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Effects of antimitotic agents on cultured adrenal chromaffin cells: Implications for microtubule involvement with adrenal nicotinic receptors

Lopez, Isabel, Ph.D.
The Ohio State University, 1992
EFFECTS OF ANTIMITOGEN AGENTS ON CULTURED ADRENAL
CHROMAFFIN CELLS: IMPLICATIONS FOR MICROTUBULE
INVolVEMENT WITH ADRENAL NICOTINIC RECEPTORS

A Dissertation

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

By

Isabel Lopez, B.S., M.S.

* * * * *

The Ohio State University
1992

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Approved by
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To Tim and My Mom and Dad
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To my parents, Vicente and Vicki, thank you so much for your loving guidance, support and patience through the years and for your unending concern and support for my education even when it has meant time away from you.

Finally, my thanks and love go to my husband and partner Tim, whose understanding patience, help, encouragement and love during the course of this work and the past year has meant everything to me. Baby, I couldn't have done it without you, you were my inspiration.
VITA

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PUBLICATIONS (continued)


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Studies in: Neuropharmacology
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<tbody>
<tr>
<td>ACh</td>
<td>Acetylcholine</td>
</tr>
<tr>
<td>ara-C</td>
<td>Cytosine Arabino-furanoside</td>
</tr>
<tr>
<td>α-Bgt</td>
<td>alpha-Bungarotoxin</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine Serum Albumin</td>
</tr>
<tr>
<td>Ca$^{+2}$</td>
<td>Calcium</td>
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<tr>
<td>cm</td>
<td>Centimeter</td>
</tr>
<tr>
<td>cpm</td>
<td>Counts Per Minute</td>
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<td>Ci</td>
<td>Curie</td>
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<tr>
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<td>Deoxyribonucleic Acid</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethylsulfoxide</td>
</tr>
<tr>
<td>dpm</td>
<td>Disintegrations Per Minute</td>
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<tr>
<td>DMEM</td>
<td>Dulbecco's Modified Eagle's Media</td>
</tr>
<tr>
<td>D-PBS</td>
<td>Dulbecco's Phosphate Buffered Saline</td>
</tr>
<tr>
<td>EDTA</td>
<td>Disodium ethylenediamine-tetraacetate</td>
</tr>
<tr>
<td>FDU</td>
<td>5-Flurodeoxyuridine</td>
</tr>
<tr>
<td>FITC</td>
<td>Fluorescein Isothiocyanate</td>
</tr>
<tr>
<td>HEPES</td>
<td>N-[2-hydroxyethyl] piperazine-N'-[2-ethanesulfonic acid]</td>
</tr>
<tr>
<td>hr</td>
<td>hour</td>
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<td>-----------------------------</td>
</tr>
<tr>
<td>$^{125}$I</td>
<td>Iodine 125</td>
</tr>
<tr>
<td>L</td>
<td>Liter</td>
</tr>
<tr>
<td>$\mu$Ci</td>
<td>MicroCurie</td>
</tr>
<tr>
<td>$\mu$g</td>
<td>Microgram</td>
</tr>
<tr>
<td>$\mu$l</td>
<td>Microliter</td>
</tr>
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<td>Minute</td>
</tr>
<tr>
<td>M</td>
<td>Molar (moles/liter)</td>
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<tr>
<td>mAb</td>
<td>monoclonal antibody</td>
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<td>nAChR</td>
<td>Nicotinic Acetylcholine Receptor</td>
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<td>nM</td>
<td>Nanomolar</td>
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<tr>
<td>PFHM</td>
<td>Protein Free Hybridoma Media</td>
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<tr>
<td>PMSF</td>
<td>Phenylmethylsulfonyl Fluoride</td>
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<tr>
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<tr>
<td>rpm</td>
<td>Revolution Per Minute</td>
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<td>sec</td>
<td>Second</td>
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<td>TCA</td>
<td>Trichloroacetic Acid</td>
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<td>v/v</td>
<td>Volume by Volume</td>
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<td>Weight by Volume</td>
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The overly cautious scientist neither errs nor discovers

W.I.B. Beveridge
CHAPTER I
STATEMENT OF THE PROBLEM

Microtubules are major components of the neuronal cytoskeleton (Peters et al., 1976; Baas and Black, 1990). They have been implicated in a variety of cellular functions including secretion (Burgoyne, 1991; Schroer and Sheetz, 1991). This is based mainly on the ability of colchicine and vinblastine, drugs which interfere with the function of microtubules, to inhibit secretory processes (Poisner, 1973; Cooke and Poisner, 1979; Poisner and Bernstein, 1971; Lacey et al., 1968).

In adrenal chromaffin cells, microtubule drugs such as taxol and vinblastine, inhibit nicotine-mediated catecholamine release without affecting release stimulated by other secretagogue (Trifaro et al., 1972; McKay and Schneider, 1984). McKay and coworkers (1984) have also demonstrated that vinblastine interferes with histrionicotoxin binding to sites associated with the nicotinic acetylcholine receptor (nAChR) ion channel complex from Torpedo electric organ. These results suggest that taxol and vinblastine are acting at the level of the nAChR to interfere with receptor function and binding. These
studies together suggest that the action of the microtubule drugs is not on the terminal steps of exocytosis, but instead alter nAChR function by either a direct effect of the drugs on the receptor or an effect on microtubule links to the nAChRs of adrenal chromaffin cells, suggesting adrenal nAChRs are associated with the cytoskeleton. Another possibility is that microtubule drugs may possess anti-cholinergic activity (Trifaro et. al., 1972; McKay and Schneider, 1984)

Numerous membrane receptors have been demonstrated to be associated with the cytoskeletal network, including receptors for nerve growth factor (Vale and Shooter, 1982), Platelet-Derived Growth Factor (Zippel et al., 1989), transferrin (Hunt et al., 1989) and fibronectin (Horwitz et al., 1986). Association of these receptors with the cytoskeleton has been demonstrated to play an important role in receptor function. Receptor-cytoskeletal associations have also been demonstrated for muscle nAChRs (Prives, et al., 1982; Stya and Axelrod, 1983; Connolly and Oldfin, 1985). In muscle, microtubules may participate in anchoring receptors (Connolly and Oldfin, 1985).

Evidence in the literature suggests that adrenal nAChRs are associated with microtubular protein. This is based primarily on the ability of vinblastine and taxol to inhibit nAChR-stimulated secretion (McKay and Schneider, 1984; McKay et al., 1985). Therefore, if indeed microtubules play a
role in adrenal nAChR function, then other microtubule drugs
should have the same or similar effects on the function of
adrenal nAChRs and should affect adrenal microtubules under
the same or similar conditions that affect receptor
function. The hypothesis under investigation is that
adrenal nAChRs are associated with microtubules and that
this association may be important for receptor function. A
schematic representation of this hypothesis is found in Fig.
1. The major objectives of this project were to determine
1) the site and mechanism of action of various microtubule
drugs (Fig. 2) on adrenal nAChR-stimulated catecholamine
release and 2) to determine if adrenal nAChRs are associated
with microtubules.

Specific Aims
1. To determine the effects various microtubule drugs,
   have on nAChRs-mediated adrenal catecholamine release.
2. To determine if microtubule drugs block secretion
   mediated by non-nAChR mechanisms.
3. To determine if microtubule drugs alter chromaffin cell
   microtubular network under similar conditions shown to
   affect nAChR function (secretion).
4. To determine if adrenal chromaffin cells contain
   acetylated microtubules.
5. To determine if microtubule drugs alter the acetylated
   microtubular network under conditions shown to affect
Figure 1. Schematic representation of adrenal nAChRs associated with cytoskeletal components. Microtubules (MTS), Microtubule-Associated Proteins (MAPS). Adapted from Hucho, 1986).
Figure 2. Chemical structure of various microtubule drugs.
nAChRs function.

6. To determine the time-dependent effects of the microtubule drugs on nicotine-stimulated catecholamine secretion.

7. To determine if the effects of the drugs on nicotine mediated release are reversible.

8. To determine if the anticholinergic effect of the microtubule drugs is due to an interaction with the nicotine binding site.

9. To determine if adrenal nAChRs are associated with the Triton-insoluble cytoskeleton.

10. To determine if microtubule drugs alter the association of nAChRs to the Triton-insoluble cytoskeleton.
CHAPTER II
INTRODUCTION

ADRENAL CHROMAFFIN CELLS

Physiology

The adrenal medulla is an endocrine gland. Secretory products released by the adrenal medulla help in the regulation of involuntary functions such as heart rate, intestinal motility and pupillary dilation. At birth it is comprised of primitive sympathetic cells which differentiate into chromaffin cells (pheochromocytes). Chromaffin cells synthesize, store and secrete a complex mixture containing epinephrine, norepinephrine and a variety of other proteins and peptides such as enkephalins, chromogranins and neuropeptides (Bank et al., 1965; Douglas et al., 1965; Aunis et al., 1980; Viveros et al., 1983; Livett, 1984).

Adrenal chromaffin cells are homologous to sympathetic neurons. Both types of cells are derived from the neural crest, synthesize and release catecholamines, receive synaptic input from preganglionic cholinergic neurons and are stimulated by the activation of nicotinic acetylcholine receptors (nAChRs). Chromaffin cells also share common subcellular features with many neuronal cells. One such
feature is the storage of secretory products in membrane-bound granules. In culture, these secretory cells have been referred to as paraneurons, a term that includes cells generally and traditionally not considered neurons and yet regarded as relatives of neurons on the basis of their structure and function (Trifaro, 1983, 1989). When chromaffin cells are grown in culture, they extend axon-like processes, giving the cells a neuronal appearance (Unsiker, et al., 1980; Trifaro et al., 1980; Livett, 1984).

In almost all species, chromaffin cells possess both nicotinic and muscarinic receptors which can stimulate catecholamine secretion (Douglas and Poisner, 1965). However, in some species (e.g., rat chromaffin cells; Brandt et al., 1976), secretion is mediated by the activation of muscarinic receptors while in others (e.g., bovine; Livett, 1983) only stimulation of nicotinic receptors will elicit secretion. Other receptors present on chromaffin cells which elicit release of catecholamines include angiotensin II (Bunn and Marley, 1989), VIP (Wilson, 1988), bradykinin (O'Sullivan and Burgoyne, 1989; Plevin and Boarder, 1988), histamine (O'Sullivan and Burgoyne, 1989; Chaliss, et al., 1991) and prostaglandin E₂ (Ito, et al., 1991).

**Catecholamine Secretion Studies**

Activation of chromaffin cell nAChRs by the neurotransmitter, acetylcholine (ACh), leads to an inward
flux of Na\(^+\) and to a lesser extent Ca\(^{2+}\) through the nAChR-associated ion channel (Douglas, et al., 1967). This results in a slight depolarization of the cell membrane, sufficient to activate the voltage-sensitive fast Na\(^+\) channels (Douglas, et al., 1967; Kidokoro, et al., 1982). Opening of both the receptor ion channel and the voltage sensitive fast Na\(^+\) channels allows influx of Ca\(^{2+}\) (Kilpatrick et al., 1981; Corcoran et al., 1983) and a concomitant increase in the concentration of intracellular Ca\(^{2+}\). This increase in intracellular Ca\(^{2+}\) is the primary stimulus for exocytosis of chromaffin granules containing catecholamines (Kilpatrick et al., 1982; Burgoyne et al., 1982). The molecular mechanism involved in changing the Ca\(^{2+}\) signal into the mechanics of granule fusion with the cell membrane and release of the granule contents is not known. Current advances in stimulus-secretion coupling suggest the involvement of Ca\(^{2+}\) binding proteins (eg., calmodulin, calpactin, synexin), Ca\(^{2+}\) dependent kinases (protein kinase C), cytoskeletal elements and their associated proteins, guanosine nucleotide binding proteins as well as plasma membrane and granule-associated proteins (Marley, 1988; Burgoyne 1991).

Catecholamine secretion can also be elicited with high extracellular K\(^+\) (Baker and Rink, 1975) or by using a Ca\(^{2+}\) selective ionophore [e.g., calcimycin (A23187), Burgoyne, 1984]. Depolarization by excess K\(^+\) bypasses the nicotinic
receptor and directly activates the voltage-sensitive Na\(^+\) channel. K\(^+\)-induced secretion is also sensitive to extracellular Ca\(^{+2}\) and can be blocked by Ca\(^{+2}\) channel antagonists such as verapamil (Kidokoro, et al., 1982).

Catecholamine release in chromaffin cells will progressively decline if the cells are continuously exposed to nicotinic agonists (Boska and Levitt, 1984; Boska and Levitt, 1984b). Previous studies by Sasakawa and coworkers (1985) have shown that pretreatment of cells with nicotinic agonists had no effect on K\(^+\)-induced Ca\(^{+2}\) influx. Their results suggest that the loss of sensitivity to nicotinic agonists was due to receptor desensitization instead of inactivation of Ca\(^{+2}\) channels. Furthermore, low concentrations of substance P have been shown to protect the nicotinic response from desensitization without affecting inactivation of K\(^+\)-induced catecholamine secretion (Boska and Levitt, 1984). These results indicate that desensitization can also occur at the level of the nicotinic receptor.

Mechanisms involved in secretion are very similar between secretory cells (i.e., exocrine, endocrine and neuronal cells). Many cells possess a regulated secretory pathway (Trifaro, 1977). This pathway involves the concentration and packaging of material to be exported into storage granules and transport of the vesicles to the site of release via microtubule-mediated transport. The
molecular mechanisms involved in the late stages of secretion, such as the movement of vesicles to the inner surface of the plasma membrane, docking of the vesicles on the plasma membrane and exocytotic membrane fusion and fission remain unknown.

In endocrine cells, the vesicles are prevented from reaching the plasma membrane prior to stimulation (Burgoyne and Cheek, 1987; Orci, 1972). Studies by Kondo et al. (1982) have demonstrated that the secretory granules of adrenal chromaffin cells are embedded in a three-dimensional cytoskeletal network. This suggests that an association between secretory granules and the cytoskeleton could be important in the regulation of exocytosis, either by holding vesicles at the plasma membrane ready to be released or by preventing access of the secretory granules to the plasma membrane until the cell is stimulated, as has been suggested in adrenal chromaffin cells (Trifaro et al., 1983; Burgoyne et al., 1986).

Much of the evidence acquired for the involvement of cytoskeletal elements in secretion has been gathered from adrenal chromaffin cells. Additionally, adrenal chromaffin cells have been used to study the interactions of cytoskeletal proteins with secretory vesicles (Burgoyne, 1987; Trifaro, 1989). Among the cytoskeletal proteins involved in transporting granules from the Golgi network to their site of release and maintaining the vesicles near
exocytosis sites are the cytoskeletal elements, microtubule proteins (discussed below) and actin. The cytoskeletal elements that interact with vesicles and impair their movement are believed to be different from microtubules and are thought to be mainly actin, caldesmon and fodrin (Koideli, 1986).

Actin binds to granule membranes through actin binding proteins such as α-actinin, fodrin and caldesmon. These proteins are extrinsic components of the secretory granule membrane. It has been demonstrated that binding of actin to secretory granule membranes, as has been observed in chromaffin granules, is increased at nM concentrations of Ca\(^{+2}\), resulting in the formation of a crosslinked gel (Burgoyne et al., 1982; Trifaro et al., 1983). This gelation increases the viscosity of the cytoplasm, and thus serves to dock the vesicles at the plasma membrane in preparation for exocytosis. Formation of this actin network gel can be inhibited by micromolar concentrations of Ca\(^{+2}\) (Stendahl et al., 1980), thus providing a mechanism to control granule exocytosis. It has been demonstrated in vitro that upon cell stimulation, intracellular Ca\(^{+2}\) concentration increases which leads to the release of secretory granules from the actin cytoskeleton, thus enabling the granules to interact with the plasma membrane or plasma membrane cytoskeleton and to undergo exocytosis (Burgoyne et al., 1982). Thus the assembly/disassembly of
actin may play an important role in the mechanism of exocytosis.

**MICROTUBULES**

**Structure and Dynamics**

Microtubules are widely distributed in eukaryotic cells, particularly nerve cells (Borisy and Taylor, 1967; Feit et al., 1971). The major protein constituent of microtubules is tubulin, a heterodimer composed of α- and β-subunits (Bryan and Wilson, 1971; Luduena et al., 1964, 1981). In addition to tubulin, microtubules contain a heterogeneous class of proteins referred to as microtubule associated proteins (MAPs), which promote tubulin assembly and affect the stability of polymerized microtubules (Black, 1986). Microtubules play a role in a number of important and diverse cellular processes such as cell division, development and maintenance of cell morphology, cell motility and intracellular transport and exocytosis (Fulton, 1981; Manfredi and Horwitz, 1984; Avila, 1990).

Microtubules are slender, hollow tubules about 25 nm (20-27 nm) in diameter. They are unbranching and appear to have a certain amount of rigidity, but at the same time have enough elasticity to bend. In cross section, they appear to be made up of 13 subunits which are arranged in parallel, longitudinal rows (Dustin, 1984). Microtubules are composed of two protein monomers of identical molecular weight.
(54,000 daltons), α- and β-tubulin, which are present throughout the cytoplasm (Dustin, 1984). The two tubulins form dimers which in turn polymerize to form the longitudinal parallel strand protofilaments. This polymerization requires Mg\(^{2+}\), GTP and MAPs (Manfredi and Horwitz, 1984; Avila, 1990). Within each microtubule, α- and β-tubulin are arranged in a helical pattern. It has been suggested that MAPs may be involved with regulation of polymerization (Black, 1986).

Many of the functions performed by microtubules depend on their ability to polymerize and depolymerize. These processes can be blocked by agents that prevent microtubule assembly or disassembly (discussed below). In order to better understand how microtubule drugs may affect these processes, it is important to understand the mechanism involved in microtubule dynamics. Formation of microtubules involves two stages, initiation and elongation (Weisenberg and Deery, 1976; Erickson and Voter, 1986). Initiation appears to involve formation of a ring-like structure to which tubulin subunits can be added. In vivo, initiation is still not well understood; however, microtubule formation appears to occur around microtubule organizing centers (MTOC), such as centrioles (Brinkley et al., 1981; Erickson and Voter, 1986).

Microtubules elongation occurs by incorporation of tubulin monomers to one end of the microtubule. The two
ends of the filament are termed the plus and minus ends, based on relative rates of tubulin polymerization. The plus end is the rapidly polymerizing end, whereas, the minus end polymerizes at a much slower rate (Mitchison and Kirschner, 1984). Addition of tubulin dimers to the growing end of the microtubule is followed by a delayed GTP hydrolysis. However, tubulin polymerization is not dependent on GTP hydrolysis. If tubulin molecules are being added to the polymer end faster than GTP hydrolysis, a GTP cap will form at the growing end which stabilizes the growing microtubule (Mitcheson and Kirschner, 1984). Since tubulin-GTP complexes bind to each other with greater affinity, the molecules will continue to grow, contributing to the population in the growing phase. On the other hand, if polymerization slows down, GTP hydrolysis to GDP occurs, resulting in a rapid and complete depolymerization of the microtubule (Mitcheson and Kirschner, 1984).

As cells grow and differentiate, two populations of microtubules appear: a dynamic (labile) population and a stable population (Schulze and Kirschner, 1987). Dynamic microtubules can be disrupted by depolymerization with drugs such as demecolcine, nocodazole or colchicine (Schulze and Kirschner, 1987). Stable microtubules are more resistant to disruption by some microtubule drugs (e.g., colchicine). Dynamic microtubules are characteristic of cells undergoing an internal reorganization such as constantly dividing cells.
or migrating cells. Stable microtubules are more prominent in highly differentiated cells, such as neurons, which have lost their ability to divide (Brady et al., 1984). In these cells, the microtubules become permanent features, i.e., they are essentially nondynamic, neither growing nor shrinking appreciably (Sahenk and Brady, 1987). The exact mechanism by which microtubules are stabilized is not known, although it has been suggested that posttranslational modifications of the microtubule protein or interaction of microtubules with certain microtubule-associated proteins (MAPs) may play a role (Hamel et al., 1983; Matus, 1988).

**Posttranslational Modifications of Microtubules.**

Although much work has been done on posttranslational modifications of tubulin, little is still known regarding the *in vivo* role of these modifications. It has been suggested that these modifications may act as signals for the binding of specific MAPs that stabilize microtubules and modulate the properties of the microtubule network in response to changes in the internal or external milieu (Black and Keyser, 1987; Piperno et al., 1987). Microtubules can undergo at least three types of posttranslational modifications: tyrosinylation, acetylation and phosphorylation. The first posttranslational modification described for tubulin was tyrosination/detyrosination (Berra et al., 1988; Gunderson
et al., 1984, 1987). In this type of modification, a tyrosine residue is inserted at the carboxy terminus of its α-chain by a tyrosine ligase or is released via a carboxypeptidase (Berra et al., 1988). Tubulin can also be modified by phosphorylation both in vivo and in vitro (Gard and Kirschner, 1985; Diaz-Nido et al., 1990).

Acetylation of α-tubulin is another form of posttranslational modification. This form of tubulin modification was first observed in *Chlamydomonas* during flagellar regeneration (L'Hernault and Rosenbaum, 1985). The availability of a monoclonal antibody, 6-11B-1, which specifically recognizes the acetylated form of tubulin, has facilitated the study of this posttranslational modification (Piperno and Fuller, 1985). Acetylated microtubules are more resistant to depolymerization by microtubule drugs such as colchicine and nocodazole (Piperno et al., 1987; Falconer et al., 1989). This type of microtubule is prominent in neurons (Black and Keyser, 1987). The enzyme, tubulin acetyltransferase, acetylates the ε-amino group of a lysine residue in α-tubulin. Acetylation probably represents a modification of preformed microtubules, since it is inhibited by microtubule depolymerizing drugs in cultured neuronal cells. In fact, the tubulin polymer is a better substrate than the dimer for α-tubulin acetyltransferase. Acetylated microtubules comprise 10 - 40 % of the total cytoplasmic microtubule network, although exceptions exist
in which few or none are found (Maruta et al., 1986). In most instances, stable microtubules containing acetylated tubulin colocalize with detyrosinated tubulin, but the distribution and extent of each modification is not always identical.

**Microtubule-Associated Proteins (MAPs)**

MAPs are a group of heterogeneous phosphoproteins originally identified as brain proteins that co-purify with tubulin through several cycles of temperature-dependent assembly and disassembly cycles (Olmsted, 1987; Valle et al., 1987). These proteins stabilize assembled microtubules and are involved in modulating tubulin polymerization into microtubules (Olmsted, 1987; Lewis et al., 1989; Cross et al., 1991). These functions are mediated by specific interactions of MAPs with tubulin. MAPs are divided into two major classes based on their molecular weight: a group of high molecular weight proteins, known as MAP1 and MAP2, and a group of 50 to 70 kD proteins, referred to as tau factors (Olmsted, 1987). Both classes of MAPs have two domains. One domain binds microtubules. The other domain is thought to be involved in linking microtubules to other cytoskeletal components (actin-filaments, neurofilaments) and cellular organelles (Olmsted, 1987). Other MAPs have also been identified and it has been suggested that some of these MAPs play a structural role in stabilizing
microtubules, in linking microtubules to other cellular organelles and in transporting organelles along microtubules.

**Function of Microtubules**

In various secretory systems, microtubule-directed transport plays an important role in the intracellular movement of organelles. For example, in mast cells, movement of histamine granules is directed by microtubules (Gillespie et al., 1968). Similarly, the insulin-producing \( \beta \) cells of the islets of Langerhans have numerous microtubules which direct alignment of granules into rows near the cell surface (Lacey et al., 1968). Exocytosis of these granules is not affected by microtubule disrupting drugs. However, it has been demonstrated that colchicine inhibits the secondary or slower phase of insulin release, which suggests movement of the secretory granules to release sites is dependent on microtubules. This mechanism of secretion applies to many secretory cells.

In neurons, where the sites specialized for exocytosis are usually very distant from the cell body, microtubule transport is very important (Schroer and Sheetz, 1991). Microtubules are also necessary for the extension and maintenance of neuronal processes and serve as tracks for axoplasmic flow (Vale, 1987). Electron micrographs show associations between organelles presumably being transported
by microtubules (Allen et al., 1987; Allen, 1987). It has been demonstrated that a single microtubule can support organelle movement in both directions and can carry small vesicles and mitochondria throughout the cytoplasm (Hayden et al, 1984; Schnapp et al., 1985; Vale, 1987). The squid giant axon has been extensively used to study microtubule-mediated transport. Examination of extruded axoplasm from this axon by video-enhanced light microscopy has shown organelles moving along filamentous tracks (Vale, 1987). The use of immunofluorescent techniques and electron microscopy have demonstrated that these tracks are single microtubules (Vale, 1987; Brady et al., 1982). In neurons, microtubule transport is important for targeting secretory vesicles to their sites of release.

**Microtubule Drugs**

To evaluate the role of microtubules in the transport of secretory vesicles to the cell surface and in exocytosis, most studies have employed the use of microtubule disrupting drugs such as colchicine, the vinca alkaloids (vinblastine and vincristine), demecolcine (colcemide) or podophyllotoxin or the microtubule stabilizing drug, taxol, to interfere with secretion. Colchicine, podophyllotoxin and demecolcine alter microtubules by binding to free tubulin which prevents further addition of tubulin to microtubules, leading to microtubule depolymerization. At high concentrations, the
vinca alkaloids disrupt microtubules by inducing the formation of paracrystalline aggregates of tubulin. Taxol has the opposite effect; it binds to microtubules and enhances assembly of free tubulin into microtubules, preventing depolymerization.

In a variety of cells including fibroblasts, hepatocytes, mammary gland cells and exocrine and endocrine cells, disruption of microtubules with microtubule drugs will alter secretion. It has been observed that release of secretory granules made prior to drug treatment and that lie at the cell surface is usually not affected. However, it has been demonstrated that secretion of newly synthesized materials is inhibited, suggesting that microtubules are required for transport of these proteins to the surface.

**Microtubules in Adrenal Chromaffin Cells**

In chromaffin cells, cytoskeletal elements such as microtubules, actin) and their regulatory proteins have been isolated and characterized (Trifaro, 1977; Bader et al., 1984). Indirect immunofluorescence techniques using antibodies raised against tubulin have localized the distribution of the microtubule network; microtubules are found in the cell body and along the neurites. Early work by Poisner and Bernstein (1971), using the microtubule disrupting drugs colchicine, vinblastine and vincristine, suggested that microtubules were involved in the secretory
mechanisms of chromaffin cells. Bader and coworkers (1984) demonstrated that treatment of chromaffin cells with colchicine induced depolymerization of microtubules and a return of secretory granules located at the tip of neurites to the cell body, which was followed by a complete retraction of the neurites. These results supported a role for microtubules in the transport of secretory granules and in neurite outgrowth and maintenance. High affinity binding sites for tubulin have been demonstrated on chromaffin granules, providing further evidence for an interaction between secretory granules and microtubules (Trifaro, 1978).

Studies conducted by Trifaro and colleagues (1972) indicated that, although microtubules might be involved in transport of chromaffin granules to the cell periphery, they were not involved in the final steps of secretion. This was based on the fact that exocytosis of catecholamines induced by depolarizing concentrations of K⁺ were not blocked by either colchicine or vinblastine. These studies suggest that the effect of colchicine and vinblastine on secretion were due to an anticholinergic action of the drugs (Trifaro et al., 1972). Other studies employing the microtubule drugs taxol, colchicine and vinblastine have also demonstrated a selective inhibitory effect on ACh stimulated catecholamine release from cultured adrenal chromaffin cells (McKay and Schneider, 1984). In these studies it was also shown that the microtubule drugs do not block catecholamine
secretion induced by other agents such as depolarizing concentrations of K\(^+\), veratridine or barium (McKay and Schneider, 1984). Therefore, in adrenal chromaffin cells, microtubules may play a role in transport of secretory granules to their site of release, but they are not involved in the terminal steps of a common secretory pathway.

**NEURONAL nAChRs**

nAChRs are found in neuromuscular junctions and on neuronal tissues (sympathetic ganglia, brain and adrenal medulla). Although muscle-type nAChRs are fairly well characterized, less is known about adrenal nAChRs and other neuronal-type nAChRs. Pharmacological similarities between neuronal and muscle nAChRs have been reported (Lipscomb and Rang, 1988). Other studies though, have indicated that these two receptor types differ in their sensitivities to various nAChR antagonists and neurotoxins (Galzi et al., 1991). It is also becoming clear that neuronal nAChRs differ from muscle nAChRs in both structure and regulation (Galzi et al., 1991).

**Structure**

Nicotinic cholinergic transmission occurs in the central nervous system, autonomic ganglia and adrenal medulla (Clarke et al., 1984; Clarke et al., 1985; Smith et al. nervous system were based on \(\alpha\)-Bgt binding to brain
membranes. However, this toxin was not a useful tool for these studies since it did not block ACh-induced ion conductance in the CNS (Oswald and Freeman, 1981; Morley and Kemp, 1981; Clark et al., 1985; Marks et al., 1986). It does not bind to brain [\(^{3}\text{H}\)]nicotine or [\(^{3}\text{H}\)]ACh-binding sites (Patrick and Stallcup, 1977; Clark et al., 1985) or compete for nicotinic binding sites in rat brain membranes (Romano and Goldstein, 1980). Studies by various groups suggest that \(\alpha\)-Bgt in brain binds to a pharmacologically distinct subtype of neuronal nicotinic receptor (Couturier et al., 1990; Schoepfer et al., 1990).

Pharmacological characterization of neuronal nAChRs has been facilitated by a new toxin, neuronal bungarotoxin or bungarotoxin 3.1 (Bgt 3.1). Bgt 3.1 has been shown to block nicotinic cholinergic transmission in chick ciliary ganglion and sympathetic ganglia (Chiappinelli, 1983; Chiappinelli et al., 1985). Affinity labeling studies using Bgt 3.1 have identified a 59 kD putative nAChR subunit in chick ciliary ganglia (Halvorsen and Berg, 1987); similarly, a putative neuronal nAChR that binds mAb 35 and Bgt 3.1 has been identified on bovine chromaffin cells (Higgins and Berg, 1987).

Another probe for identifying nAChRs in neuronal tissue has been of anti-nAChR monoclonal antibodies (Whiting et al., 1987). A monoclonal antibody (mAb 35) directed against the main immunogenic region of the \textit{Torpedo} nAChR \(\alpha\) subunit
was found to cross-react with a component in chick ciliary ganglia (Jacob et al., 1984; Smith et al., 1985). Using mAb 35, a putative chick brain nAChR was isolated and found to contain two proteins of 49 and 58 kD (Whiting and Lindstrom, 1987a). The purified receptor from chick brain was then used to raise another monoclonal antibody (mAb 270) which cross reacted with a rat brain component (Whiting and Lindstrom, 1987b). mAb 270 has been used to isolate a putative neuronal nAChR from rat brain containing two different proteins with molecular weights of 51 and 79 kD (Whiting and Lindstrom, 1987a, 1987b). The 49 and 58 kD proteins from chick brain and the 51 and 79 kD proteins from rat brain were designated as neuronal nAChR α and β subunits, for each species (Smith et al., 1985; Whiting and Lindstrom, 1987a, 1987b).

These proteins purified from brain are referred to as α and β based on molecular weight and cross reactivity with subunit-specific antibodies raised against Torpedo nAChRs (Whiting and Lindstrom, 1986). However, when these purified subunits from chick or rat brain were incubated with the ACh affinity analog, 4-(N-maleimido)benzyl-trimethylammonium (MBTA), which affinity labels the α subunit of Torpedo and muscle AChRs, only the β subunit was labeled (Whiting and Lindstrom, 1987b). From these studies it was concluded that the β subunit from brain contains the ACh-binding site and was more closely related to the α subunit of Torpedo and
muscle nAChR. Therefore, the terminology was changed: the 51 and 79 kD proteins were designated as the α subunit and the 49 and 58 kD proteins as β subunit. Whiting and Lindstrom (1986) first designated the stoichiometry for these receptors as $\alpha_2\beta_3$. The proposed $\alpha_2\beta_3$ stoichiometry has been accepted since it is analogous to that of the nAChR from Torpedo electric organ and muscle with two identical ligand binding subunits in a total of five subunits (Whiting et al., 1987a, Whiting and Lindstrom, 1987b). Studies by Cooper and coworkers (1991) have also demonstrated that functional neuronal nAChRs are pentameric complexes composed of two α and three non-α (nα, β or structural) subunits. Several lines of evidence indicate that neuronal nAChRs are made up of only two subunits, an α subunit which binds agonists and a β (structural) subunit (Whiting and Lindstrom, 1987b; Bertrand et al., 1990; Boulter, 1990).

Molecular techniques have also contributed to the identification of neuronal nAChRs subunits. Expression of cDNAs for the structural subunits and the ACh-binding subunits in Xenopus oocytes produces functional AChRs (Boulter et al., 1987). Analysis of the cDNA sequences for subunits of neuronal nAChRs demonstrates the presence of four hydrophobic domains which correspond to those of muscle nAChR (Boulter et al., 1987). The amino acid sequence of the second hydrophobic domain is the most conserved (Lindstrom et al., 1989). All subunits of neuronal nAChRs
have an N-glycosylation site and cysteine residues corresponding to those found on the muscle nAChR α subunit (α192-193), which are characteristic of binding subunits and accounts for their MBTA reactivity (Tzartos et al., 1988; Das et al., 1989; Schoepfer et al., 1989; Fronasari et al., 1990). All of the subunits in neuronal nAChRs contain a long hydrophilic domain of variable length, located at C-terminal of M₃. This region corresponds to nonhomologous highly immunogenic sequences on the cytoplasmic surface of muscle nAChRs (Ratnam et al., 1986; Schoepfer et al., 1989). It has been proposed that this cytoplasmic loop, as well as other cytoplasmic domains, like muscle, might interact with cytoskeletal elements to anchor the nAChRs at their functional locations (Schoepfer et al., 1989).

**Pharmacology**

Molecular and electrophysiological techniques have contributed in identifying several genes encoding α and β subunits of neuronal nAChRs. In the brain and peripheral ganglia these genes have distinct, yet overlapping patterns of expression. A number of neuronal nAChRs with distinct functional and pharmacological properties can be made in oocytes by pairwise combination of different α and β subunits (Boulter, et al., 1987; Deneris et al., 1989; Duvoisin et al., 1989; Papke et al., 1989; Bertrand et al., 1990; Boulter et al., 1990; Couturier et al., 1990; Sawrok
Expression studies in Xenopus oocytes have shown that the following combinations of subunits form functional nicotinic receptors: \( \alpha_2/\beta_2; \alpha_3/\beta_2 \) and \( \alpha_4/\beta_2 \) (Boulter et al., 1987; Wada et al., 1988). Different combinations of \( \alpha \) and \( \beta \) subunits form pharmacologically distinct neuronal nAChR subtype, which posses varying affinities for agonists and antagonists (Luetje and Patrick, 1991; Deneris et al., 1991). Antagonists such as hexamethonium, decamethonium and tubocurarine and other cholinergic antagonists have been shown to block receptor activation (Bertrand et al., 1990; Couturier et al., 1990; Luetju et al., 1990; Luetje and Patrick, 1991). However, the agonists, cytisine and 1,1 dimethyl-4-phenylpiperazinium (DMPP) and the antagonists, Bgt 3.1 and neosurugatoxin are more potent agonists of neuronal nAChRs than muscle type nAChRs (Couturier et al., 1990; Bertrand et al., 1990; Luetju et al., 1990; Luetje and Patrick, 1991).

Neuronal nAChRs contain two ACh-binding sites (Whiting and Lindstrom, 1986; Lindstrom et al., 1987), like Torpedo and muscle nAChRs, and these sites interact in a positive cooperative manner. The subunits of neuronal nAChRs also associate to form a ligand gated ion channel (Lindstrom et al., 1987), and the electrophysiological properties of the channel are not much different from those of muscle AChRs (Lipton et al., 1987; Sakmann et al., 1983). Like muscle
nAChRs, noncompetitive antagonists, also block neuronal nAChR ion permeability (Ramoa et al., 1990; Rapier et al., 1987). Among the noncompetitive antagonists that also block neuronal nAChRs are histrionicotoxin (Eldefrawi et al., 1977), phencyclidine (Albuquerque 1980), MK-801 (Clineschmidt et al., 1982), hexamethonium and decamethonium (Bertrand et al., 1990), tubocurarine (Wada et al., 1988) and neosurugatoxin (Luetje et al., 1990).

**Neuronal nAChRs on Adrenal Chromaffin Cells**

nAChRs on chromaffin cells resemble ganglionic nAChRs of the autonomic nervous system since both are derived embryologically from the neural crest. The unavailability of suitable probes had prevented the identification and biochemical characterization of nAChRs present in chromaffin cells. α-Bgt, which blocks the function of Torpedo and muscle nAChRs (Fambrough, 1979), has no effect on neuronal nAChRs detected in PC12 cells, chromaffin cells, autonomic ganglia and the central nervous system (Kilpatrick et al., 1981; Deneris et al., 1991).

Bgt 3.1 and mAb 35, two probes which have been useful in identifying neuronal type nAChRs, have been shown to recognize nAChRs on bovine adrenal chromaffin cells (Higgins and Berg, 1987). mAb 35 cross reacts with the functional nAChRs of chicken ciliary ganglion neurons (Halvorsen and Berg, 1986) and bovine adrenal chromaffin cells (Higgins and
Berg, 1987). This antibody binds to the main immunogenic region (a highly conserved determinant of unknown function which is very conformational dependent) of the muscle nAChR (Ralston et al., 1987). Antibodies to the main immunogenic region of muscle and electric organ nAChR α subunit have been shown to induce clustering, internalization and degradation of muscle nAChRs (Merlie et al., 1979). Prolonged exposure to mAb 35 will specifically modulate chromaffin nAChR function and cause a decrease in the number of nAChRs (Higgins and Berg, 1988). These observations provide evidence that the mAb 35 binding component on chromaffin cells is part of the nAChR.

The second probe, Bgt 3.1, has been shown to block function of neuronal nAChRs expressed in autonomic ganglia (Jacob et al., 1984; Higgins and Berg, 1987) and in adrenal chromaffin cells (Higgins and Berg, 1987). Reports suggest that nAChRs on chromaffin cells are pharmacologically similar to other neuronal nAChRs (Higgins and Berg, 1987). They are activated by agonists such as nicotine, acetylcholine and carbachol and blocked by Bgt 3.1, hexamethonium, decamethonium, d-tubocurarine and other antagonists shown to block neuronal nAChRs (Higgins and Berg, 1988b). It has been demonstrated that adrenal chromaffin nAChRs are desensitized by cholinergic agonists (Clapman and Neher, 1984; Higgins and Berg, 1988b).

An α-Bgt binding site has been demonstrated on adrenal
chromaffin cells. Studies have indicated that the α-Bgt binding component is distinct from the nAChRs on the cells, since α-Bgt does not block receptor activation by agonists (Kilpatrick, 1981; Higgins and Berg, 1988b).

Results obtained from binding and functional studies conducted to characterize adrenal chromaffin nAChRs suggest that adrenal nAChRs are indeed representatives of neuronal nAChRs (Higgins and Berg, 1988). These receptors like neuronal type nAChRs differ from muscle and electric organ nAChRs in both structure and regulation (Whiting and Lindstrom, 1986; Halvorsen and Berg, 1987; Higgins and Berg, 1987, 1988).

RECEPTOR-CYTOSKELETON ASSOCIATIONS

Recognition of a stimulus at cell surfaces and transmission of signals from the external environment across the plasma membrane are key steps in activation of many cells. Association of surface receptors with the cytoskeleton is widely believed to be an early event resulting from cellular activation (Nicolson, 1976). Numerous membrane receptors have been demonstrated to be associated with the cytoskeletal network, including receptors for nerve growth factor (Vale and Shooter, 1982), PDGF (Zippel et al., 1989), transferrin (Hunt et al., 1989) and fibronectin (Horwitz et al., 1986). Association of these receptors with the cytoskeleton has been demonstrated
to play an important role in receptor function. Receptor-cytoskeletal linkages are important for segregation of receptors and ion channels in specific membrane regions (Henis, 1989), signal transduction mechanisms (Carraway and Carraway, 1989) and in receptor-mediated endocytosis (Devreotes and Fambrough, 1975; Heinemann et al., 1977; Higuchi et al., 1981).

Receptor-cytoskeletal associations have also been demonstrated for muscle nAChRs. In postsynaptic regions of both electric organ and muscle, nAChRs accumulate at very high densities with little lateral diffusion in and out of this area. nAChRs are transmembrane proteins that are inherently capable of diffusion within the plane of the membrane; therefore, these receptors must be anchored at the synaptic site by interactions with other proteins. In adult muscle, the nACh are clustered in the postsynaptic membrane of the neuromuscular junction. Prives et al. (1982) demonstrated using cultured muscle cells that as these cells age in culture the proportion of nAChRs forming clusters increase and the receptors became more resistant to extraction with Triton, a treatment which leaves the internal cytoskeleton essentially intact. Similarly, Stya and Axelrod (1983a) have shown a direct correlation between the mobility of nAChRs in the plane of the membrane and their extractability by Triton. In these studies on cultured muscle cells, receptor clusters which were immobile
were also resistant to Triton-extraction, suggesting an association with the cytoskeleton.

Various proteins at the synaptic site have been implicated in being involved in anchoring nAChRs (Froehner, 1986). An intracellular 43 kD peripheral protein (known as the 43K protein) has been shown to colocalize in equimolar concentrations with nAChRs (LaRochelle and Froehner, 1986) from electric organ (Sealock et al., 1984) and skeletal muscle (Froehner et al., 1981). Recent studies by Froehner and coworkers (1990), using a Xenopus oocyte expression system, demonstrated that nAChRs are diffusely distributed when expressed in Xenopus oocytes; however, coexpression with the 43K protein results in clustering of nAChRs. In these studies, it was also shown that the 43K protein was capable of forming clusters when expressed in the absence of nAChRs. Similar results were obtained by Phillips and coworkers (1991), using a quail fibroblast cell line to express cDNAs for muscle nAChRs and the 43K protein. These researchers also observed that when nAChRs were expressed in the absence of the 43K protein, the receptors fail to cluster. However, when nAChRs were coexpressed with the 43K protein, clustering was observed on the cell surface. Patches on the cell surface were also observed when the 43K protein was expressed alone. These observations together suggest that the 43K protein might participate in aggregation of nAChRs and possibly link the nAChRs to the
cytoskeleton to maintain their localization, as in postsynaptic membranes (Froehner et al., 1990, Phillips et al., 1991).

Various studies have provided evidence that microtubules may participate in anchoring receptors. For example, Connolly and Oldfin (1985) using cultured chick muscle cells, have demonstrated that treatment of these cells with a microtubule stabilizing drug, taxol, induces aggregation of nAChRs. In other studies, Connolly (1984) has shown that treatment of muscle cells with microtubule disrupting drugs such as demecolcine and nocodazole, blocks formation of nAChRs clusters. These results were consistent with earlier studies by Stya and Axelrod (1983b), which demonstrated that the microtubule disrupting drug, colchicine, blocked formation of nAChR clusters on rat myotubules. Therefore, these studies provide evidence that microtubules might bind to nAChRs.

Interactions of membrane receptors with cytoskeletal proteins are believed to play important roles in membrane functions such as ion transport and signal reception (Freedman, 1981). Young and Poo (1983), using Xenopus muscle cells, demonstrated that the time the nAChR associated ion channel remained open was longer for clustered than nonclustered receptors. These studies also imply that an association of nAChRs with the cytoskeleton is important for nAChR topography within the cell membrane and
for nAChRs channel function.

Similarly, studies have implicated a role for microtubules in neuronal nAChRs. For example, it has been shown that in adrenal chromaffin cells microtubule drugs such as taxol, colchicine and vinblastine inhibit nicotine mediated catecholamine release without affecting release stimulated by other secretagogues (McKay and Schneider, 1984). Additional studies by McKay and Schneider (1985) have shown that taxol and vinblastine inhibit ACh induced Ca\(^{2+}\) uptake in adrenal chromaffin cells without affecting K\(^+\) stimulated uptake. McKay and coworkers (1984) have also demonstrated that vinblastine interferes with histrionicotoxin binding to sites associated with the nAChR ion channel complex from Torpedo electric organ. These results suggest that these microtubule drugs are acting at the level of the nAChR to interfere with receptor function and binding. Furthermore, McKay (1988) has shown that taxol alters the microtubular network of adrenal chromaffin cells. These studies together suggest that the action of the microtubule drugs are not on the terminal steps of exocytosis, but instead alter nAChR function by either a direct effect of the drugs on the receptor or an effect on microtubule links to the nAChRs of adrenal chromaffin cells. Therefore, if indeed microtubules play a role in adrenal nAChR function, then other microtubule drugs should have the same or similar effects on the function of adrenal nAChRs.
and should affect adrenal microtubules under the same or similar conditions that affect receptor function. The hypothesis under investigation is that adrenal nAChRs are associated with microtubules and that this association may be important for receptor function.
CHAPTER III
MATERIALS AND METHODS

MATERIALS.

Chemicals and Antibodies

DL-[7-\textsuperscript{3}H(N)]norepinephrine (specific activity, 8-15 Ci/mmol), L-[N-methyl-\textsuperscript{3}H]nicotine, mouse anti-tubulin antibodies, fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse immunoglobulins and FITC-conjugated goat anti-rat immunoglobulins were obtained from DuPont New England Nuclear Corporation, Boston, MA. The antibody, 6-11B-1, specific for acetylated tubulin, was a generous gift from Dr. Gianni Piperno (The Rockefeller University, N.Y.). The cell line producing, the monoclonal antibody mAb35, was obtained from the American Type Culture Collection, Rockville, MD. The following chemicals were obtained from the Sigma Chemical Company, St. Louis, MO: nicotine hydrogen tartrate, histamine hydrochloride, veratridine, podophyllotoxin, demecolcine (colcemid), nocodazole, poly-L-lysine hydrobromide (MW range 77,000 - 79,000), L-ascorbic acid, Trizma hydrochloride (Tris [hydroxymethyl] aminomethane hydrochloride), Trizma base (Tris [hydroxymethyl] aminomethane base), dimethylsulfoxide (DMSO, 99 % pure). Tubulozole hydrochloride was obtained from
Research Biochemicals Incorporated, Natick, MA. Vinblastine and vincristine were gifts from the Eli Lilly Company, Indianapolis, IN. Taxol was obtained from the National Products Branch, Division of Cancer Treatment, National Cancer Institute (Bethesda, MD) and was dissolved in DMSO. ScintiVerse E liquid scintillation cocktail was acquired from Fisher Scientific, Cincinnati, OH. All other reagents used were of analytical grade.

Cell Culture Supplies

Dulbecco's Phosphate Buffered Saline (D-PBS) 10X, powdered Dulbecco's Modified Eagle Medium (DMEM, low glucose and high glucose) and Protein Free Hybridoma Media (PFHM-II) were obtained from Gibco-BRL Laboratories, Grand Island, NY. The following chemicals were obtained from the Sigma Chemical Company, St. Louis, MO: antibiotic/antimycotic 100X solution (amphotericin B, 25 μg/ml; penicillin, 10,000 U/ml; streptomycin, 5,000 μg/ml), L-glutamine, gentamicin sulfate, Percoll, 5-fluorodeoxyuridine (FDU), cytosine arabinofuranoside (Ara-C), HEPES (N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]), fetal calf serum (FCS, heat inactivated) and deoxyribonuclease (DNase, Type IV) and bovine serum albumin, fraction V powder (BSA). Collagenase Type I, (activity 196 - 210 U/mg) was acquired from Worthington Biochemical Corporation, Freehold, NJ. Sterivex-GS filter units (0.22 μm) were obtained from
Millipore Corporation, Medford, MA. Disposable syringe filters (0.22 μm) were obtained from PGC Scientifics Co., Gaithersburg, MD. Falcon Primaria tissue culture plates (6-well plates, 35mm), Corning 24 well (16 mm well) and 12 well (25 mm well) plates, culture flasks (T25, T75 and T150 cm²) and Spectra mesh (60, 105 and 210 μm) were obtained from Fisher Scientific, Cincinnati, OH.

METHODS

Adrenal Chromaffin Cell Isolation

Adrenal chromaffin cells were isolated from bovine adrenal glands obtained immediately after slaughter from Herman Falter Packing Company, Columbus, OH, and used within 30 min of their removal. The glands were kept on ice during transport to the laboratory. Adrenal chromaffin cells were isolated from the intact gland under sterile conditions as described by McKay and Schneider (1984). After removing all the fatty tissue, 1 mm incisions were made over the entire surface of the gland to facilitate perfusion. A 3 ml syringe was used to gently inject 3 ml of sterile, Ca²⁺-free, Locke's solution (containing: 0.14 M NaCl, 5.4 mM KCl, 1.0 mM NaH₂PO₄, 11 mM glucose, 250 ng/ml amphotericin B, 100 U/ml penicillin and 50 μg/ml streptomycin, 15 mM HEPES, pH 7.3 - 7.5) gently injected into the central vein of the gland. This procedure was repeated three times, to aid in the removal of residual
peristaltic pump was set up to perfuse the glands with Locke's solution. The tubing to be used in the perfusion was kept in 70 % ethanol overnight, then rinsed with 50 ml of Locke's solution prior to use. One end of the tubing was inserted into the adrenal vein and the other end was immersed in Locke's solution. Three to four glands were then placed on a gauze covered, wire mesh on a ring stand, suspended over a 1000 ml beaker (Fig. 3A). The glands were covered with a piece of gauze and moistened with Locke's solution. Glands were then perfused through the adrenal vein with 500 mls of Locke's solution at room temperature.

Following the perfusion with Locke's solution, a 3 ml syringe was used to gently inject 3 mls of sterile Locke's solution containing 0.05 % collagenase, into the central vein of the gland. This procedure was repeated one more time to aid in the digestion of the medulla. The glands were then placed into a 100 ml beaker containing enough 0.05 % cold collagenase solution to cover the glands. The tubing was removed from the Locke's solution and placed into the 100 ml beaker. The glands were perfused with the collagenase solution for 20 to 30 min (Levitt, 1984; McKay and Schneider, 1984).

After the collagenase perfusion, the glands were
Figure 3. A. Set up of the perfusion apparatus. B. Cross-section of the adrenal gland, exposing the digested adrenal medulla (arrow).
Figure 3 continued
removed from the collagenase solution and an incision made around the perimeter of each gland. The medulla was carefully separated from the cortex by a peeling motion. When all the medulla was separated from the cortex (Fig. 3B), it was placed in a mincing bowl containing a small amount of cold Locke's solution.

Following separation of all medullae, the tissues were minced (1 - 2 mm²) with 2 small scissors for several minutes. The minced tissue was then transferred to a 50 ml sterile centrifuge tube, 20 - 25 mls of collagenase solution added and the tissue incubated for 60 min at 37°C in a shaking waterbath (Precision Dubnoff Metabolic Shaking Incubator, setting 4.5). After the digestion period, the tissue was separated from the collagenase solution by filtration through 250 μm nylon mesh. The filtrate containing dissociated cells was collected in a sterile 50 ml centrifuge tube. Cold Locke's solution was added to the tube (through the nylon mesh) to bring the final volume to 50 ml. The tube was then centrifuged at 800 rpm (75 xg) for 10 min in a bench top centrifuge (IEC-HN-SII Centrifuge). The supernatant was carefully aspirated and discarded, and the cell pellet was gently resuspended in 10 ml of Locke's solution and stored on ice. The undigested tissue remaining on the nylon filter was placed in a sterile 50 ml centrifuge tube, 20 - 25 ml of fresh collagenase solution added and the digestion, filtration, and centrifugation processes were
repeated, as described above.

The cell suspensions from the first and second digestions were pooled in a 50 ml centrifuge tube. Locke's solution supplemented with 0.5 % BSA was added to bring the final volume to approximately 50 ml. The cell suspension was centrifuged at 800 rpm (75 xg) for 10 min. The cell pellet was gently resuspended in 10 mls of Locke's solution with BSA and filtered through a 100 μm nylon mesh. The cell filtrate was collected in a sterile 50 ml centrifuge tube. Cold Locke's solution with BSA was added to the tube (through the nylon mesh) to bring the final volume to 39 mls. The cell suspension was added to Percoll (pH adjusted to approximately 7.2 - 7.4 prior to use), and this cell suspension was centrifuged at 17,500 rpm (38,000 xg) in a Sorvell refrigerated centrifuge (Sorvell RC-5B refrigerated superspeed centrifuge, Dupont, N.Y.) at 4°C for 40 min. The utilization of this Percoll gradient technique to separate live from dead cells greatly enhanced the viability of the final cell preparation. Following centrifugation in the Percoll gradient, the upper layer of dead cells was aspirated and the remaining viable cells were resuspended in Locke's with 0.5 % BSA. The cells were then centrifuged at 1000 rpm (110 xg) for 15 min. After this centrifugation, the supernatant was discarded and the cells resuspended in a given volume of Locke's with 0.5 % BSA. The number of cells in suspension and their viability were determined using a
vital dye exclusion test with 0.4 % erythrosin B. Live cells with intact membranes excluded the dye while dead cells appeared red from an accumulation of erythrosin B dye. The number of cells was determined using a Bright-Line Hemocytometer, Reichert Scientific Instruments, Buffalo, NY. Cell viability was generally 88 - 97 %. The cell suspension was then brought to a final volume of 50 ml with DMEM supplemented with 10 % FCS, glutamine (200 μM), penicillin (100 U/ml), streptomycin (50 μg/ml), amphotericin B (0.25 μg/ml), 5-fluorodeoxyuridine (10 μM), gentamicin sulfate (40 μg/ml), and centrifuged at 800 rpm (75 xg) for 10 min. The cell pellet was gently resuspended with 8 ml of supplemented DMEM and filtered through 60 μm nylon mesh. The filtrate was collected in a sterile 150 ml beaker. The cell suspension was then diluted to 1 x 10^6 cells/ml with supplemented DMEM.

Cells to be used in secretion expriments were plated in 24-well culture plates containing supplemented DMEM culture media (1 ml/well). These isolated chromaffin cells were plated at a density of 1 - 2 x 10^5 cells/well using an Eppendorf Repeat Pipetter. Cells to be used for immunocytochemistry were plated in 6-well (35 mm, 3 ml supplemented DMEM/well) culture plates containing glass coverslips (18 x 18 mm) at a density of 1 - 2 x 10^5 cells/well. Cells to be used for binding experiments were plated in 12-well (25 mm, 1.5 ml supplemented DMEM/well)
culture plates at a density of 1 × 10^6 cells/well. After plating the chromaffin cells, the plates were placed in a humidified, 37°C, 5% CO₂ incubator (Precision Scientific, Chicago, IL).

Catecholamine Secretion Studies.

**Loading Cells with [³H]Norepinephrine**

In order to measure catecholamine secretion from cultured bovine adrenal chromaffin cells, the cells were first loaded with [³H]norepinephrine. This radiolabelled catecholamine is taken up into the chromaffin cells by a neuronal uptake I-like mechanism (McKay, 1989) and stored in granules prior to release from the cells. The general procedure for loading the cells with [³H]norepinephrine are described by McKay and Schneider (1984). Modifications of this method which were necessary for some of the procedures found in this work will be outlined below.

The culture medium was aspirated from the 24-well culture plates and the cells washed once with 1 ml of a physiological salt solution (PSS, containing 140 mM NaCl, 4.4 mM KCl, 1.2 mM KH₂PO₄, 3.6 mM NaHCO₃, 1.2 mM MgSO₄, 2 mM CaCl₂, 10 mM glucose, 5 mM HEPES and 0.5% BSA, pH 7.3 - 7.35). After washing the cells, 200 μl of loading buffer was added to each well. The loading buffer was prepared by adding ascorbic acid (0.01%) and [³H]norepinephrine (0.1
μM) to PSS. The plates were then placed in a humidified, 37°C incubator for 1 - 2 hr. After the incubation period, the cells were washed three times (15 min each) with 1 ml of PSS to remove any unincorporated radioactivity.

**Catecholamine Secretion Experiments**

Catecholamine secretion experiments were performed in PSS which contained 2 mM Ca²⁺ and 0.5 % BSA. In experiments where chromaffin cells were stimulated with 56 mM KCl, the amount of Na⁺ in the buffer was decreased to maintain isotonicity. Similarly, when BaCl₂ was used to stimulate cells, the buffer composition was changed to Ba²⁺, 2 mM; NaCl, 140 mM; KCl, 5.6; MgCl₂, 1.2 mM; glucose, 10 mM; HEPES, 5mM (pH 7.2-7.4); and 0.5 % BSA. Throughout these studies, triplicate wells were used for each treatment group.

In experiments in which the effects of microtubule drugs on nicotine-stimulated catecholamine release were investigated, the cells were pretreated at room temperature with these compounds for 30 min prior to stimulation which occurred in the continued presence of the drug. For time course studies, cultured bovine adrenal chromaffin cells were incubated at room temperature for different time periods (0, 5, 10, 30, 60 and 180 min) with the microtubule drug at a concentration approximating IC₈₀ values. In studies in which the reversibility of the effects of
microtubule drugs was examined, the cells were pretreated with approximate IC_{80} concentrations of the agent for 1 hr. After the treatment period, the drug solution was removed and the cells were washed for different time periods (120, 60, 30, 10, 2 and 0 min). After the washes, the cells were stimulated with nicotine (10 μM) in the absence of the microtubule drug, except for the group of cells where the drug was not removed (0 min). Basal (PSS alone) and control nicotine-stimulated (10 μM nicotine in PSS) cells were preincubated with PSS for 30 min during the pretreatment period. Cells used to measure basal release were also kept in PSS during the 10 min stimulation period.

In studies where the selectivity of mAb 35 for nAChRs was tested, cells were incubated with 10 and 100 nM mAb 35 for 20 hrs. At the end of the incubation period, the cells were loaded with [^{3}H]norepinephrine, in the presence of either 10 nM or 100 nM mAb35, for 1 hr. After the loading period, the cells were washed three times (15 min each) with 1 ml of PSS in the presence of mAb 35 (cells were exposed to mAb 35 for a total of 24 hr). The cells were then stimulated with either nicotine (10 μM) or 56 mM K^+, in the absence of mAb 35.

For all secretion experiments, after the stimulation period, the medium from each well was transferred to 7 ml glass vials containing 5 ml scintillation cocktail, mixed well and counted by liquid scintillation spectroscopy. The
radioactivity remaining in the cells was determined by extracting the cells with 8 \% (w/v) TCA (0.5 ml per well) for 15 min. The cell extract was placed in glass vials, mixed with 5 ml of scintillation cocktail and counted for 10 min by liquid scintillation spectroscopy. The sum of the secreted and the TCA-extractable radioactivity represented total incorporated [\(^3\)H]norepinephrine. Results were expressed either: 1) as the fraction of the total incorporated [\(^3\)H]norepinephrine released under the treatment conditions (i.e., secreted [\(^3\)H]norepinephrine divided by total incorporated [\(^3\)H]NE) or 2) as % control (i.e. the fractional amount released under each treatment condition divided by the fraction released by secretagogue).

**Tubulin Immunocytochemistry**

**General Procedure**

An indirect immunofluorescence technique was used to investigate drug effects directly on the adrenal microtubular network, as described by McKay (1989). DMEM was aspirated, and the cells in each well of a 6-well plate were pretreated with the drugs to be tested. After treatment, the coverslips (with attached cells) were removed from the 6-well plate and labeled on the lower right hand corner with a diamond pen. The coverslips were then washed one time by placing into a Columbia jar containing Dulbecco's phosphate buffered saline (D-PBS) for 5 min.
Following the wash, the D-PBS was aspirated off and enough 3.7 % formaldehyde solution added to the coverslips in the Columbia jar to cover them. The cells were incubated in the fixing solution for 30 min. After fixation, the formaldehyde solution was aspirated from the Columbia jar and a 0.2 % Triton X-100 in 3.7 % formaldehyde solution added. The cells were extracted for 10 min at room temperature. At the end of the extraction period the coverslips were placed on a coverslip rack and washed extensively by dipping the rack 15 - 20 times into a 150 ml beaker containing fresh D-PBS solution. The coverslips were then placed back into a clean Columbia jar with fresh D-PBS and incubated for 10 min. The cells were then incubated in 50 % acetone for 3 min, followed by a 5 min incubation in 100 % acetone and once again incubated in 50 % acetone for 3 min. At the end of the incubation period, the acetone was aspirated, the cells rinsed twice in D-PBS, and then incubated for 10 min in D-PBS.

Following the wash, each coverslip was individually removed from D-PBS and any excess buffer removed by tilting the coverslip and blotting the edges with a tissue. Mouse anti-tubulin antibody (1/10 - 1/20 dilution) was added directly to the cells, approximately 20 - 40 µl, making sure that the entire coverslip was covered with the antibody solution. The coverslip was then placed on top of the 6-well plate. The same procedure was repeated for the other
coverslips. The coverslips were then placed in a moist chamber and incubated for 60 min.

After incubation with the primary antibody, the coverslips were removed from the moist chamber and each coverslip carefully rinsed with D-PBS using a squirt bottle to wash off excess antibody. The coverslips were then washed extensively by placing into the coverslip rack, dipped 15 - 20 times into a 150 ml beaker containing fresh D-PBS twice, placed back into a Columbia jar with fresh D-PBS and incubated for 10 min in fresh D-PBS. Following the wash, each coverslip was individually removed from D-PBS and any excess buffer removed by tilting the coverslip and blotting the edges with a tissue. FITC-conjugated goat anti-mouse immunoglobulins (1/150 - 1/200 dilution) was added directly to the cells, approximately 20 - 40 µl, making sure that the entire coverslip was covered with the antibody solution. Each coverslip was then placed on the cover of a 6-well plate, then placed in a moist chamber and incubated for 60 min.

At the end of the incubation period with the FITC-labeled secondary antibody, the cells were extensively washed as described above. After the 10 min wash in D-PBS, the coverslips were removed from the Columbia jar with forceps, the edges blotted dry with a tissue and wet mounted (cells facing down) onto a 10 µl drop of mounting media (9:1, glycerol:D-PBS). Clear nail polish was applied around
the edges of the coverslip to seal the edges. The specimens were examined with a Nikon Labophot microscope equipped with epifluorescent optics and photographed using Tri-X-Pan 400 ASA Kodak black and white print film.

**Acetylated Tubulin Studies**

The indirect immunofluorescence procedure used in these studies was identical to that described above with a few minor changes. After fixing, extracting and incubating the cells in acetone, the cells were washed twice in D-PBS containing 1% BSA for 10 min, to decrease the amount of nonspecific antibody binding. The cells were then incubated in D-PBS containing BSA for 30 min. The anti-acetylated tubulin antibody (6-11B-1) was then added directly to the coverslips and incubated as described for the anti-tubulin antibody above. The rest of the procedure is as described above.

**[^H]Nicotine Binding Studies**

**Rat Brain Membrane Preparation.**

Membranes were prepared from Sprague-Dawley rat brain tissue, according to the method of Romano and Goldstein (1980). Briefly, whole brains were removed, rinsed with ice-cold buffer containing Na₂HPO₄, 8mM; KH₂PO₄, 1.5 mM; KCl, 3 mM; NaCl, 120 mM; disodium ethylenediaminetetraacetate (EDTA), 2mM; HEPES, 20 mM (pH 7.4); 5 mM
iodoacetamide; 0.1 mM phenylmethylsulfonyl fluoride (PMSF), several times to remove any blood and homogenized. The homogenate was centrifuged (25000 xg, 0°C) for 20 min. The pellet was resuspended in distilled, deionized water and kept on ice for 60 min before centrifugation as described above. The pellet was then resuspended in the assay buffer to a final concentration of 1 mg/ml. The assay buffer was the same as the buffer used to prepare the membranes with the addition of 1 mM MgCl₂ and 2 mM CaCl₂ and the elimination of EDTA and PMSF. The protein concentration was then determined by the Bradford method (Bradford, 1976) using a commercial Bradford reagent (Bio-Rad Laboritories, Cambridge, MA). Bovine serum albumin (BSA) was used as the protein standard.

[^3H]Nicotine Binding to Rat Brain Membrane Fragments.

To determine the effects of microtubule drugs on[^3H]nicotine binding, binding assays were performed using the procedure of Lippiello and Fernandez (1982) with slight modification. Membranes (450 μg protein) were incubated with[^3H]nicotine (15 nM) and 1 μM α-Bgt in the absence or presence of the microtubule drugs in a total volume of 500 μl at room temperature for 60 min. Incubations were terminated by adding approximately 4 ml of ice-cold assay buffer (described above) and rapidly filtering the mixture through Whatman GF/B filters, using a Brandel binding
manifold (Brandel Research and Development Laboratories, Inc., Gaithersburg, MD). The filters were soaked in 0.1 %
polylysine for over 3 hr at 5°C to reduce nonspecific
binding (Romano and Goldstein, 1980). After the initial
filtration of the assay mixture, filters were continuously
washed with ice-cold assay buffer for 15 sec. The filters
were then placed in counting vials and mixed vigorously with
7 ml of ScintiVerse E. Samples were counted in a Beckman LS
6800 liquid scintillation counter at 30 - 40 % efficiency.
Nonspecific binding was determined in the presence of excess
carbachol (1 mM).

[^3H]Nicotine Binding to Intact Adrenal Chromaffin Cells

After isolation, chromaffin cells that were going to be
used for whole cell[^3H]nicotine binding studies were plated
onto Corning 12 well (25 mm) plates, containing DMEM, (1.5
ml/well), supplemented with 10 % (v/v) FCS and 200 µM, at a
density of 1x10^6 cells/well. After plating the culture
plates were placed in a humidified 37°C, 95 %/5 % CO₂
incubator. Cultures were used for experiments 3 - 5 days
after plating.

[^3H]Nicotine binding to intact adrenal chromaffin cells
was determined as described by Higgins and Berg (1988).
Culture plates were removed from the incubator, the culture
medium aspirated from the wells and the cells washed once
with 1 ml of DMEM. The culture media was then aspirated and
replaced with 500 μl of DMEM containing 10 nM [³H]nicotine and 1 μM α-Bgt. The cells were then placed in a humidified, 37°C incubator for 70 min. At the end of the incubation period the media was aspirated using a Millipore vacuum pressure pump, Bedford, MA., (vacuum set at 22 in of mercury) and the cells quickly washed 4 times (<10 sec, total wash time) with 1 ml of rinse buffer (pH 7.4, 5 mM HEPES, 137 mM NaCl, 5.4 mM KCl, 0.8 mM MgSO₄, 0.9 mM Na₂HPO₄, 0.4 mM KH₂PO₄, 1.8 mM CaCl₂, 5.6 mM glucose and 2 mg/ml BSA) at 37°C. This procedure was repeated for the remaining wells. Cells were then dissolved by incubating the cells in 500 μl of a 1 M NaOH solution for 60 min. At the end of the incubation period 100 μl aliquots from each well were added to 5 ml polystyrene plastic test tubes and assayed for protein by the method of Lowry et al. (1951) utilizing BSA as the standard. The remaining 400 μl aliquot of lysate was neutralized with 4 M HCL, mixed with scintillation cocktail and analyzed by liquid scintillation counting using a Beckman 6800 liquid scintillation counter.

[^125I]mAb 35 Studies

Growth and Maintenance of mAb 35 Secreting Hybridoma Cell Line

The hybridoma cell line secreting mAb 35, a monoclonal antibody reactive with nAChRs, was obtained from ATCC. Upon arrival, the cell suspension containing 3 x 10⁶ cells/ml was added to a T25 flask and diluted (1:25) in DMEM (high
glucose) containing 10% fetal calf serum, 2 mM glutamine, 50 µg/ml gentamycin, penicillin (100 U/ml), streptomycin (50 µg/ml), and amphotericin B (0.25 µg/ml), as directed in the product sheet.

Hybridoma cells were maintained in culture as suggested by Harlow and Lane (1988). The hybridomas were examined daily under a microscope for bacterial or fungal growth and for cell growth. Every 2 - 3 days or when the cell density appeared high enough Protein Free Hybridoma Media (PFHM-II) was added to the T25 flask to maintain a cell density between 1 x 10^5 and 1 x 10^6. This process allowed gradual dilution of the DMEM with PFHM-II. When the cell density was high and the culture media reached the neck of the T flask, the hybridomas were split by taking aliquots of the cell suspension and transferring it to a larger T flask (T75 or T100). PFHM-II was added to maintain the cell density between 1 x 10^5 and 1 x 10^6 cells. This procedure of diluting or splitting the hybridoma cells was repeated until a desired amount of supernatant containing mAb 35 was obtained.

**Purification of mAb 35**

mAb 35 was purified by ammonium sulfate precipitation from hybridoma culture media as described by Harlow and Lane (1988). Briefly, 100 ml of tissue culture supernatant were centrifuged at 5000 rpm (3000 xg) for 30 min in a
refrigerated centrifuge (Sorvall, RC-5B, refrigerated superspeed centrifuge, Dupont, N.Y.). The supernatant was transferred to a clean 400 ml beaker with a stir bar and placed on a magnetic stirrer. While the antibody solution stirred gently, 66.7 mls of saturated ammonium sulfate (761 g/L, pH 7.3 - 7.4 with NH₄OH) were slowly added to bring the final concentration to 40 % saturation. The beaker with the antibody-containing solution was then placed in a refrigerator at 4°C and left covered overnight.

The ammonium sulfate precipitated antibody was centrifuged at 5000 rpm (3000 xg) for 30 min in a Sorvall refrigerated centrifuge, to remove the precipitated antibody from the media. After centrifugation, the supernatant was discarded and the pellet was resuspended in 0.1 volume (10 mls) of the starting supernatant in D-PBS (pH 7.2). The antibody solution was then transferred to a 20 cm dialysis tubing (Spectra/Por membrane, MWCO 50,000, Fisher Scientific Co., Cincinnati, OH), placed in 2 L of D-PBS containing 0.02 % sodium azide and dialyzed against 2 changes of D-PBS overnight at 4°C. The antibody solution was then removed from the tubing and placed in a centrifuge tube and spun at 5000 rpm (3000 xg) for 30 min in a Sorvall refrigerated centrifuge, to remove any remaining debris. The concentration of the antibody was then determined by the Bradford method (Bradford, 1976) using a commercial Bradford reagent (Bio-Rad Laboratories, Cambridge, MA).
Immunoglobulin (IgG) was used as the standard. The protein concentration was usually 1 - 2 mg/ml. The antibody solution was then aliquoted and stored below 0°C.

Iodination of mAb 35

An aliquote (333 μl) of purified mAb 35 (1.5 mg/ml) was radioiodinated to a specific activity of 44 μCi/ml (or 22 Ci/mm) using the chloramine T technique as described by Lindstrom et al. (1981) for iodination of α-Bgt. This procedure was performed by Dr. Michaelle V. Darby, at The Ohio State University Comprehensive Cancer Center Radiation Laboratory.

[^125I]mAb 35 Binding to Intact Adrenal Chromaffin Cells

[^125I]mAb 35 binding to intact adrenal chromaffin cells was determined as described by Higgins and Berg (1987). Culture plates were removed from the incubator, the culture medium aspirated from the wells and replaced with 500 μl of DMEM containing 5 nM ^125IImAb 35. The cells were then placed in a humidified 37°C, 5 % CO₂ incubator for 60 min. At the end of the incubation period the media was aspirated using a vacuum pump (Millipore vacuum pressure pump, set at 22 inches of Hg). The cells were quickly washed (<10 sec) 4 times with 1 ml of rinse buffer containing 137 mM NaCl, 5.4 mM KCl, 0.8 mM MgSO₄, 0.9 mM Na₂HPO₄, 0.4 mM KH₂PO₄, 1.8 mM CaCl₂, 5.6 mM glucose, 5 mM HEPES (pH 7.4) and 2 mg/ml BSA
This procedure was repeated for the remaining wells. Cultures were then dissolved in 500 μl of a 1 M NaOH solution for 60 min. At the end of the 60 min the cells were scraped in the NaOH solution with a rubber policeman to remove any residual cells remaining attached to the plate. The dissolved cells were then added to 7 ml glass vials and analyzed for $^{125}$I with a Beckman Model 8000 gamma counter. Nonspecific binding, determined by adding 0.5 μM of unlabeled antibody to the incubation media, usually constituted 14 - 26 % of the total binding and was subtracted to yield specific binding.

[^125]mAb 35 Binding to Triton-Extracted Adrenal Chromaffin Cells

After labelling the cultures with $[^{125}\text{I}]$mAb 35, cells were Triton-extracted as described by Prives et al. (1982). Briefly, the labelled cells were washed once with a 1 ml volume of Buffer A, pH 7.4, containing 0.3M sucrose, 50 mM NaCl, 1 mM MgCl$_2$ and 10 mM HEPES. Buffer A was then aspirated and the cells extracted for 5 min with 500 μl of Buffer A containing 0.5 % Triton X-100 at room temperature. After the extraction period, the Triton buffer containing the Triton-soluble cell components was removed from the cells and transferred to 7 ml glass vials. The radioactivity remaining on the wells was determined by dissolving the Triton-insoluble cell fraction with 500 μl of a 1 M NaOH for 1 hr and scraped in NaOH. The dissolved
Triton-insoluble cell components were then added to 7 ml glass vials and analyzed for $^{125}\text{I}$ with a Beckman Model 8000 gamma counter.

In experiments in which the effect of microtubule drugs on $[^{125}\text{I}]{\text{mAb}}$ 35 binding to Triton-extracted cells were examined, $[^{125}\text{I}]{\text{mAb}}$ 35 binding was performed in the presence or absence of the microtubule drugs. $[^{125}\text{I}]{\text{mAb}}$ 35 binding was performed as mentioned above. After binding of $[^{125}\text{I}]{\text{mAb}}$ 35, the cells were extracted and the amount of radioactivity associated with the Triton-insoluble cell component determined using a gamma counter, as described above.

**Calculations and Statistics**

Secretion data are presented as arithmetic means ± SEM except for IC$_{50}$ values which are presented as geometric means (95% confidence limits); n represents the number of observations carried out in triplicate. Inhibition curves were generated by a nonlinear least squares fit for a general sigmoid curve (InPlot 3.1, GraphPad, San Diego, CA.). Data were analyzed by Dunnett's Test with significance at the $p < 0.05$ level. The data acquired in binding studies are presented as arithmetic means ± SEM; n represents the number of observations carried out in duplicates or triplicate. Data were analyzed by 2 Way ANOVA Test with significance at the $p < 0.05$ level.
CHAPTER IV
RESULTS

Effects of microtubule drugs on nicotine-stimulated $[^3\text{H}]$norepinephrine release from adrenal chromaffin cells

Drugs known to alter microtubule function, such as taxol and vinblastine, have been shown to inhibit nicotine-stimulated catecholamine release (McKay and Schneider, 1984; McKay et al., 1985). In order to further characterize the effects of other microtubule drugs on nAChR-stimulated catecholamine release in cultured bovine adrenal chromaffin cells, the concentration-response effects of other microtubule drugs on nAChR-stimulated catecholamine release were examined. Vincristine, tubulozole, podophyllotoxin and demecolcine inhibited nicotinic receptor-stimulated release of $[^3\text{H}]$norepinephrine in a concentration-dependent manner (Fig. 4) with IC$_{50}$ values of 3 (1-10), 5 (2-10), 8 (4-15) and 19 (9-39) $\mu$M, respectively (Table 1). Catecholamine release stimulated by 10 $\mu$M nicotine was almost completely abolished by all four compounds at a concentration of 100 $\mu$M.

Effects of microtubule drugs on catecholamine secretion mediated by various noncholinergic secretagogues

McKay and coworkers (1984, 1985) have previously
Table 1. Comparison of IC$_{50}$ values of various microtubule drugs for inhibition of adrenal nicotine-stimulated catecholamine release and for inhibition of in vitro tubulin polymerization.

<table>
<thead>
<tr>
<th>Drug</th>
<th>Catecholamine Release IC$_{50}$ (µM)$^a$</th>
<th>Tubulin Polymerization IC$_{50}$ (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vincristine</td>
<td>2.8 (0.8-10)</td>
<td>0.1$^b$, 2.0$^c$</td>
</tr>
<tr>
<td>Tubulozole</td>
<td>4.6 (2-10)</td>
<td>0.3$^d$, 4.0$^e$</td>
</tr>
<tr>
<td>Podophyllotoxin</td>
<td>8.1 (4.4-14.7)</td>
<td>0.5$^f$, 1.0$^g$</td>
</tr>
<tr>
<td>Demecolcine</td>
<td>19 (9.3-39.4)</td>
<td>NK$^h$</td>
</tr>
</tbody>
</table>

$^a$Values were derived from concentration-response curves.  
$^b$Himes et al., 1977  
$^c$Owellen et al., 1972 (porcine brain tubulin)  
$^d$Van Ginckel et al., 1984 (rat brain)  
$^e$Duanwu et al., 1989 (L1210 murine leukemia tumor cell line)  
$^f$Jordan et al., 1983  
$^g$Hoebeke et al., 1975  
$^h$Not Known
FIGURE 4. The concentration-response effects of several microtubule drugs on nAChR-stimulated adrenal catecholamine release. Cultured adrenal chromaffin cells were pretreated for 30 min with increasing concentrations of vincristine (filled diamonds), tubulozole (filled triangles), podophyllotoxin (filled squares) or demecolcine (filled circles) prior to stimulation with 10 μM nicotine in the continued presence of the drug. Results are expressed as a percentage of the nicotine-stimulated control response. Basal [3H]norepinephrine fractional release was 0.057 ± 0.005 and nicotine stimulated fractional release was 0.207 ± 0.014. Values represent means ± SEM of 8 to 10 experiments performed in triplicate.
demonstrated that the inhibitory effects of taxol and vinblastine on adrenal catecholamine secretion are selective for release stimulated through activation of nAChRs. To determine whether other microtubule drugs show similar selectivity, the effects of tubulozole, demecolcine, podophyllotoxin and taxol on adrenal catecholamine release stimulated by noncholinergic secretagogues (Ba\(^+2\), K\(^+\), veratridine and histamine) were investigated. Tubulozole (10 \(\mu\)M), demecolcine (100 \(\mu\)M), podophyllotoxin (100 \(\mu\)M) and taxol (10 \(\mu\)M) significantly inhibited nicotine-stimulated \([3H]\)norepinephrine release by 63 %, 82 %, 76 % and 61 %, respectively (Table 2). However, at these same concentrations the microtubule drugs did not affect release stimulated by 56 mM KCl, 2 mM Ba\(^+2\) or 50 \(\mu\)M veratridine. The ability of these drugs to interfere with histamine-mediated \([3H]\)norepinephrine release was also investigated to determine if the effect of the microtubule drugs was specific for release stimulated by receptor mechanisms. None of the microtubule drugs significantly altered histamine-stimulated catecholamine release (Fig. 5). Basal release of \([3H]\)norepinephrine was not significantly affected by any of the microtubule drugs tested (Table 3).

**Effects of microtubule drug treatment time on nicotine-stimulated catecholamine secretion**

Since it appears that microtubule drugs only inhibit catecholamine release mediated by nAChR in cultured bovine
Table 2. Selective inhibition of nAChR-stimulated adrenal catecholamine release by microtubule drugs.\textsuperscript{a}

<table>
<thead>
<tr>
<th>Catecholamine Release (fractional)</th>
<th>10 μM Nicotine</th>
<th>56 mM KCl</th>
<th>2 mM Barium</th>
<th>50 μM Veratridine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basal (nonstimulated)</td>
<td>0.059±0.011</td>
<td>0.041±0.008</td>
<td>0.071±0.016</td>
<td>0.036±0.007</td>
</tr>
<tr>
<td>Secretagogue</td>
<td>0.165±0.016</td>
<td>0.122±0.016</td>
<td>0.417±0.048</td>
<td>0.074±0.010</td>
</tr>
<tr>
<td>+ Taxol, 10 μM</td>
<td>0.100±0.011\textsuperscript{c}</td>
<td>0.125±0.023</td>
<td>0.393±0.037</td>
<td>ND\textsuperscript{b}</td>
</tr>
<tr>
<td>+ Tubulozole, 10 μM</td>
<td>0.098±0.010\textsuperscript{c}</td>
<td>0.087±0.013</td>
<td>0.328±0.020</td>
<td>0.049±0.047</td>
</tr>
<tr>
<td>+ Demecolcine, 100 μM</td>
<td>0.078±0.013\textsuperscript{c}</td>
<td>0.101±0.015</td>
<td>0.416±0.043</td>
<td>0.072±0.010</td>
</tr>
<tr>
<td>+ Podophyllotoxin 100 μM</td>
<td>0.085±0.016\textsuperscript{c}</td>
<td>0.094±0.017</td>
<td>0.390±0.031</td>
<td>0.055±0.008</td>
</tr>
</tbody>
</table>

\textsuperscript{a}Cultured adrenal chromaffin cells were pretreated for 30 min with the drug prior to stimulation in the continued presence of the drug. Results are expressed as a fraction of the total incorporated \textsuperscript{3}H]norepinephrine release during the 10 min stimulation period. Values represent means ± SEM of 5 to 7 experiments.

\textsuperscript{b}Not determined

\textsuperscript{c}Significantly different from the corresponding secretagogue-stimulated controls (p < 0.05).
Table 3. Effects of microtubule drugs on basal (nonstimulated) catecholamine release.\(^a\)

<table>
<thead>
<tr>
<th>Drug</th>
<th>Basal Catecholamine Release(^a)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basal (control)</td>
<td>0.022 ± 0.004</td>
</tr>
<tr>
<td>+ Demecolcine (100 µM)</td>
<td>0.028 ± 0.005</td>
</tr>
<tr>
<td>+ Podophyllotoxin (100 µM)</td>
<td>0.032 ± 0.003</td>
</tr>
<tr>
<td>+ Taxol (10 µM)</td>
<td>0.045 ± 0.004</td>
</tr>
<tr>
<td>+ Tubulozole (10 µM)</td>
<td>0.045 ± 0.008</td>
</tr>
<tr>
<td>+ Vincristine (10 µM)</td>
<td>0.032 ± 0.003</td>
</tr>
</tbody>
</table>

\(^a\)Cultured adrenal chromaffin cells were treated for 30 min in the absence (control) and presence of the indicated microtubule drug. The amount of \(^3\)H\)norepinephrine was measured during a 10 min period. Results are expressed as a fraction of the total incorporated \(^3\)H\)norepinephrine released. Values represent means ± SEM of 5 to 7 experiments. None of the treatments were statistically different from control (\(p < 0.05\)).
FIGURE 5. Effects of various microtubule drugs on \[^{3}H\]norepinephrine release stimulated by 10 μM nicotine or 500 μM histamine in cultured bovine adrenal chromaffin cells. Cells were pretreated for 30 min with 100 μM demecolcine (DEM), 100 μM podophyllotoxin (POD), 100 μM nocodazole (NOC), 10 μM taxol (TAX), 10 μM tubulozole (TUB) or 10 μM vincristine (VCR) prior to stimulation with agonist in the continued presence of the drug. Results are expressed as a percentage of the respective secretagogue-stimulated control response. Basal \[^{3}H\]norepinephrine fractional releases were 0.059 ± 0.011 and 0.045 ± 0.005 for nicotine and histamine treatment groups, respectively; stimulated fractional releases were 0.224 ± 0.02 and 0.206 ± 0.011, respectively. Results significantly different from control are marked by an asterisk (p < 0.05). Values represent means ± SEM of 6 to 10 experiments performed in triplicate.
adrenal chromaffin cells, it was of interest to further characterize the manner by which these compounds elicit this effect. One way of characterizing this effect is by examining the length of time required for the microtubule agents to inhibit nAChR-mediated [\(^3\)H]norepinephrine release. Cells were pretreated for various time periods (0, 5, 10, 30, 60, 120 min) with concentrations of microtubule drug (100 \(\mu\)M demecolcine, 50 \(\mu\)M podophyllotoxin, 50 \(\mu\)M vincristine, 50 \(\mu\)M tubulozole and 10 \(\mu\)M taxol) which produced about 80% inhibition (IC\(_{80}\)), followed by stimulation with 10 \(\mu\)M nicotine in the presence of each drug. Maximum inhibition for all drugs (80 - 90% inhibition) occurred quickly, within 10 - 30 min (Fig. 6). In fact, an inhibition of 55 - 80% can be obtained with these drugs upon simultaneous addition of the microtubule drug with nicotine.

Reversal of the effects of the microtubule drugs on nicotine-stimulated catecholamine release

It was also important to determine whether the inhibitory effects of these microtubule agents on nAChR-modulated catecholamine release could be reversed. Cultured bovine adrenal chromaffin cells were preincubated for 60 min at 37°C with 100 \(\mu\)M demecolcine, 50 \(\mu\)M podophyllotoxin, 50 \(\mu\)M vincristine, 50 \(\mu\)M tubulozole or 10 \(\mu\)M taxol. The cells were then incubated in drug-free buffer for various time periods (2, 10, 30, 60 or 120 min) with 3 buffer changes,
FIGURE 6. Time-dependent effects of microtubule drugs on nAChR-stimulated $[^3H]$norepinephrine release. Cells were pretreated for the indicated times with 100 $\mu$M demecolcine (DEM), 50 $\mu$M podophyllotoxin (POD), 50 $\mu$M tubulozole (TUB) or 50 $\mu$M vincristine (VCR) prior to stimulation with 10 $\mu$M nicotine in the continued presence of the drug. Results are expressed as a percentage of the inhibition of the control 10 $\mu$M nicotine-stimulated response. In the absence of any microtubule drug, basal $[^3H]$norepinephrine fractional release was 0.056 ± 0.003 and the fractional release caused by 10 $\mu$M nicotine was 0.176 ± 0.008. Values represent means ± SEM of 6 experiments performed in triplicate.
followed by stimulation with 10 μM nicotine for 10 min in the absence of drug. Figure 7 illustrates the reversibility of the inhibitory effects of these microtubule drugs on nicotine-stimulated [3H]norepinephrine release. Within 2 min of removal of the microtubule drug, a reversal of the inhibition of [3H]norepinephrine release is seen for all the microtubule agents. With a 30 min washout period, all the microtubule drugs had approximately a 2-fold greater stimulation of [3H]norepinephrine release by nicotine when compared to cells in which the drugs had not been washed out. Over ≥80 % of nAChRs function returns when chromaffin cells are drug-free for 60 min or longer.

Effect of chronic treatment with microtubule drugs on adrenal catecholamine release

Because of the relatively rapid onset and reversibility of the inhibitory action of microtubule drugs on nAChR-mediated catecholamine release, it was of interest to determine if longer treatment times with lower concentrations of microtubule drugs can still inhibit secretion. Cells were exposed with approximately IC10 concentrations of the microtubule drugs for a 22 hr period, loaded with [3H]norepinephrine and stimulated in the presence of the microtubule drug for a total preincubation time with the drug of 24 hr. Vincristine (0.1 μM), podophyllotoxin (0.3 μM) and demecolcine (0.8 μM) caused small, but significant, decreases of 28 %, 34 % and 30 % on
FIGURE 7. Reversal of the effects of microtubule drugs on nAChR-stimulated [^3H]norepinephrine release from cultured bovine adrenal chromaffin cells. Cells were pretreated for 1 hr with 100 μM demecolcine (DEM), 50 μM podophyllotoxin (POD), 10 μM taxol, 50 μM tubulozole (TUB) or 50 μM vincristine (VCR), washed with PSS and incubated in PSS (in the absence of microtubule drug) for the indicated times prior to stimulation with 10 μM nicotine. Results are expressed as a percentage of the control 10 μM nicotine-stimulated response. In the absence of any microtubule drug, basal [^3H]norepinephrine fractional release was 0.074 ± 0.005 and the fractional release caused by 10 μM nicotine was 0.237 ± 0.013. Values represent means ± SEM of 5 to 6 experiments performed in triplicate.
nicotine-stimulated $[^3]$H]norepinephrine release, but did not affect release stimulated by 56 mM KCl (Fig. 8). Vinblastine (0.1 $\mu$M) and tubulozole (0.2 $\mu$M), also, decreased $[^3]$H]norepinephrine release, but the effect was not significant (Fig. 8). None of the microtubule drugs affected basal catecholamine release under these chronic treatment conditions (Table 4).

**Effects of microtubule drugs on the microtubular network of adrenal chromaffin cells**

To determine if these agents also alter adrenal microtubules under similar conditions in which they inhibit nAChR-mediated secretion of $[^3]$H]norepinephrine, the direct effects of the microtubule drugs on the microtubular network of cultured adrenal chromaffin cells were examined using immunocytochemical techniques (Fig. 9, A-F and Fig. 10, A-F). When cultured adrenal chromaffin cells were stained with anti-tubulin antibodies and examined under a fluorescence microscope, control cells had an evenly dispersed microtubule network radiating throughout the cell bodies and down the length of the neurites (Fig. 9A). Cells treated for 60 min with the vinca alkaloid, vincristine (10 $\mu$M), lack a discernable microtubular network but instead contained tubulin paracrystals in the cytosol (Fig. 9B). These cells were also less fluorescent than control cells. In cultured bovine adrenal chromaffin cells treated for one hr with taxol (10 $\mu$M), highly fluorescent parallel arrays
Table 4. Effects of a 24 hr treatment with low concentrations of microtubule drugs on basal (nonstimulated) catecholamine release.\(^a\)

<table>
<thead>
<tr>
<th>Basal (control)</th>
<th>Basal Catecholamine Release(^a)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.076 ± 0.009</td>
</tr>
<tr>
<td>+ Demecolcine (0.8 μM)</td>
<td>0.073 ± 0.007</td>
</tr>
<tr>
<td>+ Podophyllotoxin (0.3 μM)</td>
<td>0.077 ± 0.006</td>
</tr>
<tr>
<td>+ Tubulozole (0.2 μM)</td>
<td>0.097 ± 0.022</td>
</tr>
<tr>
<td>+ Vinblastine (0.1 μM)</td>
<td>0.082 ± 0.006</td>
</tr>
<tr>
<td>+ Vincristine (0.1 μM)</td>
<td>0.094 ± 0.010</td>
</tr>
</tbody>
</table>

\(^a\)Cultured adrenal chromaffin cells were treated for 24 hr in the absence (control) or presence of the indicated microtubule drug. The amount of \(^3\)H]norepinephrine was determined during a 10 min period. Results are expressed as a fraction of the total incorporated \(^3\)H]norepinephrine released. Values represent means ± SEM of 5 to 7 experiments. None of the treatments were statistically different from control (\(p < 0.05\)).
FIGURE 8. Effect of chronic treatment with microtubule drugs on adrenal nicotine- and K⁺-stimulated \[^{3}H\]norepinephrine release. Cells were pretreated with 0.1 μM vinblastine (VBL), 0.1 μM vincristine (VCR), 0.2 μM tubulozole (TUB), 0.3 μM podophyllotoxin (POD) or 0.8 μM demecolcine (DEM) for 24 hr prior to stimulation with either 10 μM nicotine or 56 mM KCl in the continued presence of the drug. Results are expressed as a percentage of the respective secretagogue-stimulated control response. Basal \[^{3}H\]norepinephrine fractional release was 0.069 ± 0.001 and stimulated fractional releases were 0.232 ± 0.011 and 0.140 ± 0.008 for nicotine and KCl, respectively. Results significantly different from control are marked by an asterisk (p < 0.05). Values represent means ± SEM of 6 to 12 experiments performed in triplicate.
FIGURE 9. Effects of various microtubule drugs on the immunological staining of cultured adrenal chromaffin cells by anti-tubulin antibodies. A: Control cells. B to F: Cells treated for 60 min with 10 μM vincristine (B), 10 μM tubulozole (C), 100 μM podophyllotoxin (D), 100 μM demecolcine (E) and 10 μM taxol (F). Calibration bar = 20 μm. Results are representative of 4 to 7 experiments.
Figure 9 continued
Figure 9 continued
were seen scattered throughout the cytosol. The neurites of these cells are difficult to discern due to reduced fluorescence (Fig. 9F). One hr treatment with podophyllotoxin (100 μM), tubulozole (10 μM) and demecolcine (100 μM) also results in disrupted microtubular networks (Fig. 9, C-E). Cells treated with these drugs exhibit decreases in total cell fluorescence, making it difficult to detect neurites, and uneven distributions of fluorescence in the cytoplasm. A more pronounced effect of the drugs on the microtubular network can be seen in cells treated with these agents for 3 h (Figure 10, A-F).

**Time-course for the appearance of vinblastine-induced paracrystals**

Small alterations in the chromaffin cell microtubular network, produced by treatment with the microtubule drugs are difficult to detect due to the qualitative nature of tubulin immunofluorescence. However, to determine how early these drugs could alter microtubules, the time-dependent effects of vinblastine were investigated. Vinblastine was used in these studies since its effects are manifested by formation of easily detectable tubulin paracrystals. The time-course for the effects of vinblastine on adrenal microtubules is seen in Fig. 11, B-D. Cultured chromaffin cells were treated with 10 μM vinblastine for 15, 60 and 180 min. In cells treated for 15 min, several tubulin paracrystals can be noticed scattered throughout the
FIGURE 10. Effects of various microtubule drugs on the immunological staining of cultured adrenal chromaffin cells by anti-tubulin antibodies. A: Control cells. B to F: Cells treated for 180 min with 10 μM vincristine (B), 10 μM tubulozole (C), 100 μM podophyllotoxin (D), 100 μM demecolcine (E) and 10 μM taxol (F). Calibration bar = 20 μm. Results are representative of 4 to 7 experiments.
Figure 10 continued
FIGURE 11. Time-course for the appearance of vinblastine-induced paracrystals in cultured adrenal chromaffin cells by anti-tubulin antibodies. A: Control cells. B to D: Cells treated with vinblastine for 15 min (B), 60 min (C) and 180 min (D). Calibration bar = 10 μm. Results are representative of 4 to 8 experiments.
Figure 11
Figure 11 continued
cytoplasm and along the neurites. A progressive increase in the number of tubulin paracrystals can be observed in the cytosol as the treatment times increases. After 180 min of treatment, the paracrystals are quite obvious and spread throughout the adrenal cells. Therefore, with increasing treatment time with vinblastine from 15 min, when only a few paracrystals can be seen, up to a 3 hr exposure when many are seen, there appears to be a time-dependent effect on the disruption of the microtubular network.

**Effect of microtubule drugs on the acetylated tubulin population of adrenal chromaffin cells**

Various populations of microtubules have been identified on the basis of posttranslational modifications. One such population of microtubules are acetylated microtubules. These microtubules have been shown to belong to a stable group of microtubules, i.e., the rate of depolymerization of these microtubules is slower than unstable microtubules which are constantly polymerizing and depolymerizing. To determine if the microtubule drugs are altering a more stable population of microtubules, represented by acetylated microtubules, adrenal chromaffin cells were treated with microtubule drugs, stained with 6-11B-1 (anti-acetylated tubulin antibodies) and examined by fluorescence microscopy. Control cells contained an evenly dispersed acetylated microtubular network, radiating from the perinuclear region and extending throughout the cell
bodies. However, the density of the microtubular network was not as great as that seen with the tubulin antibodies. In some cells the microtubular network extended down the neurites (Fig. 12A). In cells treated for 60 min with 100 μM demecolcine, 100 μM podophyllotoxin and 10 μM tubulozole (Fig. 12, B-D), the microtubules containing acetylated tubulin had a wiry appearance rather than an evenly dispersed microtubular network as seen in untreated cells. The total fluorescence was less in microtubule drug-treated cells than in control cells. Some cells that were treated with 10 μM taxol had highly fluorescent parallel wiry arrays scattered in the perinuclear region of the cells (Fig. 12E). In other taxol-treated cells, less fluorescence was noted and only a few microtubules were observed scattered throughout the cytoplasm that had a wiry, curved appearance. The neurites of these cells are difficult to discern due to reduced fluorescence (Fig. 12E). Many cells treated with the vinca alkaloid, vinblastine (10 μM), had paracrystals present in the cytosol (Fig. 12F). However, other vinblastine-treated cells did not have paracrystals but, like demecolcine- and podophyllotoxin-treated cells, the acetylated microtubules were curved and wiry in appearance. The overall fluorescence in cells treated with vinblastine was less than control cells. Similar results were obtained for vincristine (10 μM) treated cells (Fig. 12G). The acetylated microtubules present were wiry as in other
FIGURE 12. Effects of various microtubule drugs on the immunological staining of cultured adrenal chromaffin cells by anti-acetylated tubulin antibodies (6-11B-1). A: Control cells. B to G: Cells treated for 60 min with 100 μM demecolcine (B), 100 μM podophyllotoxin (C), 10 μM tubulozole (D), 10 μM taxol (E), 10 μM vinblastine (F) and 10 μM vincristine (G). Calibration bar = 20 μm. Results are representative of 3 to 5 experiments.
Figure 12 continued
Figure 12 continued
Figure 12 continued
Effect of microtubule drugs on $[^3H]$nicotine binding to rat brain membrane fragments

Microtubule drugs inhibit only nAChR-mediated catecholamine release, as demonstrated above. Therefore, it is possible that these drugs can act either as cholinergic antagonists or as microtubule disrupting agents. To determine if the action of the microtubule drugs on nAChRs is due to interference with agonist binding, the ability of these compounds to interfere with $[^3H]$nicotine binding to nAChR binding sites was examined. Membrane fragments from rat brain were used as a plentiful source of neuronal nAChRs. Competition curves of specific $[^3H]$nicotine binding by the nAChR agonists, nicotine and carbachol, are depicted in Fig. 13. Both nicotine ($appK_i = 14$ nM) and carbachol ($appK_i = 530$ nM) inhibited $[^3H]$nicotine binding, demonstrating the presence of nAChRs in this preparation. At concentrations of the microtubule drugs known to inhibit 70 - 80 % of nicotine mediated release (50 $\mu$M concentrations of each), demecolcine, podophyllotoxin, tubulozole, vincristine and nocodazole did not block $[^3H]$nicotine binding (Fig. 14). In addition, 50 $\mu$M adiphenine (a noncompetitive nAChR ion channel blocker), failed to block $[^3H]$nicotine binding. These results suggest that the microtubule drugs are not acting as nAChR antagonists in rat brain membrane fragments. It should be pointed out, though,
FIGURE 13. Drug displacement curves of specific $[^3$H$]$nicotine binding by unlabelled nicotine and carbachol. Aliquots of membrane protein were incubated with $[^3$H$]$nicotine (15 nM) in the presence and absence of various concentrations of unlabelled nicotine and carbachol for 60 min at room temperature. Carbachol (1 mM) was used to determine nonspecific binding which was 52% of total binding. Control specific $[^3$H$]$nicotine binding was 7200 ± 1800 CPM/mg protein (mean ± SEM). Results are expressed as a percentage of specific $[^3$H$]$nicotine binding. Values represent the mean ± SEM of 3 to 4 experiments performed in duplicate.
FIGURE 14. Effect of various microtubule drugs on 
$[^3H]$nicotine binding in rat brain membrane fragments. 
Aliquots of membrane protein were incubated with 
$[^3H]$nicotine (10 nM) for 60 min at room temperature in the presence of 50 μM concentrations of demecolcine (DEM), podophyllotoxin (POD), tubulozole (TUB), vincristine (VCR), nocodazole (NOC) or adiphenine (ADI). Carbachol (1 mM) was used to determine nonspecific binding which was 52 % of total binding. Control specific binding was 5300 ± 900 CPM/mg protein. Results are expressed as a percentage of the control specific $[^3H]$nicotine binding. Values represent the mean ± SEM of 8 to 10 experiments performed in duplicate.
that this rat brain preparation lacks an intact cytoskeleton.

*Effect of microtubule drugs on \(^{[3]H}\)nicotine binding to nAChRs in intact adrenal chromaffin cells*

In membrane fragments which lack an intact microtubular network, microtubule drugs had no effect on \(^{[3]H}\)nicotine binding. Therefore, to determine if an intact microtubular network is necessary for the action of the microtubule drugs, \(^{[3]H}\)nicotine binding studies were conducted on intact adrenal chromaffin cells. Additionally, the effects of various cholinergic drugs (carbachol, hexamethonium, mecamylamine, d-tubocurarine and adiphenine) on \(^{[3]H}\)nicotine binding were also examined. A 50 μM concentration of carbachol, hexamethonium, mecamylamine, d-tubocurarine and adiphenine significantly inhibited \(^{[3]H}\)nicotine binding by 85 %, 73 %, 66 %, 53 % and 64 %, respectively (Fig. 15). The effects of various microtubule drugs, at concentrations shown to inhibit 90 % of nicotine-stimulated catecholamine release, on \(^{[3]H}\)nicotine binding in intact cultured bovine adrenal chromaffin cells were also examined. At 50 μM concentrations, demecolcine, podophyllotoxin, taxol, vinblastine and vincristine significantly inhibited \(^{[3]H}\)nicotine binding by 55 %, 50 %, 48 %, 62 % and 39 %, respectively (Fig. 15). Since it has been shown that cold temperature (0°C) causes disruption of microtubules (Penningroth and Kirschner, 1977), binding was also
FIGURE 15. The effect of various microtubule drugs on $[^3H]$nicotine binding to intact primary cultures of adrenal chromaffin cells. Cell cultures (1x10^6 cells/well) were incubated with 10 nM $[^3H]$nicotine for 70 min at 37°C in the presence or absence of demecolcine (DEM), podophyllotoxin (POD), taxol (TAX), tubulozole (TUB), vinblastine (VBL), vincristine (VCR), carbachol (CCH), hexamethonium (C6), mecamylamine (MMA), d-tubocurarine (d-TC) or adiphenine (ADI). Unlabeled nicotine (50 μM or 1 mM) was used to determine nonspecific binding which was 56% of total binding. The mean value for control binding was 9600 ± 2300 CPM/mg protein. Results significantly different from specific binding in the absence of drug, are marked by asterisks (** p < 0.01, * p < 0.05). Values represent the mean ± SEM of 3-12 experiments performed in triplicate.
conducted at 0°C to examine the effects on $[^3$H]nicotine binding. The results obtained suggest that cold temperature has no effect on binding, however, if indeed alterations in the microtubular network interferes with $[^3$H]nicotine binding, as observed with the microtubule drugs, then it is possible that the conditions used in these studies were not adequate enough to disrupt microtubules or to detect an effect on binding (Fig. 15).

Noncompetitive inhibition of nicotine-stimulated catecholamine release by microtubule drugs

In bovine adrenal chromaffin cells, taxol, vinblastine and colchicine have been shown to exhibit properties similar to those of noncompetitive inhibitors of the nAChR-associated ion channel (McKay et al., 1985). Therefore, to determine the type of inhibitory action that demecolcine, podophyllotoxin, tubulozole and vincristine have on adrenal nAChRs, the concentration-response relationship of nicotine on catecholamine secretion was determined in the presence and absence of the microtubule drugs. In the absence of microtubule drugs, maximum stimulation of catecholamine release occurred with approximately 50 μM nicotine and half-maximum stimulation occurred with 5 μM nicotine (Fig. 16, Table 5). In the presence of a fixed concentration of demecolcine (20 μM), podophyllotoxin (5 μM), tubulozole (5 μM) and vincristine (5 μM), no changes in the concentration of nicotine eliciting half-maximum response (EC$_{50}$ values)
Table 5. Effects of microtubule drugs on the concentration-response effects of nicotine on adrenal catecholamine release.

<table>
<thead>
<tr>
<th>Drug</th>
<th>EC$_{50}$ (μM)</th>
<th>E$_{max}$ $^{b}$ (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nicotine</td>
<td>5.0 ± 0.5</td>
<td>0.176 ± 0.018</td>
</tr>
<tr>
<td>+ Demecolcine (20 μM)</td>
<td>4.4 ± 0.4</td>
<td>0.116 ± 0.015$^{c}$</td>
</tr>
<tr>
<td>+ Podophyllotoxin (5 μM)</td>
<td>4.3 ± 0.3</td>
<td>0.115 ± 0.014$^{c}$</td>
</tr>
<tr>
<td>+ Tubulozole (5 μM)</td>
<td>6.2 ± 1.0</td>
<td>0.109 ± 0.016$^{c}$</td>
</tr>
<tr>
<td>+ Vincristine (5 μM)</td>
<td>7.0 ± 3.3</td>
<td>0.109 ± 0.016$^{c}$</td>
</tr>
</tbody>
</table>

$^{a}$Half maximum effective concentrations (EC$_{50}$) of nicotine (μM) were calculated as described in Material and Methods from the data found in Fig. 16. Values represent means ± SEM (n=5-7).

$^{b}$E$_{max}$ is the maximum response obtained for nicotine response in the presence or absence of the microtubule drug.

$^{c}$Significantly different from the corresponding secretagogue-stimulated controls (p < 0.05).
FIGURE 16. The concentration-response effects of nicotine on adrenal catecholamine release in the absence and presence of various microtubule drugs. Cultured adrenal chromaffin cells were pretreated for 30 min with either 20 μM demecolcine (DEM), 5 μM podophyllotoxin (POD), 5 μM tubulozole or 5 μM vincristine (VCR) prior to stimulation with increasing concentrations of nicotine in the continued presence of the drug. Control cells were treated identically except in the absence of any microtubule drug. Results are expressed as a percent of 10 μM nicotine-stimulated [3H]norepinephrine released during a 10 min stimulation period. In the absence of any microtubule drug, basal [3H]norepinephrine fractional release was 0.037 ± 0.002 and the fractional release caused by 10 μM nicotine was 0.200 ± 0.004. Values represent means ± SEM of 6 to 7 experiments performed in triplicate.
were observed (Table 5). However, the maximum amount of 

$[^3H]$norepinephrine secretion ($E_{\text{max}}$) was reduced by 53 %, 59 %, 45 % and 43 % by demecolcine, podophyllotoxin, tubulozole and vincristine, respectively (Table 5). The type of inhibition of the microtubule drugs appears to be noncompetitive.

**Effect of Triton-extraction on $[^{125}\text{I}]$mAb 35 binding to adrenal chromaffin cells**

Evidence exists for the association of muscle nAChRs with the cytoskeleton (Prives et al., 1982; Stya and Axelrod, 1983; Phillips et al., 1991; Froehner et al., 1991). Results acquired from earlier studies and work done in our laboratory suggests that adrenal nAChRs might also be associated with the Triton-insoluble cytoskeleton.

mAb 35 is a monoclonal antibody raised against nAChRs from *Electrophorus* electric organ, that recognizes the main immunogenic region (MIR) of muscle and electric organ AChRs (Tzartos, 1981) and cross reacts with chick ciliary ganglion nAChRs (Jacob et al., 1984) and the nAChRs from adrenal chromaffin cells (Higgins and Berg, 1987). Hybridoma cells, which secrete monoclonal antibodies (mAb 35) reactive with nAChRs, were grown and maintained in culture as described by Harlow and Lane (1988) until a desired amount of supernatant containing mAb 35 was obtained. mAb 35 was purified by ammonium sulfate precipitation from hybridoma culture media (Harlow and Lane, 1988). To provide evidence that the
purified antibody was selective for adrenal chromaffin nAChRs, the ability of mAb 35 to modulate the secretory response of chromaffin cells to nicotine and depolarizing concentrations of K⁺ were examined using concentrations of mAb 35 reported by Higgins and Berg (1987) to attenuate nicotine-stimulated release by 40% and 90%. Exposure of chromaffin cells to 10 nM and 100 nM of mAb 35 for 24 hr significantly inhibited nicotine-stimulated release by 70% and 90%, respectively. However, these same concentrations of mAb 35 did not affect catecholamine release by 56 mM KCl (Fig. 17). These results verify that the monoclonal antibody purified (mAb 35) is selective for nAChRs.

In these studies mAb 35 was radioiodinated and used as a probe to detect adrenal nAChRs associated with the Triton-insoluble cytoskeleton. Triton is a mild detergent that removes lipids and soluble proteins and leaves the internal cytoskeleton and associated proteins essentially intact (Ben-Ze'ev et al., 1979). Therefore, to determine if adrenal nAChRs are indeed associated with the cytoskeleton, [125I]mAb 35 was used to label nAChRs. The degree of association of nAChRs with the cytoskeleton was determined as described in the Material and Methods section. In cells which were not Triton-extracted, 19 fmole of [125I]mAb 35 labeled nAChRs were associated with surface sites (Fig. 18, Table 6). After Triton-extraction 7 fmole of nAChRs remained associated with the Triton-insoluble cytoskeleton.
FIGURE 17. Effect of 24 hr exposure with mAb 35 on secretagogue-stimulated adrenal [³H]norepinephrine release. Cells were exposed to either 10 nM or 100 nM mAb 35 for 24 hr prior to stimulation with either 10 µM nicotine or 56 mM KCl in the absence of mAb 35. Results are expressed as a fraction of the total incorporated [³H]norepinephrine released during the 10 min stimulation period. Basal [³H]norepinephrine fractional release was 0.075 ± 0.005, whereas stimulated fractional releases were 0.212 ± 0.006 and 0.078 ± 0.012 for 10 µM nicotine and 56 mM KCl, respectively. Values represent means ± SEM of 2 experiments performed in triplicate. Results significantly different from the corresponding secretagogue-stimulated control are marked by an asterisk (p < 0.05).
Table 6. $[^{125}\text{I}]\text{mAb 35}$ binding to Triton-extracted cultured adrenal chromaffin cells.\(^{a}\)

<table>
<thead>
<tr>
<th></th>
<th>$[^{125}\text{I}]\text{mAb 35}$ Bound (fmole/10^6 cells)</th>
<th>Receptors per cell</th>
<th>% Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control Cells (unextracted)</td>
<td>19</td>
<td>5909</td>
<td>100</td>
</tr>
<tr>
<td>Triton-Insoluble Cell Extract</td>
<td>7(^{b})</td>
<td>2177(^{b})</td>
<td>36(^{b})</td>
</tr>
</tbody>
</table>

\(^{a}\)Cultured adrenal chromaffin cells (1x10^6 cells/well) were incubated with $[^{125}\text{I}]\text{mAb 35}$ (5 nM) for 60 min at 37°C. After washing unbound $[^{125}\text{I}]\text{mAb 35}$, the cells were extracted for 5 min with buffer containing 0.5 % Triton. Unlabeled mAb 35 (0.5 μM) was used to determine nonspecific binding which was 20 % of total binding. The mean value for control was 652 ± 40 CPM/1x10^6 cells. Values represent the mean ± SEM of 3 experiments performed in triplicate and were analyzed by Two Way ANOVA. The amount of protein per well was 0.22 ± 0.01 mg. 

\(^{b}\)Significantly different from control ($p < 0.01$).
FIGURE 18. \[^{125}\text{I}]\text{mAb 35 binding to Triton-extracted cultured adrenal chromaffin cells. Cultured cells (1x10}^6 \text{ cells/well) were incubated with [}^{125}\text{I}]\text{mAb 35 (5 nM) for 60 min at 37°C. After washing unbound [}^{125}\text{I}]\text{mAb 35, the cells were} extrated for 5 min with buffer containing 0.5 \% Triton. Unlabelled mAb 35 (0.5 \mu\text{M}) was used to determine nonspecific binding which was 20 \% of total binding. The mean value for control was 652 \pm 40 \text{ CPM/1x10}^6 \text{ cells. Values represent the mean \pm SEM of 3 experiments performed in triplicate. Results significantly different from control (total specific binding in the absence of drug) are marked by an asterisk (** } p < 0.01, \text{ Two Way ANOVA). The amount of protein per well was 0.22 \pm 0.01 \text{ mg.} \]
These results suggest that 36% of the total surface receptors are associated with the Triton-insoluble cytoskeleton.

Influence of microtubule drugs on the association of adrenal nAChRs to Triton-extracted adrenal chromaffin cells

To determine if an alteration in microtubules alters nAChRs association with the cytoskeleton, the effect of microtubule drugs on the Triton-extractability of nAChRs were investigated. Additionally, the effects of various cholinergic ligands (nicotine, mecamylamine and adiphenine) on $[^{125}\text{I}]\text{mAb 35}$ binding were also examined. At concentrations of nicotine (10 μM), mecamylamine (1 μM) and adiphenine (5 μM) shown to inhibit 80% of nicotine-stimulated catecholamine release in chromaffin cells, only nicotine and mecamylamine significantly decreased $[^{125}\text{I}]\text{mAb 35}$ binding (24% and 51%, respectively), Fig. 19, Table 7. The noncompetitive nAChR ion channel blocker, adiphenine, had no significant effect on the amount of $[^{125}\text{I}]\text{mAb 35}$ (7 fmole) associated with the Triton-insoluble cytoskeletal component (Fig. 19, Table 7). At concentrations of the microtubule drugs known to inhibit 70–80% of nicotine-stimulated secretion, demecolcine podophyllotoxin, vinblastine, vincristine and taxol significantly decreased the Triton-insoluble bound $[^{125}\text{I}]\text{mAb 35}$ by 37%, 46%, 33%, 38% and 43%, respectively (Fig. 19, Table 7). Although tubulozole inhibited $[^{125}\text{I}]\text{mAb 35}$ binding by 24% it was not
Table 7. Effect of microtubule drugs on $[^{125}\text{I}]$mAb 35 bound to Triton-extracted adrenal chromaffin cells.$^a$

<table>
<thead>
<tr>
<th></th>
<th>$[^{125}\text{I}]$mAb 35 Bound (fmole/1x10$^6$ cells)</th>
<th>Receptor per cell ($\times 10^3$)</th>
<th>% Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unextracted Cells</td>
<td>17 ± 0.70</td>
<td>5.6 ± 0.1</td>
<td></td>
</tr>
<tr>
<td>Control, Triton-Insoluble Cell Extract</td>
<td></td>
<td></td>
<td>100</td>
</tr>
<tr>
<td>+ Nicotine (10 $\mu$M)</td>
<td>5 ± 0.3$^b$</td>
<td>1.7 ± 0.09$^b$</td>
<td>76 ± 6$^b$</td>
</tr>
<tr>
<td>+ Mecamylamine (1 $\mu$M)</td>
<td>4 ± 0.4$^b$</td>
<td>1.1 ± 0.10$^b$</td>
<td>49 ± 5$^b$</td>
</tr>
<tr>
<td>+ Adiphenine (5 $\mu$M)</td>
<td>7 ± 0.6$^b$</td>
<td>2.2 ± 0.20$^b$</td>
<td>95 ± 6$^b$</td>
</tr>
<tr>
<td>+ Demecolcine (50 $\mu$M)</td>
<td>5 ± 0.5$^b$</td>
<td>1.4 ± 0.16$^b$</td>
<td>63 ± 5$^b$</td>
</tr>
<tr>
<td>+ Podophyllotoxin (50 $\mu$M)</td>
<td>4 ± 0.7$^b$</td>
<td>1.2 ± 0.20$^b$</td>
<td>54 ± 7$^b$</td>
</tr>
<tr>
<td>+ Taxol (30 $\mu$M)</td>
<td>5 ± 0.1$^b$</td>
<td>1.5 ± 0.02$^b$</td>
<td>67 ± 2$^b$</td>
</tr>
<tr>
<td>+ Tubulozole (50 $\mu$M)</td>
<td>6 ± 1.5$^b$</td>
<td>1.7 ± 0.50$^b$</td>
<td>76 ± 20$^b$</td>
</tr>
<tr>
<td>+ Vinblastine (50 $\mu$M)</td>
<td>5 ± 0.6$^b$</td>
<td>1.4 ± 0.20$^b$</td>
<td>62 ± 8$^b$</td>
</tr>
<tr>
<td>+ Vincristine (50 $\mu$M)</td>
<td>4 ± 0.3$^b$</td>
<td>1.3 ± 0.10$^b$</td>
<td>57 ± 3$^b$</td>
</tr>
</tbody>
</table>

$^a$Cultured adrenal chromaffin cells (1x10$^6$ cells/well) were incubated with $[^{125}\text{I}]$mAb 35 (5 nM) for 60 min at 37°C in the presence or absence of microtubule drug, receptor agonist, antagonist or receptor ion channel blocker. After washing unbound $[^{125}\text{I}]$mAb 35, the cells were extracted for 5 min with buffer containing 0.5 % Triton. Unlabeled mAb 35 (0.5 $\mu$M) was used to determine nonspecific binding, which was 20 % of total binding. The mean value for control was 249 ± 9 CPM/1x10$^6$ cells. Values represent the mean ± SEM of 3 experiments performed in triplicate and were analyzed by Two Way ANOVA. The amount of protein per well was 0.22 ± 0.01 mg.

$^b$Significantly different from control ($p < 0.01$).
FIGURE 19. Effect of microtubule drugs on $^{125}$I-mAb 35 binding to Triton-extracted adrenal chromaffin cells. Cultured adrenal chromaffin cells (1x10^6 cells/well) were incubated with 50 μM $^{125}$I-mAb 35 for 60 min at 37°C in the presence or absence of demecolcine (DEM), podophyllotoxin (POD), taxol (TAX), tubulozole (TUB), vinblastine (VBL), vincristine (VCR), carbachol (CCH), hexamethonium (C6), mecamylamine (MMA), d-tubocurarine (d-TC) or adiphenine (ADI). After washing unbound $^{125}$I-mAb 35, the cells were extracted for 5 min with buffer containing 0.5 % Triton. Nonspecific binding was 20 % of total binding. The mean value for control Triton-insoluble bound $^{125}$I-mAb 35 was 249 ± 9 CPM/1x10^6 cells. Values represent the mean ± SEM of 3 experiments performed in triplicate. Results significantly different from control are marked by asterisks (** p < 0.01). The amount of protein per well was 0.22 ± 0.01 mg.
Effect of microtubule drugs on $[^{125}\text{I}]\text{mAb 35}$ binding to intact adrenal chromaffin cells

In the previous study, cholinergic ligands (nicotine and mecamylamine) and microtubule drugs decreased the association of $[^{125}\text{I}]\text{mAb 35}$ with the Triton-insoluble cytoskeleton. Therefore, to determine if the reduction in binding was due to an inhibitory effect of these compounds on $[^{125}\text{I}]\text{mAb 35}$ binding, binding studies were conducted on intact adrenal chromaffin cells. In these studies, none of the drugs (10 μM nicotine, 1 μM mecamylamine, 50 μM demecolcine, 50 μM podophyllotoxin, 50 μM tubulozole, 30 μM taxol, 50 μM vinblastine or 50 μM vincristine) affected $[^{125}\text{I}]\text{mAb 35}$ binding to intact (non-extracted) chromaffin cells (Table 8).
Table 8. Effect of microtubule drugs on $^{125}\text{I}]$mAb 35 binding to cultured adrenal chromaffin cells$^a$

<table>
<thead>
<tr>
<th></th>
<th>$^{125}\text{I}]$mAb 35 Bound (fmole/1x10⁶ cells)</th>
<th>Receptor per cell (x 10³)</th>
<th>% Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (unextracted)</td>
<td>15 ± 2</td>
<td>4.7 ± 0.6</td>
<td>100</td>
</tr>
<tr>
<td>+ Nicotine (10 μM)</td>
<td>16 ± 2</td>
<td>4.8 ± 0.5</td>
<td>107 ± 18</td>
</tr>
<tr>
<td>+ Mecamylamine (1 μM)</td>
<td>15 ± 1</td>
<td>4.5 ± 0.1</td>
<td>99 ± 14</td>
</tr>
<tr>
<td>+ Demecolcine (50 μM)</td>
<td>19 ± 4</td>
<td>5.8 ± 1.1</td>
<td>126 ± 27</td>
</tr>
<tr>
<td>+ Podophyllotoxin (50 μM)</td>
<td>18 ± 3</td>
<td>5.7 ± 1.0</td>
<td>121 ± 18</td>
</tr>
<tr>
<td>+ Taxol (30 μM)</td>
<td>17 ± 1</td>
<td>5.2 ± 0.5</td>
<td>112 ± 8</td>
</tr>
<tr>
<td>+ Tubulozole (50 μM)</td>
<td>15 ± 2</td>
<td>4.8 ± 0.5</td>
<td>102 ± 14</td>
</tr>
<tr>
<td>+ Vinblastine (50 μM)</td>
<td>17 ± 3</td>
<td>5.3 ± 1.0</td>
<td>111 ± 16</td>
</tr>
<tr>
<td>+ Vincristine (50 μM)</td>
<td>17 ± 2</td>
<td>5.3 ± 0.7</td>
<td>114 ± 15</td>
</tr>
</tbody>
</table>

$^a$Cultured adrenal chromaffin cells (1x10⁶ cells/well) were incubated with $^{125}\text{I}]$mAb 35 (5 nM) for 60 min at 37°C in the presence or absence of microtubule drugs, receptor agonist or antagonist. Unlabeled mAb 35 (0.5 μM) was used to determine nonspecific binding, which was 20% of total binding. The mean value for control was 652 ± 40 CPM/1x10⁶ cells. Values represent the mean ± SEM of 3 experiments performed in triplicate and were analyzed by Two Way ANOVA. None of the treatments were statistically different from control ($p<0.05$). The amount of protein per well was 0.22 ± 0.01 mg.
Chromaffin cells contain a cytoskeletal network made up microtubules and other filaments. In various cells these proteins have been implicated in playing a role in cellular secretion. This is based on the ability of microtubule drugs, such as colchicine and vinblastine, to inhibit specific exocytotic processes. In adrenal chromaffin cells, evidence exists that nAChRs are associated with cytoskeletal proteins and that this association may be important for receptor function (McKay and Schneider, 1984; McKay, 1988). This is based on the effects of taxol and vinblastine on catecholamine release from cultured adrenal chromaffin cells. The precise mechanism by which these drugs inhibit receptor function is not established. Therefore, the major objectives of these studies were to determine 1) the site and mechanism of action of the microtubule drugs, 2) if adrenal nAChRs are associated with microtubules and 3) if this association plays a role in receptor function.

It is well documented that the microtubule drugs taxol, colchicine and vinblastine inhibit adrenal catecholamine release (Poisner and Bernstein, 1971; Douglas and Sorimachi, 1972; Trifaro, et al., 1972; McKay and Schneider, 1984;
Several steps in the secretory process are potential targets for these drugs. Classically, drugs which interfere with microtubular function have been shown to inhibit secretion by interfering with the release of secretory granules. This inhibitory effect has been demonstrated for the release of insulin from β-cells of the pancreas (Lacey et al., 1968) and histamine release from mast cells (Gillespie et al., 1968) and basophils (Levy and Carlton, 1969). In adrenal chromaffin cells, vinblastine and taxol inhibit adrenal catecholamine release by mechanisms which do not involve the more terminal steps in stimulus-secretion coupling, secretory granule transport or exocytosis (McKay and Schneider, 1984). The site of action of taxol and vinblastine appears to be at the level of the adrenal nAChR. However, the mechanism by which these drugs inhibit nAChR function has not been determined. Reports in the literature suggest that vinblastine possesses anti-nAChR activity in neuronal tissues (Trifaro, 1972; McKay and Schneider, 1984; McKay et al., 1985). Several studies support the possibility that taxol, colchicine and vinblastine possess anticholinergic activity (Trifaro et al., 1972; McKay and Schneider, 1984; McKay et al., 1987). Other studies suggest that the microtubule drugs affect receptor function by an indirect action on microtubules (McKay and Schneider, 1984; McKay, et al., 1985; McKay, 1988).
In the present study, the action of other microtubule drugs on nicotine-stimulated catecholamine release was investigated. The results of these studies demonstrate that all of the microtubule drugs (demecolcine, podophyllotoxin, tubulozole and vincristine) inhibit release. The effect of the microtubule drugs on secretion stimulated by other secretagogues was then examined. All of the microtubule drugs selectively inhibited nicotine stimulated catecholamine release. The microtubule drugs had little or no effects on secretion stimulated by 1) the nonreceptor-mediated secretagogues, K⁺, Ba⁺², or veratridine or 2) by the receptor-mediated secretagogue, histamine. None of the drugs affected basal release of catecholamines. McKay and Schneider (1984) have reported similar effects for taxol and vinblastine on adrenal catecholamine release. Douglas et al. (1965) have demonstrated that a common secretory pathway exists in chromaffin cells for different secretagogues, including ACh and depolarizing concentrations of K⁺. Therefore, if, indeed, a common secretory pathway exists, then the site of action of the microtubule drugs must not be at the terminal steps of exocytosis since microtubule drugs do not attenuate secretion stimulated by non-nAChR mechanisms.

In these studies, the demonstration that none of the microtubule drugs affected Ba⁺²-stimulated release suggests that they do not directly alter secretory steps which might
involve calcium. \( \text{Ba}^{+2} \) stimulates adrenal catecholamine release by entering the cells through voltage operated calcium channels (VOC) and displacing calcium from intracellular storage sites (Hamano et al., 1991). In addition, the fact that the microtubule drugs did not alter release by depolarizing concentrations of \( K^+ \), a step which precedes opening of the VOC, suggests that the site of action of these drugs is at a step prior to cell depolarization. The lack of an inhibitory effect of the microtubule drugs on veratridine-stimulated catecholamine secretion also implies that the drugs are not directly affecting tetrodotoxin-sensitive \( Na^+ \) channels, but supports an action of the drugs at a step prior to depolarization.

These studies demonstrate that the microtubule drugs are inhibiting nAChR-stimulated release by interfering with signal transduction at the receptor level. Several lines of evidence support the hypothesis that the inhibitory effects of the microtubule drugs on nAChR function involves an action on microtubules. Firstly, all the microtubule drugs have similar effects, yet they are all structurally different and they interfere with microtubule function by different mechanisms. In addition, the IC\(_{50}\) values at which podophyllotoxin, tubulozole and vincristine inhibit adrenal catecholamine release are close to the IC\(_{50}\) concentrations which have been reported for each drug to inhibit in vitro tubulin polymerization, 0.5 - 1 \( \mu \text{M} \) (Hoebke and Van Nijen,
1975; Jordan and Farsell, 1983), 0.3 - 4 μM (Van Ginckel et al., 1984; Duanmy et al., 1989) and 0.1 - 2 μM (Owellen et al., 1972; Himes et al., 1977), respectively. It is interesting to note that the IC₅₀ values for all the microtubule drugs are very close to values reported by McKay and Schneider (1984) for the inhibition of ACh-stimulated catecholamine release by taxol and vinblastine. These results would suggest, then, that the effect of these (structurally different) microtubule drugs on catecholamine release is due to an action on microtubules, since they all have similar abilities to interact with microtubules.

The rapid inhibitory effect on nAChR function observed in these studies, as well as the rapid reversal of drug action could also be explained by an action of the drugs on microtubules at or near the plasma membrane. It is well documented that the action of microtubule drugs on tubulin is quick and reversible (Manfredi and Horwitz, 1984; Hamel, 1990). In vitro tubulin binding constants reported for demecolcine, podophyllotoxin and vincristine are 7 - 14 μM (Banerjee and Bhattacharyya, 1979; Ray et al., 1981), 0.3 - 0.7 μM (Flavin and Slaughter, 1974; Wilson et al., 1978) and 2 - 17 μM (Owellen et al., 1972; Wilson, 1975), respectively. In the present study, maximum inhibitory effects produced by the microtubule drugs were observed within 10 min for demecolcine, vincristine, podophyllotoxin and tubulozole. Furthermore, the inhibitory effects of the
microtubule drugs were quickly reversible. Within 2 min after removal of the drug, a reversal of the inhibition of catecholamine release was observed. McKay and Schneider (1984) have also reported maximum inhibitory effects for taxol and vinblastine on nAChR-mediated catecholamine release to occur as early as 5 min. These results are similar to those reported for vincristine (< 5 min, Wilson, 1975), demecolcine (20 - 30 min, Banerjee et al., 1979; Ray et al., 1981), podophyllotoxin (< 30 min, Kelleher, 1977) and tubulozole (< 15 - 60 min, Geuens et al., 1985) for dissociation from tubulin.

Adrenal chromaffin cells, like most cells, contain an extensive microtubular network (Bader et al., 1984). In these studies, all of the microtubule drugs have a direct effect on adrenal chromaffin cell microtubular network, and these effects are similar to those reported in other cells (Melmed et al., 1981; Manfredi and Horwitz, 1984; Mraz, 1985; Fujiwara and Tilney, 1975). If the receptor-related actions of the microtubule drugs involves microtubules, then it is important to establish that these drugs directly alter adrenal microtubules under similar conditions that affect receptor function. In the present study a 60 min treatment with concentrations of microtubule drug that inhibit receptor function was sufficient to detect the effects of the drugs on the adrenal microtubular system. Longer treatment times with the drugs demonstrated a greater effect
on the microtubular network. Treatments for shorter time periods were not conducted because of the qualitative nature of tubulin immunofluorescence technique; it is very difficult to detect small changes in the microtubular network. Since the functional effects of the microtubule drugs occur rapidly it was of interest to determine how early microtubule drugs alter microtubules. Therefore, the time-dependent effects of vinblastine were investigated. Vinblastine was used in these studies since its effects are manifested by formation of easily detectable paracrystals. Vinblastine-induced paracrystals could be detected as early as 15 min. An effect of microtubule drugs on the microtubular network as early as 15 min would agree well the time period the effects of the microtubule drugs on secretion occur. These results demonstrated that the microtubule drugs affect the adrenal microtubular network using similar drug treatment conditions which affect nAChR function, further supporting an action of the drugs on microtubules.

Several different populations of microtubules have been identified within neurons and PC12 cells (Gozes and Sweekner, 1981; Black and Keyser, 1987). The heterogeneity of microtubules is due to posttranslational modifications. One such population of microtubules are acetylated microtubules. Acetylated microtubules comprise 10 - 40 % of the total cytoplasmic microtubule population (Maruta et al.,
1986). Various studies have shown that acetylation of microtubules increases the stability of microtubules, i.e., microtubules are more resistant to conditions which cause microtubule depolymerization (L'Hernault and Rosenbaum, 1983; Piperno and Fuller, 1985). The availability of a monoclonal antibody, 6-11B-1, which specifically recognizes the acetylated form of tubulin, has made it possible to study the distribution of microtubules containing acetylated tubulin and the effect of drugs on this population of microtubules (Piperno and Fuller, 1985). Immunofluorescence studies by Jasmin et al. (1990), using cryostat sections of chick anterior latissimus dorsi muscle, demonstrated that areas underneath motor plates which were labelled with FITC-conjugated α-Bgt were similar to areas labeled by the 6-11-B-1 antibody. In these studies it was suggested that one possible role for microtubules localized in subsynaptic areas was in the transport of nAChRs to the endplated surface.

To determine if adrenal chromaffin cells contain acetylated tubulin and if microtubule drugs alter this population of microtubules, cells were treated with the microtubule drugs, stained with 6-11B-1 (anti-acetylated tubulin antibodies) and examined by immunofluorescence microscopy. The results obtained from this study demonstrated that all of the microtubule drugs altered the acetylated microtubular network and suggest that the effect
of these drugs on nAChRs could be due to an alteration in the acetylated microtubule population.

Another possibility for the effect of microtubule drugs on nAChR function is an anticholinergic action. Various reports in the literature demonstrate that taxol and vinblastine possess anti-nAChR activity (Trifaro et al., 1972; McKay and Schneider, 1984; McKay et al., 1985, McKay, 1988). In these studies the selectivity of the microtubule drugs for cholinergically mediated catecholamine secretion by isolated chromaffin cells, suggests that the inhibitory effect of the microtubule drugs on nAChR function might be due to a cholinergic action of the drugs. One approach to investigate this possibility is to determine whether the microtubule drugs interfere with binding of nicotine to nAChRs. To investigate the interaction of microtubule drugs with nAChRs, [3H]nicotine binding to membrane fragments from bovine adrenal medulla and cultured chromaffin cells was attempted. However, under the conditions used in these binding studies, specific binding could not be obtained from adrenal tissue or cell homogenates. There are various factors in the preparation of the membrane fragments that could render the nAChRs labile. A decrease in the number of nAChRs density could impede measurement of [3H]nicotine binding. It should also be pointed out that [3H]nicotine binding to membranes prepared from adrenal medullary tissue has not been reported in the literature. Binding of
[³H]nicotine to membrane fragments from cultured adrenal chromaffin cells was also attempted. Higgins and Berg (1988) have been the only group to report binding to membrane fragments from cultured adrenal chromaffin cells. However, attempts to duplicate their results have failed. Higgins and Berg (1988) investigated binding of various nAChR agonists and antagonists to membrane fragments from cultured bovine chromaffin cells and demonstrated that the affinity of these compounds were similar to those reported for [³H]nicotine binding to rat brain membrane fragments, suggesting a pharmacological similarity between adrenal and brain neuronal nAChRs.

Since [³H]nicotine binding to adrenal chromaffin membrane fragments could not be accomplished in our laboratory, membrane fragments from rat brain were used as a plentiful source of neuronal nAChRs. In rat brain homogenates, both carbachol and nicotine inhibited [³H]nicotine binding with appKᵢ values close to those reported for high affinity nicotine binding to rat brain membrane (Romano and Goldstein, 1980; Lippiello and Fernandez, 1986) and chromaffin cell membranes fragments (Higgins and Berg, 1988), confirming the presence of nAChRs. The effects of microtubule drugs on [³H]nicotine binding were then examined. At concentrations of microtubule drugs known to inhibit approximately 80% of nicotine-stimulated catecholamine release, none of the microtubule drugs
(demecolcine, podophyllotoxin, tubulozole, vincristine or nocodazole) inhibited $[^3H]$nicotine binding. These results suggest that in rat brain membrane fragments, the microtubule drugs do not affect $[^3H]$nicotine binding and therefore, do not appear to be nAChR antagonists. It should be pointed out that these rat brain preparations lack an intact microtubule network. Similarly, McKay and coworkers (1985) have reported that the microtubule drugs taxol, vinblastine and colchicine do not inhibit $[^{125}I] \alpha$-Bgt binding to nAChRs from Torpedo electric organ. These results suggest that microtubule drugs are not classic nAChR antagonists and in order to see an action of the microtubule drugs on nAChRs an intact cytoskeletal network may be necessary.

$[^3H]$Nicotine binding studies were also conducted on cultured adrenal chromaffin cells. In this preparation, the cells are viable and nAChRs would remain coupled to the microtubular network. The microtubule drugs, demecolcine, podophyllotoxin, taxol, vinblastine and vincristine inhibited $[^3H]$nicotine binding. Although tubulozole also blocked $[^3H]$nicotine binding, the effect was not significant. These results suggest that in order for the microtubule drugs to affect $[^3H]$nicotine binding an intact cytoskeleton is necessary.

To directly determine if adrenal nAChRs are linked to the cytoskeleton, adrenal nAChRs were labelled using a
radiolabeled antibody, $[^{125}\text{I}]{\text{mAb}}$ 35. mAb 35, is a monoclonal antibody (mAb), raised against nAChR from *Electrophorus* electric organ that recognizes the main immunogenic region (MIR) of muscle and electric organ AChRs (Tzartos et al., 1981) and cross reacts with chick ciliary ganglion AChRs (Jacob et al., 1984) and nAChRs of bovine adrenal chromaffin cells (Higgins and Berg, 1987). After establishing that mAb 35 interacts specifically with adrenal nAChRs, chromaffin cells were incubated with 5nM $[^{125}\text{I}]{\text{mAb}}$ 35 for 1 hr prior to Triton-extraction. Under the conditions used in these studies, Higgins and Berg (1988b) have demonstrated that receptor internalization is minimum. The results obtained in the present study indicated that 36% of surface nAChRs remain associated with the Triton-insoluble cytoskeleton. Similar findings have been reported for muscle nAChRs (Stya and Axelrod, 1983a).

After establishing that a population of nAChRs remains associated with the Triton-insoluble cytoskeleton, the effects of various microtubule drugs (demecolcine, podophyllotoxin, taxol, tubulozole, vinblastine and vincristine) on the extractibility of the nAChRs were investigated. Triton is a mild detergent that removes lipids and soluble proteins and leaves the internal cytoskeleton and associated proteins essentially intact (Ben-Ze'ev et al., 1979). In these studies all of the microtubule drugs decreased the number of receptors
associated with the cytoskeleton as measured by the amount of $[^{125}\text{I}]m\text{Ab\ }35$ bound.

It is of interest to note that all the microtubule drugs decreased nAChR association with the Triton-insoluble cytoskeleton and $[^{3}\text{H}]\text{nicotine}$ binding to intact adrenal chromaffin cells to a similar extent, approximately 50%. Since both nicotine and mAb 35 bind to different sites of the nAChRs, these results suggest that the microtubule drugs produce an alteration of the microtubular network that results in a major conformational change in the nAChR. Adiphenine, the noncompetitive blocker, had no effect on the extractability of nAChRs. A decrease in the number of receptors associated with the cytoskeleton was observed in cells treated with nicotine and mecamylamine. It is not known why this occurred; however, a possible explanation may be that an interaction of drugs with the binding site causes a conformational change in the nAChR, altering receptor-cytoskeleton association and facilitating the extractability of nAChRs.
CHAPTER VI
SUMMARY AND CONCLUSIONS

Cytoskeletal links with cell surface receptors occurs in a number of receptor systems and these linkages are involved with receptor topography and mobility and have been implicated in signal transduction mechanisms (Nicolson, 1976; Carraway and Carraway, 1989). Receptor-cytoskeletal associations have also been demonstrated for muscle nAChRs (Prives, et al., 1982; Stya and Axelrod, 1983a, 1983b; Connolly and Oldefin, 1985). In adrenal chromaffin cells, evidence exists that nAChRs are associated with cytoskeletal proteins and that this association may be important for receptor function (McKay and Schneider, 1984; McKay, 1988).

In the present studies, the effects of several microtubule drugs on cultured adrenal chromaffin cells were investigated. The drugs used in these studies are structurally different but have one common feature: each drug interferes with microtubule function. All the microtubule drugs 1) selectively inhibited nAChR function, 2) inhibited nAChR function at concentrations reported for inhibition of tubulin polymerization and 3) altered microtubule networks under similar conditions at which they
inhibit nAChR function. Taken together, these studies suggested that the inhibitory effects of the microtubule drugs on nAChR-stimulated catecholamine release may be due to an action of the drugs on microtubules. These results support the hypothesis that adrenal nAChRs are functionally linked to the microtubular network.

To directly explore the possibility that adrenal nAChRs are associated with the intracellular cytoskeletal network, detergent extraction experiments were conducted. The results of these studies demonstrated 1) that a population of adrenal nAChRs are associated with the Triton-insoluble cytoskeleton and 2) that microtubule drugs can alter the association of nAChRs with the cytoskeleton. Most importantly, the treatment conditions which cause dissociation of nAChRs from the cytoskeleton are the same that inhibit nAChR function and decrease agonist binding to cultured adrenal chromaffin cells. In conclusion, these studies support the hypothesis that there is an association of adrenal nAChRs with microtubules and suggest that the mechanism by which the microtubule drugs inhibit nAChR function is by inducing dissociation of nAChRs from the microtubular network. Furthermore, acetylated microtubules may be involved in localizing and anchoring of adrenal nAChRs on the cell membrane as has been implicated for muscle nAChRs (Jasmin et al., 1990).
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