EXPRESSION OF MULTIPLE POPULATIONS OF NICOTINIC ACETYLCOLINE RECEPTORS IN BOVINE ADRENAL CHROMAFFIN CELLS

DISSERTATION

Presented in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy in the Graduate School of The Ohio State University

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The importance of the role of nAChRs in physiological and pathological states is becoming increasingly clear. It is apparent that there are multitudes of nAChR subtypes with different expression patterns, pharmacologies and functions that may be important in various disease states. Therefore, a greater understanding of nAChR subtypes is essential for potential pharmacological intervention in nAChR systems. Bovine adrenal chromaffin cells are a primary culture of a neuronal type cell that express ganglionic types of nAChRs whose activation can be related to a functional response. While much is known about the outcome of functional activation of adrenal nAChRs, little work has been done in characterizing populations of nAChRs in adrenal chromaffin cells. These studies characterize the pharmacology and regulation of populations of nAChRs found in bovine adrenal chromaffin cells. The primary findings of this research include 1) the characterization of an irreversible antagonist of adrenal nAChRs, 2) the discovery of spare nAChRs in bovine adrenal chromaffin cells, 3) the pharmacological characterization of mAb35 insensitive nAChRs which may account for as many as 45% of nAChRs in bovine adrenal chromaffin cells, 4) the finding that disulfide bonds do not play a critical role in the function of adrenal nAChRs which is contrary to their importance in other tissues, 5) the use of (+)-tubocurarine in a receptor protection assay
to identify a subpopulation of nAChRs, 6) the characterization of the role of protein synthesis, glycosylation, and phosphorylation in the turnover of nAChRs, and 7) the identification and partial sequencing of a bovine β4 nAChR transcript expressed in adrenal chromaffin cells.
This work is dedicated to Amelia Elisabeth Wenger,

whose mere possibility allowed me

to finish what I had started.
ACKNOWLEDGMENTS

I wish to sincerely thank Dr. Dennis McKay who guided me through this long process and never gave up on me even when I disappeared into another profession. I can not overstate the importance of the love of my wonderful wife Jane and my family in the completion of this long task. Their interest, support, understanding, and, yes, even nagging pushed me the rest of the way through. Finally, I would also like to thank the excellent staff of the College of Pharmacy and my dissertation committee, Dr. R. Thomas Boyd, Dr. Popat N. Patil, and Dr. Lane J. Wallace, for teaching me the nuances of this amazing science and for allowing me to complete my studies.
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STATEMENT OF THE PROBLEM

Nicotine is the major biologically active alkaloid found in tobacco and is primarily responsible for establishing and reinforcing the addiction to tobacco products. Many of the pleasurable characteristics associated with smoking are likely the result of transient high levels of nicotine activating nicotinic acetylcholine receptors (nAChRs) found in the brain. These neuronal nAChRs have also been implicated in the symptomology of Alzheimer’s disease and Parkinson’s disease. As well, there is recent interest in developing drugs that act upon nAChRs for the treatment of pain and anxiety and to increase cognitive ability. Unfortunately, no less than 12 genes have been cloned and sequenced for neuronal nAChR subunits, leaving open the possibility that a daunting number of nAChR subtypes exist. Furthermore, studies in oocyte expression systems have found that the specific combination of subunits that make up the nAChR affects its channel characteristics and pharmacology. Researchers have identified multiple subtypes of neuronal nAChRs within the central and peripheral nervous systems, so the utilization of subtype specific agents would be necessary in order to decrease potential side effects. Identifying nAChRs subtypes has been complicated by their low numbers within neuronal tissues, difficulty in obtaining the tissue, and a lack of functional assays for nAChR activation. With their neural crest origins, ease of isolation, and easily measured secretory function, bovine adrenal chromaffin cells have been used as a model for sympathetic neurons and may be useful for the testing of subtype specific agents as well as the discovery of the properties of neuronal nAChR subtypes and the processes that influence subtype function and turnover. However very little is known about subtype(s)
nAChRs found in bovine adrenal chromaffin cells. Studies from ganglionic tissues have found the presence of α3, α5, α7, β2, and β4 subunits as well as multiple populations of nAChRs. Previous studies from our laboratory have found that the anti-nAChR monoclonal antibody, mAb35, was unable to abolish nAChR-mediated secretory function completely, even after greater than 24hrs of treatment. These findings as well as the discovery of multiple populations of nAChRs in related cell types have lead to our hypothesis that bovine adrenal chromaffin cells contain multiple subtypes of nAChRs involved in the secretory response and that these receptors contain the α3, α5, β4 and possibly the β2 subunits. The following specific aims will be addressed related to this hypothesis.

1) Characterize the effects of an irreversible inhibitor of nAChRs in bovine adrenal chromaffin cells. The hypothesis being tested is that under reducing conditions brACh will irreversibly inhibit the function of bovine adrenal nAChRs. The loss of receptor function will be long lasting, and protein synthesis will be required for the return of receptor function.

2) Determine the extent of the nAChR reserve present in bovine adrenal chromaffin cells. Any nAChR reserve will be detected by first irreversibly inactivating nAChR with submaximal concentrations of brACh. The effect of this treatment on the concentration response relationship of several nAChR agonists will be determined. The hypothesis is that bovine adrenal chromaffin cells contain a small but detectable nAChR reserve.

3) Use monoclonal antibodies to identify the presence and extent of subpopulations of nAChRs in bovine adrenal chromaffin cells. The monoclonal antibody mAb35 will be used to induce slowly developing and incomplete loss of nAChR function. The
concentration response relationship of several nAChR agonists for mAb35 insensitive nAChRs will be determined and compared to untreated nAChRs. The hypothesis is that mAb35 insensitive receptors will have a different pharmacological profile than untreated nAChRs.

4) Use receptor protection assays to identify the presence of nAChR subpopulations in bovine adrenal chromaffin cells. The hypothesis is that nAChR agonists and antagonists will show some subtype selectivity and preferentially protect populations of adrenal nAChRs from irreversible inactivation. This will eliminate some nAChR populations and allow for the study of the functional pharmacology of the protected receptors.

5) Determine the effects of disulfide bond reduction on nAChR-mediated catecholamine release and apparent affinities of nAChR agonists and antagonists in bovine adrenal chromaffin cells. The hypothesis is that reduction of disulfide bonds will alter the pharmacological profile of bovine adrenal nAChRs. Furthermore the effect may be subtype specific and allow for the characterization of nAChR subpopulations.

6) Determine the biochemical processes involved in the turnover of nAChRs in bovine adrenal chromaffin cells. The hypothesis being tested is that protein synthesis, glycosylation, and phosphorylation events play an important role in the regulation of bovine adrenal nAChRs.

7) Clone and sequence nAChR subunits expressed in bovine adrenal chromaffin cells. The hypothesis is that bovine adrenal chromaffin cells contain the α3, α5, β4, and possibly the β2 nAChR subunits.
CHAPTER 1

INTRODUCTION

1.1. Nicotinic acetylcholine receptors in health and disease

Nicotinic acetylcholine receptors can be found throughout the central nervous system (CNS), autonomic ganglia, and adrenal medulla and have been implicated in several disease states associated with the CNS and the peripheral nervous system (PNS). Perhaps the most widespread problem is nicotine addiction. Understanding the nAChR’s role in establishing and maintaining the addiction to tobacco products is vital. Nicotine’s most important effects likely occur in the CNS where it causes the release of dopamine in the nucleus accumbens (Dani et al. 2001). This action has been implicated in the addiction process. There is also an upregulation of nAChRs in the brains of smokers (Perry et al. 1999). However, not all of nicotine’s effects are harmful. Nicotine has antinociceptive effects although its toxicity prevents it from being used as a therapeutic medication. The advent of subtype selective agonists has opened up the possibility of using nAChRs as a novel approach to managing pain (for review see Flores 2000). Several other disease states have links to nAChRs. A form of epilepsy called autosomal dominant frontal lobe epilepsy has been linked to a defect in a nAChR subunit (Steinlein et al. 1995). Furthermore, there are losses of nAChR binding sites in the brains of
patients with Alzheimer’s disease and Parkinson’s disease (for review see Paterson and Nordberg 2000; Nordberg 2001). While the effect and mechanism of these loses are unknown, they do correlate with the progression of the disease.

The full mechanism of nicotine’s action and the role of nAChRs in the body are likely complex due to the nature of nAChRs. For example, nAChRs have been found in presynaptic, postsynaptic, extrasynaptic and perisynaptic locations. Nicotine can activate and open the nAChR ion channel while high concentrations can desensitize the receptor and close the ion channel. Additionally, there are several nAChR subtypes, which have different distributions, regulation and pharmacology. The wide spread distribution of the cholinergic system and the lack of specificity of current cholinergic agonists and antagonists complicate the treatment of diseases. This leads to unwanted side effects that may affect patient compliance or may possibly even be life threatening. With twelve possible neuronal nAChR genes from which to choose, there are many potential subtypes of the nAChR, and each subtype potentially has a different pharmacological profile. Discovering what subtypes are present in a tissue or cell type would be an important step in understanding the etiology of cholinergic diseases and then in optimizing drug use or even designing subtype specific drugs to treat the disease while lowering the incidence of side effects.

1.2 nAChR diversity

Neuronal nicotinic acetylcholine receptors (nAChR) are members of the superfamily of ligand-gated ion channels. This superfamily includes GABA_A, 5-HT_3,
and glycine receptors which are multimeric proteins whose subunits are arranged around a central ion channel. For many years the nAChR found at the neuromuscular junction has been the prototype for members of this superfamily. The presence of large quantities of the protein in the electric organ of the electric eel coupled with the discovery of a nearly irreversible antagonist, α-bungarotoxin, have allowed for the isolation and characterization of the receptor complex. The muscle nAChR is a pentamer with the stoichiometry of $\alpha_2\beta\gamma\delta$ (for review see Séguéla et al. 1993). Neuromuscular junction nAChRs are developmentally regulated with an ε subunit replacing the γ subunit later in development (Mishina et al. 1986).

Nicotinic acetylcholine receptors are also found in the CNS, autonomic ganglia, and adrenal chromaffin cells where they mediate a variety of cellular functions including direct synaptic transmission, modulation of synaptic transmission, regulation of neurite outgrowth, and contribute to cytoprotective effects (Lindstrom 1997). These receptors differ from neuromuscular junction nAChRs in pharmacology and subunit composition and are referred to as neuronal nAChRs. Characterization of these receptors had been hampered by the fact that although many of these tissues contained α-bungarotoxin binding sites, the binding of the toxin had little to no effect on function. However, the finding that mAb35, a monoclonal antibody raised against the muscle type nAChR found in the electric organ of the eel Torpedo californicus, cross reacted with neuronal type receptors and inhibited function lead researchers to the possibility of using muscle cDNAs to probe the neuronal system. By probing cDNA libraries from PC12 cells under low stringency conditions with the α subunit of the neuromuscular junction nAChR,
researchers cloned and sequenced the α3 gene (Wada et al. 1988). Now at least 12 distinct genes (α2-10 and β2-4) encoding proteins for neuronal nAChR subunits have been cloned and sequenced (Maruta et al. 1986; Boulter et al. 1986; Goldman et al. 1987; Boulter et al. 1987; Boulter et al. 1990; Criado et al. 1992; Séguéla et al. 1993; Elgoyhen et al. 1994).

1.3 nAChR Subunit Structure

Neuronal nAChR subunits share a high degree of homology (39% - 70%) amongst themselves and the subunits of the muscle nAChR. nAChR genes code for proteins of about 424 - 600 amino acids in length. Both the C-terminus and N-terminus are extracellular with the N-terminus containing about 200 amino acids. There are four proposed transmembrane regions. These regions show very high homology among subunits. The cytoplasmic loop between transmembrane regions three and four shows the greatest amount of diversity in amino acid sequence and accounts for most of the diversity in length found between subunits (for review see, (Lindstrom 1997).

Two cysteines corresponding to positions 128 and 142 of the α1 gene are found in all nAChR subunits. Two additional cysteines analogous to those found at positions 192 and 193 of the α1 subunit are found in neuronal α subunits and are diagnostic for α subunits. These vicinal cysteines found at positions 192 and 193 form a disulfide bond that can be readily reduced by mild reducing agents and are believed to be part of the agonist binding site. The characteristics of nAChRs are affected by the state of these important disulfide bonds, with subtypes of nAChRs showing varying degrees of
susceptibility to reduction. Following treatment with the mild reducing agent
dithiothreitol (DTT), the affinity agent 4-(N-maleimido)benzyltrimethylammonium
(MBTA) labeled these cysteines. This treatment breaks the disulfide bonds between the
viscinal cysteines producing a binding site that is susceptible to electrophilic attack by
quaternary ammonium moieties like those in MBTA. The quaternary ammonium moiety
resembles the cholinergic ligand and reacts with the negative subsite of the active site of
the nAChR.

Since the $\alpha$ subunits contain the viscinal cysteines believed to be a part of the
agonist binding site, they are sometimes called the agonist binding subunits. $\beta$ subunits
do not contain the vicinal cysteines and are thus called structural subunits. The terms
agonist binding and structural subunits may be misleading as the $\beta$ subunit influences the
pharmacology of the neuronal nAChR (Luetje and Patrick 1991; Wheeler et al. 1993).
This situation is analogous to that found in the neuromuscular junction where the ligand
is believed to bind to the $\alpha$ subunit but also interacts with the other subunits (Pederson

1.4 Neuronal nAChR Subtypes

Oocyte expression studies have led to the understanding that the combination of
nAChR subunits present in the receptor affects its characteristics including pharmacology
(Luetje and Patrick 1991; Gerzanich et al. 1994; Elgoyhen et al. 1994). In these
expression studies, the mRNA of the various subunits are microinjected into an oocyte.
The oocyte then translates the mRNA into protein. In this case, some subunit
combinations can assemble into functional membrane channels whose characteristics can be examined via the patch clamp method. Using single and pair-wise subunit combinations these studies have yielded a wealth of information concerning subunit combinations that yield functional ion channels and their respective channel characteristics and pharmacology. For instance, the $\alpha_7$ and $\alpha_8$ subunits are capable of forming functional homoligomeric ion channels that are described as being large (high) conductance, rapidly desensitizing, cation channels (Gerzanich et al. 1994). The $\alpha_9$ subunit also forms homomeric channels although its sequence homology and pharmacology distinguish it from the $\alpha_7$ and $\alpha_8$ homomers (Elgoyhen et al. 1994). The $\alpha_2$, $\alpha_3$, and $\alpha_4$ subunits require co-expression with either the $\beta_2$ or $\beta_4$ subunits while the $\alpha_5$, $\alpha_6$ and $\beta_3$ subunits do not form functional receptors alone or in pair-wise combination with other subunits (Luetje and Patrick 1991). These studies suggest that, with the exception of $\alpha_7$, $\alpha_8$, and $\alpha_9$ homomers, neuronal nAChRs are $\alpha/\beta$ heteromers. Furthermore, oocyte expression studies suggest that neuronal nAChRs are pentameric with an $\alpha_2\beta_3$ stoichiometry (Anand et al. 1991). Transmembrane region two is believed to line the lumen of the ion channel (Revah et al. 1991). Unfortunately, these procedures do not address the in vivo situation, and so the true arrangement(s) of the neuronal nAChR remains to be elucidated.

As mentioned earlier, both the $\alpha$ and $\beta$ subunits contribute to the pharmacology of the neuronal nAChR. Each combination of $\alpha$ and $\beta$ subunits displays a different rank-order of potencies to nicotinic agonists such as nicotine, acetylcholine, 1,1-dimethyl-4-
phenylpiperazinium (DMPP), and cytisine (Luetje and Patrick 1991). For instance, nicotine is more potent than acetylcholine in the $\alpha_2\beta_2$ combination and equipotent in the $\alpha_4\beta_2$ combination. The $\alpha_3\beta_2$ combination is sensitive to acetylcholine but shows little response to nicotine. The differential sensitivity to nicotine between the $\alpha_2$ and $\alpha_3$ combinations appears to be at least partially determined by amino acids 1-84 and 195-215 with amino acid 198 being of special importance (Luetje et al. 1993). However, agonist potency does not only rely solely upon the $\alpha$ subunit. While nicotine has very little effect on the $\alpha_3\beta_2$, it produces a robust response when applied to oocytes expressing the $\alpha_3\beta_4$ combination (Luetje and Patrick 1991). Additionally, sensitivity to cytisine appears to depend largely upon the $\beta$ subunit. Cytisine is more potent than nicotine, acetylcholine, or DMPP when it is applied to oocytes expressing $\alpha_2$, $\alpha_3$, or $\alpha_4$ in combination with $\beta_4$. In contrast, cytisine is acts as a partial agonist with little or no efficacy when the same $\alpha$ subunits are expressed with the $\beta_2$ subunit. Although initially the $\alpha_5$ subunit was not able to form functional in pairwise combinations with other subunits, it has subsequently been found to combine with either $\alpha_3\beta_2$ receptors or $\alpha_3\beta_4$ receptors in oocytes. The resulting receptors had altered pharmacologies when compared to the pairwise combinations of $\alpha_3\beta_2$ and $\alpha_3\beta_4$. Additionally they desensitized more rapidly and gated more Ca$^{2+}$ (Gerzanich et al. 1998).

A similar situation exists for antagonists. Two snake toxins, $\alpha$-bungarotoxin and neuronal-bungarotoxin ($n$-bungarotoxin, toxin F, $k$-bungarotoxin, and bungarotoxin 3.1) isolated from *Bungaris* multicipunctus, have helped to further define the pharmacology of
nAChRs. While α-bungarotoxin binds tightly to and inhibits the function of the neuromuscular junction nAChRs, it binds but does not appear to affect function in the CNS and PNS. α-Bungarotoxin inhibits α7, α8 and α9 homoligomers expressed in oocytes and has no effect on other functional combinations (Corriveau and Berg 1993; Amar et al. 1993; Elgoyhen et al. 1994). More recently, the properties of n-bungarotoxin have been explored. Like α-bungarotoxin, n-bungarotoxin is an antagonist of the nAChR, although, as its name suggests, it primarily acts upon neuronal type receptors. Still, the name neuronal-bungarotoxin may be misleading since the toxin does not act equally well on all subtypes of neuronal nAChR. Data from the oocyte expression system have revealed that β4 containing receptors are insensitive to the toxin and the sensitivity to β2 containing receptors depends upon the α subunit (α3 > α4 > α2; Wheeler et al. 1993; Luetje et al. 1990). Furthermore, the construction of chimeric subunits has revealed amino acid residues 84-121, 121-181, and 195-215 and in particular the gln residue at position 198 of the α3 subunit are important for n-bungarotoxin sensitivity (Wheeler et al. 1993). In a separate study, investigators found that the first 80 residues of the α subunit were important for n-bungarotoxin sensitivity (Luetje et al. 1993). Interestingly, α9 homoligomers are inhibited by n-bungarotoxin as well as α-bungarotoxin (Elgoyhen et al. 1994). Several other toxins including neosuragatoxin, lophotoxin, and the α-conotoxins, show differential effects on nAChRs expressed in oocytes (Luetje et al. 1990).
Despite all of the data obtained from *Xenopus* oocytes, the *in vivo* composition of neuronal nAChRs is still largely unknown. Channel characteristics of nAChRs *in vivo* seldom correlate well with the characteristics of the expressed channels. This could be due to many different factors. For instance, the post-translation modifications done in the oocyte may be different than the *in vivo* situation. Also, experimental conditions which can effect channel characteristics often vary from study to study. Moreover, there may be multiple channel types present in vivo that contribute to the overall response.

1.5 Heterogeneous Population of Neuronal nAChRs

It is becoming increasingly evident that nAChR populations within a given neuronal tissue may be heterogeneous. In chick ciliary ganglia at least 3 populations of nAChRs have been immunologically identified. These include a large population of α7-containing receptors which gate Ca\(^{2+}\) in response to nicotine and are inhibited by α-bungarotoxin (Vijayaraghavan et al. 1992; Pugh and Berg 1994; Zhang et al. 1994). These cells also contain a smaller population of α3α5β4 receptors that cross-react with the monoclonal antibody, mAb35 (Conroy et al. 1992; Vernallis et al. 1993). Additionally, a small population of nAChRs that bind mAb35 also contain the β2 subunit (Conroy and Berg 1995). Patch clamp techniques have also been used to identify multiple populations of nAChRs within a tissue. In chick sympathetic ganglia, four nAChR channel conductances have been identified (Listerud et al. 1991). In cultured hippocampal neurons, four classes of nicotine-mediated currents (types IA, IB, II, and III) can be demonstrated based upon channel characteristics and pharmacological profiles.
(Alkondon and Albuquerque 1993; Lukas 1995). These findings indicate that nAChRs may be formed by as many as three different subunits and that neurons can contain more than one subtype of nAChR.

1.6 Biochemical Regulation of nAChRs

A basic understanding of the mechanisms involved with nAChR regulation are of critical importance since these processes may underlie certain disease states and may significantly effect the clinical usefulness of cholinergic drugs. The properties of nAChRs are, in part, due to the particular combination of known nAChR subunits. However other processes, including receptor desensitization, up- or down-regulation, and tolerance and dependence can also affect the functional properties of receptors and/or affect the number of receptors present. These processes have a temporal component, i.e. they occur at different rates: 1) loss of receptor function via desensitization occurs rapidly, usually within seconds or minutes, 2) receptor up- or down-regulation develops over several hours, 3) tolerance and dependence generally takes days or weeks to develop. Several mechanisms may underlie these processes, including covalent modifications of receptors and alterations in receptor internalization, receptor degradation, and/or changes in receptor synthesis.

Nicotinic acetylcholine receptors of the neuromuscular junction have been the prototype member of the family of ligand gated ion channels. A great deal of research has been done concerning the receptor function and its regulation. In general, the nAChR exists in several distinct states that can be characterized by affinity of the binding site as
well as the condition of the ion channel. In the first state, the agonist binds to the nAChR with low affinity, and the ion channel is gated open allowing the passage of Na\(^+\) ions as well as a considerable amount of Ca\(^{2+}\) ions. However the receptor will quickly desensitize in 2 stages in the continued presence of the agonist. In the first stage of desensitization, the binding site has an intermediate affinity for the ligand and the channel is closed. The intermediate state of desensitization is also characterized by rapid recovery from desensitization when the agonist is removed. If the agonist remains for prolonged periods of time the receptor can enter into a deeply desensitized state. This state has the highest affinity for the ligand but, in contrast to the intermediate state, recovery from the deep state takes substantially longer. In general, the longer the agonist is applied, the greater the number of receptors in the deep desensitized state and the longer the recovery from desensitization will take (For review see Quick and Lester 2002).

Given the time course of desensitization and recovery from desensitization, it seems likely that protein phosphorylation events play an important role in both the induction of and recovery from desensitization. Many neuronal nAChR subunits contain consensus sites for phosphorylation by cAMP dependent protein kinase (PKA) and calcium dependent protein kinase (PKC) in the cytoplasmic loop between TM3 and TM4. The location of the residues makes them ideal targets for intracellular modification and regulation of nAChRs via second messenger pathways. For instance, both PKC and PKA have been shown to phosphorylate the \(\alpha 4\) subunit, while PKA dependent events phosphorylate the \(\alpha 3\) and \(\alpha 7\) subunits (Vijayaraghavan et al. 1990;
Wecker and Rogers 2003). In muscle nAChRs the level of phosphorylation affects the kinetics of the induction of desensitization (Huganir et al. 1986; Hopfield et al. 1988; Hoffman et al. 1994).

Similar to the muscle nAChR model, recovery from desensitization of neuronal nAChRs appears to proceed in two phases. A fast phase and a slow phase can be distinguished by their dependence on extracellular calcium. The fast phase of recovery is not calcium dependent, while the slow phase of recovery is calcium dependent. Data also suggest that the longer the agonist application the greater the percentage of receptor that must undergo the slow phase. After short pulses of agonist, more receptors recover in the fast phase (Fenster et al. 1999a).

Phosphorylation events have also been implicated in recovery from desensitization. Recovery of α4β2 nAChR function following prolonged agonist treatment is enhanced by activation of PKC and retarded by PKC inhibition (Fenster et al. 1999a). This effect was also seen for PKC and PKA activation in rat adrenal chromaffin cells (Khiroug et al. 1998). Furthermore, deactivation or inhibition of PKC alone can cause a functional deactivation of nAChRs similar to desensitization (Eilers et al. 1997). Additionally, inhibition of phosphatases, including calcineuron, also enhances recovery from prolonged agonist treatment (Eilers et al. 1997; Khiroug et al. 1998; Fenster et al. 1999a). Over all these studies support a model where long-term desensitization is characterized by low levels of phosphorylation, while activatable receptors have relatively higher levels of phosphorylation. Numerous biochemical pathways can be
recruited to modify the level of phosphorylation in the system. However these studies do not attempt to identify in what portion of the biochemical pathway that the phosphorylation events are taking place.

Aside from desensitization, protein kinase activities are also likely candidates for controlling other properties of nAChR such as receptor clustering and receptor up- or down-regulation. In muscle, the protein agrin, which is released from the inervating presynaptic fiber, causes the tyrosine phosphorylation of nAChRs through the action of protein tyrosine kinases. This promotes nAChR aggregation and attachment to the cytoskeleton beneath the forming neuromuscular junction. Additionally, protein tyrosine kinases have also been implicated in the regulation of ganglionic type nAChRs. In chick ciliary ganglion cells, inhibition of protein tyrosine kinases causes a slowly developing loss of mAb35 binding but does not affect α-bungarotoxin binding. This effect does not seem to involve increased degradation of surface receptor and preferentially affects surface receptors over intracellular receptors (Haselbeck and Berg 1996). nAChRs are also regulated by phosphorylation of serine and threonine residues. For instance, PKC phosphorylates serine residues on the muscle δ subunit. The phosphorylation of serine residues appears to cause the disaggregation of nAChR clusters (Nimnual et al. 1998).

Serine/threonine protein kinases have been implicated in turnover of neuronal nAChRs. Many subtypes of neuronal nAChR can be upregulated when treated for long periods of time with nAChR agonists. The principal brain nAChR, α4β2, is upregulated by chronic exposure to nicotine in a variety of cell types and cell lines. Other subtypes of
nAChR can be upregulated in response to chronic nicotine treatment, including the $\alpha 7$ subtype and $\alpha 3^*$ subtypes (Wang et al. 1998; Ridley et al. 2001). The $\alpha 3^*$ subtypes appear to require the coexpression of the $\beta 2$ subunit but not the $\beta 4$ subunit (Wang et al. 1998). This process is not well understood but it appears to involve posttranslational modifications since it readily occurs in transfected cell types that do not normally express nAChRs (Peng et al. 1994; Rothhut et al. 1996). Upregulation by chronic nicotine treatment does not cause a change in the levels of mRNA for the affected subunits and is resistant to treatment by cylcoheximide. This further indicates that posttranslational mechanisms are at work.

The exact mechanism for nAChR upregulation is not known; however, there is evidence that chronic nicotine treatment stabilizes the receptor in the membrane, slowing down its degradation (Peng et al. 1994). Some studies also indicate that the receptors fold and combine more efficiently under prolonged nicotine treatment (Harkness and Millar 2002). Both cases would result in a shift of the receptors from intracellular pools to the surface of the cell. There appears to be a link between the need for desensitization and upregulation since the EC$_{50}$ for upregulation is similar to the IC$_{50}$ for desensitization in $\alpha \beta$ receptors expressed in oocytes (Fenster et al. 1999b). The same study found that mutant nAChRs that stay chronically desensitized even in the absence of nicotine were also able to upregulate. PKC inhibition by chronic phorbol esters treatment which causes inhibition of recovery from desensitization (Fenster et al. 1999b) can also cause upregulation (Gopalakrishnan et al. 1997). This role of desensitization in upregulation is not clear since evidence from other systems shows a large difference between the
concentration of nicotine needed for upregulation and the concentration of nicotine that causes desensitization. The link between desensitization and upregulation also supports evidence that upregulated receptors are functionally inactive and offers an explanation for nicotine tolerance and addiction. However other findings indicate that upregulated receptors may actually have increased function. Additionally there is evidence that nicotine can be intracellularly sequestered in oocytes, leading to prolonged release of nicotine after wash out and possibly explaining the loss of function of upregulated nAChRs in oocytes (Jia et al. 2003).

Other methods can be used to upregulate nAChRs. However, these methods may utilize a mechanism distinct from the mechanism of chronic nicotine induced upregulation. Chronic treatments with KCl can upregulate $\alpha$-bungarotoxin binding sites in hippocampal cell cultures. Verapamil and the calcium calmodulin dependent protein kinase (CAM kinase II) inhibitor, KN-62, can block this effect. Neither verapamil nor KN-62 had any effect on chronic nicotine induced upregulation of $\alpha$-bungarotoxin binding sites. However KN-62 by itself could upregulate [$^3$H]-epibatidine binding sites in a neuroblastoma cell line (Ridley et al. 2001). Activation of PKA has also been found to cause the upregulation of $\alpha 4\beta 2$ receptors. For PKA there seems to be an increase in both the number of intracellular as well as extracellular receptors, although there is a shift in the proportion of extracellular receptors. Contrary to upregulation induced by chronic nicotine treatment, PKA activation does cause a small increase in $\alpha 4$ mRNA levels but not in the $\beta 2$ mRNA levels. This is surprising since these studies were conducted in transfected cells, and the expression of the nAChR subunits was under the control of a
dexamethasone response element. Finally PKA induced upregulation is additive with upregulation caused by chronic nicotine treatment. Taken together, these findings imply that the two forms of upregulation proceed via different mechanisms. (Rothhut et al. 1996).

Upregulation can also occur in cell types that contain ganglionic nAChRs. Upregulation of receptors proceeds though a PKA dependent mechanism. PKA deficient PC12 cells do not show increase in nicotine binding after prolonged incubation with high levels of nicotine (Madhok et al. 1995). Activation of PKA also increases nicotine binding (Madhok et al. 1994). The exact mechanism of this upregulation is unknown. Activation of PKA by forskolin and deactivation of PKC by chronic phorbol ester treatment has been shown to enhance the promoter activity of the α3 gene (Boyd 1996). The α3 subunit can be phosphorylated by a cAMP dependant mechanism (Vijayaraghavan et al. 1990).

To date, chronic nicotine treatment has not been shown to upregulate nAChRs in bovine adrenal chromaffin cells. However, the number of α-bungarotoxin binding sites can be increased by treatment over several days with tubocurarine, mecamylamine, high K⁺, or phorbol esters (Quik et al. 1987; Geertsen et al. 1990; Geertsen et al. 1992). This increase in α-bungarotoxin binding sites has no effect on internal Ca²⁺ levels. Additionally activation of PKA does not affect channel characteristics, mAb35 binding, or nAChR mediated secretion (Dubin et al. 1992). Forskolin, a PKA activator, inhibits
nAChR mediated events in chromaffin cells but this effect does not appear to be related to PKA activity. The effect is rapidly reversible, and inactive analogues of forskolin cause the same effect (Gandia et al, 1997).

1.7 Adrenal nAChRs

Adrenal chromaffin cells share many important features and a common embryological origin with sympathetic neurons. Their ease of isolation and established culturing conditions make them a good model for sympathetic neurons (for review see Livett 1984). It is well established that adrenal chromaffin cells contain nicotinic receptors of the neuronal type. Activation of adrenal nAChRs leads to the release of a variety of secretory products, including epinephrine. Despite their physiological importance, very little information is available on adrenal nAChRs. Patch clamp and binding studies have indicated that the number of nAChRs on chromaffin cells is relatively low (Lee et al. 1992; Maconochie and Knight 1992; Free and McKay 2003). Binding studies show that adrenal nAChRs should be classified as $\alpha_3\beta_4^*$ receptors as their characteristics are similar to $\alpha_3\beta_4^*$ in other tissues and in cells expressing bovine $\alpha_3\beta_4$ (Free et al. 2002; Free and McKay 2003). Pharmacological studies support the presence of multiple populations of adrenal nAChRs. One population binds $\alpha$-bungarotoxin and probably is not involved with secretion as the toxin has no effect on secretion or on internal Ca$^{2+}$ concentrations (Wilson and Kirshner 1977; Afar et al. 1994).

Another population of nAChRs is present which interacts with the nAChR antibody, mAb35. Our laboratory has demonstrated that adrenal chromaffin cells contain
mAb35 binding sites (Lopez and McKay 1997) and that mAb35 potently and specifically reduces nAChR-stimulated catecholamine release (Gu et al. 1996). These effects of mAb35 develop slowly and are slowly reversible, suggesting that mAb35 induces nAChR down-regulation. The ability of mAb35 to modulate adrenal nAChRs has led to their identification as mAb35-nAChRs (Gu et al. 1996); a similar classification has been used for nAChRs found on chick ciliary ganglion neurons (Halvorsen and Berg 1990; Conroy et al. 1992; Vernallis et al. 1993). Even after prolonged treatment, mAb35 only eliminates approximately 60% of nAChR mediated function. This leaves the possibility that another population of functionally relevant nAChRs mediates the residual mAb35 insensitive response.

All together, this evidence suggests that there are at least three subtypes of nAChRs present on adrenal chromaffin cells. One is α-bungarotoxin sensitive and presumably contains α7 subunits. The function of this subtype is unknown. The other population is mAb35 sensitive and mediates secretion. A third subpopulation is mAb35 insensitive and is also important for functional response. The subunit composition of mAb35-nAChRs and other nAChR subtypes possibly involved with adrenal secretion are unknown. At the time that this research was proposed only the α3 and α7 nAChR subunits had been cloned and sequenced in bovine adrenal chromaffin cells (Criado et al. 1992; Garcia-Guzman et al. 1995). However since then, the α5 and β4 subunits have also been cloned and sequenced (Campos-Caro et al. 1997; Wenger et al. 1997). This is similar but not identical to the subunits found in related ganglionic tissues. In the related rat PC12 cell line, α3, α5, α7, β2, β3, and β4 mRNA have been detected (Boulter et al.
1987; Boulter et al. 1990; Rogers et al. 1992). Similarly, the α3, α5, α7, β2, and β4 mRNAs have been found in the parasympathetic chick ciliary ganglia (Corriveau and Berg 1993).
CHAPTER 2

MATERIALS AND METHODS

2.1 Materials

(-) Nicotine hydrogen tartrate, cytisine, acetylcholine chloride, 1,1-dimethyl-4-phenylpiperizinium iodide, neostigmine bromide, d-tubocurarine chloride, hexamethonium bromide, decamethonium bromide, pentolinium di(L(+)-tartrate), mecamylamine hydrochloride, adiphinine hydrochloride, tetracaine hydrochloride, amantidine hydrochloride, cyclohexamide, puromycin hydrochloride, tunicamycin, colchicine and staurosporine were obtained from the Sigma Chemical Company (St. Louis, MO). (±) Epibatidine dihydrochloride, bromoacetylcholine bromide, H-7, and H-8 were purchased from Research Biochemicals Incorporated (Natick, MA). K252a and chelerythrine chloride were obtained from LC Laboratories (Woburn, MA). Vinblastine was a generous gift from Eli Lilly and Company (Indianapolis, IN). Taxol was obtained from the National Products Branch, Division of Cancer Treatment, National Cancer Institute (Bethesda, MA). Vinblastine was dissolved in a 0.9% saline solution. Taxol, staurosporine, H-7, and H-8 were dissolved in 100% dimethylsulfoxide. K252a was dissolved in 100% ethanol.
2.2 Isolation and primary culture of bovine adrenal chromaffin cells

Bovine adrenal glands were obtained from Herman Falter Packing Company (Columbus, OH). Surgical tools were purchased from Roboz Surgical Instruments Company (Rockville, MD). Conical centrifuge tubes, 15 and 50 mL, as well as 100 mM culture dishes were purchased from Corning Glass Works (Corning, NY). Falcon Primaria 6, 12 and 24 well plates were obtained from Becton Dickinson and Company (Lincoln Park, NJ). Cell suspensions were filtered through various sizes of polypropylene or nylon mesh filters purchased from Spectrum (Houston, TX). Sterile filtration of bulk solutions was performed using a Millipore peristaltic pump and Sterivex-GP 0.22 µ filter units purchased from the Millipore Corporation (Bedford, MA). Small volumes of solution were syringe sterilized using MFS 0.20 µM syringe filter units obtained from Life Science Products, Incorporated (Denver, CO). Adrenal gland perfusions were performed using a Buchler (Haake Buchler Instruments, Saddle Brook, NJ) multistatic four channel pump fitted with Tygon tubing (Norton Performance Plastics, Akron, OH). Low-speed centrifugation was performed in a IEC HN-SII bench top centrifuge (International Equipment Company, Needham Heights, MA). High-speed centrifugation was performed in a Sorvall RC5B Plus high-speed centrifuge equipped with a SS-34 rotor (DuPont Company, Wilmington, DE). Collagenase type 1 (239 U/mg) and DNase 1 (2690 U/mg) were purchased from Worthington Biochemical Corp (Freehold, NJ). Dulbecco's Modified Eagles Medium (DMEM) as well as DMEM/F12 medium, penicillin/streptomycin solution (10,000 U/ml penicillin/ 10,000 µg/ml streptomycin), and Fungizone (12.5 µg/ml amphotericin B) were purchased from Gibco/BRL (Grand Island, NY). Fetal calf serum (FCS) was obtained from Atlanta
Biologica!s (Norcross, GA). Bovine serum albumin (BSA), l-glutamine, putrascine, sodium selenite, water-soluble progesterone, bovine insulin, and 5-fluro-2'-deoxyuridine were purchased from Sigma Chemical Company (St. Louis, MO). All other reagents were of analytical grade.

Adrenal chromaffin cells were dissociated from intact glands and plated, as previously described (Maurer and McKay 1994). Bovine adrenal glands were placed on ice immediately upon removal from the animal. Within one hour of removal, four glands were excised from surrounding connective and fat tissue. Using a surgical blade, small incisions (approx. 5mm) were made across the entire surface of the cortex of the each gland. The adrenal vein of each gland was canulated, and the glands were reverse perfused with approximately 250 - 300 ml of cold Locke's solution. The glands were next perfused with 0.05% collagenase type I and 0.004% DNase I in Locke's solution at room temperature for 40 min. Following the collagenase/Locke's perfusion the glands were incubated a further 20 min in collagenase at room temperature. The medulla of the glands were carefully separated from the cortex and minced thoroughly in ice cold Locke's solution. The Lockes's solution was decanted, and the minced tissue was transferred into a 50 ml conical centrifuge tube containing collagenase solution and incubated 30 min at 37 °C with gentle shaking. Undigested tissue was separated from dissociated cells by passage through a 210 µ mesh filter. The dissociated cells were pelleted by centrifugation (800 x g), and the collagenase solution was aspirated off.
The cells were resuspended in Locke's solution containing 0.5% BSA. The dissociated cells were further filtered through 105 µ mesh filter, pelleted and finally resuspended to a volume of 39 ml in Locke's solution containing 0.5% BSA. The cell suspension was added to 32 ml of Percol/Locke’s and centrifuged at 4°C for 40 min at 28,200 x g. The top layer of dead cells was aspirated off prior to the careful removal of the central purified chromaffin cell layer with a pipette. The Percoll was removed by dilution in Locke's with BSA followed by centrifugation (800 x g) and aspiration of the supernatant. After repeating, the pellet was resuspended in Locke’s with BSA. The number of chromaffin cells was determined on a hemocytometer, while the viability of the cells was determined via vital dye staining using 0.4% erythrosin B in 0.9% NaCl (5:1). Finally, the cells were pelleted and resuspended in DMEM and filtered through a 60 µ nylon mesh filter prior to plating.

Cells were plated at a density of 1-2x10^5 cells per well on 24-well culture plates for secretion studies or 10^n cells per 100 mm dish for RNA studies. Two days after plating, media were replaced with a modified serum-free N2+ medium previously described by our laboratory (Maurer and McKay 1994). N2+ medium was comprised of DMEM/F12 medium (1/1), 5µg/ml insulin, 100µM putriscine, 20nM progesterone, and 30nM sodium selenite. DMEM and N2+ media were supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, 250 ng/ml amphotericin B, and 10 µM 5-fluoro-2'-deoxyuridine. DMEM also contained 9% FCS. One day prior to experimentation, the culture medium was removed and replaced with N2+ medium free of amphotericin B and 5-fluoro-2'-deoxyuridine. Cells were used 4-7 days after isolation.
2.3 Catecholamine secretion studies

DL-[\textsuperscript{3}H]-Norepinephrine (specific activity, 12.0-15.0 Ci/mmol) was purchased from Dupont-New England Nuclear Corporation (Boston, MA). Ascorbic acid was obtained from Sigma Chemical Company (St. Lious, MO). 4(-2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) was purchased from Fischer Scientific (Pittsburgh, PA). All other reagents were of analytical grade or as described previously.

A [\textsuperscript{3}H]-norepinephrine ([\textsuperscript{3}H]NE) assay was used to monitor catecholamine release from cultured cells (McKay and Schneider 1984). Cells were incubated with 0.1 µM [\textsuperscript{3}H]NE in a physiological saline solution (PSS) containing 140 mM NaCl, 4.4 mM KCl, 1.2 mM MgSO\textsubscript{4}, 3.6 mM NaHCO\textsubscript{3}, 1.2 mM KH\textsubscript{2}PO\textsubscript{4}, 2 mM CaCl\textsubscript{2}, 10 mM glucose, 5 mM HEPES (pH 7.2-7.4) and 0.5% bovine serum albumin (BSA) and extensively washed prior to all treatments. The amount of radioactivity released following a 5 min incubation with secretagogue (stimulated release) or without secretagogue (basal release) was determined using liquid scintillation spectroscopy. The radioactivity remaining in the cells was then extracted with 8% trichloroacetic acid (TCA) and counted. The sum of the secreted and the TCA-extractable radioactivity represented total incorporated [\textsuperscript{3}H]NE. Results were expressed either 1) as a percentage of total (% total) incorporated [\textsuperscript{3}H]NE released under the treatment conditions (\textit{i.e.}, secreted [\textsuperscript{3}H]NE divided by total incorporated [\textsuperscript{3}H]NE x 100) or 2) as a percentage of the net stimulated control response (% control) where basal (nonstimulated) release was subtracted from all groups (\textit{i.e.}, treatment group release minus basal release divided by control nicotine-stimulated release minus basal release x 100).
2.4 Alkylation of nAChRs

DL-dithiothreitol and 5,5'-dithio-bis(2-nitrobenzoic acid) were obtained from Sigma Chemical Company (St. Louis, MO). All other reagents were of analytical grade or as described previously.

Adrenal nAChRs were irreversibly inactivated via alkylation with brACh using techniques modified from Gardette et al., (Gardette et al. 1991) as previously described by our laboratory(Wenger et al. 1997). The cells were first treated with 1 mM dithiothreitol (DTT) in PSS (pH 8) for 15 min at 37°C in order to reduce nAChR disulfide bonds. Cells were then washed (1 ml PSS/well, 15 min), followed by treatment with brACh for 6 min at room temperature. After washing (1 ml PSS/well, 5 min), the disulfide bonds were reoxidized by incubating the cells with 1 mM 5,5'-dithio-bis(2-nitrobenzoic acid) (DTNB) in PSS for 15 min at 37°C. The ability of a variety of pharmacological agents to interfere with the alkylation process (i.e., provide protection from brACh-induced alkylation) was assessed by including the pharmacological agent in both the first wash and the brACh treatment periods. After a final wash (1 ml PSS/well, 5 min), the cells were stimulated. In control studies, in the absence of alkylation, pretreatment with the nAChR inhibitors, followed by normal washing procedures, produced no residual inhibitory effects (data not shown).

2.5 Isolation and purification of mAb35

A hybridoma cell line which secretes mAb35, a monoclonal antibody directed against the main immunogenic region of nAChRs, was obtained from the American Type
Culture Collection (Rockville, MD). The cells were cultured and the antibody was concentrated and purified using techniques described previously (Gu et al. 1996).

2.6 Reverse transcription/polymerase chain reaction (RT/PCR), cloning, sequencing and Northern analysis.

Trizol Reagent, DNase I (amplification grade), Superscript Preamplification System, and PCR Reagent System were purchased from Life Technologies (Grand Island, NY). PCR primers were synthesized by National Biosciences Incorporated (Plymouth, MN). The TA cloning kit was purchased from Invitrogen (San Diego, CA). A Sequenase sequencing kit was obtained from United States Biochemical (Cleveland, OH). $^{35}$S-nuclide, and $[^{32}P]$dCTP were obtained from Dupont-New England Nuclear Corporation (Boston, MA). All other reagents were of molecular grade.

DNA amplification by PCR was performed in an MJ Research thermocycler (MJ Research, Inc., Boston, MA), and blots were hybridized to Genescreen Plus hybridization membranes (Dupont-New England Nuclear Corporation, Boston, MA). Additional analysis of sequences was performed using the GeneWorks 2.1 program (IntelliGenetics, Mountain View, CA).

Total RNA was isolated from adrenal medulla using Trizol Reagent according to the procedure of Chomczynski and Sacchi (Chomczynski and Sacchi 1987). Prior to use as a template for reverse transcription, the RNA was treated with 1 unit of DNase I, Amplification Grade. The reverse transcription reaction was performed in a 20 µl
volume and contained 500 µM dNTPs, 10 mM DTT, 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 2.5 mM MgCl₂, 200 units Superscript II, and 100 ng random hexamers. RNA and random primers were heat denatured and reverse transcribed for 10 min at 25°C, then for 50 min at 42°C followed by treatment with 2 units of RNase H at 37°C for 20 min.

Single-stranded cDNA was amplified by PCR in a final volume of 100 µl containing 1.5 mM MgCl₂, 50 mM KCl, 20 mM Tris-HCl (pH 8.4), 500 nM of each primer, 500 µM dNTPs, and 5 units of Taq polymerase overlaid with mineral oil. The design of degenerate PCR primers was based upon homologous sequences of transmembrane regions three and four (TM3 and TM4) of the bovine α3, rat α3, rat β2, and rat β4 subunit genes. The TM3 primer sequence was 5’-GTMACCYTYTCCATYGTCA 3’; the TM4 primer sequence was 5’-CGRTCTAYSACCATSGCMAC 3’ where M = A or C, Y = C or T, S = G or C, and R = A or G. Amplification was performed as follows: samples were heated to 94°C for 5 min after which the temperature was lowered to 80°C. Taq polymerase was then added, and the reaction proceeded for 36 cycles (94°C for 1 min, 52°C for 1 min, 72°C for 1.5 min) followed by incubation for 10 min at 72°C to complete the extension. PCR products were cloned into the vector, pCRII, using the TA Cloning Kit according to manufacturer’s instructions and sequenced. Sequencing was performed using the chain termination method (Sanger et al., 1977) with Sequenase T7 polymerase according to manufacturer instructions. A BLAST (Altschul et al., 1990) search of non-redundant Genbank and EMBL sequences was used to identify similar sequences.

RNA was purified from cultured bovine adrenal chromaffin cells using Trizol Reagent according to the procedure of Chomczynski and Sacchi (Chomczynski and
Sacchi 1987). Northern blot analysis was performed using 1% (w/v) agarose gels containing 7.4% (v/v) formaldehyde in 20 mM 3-(N-Morpholino)-propanesulfonic acid (MOPS), 1 mM ethylenediaminetetraacetic acid Na$_2$ (EDTA), and 5 mM sodium acetate at pH 7. After electrophoresis, the RNA was transferred to Gene Screen Plus (Dupont-New England Nuclear Corporation, Boston, MA) in 10X SSC (1X SSC=0.15 M sodium chloride and 0.015 M sodium citrate) according to the manufacturer's instructions. The EcoRI insert from the pCRII vector containing either the bovine cDNA 4 or the bovine \( \alpha_3 \) cDNA were $^{32}$P-labeled with $[^{32}P]$-dCTP (3000 Ci/mmol) by random priming (Feinberg and Vogelstein 1983) and hybridized to Gene Screen Plus in 5X SSPE (1X SSPE contains 0.15 M NaCl, 0.01 M NaH$_2$PO$_4$, and 1 mM EDTA, pH 7.4), 50% deionized formamide, 5X Denhardt's solution (1X Denhardt's solution contains 0.2 mg/ml polyvinylpyrrolidone, 0.2 mg/ml BSA, 0.2 mg/ml Ficoll 400), 1% sodium dodecyl sulfate (SDS), 10% dextran sulfate, and 100 \( \mu \)g/ml of salmon sperm DNA) at 42°C. The filters were washed in 2X SSPE at room temperature, 2X SSPE, 2% SDS at 65°C, and 0.1X SSPE at room temperature. Autoradiograms were developed after exposure to Kodak XAR-5 film at -70°C with a Dupont Cronex intensifying screen.
2.7 Calculations and Statistics

Results were calculated from the number of observations (n) performed in duplicate or triplicate. EC\textsubscript{50} and E\textsubscript{max} values were obtained by averaging values generated from sigmoid, nonlinear regression analyses (Inplot 3.1, GraphPad, San Diego, CA) of individual concentration-response curves. At high agonist concentrations, a reduction in secretory response is often seen; these data were omitted during nonlinear curve fitting. Results are expressed as arithmetic means ± SE, except for EC\textsubscript{50} values, which are expressed as geometric means (95% confidence limits). Statistical analysis was performed using Dunnett's multiple comparison procedure at a 0.05 level of significance.
CHAPTER 3

RESULTS

3.1 Alkylation of adrenal nAChRs

The first set of studies that we performed involved the characterization of the effects of an irreversible inhibitor of nAChRs on bovine adrenal chromaffin cells. Irreversible receptor antagonists are often used to investigate the presence of spare receptors. These antagonists produce increases in EC₅₀ values and decreases in Eₘₐₓ values, which are characteristics of systems containing receptor reserves. Previous studies have documented the utility of brACh treatment in the study of nAChRs (Leprince 1983; Listerud et al. 1991; Gardette et al. 1991). BrACh is an analog of the endogenous neurotransmitter acetylcholine. Not surprisingly, brACh acts as a nAChR agonist. However, under reducing conditions, brACh irreversibly deactivates nAChRs resulting in the inhibition of nAChRs. This effect is presumably due to an interaction with the pair of cysteines analogous to cys 192 and 193 of the alpha 1 subunit that normally form a disulfide bridge in the native protein. Under treatment with DTT, a mild reducing agent, these bonds are broken and alkylating agents are able to irreversibly bind to the sulfhydryl groups. The result is blockade of agonist/antagonist binding as well as
irreversible inactivation of the receptors. For these reasons, we initially characterized the effects of brACh treatment on nAChR-mediated secretory function in bovine adrenal chromaffin cells.

We have found that brACh is effective as a nAChR agonist (Figure 1) with an EC$_{50}$ of approximately 4 µM. However, when brACh is used under alkylating conditions (see methods), an immediate loss of nicotine-stimulated adrenal catecholamine release occurs. These effects are concentration-dependent (IC$_{50}$, ~ 0.3 µM). At concentrations greater than 10 µM, brACh eliminates approximately 90% of control nAChR-stimulated release (Figure 2). brACh has no effect on 56 mM KCl-stimulated release under conditions that reduce nicotine-stimulated release (data not shown). The reduction in nAChR-stimulated release is not immediately reversible (Figure 3). nAChR-stimulated secretion slowly returns with an approximate functional recovery rate of 2% per hour (Figure 3). Recovery of nAChR-stimulated function can be blocked by cyclohexamide (1 µg/ml) treatment (data not shown), suggesting the return of functional receptors involves protein synthesis and supporting the irreversible nature of brACh treatment.
Figure 1. **BrACh-induced stimulation of bovine adrenal nAChRs: concentration-dependent effects.** Cultured bovine adrenal chromaffin cells were stimulated for 5 min with various concentrations of brACh, and catecholamine release during this stimulation period was determined. Results are expressed as a percentage of total catecholamine content. Nonstimulated (basal) catecholamine release was 3.0 ± 0.7% (dotted line). Values represent means ± SE (n = 5 - 6).
Figure 2. BrACh-induced inactivation of bovine adrenal nAChRs: concentration-dependent effects. brACh can be used as a nAChR agonist or, under alkylating conditions (see methods), can be used to inactivate nAChRs. Cultured bovine adrenal chromaffin cells were treated under alkylating conditions with various concentrations of brACh. The effects of brACh-induced nAChR inactivation on 10 µM nicotine-stimulated catecholamine release (5 min) were determined. Results are expressed as a percentage of total catecholamine content. Values for nonstimulated (dotted line) and 10 µM nicotine-stimulated (dashed line) catecholamine release were 1.5 ± 0.5% and 19.8 ± 1.9%, respectively. Values represent means ± SE (n = 6).
Figure 3. Time course for recovery from the inhibitory effects of brACh on adrenal catecholamine release. Cultured chromaffin cells were treated with 100 μM brACh under alkylating conditions. Following this treatment, the cells were washed and placed in N2+ medium. Recovery of nAChR-mediated secretory function was then assessed. At the indicated times after nAChR inactivation, cells were stimulated for 5 min with 10 μM nicotine and catecholamine release during this stimulation period was determined (closed squares). Results are expressed as a percentage of total catecholamine content. Nontreated, 10 μM nicotine-stimulated (closed circles) and nonstimulated (closed triangles) groups were run in parallel. Values represent means ± SE (n = 4 - 6).
3.2 Identification of a nAChR reserve

While it is recognized that subunit composition influences the pharmacology of nAChRs, the presence of receptor reserves (spare receptors) may also alter pharmacological profiles of nAChR populations. Little or no data are available on the presence of spare nAChRs in neuronal tissues, and relatively little is known about the type or number of nAChRs present on adrenal chromaffin cells. The following studies were designed to investigate the presence and composition of adrenal nAChR reserves.

In the next series of experiments, bromoacetylcholine (brACh) was used to irreversibly inactivate nAChRs and then the concentration-response profiles of the nAChR agonists, nicotine, epibatidine and cytisine were determined. As seen in figures 4 - 6, and table 1, the EC50 values for nicotine, epibatidine and cytisine are approximately 4.0 μM, 8.5 nM and 41.0 μM, respectively. With increasing concentrations of brACh, shifts to the right in the concentration-response curves of nicotine and epibatidine occur (Figures 4 - 5, Table 1). At a concentration of 100 nM, brACh causes a small increase in the EC50 value of nicotine and at 1 μM brACh, an increase in the EC50 value of approximately 3.7 fold is seen (Figure 4, Table 1). Similarly, 1 μM brACh causes an increase in the EC50 value of epibatidine of approximately 4.1 fold (Figure 5, Table 1). At 10 μM brACh, there is a further increase in EC50 values for nicotine and epibatidine however the values are not significantly different than the EC50 values generated after 1 μM brACh treatment (Figures 4 and 5, Table 1). Additionally, curve fitting of the depressed values is difficult and introduces considerable variability. Unlike the observed shifts with nicotine and epibatidine, 1 μM brACh increases the EC50 value of cytisine by
approximately 2 fold (Figure 6, Table 1). Finally, 100 nM brACh has no significant
effect on the $E_{\text{max}}$ values of the agonists. However, at 1 µM brACh, the $E_{\text{max}}$ values for
nicotine, epibatidine, and cytisine are reduced by 44%, 35%, and 38% respectively
(Figures 4 - 6, Table 1). Increasing the concentration of brACh to 10µM causes a further
reduction in the $E_{\text{max}}$ value for each agonist (Figures 4 - 6, Table 1).
Figure 4. The concentration-response effects of nicotine after brACh-induced nAChR inactivation. Cultured adrenal chromaffin cells were either not treated (closed squares) or treated under alkylating conditions with 100 nM (upright triangles), 1 µM (inverted triangles), or 10 µM (closed diamonds) brACh. Cells were washed and then stimulated for 5 min with the indicated concentrations of nicotine, and catecholamine release during this stimulation period was determined. Results are expressed as a percentage of net stimulated control response. Control stimulated values (nontreated, 10 µM nicotine) were 15.9 ± 1.0% of total catecholamine content. Basal (nonstimulated) values were 0.8 ± 0.1% of total catecholamine content. Values represent means ± SE (n = 3 - 5).
Figure 5. The concentration-response effects of epibatidine after brACh-induced nAChR inactivation. Cultured adrenal chromaffin cells were either not treated (closed squares) or treated under alkylating conditions with 100 nM (upright triangles), 1 µM (inverted triangles), or 10 µM (closed diamonds) brACh. Cells were washed and then stimulated for 5 min with the indicated concentrations of epibatidine, and catecholamine release during this stimulation period was determined. Results are expressed as a percentage of net stimulated control response. Control stimulated values (nontreated, 10 µM nicotine) were 17.8 ± 0.8% of total catecholamine content. Basal (nonstimulated) values were 1.1 ± 0.2% of total catecholamine content. Values represent means ± SE (n = 3 - 4).
Figure 6. The concentration-response effects of cytisine after brACh-induced nAChR inactivation. Cultured adrenal chromaffin cells were either not treated (closed squares) or treated under alkylating conditions with 100 nM (upright triangles), 1 µM (inverted triangles), or 10 µM (closed diamonds) brACh. Cells were washed and then stimulated for 5 min with the indicated concentrations of cytisine, and catecholamine release during this stimulation period was determined. Results are expressed as a percentage of net stimulated control response. Control stimulated values (nontreated, 10 µM nicotine) were 20.6 ± 0.5% of total catecholamine content. Basal (nonstimulated) values were 0.7 ± 0.1% of total catecholamine content. Values represent means ± SE (n = 3 - 4).
<table>
<thead>
<tr>
<th>Treatment</th>
<th>Agonist</th>
<th>EC$_{50}$ values$^b$</th>
<th>E$_{max}$ Values$^b$ (% control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>--</td>
<td>Nicotine</td>
<td>3.6µM (2.9-4.5) 100.1 ± 8.5 (n=5)</td>
<td></td>
</tr>
<tr>
<td>100nM brACh</td>
<td>Nicotine</td>
<td>5.7µM (3.7-8.9) 102.7 ± 4.1 (n=4)</td>
<td></td>
</tr>
<tr>
<td>1µM brACh</td>
<td>Nicotine</td>
<td>13.3µM (8.0-22.2)* 56.4 ± 6.3* (n=5)</td>
<td></td>
</tr>
<tr>
<td>10µM brACh</td>
<td>Nicotine</td>
<td>28.3µM (8.5-93.7)* 30.5 ± 6.7* (n=3)</td>
<td></td>
</tr>
<tr>
<td>--</td>
<td>Epibatidine</td>
<td>8.6nM (5.5-13.5) 114.7 ± 3.5 (n=4)</td>
<td></td>
</tr>
<tr>
<td>100nM brACh</td>
<td>Epibatidine</td>
<td>14.2nM (3.6-55.3) 103.3 ± 1.4 (n=3)</td>
<td></td>
</tr>
<tr>
<td>1µM brACh</td>
<td>Epibatidine</td>
<td>35.2nM (10.3-120.4)* 74.5 ± 12.5* (n=4)</td>
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</tr>
<tr>
<td>10µM brACh</td>
<td>Epibatidine</td>
<td>122.2 (28.3-523.2)* 44.4 ± 5.9* (n=3)</td>
<td></td>
</tr>
<tr>
<td>--</td>
<td>Cytisine</td>
<td>31.5µM (24.4-40.6) 79.0 ± 4.1 (n=6)</td>
<td></td>
</tr>
<tr>
<td>100nM brACh</td>
<td>Cytisine</td>
<td>43.8µM (35.5-54.1) 81.3 ± 5.9 (n=4)</td>
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</tr>
<tr>
<td>1µM brACh</td>
<td>Cytisine</td>
<td>62.7µM (34.2-115.1)* 49.2 ± 9.0* (n=6)</td>
<td></td>
</tr>
<tr>
<td>10µM brACh</td>
<td>Cytisine</td>
<td>ND$^c$</td>
<td>ND$^c$</td>
</tr>
</tbody>
</table>

$^a$ Chromaffin cells were treated with the indicated concentration of brACh and stimulated with the indicated agonists.

$^b$ EC$_{50}$ values are expressed as geometric means (95% confidence intervals) and E$_{max}$ values are expressed as arithmetic means ± SE as determined by nonlinear regression of individual concentration-response curves.

$^c$ Value could not be determined from curve.

* Denotes significant differences (P<.05) from corresponding secretagogue control group.

**Table 1.** Changes in EC$_{50}$ values and E$_{max}$ values of several nAChR agonists after brACh treatment.$^a$
3.3 Identification of nAChR subpopulations using monoclonal antibodies

The studies described above demonstrate that adrenal chromaffin cells contain a receptor reserve among the population of nAChRs involved in secretion. Previous studies from our laboratory indicate that more than one subtype of nAChR is involved in secretory function and suggest that mAb35-nAChRs are the principal receptors mediating secretory responses (Gu et al. 1996). Evidence for the presence of mAb35-nAChRs is supported by the ability of mAb35 treatment to cause down-regulation of a population of adrenal nAChRs. The time course for the effects of mAb35 suggests a gradual disappearance of surface receptors (Gu et al. 1996). If true, then mAb35 treatment should produce shifts in agonists’ concentration-response curves with increasing treatment times as the antibody eliminates receptor reserves. To investigate these possibilities, we used a similar approach as the one described above for brACh. In these studies, the concentration-response relationships of nicotine, epibatidine, and cytisine at various times following treatment with maximal inhibitory concentrations (50 nM) of mAb35 (Gu et al. 1996) were determined. mAb35 treatment produces a time-dependent shift in the agonist concentration-response curves for nicotine and epibatidine (Figures 7 and 8, Table 2). After 6 hr, the EC50 value for nicotine increases by approximately 2 fold (Table 2). After 24 and 48 hrs, no further increase in the EC50 value of nicotine is seen; however, the Emax value is reduced by approximately 26% after 24 hr and by approximately 31% after 48 hr (Figure 7, Table 2). Like nicotine, a 6 hr treatment with mAb35 increases the EC50 value of epibatidine by approximately 2 fold (Figure 8, Table 2). After 24 and 48 hr, no further increases in the EC50 value for epibatidine are seen; however, the Emax value is reduced by approximately 22% after 24 hr and by 26% after 48 hr (Figure 8, Table 2). mAb35 pretreatment has no significant effect on the EC50 value of cytisine, even after 48 hr of treatment (Figure 9, Table 2). However, in as little as 6 hr, mAb35 pretreatment reduces the Emax value for cytisine by approximately 56%
After 24 and 48 hr, the $E_{\text{max}}$ value for cytisine is reduced by approximately 63% and 64% respectively (Figure 9, Table 2). No further reductions in $E_{\text{max}}$ values for the agonists are seen after 72 hr of mAb35 treatment (data not shown). These results with mAb35 parallel those observed with brACh treatment, providing evidence that mAb35 causes the down-regulation and loss of a population of nAChRs.

As previously demonstrated (Gu et al. 1996), mAb35 treatment does not totally eliminate secretory responses to nicotine, suggesting an additional population of nAChRs (non-mAb35-nAChRs). Evidence exists that nAChRs with different subunit composition, show different sensitivities to pharmacological agents (Luetje and Patrick 1991; Harvey and Luetje 1996). In the next series of experiments, sensitivities of residual nAChRs (i.e., nAChRs remaining after mAb35 treatment) to several nAChR antagonists were investigated. As demonstrated in Table 3, residual nAChR-stimulated release (i.e., release remaining after 24 hr mAb35 treatment) remains sensitive to a variety of nAChR antagonists. Using ~ IC$_{60}$ concentrations, hexamethonium (15 µM) appears to be more effective at inhibiting residual release while there was no significant change in the effectiveness of either d-tubocurarine (2 µM) or pentolinium (0.5 µM).

Besides not completely inhibiting agonist-induced catecholamine release, mAb35 only caused a 2 fold increase in the EC$_{50}$ values for nicotine and epibatidine (Table 2). This is somewhat smaller than the 4 fold increase in the EC$_{50}$ values for nicotine and epibatidine seen after 1 µM brACh treatment (Table 1). This raises the possibility that there is a receptor reserve of mAb35 insensitive nAChRs. In this case, these receptors would achieve a smaller maximal response than the mAb35 sensitive nAChRs. To address this possibility the effects of increasing concentrations of brACh on the nicotine concentration-response profile were determined for mAb35 insensitive nAChRs.
Treatment with increasing concentrations of brACh caused no significant change in the EC$_{50}$ of nicotine for mAb35 insensitive nAChRs. There was a significant decrease in the E$_{\text{max}}$ for nicotine on mAb35 insensitive nAChRs following treatment with concentrations of brACh as low as 0.1 µM (Figure 10). This is consistent for a system that does not contain a receptor reserve.
Figure 7. The concentration-response effects of nicotine after mAb35-induced nAChR modulation. Cultured adrenal chromaffin cells were either not treated (closed squares) or treated for 6 hr (upright triangles), 24 hr (inverted triangles), or 48 hr (closed diamonds) with 50 nM mAb35. Cells were washed and then stimulated for 5 min with the indicated concentrations of nicotine, and catecholamine release during this stimulation period was determined. Results are expressed as a percentage of net stimulated control response. Control stimulated values (nontreated, 10 µM nicotine) were 13.6 ± 0.5% of total catecholamine content. Basal (nonstimulated) values were 0.9 ± 0.1% of total catecholamine content. Values represent means ± SE (n = 3 - 5).
Figure 8. The concentration-response effects of epibatidine after mAb35-induced nAChR modulation. Cultured adrenal chromaffin cells were either not treated (closed squares) or treated for 6 hr (upright triangles), 24 hr (inverted triangles), or 48 hr (closed diamonds) with 50 nM mAb35. Cells were washed and then stimulated for 5 min with the indicated concentrations of epibatidine, and catecholamine release during this stimulation period was determined. Results are expressed as a percentage of net stimulated control response. Control stimulated values (nontreated, 10 µM nicotine) were 16.9 ± 0.5% of total catecholamine content. Basal (nonstimulated) values were 1.8 ± 0.3% of total catecholamine content. Values represent means ± SE (n = 3 - 5).
Figure 9. The concentration-response effects of cytisine after mAb35-induced nAChR modulation. Cultured adrenal chromaffin cells were either not treated (closed squares) or treated for 6 hr (upright triangles), 24 hr (inverted triangles), or 48 hr (closed diamonds) with 50 nM mAb35. Cells were washed and then stimulated for 5 min with the indicated concentrations of cytisine, and catecholamine release during this stimulation period was determined. Results are expressed as a percentage of net stimulated control response. Control stimulated values (nontreated, 10 µM nicotine) were 13.5 ± 0.6% of total catecholamine content. Basal (nonstimulated) values were 1.1 ± 0.1% of total catecholamine content. Values represent means ± SE (n = 3 - 5).
<table>
<thead>
<tr>
<th>Treatment</th>
<th>Agonist</th>
<th>EC&lt;sub&gt;50&lt;/sub&gt; Values&lt;sup&gt;b&lt;/sup&gt;</th>
<th>E&lt;sub&gt;max&lt;/sub&gt; Values&lt;sup&gt;b&lt;/sup&gt; (% control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>--</td>
<td>Nicotine</td>
<td>4.2µM (3.8-4.6)</td>
<td>109.2 ± 3.2 (n=4)</td>
</tr>
<tr>
<td>50nM mAb35 6hr</td>
<td>Nicotine</td>
<td>8.3µM (6.7-9.9)*</td>
<td>96.6 ± 6.7 (n=4)</td>
</tr>
<tr>
<td>50nM mAb35 24hr</td>
<td>Nicotine</td>
<td>7.6µM (5.9-9.8)*</td>
<td>80.3 ± 3.2* (n=4)</td>
</tr>
<tr>
<td>50nM mAb35 48hr</td>
<td>Nicotine</td>
<td>7.3µM (4.8-11.2)*</td>
<td>75.4 ± 3.0* (n=4)</td>
</tr>
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<td>--</td>
<td>Epibatidine</td>
<td>8.3nM (3.5-19.6)</td>
<td>108.8 ± 1.7 (n=3)</td>
</tr>
<tr>
<td>50nM mAb35 6hr</td>
<td>Epibatidine</td>
<td>18.8nM (14.3-24.7)*</td>
<td>99.1 ± 2.1 (n=3)</td>
</tr>
<tr>
<td>50nM mAb35 24hr</td>
<td>Epibatidine</td>
<td>16.0nM (11.3-22.7)*</td>
<td>85.3 ± 4.8* (n=3)</td>
</tr>
<tr>
<td>50nM mAb35 48hr</td>
<td>Epibatidine</td>
<td>15.3nM (10.4-22.4)*</td>
<td>80.3 ± 4.4* (n=3)</td>
</tr>
<tr>
<td>--</td>
<td>Cytisine</td>
<td>56.0µM (22.3-140.8)</td>
<td>92.0 ± 7.0 (n=5)</td>
</tr>
<tr>
<td>50nM mAb35 6hr</td>
<td>Cytisine</td>
<td>74.5µM (60.4-91.8)</td>
<td>40.3 ± 7.0* (n=5)</td>
</tr>
<tr>
<td>50nM mAb35 24hr</td>
<td>Cytisine</td>
<td>39.3µM (26.2-58.8)</td>
<td>34.2 ± 9.7* (n=5)</td>
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<tr>
<td>50nM mAb35 48hr</td>
<td>Cytisine</td>
<td>47.1µM (37.7-58.7)</td>
<td>33.1 ± 7.1* (n=5)</td>
</tr>
</tbody>
</table>

<sup>a</sup> Chromaffin cells were treated with 50 nM mAb35 for the indicated times prior to stimulation with the indicated agonists.

<sup>b</sup> EC<sub>50</sub> values are expressed as geometric means (95% confidence intervals) and E<sub>max</sub> values are expressed as arithmetic means ± SE as determined by nonlinear regression of individual concentration-response curves.

* Denotes significant differences (P<.05) from corresponding secretagogue control group.

Table 2. Changes in EC<sub>50</sub> values and E<sub>max</sub> values of several nAChR agonists after mAb35 treatment.
<table>
<thead>
<tr>
<th>Pretreatment</th>
<th>Treatment</th>
<th>Catecholamine Release</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>% total&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>DMEM</td>
<td>Control buffer (basal)</td>
<td>1.5 ± 0.4</td>
</tr>
<tr>
<td>DMEM</td>
<td>10μM nicotine</td>
<td>16.0 ± 0.8</td>
</tr>
<tr>
<td>DMEM</td>
<td>10μM nicotine + 15μM hexamethonium</td>
<td>9.9 ± 0.9</td>
</tr>
<tr>
<td>DMEM</td>
<td>10μM nicotine + 2μM d-tubocurarine</td>
<td>6.0 ± 0.9</td>
</tr>
<tr>
<td>DMEM</td>
<td>10μM nicotine + 0.5μM pentolinium</td>
<td>6.0 ± 0.3</td>
</tr>
<tr>
<td>DMEM + 50nM mAb35</td>
<td>30μM nicotine</td>
<td>11.9 ± 0.6</td>
</tr>
<tr>
<td>DMEM + 50nM mAb35</td>
<td>30μM nicotine + 15μM hexamethonium</td>
<td>5.0 ± 0.3</td>
</tr>
<tr>
<td>DMEM + 50nM mAb35</td>
<td>30μM nicotine + 2μM d-tubocurarine</td>
<td>4.2 ± 0.4</td>
</tr>
<tr>
<td>DMEM + 50nM mAb35</td>
<td>30μM nicotine + 0.5μM pentolinium</td>
<td>3.8 ± 0.4</td>
</tr>
</tbody>
</table>

<sup>a</sup> Cultured adrenal chromaffin cells were pretreated, as indicated, for 24 hr and then stimulated with concentrations of nicotine that produce maximal stimulation under the given pretreatment conditions.

<sup>b</sup> Cultured adrenal chromaffin cells were treated for 5 min in the absence or presence of nicotine and the indicated inhibitors at concentrations reported to inhibit catecholamine release by ~60%.

<sup>c</sup> Results are expressed as a percentage of the total incorporated [3H]NE that was released during a 5 min stimulation period. Values represent means ± SE (n=6-8).

<sup>d</sup> Results are expressed as a percentage of corresponding control net stimulated responses.

<sup>*</sup> Denotes significant differences (P<.05) from corresponding drug-treated group without mAb35-pretreatment.

Table 3. Sensitivity of residual adrenal nAChRs to nAChR antagonists after mAb35-induced nAChR modulation<sup>a</sup>
Figure 10. Effects of submaximal concentrations of brACh on mAb35 insensitive nAChRs. Cultured adrenal chromaffin cells were treated for 24 hrs with 50 nM mAb35. The cells then were either left untreated (circles) or alkylated with 0.1 µM brACh (squares) or 1 µM brACh (triangles). Cells were washed and then stimulated for 5 min with the indicated concentrations of nicotine and catecholamine release during this stimulation period was determined. Results are expressed as a percentage of net stimulated control response. Values represent means ± SE (n = 3 - 5).
3.4 Identification of nAChR subpopulations using receptor protection assays

When used under alkylating conditions, brACh reduces nAChR-stimulated secretion in a concentration-dependent manner. It has been shown in other receptor systems that receptor agonists or antagonists can be used to interfere with the binding of receptor alkylating agents, thus protecting receptors from irreversible inactivation (Furchgott 1954; Grider and Makhlof 1991; Oriowo et al. 1992). Since brACh presumably interacts at the agonist binding site in reduced nAChRs, it is reasonable to assume that drugs that act at the ACh binding site of nAChRs would be able to offer protection from alkylation by brACh. Receptor protection assays, using receptor subtype-specific agonists and antagonists, have been employed to elucidate the role of different subpopulations of receptors (Furchgott 1954; Grider and Makhlof 1991; Oriowo et al. 1992). This approach allows for the study of the pharmacology of protected receptor subtypes without the interfering effects of other subtypes. In the following studies, we use receptor protection assays to identify subpopulations of nAChRs on cultured adrenal chromaffin cells.

In the following studies, the ability of nAChR antagonists (pentolinium, mecamylamine, tubocurarine, hexamethonium, and decamethonium) and noncompetitive nAChR blockers (adiphenine, tetracaine, and amantadine) to provide protection from alkylation by brACh was investigated. Under alkylating conditions, brACh (30 μM) reduced nicotine-stimulated catecholamine release by 79.0% ± 4.3% (Figure 11) and 82.3% ± 2.3% (Figure 12). When present during alkylation, tubocurarine (100 μM) was able to provide significant protection; nicotine-stimulated secretion was reduced by only
26.2% ± 3.4% (Figure 11). Additionally, both tetracaine (100 µM) and amantadine (500 µM) produced a small, but significant, amount of protection (Figure 12). Mecamylamine (50 µM), pentolinium (50 µM), hexamethonium (500 µM), decamethonium (500 µM), and adiphenine (50 µM) afforded no protection from alkylation (Figures 11 and 12). The concentration-dependency of the protective effects of tubocurarine is illustrated in figure 13. Tubocurarine provided protection at concentrations ranging from 1 µM to 30 µM, with an EC50 value of 4.0 µM (2.5 µM - 6.5 µM). Maximum protective effects were achieved at 30 µM tubocurarine.

Although tubocurarine produces dramatic protection from brACh-induced alkylation, it does not provide complete (i.e., 100%) protection, even at high concentrations (Figures 11 and 13). Interestingly, the degree of protection afforded by the noncompetitive inhibitors, tetracaine and amantadine, is similar to the amount of brACh inhibition that is resistant to protection by tubocurarine (Figure 11 and 12). To address the question of whether the receptor populations protected by tubocurarine and the noncompetitive inhibitors are different, protection studies were performed by combining tubocurarine with amantadine. As seen in Figure 14, no increase in protection can be demonstrated when both agents are present as compared with tubocurarine alone, suggesting that both agents interact with similar nAChR populations. Similar results were obtained when tetracaine was combined with tubocurarine (data not shown).

Since competition for the site of alkylation is potentially important in achieving receptor protection, high concentrations of brACh may reduce the amount of protection
that is observed. In the next series of experiments, intermediate (1 µM) and low (0.5 µM) concentrations of brACh were used for alkylation, and the effects of nAChR inhibitors were tested for the ability to protect against alkylation. Intermediate and low concentrations of brACh reduced 10 µM nicotine-stimulated catecholamine release by 50.1% ± 2.6%, and 30.0% ± 5.5%, respectively (Table 4). Of the inhibitors tested, only tubocurarine (100 µM) was able to significantly protect against alkylation using intermediate concentrations of brACh (nicotine-stimulated secretion was reduced by 28.5% ± 6.4%). No inhibitor tested was able to significantly protect against alkylation using the lowest concentration of brACh (Table 4).

The next series of experiments were designed to address whether tubocurarine-protected receptors represent a subpopulation of adrenal nAChRs by investigating the pharmacological profile of tubocurarine-protected receptors. The nicotine concentration-response profiles for untreated and tubocurarine-protected nAChRs are found in Figure 15 and Table 5. When compared to untreated cells, the nicotine concentration-response curve for tubocurarine-protected cells was shifted to the right, suggesting a decrease in the affinity of nicotine for tubocurarine-protected receptors. The EC50 for nicotine increased significantly from 4.0 µM (2.5 µM - 6.2 µM) in untreated cells to 9.1 µM (7.2 µM - 11.4 µM) in tubocurarine-protected cells (Figure 15, Table 5). There was no significant change in the Emax for untreated (20.7% ± 1.9%) and tubocurarine-protected (20.8% ± 1.8%) cells (Figure 15, Table 5). To further investigate whether tubocurarine-protected nAChRs represent a subpopulation of nAChRs with a higher affinity for tubocurarine, we next determined the concentration-dependency of tubocurarine on
nAChR-stimulated release from untreated cells and tubocurarine-protected cells. When compared to untreated cells, the tubocurarine concentration-response curve in tubocurarine-protected cells shifted to the left, suggesting an increase in the affinity of tubocurarine for tubocurarine-protected receptors (Figure 16). The IC₅₀ for tubocurarine decreased significantly from 660 nM (490 nM - 890 nM) in untreated cells to 310 nM (220 nM - 430 nM) in tubocurarine-protected cells (Figure 16, Table 5).
Figure 11. Ability of nAChR inhibitors to protect nAChRs from alkylation using 30 µM brACh. Cultured adrenal chromaffin cells were alkylated (30 µM brACh) in the presence of 50 µM mecamylamine (MEC), 50 µM pentolinium (PEN), 100 µM tubocurarine (dTC), 500 µM hexamethonium (HEX), or 500 µM decamethonium (DEC), as indicated. The cells were then stimulated for 5 min with 10 µM nicotine. Catecholamine release during this period was determined. Results are expressed as a percentage of the total catecholamine content (% total). Values represent means ± SE and were analyzed by Dunnett’s multiple comparison procedure (n = 5). Results significantly different from unprotected, 30 µM brACh treated values are indicated by asterisks (p < 0.05). Basal release (unstimulated; dotted line) was 0.9% ± 0.1%.
Figure 12. Ability of noncompetitive nAChR inhibitors to protect nAChRs from alkylation using 30 µM brACh. Cultured adrenal chromaffin cells were alkylated (30 µM brACh) in the presence of 50 µM adiphenine (ADI), 100 µM tetracaine (TET), or 500 µM amantadine (AMA), as indicated. The cells were then stimulated for 5 min with 10 µM nicotine. Catecholamine release during this period was determined. Results are expressed as a percentage of the total catecholamine content (% total). Values represent means ± SE and were analyzed by Dunnett’s multiple comparison procedure (n = 5 - 6). Results significantly different from unprotected, 30 µM brACh treated values are indicated by asterisks (p < 0.05). Basal release (unstimulated; dotted line) was 1.3% ± 0.3%. 
Figure 13. The concentration-dependency of the protective effects of tubocurarine. Cultured adrenal chromaffin cells were alkylated (30 µM brACh) in the presence of the indicated concentrations of tubocurarine. The cells were then stimulated for 5 min with 10 µM nicotine. Results are expressed as a percentage of the total catecholamine content (% total). Values represent means ± SE (n = 6). Basal release (unstimulated; dotted line) was 0.9% ± 0.03%. Control, nonalkylated, 10µM nicotine-stimulated release (dashed-dotted line) was 21.6% ± 2.7%. Alkylation (30 µM brACh) in the absence of tubocurarine (dashed line) reduced 10 µM nicotine stimulated release to 3.7% ± 0.4%.
Figure 14. Ability of tubocurarine plus amantadine to protect nAChRs from alkylation by brACh. Cultured adrenal chromaffin cells were alkylated (30 µM brACh) in the presence of 100 µM tubocurarine (dTC), 500 µM amantadine (AMA), or both 100 µM tubocurarine and 500 µM amantadine (dTC + AMA), as indicated. The cells were then stimulated for 5 min with 10 µM nicotine. Catecholamine release during this period was determined. Results are expressed as a percentage of the total catecholamine content (% total). Values represent means ± SE (n = 6) and were analyzed by Dunnett’s multiple comparison procedure. Results significantly different from unprotected, 30µM brACh treated values are indicated by asterisks (p < 0.05). Basal release (unstimulated; dotted line) was 1.5% ± 0.2%.
Cultured adrenal chromaffin cells were alkylated using either 1µM or 0.5µM brACh in the presence of the indicated drugs. The cells were then stimulated for 5 min with 10 µM nicotine. Results are expressed as a percentage of the total catecholamine content (% total). Values represent means ± SE (n = 4).

1 Cultured adrenal chromaffin cells were alkylated using either 1µM or 0.5µM brACh in the presence of the indicated drugs. The cells were then stimulated for 5 min with 10 µM nicotine. Results are expressed as a percentage of the total catecholamine content (% total). Values represent means ± SE (n = 4).

2NA, not applicable.

*Significant difference from corresponding unprotected, alkylated group (p < 0.05; Dunnett’s test).

** Significant difference from corresponding unprotected, nonalkylated, nicotine-stimulated control group (p < 0.05; Student’s t-test).

Table 4. Ability of nAChR inhibitors to protect nAChRs from alkylation: studies using intermediate and low concentrations of brACh for alkylation1.
Figure 15. Nicotine concentration-response profile of tubocurarine-protected nAChRs. Cultured adrenal chromaffin cells were either not treated (closed square), alkylated (30 µM brACh) in the presence of 100 µM tubocurarine (upright triangles), or alkylated (30 µM brACh) in the absence of tubocurarine (inverted triangles). Cells were then stimulated for 5 min with the indicated concentrations of nicotine. Catecholamine release during this period was determined. Results are expressed as a percentage of net stimulated control response (% control). Control stimulated values (nontreated, 10 µM nicotine) were 21.8 ± 1.1% of total catecholamine content. Basal (nonstimulated) values were 1.3 ± 0.1% of total catecholamine content. Values represent means ± SE (N = 3 - 5).
Figure 16. Tubocurarine concentration-response profile of tubocurarine-protected nAChRs. Cultured adrenal chromaffin cells were divided into two groups. The first group was alkylated (30 µM brACh) in the presence of tubocurarine (100 µM; square) and the second group was not (circle). Following a 15 min pretreatment with the indicated concentrations of tubocurarine, both groups of cells were stimulated for 5 min with 10 µM nicotine in the continued presence of tubocurarine. Catecholamine release during this period was determined. Results are expressed as a percentage of the total catecholamine content (% total). Control, nonalkylated, 10 µM nicotine-stimulated release (dotted line) was 20.5% ± 1.7%. Alkylation (30 µM brACh) in the presence of tubocurarine (100 µM) reduced the 10 µM nicotine-stimulated control value (dashed line) to 14.4% ± 1.8%. Values represent means ± SE (n = 4).
### Protection Drug EC<sub>50</sub> or IC<sub>50</sub> E<sub>max</sub>

<table>
<thead>
<tr>
<th>Protection</th>
<th>Drug</th>
<th>EC&lt;sub&gt;50&lt;/sub&gt; or IC&lt;sub&gt;50&lt;/sub&gt;</th>
<th>E&lt;sub&gt;max&lt;/sub&gt;</th>
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<tr>
<td>No</td>
<td>nicotine&lt;sup&gt;1&lt;/sup&gt;</td>
<td>4.0 µM (2.5 – 6.2)</td>
<td>20.7 ± 1.9</td>
</tr>
<tr>
<td>Yes</td>
<td>nicotine&lt;sup&gt;1&lt;/sup&gt;</td>
<td>9.1 µM (7.2 – 11.4)*</td>
<td>20.8 ± 1.8</td>
</tr>
<tr>
<td>No</td>
<td>Tubocurarine&lt;sup&gt;2&lt;/sup&gt;</td>
<td>660 nM (490 – 890)</td>
<td>NA&lt;sup&gt;3&lt;/sup&gt;</td>
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<tr>
<td>Yes</td>
<td>Tubocurarine&lt;sup&gt;2&lt;/sup&gt;</td>
<td>310 nM (220 – 430)*</td>
<td>NA&lt;sup&gt;3&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>1</sup> EC<sub>50</sub> and E<sub>max</sub> values interpolated from the curves in Figure 15
<sup>2</sup> IC<sub>50</sub> values interpolated from the curves in Figure 16
<sup>3</sup> NA not applicable

* Significant difference from the corresponding unprotected control groups (p < 0.05, Student’s t-test)

**Table 5. Pharmacology of tubocurarine-protected nAChRs**
3.6 Effects of disulfide bond reduction on nAChR pharmacology

As described above, no agonist or antagonist, with the exception of tubocurarine, tested was able to produce a significant amount of protection from brACh-induced loss of secretory function. There are several possibilities that may explain these results. One possibility is that the agonist binding site of the receptor has been altered by the alkylation process such that the site has altered affinities for agonists and/or antagonists. Of primary importance would be changes that result in an increased affinity for brACh and/or a decreased affinity for other nicotinic agents. To examine this hypothesis, we initially screened a variety of nAChR antagonists and agonists for changes in apparent affinity following DTT treatment. Therefore, we ran the same tests using IC_{60} concentrations of each antagonist. Following DTT pretreatment, the inhibitors appeared to have lost some affinity. Although the loss is small (~15 – 20% control), it is significant for mecamylamine and hexamethonium despite very small n values (Table 6).

To further explore changes in affinity of the nicotine binding site, full concentration-response profiles were determined for nicotine, acetylcholine, epibatidine, and cytisine. After treatment with 1 mM DTT there is approximately a 3.4 fold shift to the right in the concentration-response curve for epibatidine (Figure 17, Table 7). Similarly, after 1 mM DTT treatment, the concentration-response curve for acetylcholine shifts to the right by approximately 1.7 fold (Figure 18, Table 7). DTT treatment had no significant effect on the EC_{50} values for either nicotine or cytisine (Figures 19 and 20, Table 7). Similarly, DTT treatment had no significant effect on the E_{max} values for epibatidine, acetylcholine, nicotine or cytisine (Figures 17 - 20, Table 7). A second explanation for the apparent lack of protective effects would be nAChR alterations that increase the affinity of the receptor for brACh. To further explore this possibility, we determined the full concentration-response profile for brACh both with and without 1
mM DTT. Contrary to the results using nAChR agonists, DTT pretreatment shifted the concentration-response profile of brACh to the left by greater than 10 fold and increased the $E_{\text{max}}$ of brACh by approximately 44% (Figure 21, Table 7).

In other systems, the experimental protocols measured endpoints associated with the nAChR (i.e. binding or Na$^+$ uptake). In our system, the measured endpoint ([3H]-NE release) is downstream from nAChR activation at the extreme end of the stimulus-secretion-coupling pathway. It is conceivable that DTT alters disulfide bonds of additional proteins within the stimulus-secretion-coupling pathway and that these alterations serve to enhance the secretory response and mask reductions in nAChR function. To address this possibility, the effects of DTT treatment on KCl-stimulated catecholamine release was determined. Our results show that DTT pretreatment increases the $E_{\text{max}}$ of KCl-stimulated catecholamine release by approximately 26% but has no significant effect on the EC$_{50}$ (Figure 22, Table 7).
1 Adrenal chromaffin cells where either pretreated with PSS or 1 mM DTT for 15 min prior to incubation with the indicated inhibitor and stimulation with 10 µM brACh in the continued presence of the inhibitor.

2 Results are expressed as net control stimulated release where the control was untreated, 10µM brACh stimulated release.

* significant difference from appropriate PSS pretreated group (p< 0.05; Student’s t-test)

<table>
<thead>
<tr>
<th>Pretreatment¹</th>
<th>Inhibitor</th>
<th>Catecholamine release (% 10 µM brACh)²</th>
</tr>
</thead>
<tbody>
<tr>
<td>PSS</td>
<td>0.8 µM d-tubocurarine</td>
<td>37.4 ± 2.4 (n = 2)</td>
</tr>
<tr>
<td>1 mM DTT</td>
<td>0.8 µM d-tubocurarine</td>
<td>49.2 ± 4.4 (n = 2)</td>
</tr>
<tr>
<td>PSS</td>
<td>0.2 µM mecamylamine</td>
<td>43.8 ± 1.5 (n = 2)</td>
</tr>
<tr>
<td>1 mM DTT</td>
<td>0.2 µM mecamylamine</td>
<td>59.1 ± 0.7* (n = 2)</td>
</tr>
<tr>
<td>PSS</td>
<td>20 µM hexamethonium</td>
<td>45.1 ± 0.6 (n = 2)</td>
</tr>
<tr>
<td>1 mM DTT</td>
<td>20 µM hexamethonium</td>
<td>65.1 ± 3.5* (n = 2)</td>
</tr>
</tbody>
</table>

Table 6. Effects of DTT pretreatment on the affinity of nAChR inhibitors
Figure 17. Effects of DTT on the concentration-response profile of epibatidine. Cultured adrenal chromaffin cells were either not treated (closed squares) or treated for 15 min with 1mM DTT (open squares). Cells were washed and then stimulated for 5 min with the indicated concentrations of epibatidine, and catecholamine release during this stimulation period was determined. Results are expressed as a percentage of total catecholamine content (% total). Values represent means ± SE (n = 4).
Figure 18. Effects of DTT on the concentration-response profile of ACh. Cultured adrenal chromaffin cells were either not treated (closed squares) or treated for 15 min with 1mM DTT (open squares). Cells were washed and then stimulated for 5 min with the indicated concentrations of ACh, and catecholamine release during this stimulation period was determined. Results are expressed as a percentage of total catecholamine content (% total). Values represent means ± SE (n = 5).
Figure 19. Effects of DTT on the concentration-response profile of nicotine.
Cultured adrenal chromaffin cells were either not treated (closed squares) or treated for
15 min with 1mM DTT (open squares). Cells were washed and then stimulated for 5 min
with the indicated concentrations of nicotine, and catecholamine release during this
stimulation period was determined. Results are expressed as a percentage of total
catecholamine content (% total). Values represent means ± SE (n = 5).
Figure 20. Effects of DTT on the concentration-response profile of cytisine. Cultured adrenal chromaffin cells were either not treated (closed squares) or treated for 15 min with 1mM DTT (open squares). Cells were washed and then stimulated for 5 min with the indicated concentrations of cytisine, and catecholamine release during this stimulation period was determined. Results are expressed as a percentage of total catecholamine content (% total). Values represent means ± SE (n = 5).
Figure 21. Effects of DTT on the concentration-response profile of brACh. Cultured adrenal chromaffin cells were either not treated (closed squares) or treated for 15 min with 1 mM DTT (open squares). Cells were washed and then stimulated for 5 min with the indicated concentrations of brACh, and catecholamine release during this stimulation period was determined. Results are expressed as a percentage of total catecholamine content (% total). Values represent means ± SE (n = 3 - 6).
**Figure 22. Effects of DTT on KCl-stimulated secretory response.** Cultured adrenal chromaffin cells were either not treated (closed squares) or treated for 15 min with 1mM DTT (open squares). Cells were washed and then stimulated for 5 min with the indicated concentrations of KCl, and catecholamine release during this stimulation period was determined. Results are expressed as a percentage of total catecholamine release (% total). Values represent means ± SE (n = 6).
<table>
<thead>
<tr>
<th>Pretreatment</th>
<th>Agonist</th>
<th>$\text{EC}_{50}^{1}$</th>
<th>$\text{E}_{\text{max}}$ (% total)$^{1}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>Epibatidine</td>
<td>5.0nM (3.2 - 7.8)</td>
<td>21.1 ± 1.0</td>
</tr>
<tr>
<td>1 mM DTT</td>
<td>Epibatidine</td>
<td>17.0nM (10.1-27.5)*</td>
<td>23.0 ± 1.0</td>
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<tr>
<td>None</td>
<td>ACh</td>
<td>11.3µM (8.0 - 15.9)</td>
<td>20.8 ± 0.5</td>
</tr>
<tr>
<td>1 mM DTT</td>
<td>ACh</td>
<td>18.7µM (14.2 - 24.9)*</td>
<td>22.9 ± 1.0</td>
</tr>
<tr>
<td>None</td>
<td>Nicotine</td>
<td>4.6µM (3.4 - 6.1)</td>
<td>20.7 ± 0.9</td>
</tr>
<tr>
<td>1 mM DTT</td>
<td>Nicotine</td>
<td>5.7µM (4.0 - 8.0)</td>
<td>22.5 ± 1.8</td>
</tr>
<tr>
<td>None</td>
<td>Cytisine</td>
<td>43.5µM (34.6 - 47.5)</td>
<td>16.5 ± 2.3</td>
</tr>
<tr>
<td>1 mM DTT</td>
<td>Cytisine</td>
<td>67.1µM (47.5 - 95.1)</td>
<td>15.4 ± 3.0</td>
</tr>
<tr>
<td>None</td>
<td>BrACh</td>
<td>5.0µM (3.5 - 7.2)</td>
<td>18.3 ± 2.7</td>
</tr>
<tr>
<td>1 mM DTT</td>
<td>BrACh</td>
<td>126nM (76 - 210)*</td>
<td>26.4 ± 1.1*</td>
</tr>
<tr>
<td>None</td>
<td>KCl</td>
<td>24mM (19 - 30)</td>
<td>10.4 ± 0.7</td>
</tr>
<tr>
<td>1 mM DTT</td>
<td>KCl</td>
<td>21mM (17 - 24)</td>
<td>13.1 ± 0.8*</td>
</tr>
</tbody>
</table>

$^{1}$EC$_{50}$ and E$_{\text{max}}$ values interpolated from the curves in figures 17 - 22.

* Significant difference from corresponding unprotected, control groups (p < 0.05, Student’s t-test).

Table 7. EC$_{50}$ and E$_{\text{max}}$ values for agonists after treatment with DTT.
3.6 Biochemical regulation of nAChR turnover

Little is known about adrenal nAChR regulation. Previous studies have used the monoclonal antibody, mAb35, to modulate nAChRs and the associated secretory response. In these studies we have used irreversible alkylation by brACh to reduce nAChR function. mAb35 causes a slowly developing (~ 24 hr) loss of about 50% of nAChR mediated secretory function. In contrast, under alkylating conditions, brACh causes a rapid loss of > 90% of nAChR-mediated secretory response. While the time course and extent of each effect is different, secretory function lost to both mAb35 induced down-regulation and alkylation by brACh recovers at a similar rate (~ 2% per hour). These results suggest that similar processes underlie recovery from both brACh and mAb35 treatment. In these studies, the roles of protein synthesis, glycosylation, and protein phosphorylation in recovery from brACh and mAb35 induced loss of nAChR-mediated secretory responses were investigated.

In the first set of studies, brACh treatment was used in reducing conditions to alkylate existing nAChRs. The cells were then allowed to recover from alkylation for 24 hr in the presence of drugs that inhibit various biochemical processes likely to be important in the turnover of nAChRs. Initially the roles of protein synthesis and glycosylation in recovery from brACh induced down-regulation were investigated. When used under reducing conditions, 100 µM brACh rapidly reduced nAChR-stimulated adrenal catecholamine release to approximately 5 – 20% of control values (untreated, 10µM nicotine-stimulated). Within 24 hr after brACh treatment, nAChR-stimulated secretion returned to approximately 55-94% of control values. When the cells were incubated in the presence of the protein synthesis inhibitors, cyclohexamide (5 µg/ml) or puromycin (10 µg/ml) for 24 hr following a 100 µM brACh treatment, nAChR-stimulated secretion recovers to only 26.2 ± 8.1% and 22.8 ± 3.9% of
control, respectively (Figure 23). These values are significantly less than untreated recovery from alkylation (93.3 ± 5.6% and 94.6 ± 6.7% of control, respectively; Figure 23). Additionally, these results are significantly different from the effects of a 24 hr pretreatment with cyclohexamide or puromycin on untreated, nAChR-stimulated secretion (64.4 ± 7.2% and 102.9 ± 11.3% of control, respectively; Figure 23). These results implicate the involvement of protein synthesis in the recovery from brACh treatment. In a similar manner, when the cells are incubated in the presence of tunicamycin (1 µg/ml), a glycosylase inhibitor, for 24 hr following a 100 µM brACh treatment, nAChR-stimulated secretion recovers to only 36.1 ± 4.3% of control (Figure 23). This value is significantly less than untreated recovery from alkylation (55.6 ± 2.3% of control; Figure 23). Additionally, this result is significantly different from the effects of a 24 hr pretreatment with tunicamycin on untreated, nAChR-stimulated secretion (91.7 ± 3.4% of control; Figure 23). This finding implicates the involvement of glycosylation in the recovery from brACh treatment.

Phosphorylation events have been shown to alter the characteristics of nAChRs. In order to test the importance of protein phosphorylation on turnover of bovine adrenal nAChRs, several protein kinase inhibitors were tested for their ability to affect recovery from 100 µM brACh treatment. When present during the recovery period (24 hr) after 100 µM brACh treatment, the nonspecific protein kinase inhibitor, K252a (1.0 µM), and the PKC specific inhibitor, chelerythrine chloride (5 µM), reduced recovery of the functional response to 28.1 ± 3.3% and 76.0 ± 17.0% of control, respectively (Figure 24). Normal (untreated, 24 hr) recovery of functional response was 109.4 ± 5.1 and 107.4 ± 5.3% of control, respectively (Figure 24). K252a (1.0 µM) but not chelerythrine chloride had a small but significant effect on control nicotine stimulated release (Figure 24). The nonspecific protein kinase inhibitors, staurosporine (1 nM) and H-7 (50 µM), as well as
the PKA specific protein kinase inhibitor, H-8 (50µM), were also tested for their effects on recovery from 100 µM brACH treatment but their results were not significantly different from untreated 24 hr recovery (Figure 25). All of the protein kinase inhibitors tested thus far are known to inhibit protein serine/threonine kinases. However, protein tyrosine phosphorylation has been shown to affect muscle nAChR clustering and also has been shown to decrease nAChRs in chick ciliary ganglia. To test the role of protein tyrosine phosphorylation on nAChR turnover, the effects of the protein tyrosine kinase inhibitors, herbimycin and lavendustin, on recovery from 100 µM brACH treatment were determined. Neither herbimycin nor lavendustin had any significant effect on recovery from 100 µM brACH treatment (Figure 26). These results implicate protein kinase involvement in the recovery from brACH treatment.

As stated above, the monoclonal antibody mAb35 causes a slowly developing loss of nAChR mediated secretory function in adrenal chromaffin cells (Gu et al. 1996). The mAb35 induced loss of secretory function slowly recovers at a rate similar to recovery from alkylation by brACH. This implies that recovery from mAb35 induced loss of secretory function and brACH induced loss of secretory function proceed through similar mechanisms. In order to test this hypothesis, adrenal chromaffin cells were treated for 24hrs with 50 nM mAb35. A 24 hr incubation of with mAb35 causes approximately a 42 – 62% loss of control nicotine stimulated catecholamine release (Figures 27 and 28). The cells were then allowed to recover for 24hrs in the absence of the antibody. During the recovery period the cells recovered to approximately 72 – 90% of control nicotine stimulated release (Figures 27 and 28). When present during the recovery period, the protein synthesis inhibitors cyclohexamide (1µg/ml) and puromycin (10µg/ml) reduced recovery from mAb35 down-regulation to 27.1 ± 2.4 and 43.0 ± 3.5% of control nicotine stimulated release (Figure 27). Similar to the results seen with
recovery from brACh treatment, a 24hr treatment with 1 µM cyclohexamide alone but not puromycin alone caused a significant loss of nicotine stimulated catecholamine release (Figure 27). Unlike recovery from alkylation, tunicamycin had no significant effect on recovery from mAb35 induced down-regulation of nicotine stimulated release (Figure 27). The protein kinase inhibitors, K252a (1µM) but not chelerythrine chloride (5µM) significantly reduced recovery from mAb35 down-regulation to 43.4 ± 1.8% of control nicotine stimulated release (Figure 28). Overall these results demonstrate that protein synthesis and protein phosphorylation but not glycosylation are important in recovery from mAb35 induced down-regulation of nicotine stimulated catecholamine release.
Figure 23. Effects of protein synthesis inhibitors and glycosylase inhibitors on recovery from brACH-induced loss of secretory function. Cultured chromaffin cells were treated with 100 µM brACH under alkylating conditions. Following this treatment, the cells were washed and placed in N2+ medium or N2+ media containing 5 µg/ml cyclohexamide, 10 µg/ml puromycin, or 1 µg/ml tunicamycin, as indicated. The cells were then washed in PSS for 5 min and stimulated for 5 min with 10 µM nicotine. Catecholamine release during this stimulation period was determined. Results are expressed as a percent of the net stimulated control response (% control). Untreated nonstimulated release (basal) was 2.4 ± 0.5% while untreated 10µM nicotine stimulated release was 20.2 ± 1.2% of total catecholamine content. Values represent means ± SE (n = 4 - 8).
**Figure 24. The effects of the protein kinase inhibitors, K252a and chelerythrine chloride, on recovery from brACh induced loss of secretory function.** Cultured chromaffin cells were treated with 100 μM brACh under alkylating conditions. Following this treatment, the cells were washed and placed in N2+ medium or N2+ media containing 1 μM K252a or 5 μM chelerythrine chloride, as indicated. The cells were then washed in PSS for 5 min and stimulated for 5 min with 10 μM nicotine. Catecholamine release during this stimulation period was determined. Results are expressed as a percent of the net stimulated control response. Untreated nonstimulated release (basal) was 0.8 ± 0.1 while nontreated 10μM nicotine stimulated release (control) was 10.9 ± 0.6. Values represent means ± SE (n = 8)
Figure 25. The effects of the protein kinase inhibitors, staurosporine, H-7 and H-8 on recovery from brACh induced loss of secretory function. Cultured chromaffin cells were treated with 100 µM brACh under alkylating conditions. Following this treatment, the cells were washed and placed in N2+ medium or N2+ media containing 1 nM staurosporine, 50 µM H-7, or 50 µM H-8, as indicated. The cells were then washed in PSS for 5 min and stimulated for 5 min with 10 µM nicotine. Catecholamine release during this stimulation period was determined. Results are expressed as a percent of the net stimulated control response. Untreated nonstimulated release (basal) was 1.0 ± 0.1 while nontreated 10µM nicotine stimulated release (control) was 10.9 ± 0.5% of total catecholamine content. Values represent means ± SE (n = 7 - 8)
Figure 26. The effects of protein tyrosine kinase inhibitors on recovery from brACH induced loss of secretory function. Cultured chromaffin cells were treated with 100 µM brACH under alkylating conditions. Following this treatment, the cells were washed and placed in N2+ medium or N2+ media containing herbimycin (Herb) or lavendustin (Lave), as indicated. The cells were then washed in PSS for 5 min and stimulated for 5 min with 10 µM nicotine. Catecholamine release during this stimulation period was determined. Results are expressed as a percent of the net stimulated control response (% control). Untreated nonstimulated release (basal) was 2.6 ± 0.4 while untreated nicotine stimulated release (control) was 11.7 ± 0.5. Values represent means ± SE (n = 5-7)
Figure 27. The effects of protein synthesis inhibitors and glycosylase inhibitors on recovery from mAb35-induced loss of secretory function. Cultured chromaffin cells were treated for 2 consecutive 24 hr periods in either N2+ media or N2+ media containing 50 nM mAb35, 1 µg/ml cyclohexamide (open bars), 10 µg/ml puromycin (hatched bars), and/or 1µg/ml tunicamycin (filled bars) as indicated. Following this treatment, the cells were then washed in PSS for 5 min and stimulated for 5 min with 10 µM nicotine. Catecholamine release during this stimulation period was determined. Results are expressed as a percent of the net stimulated control response. Untreated nonstimulated release (basal) was 4.1 ± 0.6 while nontreated nicotine stimulated release (control) was 19.2 ± 0.8. Values represent means ± SE (n = 5 - 6).
Figure 28. The effects of the protein kinase inhibitors, K252a and chelerythrine chloride on mAb35-induced loss of secretory function. Cultured chromaffin cells were treated for 2 consecutive 24 hr periods in either N2+ media or N2+ media containing 50 nM mAb35, 1 µM K252a (K252) and/or 5 µM chelerythrine chloride (CC) as indicated. Following this treatment, the cells were then washed in PSS for 5 min and stimulated for 5 min with 10 µM nicotine. Catecholamine release during this stimulation period was determined. Results are expressed as a percent of the net stimulated control response. Untreated nonstimulated release (basal) was 1.2 ± 0.1 while nontreated nicotine stimulated release (control) was 14.8 ± 0.5. Values represent means ± SE (n = 5).
3.7 Identification of nAChR subunits

The subunit composition of mAb35-nAChRs in chromaffin cells is not known. In the chick ciliary ganglion (autonomic, parasympathetic neurons), mAb35-nAChRs are composed of α3, α5, β4 and sometimes β2 subunits (Conroy et al. 1992; Vernallis et al. 1993; Conroy and Berg 1995). Chromaffin cell nAChRs may be of similar composition. In order to complement studies of the pharmacology, turnover, and expression of nAChRs in chromaffin cells, there is a need to clone and characterize additional nAChR subunits. We used knowledge of subunit sequences from other species to design a strategy to clone additional chromaffin nAChR subunits. Each nAChR subunit thus far identified has four putative transmembrane (TM) regions. The sequences of these regions are conserved between nAChRs. The sequence between TM3 and TM4 forms an intracellular loop region which varies in length and sequence between nAChR subunits (for review see Sargent 1993) and is much less conserved than the TM regions.

Oligonucleotide primers were designed to amplify sequences related to nAChR TM3 and TM4 regions after reverse transcription of bovine adrenal RNA. Using RT-PCR, we amplified bovine cDNA sequences with homology to nAChR sequences. When the bovine cDNAs were cloned and sequenced, two distinct classes of PCR products were identified. One was identical to a bovine α3 cDNA previously identified in bovine adrenal chromaffin cells (Criado et al. 1992). Another cDNA was sequenced and was distinct from the bovine α3 sequence. This sequence, bovine cDNA 4, was used to search Genbank and EMBL data bases for similar sequences. The sequence with the greatest similarity to bovine cDNA 4 were from the TM3 - TM4 regions of nAChR genes. The Genbank search produced sequences with the highest homology to human and rat β4 subunits (Figure 29). The bovine cDNA 4 clone was also aligned pairwise with TM3 - TM4 regions of human β4, rat β4, and rat β2 nAChR subunits. The bovine cDNA 4 sequence was identical to 81% of human β4, and 76% of rat β4, but only 56% of
rat β2 TM3 - TM4 nucleotide sequences. The bovine cDNA 4 was used to probe a
Northern blot containing cultured bovine adrenal chromaffin RNA. One major and one
minor band of about 3 kb were observed (Figure 30). This pattern is distinct from that
observed using bovine (Figure 30) or rat α3 (Gu et al. 1996) cDNAs as probes.
Figure 29. Alignment of bovine cDNA 4 with nAChR gene sequences. Single-stranded cDNA was synthesized by reverse transcription with random primers and amplified using degenerate PCR primers based upon transmembrane sequences (TM) of nAChRs. The cDNA was cloned into pCRII and sequenced (see methods). The bovine cDNA 4 was used for a BLAST (Altschul et al., 1990) search of non-redundant Genbank and EMBL sequences. The two sequences with the highest degree of similarity are both TM3 - TM4 regions from nAChRs. The bovine cDNA 4 was aligned with the TM3 - TM4 regions of the human and rat β4 nAChR subunit genes using the GeneWorks 2.1 program.
Figure 30. Northern blot analysis of bovine nAChR α3 subunit and bovine cDNA 4 RNAs. Ten micrograms of total bovine adrenal RNA from cultured cells was electrophoresed in a 1% agarose gel and transferred to Gene Screen Plus (see methods). The Northern blots were probed with the designated random primed 32P-labeled probes. Autoradiograms were developed after exposure to Kodak XAR-5 film at -70°C with a Dupont Cronex intensifying screen. 18S (1.8 kb) and 28S (4 kb) ribosomal RNAs are designated by bars.
CHAPTER 4

DISCUSSION

4.1 Adrenal chromaffin cells contain spare receptors

In these studies we have used brACh, an analog of the endogenous neurotransmitter, acetylcholine, to alkylate adrenal chromaffin nAChRs. We have demonstrated that brACh is an effective agonist on adrenal chromaffin nAChRs. However, when used under reducing conditions, brACh irreversibly binds nAChRs, thus interfering with binding to or function of nAChRs (Wolosin et al. 1980; Leprince 1983; Stitzel et al. 1988). This effect is thought to be due to an interaction of brACh with a pair of vicinal cysteines on α subunits that normally form a disulfide bridge in the native protein. Treatment of nAChRs with DTT, a reducing agent, causes these disulfide bonds to break, allowing alkylating agents to irreversibly bind to the sulfhydryl groups (Kao et al. 1984; Kao and Karlin 1986). In adrenal chromaffin cells, under reducing conditions, brACh inhibits nAChR-mediated catecholamine release (IC_{50}, ~300 nM). This loss of nAChR-mediated function is long lasting (24-48 hr) and is prevented in the presence of a protein synthesis inhibitor. Taken together, these studies support the irreversible nature of the interaction of brACh with adrenal chromaffin nAChRs.
In these studies, we have used brACh, as a tool to investigate adrenal nAChR populations. We have shown that, under alkylating conditions, brACh reduces nicotine-stimulated catecholamine secretion in bovine adrenal chromaffin cells. This effect is receptor-specific (i.e., alklylation with brACh has no affects on catecholamine release induced by depolarizing concentrations of KCl). We have also found that the effects of brACh are slowly reversible with full recovery of nAChR-mediated secretory function occurring within 24-48 hr. In the presence of the protein synthesis inhibitor, cyclohexamide, recovery of nAChR-mediated secretory function does not occur. These characteristics are consistent with the actions of brACh in a variety of muscle and neuronal nAChR preparations in which brACh covalently binds to nAChR subunits producing irreversible nAChR inactivation (Moore and Raftery 1979; Kao and Karlin 1986).

In these studies, we have documented the presence of a nAChR reserve (i.e., spare receptors) using brACh to irreversibly inactivate adrenal nAChRs. According to spare receptor theory, as an irreversible antagonist eliminates a receptor reserve, the concentration-response curve of a full agonist shifts to the right. This produces an apparent increase in the EC\textsubscript{50} value for the agonist but does not decrease its E\textsubscript{max}. When the irreversible antagonist has totally eliminated the receptor reserve, further elimination of functional receptors decreases the E\textsubscript{max} value for the agonist but has no further effect on the EC\textsubscript{50} (for review, (Ruffolo 1982). In our studies, as nAChRs are inactivated with increasing concentrations of brACh, there are progressive increases in EC\textsubscript{50} values, followed by progressive decreases in E\textsubscript{max} values for nicotine and epibatidine. These
characteristics support the presence of a population of spare nAChRs. The magnitude of our nAChR reserve is difficult to estimate accurately because knowledge of K\textsubscript{d} values is required. Theoretically, fractional receptor occupancy equals the concentration of the ligand divided by the sum of the ligand's K\textsubscript{d} plus the concentration of the ligand. However, K\textsubscript{d} values obtained from nAChR binding studies may not be an accurate reflection of the relationship between nAChR affinity and functional responses. Contrary to the expected, nicotine's K\textsubscript{d} value (nanomolar) is several orders of magnitude lower than nicotine's EC\textsubscript{50} value (micromolar) for a variety of functional responses (Lee et al. 1992); Table 1). These lower K\textsubscript{d} values are believed to represent binding to desensitized, higher affinity states of nAChRs (Sine and Taylor 1979). K\textsubscript{d} values can be calculated from functional data using Furchgott analysis (Furchgott 1954). However, this type of analysis is difficult to perform due to the relatively small changes in EC\textsubscript{50} values in our studies. For our determination of receptor occupancy, we have extrapolated apparent K\textsubscript{d} values. Theoretically, when the receptor reserve has been eliminated, the E\textsubscript{max} value should begin to decrease and the EC\textsubscript{50} value should no longer increase; at this point, the EC\textsubscript{50} value should equal the agonist's K\textsubscript{d}. From Table 1, we have estimated the K\textsubscript{d} values of nicotine and epibatidine to be 13 µM and 35 nM, respectively. Using these K\textsubscript{d} values in the receptor occupancy equation stated above, receptor occupancy at maximum functional response for nicotine (10 µM) and epibatidine (30 nM) are 43% and 46%, respectively (Figure 31). Accordingly, when considering nAChRs involved with secretion, bovine adrenal chromaffin cells possess more than two times the number nAChRs than are required for maximum secretory responses. Binding studies have shown that bovine adrenal chromaffin cells contain approximately 11,300 [\textsuperscript{3}H]-
epibatidine binding sites per cell (Free and McKay 2003). This means that only approximately 5000 binding sites per cell are needed to achieve a full functional response.

To further investigate adrenal nAChR populations involved in secretory function, we have used the nAChR antibody, mAb35. When compared with brACh treatment, mAb35 causes a more slowly developing and less complete down-regulation of nAChR-mediated secretory function (Gu et al. 1996). We show in these studies that the mAb35-induced loss of nAChR-mediated secretory function is characterized by a shift in the concentration-response curve to the right, as well as, a decrease in $E_{\text{max}}$. The time course of this effect is consistent with a scenario where the antibody slowly down-regulates nAChRs (i.e., removes the receptor from the cell surface). The resulting loss of receptors can be seen as a shift in $EC_{50}$ followed by a reduction in $E_{\text{max}}$ as the receptor reserve is eliminated. Furthermore, the maximum attainable reduction in $E_{\text{max}}$ with mAb35 in our studies is 30%. Since full secretory responses are reached with only 45% receptor occupancy, this seemingly minor mAb35-induced reduction in $E_{\text{max}}$ likely represents a greater than 55% reduction in the total population of adrenal nAChRs involved with secretion. Although these calculations assume that nAChR populations are functionally similar, these studies provide further evidence that nAChR reserves exist and that mAb35-nAChRs are the principal receptors mediating adrenal catecholamine release.

In these studies, we have also used the nAChR agonist, cytisine, to study adrenal nAChR populations involved in secretion. Cytisine has been shown to have activity on
β4-containing nAChRs and little or no activity on β2-containing nAChRs (Luetje and Patrick 1991; Papke and Heinemann 1993). We have shown that cytisine stimulates adrenal catecholamine release (EC₅₀, ~ 41 µM); however, cytisine is somewhat less efficacious than either nicotine or epibatidine (80% - 90% of the response attainable with either nicotine or epibatidine). Our data are consistent with cytisine acting as a partial agonist on adrenal nAChRs. Classically, a partial agonist can achieve full receptor occupancy but is unable to fully activate the receptor (Figure 31). This leads to a less than full functional response. In support of this, reductions in nAChR pools by either brACh or mAb35 produce progressive reductions in cytisine's Eₘₐₓ values and have little effect on cytisine's EC₅₀ values. These are characteristics associated with partial agonism. A partial agonist can not achieve a full functional response even at full receptor occupancy so the functional deletion of any receptor by an irreversible antagonist will only result in a reduction of the agonist’s Eₘₐₓ but will not affect the agonist’s EC₅₀. mAb35 treatment alters the concentration-response effects of cytisine to a much greater extent than that seen with nicotine and epibatidine. These findings are also consistent with the actions of cytisine as a partial agonist.

It is possible that cytisine may be acting as a full agonist on a subpopulation of nAChRs, the majority of which coincides with mAb35-nAChRs. Our data are unable to distinguish between these two possibilities. However in binding studies, a partial agonist is indistinguishable from a full agonist. Cytisine can fully inhibit the binding of [³H]-epibatidine in adrenal membrane preparations (Free and McKay 2003). If cytisine were a full agonist acting on a subpopulation of nAChRs then that subpopulation would
represent less than 50% of the total functional nAChRs given that cytisine can only produce a 80-90% functional response. Therefore, it would only be expected to inhibit less than 50% of [³H]-epibatidine binding. Based on these results then, it seems that cytisine is acting more like a classical partial agonist. However, this does not mean that cytisine acts equally on all populations of nAChRs. For instance cytisine could be acting as a full agonist affect on one population of nAChR but a partial agonist on another population of nAChRs.

These studies demonstrate that a receptor reserve exists for nAChR-mediated adrenal catecholamine secretion. This represents the first time receptor reserves for nAChRs involved with secretion have been documented on neuronal tissues and has important functional and pharmacological implications. Spare receptors influence concentration-response relationships of agonists and antagonists (Zhu 1991). Due to the presence of spare receptors, reductions in nAChRs may lead to alterations in the pharmacological profile of remaining nAChRs. In these studies, we also provide supporting evidence that the total population of nAChRs regulating secretory function may be heterogeneous, with mAb35-nAChRs representing no less than 55% of the total nAChR population involved with secretion. This observation may also have important functional and pharmacological implications. Finally, the presence of cytisine-sensitivity and our observation of a β4-related subunit transcript in adrenal chromaffin cells support the presence of the β4 subunit in adrenal nAChRs involved with secretion.
Figure 31. Occupancy-response curve for nAChR in bovine adrenal chromaffin cells. Data from untreated agonist concentration response curves from Figures 4-6 was transformed and replotted to generate the occupancy response curves. Receptor occupancy was calculated using the equation, % receptor occupancy = 100 * [ligand]/([ligand] + K_d). The EC_{50} for each agonist when E_{max} was less than 100% was used as an estimate for the nondesensitized K_d value as was taken from data on Table 1.
4.2 Receptor protection assays detect a subpopulation of nAChR

These studies represent the first attempt to differentiate neuronal nAChRs using receptor protection assays. The rationale for our approach is based on studies in other systems where receptor protection assays, involving selective receptor agonists and antagonists, have been used to dissect out different receptor subpopulations (Furchgott 1954; Grider and Makhlof 1991; Oriowo et al. 1992). Reports that neuronal cells possess multiple nAChRs populations (Listerud et al. 1991; Conroy et al. 1992; Vernallis et al. 1993; Alkondon and Albuquerque 1993; Conroy and Berg 1995) and that nAChR subtypes, as distinguished by subunit contribution, possess distinct pharmacological properties (Luetje et al. 1990; Luetje and Patrick 1991; Cachelin and Rust 1994; Cachelin and Rust 1995) support our approach.

Theoretically, protection from alkylation would best be achieved using a drug that interacts competitively with the agonist’s binding site. Initially, protection studies were performed using the nAChR agonists, nicotine, cytisine, acetylcholine, and epibatidine. However, due to the agonist concentrations used and the need for a brief pretreatment to establish the protective blockade prior to co-incubation with brACh, significant reduction in the nAChR-stimulated secretory response (i.e., receptor desensitization) occurred even in the absence of alkylation (data not shown). Since this desensitized secretory response is not reversible by the end of the alkylation reaction, interpretation of the protective effects of agonists is difficult. Competitive antagonists would be the next logical choice to use as the protective agent in protection studies. As antagonists, these drugs do not cause receptor desensitization and would interact with the site of alkylation. However the
classification of a nAChR antagonist as either competitive or noncompetitive is complex and depends on the species, tissue, and receptor subtype (Nooney et al. 1992; Cachelin and Rust 1994). For this reasons we screened a variety of nAChR antagonists for their ability to protect nAChR from alkylation by brACh.

In these studies, we found that most nAChR antagonists and noncompetitive nAChR blockers (i.e., hexamethonium, decamethonium, mecamylamine, pentolinium and adiphenine) were unable to protect against brACh-induced alkylation despite the use of maximum inhibitory concentrations (IC$_{100}$) of the inhibitors (McKay and Sanchez 1990; Nooney et al. 1992; McKay and Burkman 1993). One possible explanation is that the mechanism of action for these inhibitors does not involve antagonism at the site of alkylation (i.e., the acetylcholine binding site). Secretion and patch clamp studies in bovine adrenal chromaffin cells support a noncompetitive mechanism of action for hexamethonium, decamethonium, and mecamylamine (Kilpatrick et al. 1981; Nooney et al. 1992). Hexamethonium does block [$^3$H]-epibatidine binding in adrenal chomaffin cells but its $K_i$ value is $>$1mM (Free et al. 2002) which is significantly greater than its IC$_{50}$ value of 16.2µM (McKay and Burkman 1993). Since the $K_i$ value is substantially greater than the IC$_{50}$, hexamethonium likely primarily interacts with nAChRs in a noncompetitive fashion. Additionally, mecamylamine was only able to inhibit approximately 30% of [$^3$H]-epibatidine binding in adrenal chromaffin cells even at concentrations greater than the 50 µM used in these studies (Free et al. 2002). Due to their inability to protect, our studies also support a noncompetitive interaction of hexamethonium, decamethonium, and mecamylamine for adrenal nAChRs. It is likely,
then, that these noncompetitive antagonists do not protect nAChRs from alkylation because their site of action does not sufficiently interfere with or alter the site of alkylation. It is possible, though, that some noncompetitive inhibitors could potentially provide protection if allosteric effects alter the site of alkylation. This is supported by our finding that the noncompetitive nAChR blockers, tetracaine and amantadine, provide a small, but significant amount of protection from alkylation.

In our studies, tubocurarine was the only nAChR antagonist to produce dramatic protection. Tubocurarine’s EC<sub>50</sub> value of 4 µM as a protective agent is similar to the IC<sub>50</sub> values for its inhibitory effects on acetylcholine-stimulated (3 µM) (McKay and Burkman 1993) and nicotine-stimulated (0.7 µM; table 2) catecholamine release from cultured adrenal chromaffin cells. Tubocurarine’s interaction with nAChRs is complex (Nooney et al. 1992; Cachelin and Rust 1994). Tubocurarine has been reported to be either a competitive inhibitor or an ion channel blocker, depending on the species and/or the type of nAChR. Binding studies, using a membrane preparation from bovine adrenal chromaffin cells, found that tubocurarine was able to block [³H]-nicotine binding, but only at high concentrations (IC<sub>50</sub>, ~0.1 mM)(Lee et al. 1992). However, binding studies using adrenal membrane preparations show that tubocurarine was able to inhibit [³H]-epibatidine binding with a K<sub>i</sub> of .397 µM. Patch clamp studies in bovine adrenal chromaffin cells show that tubocurarine not only behaves as an antagonist, but also has weak agonist properties (Nooney et al. 1992). These findings implicate an action of tubocurarine at the agonist binding site and may explain its protective activity.
In addition to the issue of mechanism of inhibition, these results also raise questions concerning the integrity of the agonist binding site after DTT treatment. The protective effects of tubocurarine and two noncompetitive blockers may involve changes in receptor conformation induced by DTT treatment. Under alkylating conditions, where disulfide bonds are broken, the conformation of the receptor may be altered, thus affecting binding affinities and/or access to binding sites. This possibility is supported by studies in rat brain where DTT increased the $K_d$ and decreased the $B_{max}$ for nicotine (Stitzel et al. 1988).

The question remains as to whether the population of tubocurarine-protected nAChRs represents a subpopulation of adrenal nAChRs. Several observations support the existence of at least two subpopulations of adrenal nAChRs, which can be differentiated using tubocurarine. We have shown that the maximum amount of protection afforded by tubocurarine does not produce protection for all adrenal nAChRs. The selective protective property of tubocurarine is supported by studies which have shown that the effects of tubocurarine on $\beta 4$ -containing nAChRs differ dramatically when compared to $\beta 2$-containing nAChRs (Cachelin and Rust 1994). Finally, we have also shown that the apparent affinity of nicotine has decreased and that the apparent affinity of tubocurarine has increased in chromaffin cells possessing only tubocurarine-protected nAChRs, implicating a second population of nAChRs with a differing pharmacology. It cannot be ruled out, however, that the observed changes in apparent
affinities are due to the elimination of a nAChR reserve after alkylation. It is well
documented that the loss of a receptor reserve can produce changes in apparent affinities
of agonists and antagonists (Zhu 1991).

A major problem of these studies is the selectivity of the agents used for the
protection assays. The development of more selective drugs for nAChR subtypes may
provide the tools needed to dissect subtypes in receptor protection assays. With the
availability of subtype selective agents, receptor protection assays should provide a
valuable tool to study neuronal cells containing heterogeneous populations of nAChRs.

4.3 Disulfide integrity is not required for nAChR function

Neuronal nAChR α subunits contain a pair of viscinal cysteines analogous to cys
192-193 of the α1 subunit, which are considered to be an important part of the agonist
binding site. As these cysteines are side by side it is assumed that the disulfide bond
between them would be under a considerable amount of strain so that the reduction of the
disulfide bond would cause changes in the characteristics of the binding site. In many
systems, reduction with the reducing agent DTT significantly alters the characteristics of
nAChRs. Studies in the eel electroplax show that treatment with DTT prevents nicotinic
responses (Karlin and Bartels 1966). In muscle, DTT increases the EC\textsubscript{50} for nAChR
agonists but does not decrease the maximum response.

In neuronal systems, the effects of DTT are not quite as clear. In most cases the
disruption of disulfide bonds causes a loss in function or a changes in binding parameters
consistent with less effective binding. In PC12 cells, DTT caused a substantial loss of carbachol mediated $^{22}\text{Na}^+$ uptake (Leprince 1983). The effects of DTT depend not only on the tissue tested but also on the subtype of receptor. In mouse brain, DTT pretreatment caused a loss of >80% of nicotine binding. This effect could be accounted for by both changes in affinity and a loss in $B_{\text{max}}$. This likely represents reduction of the $\alpha_4$ containing receptors as the $\alpha_4\beta_2$ receptors are the major brain nAChR subtype. However the same treatment caused only a small loss in $\alpha$-bungarotoxin binding due to a modest increase in $K_d$ (Stitzel et al. 1988). This most likely represents the reduction of $\alpha_7$ containing nAChRs. In other cases DTT has no significant effect on neuronal nAChR properties. In BC3H-11 cells, DTT had no effect on the nAChR channel characteristics; however subsequent alkylation with NEM caused a drastic reduction in mean channel open time (Bouzat et al. 1991). Neither DTT treatment nor subsequent alkylation was found to interfere with the inhibitory nicotinic response seen in rat dorsolateral septal neurons (Sorenson and Gallagher 1993). It may be possible to elucidate nAChR subpopulations by their susceptibility to disulfide bond reduction caused by mild reducing agents.

In our system, we have shown that DTT has modest effects in the apparent affinities and efficacies of nicotine, cytisine, and acetylcholine and on the effects of nAChR inhibitors. This effect was not simply a lack of reduction of disulfide bonds by our DTT treatment paradigm since the concentration and time of treatment used was sufficient to alkylate receptors with brACh. Furthermore the oxidizing agent DTNB could reverse the modest effects of DTT treatment on the epibatidine concentration.
response curve (data not shown). This implies that the integrity of disulfide bonds is not critical in the maintenance of the binding site of nAChRs in bovine adrenal chromaffin cells. The major exception is brACh, where DTT pretreatment increases the apparent affinity approximately 10 fold and increases the efficacy of brACh. Presumably the increase in affinity is due to the covalent attachment of brACh to the reduced receptor. The >3 fold increase in the apparent affinity of epibatidine is also interesting and raises questions as to why one nAChR agonist would show more dramatic effects than the others. It should be noted that epibatidine is approximately 3 orders of magnitude more potent than nicotine in adrenal chromaffin cells. Its high affinity may render it more susceptible to subtle changes in the agonist binding site, thus explaining the modest effect of DTT.

Not all of the effects of DTT on adrenal secretion are related to reductions of nAChRs. DTT also increases secretory responses mediated by depolarizing concentrations of KCl, a procedure that bypasses the nAChR in stimulus secretion coupling. The non receptor mediated effects of DTT are likely due to reductions of the intracellular secretory machinery as the membrane impermeable oxidizing agent DTNB was unable to reverse the effects of DTT on high KCl stimulated secretory function. It is unclear as to what extent enhanced KCl-stimulated release may mask a loss in nAChR function or if it is sufficient to account for the increased efficacy of brACh on DTT-treated cells. This effect could prove to be a characteristic of adrenal nAChRs.
4.4 Protein synthesis, glycosylation and phosphorylation are important in nAChR turnover

In these studies we use the alkylating agent brACh to examine the biochemical processes involved in turnover. Recovery from alkylation by brACh could conceivably be accomplished by several mechanisms. The alkylating unit could be chemically removed from the receptor thereby restoring function, new receptors could be synthesized by the cell, or a supply of ready made nAChRs could be used to replace alkylated receptors. In these studies the protein synthesis inhibitors puromycin and cyclohexamide prevent the recovery nAChR mediated secretory function following alkylation by brACh. These findings support a role for the synthesis of new receptors and argue against the chemical removal of the alkylating agent. Intracellular pools of nAChRs have been detected in other cell types, and recent studies have discovered the presence of an intracellular population of nAChRs in bovine adrenal chromaffin cells. The exact composition of these receptors is unknown, but they have binding properties similar to surface nAChRs. If this pool of internal receptors is used to regulate the number of surface receptors, then replacement of alkylated receptors could conceivably occur in the presence of protein inhibition. However recovery from alkylation is nearly completely inhibited in the presence of protein synthesis inhibitors (Figure 23). This finding implicates the need for constitutive synthesis of new receptors to replace alkylated receptors on the cell surface. Another possibility is that protein synthesis inhibitors affect a high turnover protein that is critical to the synthesis and assembly of functional nAChRs on the surface of the cell. Our laboratory has also shown that protein synthesis inhibitors reduce the number of $[^3]$H-epibatidine binding sites both on the cell surface and
internally. Furthermore, protein synthesis inhibitors reduce the amount of nAChR protein. Taken together, these data indicate that nAChR turnover relies on constitutive de novo protein synthesis with little input from the intracellular pool of receptors. The relevance of the intracellular pool of receptors remains to be elucidated.

Protein glycosylation has been implicated in the folding of proteins in the ER and in stabilizing proteins on the cell’s surface. nAChRs contain putative N-linked glycosylation sites. Our studies show that a glycosylase inhibitor, tunicamycin, can inhibit recovery from alkylation by brACh. This finding implicates a role for glycosylation in the turnover of nAChRs. Glycosylation is likely important for the formation of many proteins, and some of these proteins may be part of a nAChR regulatory pathway. Therefore we can not determine if tunicamycin is exerting its effect by altering glycosylation of nAChRs or through other mechanisms. However our lab does have evidence that tunicamycin affects the glycosylation of the β4 protein (data not shown).

Our data indicate that phosphorylation events play a role in nAChR regulation since the nonspecific protein kinase inhibitor K252a significantly reduced nAChR mediated secretion following alkylation by brACh. It should be noted that a 24 hour treatment with K252a also depressed nicotine stimulated release even in cells that had not been alkylated by brACh. However the reduction in nicotine stimulated release during the recovery period was significantly greater than the reduction seen in nonalkylated cells. The exact mechanism of action of K252a was not investigated in these studies. It
is possible that K252a acts upon a portion of the stimulus secretion coupling pathway
downstream of nAChR activation. However it seems that at least a portion of the effect
of K252a is receptor related since its effect on alkylated cells is greater than its effect on
nonalkylated cells. This same rationale also argues against K252a acting directly on the
nAChR as an antagonist. Furthermore, our procedure uses several washes prior to
stimulation with agonists making it likely that the K252a would be washed out prior to
stimulation.

In addition to K252a, the PKC specific protein kinase inhibitor, chelerythrine
chloride, also reduced recovery from alkylation by brACh. The level of reduction was
not as great as K252a, but chelerythrine chloride did not significantly affect nicotine
stimulated release in nonalkylated cells. Chelerythrine has been shown to inhibit nAChR
currents in PC12 cells, but this effect does not depend on the inhibition of PKC and is
rapidly reversible (Shi and Wang 1999). PKC has been shown to affect many of the
properties of nAChRs in other systems. For instance activation of PKC promotes the
recovery from functional deactivation caused by prolonged agonist treatment (Eilers et al.
1997; Khiroug et al. 1998; Fenster et al. 1999a). The mechanisms at work in recovery
from prolonged agonist treatment are not likely to be the same as the mechanisms
involved in recovery from alkylation as the time courses of the two effects are different.
In muscle, PKC phosphorylates serine residues on the muscle δ subunit. The
phosphorylation of serine residues appears to cause the disaggregation of nAChR clusters
(Nimnual et al. 1998). Similar to muscle receptors, evidence suggests that a population
of adrenal nAChRs is attached to the cytoskeleton (Lopez and McKay 1997).
Furthermore immunocytochemical techniques using mAb35 show a punctate fluorescence, suggesting that mAb35 sensitive nAChRs are clustered (Gu et al. 1996). So similar mechanisms may be important for the regulation of adrenal nAChRs. The mechanism of action of PKC’s effect on recovery from alkylation will require additional studies.

The role of PKA in nAChR turnover was not directly tested in these studies. However in other studies, activation of PKA did not affect channel characteristics, mAb35 binding, or nAChR mediated secretion in adrenal chromaffin cells (Dubin et al. 1992). Forskolin, a PKA activator, inhibits nAChR mediated events in chromaffin cells but this effect does not appear to be related to PKA activity. The effect is rapidly reversible and also inactive analogues of forskolin cause the same effect (Gandia et al, 1997).

Protein tyrosine phosphorylation events have been shown to affect the properties of nAChRs. In muscle, the protein agrin, which is released from the enervating presynaptic fiber, causes the tyrosine phosphorylation of nAChRs through the action of protein tyrosine kinases. This promotes nAChR aggregation and attachment to the cytoskeleton beneath the forming neuromuscular junction. Since bovine adrenal chromaffin nAChRs have been shown to be attached to the cytoskeleton, similar mechanisms may be important for the regulation of adrenal nAChRs (Lopez and McKay 1997). Additionally, protein tyrosine kinases have also been implicated in the regulation of ganglionic type nAChRs. In chick ciliary ganglion cells, inhibition of protein tyrosine
kinases causes a slowly developing loss of mAb35 binding but does not affect α-bgt binding. This effect does not seem to involve increased degradation of surface receptor and preferentially affects surface receptors over intracellular receptors (Haselbeck and Berg 1996). In our studies, protein tyrosine kinase inhibitors have no significant affect either on unalkylated nAChR mediated secretion or on recovery from nAChR loss of function induced by alkylation by brACh. This suggests that protein tyrosine phosphorylation has little affect on the regulation of adrenal nAChRs even though adrenal chromaffin cells contain a ganglionic type of nAChR. However, these studies were performed at a single concentration of nicotine. The presence of spare receptors means that even a significant loss of nAChR may not yield a significant change in catecholamine release. To further address this issue, whole concentration response profiles both before and after recovery in the presence of the protein kinase inhibitors would have to be undertaken. Alternately, binding studies would be better suited to address this problem.

Recovery from antibody induced down-regulation (mAb35) appears to follow similar biochemical pathways. Like recovery from alkylation by brACh, recovery from mAb35 treatment requires constuitive protein synthesis. Also similar is the role of protein kinases in recovery from where K252a was able to inhibit functional recovery from mAb35 treatment. Despite these similarities there were differences. The glycosylase inhibitor, tunicamycin, as well as the PKC inhibitor, chelerythrine chloride,
had no significant effect on recovery from mAb35 treatment. This implies that although there are similarities in the recovery from the two forms of down-regulation of function, the two processes are not identical.

Overall these results implicate a role for constitutive protein synthesis, glycosylation, and phosphorylation (most likely mediated by PKC) as important biochemical events involved in the turnover of nAChRs in bovine adrenal chromaffin cells. Additionally, recovery from mAb35 induced downregulation also appears to follow similar but not identical biochemical pathways as recovery from alkylation by brACh. These studies represent a starting point for further studies into the biochemical regulation of nAChRs.

4.5 β4 subunits are expresses in bovine adrenal chromaffin cells

As described above, cytisine, a nAChR agonist in bovine adrenal chromaffin cells, has been shown to have activity on β4-containing nAChRs (Luetje and Patrick 1991). This suggests that β4 nAChR subunits may be present in these cells. In these studies, we present evidence that a β4 subunit is expressed in bovine adrenal chromaffin cells. Bovine cDNA 4 likely represents a segment of the bovine β4 nAChR subunit corresponding to the large cytoplasmic loop between TM3 and TM4. This clone has the highest degree of sequence similarity with analogous regions of β4 nAChR subunits from human (81%) and rat (76%). Bovine cDNA 4 has a lesser degree of sequence similarity with the analogous region of rat β2 (56%), which is comparable to the degree of sequence
identity between analogous regions of rat β4 and rat β2 nAChR subunits (54%). It is not surprising that β4 transcripts might be present in adrenal chromaffin cells since β4 subunits are found in several tissues of similar embryonic origin. In chick ciliary ganglia, the β4 subunit is associated with a synaptic-type nAChR that also contains the α3, α5 and sometimes β2 subunits (Conroy et al. 1992; Vernallis et al. 1993; Conroy and Berg 1995). In the rat PC12 cell line, α3, α5, β2 and β4 transcripts have been found (Boulter et al. 1990). Rat superior cervical ganglia also express the β4 subunit and contain nAChRs that can be activated by cytisine with an EC50 of approximately 20 µM (Mandelzys et al. 1995), which is similar to the EC50 for cytisine in our studies. While comparing pharmacological/sequence data across species can be problematic, our studies suggest that bovine adrenal chromaffin cells contain a β4 subunit in nAChRs involved in secretion. Subsequent to the proposal of this research, the α5 and β4 subunits were cloned and sequenced in bovine adrenal chromaffin cells (Campos-Caro et al. 1997; Wenger et al. 1997).


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