This is to certify that the

thesis entitled

EFFECTS OF DITHIOBIURET ON THE SYNTHESIS AND RELEASE OF DOPAMINE AND ACETYLCHOLINE FROM PHEOCHROMOCYTOMA (PC12) CELLS

presented by

Lynne Marie Ireland

has been accepted towards fulfillment of the requirements for

M.S. degree in Pharmacology/Toxicology

William D. Atchison

Major professor

Date 3-31-94

EFFECTS OF DITHIOBIURET ON THE SYNTHESIS AND RELEASE OF DOPAMINE AND ACETYLCHOLINE FROM PHEOCHROMOCYTOMA (PC12) CELLS

By

Lynne Marie Ireland

A THESIS

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

MASTER OF SCIENCE

Department of Pharmacology and Toxicology

1994

ABSTRACT

EFFECTS OF DITHIOBIURET ON THE SYNTHESIS AND RELEASE OF DOPAMINE AND ACETYLCHOLINE FROM PHEOCHROMOCYTOMA (PC12) CELLS

By

Lynne Marie Ireland

Chronic administration of dithiobiuret (DTB) causes delayed-onset neuromuscular weakness in rats. Electrophysiological and biochemical studies suggest DTB inhibits quantal acetylcholine (ACh) release from motor nerve terminals. The effects on non-cholinergic neurotransmission are unknown. To determine the specificity of action of DTB, pheochromocytoma (PC12) cells were used to compare the effects of DTB on the content and release of ACh and dopamine (DA). DTB reduced evoked release of ACh without altering cellular ACh or choline levels, suggesting that DTB acts on mechanisms involved in ACh release. α-Latrotoxin-stimulated release of ACh was not inhibited by DTB. At low concentrations, DTB pretreatment enhanced α-latrotoxin-stimulated release of ACh suggesting an alteration of vesicle docking or fusion with the plasma membrane. DTB also reduced evoked release of DA and inhibited DA synthesis, resulting in a decrease in the readily releasable pool of DA.

To my parents, Jim and Claudia Ireland and my grandparents, Claude and Lucile Smith for their love and support of my graduate education

ACKNOWLEDGMENTS

I would like to thank the members of my thesis committee, Drs. Barman, Braselton, Cobbett, Galligan and Atchison for their guidance and time. I am grateful for the critical review of the various drafts of my thesis by Drs. Hare, Cobbett, Atchison and Michael Denny. I would like to acknowledge Dr. Atchison for providing the laboratory and funding for this work. I also need to thank several laboratories for allowing me to borrow supplies or equipment: Drs. Braselton, Cobbett, Contreras, Galligan, Roth, Lookingland and Moore. I would also like to thank Drs. Barman, Lookingland and Atchison for writing good letters of recommendation for my career in science.

I am grateful for the helpful suggestions and the friendships of Mike Denny, Michael Hare, Sandy Hewett, Ravindra Hajela, Laura Huelskamp, Annette McLane, Sue Marty, Sue Stejskal, Jay Sirois, Aizhen Yao, Chunhong Yan, and Yukun Yuan. I would like to thank Rob Angus, Paul Bertrand, Misty Eaton, Annette Fleckenstein, and Anne Marie Yunker for being good friends. I would also like to thank Diane Hummel and Mickie Vanderlip for their help and Nelda Carpenter for her valuable friendship.

I would especially like to thank my parents and grandparents for their love and support. They always believed in me even when I did not. I will forever be grateful for all the wonderful things my parents and grandparents did for me. I would also like to thank Mike Denny for his love and friendship. Mike is the best human being (and biochemist) I have ever known.

TABLE OF CONTENTS

APPENDIX

LIST OF FIGURES

4A

αL'

AC

ACI

ACI

BTX

[Ca²

[Ca²

CAT

DA -

DME

DMS

DOP

DOP

DTB

EDT

EPP

HC-3

LIST OF ABBREVIATIONS

4AP - 4-aminopyridine

αLTX - alpha-latrotoxin

ACh - acetylcholine

AChE - acetylcholine esterase

AChR - acetylcholine receptors

BTX - botulinum neurotoxins

$[Ca^{2+}]_e$ - extracellular calcium ion concentration

$[Ca^{2+}]_i$ - intracellular calcium ion concentration

CAT - choline acetyltransferase

DA - dopamine

DMEM - Dulbecco's modified Eagles' medium

DMSO - dimethyl sulfoxide

DOPA - dihydroxyphenylalanine

DOPAC - dihydroxyphenylacetic acid

DTB - dithiobiuret

EDTA - ethylenediamine tetraacetic acid

EPP - end plate potential

HC-3 - hemicholinium-3

HEPES - N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]

HKB - high K^+ buffer

HPLC-EC - high performance liquid chromatography coupled to electrochemical detection

LDCV - large dense core vesicle

LDH - lactate dehydrogenase

LKB - low K^+ buffer

m - mean quantal content

MEPP - miniature end plate potential

n - total releasable store

nAChR - nicotinic acetylcholine receptor

NAD^+ - nicotinamide adenine dinucleotide, oxidized form

NADH - nicotinamide adenine dinucleotide, reduced form

NE - norepinephrine

NGF - nerve growth factor

p - statistical probability that a given quantum will be released

PC12 cells - pheochromocytoma cells

PTP - post tetanic potentiation

SB - sucrose buffer

SCV - small clear vesicles

SH - sulfhydryl bond

TH - tyrosine hydroxylase

INTRODUCTION

I. HUMAN NEUROMUSCULAR DISEASES - DISRUPTED ACh RELEASE

Synaptic transmission at the neuromuscular junction involves several steps resulting in the release of the neurotransmitter acetylcholine (ACh) from motor nerve terminals, followed by the activation of acetylcholine receptors (AChR) on the muscle membrane, culminating in muscle contraction. Some human neuromuscular diseases result from abnormalities in cholinergic synaptic transmission. Such diseases can be put into two categories, postsynaptic dysfunction and presynaptic dysfunction.

A postsynaptic disorder is characterized by a decrease in the ability of muscle to respond to normal motor nerve activity. An example of a neuromuscular disorder attributed to postsynaptic dysfunction is myasthenia gravis. Muscle biopsy specimens from patients with myasthenia gravis were found to contain a low number of AChR and to have distorted morphology of the postsynaptic membrane (Engel *et al.*, 1988). These changes in the muscle membrane play a role in the postsynaptic dysfunction of myasthenia gravis.

A presynaptic defect in transmission involves dysfunction of the motor nerve. Two disorders, *Lambert-Eaton myasthenic syndrome* and *familial infantile myasthenia*, result from aberrations in presynaptic function (Engel,

1988). The most common symptoms of *Lambert-Eaton myasthenic syndrome* are muscle weakness, dry mouth, urinary hesitancy, and constipation. These symptoms of *Lambert-Eaton myasthenic syndrome* are caused by reduced ACh release from motor nerve terminals or nerves terminating on secretory glands (Lambert and Elmqvist, 1971). *Familial infantile myasthenia* is characterized by diminished nerve-evoked muscle contractions resulting from a progressive decrease in the amount of quantal ACh released from motor nerves (Mora *et al.*, 1987). Newborns with *familial infantile myasthenia* show increased fatigability on exertion, feeding difficulty, ptosis (droopy eyelids), and episodes of apnea (Engel, 1988). The apnea can cause sudden death or brain injury in infants.

II. NEUROMUSCULAR WEAKNESS - 2,4-DITHIOBIURET

2,4-Dithiobiuret (DTB) is a thiourea derivative with moderate reducing capabilities (Preisler and Bateman, 1947). It can cause delayed-onset neuromuscular weakness when administered chronically to rats (Astwood *et al.*, 1945; Atchison *et al.*, 1981) or rabbits (Seifter *et al.*, 1948) and can result in death, presumably due to paralysis of the respiratory muscles (Astwood *et al.*, 1945). Several alterations in neuromuscular function in rats or rabbits treated chronically with DTB are similar to those seen in human neuromuscular disorders. Rabbits treated chronically with DTB show flaccid skeletal muscle weakness, decreased food intake, difficulty urinating, and constipation (Seifter *et al.*, 1948). Signs of DTB-induced neuromuscular weakness in rats also include altered gastrointestinal function (Atchison and

Peterson, 1981).

DTB-induced neuromuscular weakness is caused by disruption of synaptic transmission at the neuromuscular junction (Atchison *et al.*, 1982). Following chronic treatment of rats with DTB, isolated nerve-muscle preparations were stimulated and the muscle responses were recorded by using electrophysiological techniques. DTB did not alter the contractile response of the muscle (Atchison *et al.*, 1981), muscle membrane potential, or muscle input resistance (Weiler *et al.*, 1986) after direct electrical stimulation.

Indirect muscle stimulation involves stimulating the motor nerve to release ACh; the muscle nicotinic AChR (nAChR) responds to ACh by allowing Na^+ and K^+ ions to pass through the nAChR-ion channel. The movement of Na^+ and K^+ ions causes the muscle membrane near the motor nerve end-plate to depolarize. This induces a change in membrane potential that brings the muscle membrane to threshold for generating an action potential, leading to muscle contraction. The change of membrane potential in response to evoked ACh release is called the end-plate potential (EPP). The EPP amplitude is related to the amount of ACh released upon electrical stimulation of the nerve. Spontaneous quantal release of ACh from the motor nerve is measured as a miniature end plate potential (MEPP). Total spontaneous ACh release equals the amount of quantal ACh release measured as MEPPs plus the amount of nonquantal ACh which diffuses across the plasma membrane (Polak *et al.*, 1981). The frequency of spontaneous quantal release of ACh from motor nerves (MEPP frequency), is dependent on the concentration of intracellular

Ca^{2+} ($[Ca^{2+}]_i$) and can be increased by elevating extracellular K^+ concentration (Elmqvist and Feldman, 1966).

EPPs and MEPPs measured in nerve-muscle preparations from DTB-treated rats have slower rise and decay times than normally found at the neuromuscular junction which may indicate a change in post-synaptic nAChR function, or an increase in the diffusion pathlength of ACh to the postsynaptic nAChR (Atchison, 1989). Work by Spitsbergen (1991) suggested that acute exposure of a nerve-muscle preparation to DTB decreased the decay of synaptic end-plate currents, which is the amount of ion flow through the nAChR at the end-plate. This finding suggests that DTB alters the duration for which ACh remains bound to the postsynaptic ACh receptor or decreases the open time for ACh receptor-gated ion channels (Spitsbergen and Atchison, 1990). DTB-induced neuromuscular weakness in rats may involve alterations in postsynaptic nAChR function; however, the predominate effects of DTB appear to occur at a presynaptic site (Atchison *et al.*, 1982; Weiler *et al.*, 1986; Atchison, 1989).

III. DTB - DISRUPTED ACh RELEASE

The presynaptic effects of DTB have also been studied using nerve-muscle preparations removed from DTB-treated rats. Electrical stimulation of the sciatic nerve produced smaller twitch tension in *gastrocnemius* muscle from DTB-treated rats as compared to control rats (Atchison *et al.*, 1981). Sciatic nerve-*gastrocnemius* preparations from DTB-treated rats also displayed shorter duration of post-tetanic potentiation (PTP) and decreased tension after

the PTP period. PTP is a phenomenon believed to be due to increased mobilization and release of neurotransmitter (Gage and Hubbard, 1966). The effects of DTB treatment on synaptic transmission at motor end-plates were examined using electrophysiological recording techniques in peroneal nerve-*extensor digitorum longus* muscle preparations. Both EPP and MEPP amplitude, and MEPP frequency were decreased in nerve-muscle preparations taken from DTB-treated rats (Weiler *et al.*, 1986; Atchison, 1989). Increasing extracellular $[K^+]$, thus increasing $[Ca^{2+}]_i$, did not return MEPP frequency to control values (Atchison, 1989), suggesting that DTB alters spontaneous quantal ACh release *via* a mechanism which is Ca^{2+}-independent or is not reversible by increases in $[Ca^{2+}]_i$. Spontaneous nonquantal ACh release was not affected by chronic treatment with DTB (Weiler *et al.*, 1986).

The effects of acute treatment with DTB on neuromuscular transmission were compared to the effects of chronic DTB treatment. Nerve-muscle preparations removed from rats 1 hr after treatment with a single large dose of DTB showed decreased EPP and MEPP amplitudes, and MEPP frequency (Spitsbergen and Atchison, 1990). However, the acute DTB treatment did not cause neuromuscular weakness. To examine the early effects of DTB, nerve-muscle preparations removed from control rats were perfused with bathing medium containing DTB. Bath application of DTB on nerve-muscle preparations caused similar decreases in neuromuscular transmission as did acute administration of DTB (Spitsbergen and Atchison, 1990). However, an initial transient increase in EPP and MEPP amplitude and MEPP frequency

was observed immediately after bath application of DTB.

Giant MEPPs, thought to reflect the spontaneous release of multiple packets of ACh (Publicover and Duncan, 1981), were observed with increased frequency in rats treated chronically (Atchison, 1989) or acutely with DTB (Spitsbergen and Atchison, 1990). These large MEPPs characteristically have slow rise and decay times compared to normal MEPPs. Diamide, a thiol-reactive agent, markedly increases the occurrence of giant MEPPs at the frog neuromuscular junction (Publicover and Duncan, 1981). It was concluded that diamide does not alter Ca^{2+} influx through voltage-dependent Ca^{2+} channels or affect mitochondrial function. Instead, it was proposed that diamide affects the quantal release system by altering vesicle protein sulfhydryl (-SH) groups resulting in an increase frequency of vesicle-vesicle fusion prior to vesicle-plasma membrane fusion. The release of fused, large vesicles may lead to the presence of giant MEPPs (Publicover and Duncan, 1981). DTB, which contains two thio groups, is capable of forming disulfide bridges with proteins and may act in a similar manner to diamide to increase in occurrence of giant MEPPs and to alter quantal ACh release at the neuromuscular junction.

At the neuromuscular junction, the amount of vesicular ACh released upon nerve stimulation is called the mean quantal content (m). The m was significantly depressed in nerve-muscle preparations from rats treated chronically (Weiler *et al.*, 1986; Atchison, 1989) but not acutely with DTB (Spitsbergen and Atchison, 1990). The m is the product of the total releasable store (n) and the average probability (p) that a given quantum will be released:

$m = np$ (del Castillo and Katz, 1957a). The DTB-induced decrease in m was found to be due to a change in the releasable ACh store; the probability of release of a quantum of neurotransmitter remained unchanged (Atchison, 1989). The p is thought to be related to $[Ca^{2+}]_i$ (Katz, 1966). Since DTB-treatment does not change p, DTB may act through a Ca^{2+}-independent mechanism which alters vesicular release of ACh at the neuromuscular junction.

Drugs were used to study the mechanisms involved in the prejunctional effects of DTB at the neuromuscular junction. Hemicholinium-3 (HC-3), a relatively specific inhibitor of the high affinity choline uptake system (Collier and MacIntosh, 1969), causes neuromuscular fatigue by reducing the availability of intracellular choline and thus decreasing the ACh stores in the motorneuron. DTB attenuated the neuromuscular fatigue induced by HC-3 (Atchison *et al.*, 1982). This action of DTB is likely due to a reduction in m resulting in a decrease in the amount of ACh released into the synaptic cleft. 4-Aminopyridine (4AP), a blocker of voltage-dependent K^+ channels (Thompson and Aldrich, 1980), increases the release of ACh at the neuromuscular junction resulting in facilitation of contractile responses (Pelhate and Pichon, 1974; Molgo *et al.*, 1977). Block of voltage-dependent K^+ channels prolongs the duration of the action potential. This in turn lengthens the time of activation of the voltage-dependent Ca^{2+} channel, allowing increased Ca^{2+} influx thus increasing p (Katz, 1966). 4AP only partially restored contractile responses in DTB-treated preparations (Atchison *et al.*, 1982). However, increasing

extracellular [Ca^{2+}] ([Ca^{2+}]$_e$) does not restore *m* of DTB-treated preparations to control; suggesting that DTB affects cholinergic release mechanisms independent of the influx of Ca^{2+} through voltage-sensitive Ca^{2+} channels (Atchison *et al.*, 1982).

The effects of chronic DTB treatment were studied by using the peroneal nerve-*extensor digitorum longus* muscle preparation to examine ACh release and metabolism from motor nerve terminals (Weiler *et al.*, 1986). The nerve-muscle preparation was perfused with medium containing [$^2H_4$]choline for 15 min to create a pool of [$^2H_4$]ACh vesicles within the terminal of the nerve. The preparation was then perfused with medium to wash away extracellular [$^2H_4$]choline. The release perfusates were analyzed for ACh and choline release by using the gas chromatography/mass spectrometry technique of Jenden *et al.* (1973). Spontaneous release of ACh from the nerve-muscle preparations of DTB-treated rats was not significantly different from that of control preparations (Weiler *et al.*, 1986). However, evoked release of ACh was significantly reduced and this reduction was correlated with the decrease in *m* determined from electrophysiological analysis of EPPs. The tissue concentration of newly synthesized [$^2H_4$]ACh was 50% lower in the nerve-muscle preparations from DTB-treated rats; however, overall ACh content was not affected. This suggests that DTB disrupts ACh release without altering overall ACh content at the motor nerve terminal, resulting in dysfunction of neuromuscular transmission.

I

a

ir

th

wa

Ra

an

(C1

cha

194

in r

obs

as

trea

trea

rats

exh

glar

sym

neu

imp

IV. DTB - NON-CHOLINERGIC SYNAPTIC TRANSMISSION

While neuromuscular transmission is impaired by DTB, sensory systems appear to be unaltered by DTB treatment (Altschul, 1947). Rats with DTB-induced neuromuscular weakness responded to painful stimuli as measured by the tail-flick reflex (Atchison and Peterson, 1981). The latency for the tail flick was unaffected although the intensity of the muscle contraction was decreased. Rats treated chronically with DTB showed neuromuscular weakness without any disruption of thermal sensitivity, auditory thresholds, or pattern vision (Crofton *et al.*, 1991). Rats treated chronically with DTB also showed no change in electroencephalographic measurements of cortical activity (Altschul, 1947). However, recent work studying the effects of DTB on cognitive function in rats showed that DTB enhances working memory (Bushnell, 1994). These observations suggest that the central nervous system and ocular components as well as peripheral pain responses remain intact during chronic DTB treatment.

Other cholinergic functions which appear to be affected by DTB treatment involve the enteric and autonomic nervous systems. DTB-treated rats had mucoid feces and delayed onset diuresis; some rats treated with DTB exhibited chromodacryorrhea, a porphyrin pigment secreted by the Harderian glands of the eye (Astwood *et al.*, 1945; Atchison and Peterson, 1981). These symptoms suggest that DTB affects cholinergic function other than at neuromuscular junctions. DTB-treated rats do not display symptoms of impaired non-cholinergic neurotransmission (Astwood *et al.*, 1945; Atchison

an

cho

inh

fun

in r

on

neu

effe

purp

of ac

dete

dete

disti

a sir

deter

inhib

V. PC

DTB

neuro

releas

depen

and Peterson, 1981). Because of a lack of evidence, the effect of DTB on non-cholinergic neurotransmission is unknown.

Results of electrophysiological and biochemical studies suggest that DTB inhibits quantal ACh release from motor nerve terminals and alters nAChR function in the muscle membrane. The specificity of DTB-induced dysfunction in neurotransmission for cholinergic synapses may be due to the effects of DTB on ACh release and/or the effects of DTB on AChR. By using the neuromuscular junction, it may be impossible to distinguish the presynaptic effects from the postsynaptic effects of DTB on synaptic transmission. The purpose of the work described here was to study the presynaptic mechanism of action of DTB-induced decrease in evoked neurotransmitter release and to determine if DTB acts specifically to reduce cholinergic neuronal activity. To determine if DTB acts specifically on ACh release, the effects of DTB on two distinct neurotransmitter release mechanisms were compared. We chose to use a simple one cell model which releases two transmitters to facilitate the determination of a specific target or mechanism involved in DTB-induced inhibition of neurotransmitter release.

V. PC12 CELLS - A MODEL FOR NEURONAL TRANSMITTER RELEASE

Pheochromocytoma (PC12) cells can be used to compare the effects of DTB on the content and release of ACh simultaneously with a non-cholinergic neurotransmitter. PC12 cells, a clonal line from a rat pheochromocytoma, release ACh, dopamine (DA), norepinephrine (NE) and ATP in a Ca^{2+}-dependent manner (Greene and Tischler, 1976; Greene and Rein, 1977a).

Reserpine (1 μM) treatment for 21 hr depleted cellular catecholamine levels and therefore evoked DA release (Greene and Rein, 1977a) without altering cellular ACh levels in PC12 cells (Schubert and Klier, 1977). Using sucrose density gradient analysis, it was confirmed that catecholamines and ACh were stored in separate storage vesicles (Schubert and Klier, 1977). Thus, PC12 cells can release catecholamines and ACh by means of separate exocytotic secretion mechanisms (Greene and Rein, 1977a; Schubert and Klier, 1977).

Upon exposure to nerve growth factor (NGF) PC12 cells cease to divide and develop neurites (Greene and Tischler, 1976). Total RNA and protein levels increase and choline acetyltransferase (CAT) and tyrosine hydroxylase (TH) activities are elevated in differentiated PC12 cells (Greene and Rein, 1977b). CAT and TH are enzymes involved in the synthesis of ACh and DA, respectively. NGF-treated PC12 cells contain more DA and ACh than do undifferentiated cells (Greene and Rein, 1977b).

NGF treatment also increases the ACh sensitivity of PC12 cells as measured by a change in membrane potential (Dichter *et al.*, 1977). Biochemical and pharmacological evidence indicates that PC12 cells have both muscarinic AChR and a ganglionic-type nicotinic AChR (Greene and Rein, 1977c; Jumblatt and Tischler, 1982). Catecholamine release from PC12 cells is stimulated by nicotinic (Greene and Rein, 1977c) and muscarinic agonists (Rabe *et al.*, 1987). Muscarinic receptor stimulation increases inositol phospholipid metabolism in PC12 cells (Horwitz, 1989) and elevates $[Ca^{2+}]_i$ (Rabe *et al.*, 1987). The nAChR channels in PC12 cells are permeant to Ca^{2+},

and in normal media conditions allow significant (5% of total) ACh-gated current to be carried by Ca^{2+} ions (Sands and Barish, 1991). Stimulation of nAChR evokes neurotransmitter release from PC12 cells through a Ca^{2+}-dependent and tetrodotoxin insensitive mechanism (Greene and Rein, 1977c).

A. ACh Synthesis in PC12 Cells

ACh synthesis involves the uptake of choline from the media. The uptake of choline by PC12 cells is Na^+-independent and somewhat insensitive to HC-3 (IC_{50}=50 μM) (Guroff, 1985). Choline is acetylated by the cytosolic enzyme CAT which uses acetyl Coenzyme A as the source of the acetyl group. The cytosolic ACh is taken up by the vesicular ACh transporter of cholinergic vesicles; the ACh transporter does not transport any other classical neurotransmitter into cholinergic vesicles (Clarkson *et al.*, 1993). Approximately 35% of total cellular ACh in the PC12 cells is found in the vesicles (Rebois *et al.*, 1980). Released and cytosolic ACh is metabolized by acetylcholinesterase (AChE). PC12 cells contain high levels of cytoplasmic AChE activity, and as such, turnover of soluble (non-vesicular) ACh is rapid (Melega and Howard, 1984). Within a 30 min period, turnover of 75% of the cytosolic [^{3}H]ACh and 20% of the vesicular [^{3}H]ACh will occur (Melega and Howard, 1984).

B. Catecholamine Synthesis in PC12 Cells

PC12 cells take up tyrosine from the culture medium and tyrosine hydroxylase (TH) converts it into dihydroxyphenylalanine (DOPA). DOPA is then decarboxylated by DOPA-decarboxylase to yield DA. DA is taken up into

catecholaminergic vesicles where DA may then be converted to NE by the vesicular enzyme DA-β-hydroxylase (Johnson and Scarpa, 1976). PC12 cells produce a large excess of DA relative to NE due to an insufficiency of ascorbic acid, a necessary cofactor for DA-β-hydroxylase (Guroff, 1985). Approximately 60% of the total cellular content of DA and NE is in vesicles (Schubert and Klier, 1977). PC12 cells contain monoamine oxidase which metabolizes non-vesicular DA into dihydroxyphenylacetic acid (DOPAC) (Greene and Rein 1977a).

VI. NEUROTRANSMITTER RELEASE MECHANISMS

Vesicular release of neurotransmitter is dependent upon a specific sequence of events within the nerve terminal. Action potentials invade the nerve terminal and depolarize the plasma membrane, causing activation of voltage-dependent Ca^{2+} channels. The influx of Ca^{2+} results in localized increases in $[Ca^{2+}]_i$ which stimulates vesicles to move towards and fuse with the plasma membrane and release neurotransmitter into the synaptic cleft (Katz and Miledi, 1966). The exact mechanisms involved in Ca^{2+}-triggered neurotransmitter release are not known, but are the subject of intense study.

The study of vesicular release mechanisms has concentrated on the interaction between vesicular proteins and surrounding cytosolic, cytoskeletal, and plasma membrane proteins implicated in vesicle trafficking, docking and fusion with the plasma membrane. Synapsins, Rab3, synaptobrevins, synaptotagmin and synaptophysin are all vesicular proteins involved in

tr

to

m

an

ha

(B

tha

an

ne

al.,

via

199

syn

neu

vesi

vesi

cate

in re

relat

and

transmitter release (Südhof and Jahn, 1991). Vesicular trafficking is thought to involve synapsins binding to cytoskeletal components (F-actin) which maneuver the vesicles close to the active zone for imminent release (Bähler and Greengard, 1987). Rab3A and Rab3B are small GTP-binding proteins that have been implicated in vesicular trafficking and neurotransmitter release (Balch, 1990). Synaptobrevin is an integral synaptic vesicle membrane protein that may be involved in vesicular docking with the plasma membrane (Chin and Goldman, 1992). Botulinum toxin-B is a zinc endopeptidase that blocks neurotransmitter release by proteolytic cleavage of synaptobrevin (Schiavo *et al.*, 1992).

After release of neurotransmitters, the empty vesicles are endocytosed *via* clathrin-coated pits and reloaded with neurotransmitter (Südhof and Jahn, 1991). A Mg^{2+}-ATPase generates an electrochemical proton gradient across the synaptic vesicle membrane which drives neurotransmitter uptake by specific neurotransmitter transporters (Maycox *et al.*, 1990). After reloading, the vesicles are available to release transmitter once again.

PC12 cells contain both large dense-core vesicles (LDCV) and small clear vesicles (SCV) (Greene and Rein, 1977b). The LDCV may contain catecholamines, and the SCV may contain ACh. The SCV increase in number in response to NGF; it is not known whether the increase in SCV number is related to the increase in ACh levels in NGF-differentiated PC12 cells (Cutler and Cramer, 1990).

(W

sy

Sy

no

Th

(C

fou

Sü

int

syr

int

ves

put

App

cate

cate

anti

regu

syna

Synaptophysin (p38) purifies with SCV and not with LDCV of PC12 cells (Wiedenmann *et al.*, 1987; Cutler and Cramer, 1990). This suggests that synaptophysin is specific for the cholinergic vesicles in PC12 cells. Synaptophysin is present only in the membranes of SCV in rat brain which do not contain peptide neurotransmitters or catecholamines (Knaus *et al.*, 1990). The SCV from PC12 cells have a similar density to that of rat brain SCV (Cutler and Cramer, 1990). Synaptophysin is a synaptic vesicle protein with four transmembrane regions and two intravesicular loops (Johnston and Südhof, 1990). Synaptophysin monomers are linked together by unstable intramolecular disulfide bonds to form homopolymers. The structure of synaptophysin and its interaction with other proteins are dependent on the integrity of its intramolecular disulfide bonds (Johnston and Südhof, 1990).

Synaptotagmin may also be involved in docking and fusion of the vesicles. Synaptotagmin has a Ca^{2+}-regulatory binding domain and may be the putative Ca^{2+}-binding trigger for neurotransmitter release (Perin *et al.*, 1991). Apparently, synaptotagmin (p65) is not essential for secretion of catecholamines or ATP from PC12 cells (Shoji-Kasai *et al.*, 1992). However, catecholamine release from PC12 cells could be blocked by microinjection of antibodies against synaptotagmin, suggesting that this vesicular protein is a regulator of neurotransmitter release (Elferink *et al.*, 1993). Without synaptotagmin, cells may have only constitutive neurotransmitter release.

The study of neurotransmitter release mechanisms operative in PC12

cells has relied, in part, on traditional pharmacological methods. In particular, neurotoxins with known mechanisms of action have been useful in studying the mechanisms of vesicular neurotransmitter release. Botulinum neurotoxins (BTX) inhibit Ca^{2+}-dependent NE release from permeabilized PC12 cells (Lomneth *et al.*, 1991) by specifically cleaving synaptobrevin which impairs neurotransmitter release (Schiavo *et al.*, 1992). Black widow spider toxin treatment causes a massive increase of cytosolic calcium followed by the secretion of 30% of the total cellular stores of DA and NE in PC12 cells (Grasso *et al.*, 1980). When EGTA is used to chelate the extracellular Ca^{2+}, toxin treatment has no effect on neurotransmitter release suggesting the toxin induces Ca^{2+} influx rather than release of intracellular Ca^{2+} stores. α-Latrotoxin (αLTX), the major toxin in the venom of *Latrodectus* genus spiders (black widow spiders), also causes a rapid influx of Ca^{2+} and Na^{+} ions which is followed by release of catecholamines from PC12 cells (Grasso *et al.*, 1982). EGTA treatment reduces but does not block αLTX-induced release (Meldolesi *et al.*, 1984). The maximum release of [^{3}H]DA induced by α-LTX is 60% of the total cellular [^{3}H]DA, (*i.e.* the total releasable pool of [^{3}H]DA), loaded into PC12 cells (Saito *et al.*, 1985). The mechanism of action of αLTX requires binding of the toxin to a high-affinity receptor on the plasma membrane of PC12 cells (Meldolesi *et al.*, 1984). The αLTX receptor may be one of the plasma membrane proteins known as neurexins (Ushkaryov *et al.*, 1992). Neurexins are thought to be the cell surface membrane target for synaptotagmin, and as

such, may be involved in vesicle fusion with the plasma membrane (Petrenko *et al.*, 1991). It has been suggested that αLTX induces a change in the conformation of synaptotagmin-neurexin interaction which mimics that induced by an influx of Ca^{2+} (Bennet and Scheller, 1993).

DTB may be used as a tool to study the mechanism of neurotransmitter release in PC12 cells. **It is hypothesized that DTB specifically affects evoked release of ACh rather than non-cholinergic neurotransmitter release because of specific actions of DTB on components of the cholinergic system that are not operative or not present in non-cholinergic systems.** The components determined to be only in the membranes of cholinergic vesicles in PC12 cells, are the vesicular ACh transporter and synaptophysin. Because both the cholinergic and catecholaminergic systems reside in PC12 cells, these cells can be used to study the effects of DTB on neurotransmitter release mechanisms and thus facilitate the comparisons. Experiments designed to test the hypothesis involve measuring ACh and DA release from PC12 cells, determining cellular neurotransmitter content in PC12 cells, and comparing the effects of DTB on ACh and DA synthesis, storage and release.

MATERIALS AND METHODS

I. TISSUE CULTURE

PC12 cells, a rat pheochromocytoma cell line, of passages 13-15 from our receipt, were maintained (6 X 10^6 cells/plate) on poly-L-lysine coated culture dishes (100 mm) in Dulbecco's modified Eagles' medium (DMEM) supplemented with 10% horse serum and 5% fetal bovine serum. PC12 cells were differentiated (Tischler and Greene, 1975) by addition of 50 ng/ml nerve growth factor (NGF; Bioproducts for Scientists - Harlan) one day following plating. The cells were grown in sera supplemented DMEM containing NGF for 10 days; the medium was changed every other day. Medium supplemented with 300 µM choline chloride was added to the plates 24 hr before experiments were started; all experiments were performed on day 10 of NGF treatment. The high choline chloride concentration was used to drive the synthesis of ACh (Cohen and Wurtman, 1975) in our PC12 clone. Acute choline supplementation of discrete brain regions enhances evoked ACh release from these regions by providing excess substrate to choline acetyltransferase for ACh synthesis when release is stimulated (Wecker, *et al.*, 1989 and Farber, *et al.*, 1993). Choline supplementation may influence K^+-depolarization induced ACh release from brain slices *via* a direct mechanism as well (Weiler *et al.*, 1983).

II. CELL VIABILITY MEASUREMENT

PC12 cell viability was measured to assess the toxic affects of DTB or the adverse effects of the different manipulations on PC12 cell integrity. PC12 cell viability was measured using trypan blue exclusion or lactate dehydrogenase (LDH) leakage.

Trypan blue dye is excluded by live cells whereas dead or dying cells allow the diffusion of the dye into the cell and stain blue (Tolnai, 1975). Following 24 hr incubation with medium (±DTB), the medium was removed and replaced with 5 ml of a low [K^+] buffer (described below) containing 0.01% trypan blue. The total number of cells as well as number of dead (blue) cells were counted. Viability is expressed as percent of live cells/total cells counted.

LDH is a cytosolic enzyme which reduces pyruvate to lactate, and this reduction is coupled with the oxidation of nicotinamide adenine dinucleotide, reduced form (NADH) to NAD^+ (Henry, 1968). Damaged cells allow LDH to leak out into the medium and the amount of LDH activity in the medium is proportional to the number of dead cells. LDH activity can be measured by monitoring the oxidation of NADH to NAD^+ fluorometrically. LDH converts pyruvate to lactate causing the oxidation of NADH to NAD^+ with a resultant decrease in fluorescence intensity. Changes in NADH fluorescence were monitored at the emission wavelength of 450 nm following excitation at 360 nm, pathlength 1 cm. Minimal NAD^+ fluorescence was measured by using these excitation and emission wavelengths. Baseline NADH fluorescence was monitored for 60 sec at which point a 50 µl aliquot of medium was added to a

cuvette containing 0.2 mg NADH, 2.85 ml potassium phosphate buffer (0.1 mol/l, pH 7.5), and 0.1 ml sodium pyruvate (22.7 mmol/l in phosphate buffer). To measure the cellular LDH activity, the PC12 cells were collected, homogenized, and centrifuged at 1000 X *g* to remove particulates. A 50 µl aliquot of the cell supernatant was added to a cuvette containing NADH buffer as described above. To calculate the number of LDH units, the rate of change in NADH fluorescence of the samples was compared to the fluorescence of NADH standards to determine the rate of NADH conversion to NAD^+. The change in NADH fluorescence over time is directly related to the number of units of LDH from the aliquot. For example, the rate of change in fluorescence intensity in a control sample of medium was -1836 (min^{-1}) and the standard curve for NADH had a slope of -23314 (l*cm/µmole). By multiplying (sample rate)(1/standard slope)(pathlength)(cuvette volume)(1/aliquot volume) [(-1836/min)(1 µmole/-23314 l*cm)(1 cm)(3 X 10^{-3} l)(1/0.05 ml)] the total LDH activity, in Units/ml (µmoles/ml min), in the 3 ml cuvette could be ascertained. One Unit of an enzyme is defined as that amount which will convert 1 µmole of substrate per minute.

III. DTB EXPOSURE

DTB (Ash Stevens, Detroit, MI) was dissolved in dimethyl sulfoxide (DMSO) prior to addition to aqueous solutions. The final concentration of DMSO in aqueous solutions was ≤ 0.1% (v/v), and did not alter PC12 cell morphology, viability, neurotransmitter content or release.

For chronic exposure, the cells were treated with medium containing

DTB for 24 hr. In experiments to determine the acute effects of DTB, DTB was added to serum-free release buffers as described below.

IV. NEUROTRANSMITTER RELEASE EXPERIMENTS

To assess the effects of DTB on neurotransmitter release, PC12 cells were incubated with a release buffer to collect and measure the levels of both ACh and DA. Medium (±DTB) was removed and replaced with 5 ml of warm (37°C) low [K^+] release buffer (LKB) containing (mM): 100 NaCl, 25 $NaHCO_3$ or 25 N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid] (HEPES), 5.6 d-glucose, 4.8 KCl, 1.2 $MgCl_2$, 1.3 $CaCl_2$ at a pH = 7.3. Neostigmine bromide (50 µM) or physostigmine (50 µM), inhibitors of acetylcholine esterase (AChE), was added to inhibit the breakdown of ACh to choline and acetate. The cells were incubated for 3 hr at 37°C in 10% CO_2. The LKB was then removed and replaced with 5 ml of warm (37°C) high [K^+] release buffer (HKB) to evoke release of neurotransmitter. HKB consists of (mM): 45.75 NaCl, 25 $NaHCO_3$ or 25 HEPES, 5.6 d-glucose, 56 KCl, 1.2 $MgCl_2$, 1.3 $CaCl_2$, pH = 7.3. After 5 min, the HKB was collected, filtered and prepared for analysis of neurotransmitter content.

A. Measurement of Neurotransmitters by High Performance Liquid Chromatography - ACh and Choline

ACh and choline, synthesized and released by PC12 cells, were measured by high performance liquid chromatography coupled to electrochemical detection (HPLC-EC). Release samples were flash frozen in a dry ice/acetone bath and stored at -20°C until HPLC-EC analysis. A Beckman

pu
ar
ch
en
th
us
co
en
do
sub
O_2
ele
ele
det
wer
amo
grea

The
Gils
was
sulf

pump and injector delivered the mobile phase (35 mM Na_2HPO_4, pH 8.5) to an analytical column (Bioanalytical Systems, Inc.) which separates ACh from choline. The effluent from the analytical column passes directly through an enzymatic column consisting of choline oxidase and AChE covalently bound to the column packing. A reversible AChE inhibitor, like neostigmine, can be used to prevent ACh breakdown during storage of a sample; however, the concentration of neostigmine must remain low so that the activity of the enzymatic column AChE is not inhibited as well. The column AChE breaks down ACh into choline; this choline as well as the precursor choline is subsequently converted to betaine and H_2O_2 by choline oxidase using dissolved O_2 as a cofactor (Ikuta *et al.*, 1977). The H_2O_2 is oxidized by a dual platinum electrode, causing a change in the electrical potential between the platinum electrode and a reference electrode. This potential difference is recorded by a detector (BAS model LC-2A) connected to a chart recorder (Linear). The peaks were then measured by hand and compared to standards to determine the amount of ACh and choline content in the injected sample. Quantities of ACh greater than 0.3 ng/40 µl injection can be detected by the HPLC-EC technique.

B. HPLC - DA, NE, and DOPAC

DA, NE and DOPAC were measured using reverse phase HPLC-EC. The HPLC-EC system consisted of a Gilson pump, a rheodyne injector, and a Gilson module interfaced with a Microsoft computer system. The mobile phase was made of 22% methanol (v/v) with 84 mM NaH_2PO_4, 2.6 mM sodium octyl sulfate, 0.1 mM ethylenediaminetetraacetic acid (EDTA), and 0.25 mM

triethylamine HCl, pH = 3.35. DA, NE and DOPAC were separated using an analytical column (C18-reverse phase, BAS). The effluent then passed over a glassy-carbon electrode from which DA, NE and DOPAC were oxidized, creating a change in potential between the carbon electrode and a reference electrode. This was detected by a LC-4B detector (BAS) and analyzed by a computerized peak integrator. The change in potential was related to the amount of oxidized DA, NE, or DOPAC which passed over the electrode. A single neurotransmitter release sample was analyzed for both catecholamines and ACh. Comparisons of the effect of DTB on both catecholaminergic and cholinergic transmitter release were made from the same PC12 cells.

C. RADIOLABELING NEUROTRANSMITTERS

[^{3}H]ACh can be used to study the effects of DTB on ACh release from PC12 cells, especially if DTB alters a particular store (newly-synthesized versus old) of ACh. Cells were washed once with LKB (37°C) containing 50μM physostigmine prior to incubating the cells with LKB containing 0.4 μCi/ml (5.2 nM) [^{3}H]choline chloride with a specific activity of 79.2 Ci/mmol (NEN Radiochemicals - Dupont). Cells were incubated for 3 hours at 37°C with 10% CO_2 to allow for the uptake of [^{3}H]choline and synthesis of [^{3}H]ACh. The [^{3}H]ACh represents newly-synthesized (synthesized within 3 hr) ACh. The specific activity of [^{3}H]ACh synthesized in PC12 cells was calculated as the mole fraction of labelled ACh over the total ACh content in PC12 cells {[^{3}H]ACh/([^{3}H]ACh + [^{1}H]ACh)} (Melega and Howard, 1981). The specific activity of the newly-synthesized [^{3}H]ACh in the PC12 cells was 0.09 (mole

fraction). The total pool of ACh was labelled with [^{3}H]ACh by incubating the cells in medium containing 0.4μCi/ml (5.2 nM) [^{3}H]choline chloride for 24 hr prior to treating the cells with various concentrations of DTB and collecting neurotransmitter samples as described below. The specific activity of the [^{3}H]ACh which represents the total pool of ACh in PC12 cells was 9 X 10^{-4} (mole fraction).

After the incubation with [^{3}H]choline, the PC12 cells were washed once with LKB to remove excess [^{3}H]choline chloride. The cells were then exposed to 5 ml of HKB containing 50μM physostigmine for 5 min. The release sample was collected and [^{3}H]choline was separated from [^{3}H]ACh by the method of Goldberg and McCaman (1973). Choline was converted to phosphocholine by choline kinase and ACh was separated from phosphocholine by an aqueous-organic extraction. One ml of phosphorylation buffer containing 0.02U choline kinase, 20 mM $MgCl_2$, 20 mM ATP, and 20mM Na_2HPO_4 (pH = 7.9) was added to one ml of release sample and incubated for 15 min at 37°C. One ml of tetraphenylboron (10 mg/ml) in 3-heptanone was then added to extract ACh into the organic phase while the phosphorylated choline remained in the aqueous phase. To determine the radioactivity attributed to non-phosphorylated [^{3}H]choline in the organic phase, 200 U AChE was added to a high [K^+] release sample and considered void of [^{3}H]ACh (Goldberg and McCaman, 1973). Thus, any radioactivity in this sample containing AChE could be attributed to non-phosphorylated [^{3}H]choline. Toluene-based scintillation fluid (10ml) was carefully added to the vial without disturbing the

aqueous layer and the amount of [^{3}H]ACh in the organic phase was determined using a Searle 6680 Mark III liquid scintillation counter.

V. CELLULAR NEUROTRANSMITTER EXPERIMENTS

DTB-induced disruption of ACh release could result from alterations in cellular regulation of ACh content or release. Alterations in the amount of released [^{3}H]ACh could be the result of altered ACh metabolism, disruption in neurotransmitter packaging, or inhibition of vesicular transport. Indeed, DTB alters the vesicular pool of ACh at the motor nerve terminal and reduces the tissue concentration of newly synthesized $^2H_4$-ACh (Weiler *et al.*, 1986).

A. [^{3}H]Choline Chloride Uptake

To determine if DTB inhibited choline uptake, the uptake of [^{3}H]choline by PC12 cells was measured. NGF-treated PC12 cells plated on 150 cm^3 flasks were washed once with LKB and then dislodged mechanically off the bottom of the flask. The cells were collected and centrifuged at 1000 X *g* for 5 min and the pellet was resuspended in 5 ml LKB (37°C) containing 0.4 µCi/ml (5.2 nM) [^{3}H]choline chloride, 10 µM choline chloride, and 0-1000 µM DTB. For some experiments, a parallel set of cells was collected and resuspended in LKB containing 0.4 µCi/ml [^{3}H]choline chloride, 10 µM choline chloride, 0-1000 µM DTB, and 500 µM HC-3. The cells were then incubated for various time points (0-30min) in a 37°C shaking water bath. After the incubation, the cells were kept on ice until centrifuged at 1000 X *g* for 5 min, the radioactive buffer was removed, and the cells were resuspended in LKB. To wash away excess [^{3}H]choline chloride, the cells were centrifuged and resuspended in fresh buffer

three times. After the last centrifugation, the cells were resuspended in only 1 ml of LKB and placed into scintillation vials. Aqueous-based scintillation fluid (10 ml), which dissolved the cells, was added to the vials and the amount of [^{3}H]choline chloride in the cells was measured using a Searle 6680 Mark III liquid scintillation counter.

B. PC12 Cell Fractionation

To determine if the vesicular pool of ACh from PC12 cells was affected by DTB treatment, the cells were collected and the vesicular fraction analyzed for neurotransmitter content. The medium was removed from the dishes and the plates washed once with LKB. A sucrose buffer (SB) consisting of 0.32 M sucrose, 10 mM $NaHCO_3$ and 50 µM neostigmine at a pH = 7.3, was utilized to separate cytosolic (soluble) neurotransmitter from vesicular (bound) neurotransmitter. Cold SB (5 ml) was placed on the cells, which were then scraped off the plate and collected into centrifuge tubes and kept on ice. Soluble and bound neurotransmitter were isolated by the method of Melega and Howard (1984). The cells were homogenized, then centrifuged at 4°C at 1000 X *g* for 10 min. The pellet (P_1) consisting of plasma membrane, nuclei, and contaminating whole cells, was kept for protein determination (Lowry *et al.*, 1951). The supernatant (S_1) was removed and centrifuged at 20,000 X *g* for 30 min. The supernatant (S_2) from the 20,000 X *g* spin contains cytosolic neurotransmitter. For analysis of neurotransmitter content by HPLC-EC, the cytosolic fraction was removed, acidified using 0.2 M $HClO_4$ and rapidly frozen. The pellet (P_2), containing the vesicular fraction, was resuspended in 0.5 ml

0.2M $HClO_4$ and frozen at -20°C until HPLC-EC analysis. Vesicular samples were thawed, sonicated and filtered just prior to HPLC-EC analysis. For analysis of cellular [^{3}H]choline and [^{3}H]ACh, the soluble and vesicular fractions were assayed for [^{3}H]choline and [^{3}H]ACh by the method of Goldberg and McCaman (1973) modified as described above.

C. Neurotransmitter Metabolism

To determine if catecholamine metabolism was altered by DTB treatment, DOPAC concentrations were measured in the PC12 cells as well as in release samples. DOPAC is a major metabolite of DA, and as such, changes in DOPAC concentration reflect changes in DA metabolism or synthesis (Roth *et al.*, 1976). For example, decreased DA levels and increased DOPAC levels would suggest an increase in DA metabolism. Conversely, if both DA and DOPAC levels were decreased this would suggest a decrease in DA synthesis. ACh metabolism was not determined.

VI. STATISTICS

Data (n = 3-7) were analyzed by using analysis of variance and when necessary, Dunnett's post-hoc test (Dunnett, 1955). Values were considered significant if $p < 0.05$.

RESULTS

I. CHARACTERIZATION OF PC12 CELL NEUROTRANSMITTER RELEASE

PC12 cells release both DA and ACh in a manner that is dependent on extracellular Ca^{2+} concentration and on membrane potential (Greene and Rein, 1977a). To determine if the PC12 cell clone utilized in the present experiments released both ACh and DA by classical mechanisms, cells were incubated in LKB or HKB for 5 min to elicit ACh and DA release. ACh and DA release were significantly higher in HKB than in LKB (Fig. 1). K^+-evoked release was Ca^{2+} dependent since removal of extracellular Ca^{2+} reduced high [K^+]-evoked release of ACh and DA from PC12 cells (Fig. 2).

K^+-evoked release of ACh from this clone of PC12 cells was greatly enhanced if the medium was supplemented with 300 µM choline chloride. Apparently, the excess choline provided sufficient precursor to stimulate synthesis and enhance K^+-evoked release of ACh to the levels detectable by HPLC-EC. Choline supplementation also increased K^+-evoked DA release. PC12 cells cultured in medium supplemented with 300 µM choline chloride released 700 ± 102 ng DA/mg protein in response to 56 mM K^+. In contrast, cells incubated with medium containing the standard concentration of 30 µM choline chloride released only 162 ± 26 ng DA/mg protein. Choline

Figure 1. High [K^+]-evoked release of neurotransmitters from PC12 cells as a function of time. PC12 cells were incubated with 5 ml warm (37°C) HKB (solid line) or LKB (dashed line) for various times. The buffer was then collected and assayed for (A) [^{3}H]ACh or (B) DA. Asterisks indicate values that are significantly different from LKB ($p < 0.05$). Values are the mean ± SEM of 4 experiments.

Figure 1

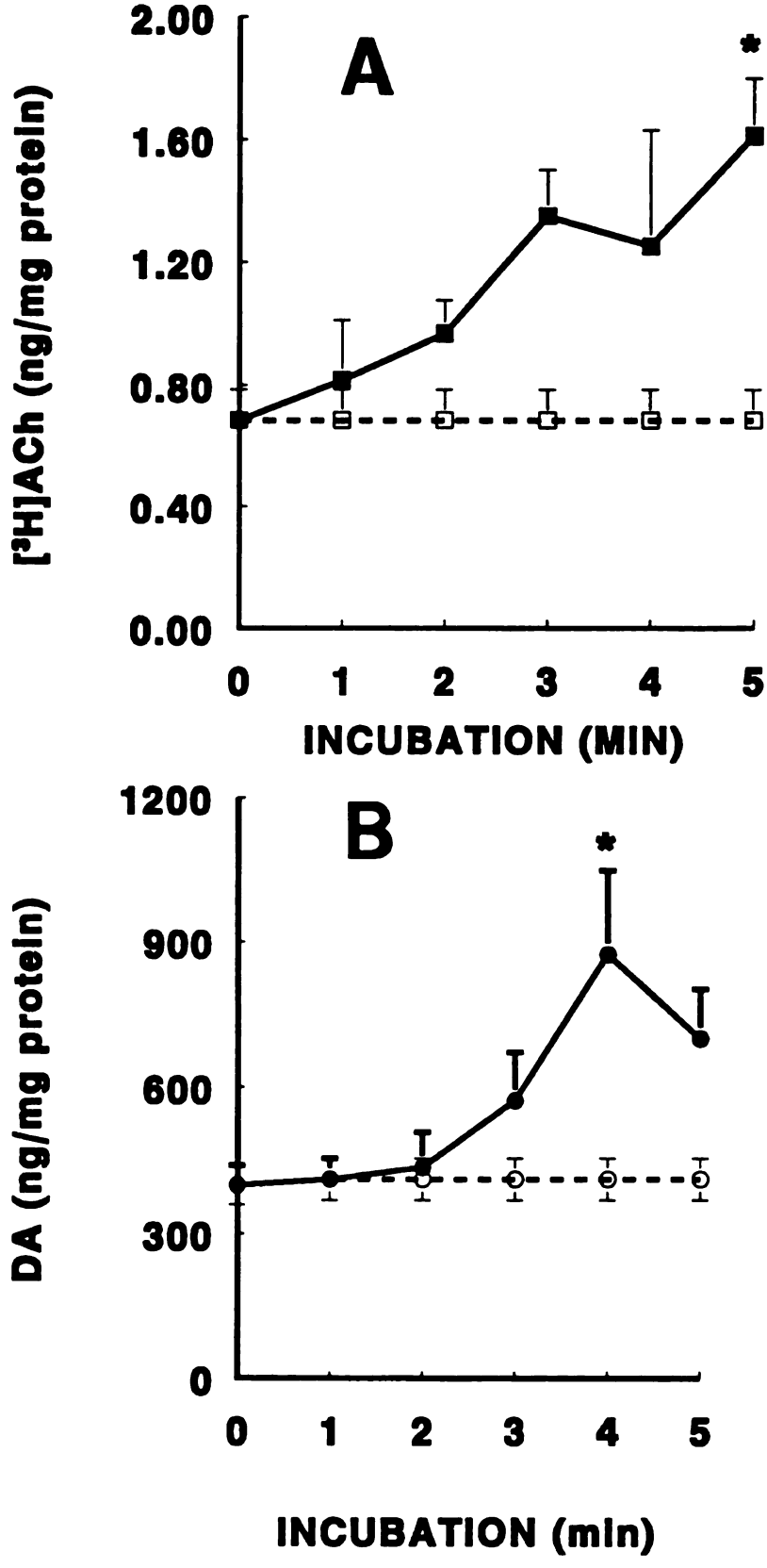
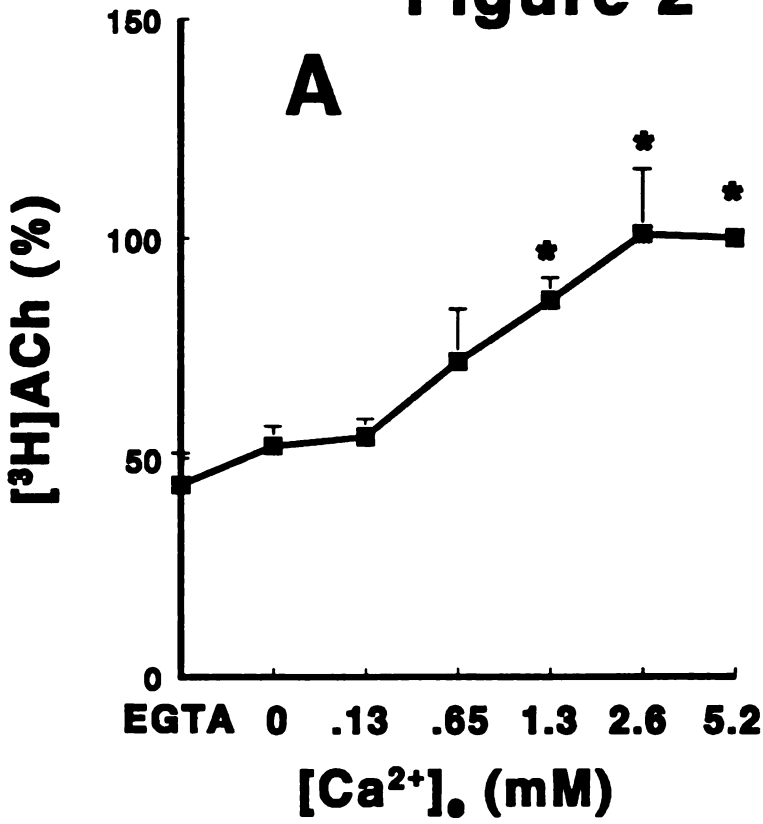
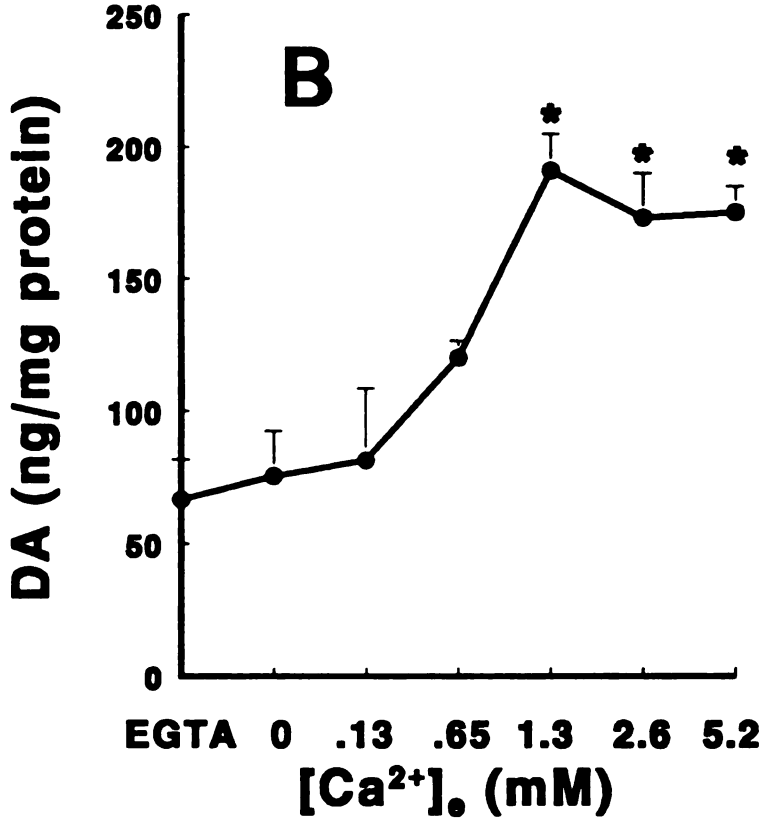

A
B
2.00
1.60
1.20
0.80
0.40
0.00
[³H]ACh (ng/mg protein)
0
1
2
3
4
5
INCUBATION (MIN)
1200
900
600
300
0
DA (ng/mg protein)
INCUBATION (min)
*

Figure 2. High [K^+]-evoked release of neurotransmitters from PC12 cells as a function of extracellular Ca^{2+} concentration. PC12 cells were incubated for 5 min with HKB containing various Ca^{2+} concentrations. The buffer was collected and assayed for (A) [^{3}H]ACh or (B) DA. The [^{3}H]ACh is expressed in % of evoked [^{3}H]ACh released at 5.2 mM $[Ca^{2+}]_e$, because the basal levels of [^{3}H]ACh were different between each n. Asterisks indicate values that are significantly different from the EGTA treated ($[Ca^{2+}]_e$ = 100 nM) group ($p < 0.05$). The error bar was not reported in (A) because the standard error of the mean was too small to be distinguished. Values are the mean ± SEM of 4 experiments.

Figure 2


A
150
100
50
0
[³H]ACh (%)
EGTA 0 .13 .65 1.3 2.6 5.2
[Ca²⁺]ₑ (mM)


B
250
200
150
100
50
0
DA (ng/mg protein)
EGTA 0 .13 .65 1.3 2.6 5.2
[Ca²⁺]ₑ (mM)

supp

relea

II. I

evalu

diffe

num

PC12

enzy

incre

cellu

24 h

III. T

DTB

300

high

50%

dete

new

com

PC1

supplementation did not alter the effects of DTB on K^+-evoked DA or ACh release from PC12 cells.

II. PC12 CELL VIABILITY

DTB cytotoxicity (0-1000 μM) on differentiated, confluent PC12 cells was evaluated by the trypan blue exclusion assay. There was no significant difference in the percent of live cells/total cells counted (Fig. 3) or the cell number between control and DTB-treated groups following a 24 hr exposure. PC12 cell viability was also assessed by measuring the level of the cytosolic enzyme LDH in the medium. DTB treatment (0-1000 μM) for 24 hr did not increase LDH activity in the medium (Fig. 4A). Moreover, DTB did not alter cellular LDH levels (Fig. 4B). Thus, concentrations of DTB up to 1000 μM for 24 hr did not appear to affect PC12 cell viability or cell number.

III. THE EFFECT OF DTB ON SYNTHESIS AND RELEASE OF ACh FROM PC12 CELLS

The effect of DTB on endogenous (native) ACh release was examined in DTB-treated PC12 cells. PC12 cells were incubated with medium containing 300 μM choline and various concentrations of DTB for 24 hr. DTB inhibited high [K^+]-evoked release of endogenous ACh from PC12 cells by approximately 50% (Fig. 5). By using radiolabelling techniques, it should be possible to determine whether DTB inhibits specifically the high [K^+]-evoked release of newly-synthesized [^{3}H]ACh over older stores of ACh. However, the comparisons of the effects of DTB on newly-synthesized ACh released from PC12 cells and motor nerve terminals at neuromuscular junctions may be

Figure 3. Trypan blue exclusion of PC12 cells after treatment with DTB for 24 hr. PC12 cells were washed with LKB, and incubated with LKB containing 0.01% trypan blue (v/v). Cells stained blue were considered dead, clear cells were counted as live. Viability was expressed as (number of live cells)/(total cells counted) X 100. Values are the mean ± SEM of 7 experiments.

Figure 3

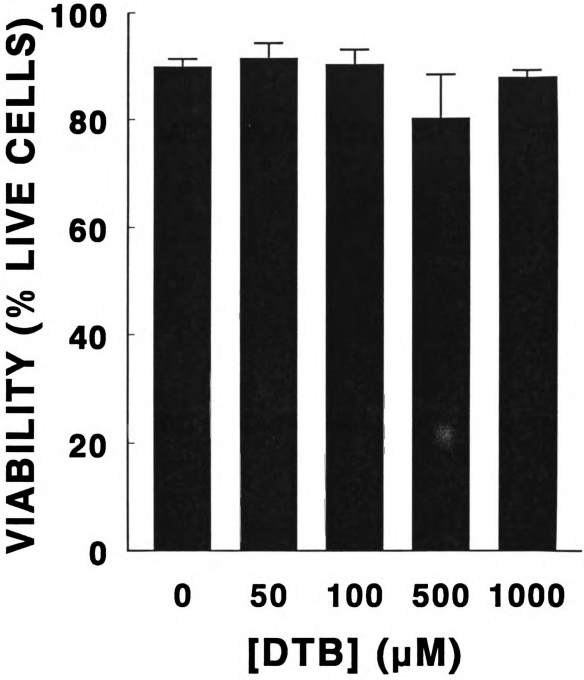
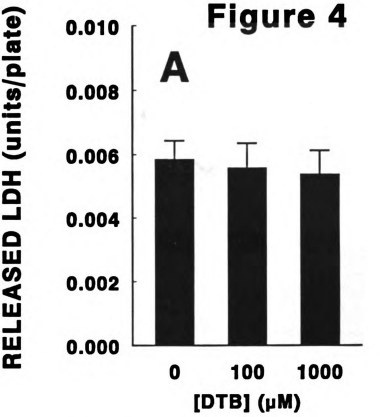
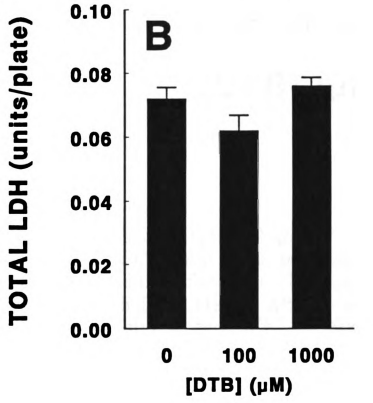


Figure 4. LDH distribution in PC12 cells after treatment with (0-1000 μM) DTB for 24 hr. (A) Medium (50 μL) was removed from the cells and added to a vial containing NADH, sodium pyruvate, and potassium phosphate buffer. LDH activity in the medium was monitored as decrease in NADH fluorescence. LDH activity released into the medium is expressed as Units/ml of medium on the plate. (B) Cells were collected, homogenized, and centrifuged to remove particulates. Cellular LDH content was then determined by measuring the LDH activity in 50 μL of the cell supernatant. Total LDH activity is equal to the amount of LDH activity in the cell homogenate plus that released into the medium. Total LDH activity is expressed as Units/plate of PC12 cells. Values are the mean ± SEM of 4 experiments.

Figure 4


A
RELEASED LDH (units/plate)
0.010
0.008
0.006
0.004
0.002
0.000
0
100
1000
[DTB] (μM)


B
TOTAL LDH (units/plate)
0.10
0.08
0.06
0.04
0.02
0.00
0
100
1000
[DTB] (μM)

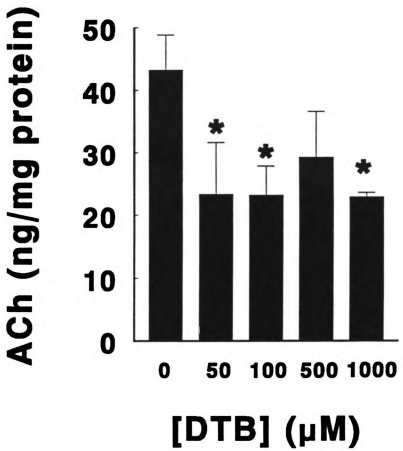


Figure 5. The effect of DTB on high [K^+]-evoked ACh release from PC12 cells. PC12 cells were incubated with DTB (0-1000 μM) containing medium for 24 hr. The medium was removed, and replaced with 5 ml HKB. After 5 min, the buffer was collected and analyzed for ACh content using HPLC-EC. Asterisks indicate values that are significantly different from control ($p < 0.05$). Values are the mean ± SEM of 4 experiments.

complicated by the fact that PC12 cells do not preferentially release newly-synthesized ACh over older stores of ACh (Melega and Howard, 1981). Nevertheless, after a 24 hr exposure to 500 and 1000 μM DTB, release of [^{3}H]ACh was reduced to approximately 33% and 10% of controls (Fig. 6). PC12 cells labelled with [^{3}H]choline chloride for 24 hr followed by treatment with (0-1000 μM) DTB had reduced K^+-evoked release at all DTB concentrations; however, spontaneous [^{3}H]ACh release was not significantly affected (Fig. 7).

The decrease in K^+-evoked ACh could be due to changes in ACh content within the cells. Alterations in cellular ACh content could result from changes in precursor availability. For example, the uptake of choline could be impaired. To test for this possibility, the uptake of [^{3}H]choline was assessed in the presence or absence of DTB. Total [^{3}H]choline uptake was unaltered after acute DTB treatment (Fig. 8). The effects of DTB on HC-3 sensitive, high affinity choline uptake was tested in PC12 cells because choline uptake at motor nerve terminals occurs predominately *via* the HC-3 sensitive high affinity transporter (Collier and MacIntosh, 1969) and is therefore relevant when comparing the effects of DTB on motor nerve terminals and PC12 cells. Acute DTB (10 μM) treatment increased HC-3 sensitive [^{3}H]choline uptake by 10 min (Fig. 9). However, the [^{3}H]choline content in PC12 cells after 3 hr (Fig. 10A) or 24 hr (Fig. 10B) incubation with [^{3}H]choline chloride was unaffected by DTB treatment.

The availability of choline for ACh synthesis may be altered by DTB treatment if the cellular distribution of choline was affected by DTB. Since the

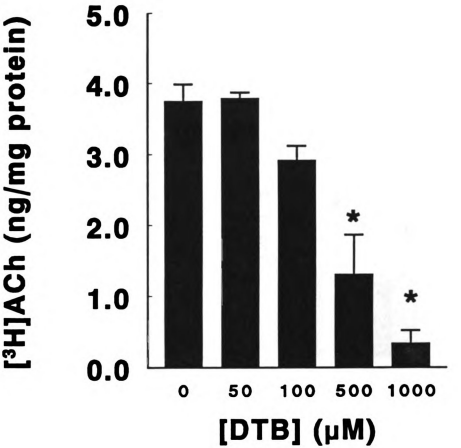


Figure 6. The effect of DTB on high [K^+]-evoked release of newly-synthesized [^{3}H]ACh. PC12 cells were treated with 300 μM choline and various concentrations of DTB for 24 hr. The cells were then incubated with LKB containing 0.4 μCi/ml [^{3}H]choline chloride for 3 hr. The cells were then incubated with 5 ml HKB. After 5 min, the buffer was collected and analyzed for [^{3}H]ACh content. Asterisks indicate values that are significantly different from control ($p < 0.05$). Values are the mean ± SEM of 4 experiments.

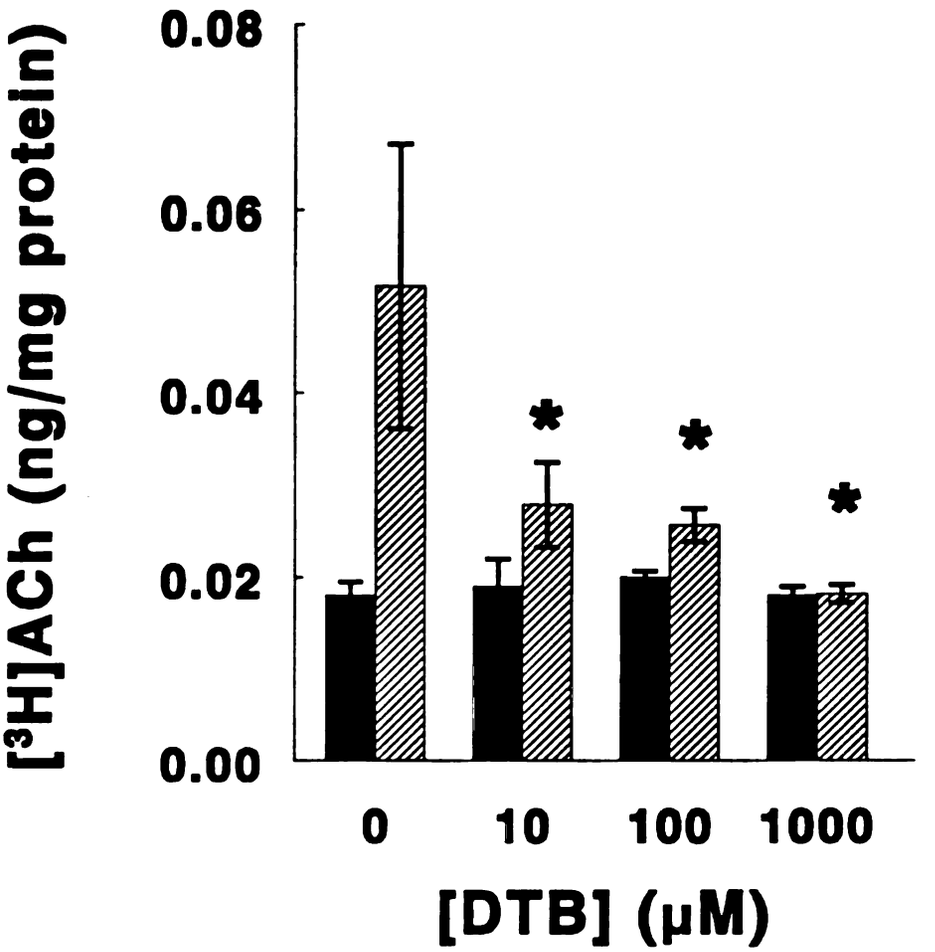


Figure 7. The effect of DTB on high [K^+]-evoked and spontaneous [^{3}H]ACh release from PC12 cells. Cells were incubated with medium containing 300 μM choline chloride for 24 hr. The medium was removed and replaced with fresh medium containing 0.4 μCi/ml [^{3}H]choline chloride. After 24 hr, DTB (0-1000 μM) medium was added for an additional 24 hr incubation. To measure [^{3}H]ACh release, the cells were incubated with HKB (hatched bars) or LKB (solid bars). After 5 min, the buffer was then collected and analyzed for [^{3}H]ACh content. Asterisks indicate values that are significantly different from control ($p < 0.05$). Values are the mean ± SEM of 3 experiments.

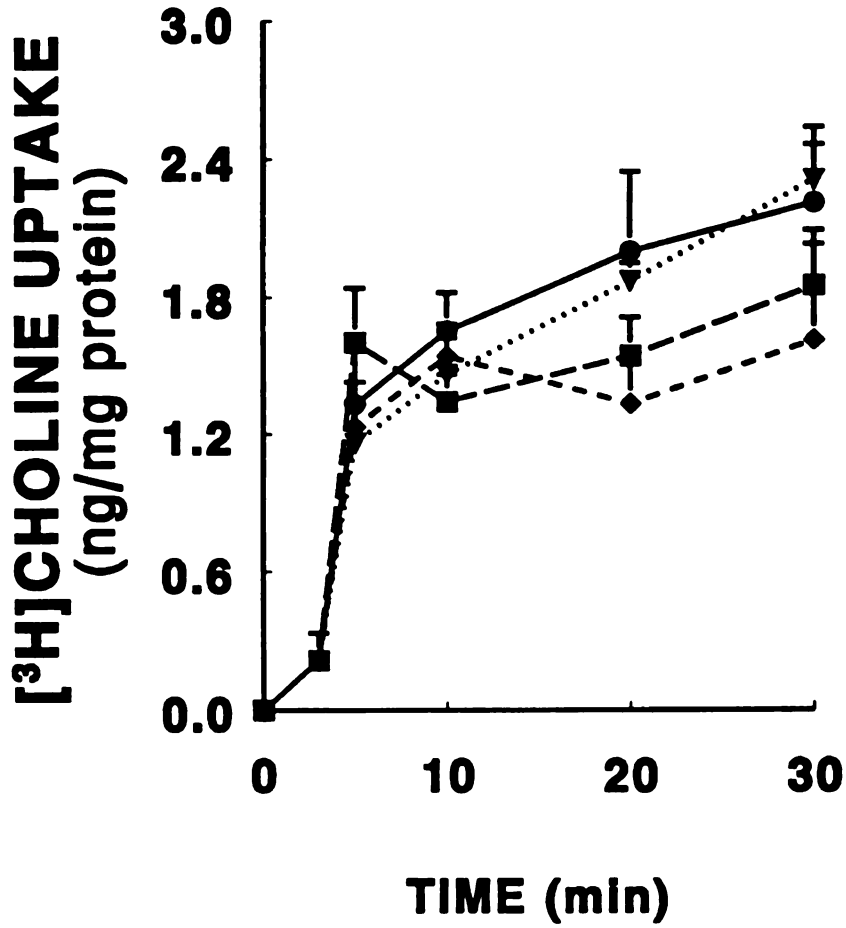


Figure 8. The effect of DTB on total [^{3}H]choline uptake into PC12 cells. PC12 cells were collected in 5 ml LKB containing [^{3}H]choline chloride (0.4 µCi/ml) and various concentrations of DTB (µM). The cell suspension was incubated at 37°C for various times and then placed on ice until the cells were collected by centrifugation, washed twice to remove excess [^{3}H]choline, and analyzed for cellular [^{3}H]choline content. Values are the mean ± SEM of 7 experiments.

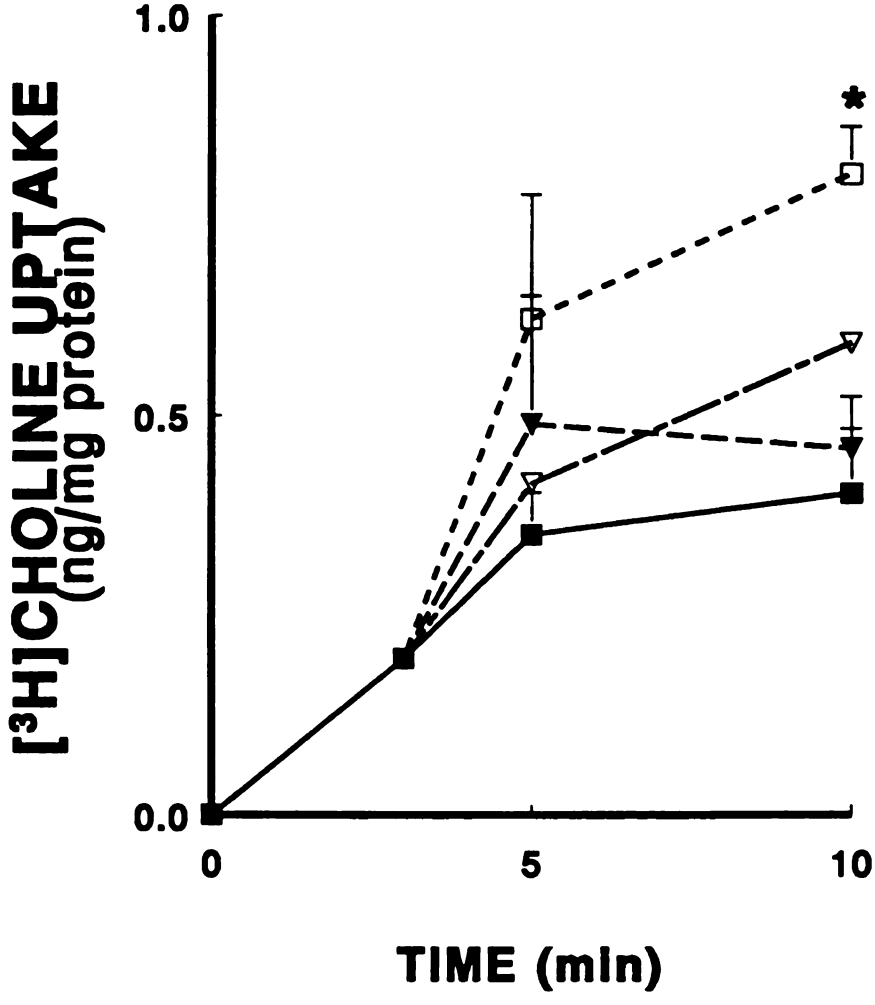
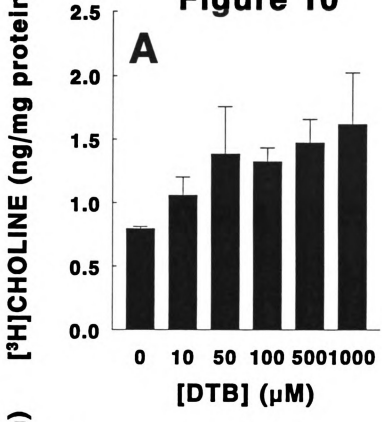
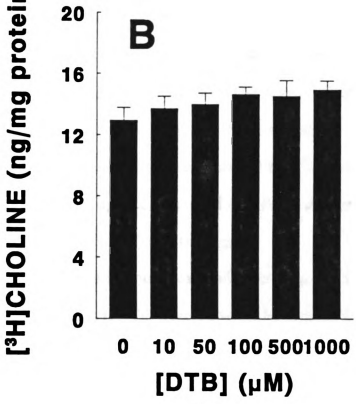


Figure 9. The effect of DTB on HC-3 sensitive [^{3}H]choline uptake into PC12 cells. PC12 cells were collected in 5 ml LKB containing [^{3}H]choline chloride (0.4 µCi/ml) with 500 µM HC-3 and various concentrations of DTB (
µM). The cell suspension was incubated at 37°C for various times up to 10 min. The cells were then collected by centrifugation, washed twice to remove excess [^{3}H]choline, and analyzed for cellular [^{3}H]choline content. The asterisk indicates a value that is significantly different from control ($p < 0.05$). Values are the mean ± SEM of 4 experiments.

Figure 10. The effect of DTB on [^{3}H]choline content in PC12 cells. PC12 cells were incubated with DTB (0-1000 μM) for 24 hr. (A) The medium was removed and replaced with [^{3}H]choline chloride (0.4 μCi/ml) in LKB for 3 hr. (B) PC12 cells were incubated with DTB and [^{3}H]choline chloride (0.4 μCi/ml) in medium for 24 hr. Cells were collected and analyzed for [^{3}H]choline content. Values are the mean ± SEM of 7 experiments.

Figure 10


A
[³H]CHOLINE (ng/mg protein)
2.5
2.0
1.5
1.0
0.5
0.0
0 10 50 100 5001000
[DTB] (μM)


B
[³H]CHOLINE (ng/mg protein)
20
16
12
8
4
0
0 10 50 100 5001000
[DTB] (μM)

majority of choline in PC12 cells is incorporated into the plasma membrane as phosphocholine (Melega and Howard, 1981), changes in choline distribution would be reflected by alterations in the amount of choline incorporation into the plasma membrane. DTB treatment did not alter the amount of [^{3}H]choline incorporation into the cell membranes of PC12 cells (Fig. 11). These results suggest precursor availability and distribution were unaltered by DTB.

To determine if the inhibition of K^+-evoked ACh release was due to a decrease in ACh synthesis, cellular levels of ACh were measured using HPLC-EC and radiolabelling techniques. DTB (100 or 1000 µM) did not alter endogenous ACh or [^{3}H]ACh levels in PC12 cells compared to controls (Fig. 12). DTB also did not alter the distribution of ACh within PC12 cells since the ratio of vesicular to cytosolic ACh was not altered by DTB treatment. DTB did not alter newly-synthesized [^{3}H]ACh levels in either the cytosolic (Fig. 13A) or the vesicular fractions (Fig. 13B). After a 24 hr exposure to DTB (10-1000 µM) and 0.4 µCi/mL [^{3}H]choline chloride, the levels of cytosolic (Fig. 14A) and vesicular [^{3}H]ACh fractions (Fig. 14B) were similar to control. Thus, the reduction in K^+-evoked ACh release caused by DTB could not be attributed to a decrease in choline uptake, impaired ACh synthesis or disruption of vesicular storage of ACh. These results suggest that DTB may interfere with mechanisms involved in the release of ACh from PC12 cells.

To determine if DTB affected vesicular trafficking to or fusion with the plasma membrane, the neurotoxin αLTX was utilized to study vesicular ACh release at a step beyond Ca^{2+} triggering. PC12 cells were treated for 24 hr

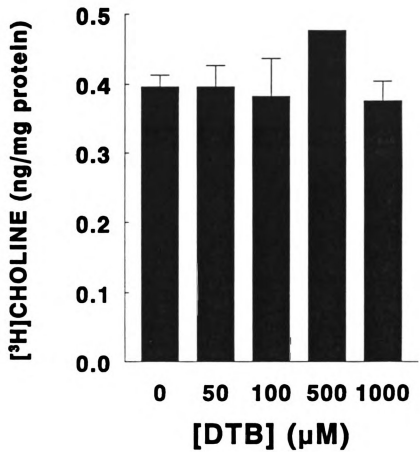
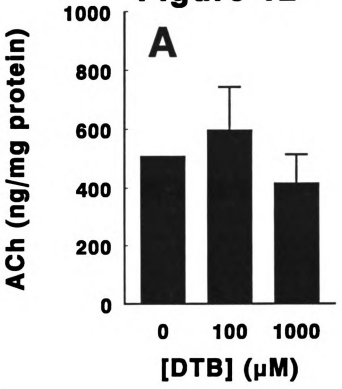


Figure 11. The effect of DTB on [^{3}H]choline incorporation into PC12 cell membranes. PC12 cells were incubated with medium containing DTB (0-1000 μM) and 0.4 μCi/ml [^{3}H]choline chloride for 24 hr. Cells were then collected in LKB, homogenized, and centrifuged at 1000 X g for 10 min. The membrane fraction was collected and analyzed for [^{3}H]choline content. Error bars were not reported if the standard error of the mean was too small to be distinguished. Values are the mean ± SEM of 7 experiments.

Figure 12. The effect of DTB on endogenous ACh and [^{3}H]ACh levels in PC12 cells. PC12 cells were treated with DTB (0, 100, 1000 μM) for 24 hr. (A) The cells were collected in 5 ml SB, homogenized, and centrifuged at 1000 X g for 10 min. The supernatant (S_1) was analyzed for ACh content by HPLC-EC. (B) Following DTB treatment, the cells were incubated with LKB containing [^{3}H]choline chloride (0.4 μCi/ml) for 3 hr. The cells were collected as described in the material and methods section, then analyzed for [^{3}H]ACh content. Error bars were not reported if the standard error of the means were too small to be distinguished. Values are the mean ± SEM of 4 experiments.

Figure 12


A
ACh (ng/mg protein)
1000
800
600
400
200
0
0
100
1000
[DTB] (μM)

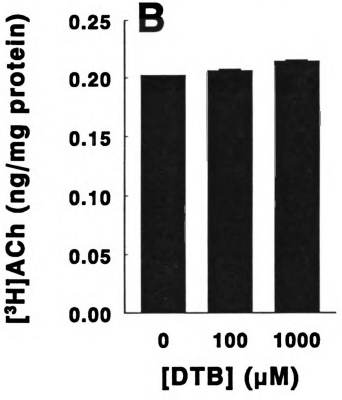

B
[3H]ACh (ng/mg protein)
0.25
0.20
0.15
0.10
0.05
0.00
0
100
1000
[DTB] (μM)

Figure 13. The effect of DTB on newly-synthesized [^{3}H]ACh levels in PC12 cells. PC12 cells were treated with DTB (0-1000 μM) for 24 hr and then incubated with [^{3}H]choline chloride (0.4 μCi/ml) for 3 hr. The cells were collected as described in Fig. 11. The supernatant (S_1) was centrifuged at 20,000 X g for 30 min. The pellet was collected for the vesicular fraction and the S_2 was collected for the soluble fraction. The fractions were then analyzed for [^{3}H]ACh content in the (A) cytosolic fraction and the (B) vesicular fraction of the cells. If error bars are not depicted the standard error of the means was too small to be distinguished. Values are the mean ± SEM of 6 experiments.

Figure 13

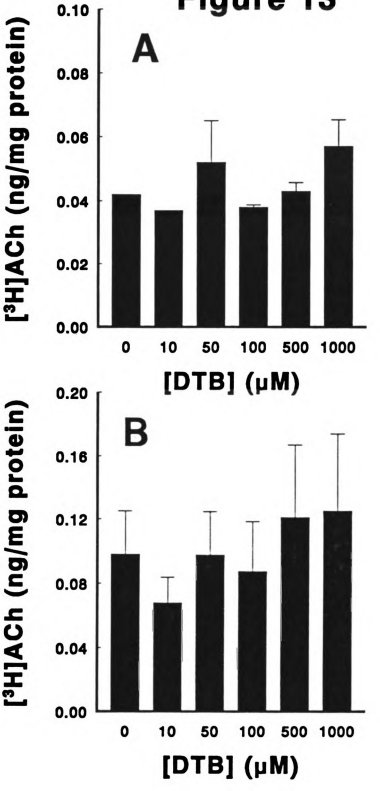
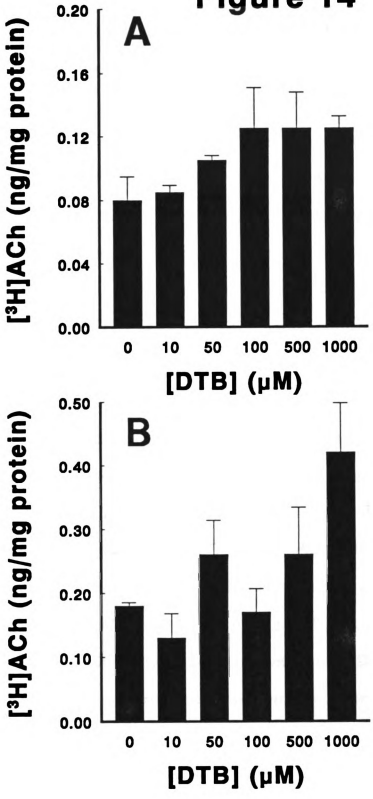

A
[3H]ACh (ng/mg protein)
0.10
0.08
0.06
0.04
0.02
0.00
0
10
50
100
500
1000
[DTB] (μM)
B
[3H]ACh (ng/mg protein)
0.20
0.16
0.12
0.08
0.04
0.00
0
10
50
100
500
1000
[DTB] (μM)

Figure 14. The effect of DTB on cellular [^{3}H]ACh levels in PC12 cells. PC12 cells were incubated with medium containing DTB (0-1000 µM) and [^{3}H]choline chloride (0.4 µCi/ml) for 24 hr. The cells were collected and fractionated, then analyzed for [^{3}H]ACh content in the (A) cytosolic fraction and the (B) vesicular fraction of the cells. In (B), the analysis of variance value for p was 0.04, however post-hoc analysis (Tukey's test) determined that this difference was between the means ± SEM of the 10 µM and 1000 µM treated groups. Values are the mean ± SEM of 4 experiments.

Figure 14


A
[3H]ACh (ng/mg protein)
0.20
0.16
0.12
0.08
0.04
0.00
0
10
50
100
500
1000
[DTB] (μM)
B
[3H]ACh (ng/mg protein)
0.50
0.40
0.30
0.20
0.10
0.00
0
10
50
100
500
1000
[DTB] (μM)

with DTB, incubated with LKB containing 0.4 μCi/mL [^{3}H]choline for 3 hr, then incubated with a 5 min incubation with LKB containing αLTX. This protocol is the same as that used to determine the effects of DTB on K^+-evoked newly-synthesized [^{3}H]ACh release from PC12 cells (Fig. 6). αLTX stimulated [^{3}H]ACh release from PC12 cells. Prior treatment with DTB (50 and 100 μM) enhanced αLTX-stimulated release (Fig. 15). At these same DTB concentrations, K^+-evoked release of newly-synthesized [^{3}H]ACh was unaltered (Fig. 6). Conversely, K^+-evoked release of newly-synthesized [^{3}H]ACh was reduced at 1000 μM DTB (Fig. 6) while αLTX-stimulated release of [^{3}H]ACh from 1000 μM DTB-treated PC12 cells was similar to the control αLTX group.

IV. THE EFFECT OF DTB ON SYNTHESIS AND RELEASE OF DA FROM PC12 CELLS

The effects of DTB on ACh release were compared with the effects of DTB on K^+-evoked release of DA from PC12 cells. Treatment of PC12 cells with 1000 μM DTB for 24 hr inhibited K^+-evoked release of DA (Fig. 16A) while spontaneous release of DA was unaffected (Fig. 16B).

In contrast to cellular ACh content, which was unaffected by DTB, the cytosolic (Fig. 17A) and vesicular (Fig. 17B) DA content of PC12 cells was decreased significantly by 24 hr treatment with DTB. Cytosolic DA content was reduced to a greater extent than the vesicular DA content by DTB treatment (Fig 17).

The decrease in cellular DA levels may be due to a decrease in catecholamine synthesis and/or an increase in DA metabolism. DA metabolism

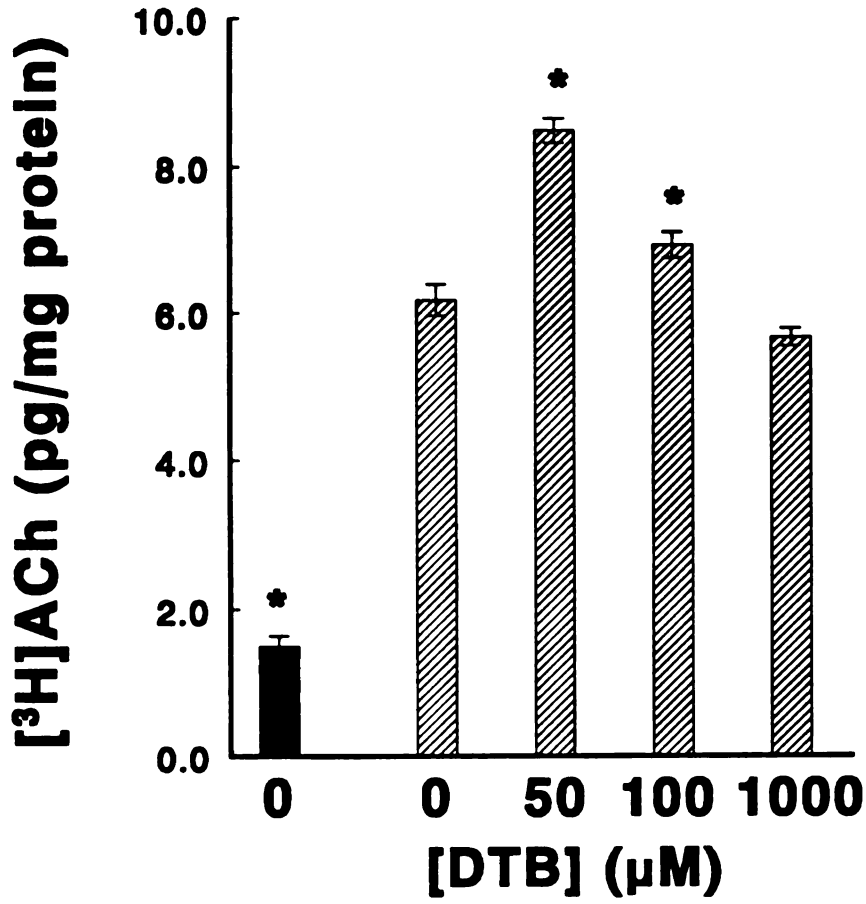
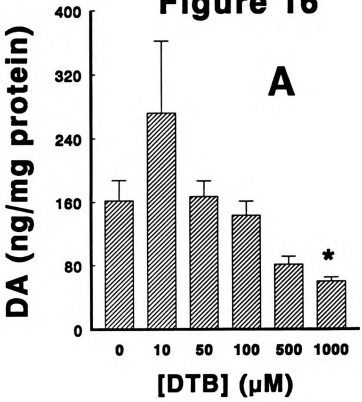


Figure 15. The effect αLTX on [^{3}H]ACh release from PC12 cells treated with DTB (0-1000 μM) for 24 hr. Cells were incubated with LKB containing [^{3}H]choline chloride for 3 hr. Fresh LKB containing 1 nM αLTX was placed on the cells and incubated for 5 min. The buffer was collected and analyzed for [^{3}H]ACh content. αLTX-stimulated [^{3}H]ACh release (hatched bars) was significantly greater than spontaneous [^{3}H]ACh release (solid bar). The asterisks indicate a value that is significantly different from αLTX group ($p < 0.05$). Values are the mean ± SEM of 4 experiments.

Figure 16. The effect of DTB on high [K^+]-evoked and spontaneous DA release from PC12 cells. PC12 cells were treated with DTB (0-1000 μM) for 24 hr after which the medium was replaced with 5 ml (A) HKB or (B) LKB and incubated for 5 min. The buffer was collected and analyzed for DA by HPLC-EC as described in the methods. The asterisk indicates a value that was significantly different from control ($p < 0.05$). Values are the mean ± SEM of 6 experiments.

Figure 16


A
DA (ng/mg protein)
400
320
240
160
80
0
*
0
10
50
100
500
1000
[DTB] (μM)

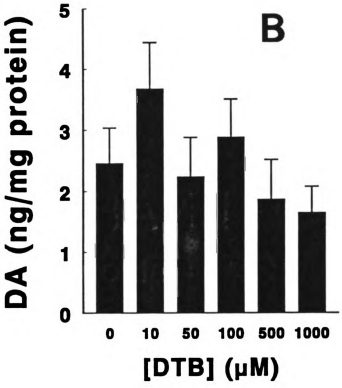
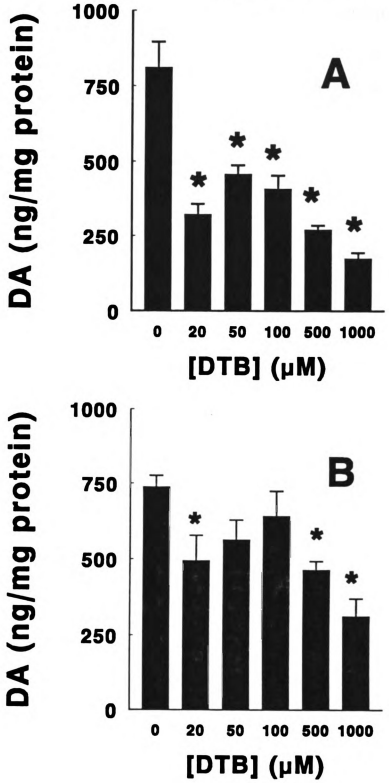

B
DA (ng/mg protein)
5
4
3
2
1
0
0
10
50
100
500
1000
[DTB] (μM)

Figure 17. The effect of DTB on cellular DA content in PC12 cells. Cells were treated with DTB (0-1000 μM) for 24 hr. The cells were collected and fractionated as described in the methods, then analyzed for DA content in the (A) cytosolic fraction and the (B) vesicular fraction. Asterisks indicate values that are significantly different from control ($p < 0.05$). Values are the mean ± SEM of 6 experiments.

Figure 17


DA (ng/mg protein)
1000
750
500
250
0
A
*
*
*
*
*
0 20 50 100 500 1000
[DTB] (μM)
DA (ng/mg protein)
1000
750
500
250
0
B
*
*
*
0 20 50 100 500 1000
[DTB] (μM)

can be measured by monitoring cellular levels of the metabolite DOPAC using HPLC-EC. After 24 hr treatment with DTB, cellular DOPAC levels were either less than or equal to control levels (Fig. 18) which is inconsistent with increased DA metabolism. Thus, it appears that DTB inhibits DA synthesis in PC12 cells.

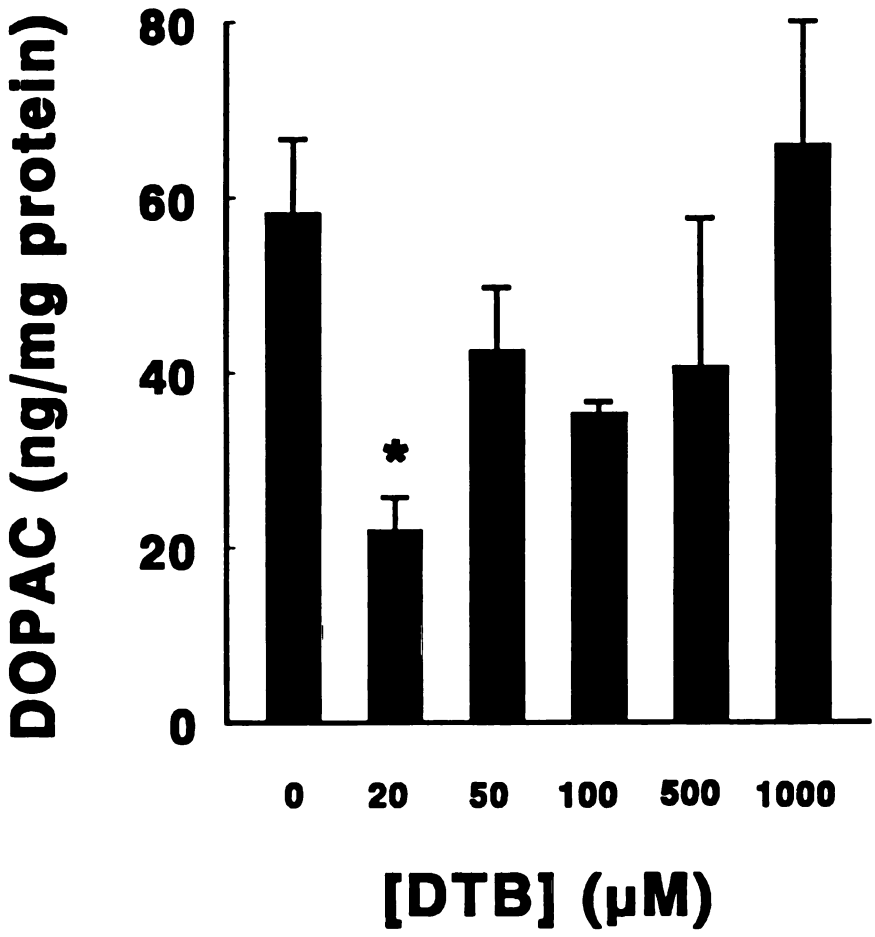


Figure 18. The effect of DTB on DA metabolism in PC12 cells. DOPAC is the major metabolite of DA; as such, the levels of DOPAC within the cell can be used as an index of the degradation of DA. PC12 cells were treated with DTB (0-1000 μM) for 24 hr. The cells were collected as described in Fig. 11, then analyzed for DOPAC content by HPLC-EC. The asterisk indicates a mean value that is significantly different from control ($p < 0.05$). Values are the mean ± SEM of 6 experiments.

DISCUSSION

I. CHARACTERIZATION OF PC12 CELL NEUROTRANSMITTER RELEASE

PC12 cells were used to compare the effects of DTB on cholinergic and catecholaminergic transmitter release. [K^+]-evoked release of ACh from PC12 cells was below the detectable limit of HPLC-EC sensitivity unless the medium was supplemented with 300 µM choline chloride prior to performing the experiments. Apparently, the PC12 cell line used in our experiments does not synthesize sufficient ACh under standard culturing conditions (30 µM choline) to support [K^+]-evoked release. Acute choline supplementation *in vivo* enhances evoked release of ACh by providing excess substrate to choline acetyltransferase for ACh synthesis when release is stimulated (Wecker *et al.*, 1989; Farber *et al.*, 1993).

Choline supplementation also stimulated catecholamine synthesis and enhanced [K^+]-evoked release of DA. PC12 cells cultured in medium supplemented with 300 µM choline chloride released four-fold more DA in response to 56 mM [K^+] than cells incubated in standard (30 µM choline) medium. The facilitatory effect of choline supplementation on [K^+]-evoked release of DA may be due to an agonistic action on the nicotinic and/or muscarinic ACh receptors. For instance, choline supplementation enhances the

synthesis and release of ACh. Released ACh then activates ACh receptors to enhance [K^+]-evoked release of DA possibly by increasing $[Ca^{2+}]_i$ (Inoue and Kenimer, 1988; Courtney *et al.*, 1991) or by altering cyclic AMP levels which may mediate release of DA from PC12 cells (Braizer and Weiner, 1985; Courtney *et al.*, 1991). In addition, activation of nAChR stimulates the Ca^{2+}-dependent phosphorylation of TH, and thus increases overall DA synthesis resulting in elevated levels of [K^+]-evoked release of DA (Nose *et al.*, 1985). Moreover, choline is a weak agonist of AChR (del Castillo and Katz, 1957b), and a 300 µM concentration may be sufficient to activate a substantial proportion of the AChR to enhance DA synthesis and release.

[K^+]-evoked release of DA and [^{3}H]ACh was 2-3 fold greater than that measured in LKB after a 5 min incubation. The depolarization-induced increase in DA and ACh release from PC12 cells was Ca^{2+}-dependent. Lowering of extracellular Ca^{2+} concentrations to less than 0.13 mM reduced [K^+]-evoked release of DA and ACh to levels measured in LKB. Therefore, 56 mM [K^+] stimulates the Ca^{2+}-dependent release of vesicular stores of DA and ACh from PC12 cells (Greene and Rein, 1977b). The increase in release was not due to non-specific leak of neurotransmitter from the cells following [K^+]-induced membrane-damaged cells. This is supported by assessing the cell viability after high [K^+] exposure using trypan blue exclusion. High [K^+] exposure for 5 min did not alter cell viability. PC12 cell viability is not altered by up to 30 min incubation with 55mM [K^+] (Carrol *et al.*, 1992).

DTB treatment also did not alter PC12 cell viability. Therefore, DTB-

induced changes in neurotransmitter metabolism or release were not likely due to decreased cell viability or number. DTB-induced changes in release of ACh from motor nerve terminals occur before evidence of axonal degeneration suggesting that the decrease in release of ACh is not attributed to nerve cell death (Kemplay, 1984).

II. EFFECTS OF DTB ON ACh SYNTHESIS, STORAGE, AND RELEASE

Electrophysiological studies performed on nerve-muscle preparations indicated that DTB treatment reduces quantal release of ACh from motor nerve terminals (Atchison *et al.*,1981; Atchison *et al.*,1982). DTB-treated rats show symptoms possibly of altered cholinergic function at the Harderian glands resulting in chromodacryorrhea (Astwood *et al.*, 1945) and from mucous secreting cells in the gastrointestinal tract causing mucoid feces (Atchison and Peterson, 1981). DTB treatment also reduced [K^+]-evoked release of ACh from PC12 cells. Thus, DTB is capable of reducing evoked release of ACh from several different cholinergic cell types.

DTB reduces spontaneous quantal release of ACh from motor nerve terminals (Atchison, 1989). However, spontaneous non-quantal release of [$^2H_4$]ACh from motor nerve terminals was not affected by DTB treatment (Weiler *et al.*, 1986). Consistent with these observations, spontaneous release of [^{3}H]ACh from PC12 cells was unaffected by DTB treatment. More than 90% of the spontaneous release of ACh from motor nerve terminals is non-quantal (Polak *et al.*, 1981). If such is the case in PC12 cells, then spontaneous non-quantal release of ACh from PC12 cells was unaffected by DTB treatment.

Vesicular ACh is the source of quantal release of ACh from cholinergic cells (del Castillo and Katz, 1957a). ACh is synthesized from choline by CAT, the activity of which is limited only by the levels of choline in the cytoplasm (Cohen and Wurtman, 1975). Alterations in choline uptake or availability in the cytoplasm may be reflected in altered quantal release of ACh from cholinergic cells. DTB did not reduce the rate of [^{3}H]choline uptake, or the cellular [^{3}H]choline content in PC12 cells or at the neuromuscular junction (Weiler *et al.*, 1986). [^{3}H]Choline distribution, assessed by measuring [^{3}H]choline incorporation into the plasma membrane, also remained unaltered after DTB treatment. These results suggest that DTB did not alter precursor availability in PC12 cells.

The inhibitory effects of DTB on evoked release of ACh at the neuromuscular junction are not due to a decrease in ACh content (Weiler *et al.*, 1986). DTB also did not alter cellular ACh levels in PC12 cells. The reduction in EPP and MEPP amplitude from nerve-muscle preparations removed from DTB-treated rats was due to a decrease in mean quantal content (m) (Atchison, 1989). This decrease in m could be due to alterations in vesicular stores of ACh and/or a reduction in the readily releasable pool of ACh (n) (Atchison, 1989). It was important to determine if DTB reduced evoked release of ACh by altering the vesicular stores of ACh. The relative distribution of ACh in the cytosol and vesicular fractions of PC12 cells was unaltered by DTB treatment. Thus, reduction in evoked release by DTB was not due to a decrease in the vesicular stores of ACh. However, the effect of DTB on the readily releasable

pool of ACh (*n*) from PC12 cells could not be determined. Consistent with observations using the neuromuscular junction (Weiler *et al.*, 1986), DTB did not alter the ACh and choline content of the cells. It is likely then, that the effects of DTB are mediated by actions of DTB on the mechanisms of ACh release rather than on choline uptake, ACh synthesis, or vesicular storage.

It has been postulated that DTB affects the cholinergic release mechanisms at some point after the influx of Ca^{2+} through voltage-dependent Ca^{2+} channels (Atchison *et al.*, 1982). To determine if DTB alters evoked release of ACh at a step beyond Ca^{2+} influx, the neurotoxin, αLTX was used to stimulate release of ACh from PC12 cells. αLTX is believed to stimulate neurotransmitter release by inducing a conformational change in the synaptotagmin/neurexin complex, similar to that induced by an influx of Ca^{2+}, which causes vesicle fusion with the plasma membrane (Petrenko *et al.*, 1991; Bennet and Scheller, 1993).

αLTX can overcome the inhibitory effects of DTB on stimulated release from PC12 cells and of BTX on stimulated release of ACh from motor nerve terminals (Cull-Candy *et al.*, 1976). BTX and DTB both inhibit quantal ACh release from neuromuscular junctions (Cull-Candy *et al.*, 1976; Atchison *et al.*, 1982). DTB and BTX-poisoned motor nerve terminals lose the ability to release neurotransmitter even though Ca^{2+} influx in response to membrane depolarization appears to remains intact (Simpson, 1986; Atchison *et al.*, 1982; Atchison, 1989). Both BTX and DTB treatment increase the frequency of giant MEPPs (Cull-Candy *et al.*, 1976; Atchison, 1989). The release of fused, large

vesicles may lead to the presence of giant MEPPs (Publicover and Duncan, 1981). Motor nerve terminals from rats treated for 7 days with DTB showed numerous vesicles of different sizes, including some very large vesicle-like structures (Sahenk, 1990). BTX inhibits vesicular release of ACh by cleaving synaptobrevin (Schiavo *et al.*, 1992). DTB, which contains two thio groups, is capable of forming disulfide bridges with proteins and may act to similarly target a particular synaptic vesicle protein to increase in occurrence of giant MEPPs and to alter quantal ACh release at the neuromuscular junction.

Prior treatment of PC12 cells with 50 and 100 μM DTB, actually enhanced αLTX-stimulated release of [^{3}H]ACh. DTB had no effect on [K^+]-evoked release of newly synthesized [^{3}H]ACh at the same concentrations. The increased αLTX-stimulated release of ACh suggests that more vesicles are docked and primed for fusion with the plasma membrane (Fig. 19). If DTB attenuates Ca^{2+} activated vesicle fusion with the plasma membrane, then an increase may occur in the number of docked vesicles primed for release. αLTX could then stimulate the readily releasable pool which has been augmented by prior treatment with the lower concentrations of DTB. Motor nerve terminals from rats treated for 7 days with DTB appeared to have a large number of synaptic vesicles (Sahenk, 1990).

At high concentrations, DTB (1000 μM) effectively reduces [K^+]-evoked release of newly-synthesized [^{3}H]ACh and no longer enhances αLTX stimulated release. DTB (1000 μM) may inhibit vesicle fusion with the plasma membrane, to the point at which αLTX-stimulated release is no longer enhanced (Fig. 19).

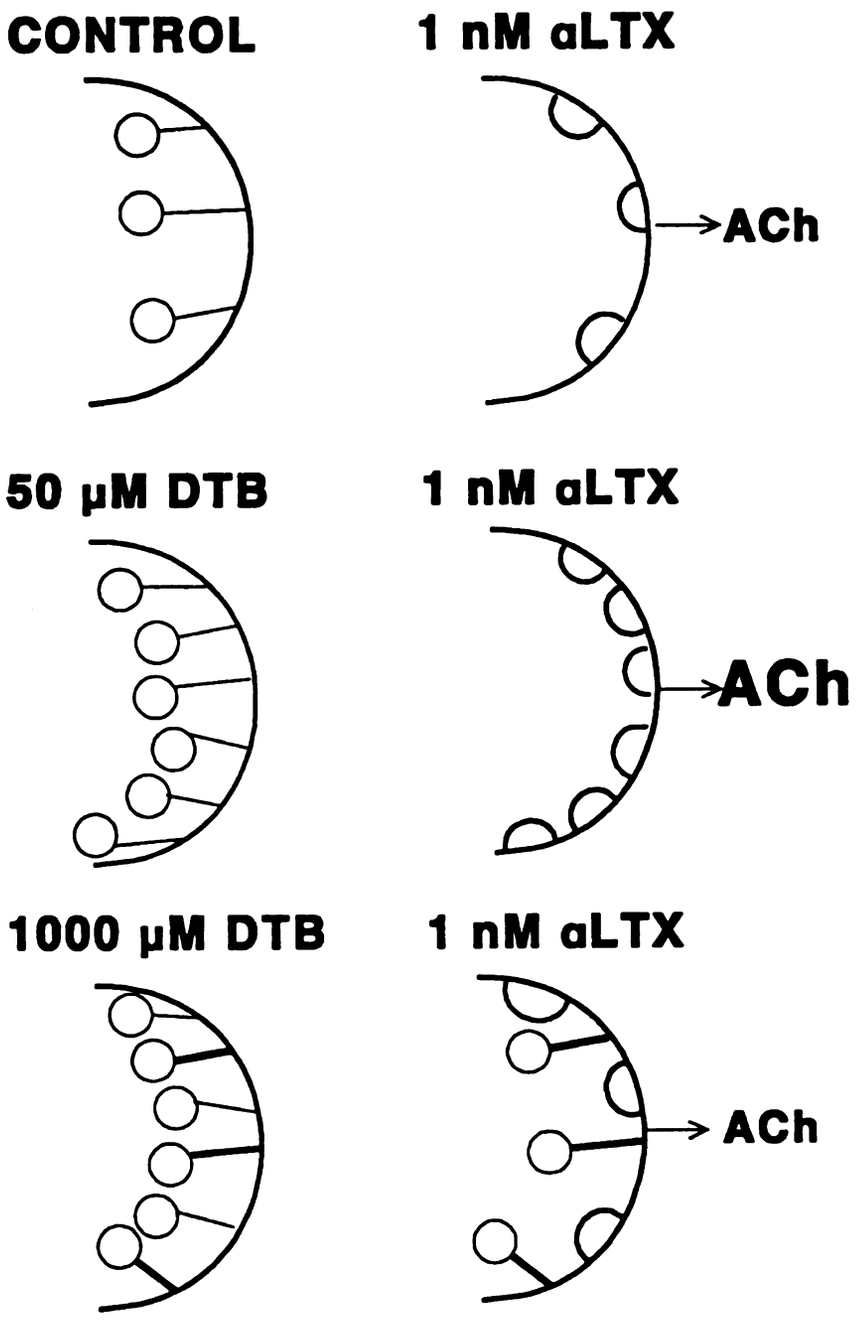


Figure 19. The proposed mechanism of action of DTB on αLTX-stimulated release of ACh from PC12 cells. If 50 μM DTB inhibits vesicle fusion with the plasma membrane, then the number of vesicles docked at the plasma membrane could increase. This effectively increases the readily releasable pool of ACh. αLTX is capable of stimulating the release of the total releasable pool of ACh and 50 μM DTB pretreatment would enhance αLTX-stimulated release. However, 1000 μM DTB may be capable of decreasing the readily releasable pool by completely inhibiting some of the vesicles from fusing with the plasma membrane. This results in the apparent return of αLTX-stimulated ACh release to similar levels in control and 1000 μM DTB treated groups.

αLTX stimulates the total releasable pool of neurotransmitters from PC12 cells (Saito *et al.*, 1985). High K^+ is less effective at stimulating neurotransmitter release from PC12 cells. Only 40% of the total releasable pool of ACh is released after 5 min incubation with 56 mM K^+ (Melega and Howard, 1984). If 1000 μM DTB reduces the readily releasable pool of ACh by altering vesicle fusion with the plasma membrane, then K^+-evoked release would be reduced to a greater extent than αLTX stimulated release (Fig. 20).

K^+-evoked release of ACh from PC12 cells consists of the amount of spontaneous release of ACh plus the amount of evoked release of ACh within the 5 min incubation. The effects of DTB on stimulated vesicular ACh release may be masked by unaltered spontaneous release. To determine how much spontaneous release of ACh is contributing to total K^+-evoked release of ACh, the vesicular pool of ACh can be depleted by using the vesicular ACh transporter inhibitor, vesamicol.

The effects of DTB on evoked release of ACh are concentration dependent. DTB, at low concentrations, enhanced αLTX-stimulated release of ACh while K^+-evoked release of ACh remained unaltered. At high concentrations, DTB inhibited K^+-evoked release of ACh while αLTX-stimulated release of ACh remained unaltered. The affects of DTB on cholinergic neurotransmitter release is biphasic, depending on the concentration of DTB at or in PC12 cells. A similar response was noted with bath application of DTB on nerve-muscle preparations removed from rats. An initial transient increase in EPP amplitude and MEPP frequency was observed

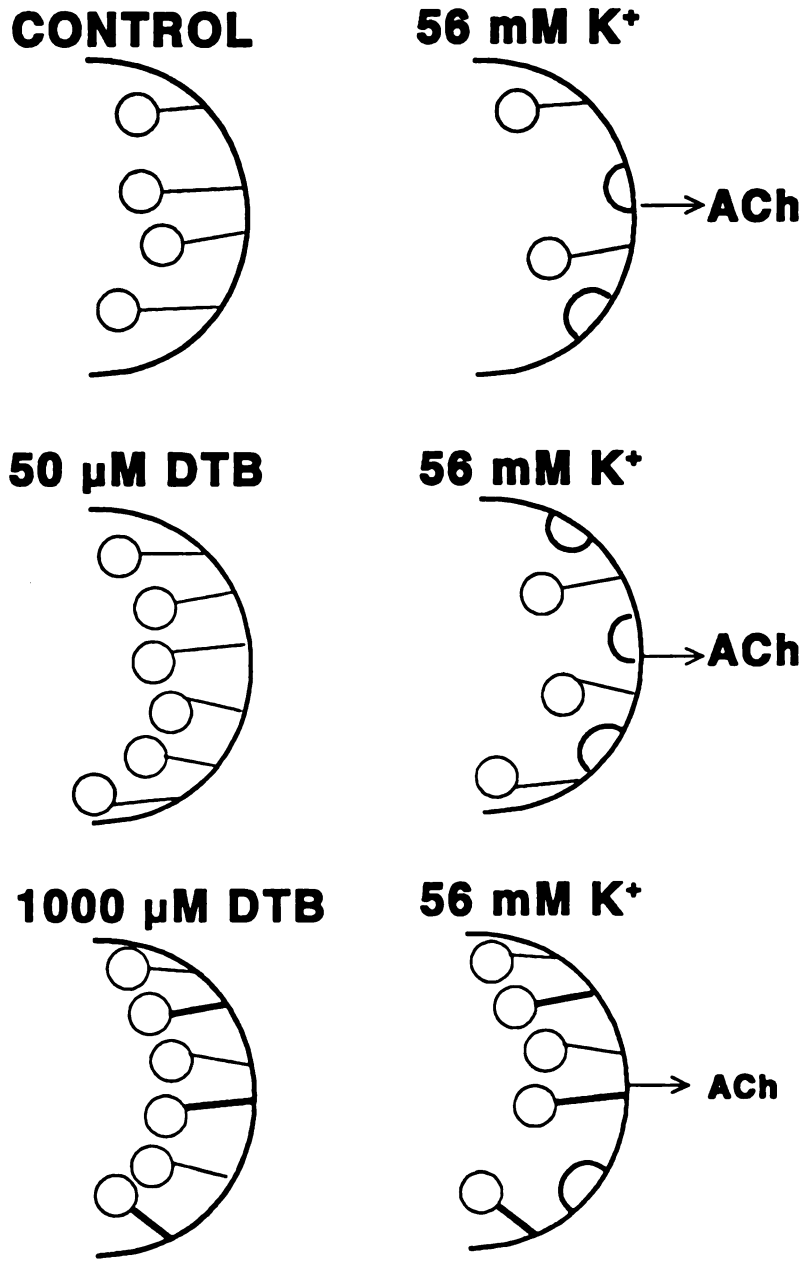


Figure 20. The proposed mechanism of action of DTB on K^+-evoked release of ACh from PC12 cells. If 50 µM DTB inhibits vesicle fusion with the plasma membrane, then the number of vesicles docked at the plasma membrane could increase. This effectively increases the readily releasable pool of ACh. However, 56 mM K^+ is capable of stimulating the release of only 40% of the total releasable pool of ACh. Thus, 50 µM DTB does not significantly alter the amount of K^+-evoked release of ACh from PC12 cells. Whereas, 1000 µM DTB may be capable of decreasing the readily releasable pool by completely inhibiting some of the vesicles from fusing with the plasma membrane. Thus K^+-evoked release of ACh would be significantly decreased by pretreatment with 1000 µM DTB.

immediately after bath application of 1850 μM DTB (Spitsbergen, 1991). However after 10 min perfusion with DTB, EPP amplitude and MEPP frequency were decreased suggesting that the inhibitory effects of DTB on quantal release of ACh depended on the accumulation of DTB at the motor nerve terminal (Spitsbergen, 1991).

III. EFFECTS OF DTB ON DA SYNTHESIS, STORAGE AND RELEASE

DTB treated rats showed symptoms of altered cholinergic function but non-cholinergic neurotransmission appeared to remain unaffected by the neurotoxicant (Altschul, 1947; Atchison and Peterson, 1981; Crofton *et al.*, 1991). However, the effects of DTB on non-cholinergic neurotransmitter release have not been studied. Based on the observations of rats treated with DTB, our hypothesis was that DTB would alter evoked release of ACh preferential to attenuation of non-cholinergic neurotransmitter release. That is, DTB would reduce the amount of evoked release of ACh from PC12 cells, but would have little or no effect on evoked release of DA. Thus, the effects of DTB on catecholamine release and on ACh release were compared. As was the case with release of ACh, DTB reduced [K^+]-evoked release of DA from PC12 cells. Following a similar rationale presented for the ACh experiments, we investigated at what level the perturbation in release of DA may occur. The effect of DTB on the cytosolic and vesicular DA levels was measured and compared to control levels. DTB-treated cells contained less than 75% of control DA levels. Reductions in DA content in DTB-treated cells were observed in both the cytosolic and vesicular pools. Thus, the DTB-induced

decrease in evoked release of DA could be due to a decrease in the available pool of releasable DA.

The effect of DTB on cellular DA levels in PC12 cells was unexpected because there were no symptoms of disturbances in catecholaminergic transmission in rats treated with DTB (Atchison and Peterson, 1981; Crofton *et al.*, 1991). DTB treatment did not alter the acoustic startle response which is sensitive to a wide variety of neuroactive chemicals and other modifications of CNS function (Davis, 1980; Crofton *et al.*, 1991). Catecholaminergic function may appear not to be altered by DTB because the reduction in cellular content of catecholamines is not sufficient to attenuate the postsynaptic response. For instance, in Parkinson's disease, symptoms of the disease do not appear until 80-90% of the dopaminergic neurons in certain areas of the brain degenerate (Birkmayer and Hornykiewicz, 1976). Thus, it is possible that DTB-induced decreases in catecholamines occur *in vivo*, but the appearance of symptoms does not develop.

The effect of DTB on DA synthesis could be mediated indirectly by effects on ACh receptors. DTB may interact with PC12 cell ACh receptors in a manner analogous to the effects of DTB on the nAChR at the neuromuscular junction in rats (Spitsbergen and Atchison, 1990). DTB may react with sulfhydryl groups located on the nAChR causing alterations in the kinetics of the end-plate current at the rat neuromuscular junction (Spitsbergen and Atchison, 1990). In PC12 cells, such alterations in the ganglionic-type nAChR (Jumblatt and Tischler, 1982) may result in reducing the nAChR-ion influx,

phosphorylation of TH, and consequently attenuating DA synthesis. If DTB reduces cellular catecholamine content through alterations in nAChR function, then this effect would occur *in vivo* only with catecholaminergic neurons expressing nAChR, such as sympathetic ganglionic neurons (Jan and Jan, 1983; Mathie *et al.*, 1990). However, the observation that choline supplement did not effect the DTB-induced decrease in PC12 cell DA content suggests that DTB may not be acting through alterations in AChR function.

The DTB-induced decrease in cellular DA content in PC12 cells may involve increased metabolism of DA. If this is the case, then the levels of the major DA metabolite DOPAC would be elevated (Roth *et al.*, 1976). The amount of DOPAC in PC12 cells treated with DTB was less than or equal to that in untreated cells suggesting that DA metabolism was not elevated in DTB-treated cells. Thus, the decrease in cellular DA concentrations was likely due to a decrease in catecholamine synthesis as opposed to an increase in DA metabolism.

IV. CONCLUSIONS

PC12 cells treated with DTB did not have sufficient cytosolic or vesicular DA to support evoked release of DA. The preponderate effects of DTB on DA synthesis precluded determining if DTB inhibits release of DA as well. However, no evidence of catecholamine neurotransmission dysfunction has been observed in animals treated chronically with DTB (Altschul, 1947; Atchison and Peterson, 1981; Crofton *et al.*, 1991). DTB appeared to disrupt cholinergic release mechanisms without altering cellular ACh content or

distribution in PC12 cells. These results are consistent with the effects of DTB on cholinergic neurotransmission.

DTB may inhibit a critical event in the fusion process of cholinergic vesicles. The appearance of giant MEPPs in nerve-muscle preparations removed from DTB treated rats (Atchison, 1989) is thought to occur when multiple vesicles fuse together prior to fusion with the plasma membrane (Publicover and Duncan, 1981). Giant MEPPs may appear because DTB alters the vesicular fusion process. αLTX could overcome the DTB effect which suggests that DTB may act at a step after the influx of Ca^{2+} and before the conformational change of synaptotagmin/neurexin (Petrenko *et al.*, 1991; Bennet and Scheller, 1993). The enhancement of αLTX stimulated release of newly-synthesized [^{3}H]ACh by DTB suggests that vesicle fusion with the plasma membrane may be altered. It is possible that DTB targets a vesicular protein found only in small clear (cholinergic) vesicles, like synaptophysin, which is important in the docking and/or fusion of vesicles with the plasma membrane. The structure of synaptophysin and its interaction with other proteins are dependent on the integrity of its intramolecular disulfide bonds (Johnston and Südhof, 1990). The two thio groups of DTB could disrupt the disulfide bonds and alter the structure and function of synaptophysin. We can postulate that DTB-induced reduction in [K^+]-evoked release of ACh from PC12 cells may involve the inhibition of a specific cholinergic vesicular protein, such as synaptophysin, which is important for quantal release of ACh. If DTB reduces release of ACh from motor nerve terminals by altering vesicular

fusion, then detailed analysis at the electronmicroscopic level of active zones and, in particular, synaptic vesicles may be beneficial in determining the mechanism involved in disruption of cholinergic release by DTB. Electronmicroscopic studies suggests that DTB alters vesicle size (Sahenk, 1990) and possibly vesicular trafficking and fusion.

APPENDIX

APPENDIX

I. PC12 CELLS DIFFERENTIATE WHEN CO-CULTURED WITH A MOUSE CLONAL MUSCLE CELL LINE.

The neuromuscular junction consists of a motor nerve terminal, muscle cells, and Schwann cells. Consequently, it is difficult to ascertain the role of a single cell type in specific effects attributed to alterations in the function of neuromuscular transmission. A simple model of the neuromuscular junction would involve one motor nerve terminal synapsing on one muscle cell. Primary co-cultures of neurons with muscle cells provide an *in vitro* model of the neuromuscular junction but primary cells last only up to 10 days in culture. Therefore, clonal cell lines have been used to create a simple *in vitro* model of the neuromuscular junction in isolation, which can be easily replicated. The neuroblastoma x glioma hybrid cells, NG108-15, forms functional synapses with differentiated G8 myotubes as determined using electrophysiological techniques (Christian *et al.*, 1977). The clonal striated myotubes, G8, were derived from a myogenic cell line which arose spontaneously from cultured mouse hindlimb muscle cells (Christian *et al.*, 1977). G8 cells adhere to plastic culture dishes and become confluent within 7 days. When confluent, G8 cells form parallel arrays of spindle-shaped mononucleated cells which fuse, forming

striated multinucleated myotubes that resemble morphologically normal mouse myotubes (Christian *et al.*, 1977). Well-differentiated G8 myotubes contract spontaneously in DMEM containing 0.5% horse serum and 0.5% fetal bovine serum.

PC12 cells synapse on L6 clonal rat skeletal muscle line when co-cultured. MEPPs were measured in L6 cells which were presumably due to ACh release from PC12 cells (Schubert *et al.*, 1977). Our goal was to form functional synapses between the neuron-like PC12 cells and differentiated G8 cells in culture. This co-culture may be used as a simple *in vitro* model of a neuromuscular junction in isolation.

The co-culture procedure involved growing the G8 cells to confluency in DMEM supplemented with 10% horse serum and 5% fetal bovine serum, then differentiating the G8 cells by replacing the media with DMEM supplemented with only 0.5% horse and 0.5% fetal bovine serum. By 7 days, approximately 5% of the cells form multinucleated myotubes. The myotubes were dissociated with a solution of 0.125% trypsin with 1 mM EDTA (GibcoBRL) in phosphate buffered saline (pH 7.4; 1 mM Na_2HPO_4, 0.32 mM KH_2PO_4, 1.1 mM NaCl), collected, and centrifuged at 50 X *g* for 2 min. Differentiated G8 myotubes formed a pellet while most of the undifferentiated cells remained in the supernatant and were discarded. The pellet, which was 90% differentiated G8 cells and 10% undifferentiated G8 cells, was resuspended in DMEM supplemented with 0.5% serum and the cells were plated at a density of 1 X 10^4 cells/ml. After 24 hr, undifferentiated PC12 cells (1 X 10^5 cells/ml) were

added to the G8 culture plates. The PC12 cells were co-cultured for 4 days with the G8 cells.

PC12 cells cultured with G8 cells differentiated without the addition of NGF (Fig. 19). The PC12 cells appeared to grow neurites toward and contact undifferentiated G8 cells. PC12 cell neurites never appeared to 'synapse' on differentiated G8 cells even though neurites would pass under and over the large multinucleated myotubes to reach an undifferentiated G8 cell. Since PC12 cells would only form contacts with undifferentiated G8 cells, we tried to measure a response (MEPP) to ACh released from PC12 cells in an undifferentiated G8 cell using electrophysiological techniques. Unfortunately, undifferentiated G8 cells were so flat that they could not be impaled with intracellular microelectrodes. In summary, PC12 cells did not synapse on differentiated G8 muscle cells, and the nature of the contacts between PC12 cells and undifferentiated G8 cells could not be evaluated electrophysiologically.

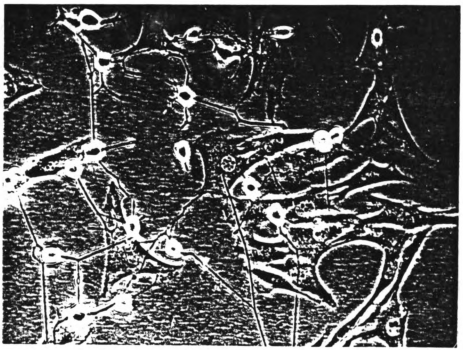


Figure 21. A light micrograph of PC12 cells co-cultured with undifferentiated G8 muscle cells. G8 muscle cells were plated at a low density in DMEM containing 0.5% horse and fetal bovine serum. After 24 hr, PC12 cells were plated on top of the G8 cells at a high density. After 4 days in co-culture, the PC12 cells differentiated without NGF treatment.

II. MEASURING ACh USING THE GAS CHROMATOGRAPHY-MASS SPECTROMETRY METHOD.

[^{1}H]ACh can be measured using gas chromatography coupled to mass spectrometry (Jenden *et al.*, 1973). The reaction depends upon the demethylation of the tertiary amine of ACh by bezenethiolate ion; this converts the nonvolatile ACh to the demethylated derivative which is volatile and can be measured using gas chromatography-mass spectrometry (Jenden and Hanin, 1974). Neurotransmitter release samples collected from PC12 cells as described in the Materials and Methods section were thawed and 1 ml was placed into 25 ml glass conical centrifuge tubes. Cold 1N formic acid-acetone solution (10 ml; 15/85 v/v) was added to the tubes to denature proteins and prevent enzymatic degradation of ACh. Samples were washed twice with diethyl ether (20ml) to extract the formic acid. Diethyl ether in the organic phase was evaporated in a stream of dry N_2 gas. 1 M tris-(hydroxymethyl) methylaminopropane sulphonic acid (10 ml, pH 9.2) mixed into the sample improved ACh extraction into the organic phase. 5 ml of 1 mM dipicrylamine in dichloromethane was added to precipitate and isolate ACh and choline, mixed vigorously, then centrifuged at 1000 X *g* for 2 min. The aqueous layer was discarded and the remaining organic layer was transferred to a clean conical centrifuge tube and dried completely under a stream of N_2 gas. 500 µl of 5 mM silver-p-toluene sulfonate in acetonitrile was mixed with the dried solid. Then, 50 µl propionyl chloride was added to propionate choline which makes the separation of ACh from propionyl choline possible by gas

chromatography. The sample was mixed, kept at room temperature for 5 min, and then centrifuged at 1000 X *g* for 2 min to remove any insoluble silver reineckate salts leaving the soluble p-toluene sulfonate salts of ACh and choline. The supernatant was transferred to a clean tube, and dried in a stream of dry N_2 gas. 0.5 ml of hot (80°C) 50 mM sodium benzenethiolate dissolved in methyl ethyl ketone containing 25 mM benzenethiol was added to the dried precipitate containing propionyl choline and ACh, the air in the tube was purged with N_2 gas, the tube was capped and incubated in a water bath at 80°C for 45 min. The samples were left to cool to room temperature before addition of 100 µl cold 0.5 M citric acid to partition the demethylated ACh and choline into the aqueous phase. The sample was washed with 1 ml diethyl ether twice followed by a wash using 2 ml pentane to remove excess benzenethiol and methylphenylsulfide. The remaining traces of the pentane were evaporated with N_2 gas. 100 µl of a solution containing 7.5 M ammonium hydroxide and 2 M ammonium citrate (pH 9.5) was added to the remaining aqueous layer to enhance the extraction of the demethylated products into the organic phase. 50 µl of methylene chloride was added and mixed vigorously for 2 min then centrifuged at 1000 X *g* for 2 min. The methylene chloride layer was injected (1-2 µl) into a gas chromatograph equipped with a 30 m DB4 (J&W Scientific) capillary column kept at 40°C coupled to a Hewlett-Packard 5987B mass spectrometer. ACh and propionyl choline were separated by an isothermal (40°C) program yielding retention times of 4 min and 9 min, respectively. The gas effluent from the capillary column flows into the mass

spectrometer for characterization of gas chromatographic peaks. A standard containing 1 mg/ml ACh and choline was derivatized in parallel with PC12 cell samples to determine the final yield of ACh and propionyl choline. A representative chromatograph of the standard (1 µl) injected into the gas chromatograph (Fig. 20) coupled to the mass spectrometer (Fig. 21). Although we were able to measure [^{1}H]ACh in the standards, no ACh could be recovered from either the neurotransmitter release samples or cell extract samples of PC12 cells.

III. MEASUREMENT OF $[Ca^{2+}]_i$ IN PC12 CELLS USING FURA-2

The concentration of intracellular Ca^{2+} in PC12 cells was measured using the Ca^{2+}-selective fluorescent indicator fura-2 (Grynkiewicz *et al.*,1985). The procedure for loading PC12 cells with fura-2 was a modification of that reported previously (Fanó *et al.*, 1993). Six flasks of PC12 cells (6 X 10^6 cells/ml) were differentiated for 10 days with 50 ng/ml NGF, and dislodged by agitation of the flasks. The cells were collected by centrifugation at 1000 X *g* for 5 min, and resuspended in LKB containing 250 µM sulfinpyrazone, an inhibitor of organic-anion transport systems that reduces excretion of fura-2 from PC12 cells (DiVirgilio *et al.*, 1988). The cells were washed twice with LKB containing sulfinpyrazone and resuspended to a final concentration of 2 mg protein (cells)/ml buffer. The cell suspension was split into at least three aliquots; one for intrinsic fluorescence measurement, one with fura-2 alone, and the other(s) for fura-2 + DTB treatment. Fura-2 is loaded into cells in its cell permeant form, fura-2AM. Cellular esterases cleave the

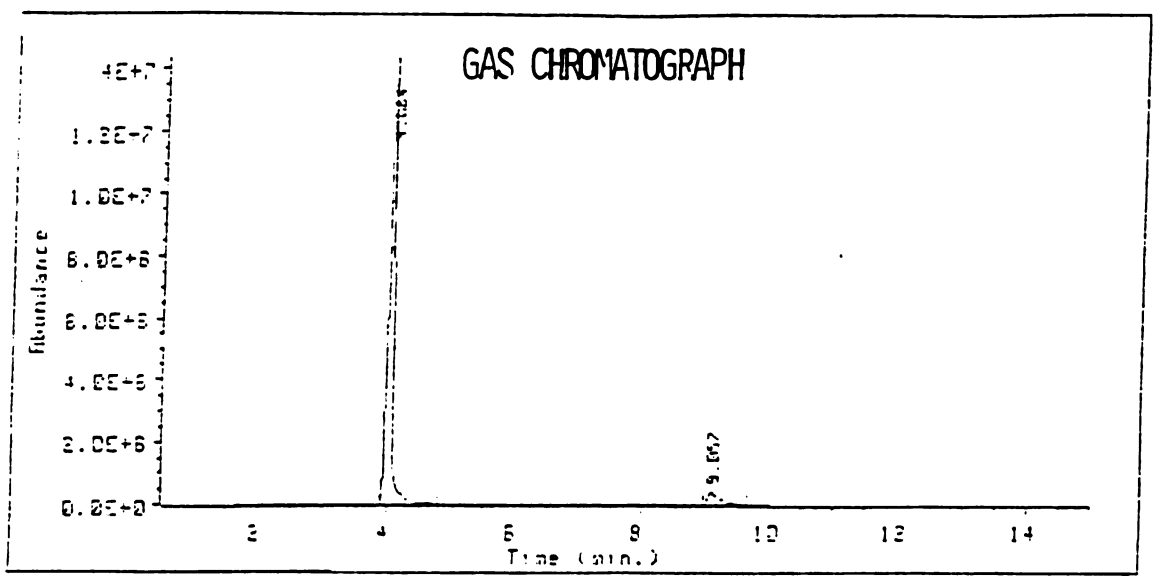


Figure 22. The gas chromatograph of ACh and choline standard. One µl of a 1 mg/ml ACh and choline standard solution was injected into a gas chromatography column set at a temperature of 40°C. The ACh and choline standard was derivatized as described in the appendix. The retention time for ACh and choline was 4.064 and 9.057, respectively.

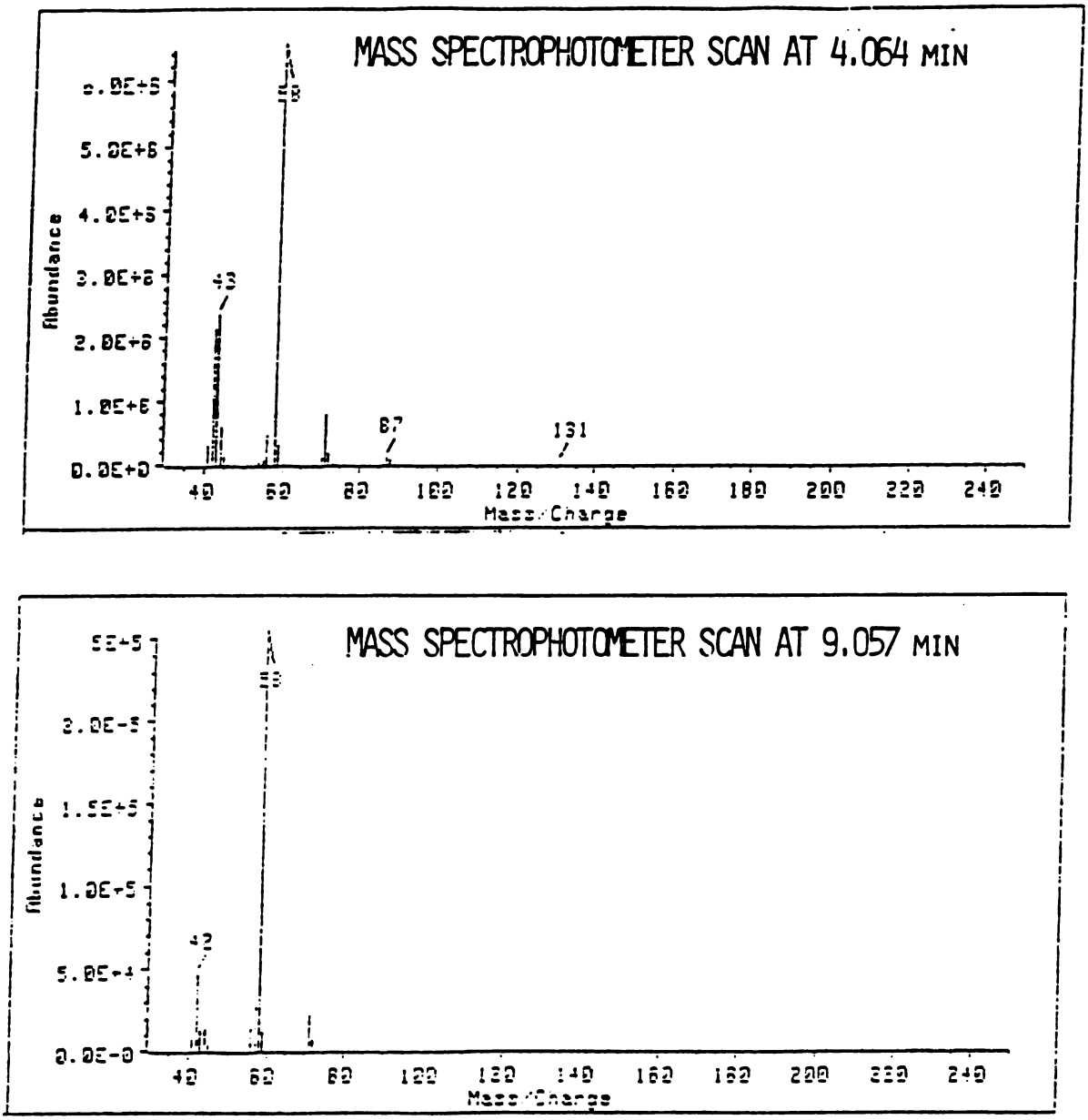


Figure 23. The mass spectrograph of ACh (4.064 min) and choline (9.057 min) standard following separation by gas chromatography. The effluent of an 1 µl sample of a 1 mg/ml ACh and choline standard injected into a gas chromatography column was analyzed by mass spectrometry. ACh has a major peak at 58 m/e with minor peaks at 43, 72, 87, 131 m/e. Propionyl choline has a major peak at 58 m/e with minor peaks at 42 and 72 m/e.

acetoxymethylester groups to convert the dye to its Ca^{2+}-sensitive, cell-impermeant form. PC12 cells were incubated with 3 μM fura-2AM in 0.1% DMSO (v/v) at 37°C for 30 min in a shaking water bath. Cells used for measurement of intrinsic fluorescence were incubated with 0.1% DMSO without fura-2AM. After the incubation, 10 ml of LKB containing sulfinpyrazone was added to dilute out any unhydrolyzed fura-2AM. The cells were centrifuged at 1000 X *g* for 2 min and resuspended in fresh LKB containing sulfinpyrazone to a final concentration of 2 mg protein/ml. The samples were incubated at 37°C in the shaking water bath until used. 2 ml aliquots of suspended PC12 cells were transferred to a polystyrene cuvette containing a magnetic stir bar and place into a spectrofluorometer (SPEX Industries, Edison, NJ) equipped with a thermally jacketed cuvette holder at 37°C. The emission intensity of the fura-2 was monitored at 505 nm following excitation at 340 and 380 nm and reported as a time based scan versus the ratio of 340 to 380 nm intensity (counts per second) as described previously (Denny *et al.*, 1993). PC12 cells were treated acutely with 500 μM DTB in the serum-free LKB containing sulfinpyrazone. After establishing a two min baseline of fura-2 fluorescence intensity, 500 μM DTB was added to the cells. DTB caused an immediate decrease in both 340 and 380 nm fluorescence intensity as well as a decrease in the ratio of the fluorescence intensity at the excitation wavelengths of 340 and 380 nm (Fig. 22). DTB did not alter the rate of change of fluorescence intensity change or ratio over time (Fig. 22). The gradual rise in 340/380 nm ratio may actually represent leakage of fura-2 from

the PC12 cells rather than an increase in $[Ca^{2+}]_i$ even though sulfinpyrazone was included in all buffers (Fig. 22). In conclusion, 500 μM DTB did affect fura-2 fluorescence, however, this was not consistent with a DTB-induced increase in $[Ca^{2+}]_i$.

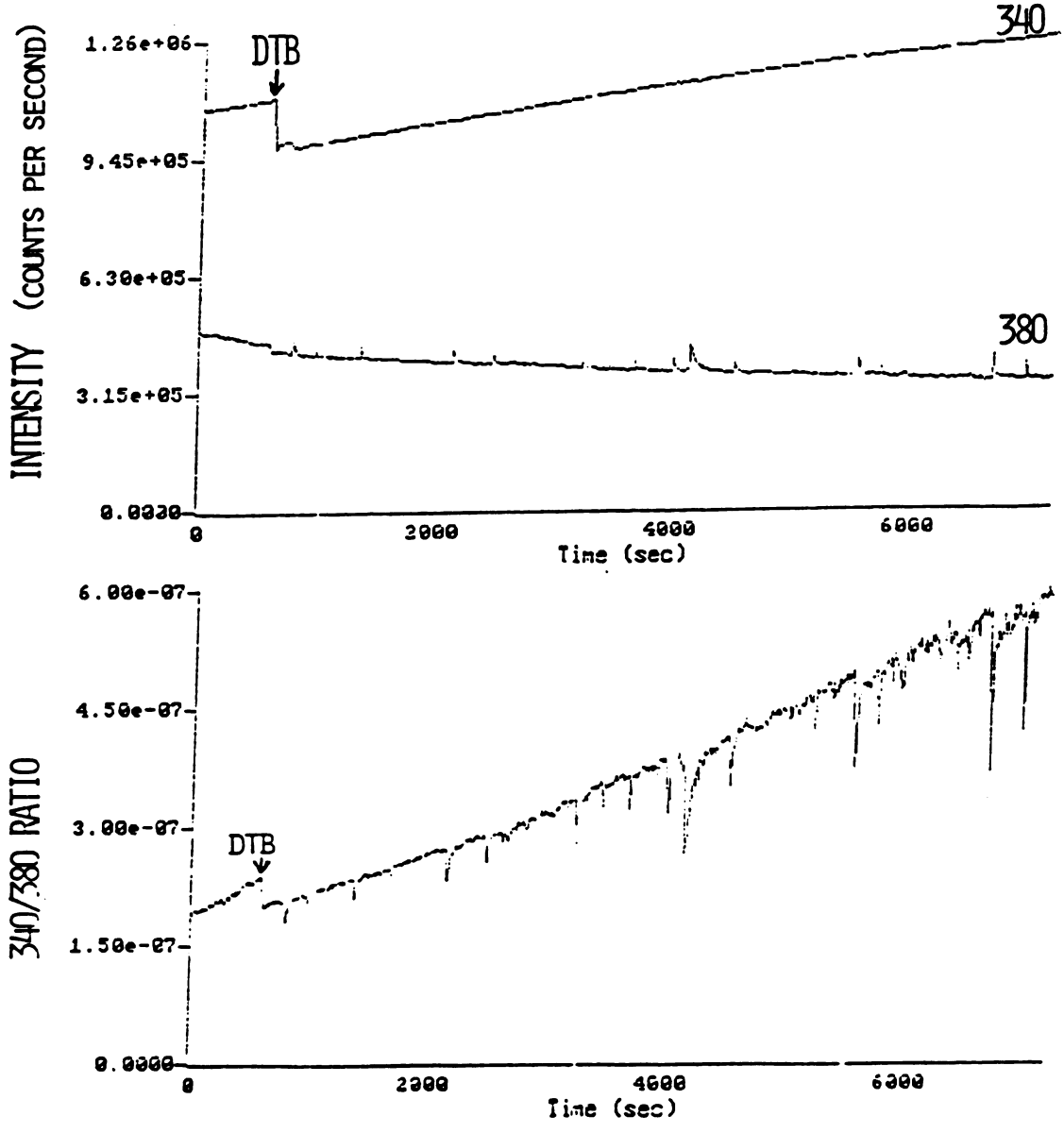


Figure 24. Measurement of $[Ca^{2+}]_i$ in PC12 cells using fura-2. PC12 cells were loaded with 3 µM fura-2AM for 30 min at 37°C, washed once, and resuspended at 2 mg protein (cells)/ml of LKB containing 250 µM sulfinpyrazone. A 2 ml sample was placed in a cuvette and the fluorescence intensity of the fura-2 in PC12 cells was monitored at 505 nm following excitation at 340 and 380 nm and reported as a time based scan versus the 340 to 380 fluorescence intensity (counts per second). The intracellular Ca^{2+} was monitored using the ratio of 340/380 fluorescence intensity as a function of time. The baseline fluorescence was monitored for 2 min, then 500 µM DTB was added to the cuvette. DTB caused a rapid drop in fura-2 fluorescence but did not alter the rate of change in fluorescence intensity over time.

LIST OF REFERENCES

LIST OF REFERENCES

Altschul, S. (1947) Effects of dithiobiuret on the central nervous system. *Proc. Soc. Exp. Biol. Med.* **66**, 448-451.

Astwood, E.B., Hughes, A.M., Lubin, M., VanderLaan, W.P. and Adams, R.D. (1945) Reversible paralysis of motor function in rats from the chronic administration of dithiobiuret. *Science* **102**, 196-197.

Atchison, W.D. (1989) Alterations of spontaneous and evoked release of acetylcholine during dithiobiuret-induced neuromuscular weakness. *J. Pharmacol. Exp. Ther.* **249**, 735-743.

Atchison, W.D. and Peterson, R.E. (1981) Potential neuromuscular toxicity of 2,4-dithiobiuret in the rat. *Toxicol. App. Pharmacol.* **57**, 63-68.

Atchison, W.D., Lalley, P.M., Cassens, R.G., and Peterson, R.E. (1981) Depression of neuromuscular function in the rat by chronic 2,4-dithiobiuret treatment. *Neurotoxicology* **2**, 329-346.

Atchison, W.D., Mellon, W.S., Lalley, P.M. and Peterson, R.E. (1982) Dithiobiuret-induced muscle weakness in rats: evidence for a prejunctional effect. *Neurotoxicology* **3**, 44-54.

Bähler, M. and Greengard, P. (1987) Synapsin I bundles F-actin in a phosphorylation-dependent manner. *Nature (Lond.)* **326**, 704-707.

Balch, W.E. (1990) Small GTP-binding proteins in vesicular transport. *Trends Biochem. Sci.* **15**, 473-477.

Bennet, M.K., and Scheller, R.H. (1993) The molecular machinery for secretion is conserved from yeast to neurons. *Proc. Natl. Acad. Sci. USA* **90**, 2559-2563.

Birkmayer, W., and Hornykiewicz, O. (1976) *Advances in Parkinsonism: Biochemistry, Physiology, Treatment. Fifth International Symposium on Parkinson's Disease*. Basel: Roche, Vienna.

Braizer, L., and Weiner, N. (1985) Regulation of dopamine release from PC12 cell cultures during stimulation with elevated potassium or carbachol. *J. Neurochem.* **44**, 495-501.

Bushnell, P.J. (1994) Cognitive and motro effects of repeated 2,4-dithiobiuret injection in rats. *The Toxixologist* **14**, 94.

Carrol, J.M., Toral-Barza, L., and Gibson G. (1992) Cytosolic free calcium and gene expression during chemical hypoxia. *J. Neurochem.* **59**, 1836-1843.

Chin, G.J., and Goldman, S.A. (1992) Purification of squid synaptic vesicles and characterization of the vesicle-associated proteins synaptobrevin and Rab3A. *Brain Res.* **571**, 89-96.

Christian, C.N., Nelson, P.G., Peacock, J. and Nirenberg, M. (1977) Synapse formation between two clonal cell lines. *Science* **196**, 995-998.

Clarkson, E.D., Bahr, B.A., and Parsons, S.M. (1993) Classical non-cholinergic neurotransmitters and the vesicular transport system for acetylcholine. *J. Neurochem.* **61**, 22-28.

Cohen, E.L. and Wurtman, R.J. (1975) Brain acetylcholine: increase after systemic choline administration. *Life Sciences* **16**, 1095-1102.

Collier, B., and MacIntosh, F.C. (1969) The source of choline for acetylcholine synthesis in a sympathetic ganglion. *Can. J. Physiol. Pharmacol.* **47**, 127-135.

Courtney, N.D., Howlett, A.C., and Westfall, T.C. (1991) Regulation of nicotine-evoked dopamine release from PC12 cells. *Life Sciences* **48**, 1671-1678.

Crofton, K.M., Dean, K.F., Hamrick, R.C., and Boyes, W.K. (1991) The effects of 2,4-dithiobiuret on sensory and motor function. *Fundam. Appl. Toxicol.* **16**, 469-481.

Cull-Candy, S.G. Lundh, H. and Thesleff, S. (1976) Effects of botulinum toxin on neuromuscular transmission in the rat. *J. Physiol. (Lond.)* **260**, 177-203.

Cutler, D.F. and Cramer, L.P. (1990) Sorting during transport to the surface of PC12 cells: Divergence of synaptic vesicle and secretory granule proteins. *J. Cell Biol.* **110**, 721-730.

Davis, M. (1980) Neurochemical modulation of sensory-motor reactivity: Acoustic and tactile startle reflexes. *Neurosci. Biobehav. Rev.* **4**, 241-263.

del Castillo, J. and Katz, B. (1957a) Quantal components of the end-plate potential. *J. Physiol. (Lond.)* **124**, 560-573.

del Castillo, J., and Katz, B. (1957b) Interaction at end-plate receptors between different choline derivatives. *Proc. Roy. Soc. B*, **146**, 369-381.

Denny ,M.F., Hare, M.F. and Atchison, W.D. (1993) Mehtylmercury alters intrasynaptosomal concentrations of endogenous polyvalent cations. *Toxicol. Appl. Pharmacol.* **122**, 222-232.

Dichter, M.A., Tischler, A.S., and Greene, L.A. (1977) Nerve growth factor-induced increase in electrical excitability and acetylcholine sensitivity of a rat pheochromocytoma cell line. *Nature (Lond.)* **269**, 501-504.

DiVirgilio, F., Fasolato, C. and Steinberg, T.H. (1988) Inhibitors of membrane transport system for organic anions block fura-2 excretion from Pc12 and N2A cells. *Biochem. J.* **256**, 959-963.

Dunnett, C.W. (1955) A multiple comparisons procedure for comparing several treatments with a control. *J. Amer. Statist. Ass.* **50**, 1096-1121.

Elferink, L.A., Peterson M.R., and Scheller, R.H. (1993) A role for synaptotagmin (p65) in regulated exocytosis. *Cell* **72**, 153-159.

Elmqvist, D. and Feldman, D.S. (1966) Influence of ionic environment on acetylcholine release from the motor nerve terminals. *Acta Physiol. Scand.* **67**, 34-42.

Engel, A.G. (1988) Changes in end-plate structure in neuromuscular transmission disorders, in *Neuromuscular Junction* (Sellin, L.C., Libelius, R. and Thesleff, S., eds), pp.415-428. Elsevier Science Publishers.

Fanó, G., Mariggió, M.A., Angelella, P., Nicoletti, I., Antonica, A., Fulle, S. and Calissano, P. (1993) The S-100 protein casues an increase of intracellular calcium and death of PC12 cells. *Neuroscience* **53**, 919-925.

Farber, S.A., Kischka, U., Marshall, D.L., and Wurtman, R.J. (1993) Potentiation by choline of basal and electrically evoked acetylcholine release, as studied using a novel device which both stimulates and perfuses rat *corpus striatum*. *Brain Res.* **607**, 177-184.

Gage, P.W., and Hubbard, J.I. (1966) An investigation of the post-tetanic potentiation of end-plate potentials at a mammalian neuromuscular junction. *J. Physiol. (Lond.)* **184**, 353-375.

Goldberg, A.M. and McCaman, R.E. (1973) Determination of picomole concentrations of ACh in brain. *J. Neurochem.* **20**, 1-8.

Grasso, A., Pelliccia, M., and Alemà, S. (1982) Characterization of α-latrotoxin interaction with rat brain synaptosomes and PC12 cells. *Toxicon* **20**, 149-156.

Grasso, A. Alemà, S. Rufini, S. and Senni M.I. (1980) Black widow spider toxin-induced calcium fluxes and transmitter release in a neurosecretory cell line. *Nature (Lond.)* **283**, 774-776.

Greene, L.A. and Rein, G. (1977a) Release, storage and uptake of catecholamines by a clonal cell line of nerve growth factor (NGF) responsive pheochromocytoma cells. *Brain Res.* **129**, 247-263.

Greene, L.A. and Rein, G. (1977b) Synthesis, storage and release of acetylcholine by a noradrenergic pheochromocytoma cell line. *Nature (Lond.)* **268**, 349-351.

Greene, L.A. and Rein, G. (1977c) Release of [^{3}H]norepinephrine from a clonal line of pheochromocytoma cells (PC12) by nicotinic stimulation. *Brain Res.* **138**, 521-528.

Greene, L.A. and Tischler, A.S. (1976) Establishment of a noradrenergic clonal line of rat adrenal pheochromocytoma cells which respond to nerve growth factor. *Proc. Nat. Acad. Sci. USA* **73**, 2424-2428.

Grynkiewicz G., Poenie M., and Tsien R.Y. (1985) A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. *J. Biol. Chem.*, **260**, 3440-3450.

Guroff, G. (1985) PC12 cells as a model of neuronal differentiation, in *Cell Culture in the Neurosciences* (Bottenstein, J.E., and Sato, G., eds), pp. 245-272. Ptenum Publishing Corp..

Henry, R.J. (1968) *Clinical Chemistry - Principles and Techniques,* pp. 509-510, 122-137. Harper & Row, New York.

Horwitz, J. (1989) Muscarinic receptor stimulation increases inositol-phospholipid metabolism and inhibits cyclic AMP accumulation in PC12 cells. *J. Neurochem.* **53**, 197-204.

Ikuta S., Imamura, S., Misabi, H., and Horiuti, Y. (1977) Purification and characterization of choline oxidase from *arthrobacter globiformis*. *J. Biochem.*82, 1714-1749.

Inoue, K. and Kenimer, J.G. (1988) Muscarinic stimulation of calcium influx and norepinephrine release in PC12 cells. *J. Biol. Chem.* **263**, 8157-8161.

Jan, Y.N. and Jan, L.Y. (1983) A LHRH-like peptidergic neurotransmitter capable of 'action at a distance' in autonomic ganglia. *Trends Neurosci.* **6**, 320-325.

Jenden, D.J., Roch, M., and Booth R. (1973) Simultaneous measurement of endogenous and deuterium labelled tracer variants of choline and acetylcholine in subpicomole quantities by gas chromatography mass spectrometry. *Anal. Biochem.* **55**, 438-448.

Jenden, D.J. and Hanin, I. (1974) Gas chromoatographic microestimation of choline and acetylcholine after N-demethylation by sodium benzenethiolate, in *Choline and acetylcholine: Handook of chemical assay methods* (Hanin, I. ed.) pp. 135-150. Raven Press, New York.

Johnson, R.G. and Scarpa, A. (1976) Internal pH of isolated chromaffin vesicles. *J. Biol. Chem.* **251**, 2189-2191.

Johnston, P.A. and Südhof, T.C. (1990) The multisubunit structure of synaptophysin. Relationship between disulfide bonding and homo-oligomerization. *J. Biol. Chem.* **265**, 8869-8873.

Jumblatt, J.E. and Tischler, A.S. (1982) Regulation of muscarinic ligand binding sites by nerve growth factor in PC12 pheochromocytoma cells. *Nature (Lond.)* **297**, 152-154.

Katz, B. (1966) *Nerve, Muscle and Synapse*, p. 135. McGraw-Hill, New York.

Katz, B. and Miledi, R. (1969) Spontaneous and evoked activity of motor nerve endings in calcium ringer. *J. Physiol. (Lond.)* **203**, 689-706.

Kemplay, S. (1984) Effects of dithiobiuret intoxication on motor end plates in sternocostalis and hindlimb muscles of female rats. *Acta Neuropathol (Berl)* **65**, 77-84.

Knaus, P., Marquèze-Pouey, B., Scherer, H., and Betz, H. (1990) Synaptoporin, a novel putative channel protein of synaptic vesicles. *Neuron* **6**, 453-462.

Lambert, E.H. and Elmqvist, D. (1971) Quantal components of end-plate potentials in myasthenic syndromes. *Ann. NY Acad. Sci.* **183**, 183-199.

Lomneth, R., Martin, T.F.J., and DasGupta, B.R. (1991) Botulinum neurotoxin light chain inhibits norepinephrine secretion in PC12 cells at an intracellular membranous or cytoskeletal site. *J. Neurochem.* **57**, 1413-1421.

Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**, 265-275.

Mathie, A., Colquhoun, D., and Cull-Candy, S.G. (1990) Rectification of currents activated by nicotinic acetylcholine receptors in rat sympathetic ganglion neurones. *J. Physiol.* **427**, 625-655.

Maycox, P.R., Hell, J.W., and Jahn R. (1990) Amino acid neurotransmission: spotlight on synaptic vesicles. *Trends Neurosci.* **13**, 83-87.

Meldolesi, J., Huttner, W.B., Tsien, R.Y., and Pozzan, T. (1984) Free cytoplasmic Ca^{2+} and neurotransmitter release: Studies on PC12 cells and synaptosomes exposed to αlatrotoxin. *Proc. Natl. Acad. Sci. USA* **81**, 6535-6538.

Melega, W.P. and Howard, B.D. (1981) Choline and acetylcholine metabolism in PC12 secretory cells. *Biochemistry* **20**, 4477-4483.

Melega, W.P. and Howard, B.D. (1984) Biochemical evidence that vesicles are the source of the acetylcholine released from stimulated PC12 cells. *Proc. Natl. Acad. Sci. USA* **81**, 6535-6538.

Molgo, J., Lemeignan, M., and Lechat, P. (1977) Effects of 4-aminopyridine at the frog neuromuscular junction. *J. Pharmacol. Exp. Ther.* **203**, 653-663.

Mora, M., Lambert, E.H., Engel, A.G. (1987) Synaptic vesicle abnormality in familial infantile myasthenia. *Neurology* **37**, 206-214.

Nose, P.S., Griffith, L.C., and Schulman, H. (1985) Ca^{2+}-dependent phosphorylation of tyrosine hydroxylase in PC12 cells. *J. Cell Biol.* **101**, 1182-1900.

Pelhate, M. and Pichon, Y. (1974) Selective inhibition of potassium current in the giant axon of the cockroach. *J. Physiol. (Lond.)* **242**: 90P-91P.

Perin, M.S., Brose, N., Jahn, R., and Südhof, T.C. (1991) Domain structure of synaptotagmin (p65). *J. Biol. Chem.* **266**, 623-629.

Petrenko, A. G., Perin, M.S., Davletox, B.A., Ushkaryov, Y.A., Geppert, M., and Südhof, T.C. (1991) Binding of synaptotagmin to the α-latrotoxin receptor implicates both in synaptic vesicle exocytosis. *Nature (Lond.)* 353: 65-68.

Polak, R.L., Sellin, L.C., and Thesleff, S. (1981) Acetylcholine content and release in denervated or botulinum poisoned rat skeletal muscle. *J. Physol. (Lond.)* **319**, 253-259.

Preisler, P.W. and Bateman, M.J. (1947) Oxidation-reduction potentials of thiol-disulfide systems. II. Dithiobiuret -3,5-diimino-1,2,4-dithiazoline. *J. Amer. Chem. Soc.* **69**, 2632-2635.

Publicover, S.J. and Duncan, C.J. (1981) Diamide, temperature and spontaneous transmitter release at the neuromuscular junction: Stimulation of exocytosis by a direct effect on membrane fusion? *Eur. J. Pharmacol.* **70**, 203-211.

Rabe, C.S., Delorme, E., and Weight, F.F. (1987) Muscarine-stimulated neurotransmitter release from PC12 cells. *J. Pharmacol. Exp. Ther.* **243**, 534-541.

Rebois, R.V., Reynolds, E.E., Toll, L., and Howard, B.D. (1980) Storage of dopamine and acetylcholine in granules of PC12, a clonal pheochromocytoma cell line. *Biochemistry* **19**, 1240-1248.

Roth, R.H., Murrin, L.C., and Walters, J.R. (1976) Central dopaminergic neurons: effects of alterations in impulse flow on the accumulation of dihydroxyphenylacetic acid. *Eur. J. Pharmacol.* **36**, 163-171.

Saito, I., Dozio, N., Meldolesi, J. (1985) The effect of α-latrotoxin on the neurosecretory PC12 cells differentiated by treatment with nerve growth factor. *Neuroscience* **14**, 1163-1174.

Sands, S.B. and Barish, M.E. (1991) Calcium permeability of neuronal nicotinic acetylcholine receptor channels in PC12 cells. *Brain Res.* **560**, 38-42.

Sahenk, Z. (1990) Distal terminal axonopathy produced by 2,4-dithiobiuret: effects of long-term intoxication in rats. *Acta Neuropathol.* **81**, 141-147.

Schiavo, G., Benfenati, F., Poulain, B., Rossetto, O., Polverino de Laureto, P., DasGupta, B.R., and Montecucco, C. (1992) Tetanus and botulinum-B neurotoxins block neurotransmitter release by proteolytic cleavage of synaptobrevin. *Nature (Lond.)* **359**, 832-835.

Schubert, D. and Klier, F.G. (1977) Storage and release of acetylcholine by a clonal cell line. *Proc. Natl. Acad. Sci. USA* **74**, 5184-5188.

Schubert, D., Heinemann, S. and Kidokoro, Y. (1977) Cholinergic metabolism and synapse formation by a rat nerve cell line. *Proc. Natl. Acad. Sci USA* **74**,2579-2583.

Seifter, S., Harkness, D.M., Muntwyler, E., and Seifter, J. (1948) The effect of dithiobiuret (DTB) on the electrolyte and water content of skeletal muscle, and on carbohydrate metabolism. *J. Pharmacol. Exp. Ther.* **93**, 93-100.

Shoji-Kasai, Y. Yoshida, A., Sato, K., Hoshino, T., Ogura, A., Kondo, S., Fujimoto, Y., Kuwahara, R., Kato, R., Takahashi, M. (1992) Neurotransmitter release from synaptotagmin-deficient clonal variants of PC12 cells. *Science* **256**, 1820-1823.

Simpson, L. (1986) Molecular pharmacology of botulinum toxin and tetanus toxin. *Ann. Rev. Pharmacol. Toxicol.* **26**, 427-454.

Spitsbergen, J. (1991) Modification of ionic conductance at the neuromuscular junction following exposure to the paralytic agent 2,4-dithiobiuret. Thesis p. 66.

Spitsbergen, J. and Atchison, W.D. (1990) Acute alterations in murine neuromuscular transmission following exposure to a nonparalytic dose of dithiobiuret. *Toxicol. Appl. Pharmacol.* **102**, 68-79.

Südhof, T.C., and Jahn, R. (1991) Proteins of synaptic vesicles involved in exocytosis and membrane recycling. *Neuron* **6**, 665-677.

Thompson, H. and Aldrich, R.W. (1980) Membrane potassium channels, in *The Cell Surface and Neuronal Function* (Cotman, C.W., Poste, G. and Nicolson, G.L., eds) pp. 49-85. Elsevier, New York.

Tischler, A.S. and Greene, L.A. (1975) Nerve growth factor-induced process formation by cultured rat pheochromocytoma cells. *Nature (Lond.)* **258**, 341-342.

Tolnai, S. (1975) A method for viable cell count. *Tissue Cult. Assoc. Man.* **1**, 37-38.

Ushkaryov, Y.A., Petrenko, A.G., Geppert M., and Südhof, T. (1992) Neurexins: synaptic cell surface proteins related to the α-latrotoxin receptor and laminin. *Science* **257**, 50-56.

Wecker, L., Cawley, G., and Rothermel, S. (1989) Acute choline supplementation *in vivo* enhances ACh synthesis *in vitro* when neurotransmitter release is increased by potassium. *J. Neurochem.* **52**, 568-575.

Weiler, M.H., Bak, I.J., and Jenden, D.J. (1983) Choline and acetylcholine metabolism in rat neostriatal slices. *J. Neurochem.* **41**, 473-480.

Weiler, M.H., Williams, K.D., and Peterson, R.E. (1986) Effects of 2,4-dithiobiuret treatment in rats on cholinergic function and metabolism of the *extensor digitorum longus* muscle. *Toxicol. Appl. Pharmacol.* **84**: 220-231.

Wiedenmann, B. Rehm, H. and Franke, W.W. (1987) Synaptophysin, an integral membrane protein of vesicles present in normal and neoplastic neuroendocrine cells. *Ann. NY Acad. Sci.* **493**, 500-503.