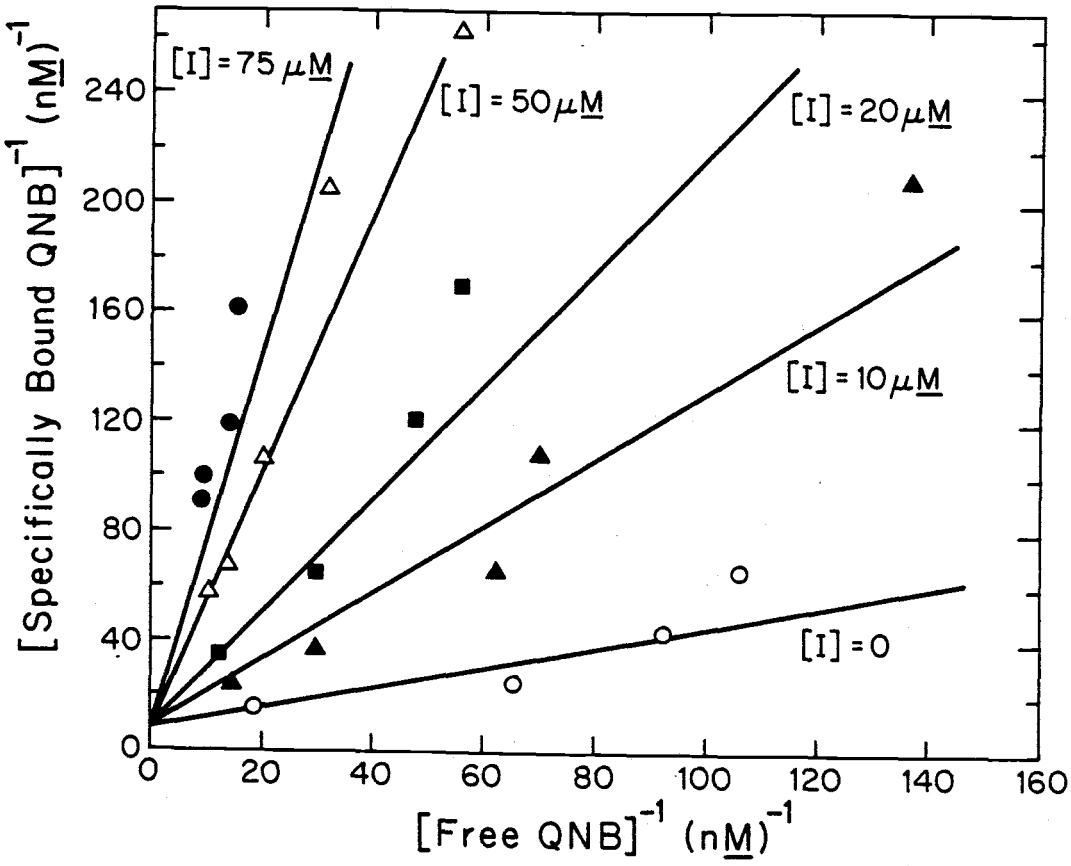


Fig. 5 Octyl guanidine titration of {^{3}H}L-QNB specifically bound to the membrane-bound mAcChR. Final concentration of L-QNB sites equaled 100 pM (.053 mg/ml protein concentration). Each data point represents the average of two replicates. The line through the data was computed from a least-squares fit to Equation 1, which gave the following values: K_L (4.4 ± 1.1) x 10^{-11}M; K_i, (4.5 ± 0.9) x 10^{-6}M and {R_o}, (1.21 ± 0.20) x 10^{-10}M.

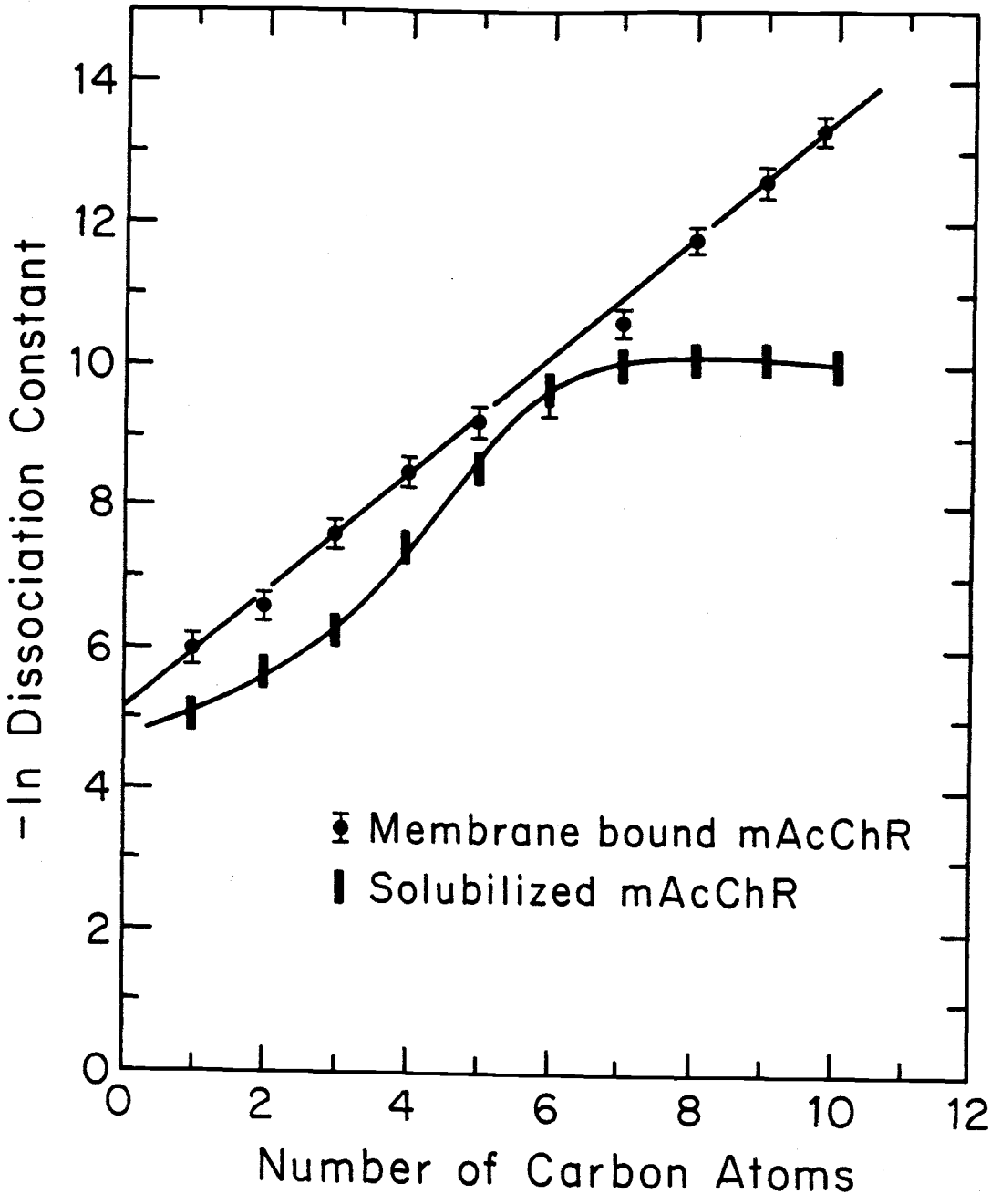


Fig. 6 $-\ln K_i$ for alkyl guanidines *versus* alkyl chain carbon number. A least-squares linear regression analysis gave a ΔG per methylene carbon equal to $-(473 \pm 30)$ cal/mole from the slope of the line for the membrane-bound mAcChR. The curve through the data points for the solubilized mAcChR was not theoretically derived.

Table III. Summary of Equilibrium Dissociation Constants for the Membrane-Bound and Solubilized Atrial mAcChR[a].

compound	Equilibrium Dissociation Constants (M) membrane-bound[b]	solubilized[c]
methyl guanidine	$(2.5 \pm 0.5) \times 10^{-3}$	$(6.8 \pm 1.4) \times 10^{-3}$
ethyl guanidine	$(1.3 \pm 0.3) \times 10^{-3}$	$(3.5 \pm 0.7) \times 10^{-3}$
propyl guanidine	$(5.0 \pm 1.0) \times 10^{-4}$	$(1.8 \pm 0.4) \times 10^{-3}$
butyl guanidine	$(2.1 \pm 0.4) \times 10^{-4}$	$(5.8 \pm 1.2) \times 10^{-4}$
pentyl guanidine	$(1.0 \pm 0.2) \times 10^{-4}$	$(9.5 \pm 1.9) \times 10^{-5}$
hexyl guanidine	$(7.3 \pm 1.5) \times 10^{-5}$	$(6.8 \pm 1.4) \times 10^{-5}$
heptyl guanidine	$(7.3 \pm 1.5) \times 10^{-5}$	$(4.6 \pm 0.9) \times 10^{-5}$
octyl guanidine	$(7.7 \pm 1.5) \times 10^{-6}$	$(4.0 \pm 0.8) \times 10^{-5}$
nonyl guanidine	$(3.8 \pm 0.8) \times 10^{-6}$	$(4.0 \pm 0.8) \times 10^{-5}$
decyl guanidine	$(1.6 \pm 0.3) \times 10^{-6}$	$(4.7 \pm 0.9) \times 10^{-5}$
H_{10}-Histrionicotoxin	$(6.9 \pm 1.4) \times 10^{-6}$	$(1.5 \pm 0.3) \times 10^{-5}$

[a]All dissociation constants were determined as described in Data Evaluation using Equation 2. These data were presented as the mean ± SE (n = 3).

[b]pH = 7.4, 50 mM sodium phosphate, 1mM EDTA, 0.02% NaN_3; 21°C.

[c]pH = 7.4, 10 mM sodium phosphate, 1mM EDTA, 0.02% NaN_3, 0.4% (w/v) digitonin, 0.08% (w/v) cholic acid; 21°C.

ten carbons. A free energy of association per methylene carbon of the aliphatic chain of $-(473 \pm 30)$ cal/mole was calculated assuming that $\Delta G = RT \ln K_i$.

An identical series of experiments was performed for the solubilized preparation. The data shown in Figure 7 for the butyl guanidine titration of $\{^3H\}$L-QNB was representative of the competitive behavior observed for guanidines with side chain carbon numbered from one to seven. Octyl, nonyl and decyl guanidine titrations, however, were not competitive with respect to $\{^3H\}$L-QNB. The nonyl guanidine titration, shown in Figure 8, was an example of an apparently cooperative displacement of $\{^3H\}$L-QNB that did not fit the competitive model at the higher ligand concentrations examined. The dissociation constants for octyl, nonyl and decyl guanidine were determined by fitting the data obtained at low inhibitor concentrations where the law of mass action was obeyed. Analysis of this data in the form of a Hill plot (Hill, 1910) showed a limiting slope of approximately 2 at the higher ligand concentrations (plot not shown). A control experiment in which a high concentration (1 mM) of nonyl guanidine was incubated with the solubilized mAcChR for 1 hour at 25°C followed by removing the ligand by dialysis showed that there was no irreversible loss of $\{^3H\}$L-QNB binding.

For the solubilized receptor, decreasing

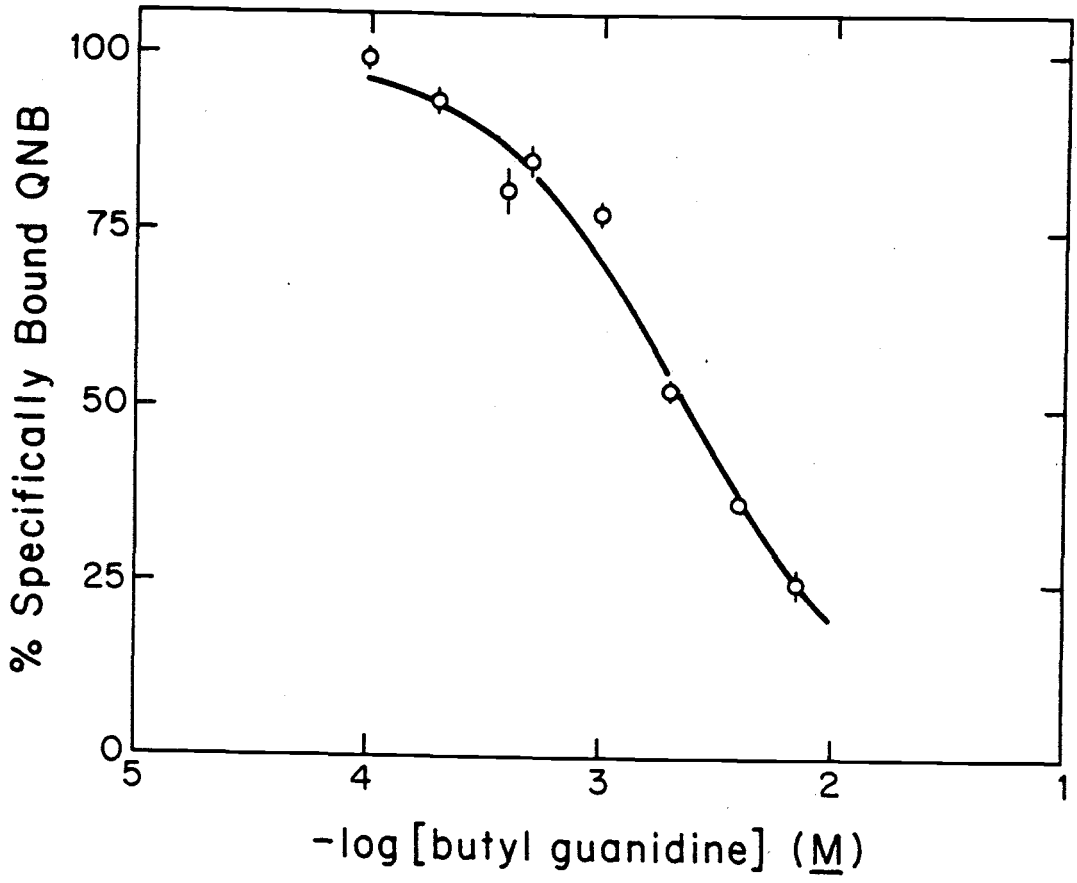


Fig. 7 Butyl guanidine titration of {^{3}H} L-QNB specifically bound to the solubilized mAcChR. Final concentrations equaled: 6.5×10^{-10}M {^{3}H}L-QNB, 7.7×10^{-10}M L-QNB sites, 0.8 mg/ml protein concentration. Data were evaluated using Equation 2 to give a dissociation constant (K_i) of (5.8 ± 1.2) x 10^{-4}M. A dissociation constant for L-QNB (K) of (2.54 ± 10) x 10^{-10}M (Herron et al., 1982) was used to calculate the curve through the data points.

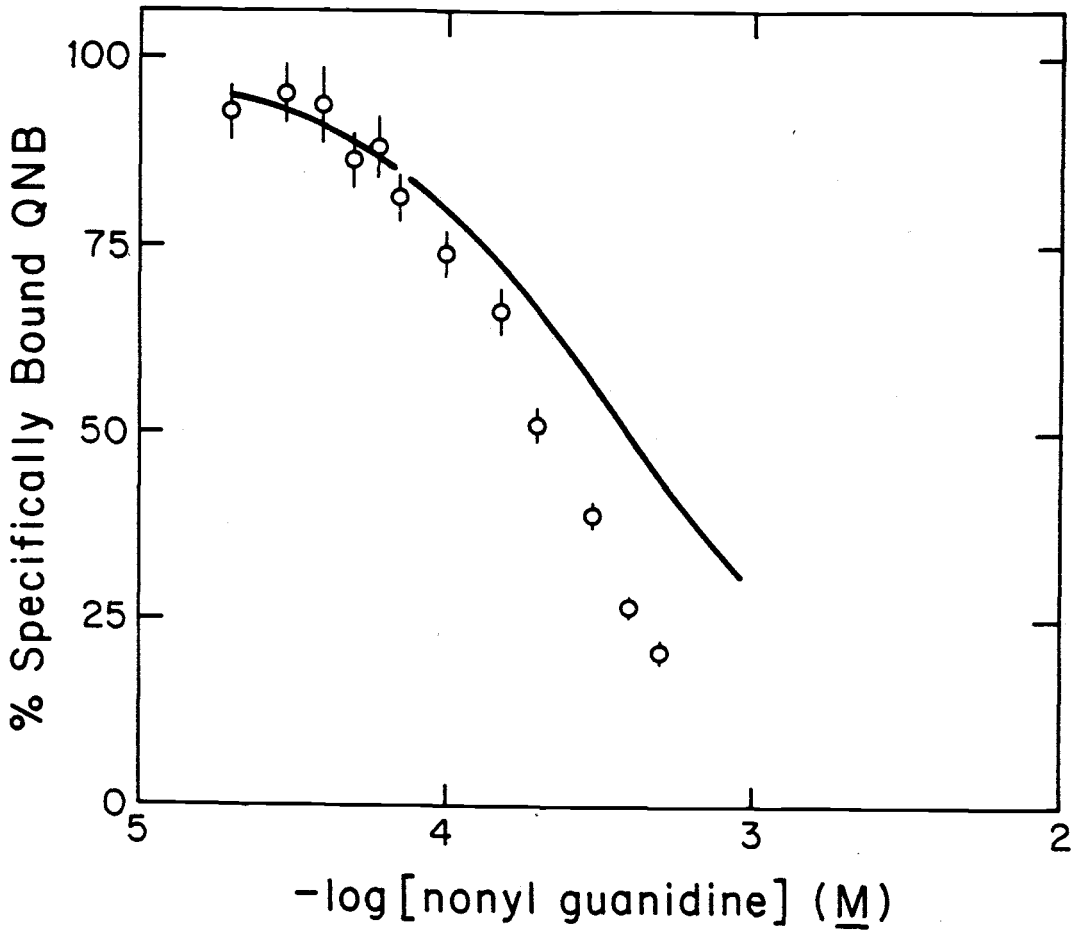


Fig. 8 Nonyl guanidine titration of {^{3}H}L-QNB specifically bound to the solubilized mAcChR. Final concentrations equaled: 6.1 x 10^{-10}M {^{3}H}L-QNB, 9.1 x 10^{-10}M L-QNB sites, 0.59 mg/ml protein concentration. Data were evaluated using Equation 2 to give a dissociation constant (K_i) for nonyl guanidine of (4.0 ± 0.8) x 10^{-5}M. [1]The curve through the data points was calculated as in Figure 3, except that the four points with the highest ligand concentration were not included in the fit.

dissociation constants were also observed with increasing alkyl chain length up to 7 carbons (see Figure 6). The plot of $-\ln K_i$ versus carbon chain length appeared sigmoidal with limiting ΔG's per methylene carbon of -400 cal/mol and 0 cal/mol and a maximum slope of -650 cal/mol through carbon numbers 3 to 6.

To test the effects of alkyl guanidines on the properties of the membranes and detergent-lipid-protein vesicles, we examined the fluorescence enhancement of ANS and N-phenyl-1-naphthylamine with increasing alkyl guanidine concentration. For both the membrane-bound and solubilized preparations under the same conditions of the $\{^3H\}$L-QNB binding experiments, the increase in fluorescence of ANS with increasing alkyl guanidine concentration appeared to follow the law of mass action for a single class of sites. The data in Figure 9 for hexyl guanidine is shown as an example. The apparent dissociation constant for hexyl guanidine obtained from fitting the data in Figure 9 to Equation 4 was $(2.5 \pm 0.1) \times 10^{-3}$ M whereas the value listed in Table III, obtained by competition with $\{^3H\}$L-QNB equaled $(6.8 \pm 1.4) \times 10^{-5}$ M. The apparent dissociation constants calculated from fluorescence measurements for all alkyl guanidines studied were consistently about two orders of magnitude higher than dissociation constants obtained by $\{^3H\}$L-QNB binding inhibition. No

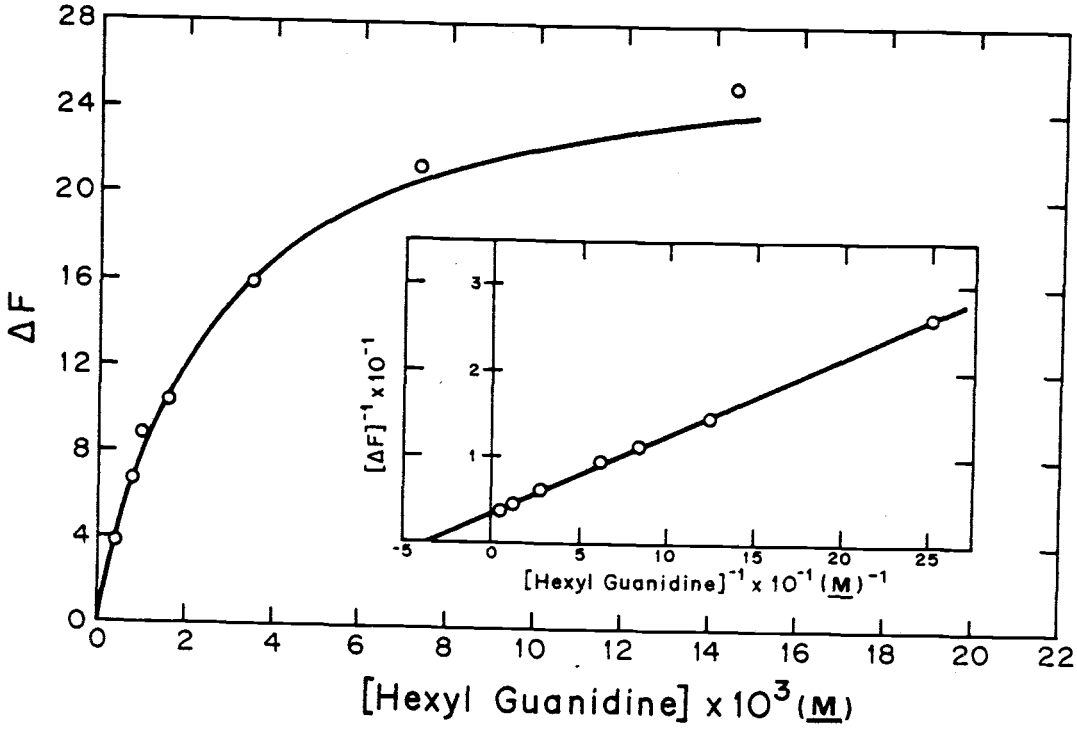


Fig. 9 Hexyl guanidine titration of ANS fluorescence in the solubilized preparation. Data were fit to Equation 4 which gave an apparent dissociation constant for hexyl guanidine (K_{app}) equal to (2.5 ± 0.1) x 10^{-3}M and ΔF_{max} = 28.1 ± 0.9.

fluorescence enhancement was observed for an identical series of experiments using N-phenyl-1-naphthylamine as a probe. Control experiments indicated that neither ANS nor N-phenyl-1-naphthylamine displaced $\{^3H\}$L-QNB from either the membrane-bound or solubilized receptor at concentrations up to 50 μM.

H_{10}-HTX appeared to displace $\{^3H\}$L-QNB competitively from a single class of sites in both the membrane-bound and solubilized preparations, with similar dissociation constants of $(6.9 \pm 1.4) \times 10^{-6}$ M and $(1.5 \pm 0.3) \times 10^{-5}$ M, respectively.

Discussion

All alkyl guanidines tested appeared to displace $\{^3H\}$L-QNB in a competitive manner from the membrane-bound mAcChR (Figure 5, Table III). The free energy change per methylene carbon calculated from the plot of $-\ln K_i$ *versus* alkyl chain carbon number (Figure 6) was (-473 ± 30) cal/mol at 21° C. This value was similar to that found (-590 cal/mol) by Reed and Trzos (1979) in their studies of alkyl guanidine competition for the tetrodotoxin binding site of the potential dependent sodium channel in eel electroplax membranes. The free energy change found above was somewhat lower than the reported value of (-850 ± 40) cal/mol for the contribution of methylenes to the partitioning of amphiphiles between aqueous and hydrocarbon phases (Tanford, 1980). However, the increase in affinity observed with increasing hydrophobicity as well as the similarity of the magnitude of the free energy change (about 60% of the reported value) do seem to be consistent with the notion that alkyl guanidines bind to a hydrophobic site on the membrane-bound mAcChR. Although the binding energy per methylene carbon was similar for both the membrane-bound mAcChR and the potential dependent sodium channel, the dissociation constants for alkyl guanidines were about ten fold lower for the membrane-bound mAcChR. This may indicate an increased contribution to the overall binding

energy either from the guanidinium group or protein conformational changes subsequent to binding. Alkyl guanidines with carbon numbers less than six bind ten to twenty fold tighter to the membrane-bound mAcChR than to the membrane-bound nAcChR from Torpedo californica (Miller, 1979). In the latter system, the alkyl guanidines bound competitively versus $\{^3H\}$ H_{12}-HTX, but not α-bungarotoxin (Miller, 1979), indicating that they were not binding to the same site as nicotinic agonists and antagonists (Elliot and Raftery, 1979). The simplest interpretation of the competition between guanidines and the antagonist $\{^3H\}$L-QNB was that both ligands bind to the same site on the membrane-bound mAcChR. However, the possibility that alkyl guanidines bind to a second site causing a conformational change that precludes $\{^3H\}$L-QNB binding cannot be discarded at this time. Mass action considerations prevent a direct determination of $\{^3H\}$ H_{12}-HTX displacement by alkyl guanidines and muscarinic agonists since the concentrations of mAcChR is approximately three orders of magnitude lower than the dissociation constant for H_{10}-HTX. Similarily, since these studies were done using only radiolabeled antagonist, it is not possible to state definitively that H_{10}-HTX or the alkyl guanidines would displace agonists from the mAcChR. However, since $\{^3H\}$L-QNB was competitively displaced by agonists in both the membrane-bound

(Schimerlik and Searles, 1980) and solubilized preparations (Herron et al., 1982) as well as by H_{10}-HTX and alkyl guanidines, the simplest explanation of this behavior would be that all of these ligands either directly or indirectly (through conformational changes) appear to affect the same binding site.

The interactions of alkyl guanidines with the solubilized mAcChR were more difficult to interpret. At carbon chain lengths equal to or less than seven, competitive inhibition of $\{^3H\}$L-QNB binding was observed (Figure 7); however, at carbon chain lengths greater than seven, the displacement of $\{^3H\}$L-QNB appeared cooperative in nature (Figure 8). Although the reason for this behavior was not known, several possible explanations have been considered. The first possibility was that higher concentrations of the more hydrophobic alkyl guanidines either caused irreversible loss of $\{^3H\}$L-QNB binding activity or interfered with the DEAE disc assay. Incubation of the solubilized mAcChR with 1 mM nonyl guanidine for 1 hour at room temperature followed by dialysis did not result in a loss of $\{^3H\}$L-QNB binding sites, indicating that any deleterious effects on mAcChR L-QNB binding activity must be completely reversible. Control experiments, where the higher concentrations of guanidines used in these titrations were added immediately prior to pipetting the receptor solution onto the

DEAE discs, indicated that the alkyl guanidines did not interfere with the DEAE disc assay. A second alternative was the solubilization of the mAcChR exposed additional hydrophobic binding sites. The binding of alkyl guanidines to these additional sites must then be conformationally coupled to {^{3}H}L-QNB binding site in order to cause the apparently cooperative displacement. The additional hydrophobic binding sites may be heterogeneous and, in addition to the presence of mixed micelles, may cause the apparently cooperative behavior. However, earlier results from this laboratory (Herron et al., 1982) indicated that agonists, antagonist, and local anasthetics all competitively displaced {^{3}H}L-QNB from a single homogenous class of binding sites in the digitonin/cholate solubilized preparation. A third alternative was that the alkyl guanidines with carbon chain length greater than seven preferentially partition into the detergent-lipid-protein micelle, either resulting in higher local concentrations near the muscarinic ligand binding site or altering the surface charge characteristics of anionic lipids and detergent (e.g. cholate). Since ANS binds to membranes in the region of the phospholipid head groups (Slavik, 1982), it was used as a probe to study nonspecific effects of alkyl guanidines on the membrane-bound and solubilized mAcChR. The observation that the apparent dissociation constants of

alkyl guanidines calculated from the fluorescence measurements were consistently two orders of magnitude higher than those determined from the inhibition of $\{^3H\}$L-QNB binding may indicate that nonspecific alterations in the surface charge or the detergent system did not occur in the concentration ranges that affected $\{^3H\}$L-QNB binding. If the enhancement of ANS fluorescence were due to alterations in lipid structure occurring in the hydrophobic interior of the membrane or vesicle, the neutral dye N-phenyl-1-naphthylamine might respond similarly to ANS since their chromophoric groups are identical. Since N-phenyl-1-naphthylamine did not show any change in fluorescence intensity, the ANS fluorescence changes were tentatively attributed to surface interactions between the positively charged alkyl guanidine and negatively charged lipid moieties such as phosphate. Subtle effects of alkyl guanidines on the properties of the detergent solubilized mAcChR would probably not be reflected in our fluorescence titrations.

The sigmoidal behavior of the plot of $-\ln K_i$ versus methylene carbon number (Figure 6) is not currently understood. The transition observed between carbon numbers three and six may relate to alterations in either the detergent properties or the partition coefficients of the guanidines into the lipid-detergent phase surrounding the mAcChR. On the other hand, the lack of

increase in binding affinity observed from seven to ten carbons may be an indication of a size limitation on the solubilized guanidine binding site that was not evident in the membrane-bound state. It should be emphasized that the dissociation constants of octyl, nonyl and decyl guanidine were estimated from the concentration range over which they did not show apparently cooperative behavior. This approach may result in errors in the determination of the dissociation constant for these molecules since the mechanism by which their behavior can be analytically explained is not yet known. Regardless, all dissociation constants reported were lower for the membrane-bound mAcChR than for the solubilized preparation. These results were consistent with results from this laboratory (Herron et al., 1982) where antagonists were shown to bind one to five fold more tightly to the membrane-bound than to the solubilized preparation of the atrial mAcChR.

H_{10}-HTX displaced {^{3}H}L-QNB competitively from a single class of sites for both the membrane-bound and solubilized preparation of the atrial mAcChR. These results were in contrast to those of Burgermeister et al. (1978) who found that H_2-HTX inhibited {^{3}H} scopolamine binding to neuroblastoma cell line N1E-115 mAcChRs in a noncompetitive manner. We obtained similar dissociation constants for both membrane-bound $(6.9 \pm 1.4) \times 10^{-6}$ M

and solubilized (1.5 ± 0.3) x 10^{-5} M atrial mAcChRs. These values were lower than the IC_{50} Value of 70 μM found for H_2-HTX in the neuroblastoma cell line (Burgermeister et al., 1978); however, they were 20-50 fold higher than the values reported for H_{12}-HTX binding to the nAcChR (Elliot and Raftery, 1979).

In summary, H_{10}-HTX appeared to bind competitively to the antagonist binding site of both the membrane-bound and solubilized mAcChR indicating a different site of action than previously observed for the nAcChR (Elliot and Raftery, 1979; Albequerque, et al., 1973). Alkyl guanidines with carbon chain lengths from one to ten also bound to the membrane-bound mAcChR competitively versus $\{^3H\}$L-QNB. Interactions of these compounds with the solubilized mAcChR were more complex, resulting in apparently cooperative inhibition of $\{^3H\}$L-QNB binding at higher alkyl carbon chain lengths. The energetic contribution to binding was a logarithmic function of methylene carbon number for the membrane-bound mAcChR but showed sigmoidal behavior for the solubilized protein. The simplest interpretation consistant with these results was that the alkyl guanidines acted as sensitive probes of the antagonist binding site of the mAcChR, reflecting alterations in environment that occurred upon solubilization. The above results, in addition to previous studies in the potential dependent sodium

channel (Reed and Trzos, 1979) and nAcChR (Miller, 1979; Elliot and Raftery, 1979; Albequerque *et al*., 1973) indicate that these molecules may prove useful as *in vitro* probes of anionic receptor binding sites located in a hydrophobic environment.

PHOTOAFFINITY LABELING OF THE SOLUBILIZED MUSCARINIC ACETYLCHOLINE RECEPTOR FROM PORCINE ATRIA

Photoaffinity Labeling of Proteins

Photoaffinity labeling is a specialized way to chemically modify proteins. Photoaffinity labeling falls into the general catagory of affinity labeling (Jakoby and Wilchek, 1977, a type of chemical modification of proteins (Means and Feeney, 1971) which takes advantage of specific ligand-receptor interaction (through a reaction rate acceleration) to selectively label a protein active site. Unlike group specific reagents, affinity labels tend to form covalent bonds with proteins mostly at the active site due to selective and reversible binding before the bond is formed,

$$L + R \rightleftharpoons LR \rightarrow L\text{-}R$$

where L=ligand, R=receptor, LR=ligand-receptor complex and L-R=covalent ligand-receptor complex. Thus most affinity labels are electrophilic ligand analogs capable of reacting with nucleophilic amino acids near or at the protein active site by alkylation (aziridium, α-haloketones), acylating (ketenes) or diazo coupling. Un-

fortunately, many affinity labels react more rapidly with water and are destroyed hydrolytically before labeling the active site (Bayley and Knowles, 1977). In contrast, photoaffinity labels contain light sensitive groups such as diazo and azide functions, although they are ligand analogs. Therefore, photoaffinity labels like affinity labels, reversibly and selectively bind to the active site. However, light activation in the proper spectral range is required before the reactive intermediate is generated and the final covalent protein-ligand complex is formed,

$$L + R \rightleftharpoons LR \xrightarrow{\text{light}} L^*R \rightarrow L\text{-}R$$

where L* is the photoactivated ligand.

The theoretical basis of photoaffinity labeling has been the subject of several reviews (Bayley and Knowles, 1977; Chowdhry and Westheimer, 1979). Diazo and azido groups form extremely reactive intermediates, carbenes and nitrenes, upon ultraviolet light activation (Lwowski, 1970; Reiser and Wagner, 1971). These intermediates are much more reactive than the typical electrophiles of affinity labels and are capable of insertion reactions with C-H, O-H and N-H bonds, and addition to multiple bonds, aromatic systems, and non-bonding electron pairs (Knowles, 1972; Lwowski, 1970). Thus the reaction

can be nonselective and where no available nucleophilic group at the active site is present for a noncovalent bond to form. Ideal photoaffinity reagents can react with ubiquitous hydrophobic regions of proteins. This can be especially useful for receptor sites that may not be catalytically important, such as modifier sites on enzymes, hormone and neurotransmitter receptor sites and immunoglobulins (Chowdry and Westheimer, 1979). Photoaffinity reagents have the potential for both high reactivity and activation, only under those conditions defined by the experimenter, at the active site. Because the ligand and receptor are in reversible equilibrium in the dark without reaction, it is possible to thoroughly determine activities and equilibrium binding parameters of the ligand itself and compare it to that for the parent compound. It is essential that the analog mimic the biological activity of the parent compound.

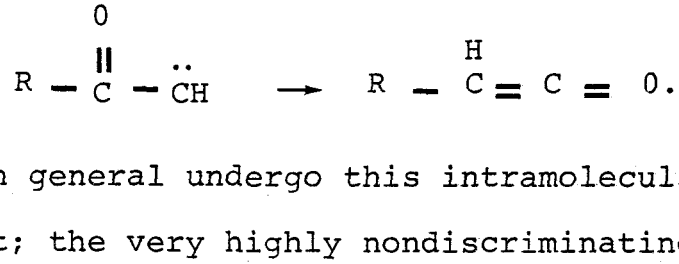
Photoaffinity labeling can provide information about the structure of binding and catalytic sites of isolated proteins and receptors at various stages of purification in soluble and membrane-bound forms (Hanstein, 1979).

The requirements of a good photoaffinity label are based upon a highly reactive species generated by irradiation of a chemically stable precursor. Obviously, photoaffinity labels must be stable in the dark in aqueous

solution at pHs appropriate for the protein system of interest. The compound should be readily synthesized and susceptible to photolysis conditions unharmful to the system. Preferably, the reactive intermediate should have a very short half-life and not be susceptible to intramolecular rearrangements which decrease reactivity. If the analog is to be prepared in a radioactive form, the radioactivity should be introduced so that it will remain with the covalently bonded portion of a labile analog. Most importantly, the modified ligand must retain the overall conformation and biological specificity of the parent compound. In practice, the most synthetically accessible compounds are most often tried, and further work is based upon these results (Hanstein, 1979; Bayley and Knowles).

Although none of the photolabile groups currently in use meet all of the above requirements, the most promising reagents for good photoaffinity labels are carbene and nitrene precursors. Bayley and Knowles (1977) have reviewed examples of carbene and nitrene precursor analogs of natural ligands. Carbenes are most often generated from diazoacetyl or diazomalonyl derivatives. The first report of photoaffinity labeling involved a diazoacetyl compound (Singh, et al., 1962) which proved susceptible to the Wolff rearrangement

upon photolysis, generating the ketene (Hanstein, 1979; Chowdry and Westheimer, 1979; Bayley and Knowles, 1977),



Carbenes in general undergo this intramolecular rearrangement; the very highly nondiscriminating reaction of the carbene is destroyed and replaced by an acylating agent reacting only with nucleophiles. In general, carbenes are not as stable as nitrenes, however electron withdrawing groups adjacent to the diazo group stabilize the parent compounds (Bayley and Knowles, 1977).

Nitrenes are not as reactive and less indiscriminate than carbenes (Knowles, 1972). The most useful nitrene precursors are aryl azides. Most aryl azides are stable at room temperature, but are inactivated by heating. Alkyl azides are subject to intramolecular rearrangements to imines upon photolysis and are not useful reagents. Aryl azides are less susceptible to rearrangement, and relatively easily synthesized from the aryl nitro or amine precursor. The readily available 2,-nitro, 4-azidophenyl fluoride (FNAP) (Jeng and Guillory, 1975) can easily be incorporated into a ligand and has the further advantage of an electron-withdrawing substituent which increases nitrene reactivity (Bayley and Knowles, 1977). However, this increase in reactivity is

coupled with an undesirable increased electrophilicity of the nitrene, resulting in a more preferred reaction with water compared with receptor C-H bonds (Chowdhry and Westheimer, 1979; Bayley and Knowles, 1977).

The two most prevalent drawbacks of the techniques of photoaffinity labeling are either very low labeling efficiencies or large amounts of non-specific labeling. Highly selective labeling is therefore a requirement. This can be determined by the chemistry of the probe and its reactive intermediates, as discussed above, and the conditions of the experiment. Labeling membrane proteins can present some unique difficulties. Partition equilibria, membrane impermeability, and degree of lipophilicity of the reagent, and large amounts of lipids near the protein(s) of interest are pertinent to a successful membrane protein photoaffinity labeling experiment (Hanstein, 1979).

Non-specific labeling can result from side reactions of the probe that ultimately compete with the reaction of the intended activated intermediate at the receptor site. The amount of non-specific labeling is measured in the presence of the parent compound or inhibitiors of the binding reaction. The probe can react with the solvent to yield unproductive labeling and thus low labeling efficiencies. Proteins, lipids and detergents in high

concentrations but not involved with the active or receptor site can be non-specifically labeled. This behavior can be minimized by using less hydrophobic ligands for example in membrane systems, and lowering the temperature of the solution. Also, photolysing under conditions where $R_o >> K_d$ (R_o=total receptor sites; K_d= equilibrium dissociation constant) decreases the proportion of free/bound label. This condition is acceptable for the purpose of identifying the protein bearing the ligand site. However, it is necessary to label most of the sites for labeled peptide sequencing and amino acid composition determinations (Hanstein, 1979). Theoretically, non-specific labeling and low labeling efficiencies result when the lifetime of the reactive intermediate is long enough compared to its dissociation rate constant to dissociate from the receptor before insertion. Under conditions where R_o is low compared to K_d, non-specific binding can be minimized by photolysis in the presence of scavengers. Scavengers are believed to react with photoactivated reagent present only in the solution and not bound to the active site (Ruoho et al., 1973). Control experiments with scavengers are used to distinguish true photoaffinity labeling from affinity labeling by a long-lived activated nitrene (pseudo-photoaffinity labeling), which diffuses in and out of the active site before reacting. Commonly used scavengers

are paraaminobenzoic acid, glutathione, TRIS, and soluble proteins.

In general, four control experiments must accompany any photolabeling experiment (Bayley and Knowles, 1977). (1) The reagent must not label any constituent in the dark, a test for reagent stability. (2) Prephotolyzed probe must not bind covalently to the preparation. This binding would be an indication of long-lived decomposition products or generation of affinity labels upon photolysis. (3) The stability of the protein system to photolysis must be measured. (4) The labeling must be protectable by the parent compound to be considered specific. The specificity of the labeling should be further characterized by demonstrating a saturable binding curve, which is good evidence for the presence of a reversible ligand-receptor complex prior to photolysis. A K_{app} may be measured in this way, and should be comparable to the binding constant as measured by another assay.

Introduction

Structural characterization of the mAcChR has been limited by difficulties in solubilization and purification. Until recently, few structural studies have been reported. The subunit molecular weight of $\{^3H\}$PrBCM[1] covalently labeled membrane-bound mAcChRs from smooth muscle, brain (Birdsall et al., 1979; Venter, 1983) and heart (Venter, 1983) have recently been estimated to be near 80,000 M_r by SDS gel electrophoresis. Venter (1983) has recently published molecular weights consistent with gel electrophoresis results (Birdsall et al., 1979) for brain and smooth muscle mAcChRs using radiation inactivation-target size analysis. No molecular weight for the cardiac receptor was reported using this method. Target size analysis of other receptor systems has indicated that this technique can detect functional oligomers in situ. Therefore Venter (1983) has proposed that the muscarinic receptor in brain and smooth muscle is

---

[1]Abbreviations used: $\{^3H\}$PrBCM, tritiated form of propylbenzilylcholine mustard; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; M_r HAc, acetic acid; MeOH, methanol; EtOH, ethanol; K_d, equilibrium dissociation constant; K_{app}, apparent equilibrium dissociation constant; WGA, wheat germ agglutitin; PMSF, phenylmethanesulfonyl fluoride; EDTA, ethylenediaminetetraacetic acid.

composed of an 80,000 M_r monomer.

Haga (1980) reported a molecular weight of 86,000 for a Lubrol PX solubilized {^{3}H}QNB-receptor complex from brain using sucrose gradient centrifugation in H_2O and D_2O. Amitai et al. (1982) reported a single labeled peak at molecular weight 86,000 in their photoaffinity labeling studies of membrane-bound rat cerebral cortex mAcChRs and two peaks at 160,000 and 86,000 daltons in rat atria using an aryl azide derivative of N-methyl-4-piperidyl benzilate. Avissar et al.(1983), using the same probe, report the appearance of a 160,000 M_r labeled peptide (in addition to the 86,000 M_r peptide) in the presence of Mn^{2+} in the same cortex preparation. These authors suggest that the 86,000 and 160,000 dalton components may correspond to receptor states that exhibit low and high affinity for agonists respectively. However, specific agonist protection of these peaks in the proper concentration range was not ascertained. Birdsall et al.(1978), in their study of brain mAcChRs reported two major populations of agonist binding sites which did not interconvert during binding experiments. The results of Avissar (1983) may present structural evidence for interconvertible high and low affinity agonist binding states of brain mAcChRs.

We report the first covalent labeling of soluble

mAcChRs from any tissue. Using an aryl azide analog of atropine as a photoaffinity label. we report specific covalent labeling of porcine cardiac mAcChRs in a broad molecular weight range between 70-79,000 M_r using gel electrophoresis. The results of Avissar et al.(1983) have not been confirmed by this laboratory because our attempts at the synthesis of their probe (which has not been published) have been unsuccessful.

Experimental Procedures

Materials

Bovine serum albumin and other proteins used for molecular weight calibration, sodium azide, digitonin (Lot. No. 92F-0661), cholic acid, carbamylcholine, phenylmethanesulfonyl fluoride, N,N´-methylene-bis-acrylamide, N,N,N´,N´-tetramethylethylenediamine, atropine sulfate and tropine were purchased from Sigma Chemical Company; 2.4 cm DEAE (DE 81) and GF/B filters, from Whatman. Sodium dodecyl sulfate and acrylamide were purchased from Bio-rad Laboratories as electrophoresis purity grade reagents. p-Aminophenylacetic acid was obtained from Aldrich; $\{^3H\}$ methyl iodide (10.0 Ci/mmole) from Amersham; $\{^3H\}$L-QNB (33.1 Ci/mmole), purchased from New England Nuclear, cochromatographed with a non-radiolabeled standard (the generous gift of Dr. W.E. Scott, Hoffman-LaRoche, Inc.) on Merck silica gel 60 plates in chloroform:methanol:water:acetic acid (65:25:5:5); R_f=0.58) and acetone:methanol:diethanolamine (10:10:0.3; R_f=0.38) solvent systems. Ninety-five percent of the radioactivity was found in the QNB spot.

Synthesis of azide derivatives of atropine.

Precoated Silica Gel 60 F 254 preparative and analytical TLC plates from Merck (20 x 20 mm, 2 mm

and 0.2 mm thick, respectively) were visualized by UV light at 254 nm and a potassium iodoplatinate spray solution (Krebs et al., 1969). All light sensitive reactions were done in the dark or with reaction vessels covered with foil unless otherwise noted. TLC solvent systems: A, chloroform:methanol:acetic acid:water (65:25:5:5), B, chloroform:methanol (4:1), C, 1-butanol: acetic acid:water (66:17:17).

p-Azido atropine (see Figure 10). The p-azidophenyl-acetate ester of tropine was synthesized by a modification of the method described by Moreno-Yanes and Mahler (1980). 20 g (132 mmoles) p-aminophenylacetic acid was stirred into 27 mls conc. H_2SO_4 in 200 mls H_2O and cooled with an ice-salt bath until 0- -5°C. 10.95 g (159 mmoles, 20% excess) $NaNO_2$ in 130 mls H_2O was added dropwise to the slurry with stirring. The solution was kept cold and stirred for another 20 minutes before adding a few drops of saturated urea solution to react with excess $NaNO_2$. After stirring for another 10 minutes, 17.22 g (265 mmoles) NaN_3 in 130 mls H_2O was added dropwise with vigorous stirring. The resulting redish foam was collected by filtration, washed with cold H_2O, and air dried. The p-azidophenylacetic acid product was crystallized from hot 95% ethanol and dried over P_2O_5 to give 15.1 grams (68% yield). The product migrated as a single spot using solvent system B; R_f=0.79. A

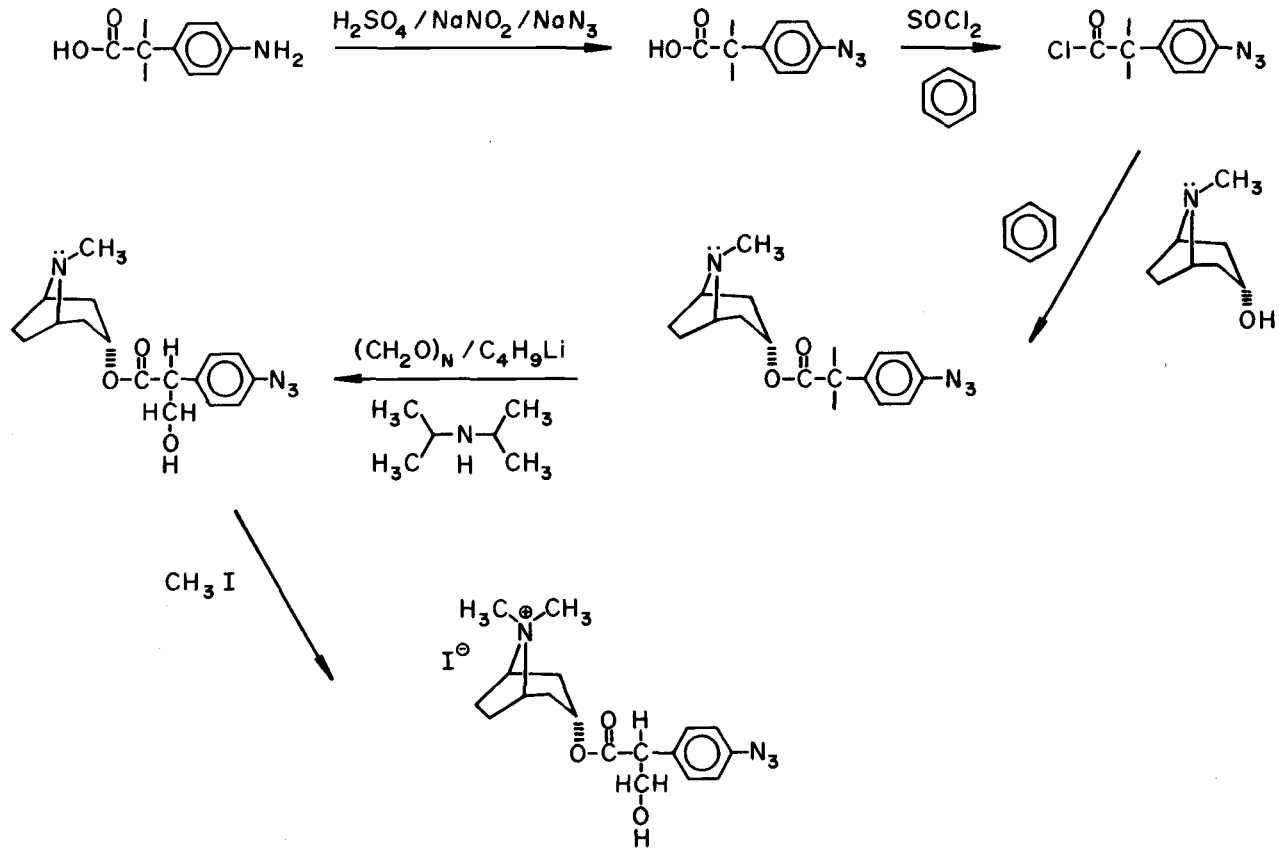


Fig. 10 Synthesis of p-azidoatropine methyl iodide. Details of the synthesis were described in the methods section.

second crop of crystals from 95% EtOH may be obtained to increase the overall yield. 17 g (112 mmoles) p-azidophenylacetic acid was added to 110 mls thionyl chloride (10-fold excess) and refluxed for 1 hour. The thionyl chloride was flash evaporated and the dark red-brownish oil was washed 4 times with 10 ml aliquots of dry benzene. Equimolar tropine (15 g) was dissolved in 80 mls dry benzene and added dropwise into the acid chloride refluxing in 120 mls dry benzene. The reaction was refluxed ½ hour after tropine addition and allowed to sit overnight at room temperature with stirring. The brown precipitate was collected by filtration and dissolved in H_2O. Na_2CO_3 was added to pH 9 and the ester product was extracted with 3 x 100 ml aliquots of ether. The ether was then back extracted with a saturated NaCl solution. The ether was then filtered and flash evaporated to dryness; the resulting dark brownish oil was dried over P_2O_5 for 2 days; one major spot, R_f=0.29 was obtained using solvent system B. Yield=35%. The hydroxymethylation was performed in a 3-neck round-bottomed flask equipped with a stir bar. A separate round-bottomed flask containing 10 g of paraformaldehyde (dried overnight over P_2O_5) was connected by teflon tubing to one neck of the reaction flask. All connections were sealed with rubber septum stoppers. The other 2 necks of the reaction flask were used for a nitrogen

gas inlet-out and for adding reactants by syringe, respectively. The sealed reaction flask with paraformaldehyde connection in place was cooled to $0^{o}C$ and flushed continuously with N_2 gas. The flow of gas was maintained for the entire reaction. 4.8 mls (1 equivalent) diisopropylamine and 50 mls dry THF were added by syringe to the reaction flask and allowed to cool on ice with stirring. 20.6 mls (1 equivalent) butyl lithium were added very slowly and cooled on ice for 15 minutes and then on a dry ice-acetone bath for 15 minutes. 10 g of the p-azidophenylacetate ester of tropine dissolved in a minimum volume of dry THF was added by syringe, washed in with an additional 150 mls dry THF, and allowed to sit for 20 minutes. An oil bath was then placed under the paraformaldehyde flask and heated to $150^{o}C$. The resulting formaldehyde gas was bubbled through the teflon tube into the reaction volume for 1-2 hours, until a film of paraformaldehyde formed over the surface of the reaction. During the hydroxymethylation, the color changed from dark red brown to lighter red. The paraformaldehyde was removed from the heat and the reaction mixture was allowed to slowly equilibrate to room temperature overnight. Na_2CO_3 was then added to pH 9 and the solution was extracted with 3 x 100 ml aliquots ether. The ether was back extracted with saturated NaCl solution and dried over Na_2SO_4. TLC analysis using solvent system A

showed the crude product to be an approximately equimolar mixture of the p-azidophenylacetate ester of tropine (R_f=0.54 and its hydroxymethylated derivative (R_f=0.40) and small amounts of other unknown products. The ether was flash evaporated and the product was purified by preparative TLC in solvent system A. The product was eluted from the gel with the same solvent solution, flash evaporated to dryness, dissolved in ether and dryed over Na_2SO_4; TLC analysis showed a single spot in solvent system A (R_f=0.40) and C (R_f=0.33). The ether was flashed to dryness and the compound (red brown powder) was stored at -20°C dessicated in a foil wrapped vial. Yield=40%. ^{1}H-NMR ($CDCl_3$) (see Figure 11): δ7(4H, q, aromatic protons), 4.9 (1H, t, C_3 methine in tropane skeleton), 4.1 (1H, s, O<u>H</u>), 3.5-4.0 (3H, m, -C<u>H</u>-C<u>H</u>$_2$-OH), 2.9 (2H, broad s, C_1 and C_5 methines on tropane skeleton), 2.18 (3H, s, >N-<u>C</u>H$_3$), 1-2 (10H, m, four methylenes in tropane skeleton). The 80 MH spectrum was taken at the Oregon State University NMR Spectroscopy Laboratory, Dept. of Chemistry, using a Varian FT80A spectrometer.

p-Azidoatropine methyl iodide. The free base of p-azidoatropine was methylated in $CHCl_3$ by stirring with equimolar CH_3I for 3 days at 4°C. The white precipitate was collected by filtration, washed with $CHCl_3$ and dissolved in 95% EtOH and a small amount of H_2O. The quaternary amine product was separated from the

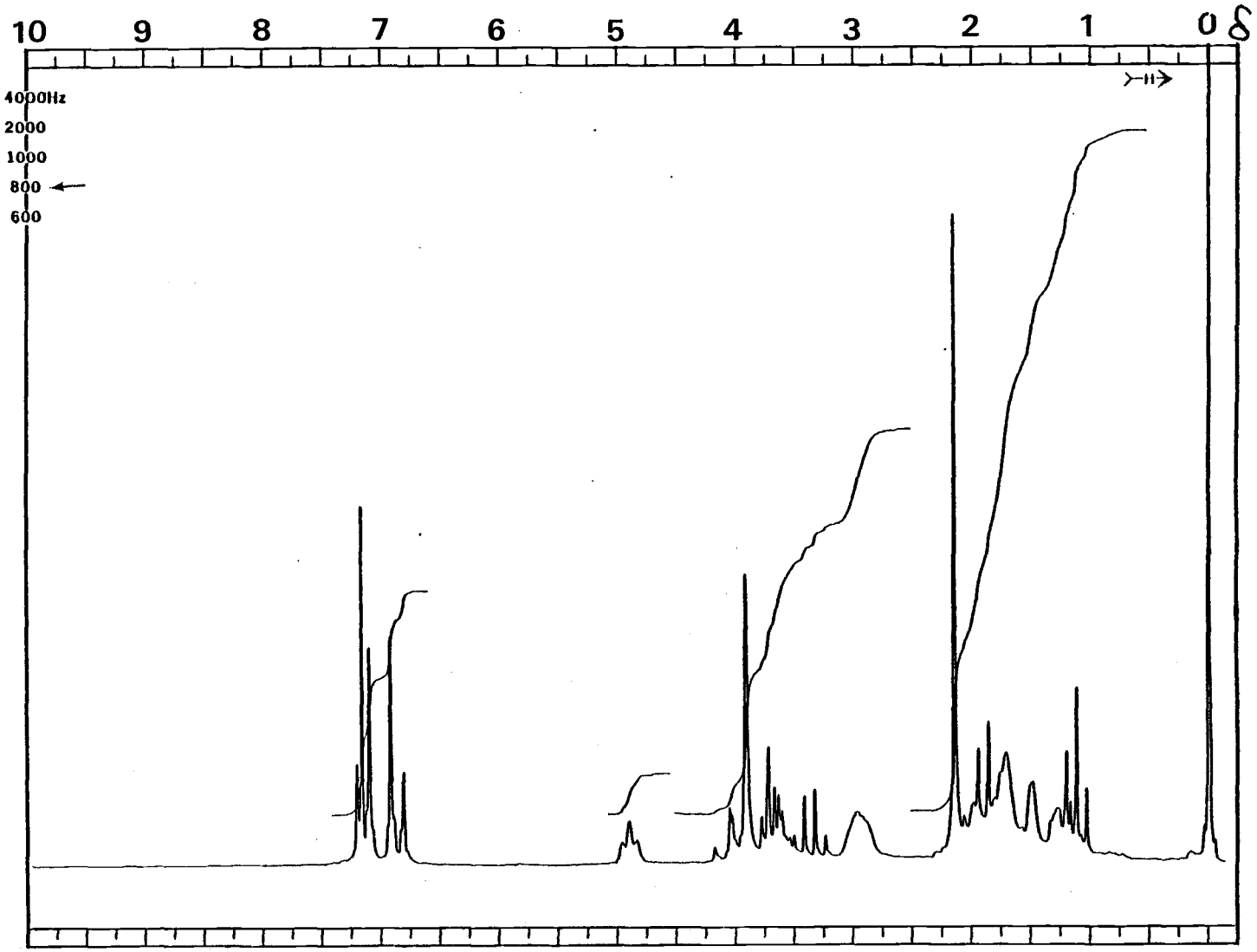


Fig. 11 ^{1}H-NMR spectrum of p-azidoatropine

unreacted tertiary compound by preparative TLC using solvent system A:R_f=0.15. Elemental analysis, M.H.W. Laboratories, Phoemix, Arizona U.S.A.; $\pm$0.3%. ($C_{18}H_{25}O_3N_4I$) calc. C (45.8), H (5.3), O (10.2), N (11.9), I (26.9); found C (45.6), H (5.5), O (10.3), N (11.8), I (26.8). m.p.=183-188 (decomp.) IR_{max}^{KBR} cm^{-1}: 3400 (broad, H bonding), 2100 (sharp, azide), 1735 (sharp, ester), 1575 (sharp, phenyl ring), see Figure 12 (Beckman Acculab 7). UV-Vis spectrum (Figure 13) showed a characteristic phenyl azide absorption maxima at 252 nm, which was diminished after exposure to UV light.

{^{3}H}p-Azidoatropine methyl iodide. A solution of 3.6 mgs (11 μmoles) free base in 450 μls $CHCl_3$ was added to 10 μmoles {^{3}H}-methyl iodide (10.0 Ci/mmole) cooled with liquid N_2. The reaction was sealed after reaching room temperature. After 6 days at 4°C the reaction mixture was applied to a preparative TLC plate and chromatographed using solvent system A as described above. The radioactive product was eluted with solvent system A, flash evaporated and brought to a volume of 7 mls 95% EtOH stored in a foil wrapped vial at -20°C over $CaSO_4$. Analytical TLC analysis indicated that the radioactive product cochromatographed with the cold compound in solvent system A (R_f=0.17) and B (R_f=0.15) with 92% of the radioactivity in the single

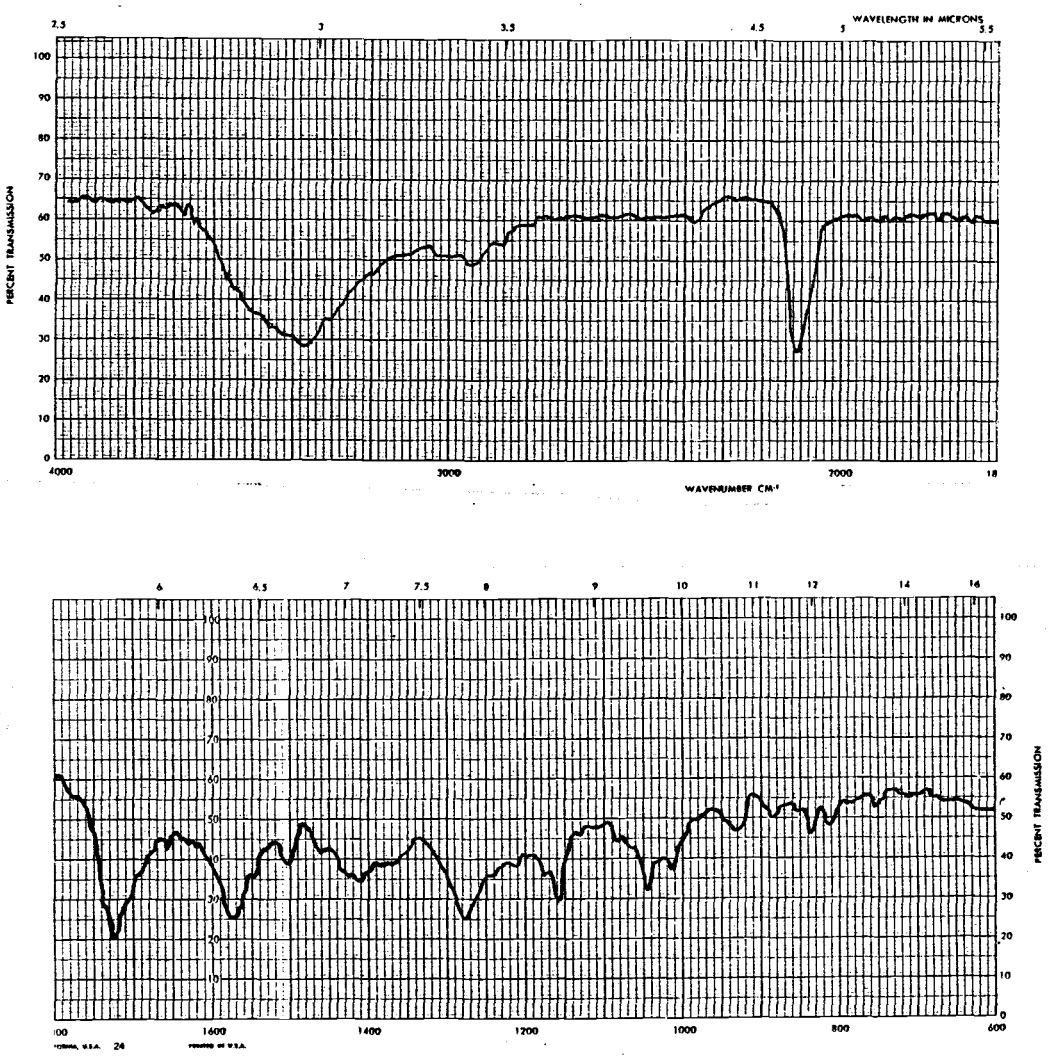


Fig. 12 IR spectrum of p-azidoatropine methyl iodide.

Fig. 13 Effect of photolysis on the UV-VIS absorption spectrum of atropine azide. A 21.5 μM solution of atropine azide in H_2O (1 cm path length) was irradiated for 0 (solid line) and 10 (dashed line) minutes with a Model UVSL-25 Mineralight Lamp (Ultraviolet Products Inc.) (UV max 254) at a distance of 1 cm, λmax, 227, 252; ε, 11,700, 11,400.

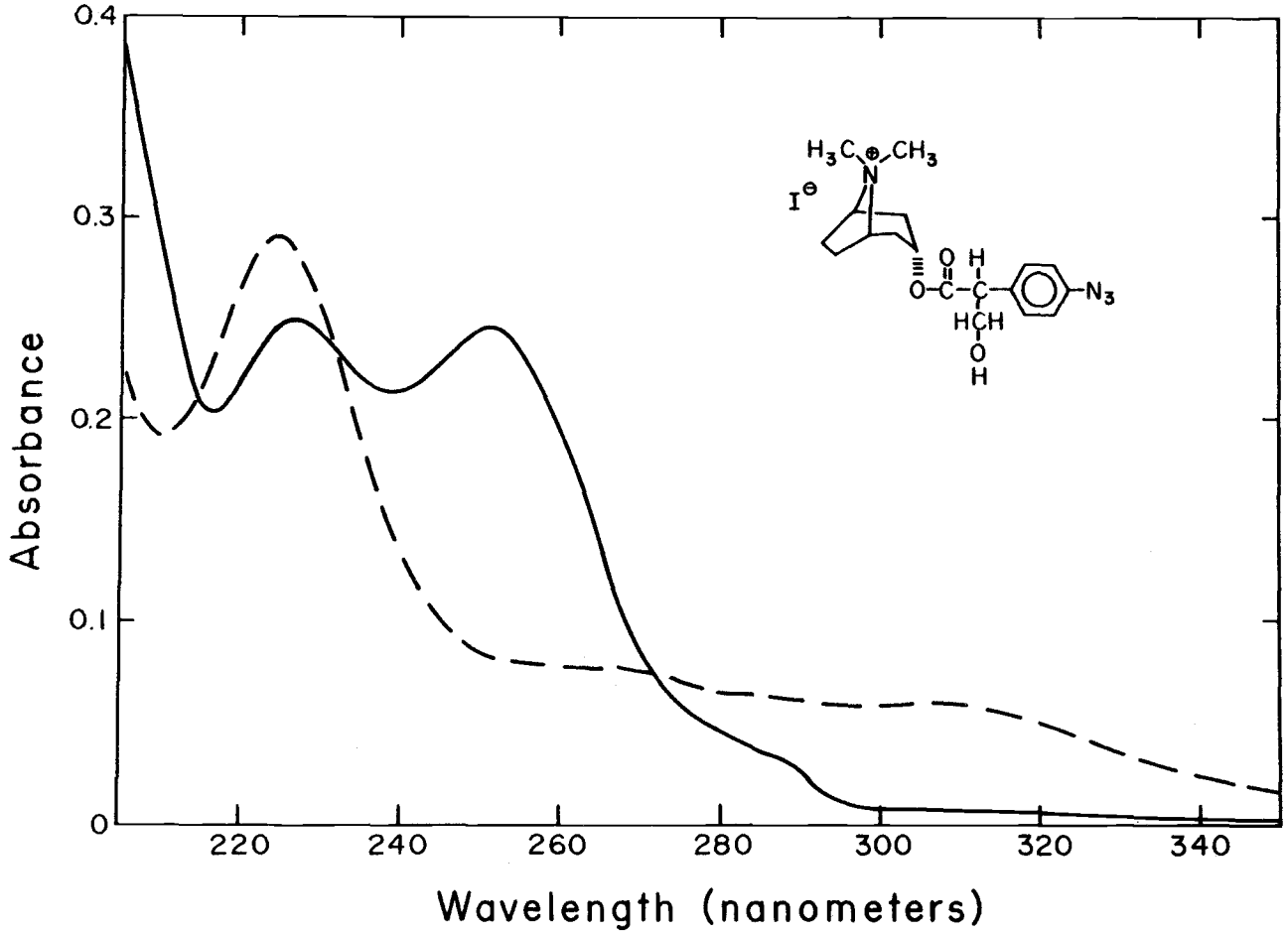

0.4
0.3
0.2
0.1
0
Absorbance
220
240
260
280
300
320
340
Wavelength (nanometers)
H_3C CH_3
N
$I^{\ominus}$
O H
O-C-C
HCH
O
H
N_3

Figure 13

spot. Yield=12%. The product was very stable under the storage conditions; no change in the TLC analysis was observed over one year.

Preparation of membrane-bound and solubilized mAcChR

Atrial membranes were prepared as described by Peterson and Schimerlik (1982) except that $NaClO_4$ and sucrose were omitted from the homogenizing and resuspension buffers; PMSF was present in all steps of the membrane preparation. 0.4% (w/v) digitonin and 0.08% (w/v) cholate were used together to solubilize atrial membranes by a modification of the method of Cremo et al. (1981) as described by Herron and Schimerlik (1983). PMSF was not present in the detergent buffers. A typical solubilization yielded a protein concentration of 2-3 mg/ml and 8-12 nmol {^{3}H} L-QNB sites/protein. The solubilized receptor was further purified in the same detergent system by a preparative WGA affinity chromatography method described by Herron and Schimerlik (1983); resulting fractions were approximately 0.3-0.4 mg protein/ml and 130 nMol {^{3}H}L-QNB sites/g protein. All receptor purifications were performed with 25 mM imidazole, 1 mM Na_2EDTA, pH 7.4 (buffer A) in the absence or presence of 0.4% (w/v) digitonin, .08% (w/v) cholate (buffer B). To change the buffer to 10 mM PO_4, 1 mM Na_2EDTA, pH 7.4 (buffer C without detergents for membranes, buffer D with detergents present as above), membrane-bound receptor

preparations were centrifuged and resuspended 3 times and solubilized fractions were either dialysed against 1/10 x detergent in buffer C or passed over a P_{10} (BioGel) column in buffer D.

$\{^3H\}$L-QNB binding assays and equilibrium titrations

Membrane-bound and solubilized mAcChR concentration was quantitated in terms of $\{^3H\}$L-QNB sites using a DEAE filter disc assay developed by Schmidt and Raftery (1973) for the nAcChR and modified in this laboratory for use with the digitonin/cholate mixed detergent system (Cremo et al., 1981). The DEAE disc assay was also used for equilibrium titrations with the solubilized mAcChR. The method of Yamamura and Snyder (1974) was used for equilibrium titrations with the membrane-bound receptor.

Data evaluation

Equilibrium titration curves measuring $\{^3H\}$L-QNB displacement were analyzed by using weighted least-squares fit to Equation 1.

$$\frac{\{I_o\}}{\{R_o\}\left[1 - \frac{\overline{RQ}_o}{RQ_o}\right]} - 1 = \bar{j} = \frac{\{Q\}}{RQ} \quad \frac{K_i}{K} \qquad (1)$$

The value of the dissociation constant (K_i) for a

given competitive inhibitor was calculated from the slope of the plot of $\bar{j}$ function (Best-Belpomme and Dessen, 1973) versus {Q}/{RQ} where {Q} equals the free {^{3}H}L-QNB at fixed levels of total inhibitor at differing {I_o}, {RQ}equals specifically bound {^{3}H}L-QNB, and $\overline{RQ}$ and $\overline{RQ}_o$ equal the fraction saturation L-QNB sites in the presence of total inhibitor concentration {I_o} and in the absence of inhibitor, respectively. Thus, from the slope of the plot of $\bar{j}$ versus {Q}/{RQ} and the value of K, the dissociation constant for L-QNB, the K_i values could be computed for the inhibitor.

Photoaffinity labeling

Aliquots of mAcChR preparations indicated in figure legends were placed in 1 ml plastic beakers and incubated at room temperature for ½ hour in the presence or absence of competing reagents. {^{3}H} p-azido atropine methyl iodide was added in the dark and equilibrated under conditions specified in figure legends. Samples were irradiated on ice with a Model UVSL-25 Mineralight lamp (Ultraviolet Products, Inc.) at 254 nm, located 3 cm from the surface of the solution. The optical density of the samples was never greater than 1.0. The photolysed samples were then denatured immediately with SDS and β-mercaptoethanol in electrophoresis sample buffer according to Laemmli (1970), unless otherwise specified.

Denaturing gel electrophoresis

Electrophoresis with 7.0% polyacrylamide containing 0.1% SDS were performed as described by Laemmli (1970). Gels were poured in tubes 1 cm wide and to various lengths between 80 and 120 cms with a 2.0 cm, 3% stacking gel and electrophoresed at 4°C. Gels with photolabeled samples were sliced automatically into 1 mm pieces; only the running gels were sliced. Gels were not stained and destained before slicing unless otherwise noted. Each slice was oxidized in a 10 ml glass scintillation vial with a sealed plastic cone cap with 0.75 ml 30% H_2O_2 at 75°C overnight before counting in 6.5 mls toluene-triton fluor. Additional gels used to visualize protein patterns of identical photolabeled samples and molecular weight standards were marked at the dye front and stained for 2 hours with Coomassie blue, destained with 7.5% HAc, 5% MeOH, and scanned densitometrically at 600 nm with a Cary 219 spectrophotometer gel scanning accessory. All molecular weight standards were prepared as other samples, with respect to detergent content and volumes loaded.

Results

Reversible interaction of p-azidoatropine methyl iodide with mAcChR {^{3}H}L-QNB binding sites.

Titration curves of specifically bound {^{3}H}L-QNB *versus* p-azidoatropine methyl iodide for the membrane-bound, solubilized and WGA partially purified solubilized mAcChRs in phosphate buffer are shown in Figures 14, 15 and 16, respectively. The photoaffinity label appeared to displace {^{3}H}L-QNB in a competitive manner from a single class of sites for the three mAcChR preparations examined. The results of these experiments and an identical set of experiments using buffer A & B (imidazole) were tabulated in Table IV. Titrations in the presence of imidazole (data not shown), like those shown for phosphate (Figures 14, 15 and 16) were competitive *versus* {^{3}H}L-QNB. However, the K_ds for the photoaffinity label in the presence of imidazole were approximately 10-fold higher than in phosphate in all preparations studied (see Table IV). For each buffer, however, the data indicate that the K_ds for the three preparations were not significantly different. On the basis of these results, phosphate buffers were routinely used for photolabeling experiments. However, in attempts to reduce non-specific labeling, some photolabeling experiments were done in the presence of imidazole.

Fig. 14 p-azidoatropine methyl iodide titration of specifically bound {^{3}H}L-QNB to membrane-bound mAcChR. The atropine azide concentration was varied at constant concentrations of mAcChR (66 p$\underline{M}$ L-QNB sites) and {^{3}H}L-QNB (179 pM) and equilibrated in the dark for 1 hour at room temperature in buffer C. After removing 100 µl aliquots to measure total {^{3}H}L-QNB concentration, duplicate 2 ml aliquots were pipetted onto GF/B glass fiber filter discs mounted on a filtration manifold and washed once with 7 mls ice-cold buffer C to remove free {^{3}H}-L-QNB. Specifically bound receptor was calculated after correction for nonspecifically bound label determined as above but in the presence of 10 µ$\underline{M}$ atropine. Data were evaluated by a weighted least squares fit to Eq. (1) to give a value of $(6.1 \pm 0.4) \times 10^{-8}$M for the K_d of p-azidoatropine methyl iodide. The curve through the data points was calculated from the law of mass action using $(4.1 \pm 0.7) \times 10^{-11}$M as the dissociation constant for L-QNB, the above value for p-azidoatropine methyl iodide, and the experimentally determined concentrations of free {^{3}H}L-QNB at each data point.

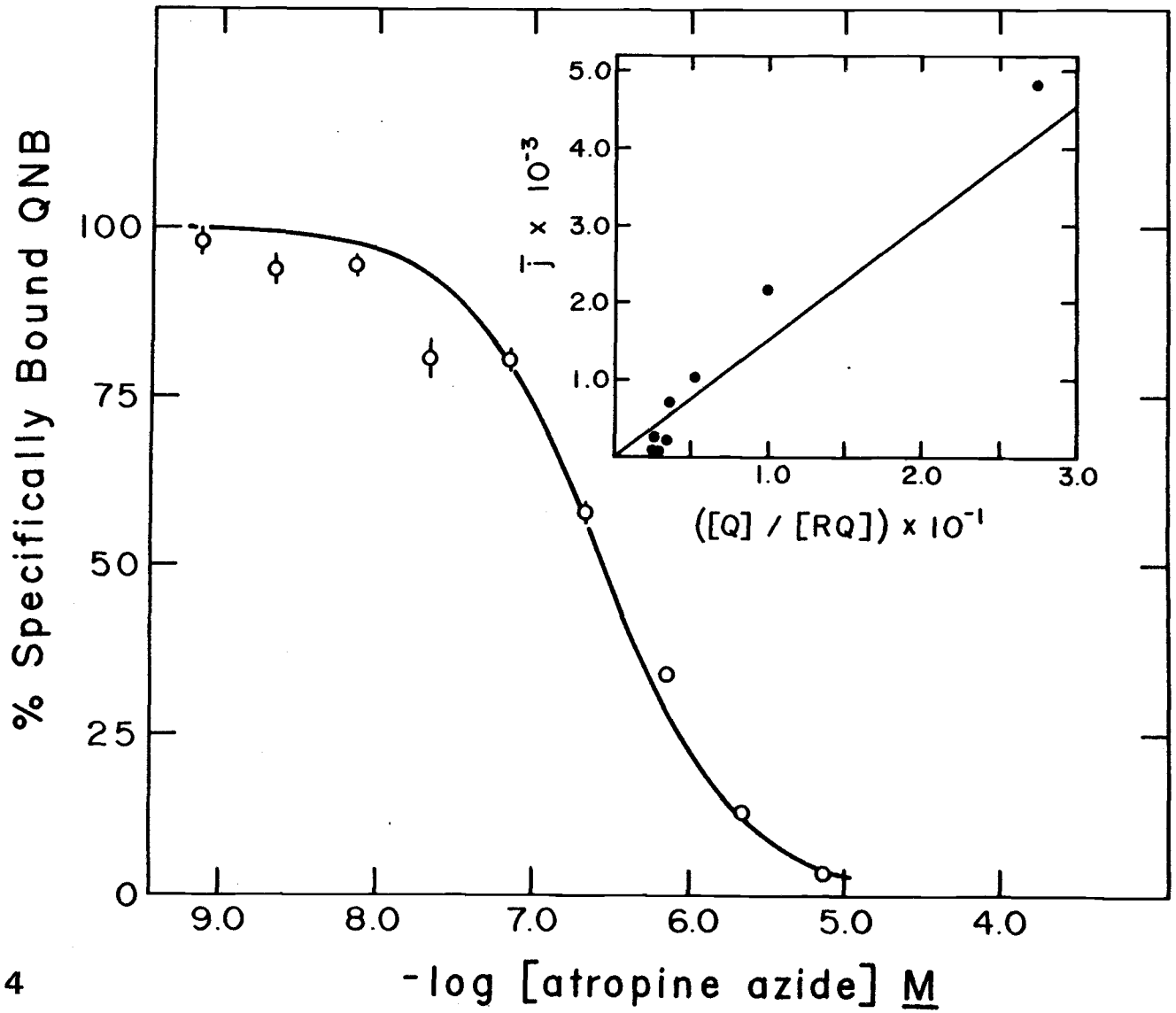

% Specifically Bound QNB
100
75
50
25
0
9.0
8.0
7.0
6.0
5.0
4.0
-log [atropine azide] M
$\bar{J} \times 10^{-3}$
5.0
4.0
3.0
2.0
1.0
1.0
2.0
3.0
([Q] / [RQ]) x 10^{-1}

Figure 14

Fig. 15 p-Azidoatropine methyl iodide titration of specifically bound {^{3}H}L-QNB to digitonin/cholate solubilized mAcChR. Methods and data evaluation were as described in Figure 14 except that samples were equilibrated in buffer D and the DEAE disc assay was used to separate bound from free {^{3}H}L-QNB. mAcChR and {^{3}H}L-QNB concentrations were 0.4 nM {^{3}H}L-QNB sites and 1.1 nM respectively. A value of $(1.28 \pm 0.22) \times 10^{-7}$M was obtained for the dissociation constant for p-azidoatropine methyl iodide using $(2.5 \pm 0.4) \times 10^{-10}$M as the dissociation constant for L-QNB.

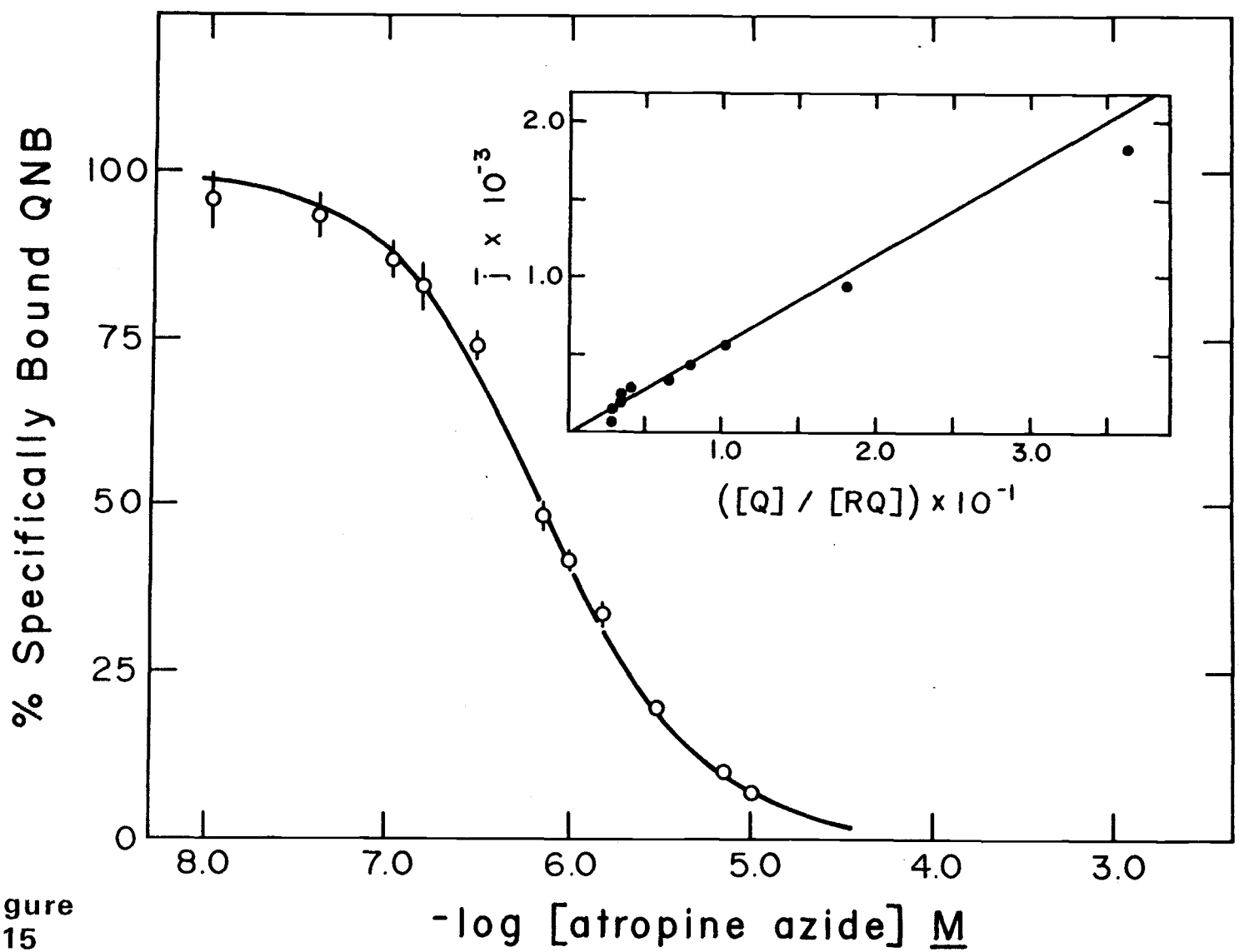

% Specifically Bound QNB
100
75
50
25
0
8.0
7.0
6.0
5.0
4.0
3.0
-log [atropine azide] M
1/I x 10^-3
2.0
1.0
1.0
2.0
3.0
([Q] / [RQ]) x 10^-1

Figure 15

Fig. 16 p-Azidoatropine methyl iodide titration of specifically bound {^{3}H}L-QNB to WGA affinity partially purified mAcChR. Methods and data evaluation were as described in Figure 15. mAcChR and {^{3}H}L-QNB concentrations were 1.7 nM and 1.0 nM respectively. A value of $(7.6 \pm 1.2) \times 10^{-8}$M was obtained for the dissociation constant for p-azidoatropine methyl iodide using $(2.5 \pm 0.4) \times 10^{-10}$M as the dissociation constant for L-QNB.

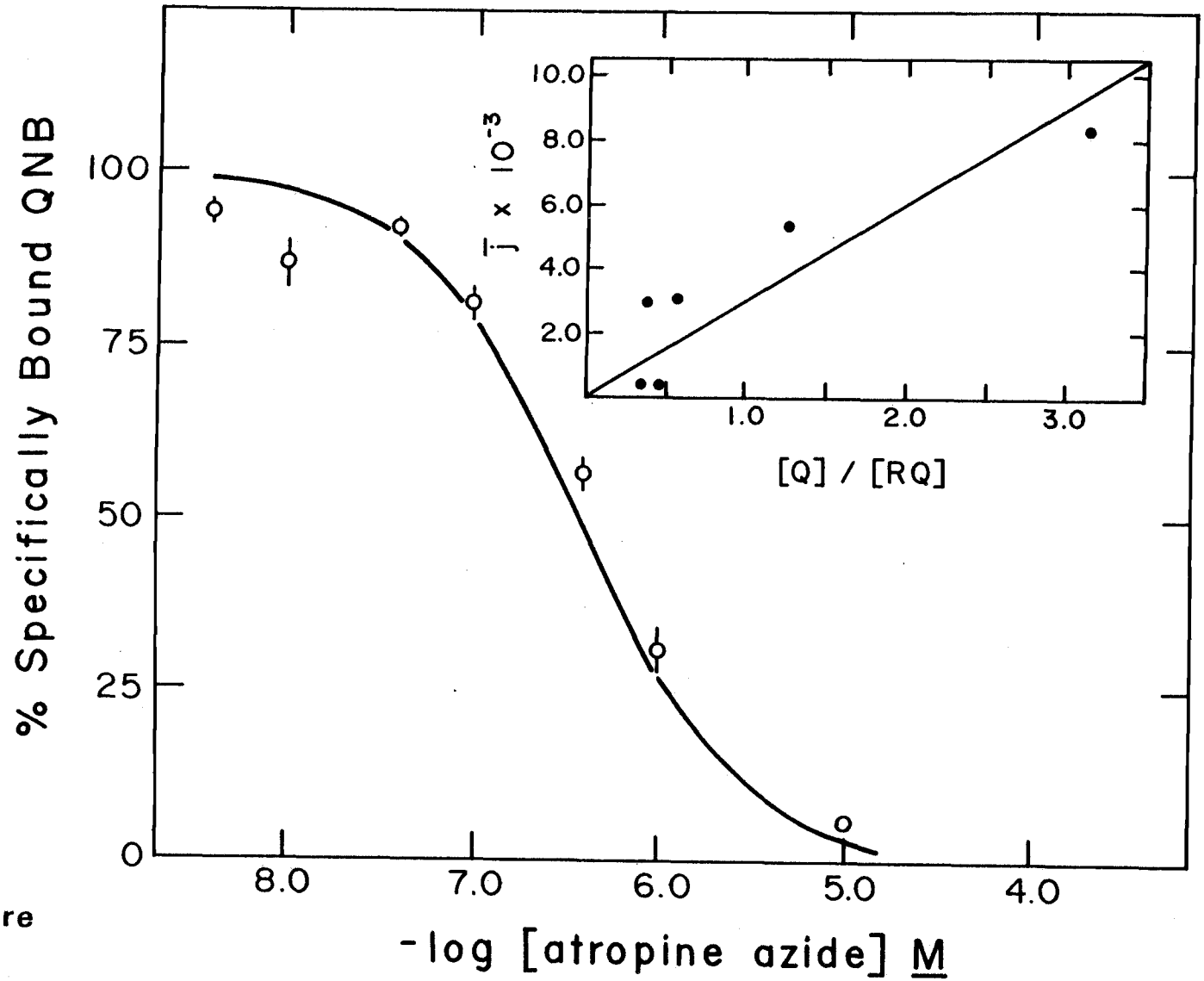

% Specifically Bound QNB
100
75
50
25
0
8.0
7.0
6.0
5.0
4.0
-log [atropine azide] M
1/I x 10^-3
10.0
8.0
6.0
4.0
2.0
1.0
2.0
3.0
[Q] / [RQ]

Figure 16

Table IV. Summary of Equilibrium Dissociation Constants[a] for p-Azidoatropine Methyl Iodide

Buffer	mAcChR Preparation					
	membrane-bound	n[d]	solubilized	n	WGA partially purified	n
phosphate[b]	$(7.0 \pm 1.3) \times 10^{-8}$ M[e]	2	$(7.8 \pm 4.4) \times 10^{-8}$ M	3	$(7.6 \pm 1.2) \times 10^{-8}$ M	1
imidazole[c]	$(8.4 \pm 3.0) \times 10^{-7}$ M	2	$(6.2 \pm 0.6) \times 10^{-7}$ M	1	$(1.82 \pm 0.6) \times 10^{-6}$ M	1

[a]Determined by competition with $\{^3H\}$L-QNB at 21°C.

[b]Buffer C for membrane-bound and D for soluble preparations.

[c]Buffer A for membrane-bound and B for soluble preparations.

[d]n=number of independent determinations.

[e]Except where n=1, each value was the mean of separate experiments, ± s.d. Where n=1, uncertainties are determined as described in Data Evaluation.

Covalent labeling of mAcChR with p-azidoatropine methyl iodide.

Denaturing SDS polyacrylamide gel electrophoresis was used exclusively to assay for covalent interaction of {^{3}H} labels with the mAcChR. Figure 17 shows the distribution of radioactive label in a typical gel of {^{3}H} p-azidoatropine methyl iodide labeled WGA partially purified mAcChR. A densitometric scan of an identical sample stained with Coomassie blue is shown in Figure 18. Non-specific labeling with the azide derivative was measured in the presence of 110 nM L-QNB. Saturation of L-QNB binding sites was predicted at 110 nM L-QNB, where the K_d for L-QNB in the WGA partially purified fraction = 250 pM (see Figure 15). The results in Figure 17 indicate that one or more proteins migrating in the broad molecular weight range 70-79,000 M_r (R_f=0.33-0.40) were specifically labeled by photolysis in the presence of {^{3}H} p-azidoatropine methyl iodide. A large amount of radioactivity was also incorporated into one or more proteins migrating in the 90-92,000 M_r region, however incorporation was only partially blocked in the presence of 110 nM L-QNB. Background levels (approximately 500 counts/minute) of radioactivity were observed between R_f=0.2 and 0.95 in a control experiment (data not shown) where the tritiated derivative was prephotolysed before incubating and photolysing in the presence of the

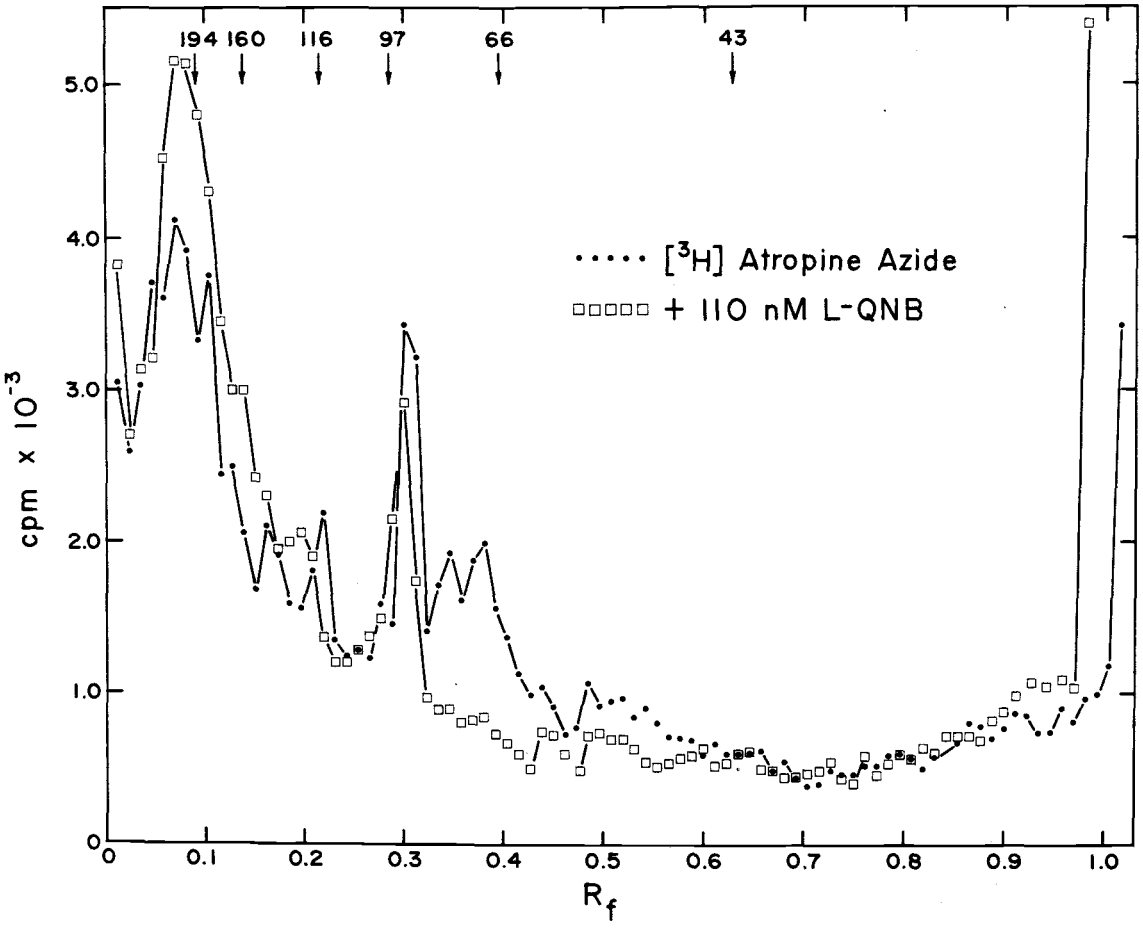


Fig. 17 SDS-PAGE of WGA partially purified mAcChR covalently labeled with p-azidoatropine methyl iodide. 0.5 ml receptor aliquots (0.6 mg protein/ml, 44 nM {^{3}H}L-QNB binding sites in 1/10 x detergent in buffer C were incubated on ice in the dark with 150 nM {^{3}H} azide label for 24 hours in the absence (●—●) or presence (□—□) of 110 nM L-QNB. The samples were removed from ice and incubated for 1 hour at 21°C before equilibrating again to 4°C and photolysing for 5 minutes with slow stirring at 4°C. Molecular weight markers were ovalbumin (43K), bovine serum allbumin (66K), phosphorylase A (97K), β-galactosidase (116), debranching enzyme (160K) and myosin (194K).

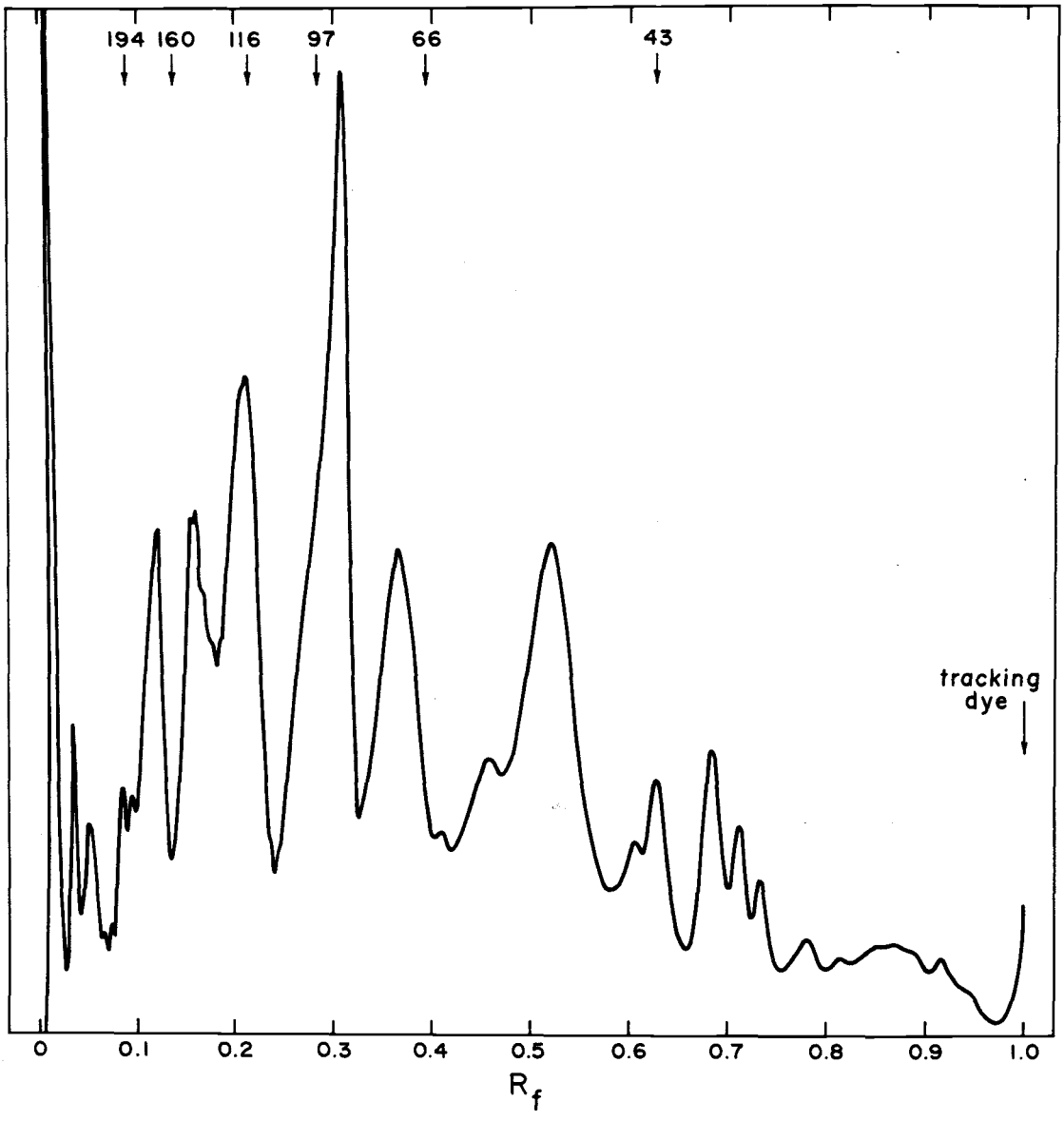


Fig. 18 Scan at 590 nM of a Coomassie blue stained SDS-PAG of WGA partially purified mAcChR. The protein sample was identical to that described in Figures 17 and 19, except that the protein was not labeled.

receptor preparation. This control experiment also showed a large amount of label migrating at the top of the gel (R_f=0-0.15) identical to that seen in Figure 17. This peak probably represents non-covalently bound photolysed probe because background levels (approximately 500 cpm/slice) of radioactivity were observed in this region if samples were prepared exactly as in Figure 17 but dialysed against 1/10 x detergent in buffer C before gel electrophoresis (data not shown). This treatment however, did not alter the distribution of radioactivity in the R_f=0.2-0.95 region indicating that the label in this region was not dialysable and was probably covalently bound to protein. If protein was omitted from the same experiment described in Figure 17, only the large peak at R_f=0-0.15 was observed, indicating that the label found in the presence of protein in the R_f=0.2 to 0.95 region (see Figure 17) was not due to tritiated photolysis products non-covalently bound to protein migrating in this region. A large amount of radioactivity comigrating with the dye front to the leading schleiren line (region of gel not shown) was present in the experiments described in Figure 17 and in the 2 control experiments (prephotolysis and protein omitted) described above. A third control experiment where samples prepared as in Figure 17 were not photolysed, showed background levels across the gel and

a large peak corresponding to the dye front to leading schleiren line region. This radioactivity could only be partially reduced by dialysing samples before electrophoresis. The results of these 3 control experiments indicate that the labeling at the dye front to the leading schleiren line may represent covalently and noncovalently labeled lipids, detergent and low molecular weight proteins, and some non-photolysed probe. Labeling in this region was not effected by L-QNB or atropine preincubations under conditions for determining non-specific labeling (nanomolar to μ molar range) and was only reduced with high concentrations (mM) of these 2 compounds. These control experiments indicate that the distribution of radioactivity in this region of the gel probably does not contain useful information about specific protein labeling.

{^{3}H} L-QNB binding activity was found to be very stable under the photolysis conditions described in the methods section. After 10 minutes photolysis at 4°C, the following loss in activities relative to a non-photolysed sample were observed: membrane-bound, 3%, soluble extract, 10%, and WGA partially purified, 0%. Photolytically induced protein crosslinking was not detected as protein at the top of the gel in gel protein staining patterns of the samples described above.

The nature of the specificity of covalent labeling

observed in the 90-92,000 M_r and 70-79,000 M_r regions was demonstrated by a titration of {^{3}H} p-azidoatropine methyl iodide labeling with varying concentrations of L-QNB (Figure 19). Under the experimental conditions, (L-QNB sites >> K_d for L-QNB), a linear titration curve (solid line, Figure 19) was predicted (assuming simple competitive inhibition) with a break point equal to the total number of L-QNB sites determined by DEAE disc assay (44nM). The behavior found for the label in the 70-79,000 M_r band (●) was consistant with a break point of 44nM L-QNB sites. However, these data were not sufficient to determine an exact break point to give the number of {^{3}H} p-azidoatropine methyl iodide sites directly. The data (■) for the 90-92,000 M_r band did not show saturable protection by L-QNB. The approximately linear dependence of labeling (— — —) on {L-QNB} in this range indicated its probable non-specific nature, unrelated to mAcChR L-QNB binding sites.

A titration of specific covalent labeling in the 70-79,000 M_r region <u>versus</u> {^{3}H} p-azidoatropine methyl iodide was shown in Figure 20. The titration curve appeared to saturate at high azide concentrations, consistent with the formation of a reversible azide-protein complex prior to nitrene formation and covalent attachment of the analog. A K_{app} of 85 nM was obtained by this method, which was in excellent agreement with the value

Fig. 19 Titration of {^{3}H} atropine azide labeling by L-QNB in the 70-79,000 M_r (●) and 90-92,000 M_r (■) regions. Data points for 0 nM and 110 nM L-QNB were calculated from the gel profiles shown in Figure 17. The other 3 data points were calculated from gels electrophoresed in the same experiment, however these data were not shown in Figure 17. Counts/minute for the 71-79,000 M_r and 90-92,000 M_r bands were summed and normalized by substracting the summed base-line average counts/minute (R_f=0.6-0.8) for each {L-QNB}.

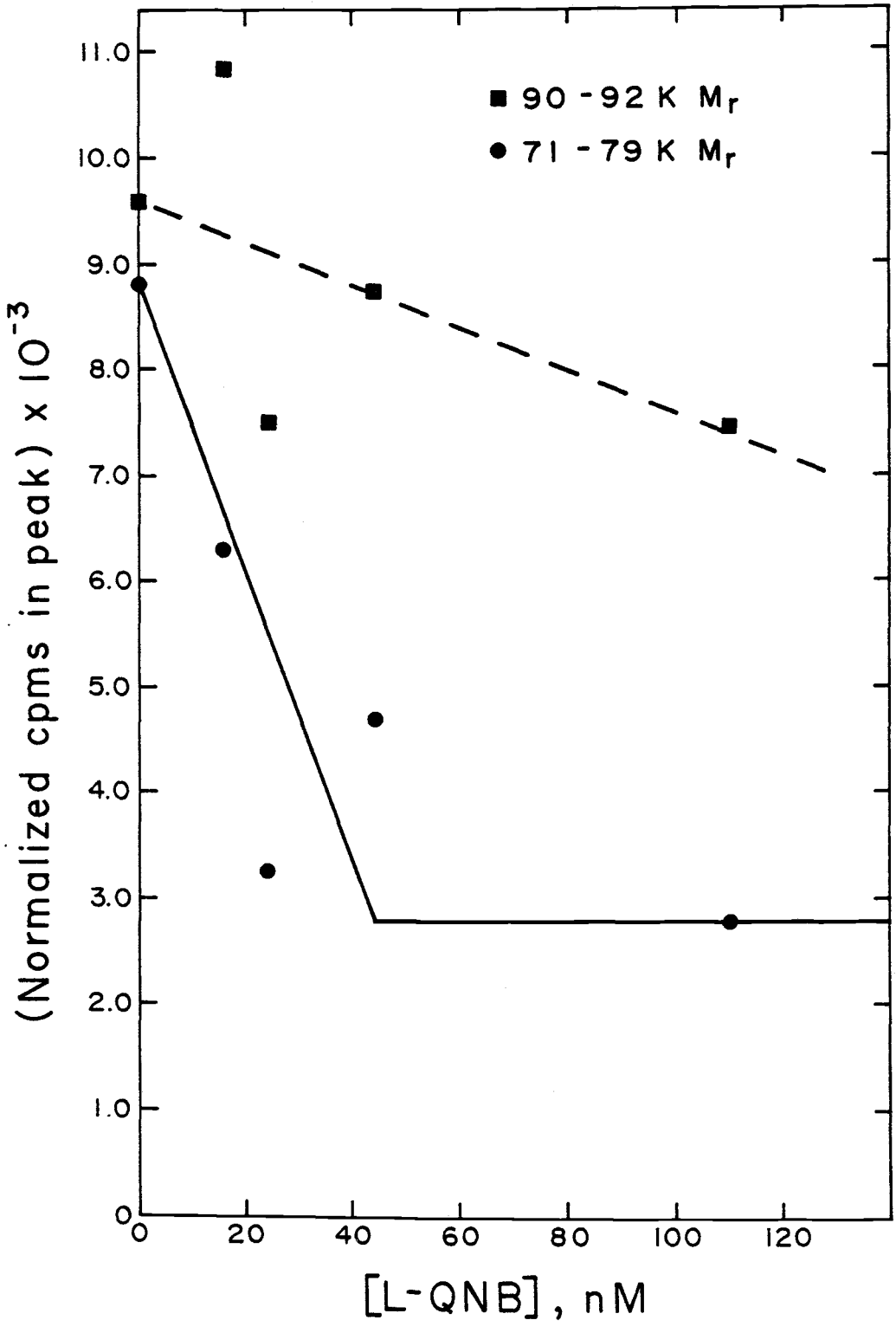

11.0
10.0
9.0
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0
(Normalized cpms in peak) x 10^-3
90 - 92 K Mr
71 - 79 K Mr
0
20
40
60
80
100
120
[L-QNB], nM

Figure 19

Fig. 20 Dependence of {^{3}H} p-azidoatropine methyl iodide specific labeling in the 70-79,000 M_r region on {^{3}H} atropine azide. The protein sample and labeling procedure were as described in Figure 17. Non-specific labeling at each azide concentration was measured in the presence of 110 nM L-QNB. Counts/minute for each gel were normalized as described in Figure 19. Specific labeling was calculated by subtracting the normalized counts/minute across the 70-79,000 M_r region in the presence 110 nM QNB from that value in the absence of L-QNB. The data were analysed (see insert) by using

$$C = \frac{C_o L_o}{K_{app} + L_o}$$

where C = specific c.p.m. measured at total ligand concentration L_o and C_o = specific c.p.m. observed at saturation with L. C_o = 2560 c.p.m. and K_{app} = (8.5×10^{-8})M were determined from the slope and Y-intercept of a least-squares fit of the plot of 1/C <u>versus</u> $1/L_o$.

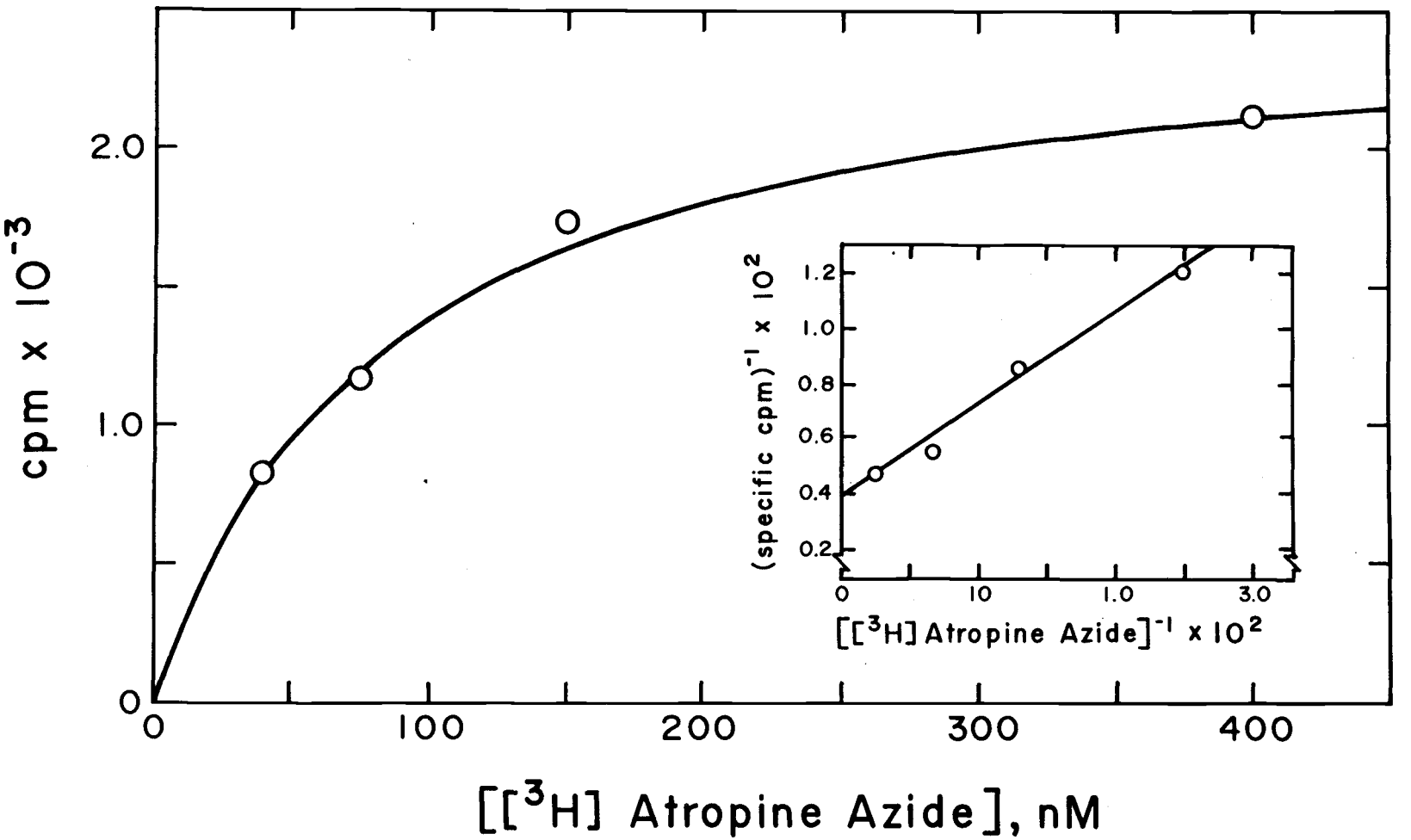

cpm x 10^{-3}
2.0
1.0
0
0
100
200
300
400
[[^{3}H] Atropine Azide], nM
(specific cpm)$^{-1}$ x 10^2
1.2
1.0
0.8
0.6
0.4
0.2
0
10
1.0
3.0
[[^{3}H] Atropine Azide]$^{-1}$ x 10^2

Figure 20

obtained by competition of the cold analog for {^{3}H}L-QNB binding sites (Figure 16, Table IV). Labeling in the 90-92,000 M_r region (data not shown) was not specific and showed no dependence on the concentration of the titrated probe.

Whereas the protein species migrating in the 90-92,000 M_r range were not identified, the labeling in the 70-79,000 M_r (Figure 17) was tentatively assigned to the mAcChR ligand binding peptide(s) on the basis of the excellent agreement between the reversible binding data (Figure 16) and the covalent binding data (Figures 17, 19, 20) for this photoaffinity label. The non-specific labeling in the 90-92,000 M_r range comigrates with a major protein peak observed by Coomassie blue staining (Figure 18). At the level of purification of the WGA partially purified fraction (assuming a M_r at 75,000, one {^{3}H}L-QNB binding site/molecule) the mAcChR represents less than 1% of the total protein and cannot be visualized with Coomassie blue stained gels.

The double peak shown in Figure 17 in the 70-79,000 M_r range was not always resolved in replicate experiments. Usually, the double nature of the broad peak was defined by the data from one, 1 mm gel slice. The uncertainty in the definition of R_f could be as much as $\pm$ 1 gel slice in these experiments and a frame shift resulting from slicing different gels could account for the

irreproducible appearance of a doublet. Because these gels were not stained before slicing and counting, no absolute reference to protein staining position was available for each gel; protein staining positions were determined from a separate gel loaded with an identical sample that was not sliced, but stained and destained. Staining and destaining each gel was avoided because control experiments using analytical TLC indicated that the p-azidoatropine methyl iodide ester linkage was unstable under destaining conditions (7.5% HAc, 5% MeOH, 21°C, 2-3 days). No specific labeling was observed with stained-destained gels. Labeling was reduced to background levels across the gel except for the 90-92,000 M_r region. These results indicated that the probe bound in this region was somehow selectively protected from ester cleavage.

Very small amounts or no specific labeling was detected in the 70-79,000 M_r region if samples were photolabeled in the presence of 25 mM imidazole. This result probably reflects the results from Table IV where the K_d for the azide probe was 10-fold higher in imidazole buffers. However, a control experiment where imidazole was added immediately before photolabeling to a sample equilibrated with probe in phosphate indicated that imidazole also acted as a scavenger to reduce non-specific labeling across the gel, and increased the level of

specific labeling in the 90-92,000 M_r range relative to the 70-79,000 M_r range. The reason for this result is not known.

Discussion

The p-azido analog of atropine methyl iodide has been synthesized in a tritiated form to a specific activity of 10 Ci/mmole. The results of IR, ^{1}H-NMR and UV-VIS spectra and elemental analysis were consistent with the proposed structure shown in Figure 10.

The reversible interactions of this photoaffinity label with mAcChR enriched preparations were measured in the dark by competition with {^{3}H}L-QNB binding. Direct reversible binding studies using the tritiated probe were avoided due to difficulties with assay procedures, and cost considerations in the case of equilibrium dialysis. The dissociation rate constant of this probe appeared to be too fast to measure by the DEAE filter disc assay. Studies using the GF/B filter assay (Yamamura and Snyder, 1974) for the membrane-bound receptor were hampered by large amounts of nonspecifically bound label.

p-Azidoatropine methyl iodide appeared to displace {^{3}H}L-QNB in a competitive manner from a single class of sites for the membrane-bound, solubilized extract and WGA partially purified receptor preparations (Figures 14, 15 and 16, respectively). The K_d for all three preparations was approximately 75 nM in phosphate and 750 nM in imidazole buffer (Table IV). Investigators in this

laboratory have reported a K_d=1nM for the parent compound, atropine, for the digitonin/cholate solubilized (Herron and Schimerlik, 1982) and membrane-bound mAcChR (Schimerlik and Searles, 1980). Therefore, assuming a synthetic yield of equal proportions of D and L isomers, substitution at the para position of the phenyl ring with an azide increases the K_d 75-fold. The K_d in phosphate buffer for the azide derivative described here was approximately 6-fold lower than that reported for the p-azidophenylacetate ester of tropine binding to rat brain membrane fractions (Moreno-Yanes and Mahler, 1980).

The reasons for the buffer dependence of the K_d for this compound were not known. However, results from this laboratory indicate that the 10-fold larger K_ds in the presence of imidazole were found for all agonists and antagonists tested (Peterson and Schimerlik, 1983). Titrations of receptor preparations prepared in phosphate buffers indicated that imidazole acts as an inhibitor of $\{^3H\}$L-QNB binding with half-maximal inhibition at approximately 20 mM. Phosphate and imidazole (25 mM) buffered preparations were compared by a Scatchard analysis (Scatchard, 1949) of $\{^3H\}$L-QNB binding. Only the K_d and not the total number of $\{^3H\}$L-QNB binding sites was altered (K_d=250 pM, phosphate; K_d= 1000 pM, imidazole; Peterson and Schimerlik, 1983). The effect of imidazole was found to be completely reversible by dialysis into

phosphate buffers.

The nature of the covalent interaction upon photolysis of the p-azidoatropine analog with the WGA partially purified mAcChR L-QNB binding site(s) was examined by SDS gel electrophoresis. Photoinactivation of the azide resulted in specific covalent interaction with protein(s) determined, by gel electrophoresis, to be in the 70-79,000 M_r range. A relatively large amount of label was also covalently, but non-specifically incorporated into the 90-92,000 M_r range. The covalent nature of the ligand-protein interaction was demonstrated by the resistance of label to dialysis before electrophoresis. Control experiments indicated that the covalent binding was probably not due to long-lived decomposition products resulting from photolysis of the azide, but direct interaction of the photoactivated azide (nitrene) at the mAcChR binding site(s).

We have shown that the labeling in the 70-79,000 M_r region was specific for the mAcChR; increasing concentrations of L-QNB reduced this labeling, consistent with the number of L-QNB sites determined by another method (DEAE disc assay) and a K_{app}=85 nM obtained by the electrophoresis assay was consistent with the results of reversible binding experiments (Figure 16). These results were in general agreement with previously published data on {^{3}H}PrBCM-labeled mAcChRs from a number

of tissues (Haga, 1980; Birdsall, et al., 1979). Recently, Venter (1983) has reported (81,000 ± 3,000) M_r and (78,000 ± 1,800) M_r for $\{^3H\}$PrBCM mAcChR labeling in canine and rat heart membranes respectively, by gel electrophoresis. Target size analysis of these tissues indicated that the intact membrane-bound receptors have these M_rs (Venter, 1983). As with the tritiated azide, we found specific labeling in one broad peak in the 70-79,000 M_r region for the WGA affinity partially purified mAcChR from porcine atria using $\{^3H\}$PrBCM (data not illustrated). The labeled peak(s) specific for the mAcChR using the two tritiated labels was always very broad, often spanning 6-7, 1mm gel slices (Figure 17). Venter (1983) and Birdsall et al. (1979) published very similar broad gel profiles in mAcChR $\{^3H\}$PrBCM labeling of brain, heart and guinea pig ileum smooth muscle, which seemed to be inconsistent with the resolution of the gel method. These results could be due to heterogeneously labeled peptide. Work in this laboratory has indicated that partially purified soluble mAcChRs from pig atria are sialoglycoproteins (Herron and Schimerlik, 1983). Therefore, broad bands on gels may be due to carbohydrate heterogeneity of the receptor. Birdsall et al. (1979) found no anomalous behavior in their study of mAcChRs by Ferguson plots (Ferguson, 1964). This would be consistent for a glycoprotein with a small

percentage of total weight representing carbohydrates (Segrest and Jackson, 1972). In fact, we have not observed a strong dependence of M_r on percent acrylamide from 10% to 6% as would be expected for anomalous SDS binding to large carbohydrate moieties.

In their work using the antagonist {^{3}H}N-methyl-4-piperidyl p-azidobenzilate (azido-4NMPB) to covalently label mAcChRs in rat atrial membranes, Amitai et al. (1982) reported specific labeling in two sharp bands at 160,000 M_r and 86,000 M_r using gel electrophoresis. Amitai et al. (1982) have suggested that the 86,000 M_r species represents the low affinity agonist binding form of the mAcChR, and that antagonists do not distinguish between these two forms. We could not detect specific labeling with our antagonist photolabel at 160,000 M_r in samples that were dialysed to remove free photolysed label that might have masked specific label in this molecular weight range. However, these results are preliminary. It is not clear whether the specific labeling at 86,000 M_r observed by Amitai et al. (1982) corresponds to our result of 70-79,000 M_r or to perhaps our non-specific labeling at 90-92,000 M_r. Labeling at 160,000 M_r by the antagonist {^{3}H}PrBCM has not been reported (Birdsall et al., 1979; Venter, 1983; Fewtrell and Rang (1973). The preparation used here exhibited low affinity agonist binding characteristics. At this time, however,

our results are inconclusive as to high affinity agonist binding characteristics. We propose to test for agonist interactions in our preparations by titrating {^{3}H} p-azidoatropine methyl iodide labeling with a muscarinic agonist. According to the model of Amitai et al. we would expect labeling in the 160,000 M_r range in addition to the 80,000 M_r range with the antagonist probe p-azidoatropine methyl iodide in our membrane-bound preparations. Schimerlik and Searles (1980) reported biphasic agonist titration curves for agonist binding to the membrane-bound mAcChR from porcine atria, corresponding to high and low affinity binding sites. Herron and Schimerlik (1982) however, found only low affinity agonist sites in their study of the digitonin/cholate solubilized mAcChR from the same tissue.

To this date we have not detected specific labeling in digitonin/cholate extract or membrane-bound photolabeled preparations. Although the results are preliminary, we are hampered by the non-specific labeling resulting from high tritiated probe concentrations (up to 40-fold over the concentration of L-QNB sites and 4-fold over the K_d for the azide) required to obtain detectable labeling by the gel electrophoresis method. The DEAE disc and GF/B assays have not proved useful, probably due to non-specific labeling of the {^{3}H} azide to the filters. For the WGA partially purified mAcChR, we routinely obtained a 3.5-

4.0% labeling efficiency (phosphate buffer) for the azide, by measuring specific c.p.m.s incorporated into the 70-79,000 M_r band using the gel electrophoresis assay. These calculations were based upon a K_d for the tritiated probe, the concentration of L-QNB sites as determined by the DEAE disc assay, 1 azide binding site/L-QNB site, and a 25% counting efficiency. This labeling efficiency affords labeling well above detectable limits in these relatively pure preparations where the probe concentrations are only 2-3 fold over the site concentration. We have not been able to detect inhibition of $\{^3H\}$L-QNB binding by photolabeling the extract or membranes in the presence of cold probe using the DEAE disc assay or GF/B assay. A labeling efficiency in the membranes and extract of 3-4% would then not be inconsistent with these results. An inhibition of 3-4% would be difficult to reproducibly detect by either the DEAE or GF/B filter assays.

We do not know the nature of the non-specific labeling observed in the 90-92,000 M_r range in the WGA partially purified receptor. Identical experiments using the $\{^3H\}$PrBCM covalent label also showed some non-specific labeling at this molecular weight. The 90-92,000 M_r band copurifies through every step of our purification of the solubilized receptor (unpublished results). In fact, in our most pure preparations, this is the major contaminant

as determined by protein silver staining (Merril et al., 1981; Oakley et al., 1980) of polyacrylamide gels. It is possible that the azide label is only incorporated into a minor protein contaminant happening to comigrate with another abundant protein. Perhaps such a protein is proximal to the azide binding site and its labeling is not blocked by L-QNB due to the activation of the azide to the nitrene. Of course, any explanation is speculative in nature at the time. However, the fact that this protein does seem to have an affinity for muscarinic ligands may hamper our attempts at purification by affinity chromatography. A study of conditions under which specific labeling in this band is enhanced or non-specific labeling minimized, may provide interesting information as to its nature.

In conclusion, we have reported specific labeling of soluble mAcChR L-QNB binding sites, using a phenyl azide analog of atropine, {^{3}H} p-azidoatropine methyl iodide. This is the first report of photoaffinity labeling of this receptor in soluble form. We propose to use this probe in the structural and functional characterization of more highly purified soluble preparations.

BIBLIOGRAPHY

Adams, R.G. (1969) J.Lipid Res. 10, 473.

Aguilar, J.S., Criado,M. and DeRobertis,E. (1980) Eur. J. Pharm. 63, 251,-257.

Alberts,P. and Bartfai, T. (1976) J. Biol. Chem. 251, 1543-1547.

Alburquerque, E.X., Barnard, E.A., Chiu, T.H., Lapa, A.J., Dolly, J.O., Jansson, S-E., Daly,J. and Witkop,B. (1973) Proc. Natl. Acad. Sci. U.S.A. 70,949-953.

Amitai, G., Avissar, S., Balderman, D. and Sokolovsky, M. (1982) Proc. Natl. Acad. Sci. U.S.A. 79, 243-247.

Aronstam,R.S., Abood, L.G. and Baumgold, J. (1977) Biochem. Pharmacol. 26, 1689-1695.

Aronstam, R.S., Schuessler, D.C. Jr., and Eldefrawi, M. E. (1978) Life Sci. 23, 1377-1382.

Avissar, S., Amitai, G. and Sokolovsky, M. (1983) Proc. Natl. Acad. Sci. U.S.A. 80, 156-159.

Barlow, R.B. (1964) Introduction to Chemical Pharmacology, 2nd Ed., Methuen, London.

Bayley, H. and Knowles, J.R. (1977) In Methods in Enzymology (W.B. Jakoby and M. Wilchek, Ed.) 46, 69.

Beld, A.J., and Ariens, E.J. (1974) Eur. J. Pharmacol. 25, 203-209.

Berrie, C.P., Birdsall, N,J.M., Burgen, A.S.V. and Hulme, E.C. (1979) Biochem. Biophys. Res. Comm. 87, 1000-1005.

Best-Belpomme, M. and Dessen, P. (1973) Biochmie 55, 11-16.

Bevington, P.R. (1964) Data Reduction and Error Analysis for the Physical Sciences, McGraw-Hill, New York, Chpts. 3 and 4.

BIBLIOGRAPHY

(continued)

Birdsall, N.J.M., Burgen, A.S.V. and Hulme, E.C. (1979) Br. J. Pharmacol. 66, 337-342.

Birdsall, N.J.M. and Hulme, E.C. (1976) J. Neurochem. 27, 7-16.

Birdsall, N.J.M., Burgen, A.S.V. and Hulme, E.C. (1978) Mol. Pharmac., 14, 723-736.

Bolton, T.B. (1979) Physio. Rev. 59, 606-718.

Burgen, A.S.V., Hiley, C.R. and Young, T.M. (1974) Br. J. Pharmac. 50, 145-151.

Burgen A.S.V. and Terroux, K.G. (1953) J. Physiol. 120, 449-464.

Burgermeister, W., Klein, W.L., Nirengerg, M. and Witkop, B. (1978) Mol. Pharmacol. 14, 751-767.

Carson, S., Godwin, S., Massoulie, J. and Kato, G. (1977) Nature 266, 176-178.

Cavey, D., Vincent, J.P. and Lazdunski, M. (1977) FEBS Lett. 84, 110-114.

Chowdhry, V. and Westheimer, F.H. (1979) Ann. Rev. Biochem., 48, 293-325.

Cleland, W.W. (1967) Adv. Enzymol. Relat. Areas Mol. Biol. 29, 1-32.

Cohen, N.M., Schmidt, D.M., McGlennen, R.C. and Klein, W.L. (1983) J. of Neurochem. 40, 547-553.

Colatsky, T.J. and Tsien, R.W. (1979) Nature, 278, 265-268.

Conti-Tronconi, B.M. and Raftery, M.A. (1982) Ann. Rev. Biochem. 51, 491-530.

Cooper, C. and Lehninger, A.L. (1956) J. Biol. Chem. 219, 489-505.

BIBLIOGRAPHY
(continued)

Cremo, C.R., Herron, G.S. and Schimerlik, M.I. (1981) Anal. Biochem. 115,331-338.

Cremo, C.R. and Schimerlik, M.I. (1982) Fed. Proc., Fed. Amer. Soc. Exptl. Biol. 41, 1211.

Cuatrecasas, P. (1974) Ann. Rev. Biochem. 43, 169-214.

Cuatrecasas, P. (1972) Proc, Natl. Acad. Sci. U.S.A. 69, 318-322.

Dale, H.H. (1914) J. Pharmacol. Exp. Ther. 6, 147-190.

Desbuquois, B. and Aurbach, G.D. (1971) J. Clin. Endocrinol. 33, 732-738.

Donald, D.E. and Shepard, J.T. (1978) J. Mol. and Cell. Cardiology 10, 449-469.

Elliot, J. and Raftery, M.A. (1979) Biochem. 18, 1868-1874.

Ferguson, K. A. (1964) Metabolism, 13, 985.

Fewtrell, C.M.S. and Rang, H.P. (1973) In Drug Receptors (H.P. Rang, Ed.) Macmillan, London, 211-226.

Fields, J.Z. Roeske, W. R. and Yamamura, H. I. (1978) J. Biol. Chem. 253, 3251-3258.

Fish, W.W. (1975) In Methods in Membrane Biology, Vol. 4 (E.D. Korn, Ed.) Plenum Press, New York, 189-276.

Fisher, S.K. and Agranoff, B.W. (1980) J. of Neurochem. 34, 1231-1240.

Fraser, C.M. and Venter, J.C. (1982) Biochem. Biophys. Res. Comm. 109, 21-29.

BIBLIOGRAPHY

(continued)

Fried, M. and Chun, P.W. (1971) In Methods in Enzymology (W.B. Jakoby, Ed.) Academic Press, New York, 22, 238-248.

Friedman, H.L. (1967) In Drugs Affecting the Peripheral Nervous System (A. Burger, Ed.) Marcel Dekkey, Inc., New York, 79-131.

Galper, J.B., Klein, W. and Catterall, W.A. (1977) J. Biol. Chem., 252, 8692-8699.

Galper, J.B. and Smith, T.W. (1980) J. Biol. Chem. 255, 9571-9579.

George, W.J., Polson, J.B., O'Toole, J.B. and Goldberg, N.D. (1970) Proc. Natl. Acad. Sci. U.S.A. 66, 398-403.

Giles, W. and Noble, S.J. (1976) J. Physiol. 261,103-123.

Gorissen, H., Aerts, E.J., and Laduron, P. (1978) FEBS Lett. 96, 64-68.

Greengard, P. (1976) Nature, 260, 101-108.

Griffin, H.D., Hawthorne, J.H. and Sykes, M. (1979) Biochem. Pharmacol. 28, 1143-1147.

Haga, T. (1980) FEBS Lett. 113, 68-73.

Halvorsen, S.W. and Nathanson, N.M. (1981) J. Biol. Chem. 256, 7941-7948.

Hanstein, W.G. (1979) Methods in Enzymology, 26, 653-683.

Hartzell, H.C. (1981) Nature 291, 539-543.

Hartzell, H.C. (1980) J. Cell. Biol. 86, 6-20.

Hartzell, H.C., Kuffler, S.W., Stickgold, R. and Yoshikami, D. (1977) J. Physiol. Lond. 271, 817-846.

BIBLIOGRAPHY
(continued)

Hartzell, H.C. and Titus,L. (1982) J. Biol. Chem. 257, 2111-2120.

Hawthorne, J.N. (1982) Nature 295, 281-282.

Heidmann, T. and Changeaux, J-P. (1978) Ann. Rev. Biochem. 47, 317-357.

Herron, G.S., Miller, S., Manley, W-L. and Schimerlik, M.I. (1982) Biochem. 21, 515-520.

Herron, G.S. and Schimerlik, M.I. (1983) J. Neurochem., in press.

Higgins, C.B., Vatner, S.F. and Braunwald, E. (1973) Pharmacological Reviews, 25, 119-155.

Hill, A.V. (1910) J. Physiol. Lond. 40, iv.

Hino, N. and Ochi, R.J. (1980) J. Physiol. Lond. 307, 183-197.

Hokin, M.R. and Hokin, L.E. (1953) J. Biol. Chem. 203, 967-977.

Horn, J.P. and Dodd, J. (1981) Nature 292, 625-627.

Hopple, C. and Cooper, C. (1968) Biochem. J. 107, 367-375.

Hubbard, R. (1954) J. Gen. Physiol.37, 381-399.

Hurko, O. (1978) Archiv. Biochem. Biophys. 190, 435-445.

Ikemoto,Y. and Goto, M. (1978) In Recent Advances in Studies on Cardiac Structure and Metabolism (T. Kobayashi, T. Sano, N.S. Dhalla, Ed.) Univ, Park Press, Baltimore, 11, 57-61.

Jakoby, W.B. and Wilchek, M. (1977) Methods in Enzymology,46.

Jeng, S.J. and Guillory, R.J. (1975) J. Supramol. Struct. 3, 448.

BIBLIOGRAPHY

(continued)

Katz, B. (1966) in Nerve, Muscle, and Synapse, McGraw-Hill, Inc., New York.

Katz, A.M. (1977) In Physiology of the Heart, Raven Press, New York.

Kehoe, J. and Marty, A. (1980) Ann. Rev. Biophys. Bioeng. 9, 437-465.

Knowles, J.R. (1972) Acct. Chem. Res. 5, 155-160.

Krebs, K.G. Heusser,D. and Wimmer, H. (1969)In Thin Layer Chromatography, (E. Stahl, Ed.) Springer-Verlag, Berlin, 883.

Kuo, J.F., Lee, T.P., Reyes, P.L., Walton, K.G. Donnelly, T.E. and Greengard, P. (1972) J. Biol. Chem. 247, 16-22.

Laemmli, U.K. (1970) Nature, 227, 680-685.

Lee, T-P., Kuo, J.F. and Greengard, P. (1972) Proc. Natl. Acad. Sci. U.S.A. 69, 3287-3291.

Levy, M.N. (1971) Circ. Res. 29, 437-445.

Lincoln, T.M.,and Keely, S.L. (1981) Biochim. et Biophys. Acta, 676,230-244.

Linden, J. and Brooker, G (1979) Biochem.Pharmacol. 28, 3351-3360.

Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265-275.

Lwowski, W. (1970) In Nitrenes, Wiley (Interscience), New York.

Magazanik, L.G. (1976) Ann. Rev. Pharm. and Tox. 16, 161-175.

Mayer, S.E. (1980) In The Pharmacological Basis of Theraputics (A.G. Gilman, A. Gilman and L.S. Goodman, Ed.) Macmillan, New York, Chpt. 4.

BIBLIOGRAPHY
(continued)

Means, G.E. and Feeney, R.E. (1971) Chemical Modification of Proteins, Holden-Day, San Francisco.

Miller, J. (1979) Ph.D. Thesis, The California Institute of Technology.

Morello, R., Begenisich, T., Trzos, W. and Reed, J.K. (1980) Biophys. J. 32, 435-440.

Moreno-Yanes, J.A. and Mahler, H.R. (1980) Biochem. Biophys. Res. Comm. 92, 610-617.

Muscholl, C. (1980) Am. J. Physiol. 239, H713-720.

Nachmansohn,D. and Neumann, E. (1975) In Chemical and Molecular Basis of Nerve Activity, Academic Press, New York.

Nathan, D. and Beeler, G.W. (1975) J. Mol. Cell. Cardiol. 7, 1-15.

Nathanson, N.M. (1982) Trends in Neurosci., Nov., 401-404.

Nathanson, N.M., Klein, W.L. and Nirenberg, M. (1978) Proc. Natl. Acad. Sci. U.S.A. 75, 1788-1791.

Nawrath,H. (1976) Nature, 262, 509-511.

Nawrath, H. (1977) Nature, 267, 72-74.

Neher, E. and Stevens, C.F. (1977) Ann. Rev. Biophys. Bioeng. 6, 345-381.

Noble, D. and Twein, R.W. (1969) J. Physiol. Lond. 200, 205-231.

Peterson, G.L. (1977) Anal. Biochem. 83, 346-356.

Peterson, G.L. and Schimerlik. M.I. (1982) Biochem. J. 202, 475-481.

Peterson, G.L and Schimerlik, M.I. Unpub. results.

BIBLIOGRAPHY

(continued)

Purves, R.D. (1976) Nature, 261, 149-151.

Purves, R.D. (1978) In Cell Membrane Receptors for Drugs and Hormones: A Multidisciplinary Approach (R.W. Straub and L. Bolis, Ed.) Raven Press, New York, 67-69.

Putney, J.W., Weiss, S.J., Van De Walle, C.M. and Haddas, R.A. (1980) Nature, 284, 345-347.

Raftery, M.A., Vandlen, R.L., Reed, K.L. and Lee, T. (1975) Symposia on Quantitative Biology, Vol. XL, 193-202.

Rang, H.P. (1975) Quart. Rev. Biophys. 7, 283-399.

Reed, J.K. and Trzos, W. (1979) Arch. Biochem.Biophys. 195, 414-422.

Reiser, A. and Wagner, H.M. (1971) In The Chemistry of the Azido Group, (S. Patai,Ed.) Wiley (Interscience), New York.

Repke,H. and Matthies, H. (1980) Pharmazie 35, 233-234.

Reuter, H. (1979) Ann. Rev. Physiol. 41, 413-424.

Reuter, H. (1983) Nature, 301, 569-574.

Reuter, H. and Scholz, H (1977) J. Physiol. Lond. 264, 49-62.

Rinaldi, M.L., Le Peuch, C.J. and Demaille, J.G. (1981) FEBS Lett. 129, 277-281.

Rosenberger, L.B., Roeske, W.R. and Yamamura, H.I. (1979) Eur. J. Pharmacol. 56, 179-180.

Rosenqvist, E., Michaelsen, T.E. and Vistres, A.J. (1980) Biochim. Biophys. Acta. 600, 91-102.

RueB, K.-P., and Lieflander, M. (1979) Biochem. Biophys. Res. Comm. 88, 627-633.

Ruoho, A.E., Kiefer, H., Roeder, P.E. and Singer, S.J. (1973) Proc, Natl. Acad. Sci. U.S.A., 70, 2567-2571.

BIBLIOGRAPHY

(continued)

Salmon, D.M. and Honeyman, T.W. (1980) Nature, 284, 344-345.

Scatchard, G. (1949) Ann. N.Y. Acad.Sci. 51, 660-671.

Schimerlik, M.I. and Searles, R.P. (1980) Biochem. 19, 3407-3413.

Schmidt, J. and Raftery, M.A. (1973) Anal. Biochem, 52, 349-354.

Schnaitman, C., Erwin, V.G. and Greenawalt, J.W. (1967) J. Cell. Biol. 32, 719.

Segrest, J.P. and Jackson, R.L. (1972) In Methods In Enzymology 28, 54-63.

Singh, A., Thorton, E.R. and Westheimer, F.H. (1962) (1962) J. Biol. Chem. 237, PC 3006.

Slavik, J. (1982) Biochim. Biophys. Acta 694, 1-25.

Sokolovsky, M. and Bartfai, T. (1981) Trends in Biochem. Sci., 303-305.

Sperelakis, N. (1979) Ann. Rev. Physiol. 41, 441-457.

Strain, W.H. (1943) The Steriods in Organic Chemistry (H. Gilman, Ed.) John Wiley and Sons, Inc., New York, Vol. II, 1454-1457.

Strosberg, A.D., Vauguelin, G., Durieu-Trautmann, O., Delavier-Klutchko, C, Bottari, S. and Andre, C. (1980) Trends In Biochem. Sci., 11-14.

Takeuchi, Y., Koga, K., Shioiri, T. and Yamada, S.-I. (1971) Chem. Pharm. Bull. 19, 2603-2608.

Tanford, C. (1980) The Hydrophobic Effect, 2nd ed., 17.

Ten Eick, R., Nawrath, H., McDonald, T.F., Trautwein, W. (1976) Pfluegers Arch. 361,207-13.

The Merck Index (1976) (M. Windholz, Ed.) 9th Ed. Merck & Co., Inc., Rahway, N.J., 417-418.

BIBLIOGRAPHY

(continued)

Trautwein, W. and Dudel, J. (1958) Pflugers Arch. 226, 324-334.

Trautwein, W., Kuffler, S.W. and Edwards, C. (1956) J. Gen. Physiol. 40, 135-145.

Tsein, R.W. (1977) Adv. Cuclic Nucleotide Res. 8, 363-420.

Tyson, C.A., Zande, H.V. and Green, D.E. (1976) J. Biol. Chem. 251, 1326-1332.

Vanhoutte, P.M. and Levy, M.N. (1980) Am. J. Physiol. 238, H275-281.

Vassalle, M. (1979) Ann. Rev. Physiol. 41, 425-40.

Venter, J.C. (1983) J. Biol. Chem. 258, 4842-4848.

Waelbroeck, M., Robberecht,P., Chatelain, P.,and Christophe, J. (1982) Mol. Pharmacol. 21, 581-588.

Wei, J.W. and Sulakhe, P.V. (1980) Eur. J. Pharmacol. 62, 345-347.

West, T.C. (1972) In Electrical Phenomenon in the Heart (W.C. DeMello, Ed.) Academic Press, New York, 191-233.

Yamamura, H.I. and Snyder, S.H. (1974) Proc. Natl. Acad. Sci. U.S.A. 71, 1725-1729.