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CHARACTERIZATION AND REGULATION OF ADRENAL NEURONAL NICOTINIC ACETYLCOLLIN RECEPTORS

Dissertation

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

By

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ABSTRACT

A unifying factor of neuronal nAChR related disease is the change in receptor expression levels. Therefore it is of vital importance to understand how cells regulate nAChR receptor expression, so that the process can be altered. \( \alpha_3 \) Containing neuronal nAChRs have been implicated in a growing number of physiological and pathological roles. These studies characterize the bovine adrenal medulla as a model system for the study of ganglionic \( \alpha_3 \beta_4^* \) nAChR and use bovine adrenal chromaffin cells to understand mechanisms underlying the turnover and regulation of neuronal nAChRs. The primary contributions of this study are 1) the characterization, through the use of radiolabeled ligand binding assays and protein analysis, of bovine adrenal chromaffin cells as a native preparation expressing \( \alpha_3 \beta_4^* \) nAChR, 2) the identification of a substantial pool of fully assembled intracellular \( \alpha_3 \beta_4^* \) nAChRs expressed in intact adrenal chromaffin cells that may play a role in nAChR regulation and turnover, 3) the finding of unique differences in the agonist binding site of \( \alpha_3 \beta_4^* \) nAChRs versus other nAChR subtypes, namely the importance of the disulfide bonds found adjacent to the agonist binding site which are essential for activation of most nAChRs, but appear to play only a minor role in \( \alpha_3 \beta_4^* \) nAChR agonist interactions, 4) the discovery that nAChR protection assays present a novel mechanism to separate different populations of neuronal nAChRs on the same
tissue, 5) the demonstration that bovine adrenal chromaffin cells, coupled with several down-regulation paradigms, can be used to investigate mechanisms of \( \alpha_3\beta_4^* \) nAChR turnover, and 6) the finding that \( \alpha_3\beta_4^* \) nAChRs undergo constitutive receptor turnover, regulated by \textit{de novo} protein synthesis. These findings provide significant evidence that bovine adrenal chromaffin cells are a useful model for the investigation ganglionic \( \alpha_3\beta_4^* \) nAChR regulation in a native system. These data also present a mechanistic model for neuronal nAChR regulation based on constitutive nAChR turnover.
Dedicated to my entire family
for their unwavering support throughout my education
and to Grams and Sefie for the backin'.
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[^3H]Epibatidine binding to bovine adrenal medulla: Evidence for α3β4* nicotinic


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# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABSTRACT</td>
<td>ii</td>
</tr>
<tr>
<td>ACKNOWLEDGEMENTS</td>
<td>v</td>
</tr>
<tr>
<td>VITA</td>
<td>vi</td>
</tr>
<tr>
<td>LIST OF TABLES</td>
<td>xii</td>
</tr>
<tr>
<td>LIST OF FIGURES</td>
<td>xiii</td>
</tr>
<tr>
<td>STATEMENT OF THE PROBLEM</td>
<td>1</td>
</tr>
</tbody>
</table>

## CHAPTERS

1. Introduction ........................................... 5  
   1.1 Nicotinic acetylcholine receptors in health and disease  ......... 5  
   1.2 Nicotinic receptor classification and characterization .......... 10  
   1.3 Regulation of expression of neuronal nicotinic receptors ........ 14  
   1.4 \( \alpha 3^\star \) neuronal nicotinic receptors ........................ 19  
   1.5 Adrenal chromaffin cells ...................................... 21  

2. Materials and methods ................................... 24  
   2.1 Materials ............................................. 24  
   2.2 Isolation and culture of bovine adrenal chromaffin cells ........ 25  
   2.3 Adrenal medulla membrane preparation .......................... 26  
   2.4 Catecholamine secretion assays ............................... 26  
   2.5 Receptor disulfide reduction and alkylation ..................... 28  
   2.6 Receptor protection assays .................................. 29  
   2.7 \[^{3}\text{H}]\text{Epibatidine binding to an adrenal membrane preparation} 30  
   2.8 \[^{3}\text{H}]\text{Epibatidine binding to intact adrenal chromaffin cells} ... 32  

*Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.*
2.9 nAChR down-regulation paradigms ................................................. 33
2.10 Western analyses of nAChR protein ............................................... 34
2.11 Northern analyses of nAChR mRNA ............................................. 35

3. Results .................................................................................................. 37

3.1 Characterization of adrenal nAChRs

3.1.1 Radiolabeled binding techniques to characterize nAChRs of the adrenal medulla .......................................................... 37

3.1.2 Western analyses to characterize nAChRs of the adrenal medulla ........................................................................ 45

3.1.3 [³H]Epibatidine binding studies to surface and intracellular nAChRs in intact adrenal chromaffin cells .................................. 48

3.1.4 Disulfide integrity of the agonist binding site is not essential for function of adrenal α3β4* nAChRs ........................................ 59

3.1.5 Receptor protection assays to separate multiple populations of nAChRs in one tissue ......................................................... 73

3.2 α3β4* nAChR turnover and regulation

3.2.1 Catecholamine secretion assays to examine nAChR regulation .................................................................................. 90

3.2.2 nAChR mRNA remains constant throughout receptor down-regulation and recovery ..................................................... 100

3.2.3 [³H]Epibatidine binding assays to examine nAChR regulation .................................................................................. 102

3.2.4 Puromycin treatment decreases β4 nAChR protein levels .......................................................................................... 108
4. Discussion .................................................................111

4.1 Overview of findings .................................................111

4.2 Adrenal chromaffin cells express α3β4* nAChRs ..............113

4.3 Chromaffin cells express a substantial population of intracellular α3β4* nAChRs ........................................120

4.4 Adrenal chromaffin α3β4* nAChRs lack the necessity for disulfide integrity of the agonist binding site ..........124

4.5 Receptor protection assays can be utilized to investigate multiple nAChR populations ..................................130

4.6 Receptor down-regulation paradigms can be used to study α3β4* nAChRs turnover ........................................135

4.7 Adrenal α3β4* nAChRs undergo constitutive turnover governed by de novo protein synthesis .................137

BIBLIOGRAPHY .................................................................142

APPENDICES: WORK COMPLETED THROUGH COLLABORATIVE INVESTIGATIONS

APPENDIX A. Ring E analogs of methyllycaconitine (MLA) as novel nicotinic antagonists ...........................................156

APPENDIX B. Structure-activity studies with ring E analogues of methyllycaconitine: evidence for activity on α3*-nicotinic acetylcholine receptors ...........................................165
LIST OF TABLES

Table 1.  Comparison of the effects of several cholinergic drugs on binding and secretion .................................................................41

Table 2.  Effects of nAChR reduction and reoxidation on EC₅₀ and Eₘₐₓ values for several nAChR agonists ........................................63

Table 3.  Effects of nAChR reduction on IC₅₀ values for several nAChR antagonists on brACh-induced secretion .............................64

Table 4.  Effects of DTT pretreatment on the ability of nAChR antagonists to inhibit brACh-induced catecholamine secretion ..............78

Table 5.  Ability of nAChR inhibitors to protect nAChRs from alkylation: studies using intermediate and low concentrations of brACh for alkylation .................................................................79

Table 6.  Pharmacology of tubocurarine-protected nAChRs ......................................................81

Table 7.  Comparison of inhibition constants of several cholinergic drugs..119

Table 8.  Yields and nicotinic antagonist properties of target compounds..162

Table 9.  Comparison of effects of MLA and ring E analogues on subtypes of nAChRs .................................................................180

Table 10. Effects of ring E analogues on [³H]epibatidine binding to adrenal chromaffin cells ..........................................................181
LIST OF FIGURES

Figure 1. nAchR structure and subunit arrangement.........................................12
Figure 2. Schematic representation of the known endogenous mammalian subtypes of nAChRs.................................................................13
Figure 3. [3H]Epibatidine binding kinetics to adrenal medullary membranes.................................................................................................42
Figure 4. Saturation binding of [3H]epibatidine to adrenal medullary membranes.........................................................................................43
Figure 5. Concentration-response effects of cholinergic drugs on [3H]epibatidine binding to adrenal membranes..................................................44
Figure 6. Western analysis of mAb35 immunoreactive proteins............................47
Figure 7. Effects of nAChR alkylation and permeant (nicotine) and impermeant (carbachol, DMPP) cholinergic agents on [3H]epibatidine binding........................................................................53
Figure 8. Effects of carbachol on [3H]epibatidine binding to control and alkylated adrenal chromaffin cells.................................................................54
Figure 9. Alkylation of adrenal membrane preparations........................................55
Figure 10. Association studies on intact adrenal chromaffin cells in culture.................................56
Figure 11. Saturation studies on intact adrenal chromaffin cells in culture.................................57
Figure 12. Homologous epibatidine competition binding studies on adrenal chromaffin cells in culture.................................................................58
Figure 13. The effects of DTT treatment on nicotine, acetylcholine, and cytisine stimulated adrenal catecholamine secretion.................................65
Figure 14. The effects of DTT treatment on epibatidine stimulated adrenal catecholamine secretion

Figure 15. The effects of DTT treatment on brACh stimulated adrenal catecholamine secretion

Figure 16. The effects of DTT treatment on the ability of nAChR antagonists to inhibit brACh-stimulated adrenal catecholamine secretion

Figure 17. Concentration-response effects of DTT on brACh-stimulated secretion, on epibatidine-stimulated secretion and on nAChR alkylation

Figure 18. The effects of DTT treatment on basal and KCl-stimulated adrenal catecholamine secretion

Figure 19. The effects of DTT treatment on KCl-stimulated adrenal catecholamine secretion

Figure 20. The inability of DTNB to reverse the non-receptor mediated effects of DTT

Figure 21. Ability of nAChR antagonists to protect nAChRs from alkylation

Figure 22. Ability of nAChR inhibitors to protect nAChRs from alkylation

Figure 23. The concentration-dependency of the protective effects of tubocurarine

Figure 24. Ability of tubocurarine and amantadine to protect nAChRs from alkylation by brACh

Figure 25. Inability of α7 nAChR selective antagonists to protect nAChRs from alkylation by brACh

Figure 26. Nicotine concentration-response profile of tubocurarine-protected nAChRs

Figure 27. Tubocurarine concentration-response profile of tubocurarine-protected nAChRs

Figure 28. Effects of puromycin on recovery of nAChR mediated catecholamine secretion following nAChR alkylation
Effects of actinomycin D on recovery of secretory function after nAChR down-regulation via alkylation.................................94

Effects of puromycin on recovery of nAChR mediated catecholamine release from mAb35 induced nAChR down-regulation.................................................................95

Effects of actinomycin D on recovery of secretory function after mAb35 induced nAChR down-regulation.................................................................96

Effects of DTT on recovery of secretory function after mAb35 induced nAChR down-regulation.................................................................97

Time dependent effects of puromycin on nicotine-stimulated adrenal secretion.................................................................98

Time dependent recovery of nicotine-stimulated secretion after down-regulation with puromycin.................................................................99

Effects of alkylation induced down-regulation and recovery on nAChR subunit mRNA levels.................................................................101

Effects of alkylation and recovery from alkylation on adrenal nAChR binding to both surface and intracellular nAChRs........105

Effects of alkylation and recovery from mAb35 induced down-regulation on adrenal on nAChR binding to both surface and intracellular nAChRs .................................................................106

Effects of puromycin treatment on adrenal nAChRs......................107

Effect of puromycin treatment on β4 nAChR protein.................110

Structures of a ring E analog of MLA, MLA, and ring A/E analog..163

Synthesis Scheme for ring E analogs of MLA.............................164

Structures of MLA and Ring E analogues..................................182

Effects of ring E analogues on nAChR-stimulated secretion from bovine adrenal chromaffin cells.................................................................183

Effects of hydrolysis products of the N-phenpropyl substituted ring E analogue of MLA on nAChR-stimulated adrenal secretion......184
Figure 45. Effects of the ring E analogues on $[^{125}\text{I}]\alpha$BGT binding to rat brain membranes. 185
STATEMENT OF THE PROBLEM

Neuronal nicotinic acetylcholine receptors are ligand gated ion channels found in both the peripheral and central nervous system. Despite the importance of neuronal nicotinic acetylcholine receptor (nAChR) regulation in several disease states, very little is known about the mechanisms underlying nAChR expression. This likely stems from a variety of complications including the lack of a suitably characterized model to study neuronal nAChRs in native tissue, the fact that most tissues that endogenously express neuronal nAChRs express more than one subtype of nAChRs, and the lack of subtype specific ligands. One of the most interesting nAChR subtypes that has been clearly implicated with disease is also the least characterized, those containing the α3 nAChR subunit.

A central goal of this thesis is to develop and characterize the bovine adrenal gland medulla as a model system for the study of ganglionic neuronal nAChRs and to use bovine adrenal chromaffin cells to understand mechanisms underlying the turnover and regulation of neuronal nAChRs.
The overall goals of this dissertation are to characterize the nAChRs expressed in adrenal chromaffin cells and to determine mechanisms underlying their regulation.

The specific aims are as follows:

1. Characterize the subunit composition of the nAChR expressed in bovine adrenal chromaffin cells using radioligand binding techniques to a bovine adrenal membrane preparation. Adrenal nAChRs will be characterized by conducting binding kinetic studies, saturation analysis, and pharmacological affinity determination for a number of different ligands. This data is then compared to similar data of known nAChR subtypes. The hypothesis is that bovine adrenal chromaffin cells express $\alpha_3\beta_4^*$ nAChRs and these receptors are the primary mediators of catecholamine release from the adrenal medulla.

2. Identify the expression levels and locale of nAChR using radiolabeled ligand binding to label adrenal nAChRs expressed in intact cultured chromaffin cells. The hypothesis is that adrenal chromaffin cells contain both surface and intracellular nAChRs that are involved in nAChR expression and regulation.

3. Determine the importance of disulfide integrity at the agonist binding site of adrenal nAChRs. The hypothesis is that receptor reliance on disulfide integrity varies between subtypes of nAChRs. In most subtypes reduction of
the vicinal cystines located adjacent to the agonist binding site destroys
receptor functional responses.

4. Investigate the use of nAChR protection assays to distinguish multiple
populations of neuronal nAChRs on bovine adrenal chromaffin cells. The
hypothesis is that receptors can be protected from irreversible blockade
duced by nAChR alkylation, by ‘protecting’ them with nAChR subtype
specific compounds. Compounds added during the alkylation procedure
should prevent the receptors from being alkylated. If subtype specific agents
are used, then particular nAChR subtypes can be studied by eliminating the
other subtypes that are present of the same tissue. The hypothesis is that
subtype specific compounds can be used to protect populations of adrenal
nAChRs.

5. Characterize processes involved with nAChR turnover by exploiting two
paradigms that cause nAChR down-regulation. Following receptor down-
regulation, drugs will be investigated for their ability to inhibit nAChR recovery
following down-regulation. The hypothesis is that compounds that interfere
with processes involved with nAChR synthesis or trafficking will prevent
nAChR recovery.

6. Determine any changes in Messenger RNA levels of nAChR subunits during
nAChR down-regulation and recovery to examine changes in gene

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transcription during nAChR turnover. The hypothesis is that changes in mRNA levels do not play a primary role in the regulation of expression of adrenal nAChRs during receptor down-regulation and recovery.

7. Demonstrate the importance of de novo protein synthesis in the regulation and maintenance of adrenal nAChRs. The hypothesis being tested is that these nAChRs undergo a constitutive turnover requiring constant de novo protein synthesis.

8. Probe the importance of a large intracellular pool of nAChRs. The hypothesis is that the large pool can be separated into several distinct populations, each which is individually regulated and involved in nAChR expression.
CHAPTER 1

INTRODUCTION

1.1 Nicotinic acetylcholine receptors in health and disease

Nicotine elicits a complex modulation of CNS function. However, the exact role of nicotinic acetylcholine receptors (nAChRs) in the brain remains to be determined. Nicotine, through stimulation of primarily presynaptic nAChRs, causes the release of a variety of neurotransmitters in the CNS including acetylcholine, dopamine, serotonin, GABA, and glutamate (McGehee and Role, 1995), and is therefore, thought to augment or gate a number of neuronal systems. It is clear that many neuronal nicotinic subtypes do not function in the classic post-synaptic, direct excitatory manner of neuromuscular transmission. Instead neuronal nAChRs are found not only in post-synaptic, but also in pre-, peri-, and extra synaptic locations. Here nAChRs influence physiological actions in a variety of ways. It has been proposed that the function of nAChRs in the CNS is to modify neuron excitability (McGehee and Role, 1995) in an effort to produce optimal neuron performance (Paterson and Nordberg, 2000). Modulation of neuronal excitability is likely to be important in a number of behaviors and cognitive processes, particularly learning and memory.
In addition to the CNS, neuronal nAChRs are found on postsynaptic neurons in parasympathetic ganglia, sympathetic ganglia, and the adrenal medulla. In autonomic ganglia the primary electrophysiological event following preganglionic stimulation is the depolarization of postsynaptic sites through activation of neuronal nAChRs, resulting in an excitatory postsynaptic potential. Therefore, neuronal nAChRs are the primary mediators of autonomic transmission in the peripheral ganglia, playing an important role in nearly all neurotransmission of the peripheral nervous system.

Since neuronal nAChRs play such a prominent role in neurotransmission, it is not surprising that several disease states are associated with these receptors. The most direct medical implication of neuronal nAChRs is their role as the intermediaries of tobacco addiction. The precise mechanisms that lead to nicotine addiction are poorly understood, however, it is known to involve brain monoamines. Nicotine increases the release of dopamine in the nucleus accumbens, an action that is known to play a central role in the reinforcing effect of drugs particularly in the acquisition phase (Dani et al., 2001). In addition, neuronal nAChR binding sites are upregulated in brain slices from smokers vs. that of matched controls (Perry et al., 1999). This change in the number of nAChRs likely plays a role in the addiction process (Buisson and Bertrand, 2002).

Alzheimer's disease is a serious neurodegenerative disease characterized by progressive dementia that affects almost 1 in every 10 individuals over the age of 65. One of the most severe neurochemical abnormalities seen in
Alzheimer's disease is the loss of cholinergic innervation in the cerebral cortex and hippocampus. Consistent losses of nAChRs have been measured both in vitro with receptor binding studies and in vivo with neuronal imaging. These losses significantly correlate with cognitive impairments in Alzheimer's patients (for review see Nordberg, 2001). While the underlying mechanism of this loss is unknown, several nAChR subtypes are involved, since there is a loss of \( \alpha 3, \alpha 4, \) and \( \alpha 7 \) protein (Guan et al., 2000). Interestingly there are no corresponding changes in the expression of mRNA levels for the \( \alpha 3 \) or \( \alpha 4 \) subtypes (Terzano et al., 1998). These finding may implicate postranscriptional regulation in the underlying changes in nAChR number seen in this disease.

Parkinson's disease is another major neurodegenerative disease, involving the dopaminergic neurons of the substantial nigra. Similar to Alzheimer's disease, there is a loss of cholinergic cells in the basal forebrain accompanied by a significant reduction in the number of nicotine binding sites (for review see Paterson and Nordberg, 2000). This loss closely correlates with the progression of the disease.

While it is not yet known exactly where in the progression of neurodegenerative disease that neuronal nAChRs are involved, a form of epilepsy is directly caused by a change in nAChRs. Autosomal dominant frontal lobe epilepsy, is a form of epilepsy that causes brief seizures to occur during light sleep. It has been found that this condition is associated with a mutation in the \( \alpha 4 \) neuronal nAChR subunit gene (Steinlein et al., 1995). On the other end of the spectrum, several disease states are just beginning to be associated with
nAChRs. These include both schizophrenia and Tourette's syndrome. Schizophrenia was first thought to involve nAChRs because of the large percentage of schizophrenic individuals (90% of the schizophrenic population) who smoke tobacco. Recently studies of postmortem schizophrenic brains have shown a decrease in both nAChR binding sites (Freedman et al., 1995) and nAChR protein (Guan et al., 1999). These findings lead to the hypothesis that patients may be trying to self medicate by smoking (Paterson and Nordberg, 2000) to overcome insufficiency in nAChRs.

Nicotine has long been known to produce analgesic effects in several models of pain, however, its toxicity prevents it from being a viable medication. However, discovery of subtype selective compounds has caused a resurgence in the investigation of nAChR compounds for pain therapy (for review see (Flores, 2000). The subtype of nAChR involved in pain transmission remains the focus of ongoing studies.

Finally, neuronal nAChRs are curiously being found in non-neuronal cells. This has stirred a search for the purpose of these ligand gated ion channels on cell types where they would not traditionally be found (Conti-Fine et al., 2000). Non-neuronal tissues expressing neuronal nAChRs include human bronchial epithelial cells, human blood vessel endothelial cells, and human skin keratinocytes (for review see (Conti-Fine, Navaneetham, Lei, and Maus, 2000). Interestingly all of these tissues appear to express the same nAChR subtype, likely composed of α3, β4, α5, and β2 subunits. While the physiological role of neuronal nAChRs in non-neuronal tissues are just beginning to be dissected, it is 

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likely that these nAChRs are also mediators of nicotine toxicity and may play additional roles in nAChR related disease.
1.2 Nicotinic acetylcholine receptor classification

Cholinoceptors, activated by the endogenous ligand acetylcholine, consist of both the muscarinic and nicotinic receptors. While the muscarinic acetylcholine receptor is a metabotropic G-protein linked receptor, the nicotinic acetylcholine receptor (nAChR) is an ionotropic ligand-gated ion channel. nAChRs are classified in the four transmembrane receptor family based on their protein structure containing four transmembrane domains, similar to some serotonin receptors, glycine receptors, and GABA<sub>A</sub> receptors. nAChRs are pentameric complexes made up of five individual glycoproteins that can be subclassified as either muscle nAChRs or neuronal nAChRs (Figure 1). Molecular cloning has resulted in the identification of the muscle nAChR subunits α1, β1, δ, and ε. While structurally related, the neuronal nAChRs are a much more diverse group. Currently, nine types of neuronal nAChR α genes (α2 - α10) and three types of neuronal nAChR β genes (β2 - β4) have been cloned (for review, (Lindstrom, 1997)). The neuronal subtypes generally assemble as either two α subunits and three β subunits, or homomers of α subunits (α7, α8, α9 have been shown to form homomers). The diversity of nAChR subunits allows for the possibility of myriad of subtypes based upon subunit composition. In vitro, numerous combinations of neuronal nAChR subunits have been reported to form functional nAChRs (e.g., (Elgoyhen et al., 1994; Gerzanich et al., 1994; Luetje and Patrick, 1991)). Various combinations of these subunits result in a diverse receptor population and physiology. In vivo, the characterization of only a few
nAChR subtypes are well established, including \( \alpha 3\beta 4^*, \alpha 3\alpha 5\beta 4, \alpha 3\alpha 5\beta 2\beta 4, \)
\((\alpha 4)_2(\beta 2)_3, \alpha 4\alpha 5\beta 2, \alpha 7^*, \alpha 7\alpha 8^*, \alpha 8^*, \) and \( \alpha 9^* \) (Lukas et al., 1999), Figure 2.

The pharmacological properties of nAChRs are dictated by subunit composition. Expression of various subtypes in recombinant systems such as Xenopus oocytes (for example, (Luetje and Patrick, 1991; Parker et al., 1998) or cell lines (for example, (Xiao et al., 1998), has allowed for the characterization of the pharmacological properties of several subtypes. These data coupled with subtype selective ligands have provided a comparison tool for the classification of receptors expressed in native tissue. Therefore, in addition to molecular biological techniques, receptors expressed in native tissues can be characterized based on pharmacological properties.
Muscle

Neuronal

\( \alpha_2 - \alpha_{10} \) and \( \beta_2 - \beta_4 \)

Figure 1. nAChR structure and subunit arrangement
Figure 2: Schematic representation of the known endogenous mammalian subtypes of neuronal nAChRs.
1.3 Regulation of expression of neuronal nAChRs

Despite the importance of understanding nAChR regulation, very little is known about the assembly and trafficking of neuronal nAChRs. The functional protein making up the nAChR is a structural oligomer made up of particular subunit polypeptides that are correctly folded intra-cellularly into the proper conformation for function. This folding process typically takes place in the endoplasmic reticulum, or in some cases the golgi apparatus. This process involves highly specific folding, recognition, and association of the polypeptides to produce hetero- or homooligomers that can then be transported to the cell surface.

Studies using muscle cells have shown that receptor subunits undergo several post-translational modifications prior to assembly. These have been shown to include N-linked core glycosylation, disulfide bond formation, fatty acid acylation, and phosphorylation. Good evidence exists for the importance of phosphorylation/de-phosphorylation events in the regulation of muscle nAChR assembly (Ross et al., 1987). In recent studies the use of transfected cells has allowed for the study of various aspects of subunit maturation. These include the order in which the subunits assemble and the location of putative recognition sites for subunit-subunit interactions. These subunit-subunit interactions are of particular importance in that they may dictate the agonist binding site, which has been shown to exist at the subunit interface. However, even with these new abilities to investigate assembly, the true mechanisms involved in formation of a precise stoichiometry and order of subunits are still poorly understood.
Several processes have been described that regulate neuronal nAChR expression and directly influence their functional activity. These processes have a temporal component. Loss of receptor function via desensitization occurs rapidly, usually within seconds or minutes. Receptor down-regulation develops over several hours. Receptor up-regulation occurs with some neuronal nAChR subtypes and also develops over several hours. Finally tolerance and dependence generally take days to weeks to develop. Several mechanisms are likely involved with these processes including alterations in receptor internalization, receptor recycling, receptor degradation and/or receptor synthesis.

Very little information is available on the synthesis, assembly, trafficking and surface expression of neuronal nAChRs. It is postulated that neuronal nAChRs assemble and traffic in a manner similar to their muscle counterpart. However, neuronal nAChRs are a much more diverse group than the traditional excitatory muscle receptors, and we are just beginning to understand the intricacies of their regulation.

One means for cells to regulate receptor expression is at the transcriptional level. Transcriptional regulation of neuronal nAChR subunits has been difficult to study since individual nAChR subunit gene expression occurs in distinct yet overlapping temporally and spatially restricted patterns within both the CNS and PNS. Both negative and positive regulations have been seen in neuronal nAChRs. Molecular evidence with respect to how these different subunits are regulated in their transcription is lacking. The segregation of nAChR
gene expression suggests that transcriptional regulation and control may play a role in what subtype of receptor is expressed in which area of the body, region of the brain, or even part of the cell. Although the evidence of specific control elements and their DNA binding proteins are limited, the rat β4 nAChR subunit (Du et al., 1997), α7 nAChR subunit, (Nagavarapu et al., 2001) have been investigated. In additional studies, members of the Brn-3 POU family have been shown to be involved in activation or repression of the α3 subunit gene. The α3, β4, and α5 nAChR subunits are tightly linked in the rat genome, suggesting that the genes may be co-regulated to ensure proper expression in appropriate developmental stages and bodily locations.

It is interesting to postulate that different regulatory proteins or transcription factors account for regulation of which subunit genes are transcribed in a particular set of neurons or stage of development. As more research is conducted, it may prove possible to link certain regulatory proteins selectively to particular subunit genes.

While it appears that transcriptional regulation does play a vital role in both developmental and basal levels of nAChR expression, very little evidence supports transcriptional regulation playing a role in changes in receptor expression during disease. Messenger RNA levels do not change for nAChRs in diseases such as Alzheimer’s (Nordberg, 2001) or nicotine addiction (Marks et al., 1992), demonstrating the primary mechanism of change in nAChR expression is likely via post- transcriptional modifications.
Several post-transcriptional modifications have been suggested to influence neuronal nAChR expression. At least three separate locations in the receptor expression/turover process could potentially be regulated to affect the number of nAChRs expressed on the cell surface. These include alterations in nAChR assembly (Mitra et al., 2001), nAChR transport to the cell surface (Rothhut et al., 1996; Keller et al., 2001), or nAChR stabilization in the cellular membrane (Peng et al., 1994). Which of these processes is rate limiting remains to be determined and most likely depends on the disease condition. For example, stabilization in the cellular membrane is thought to occur during prolonged agonist exposure resulting in nAChR upregulation (Peng et al., 1997; Peng, Gerzanich, Anand, Whiting, and Lindstrom, 1994), a condition seen in tobacco addiction but not other nAChR related disease.

The most intriguing way to influence nAChRs, alterations in trafficking to the cell surface, is also the mechanism we know the least about. Several processes have been identified as essential for surface trafficking of nAChRs, including receptor glycosylation and phosphorylation mechanisms (Rothhut, Romano, Vijayaraghavan, and Berg, 1996; Sava et al., 2001). In addition, it is likely that chaperone proteins like calnexin (Gelman et al., 1995) and structural/trafficking proteins like rapsyn play a role in the expression of nAChRs. Alterations at any of these points in the trafficking process could account for changes in the levels of surface expression.

The relative importance of intracellular proteins interacting with nAChRs is just beginning to be understood. All nAChRs contain large intracellular loops that
represent the regions of greatest diversity between receptor subunits. They differ in both sequence and length. Limited information is available about the function of these loops, but several have been shown to contain putative phosphorylation sites, and are essential for dictating cellular localization of receptors, particularly those containing an \( \alpha_3 \) subunit (Williams et al., 1998). Furthermore, it has been demonstrated that several interesting intracellular proteins have been associated with these loops. These proteins have been the target of some investigations relating to the muscle nAChR (for example, Phillips, 1995) and (Fuhrer et al., 1999), but remain to be clearly established as modulators of neuronal nAChR expression. Regardless, the interaction of the nAChR with intracellular proteins likely plays a role in their trafficking and regulation and present interesting regulatory targets.
1.4 $\alpha^3*$ Nicotinic acetlycholine receptors

The majority of information known about ganglionic nAChRs is from studies using the ciliary ganglia of the chicken. This tissue expresses multiple subtypes of nAChRs including those containing $\alpha_3$, $\beta_4$, and $\alpha_5$ subunits and a homomer containing only the $\alpha_7$ subunit (Halvorsen and Berg, 1990). Native subtypes containing $\alpha_3$ subunits are probably the least characterized in terms of precise subunit composition and functional roles. In the peripheral nervous system, $\alpha_3$-containing nAChRs are found on postsynaptic neurons in autonomic ganglia and cells of the adrenal medulla.

The subtypes believed to be expressed in autonomic ganglia are $\alpha_3\beta_4^*$, $\alpha_3\alpha_5\beta_4$, and $\alpha_3\alpha_5\beta_2\beta_4$ (Lukas, Changeux, le Novère, Albuquerque, Balfour, Berg, Bertrand, Chiappinelli, Clarke, Collins, Dani, Grady, Kellar, Lindstrom, Marks, Quik, Taylor, and Wonnacott, 1999) and they play prominent roles in autonomic neurotransmission and adaptive responses to stress. $\alpha_3$-Containing nAChRs are also distributed in several brain regions, (for reviews, see (Jones et al., 1999;Picciotto et al., 2000;Cordero-Erausquin et al., 2000)) where they may regulate neurotransmitter release (Sershen et al., 1997). The physiological significance of the $\alpha_3^*$ nAChRs is demonstrated by the lethality seen in $\alpha_3$ knockout mice (Xu et al., 1999). Lack of the $\alpha_3$ nAChR been shown to cause autonomic dysfunction of the bladder and eye (Xu, Gelber, Orr-Urtreger, Armstrong, Lewis, Ou, Patrick, Role, De Biasi, and Beaudet, 1999),and megacystis-microcolon-intestinal hypoperistalsis syndrome (Richardson et al., 2001). In addition antibodies to the $\alpha_3$ nAChR have been found in autonomic
neuropathy (Vernino et al., 2000). Interestingly, a consistent loss of α3 protein is found in Alzheimer’s Disease temporal cortex and hippocampi as compared to control (Guan, Zhang, Ravid, and Nordberg, 2000). However, there is no corresponding loss of mRNA for the α3 subunit in Alzheimer’s diseased brain (Terzano, Court, Fornasari, Griffiths, Spurden, Lloyd, Perry, and Clementi, 1998).

Despite α3 nAChRs broad range of effects, very little is known about the pharmacology and physiology of this subtype. The α3β4 nAChR has a much lower affinity for most nicotinic receptor agonists as compared to the primary CNS subtype the α4β2 nAChR. This has hindered the study of these receptors due a lack of a suitable high affinity ligand. Classic cholinergic radioligands like [3H]nicotine, which are very useful for labeling α4 nAChRs, are not useful for labeling of α3 containing nAChRs since their much lower affinity would require very high concentrations at which non-specific binding would obscure specific binding (Xiao, Meyer, Thompson, Surin, Wroblewski, and Kellar, 1998). These obstacles have been partially overcome with the finding that the natural alkaloid epibatidine, has high affinity for ganglionic nAChRs (Badio and Daly, 1994).

Another obstacle with studying α3* nAChR is that the tissues where they are located in substantial numbers (autonomic ganglia and adrenal gland) are poorly characterized in terms of subtype composition and multiple populations of nAChRs. The characterization of a native receptor population containing the α3 subunit would allow for further investigation of this subtype in its native environment.
1.5 Adrenal chromaffin cells

The adrenal medulla is thought of as part of the sympathetic nervous system, sharing several characteristics with sympathetic neurons (Marley and Prout, 1965). The splanchnic nerve through which stimulation results in the release of acetylcholine innervates the adrenal gland. Acetylcholine released from the splanchnic nerve acts on cholinoreceptors of the adrenal medulla including the nAChR. Activation of these receptors elicits a number of biochemical responses including secretion of the catecholamines epinephrine and norepinephrine. Catecholamines released in this manner enter directly into the blood stream where they travel to affect distant target organs and stimulate physiological responses. Therefore, adrenal chromaffin cells share several similarities to sympathetic ganglion fibers in that preganglionic cholinergic nerves innervate them, they release catecholamines upon cholinergic stimulation, and they possess similar biochemical processes.

Chromaffin cells contain tyrosine hydroxylase, aromatic L-amino acid decarboxylase, dopamine β-hydroxylase, and phenylephrine N-methyltransferase, the enzymes needed for catecholamine synthesis (Ungar and Phillips, 1983). In addition, similar to sympathetic neurons, chromaffin cells contain membrane bound organelles that store and release catecholamines (Douglas, 1968) and an assortment of other products including enkephalins, chromogranin A, somatostatin, and other neurotropic factors (Winkler, 1976). Therefore, the primary culture of these chromaffin cells can serve as a good
model for sympathetic neurons and has been long exploited for the study of stimulus secretion coupling.

Stimulus secretion coupling (Douglas, 1968), a model derived originally from the excitation contraction coupling of muscle cells, describes the events occurring after adrenal chromaffin cells are exposed to a stimulus. Acetylcholine acts on nAChRs resulting in an opening of this ligand gated ion channel allowing influx of Na⁺ and Ca²⁺. Entry of these ions slightly depolarizes the cell membrane, activating voltage gated Na⁺ channels resulting in further Na⁺ influx. This influx then activates voltage gated Ca²⁺ channels resulting in a Ca²⁺ flow into the cell and activation of the cellular mechanisms of catecholamine release (Baker and Knight, 1984). As evident from above, this entire process is triggered by the single event of nAChR activation. It is also important to note that the activation of the nAChR can be bypassed to result in catecholamine release by directly depolarizing the cellular membrane with KCl (Douglas et al., 1967). This provides a valuable tool to verify where in the stimulus secretion coupling pathway a particular effect is taking place.

Since stimulation of nAChRs is the primary mediator of catecholamine release in chromaffin cells, these cells provide an excellent model to investigate changes in nAChRs since changes in cellular function can be directly correlated with changes in nAChRs. Historically, bovine adrenal medullary cells have been used primarily as a model for the study of neurosecretory mechanisms (Livett et al., 1983). Although the potential exists for the use of bovine adrenal cells to study native neuronal nAChRs, few studies have been reported (Higgins and
Berg, 1988a; Lee et al., 1992). This is likely due to the lack of knowledge about
the precise composition of the nAChRs mediating adrenal secretion.

Several genes coding neuronal nAChR subunits have been cloned from
bovine adrenal cells including the α3, α5, α7 and β4 subunits (Criado et al.,
1992; Garcia-Guzman et al., 1995; Campos-Caro et al., 1997; Wenger et al.,
1997). There exist very few native models of α3 containing nAChRs. These cells
could prove valuable for examining the regulation of nAChRs in a native system,
however, the model must first be characterized. Studies from our laboratory
suggest that the primary mediator of catecholamine release from chromaffin cells
is the mAb35-nAChR (Gu et al., 1996). This finding is based on the ability of the
monoclonal antibody mAb35, originally raised against the main immunogenic
region of the muscle nAChR (Lindstrom et al., 1981), to downregulate nAChR
function in the adrenal medulla. However, it is unknown which nAChR subunits
this antibody cross reacts with in bovine chromaffin tissue. Furthermore, mAb35
treatment results in only a partial downregulation of nAChR mediated function,
indicating that it may not recognize all functional nAChRs. Therefore, the
composition of the nAChRs expressed in this tissue remains unclear. To utilize
bovine adrenal chromaffin cells as a model to examine α3 containing nAChR
turnover, the composition of the receptors expressed in this tissue must be
characterized.
CHAPTER 2

MATERIALS AND METHODS

2.1 Materials

(-) Nicotine hydrogen tartrate, d-tubocurarine chloride, amantadine hydrochloride, tetracaine hydrochloride, adiphenine hydrochloride, pentolinium ditartrate, mecamylamine hydrochloride, decamethonium bromide, hexamethonium bromide, (±)epibatidine 2HCl, cytisine, acetylcholine chloride (ACh), carbamylcholine chloride (carbachol), cytisine, dihydro-β-erythrodine hydrobromide (DHβE), 1,1-dimethyl-4-phenyl-piperazinium (DMPP), iodoacetamide, phenethylsulfonyl fluoride (PMSF), and polyethyleneimine (PEI), puromycin, neostigmine bromide, 5,5'-dithio-bis(2-nitrobenzoic acid) (DTNB), Bradford protein assay, and components of N2+ media were obtained from the Sigma Chemical Company (St. Louis, MO). Bromoacetylcholine bromide (brACh) and α-bungarotoxin (α-BGT) were purchased from Research Biochemicals International (Natick, MA). Methyllycaconitine citrate (MLA) was purchased from Tocris (Ballwin, MO). Dithiothrietol (DTT) was purchased from Amersham Pharmacia Biotech, Inc. (Piscataway, NJ). Dulbecco's Modified Eagle Medium (DMEM), DMEM/F12, and penicillin G sodium/streptomycin sulfate solution were obtained from GibCO/BRL (Grand Island, NY). DL-[3H]norepinephrine ([3H]-NE) (specific activity, 12.0-15.0 Ci/mmol), and (±)[5,6-
bicycloheptyl-[3H]-epibatidine (specific activity, 66.6 Ci/mmol) were purchased from Dupont-New England Nuclear Corporation (Boston, MA). Whatman GF/B filters were purchased from Brandel Laboratories, Inc. (Gaithersburg, MD). Polyclonal anti-nAChR anti-sera to the intracellular loop of the bovine β4 nAChR (PCβ4) was developed through a collaboration of Dr. Dennis McKay (The Ohio State University), Dr. Thomas Boyd (The Ohio State University), and Dr. Paul Gottlieb (University of Texas). Immunizations were performed by Cocalico Biologicals, Inc. (Reamstown, PA). Goat anti-rat IgG fluorescein-conjugated antibodies were purchased from ICN Biomedicals, Inc. (Aurora, OH). mAb35 (anti-acetylcholine receptor monoclonal antibody) was obtained from a hybridoma cell line purchased from American Type Culture Collection (Rockville, MD). The cells were cultured and the antibody was concentrated and purified using techniques previously described by our laboratory (Gu, Wenger, Lopez, McKay, Boyd, and McKay, 1996).

2.2 Isolation and Primary Culture of Bovine Adrenal Chromaffin Cells

Adrenal chromaffin cells were dissociated from intact glands and plated in supplemented DMEM, as previously described by our laboratory (Maurer and McKay, 1994). Cells were plated in DMEM media supplemented with 10% fetal calf serum, 250 ng/ml amphotericin B, 100 U/ml penicillin, 100 µg/ml streptomycin, 2 mM L-glutamine, and 10 µM 5-fluoro-2'-deoxyuridine. Two days after plating, media were replaced with serum-free, N2+ medium, as previously described by our laboratory (Maurer and McKay, 1994). Cells were plated on 24
well plates at a density of 1-2x10^5 cells per well for catecholamine release assays and 5x10^5 cells per well for intact cell binding. One day prior to experimentation, culture media were removed and replaced with N2+ media free of amphotericin B and 5-fluoro-2'-deoxyuridine. Cells were used 4-7 days after isolation.

2.3 Adrenal medulla membrane preparation

Bovine adrenal medullae were dissected from intact adrenal glands and prepared as previously described by our laboratory (Free et al., 2002). Briefly, medullae were immediately placed in ice-cold preparation buffer consisting of 300 mM sucrose, 50 mM Tris (pH 8.8), 1 mM EDTA, 1 mM EGTA, 5 mM iodoacetamide, and 0.1 mM PMSF. Medullary tissue was minced and then homogenized in ice-cold buffer (1:1, weight:volume) using a polytron. The homogenate was centrifuged for 50 min at 82,000 x g at 4 °C. Pellets were then resuspended in assay/rinse buffer (pH 7.4) consisting of 120 mM NaCl, 5 mM KCl, 8 mM Na2HPO4, 2 mM EDTA, 2 mM EGTA, 5 mM HEPES, 5 mM iodoacetamide, and 0.1 mM PMSF. The homogenate was centrifuged for 25 min at 82,000 x g at 4 °C and resuspended in assay/rinse buffer. Protein concentrations were determined using the Bradford protein assay with bovine serum albumin as the standard.

2.4 Catecholamine Secretion Studies

A [3H]norepinephrine ([3H]NE) assay was used to monitor catecholamine release from cultured cells (McKay and Schneider, 1984). Cells were incubated (60-
90min) with 0.1 µM [³H]NE in a physiological salt solution (PSS) containing 140 mM NaCl, 4.4 mM KCl, 1.2 mM MgSO₄, 3.6 mM NaHCO₃, 1.2 mM KH₂PO₄, 10 mM glucose, 2 mM CaCl₂, 5 mM HEPES (pH 7.2-7.4) and 0.5 % bovine serum albumin (BSA). The buffer was then aspirated and the cells washed (1ml/wash) at least 3 times (15 min each) prior to stimulation. 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 [³H]NE. Typically 10µM nicotine was used to stimulate maximum catecholamine release. When KCl was used as a stimulant, the NaCl concentration in the PSS was reduced accordingly to maintain isotonicity. When ACh or brACh were used as stimulants, 10 µM neostigmine was included in the medium. Results were expressed either 1) as a percentage of total (% total) incorporated [³H]NE released under the treatment conditions (i.e., secreted [³H]NE divided by total incorporated [³H]NE times 100), 2) as a percentage of the inhibition of control nicotine-stimulated release (% inhibition) where basal was subtracted from all groups (i.e., 100 minus the treatment group release minus basal release divided by control nicotine-stimulated release minus basal release times 100) or 3) as a percentage of net release (% net release) (i.e., %total release minus basal release). Data were calculated from the number of observations (n) performed in duplicate or triplicate. EC₅₀ and Eₘₐₓ values were obtained by averaging values.
generated from nonlinear regression analyses (Prizm, GraphPad, San Diego, CA) of individual concentration-response curves. At high agonist concentrations, a reduction in secretory response (i.e., receptor desensitization) was typically seen; these data were omitted during nonlinear curve fitting. Results are expressed as arithmetic means ± SEM, except for EC$_{50}$ values which are expressed as geometric means (95% confidence limits). Where appropriate, experimental values were compared using Student’s $t$ test ($p<0.05$) or Dunnett’s multiple comparison test ($p<0.05$).

2.5 Receptor disulfide reduction and alkylation

In some studies the effects of nAChR disulfide reduction on nAChR-stimulated catecholamine release were investigated. For these studies, cultured bovine chromaffin cells were loaded with [$^{3}$H]NE, as described above. Following the washing steps, cells were incubated with 1mM DTT in PSS (pH 8) for 15 min at 37°C to reduce disulfide bonds. Cells were then washed for 5 min with PSS prior to all other treatments. When the wash was omitted and DTT remained present throughout the stimulation period, no differences were observed in the secretory response when compared to the washed cells. For receptor alkylation studies, adrenal nAChRs were alkylated with brACh using techniques previously described by our laboratory (Wenger, Bryant, Boyd, and McKay, 1997; Free and McKay, 2001) as modified from (Gardette et al., 1991). Briefly, cells were treated with 1 mM DTT for 15 min, washed with PSS (5 min), treated with 10 μM brACh for 6 min, washed with PSS (5 min), treated with 1 mM DTNB (15 min) and then
washed (5 min) prior to stimulation. Neostigmine (10 μM) was added to all solutions containing brACh. For studies using an adrenal membrane preparation, nAChRs were alkylated by modifications of this technique. Adrenal membranes in PSS (1.0 – 1.8 mg/ml) were centrifuged using a microcentrifuge (5 min/18000 rcf). Pellets were resuspended in 30 mM DTT in PSS (pH 8) in order to reduce nAChR disulfide bonds. After 15 min at 37 °C, membranes were centrifuged and the pellets washed once by resuspension in 1 ml PSS, followed by centrifugation. Membranes were then resuspended in 100 μM brACh and 1μM neostigmine in PSS for 6 min at room temperature, followed by centrifuging and washing, as described above. Disulfide bonds were reoxidized by resuspending the membranes in 1 mM DTNB in PSS for 15 min at 37 °C. Membranes were centrifuged and pellets washed for 5 min in PSS. Membranes were then centrifuged and pellets were resuspended in membrane binding buffer for use in the membrane binding assay, see below (Free, Bryant, McKay, Kaser, and McKay, 2002). Non-alkylated membranes were processed in parallel with the alkylated membranes, without the addition of reducing, alkylating, or reoxidizing agents.

2.6 Receptor protection assays

Adrenal nAChRs were irreversibly inactivated via alkylation with brACh using techniques modified from Gardette et al. (Gardette, Listerud, Brussaard, and Role, 1991), as described above and previously by our laboratory (Wenger, Bryant, Boyd, and McKay, 1997; Free and McKay, 2001). For the receptor
protection assays, the nAChR antagonist to be tested was added to the wash preceding brACh treatment, as well as to the solution containing brACh. In control studies, pretreatment with the pharmacological agent, followed by normal washing procedures, produced no residual inhibitory effects (data not shown). In addition, 1) reduction and re-oxidation (without alkylation) of adrenal nAChRs did not affect 10 μM nicotine-stimulated catecholamine secretion and 2) nicotine's EC₅₀ value for reduced nAChRs was not significantly different from untreated controls (Free et al., 2000).

2.7 [³H]Epibatidine binding to an adrenal membrane preparation

Binding studies were conducted as previously described (Free, Bryant, McKay, Kaser, and McKay, 2002) to an adrenal membrane preparation obtained as described above. Adrenal membranes (500-900 μg per assay tube) were incubated at room temperature for 60 min in 500 μl of binding buffer containing [³H]epibatidine. αBGT (1 μM) was added to the binding buffer to eliminate binding to αBGT binding sites. After the 60 min incubation, the assay mixtures were filtered and rapidly washed 4 times (~4 ml/wash) using a binding manifold (Brandel Laboratories, Inc., Gaithersburg, MD). Prior to the binding reaction, filter membranes were soaked >5 hrs in 5% PEI in water (Lee, Miwa, Kochimura, and Ito, 1992) to reduce nonspecific binding to the filter paper. No specific binding to PEI-treated filters was seen (data not shown). The filters with adrenal membranes were added to 4.5 ml of Scintiverse E (Fisher Scientific Co., Pittsburgh, PA) and allowed to sit overnight prior to counting via liquid scintillation.

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spectroscopy. Nonspecific binding was determined in the presence of 300 μM nicotine. Specific binding was determined by subtracting nonspecific binding (typically 5-10%) from total binding. Total [3H]epibatidine binding represented <10% of the total radioligand added.

Each observation (n) was performed in duplicate. Experiments were performed on at least three different membrane preparations. \( K_d \) and \( B_{\text{max}} \) values were determined from saturation binding assays by non-linear regression fit of the data to a model incorporating a single class of non-interacting binding sites (Prizm, GraphPad, San Diego, CA). Data from dissociation binding experiments were fitted to an exponential decay equation to determine the dissociation rate constant, \( k_{\text{off}} \). Data from association binding experiments were fitted to an exponential association equation to determine the observed association rate constant, \( k_{\text{on}} \). The association rate constant, \( k_{\text{on}} \), was calculated using the equation \( k_{\text{on}} = (k_{\text{ob}} - k_{\text{off}}) / [^{3}\text{H-epibatidine}] \). IC\textsubscript{50} values and Hill coefficients were obtained by averaging values generated from nonlinear regression analyses (Prizm, GraphPad, San Diego, CA) of individual concentration-response curves. Dissociation constants (\( K_i \)) for each drug were calculated using the Cheng-Prusoff relationship, \( K_i = IC_{\text{50}} / \{1+([^{3}\text{H-epibatidine}]/K_d \) of the \([^{3}\text{H}]\text{epibatidine})\} \) (Cheng and Prusoff, 1973). The \( K_d \) value of \([^{3}\text{H}]\text{epibatidine} \) used in this equation was 0.5 nM.
2.8 \[^{[H]}\text{Epibatidine binding to intact bovine adrenal chromaffin cells}\]

Adrenal chromaffin cells were isolated from bovine adrenal glands and cultured, as previously described above. Cells were used experimentally 4-7 days after isolation. Initially, cells (5 X 10^5 cells per culture well) were washed for 5 min in a binding buffer. This buffer is identical the physiological salt solution (PSS) used by our laboratory in functional assays on cultured chromaffin cells (see above). (Free and McKay, 2001)Cells were then incubated for 60 min at room temperature in binding buffer containing the indicated concentration of \[^{[H]}\text{Epibatidine, and 1}\mu\text{M aBGT to eliminate binding to aBGT binding sites. After the 60 min incubation, buffer was aspirated and the cells were rapidly washed with binding buffer (4 times, 1ml/wash). Cells were extracted in 1M NaOH (500 \mu l) for at least 60 min, followed by scraping. The cellular extracts were neutralized and counted using liquid scintillation spectroscopy. Non-specific binding was determined in the presence of 300 \mu M nicotine and typically represented 50 - 55% of the total binding. When binding to only surface nAChRs was investigated, nonspecific binding was determined in the presence of 5 mM carbachol, an impermeant cholinergic receptor agonist. Under these conditions nonspecific binding usually represented 65 -75 % of total binding. This higher value likely represents nonspecific binding and specific intracellular binding (see Results section).

Data were analyzed from the number of observations (n) performed in duplicate. Experiments were performed on cells from 2-4 different cell isolations. \(K_d\) and \(B_{\text{max}}\) values were determined from saturation binding assays by non-linear

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regression fit of the data to a model incorporating a single class of non-
interacting binding sites (Prizm, GraphPad, San Diego, CA). Data from
equilibrium binding experiments were fitted to an exponential association
equation to determine the t_{1/2} for association. For the homologous competition
studies, IC_{50} values were obtained from nonlinear regression analyses of
concentration-response curves using either a one site or a two site model (Prizm,
GraphPad, San Diego, CA). K_d and B_{max} values were derived from these data
using the equations, \( K_d = IC_{50} - [^3H\text{-epibatidine}] \) and \( B_{max} = \frac{(top - bottom)}{[ ^3H\text{-epibatidine}] / (K_d + [ ^3H\text{-epibatidine}])} \), respectively (GraphPad, San Diego, CA)

2.9 nAChR Downregulation Paradigms

For nAChR turnover studies, two paradigms were used to downregulate
nAChRs, receptor alkylation with bromoacetlycholine and receptor antigenic
modulation with the anti-nAChR antibody mAb35. For alkylation experiments,
cultured cells were alkylated as previously described above with minor
modifications. Cells were removed from the incubator, media was aspirated, and
cells were washed one time with sterile PSS. Cells were then treated (15 min at
37° C with 1mM DTT, which had been prepared in PSS (pH 8) and then sterilized
via passage through a 0.22μm syringe filter. Cells were then washed 5min in
sterile PSS and then incubated (6 min) with 100μM brACh prepared in sterile
PSS. Following washing (5 min with sterile PSS) cells were incubated in 1 mM
DTNB (15min at 37° C), which had been prepared in PSS and then sterilized via
passage through a 0.22μm syringe filter. Cells were then washed two times (5
min each in sterile PSS) and placed back into media. In some studies compounds were added to the cells during the recovery process, for these studies the compounds were added directly to the media.

When adrenal nAChRs were down regulated with the monoclonal antibody, mAb35 (50 nM), the antibody was diluted in media and subsequently added directly to the cells. In some studies cells were allowed to recover from mAb35 induced down regulation. For these studies, after 24 hours in the presence of 50nM mAb35, cells were washed 2 times for 15 min each in N2+ media. Cells were then placed back in N2+ media to recover. When inhibitors of the recovery process were tested they were added directly to the N2+ media.

2.10 Western analysis of nAChR protein

Adrenal chromaffin cells grown on 35mm plates (2.5X10^6 cells per plate) were washed rapidly one time with PBS. Cells were then incubated for 5 min in Mammalian Protein Extraction Buffer (M-PER™, Pierce, Rockford, Illinois). Equivalent amounts of protein were separated in 10 % SDS-polyacrylamide gels and then electroblotted onto nitrocellulose membranes (Hybond™, Amersham Pharmacia Biotech, UK). The blots were probed overnight at 4°C with a 1:2500 dilution of PCβ4 obtained as described above. Bound antibodies were detected using a 1:2500 dilution of an anti-rabbit IgG linked to horseradish peroxidase (Amersham Pharmacia Biotech, Piscataway, NJ). Western blots were probed and detected according to the manufacturers instructions, using enhanced chemiluminescence (ECL™, Amersham Pharmacia Biotech, Little Chalfont, UK).
Blots were visualized using a Biochemi™ Imaging System (UVP, Inc. Upland, CA). Molecular weights were determined using LabWorks™ imaging software (UVP, Inc. Upland, CA).

2.11 Northern analysis of nAChR mRNA

RNA was isolated from chromaffin cells plated on 60 mm dishes according to the manufacturer’s instructions using Trizol® (GIBCO/BRL, Grand Island, NY). Northern blot analysis was performed using 1 % (w/v) agarose gels containing 7.4 % (v/v) formaldehyde in 20 mM MOPS, 1 mM EDTA, and 5 mM sodium acetate at pH 7.0. Equal amounts of RNA were run on each lane of the gel. After electrophoresis, the RNA was transferred to GeneScreen Plus (Dupont-New England Nuclear Corporation, Boston, MA) in 10 X SSC according to the manufacturer’s instructions. Bovine α3, β4, α5 cDNA (a generous gift from Dr. M. Criado, Instituto de Neurociencias, Centro Mixto CSIC-Universidad Miguel Hernandez, Alicante, Spin), and mouse GAPDH (Ambion, Austin, TX). cDNAs were labeled with α-32P-dCTP using the Prime-lt Rm T™ random primer labeling kit (Stratagene, La Jolla, CA). The 32P-labeled probes were hybridized to the GeneScreen Plus membrane in 5 X SSPE, 50 % deionized formamide, 5 X Denhardt’s Solution, 1 % SDS, 10 % Dextran Sulfate, and 100 μg/ml of salmon sperm DNA at 42°C. The filters were washed in 2 X SSPE at room temperature, 2 X SSPE, 2 % SDS at 65°C, 0.1 X SSPE for 45 minutes, and 0.1 % SDS at room temperature for 15 minutes. The blots were exposed to X-ray (Kodak XAR-5) film at -70°C with an intensifying screen. A Molecular Dynamics
phosphoimager was used to quantify the signal intensities in each lane. The mRNA signals were normalized to the GAPDH signals to measure relative changes in mRNA levels.
CHAPTER 3
RESULTS

3.1 Characterization of Adrenal nAChRs

3.1.1 Radiolabeled binding techniques to characterize nAChRs of the adrenal medulla

In these studies, $[^{3}H]$epibatidine is used as the radioligand to characterize nicotinic acetylcholine receptors (nAChRs) from bovine adrenal medulla. Recently, $[^{3}H]$epibatidine has been used to characterize neuronal nAChRs. Although its affinity for $\alpha_4\beta_2$ nAChRs is higher than for $\alpha_3\beta_4$ nAChR (Parker, Beck, and Luetje, 1998), $[^{3}H]$epibatidine’s usefulness as a radioligand for $\alpha_3\beta_4$ nAChRs is well documented when these receptors are expressed in cell lines (Xiao, Meyer, Thompson, Surin, Wroblewski, and Kellar, 1998; Stauderman et al., 1998).

Epibatidine has high affinity for neuronal nAChRs (Gerzanich et al., 1995; Stauderman, Mahaffy, Akong, Velicerebi, Chavez-Noriega, Crona, Johnson, Elliot, Gillespie, Reid, Adams, Harpold, and Corey-Naeve, 1998; Xiao, Meyer, Thompson, Surin, Wroblewski, and Kellar, 1998) and is a potent secretagogue of adrenal catecholamines (Wenger, Bryant, Boyd, and McKay, 1997). Epibatidine binding sites have been found in rat adrenal tissue (Houghtling et al., 1995; Di Angelantonio et al., 2000). In the studies reported here, $[^{3}H]$epibatidine, was
used as the radioligand to characterize bovine adrenal nAChRs. As seen in Figure 3, specific binding increases as a function of time and reaches equilibrium within 30 min at room temperature. The addition of 300 µM nicotine after 60 min of binding causes a displacement of [³H]epibatidine binding (Figure 3, inset). The association rate constant, $k_{on}$, and the dissociation constant, $k_{off}$, were calculated to be 0.26 nm⁻¹ min⁻¹ and 0.04 min⁻¹, respectively. The equilibrium dissociation constant, $K_d$, was calculated to be 0.2 nM using the equation, $K_d = k_{off}/k_{on}$.

Specific binding of [³H]epibatidine is saturable with a $K_d$ of 0.52 ± 0.06 nM, fits a model for a single site with a Hill coefficient of 1.05 and represents >90% of the total binding throughout the concentration range used (Figure 4). The $B_{max}$ for [³H]epibatidine binding from nonlinear regression is 34.0 ± 3.7 fmol/mg protein.

Nicotinic agonists and antagonists were investigated for their ability to compete with [³H]epibatidine for binding (Figure 5, Table 1). Hill coefficients were always near unity, indicating no cooperative interactions among sites. [³H]Epibatidine binding was competitively inhibited by a variety of cholinergic agents with the following rank order of potency: epibatidine >> nicotine ≥ cytisine = d-tubocurarine > DMPP > carbachol >> decamethonium. Complete inhibition with mecamylamine and decamethonium were not obtainable at concentrations up to 1 mM. The affinities of cholinergic agonists and antagonists for adrenal nAChRs are very close to those reported for other tissues including those known to express α3β4 nAChRs (Xiao, Meyer, Thompson, Surin, Wroblewski, and Kellar, 1998).
Binding IC50 values were positively correlated to functional IC50 values for the agonists (Table 1), with the exception of the partial agonist, cytisine (Wenger, Bryant, Boyd, and McKay, 1997). Comparison of the IC50 values from binding and functional studies for the antagonists (Table 1) support previously reported data that d-tubocurarine acts as a competitive antagonist and decamethonium, hexamethonium and mecamylamine are noncompetitive.

Ki values were determined for a variety of cholinergic agents using the Cheng-Prusoff equation (Cheng and Prusoff, 1973). The determined values for epibatidine ($K_i, 0.3 \text{nM}$), nicotine ($K_i, 0.2 \mu \text{M}$), cytisine ($K_i, 0.4 \mu \text{M}$), carbachol ($K_i, 4.6 \mu \text{M}$), DHβE ($K_i, 31.8 \mu \text{M}$), d-tubocurarine ($K_i, 0.4 \mu \text{M}$), DMPP ($K_i, 0.8 \mu \text{M}$), and decamethonium ($K_i, 209 \mu \text{M}$), closely match those of Ki values determined in a recombinant cell line expressing rat $\alpha 3\beta 4$ nAChRs (Xiao, Meyer, Thompson, Surin, Wroblewski, and Kellar, 1998).

Differential effects were observed with other nAChR antagonists. No displacement of binding was seen with the $\alpha 7$ selective nAChR antagonist $\alpha$-bungarotoxin. Mecamylamine gave interesting results resulting in a partial displacement of binding that reached a plateau at approximately 40% inhibition. This may indicate the presence of a mecamylamine insensitive population of $[^3\text{H}]$epibatidine binding sites.

These binding studies document the utility of this adrenal medullary membrane preparation and $[^3\text{H}]$epibatidine for the study of native adrenal nAChRs and, based on saturation and competition experiments, support the classification (Lukas, Changeux, le Novere, Albuquerque, Balfour, Berg, 39

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Bertrand, Chiappinelli, Clarke, Collins, Dani, Grady, Kellar, Lindstrom, Marks, Quik, Taylor, and Wonnacott, 1999) of adrenal nAChRs as α3β4* nAChRs.

These studies also support the use of adrenal nAChRs as a model for the study of native α3β4* nAChRs.
<table>
<thead>
<tr>
<th>CHOLINERGIC</th>
<th>Binding IC50 (μM)</th>
<th>Functional EC50 or IC50 (μM)</th>
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</thead>
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<td>d-Tubocurarine</td>
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<td>1.4&lt;sup&gt;c&lt;/sup&gt;</td>
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<td>17.5&lt;sup&gt;b&lt;/sup&gt;</td>
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<td>Hexamethonium</td>
<td>&gt; 1000</td>
<td>16.2&lt;sup&gt;b&lt;/sup&gt;</td>
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</table>

Table 1. Comparison of Effects of Several Cholinergic Drugs on Binding and Secretion. <sup>a</sup> Secretion data from Free et al. (2000). <sup>b</sup> Secretion data from McKay and Burkman (McKay and Burkman, 1993). <sup>d</sup> EC<sub>50</sub> and IC<sub>50</sub> values are expressed as means ± SEM (n=3-4).
Figure 3. [³H]Epibatidine binding kinetics to adrenal medullary membranes. Membranes were incubated at room temperature for the indicated times with 1nM [³H]epibatidine and 10⁻⁶M αBGT, in the absence (total binding) or presence (non-specific binding) of 300 µM nicotine. Values represent means ± SEM (n = 4). Inset: Dissociation curve. Membranes were incubated at room temperature for 60 min with 1nM [³H]epibatidine and 10⁻⁶ αBGT. After 60 min, nicotine (300 µM) was added to the reaction mixture and the incubation continued. At the indicated times, an aliquot of the reaction mixture was filtered and washed. Dashed line represents nonspecific binding. Values represent means ± SEM (n = 4).
Figure 4. Saturation binding of $[^3]$Hepibatidine to adrenal medullary membranes. Membranes were incubated at room temperature for 60 min with various concentrations of $[^3]$Hepibatidine with $10^{-6}$M $\alpha$BGT, in the absence (total binding, □) or presence (nonspecific binding, ■) of 300 $\mu$M nicotine. Specific binding (●) was determined by subtracting nonspecific from total for each value. Values represent means ± SEM (n = 4). Nonlinear regression analysis of specific binding indicates a $K_d$ value of 0.52 ± 0.06 nM and a $B_{max}$ value of 34.0 ± 3.7 fmol/mg protein. Inset: Hill plot of the mean values of specific binding from the saturation binding experiments. Linear regression analysis indicates a Hill coefficient of 1.05.
Figure 5. Concentration-response effects of cholinergic drugs on \[^{3}H\]epibatidine binding to adrenal membranes. Competition binding experiments were performed using the indicated concentrations of cholinergic agonists or antagonists. Data are expressed as a percentage of control specific binding. Values represent means \(\pm\) SEM (n = 4-5).
3.1.2 Western analysis to characterize nAChRs of the adrenal medulla

Our laboratory has previously demonstrated that the anti-nAChR monoclonal antibody, mAb35 results in down regulation of nAChR mediated functional responses such as catecholamine release (Gu, Wenger, Lopez, McKay, Boyd, and McKay, 1996; Wenger, Bryant, Boyd, and McKay, 1997). These data support the hypothesis that nAChRs primarily responsible for catecholamine release are mAb35-nAChRs. However, since mAb35 was initially raised against the main immunogenic region of the muscle nAChR (Lindstrom, Einarson, and Tzartos, 1981), its cross reactivity with bovine adrenal nAChRs is unknown. However, evidence does exist that it may recognize both the α3 and α5 nAChR subunits in chick ciliary ganglia.

To investigate the bovine nAChR subunits recognized by mAb35, Dr. R. Thomas Boyd constructed recombinant cell lines expressing several bovine nAChR subunits. Bovine nAChR α5 and α3 cDNAs were cloned into the eukaryotic expression vector pcDNA 3.1+ (Invitrogen), which allows high level protein expression in eukaryotic cells and provides the generation of stable cell lines by selection for G418 resistance. The α5 and α3 expression constructs were transfected into COS-1 cells using Lipofectamine (Life Technologies). Three days after transfection cells were selected with G418 and passaged in the continued presence of G418. Two cell lines expressing each subunit were made. Transfection was verified by measurement of appropriate mRNA from the cell lines.
These cells were cultured and cellular protein was extracted and separated on a polyacrylamide gel. Blots were probed with mAb35 and analyzed via western analysis as described in Methods. As seen in figure 6A, mAb35 immunoreactive protein of molecular weight 55-60 kD is seen in both the α3 and the α5 containing COS-1 cells. No banding is seen in control, untransfected, COS-1 cells. These data demonstrate that mAb35 cross reacts with both α5 and α3 bovine nAChR subunits.

To verify that mAb35 recognizes specific nAChRs from bovine adrenal medulla, chromaffin cell membranes were extracted, separated on a polyacrylamide gel and probed with mAb35. As seen in figure 6B, mAb35 recognizes multiple immuno-reactive proteins of molecular weight 70-75 kD, and 55-65 kD.

Taken together with the binding data presented in section 3.1.1, these data support the hypothesis that adrenal chromaffin cells express α3β4* nAChRs and that these receptors are the primary mediators of catecholamine secretion from these cells.
Figure 6. Western blot of mAb35 immunoreactive proteins. Protein extracts from A. untransfected COS-1 Cells (Cont), COS-1 Cells transfected with α5 (α5), or COS-1 cells transfected with α3 (α3), were analyzed via immunoblots as described in methods. Blots were probed with mAb35 and reacts with proteins in both α3 and α5 COS-1 cells. Arrow indicates 55-60Kd. B. Adrenal chromaffin cell extracts were also probed with mAb35 resulting in immunoreactive proteins. Arrow indicates ~65-75 kD and 55-60 kD.
3.1.3 \[^{3}\text{H}]\text{Epibatidine binding studies to surface and intracellular nAChRs in intact adrenal chromaffin cells}

In these studies \[^{3}\text{H}]\text{Epibatidine binding techniques were used to characterize surface and intracellular sites expressed in intact adrenal chromaffin cells in culture. \[^{3}\text{H}]\text{Epibatidine has been found to readily cross membranes and label intracellular sites in HEK293 cells expressing } \alpha3\beta4 \text{nAChRs (Xiao, Meyer, Thompson, Surin, Wroblewski, and Kellar, 1998). Although intracellular nAChRs in native tissue would potentially be of great importance for nAChR regulation, few studies directly address the question of whether intracellular binding sites are fully assembled nAChRs and have binding characteristics appropriate for nAChRs. In the following studies, } \[^{3}\text{H}]\text{Epibatidine binding to surface and intracellular sites are investigated using intact bovine adrenal chromaffin cells in culture. To investigate } \[^{3}\text{H}]\text{Epibatidine binding to intracellular sites, surface nAChRs were first alkylated, a procedure previously documented by our laboratory to eliminate functional adrenal nAChRs (Wenger, Bryant, Boyd, and McKay, 1997).}

First, the ability of \[^{3}\text{H}]\text{Epibatidine to cross adrenal membranes of intact bovine adrenal chromaffin cells in culture was investigated. Non-specific binding was determined in the presence of 300 } \mu\text{M nicotine, which also readily crosses cell membranes. The effects of two impermeant cholinergic agents on } \[^{3}\text{H}]\text{Epibatidine binding to intact cells are illustrated in Figure 7. Carbachol and DMPP, at concentrations } \sim1000 \text{ times their reported } K_d \text{ values for the adrenal } \alpha3\beta4^+ \text{nAChR (Free, Bryant, McKay, Kaser, and McKay, 2002), displaced only}
42.5% ± 4.2% and 34.5% ± 2.1% of specific [3H]epibatidine binding, respectively (Figure 7). The inability of these impermeant agents to displace all specific [3H]epibatidine binding suggests that a substantial population of the binding sites is intracellular.

Additional support for both surface and intracellular [3H]epibatidine binding sites is found with alkylation experiments. Irreversible loss of receptor function following alkylation with brACh has previously been reported by our laboratory (Wenger, Bryant, Boyd, and McKay, 1997; Free and McKay, 2001). As seen in Figure 8, alkylation of intact chromaffin cells reduced specific [3H]epibatidine binding by 36.9% ± 8.3%, similar to the reductions observed with carbachol and DMPP. The inability of alkylation to eliminate 100% of specific binding is not due to a population of adrenal nAChRs resistant to alkylation. Alkylation of an adrenal chromaffin membrane preparation, devoid of intracellular compartments, resulted in a complete loss of [3H]epibatidine binding sites when compared with 300 μM nicotine (Figure 9). In these studies, nicotine displaced 87.7% ± 2.6% of total [3H]epibatidine binding and alkylation produced an 84.2% ± 5.0% reduction in total [3H]epibatidine binding; therefore, alkylation eliminated 96% of specific binding to adrenal membranes.

To determine whether the site of action of carbachol and alkylation are on the same nAChR population, carbachol competition experiments were performed on both control and alkylated cells. As illustrated in Figure 8, carbachol is unable to displace any [3H]epibatidine binding to cells that had been previously
alkylated. Likewise, the number of carbachol-insensitive receptors does not change following receptor alkylation (Figure 8). These data support two pools of \(^3\text{H}\text{epibatidine}\) binding sites (surface and intracellular) and demonstrate \(^3\text{H}\text{epibatidine}\)'s ability to bind both pools. These data also document that either receptor alkylation or the use of high concentrations of the impermeant cholinergic agents, carbachol or DMPP, eliminate surface binding of \(^3\text{H}\text{epibatidine}\) to intact adrenal chromaffin cells, leaving intracellular binding unaffected.

To further characterize \(^3\text{H}\text{epibatidine}\) binding sites expressed in intact bovine adrenal chromaffin cells in culture, association and saturation studies were performed. As seen in Figure 10A, using nicotine to define specific binding to both surface and intracellular sites, \(^3\text{H}\text{epibatidine}\) binding increased as a function of time and reached equilibrium within 30 min at room temperature. Fitting this data to an exponential association equation results in a calculated \(t_{1/2}\) of 3.8 ± 0.7 min. Similar association curves were obtained using 3 mM carbachol to define specific surface binding (\(t_{1/2}\) of 7.2 min, Figure 10B) and on alkylated cells to define specific binding to intracellular sites (\(t_{1/2}\) of 4.2 ± 1.2 min, Figure 10C). These kinetic curves are similar to those reported above for \(^3\text{H}\text{epibatidine}\) binding to a bovine adrenal medullary membrane preparation (Figure 4 and (Free, Bryant, McKay, Kaser, and McKay, 2002).

Specific binding of \(^3\text{H}\text{epibatidine}\) to intact bovine adrenal chromaffin cells (using nicotine to define specific binding) was saturable with a \(K_d\) of 3.5 ± 1.1 nM and represented ~50% of the total binding throughout the concentration range.
used (Figure 11A). The $B_{\text{max}}$ for $[^3\text{H}]$epibatidine binding sites derived from nonlinear regression analysis was $4045 \pm 811 \text{ cpm/culture}$. Saturation analysis on surface sites was performed using carbachol to define specific binding to these sites (Figure 11B). Binding to surface sites was saturable with a $K_d$ of $1.9 \pm 1.1 \text{ nM}$. The $B_{\text{max}}$ for surface $[^3\text{H}]$epibatidine binding sites was $914 \pm 270 \text{ cpm/culture}$. Specific binding to intracellular sites was also saturable when surface sites were alkylated (Figure 10C). The $K_d$ value for these sites was $3.6 \pm 1.9 \text{ nM}$. The $B_{\text{max}}$ for intracellular $[^3\text{H}]$epibatidine binding sites derived from nonlinear regression was $2639 \pm 1068 \text{ cpm/culture}$. These studies support the classification of at least a portion of intracellular $[^3\text{H}]$epibatidine binding sites as nAChRs.

A second approach to further demonstrate that the intracellular binding sites represent nAChRs, homologous (epibatidine) competition studies were conducted (Figure 12). Using nicotine to define binding to both surface and intracellular sites, epibatidine's inhibition curve is biphasic, supporting two pools of specific binding sites (Figure 12A). Two site competition analysis confirms two sites: a high affinity site with an $IC_{50}$ value of $3.7 \text{ nM}$ and a low affinity site with an $IC_{50}$ value of $70 \mu\text{M}$. To establish whether any of the high affinity sites in these studies were located intracellularly, homologous competition studies using low concentrations of epibatidine were conducted on cells that were first alkylated. As illustrated in Figure 12B, a significant portion of the high affinity sites was intracellular. These high affinity, intracellular sites had an $IC_{50}$ value of $6 \text{ nM}$. Using the equation $K_d = IC_{50} - [^3\text{H}-\text{epibatidine}]$, the $K_d$ value is $5 \text{ nM}$. The $B_{\text{max}}$
value for the high affinity intracellular sites, calculated using the equation $B_{\text{max}} = \frac{(\text{top} - \text{bottom})}{\left[\frac{[^3H]-epibatidine}{(K_d + [^3H]-epibatidine)}\right]}$, is 1050 cpm/culture.
Figure 7. Effects of nAChR alkylation and permeant (nicotine) and impermeant (carbachol, DMPP) cholinergic agents on \[^3\text{H}\]epibatidine binding. Binding experiments using 2nM \[^3\text{H}\]epibatidine were performed on cultured bovine adrenal chromaffin cells. Comparison of permeant (nicotine) and impermeant (carbachol, DMPP) cholinergic agents on \[^3\text{H}\]epibatidine binding. Total binding of the radioligand was determined either in the absence (-, control) or presence of nicotine (NIC, 300 μM), carbachol (CARB, 5 mM), or DMPP (1 mM), as indicated. Some cells were alkylated using brACh (ALK) prior to the binding reaction. Values represent mean ± SEM (n = 4).
Figure 8. Effects of carbachol on \([^3]H\)epibatidine binding to control and alkylated adrenal chromaffin cells. Cells were either untreated (lightly shaded) or alkylated (darker shaded) prior to the start of the binding reaction. Total binding of the radioligand was determined either in the absence (−, control) or presence of nicotine (NIC, 300 μM) or carbachol (CARB, 5 mM). Values represent means ± SEM (n = 6).
Figure 9. Alkylation of an adrenal membrane preparation. Adrenal membranes were either not treated or alkylated as described in materials and methods with 100μM brACh. Binding was conducted using 1 nM of [3H]epibatidine. Total binding of the radioligand was determined in either the absence (-, control, and ALK) or presence of nicotine (nic, 300μM). Values represent means ± SEM (n=4).
Figure 10. Association studies on intact adrenal chromaffin cells in culture. Specific binding to total (surface and intracellular) binding sites was defined using 300 μM nicotine. Specific binding to surface binding sites was defined using 5 mM carbachol. Specific binding to intracellular binding sites was defined on alkylated cells using 300 μM nicotine. Cells incubated at room temperature for the indicated times with 1 nM [3H]epibatidine and 1 μM αBGT. Data were analyzed as described in the Materials and Methods section. Values represent means ± SEM (n = 3 - 4).
Figure 11. Saturation studies on intact adrenal chromaffin cells in culture. Specific binding to total (surface and intracellular) binding sites was defined using 300 µM nicotine. Specific binding to surface binding sites was defined using 5 mM carbachol. Specific binding to intracellular binding sites was defined on alkylated cells using 300 µM nicotine. Cell were incubated at room temperature for 60 min with various concentrations of [3H]epibatidine with 1 µM αBGT. Data were analyzed as described in the Materials and Methods section. Values represent means ± SEM (n = 5 - 7).
Figure 12. Homologous epibatidine competition binding studies on adrenal chromaffin cells in culture. A. Competition binding experiments using 1 nM $[^3]$H]epibatidine were performed to determine the effects of epibatidine on total (surface and intracellular) $[^3]$H]epibatidine binding sites. Specific binding was defined using 300 μM nicotine. B. Competition binding experiments using 1 nM $[^3]$H]epibatidine were performed to determine the effects of epibatidine on intracellular $[^3]$H]epibatidine binding sites. Specific binding was defined using 300 μM nicotine on previously alkylated cells. Values represent means ± SEM (n = 4).
3.1.4 Disulfide integrity of the agonist binding site is not essential for function of adrenal α3β4* nAChRs

The importance of disulfide bridges in muscle nicotinic receptors is well established; however, for neuronal nicotinic receptors, the effects of sulfhydryl modification are less definitive. In these studies the effects of treatment with the mild reducing agent, dithiothreitol, on adrenal nicotinic receptors are described in an effort to further characterize the adrenal nAChR.

In the following studies, the effects of DTT treatment on activation of adrenal nAChRs were investigated. As seen in Figures 13, 14, and 15, after treatment with 1 mM DTT, adrenal chromaffin cells retained the ability to be stimulated by a variety of agonists, including nicotine, acetylcholine, cytisine, epibatidine, and brACh. However, changes in apparent affinities following DTT treatment were observed with some agonists and not with others. Using nicotine or cytisine as agonists, no changes in EC50 values were seen (Figure 13A and C and Table 2). However, when adrenal cells were stimulated using either epibatidine or brACh, changes in the apparent affinity of these two agonists were observed (Figure 14 and 15 and Table 2). The concentration-response curve for epibatidine was shifted to the right after DTT treatment (Figure 14), resulting in a 3-fold increase in its EC50 (Table 2). Conversely, concentration-response curve for brACh after DTT treatment was shifted to the left (Figure 15), resulting in a 20-fold decrease in its EC50 (Table 2). In addition, although changes in the concentration-response curves for acetylcholine were not readily apparent in Figure 13B, a small, but significant increase in its EC50 value occurred after DTT
treatment (Table 2). The effects of DTT on the apparent affinities of epibatidine and brACh were prevented if, prior to the addition of the agonists, DTT treatment was followed by treatment with the oxidizing agent, DTNB (Figure 14, Table 2). Also evident in these studies is that DTT treatment increased the efficacy of acetylcholine, epibatidine and brACh (Figures 13B, 14, 15, and Table 2). Although the $E_{\text{max}}$ value of nicotine appeared to increased after DTT treatment, this effect was not statistically significant (Table 2). DTT treatment had no effect on the efficacy of the partial agonist, cytisine (Table 2).

To further explore DTT-induced changes in adrenal nAChRs, the effects of DTT treatment on the ability of nAChR antagonists to inhibit brACh-induced secretion were investigated. brACh was chosen as a stimulant in these studies because the effects of DTT on brACh-induced secretion are relatively dramatic and readily apparent, as demonstrated by an overall increase in stimulated release (Figure 16). As seen in Figure 16 and Table 3, the IC$_{50}$ values for d-tubocurarine, mecamylamine, pentolinium, and hexamethonium remained unchanged after DTT treatment, indicating no changes in apparent affinities of the antagonists.

As supportive evidence for sulfhydryl reduction of adrenal nAChRs under our treatment conditions, we have investigated the effects of DTT on adrenal nAChR alkylation (Figure 17C). We have previously demonstrated that treatment of adrenal chromaffin cells with DTT (1mM) followed by treatment with the nAChR alkylating agent, brACh, results in a loss of nAChR-stimulated release which is not readily reversible (Wenger, Bryant, et al. 1997 733 /id).
Figure 17C, these effects of DTT on alkylation of adrenal nAChRs were concentration-dependent; more importantly, though, the concentrations of DTT that allow alkylation are similar to those reported to cause sulfhydryl modification of nAChRs in other systems (Stitzel et al., 1988; Dou et al., 1994). In parallel studies, the concentration-response effects of DTT on changes in apparent binding affinities of brACh and epibatidine were also investigated. As seen in Figure 17 A and B, these effects of DTT occurred at concentrations between 0.01 and 1.0 mM which are identical to those concentrations involved with nAChR alkylation.

As described above (Figures 13B, 14, 15, and Table 2), DTT treatment appears to increase the efficacy of nAChR agonists. To investigate whether these effects of DTT are related to its effects on adrenal nAChRs or through non-receptor-mediated mechanisms, the effects of DTT on basal secretion and secretion stimulated via direct depolarization of adrenal chromaffin cells were determined. As illustrated in Figure 18, DTT treatment produced concentration-dependent increases in both basal and 56 mM KCl-stimulated release, supporting non-nAChR-related effects of DTT on secretory processes. These effects, however, occurred at DTT concentrations which were higher than those affecting adrenal nAChRs. The magnitude of the increase at each DTT concentration was similar for both basal and KCl-stimulated release (Figure 18). The effects of DTT on KCl-stimulated secretion appeared to be independent of KCl concentration; i.e., the magnitude of the DTT-induced increase was similar at different KCl concentrations (Figure 19). When the relatively impermeant
oxidizing agent, DTNB, was tested for its ability to reverse these effects of DTT on basal and KCl-stimulated release, no effects of DTNB were observed (Figure 20), supporting an additional intracellular action of DTT on catecholamine release.
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<th>Emax (% total)</th>
</tr>
</thead>
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<td>27.5% (1.7%^b)</td>
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Table 2. Effects of nAChR Reduction and Reoxidation on EC50 and Emax Values for Several nAChR Agonists. ^a The EC₅₀ and Eₘₐₓ values found in this table were derived from the curves in Figures 1 and 2. The experimental design is described in figure legends 1 and 2. EC₅₀ values are expressed as geometric means (95% confidence limits) and Eₘₐₓ values are expressed as arithmetic means SEM (n = 4). ^b Statistically different from corresponding untreated control group (p<0.05)
Table 3. Effects of nAChR Reduction on IC<sub>50</sub> Values for Several nAChR Antagonists on brACh-induced Secretion. The IC<sub>50</sub> values found in this table were derived from the curves in Figure 3. The experimental design is described in figure legend 3. IC<sub>50</sub> values are expressed as geometric means (95% confidence limits). The IC<sub>50</sub> values for DTT-treated groups are not statistically different from their corresponding, nontreated control groups (p>0.05).

<table>
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<tr>
<th>Treatment</th>
<th>Antagonist</th>
<th>IC&lt;sub&gt;50&lt;/sub&gt; (µM)&lt;sup&gt;a&lt;/sup&gt;</th>
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<td>-</td>
<td>d-Tubocurarine</td>
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<td>DTT</td>
<td>d-Tubocurarine</td>
<td>0.9 µM (0.7 - 1.0)</td>
</tr>
<tr>
<td>-</td>
<td>Hexamethonium</td>
<td>40.1 µM (24.9-64.7)</td>
</tr>
<tr>
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<td>53.7 µM (31.0-93.1)</td>
</tr>
<tr>
<td>-</td>
<td>Pentolinium</td>
<td>0.5 µM (0.3 - 0.9)</td>
</tr>
<tr>
<td>DTT</td>
<td>Pentolinium</td>
<td>1.0 µM (0.6 - 1.5)</td>
</tr>
<tr>
<td>-</td>
<td>Mecamylamine</td>
<td>0.3 µM (0.2 - 0.4)</td>
</tr>
<tr>
<td>DTT</td>
<td>Mecamylamine</td>
<td>0.4 µM (0.2 - 0.9)</td>
</tr>
</tbody>
</table>

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Figure 13. The effects of DTT treatment on nicotine, acetylcholine, and cytisine stimulated adrenal catecholamine secretion. Cultured adrenal chromaffin cells were either not treated (control groups) or treated with 1 mM DTT (DTT-treated groups). The cells were then stimulated for 5 min with either nicotine (A), acetylcholine (B) or cytisine (C) at the indicated concentrations. Catecholamine release during this 5 min stimulation period was determined. Results are expressed as a percentage of the total catecholamine content (% total). Values represent means ± SEM (n = 5).
Figure 14. The effects of DTT treatment on epibatidine stimulated adrenal catecholamine secretion. Cultured adrenal chromaffin cells were either 1) not treated (control groups), 2) treated with 1 mM DTT (DTT-treated groups) or, 3) treated with DTT followed by treatment with DTNB (DTT and DTNB treated groups). The cells were then stimulated for 5 min with epibatidine at the indicated concentrations. Catecholamine release during this 5 min stimulation period was determined. Results are expressed as a percentage of the total catecholamine content (% total). Values represent means ± SEM (n = 4).
Figure 15. The effects of DTT treatment on bromoacetylcholine stimulated adrenal catecholamine secretion. Cultured adrenal chromaffin cells were either 1) not treated (control groups), 2) treated with 1 mM DTT (DTT-treated groups) or, 3) treated with DTT followed by treatment with DTNB (DTT and DTNB treated groups). The cells were then stimulated for 5 min with bromoacetylcholine at the indicated concentrations. Catecholamine release during this 5 min stimulation period was determined. Results are expressed as a percentage of the total catecholamine content (% total). Values represent means ± SEM (n = 4).
Figure 16. The effects of DTT treatment on the ability of nAChR antagonists to inhibit bromoacetylcholine-stimulated adrenal catecholamine secretion. Cultured adrenal chromaffin cells were either not treated (control groups) or treated with 1 mM DTT (DTT-treated groups). The cells were then stimulated for 5 min with 10μM bromoacetylcholine in the presence of either pentolinium (A), d-tubocurarine (B), hexamethonium (C), or mecamylamine (D) at the indicated concentrations. Catecholamine release during this 5 min stimulation period was determined. Results are expressed as a percentage of the total catecholamine content (% total). Values represent means ± S.E.M. (n = 4). 10μM bromoacetylcholine stimulated release in the absence of antagonist is represented as 1) dashed line with DTT treatment and 2) dotted line in the absence of DTT treatment.
FIGURE 17. Concentration-response effects of DTT on brACh-stimulated secretion, on epibatidine-stimulated secretion and on nAChR alkylation. (A, B) Cells were treated with the indicated concentrations of DTT to reduce nAChRs and then stimulated with either 1 μM brACh (A) or 10 nM epibatidine (B). The dashed line in each panel represents control, stimulated (non-DTT treated) secretion for each agonist. (C) Cells were alkylated (see Methods) using the indicated concentrations of DTT and then stimulated with 10 μM nicotine. The dashed line represents control, 10 μM nicotine-stimulated (non-alkylated) secretion. Results are expressed as a percentage of the total catecholamine content (% total) released under each experimental condition. Values represent means SEM (n=4-6).
Figure 18. The effects of DTT treatment on basal and KCl-stimulated adrenal catecholamine secretion. Cultured adrenal chromaffin cells were either not treated (0 mM DTT) or treated with DTT at the indicated concentrations. Cells were then incubated with PSS (basal) or stimulated using 56 mM KCl. Dashed lines represent release from nontreated, control groups for both basal and KCL stimulated release. Results are expressed as a percentage of the total catecholamine content (% total) released. Values represent means SEM (n = 4 - 6). Asterisks (*) indicates statistical difference (p < 0.05) from nontreated control groups using Dunnett's Multiple Comparison test (A) or Student's t-test (B).
Figure 19. The effects of DTT treatment on KCl-stimulated adrenal catecholamine secretion. Cultured adrenal chromaffin cells were either not treated (control) or treated with 1 mM DTT. Cells were then incubated with KCl at the indicated concentration. The 4.4 mM KCl group is equivalent to a basal, nonstimulated, group. For both studies, results are expressed as a percentage of the total catecholamine content (% total) released. Values represent means SEM (n = 4 - 6). Asterisks (*) indicates statistical difference (p < 0.05) from nontreated control groups using Dunnett's Multiple Comparison test (A) or Student's t-test (B).
Figure 20. The inability of DTNB to reverse the non-receptor mediated effects of DTT. Cultured adrenal chromaffin cells were either untreated, treated with 10mM DTT, or treated with 10mM DTT followed by 1mM DTNB. Secretion was then measured via incubation for 5 minutes either in the absence of secretagogue (basal), or by direct membrane depolarization with 56mM KCl. Results are expressed as a percentage of the total catecholamine content (% total). Values represent means ± SEM (n = 6).
3.1.5 Receptor protection assays can separate multiple populations of nAChRs in one tissue

This series of experiments was designed to see if receptor protection assays could be used to separate multiple populations of nAChRs. Receptor protection assays may prove to be a useful approach to determine the functional contributions of specific nAChRs subtypes since 1) neuronal nAChR subtypes possess distinct pharmacological properties (Luetje and Patrick, 1991; Luetje et al., 1990; McGehee and Role, 1995) and 2) adrenal nAChRs can be irreversibly alkylated with bromoacetylcholine resulting in a complete loss of functional responses (Wenger, Bryant, Boyd, and McKay, 1997). Receptor protection assays have been used previously to elucidate the functional role of different subpopulations of receptors (Furchgott, 1954; Grider and Makhlouf, 1991; Oriowo et al., 1992). In these studies, subpopulations of receptors can be protected from irreversible inactivation using receptor subtype-specific agonists and antagonists, allowing the study of the pharmacology and functional contribution of protected receptors. These experiments investigate the application of receptor protection assays to study populations of nAChRs expressed on adrenal chromaffin cells. The competitive nAChR antagonists tubocurarine, methyllycaconitine and α-bungarotoxin, and the noncompetitive antagonists hexamethonium, decamethonium, mecamylamine, pentolinium, adiphenine, tetracaine, adiphenine, and amantadine, were examined for their ability to protect receptors from alkylation.
Our laboratory has previously shown that adrenal nAChRs can be alkylated with brACh after reduction of disulfide bonds, see above and (Wenger, Bryant, Boyd, and McKay, 1997). In these studies, under alkylating conditions, brACh (30 μM) reduced nicotine-stimulated catecholamine release by 79.0% ± 4.3% (Figure 21) and 82.3% ± 2.3% (Figure 22). 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 21). Additionally, both tetracaine (100 μM) and amantadine (500 μM) produced a small, but significant, amount of protection (Figure 22). Mecamylamine (50 μM), pentolinium (50 μM), hexamethonium (500 μM), decamethonium (500 μM), and adiphenine (50 μM) afforded no protection from alkylation (Figures 21 and 22). The concentration dependency of the protective effects of tubocurarine is illustrated in figure 23. 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).

The studies described above are important since they demonstrate that tubocurarine still binds at the agonist binding site after reduction of disulfide bonds. One possible explanation for the lack of protection of the other antagonists is their inability to interact with reduced nAChRs. DTT treatment has been reported to markedly alter the ability of nicotinic agonists to bind nAChRs in several systems (Schwartz and Kellar, 1982; Stitzel, Campbell, Collins, and Marks, 1988). To address this issue, the ability of the antagonists to inhibit brACh-stimulated release after DTT treatment was investigated. brACh was chosen for the agonist in these studies because the antagonists must block
the interaction of brACh on reduced receptors for protection to be observed. We have recently shown that brACh is an effective agonist on both native and reduced adrenal nAChRs (Free, Wenger, and McKay, 2000). As illustrated in Table 4, adrenal chromaffin cells retained the ability to be stimulated with 10 μM brACh after treatment with 1 mM DTT. More importantly, mecamylamine, pentolinium, tubocurarine, hexamethonium, decamethonium, adiphenine, tetracaine and amantadine were able to inhibit brACh-stimulated secretion after DTT treatment (Table 4). These results demonstrate that the antagonists retained their ability to interact with reduced adrenal nAChRs and support their use in protection assays.

Although tubocurarine produced dramatic protection from brACh-induced alkylation, it did not provide complete (i.e., 100%) protection, even at high concentrations (Figures 21 and 23). Interestingly, the degree of protection afforded by the noncompetitive inhibitors, tetracaine and amantadine, was similar to the amount of brACh inhibition that was resistant to protection by tubocurarine (Figure 21). 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 24, no increase in protection was demonstrated when both agents were present as compared with tubocurarine alone, suggesting that both agents interact with similar nAChR populations.

α7-nAChRs have recently been implicated in secretory events in adrenal chromaffin cells (Lopez et al., 1998); however, in order to see their involvement
in secretion, rapid secretion measurements are required since these receptors quickly desensitize. Although it is likely that under our conditions, α7-nAChR involvement with secretion would be masked (Gu, Wenger, Lopez, McKay, Boyd, and McKay, 1996), additional experiments were conducted to examine the ability of α7-selective antagonists to protect against alkylation. In receptor protection assays, the α7-selective antagonists, α-bungarotoxin and methyllycaconitine, afforded no protection from alkylation when used alone (Figure 25). Furthermore, no additional protection was seen when combined with tubocurarine (Figure 25).

Since competition for binding at the site of alkylation is potentially important for protection, high concentrations of brACh may reduce the amount of observed protection. Therefore, in the next series of experiments the nAChR inhibitors were tested for the ability to protect against alkylation using lower concentrations of brACh. Intermediate (1 μM) and low (0.5 μM) concentrations of brACh reduced 10 μM nicotine-stimulated catecholamine release by 50.1% ± 2.6%, and 30.0% ± 5.5%, respectively (Table 5). 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 5).

The next series of experiments were designed to further address whether tubocurarine-protected receptors represent a subpopulation of adrenal nAChRs.
This was addressed by investigating the pharmacological profile of tubocurarine-protected receptors. The nicotine concentration-response profiles for untreated and tubocurarine-protected nAChRs are found in Figure 26 and Table 6. When compared to untreated cells, the nicotine concentration-response curve for tubocurarine-protected cells shifted to the right, suggesting a decrease in the affinity of nicotine for tubocurarine-protected receptors. The EC\textsubscript{50} for nicotine increased significantly from 4.0 \(\mu M\) (2.5 \(\mu M\) - 6.2 \(\mu M\)) in untreated cells to 9.1 \(\mu M\) (7.2 \(\mu M\) - 11.4 \(\mu M\)) in tubocurarine-protected cells (Figure 26, Table 6). There was no significant change in the \(E_{\text{max}}\) for untreated (20.7\% ± 1.9\%) and tubocurarine-protected (20.8\% ± 1.8\%) cells (Figure 26, Table 6). To further investigate whether tubocurarine-protected nAChRs represent a subpopulation of nAChRs with a higher affinity for tubocurarine, the concentration-dependency of tubocurarine on nAChR-stimulated release from untreated cells and tubocurarine-protected cells was determined. 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. The IC\textsubscript{50} 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 27, Table 6).
<table>
<thead>
<tr>
<th>nAChR Antagonist</th>
<th>CATECHOLAMINE RELEASE (% INHIBITION)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No DTT Pretreatment</td>
</tr>
<tr>
<td>50 μM mecamylamine</td>
<td>105.2 ± 2.8</td>
</tr>
<tr>
<td>50 μM pentolinium</td>
<td>103.9 ± 2.9</td>
</tr>
<tr>
<td>100 μM tubocurarine</td>
<td>97.6 ± 1.9</td>
</tr>
<tr>
<td>500 μM hexamethonium</td>
<td>100.2 ± 1.7</td>
</tr>
<tr>
<td>500 μM decamethonium</td>
<td>75.9 ± 1.4</td>
</tr>
<tr>
<td>50 μM adiphenine</td>
<td>112.2 ± 3.3</td>
</tr>
<tr>
<td>100 μM tetracaine</td>
<td>104.0 ± 5.8</td>
</tr>
<tr>
<td>500 μM amantadine</td>
<td>90.7 ± 2.3</td>
</tr>
</tbody>
</table>

Table 4. The Effects of DTT Pretreatment on the Ability of nAChR Antagonists to Inhibit brACh-induced Catecholamine Secretion. Cultured adrenal chromaffin cells were either not treated or treated with 1 mM DTT. The cells were then stimulated for 5 min with 10 μM brACh in the presence of either mecamylamine, pentolinium, tubocurarine, hexamethonium, decamethonium, adiphenine, tetracaine or amantadine at the indicated concentrations. Control stimulations (10 μM brACh) were 30.2% ± 0.9% and 20.6% ± 0.7% with and without DTT pretreatment, respectively. Values represent means ± SEM (n = 4-6).
<table>
<thead>
<tr>
<th>Protection</th>
<th>Alkylation</th>
<th>Stimulation</th>
<th>% total</th>
<th>% reduction</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>None</td>
<td>None</td>
<td>1.2 ± 0.2</td>
<td>NA</td>
</tr>
<tr>
<td>None</td>
<td>None</td>
<td>10 μM nicotine</td>
<td>21.7 ± 1.4</td>
<td>0</td>
</tr>
<tr>
<td>None</td>
<td>1 μM brACh</td>
<td>10 μM nicotine</td>
<td>11.4 ± 0.6**</td>
<td>50.1 ± 2.6</td>
</tr>
<tr>
<td>50 μM mecamylamine</td>
<td>1 μM brACh</td>
<td>10 μM nicotine</td>
<td>12.6 ± 0.6</td>
<td>44.0 ± 2.2</td>
</tr>
<tr>
<td>50 μM pentolinium</td>
<td>1 μM brACh</td>
<td>10 μM nicotine</td>
<td>11.9 ± 0.5</td>
<td>47.5 ± 1.7</td>
</tr>
<tr>
<td>100 μM tubocurarine</td>
<td>1 μM brACh</td>
<td>10 μM nicotine</td>
<td>16.0 ± 2.3*</td>
<td>28.5 ± 6.4</td>
</tr>
<tr>
<td>500 μM hexamethonium</td>
<td>1 μM brACh</td>
<td>10 μM nicotine</td>
<td>14.7 ± 0.6</td>
<td>33.8 ± 2.4</td>
</tr>
<tr>
<td>500 μM decamethonium</td>
<td>1 μM brACh</td>
<td>10 μM nicotine</td>
<td>14.4 ± 0.3</td>
<td>34.6 ± 6.1</td>
</tr>
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<td>None</td>
<td>0.9 ± 0.1</td>
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<td>None</td>
<td>10 μM nicotine</td>
<td>24.7 ± 2.5</td>
<td>0</td>
</tr>
<tr>
<td>None</td>
<td>0.5 μM brACh</td>
<td>10 μM nicotine</td>
<td>18.0 ± 3.1**</td>
<td>30.0 ± 5.5</td>
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<td>50 μM mecamylamine</td>
<td>0.5 μM brACh</td>
<td>10 μM nicotine</td>
<td>16.0 ± 2.4</td>
<td>35.3 ± 4.4</td>
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<td>0.5 μM brACh</td>
<td>10 μM nicotine</td>
<td>18.0 ± 2.3</td>
<td>29.5 ± 2.8</td>
</tr>
<tr>
<td></td>
<td>0.5 µM brACh</td>
<td>10 µM nicotine</td>
<td>17.6 ± 2.7</td>
<td>29.8 ± 4.8</td>
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<tr>
<td>--------------------------</td>
<td>--------------</td>
<td>----------------</td>
<td>------------</td>
<td>------------</td>
</tr>
<tr>
<td>100 µM tubocurarine</td>
<td></td>
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<td></td>
</tr>
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<td>500 µM hexamethonium</td>
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<td>19.6 ± 2.9</td>
<td>22.2 ± 4.1</td>
</tr>
<tr>
<td>500 µM decamethonium</td>
<td>0.5 µM brACh</td>
<td>10 µM nicotine</td>
<td>19.4 ± 2.8</td>
<td>25.7 ± 5.6</td>
</tr>
</tbody>
</table>

Table 5. Ability of nAChR inhibitors to protect nAChRs from alkylation: studies using intermediate and low concentrations of brACh for alkylation. Cultured adrenal chromaffin cells were alkylated using either 1 µM or 0.5 µM brACh in the presence of the indicated drugs. The cells were then stimulated for 5 min with 10µM nicotine. Values represent means ± SEM (n=4). * NA, 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>
<thead>
<tr>
<th>PROTECTION</th>
<th>DRUG</th>
<th>EC$<em>{50}$ or IC$</em>{50}$</th>
<th>Emax</th>
</tr>
</thead>
<tbody>
<tr>
<td>No</td>
<td>Nicotine$^a$</td>
<td>4.0 µM (2.5 - 6.2)</td>
<td>20.7 ± 1.9</td>
</tr>
<tr>
<td>Yes</td>
<td>Nicotine$^a$</td>
<td>9.1 µM (7.2 - 11.4)*</td>
<td>20.8 ± 1.8</td>
</tr>
<tr>
<td>No</td>
<td>Tubocurarine$^b$</td>
<td>660 nM (490 - 890)</td>
<td>NAc</td>
</tr>
<tr>
<td>Yes</td>
<td>Tubocurarine$^b$</td>
<td>310 nM (220 - 430)*</td>
<td>NAc</td>
</tr>
</tbody>
</table>

Table 6. Pharmacology of tubocurarine-protected nAChRs. $^a$ EC$_{50}$ and Emax values interpolated from the curves in figure 19. $^b$ IC$_{50}$ values interpolated from the curves in figure 20. $^c$ NA, not applicable. * Significant difference from corresponding unprotected, control groups (p < 0.05, Student’s t-test).
Figure 21. Ability of nAChR antagonists to protect nAChRs from alkylation. Cultured adrenal chromaffin cells were alkylated (30 μM brACH) either alone (-) or 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. Results are expressed as a percentage of the total catecholamine content (% total). Values represent means ± SEM (n = 5 - 6) and were analyzed by Dunnett's multiple comparison procedure. Treatment groups significantly different from the unprotected, alkylated group are indicated by asterisks (p < 0.05). Basal release (dotted line) was 0.9% ± 0.1%.
Figure 22. Ability of nAChR inhibitors to protect nAChRs from alkylation. Cultured adrenal chromaffin cells were alkylated (30 μM brACh) either alone (-) or 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. Results are expressed as a percentage of the total catecholamine content (% total). Values represent means ± SEM (n = 5 - 6) and were analyzed by Dunnett's multiple comparison procedure. Treatment groups significantly different from the unprotected, alkylated group are indicated by asterisks (p < 0.05). Basal release (dotted line) was 1.3% ± 0.3%.
Figure 23. 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 ± SEM (n = 6). Basal release was 0.9% ± 0.03% (dotted line). Control, nonalkylated, 10 μM nicotine-stimulated release was 21.6% ± 2.7% (dashed line). Alkylation in the absence of tubocurarine reduced 10 μM nicotine stimulated release to 3.7% ± 0.4% (dashed-dotted line).
Figure 24. Ability of tubocurarine and 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. Results are expressed as a percentage of the total catecholamine content (% total). Values represent means ± SEM (n = 6) and were analyzed by Dunnett's multiple comparison procedure. Treatment groups significantly different from the unprotected, alkylated group are indicated by asterisks (p < 0.05). Basal release was 1.5% ± 0.2% (dotted line).
Figure 25. Inability of α7-nAChR selective antagonists to protect nAChRs from alkylation by brACh. Cultured adrenal chromaffin cells were alkylated (30 μM brACh) either alone or in the presence of 1) 100 μM tubocurarine (dTC), 2) 1 μM methyllycaconitine (MLA), 3) 1 μM α-bungarotoxin (BGT), 4) 100 μM tubocurarine and 1 μM methyllycaconitine (dTC + MLA), or 5) 100 μM tubocurarine and 1 μM α-bungarotoxin (dTC + BGT), as indicated. 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 ± SEM (n = 6-8) and data were analyzed by Dunnett’s multiple comparison procedure. Treatment groups significantly different from the unprotected, alkylated group are indicated by asterisks (p < 0.05). Basal release was 2.3% ± 0.1% (dotted line).
Figure 26. Nicotine concentration-response profile of tubocurarine-protected nAChRs. Cultured adrenal chromaffin cells were either not treated (●; Control) or alkylated (30 μM brACh) in the presence (■; dTC Protected) or absence (▲; Alkylated) of 100 μM tubocurarine. Cells were then stimulated for 5 min with the indicated concentrations of nicotine. Results are expressed as a percentage of the total catecholamine content (% total). Values represent means ± SEM (N = 3 - 5).
Figure 27. Tubocurarine concentration-response profile of tubocurarine-protected nAChRs. Cultured adrenal chromaffin cells were divided into two groups. Cultured adrenal chromaffin cells were either not treated (●; Control) or alkylated (30 µM brACh) in the presence of 100 µM tubocurarine (■; dTC Protected). The cells were then washed and treated (15 min) with the indicated concentrations of tubocurarine, followed by stimulation with 10 µM nicotine in the continued presence of tubocurarine. Results are expressed as a percentage of the total catecholamine content (% total). Values represent means ± SEM (n = 4). Control nicotine-stimulated release was 20.5% ± 1.7% (dashed line). Nicotine-stimulated release from dTC protected cells was 14.4% ± 1.8% (dotted-dashed line).
3.2 $\alpha_3\beta_4^*$ nAChR turnover and regulation

Changes in expression of neuronal nicotinic acetylcholine receptors (nAChRs) have been associated with nicotine addiction. In addition, a variety of disease states have been linked to nAChRs that may also involve changes in expression of neuronal nAChRs or changes in their distribution (Lindstrom, 1997). Several processes have been described that regulate expression of neuronal nAChRs and directly influence their functional activity. The studies presented here examine the regulation of receptor turnover and expression of naturally occurring $\alpha_3\beta_4^*$ nAChRs in a native system.

Our laboratory has previously documented that nAChR-mediated functional responses from bovine chromaffin cells can be down-regulated via two paradigms: nAChR alkylation (Wenger, Bryant, Boyd, and McKay, 1997) and antigenic modulation with an anti-nAChR antibody (Gu et al., 1991; Wenger, Bryant, Boyd, and McKay, 1997). In these studies the paradigms are employed to investigate cellular and molecular mechanisms involved with regulation of expression of $\alpha_3\beta_4^*$ nAChRs.

In addition, receptor turnover is investigated with emphasis on mechanisms involved in nAChR expression and maintenance, particularly the importance of the intracellular pool in receptor regulation. Our data supports a model of receptor turnover based on constitutive receptor expression requiring de novo protein synthesis.
3.2.1 Catecholamine secretion assays to examine nAChR regulation

A primary assay used in our laboratory is the catecholamine secretion assay on cultured adrenal chromaffin cells (see above). Using the down-regulation paradigms, secretion assays can be used to investigate processes involved in nAChR turnover and/or recovery following receptor down-regulation.

Two protein synthesis inhibitors were used to investigate the importance of protein synthesis in nAChR regulation. Puromycin is a nucleoside antibiotic that inhibits protein synthesis by causing premature chain termination by acting as an analog of the 3' terminal end of aminoacyl-tRNA. Actinomycin D is an antineoplastic antibiotic that inhibits cell proliferation and prevents protein synthesis. The mechanism of action of actinomycin D includes forming a stable complex with DNA and blocking the movement of RNA polymerase which interferes with DNA-dependent RNA synthesis. Despite their different mechanisms of action, both compounds effectively inhibit de novo protein synthesis.

To investigate the involvement of nAChR protein synthesis in the nAChR turnover process, the ability of both compounds to prevent the recovery of receptor mediated function following receptor alkylation was investigated. Alkylation of nAChRs resulted in a complete loss of functional responses, which recovered within 24 hours (Figure 28 and 29). The return of secretory responses was nearly completely prevented in the presence of the protein synthesis inhibitor, puromycin (Figure 28). Under similar treatment conditions, puromycin
alone had no effect on secretion stimulated by depolarizing concentrations of KCl supporting an effect on nAChR recovery and not on a distal step in the stimulus-secretion pathway. Similar results were obtained using another protein synthesis inhibitor, actinomycin D (Figure 29). These studies support the involvement of de novo nAChR synthesis in recovery of nAChR mediated secretion after nAChR alkylation.

Similar experiments were conducted to determine the importance of protein synthesis in recovery of nAChR mediated secretion after antigenic modulation. Cultured chromaffin cells were treated with mAb35, which resulted in a partial loss of secretory function, that also recovers with time (Figure 30, 31, and 32) (Gu, Wenger, Lopez, McKay, Boyd, and McKay, 1996). When puromycin was present in the recovery period, the return of function from mAb35 down regulation was completely inhibited (Figure 30). To investigate whether the inability of mAb35 to cause complete nAChR down regulation is due to constitutive (replenishment) turnover, puromycin was added during mAb35 treatment. As illustrated in Figure 30, the addition of puromycin does not increase the number of downregulated, functional nAChRs. Similar results were obtained when actinomycin D was included in the recovery period (Figure 31). These studies support the involvement of de novo nAChR synthesis in recovery of nAChR mediated secretion after down-regulation via antigenic modulation.

To investigate the importance of protein assembly and folding, the reducing agent dithiotreitol was included in the recovery period following down-regulation via antigenic modulation. DTT has been shown to prevent the folding
of newly assembled proteins by reducing vicinal cystines. When present during the recovery period, DTT significantly inhibits recovery from mAb35 induced antigenic modulation (Figure 32). These data further support the need for new receptor protein synthesis in the recovery process.

Since the two mechanistically different methods of down regulation show similar recovery rates (Gu, Wenger, Lopez, McKay, Boyd, and McKay, 1996; Wenger, Bryant, Boyd, and McKay, 1997), recovery may be due to a process of constitutive receptor turnover, rather than stimulated receptor synthesis. If true, protein synthesis inhibition alone, in the absence of receptor down-regulation, should lower the number of functional nAChRs. To address this hypothesis, the effects of puromycin on nicotine stimulated secretion were investigated. Puromycin treatment produced a time-dependent, rightward-shift, followed by a down-ward shift in the nicotine-concentration response curve (Figure 33), supporting a time-dependent loss of functional nAChRs. This shift was reversible, as evident by a leftward and upward shift of the curves after puromycin was removed, until full recovery of function returned (Figure 34). These data are consistent with the necessity of new protein synthesis to maintain functional receptors.
Figure 28. Effects of puromycin on recovery from alkylation. Cultured chromaffin cells were first loaded with [³H]norepinephrine, as described in the Methods section. Cells were either not treated (-, control) or alkylated using 100µM brACh (ALK). Some groups of alkylated cells were then washed and incubated for 24 hrs either in culture media (ALK + 24 hr Recovery) or culture media containing 10 µg/ml puromycin (ALK + 24 hr Recovery + PUR). Following these treatments, cells were washed (5 min with PSS) and then stimulated with 10µM nicotine. Data are expressed as a percent of total cellular [³H]norepinephrine content released during the 5 min stimulation period. Values are means ± SEM (n = 4-6). Dashed line represents basal, nonstimulated, catecholamine release.
Figure 29. Effects of actinomycin D on recovery secretory function after nAChR down-regulation. Cultured chromaffin cells were first preloaded with [3H]NE, as described in the Methods section. Cells were either not treated (N2+, -) or alkylated using 100μM brACh (ALK, -). Some groups of alkylated cells were then washed and incubated for 24 hrs either in N2+ culture media (ALK, N2+) or culture media containing 10 μg/ml actinomycin D (ALK, act-D). Following these treatments, cells were washed (5 min with PSS) and then simulated with 10μM nicotine. Data are expressed as a percent of total cellular [3H]norepinephrine content released during the 5 min stimulation period. Values are means ± SEM (n = 4-6). Dashed line represents basal, nonstimulated, catecholamine release.
Figure 30. Effects of puromycin on recovery from mAb35 induced nAChR down-regulation. Cultured adrenal chromaffin cells were either 1) not treated (−, control) or 2) treated with 10 nM mAb35 for 24 hr, 3) treated with 10 nM mAb35 for 24 hr, followed by a 24 hr recovery period, 4) treated with 10 nM mAb35 for 24 hr, followed by a 24 hr recovery period in the presence of 10μg/ml puromycin, or 5) treated with 10 nM mAb35 for 24 hr in the presence of 10μg/ml puromycin. Cells were then stimulated with 10 μM nicotine. Values represent means ± SEM (n=3–4). Dashed line represents basal, nonstimulated release.
Figure 31. Effects of actinomicin D on recovery secretory function after nAChR down-regulation. Cultured chromaffin cells were first preloaded with \([^{3}H]NE\), as described in the Methods section. Cultured adrenal chromaffin cells were either 1) not treated (-, control) or 2) treated with 10 nM mAb35 for 24 hr, 3) treated with 10 nM mAb35 for 24 hr, followed by a 24 hr recovery period, 4) treated with 10 nM mAb35 for 24 hr, followed by a 24 hr recovery period in the presence of 10\(\mu\)g/ml actinomicin D. Cells were then stimulated with 10 \(\mu\)M nicotine. Values represent means ± SEM (\(n=3-4\)). Dashed line represents the level of down-regulation caused by incubation with mAb35.
Figure 32. Effects of dithiotreitol (DTT) on recovery from mAb35 induced nAChR down-regulation. Cultured adrenal chromaffin cells were either 1) not treated (-, control) or 2) treated with 10 nM mAb35 for 24 hr, 3) treated with 10 nM mAb35 for 24 hr, followed by a 24 hr recovery period, 4) treated with 10 nM mAb35 for 24 hr, followed by a 24 hr recovery period in the presence of 1mM DTT. Cells were then stimulated with 10 μM nicotine. Values represent means ± SEM (n=3-4). Dashed line represents basal, nonstimulated release.
Figure 33. Time-dependent effects of puromycin on nicotine-stimulated adrenal secretion. Cultured adrenal chromaffin cells were first loaded with [³H]norepinephrine, as described in the Methods section. Cells were then incubated either 1) for 48 hrs in culture media (control), 2) for 24 hrs in media, followed by 24 hrs in 10 µg/ml puromycin (24 hr puromycin treated), or 3) for 48 hrs in 10 µg/ml puromycin (48 hr puromycin treated). Cells were then washed and stimulated with the indicated concentration of nicotine. Data are expressed as percent net release (total-basal)(mean ± SEM) for each concentration (n = 4 - 6).
Figure 34. Time-dependent recovery of nicotine-stimulated secretion after down-regulation with puromycin. Cells were first loaded with $[^3]$H$^N$E, as described in the Methods section. Cells were then incubated either 1) with culture media for 96 hrs (control), 2) with media for 48 hr, followed by 10 µg/ml puromycin for 48 hrs (48 hr Puromycin Treated), 3) with media for 24 hrs, followed by 10 µg/ml puromycin for 48 hrs, followed by media for 24 hrs (24 hr Recovery), or 4) with 10 µg/ml puromycin for 48 hrs, followed by media for 48 hrs (48 hr Recovery). Cells were then washed and stimulated with the indicated concentration of nicotine. Data are expressed as a percentage of net stimulated release (total-basal)(mean ± SEM) for each concentration (n = 3).
3.2.2 nAChR mRNA remains constant throughout receptor downregulation and recovery

To investigate the cells ability to respond to nAChR down-regulation by changing the expression levels of nAChRs at the molecular level, northern analysis studies were conducted. These studies investigate changes in messenger RNA levels for the nAChR subunits during the downregulation and recovery process. mRNA was collected from cultured chromaffin cells immediately following nAChR alkylation and at various times during the nAChR recovery period. As demonstrated in Figure 35, no significant changes were documented in mRNA levels of the α3 or α5 subunits after down regulation and recovery. Therefore, the recovery process does not involve changes in receptor transcription. This suggests that changes in receptor expression is more likely due to either post-translational modifications similar to those previously reported (Rothhut, Romano, Vijayaraghavan, and Berg, 1996);(Keller, Lindstrom, Ellisman, and Taylor, 2001) or modifications in nAChR trafficking like those seen in recombinant cell lines (Peng, Gerzanich, Anand, Whiting, and Lindstrom, 1994).
Figure 35. Effects of alkylation induced down-regulation and recovery on nAChR subunit mRNA levels. Cells were treated with 100μM brACh under alkylating conditions and RNA was isolated either immediately, or 1, 2, 3 or 24 hours later as described in materials and methods. RNA was quantified, and expressed using time matched untreated controls.
3.2.3[^H]Epibatidine binding assays to examine nAChR regulation.

While nAChR functional studies provide a valuable technique to investigate turnover of functional nAChRs, they are not a direct measurement in the change in receptor number. In fact, changes in receptor number may be masked by the fact that chromaffin cells express spare receptors (Wenger, Bryant, Boyd, and McKay, 1997) and a small loss in receptor number may not be noticed in functional assays. In addition, catecholamine release assays do not allow one to investigate changes in the intracellular pool of nAChRs. For these reasons, intact cell binding assays were conducted to directly measure changes in nAChR expression.

To address trafficking of the intracellular pool, cultured chromaffin cells were alkylated and allowed to recover, before during and after which specific[^H]epibatidine binding was measured to both total (surface and intracellular) and surface only nAChRs. Alkylation caused a 35% ± 4% loss (Figure 36, surface + intracellular binding) of total (surface plus intracellular) specific binding. When only surface binding was measured (Figure 36, surface binding) a complete loss of binding was seen after nAChR alkylation. No specific binding was lost from intracellular nAChRs following nAChR alkylation (Figure 36, intracellular binding). 48 hours after alkylation,[^H]epibatidine binding to total (surface + intracellular) and surface only nAChRs recovered to untreated levels.

To address the trafficking mechanisms involved with the recovery of function following downregulation by mAb35, cells were first treated with 50nM mAb35 for 24 hours or with 50nM mAb35 for 24 hours followed by a 48 hour...
incubation in the absence of mAb35. [3H]Epibatidine binding experiments were then conducted on both surface and intracellular binding sites (Figure 37). Antigenic modulation with mAb35 results in no significant loss of total (surface + intracellular) binding sites. However, a small but significant loss of receptor binding sites from the cell surface with no significant change in the number of intracellular sites. Our data appears to suggest, however, a tendency for an increase in intracellular sites following mAb35 downregulation (Figure 38). This is consistent with mAb35 resulting in an internalization of the nAChR without nAChR destruction.

To address the importance of de novo protein synthesis in maintaining both the surface and intracellular pools of nAChRs, cells were treated with puromycin for 24 hours after which both total (surface and intracellular) and surface binding was measured. Effects on intracellular binding were determined by subtracting surface binding from total binding. Puromycin treatment, alone, for 24hrs resulted in a 35.4% ± 5% decrease in the number of total (surface and intracellular) binding sites. When examining only carbachol displaceable (surface only) [3H]epibatidine binding sites, puromycin treatment alone resulted in a 34.6% ± 2.8% decrease (Figure 38). When examining intracellular only binding sites puromycin treatment results in a 33.1% ± 4.1% decrease in intracellular binding sites (Figure 38). These data indicate that both surface and intracellular nAChRs are equally sensitive to down-regulation via inhibition of protein synthesis. To examine the reversibility of this effect, cells were treated with puromycin for 24 hours, and then allowed to incubate in media without

103
puromycin for 48 hours. Following this recovery period, total, surface and intracellular sites returned to untreated control levels (Figure 38), indicating that this effect was completely reversible.
Figure 36. Effects of alkylation and recovery from alkylation on adrenal nAChR binding. Binding experiments were performed on cultured bovine adrenal chromaffin cells. Total binding of the radioligand was determined either in the absence (-, Control) or presence of excess amounts (300 μM) of nicotine. Some cells were alkylated using 100 μM brACh (ALK) prior to the binding reaction. In addition, some cells were allowed to recover by incubating in culture media for an additional 38 hrs prior to the binding reaction. Data are represented as means ± SEM (n=4). Surface specific and intracellular specific binding was defined as described in methods.
Figure 37. Effects of mAb35 induced down-regulation and recovery on adrenal nAChR binding. Cultured adrenal chromaffin cells were either not treated (-, control) or treated. Some cells were treated with 10 nM mAb35 for 24hr, washed, and placed back in media to recover for 48hr. Binding assays were then performed, as described in the Methods section using 1 nM [3H]epibatidine (~67% receptor occupancy). Values represent means ± SEM (n = 3 - 4). Surface specific and intracellular specific binding was defined as described in methods.
Figure 38. Effects of Puromycin on Adrenal nAChRs. Cultured chromaffin cells were incubated either 1) with culture media for 24 hrs (-, control), 2) with culture media containing 10 µg/ml puromycin for 24 hrs (PUR), or 3) with culture media containing 10 µg/ml puromycin for 24 hrs, followed by a 48 hr recovery period in media (PUR + 48 hr Recovery). Following the treatment period, [3H]epibatidine binding experiments (2 nM, 80% receptor occupancy) were performed on the cells using either the permeable, competitive ligand, 300µM nicotine (Surface and Intracellular Binding) to measure specific binding of the radioligand to both surface and intracellular nAChRs, or the impermeable, competitive ligand, 5mM carbachol (Surface Binding) to measure specific binding of the radioligand to surface nAChRs. Treatment effects on intracellular binding (Intracellular Binding) was determined by subtracting specific binding obtained with carbachol from specific binding obtained from nicotine. Values represents mean±SEM (n = 4).
3.2.4 Puromycin treatment decreases β4 nAChR protein levels.

To ensure that the effects of puromycin on nAChR expression are due to the inhibition of nAChR protein synthesis, western blot analysis was conducted. An antibody to the bovine β4 subunit was not available. Therefore, a novel β4 polyclonal antibody (PCβ4) (see Materials and Methods), was used for western blots. The specificity for the antibody was tested using HEK293 cells heterologously expressing individual, (α3, α5, and β4) bovine nAChR subunits and was found to be specific for the β4 subunit (unpublished observation, Susan McKay). The ability of the PCβ4 protein to recognize β4 subunit protein from bovine chromaffin cells was demonstrated through immunodepletion experiments in which mAb35 was used to immunodeplete the adrenal nAChR. This immunodepleted extract did not retain a PCβ4 immunoreactive product (unpublished observation, Susan McKay).

Cultured chromaffin cells were incubated for the indicated times in media either with or without 10 μg/ml puromycin. Protein was extracted as described in methods and equivalent amounts of protein were separated via polyacrylamide gel electrophoresis, and analyzed via western blot as described above. The representative blot in Figure 39 demonstrates that the β4 subunit polyclonal antibody reacts with several proteins within the expected range for the β4 subunit (56 kD, 52.5 kD, 51 kD, 48 kD). Immunodepletion experiment as mentioned above indicates that the 51 and 52.5 kD bands likely represent β4 subunit protein.

108

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These immunoreactive protein bands are significantly decreased following treatment of the cells with puromycin for 24 and 48 hours. As may be predicted the time point where the cells were exposed to puromycin for 48 hr shows an increased level of protein depletion as compared to the 24 hr time point. It is also of interest to note that in the 48 hr time point the other bands in the region, that do not likely represent the β4 subunit, are also significantly decreased. These data indicate that puromycin treatment for 24 hr significantly lowers the levels of β4 subunit protein. This correlates with the observed loss of nAChRs after puromycin treatment.
Figure 39. Cultured chromaffin cells were either not treated (control 24hrs, control 48hrs) or treated for 24 (puro 24hrs) or 48 (puro 48hrs) hours in the presence of 10μg/ml puromycin. Following treatment cells were washed once and extracted by scraping in mammalian protein extraction buffer® (Pierce). Samples were then analyzed via western blot as described in materials and methods. Band size was determined as described in materials and methods. Data is shown as a representative blot. The four bands in the first lane starting with the top are 56 kD, 52.5 kD, 51 kD, and 48 kD.
A unifying factor of neuronal nAChR related disease is the change in receptor expression levels. Therefore it is of vital importance for these diseases that we understand how cells regulate nAChR receptor expression, so that the process can be altered. $\alpha_3$ Containing neuronal nAChRs have been implicated in a growing number of physiological and pathological roles. Despite this relatively little is known about their regulation. This is primarily due to difficulties in studying these receptors in a native tissue. The primary contributions of this study includes 1) the characterization of adrenal chromaffin cells as a native preparation expressing $\alpha_3\beta_4^*$ nAChR, 2) the finding of unique differences in the agonist binding site of this receptor subtype versus others, 3) the discovery of a novel mechanism to separate different populations of neuronal nAChRs on the same tissue, 4) the demonstration that this preparation can be used to investigate mechanisms of $\alpha_3\beta_4^*$ nAChR turnover, and 5) The finding that $\alpha_3\beta_4^*$ nAChRs undergo constitutive receptor turnover, regulated by de novo protein synthesis.

Neuronal nAChRs are a highly diverse group of ligand gated ion channels with different subunit compositions, structures, locations, functions and therefore
likely different regulatory mechanisms. In addition, most of what is known about
nAChRs is from studies looking at these receptors expressed in recombinant cell
lines, not native tissue. While recombinant systems have provided significant
advances in the characterization of nAChR subtypes, examining receptor
regulation in these non-native systems is not a good indication of natural
processes. (Sweileh et al., 2000). Therefore, it is important to establish a system
to study nAChR regulation of neuronal nAChRs in their natural environment.

Regulation of one subtype is not representative of regulation of all
neuronal nAChRs. For instance in Alzheimer's disease there is a loss of both α3
and α7 nAChR binding sites and protein, but only α7 mRNA shows any changes
(Nordberg, 2001). Additional evidence for independent regulation of neuronal
nAChR subtypes is seen when examining receptor upregulation, where different
subtypes of nAChRs show differential upregulation (Ridley et al., 2001;Wang et
al., 1998). Therefore, it is clear that regulatory mechanisms for one nAChR
subtype may not be involved in regulation of another nAChR subtype. This
underlies the importance of studying the neuronal subtypes independently of one
another. This is complicated by the fact that most tissues that natively express
neuronal nAChRs have more than one subtype of nAChR. This necessitates the
need for a highly characterized system that naturally expresses neuronal
nAChRs that can be separated from any co-expressed subtypes to allow for
individualized study.
4.2 Adrenal chromaffin cells express $\alpha_3\beta_4^* \text{nAChRs}$

Radiolabeled ligand binding assays are classical methods for characterizing receptors. This technique is particularly suited for characterization of neuronal nAChRs expressed in native tissue, since different nAChR subtypes can be classified based on their pharmacology. This study examines the binding characteristics of adrenal nAChRs and compares them to those of $\alpha_3\beta_4$ nAChRs expressed in recombinant cell lines.

Few binding studies have been reported using bovine adrenal membranes. Wilson and Kirshner (Wilson and Kirshner, 1977) were the first to perform binding studies using bovine adrenal medulla. These investigators, however, used $[^{125}\text{I}]\alpha\text{BGT}$ as the radioligand, thereby characterizing $\alpha_7$ nAChRs, which are now known to be present and functional on bovine adrenal cells (Lopez, Montiel, Herrera, Garcia-Palomero, Mayorgas, Hernandez-Guijo, Villarroya, Olivares, Gandia, McIntosh, Olivera, and Garcia, 1998). Two additional studies have characterized adrenal nAChRs using adrenal membrane preparations from cultured bovine adrenal cells (Higgins and Berg, 1988a; Lee, Miwa, Kochimura, and Ito, 1992). Both studies used the relatively low affinity radioligand, $[^3\text{H}]\text{nicotine}$, and were limited by the amount of membranes available (from cultured cells).

$[^3\text{H}]\text{Epibatidine}$ binding to adrenal nAChRs was saturable and fitting of the data via non linear regression analysis resulted in a measured $K_d$ value of 0.5nM. This $K_d$ value is similar to $K_d$ values reported for rat adrenal gland (0.2 nM) and...
superior cervical ganglia (0.1 nM) (Di Angelantonio, Nistri, Moretti, Clementi, and Gotti, 2000), rat trigeminal ganglia (0.6 nM) (Flores et al., 1996), and rat (0.3 nM) and human (0.2 nM) α3β4 nAChRs expressed in transfected cell lines (Xiao, Meyer, Thompson, Surin, Wroblewski, and Kellar, 1998; Stauderman, Mahaffy, Akong, Velicelebi, Chavez-Noriega, Crona, Johnson, Elliot, Gillespie, Reid, Adams, Harpold, and Corey-Naeve, 1998). The analysis also results in a Bmax value of 34.0 ± 3.7 fmol/mg protein, a value similar to that reported for using [3H]nicotine binding to membranes from cultured adrenal cells (Higgins and Berg, 1988a; Lee, Miwa, Kochimura, and Ito, 1992). This comparison supports the classification of these sites as nAChRs and suggests them to be α3β4* nAChRs.

As seen in Table 7, the affinities of cholinergic agonists and antagonists for adrenal nAChRs are very close to those reported by Xiao et al. using rat α3β4 nAChRs stably expressed in a transfected cell line (Xiao, Meyer, Thompson, Surin, Wroblewski, and Kellar, 1998). Binding data from the adrenal membrane preparation shows a rank order of potency for agonists (epibatidine > nicotine = cytisine > carbachol) identical to that seen in the α3β4 transfected cell line. However, some differences occur in Kᵢ values of the antagonists, mecamylamine, DHβE, and d-tubocurarine. These discrepancies may involve displacement by noncompetitive antagonists, precise subunit composition (e.g., the possible presence of an α5 subunit in adrenal α3β4* nAChRs), species differences, or differences between native versus expressed receptors. The unusual concentration-response curve of mecamylamine suggests its ability to distinguish
different nAChR subtypes. In support of this, a recent study has demonstrated that some nAChR-mediated responses are insensitive to blockade by mecamylamine (Nayak et al., 2001). These findings underlie the importance of native systems in the characterization of nAChR subtypes. Despite these differences the closeness of the pharmacological profiles supports the characterization of adrenal nAChRs as α3β4* nAChRs.

Data was also compared to binding data previously obtained through [3H]nicotine binding assays to cultured chromaffin cells. Comparisons of our K_i values for the binding of several cholinergic drugs to previously reported K_i values from cultured bovine adrenal chromaffin cells (Higgins and Berg, 1988a; Lee, Miwa, Kochimura, and Ito, 1992) are also found in Table 7. The values obtained in our studies are more comparable to those of Lee et al. (Lee, Miwa, Kochimura, and Ito, 1992) than to those of Higgins and Berg (Higgins and Berg, 1988a). The discrepancies are not readily explainable but may be due to the source of membranes (non-cultured vs. cultured), as well as, the radioligand ([3H]epibatidine vs. [3H]nicotine). The use of the lower affinity ligand, [3H]nicotine, to characterizing α3β4 nAChRs has been recently questioned (Xiao, Meyer, Thompson, Surin, Wroblewski, and Kellar, 1998). It should be noted, however, that discrepancies are also found when comparing K_i values reported by Lee et al. (Lee, Miwa, Kochimura, and Ito, 1992) to those of Higgins and Berg (Higgins and Berg, 1988a) (Table 1).
Using [\(^{3}\text{H}\)]nicotine, two classes of nAChR binding sites on bovine adrenal membranes have been described, one of which is displaceable by \(\alpha\)BGT (Higgins and Berg, 1988a). In our studies, excess \(\alpha\)BGT (10\(^{-6}\)M) was included in the binding reaction mixtures to eliminate potential [\(^{3}\text{H}\)]epibatidine binding to \(\alpha7\) nAChRs. However, when \(\alpha\)BGT competition experiments are performed in the absence of excess \(\alpha\)BGT, no displacement of binding is observed at \(\alpha\)BGT concentrations up to 10\(^{-6}\)M. These results are not unexpected since epibatidine shows a lower affinity for \(\alpha7\) nAChRs than to heteromeric nAChRs (Gerzanich, Peng, Wang, Anand, Fletcher, and Lindstrom, 1995). The \(K_i\) for MLA (1.3 \(\mu\)M) in the presence of 10\(^{-6}\)M \(\alpha\)BGT is \(-1000\) fold higher than its reported \(K_i\) for \(\alpha7\) nAChRs (Amar et al., 1993). Under our conditions, inhibition of binding by MLA is likely due to its ability to interact with adrenal \(\alpha3\beta4^*\) nAChRs. In support of this, MLA also inhibits nAChR-stimulated adrenal catecholamine secretion at similar concentrations (Bryant et al., 2002).

These [\(^{3}\text{H}\)]epibatidine binding studies are the first to pharmacologically characterize bovine adrenal nAChRs as \(\alpha3\beta4^*\) nAChRs. These studies document the utility of the easily obtainable membrane preparation from bovine adrenal medulla and [\(^{3}\text{H}\)]epibatidine for the study of native \(\alpha3\beta4^*\) nAChRs.

Another mechanism to investigate nAChR subunits is through analysis of protein expression using western blots. Since suitable antibodies for bovine nAChRs were not available, the anti-muscle nAChR antibody mAb35 and the newly constructed (see materials and methods) polyclonal antibody PC\(\beta4\) were
utilized to probe adrenal chromaffin protein. MAb35 was raised against the main immunogenic region of the muscle nAChR, but, as shown by its ability to elicit nAChR downregulation, (above and (Gu, Wenger, Lopez, McKay, Boyd, and McKay, 1996) cross reacts with bovine adrenal nAChRs. Studies using recombinant COS cells demonstrate that mAb35 cross reacts with the bovine α3 and α5. The PCβ4 polyclonal antibody was raised against the intracellular loop of the bovine β4 subunit and was found to be specific for the β4 subunit by probing recombinant HEK293 cells.

Western analysis of chromaffin protein using the monoclonal antibody mAb35 shows the presence of immunoreactive bands. While it is not certain that these bands correspond to the α3 and α5 nAChR subunits since the antibody was not directly raised against them, the band sizes are consistent with the predicted sizes of the α3 (495 amino acids) and α5 (425 amino acids) nAChR subunits. Western analysis of chromaffin protein with PCβ4, results in bands sizes 52.5 kD and 51 kD. These sizes are consistent with the predicted size of the β4 (471 amino acids) nAChR subunit. While it is unclear why these antibodies recognize more than one band, it may be due to multiple glycosylation states of the nAChR subunit, similar to that seen in other nAChR containing tissue.

Receptor binding coupled with western analysis clearly demonstrate that adrenal chromaffin cells express α3β4*nAChRs, and they are the primary mediators of catecholamine release from the adrenal medulla. These findings
document the composition of the adrenal nAChRs and support the use of bovine adrenal chromaffin cells as a model to study $\alpha_3\beta_4^*$ nAChRs in a native system.
### Table 7. Comparison of Inhibition Constants of Several Cholinergic Drugs

<table>
<thead>
<tr>
<th>RADIOLIGAND</th>
<th>DRUG</th>
<th>Kᵢ (μM)</th>
<th>Hexamethonium</th>
<th>Decamethonium</th>
<th>DHβE</th>
<th>Carbachol</th>
<th>Mecamylamine</th>
<th>DMPP</th>
<th>Cyamine</th>
<th>d-Tubocurarine</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Epibatidine</td>
<td>0.0003 ± 0.0001</td>
<td>234 ± 23</td>
<td>3.36 ± 5.8</td>
<td>2.05 ± 0.39</td>
<td>4.89 ± 0.39</td>
<td>0.401 ± 0.037</td>
<td>0.849 ± 0.18</td>
<td>0.397 ± 0.045</td>
<td>0.415 ± 0.069</td>
</tr>
<tr>
<td></td>
<td>Nicotine</td>
<td>0.0004 ± 0.0001</td>
<td>0.76 ± 0.05</td>
<td>0.89 ± 0.05</td>
<td>1.26 ± 0.23</td>
<td>0.74 ± 0.05</td>
<td>-1.01 ± 0.08</td>
<td>-1.02 ± 0.10</td>
<td>-0.89 ± 0.03</td>
<td>0.017 ± 0.0007</td>
</tr>
<tr>
<td></td>
<td>d-Tubocurarine</td>
<td>0.0001 ± 0.0001</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
</tr>
<tr>
<td></td>
<td>Cytisine</td>
<td>0.0001 ± 0.0001</td>
<td>577</td>
<td>219</td>
<td>2.89</td>
<td>0.033</td>
<td>0.19</td>
<td>0.017</td>
<td>0.017</td>
<td>0.049</td>
</tr>
<tr>
<td></td>
<td>DMPP</td>
<td>0.0001 ± 0.0001</td>
<td>1.0</td>
<td>0.33</td>
<td>0.33</td>
<td>0.033</td>
<td>0.19</td>
<td>0.017</td>
<td>0.017</td>
<td>0.049</td>
</tr>
<tr>
<td></td>
<td>MLA</td>
<td>0.0001 ± 0.0001</td>
<td>1.0</td>
<td>0.33</td>
<td>0.33</td>
<td>0.033</td>
<td>0.19</td>
<td>0.017</td>
<td>0.017</td>
<td>0.049</td>
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*Higher concentrations of aBGT was not used. No excess aBGT was included in the reaction buffer. Hill coefficients expressed as means ± SEM (n=4-5)."
4.3 Chromaffin cells express a substantial population of intracellular α3β4* nAChRs.

The studies presented use intact adrenal chromaffin cells in culture and ligand binding techniques, coupled with nAChR alkylation, to identify two populations of [3H]epibatidine binding sites: high affinity and low affinity. The high affinity sites were localized not only on the surface, but also within chromaffin cells. These studies support the classification of these high affinity surface sites, as well as intracellular sites, as nAChRs. Using the impermeant cholinergic agonist, carbachol, to define surface nAChRs, the Kd of these receptors was 1.9 nM. This value is similar to the Kd value for [3H]epibatidine (0.5 nM) using an adrenal membrane preparation as described above (Free, Bryant, McKay, Kaser, and McKay, 2002). The affinity of epibatidine for these surface nAChRs supports the classification of these sites as α3β4* nAChRs. This Kd value, though, is ~13 times lower than the apparent Kd of epibatidine for bovine adrenal nAChR (35 nM) derived from functional data (Wenger, Bryant, Boyd, and McKay, 1997) and likely represents binding to the desensitized, higher affinity state of nAChRs (Sine and Taylor, 1979).

The anti nAChR antibody, mAb35, has been shown to bind to immunoreactive proteins in ciliary ganglia cells known to express α3-containing nAChRs (Jacob et al., 1986). These intracellular mAb35 immunoreactive proteins are localized to the rough endoplasmic reticulum, golgi complexes, coated pits, coated vesicle, multivesicular bodies and smooth-membrane
vacuoles (Jacob, Lindstrom, and Berg, 1986). The composition of the intracellular mAb35-binding components has never been established, but they may at least in part represent nAChRs. (Stollberg and Berg, 1987b) similar to what has been described for muscle nAChRs (Pestronk, 1985).

Receptor alkylation presents a novel method for investigating intracellular receptors by allowing direct and permanent elimination of binding to surface receptors. Our laboratory has previously demonstrated that alkylation of cultured adrenal chromaffin cells using brACh causes an immediate and complete loss of nAChR-stimulated adrenal neurosecretion (Wenger, Bryant, Boyd, and McKay, 1997). The reduction in nAChR-stimulated secretion is likely due to a decrease in functional surface nAChRs, as demonstrated by a rightward shift, followed by a downward shift, of nicotine's concentration-response curve as the concentration of alkylation agent is increased (Wenger, Bryant, Boyd, and McKay, 1997). Several lines of evidence support the approach that alkylation eliminates only surface nAChRs: 1) the alkylation agent, brACh, contains a quaternary ammonium ion and will not likely cross cell membranes, 2) the alkylation procedure eliminates all binding using an adrenal membrane preparation demonstrating that no alkylation-insensitive sites are present, and 3) carbachol, an impermeable agent, is unable to reduce $[^3H]$epibatidine binding to cells that have first been alkylated, providing direct evidence that carbachol and alkylation compete for the same surface nAChR binding sites.

Alkylation of surface nAChRs affords us the opportunity to characterize intracellular $[^3H]$epibatidine binding sites without the complications of surface
binding. Using this approach, saturation analysis and homologous epibatidine competition experiments were performed on intracellular binding sites, defining \( K_d \) values of 3.6 nM and 5.0 nM, respectively. These values are similar to \( K_d \) value obtained for surface nAChRs. Based on these studies, it is probable that a portion of the intracellular \(^{3}\text{H}\)epibatidine binding sites represent fully assembled nAChRs since the \( K_d \) values fall within the expected range for \( \alpha 3\beta 4^* \) nAChRs (Free, Bryant, McKay, Kaser, and McKay, 2002).

It is also likely, however, that a significant portion of the intracellular binding sites are receptor species other than fully assembled pentamers. The homologous competition studies document the presence of low affinity, high capacitance \(^{3}\text{H}\)epibatidine binding sites, displaceable by nicotine. These low affinity intracellular binding sites may represent binding to receptor intermediates, mis-formed receptors, byproducts of receptor degradation and/or other unknown species. While it has been documented in muscle cells that \( \alpha \)-bungarotoxin can bind to incompletely formed receptors (Merlie and Lindstrom, 1983), it is unknown whether epibatidine can bind neuronal nAChR intermediates.

Our binding studies document the existence of intracellular nAChRs in cells expressing native nAChRs. From saturation studies, the \( B_{\text{max}} \) values for total (surface and intracellular), surface, and intracellular nAChRs were found to be ~50,000, ~11,300 and ~33,000 sites/cell, respectively, based on a plating density of 500,000 cells per culture. Therefore, based on saturation analysis, the intracellular pool appears to contain approximately 3 fold more nAChRs than are
expressed on the surface. A $B_{\text{max}}$ value of 13,000 sites/cells for intracellular nAChRs was obtained from homologous competition studies. The discrepancy between these values is likely due to the presence of the low affinity, high capacitance, binding sites. Regardless, these values document a significant population of intracellular nAChRs.

These studies represent the first demonstration of substantial intracellular nAChRs in bovine adrenal chromaffin cells. The physiological significance of stored intracellular receptor pools remains unclear, but may involved in nAChR turnover, possibly linked to a variety of disease states involving nAChRs or nicotine addiction (Lindstrom, 1997; Paterson and Nordberg, 2000). These studies also highlight the importance of high affinity intracellular binding sites that must be considered when using $[^3]H$epibatidine or other permeant radioligands to measure binding to intact cells or tissue preparations. Likewise, in studies investigating upregulation of neuronal nAChRs, it is important to differentiate changes in both surface and intracellular nAChR pools.
4.4 Adrenal chromaffin α3β4* nAChRs lack the necessity for disulfide integrity of the agonist binding site

The α subunits of nAChRs contain disulfide bonds formed between vicinal cysteines. The importance of these disulfide bonds has been demonstrated in several muscle and neuronal tissues where receptor-mediated function, channel characteristics and/or binding parameters have been characterized following treatment with mild reducing agents. These disulfide bridges in the native protein are adjacent to the agonist binding site (for review, see (Karlin and Akabas, 1995; McCarthy et al., 1986)).

The importance of disulfide bridges in muscle nAChRs was demonstrated by Karlin and Bartels (Karlin and Bartels, 1966) who showed that dithiothreitol (DTT) treatment prevents nicotinic responses in the eel electroplax. For neuronal nAChRs, however, the importance of disulfide bridges is less definitive. In some neuronal tissues, disulfide bond reduction leads to loss of nAChR-mediated functional responses (Xie et al., 1992; Sorenson and Gallagher, 1993) and alterations in nAChR binding (Stitzel, Campbell, Collins, and Marks, 1988; Aplin and Wonnacott, 1994; Rossant et al., 1994). In a few tissues, treatment of neuronal nAChRs with a mild reducing agent has no effect (Sorenson and Gallagher, 1993; Lerner-Marmarosh et al., 1995). These differences in effects of mild reduction on neuronal nAChRs may be related to the diversity of neuronal nAChRs. This leads to the hypothesis that neuronal nAChR subtypes possess varying degrees of susceptibility to chemical modification by reduction.
Most of the studies on the effects of mild reduction of neuronal nAChR involve receptors located in the CNS containing α7 or α4 subunits. Limited information is available on the effects of sulfhydryl modification on other subtypes of neuronal nAChRs, especially those receptors in the periphery. In bovine chromaffin cells, the mRNAs for the α3 and α5 subunits are expressed (Criado, Alamo, and Navarro, 1992; Campos-Caro, Smillie, Domínguez del Toro, Rovira, Vicente-Agullo, Chapuli, Juiz, Sala, Sala, Ballesta, and Criado, 1997). Sequence analysis has determined that vicinal cysteines occur at positions 213 and 214 on the α3 subunit and at positions 191 and 192 on the α5 subunit (Criado, Alamo, and Navarro, 1992; Campos-Caro, Smillie, Domínguez del Toro, Rovira, Vicente-Agullo, Chapuli, Juiz, Sala, Sala, Ballesta, and Criado, 1997).

In contrast to effects of sulfhydryl modification of nAChRs in other systems, we have found that mild reduction of adrenal nAChRs with DTT does not prevent their activation and has little or no effect on the apparent affinities of nicotine, acetylcholine and cytisine. These results are unexpected since these cysteines form disulfide bridges which are presumed to produce a highly strained confirmation at the agonist binding site. Therefore, their reduction would be expected to produce conformational changes and dramatically interfere with agonist-receptor interactions. We have, however, demonstrated changes in apparent affinities of epibatidine and bromoacetylcholine after mild reduction. These results support the presence of disulfide bonds near the agonist binding site in adrenal nAChRs and implicate a relatively minor role for these bonds in agonist interactions.

125

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The inability of DTT treatment to inhibit nAChR activation does not appear to be due to its failure to reduce receptor-associated disulfide bonds. Under our treatment conditions, adrenal nAChRs are alkylated with bromoacetylcholine after DTT treatment (Figure 17C and (Wenger, Bryant, Boyd, and McKay, 1997) which requires disulfide bond reduction. We have also demonstrated changes in apparent affinities of epibatidine and bromoacetylcholine after DTT treatment which are reversed when adrenal nAChRs are treated with the oxidizing agent, DTNB. These observations are consistent with DTT reducing disulfide bonds and producing conformational changes that affect these compounds' abilities to bind the receptor. Finally, disulfide reduction is further supported by the similarity of the DTT concentration-response curves to those reported to cause sulfhydryl modification (Stitzel, Campbell, Collins, and Marks, 1988).

In our studies, DTT treatment produced changes in the apparent affinities of two agonists. The increase in apparent affinity of brACh is most likely due to covalent attachment of brACh to the binding site after nAChR reduction (Brown and Kwiatkowski, 1976). The observed decrease in apparent affinity of epibatidine is interesting and suggests that the binding of epibatidine is more dependent on disulfide bridges; however, the precise mechanism mediating this change is unclear. No changes in the apparent affinities of several nAChR antagonists were seen after mild reduction of adrenal nAChRs. One problem involved with interpretation of the antagonists' data is that brACh will covalently bind to reduced nAChRs, affecting its EC₅₀ (see above) and potentially interfering with antagonist competition for binding. In addition, the mechanisms by which
these antagonists block activation of adrenal nAChRs (competitive or noncompetitive) may complicate interpretation. Regardless of their mechanism of action, these data suggest that disulfide integrity is not essential for interactions of these antagonists with adrenal nAChRs.

Treatment with DTT significantly increased the $E_{\text{max}}$ values of acetylcholine, epibatidine, and bromoacetylcholine (Table 1). An increase in secretion was also observed for nicotine, but this effect was not significant, possibly due to receptor desensitization at high agonist concentrations (see Materials and Methods section). The ability of DTT to enhance secretion is likely due to an intracellular action of DTT and is independent of activation of adrenal nAChRs. We found that DTT treatment also increased non-receptor mediated adrenal catecholamine secretion. These effects were seen at DTT concentrations higher than those necessary for nAChR reduction and were not reversed by the relatively impermeable oxidant, DTNB. These results suggest that DTT has additional effects that alter intracellular events involved with secretory processes. Several studies in muscle have documented actions of sulfhydryl reagents on excitation-contraction coupling and Ca$^{2+}$ mobilization (for example, (Oba et al., 1996;Aghdasi et al., 1997). It is conceivable, then, that in adrenal chromaffin cells DTT reduces disulfide bonds of proteins involved with exocytosis and that these alterations serve to enhance both basal and stimulated secretory responses; however, our studies do not rule out other effects of DTT.

As mentioned previously, the importance of disulfide bonds in neuronal nAChRs is not definitive. One possible explanation for nonuniformity for DTT's
effects is that different subtypes of nAChRs, based on subunit composition, show varying degrees of susceptibility to reduction by DTT; i.e., the effects of DTT may depend on the types of subunits contributing to the functional receptors (α2-α9 or β2-β4) or their particular combination. In mouse and rat brain, DTT treatment causes significant reduction of binding to presumably α4β2 nAChRs (Stitzel, Campbell, Collins, and Marks, 1988; Aplin and Wonnacott, 1994). Stitzel et al. found that DTT treatment produces no significant changes in maximum α-bungarotoxin binding (presumably to α7 nAChRs) but did increase α-bungarotoxin's K0 (Stitzel, Campbell, Collins, and Marks, 1988). Sorenson and Gallagher (Sorenson and Gallagher, 1993) reported that DTT blocks excitatory responses from rat medial vestibular nucleus neurons known to contain the α2, α3, α4, and β2 mRNA; however, neither DTT treatment nor subsequent alkylation interferes with the inhibitory nicotinic response seen in rat dorsolateral septal neurons that do not contain α2, α3, or α4 mRNA. Other studies have implicated different areas of the brain showing varying susceptibilities to changes in nAChR binding following treatment with DTT (Lerner-Marmorosh, Kende, Wang, and Abood, 1995). In our studies we have demonstrated that DTT treatment does not prevent activation of bovine adrenal nAChRs, which are thought to contain α3, α5, and β4 subunits. Taken together, these results suggest that nAChRs containing α4 and/or β2 subunits are more sensitive and those receptors containing α5 and/or β4 subunits are less sensitive to the effects of sulfhydryl reduction. It should be noted, though, that interpretation of literature data is complicated by the fact that changes in affinity may be perceived as a loss of

128

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function when only one concentration of an agonist is tested. For example, in rat 
PC12 cells DTT causes a substantial loss of carbachol stimulated $^{22}$Na$^+$ uptake 
(Leprince, 1983); this loss of function may be explained by a reduced affinity of 
carbachol for its receptor.

In summary, the studies presented here document that disulfide integrity is 
not essential for activation of adrenal nAChRs. The involvement of particular 
subunits in neuronal nAChR sensitivity to reduction, however, remains to be 
established. It is well documented, though, that neuronal nAChR subtypes 
possess distinct pharmacological properties and that both $\alpha$ and $\beta$ subunits 
contribute to the pharmacological properties of each subtype (Luetje, Wada, 
Rogers, Abramson, Tsuji, Heinemann, and Patrick, 1990; Luetje and Patrick, 
1991). Therefore, it is likely that different neuronal nAChR subtypes may also 
possess varying degrees of susceptibility to chemical modification by mild 
reduction based on subunit composition. Studies using in vitro expression 
systems should provide additional insights into this problem.
4.5 Receptor protection assays can be utilized to investigate multiple nAChR populations.

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 Makhlouf, 1991; Oriowo, Nichols, and Ruffolo, 1992). Reports that neuronal cells possess multiple nAChRs populations (Alkondon and Albuquerque, 1993; Conroy and Berg, 1995; Conroy et al., 1992; Listerud et al., 1991; Vemallis et al., 1993) and that nAChR subtypes, as distinguished by subunit contribution, possess distinct pharmacological properties (Cachelin and Rust, 1994; Cachelin and Rust, 1995; Luetje and Patrick, 1991; Luetje, Wada, Rogers, Abramson, Tsuji, Heinemann, and Patrick, 1990) support our approach. In our studies, we demonstrate that the nAChR antagonist, tubocurarine, can effectively protect adrenal chromaffin cells from loss of nAChR-mediated catecholamine release caused by the alkylation of reduced nAChRs by brACh.

nAChR antagonists were used in our receptor protection studies since these agents do not produce receptor desensitization as do nAChR agonists. 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.

Our studies investigate the protective properties of a variety of nAChR antagonists, which have been traditionally classified as either neuromuscular (decamethonium, tubocurarine) or ganglionic (hexamethonium, mecamylamine, pentolinium) blocking agents. All of these agents have been demonstrated to inhibit catecholamine release from cultured bovine adrenal chromaffin cells (McKay and Burkman, 1993). Theoretically, protection from alkylation would best be achieved using a drug that interacts competitively with the agonist binding site (i.e., competitive inhibitors). However, the interactions of many of these antagonists with nAChRs are complex and their classification as either competitive or noncompetitive may depend on several factors including species, tissue and/or nAChR subtype (Cachelin and Rust, 1994; Nooney et al., 1992). For these reasons a variety of blocking agents was investigated.

Tubocurarine's EC_{50} value of 4 μM as a protective agent is similar to the IC_{50} values for its inhibitory effects on nAChR-stimulated catecholamine release from cultured adrenal chromaffin cells (McKay and Burkman, 1993), and about 10 fold higher that its Kd value for [3H]epibatidine binding to bovine adrenal membranes. Tubocurarine's interaction with nAChRs is complex; it has been reported to be either a competitive inhibitor or an ion channel blocker, depending on the species and/or the type of nAChR (Cachelin and Rust, 1994; Nooney, 131
Lambert, and Chiappinelli, 1992). Patch clamp studies in bovine adrenal chromaffin cells have shown that tubocurarine is an antagonist that also has some agonist properties (Nooney, Lambert, and Chiappinelli, 1992) which would implicate an action of tubocurarine at the agonist binding site and explain its protective activity. The lack of protective effects of hexamethonium may be explained by its noncompetitive interactions with adrenal nAChRs. Although nAChR binding studies in adrenal cells have shown that hexamethonium inhibits $[^3H]$nicotine binding with a $K_i$ value of 0.6 mM (Lee, Miwa, Kochimura, and Ito, 1992), and our binding studies with $[^3H]$epibatidine demonstrate a $K_i$ value of $>1$ mM, others have reported that hexamethonium acts through noncompetitive mechanisms in adrenal chromaffin cells (Nooney, Lambert, and Chiappinelli, 1992). Similar conclusions have been reported by Ascher et al. (Ascher et al., 1979). The noncompetitive nAChR blockers, tetracaine and amantadine, provided a small, but significant amount of protection from alkylation. Some noncompetitive inhibitors could provide protection by either blocking access to, or allosteric alterations of, the site of alkylation.

Our studies also address questions concerning the integrity of the agonist binding site after 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 (Schwartz and Kellar, 1982; Stitzel, Campbell, Collins, and Marks, 1988). Consistent with the above described disulfide integrity studies, we have found that adrenal nAChRs retain the ability to be stimulated after reduction (Table 1). Although not a common
phenomenon, other reports exist in the literature that describe nicotinic responses after nAChR reduction (Sorenson and Gallagher, 1993; Stitzel, Campbell, Collins, and Marks, 1988). Furthermore, the antagonists used in our studies still interact with adrenal nAChRs after disulfide bond reduction (Table 1). This is important because it shows that the antagonists are able to bind after the disulfide bonds are reduced and therefore should still be able to offer protection. It is essential that any compound tested in this protection assay be able to interact with reduced receptors, since the receptor is in a reduced state during the protection procedure. For this reason every compound used in the protection assay must be tested to ensure interaction with reduced nAChRs. The ability of these cholinergic agents to interact with reduced adrenal nAChRs may be explained by the subunit composition of adrenal nAChRs; different subtypes of nAChRs may show varying degrees of susceptibility to reduction (Lerner-Marmarosh, Kende, Wang, and Abood, 1995; Sorenson and Gallagher, 1993; Stitzel, Campbell, Collins, and Marks, 1988).

The identity of the population of tubocurarine-protected nAChRs remains to be established. One possibility is that, under our alkylating conditions, complete protection may not be attainable. Another possibility is that the protected nAChRs represent a subpopulation of adrenal nAChRs. Several observations support the existence of at least two subpopulations of adrenal nAChRs that can be differentiated using tubocurarine. First, we have shown that the maximum amount of protection afforded by tubocurarine does not produce protection for all adrenal nAChRs. The selective protective properties of
tubocurarine is supported by studies showing that the effects of tubocurarine on β4-containing nAChRs differ dramatically when compared to β2-containing nAChRs (Cachelin and Rust, 1995). 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 differing pharmacology. It cannot be ruled out, however, that the changes observed in apparent affinities are due to the elimination of a nAChR reserve after alkylation. We have previously reported that adrenal chromaffin cells contain a nAChR reserve (Wenger, Bryant, Boyd, and McKay, 1997) and losses of receptor reserves can produce changes in apparent affinities of agonists and antagonists (Zhu, 1991).

The studies reported here support the use of receptor protection assays as a means to differentiate functional roles of nAChR expressed in neuronal cells containing heterogeneous populations of nAChRs. A problem of these studies is the lack of availability of selective agents for use in protection assays. The development of more selective drugs for nAChR subtypes may provide the tools needed to dissect subtypes in receptor protection assays. Receptor protection assays should provide a valuable tool to study multiple populations of nAChRs.
4.6 Receptor downregulation paradigms can be used to study of \( \alpha 3\beta 4^* \) nAChR turnover mechanisms.

Two distinct mechanisms were exploited in our laboratory to study nAChR turnover by causing nAChR down regulation. These include receptor alkylation which results in a complete and immediate loss of nAChRs (see results) and receptor mediated function (see results and (Wenger, Bryant, Boyd, and McKay, 1997)). The anti-nAChR monoclonal antibody mAb35, results in a time dependent partial loss of both receptors (see results) and receptor related function (see results and (Gu, Wenger, Lopez, McKay, Boyd, and McKay, 1996). These paradigms provide a unique opportunity to investigate nAChR turnover by measuring the return of nAChRs and nAChR mediated function.

While both paradigms down regulate nAChRs, only alkylation results in a complete, immediate, and irreversible down regulation of exclusively surface receptors. On the other hand, mAb35 treatment is a much slower process that results in only a partial down regulation that takes hours to complete. Mab35 likely causes downregulation via antigenic modulation resulting in receptor internalization (Higgins and Berg, 1988b). The rapidity and completeness of the alkylation paradigm result in a more defined, and therefore more useful tool for investigating receptor turnover. For this reason the majority of the receptor turnover assays were conducted using the alkylation paradigm.

The inclusion of pharmacological compounds in the recovery process allows for the deduction of mechanisms involved with receptor recovery. The ability of a compound to prevent recovery suggests an importance in the function
blocked by that compound. Using this rationale, inhibitors of protein synthesis (puromycin and actinomycin D) were examined for their ability to prevent nAChR recovery processes. These compounds completely inhibit recovery of functional nAChR following downregulation by either paradigm, supporting the necessity of protein synthesis events in nAChR turnover.
4.7 Adrenal $\alpha_3\beta_4^*$ nAChRs undergo constitutive turnover governed by de novo protein synthesis.

Our data suggest that the regulation and maintenance of adrenal $\alpha_3\beta_4^*$ nAChRs is governed by constitutive de novo protein synthesis which results in a constant turnover of nAChRs. This model incorporates the following findings: 1) adrenal chromaffin cells contain a substantial population of fully assembled intracellular nAChRs, 2) both surface and intracellular nAChR pools are downregulated via protein synthesis blockade, 3) mRNA levels are not altered during the down regulation or recovery process, 4) recovery of the surface receptors from downregulation via two different paradigms occurs during the same time frame and back to untreated levels, 5) puromycin prevents the recovery of function after receptor downregulation.

While the precise physiological role of a fully assembled intracellular pool remains to be determined, intracellular nAChR pools in other tissues can be modulated (Stollberg and Berg, 1987a; Rothhut, Romano, Vijayaraghavan, and Berg, 1996), and are likely involved with the receptor turnover process. The intracellular $\alpha_3\beta_4^*$ nAChRs found in our tissue may consist of as many as three distinct pools; 1) 'old' nAChRs that were once on the cell surface and have since been internalized, 2) 'new' nAChRs destined for immediate surface expression, or 3) nAChRs stored as an independent intracellular pool. Experimental design can potentially separate these pools to examine their individual trafficking and importance in the turnover process.
It is unlikely that many of the intracellular receptors were once expressed on the cell surface. This hypothesis is supported by the fact that intracellular nAChRs maintain a high affinity for the radioligand. Internalized receptors would be expected to degrade more rapidly than they are internalized (Xu and Salpeter, 1999) and therefore would not likely represent a significant amount of high affinity binding. In addition, it is not likely that newly internalized old receptors would be sensitive to the blockade of new protein synthesis and the vast majority of the intracellular pool is sensitive to downregulation by puromycin. Therefore, it is unlikely that the high affinity intracellular nAChRs represent old 'recycled' receptors, internalized from surface pools.

Only a small number of intracellular receptors are targeted for immediate expression on the cellular surface. Studies investigating nAChR recovery of function and binding sites following downregulation document that de novo protein synthesis is essential for the recovery. If the previously formed intracellular receptor pool was destined for surface expression, they should traffic to the cell surface after receptor alkylation. But, puromycin completely prevents recovery of function from down regulation. These data suggest that the intracellular pool of receptors does not play a primary role in recovery after alkylation since new receptor synthesis is required. These findings are consistent with previous studies in other systems that suggest only a small percentage of the intracellular binding sites in chick ciliary ganglion neurons (Stollberg and Berg, 1987b) or muscle cells (Pestronk, 1985) are destined for surface expression.
Fully assembled intracellular α3β4* nAChRs likely represent an independent 'reserve' pool. While it is not clear what the function of a reserve pool is, it may be that cells make nAChRs inefficiently, making many more nAChRs than can be expressed at the surface. Treatment of cultured chromaffin cells results in a time dependent loss of the intracellular pool, consistent with a steady feeding and breakdown of this pool during nAChR turnover. It is also possible that cells have these reserve nAChRs to respond to events very quickly. It is plausible that under some conditions these receptors may be targeted to the surface to affect nAChR expression faster that would be possible if new synthesis were required. We do not however, have any evidence to suggest this is the case.

A more intriguing possibility is that these receptors lack the necessary signals to be targeted for surface expression and are therefore, shunted away from the trafficking pathway. This is suggested by studies showing muscle nAChRs must interact with certain regulatory elements in the endoplasmic reticulum to be directed to the cell surface (Keller, Lindstrom, Ellisman, and Taylor, 2001). Regardless our data suggests that intracellular α3β4* nAChRs do not contribute to normal nAChR turnover and likely are an independently regulated 'reserve' pool. It is interesting however to speculate that there may be a mechanism to influence nAChR expression by trafficking intracellular α3β4* nAChRs to the cell surface.
Constitutive protein synthesis maintains a steady level of receptor expression. When protein synthesis is inhibited in these cells, for a chronic period of time, there is a reduction in surface binding sites. In addition, there is a time dependent shift and loss of function in the nicotine concentration response curve when the cells are treated with puromycin. These effects are not due to a loss of cells or a generalized toxicity since, when the inhibitor is removed both receptor function and binding return to pretreatment levels. In addition, previous studies have shown that in the continued presence (48 hrs) of the protein synthesis inhibitors actinomycin D or cyclohexamide cell survival for cultured chromaffin cells is at least 70-80% (Cardenas et al., 1995). These findings support the necessity for de novo protein synthesis in the maintenance of receptor number on the cell surface, implying a constitutive turnover of nAChRs.

Puromycin treatment lowers the levels of nAChR protein. Since it is possible that in these studies puromycin is acting through a mechanism other than inhibition of nAChR protein synthesis, western analysis was conducted to demonstrate that puromycin treatment lowers the levels of β4 subunit protein under our treatment conditions. Since the β4 subunit is present in the receptors examined in this study (Free, Bryant, McKay, Kaser, and McKay, 2002), we chose to focus on changes in β4 protein during the down regulation and turnover of receptors. The polyclonal antibody recognizes several bands within the expected range for the β4 subunit, all of which are decreased in puromycin treated cells. The precise identity of each specific band is unknown but may
represent different forms/glycosylation states of the β4 subunit. While it is unclear from our studies if the other subunits are also decreased by puromycin treatment, the loss of β4 protein is a representative loss of nAChR subunits. The loss of α3 protein was not examined due to the unavailability of a suitable bovine α3 specific antibody.

A basic understanding of cellular and molecular mechanisms involved with nAChR expression, down regulation and turnover are of critical importance. These studies significantly add to the current understanding of how neuronal cells regulate nAChR number and turnover. Further investigation of this pathway will likely provide novel targets for the manipulation of receptor number such as chaperone proteins, or other post-translational modulators that underlie receptor trafficking.
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145

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APPENDICES
APPENDIX A

Ring E analogs of Methyllycaconitine (MLA) as Novel Nicotinic Antagonists

This work was completed through a collaboration between our laboratory (including R. Benjamin Free and Dennis B. McKay, Ph.D.) and the laboratory of Stephen C. Bergmeier (including David J. Lapinsky and Stephen C. Bergmeier, Ph.D.). The work was done in the College of Pharmacy, Division of Pharmacology and Division of Medicinal Chemistry, at The Ohio State University. My contributions to this work include testing of these compounds on nicotine-stimulated catecholamine release and aiding in the preparation of the manuscript. This manuscript is published in *Bioorganic & Medicinal Chemistry Letters*. Volume 9, pages 2263-2266 (1999). The manuscript is presented below in a format suitable for publication in this journal.

Citation:

We have prepared ring E analogs of the diterpenoid alkaloid methyllycaconitine. These compounds have been assayed for nicotinic activity and were found to act as functional antagonists on adrenal nicotinic receptors.

Neuronal nicotinic acetylcholine receptors (nAChR) are located throughout the central and peripheral nervous systems, which include several different regions of the brain, spinal cord, retina, ganglia, and adrenal medulla. These receptors are composed of multiple subunits that have been divided into two general classes: α subunits and β subunits. Currently, eight neuronal nAChR α subunits (α2, α3, α4, α5, α6, α7, α8, and α9) and three neuronal nAChR β subunits (β2, β3, and β4) have been described (Lindstrom, 1997). Recent evidence has documented that some neuronal tissues express not only multiple nAChR subunits, but also multiple subtypes of neuronal nAChRs, based on specific subunit composition. The existence of multiple subtypes of neuronal nAChRs has many important physiological implications (Holladay et al., 1997, McGehee, 1995). A specific pattern of sensitivity to cholinergic agonists such as acetylcholine, nicotine, dimethylphenylpiperazinium (DMPP), and cytisine has been demonstrated with various nAChR subtypes (Luetje et al., 1991). We report here our synthesis of a new class of nicotinic antagonists (Figure 40-1) based on the alkaloid methyllycaconitine (MLA, Figure 40-2) as well as preliminary biological data on a select group of compounds.

MLA is extremely interesting as a lead compound for the development of new nAChR antagonists. MLA is the most potent nonpeptide nAChR antagonist.
currently known and is reported to selectively act at α7 nicotinic receptors (Ward, et al., 1990, Wonnacott et al., 1993). Methyllycaconitine (MLA) is one member of a larger family of diterpene alkaloids (Pelletier et al. 1991). These structurally similar molecules have been isolated from plants of the genera Aconitum and Delphinium. Both of these families of plants have a long history as a source of poisons and medicinal agents. MLA and a few others are unique in that they are potent and selective ligands for the nAChR.

Several structurally less complex analogs of MLA (Figure 40-3 for example) have been synthesized both as analogs of MLA as well as part of a partial synthesis of MLA (Baillie et al., 1994, Coates et al. 1994, Coates et al. 1996, Kraus et al. 1998, Trigg et al. 1998, Grangier et al. 1998). Most of the synthetic efforts have focused on the preparation of the A/E bicyclic ring system of the alkaloid. Only one report of the biological activity of any analogs has been published (Davies et al., 1997). In this report the analog in figure 40-3 was reported to have an IC$_{50}$ of 107 μM. A related A/E/B tricyclic analog had an IC$_{50}$ of 478 nM. Reports on the SAR of MLA indicate that the succinimide moiety is important for optimal activity at the nAChR (Blagbrough et al. 1994, Coates et al. 1996). Also of importance is the methyl group on the succinimide ring (Jacyno et al. 1996).

As one can appreciate from looking at the structure of MLA, a number of analogs of MLA could conceivably be prepared retaining the essential elements of the structure. One of the simplest analogs that might be prepared are analogs
of ring E (a piperidine ring, Figure 39-1). For our initial study we have chosen to examine the effect of different groups on the nitrogen of the piperidine ring.

We have prepared a series of analogs of MLA by the route shown in Scheme 1 (Figure 41). In this initial series of analogs we wished to look at two areas. The major area of investigation is substitution on the nitrogen of the piperidine ring. A second area of investigation was methyl substitution of the succinimide ring. To this end we have prepared two different anthranilate derivatives (Figure 41-5a and 5b) for coupling to the piperidine ring. The synthesis of both Figure 41-5a and 41-5b followed reported routes. The synthesis of Figure 41-5a while straightforward was quite low yielding (Jacyno et al. 1996). The synthesis of Figure 41-5b involved fusing methylsuccinic anhydride with anthranilic acid. This procedure gave an excellent yield of the desired imide (Sheehan et al. 1951, Kraus et al. 1998). All attempts to prepare Figure 41-5a by this method were unsuccessful. They synthesis of the piperidine portion of Figure 41-1 was addressed as follows, starting form the commercially available hydroxymethylpyridine (Figure 41-6), a pyridinium salt (Figure 41-7) was prepared in excellent (74-100%) yield. The pyridinium salt was then reduced (84-100% yield) to provide the piperidine salt (Figure 41-8). A variety of palladium and platinum catalysts were examined, only PtO2 gave consistently good yields. Coupling of (Figure 41-8) to the anthranilic acid derivatives (Figure 41-5a or 41-5b) produced our target compounds. The use of 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate (TBTU) as a coupling agent proved to be the most convenient route to the desired esters.
The yields for this coupling reaction were quite inconsistent, ranging from 6% to 60%. We were able to obtain sufficient material for our biological evaluations. Our target compounds 1a-1h (Table 8) were converted to their water soluble hydrochloride salts prior to biological evaluation.

In the studies we have used cultured bovine adrenal chromaffin cells as a neuronal model to study the functional effects of the MLA analogs. These cells contain several nAChR subunit genes, including the α3, α5, α7, and β4 genes and express multiple nAChR subtypes (Criado et al. 1992, Garcia-Guzman et al. 1995, Campos-Caro et al. 1997, Wenger et al. 1997). In cultural chromaffin cells, mAb35-nAChRs, which are believe to contain α3, α5 and β4 subunits are reported to be the principal receptors that mediate adrenal catecholamine secretion (Gu et al. 1996). In addition, α7-containing nAChRs are also expressed and recently these receptors have been reported to stimulate adrenal secretion (Lopez et al. 1998).

The relative efficacies of the MLA analogs are shown in Table 8. Compound 1a, which is a direct analog of MLA, produced moderate inhibition of nAChR-stimulated catecholamine release. Shortening (1b) or increasing the length of the N-alkyl chain (1c) resulted in no significant change in antagonist activity. Placement of an oxygen in the alkyl chain (1f) which should inductively decrease the basicity of the nitrogen also produced no significant change in antagonist activity. The preparation of the N-iPr analog (1e) significantly increased inhibition activity when compared to 1a. We felt that this compound
would be an excellent compound to assay for the importance of the methyl group on the succinimide. Thus, compound 1f was prepared and showed a marked reduction in potency. This is consistent with the work of Jacyno who reported a marked decrease in activity of MLA that lacked the methyl group on the succinimide (Jacyno et al. 1996). Feeling that larger alkyl groups might produce more potent antagonists, we prepared two further analogs 1g and 1h showed excellent activity being almost as efficacious as MLA at the concentration tested.

It is important to note that all of the compounds tested were racemates and mixtures of diastereomers yet showed significant activity as antagonists. It is also significant that these compounds show activity on adrenal nAChRs in the micromolar range. These compounds have also demonstrated selectivity for nAChR-stimulated secretion; they had no effect on release stimulated by direct depolarization with elevated levels of KCl (data not shown). The preparation of diastereomerically and enantiomerically pure compounds should lead to significantly more potent compounds.

We have discovered a simple analog (Figure 40-1) of methyllycaconitine (MLA, Figure 40-2) that acts as a micromolar inhibitor at the nAChR. This should be a fertile area from which to find new selective and potent antagonists of subtypes of the nAChR. The goal of our initial work is to quickly outline a SAR (structure-activity relationship) of this new lead compound to assess structural requirements necessary for potency and selectivity. This will a rational, rapid analysis of key features in MLA to arrive at a range of lead compounds for further development, and lead to new pharmacological tools.
<table>
<thead>
<tr>
<th>Compound</th>
<th>R₁</th>
<th>R₂</th>
<th>Yield</th>
<th>Nicotine-stimulated catecholamine release (% inhibition)¹,²</th>
</tr>
</thead>
<tbody>
<tr>
<td>1a</td>
<td>Me</td>
<td>Et</td>
<td>22%</td>
<td>33.5% ± 4.3%</td>
</tr>
<tr>
<td>1c</td>
<td>Me</td>
<td>Me</td>
<td>35%</td>
<td>30.1% ± 4.4%</td>
</tr>
<tr>
<td>1c</td>
<td>H</td>
<td>NBu</td>
<td>24%</td>
<td>38.4% ± 10.1%</td>
</tr>
<tr>
<td>1d</td>
<td>Me</td>
<td>EtOEt</td>
<td>37%</td>
<td>45.9% ± 3.7%</td>
</tr>
<tr>
<td>1e</td>
<td>Me</td>
<td>IPr</td>
<td>18%</td>
<td>56.5% ± 7.2%</td>
</tr>
<tr>
<td>1f</td>
<td>H</td>
<td>IPr</td>
<td>35%</td>
<td>36.4% ± 4.8%</td>
</tr>
<tr>
<td>1g</td>
<td>Me</td>
<td>Ph(CH₂)₂</td>
<td>60%</td>
<td>37.0% ± 7.5%</td>
</tr>
<tr>
<td>1h</td>
<td>Me</td>
<td>Ph(CH₂)₃</td>
<td>15%</td>
<td>86.3% ± 4.2%</td>
</tr>
<tr>
<td>2 (MLA)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>95.2% ± 1.9%</td>
</tr>
</tbody>
</table>

Table 8. Yields and nicotinic antagonist properties of target compounds.  
¹Cultured adrenal chromaffin cells were isolated and cultured as described previously (Mauer and McKay 1994).  
²Cells were either not treated (control groups) or treated for 15 min with 50μM concentrations of the compounds to be tested.  
The cells were then stimulated for 5 min with 10 μM nicotine in the continuous presence of the compounds.  
Catecholamine release during this 5 min stimulation period was determined McKay and Schneider 1984).  
Results are expressed as a percentage of the inhibition of control nicotine-stimulated release (% inhibition).  
Values represent means ± SEM (N=3-6).
Figure 40. Structure of ring E analog of MLA (1), MLA (2), and ring A/E analog (3)
Scheme 1

(a) $R_1 = H$, succinic anhydride, toluene, Et$_3$N, reflux, Dean-Stark, 15%.
(b) $R_1 = \text{Me}$, methylsuccinic anhydride, neat, 145 °C, 0.1 mm Hg, 3 h, 69%.
(c) $R_2 = X$, CH$_3$CN, reflux, 20 h. 7a, Et-I, 90%; 7b, Me-I, 100%; 7c, Pr-Br, 76%; 7d, nBu-Br, 100%; 7e, Br-CH$_2$-I, 84%; 7f, PhCH$_2$CH$_2$-I, 74%; 7g, PhCH$_2$CH$_2$CH$_2$-I, 100%.
(d) H$_2$ (1 atm balloon), PtO$_2$ (10% w/w), EtOH. 8a, 95%; 8b, 100%; 8c, 92%; 8d, 94%; 8e, 84%; 8f, 95%; 8g, 100%.
(e) TBTU, 8a-g, 2Pr$_2$NEt, CH$_3$CN. For yields see Table 1.

Figure 41. Synthesis scheme for ring E analogs of MLA
APPENDIX B

Structure-activity Studies with Ring E Analogues of Methyllycaconitine on Adrenal $\alpha_3\beta_4^*$ Nicotinic Receptors.

This work was completed through a collaborative effort between Dr. Dennis McKay's laboratory (including Darrell L. Bryant, M.S., R. Benjamin Free, Sara M. Thomasy, Susan B. McKay, and Dennis B. McKay, PhD), and the laboratory of Dr. Stephen Bergmeier (including David J. Lapinsky, Kadiga A. Ismail, and Stephen C. Bergmeier, PhD). The work was conducted at both the College of Pharmacy at The Ohio State University and the Department of Chemistry and Biochemistry at Ohio University in Athens Ohio. My primary contributions to this work includes validation, design, and execution of the $[^3H]$epibatidine adrenal membrane binding assays. I also aided in assay design, oversight of undergraduates, and preparation of the manuscript. This manuscript is published in Neuroscience Research and is presented below in a format suitable to publication in this journal.

Citation:

ABSTRACT

The development of new agents that selectively interact with subtypes of neuronal nicotinic receptors (nAChRs) is of primary importance for the study of physiological processes and pathophysiological conditions involving these receptors. Our laboratory has evidence that simple ring E analogues of methyllycaconitine (MLA) act as antagonists to adrenal $\alpha_3\beta_4^*$ nAChRs. The following studies were designed to characterize the concentration-response effects of several ring E analogues of MLA in order to assess structural requirements involved with their inhibitory activity on $\alpha_3\beta_4^*$ nAChRs. Ring E analogues with various substitutions on the ring E nitrogen were tested for their ability to inhibit nicotinic stimulated adrenal catecholamine release and $[^3H]$epibatidine binding to an adrenal membrane preparation. Several N-alkyl derivatives inhibited secretion with IC$_{50}$ values in the low micromolar range. The N-phenpropyl analogue was the most potent of the analogues tested (IC$_{50}$, 1.1 $\mu$M) on adrenal secretion. Competition binding studies suggest a noncompetitive interaction of the analogues with adrenal nAChRs. These studies suggest that chemical modification of ring E analogues of MLA may be a useful approach in assessing structural requirements necessary for $\alpha_3\beta_4^*$ nAChRs.

Keywords: methyllycaconitine, MLA, nicotinic receptor, binding, adrenal catecholamine secretion
INTRODUCTION

Nicotinic acetylcholine receptors (nAChRs) containing α3 subunits are found on postsynaptic neurons in autonomic ganglia and chromaffin cells of the adrenal medulla. The nAChR subtypes believed to be expressed in these tissues are α3β4*, α3α5β4, and α3α5β2β4 (Lukas et al., 1999). These α3-containing nAChRs play a prominent role in autonomic neurotransmission and adaptive responses to stress. α3-Containing nAChRs have also been found in several brain regions (e.g., substantia nigra, ventral tegmental area, hippocampus, the medial habenula and interpeduncular nucleus) (for reviews, see (Jones et al., 1999; Picciotto et al., 2000; Cordero-Erausquin et al., 2000)). The precise subunit composition and the physiological role of these nAChRs in the CNS remain to be elucidated, but have been reported to be involved with the control of norepinephrine (Sershen et al., 1997) and dopamine release (Kulak et al., 1997). A diminution of α3 mRNA in regions of the aged brain of humans (Terzano et al., 1998) and rats (Charpantier et al., 1999) has also been reported, suggesting a contribution of neurons expressing α3-containing nAChRs to age associated memory impairment.

Subtypes of neuronal nAChRs can be pharmacologically differentiated by a variety of agents found in nature, including snake venom neurotoxins (Luetje et al., 1990), marine snail conotoxins (McIntosh et al., 1994; Johnson et al., 1995; Cartier et al., 1996), and the plant alkaloid, methyllycaconitine (MLA) (Macallan et al., 1988; Ward et al., 1990). MLA is a potent, nonpeptide, nAChR antagonist.
showing selectivity for α7 nAChRs. Recently, our laboratory reported that MLA inhibits nAChR-stimulated adrenal catecholamine secretion (Bergmeier et al., 1999) demonstrating activity of MLA on α3β4* nAChRs. These effects, though, occur at MLA concentrations which are approximately 1000 fold higher than concentrations that affect α7 nAChRs.

Although the nonpeptide nature of MLA lends itself to structure-activity relationship (SAR) studies, few SAR studies have been reported and are focused on α7 nAChR activity (Hardick et al., 1996; Davies et al., 1997). Our laboratory has recently described simple ring E analogues of MLA containing the succinimidoylanthranilate side chain (Figure 42). The availability of these analogues allows for the study of structural determinants of MLA which may be important for its activity on α3β4* nAChRs. The demonstration that the ring E analogues inhibit nAChR-stimulated adrenal catecholamine secretion (Bergmeier et al., 1999) suggests that the ring E moiety of MLA containing the succinimidoylanthranilate side chain may be important for the activity of MLA on adrenal nAChRs. The following studies were designed to further characterize the SAR of ring E analogues of MLA to assess structural determinants affecting activity on adrenal nAChRs.
MATERIALS and METHODS

Materials. Ring E analogues (LB1 - LB8, IB-1, AB-2) were synthesized as described by Bergmeier et al. (Bergmeier et al., 1999). Limited availability of certain analogues due to difficulties in synthesis prevented their use in some studies. (-)Nicotine hydrogen tartrate, α-bungarotoxin (αBGT), polyethyleneimine (PEI) and components of N2+ media were obtained from the Sigma Chemical Company (St. Louis, MO). Dulbecco's Modified Eagle Medium (DMEM) and DMEM/F12 (used in N2+ medium) were obtained from Life Technologies (Grand Island, NY). (+/-)[5,6-bicycloheptyl-3H]-Epibatidine (specific activity, 66.6 Ci/mmol), [125I]tyr54-α-bungarotoxin (specific activity, 145 Ci/mmol), and DL-[7-3H(N)]-norepinephrine hydrochloride (specific activity, 13.5 Ci/mmol) were purchased from Dupont-New England Nuclear Corporation (Boston, MA). Bovine adrenal glands were purchased from the Herman Falter Packing Company (Columbus, OH). Rat brains were purchased from Harlan Bioproducts for Science (Indianapolis, IN). Whatman GF/B filters were purchased from Brandel Laboratories, Inc. (Gaithersburg, MD).

Adrenal Catecholamine Secretion Studies. Bovine adrenal chromaffin cells were dissociated from intact glands and plated at a density of 1-2x10^5 cells per well on 24-well culture plates in supplemented DMEM (Maurer and McKay, 1994). Two days after plating, media were replaced with a modified, serum-free, N2+ medium (Maurer and McKay, 1994). DMEM and N2+ media were supplemented with 250 ng/ml amphotericin B, 100 U/ml penicillin, 100 µg/ml streptomycin, 2 mM L-glutamine, and 10 µM 5-fluoro-2'-deoxyuridine. One day
prior to experimentation, the culture medium was removed and replaced with medium free of amphotericin B and 5-fluoro-2'-deoxyuridine. Cells were used 4-7 days after isolation. A $[^3\text{H}]$norepinephrine ($[^3\text{H}]\text{NE}$) assay was used to monitor catecholamine release from cultured cells (McKay and Schneider, 1984). Cells were incubated with 0.1 $\mu$M $[^3\text{H}]\text{NE}$ in a physiological salt solution (PSS) containing 140 mM NaCl, 4.4 mM KCl, 1.2 mM MgSO$_4$, 3.6 mM NaHCO$_3$, 1.2 mM KH$_2$PO$_4$, 10 mM glucose, 2 mM CaCl$_2$, 5 mM HEPES (pH 7.2-7.4) and 0.5 % bovine serum albumin (BSA) and extensively washed prior to all treatments. After loading with $[^3\text{H}]\text{NE}$, cells either were not treated (control groups) or treated for 15 minutes with various concentrations of the analogues to be tested. Cells were then stimulated in PSS for 5 minutes with 10 $\mu$M nicotine in the continuous presence of the analogues. The amount of radioactivity released following a 5 min incubation with agonist (stimulated release) or without agonist (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 $[^3\text{H}]\text{NE}$. Results were expressed as a percentage of the net stimulated control response (% control) where basal (nonstimulated) release has been subtracted from all groups [i.e., (treatment group secretion minus basal secretion) divided by (control, 10 $\mu$M nicotine-stimulated secretion minus basal secretion) x 100].

$[^3\text{H}]$Epibatidine Binding to Adrenal nAChRs. Bovine adrenal medullae were dissected from the gland and immediately placed in ice-cold preparation
buffer consisting of 300 mM sucrose, 50 mM Tris (pH 8.8), 1 mM EDTA, 1 mM EGTA, 5 mM iodoacetamide, and 0.1 mM PMSF. Medullary tissue was minced in ice-cold buffer and then homogenized in ice-cold buffer (1:1, weight:volume) using a polytron. The homogenate was centrifuged for 50 min at 82,000 x g at 4 °C. Pellets were resuspended in assay/rinse buffer (pH 7.4) consisting of 120 mM NaCl, 5 mM KCl, 8 mM Na2HPO4, 2 mM EDTA, 2 mM EGTA, 5 mM HEPES, 5 mM iodoacetamide, and 0.1 mM PMSF and centrifuged for 25 min at 82,000 x g at 4 °C. Pellets were resuspended in assay/rinse buffer and protein concentrations were determined using the Bradford protein assay with bovine serum albumin as the standard. For the adrenal binding studies adrenal membranes (500-900 µg per assay tube) were incubated at room temperature for 60 min in 500 µl of assay/rinse buffer containing 1 nM [3H]epibatidine. αBGT (1 µM) was added to the assay/rinse buffer to eliminate potential binding to αBGT binding sites. After the 60 min incubation, the assay mixtures were filtered and rapidly washed 4 times (~4 ml/wash) with assay/rinse buffer using a binding manifold. Filter membranes were soaked >5 hrs in 5% PEI in water prior to filtration. The filters with adrenal membranes were added to 4.5 ml of Scintiverse E (Fisher Scientific Co., Pittsburgh, PA) and allowed to sit overnight prior to counting via liquid scintillation spectroscopy. Nonspecific binding was determined in the presence of 300 µM nicotine. Specific binding was determined by subtracting nonspecific binding (typically 5-10%) from total binding.
[
\[
^{125}\text{I}\alpha\text{BGT Binding to Rat Brain Membranes.}
\]
Binding to rat brain membranes was by modification of the technique of Marks et. al. (Marks et al., 1986). Membrane filters were soaked at least 30 min in 0.5% PEI in binding buffer, consisting of 118 mM NaCl, 4.8 mM KCl, 1.2 mM MgSO₄, 2.5 mM CaCl₂, 20 mM HEPES (pH 7.5) and 0.01% BSA. The rinse buffer was identical to the binding buffer with the addition of 0.05% PEI. Rat brain membranes (250-500 µg per assay tube) were incubated at 37°C for 4 hr in 500 µl of binding buffer containing 1 nM [\(^{125}\text{I}\alpha\text{BGT}].\) After the 4 hr incubation, the assay mixtures were filtered and rapidly washed 4 times with rinse buffer (~4 ml/wash) using a binding manifold. The filters with membranes were then counted in a gamma counter. Nonspecific binding was determined in the presence of 1 mM nicotine and was typically 45-55% of total binding.

Calculations and Statistics. Results were calculated from the number of observations (n) performed in duplicate or triplicate. IC\(_{50}\) values were obtained by averaging values generated from nonlinear regression analyses (Prizm, GraphPad, San Diego, CA) of individual concentration-response curves. Results are expressed as arithmetic means SEM, except for IC\(_{50}\) values which are expressed as geometric means (95% confidence limits).

172
RESULTS

Our laboratory has previously identified ring E analogues of MLA that inhibit nicotine-stimulated adrenal catecholamine secretion (Bergmeier et al., 1999), implicating activity of these analogues on α3β4* nAChRs. In the following studies the concentration-response effects of several ring E analogues of MLA (Figure 42) on nAChR-stimulated adrenal catecholamine secretion were evaluated. As illustrated in Figure 43 and Table 9, the N-phenpropyl analogue (LB-8) was the most potent analogue. The analogues exert no inhibitory effect on catecholamine secretion when cells were stimulated using depolarizing concentrations of KCl (data not shown), supporting a direct action of these analogues on adrenal nAChRs. In addition, the analogues have no agonist activity (data not shown).

The ester linkage of our ring E analogue, LB-8, is hydrolyzable under the appropriate conditions (nonspecific esterases or general acid/base hydrolysis). To ascertain if either of the hydrolysis products have any appreciable antagonistic properties, both the alcohol (IB-13) and the carboxylic acid (IB-14) hydrolysis products of LB-8 were prepared (Figure 44). As illustrated in Figure 44, IB-13 inhibited nicotine-stimulated catecholamine release in a concentration-dependent manner with an IC_{50} value of 49.7 (45.4 - 54.5) μM; however, IB-14 had no inhibitory activity at concentrations up to 100 μM. These results indicate that the observed inhibitory activity of LB-8 is mostly due to the 3-phenylpiperidine moiety of IB-13. These findings also implicate the importance of
distance between the piperidine ring and the phenyl group for activity at the 
\(\alpha 3\beta 4^*\) nAChR.

To further characterize the interactions of the ring E analogues with 
adrenal nAChRs, competition binding experiments were performed (Table 10). These studies show that our ring E analogues, at concentrations up to 100 \(\mu\)M, do not competitively inhibit binding to adrenal \(\alpha 3\beta 4^*\) nAChRs. MLA, on the other hand, does competitively inhibit \(^3\text{H}\)epibatidine binding.

To address the question of whether our ring E analogues retain activity on 
\(\alpha 7\) nAChRs, competition binding experiments on rat brain using \(^{125}\text{I}\)aBGT were also performed. As demonstrated in Figure 45, ring E substituted analogues, including LB-8, showed little or no inhibition of activity on \(^{125}\text{I}\)aBGT binding. LB-8 was the most potent analogue tested, with an IC\(_{50}\) value of 177 \(\mu\)M. The other ring E analogues had IC\(_{50}\) values of >300 \(\mu\)M. The analogues had no effects on \(^3\text{H}\)nicotine binding to rat brain \(\alpha 4\beta 2\) nAChRs (data not shown).
DISCUSSION

The role of nAChRs containing the α3 subunit (α3* nAChRs) is becoming increasingly important. In the PNS, nAChRs are found on postsynaptic neurons in autonomic ganglia and cells of the adrenal medulla. The subtypes believed to be present in autonomic ganglia are α3β4*, α3α5β4, and α3α5β2β4 (Lukas et al., 1999). Cells of the adrenal medulla likely express α3β4* nAChRs. In the PNS, these α3* nAChRs play a prominent role in autonomic neurotransmission and adaptive responses to stress. In the CNS, α3* nAChRs have been found in several brain regions (e.g., substantia nigra, ventral tegmental area, hippocampus, the medial habenula and interpeduncular nucleus), (for reviews, see (Jones et al., 1999; Picciotto et al., 2000; Cordero-Erausquin et al., 2000)).

The precise subunit composition and the physiological role of α3* nAChRs in the CNS remain to be elucidated. In the CNS, α3* nAChRs have been postulated to be involved with the control of norepinephrine (Sershen et al., 1997) and dopamine release (Kulak et al., 1997). A diminution of α3 mRNA in regions of the aged brain of humans (Terzano et al., 1998) and rats (Charpantier et al., 1999) has also been found, suggesting a contribution of neurons expressing α3* nAChRs to age associated memory impairment. In the spinal cord, α3* nAChRs have been linked with pain pathways (Marubio et al., 1999). Finally, the physiological importance of α3 subunits is demonstrated by the lethality that is seen with α3 knockout mice (Xu et al., 1999).
The principal receptors that mediate adrenal catecholamine secretion are believed to be α3β4* nAChRs (Wenger et al., 1997). Our laboratory has previously identified ring E analogues of MLA that inhibit nicotine-stimulated adrenal catecholamine secretion (Bergmeier et al., 1999), implicating activity of these analogues on α3β4* nAChRs. The N-phenpropyl analogue (LB-8) was the most potent analogue. LB-8's IC50 value of 11 μM is similar to those reported for other inhibitors of adrenal catecholamine release, including d-tubocurarine (2 μM), hexamethonium (16 μM) and decamethonium (18 μM) (McKay and Burkman, 1993). Other ring E analogues with smaller, less lipophilic side chains (e.g., LB-1 and LB-2) were less potent than LB-8 (Figure 43 and Table 9), implicating the importance of appropriate size groups on the basic nitrogen of the piperidine ring for inhibition of α3β4* nAChR-stimulated catecholamine release.

Decreasing the carbon side chain separating the phenyl group from the basic nitrogen of the piperidine ring also reduced the inhibitory activity of the analogues on nAChR-stimulated adrenal secretion (Table 9). The IC50 value of the N-phenethyl analogue (LB-7) was approximately 5 times less potent than the N-phenpropyl analogue (LB-8) (Table 9) suggesting the importance of the carbon side chain length separating the tertiary nitrogen from the phenyl ring with respect to potency on adrenal nAChRs. The N-phenoxyethyl analogue (IB-1), which is similar in structure to LB-8 except the N-phenylethylether linkage replaces the 3-(phenyl)propyl on the R1 position, had a similar IC50 value as LB-8; the slight difference in IC50 values between the two analogues, however, might...
be due to the ether linkage, which may decrease the basicity of the piperidine nitrogen or may inductively increase the electron density of the phenyl ring.

The importance of the methyl substituent at the 3-succinimide (R²) is demonstrated by comparing the N-isopropyl analogues, LB-4 and LB-3, as well as the N-butyl analogues, LB-5 and AB-2, on nAChR-stimulated adrenal secretion. In both instances, 2-3 fold decreases in IC₅₀ values were obtained when the succinimide ring is substituted with a methyl group (Figure 43, Table 9). These results parallel previous studies demonstrating reduced activity of MLA on α7 nAChRs in the absence of the methyl group on the succinimide ring and implicates the importance of the substituent on the succinimide ring with regards to interactions with nAChRs (Jacyno et al., 1996).

One of the hydrolysis products of LB-8 (IB-13) retained some antagonistic activity, albeit reduced when compared to LB-8, suggesting that the piperidine structure and/or the carbonyl oxygen of the ester linkage (Ward et al., 1990) contributes to binding of these ring E analogues to adrenal nAChRs. However, without the 3-methylsuccinimidoanthranilate side chain, optimal binding cannot be achieved. These results indicate that the intact ring E molecule contributes to binding to adrenal nAChRs. Similar conclusions were drawn for MLA by Hardick and associates (Hardick et al., 1995; Hardick et al., 1996) who reported the importance of the 2-(methylsuccinimido)benzoyl side chain of MLA (similar in ring structure to LB-8) for binding to α7 nAChRs. Our findings are consistent with other studies showing a concomitant decrease in α7 nAChR affinity with decreasing number of rings retained in the norditerpinoid core (Hardick et al., 177).
These findings also support earlier work suggesting that the nortiterpinoid core is important for high affinity binding to α7 nAChRs (Hardick et al., 1995).

In our studies, MLA was found to inhibit adrenal secretion at concentrations similar to those reported to inhibit acetylcholine currents in Xenopus oocytes expressing rat α3β4 nAChRs (Lopez et al., 1998). These effects of MLA on adrenal catecholamine secretion are likely due to competitive interactions with adrenal α3β4* nAChRs. The ring E analogues also inhibit nAChR-mediated adrenal chromaffin secretion. LB-8 inhibited adrenal secretion with similar potency as MLA (IC₅₀ values, 11.4 and 2.6 μM, respectively). However, these inhibitory effects appear to involve noncompetitive interactions. These findings suggest that the properties or structures important for competitive interactions with nAChRs are not retained in our ring E analogues. These results also support the importance of ring E moiety for noncompetitive interactions with adrenal α3β4* and possibly α7 nAChRs.

The most significant finding of these studies is that we have found simple analogues of MLA (e.g., LB-8) that should be useful in assessing structural requirements necessary for antagonist potency for α3β4* nAChRs. Antagonists of nAChRs containing α3 subunits may be useful in advancing our understanding of the physiological/pathological roles of these nAChRs which are found throughout the body. In addition to their important role in ganglionic transmission, α3*-containing nAChRs have been found in several regions of the CNS and
implicated with neurotransmission (Jones et al., 1999; Picciotto et al., 2000; Yeh et al., 2001). Therefore, the emergence of possibly important physiological and pathophysiological roles of α3* nAChRs, underscores the need for drugs targeted toward these receptors and the development of models expressing native nAChRs (e.g., adrenal chromaffin cells) to study these drugs.

ACKNOWLEDGMENTS

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<table>
<thead>
<tr>
<th>Compound</th>
<th>Bovine Adrenal nAChR-Stimulated Catecholamine Release, IC\textsubscript{50} Values (µM)</th>
<th>Rat Brain ([^{125}\text{I}]\text{oBGT Binding, IC}_{50}) Values (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MLA</td>
<td>2.6 (2.3 - 3.0)</td>
<td>0.01 (0.01 - 0.02)</td>
</tr>
<tr>
<td>LB-1</td>
<td>63.9 (55.6 - 73.3)</td>
<td>&gt;300</td>
</tr>
<tr>
<td>LB-2</td>
<td>62.5 (59.0 - 66.0)</td>
<td>&gt;300</td>
</tr>
<tr>
<td>LB-3</td>
<td>37.1 (28.6 - 48.1)</td>
<td>&gt;300</td>
</tr>
<tr>
<td>LB-4</td>
<td>115 (96 - 138)</td>
<td>ND</td>
</tr>
<tr>
<td>LB-5</td>
<td>65.3 (53.1 - 80.2)</td>
<td>ND</td>
</tr>
<tr>
<td>LB-6</td>
<td>59.6 (55.6 - 63.9)</td>
<td>ND</td>
</tr>
<tr>
<td>LB-7</td>
<td>53.0 (4.2 - 57.2)</td>
<td>&gt;300</td>
</tr>
<tr>
<td>LB-8</td>
<td>11.4 (10.9 - 11.9)</td>
<td>177 (150 - 209)</td>
</tr>
<tr>
<td>IB-1</td>
<td>20.2 (19.1 - 21.3)</td>
<td>&gt;300</td>
</tr>
<tr>
<td>AB-2</td>
<td>27.4 (23.6 - 31.9)</td>
<td>&gt;300</td>
</tr>
</tbody>
</table>

Table 9. Comparison of Effects of MLA and Ring E Analogues on Subtypes of nAChRs. IC\textsubscript{50} values are interpolated for the curves in Figure 43 and 45 and are expressed as geometric means (95% confidence limits).
<table>
<thead>
<tr>
<th>Compound (100 µM)</th>
<th>Specific [³H]Epibatidine Binding (% control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MLA</td>
<td>5.5 ± 0.7</td>
</tr>
<tr>
<td>LB-1</td>
<td>106.2 ± 5.6</td>
</tr>
<tr>
<td>LB-2</td>
<td>105.4 ± 3.8</td>
</tr>
<tr>
<td>LB-3</td>
<td>109.4 ± 4.5</td>
</tr>
<tr>
<td>LB-4</td>
<td>102.8 ± 5.4</td>
</tr>
<tr>
<td>LB-5</td>
<td>92.3 ± 1.8b</td>
</tr>
<tr>
<td>LB-6</td>
<td>96.6 ± 5.1</td>
</tr>
<tr>
<td>LB-8</td>
<td>123.9 ± 8.9</td>
</tr>
<tr>
<td>IB-1</td>
<td>118.0 ± 8.1</td>
</tr>
<tr>
<td>IB-13</td>
<td>117.2 ± 3.3</td>
</tr>
<tr>
<td>IB-14</td>
<td>95.0 ± 4.4</td>
</tr>
<tr>
<td>AB-2</td>
<td>85.1 ± 7.6</td>
</tr>
</tbody>
</table>

Table 10. Effects of Ring E Analogues on [³H]Epibatidine Binding to Adrenal Chromaffin Cells. Competition binding experiments were performed as described in the methods section using 100 µM concentrations of each analogue and MLA. Data represent means ± SEM (n = 3 - 5). b n=2 due to lack of availability of compound.
Figure 42. Structures of MLA and Ring E Analogues.
Figure 43. Effects of Ring E Analogues on nAChR-stimulated Secretion from Bovine Adrenal Chromaffin Cells. Cultured adrenal chromaffin cells were treated for 15 min with the analogue prior to stimulation with nicotine (10 μM) in the continued presence of the analogue. Results are expressed as a percentage of control, stimulated catecholamine release. Values represent means ± SEM (n = 3 - 5).
Figure 44. Effects of Hydrolysis Products of the N-phenpropyl Substituted ring E Analogue of MLA on nAChR-Stimulated Adrenal Secretion. (Upper Panel) Structures of the alcohol (IB-13) and the carboxylic acid (IB-14) hydrolysis products of the N-phenpropyl substituted ring E analogue of MLA (LB-8). (Lower Panel) Cultured adrenal chromaffin cells were treated for 15 min with the indicated analogue prior to stimulation with nicotine (10 μM) in the continued presence of the analogue. Results are expressed as a percentage of control, stimulated catecholamine release. Values represent means ± SEM (n = 3 - 5).
Figure 45. Effects of the Ring E Analogues on $[^{125}\text{I}]\alpha$BGT Binding to Rat Brain Membranes. Competition binding experiments were performed on rat brain homogenates as described in the Methods section using $[^{125}\text{I}]\alpha$BGT as the radioligand. Data represent means ± SEM (n = 3 - 5).