INFORMATION TO USERS

This manuscript has been reproduced from the microfilm master. UMI films the text directly from the original or copy submitted. Thus, some thesis and dissertation copies are in typewriter face, while others may be from any type of computer printer.

The quality of this reproduction is dependent upon the quality of the copy submitted. Broken or indistinct print, colored or poor quality illustrations and photographs, print bleedthrough, substandard margins, and improper alignment can adversely affect reproduction.

In the unlikely event that the author did not send UMI a complete manuscript and there are missing pages, these will be noted. Also, if unauthorized copyright material had to be removed, a note will indicate the deletion.

Oversize materials (e.g., maps, drawings, charts) are reproduced by sectioning the original, beginning at the upper left-hand corner and continuing from left to right in equal sections with small overlaps. Each original is also photographed in one exposure and is included in reduced form at the back of the book.

Photographs included in the original manuscript have been reproduced xerographically in this copy. Higher quality 6" x 9" black and white photographic prints are available for any photographs or illustrations appearing in this copy for an additional charge. Contact UMI directly to order.

UMI

A Bell & Howell Information Company
300 North Zeeb Road, Ann Arbor MI 48106-1346 USA
313/761-4700 800/521-0600

Effects of Ethanol on Muscarinic Receptor-Induced Responses in Astroglia

by

Michelle Catherine Catlin

A dissertation submitted in partial fulfillment of the
requirements for the degree of

Doctor of Philosophy

University of Washington

1999

Approved by Lisa G. Costa
Chairperson of Supervisory Committee

Mark S. Cooper

Elaine M. Faustman

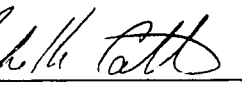
Diana L. Patterson Frost

Program Authorized
to Offer Degree Department of Environmental Health

Date Mon., March 8/99

Doctoral Dissertation

In presenting this thesis in partial fulfillment of the requirements for the Doctoral degree at the University of Washington, I agree that the Library shall make its copies freely available for inspection. I further agree that extensive copying of the dissertation is allowable only for scholarly purposes, consistent with "fair use" as prescribed in the U.S. Copyright Law. Requests for copying or reproduction of this dissertation may be referred to UMI Dissertation Services, 300 North Zeeb Road, P.O. Box 1346, Ann Arbor, MI 48106-1346, or to the author.

Signature 

Date 03/09/99

University of Washington

Abstract

EFFECTS OF ETHANOL ON MUSCARINIC RECEPTOR-INDUCED RESPONSES IN ASTROGLIA

by Michelle Catherine Catlin

Chairperson of the Supervisory Committee: Professor Lucio G. Costa
Department of Department of Environmental Health

A major and irreversible effect seen in the Fetal Alcohol Syndrome is microencephaly. The mechanism(s) underlying this effect remains unknown. Data from our laboratory demonstrate that ethanol inhibits muscarinic-induced proliferation of astroglia. An important component of the intracellular signaling cascade following muscarinic stimulation is an increase in intracellular calcium. This dissertation research tests the hypothesis that ethanol exposure during development causes central nervous system dysfunction through effects on astrocytes, specifically on their calcium response to muscarinic stimulation, thereby interfering with the normal mitogenic response to muscarinic agonists. Calcium imaging techniques were used to quantitate the carbachol-induced calcium responses in primary rat cortical astrocytes and in human 132 1N1 astrocytoma cells The effects of ethanol on these responses were also investigated. Carbachol induced a concentration-dependent increase in intracellular calcium, via the M3 receptor, which consisted of an initial spike from IP_3-sensitive stores, followed by a sustained elevation and oscillations, which were dependent upon extracellular calcium. Protein kinase C was also found to modulate these effects. Ethanol selectively inhibited these calcium responses in a concentration- and duration-dependent manner. Acute (5 min) ethanol exposure had no effect, while short-term exposure (30 min) to high concentrations (100-250 mM) partially inhibited these responses, and long-term exposure (24 h) inhibited these responses to a greater extent and at a lower concentration (10 mM). The inhibition was variable, but was more consistent at higher concentrations of ethanol. The effects persisted after ethanol removal, and responses returned to control levels after 24 h. A similar time-course of ethanol effects was seen on carbachol-induced ^3H-thymidine incorporation. Further experiments determined the calcium-dependency of

muscarinic-induced proliferation. Directly increasing calcium with ionomycin induced proliferation in these cells. Removal of extracellular calcium, or pre-incubation with nickel or cobalt, but not verapamil, nifedipine or SKF-96365, inhibited this thymidine incorporation. These data suggest that the calcium responses induced by muscarinic stimulation in astroglial cells are indirectly inhibited by ethanol and that this inhibition may mediate ethanol's inhibition of glial cell proliferation. This mechanism may be involved in the microencephaly seen in the Fetal Alcohol Syndrome.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

(n/m) ACh receptor. (nicotinic/muscarinic) acetylcholine receptor

AMPA. (±)-α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid hydrobromide

4-DAMP. 4-diphenylacetoxy-N-methylpiperidine methiodide

A/C. adenylate cyclase

ACAS. Attached-Cell Analysis and Sorter

AUC. area under the curve

BEC. blood ethanol concentration

BSA. bovine serum albumin

CaMK II. calcium- calmodulin-dependent protein kinase II

cAMP. adenosine 3',5'-cyclic monophosphate

CNS. central nervous system

CPM. counts per minute

CREB. cAMP response element binding protein

DAG. diacylglycerol

DMEM. Dulbecco's modified Eagle's medium

EGTA. ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid

FAE. Fetal Alcohol Effects

FAS. Fetal Alcohol Syndrome

FBS. fetal bovine serum

G-protein. GTP-binding protein

GABA. gamma-amino butyric acid

gcm. *glial cell missing* gene

GFAP. glial fibrillary acidic protein

HHSiD. hexahydro-sila-difenidol hydrochloride

IEG. immediate early gene

indo-1/am. indo-1 acetylmethylester

IP_2. inositol-1,4-bisphosphate

IP_3. inositol (1,4,5)-trisphosphate

IP_4. inositol (1,3,4,5)-tetrakisphosphate

NGF. nerve growth factor

NMDA. N-methyl-D-aspartate

PI. phosphatidylinositol

PIP_2. phosphatidylinositol (4,5)-bisphosphate

PKA. protein kinase A

PKC. protein kinase C

PLC. phospholipase C

PMA. phorbol 12-myristate 13-acetate

repo. *reversed polarity* gene

ROCC. receptor-operated calcium channel

VOCC. voltage-operated calcium channel

ACKNOWLEDGMENTS

I would like to acknowledge my advisor, Dr. Lucio Costa, for his support over the years. He has always, whether in Seattle or Italy, been available to advise and mentor me, maintaining focus and organization in my research. He has also been a great friend and has kept his sense of humor, which has helped me maintain my sanity.

The rest of my committee members, Dr. Mark Cooper, Dr. Terry Kavanagh, Dr. Elaine Faustman, and Dr. Diana Patterson have had valuable input, and have always been supportive and encouraging. I especially want to thank Dr. Terry Kavanagh for his encouragement, guidance, technical assistance on the ACAS, and the interest he has taken in my research; he brings not only a wealth of knowledge, but also a lot of warmth and caring to the Toxicology Program.

The members of the Costa lab have been great to work with. Janet Peters not only got the lab incredibly organized (which we appreciate to this day at times of safety inspections), but was also a pleasure to work with. I want to thank Dr. Marina Guizzetti for her scientific conversations and advice, her wonderful lab management skills, and her always pleasant demeanor. Dr. Kevin Yagle not only provided scientific advice, but also excellent movie advice and camaraderie through tissue culture problems. Hailing Lu and Min Wei, my fellow students in the lab, have been very helpful, especially with tissue culture. Lynn Fritzen provided administrative assistance whenever I needed it.

There are many people at Roosevelt who keep the facilities and equipment running smoothly. Terry Kavanagh provided immense technical assistance with my calcium measurements on the ACAS. Colin White was also invaluable in keeping the ACAS, and everything else in the building, running. Dianne Neil and Julia Tracy have maintained a clean and organized tissue culture facility, despite a chaotic amount of use.

I would not have persisted through these years without a great deal of encouragement, friendship, 'venting' capability, and chocolate, from people who not only kept me sane in the lab, but also helped me to escape it. These include Dr. Nancy Beck, Gail Gislason, Dr. Mara Seeley, Sherry Baron, Dr. Tom McHugh, Dolo Diaz, Dr. Sandy Kirchner, Pat Jansen, Dr. Lynnda Reid, Noel Hudson, Dianne Neil, and Dr. Yan Lu. Sara Griggs took care of all my graduate school and taxation questions, and her support and

cheerfulness always made my day better. Having a roof over your head is a basic need, but my housemates, Nancy Beck, Karl Seeley, and Dolo Diaz have taken housing beyond the basic need, making 2212 E. Roanoke Street very much a home.

The support and encouragement I received from all my family was priceless. I especially wish to thank Ann, for her constant encouragement and her assistance in keeping the phone companies in business, Barb, for her phone calls, airport shuttle service, and wardrobe assistance, and Brian, for being so enthusiastic about being mentioned in my Master's thesis that I had to mention him here. I would also like to thank my Aunt Marg for her friendship and always being there whenever, and for whatever, I needed her, and my Aunt Theresa for her support of education throughout my life (although she probably never imagined I would take her advice to this extreme). Shirley and George Berry have also been incredibly supportive friends through the years, and still make me feel at home on Napier Street.

This research would not have been possible without financial support from the National Institute of Alcohol and Alcohol Abuse (grant number AA-08154), the National Institute of Environmental Health Science (grant number ES-07133), and the Alcohol and Drug Abuse Institute, University of Washington.

DEDICATION

I wish to dedicate this thesis to my family, especially my parents, Frank and Noreen Catlin, who have always been extremely supportive of my education, and the decisions I have made in my life.

CHAPTER 1: INTRODUCTION

Statement of Research Problem

Ethanol is the second most widely abused drug in the world (US Public Health Service, 1990), with some reports citing that approximately 7% of adults in the United States are alcoholics and greater than 20% of hospitalized patients have a medical problem related to drinking (Diamond and Gordon, 1997). A recent editorial in *Science* further highlights the societal implications of drinking, citing a 1994 Robert Wood Johnson Foundation report indicating that alcohol abuse costs society nearly $100 billion annually (Bloom, 1997). Clinically, ethanol exposure can cause neurotoxicity following both acute and chronic intake, and *in utero* exposure can cause permanent damage in the offspring. The acute effects of ethanol in humans and the blood alcohol concentrations at which the various effects are seen, have been previously discussed (Little, 1991; Cotran *et al.*, 1994). Briefly, ethanol causes an initial euphoria, presumably through a loss of inhibitory control centers, followed by central nervous system (CNS) depression. As the dose of ethanol increases, impaired cognition, ataxia, memory loss, sedation, coma and death due to respiratory arrest can occur. A greater societal problem with respect to alcohol is the development of alcohol dependence or alcoholism. The neurotoxicological effects of chronic alcoholism consists of Wernicke's encephalopathy, which can progress to Korsakoff's syndrome, and include unsteady gait, cerebellar degeneration, and memory impairment (Cotran *et al.*, 1994).

Exposure to ethanol *in utero* can result in the Fetal Alcohol Syndrome (FAS). Although detrimental effects of ethanol on the fetus have been alluded to for centuries, FAS was not described clinically until the early 1970's (Jones *et al.*, 1973). The principal features of FAS are specific facial characteristics, pre- and post-natal growth deficiency, and central nervous system (CNS) dysfunctions (Clarren and Smith, 1978). The CNS effects are the most detrimental of ethanol's teratogenic effects and include mental retardation, behavioral effects and neurologic effects, including microcephaly. The CNS dysfunction is present at birth and persists through adolescence and well into adulthood (Day *et al.*, 1991; Streissguth *et al.*, 1991; Lemoine and Lemoine, 1992). In addition to

FAS, Fetal Alcohol Effects (FAE) have been described following exposure to lower levels of ethanol *in utero* and include decreased mental functions (Rossett *et al.*, 1981; Streissguth *et al.*, 1990; Jacobson *et al.*, 1993). Due to its numerous teratogenic effects and widespread abuse, alcohol is the leading environmental causes of mental deficiency in Western society and a major clinical problem (Hansen *et al.*, 1978). Although it has been over 20 years since FAS was first described and a great deal of epidemiological, animal and cellular research has been done on ethanol teratogenesis, the mechanisms of ethanol's neuroteratogenic effects remain elusive. This dissertation investigates possible mechanisms underlying the neuroteratogenic effects of ethanol.

In the fetus, the main CNS targets of ethanol are the hippocampus, the cerebellum and the cerebral cortex (Abel, 1985). More than half of the volume of the brain is comprised of glial cells. Astrocytes are the most abundant type of glial cell and play an important role in brain development and growth (Pellegrino *et al.*, 1993; Travis, 1994). There is evidence that cell proliferation is mediated by a rise in intracellular calcium following activation of the inositol phosphate second messenger system (Clapham, 1995), and that the binding of acetylcholine to its muscarinic receptors increases intracellular calcium and proliferation in astrocytes (Ashkenazi *et al.*, 1989; Guizzetti *et al.*, 1996). Ethanol has been shown to interfere with some aspects of this muscarinic response in tissue slices (Balduini and Costa, 1989; Balduini and Costa, 1990), mixed cortical cultures (Kovacs *et al.*, 1995), and in astrocytes (Guizzetti and Costa, 1996). This research tested the hypothesis that ethanol exposure during development causes central nervous system dysfunction through effects on astrocytes, specifically on their calcium response to muscarinic stimulation, thereby interfering with the normal mitogenic response to muscarinic agonists.

Astroglia

The two major cell types in the brain are neurons and glia. Glial cells comprise over half the volume of the brain and outnumber neurons nine to one. Although these cells were long thought to be only supporting cells or "glue" for the neuronal cells, many active functions of glial cells are being discovered as more research is carried out on these cells (Travis, 1994). Glial cells in the CNS are subdivided into microglia and

macroglial cells, which include oligodendrocytes and astrocytes (Privat *et al.*, 1995). Astrocytes are the focus of this research.

Characteristics of Astrocytes

Astrocytes, named after their stellate or star-like shape, are classified based on morphology into fibrous and protoplasmic astrocytes. These labels, however, are not useful when astrocytes are cultured *in vitro*, and a separate set of classifications has been devised based on *in vitro* morphology and cell-specific markers, separating them into type 1 astrocytes and type 2 astrocytes (see Privat, 1995). When grown in the presence of serum, type 1 astrocytes are flat, epithelial-like cells and are negative for the A2B5 antigen and positive for the RAN-2 antigen. Under similar conditions, type 2 astrocytes are process-bearing, stellate cells and are positive for the A2B5 antigen and negative for the RAN-2 antigen (Levison and Goldman, 1993). Notably, mature type 2 astrocytes are no longer mitotic so this phenotype is transient *in vitro* (Lilien and Raff, 1990). Both type 1 and type 2 astrocytes express the astrocyte-specific intermediate filament protein, glial fibrillary acidic protein (GFAP) and can be identified immunohistochemically by the presence of this protein (Levison and Goldman, 1993).

The ability to grow pure (>98%) cultures of astrocytes (McCarthy and DeVellis, 1980) has facilitated research on astrocytes and has allowed the complexity of these cells to be realized. Astrocytes have been shown to express receptors for a wide number of neurotransmitters and neuropeptides (Hosli and Hosli, 1993). The neurotransmitter receptors on astrocytes include amino acid receptors, such as γ–aminobutyric acid (GABA) and glutamate receptors, adenosine receptors, and amine receptors. The amine receptors expressed by astrocytes include adrenergic, histaminergic, dopaminergic, serotoninergic, and cholinergic receptors. Autoradiographic data and labeling with specific antibodies have demonstrated the presence of both nicotinic and muscarinic receptors in astrocyte explant cultures, with some evidence indicating that the density of nicotinic receptors is lower than that of muscarinic receptors (Hosli *et al.*, 1992; Hosli and Hosli, 1993). Immunocytochemical staining has demonstrated the presence of muscarinic receptors on astrocytes in 3 month old and 30-34 month old rat cortices (Van Der Zee *et al.*, 1993). Additional evidence from our laboratory shows the expression of muscarinic messenger ribonucleic acid (mRNA) for m2 and m3 receptors, and low levels

of m5 receptor, in cultured cortical astrocytes using reverse transcription-polymerase chain reaction studies and specific probes for the subtypes of the muscarinic receptors (Guizzetti *et al.*, 1996). Similar results were found in the human astrocytoma cell line 132 1N1 (Guizzetti *et al.*, 1996).

In addition to evidence that receptors are expressed in astrocytes, there is biochemical evidence that the receptors are functional. In astrocytes, the excitatory amino acid glutamate alters intracellular calcium levels (Cornell-Bell *et al.*, 1990; Kim *et al.*, 1994), and causes a reduction in cell proliferation (Nicoletti *et al.*, 1990; Guizzetti *et al.*, 1996). Electrophysiological studies have indicated the functionality of peptidergic, adrenergic, and cholinergic receptors (Hosli *et al.*, 1992; Hosli *et al.*, 1993). As well, there is evidence for active cholinergic receptors on astrocytes, as addition of carbachol, a stable analogue of acetylcholine, results in an increase in cellular proliferation (Ashkenazi *et al.*, 1989; Guizzetti *et al.*, 1996). Carbachol has also been shown to increase intracellular calcium and the formation of inositol phosphate in a mixed culture of cells containing 70% cortical astrocytes (Kovacs *et al.*, 1995). In human 1321N1 astrocytoma cells, carbachol causes an increase in intracellular calcium (Brown-Masters *et al.*, 1984) and induces proliferation (Guizzetti *et al.*, 1996).

In addition to cellular receptors, astrocytes have been shown to have a number of functional ion channels. Ligand activated channels have been identified on astrocytes linked to $GABA_A$ receptors and non-N-methyl-D-aspartate (NMDA) glutamate receptors, as well as stretch activated channels (Sontheimer, 1992). Voltage-activated channels, which for many years were thought to be limited to excitable cells such as neurons and muscle cells, have also been demonstrated to be on astrocytes (MacVicar, 1984). Voltage operated potassium channels, sodium channels, and calcium channels have been found on astrocytes. Voltage-operated calcium channels (VOCCs) have been subdivided pharmacologically and the three most abundant subtypes are: L-type, T-type and N-type currents channels (Hofmann *et al.*, 1994). Both L-type and T-type calcium channels are present on rat astrocytes, but no evidence has been found for the presence of N-type channels (Sontheimer, 1994). L-type calcium channels characterized as having long-lasting currents activated by high voltage (membrane potential around -30 mV) which are blocked by dihydropyridine compounds and by verapamil (Hofmann *et al.*, 1994). T-type channels are activated and inactivated at low membrane potentials and do not have any

specific blockers, making them more difficult to study (Hofmann *et al.*, 1994). It has also been demonstrated that the voltage operated currents in astrocytes are sufficient to elevate intracellular calcium (MacVicar *et al.*, 1991). It should be noted, however, that the expression of the channels is often dependent on experimental conditions such as certain serum lots and the presence of cAMP analogues (Barres *et al.*, 1989).

Another characteristic of astrocytes is the presence of gap junctions which connect them to other astrocytes and glial cells. Such junctions have been seen in both cultured astrocytes (Kettenmann *et al.*, 1983) and in astrocytes *in vivo* (Dermietzel *et al.*, 1989), and it is with these junctions that astrocytes form a coupled syncytium which has been hypothesized to be important for long range signaling (Fischer and Kettenmann, 1985)

Functions of Astrocytes

An increasing number of functions have been proposed for astrocytes as the complexity of these cells becomes evident. These functions range from regulating the composition of the extracellular milieu, to forming a network of cells which comprise a long-range pathway as important to brain function as neuronal networks.

One of the first roles of astrocytes discovered was regulation of the extracellular environment. *In situ* experiments have demonstrated that astrocytes maintain the potassium ion homeostasis necessary for proper neuron functioning and are involved in the clearance of neurotransmitters from the synaptic area (Kimelberg *et al.*, 1993). There is evidence that in addition to being involved in the uptake of neurotransmitters, astrocytes may also release neurotransmitters. Type 2 rat cerebral cortical astrocytes released ^{3}H-GABA and ^{3}H-D-aspartate, in a Na^{+}- but not Ca^{2+}-dependent manner in response to non-NMDA agonists. Exposure to 50 mM KCl was shown to cause the release of taurine, glutamate and aspartate in type 1 and type 2 astrocytes (Gallo *et al.*, 1989; Gallo *et al.*, 1991). The sodium characteristics of this release suggest that it may be mediated through a reversal of the membrane carrier proteins which are responsible for the uptake of neurotransmitters (Levi *et al.*, 1992; Martin *et al.*, 1992).

There is increasing evidence that communication occurs between astrocytes and neurons, and that astrocytes, through this communication, comprise a long-range signaling network. The presence of functional receptors, the capability to release neurotransmitters, and the formation of an astrocyte syncytium provide the mechanism by which astrocytes could signal distinct cells. The presence of such a signaling system is supported by electrophysiological data in astrocyte cultures (Murphy *et al.*, 1993), and by examining calcium signaling in pure astrocyte cultures (Cornell-Bell *et al.*, 1990), mixed neuronal-astrocyte cultures (Charles, 1994; Nedergaard, 1994), and hippocampal slices (Dani *et al.*, 1992). Astrocyte-neuronal interactions have been shown to be essential for synaptic transmission using a specific astrocytic metabolic inhibitor and studying neuronal electrophysiological responses in hippocampal slices (Keyser and Pellmar, 1994). Recently, astrocytes have been shown to receive signals from neurons. Astrocytes responded to stimulation of neurons, as assessed by electrophysiological measures, in a manner which is consistent with ligand receptors, indicating that the response is mediated by neuronal release of glutamate (Mennerick *et al.*, 1996).

Evidence is accumulating that astrocytes are integral in the development of the CNS. Astrocytes synthesize and secrete a variety of trophic factors; for example, type 1 astrocytes secrete nerve growth factor (NGF) in culture (Rudge *et al.*, 1992). Communication between astrocytes and neurons during development is supported by the increase in glial cell production of NGF seen in response to low levels of this trophic factor, which could come from developing neurons in the area (Zimmermann *et al.*, 1994). Also, the levels of expression of the S100 protein, which is specifically localized in astrocytes and oligodendrocytes in the CNS, correlates with process elongation in cortical neurons and cerebral cortical astrocyte proliferation (Marshak, 1990). Further evidence that glial S100 is involved in stimulating proliferation and development comes from the fact that S100ß, the disulfide-linked homeodimer protein of S100, increases the intracellular concentration of calcium in C6 glioma and SH-SY5Y neuroblastoma cells (Barger and Van Eldik, 1992), stimulates neurite outgrowth (Kligman and Marshak, 1985), induces *c-myc* and *c-fos* mRNA, and stimulates the proliferation of astrocytes (Selinfreund *et al.*, 1991). In addition, molecular biology studies show that antisense oligodeoxynucleotides for S100ß decrease the rate of cellular growth (Selinfreund *et al.*, 1990) and overexpression of the S100ß protein acts as a growth factor in transgenic mice (Reeves *et al.*, 1994).

In addition to this circumstantial evidence that astrocytes are involved in CNS development, more concrete evidence has been found that shows astrocytes are necessary for appropriate neuronal development. Astrocytes have been shown to promote dendritic extensions in neurons *in vitro* (Tropea *et al.*, 1988) and are important in the formation of functioning synapses (Nakanishi *et al.*, 1994). Further research has very elegantly shown a requirement for glial cells during *Drosophila* development (Hosoya *et al.*, 1995; Jones *et al.*, 1995). Two groups of researchers have identified and cloned a gene, named *glial cells missing* (*gcm*), that encodes a nuclear protein which acts as a binary switch for the development of almost all glial cells; the expression of this protein signals cells to develop into glial cells while its absence results in the cells developing into neurons (Hosoya *et al.*, 1995; Jones *et al.*, 1995). In *Drosophila* containing a mutated form of this gene, almost no glial cells are present and, although this mutation is embryo-lethal, examination of the brain prior to death shows that axons do not form their appropriate pathways, proper neuronal differentiation does not occur, and there is decreased neuronal survival in late stage mutant embryos. This indicates that glial cells are necessary for these aspects of development. Similarly, in work on another *Drosophila* mutant, termed the *reversed polarity* (*repo*) mutant, in which terminal differentiation of glia does not occur in the visual system, there was an increase in neuronal apoptosis in this area (Xiong and Montell, 1995).

With all the functions of astrocytes and their role in the development of the brain, disruption of their functions by ethanol could result in permanent effects on the function of the CNS. Therefore, these cells must be considered possible targets for ethanol neuroteratogenesis.

Cholinergic System

Acetylcholine (ACh) is a major neurotransmitter in the central nervous system. ACh binds two general subtypes of receptors, the muscarinic (mACh) receptors and the nicotinic (nACh) receptors. The nACh receptors are directly coupled to an ion-channel which is permeable to Na^+, K^+, and, to a lesser extent, Ca^{2+}. The activation of this receptor results in a depolarization across the cellular membrane and the opening of the channel, with the main result being an influx of sodium ions (Alberts *et al.*, 1989). The

nACh receptor does not appear to be involved in the proliferative response of astrocytes following cholinergic stimulation (Guizzetti *et al.*, 1996).

The mACh receptors belong to a superfamily of seven-pass transmembrane receptor proteins which are linked to a GTP-binding protein (G-protein). Initial subclassification of the muscarinic receptors was based on selective agonists and antagonists. More recently, the muscarinic receptors have been further subtyped based on deoxyribonucleic acid (DNA) sequencing, and cloned, with five subtypes having been identified to date: the *m1, m2, m3, m4* and *m5* receptors (Bonner, 1989). These five receptors have a great deal of sequence identity; the highest identity is in the transmembrane domain and the least conservation is in the third intracellular loop. This third intracellular loop is the site of interaction between the receptor and the cell signaling system, that is, the site of interaction with a G-protein. It has been demonstrated that the *m1, m3* and *m5* receptors are coupled via a G-protein to the phosphatidylinositol (PI) second messenger system, while the *m2* and *m4* subtypes are coupled to a G-protein which acts to inhibit the adenylate cyclase (A/C) second messenger system (Bonner, 1989).

Receptor-binding studies show that during rat development there is a gradual increase in the levels of muscarinic receptors to adult levels , with adult levels present by postnatal day 30 (Coyle and Yamamura, 1976; Kuhar *et al.*, 1980). Contrary to this, the breakdown of lipid phosphoinositides, a cellular response to activation of some subtypes of the muscarinic receptors, peaks at postnatal day 6-8 in rats and then decreases to adult levels. This suggests that receptor coupling to second messenger systems changes during development (Balduini *et al.*, 1987; Balduini *et al.*, 1991a). A similar developmental pattern was seen with different ages of mixed cortical cultures responding to carbachol (Kovacs *et al.*, 1995). This peak in activity corresponds to the brain growth spurt in the rat, suggesting that acetylcholine may play a special role in the synaptogenesis and cell growth that occurs during the brain growth spurt. If carbachol plays a role during development, disruption of the carbachol-mediated response by *in utero* exposure to ethanol may represent a mechanism of ethanol's neuroteratogenesis.

Second Messenger Systems

Cells have developed many systems by which external stimuli can be transduced across the membrane to the nucleus, resulting in a cellular response. Many extracellular receptors are directly coupled to ion-channels, as in the case of the nACh receptor. Others, such as some growth hormone receptors, contain intrinsic tyrosine kinases in their intracellular motifs. Whereas still others, such as the mACh receptors, are linked to internal messenger systems via membrane–bound G–proteins, as shown in Figure 1. In the inactive state, G-proteins are trimeric heterodimers, consisting of an alpha-(α), beta-(ß) and gamma-(γ) subunit, with a GDP-bound to the α-subunit. The binding of a ligand to a receptor activates the G-protein, causing dissociation of the GDP and replacement by GTP. This causes a conformational change in the α -subunit and dissociation of this subunit from the ßγ complex and from the receptor complex. The Gα subunit has intrinsic GTPase activity as a mechanism of rapidly turning off the activation state (Neer, 1995).

More than twenty isoforms of the Gα-subunit have been discovered to date in mammals and have been characterized as to the spectrum of their activity and their sensitivity to specific toxins. The isoform of this subunit determines the cellular response after a ligand binds to a specific G-protein linked receptor. The $G\alpha_S$ isoform acts on A/C causing the activation of this enzyme, whereas the family of $G\alpha_i$ proteins inhibits this enzyme. $G\alpha_q$-related proteins activate phospholipase C (PLC). The G_S protein is sensitive to uncontrolled activation by cholera toxin and the G_i proteins are sensitive to inhibition by pertussis toxin. The G_q proteins are insensitive to both cholera and pertussis toxin inhibition (Neer, 1995).

Phosphatidylinositol Signaling System and Muscarinic Receptors

As previously mentioned, the *m1*, *m3* and *m5* ACh receptor subtypes are linked to the PI second messenger system. The cellular response to this system is shown in Figure 1. The G-protein associated with these subtypes of receptors contains $G\alpha_q$-

related subunits and, therefore, activation of this receptor results in the activation of the PLC-ß isozyme (Smrcka *et al.*, 1991; Bernstein, 1992). Once activated, PLC-ß catalyses the breakdown of the membrane lipid, phosphatidylinositol (4,5)-bisphosphate (PIP_2), to two important intracellular messengers, inositol (1,4,5)-trisphosphate (IP_3) and diacylglycerol (DAG) (Alberts *et al.*, 1989).

Upon activation of PLC-ß, the intracellular concentration of IP_3 increases from basal levels of 0.1 to 0.2 μM up to 1 μM (Bradford and Rubin, 1986), and IP_3 binds to a specific receptor in nonmitochondrial calcium stores causing the release of calcium to the cytosol. Therefore, one net effect of acetylcholine binding to the *m1, m3*, and *m5* receptors is a rise in cytosolic calcium levels, an intracellular messenger which will be discussed in detail below. IP_3 is rapidly metabolized by 5-phosphatase to the inactive compound inositol-1,4-bisphosphate (IP_2). IP_2 is further dephosphorylated to regenerate free inositol for recycling to PIP_2. IP_3 can also be phosphorylated by a 3-kinase to inositol 1,3,4,5-tetrakisphosphate (IP_4) which has activity in the cell, including possible involvement in calcium signaling, although this role is controversial (Irvine, 1992). IP_4 can be further phosphorylated to generate active, higher phosphorylated inositol compounds. The relative amount of the 5-phosphatase and the 3-kinase pathways involved physiologically in the breakdown of IP_3 is still controversial (Shears, 1992) .

DAG remains located at the membrane where it activates protein kinase C (PKC), a calcium–dependent serine/threonine kinase. PKC is a family of proteins which phosphorylate serine and threonine residues. There are three different classes of PKCs, the classical PKCs (α,β,γ) which are calcium- and DAG-dependent, the new PKCs (δ,ε,η,μ) which are not stimulated by calcium, and the atypical PKCs (ζ,λ), which are not dependent upon DAG (Nishizuka, 1995). There is evidence that PKC plays a feedback role on PLC activation. A short-term (10 min) incubation with activators of PKC inhibits the activation of PLC, probably due to phosphorylation at site on PLC that is involved in the interaction of PLC with its G-protein (Balduini *et al.*, 1990; Ryu *et al.*, 1990; Davis, 1992). Evidence suggests that PKCα is the isozyme responsible for this feedback inhibition (Huwiler *et al.*, 1997). This feedback loop has been seen in a variety of cell types, including neurons (Paulsen *et al.*, 1994) and astrocytes (Drouva *et al.*, 1991; Chen *et al.*, 1995; Mangoura *et*

al., 1995) following stimulation with a variety of neurotransmitters, including carbachol (Mangoura *et al.*, 1995), although the effect of this feedback on carbachol-induced calcium responses in astrocytes remains unknown. DAG can also be further cleaved to arachidonic acid, which can be metabolized to eicosanoids and prostaglandins, all of which have a variety of actions in the cell. There is also evidence that PKC is involved in the carbachol-induced proliferation of astrocytes and astrocytoma cells, however, the role of the three isozymes expressed in these cells (PKCα, ε, and ζ) has not been clarified yet (Guizzetti *et al.*, 1998).

Adenylate Cyclase Signaling System and Muscarinic Receptors

The *m2* and *m4* acetylcholine receptors are linked to the A/C second messenger system (Coyle and Yamamura, 1976) via a G-protein which contains the $G\alpha_i$-subunit (Parker *et al.*, 1991). Upon activation, this Gα-subunit inhibits the function of membrane-bound A/C. A/C synthesizes cyclic AMP (cAMP) from ATP. cAMP activates cAMP-dependent protein kinase A (PKA) which phosphorylates serine and threonine residues in assorted proteins. This system is turned off by cAMP phosphodiesterases which hydrolyzes cAMP to adenosine 5'-monophosphate (Alberts *et al.*, 1989). Therefore, activation of *m2* and *m4* receptors decreases A/C activity, thereby decreasing the level of PKA activity.

Intracellular Calcium in Astrocytes

As previously discussed, activation of cell surface receptors can cause an increase in intracellular calcium via signal transduction systems. Calcium is a very important intracellular messenger in all cell types and at all stages of development, mediating a large number of cellular responses (Berridge, 1993) .

Localization and Regulation of Intracellular Calcium

The cytosolic calcium level in cells is maintained very low (≈ 0.1 μM or less) relative to the high levels in the extracellular milieu and specialized intracellular storage compartments (Finkbeiner, 1993). The resting levels of cell calcium in rat cortical

astrocytes range from 34 nM to 350 nM, depending on the study (Finkbeiner, 1993). This wide range could be due to spontaneous spikes which have been seen in type 1 rat cortical astrocytes (Fatatis and Russell, 1992). The human astrocytoma cell line, 1321N1, which will also be used in this research, has been reported to have a basal calcium level slightly higher than that seen in primary astrocytes, ranging from 170 to 237 nM (Finkbeiner, 1993). A 10, 000 fold calcium gradient must be maintained across the plasma membrane, making calcium the most highly regulated ion in nature (Clapham, 1995). The calcium gradient is maintained by Na^{+}/Ca^{2+} exchange, Ca^{2+}-binding proteins, movement into mitochondria, and by active calcium pumps. Calcium pumps are located on the plasma membrane and the membranes of intracellular organelles which act as calcium stores (Grover and Khan, 1992). These intracellular stores appear to be part of the endoplasmic reticulum in astrocytes (Gambetti *et al.*, 1975). Most of the calcium in the endoplasmic reticulum is bound to proteins and is not available for cell signaling until released into the cytoplasm.

Three messengers have been identified which can trigger the release of calcium from intracellular stores: IP_3 by binding to the IP_3 receptor, cyclic adenosine 5'-diphosphate ribose (cADPR) by actions at the ryanodine receptor, and nicotinic acid adenine dinucleotide phosphate (NAADP) by an unknown mechanism. The characteristics and possible functions of these systems have been reviewed (Galione, 1994; Sitsapesan *et al.*, 1995; Dousa *et al.*, 1996; Genazzani and Galione, 1997; Lee, 1997; Lee, 1998). These three signaling pathways have been shown to be independent of each other in many systems (Chini *et al.*, 1995; Lee and Aarhus, 1995; Perez-Terzic *et al.*, 1995; Genazzani and Galione, 1996; Lee, 1997; Albrieux *et al.*, 1998). Activation of the IP_3 and ryanodine receptors causes the opening of a calcium permeable channel through which calcium flows down its concentration gradient into the cell cytosol, increasing the cytosolic concentration of free calcium to several micromolar within seconds (Bootman and Berridge, 1995). Following an increase in intracellular calcium, calcium pumps remove the cytosolic calcium to return the concentration of free cytosolic calcium to resting levels.

Intracellular Calcium Responses

As previously discussed, astrocytes have functional receptors for a variety of neurotransmitters, the activation of which induces a rise in intracellular calcium. Some

of the first evidence for neurotransmitter-induced increases in intracellular calcium in astrocytes was from Pearce, who showed that astrocytes have a calcium response to glutamate (Pearce *et al.*, 1986a). The astrocytic calcium response is not homogenous, but varies between subsets of astrocytes (McCarthy and Salm, 1991) and is heterogeneous even within a pure culture of a specific type of astrocytes (Dave *et al.*, 1991). In general though, rat cortical astrocytes have been demonstrated to respond to many ligands with an increase in intracellular calcium, including glutamate and its analogues (Cornell-Bell *et al.*, 1990; Glaum *et al.*, 1990; Jensen and Chiu, 1990), ATP, adrenergic agonists (Dave *et al.*, 1991; McCarthy and Salm, 1991), and carbachol (Dave *et al.*, 1991). The human astrocytoma cell line 1321N1 has also been shown to respond with an increase in intracellular calcium following stimulation with carbachol (Noronha-Blob *et al.*, 1987).

Confocal microscopy and the advent of calcium-sensitive dyes has allowed the calcium response within individual cells to be imaged and has demonstrated that this response is very complex. The response to glutamate in hippocampal astrocytes has been well characterized and will be used as an example (Cornell-Bell *et al.*, 1990). Following stimulation with 100 μM glutamate, most cells respond with an initial spike followed by some form of calcium oscillation. These oscillations have been categorized into three types: a sustained oscillatory response, a dampened-oscillatory response and a non-oscillatory response. Experiments in calcium-free solutions show that the spike remained, but only a few oscillations were sustained. This indicates that glutamate-induced release of calcium from intracellular stores was responsible for the spike, but that the sustained elevation and calcium oscillations require influx of calcium from the extracellular buffer. A similar calcium response has been seen following carbachol treatments in rat cortical astrocyte cultures (McCarthy and Salm, 1991), and in mixed cortical cultures containing 70% astrocytes (Kovacs *et al.*, 1995), but the carbachol response has not been well characterized in pure astrocyte cultures. Cortical astrocytes, however, have been seen to not be as homogenous as hippocampal astrocytes in there response to glutamate and other ligands, as rarely do all cortical astrocytes respond to a stimulus (McCarthy and Salm, 1991).

These complex intracellular calcium responses following the activation of PLC have been seen in a wide variety of cell types, including excitable cells such as nerve cells and muscle cells, and non-excitable cells, including astrocytes, oocytes, pancreatic acinar cells, fibroblasts and lymphocytes. The characteristics of the oscillations and waves,

however, differ between cell types (Meyer, 1991). A great deal of research has gone into understanding the mechanism underlying these intracellular calcium responses and many models have been proposed to describe the generation of these responses. All of these models begin with the release of calcium from intracellular stores by the binding of IP_3 to its intracellular membrane receptor. Some groups have proposed an oscillation of IP_3 levels, possibly by calcium regulation of PLC activity, to be responsible for the oscillation of calcium levels; this theory is supported by both experimental evidence (Harootunian *et al.*, 1991) and mathematical models (Cuthbertson and Chay, 1991). However, in contradiction with this model, there is also evidence showing that calcium waves occur in the presence of a constant level of IP_3 (Clapham, 1995). In other models, the levels of IP_3 remain constant and it is calcium itself that feeds back to create the oscillations, resulting in Ca^{2+}-induced Ca^{2+} release (CICR). One such model is based on the presence of two separate pools of calcium, one sensitive to IP_3, the other IP_3-insensitive (Berridge, 1991; Dupont *et al.*, 1991). Other models propose a single pool of calcium where calcium and IP_3 together affect the calcium release (Dupont and Goldbeter, 1993). Recently, models have been proposed for calcium changes in non-excitable cells in which IP_3 receptor mediated release of calcium is modulated by calcium itself. Calcium has been shown to affect the activity of the IP_3 receptor in a bell-shaped manner. That is, at low concentrations of calcium, calcium stimulates further release of calcium through this channel, whereas at high concentrations, calcium inhibits its release (Wakui *et al.*, 1990; Wakui and Petersen, 1990; Bezprozvanny *et al.*, 1991; Finch *et al.*, 1991; Missiaen *et al.*, 1992; Taylor and Marshall, 1992). However, there is some debate that this calcium regulation of the receptor is an artifact due to the effects of the chelators used for these types of experiments (Richardson and Taylor, 1993; Combettes and Champeil, 1994), although this has been refuted (Finch and Goldin, 1994). A mechanism which supports calcium modulating the function of the IP_3 receptor has been reported. A calcium-activated phosphatase, calcineurin, is anchored to the IP_3 receptor and can regulate the phosphorylation state of this receptor, This would account for the calcium regulation of calcium release (Cameron *et al.*, 1995).

This CICR-mediated by feedback on the IP_3 receptor can account for the elementary Ca^{2+}-release that is seen in many cell types (Bootman and Berridge, 1995).

Small puffs or sparks of calcium are seen at a particular area within the cell prior to the wave generation, giving initial calcium release a quantal or graded nature (Bootman *et al.*, 1994; Cannell *et al.*, 1995; Yao *et al.*, 1995). However, in astrocytes an all-or-none or threshold type calcium response has been seen following stimulation with a variety of agonists, including carbachol (Shao and McCarthy, 1995). In this case, calcium levels went from no change to a maximal response between only a five-fold increase in the concentration of ligand, indicating that the response in astrocytes may not be graded. Therefore, the mechanism of calcium responses may vary between cell types, with astrocytes exhibiting a threshold response, in contrast to the quantal responses seen with cardiac muscle cells and *Xenopus* oocytes.

Intercellular Calcium Waves

In addition to the complex patterns of calcium spikes and oscillations within individual cells following stimulation, stimulation of astrocytes sets in motion a wave of elevated calcium which propagates beyond individual cell borders through a network of astrocytes. It has been known for many years that astrocytes in culture form a syncytium of cells connected by gap junctions (Fischer and Kettenmann, 1985). The first evidence for the existence of long-range signaling within a syncytium of astrocytes via intercellular calcium waves was in hippocampal astrocytes following exposure to glutamate (Cornell-Bell *et al.*, 1990). Since this initial finding, long-range signaling has been confirmed in similar cultures with different stimuli (Charles *et al.*, 1991; Cornell-Bell and Finkbeiner, 1991), and in other preparations, including cortical astrocyte cultures (Enkvist and McCarthy, 1992) and fresh hippocampal slices (Dani *et al.*, 1992). It was proposed that these intercellular waves might be due to transfer of a messenger through gap junctions (Cornell-Bell *et al.*, 1990) . The involvement of gap junctions is supported by the fact that compounds which close gap junctions, such as halothane and octanol, block intercellular calcium waves (Finkbeiner, 1992). It has also been shown that wave propagation involves active regeneration of the wave since the velocity and the amplitude of the waves do not change over great distance (Smith, 1992). The identity of the messenger that travels from cell to cell is yet to be determined, although both calcium and IP_3 have been suggested (Jensen and Chiu, 1993). Studies with an IP_3 receptor antagonist, heparin, in airway epithelial cells, indicate that the messenger important in intercellular calcium waves is IP_3

(Boitano *et al.*, 1992). This is supported by other work which looks at the effects of dantrolene (which does not affect IP_3-induced calcium release) and thapsigargin (which depletes IP_3-sensitive calcium stores) on calcium waves. Dantrolene did not affect the rate or distance of propagation of calcium waves, but thapsigargin inhibited calcium waves, suggesting that IP_3 is the messenger (Charles *et al.*, 1993). Sneyd and colleagues (Sneyd *et al.*, 1994) also support IP_3 as the intercellular messenger based on a mathematical model.

The fact that in astrocytes neurotransmitters can elicit intercellular calcium waves, which can then propagate calcium signals over long distances, suggests that this could be a separate signaling pathway than neuronal excitability, which could be activated by astrocytes or by neuronal activity. This communication between neurons and astrocytes has been demonstrated for electrical responses of astrocytes (Murphy *et al.*, 1993) and for bi-directional calcium signaling between astrocytes and neurons (Charles, 1994; Nedergaard, 1994). This evidence supports the theory that calcium acts as a signal among astrocytes, as well as between neurons and astrocytes, implying that astrocytes may be equal partners with neurons in information processing in the brain.

Relevance of Ethanol Concentrations

When considering *in vitro* research on the mechanisms underlying the neurotoxicity and neuroteratogenicity of ethanol it is important that the concentrations of ethanol utilized approximate those reached *in vivo*. Pharmacokinetic studies have shown that ethanol is distributed rapidly throughout the entire body, with ethanol concentrations in tissues being well approximated by the blood ethanol levels (Lee and Becker, 1992). Research in pregnant animals has further demonstrated that ethanol concentrations are the same in the maternal blood, fetal blood, and fetal brain following maternal ingestion of ethanol in the guinea pig (Clarke *et al.*, 1986a; Clarke *et al.*, 1986b). Therefore, it can be assumed that the blood ethanol concentrations (BECs) seen in clinical and animal studies are representative of the levels in the target tissues and are rational concentrations for use in *in vitro* studies.

Legally, many states consider an individual inebriated at a BEC of 100 mg% (21.7 mM), and most people will be asleep at 350-400 mg% (76.0-86.8 mM) (Snyder

and Andrews, 1996). Such an evaluation, however, has problems due to the variability of ethanol's effects among individuals, especially when dealing with chronic alcoholics who have alterations in the metabolism of ethanol due to enzyme induction and liver damage, and who have developed a tolerance to ethanol. There have been instances where individuals have survived what would normally be considered a exceptionally high or lethal level of ethanol. Survival has been seen in an adolescent with a BEC of 757 mg% (164 mM) (Morgan *et al.*, 1995) and one case as high as 1.5 g% (325.5 mM) (Johnson *et al.*, 1982), and ranges of BECs in alcoholics have been reported as 238-489 mg% (51.6-106.1 mM) (Jones and Sternebring, 1992), and 290-421 mg% (62.93-91.36 mM) (Adachi *et al.*, 1989). These instances emphasize the high levels of BEC that are sometimes obtained in the population of concern. Work in animals has also been carried out to determine appropriate levels of ethanol for study, especially for the teratogenic effects of ethanol. Work in guinea pigs demonstrated behavioral impairment and histological effects without gross maternal or fetal toxicity when maternal BECs averaged 54 mM (Abdollah *et al.*, 1993; Catlin *et al.*, 1993). In rats, similar effects were seen at 82.4 mM (West *et al.*, 1986) and 65.1 mM (Moloney and Leonard, 1984). Therefore, cellular effects of ethanol at concentrations in the 50-100 mM range are relevant to concentrations seen in clinical populations. However, due to the high levels that have been seen clinically, cellular effects at concentrations above these levels should not be totally discounted.

Cellular Effects of Ethanol

As previously mentioned, despite a large amount of research investigating ethanol's CNS effects, the mechanisms underlying these effects remain unknown, although many have been proposed. In some instances, ethanol neurotoxicity may be secondary to malnutrition; however, while some effects of alcoholism, such as Wernicke syndrome, can be reversed by dietary intervention, other, such as the Korsakoff syndrome, can not be reversed in this manner (Cotran *et al.*, 1994). A great deal of research in animals and in *in vitro* systems has, however, clearly demonstrated that ethanol can exert direct effects on cells of the nervous system. For a long time ethanol's cellular effects were thought to be mediated by non-specific actions at the level of the lipid membrane. In recent years,

however, evidence has emerged indicating that the effects of ethanol are more specific than previously thought (Tabakoff *et al.*, 1988; Weight, 1992).

Ethanol has been shown to affect various neurotransmitter receptors, including those of glutamate, acetylcholine, serotonin, and γ-amino-butyric acid, and to alter specific second messenger systems linked to these receptors (Little, 1991; Costa, 1994; Eggeman and Browning, 1996; Diamond and Gordon, 1997). Certain ion channels, particularly calcium channels, have also been shown to display a great sensitivity to the effects of ethanol. Many of these specific effects of ethanol can result in the disruption of the normal cellular calcium homeostasis and on calcium responses.

With the central role that maintenance of calcium homeostasis and calcium responses play in cellular physiology, alterations in calcium may represent an important mechanism for ethanol's neurotoxicity. Much of the research on the effects of ethanol on cellular calcium has been done in neuronal, hepatic or cardiac cells. With the discovery of integral roles of glia in the CNS comes the possibility that the function of these cells could be a target for neurotoxicants such as ethanol, and in fact, there is evidence that astrocytes may be a target for ethanol teratogenesis, and specifically the calcium levels and responses in these cells.

Effects of Ethanol on Basal Calcium Levels

As previously mentioned, the cytosolic calcium levels in cells are tightly regulated to maintain a low basal calcium concentration (Finkbeiner, 1993). Alterations of basal calcium levels, even if transient, may have detrimental effects on CNS development. A number of studies have investigated the effects of ethanol (alone) on intracellular calcium levels. Different cellular preparations have been used, as well as different exposure protocols. Furthermore, both *in vitro* and *in vivo* experiments have been carried out. For sake of clarity, these studies are discussed based on the duration of ethanol exposure (acute vs. short term vs. chronic), and the relevance of *in vitro* findings to *in vivo* situations will be pointed out. Furthermore, potential relevance of these results to glial cells, in particular astrocytes, will be discussed.

There are inconsistent data on the ability of acute ethanol exposure to induce an immediate calcium response, as can be seen in Table 1. In a study using isolated hepatocytes, a response was seen following exposure to moderate levels of ethanol (100-300 mM) (Higashi *et al.*, 1994), which was dependent upon the activation of PLC. This response in hepatocytes was transient in nature and has been consistently seen in these cells, in studies utilizing both populations of cells as well as individual cells (Hoek *et al.*, 1992). Similarly, in PC12 cells, ethanol (400 mM) induced a calcium response, although a lower concentration of ethanol (100 mM) had no effect (Rabe and Weight, 1988). A lack of effect was also seen in cerebellar granule cells and dissociated brain cells following exposure to a low concentration (50 mM) of ethanol (Dildy and Leslie, 1989; Snell *et al.*, 1994). On the other hand, ethanol (30-600 mM) caused an increase in cytosolic free calcium in rat brain synaptosomes (Daniell *et al.*, 1987; Rezazadeh *et al.*, 1989). Furthermore, in contrast to the earlier work in PC12 cells which showed effects only at high concentrations of ethanol (Rabe and Weight, 1988), more recent work in these same cells demonstrated an ethanol-induced biphasic calcium response, with an increase in calcium levels following exposures up to 80 mM ethanol, and a diminished response at concentrations above 120 mM (Belia *et al.*, 1995). This response to ethanol was inhibited by chelation of extracellular calcium, or by blockers of voltage-operated calcium channels, indicating that it is mediated by the influx of extracellular calcium. Webb *et al.* (1995) have investigated the effect of ethanol on calcium levels in rat medial septal neurons and septohippocampal neurons using imaging techniques. In medial septal neurons from embryonic day-21 rats, low concentrations of ethanol (21.7 mM) increased basal calcium in the presence, but not absence, of NGF (Webb *et al.*, 1995); however, in postnatal day 0 preparations, this concentration of ethanol had no effect on individual cell basal calcium levels (Webb *et al.*, 1996b). In septohippocampal neurons, an increase in intracellular calcium in individual cells was seen following exposure to ethanol under certain conditions, however, as was the case with the medial septal neurons, the responses to ethanol were variable, with no clear concentration-response relationship evident (Webb *et al.*, 1996a). Mironov *et al.* (1996) also demonstrated an effect of ethanol on intracellular calcium levels. Ethanol at a concentration as low as 17 mM, induced a calcium response in individual rat hippocampal cells. This response was not dependent upon extracellular calcium, but was due to release from non-mitochondrial calcium pools, and involved both IP_3 and caffeine

sensitive stores. These researchers also demonstrated that ethanol activates PKC, and that this activation was necessary for calcium release (Mironov and Hermann, 1996).

Short-term (10-30 min) *in vitro* exposure to ethanol has also been shown to have effects on intracellular calcium levels. Studies by Davidson *et al.* (Davidson *et al.*, 1988) in synaptosomes from rat forebrain, showed that a 10 min pre-incubation with ethanol (50-500 mM) increases calcium levels in a concentration-dependent manner, irrespective of the depolarized state of the cells. In lymphocytes, Fano *et al.* (1993) also demonstrated that a 10 min incubation with ethanol (20-200 mM) increased basal calcium levels, but they found a bell-shaped concentration-response curve, with a maximum response at 60 mM ethanol following a 24 h incubation. In contrast, a short incubation (10-30 min) of skeletal muscle cells with ethanol (20-200 mM) resulted in a decrease, rather than an increase, in basal calcium concentration (Cofan *et al.*, 1995), and in mixed rat cortical cultures, which were approximately 70% glial cells, a 10 min pre-incubation with ethanol (50-500 mM) had no effect on basal calcium concentration, as measured using calcium imaging techniques (Kovacs *et al.*, 1995).

Chronic exposure (4 days) of PC12 cells to 200 mM ethanol caused a PKC-dependent increase in the uptake of radiolabelled calcium as compared to untreated control cells (Messing *et al.*, 1990). Although the relevance of calcium uptake studies as a marker for increases in intracellular calcium levels has been questioned (Davidson *et al.*, 1988), similar results were seen in fetal rat cerebellar preparations, containing predominantly Purkinje neurons, following chronic ethanol treatment (75 mM, 48 or 96 hours) by directly measuring intracellular calcium levels in single cells (Zou *et al.*, 1995), as well as measurements of currents in PC12 cells after a 6-day exposure to 200 mM ethanol (Grant *et al.*, 1993). However, in other imaging studies, ethanol did not affect basal calcium levels in cultured cortical rat neurons after 4 days (100 mM) (Blevins *et al.*, 1995), and decreased basal calcium levels in PC12 cells after a 3-day exposure (Belia *et al.*, 1995). One study carried out in rat astroglial cells (a mixture of astrocytes and oligodendrocytes) found that a one-week exposure to 50 or 100 mM ethanol caused a 50 and 100% increase in intracellular calcium, respectively (Holownia *et al.*, 1997).

A few studies have looked at the changes in resting calcium levels following chronic *in vivo* ethanol treatment. In adult rats, chronic (7 days or 8 weeks) *in vivo*

exposure to ethanol did not result in any effect on resting calcium levels (Harris and Hood, 1980; Leslie *et al.*, 1983). Although few studies have investigated the effects of *in utero* exposure to ethanol on basal calcium levels, one such study, in which blood ethanol concentrations reached 146.3 mg% (30 mM) on gestational day 15, showed no effect on basal calcium levels in dissociated whole brain preparations from less than 1 day-old neonatal rats (Lee *et al.*, 1996).

As can be seen from the results of these studies, which are summarized in Table 1, there is no consistent effect of ethanol on basal cell calcium. Acute ethanol exposure was found to either induce an increase in intracellular calcium levels or to have no effect. The particular response appears to depend upon the type of cell studied, and the differentiation and developmental stage of the cells, as well as the concentration of ethanol. In addition, even within the same cell type, contrasting results have been reported, possibly because of differences in culture conditions and experimental techniques. Similarly, short-term and chronic *in vitro* ethanol treatments have shown great variability in the calcium responses, with increases, decreases, and no effects having been reported. Exposure to ethanol has not been shown to effect calcium levels following either *in utero* or *in vivo* adult exposure, however, such exposures have not been well studied and further work needs to be carried out to confirm these results. The variable results which have been reported highlight the importance of carefully studying the effects of ethanol in the cell type of interest, and of being aware of the physiology of the cell type under different culture conditions, and on the mode of exposure to ethanol. Different cell types express different subtypes of voltage-operated calcium channels, and different receptors, channels and pumps in the intracellular stores. Furthermore, even the same cell type will express these components differently depending upon its developmental stage, differentiation state, culturing conditions, with the presence or absence of serum being a major factor. For example, astrocytes express different sub-types of voltage-operated calcium channels under different culturing conditions, and that there is a great deal of heterogeneity in these cells even within a given region, let alone from different brain regions and different types of astrocytes (Verkhratsky *et al.*, 1998). It should also be noted that following short exposures to high concentrations, or longer exposures to lower concentrations, ethanol could deplete intracellular calcium stores even though the cytosolic calcium level is constant. This effect would not be detected in the studies discussed, as no study has

looked at specific subcellular locations. This mechanism could be involved in ethanol's inhibition of the release of calcium from intracellular stores, but has yet to be investigated.

Although the data is overall inconsistent, it does indicate that ethanol can affect basal calcium levels in some cell types, either through alterations in calcium influx through voltage-operated calcium channels, or through messenger systems releasing calcium from intracellular stores. Astrocytes possess voltage-operated calcium channels under certain conditions (MacVicar, 1984; Sontheimer, 1994), as well as IP_3-sensitive calcium stores (Pozzan *et al.*, 1994). There is also evidence that astrocytes possess ryanodine-sensitive stores, as they respond to stimulation by ryanodine receptor agonists (Langley and Pearce, 1994), and also appear to have caffeine-sensitive responses (Golovina *et al.*, 1996). Limited data is available on the affect of ethanol on basal cell calcium in astrocytes, with initial results indicating that at least short incubations with ethanol have no effect on resting calcium levels. Clearly, further studies are needed in these cells to elucidate any action of ethanol on basal calcium levels upon different exposure conditions.

Ethanol and Voltage Operated Calcium Channels

In addition to maintaining constant calcium levels in the resting state, cells also respond to an array of stimuli with a rise in intracellular calcium levels. A major mechanism by which intracellular calcium concentrations can be increased is by an influx of calcium from the extracellular milieu through voltage-operated calcium channels (VOCCs). Many different subtypes of calcium channels have been identified, and the characteristics and distribution of these channels have been reviewed (DeWaard *et al.*, 1996). Experimentally, five classes of VOCCs have been distinguished based on biophysical gating characteristics and distinct pharmacology: the T-, L-, N-, P(Q), and R-type VOCCs. Although these channels were once thought to be expressed only in excitable cells, there is now evidence that VOCCs are also found in what were classically considered nonexcitable cells due to their lack of ability to respond to electrical stimulation with an action potential (Verkhratsky *et al.*, 1998), including astrocytes (MacVicar, 1984; Sontheimer, 1992; Verkhratsky and Kettenmann, 1996). Because of the role that VOCCs play in calcium responses in both excitable and nonexcitable cells, these channels have been investigated as possible targets of ethanol-induced neurotoxicity.

A large number of studies have been carried out in synaptosomal preparations, brain slices or homogenates, primary neuronal cells, and various cell lines, to investigate the effects of ethanol on VOCCs, utilizing a variety of techniques and tools, including specific channel blockers, binding proteins, and electrophysiology (Leslie *et al.*, 1990). This research has focused on alterations of VOCC function following short-term ethanol exposure as a mechanism of ethanol's acute neurotoxicity, and on changes in the density and functions of these channels following chronic ethanol exposure, as a mechanism involved in the pathologies associated with alcoholism and alcohol withdrawal. This data is summarized in Table 2. Most studies demonstrate that acute ethanol exposure inhibits the depolarization-induced influx of calcium, while chronic ethanol exposure increases this influx by increasing the number of VOCCs; this increase in channel number may also play a role in alcohol withdrawal symptoms. The increase following long term exposure could be an adaptive response to the inhibition seen following short term exposure. Some differences in the effects of ethanol are seen, however, depending upon the expression of different subtypes of calcium channels, and the developmental stage of the system studied, and, therefore, it will be very important to determine the normal channel expression in astrocytes *in vivo* to correctly interpret results from *in vitro* studies. Although astrocytes have been shown to express VOCCs under certain conditions (Verkhratsky and Kettenmann, 1996), little research has been carried out investigating the physiological role of these calcium channels in these cells, or on the ability of ethanol to inhibit their function.

There is very limited data on the effects of *in utero* exposure to ethanol on these channels, and therefore, how effects on these channels are related to the FAS. *In utero* exposure to 29-32 mM ethanol was shown to affect K^+-stimulated calcium changes, although no selectivity between N-, P- or L-type channels was seen in whole brain dissociated neurons from one-day old rats (Lee *et al.*, 1996). This lack of selectivity may indicate a difference in the effect of ethanol between prenatal and adult exposure, perhaps due to the channels that are present in the adult neurons being more sensitive to the effects of ethanol (Lee *et al.*, 1996). Due to the dramatic effects ethanol has on these channels in neuronal cell types and the role these channels may play in modulating calcium levels in astrocytes, it would be important to investigate the effects of ethanol on these channels in these glial cells, especially using a developmental model.

Ethanol and Receptor Operated Calcium Channels

In addition to voltage operated calcium channels, other types of calcium channels are expressed in the CNS, including receptor operated calcium channels (ROCCs). Glutamate, the major excitatory neurotransmitter in the CNS, binds to receptors which are linked to ROCCs. One subtype of glutamate receptors, the NMDA receptor has received much attention as a target of ethanol's neurotoxicity, and there is a great deal of evidence that ethanol can disrupt these receptor-linked channels (see reviews by Crews et al., 1996; Eggeman and Browning, 1996; Hoffman and Tabakoff, 1996). However, most evidence suggests that astrocytes do not express the NMDA subtype of glutamate receptors (Blankenfeld *et al.*, 1995), and therefore, the data on the effects of ethanol on the NMDA receptor are not discussed.

Astrocytes have, however, been shown to express glutamate receptors other than NMDA receptors, including the calcium channel-linked kainate/(±)-α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid hydrobromide (AMPA) receptors (Porter and McCarthy, 1997), and these could be a target of ethanol in these cells. A limited amount of work has been done investigating ethanol's effects on the quisqualate-induced calcium responses, that are mediated by these receptors, and is summarized in Table 3. The effects of acute ethanol on individual cultured Purkinje neurons appear to be developmentally-regulated, with alcohol enhancing the quisqualate-mediated response at early developmental stages, and inhibiting the response in mature neurons (Gruol and Curry, 1995). Following chronic (8 days) ethanol treatment, these responses are inhibited in developing cerebellar neurons, as measured by calcium imaging techniques (Gruol and Parsons, 1996). These data suggest that ethanol can affect glutamate responses through receptors other than the NMDA receptor and that the response depends upon either the duration of ethanol exposure or the specific type of neuron studied. Although no work has been done on astrocytes with respect to these receptors, they should be considered as a possible target for ethanol in these cells. Furthermore, the developmental sensitivity to the effects of ethanol of these responses may be relevant to ethanol's teratogenicity.

In addition to VOCCs and ROCCs, a novel plasma membrane calcium channel was recently described in *Drosophila melanogaster* (Hardie and Minke, 1992) and in some mammalian cell types (Philipp *et al.*, 1996; Zhu *et al.*, 1996). These channel

proteins, named *trp* or *trp*-like proteins, appear to be involved in the influx of calcium, secondary to second messenger-induced calcium release from intracellular stores (Hardie and Minke, 1995; Minke and Selinger, 1996). To date, there is no information on the expression of these channels by astrocytes, nor on the effects of ethanol on these channels.

Effects of Ethanol on G-Protein-Mediated Ca^{2+} Changes

In addition to increases in cell calcium caused by calcium influx, activation of G-proteins containing the $G\alpha_{q/11}$ subunit by a variety of receptors can result in elevation of cytosolic calcium levels, as previously discussed, in the phosphatidylinositol signaling pathway (see Figure 1). Interaction with the phosphoinositide metabolism pathway would result in alterations in calcium homeostasis. A large number of studies exist on the effect of ethanol on the formation of IP_3, and these studies are summarized in Table 3. In nerve tissue, ethanol does not appear to have any stimulatory effect on phosphoinositide metabolism when present alone (Balduini and Costa, 1990). However, agonist-induced IP_3 formation is mostly inhibited by ethanol (Table 3). For example, muscarinic receptor-stimulated phosphoinositide metabolism was inhibited in cerebral cortical slices (50-500 mM) (Balduini and Costa, 1990; Balduini *et al.*, 1991b), cortical membranes (250 mM) (Candura *et al.*, 1992), mixed rat cortical cultures (approximately 70% glia and 30% neurons) (250-500 mM) (Kovacs *et al.*, 1995), and SH-SY5Y neuroblastoma cells (100 mM) (Larsson *et al.*, 1995). Interestingly, this inhibitory effect appeared to be selective for carbachol-stimulated PI hydrolysis, and to be age-dependent, with neonatal rats being more sensitive to the action of ethanol than adults (Smith *et al.*, 1986; Balduini and Costa, 1989; Balduini and Costa, 1990). Bradykinin-induced PI metabolism was also inhibited by ethanol (100 mM) in NG 108-15 neuroblastoma-glioma cells (Simonsson *et al.*, 1989). Limited studies in astrocytes have shown that acute exposure to ethanol has no effect on norepinephrine- (50-200 mM) (Ritchie *et al.*, 1988) or glutamate- (100 mM) (Smith and Bitrick, 1995) stimulated PI hydrolysis. Glutamate-induced PI metabolism was, however, inhibited following a longer exposure to ethanol (100 mM for 4 days) (Smith and Bitrick, 1995). The varying sensitivity of the neurotransmitter responses to ethanol may be due to different second messenger systems downstream from the receptor, or due to differential sensitivity of the receptor molecule itself.

A few studies have investigated the effects of ethanol on G-protein-mediated intracellular calcium release, and all of them indicate that ethanol inhibits the release of calcium from intracellular stores (Table 3). In PC12 cells, a short term incubation with a high concentration (400 mM) of ethanol inhibited muscarine-induced increase in intracellular calcium (Rabe and Weight, 1988). Similarly, acute exposure to a low concentration (25 mM) of ethanol inhibited carbachol-stimulated calcium uptake in the same cells (Koski *et al.*, 1991). Ethanol also inhibited the carbachol-induced calcium response in individual SH-SY5Y neuroblastoma cells; ethanol reduced the number of cells that responded to carbachol with an increase in intracellular calcium, although the maximum response and the rate of the calcium response were affected only in late responding cells (Larsson *et al.*, 1998). An effect of short term (10 min) ethanol exposure was also seen on muscarinic receptor-induced calcium responses in mixed cortical cultures using imaging techniques, with a significant inhibition in the size of the maximal response (Kovacs *et al.*, 1995). Similarly, inhibition of receptor-mediated calcium responses was observed in hepatocytes following stimulation with vasopressin, phenylephrine, epidermal growth factor, angiotensin II (Higashi and Hoek, 1991), and hepatocyte growth factor in individual cells (Saso *et al.*, 1996).

These findings indicate that ethanol is capable, in certain situations, to inhibit receptor-activated PI metabolism and the subsequent release of calcium from intracellular stores. The mechanism(s) by which ethanol exerts these effects is, however, still obscure.

Ethanol does not appear to directly interact at the receptor level (Ritchie *et al.*, 1988; Balduini and Costa, 1989), suggesting one or more post-receptor targets for its action. Some studies have investigated the effects of ethanol on the activity and levels of the $G\alpha_{q/11}$ G-protein subunit. Results in NG 108-15 cells indicate that chronic ethanol may act at the level of, or distal to, the G-protein, possibly at the level of the GDP/GTP exchange on G-proteins (Simonsson *et al.*, 1991; Candura *et al.*, 1992), and that ethanol decreases $G\alpha_{q/11}$ protein levels (Williams and Kelly, 1993). In addition, a reversible decrease in $G\alpha_{q/11}$ levels was also seen in rat brain following chronic (15 day) *in vivo* exposure (Pandey, 1996). In astrocytes, however, functional data indicated an action upstream from G-proteins (Smith, 1994), and chronic ethanol had no effect on the

expression of the $G\alpha_{q/11}$-subunit (Pandey *et al.*, 1996). These authors suggested that the modulation of $G\alpha_{q/11}$-subunit seen by others (Simonsson *et al.*, 1991; Williams and Kelly, 1993) may be specific for neurons.

Downstream from the G-protein, ethanol could act on PLC or at the IP_3 receptor level, to alter the calcium response induced by activation of the PI pathway. Studies in rat brain showed that acute ethanol exposure did not affect PLC levels, but chronic treatment decreased the levels of PLC-ß (Pandey, 1996), whereas, in primary astrocytes, chronic ethanol increased the levels of PLC-δ1 (Pandey *et al.*, 1996). Possible direct effects of ethanol on the IP_3 receptors have also been investigated. Ethanol initially potentiated, then inhibited, both the currents and the calcium changes evoked by caged IP_3 in oocytes (Ilyin and Parker, 1992), and, in microsomes, ethanol decreased the amplitude of the calcium released by IP_3, possibly due to selective effects on a particular subtype of IP_3 receptor (Mezna *et al.*, 1996). Chronic *in vivo* exposure to ethanol decreased the number of IP_3 receptors, but not the binding affinity of IP_3 for its receptor in rat cortex (Rodriguez *et al.*, 1996) and mouse Purkinje cells (Simonyi *et al.*, 1996). These findings suggest that ethanol may also act at the level of the IP_3 receptor to disrupt calcium responses, though this possibility has not been yet investigated in astrocytes.

Altogether, these data indicate that there are many sites in the inositol phosphate second messenger system where ethanol may act, any one of which would disrupt G-protein-mediated alterations in intracellular calcium. The limited amount of data, however, suggests that the target(s) of ethanol may differ between neurons and astrocytes, highlighting the need to study the effects of this alcohol in different cell types.

Implications of Calcium Effects and Alcohol Neurotoxicity

Although there has been limited research investigating the functional significance of calcium responses in astrocytes, it has been shown that increases in intracellular calcium can have a wide range of effects within cells in general. The role of calcium in the regulation of protein function has been known for a long time, with the activity of phosphatases, kinases, endonucleases, and many other enzymes being calcium dependent. These effects are seen in the role that calcium plays in cell death, where there is

evidence for calcium involvement in both necrosis and apoptosis (Nicotera *et al.*, 1994). Calcium is also involved in the regulation of protein synthesis through its protein regulatory actions in a variety of cells (Palfrey and Nairn, 1995). Changes in intracellular calcium levels can have long-term effects on cells through the regulation of immediate early genes (IEGs), including *c-fos, c-myc* and *c-jun*, which act as transcription factors. This calcium regulation of the expression of the IEGs can occur at the level of transcription initiation, elongation of mRNA, the stability of mRNA and at the translational level (Rosen *et al.*, 1995). There is evidence that the increased expression of the IEGs seen in many cells following increases in cytosolic calcium can involve both post-translational effects (Sheng and Greenberg, 1990; Herschman, 1991), and effects at the transcriptional level (Rosen *et al.*, 1995). For example, the transcription of *c-fos* is regulated by calcium's actions on regulatory proteins which bind to the calcium response element and the serum response element in the upstream region of this gene (Rosen *et al.*, 1995). The increase in cell proliferation following cell stimulation by a mitogen is dependent on the induction of IEGs (Kovary and Bravo, 1991a; Kovary and Bravo, 1991b), suggesting that calcium may regulate cellular proliferation through its actions on the IEGs. Calcium has been shown, indeed, to be involved in proliferation in a variety of cell types: it is necessary for proliferation of rat hepatocytes (Bennett and Williams, 1993), and the SK-N-MC human neuroblastoma and U-373 MG astrocytoma cell lines (Lee *et al.*, 1993b), and for the entry into the S-phase of the cell cycle in human fibroblasts (Takuwa *et al.*, 1995), and DDT$_1$MF-2 smooth muscle cells (Short *et al.*, 1993).

There is increasing evidence that calcium responses are important in glial cells, as recently reviewed by Verkhratsky et al. (Verkhratsky *et al.*, 1998). Astrocytes express VOCCs (Sontheimer, 1994), and a wide variety of receptors for neurotransmitters and neuropeptides (Hosli and Hosli, 1993), including receptors linked to calcium channels and calcium signaling systems (Hosli *et al.*, 1992; Hosli and Hosli, 1993; Van Der Zee *et al.*, 1993; Guizzetti *et al.*, 1996). Activation of these receptors in astrocytic cells has been shown to alter calcium levels, and initiate both intracellular and intercellular calcium waves (Brown-Masters *et al.*, 1984; Cornell-Bell *et al.*, 1990; Kim *et al.*, 1994). Although the exact functions of these intracellular calcium responses and intercellular calcium waves are not known, calcium may act as a signal between neurons and astrocytes, making astrocytes equal partners with neurons in information processing in the brain (Smith, 1992;

Verkhratsky and Kettenmann, 1996). The phosphorylation of cAMP response element binding (CREB) protein, and the activation of mitogen-activated protein kinase have been shown to be calcium-dependent in oligodendrocyte progenitor cells (Pende *et al.*, 1997). In addition, it has been recently shown, using an immobilized enzyme preparation, that the frequency of calcium oscillations is crucial in the regulation of calcium- and calmodulin-dependent protein kinase II (CaMK II), demonstrating the sensitivity of cellular enzyme function to calcium levels (DeKoninck and Schulman, 1998). Induction of IEGs has been shown in human astrocytoma cells (Blackshear *et al.*, 1987) and primary rat astrocytes(Arenander *et al.*, 1989; Yagle and Costa, 1999) following stimulation with carbachol,, and in oligodendrocytic cells upon stimulation with glutamate (Pende *et al.*, 1994). The release of interleukin 1 (Martin *et al.*, 1992), and excitatory amino acids (Parpura *et al.*, 1994; Jeftinija *et al.*, 1996) from astrocytes has also been shown to be calcium-dependent, as have changes in astrocyte morphology induced by various compounds (MacVicar, 1987; Goldman *et al.*, 1991; Stiene-Martin *et al.*, 1993). The importance of calcium in astrocytes is further underscored by the fact that this ion is thought to act as an intercellular messenger, encoding and transmitting information similar to action potentials in neural networks (Smith, 1992; Charles, 1994; Verkhratsky and Kettenmann, 1996; Verkhratsky *et al.*, 1998).

There is a large amount of research indicating the widespread importance of calcium in cellular functions. With the increasing evidence of the importance of astrocytes in the development and functioning of the central nervous system, and the potential role of calcium and calcium signaling in these actions, it is important to consider the control of calcium in astrocytes as a potential target for neurotoxicants, including ethanol. For example, one of the main neuroteratogenic effects of ethanol is microencephaly (Clarren and Smith, 1978). Astrocytes comprise a large portion of the volume of the brain and disruption of their proliferation may lead to microencephaly. Ethanol has indeed been shown to inhibit neurotransmitter-induced glial cell proliferation (Guizzetti and Costa, 1996; Resnicoff *et al.*, 1996; Guizzetti *et al.*, 1997), although the mechanism underlying this inhibition is unknown. Since calcium is thought to participate in cell proliferation (Leslie *et al.*, 1983; Kovary and Bravo, 1991b) disruption of calcium homeostasis by ethanol may be involved in this inhibitory effect.

A large number of studies exist indicating that ethanol can disrupt calcium homeostasis in the nervous system. Significant effects on calcium channels and G-protein mediated calcium responses have been reported. It is clear, from a review of the available data, that many variables influence the ultimate effect of ethanol, including the developmental stage of the brain, the duration of exposure, the cellular preparation, and the agonist coupled to a calcium response, to name only a few. Surprisingly, the potential effects of ethanol on calcium homeostasis in astrocytes has not been fully investigated, especially in response to muscarinic stimulation. Yet, it is plausible that, with the important functions of calcium responses in astrocytes, interference by ethanol may mediate some of the neuroteratogenic and neurotoxic effects of this compound.

Hypothesis

Based on the current state of knowledge on astrocytic function and muscarinic-mediated responses, the following hypothesis was formulated:

Ethanol exposure during development causes central nervous system dysfunction through effects on astrocytes, specifically on their calcium response to muscarinic receptor stimulation, thereby interfering with the normal mitogenic response to acetylcholine.

Specific Aims

In order to test this hypothesis, the following specific aims were set for this research:

1. To characterize the intracellular calcium responses seen in individual rat astrocytes and human astrocytoma cells in response to muscarinic stimulation. An intracellular fluorescent calcium dye and confocal microscopy was used to characterize calcium responses within individual cells. The calcium spiking and oscillatory behavior following muscarinic stimulation and the origins of the calcium for the various aspects of the response were investigated.

2. To determine the effect of ethanol on the muscarinic receptor-induced calcium responses in individual glial cells. The responses seen in Aim 1 were compared to those seen following treatment with various concentrations of and durations of exposure to ethanol.

3. To investigate the cellular site of action of ethanol. The intracellular target of ethanol was studied by measuring calcium responses to various stimuli, acting at different cellular levels, in the presence and absence of ethanol. In addition, the effect of ethanol on receptor density and muscarinic receptor-induced IP_3 generation were also investigated.

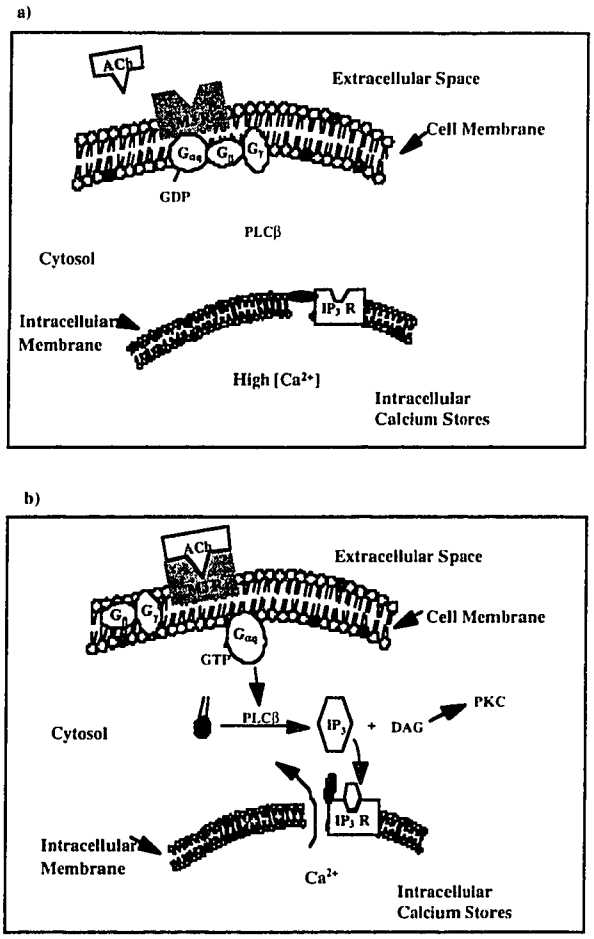


Figure 1. Acetylcholine-Induced Calcium Release Mediated by the Phosphatidylinositol Signaling System.

a) In the absence of acetylcholine (ACh) bound to its receptor (M3 R, for example), the G-protein is at rest in its trimeric state. b) Following binding of ACh to M3 R, the GDP on the α-subunit of the G-protein is replaced with a GTP, resulting in a conformational change in Gαq, its dissociation, and its activation of phospholipase C β (PLCβ). PLCβ then catalysis the hydrolysis of phosphatidylinositol (4,5)-bisphosphate (PIP_2,) to inositol(1,4,5)-trisphosphate (IP_3) and DAG. IP_3 then diffuses to its receptor (IP_3 R), where it binds, opening a calcium channel, allowing the release of calcium from intracellular stores. DAG can activate some isoforms of PKC.

Table 1. Summary of Effects of Ethanol on Basal Calcium Parameters

Exposure Type	Effect on $[Ca^{2+}]i$ *	Exposure Duration	[Ethanol]	Preparation Type (Reference)
Acute	↑	< 1 min	17-170 mM	rat hippocampal cells (Mironov and Hermann, 1996)
	↑	< 1 min	100-300 mM	hepatocytes (Hoek *et al.*, 1992; Higashi *et al.*, 1994)
	↑	< 1 min	30-600 mM	rat brain synaptosomes (Daniell *et al.*, 1987; Rezazadeh *et al.*, 1989)
	↔	< 1 min	50 mM	cerebellar granule cells (Dildy and Leslie, 1989), dissociated brain cells (Snell *et al.*, 1994)
	↔ (uptake)	< 1 min	45-720 mM	rat brain synaptosomes (Harris and Hood, 1980)
	↑	< 1 min	≤ 80 mM	PC12 cells (Rabe and Weight, 1988; Belia *et al.*, 1995)
	↑	< 1 min	22-174 mM	untreated rat neurons (Webb *et al.*, 1995)
	↓	< 1 min	22-174 mM	NGF-treated rat neurons (Webb *et al.*, 1995)
	↔	< 1 min	22-174 mM	untreated rat septohippocampal neurons (Webb *et al.*, 1996a)
	↑	< 1 min	22-174 mM	NGF-treated rat neurons (Webb *et al.*, 1996a)
Short Term	↑	10 min	50-500 mM	rat forebrain synaptosomes (Davidson *et al.*, 1988)
	↑	10 min	20-200 mM	lymphocytes (Fano *et al.*, 1993)
	↔	10 min	50-500 mM	mixed cortical cultures (Kovacs *et al.*, 1995)
	↔	1-40 min	100 mM	PC12 cells (Rabe and Weight, 1988)
	↓	10-30 min	20-200 mM	skeletal muscle cells (Cofan *et al.*, 1995)
Chronic *in vitro*	↑ (currents)	4-6 days	200 mM	PC12 cells (Messing *et al.*, 1990; Grant *et al.*, 1993)
	↑ (uptake)	4-6 days	200 mM	PC12 cells (Messing *et al.*, 1990; Grant *et al.*, 1993)
	↑	48-96 h	75 mM	fetal rat cerebellar macroneurons (Zou *et al.*, 1995)
	↑	24 h	20-200 mM, max at 60 mM	lymphocytes (Fano *et al.*, 1993)
	↑	7 days	50-100 mM	rat astroglial cells (Holownia *et al.*, 1997)
	↔	4 days	100 mM	rat cortical neurons (Blevins *et al.*, 1995)
	↔	2 weeks	40 mM	skeletal muscle cells (Cofan *et al.*, 1995)
	↓	3 days	100 mM	PC12 cells (Belia *et al.*, 1995)
Chronic *in vivo*	↔	15 days	29-32 mM (BEC)	neonatal rats (Lee *et al.*, 1996)
	↔ (uptake)	7 days	7% v/v	adult rats (Harris and Hood, 1980)
	↔ (uptake)	8 weeks	32 mM (BEC)	adult rats (Leslie *et al.*, 1983)

* Increase levels of intracellular Ca^{2+}, except when indicated; modified from Catlin et al., 1999a.

Table 2. Summary of Effects of Ethanol on Voltage-Operated Calcium Channels

Type of Exposure	Effect of Ethanol	Exposure Duration	[Ethanol]	Preparation Type (Reference)
Acute *in vitro*	↓ Ca^{2+} uptake	30 sec-10 min	45-720 mM	mouse synaptosomes (Harris and Hood, 1980)
	↓ Ca^{2+} uptake	14 min	25-150 mM	rat synaptosomes (Leslie *et al.*, 1983)
	↓ Ca^{2+}-induced dopamine release	-	50 mM	rat striatal slices (Lynch and Littleton, 1983)
	↓ K^{+}-induced uptake	25 min	50 mM	PC12 cells (Messing *et al.*, 1986)
	↓ L-type Ca^{2+} channel current	5 min	5 or 50 mM	PC12 cells (Mullikin-Kilpatrick and Treistman, 1994)
	↓ L-type Ca^{2+} channel current	5 min	25 mM	PC12 cells (Mullikin-Kilpatrick *et al.*, 1995; Mullikin-Kilpatrick and Treistman, 1995)
	↓ Ca^{2+} channel current	-	11-108 mM	dorsal root ganglion neurons (Oakes and Pozos, 1982)
	↓ Ca^{2+} channel current	15 min	300 mM	*Aplysia* neurons (Camacho-Nasi and Treistman, 1987)
	↓ Ca^{2+} channel current	15 min	50-500 mM	*Aplysia* neurons (Camacho-Nasi and Treistman, 1986)
	↓ T-type Ca^{2+} channel current	5 min	30-300 mM	N1E-115, NG108-15 cells (Twombly *et al.*, 1990)
	↓ L-type Ca^{2+} channel current	5 min	100-300 mM	N1E-115, NG108-15 cells (Twombly *et al.*, 1990)
	↑ Ca^{2+} channel current	0.5 min, 10 min	5.4 mM	murine dorsal root ganglion neurons (Huang and McArdle, 1994)
	↑ Ca^{2+} channel current	0.5 min	43.2 mM	murine dorsal root ganglion neurons (Huang and McArdle, 1994)
	↓ Ca^{2+} channel current	10 min	43.2 mM	murine dorsal root ganglion neurons (Huang and McArdle, 1994)
	↓ DHP-sensitive binding sites	10 min	100 mM	rat brain synaptosomes (Canda *et al.*, 1995)
Acute *in vivo*	↑ channel number	40 min-16 h	single 3 g/kg dose	rat cerebral cortex, hippocampus, and striatum (Ruis *et al.*, 1987)
	↓ Ca^{2+} uptake	3-5 min	single 4 g/kg dose	mouse synaptosomes (Harris and Hood, 1980)
	↔ Ca^{2+} uptake	60 min	single 4 g/kg dose	mouse synaptosomes (Harris and Hood, 1980)
Chronic *in vitro*	↑ Ca^{2+} uptake ↑ DHP-sensitive binding sites	2-10 days	200 mM	PC 12 cells (Messing *et al.*, 1990)

Table 2 (continued)

Chronic *in vitro* (continued)	↑ Ca^{2+} uptake ↑ Ca^{2+} channel current	6 days	200 mM	PC 12 cells (Grant *et al.*, 1993)
	↑ Ca^{2+} uptake ↑ DHP-sensitive binding sites	6 days	200 mM	PC 12 cells (Messing *et al.*, 1986)
	↑ Ca^{2+} uptake via L-type channels	6 days	200 mM	PC 12 cells (Greenberg *et al.*, 1987)
	↑ Ca^{2+} uptake ↑ DHP-sensitive binding sites	6 days	200 mM	PC 12 cells (Marks *et al.*, 1989)
	↑ Ca^{2+} uptake	10-12 min	100-800 mM	PC 12 cells (Skattebol and Rabin, 1987)
	↑ high threshold currents, ↓ low threshold currents	8-10 days	33 mM	fetal rat cerebellar Purkinje cells (Gruol and Parsons, 1994)
	↓ current	96 h	75 mM	fetal rat cerebellar macroneurons (Zou *et al.*, 1995)
	↑ number of L-type ↔ number of N-type	72 h	200 mM	undifferentiated NG108-15 cells (Bergamaschi *et al.*, 1995)
	↔ number of L-type ↓ number of N-type	72 h	200 mM	differentiated NG108-15 cells (Bergamaschi *et al.*, 1995)
Chronic *in vivo*	tolerance	7 days	7% v/v	synaptosomes (Harris and Hood, 1980)
	tolerance	3 weeks	75 mM (BEC)	rat brain synaptosomes (Canda *et al.*, 1995)
	tolerance	8 weeks	31.7 mM (BEC)	synaptosomes (Leslie *et al.*, 1983)
	↑ DHP-sensitive binding sites	10 days	27-29 mM (BEC)	cerebral cortex from ethanol dependent rats (Dolin and Little, 1989)
	↑ DHP-sensitive binding sites	6-10 days	inhalation of 10-22 mg/l	cortex, heart, vas deferens and skeletal muscle from ethanol dependent rats (Guppy and Littleton, 1994)
	↑ DHP-sensitive binding sites	4-10 days	inhalation of 10-15 mg/l	cortex, heart and vas deferens from ethanol dependent rats (Guppy *et al.*, 1995)
in utero	↓ K^+-stimulated Ca^{2+} uptake	15 days	29-32 mM	whole brain dissociated neurons (Lee *et al.*, 1996)

modified from Catlin et al., 1999a.

Table 3. Summary of Effects of Ethanol on G-protein Mediated Receptor Functions

Exposure Type	Effect of Ethanol	Exposure Duration	[Ethanol]	Preparation Type (Reference)
Acute *in vitro*	↓ muscarinic-induced IP_3 response	30 sec - 90 min	50-500 mM	cerebral cortical slices (Balduini and Costa, 1990; Balduini *et al.*, 1991b), cortical membranes (Candura *et al.*, 1992), mixed cortical cultures (Kovacs *et al.*, 1995), SH-SY5Y neuroblastoma cells (Larsson *et al.*, 1995)
	↓ muscarinic-stimulated Ca^{2+} uptake	0-10 min	25-100 mM	PC12 cells (Koski *et al.*, 1991)
	↓ number of cells with muscarinic-induced Ca^{2+} response	2 min	100 mM	SH-SY5Y cells (Larsson *et al.*, 1998)
	↓ size of muscarinic-induced Ca^{2+} response	10 min	250-500 mM	mixed cortical cultures (Kovacs *et al.*, 1995)
	↓ vasopressin-, phenylephrine-, epidermal growth factor-, and angiotensin II-induced Ca^{2+} response	2-3 min	6-300 mM	hepatocytes (Higashi and Hoek, 1991; Saso *et al.*, 1996)
	↓ hepatocyte growth factor-induced Ca^{2+} response	2 min	50-300 mM	hepatocytes (Higashi and Hoek, 1991; Saso *et al.*, 1996)
	↑ metabotropic glutamate receptor-induced response	5 min	33 mM	early developmental stage Purkinje neurons (Gruol and Curry, 1995)
	↓ metabotropic glutamate receptor-induced response	5 min	33 mM	mature Purkinje neurons (Gruol and Curry, 1995)
Chronic *in vitro*	↓ bradykinin-induced IP_3 response	4 days	100 mM	NG108-15 neuroblastoma-glioma (Simonsson *et al.*, 1991)
	↓ metabotropic glutamate and NE receptor-induced IP_3 response	4 days	100 mM	primary astrocytes (Smith and Bitrick, 1995)
	↓ metabotropic glutamate receptor-induced response	8-10 days	33 or 50 mM	Purkinje neurons (Gruol and Parsons, 1996)
Chronic *in vivo*	↓ muscarinic-induced changes	6 days	51-61 mM (BEC)	neonatal rats (Balduini and Costa, 1989)

modified from Catlin et al., 1999a.

CHAPTER 2: MUSCARINIC RECEPTOR-INDUCED CALCIUM RESPONSES IN ASTROGLIA

Abstract

Regulation of calcium homeostasis is known to play an important role in the nervous system. The objective of this study was to characterize and quantitate the calcium responses to cholinergic stimulation in primary rat cortical astrocytes and human 132 1N1 astrocytoma cells, and investigate the signaling pathways the mediate these responses. The fluorescent calcium probe Indo-1 AM and an ACAS (Attached Cell Analysis and Sorting) instrument were used to quantitate calcium responses in these cells. A concentration-dependent response to carbachol was seen in both cell types, however, carbachol was more potent and efficacious, and the response was more homogenous, in the cell line. The calcium response was not affected by nicotinic or muscarinic M2 receptor antagonists, but was blocked by muscarinic M3 receptor antagonists indicating an M3-mediated effect. Experiments in the absence of extracellular calcium and with EGTA, or in the presence of ryanodine or xestospongin C, demonstrated that the initial calcium spike is due to calcium release from inositol trisphosphate-sensitive calcium stores, whereas the sustained elevation and oscillations are dependent upon calcium influx. The latter is due to calcium-induced calcium entry. Protein kinase C exerts a feedback inhibition of the carbachol-induced calcium responses, and appears to be involved in maintaining the elevated calcium concentration and oscillations as, GF 109203X inhibits this phase of the calcium response.

Introduction

Acetylcholine (ACh), a major neurotransmitter in the central nervous system, binds to two general subtypes of receptors, the muscarinic (mACh) receptor and the nicotinic (nACh) receptor. The nACh receptors are directly coupled to an ion-channel which is permeable to Na^+, K^+, and, to a lesser extent, Ca^{2+}. The activation of this receptor results in a depolarization across the cellular membrane and the opening of the channel, with the main result being an influx of sodium ions (Alberts *et al.*, 1989). The mACh

receptor belongs to a family of seven transmembrane domain receptor proteins which are linked to GTP-binding proteins (G-protein). The muscarinic receptors have been cloned and further subtyped based their nucleotide sequence, with five subtypes identified to date: *m*1-*m5* (Bonner, 1989). The *m1*, *m3* and *m5* receptors are linked, via G_{α_q}, to the activation of phospholipase C (PLC)-ß (Smrcka *et al.*, 1991; Bernstein, 1992). Once activated, PLC–ß catalyses the breakdown of a membrane lipid, phosphatidylinositol (4,5)-bisphosphate (PIP_2), to two important intracellular messengers, inositol (1,4,5)-trisphosphate (IP_3) and diacylglycerol (DAG). IP_3 binds to a specific receptor in nonmitochondrial calcium stores and causes the release of calcium to the cytosol, while DAG activates protein kinase C (PKC).

Autoradiography and specific antibodies have demonstrated that astrocytes express cholinergic receptors (Hosli *et al.*, 1992; Hosli and Hosli, 1993). Specifically, primary rat astrocytes and the human astrocytoma cell line, 132 1N1, express *m2*, *m3*, and a small amount of *m5* mRNA (Guizzetti *et al.*, 1996). An increase in IP_3 generation, as well as other inositol lipids, has been seen following activation of muscarinic receptors in astrocytes (Pearce *et al.*, 1986b; Pearce *et al.*, 1988; Oliva *et al.*, 1997), astrocytoma cells(Masters *et al.*, 1984; Evans *et al.*, 1985; Ambler *et al.*, 1987), and mixed cortical cell cultures containing 70% astrocytes (Kovacs *et al.*, 1995). Additionally, PKC is activated by muscarinic stimulation of astrocytoma cells (Trilivas and Brown, 1989; Guizzetti *et al.*, 1998). There is also evidence that acetylcholine elevates intracellular calcium in mixed cortical cultures (Kovacs *et al.*, 1995), primary astrocytes, and astrocytoma cells (Masters *et al.*, 1984; Evans *et al.*, 1985; Masters *et al.*, 1985a; Masters *et al.*, 1985b; Orellana *et al.*, 1985; Noronha-Blob *et al.*, 1987; McDonough *et al.*, 1988; Pearce *et al.*, 1988; Enkvist *et al.*, 1989; Dave *et al.*, 1991; Inagaki *et al.*, 1991; McCarthy and Salm, 1991; Shao and McCarthy, 1993; Shao and McCarthy, 1995). Many of these studies, however, have looked at population responses or radioactive calcium release (Masters *et al.*, 1984; Evans *et al.*, 1985; Masters *et al.*, 1985a; Masters *et al.*, 1985b; Orellana *et al.*, 1985; Noronha-Blob *et al.*, 1987; McDonough *et al.*, 1988; Pearce *et al.*, 1988; Enkvist *et al.*, 1989), with only a limited number of studies assessing calcium signals in individual astroglia (Dave *et al.*, 1991; Inagaki *et al.*, 1991; McCarthy and Salm, 1991; Shao and McCarthy, 1993; Shao and McCarthy, 1995). Furthermore, several of these studies did not attempt to quantitate the observed responses.

The purpose of this study was, therefore, to characterize the calcium responses in astrocytic cells following stimulation of muscarinic receptors, utilizing both primary rat astrocytes and the human 132 1N1 astrocytoma cell line.

Materials and Methods

Animals and materials

Time-mated pregnant rats were supplied by B&K Universal (Kent, WA), and housed, one per cage, in the University of Washington Vivarium. All tissue culture reagents were from Gibco (Grand Island, NY) or Sigma (St. Louis, MO). Two-well coverglass chamberslides were from Nalge Nunc International (Naperville, IL), antibodies were from Accurate Chemical (Westbury, NY), and Indo-1/acetylmethylester (AM) and calcium standards were from Molecular Probes (Eugene, OR). Pharmacological agents used as specific inhibitors or antagonists were from RBI (Natick, MA), except xestospongin C which was donated by Dr. I. Pessah (University of California at Davis).

Culture of astrocytes and astrocytoma cells

Primary rat cortical astrocyte cultures were prepared by a modification of the method of McCarthy and DeVellis (McCarthy and DeVellis, 1980), as described previously (Guizzetti *et al.*, 1996). Briefly, cortices from day 21 fetuses were minced, and trypsinized by trituration in 0.125% (v/v) trypsin for 10 min at 37°C. The reaction was stopped by the addition of low glucose Dulbecco's modified Eagle's medium with calcium, magnesium, and HEPES buffer (DMEM) supplemented with 10% fetal bovine serum (FBS), 1 U/ml penicillin G and 1 mg/ml streptomycin (complete 10% medium). Cells were centrifuged at 250x*g*, resuspended in complete 10% medium and vortexed at high speed for 1 min to kill neurons. The tissue was washed three times with complete medium and centrifugation. Following washing, the tissue was triturated and passed through a 100 µm nylon filter to disperse cells and remove remaining clumps. Cell viability and number were assessed with trypan blue exclusion and Turk's solution. Astrocytes were plated at 1.5×10^5 cells/cm^2 in 75 cm^2 flasks, previously coated with poly-d-lysine (10 µg/ml, 10 ml). Within 24 hours of plating, the flasks were shaken to remove

contaminating neurons, and fresh medium was added. Flasks were maintained in a humidified atmosphere of 5% CO_2/95% air at 37°C for 9 days, and fed every 2-3 days. On day 9 in culture the flasks were shaken overnight to remove contaminating microglia, trypsinized, and reseeded for experiments. Astrocyte cultures were determined to be at least 95% pure by indirect immunofluorescence with antibodies against glial fibrillary acidic protein and neuron-specific enolase (not shown).

The immortalized human astrocytoma cell line 132 1N1 (obtained from Dr. J.H.Brown, University of California at San Diego) was maintained as previously described (Guizzetti *et al.*, 1996). Briefly, cells were maintained in 75 cm^2 flasks in DMEM with 5% FBS, 1 U/ml penicillin G and 1 mg/ml streptomycin (complete 5% medium), in a humidified atmosphere of 5% CO_2/95% air at 37°C. Flasks were fed every 2-3 days with complete 5% medium and passaged once a week. Cells were trypsinized weekly with 0.05% (v/v) trypsin and reseeded for experiments. Cells were used between the third and the seventeenth passage.

Calcium Measurements

Calcium measurements were carried out in primary rat astrocytes by a modification of the method of Kovacs (Kovacs *et al.*, 1995), as previously described (Gafni *et al.*, 1997). On day 10 of culture primary rat astrocytes were reseeded at $2.4x10^5$ cells/cm^2 in 2 well coverglass chamber slides previously coated with poly-d-lysine (10 µg/ml, 2 ml). Astrocytes were grown for 2 days in complete 10% DMEM, then rinsed with phosphate-buffered saline and serum-deprived for 2 days in DMEM supplemented with 0.1% Fraction V fatty acid-free bovine serum albumin and 1 U/ml penicillin G and 1 mg/ml streptomycin (BSA medium). Cells were rinsed twice with Kreb's bicarbonate buffer and loaded with cell permeant Indo-1/acetylmethylester (Indo-1/AM) by incubation in a 2 µM solution of Indo-1/AM in Kreb's buffer containing 1% Fraction V BSA, and 1 mM probenecid, for 30 minutes, in a humidified atmosphere of 5% CO_2/95% air at 37°C. Following loading, cells were rinsed with BSA-free Kreb's buffer containing 1 mM probenecid and incubated for 15 minutes at 37°C to allow cleavage of the Indo-1 ester. Indo-1 was excited at 351-363 nm and emission was detected

simultaneously at 405 and 530 nm to measure the fluorescence intensity of the dye bound and not bound to calcium, respectively. Fluorescence measurements were made using an attached-cell analysis and sorter (ACAS) instrument (Meridian Instruments, Okemos, MI). The ACAS consists of a motorized stage on an inverted, confocal microscope attached to an argon laser for excitation and two photomultiplier tubes for fluorescence detection. The ratio of the fluorescence at the two wavelengths was determined in individual cells as a representation of the cytosolic calcium level. Ratios were then compared to the ratio for known calcium concentrations in solution to determine the absolute calcium concentration within individual cells. The calcium concentration within 50 cells was quantitated for each experiment.

A similar protocol was followed with the human 132 1N1 astrocytoma cell line. Astrocytoma cells were reseeded at $2x10^4$ cells/cm^2 in two-well coverglass chamber slides, grown for 4 days in complete 5% DMEM and serum-deprived for 2 days in BSA medium. Cells were rinsed twice with Kreb's bicarbonate buffer and loaded with cell permeant Indo-1/AM by incubation in a 2 μM solution of Indo-1/AM in Kreb's buffer containing 1% Fraction V BSA for 60 minutes in a humidified atmosphere of 5% CO_2/95% air at 37°C. Following loading, cells were rinsed with BSA-free Kreb's buffer, and incubated for 15 minutes at 37°C to allow cleavage of the Indo-1 AM. Calcium measurements were carried out using the ACAS as described for the primary rat cortical astrocytes.

Data Analysis

For both primary rat cortical astrocytes and human 132 1N1 astrocytoma cells the intracellular calcium level was quantified in 50 cells for 323 sec (20 scans). Baseline calcium measurements were determined by scanning three times prior to the addition of the stimulant, and the calcium was monitored for 17 scans after the addition of the stimulant. Both qualitative and quantitative calcium data was collected. Pseudocolor images of the field of view showing the fluorescence intensity ratio of bound to calcium to unbound Indo-1 were generated to provide a qualitative assessment of the calcium changes within cells. Time-course plots of the concentration of calcium (μM) across-time (sec) were generated showing the calcium levels in individual cells. Background fluorescence was thresholded to zero on both detectors for this analysis. The concentration of calcium in

each of 50 cells at each time point (scan) was collected and four quantitative endpoints calculated. The basal calcium level was calculated in each cell as the average of the calcium level (μM) in the three pre-stimulus scans. The percentage of the 50 cells responding to a stimulus was determined within each experiment; a cell was considered to have responded if immediately (8 sec) following the addition of the stimulus the calcium level exceeded two standard deviations above the average basal calcium level for that individual cell. This endpoint provides a quantitative assessment of the heterogeneity of the cells with regards to their ability to respond to a given stimulus. In addition, the types of responses were assessed qualitatively. The third quantitative endpoint analyzed, the amplitude of the initial spike, was calculated as the average percentage increase in the calcium concentration above basal within 8 sec of addition of stimulus, with each cell acting as its own comparison control; only those cells which responded were used to determine the average amplitude of the response so as not to underestimate the size of the response due to non-responding cells. This endpoint represents the amount of calcium initially entering the cytosol, presumably from intracellular stores. The fourth quantitative endpoint represents the total increase in the concentration of calcium during the 277 sec monitored after the addition of the stimulus. These values were calculated as the area under the concentration versus time curve above basal (AUC, μMxsec). The AUC for each cell was then averaged across the 50 cells in each experiment to give the average AUC for each experiment. This endpoint quantifies the whole profile of the calcium response, encompassing both the initial calcium release phase, and the second phase of the calcium response.

Statistical Analysis

Statistically significant differences were tested for using either a one-way Student's t-test or a one-way analysis of variance (ANOVA) followed by Dunnett's test to determine where any differences lay.

Results

The aim of this study was to provide a quantitative characterization of the calcium responses elicited by muscarinic receptors in rat cortical astrocytes and human astrocytoma cells. Figure 2 shows computer-generated images of the two cell types prior to (a,d) and following (b,e) stimulation with 1 mM carbachol: both cell types responded

with an increase in intracellular calcium. The response in primary cells (Fig. 2c) was more heterogeneous than that of the cell line (Fig. 2f); furthermore, almost all astrocytoma cells normally responded with an increase in intracellular calcium, while many of primary cells did not. In primary astrocytes, three general responses were seen: non-responding cells, cells which responded with an increase in calcium, with calcium levels remaining elevated above baseline and oscillating, and cells in which calcium level returned to baseline levels soon after the initial response (Fig. 3). Almost all astrocytoma cells responded to 1 mM carbachol with an initial increase in calcium followed by a partial decrease, but the level still remained above basal calcium levels, and oscillations occurred (Fig. 3b). These oscillations were very similar to those seen in the primary cells, as can been seen when the axis are shown on the same scale (Fig. 3c).

Carbachol induced a concentration-dependent response in primary rat cortical astrocytes (Fig. 4a,c, and e) and human 132 1N1 astrocytoma cells (Fig. 4b, d, and f), with the percentage of cells responding, the average amplitude of the calcium spike, and the area under the curve increasing with increasing carbachol concentrations. Though the responses were qualitatively similar in the two cell types, significant quantitative differences existed. Basal calcium levels differed significantly between the two cells types, with the primary cells having a lower resting calcium level (0.07 ± 0.0053 μM) than the cell line (0.11 ± 0.010 μM, n=43 experiments, 50 cells/experiment, $p<0.001$). The concentration-response curves were shifted to the left in the astrocytoma cells, with a concentration of 0.01 mM carbachol or less eliciting a response in the cell line (Fig 4b,d,f), compared to a 10-fold higher concentration of 0.1 mM carbachol necessary to elicit any response in the primary cells (Fig 4a,c,e). Carbachol was not only more potent in the cell line, but was more efficacious, with a maximum initial peak of $575 \pm 78\%$ above basal in astrocytoma versus $150 \pm 25\%$ primary cells ($p<0.01$, n=6-8). Total AUC for the length of time investigated was also higher in astrocytoma cells (53 ± 12 compared to 15.1 ± 4.3). The qualitative similarities between the two cell types did not extend to all stimuli. For example primary astrocytes responded to glutamate (100 μM) in a manner similar to their response to carbachol, whereas astrocytoma cells did not respond to this amino acid (Table 4).

Mecamylamine (10 μM), a specific antagonist of nicotinic acetylcholine receptors, did not antagonize carbachol-induced calcium responses in astrocytoma cells, whereas atropine (10 μM), a specific muscarinic receptor antagonist, blocked this response (Fig. 5), indicating that the response is mediated by muscarinic acetylcholine receptors. Both these cell types have been shown to express mRNA for *m2*, *m3*, and low levels of *m5* muscarinic receptors (Guizzetti *et al.*, 1996). As shown in Fig. 5, carbachol-induced calcium responses were mostly inhibited by hexahydro-sila-difenidol hydrochloride (HHSiD) and 4–diphenylacetoxy-N-methylpiperidine methiodide (4-DAMP), two M3 selective antagonists, whereas methoctramine and gallamine, two M2 selective antagonists, had little effect, indicating that the M3 muscarinic receptor subtype mediates the carbachol-induced increase in intracellular calcium. Similar results were obtained in primary rat cortical astrocytes, and data with nicotinic and muscarinic antagonists are shown in Fig. 6. None of the antagonists affected basal calcium levels in either cell type (data not shown).

The increase in calcium could be due to either release of calcium from intracellular calcium stores, and/or due to influx through calcium channels in the plasma membrane. Fig. 7 shows the calcium response in primary rat astrocytes and human 132 1N1 astrocytoma cells in normal Kreb's buffer ($[Ca^{2+}]$=1.8 mM), and in calcium-free buffer containing 1 mM EGTA. In the absence of calcium, the initial calcium spike remained, however, the sustained elevation and oscillations no longer occurred. Under these conditions, calcium concentrations were back to pre-stimulus levels by the end of the experiment. These findings indicate that the initial calcium spike was not dependent upon the presence of extracellular calcium, and was due to release of calcium from intracellular stores, whereas the second phase was dependent on extracellular calcium. Since the calcium responses are mediated by the M3 muscarinic receptors, which are linked to the phosphatidylinositol signaling pathway, IP_3-sensitive stores are likely involved in the initial spike response. A 30 min incubation of 132 1N1 astrocytoma cells with 30 μM ryanodine, a concentration that blocks ryanodine-receptor-mediated calcium release (Sutko *et al.*, 1997), had no effect on the calcium response, indicating that ryanodine-sensitive stores are not involved in this response (Fig. 8a,b). We have previously shown that the recently isolated IP_3-receptor channel blockers, xestospongin A and C, inhibit the carbachol-mediated response in primary astrocytes (Gafni *et al.*, 1997). This inhibitory effect of xestospongin C was confirmed (Fig. 8c,d). The data with xestospongin C also showed that inhibition of this initial, IP_3-mediated calcium response inhibited the secondary

phase of the calcium response, indicating that the sustained elevations and oscillations, which were dependent upon the influx of extracellular calcium, were also dependent upon the release of calcium from intracellular calcium stores.

As there is evidence that the activation of protein kinase C (PKC) can modulate IP_3 and IP_3-mediated responses (Orellana *et al.*, 1985; Pearce *et al.*, 1988; Balduini *et al.*, 1990; Orellana *et al.*, 1998), the effect of a phorbol ester on muscarinic calcium responses in individual astrocytoma cells was investigated. Phorbol 12-myristate 13-acetate (PMA; 200 ng/ml), a direct activator of PKC, did not induce a calcium response within the first 5 min after addition (data not shown), nor did a 20 min incubation with PMA affect basal calcium levels (control, 120 ± 27 nM; PMA treated, 117 ± 18 nM; n=5-6). A 20 min incubation with PMA, however, inhibited carbachol-induced calcium-responses in these cells. The percentage of cells responding, the average amplitude of the initial calcium spike, and the area under the curve were significantly inhibited by incubation with PMA (Fig. 9). Bisindolymaleimide 1 (GF 109203X; 1 μM), a rather specific PKC inhibitor, did not affect the percentage of cells responding to carbachol and the average amplitude of the response, but did reverse the inhibitory effect of PMA on these two responses, indicating that the effect of PMA on carbachol-induced calcium responses is due to the activation of PKC. In contrast, GF 109203X alone appeared to inhibit (though the effect was not statistically significant) the carbachol-induced increase in the area under the curve (Fig. 10), which, unlike the other two endpoints, reflects the phase of the calcium response that is dependent upon extracellular calcium (Fig 9c). This inhibition by GF 109203X suggests a role for PKC activation in this calcium entry through the plasma membrane.

Discussion

Glial cells respond to muscarinic stimulation with an increase in intracellular calcium levels. Although this effect had been previously observed in primary astrocytes and astrocytoma cells (Masters *et al.*, 1984; Evans *et al.*, 1985; Masters *et al.*, 1985a; Masters *et al.*, 1985b; Orellana *et al.*, 1985; Noronha-Blob *et al.*, 1987; McDonough *et al.*, 1988; Pearce *et al.*, 1988; Enkvist *et al.*, 1989; Dave *et al.*, 1991; Inagaki *et al.*, 1991; McCarthy and Salm, 1991; Shao and McCarthy, 1993; Shao and McCarthy, 1995), these

responses have not been well characterized and quantitated. Intracellular calcium changes play an important role in many cellular functions, including proliferation, with the amplitude and frequency of the response determining the cellular response (see reviews by Finkbeiner, 1992; Berridge, 1993; Clapham, 1995; Berridge, 1997).

Carbachol caused an increase in cytosolic calcium levels which consisted of an initial calcium spike followed by a sustained elevation and oscillations in both primary rat cortical astrocytes and human 132 1N1 astrocytoma cells, with the second phase, but not the initial phase, requiring extracellular calcium. These responses are comparable to those others have reported in glial cells after stimulation with carbachol (Enkvist *et al.*, 1989; McCarthy and Salm, 1991), as well as other agonists, including thrombin (Ubl and Reiser, 1997), ATP (Centemeri *et al.*, 1997), glutamate (Cornell-Bell *et al.*, 1990; Dave *et al.*, 1991; Holzwarth *et al.*, 1994; Kim *et al.*, 1994), and norepinephrine (Dave *et al.*, 1991). The primary astrocytes either responded and maintained an elevated calcium concentration, responded and then returned to baseline calcium levels, or did not respond at all. In those cells which responded and then returned to baseline calcium levels the initial spike was often relatively small. It is possible that this smaller amplitude in the initial spike was not sufficient to trigger the mechanisms involved in the sustained calcium concentration and oscillations. In contrast, the response in the astrocytoma was relatively homogenous, with an initial spike followed by a decrease in the calcium concentration, but not to basal levels, and oscillations.

Approximately 66% and 89% of the primary cells responded to carbachol and glutamate, respectively, a higher percentage than has been seen previously (McCarthy and Salm, 1991). This difference could be due to serum-deprivation of our cells with BSA-medium 48 hours prior to the experiment, whereas McCarthy and Salm only partially serum-deprived (2% FBS) their cells for 30 min prior to the experiment. This would affect the differentiation of the cells and may, therefore, affect the expression of cellular receptors. We have indeed found that the density of muscarinic receptors is higher in serum-deprived cells compared to cells kept in serum (Costa, unpublished observation). In contrast to primary cells, almost 100% of the human 132 1N1 astrocytoma cells responded to carbachol. While the variability of primary astrocyte responses has been attributed to the presence of pharmacologically-distinct astrocytes in the primary cultures (McCarthy and Salm, 1991), since the cell line is of clonal origin, a homogenous population and

subsequent response would be expected. Unlike primary astrocytes, the astrocytoma cells did not respond to glutamate, suggesting the absence of functional glutamate receptors on this cell line.

As the concentration of carbachol increased, so did the number of cells responding in both cell types, possibly due to increased receptor occupancy at the higher concentrations of agonist. In both cell types there appears to be graded concentration-response for the average size of the initial spike in responding cells, a finding that is in contrast to the all-or-none responses that have previously been found in astroglia stimulated various agonists, including carbachol (Shao and McCarthy, 1995). The quantal nature of Ca^{2+}-induced Ca^{2+} release (CICR) that has been reported for intracellular calcium release may also predict an all-or-none type response (Berridge, 1997). There is evidence in the heart, however, that quantal release from intracellular stores can result in a graded overall calcium response simply by different numbers of these quantal units being triggered, depending upon the extent of stimulation the cell received (Bootman *et al.*, 1994). A similar phenomenon may occur in astroglial cells resulting in the graded response seen. Another explanation for the graded response seen in our experiments, as compared with the results of Shao and McCarthy (1995), is the difference in experimental protocols. These investigators studied the response of an individual cell to various concentrations of carbachol. In contrast, we studied the effect of different concentrations of carbachol on multiple cells and averaged the results. The variability between cells could result in a graded concentration-response curve, even if the response in any individual cell is quantal. The area under the curve, which represents the total increase in cytosolic calcium above basal during the post-stimulus period (277 sec), also showed a graded response. This endpoint, in order to provide an overall assessment of calcium entry, including any cells that may have responded late, was calculated using data from cells that did not respond; these non-responders would attenuate the overall AUC and also result in the graded concentration-response curve seen.

When comparing the calcium dynamics between the two cell types, it is evident that basal calcium levels are higher in astrocytoma cells, as are the percentage of the cells that respond, and the amplitude of both the initial spike and total increase in cytosolic calcium. The higher responses may be due to a higher receptor density, tighter coupling

between the receptors and the G-protein, such that binding of agonist to receptor results in a greater activation second messenger, or increased PLC activity.

The receptor subtype responsible for these calcium changes was investigated using selective antagonists. In both cell types, the calcium responses were due to carbachol's actions at muscarinic receptors, as these responses were not affected by the nicotinic antagonist mecamylamine, but were inhibited by the specific muscarinic antagonist atropine. The response was further shown to be mediated by the M3 muscarinic subtype, as selective antagonists at the M3 receptor, but not the M2 receptor, blocked the calcium response. This is in agreement with what was expected based on the profile of muscarinic-receptor mRNA expressed by these cells and the second messenger systems coupled to the receptor subtypes (Guizzetti *et al.*, 1996), and previous findings indicating that muscarinic-receptor-mediated calcium responses are insensitive to pertussis toxin (Masters *et al.*, 1985a; Tanabe *et al.*, 1997).

The initial calcium spike was independent of the presence of calcium in the extracellular buffer, whereas the sustained calcium elevation and oscillations seen in many of the cells required extracellular calcium. The initial spike could be due to release of calcium from IP_3-sensitive stores or ryanodine-sensitive stores. Experiments in astrocytoma cells demonstrated that blocking calcium release from ryanodine-sensitive stores had no effect on the carbachol-mediated calcium response. This indicates that ryanodine-sensitive stores are not involved in this response. Previous work in primary astrocytes with xestospongin C, a cell-permeant blocker of the IP_3-linked calcium channel (Gafni *et al.*, 1997), was confirmed in this study, further demonstrating that the initial calcium peak is due to calcium release from IP_3-sensitive stores. The inhibition of this initial calcium release also inhibited the second phase of the calcium response, indicating that the second "plateau" phase requires the initial calcium spike to trigger the influx of extracellular calcium (i.e. calcium-induced calcium entry).

There is evidence that PKC regulates G-protein mediated activation of phospholipase C and the subsequent calcium response in many cell types (Ryu *et al.*, 1990; Paulsen *et al.*, 1994; Chen and Chen, 1996). Direct activation of PKC with PMA did not cause an increase in intracellular calcium, either immediately following its addition, or after a 20 min incubation with PMA. However, a 20 min pre-incubation with PMA

inhibited the carbachol-induced calcium response in a GF 109203X-sensitive manner, indicating that this inhibition is due to the activation of PKC. Human 132 1N1 astrocytoma cells express the α, ε, and ζ isozymes of PKC (Post *et al.*, 1996), suggesting that both the PMA-sensitive PKCα and ε may mediate its feedback inhibition on the carbachol response. Evidence in bradykinin-stimulated astrocytes suggests that this feedback is due to the actions of PKCα and δ, but not ζ (Chen *et al.*, 1995), and these same researchers have found a role for PKCα, and δ in ATP-evoked responses (Chen and Chen, 1996). A possible mechanism underlying this inhibition is phosphorylation by PKC of a specific site on PLCβ that disrupts the ability of the G-protein to interact and activate PLCβ (Ryu *et al.*, 1990).

Interestingly, the PKC antagonist GF 109203X decreased the total amount of calcium entering the cells following carbachol stimulation (AUC, Fig. 10) when present alone, although it had no effect on the amplitude of the initial spike. Unlike the other quantitative endpoints, the AUC reflects not only the initial calcium spike, but also the second phase of the calcium response that requires extracellular calcium entry. Figure 10 (a,b) shows a representative experiment where this second phase of the calcium response was inhibited by GF 109203X. A similar effect of GF 109203X, and other PKC inhibitors has been seen on thrombin-induced calcium responses in C6 rat glioma cells (Ubl and Reiser, 1997), and on phenylephrine-induced calcium oscillations in rat hepatocytes (Berrie and Cobbold, 1995). This data is suggestive of a role for PKC in the sustained calcium elevation and oscillations seen following carbachol stimulation. Although little is known regarding this effect, one could speculate that PKC may phosphorylate a calcium channel involved in calcium-induced calcium influx. Phosphorylation of the *Drosophila* TRP calcium channel by a PKC has been seen (Huber *et al.*, 1998). Homologous calcium channels have been cloned in mammalian cells (Philipp *et al.*, 1996; Boulay *et al.*, 1997), and evidence is accumulating that these channels mediate the influx of calcium following activation of the phosphatidyl inositol pathway (Hardie and Minke, 1995; Minke and Selinger, 1996; Philipp *et al.*, 1996; Zhu *et al.*, 1996; Philipp *et al.*, 1998). These data indicate that investigation into the expression and function of *trp* homologs in glial cells

may elucidate some of the mechanisms underlying the complex calcium responses seen following muscarinic activation.

Altogether, these results indicate that carbachol induces a concentration-dependent increase in the cytosolic calcium concentration in glial cells via stimulation of M3 muscarinic receptors and release from IP_3-sensitive stores. The amplitude, frequency and duration of calcium responses are important intracellular signals in many cells, regulating transcription factor activation, and cell differentiation (Gu and Spitzer, 1995; Dolmetsch *et al.*, 1997). Calcium has also been shown to mediate the proliferation of many cell types, including glial cells (Lee *et al.*, 1993b; Kawanishi *et al.*, 1994; Lee *et al.*, 1994; Stanimirovic *et al.*, 1995). The characterization and quantitation of this calcium responses, therefore, will allow the study of pharmacological and toxicological agents on this response.

Acknowledgments

This study was supported by grants from NIAAA (AA-08154) and NIEHS (ES-07133).

Table 4. Effect of glutamate on intracellular calcium levels in primary rat cortical astrocytes and human 132 1N1 astrocytoma cells.

	Percentage of Cells Responding	Average Amplitude of Response (% of Control ± S.E.M.)	Area Under Curve
Primary astrocytes	88.9 ± 6.8	230.0 ± 14.7	10.1± 2.9
Astrocytoma cells	5.6 ± 3.3	113.8 ± 7.5	0 ± 0.3

Concentration of glutamate was 100 μM. Results represent the mean (± S.E.M) of five experiments. In each experiment 50 cells were analyzed.

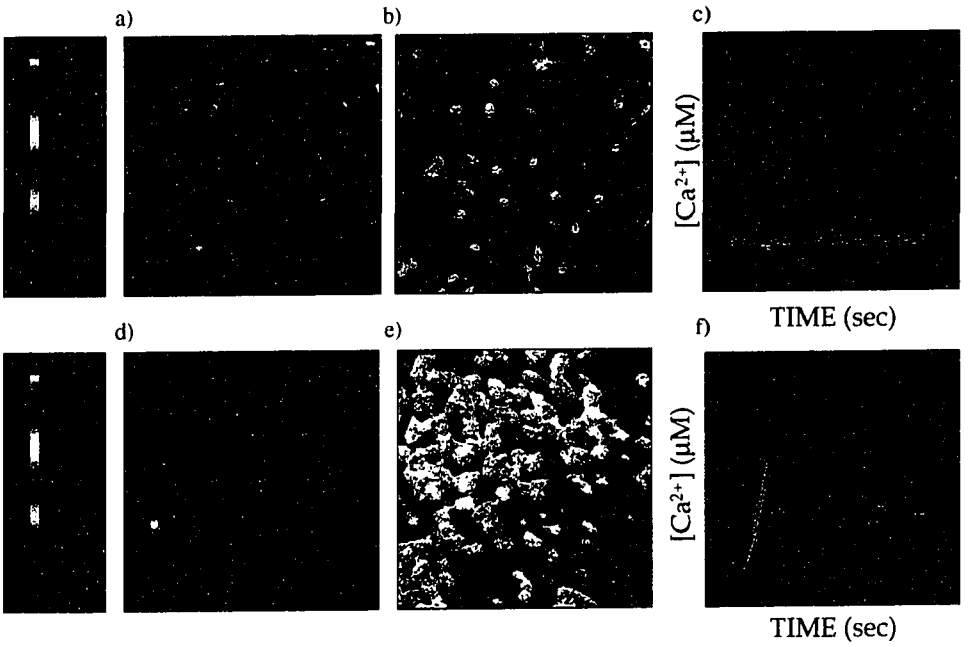


Figure 2. Carbachol-induced responses in human 132 1N1 astrocytoma cells and primary rat cortical astrocytes.

Representative pseudocolor images of calcium levels in primary astrocytes (a,b), and astrocytoma cells (d,e) loaded with 2 μm Indo-1/AM under control conditions (a,d), and 8 sec after the addition of 1 mM carbachol (b,e). The color scales on the left indicate the corresponding calcium concentration (μM) ratio. Representative time course of the carbachol-induced response in primary astrocytes (c) and astrocytoma cells (f). Each of the 50 lines indicates the calcium concentration (μM) in an individual cell, with the vertical line indicating the time of addition of 1 mM carbachol.

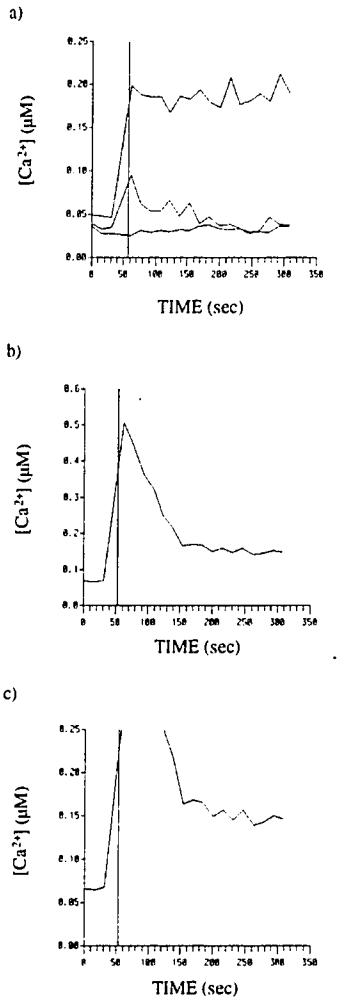


Figure 3 Characteristic responses seen in primary rat cortical astrocytes and human 132 1N1 astrocytoma cells.

Representative time course plots of individual cells which exhibit the three characteristic responses of primary astrocytes (a), and the one characteristic response of astrocytoma cells (b). This astrocytoma response is plotted with the same y-axis scale as the primary astrocyte response (c). Each line indicates the calcium concentration (μM) in an individual cell, with the vertical line indicating the time of addition of 1 mM carbachol.

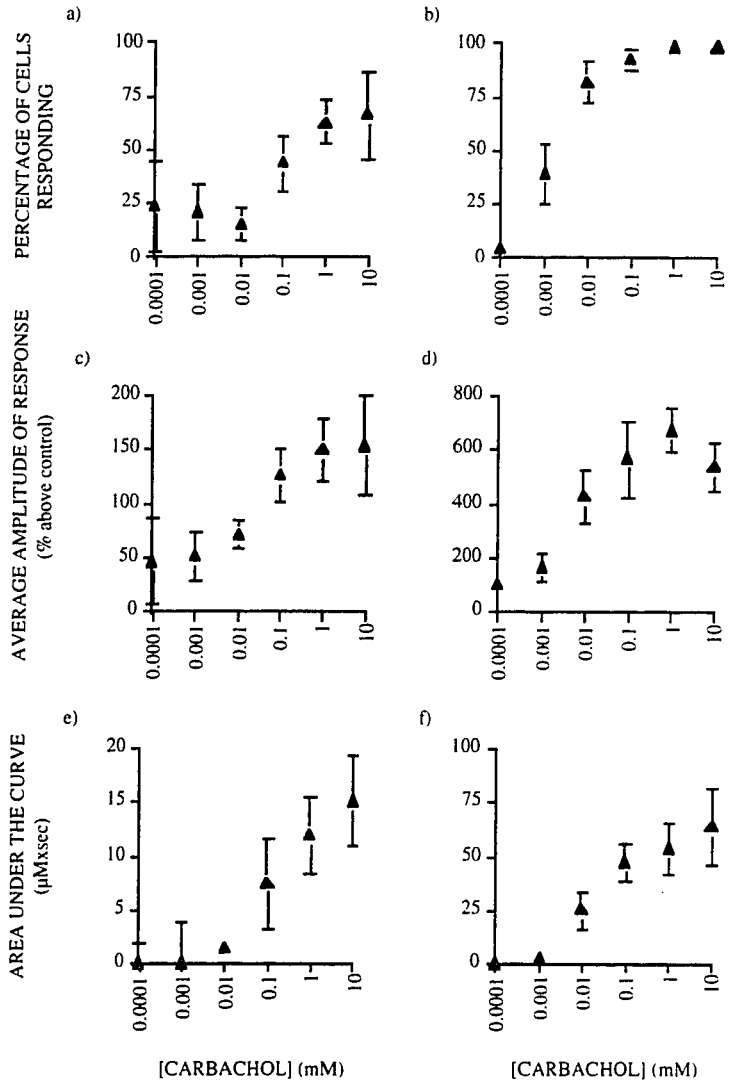


Figure 4. Concentration-response curves of carbachol-induced calcium responses in primary rat cortical astrocytes and human 132 1N1 astrocytoma cells.

Results in astrocytes (a,c,e) and astrocytoma (b,d,f) cells were analyzed to determine the percentage of cells responding to carbachol (a,b), the average amplitude of the response (c,d), and the area under the curve (AUC) (e,f). Results are expressed as the mean response ± S.E.M. (n=5-6); 50 cells were analyzed in each experiment.

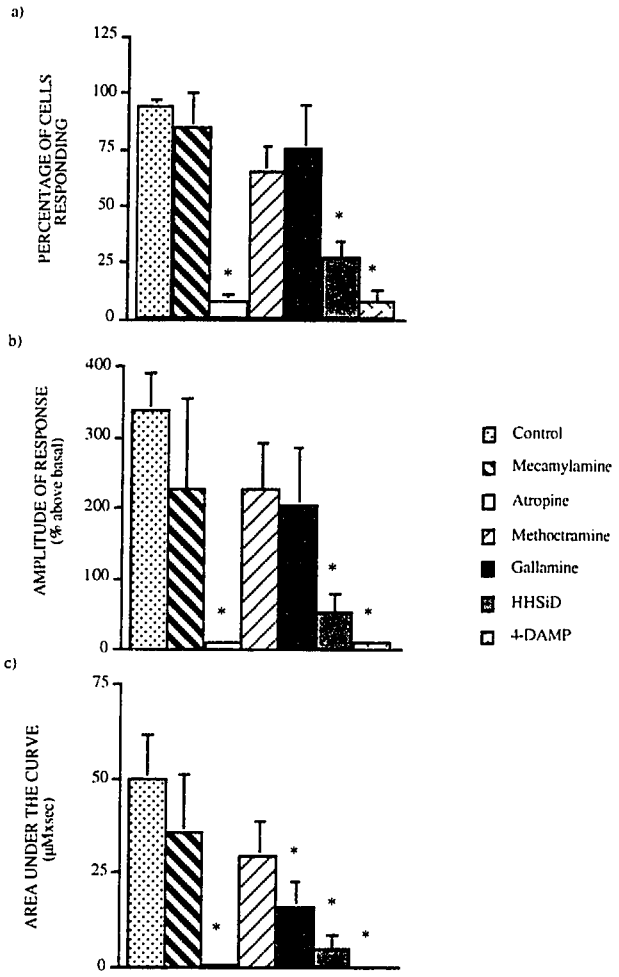


Figure 5. The carbachol-induced calcium response in human 132 1N1 astrocytoma cells is mediated by the M3 muscarinic receptor subtype.

Results are shown following stimulation with 1 mM carbachol alone, or following a 10 min pre-incubation with 10 μM atropine, 10 μM mecamylamine, 1 μM methoctramine, 1 μM gallamine, 1 μM hexahydro-sila-difenidol hydrochloride (HHSiD), or 1 μM 4-diphenylacetoxy-N-methylpiperidine methiodide (4-DAMP). Results were analyzed to determine the percentage of cells responding to carbachol (a), the average amplitude of the response (b), and the area under the curve (AUC) (c). Results are expressed as the mean percent response ± S.E.M. (n=5-7); 50 cells were analyzed in each experiment. * significantly different from control response, $p<0.05$.

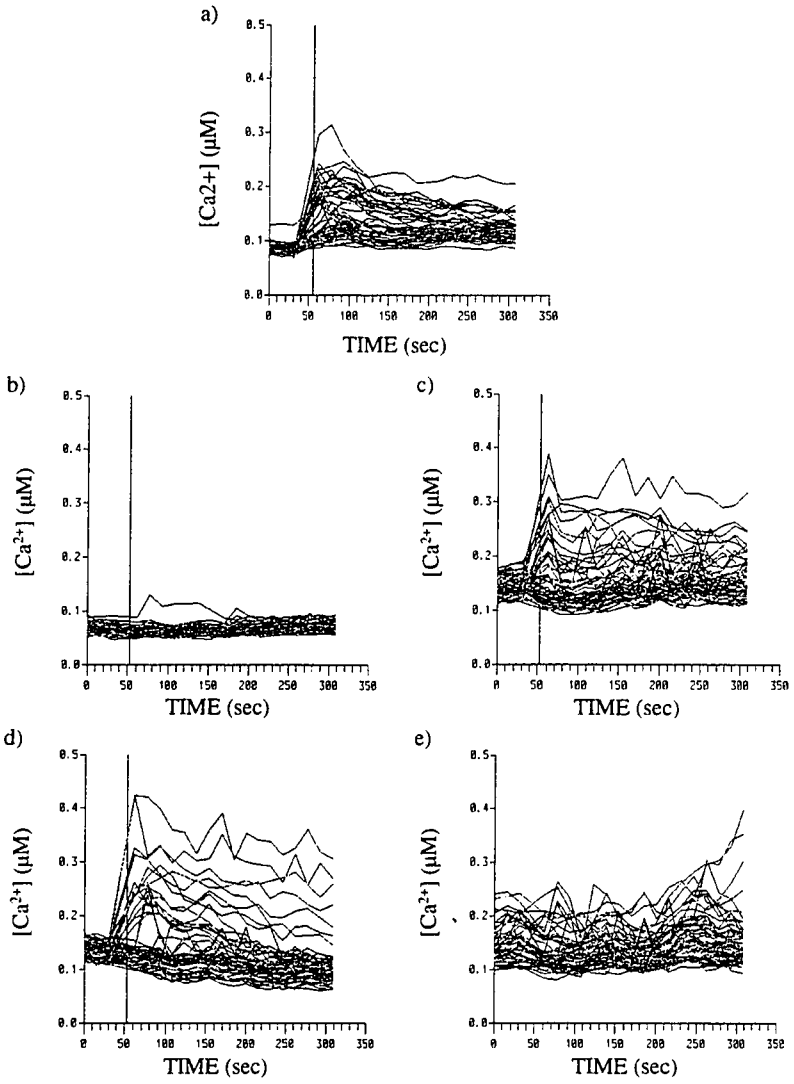


Figure 6. The carbachol-induced calcium response in primary rat cortical astrocytes is mediated by the M3 muscarinic receptor subtype.

Time courses plots of the carbachol-induced response alone (a), or following a 10 min pre-incubation with 10 μM atropine (b), 10 μM mecamylamine (c), 1 μM methoctramine (d) , or 1 μM 4–diphenylacetoxy-N-methylpiperidine methiodide (4-DAMP) (e). Each of the 50 lines indicates the calcium concentration (μM) in an individual cell, with the vertical line indicating the time of addition of 1 mM carbachol.

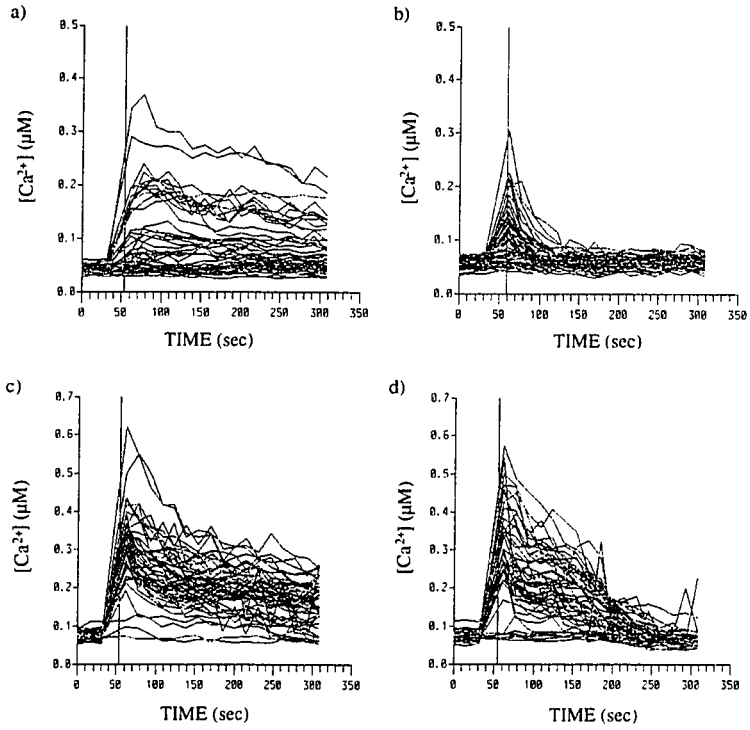


Figure 7. The initial calcium spike is due to release from intracellular calcium stores, whereas the sustained elevation and oscillations are dependent upon the presence of extracellular calcium.

Representative time course of the carbachol-induced responses in primary rat cortical astrocytes (a,b) and human astrocytoma 132 1N1 cells (c,d) in the presence of normal Krebs' buffer ($[Ca^{2+}]$=1.8 mM]) (a,c) and calcium-free Krebs' buffer containing 1 mM EGTA (b,d). Each of the 50 lines indicates the calcium concentration (μM) in an individual cell, with the vertical line indicating the time of addition of 1 mM carbachol in the primary astrocytes or 0.01 mM carbachol in the astrocytoma cells.

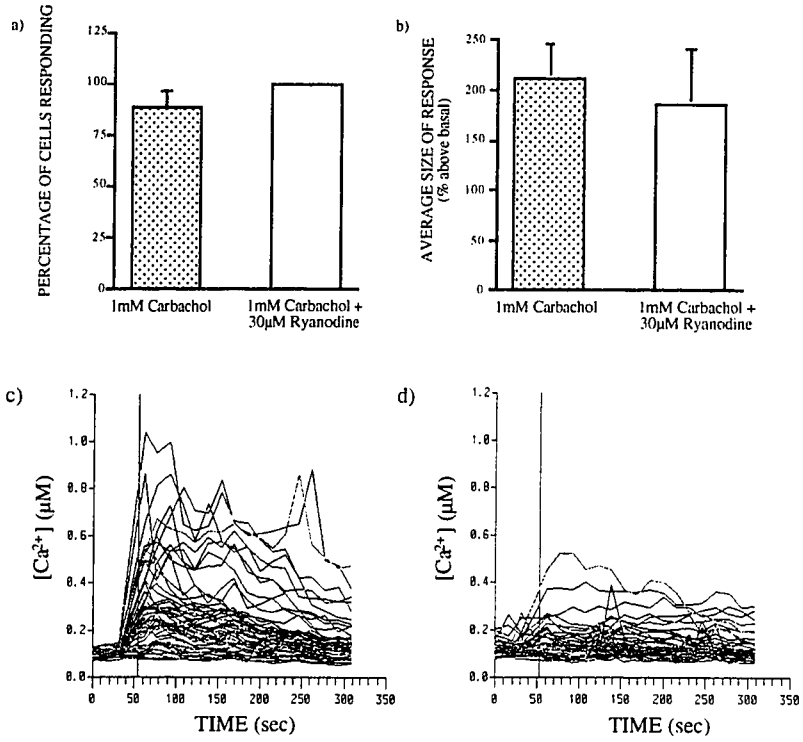


Figure 8. The initial calcium spike is due to release of calcium from IP_3-sensitive stores.

a,b) Results are shown following stimulation with 1 mM carbachol alone, or following a 30 min incubation with 30 μM ryanodine; data were analyzed to determine the percentage of cells responding to carbachol (a), and the average amplitude of the response (b). Data are expressed as the mean percent response ± S.E.M. (n=2); 50 cells were analyzed in each experiment. c,d) Representative time course in primary rat cortical astrocytes for 1 mM carbachol-induced responses alone (c), or following a 30 min incubation with 40 μM xestospongin C, which blocks IP_3-mediated calcium release.

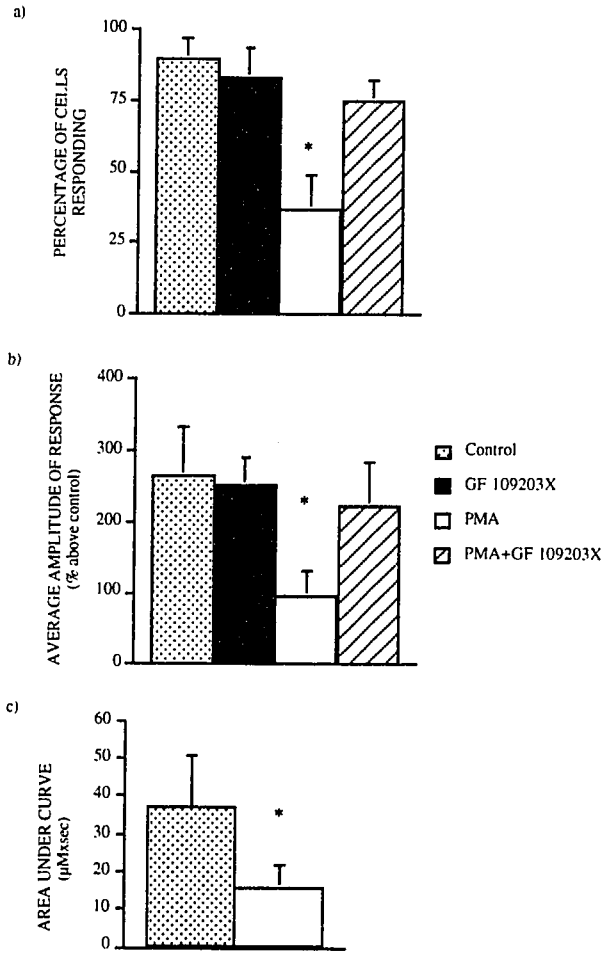


Figure 9. The carbachol-induced calcium response is inhibited by phorbol 12-myristate 13-acetate (PMA) in a bisindolylmaleimide 1 (GF 109203X) sensitive manner.

Results are in human 132 1N1 astrocytoma cells following stimulation with 1 mM carbachol alone, following a 10 min pre-incubation with 200 ng/ml PMA, and/or a 20 min pre-incubation with 1 μM GF 109203X. Results were analyzed to determine the percentage of cells responding to carbachol (a), the average amplitude of the response (b), and the area under the curve (AUC) (c). Results are expressed as the mean percent response ± S.E.M. (n=5-6); 50 cells were analyzed in each experiment. * significantly different from control response, $p<0.05$.

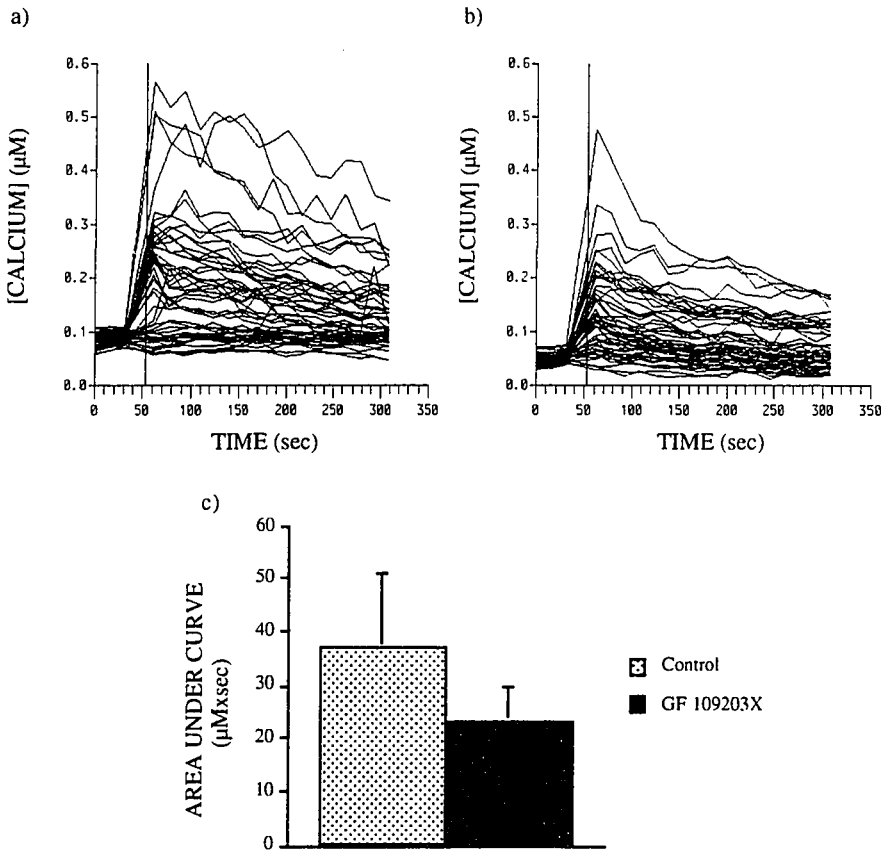


Figure 10. The sustained calcium elevation and oscillations are inhibited by bisindolylmaleimide 1 (GF 109203X).

Representative time course of the carbachol-induced responses in human astrocytoma 132 1N1 cells following stimulation with 1 mM carbachol alone (a), or following a 20 min pre-incubation with 1 μM GF 109203X (b). Each of the 50 lines indicates the calcium concentration (μM) in an individual cell, with the vertical line indicating the time of addition of carbachol. The average area under the curve (AUC) (c) was calculated. Results are expressed as the mean AUC ± S.E.M. (n=5-6); 50 cells were analyzed in each experiment.

CHAPTER 3: EFFECT OF ETHANOL ON MUSCARINIC RECEPTOR-INDUCED CALCIUM RESPONSES IN ASTROGLIA

Abstract

The effects of ethanol on muscarinic-mediated calcium responses were investigated in individual primary rat astrocytes and human 132 1N1 astrocytoma cells using indo-1/AM and image cytometry. Muscarinic-induced calcium responses were inhibited after a 30 min incubation with 100 or 250 mM ethanol. The ethanol effects were more pronounced and occurred at lower ethanol concentrations with a longer ethanol exposure, with significant inhibition seen at 10 mM ethanol following a 24 h incubation with ethanol. Thapsigargin- and glutamate-induced responses were unaffected, indicating some selectivity in this inhibition. A partial inhibition of calcium responses remained up to 12 h after ethanol removal, with responses returning to normal by 24 h after washout. Similar effects were seen in primary rat cortical astrocytes. Ethanol treatment did not affect muscarinic-receptor binding in astrocytoma cells. Ethanol inhibited ^{3}H-thymidine incorporation in the cells with a similar concentration- and timing-dependency as the calcium responses. These data suggest that ethanol inhibits muscarinic-induced calcium responses in astroglia in a concentration- and duration-dependent manner, that co-incubation with ethanol is not necessary for this effect, that the site of action of ethanol is downstream of the receptor, and that this inhibition may be involved in ethanol's inhibition of carbachol-induced thymidine incorporation.

Introduction

Glial cells have been proposed as targets in the Fetal Alcohol Syndrome (FAS) (Guizzetti et al., 1997). These cells play an active role in CNS development, secreting growth factors (Rudge *et al.*, 1992), and guiding the formation of synapses (Nakanishi *et al.*, 1994), and are essential for axonal pathway formation, neuronal differentiation and neuronal survival (Hosoya *et al.*, 1995; Jones *et al.*, 1995; Xiong and Montell, 1995). In addition, glial cells comprise more than one half the volume of the

brain. Effects on the proliferation of these cells during development, therefore, may have a large effect on the overall brain size.

Carbachol induces proliferation of primary rat cortical astrocytes and human 132 1N1 astrocytoma cells (Guizzetti *et al.*, 1996). This proliferation appears to be mediated by the M3 muscarinic receptor subtype, which is linked by $G_{\alpha q/11}$-protein to the phosphatidylinositol pathway. Ethanol inhibits muscarinic-receptor-induced proliferation in these cells in a concentration-dependent manner (Guizzetti and Costa, 1996). This inhibition may be related to the microencephaly seen in FAS, however, the mechanism underlying this inhibition is unknown. Since M3 muscarinic receptors activate the metabolism of phosphatidylinositol (4,5)-bisphosphate, inhibition of inositol-1,4,5-trisphosphate (IP_3) formation and of calcium mobilization may be involved in the effect of ethanol. There is evidence that ethanol affects the muscarinic-induced generation of IP_3. Treatment of rats with ethanol inhibited the muscarinic-receptor-induced phosphoinositide metabolism in the brain (Balduini and Costa, 1989), and incubation of brain slices (Balduini and Costa, 1990; Balduini *et al.*, 1991b), mixed rat cortical cell cultures (Kovacs *et al.*, 1995), and SY5Y cells (Larsson *et al.*, 1995) with ethanol inhibits muscarinic-receptor-induced IP_3 generation *in vitro*.

Binding of IP_3 to its specific receptors on the endoplasmic reticulum leads to the release of calcium from intracellular stores. These intracellular calcium responses have been characterized in glial cells (Catlin *et al.*, 1999c). Activation of primary rat astrocytes and human 132 1N1 astrocytoma cells with carbachol results in complex calcium responses consisting of an initial calcium spike, mediated by release from IP_3-sensitive intracellular stores, followed by a sustained calcium elevation and oscillations (Catlin *et al.*, 1999c).

Calcium is an important intracellular messenger in glial cells, that is believed to play a role in cell proliferation. Calcium responses have been shown to mediate cell cycle progression (Takuwa *et al.*, 1995), DNA synthesis (Bennett and Williams, 1993), and cell growth (Short *et al.*, 1993), including carbachol-induced proliferation in U-373 human astrocytoma cells (Lee *et al.*, 1993b; Lee *et al.*, 1993a; Lee *et al.*, 1994). In astroglial cells, both carbachol-induced proliferation and alterations in calcium homeostasis

appear to be mediated by activation of M3 muscarinic receptors (Guizzetti *et al.*, 1996; Catlin *et al.*, 1999c).

Ethanol exerts a variety of effects on calcium homeostasis in glial and neuronal cells (Catlin et al., 1999a). In particular, ethanol has been shown to inhibit muscarinic-receptor-mediated calcium responses in SY5Y neuroblastoma cells (Larsson *et al.*, 1998) and mixed rat cortical cell cultures (Kovacs *et al.*, 1995). However, the effect of ethanol on muscarinic-receptor-induced calcium responses in glial cells has not been investigated in any detail.

The objective of this research was, therefore, to investigate the effects of ethanol on muscarinic-receptor-mediated calcium responses in primary rat cortical astrocytes and human 132 1N1 astrocytoma cells, and the possible relationship between this inhibition and the decrease in astroglial proliferation seen following ethanol exposure.

Materials and Methods

Animals and materials

Time-mated pregnant rats were supplied by B&K Universal (Kent, WA), and housed, one per cage, in the University of Washington Vivarium. Use of rats was approved by the University of Washington Animal Care Committee. Two-well coverglass chamberslides were from Nalge Nunc International (Naperville, IL), antibodies were from Accurate Chemical (Westbury, NY), and Indo-1/acetylmethylester (AM) and calcium standards were from Molecular Probes (Eugene, OR). The inositol-1,4,5-trisphosphate [^{3}H]-radioreceptor assay kit, ^{3}H-N-methylscopolamine, and [methyl-^{3}H]-thymidine were from DuPont NEN Research Products (Boston, MA). The ethanol diagnostic kit was from Sigma (St. Louis, MO). All other chemicals were from Gibco (Grand Island, NY) or Sigma.

Tissue Culture

Primary rat cortical astrocyte cultures were prepared by a modification of the method of McCarthy and DeVellis (McCarthy and DeVellis, 1980), as described previously (Guizzetti *et al.*, 1996). Briefly, the cortices from day 21 fetuses were minced,

trypsinized and washed three times by centrifugation with Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), penicillin and streptomycin. The tissue was triturated, filtered and plated in 75 cm^2 flasks, previously coated with poly-d-lysine (10 µg/ml, 10 ml), at $1.5x10^5$ cells/cm^2. Within 24 hours of plating, the flasks were shaken and fresh media was added. Flasks were maintained at 37°C with 5% CO_2 for 9 days and fed every 2-3 days. On day 9 in culture the flasks were shaken overnight, trypsinized and reseeded for experiments. Following reseeding, astrocytes were grown for 2 days, then rinsed with phosphate-buffered saline and serum-deprived for 2 days in DMEM supplemented with 0.1% Fraction V fatty acid-free bovine serum albumin. Astrocyte cultures were determined to be at least 95% pure by indirect immunofluorescence with antibodies against glial fibrillary acidic protein (GFAP) and neuron-specific enolase.

The immortalized human astrocytoma cell line, 132 1N1 (provided by Dr. J. Heller-Brown, University of California at San Diego) was cultured as previously described (Guizzetti *et al.*, 1996). Briefly, cells were maintained in 75 cm^2 flasks in DMEM with 5% FBS, penicillin and streptomycin added. Flasks were fed every 2-3 days and passaged once a week, at which time cells were reseeded for experiments. Cells were grown for 4 days, and serum deprived 2 days prior to the experiment. Cells were used between the third and the seventeenth passage.

Ethanol Treatments

Evaporation of ethanol was minimized in a manner similar to that described previously for long-term ethanol exposures (Guizzetti and Costa, 1996). For experiments carried out in 24 well plates, the interwell spaces were filled with the ethanol concentration of the experiment. For 35 mm dishes and chamber slides, dishes were placed inside a 100 mm or 150 mm dish which contained the ethanol concentration of the experiment. Although there was some loss of ethanol, in the 35 mm dishes and chamber slides, 24 h after ethanol treatment the ethanol concentration was at least 65% of the original concentration. Also, for the calcium experiments, cells were placed in Kreb's buffer for the last 1-1.5 h of incubation. This buffer, prepared at the time of addition, contained ethanol at the experimental concentration, replenishing the ethanol in these experiments.

Ethanol concentrations following a 24 h incubation in 24-well plates has previously been shown to be at least 83% of the starting concentration (Guizzetti and Costa, 1996).

Calcium Measurements

Calcium measurements were carried out in primary rat astrocytes by a modification of the method of Kovacs (Kovacs *et al.*, 1995), as previously described (Gafni *et al.*, 1997; Catlin *et al.*, 1999c). On day 10 of culture, primary rat astrocytes were reseeded at $2.4x10^5$ cells/cm^2 in 2 well coverglass chamber slides previously coated with poly-d-lysine (10 μg/ml, 2 ml). Primary astrocytes were loaded with a 2 μM solution of cell permeant Indo-1/AM in Kreb's buffer containing 1% Fraction V BSA and 1 mM probenecid for 30 min, rinsed, and incubated for 15 min. Indo-1 was excited at 351-363 nm and emission was detected simultaneously at 405 and 530 nm to measure the fluorescence intensity of the dye bound and unbound to calcium, respectively, using an attached-cell analysis and sorter (ACAS) Ultima instrument (Meridian Instruments, Okemos, MI). The ratio of the fluorescence at the two wavelengths was determined in 50 cells and was compared to the ratio for calcium standards in solution to determine the absolute calcium concentration.

A similar protocol was followed in the human 132 1N1 astrocytoma cell line. Astrocytoma cells were reseeded at $2x10^4$ cells/cm^2 in chamber slides. Astrocytoma cells were loaded with a 2 μM solution of Indo-1/AM for 60 minutes, without probenecid. Calcium measurements were carried out using the ACAS as described for the primary rat cortical astrocytes.

Data were analyzed as previously described (Catlin *et al.*, 1999c). The basal calcium level, the percentage of cells responding to a stimulus (response greater than 2 standard deviations above basal calcium), the amplitude of the initial spike, and the area above basal under the concentration-versus-time curve (AUC, unitless) were calculated. Experiments were carried out in calcium-free Kreb's buffer, added immediately prior to measuring calcium, containing 1 mM EGTA, to determine the effect of ethanol on IP_3-mediated calcium responses (Catlin *et al.*, 1999c).

Receptor Binding Assay

Human 132 1N1 astrocytoma cells were reseeded at $2.4x10^5$ cells/cm^2 in 35 mm tissue culture dishes, and ^{3}H-N-methylscopolamine binding measured by a modification of the method of Ehrich et al. (1994). Following ethanol treatments, cells were rinsed twice with warm buffer, 10 µM atropine was added for 10 min to some dishes to determine non-specific binding, and 0.15 nM (equivalent to the k_D value in separate experiments) ^{3}H-N-methylscopolamine was added for 60 min at 37°C. Cells were rinsed twice with cold buffer, and incubated two times with 1 ml methanol for 45 min. Methanol was collected in scintillation vials, evaporated overnight, and after addition of 5 ml of scintillation fluid, radioactivity was counted on a Beckmann LS 5000 CE scintillation counter. Protein determinations were done for each treatment using the bicinchoninic acid protein assay (Pierce, Rockford, Il) with bovine serum albumin standards.

IP_3 Assay

Human 132 1N1 astrocytoma cells were reseeded at $2.4x10^5$ cells/cm^2 in 35 mm tissue culture dishes. Following ethanol treatments, cells were rinsed twice with Kreb's buffer containing LiCl and treated for 1 min with either buffer or carbachol (1 mM) and harvested in 500 µl of 10% trichloroacetic acid. IP_3 was assayed following the instructions in the NEN kit. Briefly, IP_3 was extracted in a 1,2-trichloro-1,2,2-trifluoroethane:trioctylamine (3:1) solution, and the aqueous layer was collected and assayed for protein using the bicinchoninic acid protein assay. One hundred microliters of the extracted solution was incubated with [^{3}H]-IP_3 and a calf cerebellum membrane preparation containing the IP_3 receptor. This mixture was incubated for one hour at 4°C to allow displacement of radiolabelled IP_3, and IP_3-receptor complex was precipitated by centrifugation. The supernatant was removed, the IP_3 bound to the receptor was resuspended in NaOH, and the radioactivity was counted in scintillation fluid on a Beckmann LS 5000 CE scintillation counter. The percent bound for each sample was compared to a standard curve to determine the absolute amount of IP_3 present.

Thymidine Incorporation

[methyl-^{3}H]-Thymidine incorporation was measured as previously described (Guizzetti and Costa, 1996; Guizzetti et al., 1996). Briefly, cells were incubated for 24 hours with carbamylcholine chloride (carbachol), with [^{3}H]-thymidine added for the last 6 hours of incubation. Cells were rinsed three times with phosphate buffered saline and DNA precipitated with trichloroacetic acid. The DNA was solubilized in 1M NaOH and a 250 µl aliquot was counted in scintillation fluid on a Beckmann LS 5000 CE scintillation counter. Thymidine incorporation is expressed as percent of basal incorporation. The carbachol-induced incorporation of [methyl-^{3}H]-thymidine in these cells has been shown previously to be accompanied by cell cycle progression, including cells in a new G_0 and G_1 phase, using bromodeoxyuridine and Hoescht staining with flow cytometry, and an increase in cell number by cell counting (Guizzetti and Costa, 1996; Guizzetti *et al.*, 1998).

Statistical Analysis

Statistically significant differences were determined by paired Students' t-tests.

Results

Carbachol-induced calcium responses had been characterized and quantitated in primary rat cortical astrocytes and human 132 1N1 astrocytoma cells (Catlin *et al.*, 1999c). In the presence of extracellular calcium, an initial increase of intracellular calcium levels (calcium spike) was followed by a sustained elevation of calcium levels. Addition of EGTA to the incubation medium abolishes the sustained elevation of calcium, indicating its dependence upon entry of calcium from the extracellular milieu. To investigate the effect of ethanol on the IP_3-induced calcium mobilization, all present experiments were carried out in the presence of EGTA. Under these conditions, the control responses in both cell types were similar: carbachol caused an immediate spike in the intracellular calcium concentration, increasing cytosolic calcium from a basal concentration of 70±5 nM to 340±96 nM (n=16) in human 132 1N1 astrocytoma cells, and from 60±10 nM to 130±25 nM (n=8) in primary rat cortical astrocytes. As expected, the

cytosolic calcium concentration returned to basal levels by the end of the data collection period (Fig. 11).

The effect of ethanol on three aspects of the carbachol-stimulated increase in cytosolic calcium were considered: the total number of cells responding to the stimulation with an increase in calcium, the amplitude of the calcium spike, which reflects IP_3-induced calcium release, and the area under the curve (AUC), which normally reflects the entry of calcium from the extracellular milieu. In the presence of calcium the sustained elevation and oscillations due to calcium entry are normally reflected in the AUC. This sustained increase and oscillations are not present in the presence of EGTA as in these experiments, however, in some experiments ethanol delayed the calcium response, therefore, the area under the curve was quantitated to take this effect into account. A 30 min incubation with 10-250 mM ethanol had no effect on basal calcium levels (data not shown), and had limited effects on the carbachol-induced calcium response. The AUC was inhibited by 100 mM ethanol (Fig. 12e), and both the amplitude of the calcium spike and the area under the curve were inhibited by 250 mM ethanol (Fig. 12c,e), while the percentage of cells responding to carbachol was not significantly affected by a 30 min exposure to the concentrations of ethanol tested (Fig. 12a). Similar results were seen in primary rat cortical astrocytes following a 30 min incubation with ethanol, with the amplitude of the response decreased at 250 mM ethanol (Fig. 12d). In cortical astrocytes, calcium levels went below baseline levels in many cells, resulting in negative values for the area under the curve, therefore, this endpoint was not used in these cells.

Different incubation times with ethanol were then investigated in astrocytoma cells to determine whether the length of exposure to ethanol affects the carbachol-induced calcium response (Fig. 13). Calcium responses were measured after a 5 min, 30 min, 6 h or 24 h incubation with 250 mM ethanol, with the ethanol present at the time of carbachol addition. None of these treatments had any effect on basal calcium levels in this cell line. Basal calcium levels were 100±19 nM in control cells and 110±18 nM in cells treated for 24 h with 250 mM ethanol (n=7). The duration of exposure did, however, alter the effect of ethanol on muscarinic-induced calcium responses. The amplitude of the calcium spike and the area under the curve were not affected by a 5 min incubation with 250 mM ethanol, but a 30 min or longer incubation

significantly decreased these responses. The degree of inhibition was duration-dependent, with a stronger and more consistent inhibition after a 24 h exposure to ethanol. The percentage of cells responding was only affected following a 24 h exposure to ethanol. In addition, the concentration of ethanol required to inhibit carbachol-induced calcium responses decreased with a longer exposure to ethanol (Fig. 14). After a 24 h incubation, all three measured endpoints were inhibited at almost all of the ethanol concentrations, with significant effects on the percentage of cells responding and the amplitude of the response starting at 10 mM ethanol.

There was a great deal of variability in the effects of ethanol between experiments which suggested different classes or categories of inhibition by ethanol. Although the number of experiments is not large enough to statistically analyze this data categorically, information can be gained by classifying the affects of ethanol on calcium responses. Ethanol, at any given exposure, sometimes did not inhibit the calcium response, whereas in another experiment, using the same exposure protocol, a total inhibition of the calcium response was seen. Intermediate effects were also seen, as was a delay in the calcium response. As the concentration of ethanol or the duration of ethanol exposure increased, however, the inhibition by ethanol was more consistent.

In order to gain insight on the mechanism(s) underlying inhibition by ethanol, the specificity of this effect was investigated after 24 h incubations. As shown in Fig. 15, 100 mM ethanol had no effect on the calcium released by 250 nM thapsigargin in human 132 1N1 astrocytoma cells (Fig. 15a-c). Primary astrocytes were used to investigate the effects of ethanol on glutamate responses, as 123 1N1 astrocytoma cells do not respond to glutamate with a calcium response (Catlin *et al.*, 1999c). Exposure to 100 mM ethanol had no effect on glutamate (1 mM)-induced calcium response in these cells (Fig. 15d,e).

Since inhibition of the carbachol-induced calcium response by ethanol was dependent upon the duration of incubation with the alcohol, experiments were carried out to dctermine whether the presence of ethanol at the time of carbachol addition was necessary for an inhibitory effect to be manifested. For this purpose, astrocytoma cells were exposed to 250 mM ethanol for 24 h, rinsed, and the calcium responses to carbachol were measured. Upon removal of ethanol the percentage of cells responding rapidly (within

5 min) returned to 100% (Fig. 16a). The amplitude of the initial calcium spike (Fig. 16b) and the area under the calcium curve (Fig. 16c), however, remained inhibited following removal of ethanol, with the area significantly below control levels up to 6 h after ethanol removal, and the amplitude of the spike was still significantly decreased 12 h after ethanol was removed. Responses had fully returned to control levels by 24 h. These results indicate that the presence of ethanol together with carbachol is not necessary for the alcohol to inhibit the muscarinic receptor-induced calcium response. Furthermore, it also appears that the inhibiting effect of ethanol is slowly reversible with time.

The ability of ethanol to strongly inhibit the carbachol-induced calcium response following long-term incubation, in a reversible manner, was confirmed in primary rat cortical astrocytes (Fig. 17). A 24 h incubation with 100 mM ethanol almost totally inhibited the carbachol-induced calcium response in these cells (Fig. 17b). A 24 h incubation with 100 mM ethanol, followed by 24 h in the absence of ethanol resulted in a recovery of the carbachol response (Fig. 17c).

Possible sites of action of ethanol were investigated by determining the effect of ethanol on muscarinic receptor binding in human 132 1N1 astrocytoma cells. In preliminary experiments we determined that in control cells, ^{3}H-N-methylscopolamine labels a single population of muscarinic receptors with a receptor density (B_{max}) of 97 fmol/mg protein, and a dissociation constant (k_d) of 0.15 nM. A 24 h incubation with 100 mM or 250 mM ethanol had no affect on the binding of ^{3}H-N-methylscopolamine (0.15 nM) in these cells: binding (fmol/mg protein) was 44.6±10.5, 41.2±9.2 (100 mM ethanol), and 45.7±5.6 (250 mM ethanol) (n=3; p=0.78).

Since IP_3 generation is responsible for the initial calcium spike (Gafni *et al.*, 1997; Catlin *et al.*, 1999b), the effect of ethanol on carbachol-induced IP_3 formation was also measured. In a representative experiment, carbachol caused an approximately 10-fold increase in IP_3, from 8 to 100 pmol/mg protein. A 24 h incubation with 100 mM ethanol had no effect on basal IP_3 formation (12 pmol/mg protein), but inhibited the carbachol-induced increase (27 pmol/mg protein). This inhibition persisted following the removal of ethanol (38 pmol/mg protein).

As ethanol is known to inhibit muscarinic-receptor-induced proliferation of glial cells (Guizzetti and Costa, 1996), time-course experiments with ethanol were carried out and compared to the effects of ethanol on muscarinic-receptor-induced calcium responses. Figure 18a shows that a 24 hour co-incubation with 50 mM or 100 mM ethanol caused a strong inhibition of muscarinic-induced ^{3}H-thymidine incorporation in human 132 1N1 astrocytoma cells. If ethanol was present for 24 h before the experiment, but then removed during the carbachol incubation, there was a small decrease of inhibition, but the incorporation was still significantly inhibited. After ethanol removal a time-dependent return to the normal incorporation response was observed (Fig. 18b). Significant inhibition of thymidine incorporation was still present 6 h after removal of ethanol, however, incorporation was back to control levels by 12 to 24 h.

Discussion

Activation of muscarinic receptors leads to IP_3-mediated intracellular calcium release (Catlin *et al.*, 1999c) and an increase in cell proliferation in primary rat astrocytes and human 132 1N1 astrocytoma cells (Guizzetti *et al.*, 1996; Guizzetti *et al.*, 1998), with both of these responses mediated by the M3 muscarinic receptor subtype. Muscarinic-receptor induced proliferation in these cells is sensitive to inhibition by ethanol, but the mechanism underlying this inhibition is unknown (Guizzetti and Costa, 1996). Calcium is involved in the proliferation of many cell types (Kawanishi *et al.*, 1994; Loza *et al.*, 1995; Schmitt *et al.*, 1995; Stanimirovic *et al.*, 1995; Ouyang *et al.*, 1996; Kataoka *et al.*, 1997; Sahai *et al.*, 1997; Zeitler *et al.*, 1997; Schmid *et al.*, 1998; Smith-Thomas *et al.*, 1998), and, as a result, any effects on intracellular calcium responses may affect proliferation. Despite these important implications, the effects of ethanol on calcium responses in glial cells, and how they may relate to cell proliferation, have not been investigated. The present study shows that ethanol inhibits IP_3-mediated calcium responses in astroglia, and suggests the inhibition of these responses may be related to the ethanol-induced inhibition of glial cell proliferation.

The increase in intracellular calcium following muscarinic stimulation in primary rat cortical astrocytes and human 132 1N1 astrocytoma cells has been quantitated using image cytometry. The calcium responses found in the present study are similar to those previously seen in these cell types (Catlin *et al.*, 1999c). Ethanol had no effect on

basal calcium levels, except for an increase in the calcium level after a 24 h incubation with 250 mM ethanol in primary astrocytes. There does not appear to be any consistent effect of ethanol on basal calcium levels between different cell types. Ethanol did not increase basal calcium levels in a mixed rat cortical cell culture (10 min, up to 500 mM) (Kovacs *et al.*, 1995), cortical neurons (4 days, 100 mM) (Blevins *et al.*, 1995), or skeletal muscle cells (2 weeks, 40 mM) (Cofan *et al.*, 1995). In PC12 cells, short-term ethanol did not affect calcium levels (40 min, 100 mM) (Rabe and Weight, 1988), however, levels were increased following longer exposures (4-6 days, 200 mM) (Messing *et al.*, 1990; Grant *et al.*, 1993). Long-term ethanol exposure also increased intracellular calcium levels in cerebellar macroneurons (48-96 h, 75 mM) (Zou *et al.*, 1995), lymphocytes (24 h, 20-200 mM) (Fano *et al.*, 1993), and astroglia (7 days, 50-100 mM) (Holownia *et al.*, 1997). Overall, these data may indicate that the effects of ethanol on basal calcium levels may be dependent upon cell type, and the length and concentration of exposure. This concentration- and duration-dependency was also seen in the primary rat astrocytes in this research, where effects were seen only at the highest concentration when these cell cultures were exposed to ethanol for 24 h.

To investigate the effects of ethanol on IP_3-mediated calcium responses, the effects of various concentrations of ethanol on carbachol-induced calcium responses were studied in the absence of extracellular calcium. Inhibition of the response occurred at the higher concentrations of ethanol used (100 and 250 mM) following a short-term (30 min) incubation. A similar inhibition of calcium responses was seen previously in cortical cell cultures (Kovacs *et al.*, 1995) following the same short-term exposure to ethanol. The duration of cell exposure to ethanol affected ethanol's inhibition of carbachol-induced calcium responses. A 5 min incubation with ethanol had no effect on the calcium response, whereas a 24 h incubation with the same concentration of ethanol resulted in a strong inhibition (Fig. 13). The duration of ethanol exposure not only affected the magnitude of the ethanol inhibition, but also decreased the concentration of ethanol required to inhibit carbachol-stimulated calcium responses. Indeed, after a 24 h incubation with ethanol, a significant inhibition of the calcium response was seen at concentrations as low as 10 mM ethanol (Fig. 14), in contrast to a 30 min incubation where inhibition was not seen at concentrations less than 100 mM ethanol (Fig. 12). This dependency of ethanol inhibition on the duration of exposure suggests that ethanol does not directly alter the calcium response, but instead has some other effect(s) on the cell, that requires hours to

fully occur, and that ultimately results in the inhibition of calcium responses. This hypothesis was also supported by the finding that the presence of ethanol together with carbachol was not necessary for inhibition of the calcium response to be manifest (see below).

The calcium responses induced by thapsigargin, which inhibits the endoplasmic reticular Ca^{2+}-ATPase, and glutamate, which likely acts through the activation of mGluR5 metabotropic glutamate receptors in astrocytes (Balazs *et al.*, 1997; Condorelli *et al.*, 1997; Nakahara *et al.*, 1997), were not inhibited by a 24 h exposure to 100 mM ethanol. The lack of inhibition of the thapsigargin response indicates not only that ethanol does not indiscriminately inhibit calcium responses, since the cells are still capable of eliciting a calcium response, but also that the ethanol treatment does not interfere with the measurement of calcium signals. In addition, the response to thapsigargin, which is dependent upon an active ATPase, indicates that the cells are healthy and not energy depleted. The absence of an effect of ethanol on glutamate-induced calcium increase, on the other hand, indicates that the inhibitory action of this alcohol may not extend to all G-protein coupled receptors. It is also important to note that, in addition to suggesting a degree of selectivity by ethanol, the lack of inhibition by long-term ethanol exposure on thapsigargin- and glutamate-induced calcium responses, also indicates that the cells are viable and maintain their ability to respond to a stimulus with a calcium response throughout the time course of the experiment.

Since ethanol caused an inhibition of muscarinic-induced calcium responses in a duration-dependent manner, indicating that ethanol's inhibition of the calcium responses may be due to an indirect effect, the possibility that such an effect would persist even after the removal of ethanol was investigated. Following the removal of ethanol, an inhibition of muscarinic-mediated calcium responses indeed persisted, with a significant inhibition remaining up to 6 h or 12 h after alcohol removal. Responses returned to control levels by 24 h. Although it could be argued that the residual inhibition may be due to small amounts of ethanol that remained after thoroughly rinsing the cells, it should be noted that ethanol is a volatile compound and would be expected to evaporate long before the 6 or 12 h time-points when a significant inhibition is still seen. Furthermore, in one of the parameters examined (number of cells responding) there was a full recovery within 5 minutes after ethanol removal. Nevertheless, the possibility that residual ethanol,

"embedded" in the cell membrane may contribute to the long-term inhibition of the calcium response, may not be fully excluded.

Potential target(s) of ethanol's effects in glial cells were investigated by determining the effects of ethanol on muscarinic receptor binding and carbachol-induced IP_3 formation. Ethanol did not affect receptor binding. A similar lack of effect of ethanol on muscarinic binding has been seen following exposure to ethanol either *in vivo* (Balduini and Costa, 1989) or *in vitro* (Balduini and Costa, 1990). This does not rule out the possibility, however, that the cellular response to ethanol may disrupt the coupling between the receptor and its G-protein. Ethanol decreased the generation of IP_3 in human 132 1N1 astrocytoma cells. Inhibition of muscarinic-induced generation of IP_3 has also been seen after *in vivo* and *in vitro* exposure to ethanol (Balduini and Costa, 1989; Balduini and Costa, 1990; Balduini *et al.*, 1991b; Kovacs *et al.*, 1995; Larsson *et al.*, 1995). These data suggest that the cellular target of ethanol lies somewhere within the interaction of muscarinic receptor with $G_{\alpha q/11}$, phospholipase C, and the generation of IP_3. The effects of ethanol on this system have not been extensively investigated. A downregulation of $G_{\alpha q/11}$ and PLC-β was seen in some experiments, but not in others (Williams and Kelly, 1993; Pandey *et al.*, 1996), and effects on GTPase activity were also seen, but only *in vitro*, not *in vivo* (Singh *et al.*, 1997). Overall, the effect of ethanol on these signaling molecules seems to depend upon the cell type, and the ethanol exposure protocol. In astrocytes, no effects were seen on $G_{\alpha q/11}$ or PLC-β protein expression, and only a decrease in PLC δ was evident (Pandey, 1996). Though there is no effect on the levels of these proteins, however, ethanol may affect their function, and this remains to be more thoroughly investigated.

In addition to elucidating the cellular effects that mediate ethanol's effects on muscarinic-induced calcium responses, it is also important to determine what downstream effects this inhibition may have on the cells The cholinergic system may play an important role in development. Such a role is suggested by the pattern of expression of muscarinic receptors during development, as there is a peak in the muscarinic receptor-induced phosphoinositide response during the brain growth spurt, a developmental period when the proliferation and maturation of glial cells occurs (Balduini *et al.*, 1987). Acetylcholine and

its analogs induce an increase in ^{3}H-thymidine incorporation in primary astrocytes and human 132 1N1 astrocytoma cells, primarily through activation of M3 receptor subtypes (Ashkenazi *et al.*, 1989; Guizzetti *et al.*, 1996). The carbachol-induced increase in thymidine incorporation was accompanied by progression of the cells through the cell cycle and an increase in cell number (Guizzetti and Costa, 1996; Guizzetti *et al.*, 1998), indicating that this increased incorporation may be representative of cell proliferation. Co-incubation of these cells with ethanol and carbachol causes an inhibition of the carbachol-induced proliferation of these cells, and also inhibited the cell cycle progression (Guizzetti and Costa, 1996). In most of these experiments, the cells were exposed to ethanol over a 24 h period, with the incorporation of radioactive thymidine measured for the last 6 h. This inhibition of proliferation was seen at concentrations as low as 10-25 mM ethanol, which resulted in a 50% inhibition of thymidine incorporation. These results are very similar to the effects of ethanol on carbachol-induced calcium response, where 10 mM ethanol inhibited the amplitude of the calcium response by almost 50% after a 24 h exposure. The duration of ethanol exposure was also shown to be important in the inhibition on proliferation, as it is for muscarinic-induced calcium responses (Guizzetti and Costa, 1996). In the present study, further proliferation experiments were carried out to determine whether, following a 24 h pre-incubation with ethanol, the inhibition would persist after the removal of ethanol. This was indeed the case, with pre-incubation of ethanol inhibiting the proliferative response for up to 6 h. This time course is very similar to that seen for the inhibition of calcium responses, where the amplitude and area of the calcium responses were inhibited for 6 and 12 h, respectively. Although causality can not be inferred from this data, the correlation between the time course of ethanol's effects on calcium responses and thymidine incorporation in these cells indicates that an inhibition of calcium responses could mediate ethanol's inhibition of proliferation.

This possibility is further substantiated by the findings that calcium controls aspects of cell growth in many cells, including cell cycle progression (Takuwa *et al.*, 1995), DNA synthesis (Bennett and Williams, 1993), and cell growth (Short *et al.*, 1993). In fact, calcium responses have been implicated in carbachol-induced proliferation in U-373 human astrocytoma cells (Lee *et al.*, 1993a; Lee *et al.*, 1993b; Lee *et al.*, 1994), lending support to the hypothesis that inhibition of calcium responses by ethanol mediate ethanol's inhibition of proliferation in astroglia. Research has demonstrated that changes in the amplitude, duration, and frequency of the calcium changes, even more subtle than those

caused by ethanol, can have major implications on a cell's destiny (Gu and Spitzer, 1995; Dolmetsch *et al.*, 1997; DeKoninck and Schulman, 1998). For example, in B lymphocytes it has been shown that a different pattern of transcription factor activation is seen following a large transient increase in intracellular calcium versus a small calcium plateau (Dolmetsch *et al.*, 1997).

The present data show that ethanol inhibits muscarinic-mediated calcium responses and ^{3}H-thymidine incorporation in astroglia at similar concentrations and with a similar time-course. Evidence suggests that the carbachol-induced thymidine incorporation represents proliferation in these cells, and that ethanol inhibits this proliferation (Guizzetti and Costa, 1996). Little is known about the mechanism that underlies the microencephaly seen in the Fetal Alcohol Syndrome. It is known in the rat, however, that ethanol treatment during the third trimester equivalent, when the brain growth spurt, a time of rapid glial cell proliferation , leads to microencephaly, with an overall decrease in the brain to body weight ratio, along with more specific effects on the neocortex and cerebellum (Kelly *et al.*, 1987; Bonthius and West, 1988; Miller, 1996; Maier *et al.*, 1997). This decrease has been shown to be accompanied by a decreased number of neurons and glia (Miller, 1993). Further research studying brain area weight, DNA content, and protein content suggests that the primary, but not only, target of ethanol leading to microencephaly is the proliferating cell (Miller, 1996). The only other study investigating the cause of this microencephaly examined ornithine decarboxylase activity, and suggested that inhibition of this enzyme by ethanol is involved in the microencephaly seen following *in utero* ethanol exposure (Davidson *et al.*, 1998).

Many of these studies suggest that inhibition of glial cell proliferation may be a mechanism partially responsible for the microencephaly seen in experimental FAS. The results of this research indicate that ethanol affects muscarinic-receptor-mediated calcium responses and thymidine incorporation in glial cells, and that these two effects may be related. Although more research is needed to investigate the role of calcium in this thymidine incorporation, and to determine how this is related to glial cell proliferation, these data suggest that inhibition of calcium responses by ethanol may be one possible mechanism by which ethanol might lead to the microencephaly seen in FAS.

Acknowledgments

This study was supported by grants from NIAAA (AA-08154) and NIEHS (ES-07133).

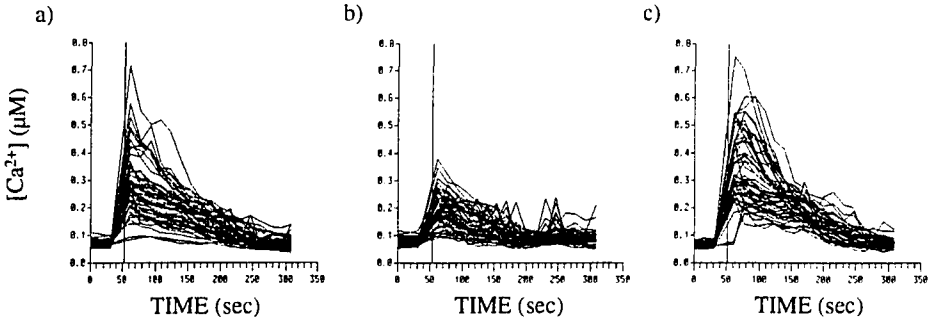


Figure 11. Concentration-versus-time curves for carbachol response in the absence, presence, or following removal of ethanol.

Time-course plots in human astrocytoma cells, in the absence of extracellular calcium, following addition of 0.01 mM carbachol. Each line shows the calcium concentration (μM) in an individual cell, with the vertical line indicating the time of addition of carbachol. Response seen with 0.01 mM carbachol alone a), following a 24 h incubation with 250 mM ethanol b), and following a 24 h incubation with 250 mM ethanol then 24 h in the absence of ethanol c).

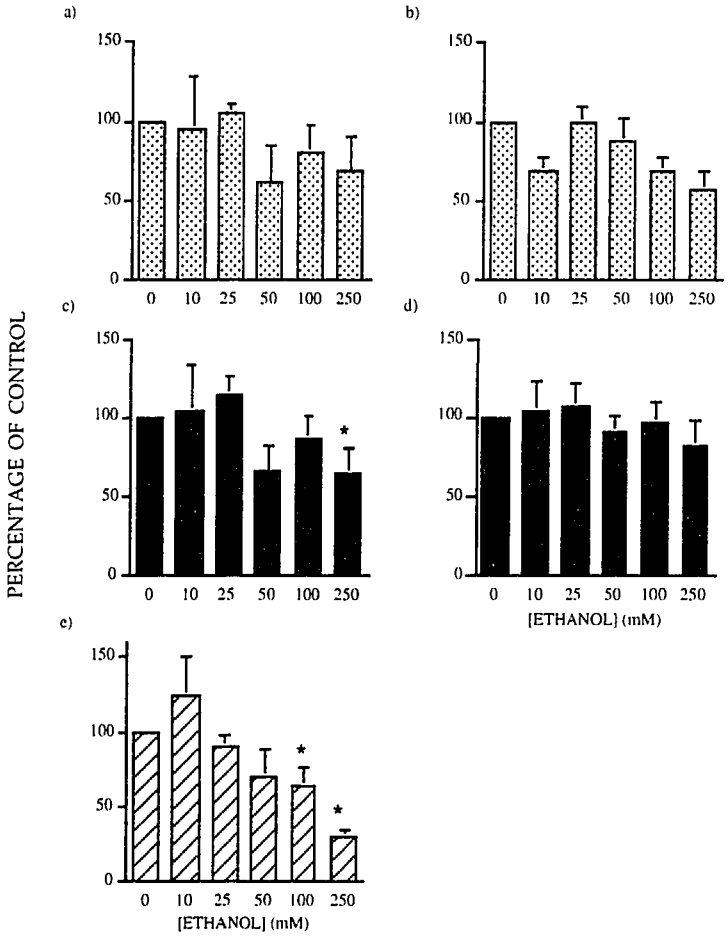


Figure 12. Effect of short-term ethanol exposure on carbachol-induced calcium responses in human 132 1N1 astrocytoma cells and primary rat cortical astrocytes.

Results are shown in astrocytoma cells following stimulation with carbachol alone, or following a 30 min incubation with the specified concentration of ethanol in astrocytoma cells (0.01 mM carbachol) a,c,e) and in primary astrocytes (1 mM carbachol) b,d). Experiments were carried out in calcium-free Kreb's buffer. Ethanol continued to be present at the time of addition of carbachol. Results were analyzed to determine the percentage of cells responding to carbachol (a,b), the average amplitude of the response (c,d), and the area under the curve (AUC) (e). Results are expressed as the mean percentage of the carbachol response (shown as 100%) $\pm$ S.E.M. (n=4-6); 50 cells were analyzed in each experiment. * significantly different from control response, $p<0.05$.

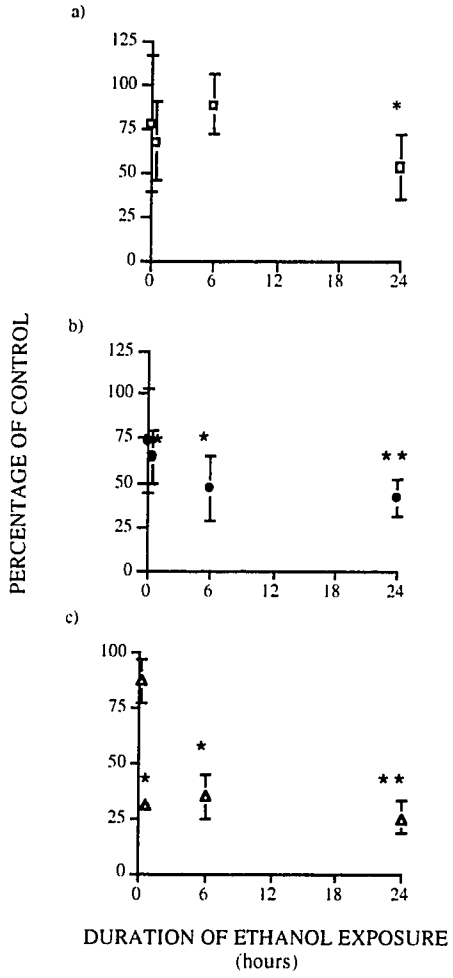


Figure 13. Effect of length of exposure to ethanol on carbachol-induced calcium responses in human 132 1N1 astrocytoma cells.

Results were analyzed following 0.01 mM carbachol exposure to determine the percentage of cells responding (a), the average amplitude of the response (b), and the area under the curve (AUC) (c) following incubation with 250 mM ethanol for the specified period of time. Experiments were carried out in calcium-free Kreb's buffer. Ethanol was present at the time of addition of carbachol. Results are expressed as the mean percentage of the 0.01 mM carbachol response (shown as 100%) ± S.E.M. (n=3-7); 50 cells were analyzed in each experiment. * significantly different from control response, $p<0.05$. ** significantly different from control response, $p<0.001$.

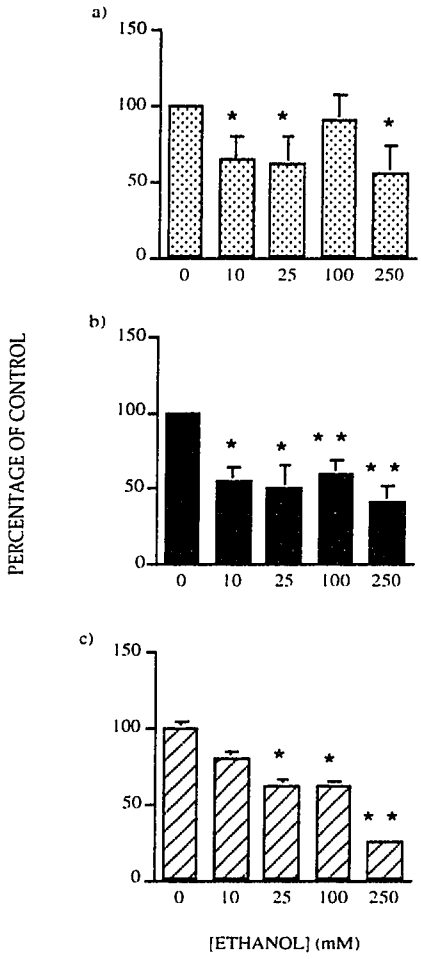


Figure 14. Effect of long-term ethanol exposure on carbachol-induced calcium responses in human 132 1N1 astrocytoma cells.

Results are shown following stimulation with 0.01 mM carbachol alone, or following a 24 hour incubation with the specified concentration of ethanol. Experiments were carried out in calcium-free Kreb's buffer. Ethanol was present at the time of addition of carbachol. Results were analyzed to determine the percentage of cells responding to carbachol (a), the average amplitude of the response (b), and the area under the curve (AUC) (c). Results are expressed as the mean percent of the 0.01 mM carbachol response (shown as 100%) $\pm$ S.E.M. (n=6-7); 50 cells were analyzed in each experiment. * significantly different from control response, $p<0.05$. ** significantly different from control response, $p<0.001$.

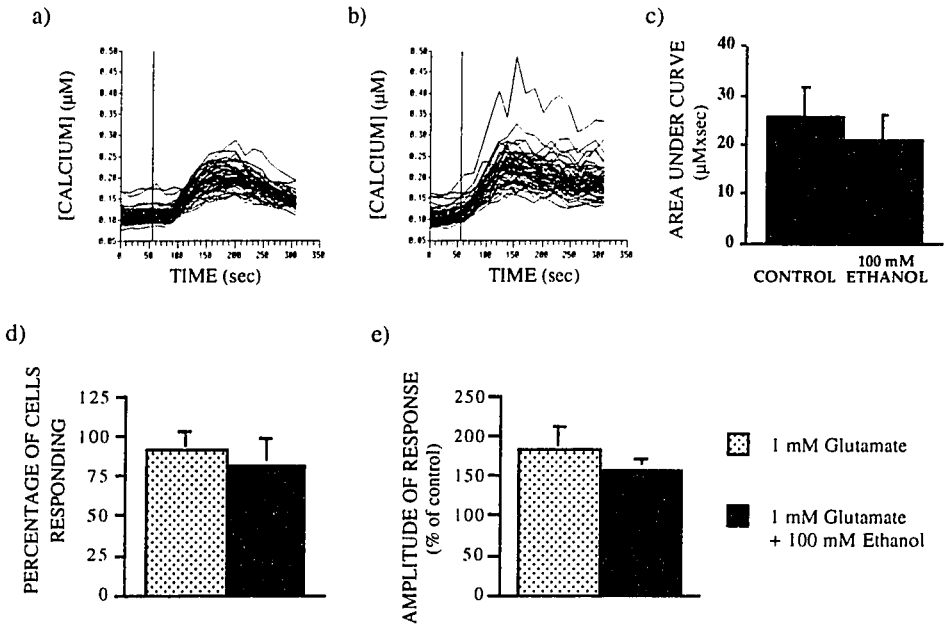


Figure 15. Effect of ethanol on non-muscarinic-receptor-mediated calcium responses.

Time-course plots in human astrocytoma cells, in the presence of extracellular calcium, following addition of 250 nM thapsigargin. Each line shows the calcium concentration of (μM) in an individual cell, with the vertical line indicating the time of addition of thapsigargin. Response seen in control solution a), and following a 24 h incubation with 100 mM ethanol b). Results were analyzed to determine the area under the curve (AUC) c). Results are expressed as the mean amount of calcium±S.E.M. (n=5); 50 cells were analyzed in each experiment. Percentage of cells responding d) and amplitude of the response e) to 1 mM glutamate in primary rat cortical astrocytes under control conditions or following a 24 h incubation with 100 nM ethanol (n=3). No statistically significant differences were seen.

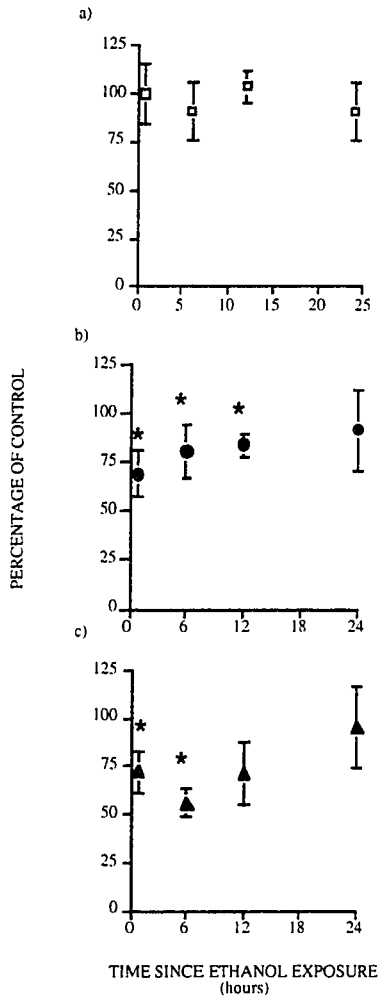


Figure 16. Inhibition of the carbachol-induced calcium response in human 132 1N1 astrocytoma cells after the removal of ethanol.

Results were analyzed following 0.01 mM carbachol exposure to determine the percentage of cells responding to carbachol (a), the average amplitude of the response (b), and the area under the curve (AUC) (c) following pre-incubation with 250 mM ethanol for 24 hours and incubation in the absence of ethanol for the specified length of time. Experiments were carried out in calcium-free Kreb's buffer. Results are expressed as the mean percent of the 1 mM carbachol response (shown as 100%) $\pm$ S.E.M. (n=6-7); 50 cells were analyzed in each experiment. * significantly different from control response, $p<0.05$.

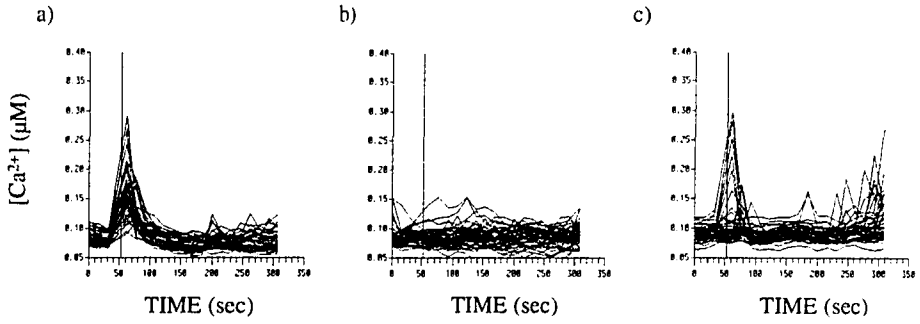


Figure 17. Effect of long-term ethanol exposure on carbachol-induced calcium responses in primary rat cortical astrocytes.

Time-course plots in rat astrocytes, in the absence of extracellular calcium, following addition of 1 mM carbachol. Each line shows the calcium concentration (μM) in an individual cell, with the vertical line indicating the time of addition of carbachol. Response seen with 1 mM carbachol alone a), following a 24 h incubation with 100 mM ethanol b), and following a 24 h incubation with 100 mM ethanol then 24 h in the absence of ethanol c).

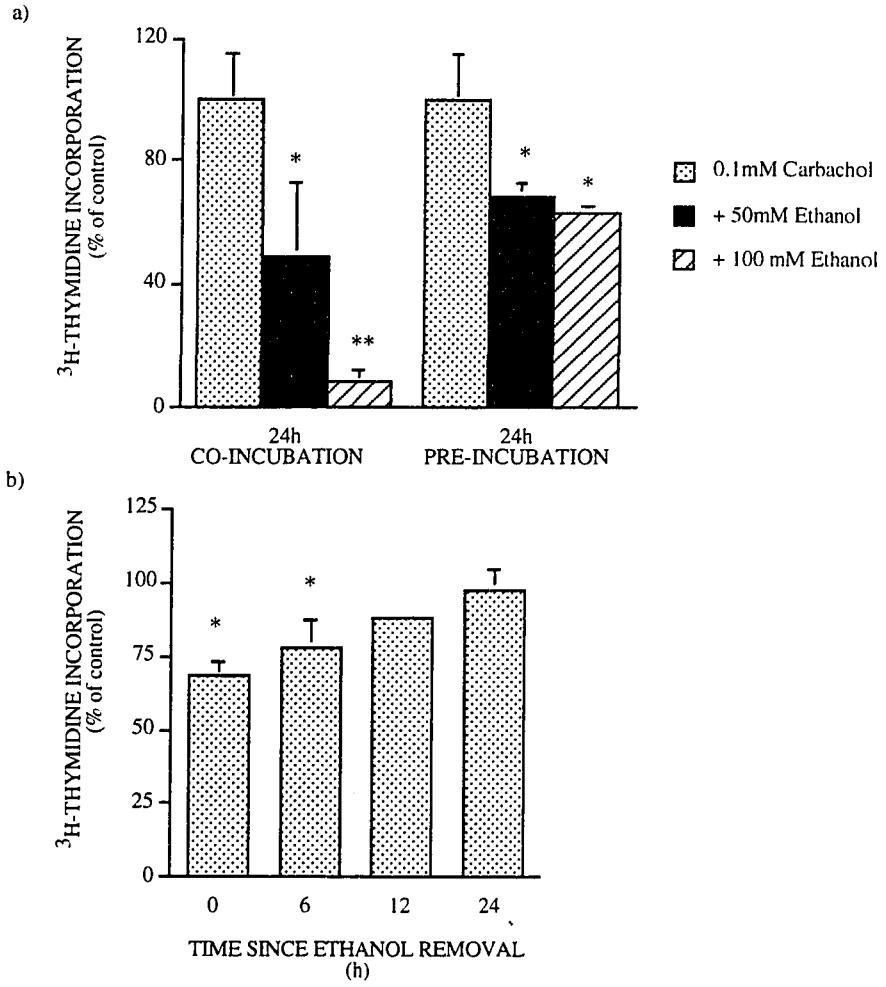


Figure 18. The effect of ethanol and on muscarinic-receptor induced proliferation in human 132 1N1 astrocytoma cells.

[Methyl-^{3}H]-thymidine incorporation into astrocytoma cells. a) Response following stimulation with 1 mM carbachol alone, or following either a 24 h co-incubation, or a 24 h pre-incubation with 50 and 100 mM ethanol. b) Response following pre-incubation with 50 mM ethanol for 24 hours and incubation in the absence of ethanol for the specified length of time. Results are expressed as the mean percent of the 1 mM carbachol response$\pm$ S.E.M. (n=3); 50 cells were analyzed in each experiment. * significantly different from control response, $p<0.05$, ** significantly different from control response, $p<0.001$.

CHAPTER 4: THE ROLE OF CALCIUM IN MUSCARINIC RECEPTOR-INDUCED PROLIFERATION OF ASTROGLIA

Abstract

Calcium is an important cellular messenger which is involved in the proliferation of many cell types. Evidence from our laboratory investigating the mechanism underlying the microencephaly seen in the Fetal Alcohol Syndrome has demonstrated that ethanol inhibits muscarinic-induced proliferation and muscarinic-induced calcium responses in glial cells at similar concentrations and with similar time courses, suggesting a relationship between these two effects in these cells. This research investigates the calcium dependency of muscarinic-induced proliferation in primary rat cortical astrocytes and human 132 1N1 astrocytoma cells. ^{3}H-thymidine incorporation was measured to indicate compounds which may affect cell proliferation. A pulse exposure (30, 60, or 90 min) to ionomycin (10 μM) increased ^{3}H-thymidine incorporation in astrocytes. Carbachol-induced ^{3}H-thymidine incorporation was decreased in the absence of extracellular calcium, however, this treatment affected basal thymidine incorporation. SKF-96365 (10 μM) and verapamil (10 μM) inhibited the carbachol-induced thymidine incorporation, but only at a concentration which also affected basal thymidine incorporation. Cobalt, appeared to inhibit carbachol-stimulated thymidine incorporation. However, this effect was not statistically significant. Nickel (100 μM) inhibited the carbachol-induced thymidine incorporation without affecting basal thymidine incorporation. Surprisingly, lanthanum and gadolinium, which are known to block calcium channels, caused a concentration-dependent increase in ^{3}H-thymidine incorporation in astrocytoma cells. The mechanism underlying this response is unknown. These results suggest that calcium entry is sufficient to initiate ^{3}H-thymidine incorporation in glial cells. Calcium entry via T-type calcium channels may be involved in muscarinic-receptor-mediated thymidine incorporation, although further research investigating possible effects of calcium channel blockers other than at calcium channels needs to be done.

Introduction

Acetylcholine is among the neurotransmitters thought to play a role in the development of the central nervous system (CNS). The ontogeny of the cholinergic system is suggestive of such a role, as the response to cholinergic stimulation peaks during the brain growth spurt, a period of rapid brain growth, when proliferation and maturation of glial cells occurs (Balduini *et al.*, 1987). Acetylcholine has also been shown to be mitogenic in a variety of cell types, including primary rat cortical astrocytes and human 132 1N1 astrocytoma cells (Ashkenazi *et al.*, 1989; Guizzetti *et al.*, 1996; Guizzetti *et al.*, 1998).

This proliferative response is due to actions at the M3 muscarinic receptor subtype (Ashkenazi *et al.*, 1989; Guizzetti *et al.*, 1996), which is linked to the activation of PLC–β through $G_{\alpha q}$ (Smrcka *et al.*, 1991; Bernstein, 1992). The activation of this enzyme leads to the breakdown of phosphatidylinositol (4,5)-bisphosphate, generating inositol (1,4,5)-trisphosphate (IP_3) and diacylglycerol (DAG); IP_3 binds to its receptor on the endoplasmic reticulum and releases calcium from intracellular stores, while DAG activates protein kinase C (PKC) (Berridge, 1995). Intracellular calcium regulates a wide variety of cellular responses (Ghosh and Greenberg, 1995). Evidence suggests that proliferation is one such response, as calcium is involved in DNA synthesis in rat hepatocytes (Bennett and Williams, 1993), cell cycle progression in human fibroblasts (Takuwa *et al.*, 1995), and the control of cell growth in smooth muscle cells (Short *et al.*, 1993). Further evidence in the human U-373 MG astrocytoma cell line implicates calcium in the carbachol-induced proliferation of these cells, as calcium channel blockers inhibited proliferation in these cells (Lee *et al.*, 1993b; Lee *et al.*, 1993a; Lee *et al.*, 1994).

In primary rat cortical astrocytes and human 132 1N1 astrocytoma cells, muscarinic stimulation results in calcium responses via the M3 muscarinic receptor subtype (Catlin *et al.*, 1999c). These responses consisted of an initial spike in intracellular calcium, mediated by the release of calcium from IP_3-sensitive stores, followed by a sustained calcium elevation and oscillations. The second phase of this response requires extracellular calcium, however, the mechanism by which calcium entry occurs is not fully understood. In non-excitable cells this secondary entry, termed "capacative calcium entry" is thought to be trigged by the emptying of the intracellular stores, however, the pathway of this calcium

entry is still unknown. Depending upon culturing conditions astrocytes express a variety of calcium channels. Both L-type and T-type voltage operated calcium channels (VOCCs) are present on rat astrocytes, but no evidence has been found for the presence of N-type channels (Sontheimer, 1994). Astrocytes also express receptor operated calcium channels (ROCCs) linked to $GABA_A$ receptors and non-NMDA glutamate receptors, as well as stretch activated channels (Sontheimer, 1992). These channels, or perhaps as channel which has yet to be identified on these cells, may be involved in cell proliferation.

Although both muscarinic-mediated proliferation and calcium response occur due to stimulation of the M3 receptor, the relationship between these two responses is not known (Guizzetti *et al.*, 1996; Catlin *et al.*, 1999c). Ethanol inhibited both of these responses, with the ethanol concentration curves and the time courses correlating well between these effects (Guizzetti *et al.*, 1997; Catlin *et al.*, 1999b). This inhibition of glial cell proliferation may be involved in the microencephaly that is seen in the Fetal Alcohol Syndrome (Guizzetti *et al.*, 1997). This research, therefore, investigates the role of calcium in muscarinic-induced proliferation to determine the pathway mediating this response, with possible implications for the mechanism underlying ethanol neuroteratogenesis.

Materials and Methods

Animals and materials

Time-mated pregnant rats were supplied by B&K Universal (Kent, WA), and housed, one per cage, in the University of Washington Vivarium. Use of rats was approved by the University of Washington Animal Care Committee and was in accordance with Washington State guidelines. Antibodies were from Accurate Chemical (Westbury, NY), and [methyl-^{3}H]-thymidine was from DuPont NEN Research Products (Boston, MA). SKF-96365 and nifedipine were from RBI (Natick, MA), and all other chemicals were from Gibco (Grand Island, NY) or Sigma (St. Louis, MO).

Tissue Culture

Primary rat cortical astrocyte cultures were prepared by a modification of the method of McCarthy and DeVellis (McCarthy and DeVellis, 1980), as described previously (Guizzetti *et al.*, 1996). Briefly, cortices from day 21 fetuses were minced, trypsinized and washed three times by centrifugation with Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS). The tissue was triturated, filtered and plated in 75 cm^2 flasks, previously coated with poly-d-lysine (10 µg/ml, 10 ml), at $1.5x10^5$ cells/cm^2. Within 24 hours of plating, the flasks were shaken and fresh media was added. Flasks were maintained at 37°C with 5% CO_2 for 9 days and fed every 2-3 days. On day 9 in culture the flasks were shaken overnight, trypsinized and reseeded in pre-coated 24 well tissue culture plates at $5.3x10^5$ cells/cm^2. Following reseeding, astrocytes were grown for 2 days, then rinsed with phosphate-buffered saline and serum-deprived for 2 days in DMEM supplemented with 0.1% Fraction V fatty acid-free bovine serum albumin (BSA). Astrocyte cultures were determined to be at least 95% pure by indirect immunofluorescence with antibodies against glial fibrillary acidic protein and neuron-specific enolase.

The immortalized human astrocytoma cell line, 132 1N1 (provided by J. Heller-Brown, University of San Diego) was maintained as previously described (Guizzetti *et al.*, 1996). Briefly, cells were maintained in 75 cm^2 flasks in DMEM with 5% FBS, penicillin and streptomycin added. Flasks were fed every 2-3 days and passaged once a week. Cells were trypsinized with 0.05% trypsin, present at room temperature for 5 minutes, and reseeded in 24 well tissue culture plates at $2.6x10^4$ cells/cm^2. Cells were grown for 4 days, and serum deprived 2 days prior to the experiment. Cells were used between the third and the seventeenth passage.

Thymidine Incorporation

Cell proliferation was measured by [methyl-^{3}H]-thymidine as previously described (Guizzetti *et al.*, 1996). After serum deprivation, cells were treated with carbamylcholine chloride (carbachol) or ionomycin. Calcium channel blockers were pre-incubated for 10 min prior to the addition of carbachol. A decreased concentration of calcium was achieved using DMEM lacking calcium chloride; calcium chloride was added

back to achieve intermediate calcium concentration. This protocol, without the addition of a calcium chelator, will not remove all extracellular calcium, but will provide a lowered concentration of calcium. For ionomycin experiments, following serum deprivation cells were incubated with ionomycin (15, 30, 60, or 90 min), then rinsed and left in BSA media. After 18 hours of treatment, [methyl-^{3}H]-thymidine (1 μCi/well) was added to the wells for 6 hours. Cells were rinsed three times with phosphate buffered saline and the DNA precipitated with trichloroacetic acid. The DNA was solubilized in 1M NaOH and a 250 μl aliquot counted in scintillation fluid on a Beckmann LS 5000 CE scintillation counter. ^{3}H-thymidine incorporation data is expressed either as percent of basal incorporation, or the CPM of ^{3}H-thymidine incorporation.

Statistical Analysis

Each treatment was done in triplicate and data are presented as the mean across experiments, expressed as either CPM or percentage of the control response. Statistically significant differences were tested for using a paired t-test, a one way, or a repeated measures ANOVA, followed by Dunnett's test to determine where any differences lay. Specific tests are stated in figure legends.

Results

Initially we wanted to confirm that calcium is involved the proliferation of astroglial cells. Figure 19 shows the response of primary rat astrocytes to pulse exposures of ionomycin. Low concentrations of ionomycin (0.1 or 1 μM) had no effect on proliferation, however, 10 μM ionomycin induced proliferation in astrocytes, with the magnitude of the response increasing with increasing exposure duration (up to 90 min). A 24 h incubation with this concentration of ionomycin resulted in cell death (data not shown).

Carbachol concentration curves in both primary rat cortical astrocytes and human 132 1N1 astrocytoma cells confirmed that carbachol induces a concentration-dependent increase in ^{3}H-thymidine incorporation in both these cell types (Fig. 20), as seen previously (Guizzetti *et al.*, 1996; Guizzetti *et al.*, 1998). The calcium-dependency of this carbachol response was investigated using nominally calcium-free medium. In the

presence of nominal calcium (normal medium, [Ca^{2+}=1.8 mM]), both basal (Fig. 21a,b) and carbachol-stimulated (Fig. 21c,d) ^{3}H-thymidine incorporation was decreased in primary astrocytes and human astrocytoma cells (Fig. 21).

Since the carbachol-induced thymidine incorporation of these cells was dependent upon extracellular calcium, calcium channel blockers were used to try to determine which calcium channels mediate this influx of calcium (Fig. 22). SKF-96365, a non-specific antagonist that acts at both VOCCs and ROCCs, inhibited carbachol-stimulated thymidine incorporation, but only at concentrations (10 μM) which inhibited the basal incorporation of thymidine. A similar effect was seen with verapamil (10 μM), a selective antagonist of the L-type calcium channel. In contrast, nifedipine, a more selective antagonist at L-type calcium channels, had no effect on either basal or carbachol-stimulated thymidine incorporation. Cobalt, a non-selective antagonist of calcium channels, did not significantly inhibit thymidine incorporation. Nickel, however, a compound that is reported to be selective for T-type channels at the concentrations used in this study, inhibited carbachol-induced thymidine incorporation without affecting basal incorporation. None of these compounds had any effect on lactate dehydrogenase release (data not shown).

Surprisingly, lanthanum, which is a non-specific blocker of calcium channels, and also has inhibitory activity toward *trp* channels, increased ^{3}H-thymidine incorporation in astrocytoma cells. Concentrations of 10 μM or higher caused a significant increase of both basal and carbachol-stimulated proliferation of these cells (Fig. 23). Similar results were seen following treatment with gadolinium, and with these compounds in primary astrocytes after treatment with these compounds (data not shown). Basal thymidine incorporation was increased by up to 500% by lanthanum (100 μM). Following a co-incubation with lanthanum and 1 mM carbachol, these compounds had a superadditive effect on ^{3}H-thymidine incorporation.

Discussion

The main conclusions from this research are that calcium is involved in the ^{3}H-thymidine incorporation of primary rat cortical astrocytes and human 132 1N1

astrocytoma cells, and that extracellular calcium entry may play a role in muscarinic-induced thymidine incorporation.

Ionomycin alone induced a concentration-dependent increase in astrocyte ^{3}H-thymidine incorporation (Fig. 19). Although there have been a few reports in the literature demonstrating an increase in proliferation by ionomycin (Jouishomme and Rigal, 1991; Rajasekar and Augustin, 1992), most studies indicate that ionomycin is not mitogenic on its own, but induces the proliferation of lymphocytes when added in conjunction with phorbol 12-myristate 13-acetate (PMA) (Roifman *et al.*, 1987; Chen *et al.*, 1996; Ouyang *et al.*, 1996). In our experiments a high concentration of ionomycin (10 μM), when added as a pulse (30-90 min), increased thymidine uptake, while shorter incubations or lower concentrations did not. Chen et al. (1996) studied the effects of a one hour exposure to 1 μM ionomycin and saw no proliferative response. This concentration of ionomycin did not produce a response in our cells either. Other studies looked at the effects of a lower concentration of ionomycin for a longer period of time and saw no effects (Roifman *et al.*, 1987; Ouyang *et al.*, 1996). Research indicates that it is not just the presence or absence of a calcium response that is important to cell function, but also the profile of the response (see review; Berridge, 1997). For example, the frequency of calcium changes regulated the activation of calcium-calmodulin kinase II in solution (CaMK II) (DeKoninck and Schulman, 1998), in the frequency of the calcium response determined which transcription factors were activated in lymphocytes (Dolmetsch *et al.*, 1997), and the amplitude and duration of calcium responses determined the differentiation of neurons (Gu and Spitzer, 1995). Ionomycin is a very non-physiological method of directly increasing intracellular calcium, but a recent report of the synthesis cell permeable IP_3 analog which activates the IP_3 receptor to release calcium will provide a mechanism to study the effects of a direct increase in intracellular calcium responses in a more physiological manner (Li *et al.*, 1998).

Muscarinic stimulation with carbachol caused an increase in ^{3}H-thymidine incorporation in both cell types that was dependent upon the presence of extracellular calcium (Fig 21). In these cells, the carbachol-induced calcium response had been shown to be biphasic, with the second phase of this response, the sustained elevation and oscillations, dependent on extracellular calcium (Catlin *et al.*, 1999c). Carbachol-induced thymidine incorporation was almost totally inhibited by the decreased extracellular calcium,

indicating that the sustained elevation and oscillations were required for this carbachol-induced incorporation. The fact that the carbachol response was almost totally inhibited by these low extracellular calcium concentrations suggests that a minimum amount of calcium entry is important for this response. Though the initial calcium spike could be inhibited with xestospongin C, a specific blocker of the IP_3 receptor-linked calcium channel, inhibition of this initial spike also inhibited the second calcium phase (Gafni *et al.*, 1997; Catlin *et al.*, 1999c), so separating out these two subcellular events would not be possible. This carbachol-induced ^{3}H-thymidine incorporation has been previously shown in our laboratory to be accompanied by progression through the cell cycle and an increase in cell number (Guizzetti and Costa, 1996; Guizzetti *et al.*, 1998). The inhibition of the thymidine incorporation, therefore, could be inhibiting this cell proliferation, however, the possibility that the inhibition is due to cytotoxicity or a decrease in DNA repair can not be ruled out. Experiments in the absence of extracellular calcium, using EGTA to chelate the calcium with the addition of magnesium to maintain cell attachment, would be interesting to confirm the requirement of extracellular calcium on glial cell proliferation.

The route of entry of this calcium was next investigated using antagonists at various calcium channels. SKF-96365, which is a non-specific blocker acting on both VOCCs and ROCCs (Wayman *et al.*, 1996; Zhu *et al.*, 1998) decreased proliferation, however, effects were also seen on basal thymidine incorporation, suggesting that this inhibition may be due to non-specific effects. Similar results were seen with verapamil, an inhibitor of L-type VOCCs (Hofmann *et al.*, 1994), that also has effects at stretch activated channels (Bialecki *et al.*, 1992). Nifedipine, at concentrations that inhibit L-type calcium channels (Malcolm *et al.*, 1996) had no effect on basal or carbachol-induced proliferation, indicating that L-type calcium channels are not involved in this response. Cobalt, which inhibits this influx in a non-specific manner (Fiekers and Konopka, 1996), appeared to inhibit proliferation, but this effect was not significant. Nickel was also used in this study. Nickel, at high concentrations (2.5 mM), is a non-specific inhibitor of calcium channels, but at low concentrations (200 μM) is thought to be specific for T-type calcium channels (Mangel *et al.*, 1996; Toner and Stamford, 1997). Nickel (100 μM) inhibited the carbachol-induced proliferation without having any effects on basal thymidine incorporation, suggesting that T-type channels may be involved. Further research with the newer calcium channel antagonist, mibefradil, which is active at L- and T-type calcium

channels, with some selectivity at the T-type, would be useful to confirm the role of T-type calcium channels in proliferation (Mishra and Hermsmeyer, 1994; Schmitt *et al.*, 1995).

One major problem with this pharmacological approach to studying calcium influx is possible actions of these blockers at sites other than calcium channels. This is particularly a problem with this experimental protocol which requires a long-term (24 h) incubation and measures an endpoint, ^{3}H-thymidine incorporation, which may be quite distant from the channel. These agents were developed and used mostly in electrophysiological studies, where the effects of short-term incubations and effects channel currents themselves were monitored. Two of the metals used in this research, cobalt and nickel, have effects on DNA repair, especially ligation and postreplicative repair (see recent review by Hartwig, 1998). Since the incorporation of ^{3}H-thymidine could be measuring DNA repair, this effect of these metals could confound these data. Related to this effect, both of these metals bind zinc finger proteins and can effect DNA binding of proteins (Predki et al., 1994). Furthermore, cobalt decreased the fidelity of DNA synthesis (Zakour *et al.*, 1981). These compounds, therefore, could have multiple effects on cell function over the course of 24 h which are not related to a calcium channel block. Although there is not much information on non-channel effects of SKF-96365 and nifedipine, nifedipine has been shown to inhibit calmodulin and phosphodiesterase (Minocherhomjee and Roufogalis, 1984). Verapamil has been used clinically due to its ability to inhibit the p-glycoprotein pump (Yusa and Tsuruo, 1989), and this effect could alter cell function over 24 h. With these many effects, results using these compounds must be interpreted with caution.

Recently, a new family of calcium channels have been found and cloned in mammalian systems, called the transient receptor potential *(Trp)* channels (Philipp *et al.*, 1996; Zhu *et al.*, 1996; Boulay *et al.*, 1997). These channels are found in high levels in the rat and mouse brain (Garcia and Schilling, 1997; Okada *et al.*, 1998), and are seen following G_q and IP_3-mediated responses, which suggests that these channels would be involved in this proliferative response. SKF-96365 (25 μM) inhibits these currents, as does a high concentration of nickel (6 mM) and low concentration of gadolinium (10 μM) (Zhu *et al.*, 1998). With the little that is known about these channels, further experiments with gadolinium and lanthanum were done to try to determine if *trp* channels might be involved in our response. Surprisingly, these two compounds caused a concentration-dependent increase in glial cell proliferation. The mechanism underlying this remains a

mystery. Though these compounds are normally used to inhibit cell proliferation, such an effect has been previously reported (Ishiyama *et al.*, 1995). The question of a role for *trp*-like channels in this proliferation, therefore, remains unanswered. If it is determined that astrocytes express these channels, more work should be done investigating their role in proliferation, as well as further characterizing the role of the T-type VOCCs.

One question with this research is in regards to the effect of the various treatments on basal ^{3}H-thymidine incorporation. Following serum deprivation, these cells are in a quiescent state (Guizzetti and Costa, 1996), and therefore, should not proliferate. These treatments did not increase LDH activity, indicating that cell death is not the cause for the decreased incorporation. ^{3}H-thymidine incorporation would also measure any thymidine incorporated into the cells for DNA repair, therefore inhibition of DNA repair may account for some of the effect on basal incorporation. Non-specific incorporation would probably also be taking place, but an explanation as to why certain treatments would decrease non-specific incorporation is not obvious.

Although a role of calcium influx in the proliferation of glial cells needs to be confirmed, this research suggests that such a role is possible. Further research looking at better indices of cell cycling and cell proliferation, combined with better manipulation of calcium levels and calcium channels, perhaps using molecular techniques, will address this important and interesting issue.

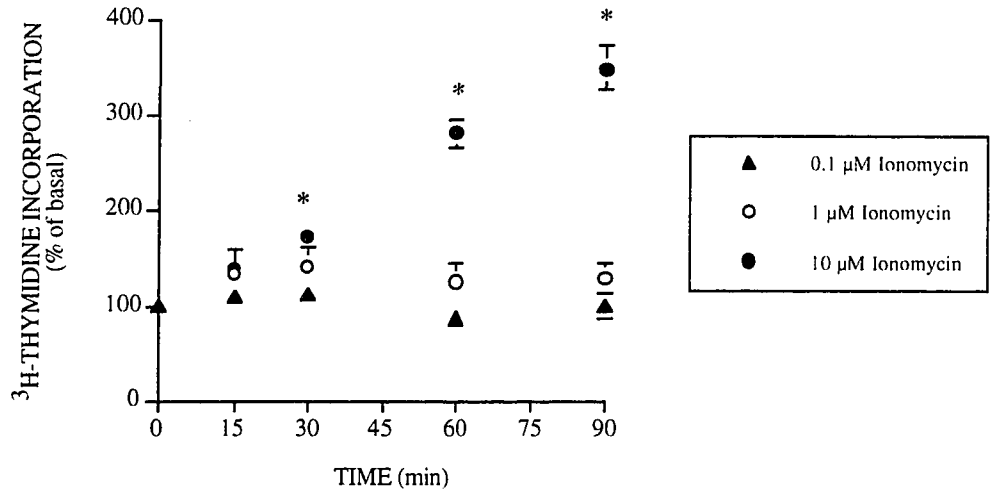


Figure 19. The effect of ionomycin on proliferation in primary rat cortical astrocytes.

[Methyl-^{3}H]-thymidine incorporation into astrocytes following a pulse exposure to 0.1 (solid triangle) (n=3-4), 1.0 (open circle) (n=4-7), or 10 μM (closed circle) (n=5-10) ionomycin. Results are expressed as the mean percent of basal incorporation ± S.E.M. Differences from control were tested for with a repeated measures ANOVA, followed by Dunnett's test. * significantly different from control response, $p<0.05$.

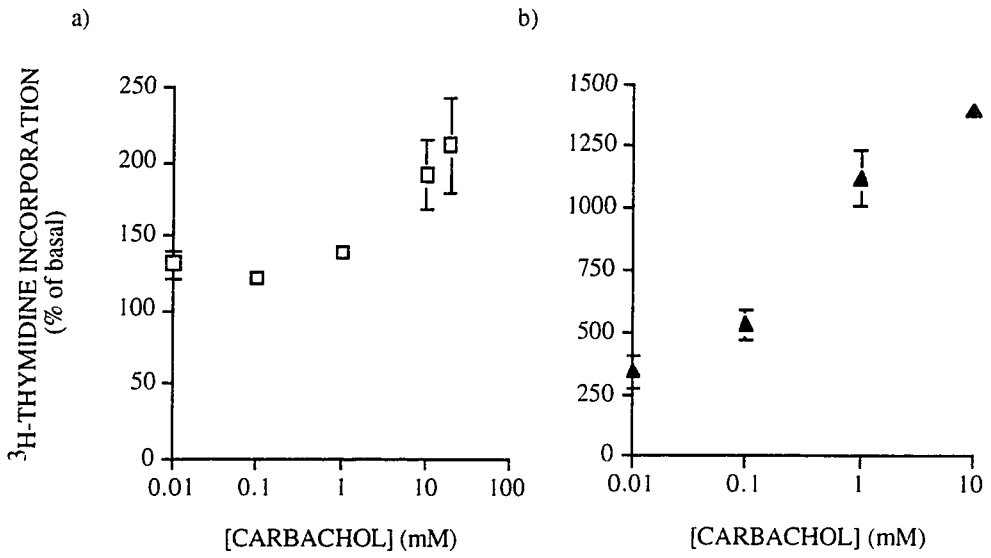


Figure 20. Concentration-response effect of carbachol on proliferation in primary rat cortical astrocytes and human 132 1N1 astrocytoma cells.

[Methyl-^{3}H]-thymidine incorporation into primary astrocytes a), and astrocytoma cells b) following exposure to specified concentration of carbachol. Results are expressed as the mean percent of basal incorporation $\pm$ S.E.M. (n>5).

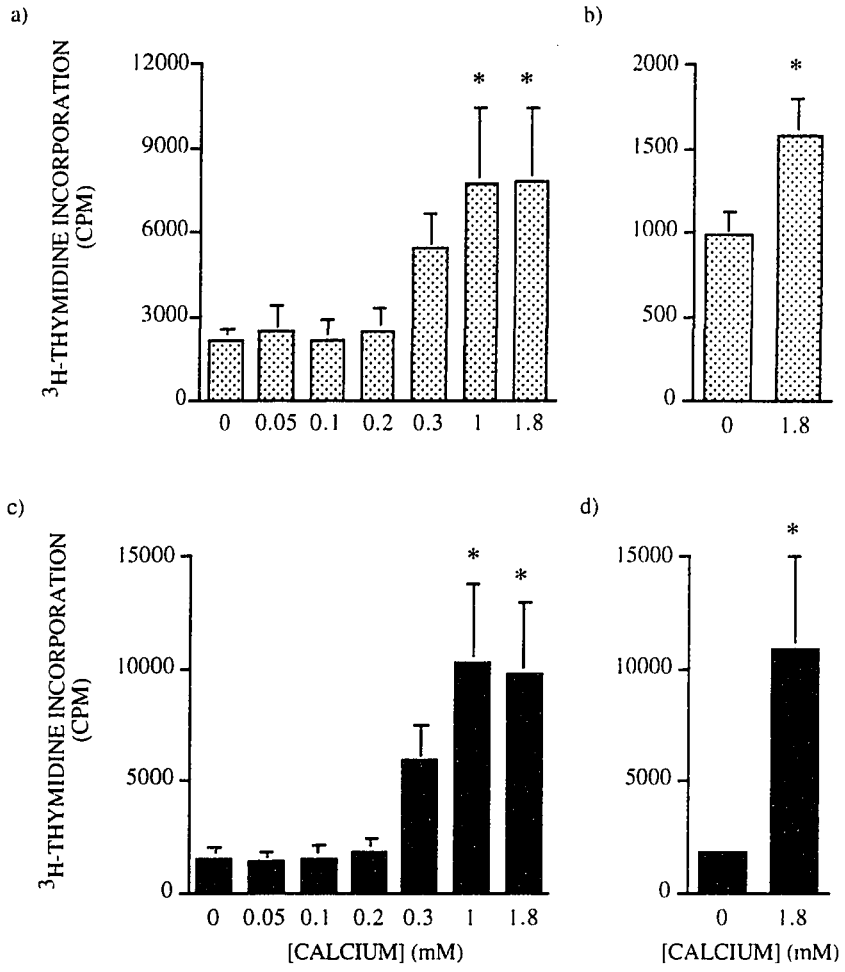


Figure 21. The dependence of carbachol-induced proliferation on extracellular calcium in primary rat cortical astrocytes and human 132 1N1 astrocytoma cells.

[Methyl-^{3}H]-thymidine incorporation into primary astrocytes a,c) (n=3-9), and astrocytoma cells b,d) (n=4); under basal a,b) and 1 mM carbachol-stimulated c,d) conditions incubated with the specified concentration of calcium. Results are expressed as CPM incorporated $\pm$ S.E.M. Differences from basal or carbachol-stimulated controls were tested for with a one way ANOVA, followed by Dunnett's test. * significantly different from control response, $p<0.05$.

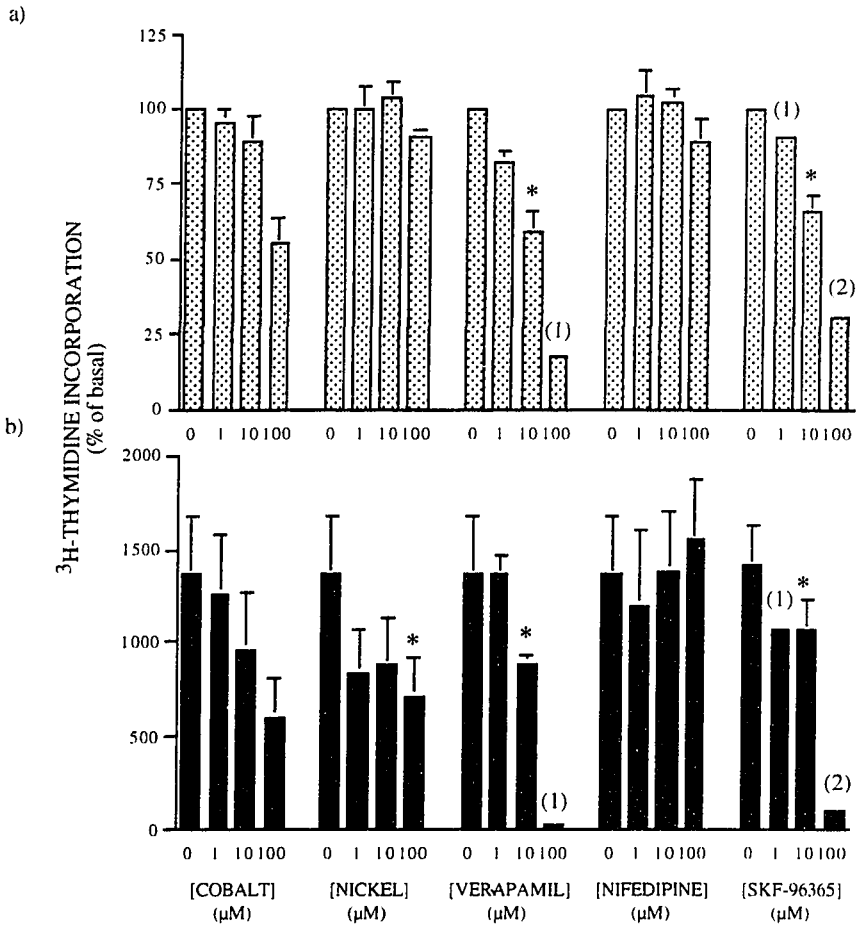


Figure 22. The effect of calcium channel blockers on proliferation in human 132 1N1 astrocytoma cells.

Basal a), and 1 mM carbachol b), stimulated [Methyl-^{3}H]-thymidine incorporation into astrocytoma cells in the presence or absence of calcium channel blockers at the specified concentrations. Blockers were added 10 min prior to the addition of carbachol to see effects on carbachol-stimulated proliferation. Results are expressed as the mean percent of basal incorporation ± S.E.M. (n=3-8, except when specified). Differences from individual basal or carbachol-stimulated controls were tested for with a one way ANOVA (or repeated measures where data was paired) followed by Dunnett's test. Data for SKF-96365 were tested with a paired t-test as there was only one concentration to be tested. * significantly different from control response, $p<0.05$.

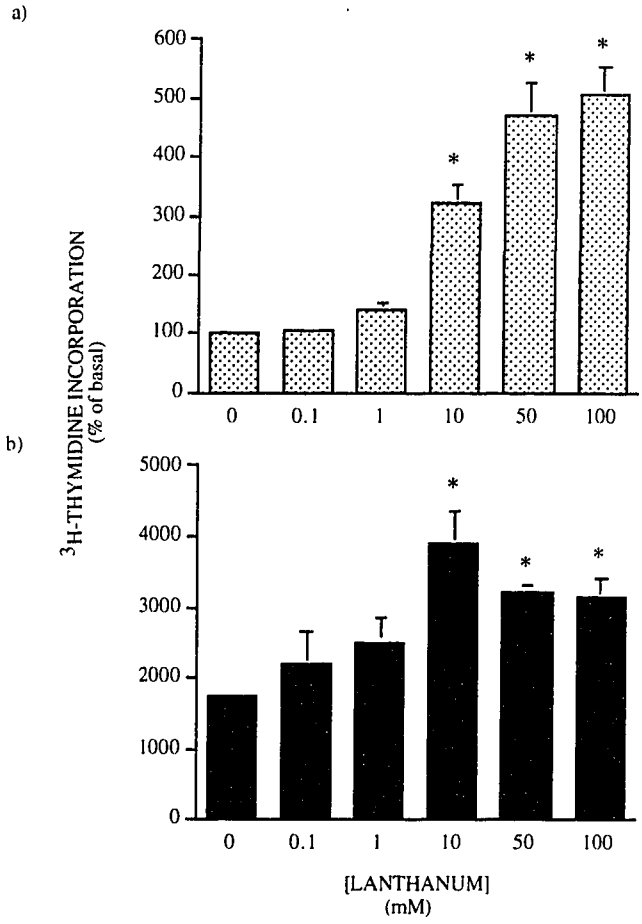


Figure 23. The effect of lanthanide metals on proliferation in human 132 1N1 astrocytoma cells.

Basal a), and 1 mM carbachol b), stimulated [Methyl-^{3}H]-thymidine incorporation into astrocytoma cells in the presence or absence of lanthanum at the specified concentrations. These calcium channel blockers were added 10 min prior to the addition of carbachol to see effects on carbachol-stimulated proliferation. Results are expressed as the mean percent of basal incorporation $\pm$ S.E.M. (n=3-5). Differences from basal or carbachol-stimulated controls were tested for with a one way ANOVA followed by Dunnett's test * significantly different from control response, $p<0.05$.

CHAPTER 5: SUMMARY & CONCLUSIONS

The CNS dysfunction that results from *in utero* exposure to ethanol has profound clinical implications, yet the mechanisms underlying this dysfunction remain elusive. To address this issue, this dissertation research focused on a general mechanism that may mediate these effects. It has been proposed that muscarinic-mediated responses in astrocytes are disrupted by ethanol, resulting in the microencephaly and mental retardation that are seen in FAS (Guizzetti *et al.*, 1997). Previous data from our laboratory showing that ethanol inhibits muscarinic-induced proliferation of primary rat cortical astrocytes and human 132 1N1 astrocytoma cells supports this hypothesis. This muscarinic agonist-induced proliferation is mediated by the M3 muscarinic receptor subtype, which, when activated, leads to an increase in intracellular calcium, a cellular signal involved in the proliferative responses in many cell types. The specific hypothesis of this research, therefore, is that ethanol exposure during development causes central nervous system dysfunction through effects on astrocytes, specifically on their calcium response to muscarinic stimulation, thereby interfering with the normal mitogenic response to muscarinic agonists.

Calcium imaging techniques were used to characterize and quantitate the carbachol-induced calcium responses in primary rat cortical astrocytes and human 132 1N1 astrocytoma cells. Carbachol induced a concentration-dependent increase in intracellular calcium in both cell types, that was mediated by M3 muscarinic receptors. This calcium response consisted of an initial calcium spike which was mediated by the release of calcium from IP_3-sensitive intracellular stores. The initial spike was followed by a sustained calcium elevation and oscillations which were dependent upon extracellular calcium. Inhibition of the initial spike with xestospongin C, a blocker of the IP_3-receptor-linked calcium channel, also inhibited the subsequent elevation and oscillations, indicating that calcium-induced-calcium release mediated this second phase of calcium release. There was a feedback inhibition on this calcium response by protein kinase C, and the activity of this enzyme also appeared to be involved in the maintenance of the sustained calcium elevation. These responses are similar to those seen in many cell types following activation with a

number of different stimuli. Quantification of these calcium signals allows the effects of toxicants on this important cellular messenger to be investigated.

Calcium has emerged as an important cellular signal which mediates proliferation in many cell types. Both the generation of IP_3 and calcium responses have been shown to be inhibited by ethanol in some cell types. The effects of ethanol on calcium were studied in astrocytes and astrocytoma cells to determine if ethanol inhibits these calcium responses, and if so, if this inhibition correlates with the inhibition of the proliferation by ethanol in these cells. Initial studies using short-term (30 min) incubation of the cells with ethanol did not show dramatic effects on the calcium responses of the cells; however, significant inhibition was seen at the two highest ethanol concentrations examined (100 and 250 mM). Interestingly, the effect was quite variable between experiments, with the same concentration of ethanol having no effect on calcium responses in one experiment, and causing a total inhibition of the response in another.

The effect of length of exposure to ethanol on the muscarinic receptor-induced calcium response was then examined. The results of these experiments indicate that the response was duration-dependent. Acute exposure (5 min) to ethanol (250 mM) had no effect on calcium responses, whereas long-term exposure (24 h) inhibited the responses to a greater extent, while requiring a lower concentration of ethanol (10 mM). Variability in the effect of ethanol was still present in these experiments, however, inhibition was more consistent with longer exposure periods and higher ethanol concentrations. These effects were selective for the muscarinic-mediated responses; no effects were seen on the thapsigargin- and glutamate-induced calcium responses. The presence of ethanol at the time of carbachol addition was not a requirement for inhibition of the calcium response; indeed, after a 24 h incubation with ethanol and its removal, the carbachol effect was still significantly inhibited. The cells, however, recovered from this inhibition with time; significant inhibition was no longer seen 6 or 12 h after ethanol removal and responses had returned to control levels by 24 h.

^{3}H-thymidine incorporation experiments were carried out to determine if the time-course and reversibility seen with ethanol's inhibition of calcium responses were also characteristic of ethanol's inhibition of DNA synthesis. Ethanol showed a similar pattern of inhibition of muscarinic-induced proliferation: after a 24 h pre-incubation with ethanol,

incorporation was inhibited up to 6 h after ethanol removal. This carbachol-induced ^{3}H-thymidine incorporation has previously been shown to by accompanied by an increase in cell number and cell cycle progression, and ethanol inhibits this cell cycle progression (Guizzetti and Costa, 1996). Ethanol's inhibition of both muscarinic-mediated calcium responses and proliferation correlated well; these inhibitions were selective for muscarinic responses, and were reversible, as well as concentration- and duration-dependent. This indicates that the inhibition is not due to overt toxicity or cell death as the cells are still able to elicit a calcium response when muscarinic-mediated responses are inhibited, and they are also able to recover the response over time. The fact that this inhibition does not occur immediately upon ethanol addition, but requires time to develop, and that it persists after ethanol removal until the cells gradually recover from the inhibition, suggests that ethanol does not directly inhibit this calcium response, but rather has an effect on some intracellular target that, in turn, leads to a decrease in the calcium responses.

Since the inhibition of muscarinic-induced glial cell proliferation and calcium responses appeared to be related, the calcium-dependency of this ^{3}H-thymidine incorporation was investigated. Ionomycin increased thymidine incorporation in these cells, confirming that calcium responses can mediate this response. The carbachol-induced response was then studied with decreased levels of extracellular calcium to see if the secondary influx of calcium was involved in the proliferation of glial cells. Incorporation was decreased under these low calcium conditions, indicating that the secondary influx of calcium was involved in glial cell thymidine incorporation. We next investigated which calcium channel mediates this entry. The non-specific VOCC and ROCC blocker SKF-96365, and the selective L-type antagonist verapamil, inhibited carbachol-induced proliferation, but only at concentrations which affected basal thymidine incorporation. The dihydropyridine nifedipine, which is selective for L-type channels had no effect on proliferation. The non-specific blocker cobalt had no significant effects on glial cell proliferation. Nickel, on the other hand, which at high concentrations is non-specific, but at lower concentrations selectively blocks T-type responses, blocked glial cell proliferation without having any effects on basal thymidine incorporation. Although these data suggest that L-type VOCCs and ROCCs are not involved in response, while T-type channels may be, these data must be interpreted cautiously, as effects of these pharmacological agents not related to their inhibition of calcium channels can not be discounted.

Other channels yet to be studied in astrocytes could be present on these cells and play a role in modulating calcium responses. Numerous cell types express *trp* or *trp*-like channels (Philipp *et al.*, 1996; Garcia and Schilling, 1997; Philipp *et al.*, 1998), which are thought to be involved in the second phase of the G_q and IP_3-induced calcium responses which we are studying (Hardie and Minke, 1995; Minke and Selinger, 1996; Zhu *et al.*, 1996). These channels are inhibited by SKF-96365, high concentrations of nickel, and also by gadolinium and low concentrations of lanthanum. We then investigated the effects of these two lanthanides on carbachol-induced proliferation in of glial cells to see if there were any indications of *trp* channel activity in these cells. These two calcium channel antagonists, however, caused an unexpected, concentration-dependent increase in glial cell proliferation. The mechanism underlying this response is not known.

Overall, these data indicate that carbachol induces complex calcium responses in astroglial cells, and ethanol inhibits these responses. There is a good correlation between the inhibition of these calcium responses and ethanol's inhibition of glial cell proliferation. This, in combination with the possible role of calcium responses in glial cell ^{3}H-thymidine incorporation, suggests that the inhibition of muscarinic-induced calcium responses may mediate ethanol's inhibition of muscarinic-stimulated glial cell proliferation. This cellular mechanism may underlie some of the microencephaly and CNS dysfunction seen in FAS. Future studies to determine the intracellular target of ethanol upstream of the generation of IP_3, as well as research into the downstream effects of these calcium responses will be of interest. The effects of ethanol on G-proteins, and their coupling to both muscarinic receptors and PLC will aid in the elucidation of this mechanism. Elucidation of the role of these calcium responses in glial cell proliferation would also be important, not only from the perspective of FAS, but also for neurooncology.

LIST OF REFERENCES

Abdollah, S., Catlin, M. C., and Brien, J. F. (1993). Ethanol neuro-behavioural teratogenesis in the guinea pig: behavioural dysfunction and hippocampal morphologic changes. *Can. J. Physiol. Pharmacol.* **71:** 776-782.

Abel, E. L. (1985). Late sequelae of fetal alcohol syndrome. In *Alcohol and the Developing Brain*, Rydberg, U., Alling, C. and Engel, J. (ed). Raven Press, New York. pp. 125-133.

Adachi, J., Mizoi, Y., Fukunaga, T., Ogawa, Y., and Imamichi, H. (1989). Comparative study on ethanol elimination and blood acetaldehyde between alcoholics and control subjects. *Alcohol. Clin. Exp. Res.* **13:** 601-604.

Alberts, B., Bray, D., Lewis, J., Raff, M., Roberts, K., and Watson, J. D. (1989). The nervous system. In *Molecular Biology of the Cell*, (ed). Garland Publishing, New York. pp. 1059-1136.

Albrieux, M., Lee, H. C., and Villaz, M. (1998). Calcium signaling by cyclic ADP-ribose, NAADP, and inositol trisphosphate are involved in distinct functions in ascidian oocytes. *J Biol Chem.* **273:** 14566-74.

Ambler, S. K., Thompson, B., Solski, P. A., Brown, J. H., and Taylor, P. (1987). Receptor-mediated inositol phosphate formation in relation to calcium mobilization: a comparison of two cell lines. *Mol. Pharmacol.* **32:** 376-383.

Arenander, A. T., de Vellis, J., and Herschman, H. R. (1989). Induction of *c-fos* and TIS genes in cultured rat astrocytes by neurotransmitters. *J. Neurosci. Res.* **24:** 107-114.

Ashkenazi, A., Ramachandran, J., and Capon, D. J. (1989). Acetylcholine analogue stimulates DNA synthesis in brain-derived cells via specific muscarinic receptor subtypes. *Nature.* **340:** 146-150.

Balazs, R., Miller, S., Romano, C., de Vries, A., Chun, Y., and Cotman, C. W. (1997). Metabotropic glutamate receptor mGluR5 in astrocytes: pharmacological properties and agonist regulation. *J. Neurochem.* **69:** 151-163.

Balduini, W., Candura, S. M., and Costa, L. G. (1991a). Regional development of carbachol-, glutamate-, norepinephrine-, and serotonin-stimulated phosphoinositide metabolism in rat brain. *Dev. Brain Res.* **62:** 115-120.

Balduini, W., Candura, S. M., Manzo, L., Cattabeni, F., and Costa, L. G. (1991b). Time-, concentration-, and age-dependent inhibition of muscarinic receptor-stimulated phosphoinositide metabolism by ethanol in the developing rat brain. *Neurochem. Res.* **16:** 1235-1240.

Balduini, W., and Costa, L. G. (1989). Effects of ethanol on muscarinic receptor-stimulated phosphoinositide metabolism during brain development. *J. Pharmacol. Exp. Ther.* **250:** 541-547.

Balduini, W., and Costa, L. G. (1990). Developmental neurotoxicity of ethanol: in vitro inhibition of muscarinic receptor-stimulated phosphoinositide metabolism in brain from neonatal but not adult rats. *Brain Res.* **512:** 248-252.

Balduini, W., Murphy, S. D., and Costa, L. G. (1987). Developmental changes in muscarinic receptor-stimulated phosphoinositide metabolism in the rat brain. *J. Pharm. Exp. Therap.* **241:** 421-427.

Balduini, W., Murphy, S. D., and Costa, L. G. (1990). Characterization of cholinergic muscarinic receptor-stimulated phosphoinositide metabolism in brain from immature rats. *J. Pharmacol. Exp. Ther.* **253:** 573-579.

Barger, S. W., and Van Eldik, L. J. (1992). S100ß stimulates calcium fluxes in glial and neuronal cells. *J. Biol. Chem.* **267:** 9689-9694.

Barres, B. A., Chun, L. L. Y., and Corey, D. P. (1989). Calcium current in cortical astrocytes: induction by cAMP and neurotransmitters and permissive effect of serum factors. *J. Neurosci.* **9:** 3169-3175.

Belia, S., Mannucci, R., Lisciarelli, M., Cacchio, M., and Fano, G. (1995). Double effect of ethanol on intracellular Ca^{2+} levels in undifferentiated PC12 cells. *Cellular Signal.* **7:** 389-395.

Bennett, A. M., and Williams, G. M. (1993). Calcium as a permissive factor but not an initiation factor in DNA synthesis induction in cultured rat hepatocytes by the peroxisome proliferator ciprofibrate. *Biochem. Pharm.* **46:** 2219-2227.

Bergamaschi, S., Battaini, F., Trabucchi, M., Parenti, M., Lopez, C. M., and Govoni, S. (1995). Neuronal differentiation modifies the effect of ethanol exposure on voltage-dependent calcium channels in NG 108-15 cells. *Alcohol.* **12:** 497-503.

Bernstein, G. (1992). Reconstitution of agonist-stimulated phosphatidylinositol 4,5-bisphosphate hydrolysis using purified m1 muscarinic receptor, Gq/11, and phospholipase C-beta 1. *J. Biol. Chem.* **267:** 8081-8088.

Berridge, M. J. (1991). Cytoplasmic calcium oscillations: a two pool model. *Cell Calcium.* **12:** 63-72.

Berridge, M. J. (1993). Inositol trisphosphate and calcium signalling. *Nature.* **361:** 315-325.

Berridge, M. J. (1995). Capacitative calcium entry. *Biochem. J.* **312:** 1-11.

Berridge, M. J. (1997). The AM and FM of calcium signalling. *Nature.* **386:** 759-760.

Berrie, C. P., and Cobbold, P. H. (1995). Both activators and inhibitors of protein kinase C promote the inhibition of phenylephrine-induced [Ca2+]i oscillations in single intact rat hepatocytes. *Cell Calcium.* **18:** 232-44.

Bezprozvanny, I., Watra, J., and Ehrlich, B. E. (1991). Bell-shaped calcium-response curves of Ins(1,4,5)P_3- and calcium-gated channels from endoplasmic reticulum of cerebellum. *Nature.* **351:** 751-754.

Bialecki, R. A., Kulik, T. J., and Colucci, W. S. (1992). Stretching increases calcium influx and efflux in cultured pulmonary arterial smooth muscle cells. *Am. J. Physiol.* **263:** L602-606.

Blackshear, P. J., Stumpo, D. J., Huang, J., Nemenoff, R. A., and Spach, D. H. (1987). Protein kinase C-dependent and -independent pathways of proto-oncogene induction in human astrocytoma cells. *J. Biol. Chem.* **262:** 7774-7781.

Blankenfeld, G. V., Enkvist, K., and Kettenmann, H. (1995). Gamma-Aminobutyric acid and glutamate receptors. In *Neuroglia*, Kettenmann, H. and Ransom, B. R. (ed). Oxford University Press, New York. pp. 335-345.

Blevins, T., Mirshahi, T., and Woodward, J. J. (1995). Increased agonist and antagonist sensitivity of *N*-methyl-D-aspartate stimulated calcium flux in cultured neurons following chronic ethanol exposure. *Neurosci. Lett.* **200:** 214-218.

Bloom, F. E. (1997). The science of substance abuse. *Science.* **278:** 15.

Boitano, S., Dirksen, E. R., and Sanderson, M. J. (1992). Intercellular propagation of calcium waves mediated by inositol trisphosphate. *Science.* **258:** 292-295.

Bonner, T. J. (1989). The molecular basis of muscarinic receptor diversity. *T. Neurosci.* **12:** 145-151.

Bonthius, D. J., and West, J. R. (1988). Blood alcohol concentration and microencephaly: a dose-response study in the neonatal rat. *Teratology.* **37:** 223-31.

Bootman, M. D., and Berridge, M. J. (1995). The elemental principles of calcium signaling. *Cell.* **83:** 675-678.

Bootman, M. D., Cheek, T. R., Moreton, R. B., Bennett, D. L., and Berridge, M. J. (1994). Smoothly graded Ca^{2+} release from inositol 1,4,5-trisphosphate-sensitive Ca^{2+} stores. *J. Biol. Chem.* **269:** 24783-24791.

Boulay, G., Zhu, X., Peyton, M., Jiang, M., Hurst, R., Stefani, E., and Birnbaumer, L. (1997). Cloning and expression of a novel mammalian homolog of Drosophila transient receptor potential (Trp) involved in calcium entry secondary to activation of receptors coupled by the Gq class of G protein. *J Biol Chem.* **272:** 29672-80.

Bradford, P. G., and Rubin, R. P. (1986). Quantitative changes in inositol 1,4,5-trisphosphate in chemoattractant-stimulated neutrophils. *J. Biol. Chem.* **261:** 15644-15647.

Brown-Masters, S., Harden, T. K., and Brown, J. H. (1984). Relationships between phosphoinositide and calcium responses to muscarinic agonists in astrocytoma cells. *Mol. Pharmacol.* **26:** 149-155.

Camacho-Nasi, P., and Treistman, S. N. (1986). Ethanol effects on voltage-dependent membrane conductances: comparative sensitivity of channel populations in *Aplysia* neurons. *Cell. Mol. Neurobiol.* **6:** 263-279.

Camacho-Nasi, P., and Treistman, S. N. (1987). Ethanol-induced reduction of neuronal calcium currents in *Aplysia*: An examination of possible mechanisms. *Cell. Mol. Neurobiol.* **7:** 191-207.

Cameron, A. M., Steiner, J. P., Roskams, A. J., Ali, S. M., Ronnett, G. V., and Snyder, S. H. (1995). Calcineurin associated with the inositol 1,4,5-trisphosphate receptor-FKBP12 complex modulates Ca^{2+} flux. *Cell.* **83:** 463-472.

Canda, A., Yu, B. H., and Sze, P. Y. (1995). Biochemical characterization of ethanol actions on dihydropyridine-sensitive calcium channels in brain synaptosomes. *Biochem. Pharmacol.* **50:** 1711-1718.

Candura, S. M., Manzo, L., and Costa, L. G. (1992). Inhibition of muscarinic receptor- and G-protein-dependent phosphoinositide metabolism in cerebrocortical membranes from neonatal rats by ethanol. *Neurotoxicology.* **13:** 281-288.

Cannell, M. B., Cheng, H., and Lederer, W. J. (1995). The control of calcium release in heart muscle. *Science.* **268:** 1045-1049.

Catlin, M. C., Abdollah, S., and Brien, J. F. (1993). Dose-dependent effects of prenatal ethanol exposure in the guinea pig. *Alcohol.* **10:** 109-115.

Catlin, M. C., Guizzetti, M., and Costa, L. G. (1999a). Effects of ethanol on calcium homeostasis in the nervous system: implications for astrocytes. *Mol. Neurobiol.* In Press.

Catlin, M. C., Guizzetti, M., and Costa, L. G. (1999b). The effects of ethanol on muscarinic-mediated responses in astroglia. (In preparation).

Catlin, M. C., Kavanagh, T. J., and Costa, L. G. (1999c). Muscarinic-receptor-induced calcium responses in astroglia. (In preparation).

Centemeri, C., Bolego, C., Abbracchio, M. P., Cattabeni, F., Puglisi, L., Burnstock, G., and Nicosia, S. (1997). Characterization of the Ca2+ responses evoked by ATP and other nucleotides in mammalian brain astrocytes. *Br. J. Pharmacol.* **121:** 1700-1706.

Charles, A. C. (1994). Glia-neuron intercellular calcium signaling. *Dev. Neurosci.* **16:** 196-206.

Charles, A. C., Dirksen, E. R., Merrill, J. E., and Sanderson, M. J. (1993). Mechanisms of intercellular calcium signaling in glial cells studied with dantrolene and thapsigargin. *Glia.* **7:** 134-145.

Charles, A. C., Merrill, J. E., Dirksen, E. R., and Sanderson, M. J. (1991). Intercellular signaling in glial cells: calcium waves and oscillations in response to mechanical stimulation and glutamate. *Neuron.* **6:** 983-992.

Chen, C. C., Chang, J., and Chen, W. C. (1995). Role of protein kinase C subtypes alpha and delta in the regulation of bradykinin-stimulated phosphoinositide breakdown in astrocytes. *Mol Pharmacol.* **48:** 39-47.

Chen, C. C., and Chen, W. C. (1996). ATP-Evoked inositol phosphates formation through activation of P_{2U} purinergic receptors in cultured astrocytes: regulation by PKC subtypes α, δ, and θ. *Glia.* **17:** 63-71.

Chen, Y.-R., Wang, X., Templeton, D., Davis, R. J., and Tan, T. H. (1996). The role of c-Jun N-terminal kinase (JNK) in apoptosis induced by ultraviolet C and γ radiation. *J. Biol. Chem.* **271:** 31929-31936.

Chini, E. N., Beers, K. W., and Dousa, T. P. (1995). Nicotinate adenine dinucleotide phosphate (NAADP) triggers a specific calcium release system in sea urchin eggs. *J. Biol.Chem.* **270:** 3216-3223.

Clapham, D. E. (1995). Calcium signaling. *Cell.* **80:** 259-268.

Clarke, D. W., Steenaart, N. A. E., and Brien, J. F. (1986a). Disposition of ethanol and activity of hepatic and placental alcohol dehydrogenase and aldehyde dehydrogenases in the third-trimester pregnant guinea pig for single and short-term oral ethanol administration. *Alcohol Clin. Exp. Res.* **10:** 330-336.

Clarke, D. W., Steenaart, N. A. E., Slack, C. J., and Brien, J. F. (1986b). Pharmacokinetics of ethanol and its metabolite, acetaldehyde, and fetolethality in the third-trimester pregnant guinea pig for oral administration of acute, multiple-dose ethanol. *Can. J. Physiol. Pharmacol.* **64:** 1060-1067.

Clarren, S. K., and Smith, D. W. (1978). The fetal alcohol syndrome. *N. Eng. J. Med.* **298:** 1063-1067.

Cofan, M., Fernandez-Sola, J., Nicolas, J. M., Poch, E., and Urbano-Marquez, A. (1995). Ethanol decreases basal cytosolic-free calcium concentration in cultured skeletal muscle cells. *Alcohol Alcohol.* **30:** 617-621.

Combettes, L., and Champeil, P. (1994). Calcium and inositol 1,4,5-trisphosphate-induced Ca^{2+} release. *Science.* **265:** 813.

Condorelli, D. F., Dell'Albani, P., Corsaro, M., Giuffrida, R., Caruso, A., Salinaro, A. T., Spinella, F., Nicoletti, F., Albanese, V., and Giuffrida Stella, A. M. (1997). Metabotropic glutamate receptor expression in cultured rat astrocytes and human gliomas. *Neurochem. Res.* **22:** 1127-1133.

Cornell-Bell, A. H., and Finkbeiner, S. M. (1991). Ca^{2+} waves in astrocytes. *Cell Calcium.* **12:** 185-204.

Cornell-Bell, A. H., Finkbeiner, S. M., Cooper, M. S., and Smith, S. J. (1990). Glutamate induces calcium waves in cultured astrocytes: long-range glial signaling. *Science.* **247:** 470-473.

Costa, L. G. (1994). Signal transduction mechanisms in developmental neurotoxicity: the phosphoinositide pathway. *Neurotoxicology.* **15:** 19-27.

Cotran, R. S., Kumar, V., and Robbins, S. L. (1994). Environmental and nutritional diseases. In *Robbin's Pathologic Basis of Disease, 5th Ed.*, Cotran, R. S., Kumar, V. and Robbins, S. L. (ed). W.B. Saunders Company, Philadelphia, PA. pp. 320-342.

Coyle, J. T., and Yamamura, H. I. (1976). Neurochemical aspects of the ontogenesis of cholinergic neurons in the rat brain. *Brain Res.* **118:** 429-440.

Crews, F. T., Morrow, A. L., Criswell, H., and Breese, G. (1996). Effects of ethanol on ion channels. *Int. Rev. Neurobiol.* **39:** 283-367.

Cuthbertson, K. S. R., and Chay, T. R. (1991). Modelling receptor-controlled intracellular calcium oscillations. *Cell Calcium.* **12:** 97-109.

Dani, J. W., Chernjavsky, A., and Smith, S. J. (1992). Neuronal activity triggers calcium waves in hippocampal astrocyte networks. *Neuron.* **8:** 429-440.

Daniell, L. C., Brass, E. P., and Harris, R. A. (1987). Effect of ethanol on intracellular ionized calcium concentrations in synaptosomes and hepatocytes. *Mol. Pharmacol.* **32:** 831-837.

Dave, V., Gordon, G. W., and McCarthy, K. D. (1991). Cerebral type 2 astroglia are heterogeneous with respect to their ability to respond to neuroligands linked to calcium mobilization. *Glia.* **4:** 440-447.

Davidson, M., Bedi, K., and Wilce, P. (1998). Ethanol inhibition of brain ornithine decarboxylase activity in the postnatal rat. *Neurotoxicol Teratol.* **20:** 523-30.

Davidson, M., Wilce, P., and Shanley, B. (1988). Ethanol increases synaptosomal free calcium concentration. *Neurosci. Lett.* **89:** 165-169.

Davis, J. S. (1992). Modulation of lutenizing hormone-stimulated inositol phosphate accumulation by phorbol esters in bovine luteal cells. *Endocrinology.* **131:** 749-57.

Day, N. L., Robles, N., Richardson, G., Geva, D., Taylor, P., Scher, M., Stoffer, D., Cornelius, M., and Goldschmidt, L. (1991). The effects of prenatal alcohol use on the growth of children at three years of age. *Alchol. Clin. Exp. Res.* **15:** 67-71.

DeKoninck, P., and Schulman, H. (1998). Sensitivity of CaM kinase II to the frequency of Ca^{2+} oscillations. *Science.* **279:** 227-230.

Dermietzel, R., Traub, O., Hwang, T. K., Beyer, E., Bennett, M. V. L., Spray, D. C., and Willecke, K. (1989). Differential expression of three gap junction proteins in developing and mature brain tissues. *Neurobiol.* **86:** 10148-10152.

DeWaard, M., Gurnett, C. A., and Campbell, K. P. (1996). Structural and functional diversity of voltage-activated calcium channels. Vol. 4. In *Ion Channels*, Narahashi, T. (ed). Plenum Press, New York. pp. 41-60.

Diamond, I., and Gordon, A. S. (1997). Cellular and molecular neuroscience of alcoholism. *Physiol. Rev.* **77:** 1-19.

Dildy, J. E., and Leslie, S. W. (1989). Ethanol inhibits NMDA-induced increases in free intracellular Ca^{2+} in dissociated brain cells. *Brain Res.* **499:** 383-387.

Dolin, S. J., and Little, H. J. (1989). Are changes in neuronal calcium channels involved in ethanol tolerance? *J. Pharmacol. Exp. Ther.* **250:** 985-991.

Dolmetsch, R. E., Lewis, R. S., Goodnow, C. C., and Healy, J. I. (1997). Differential activation of transcription factors induced by Ca^{2+} response amplitude and duration. *Nature.* **386:** 855-858.

Dousa, T. P., Chini, E. N., and Beers, K. W. (1996). Adenine nucleotide diphosphates: emerging second messengers acting via intracellular Ca^{2+} release. *Am J Physiol.* **271:** C1007-24.

Drouva, S. V., Faivre, B. A., Loudes, C., Laplante, E., and Kordon, C. (1991). Alpha 1-adrenergic receptor coupling with phospholipase-C is negatively regulated by protein kinase-C in primary cultures of hypothalamic neurons and glial cells. *Endocrinology.* **129:** 1605-13.

Dupont, G., Berridge, M. J., and Goldbeter, A. (1991). Signal-induced Ca^{2+} oscillations: properties of a model based on Ca^{2+}-induced Ca^{2+} release. *Cell Calcium.* **12:** 73-85.

Dupont, G., and Goldbeter, A. (1993). One-pool model for Ca^{2+} oscillations involving Ca^{2+} and inositol 1,4,5-trisphosphate as co-agonists for Ca^{2+} release. *Cell Calcium.* **14:** 311-322.

Eggeman, K. T., and Browning, M. D. (1996). Ethanol and the phosphorylation of ligand-gated ion channels. In *Pharmacological Effects of Ethanol on the Nervous*

System, Deitrich, R. A. and Erwin, V. G. (ed). CRC Press, Boca Raton, Fla. pp. 117-133.

Ehrich, M., Intropido, L., and Costa, L. G. (1994). Interaction of organophosphorous compounds with muscarinic receptors in SH-SY5Y human neuroblastoma cells. *J. Toxicol. Env. Health.* **43:** 51-63.

Enkvist, M. O., Holopainen, I., and Akerman, K. E. (1989). Alpha-receptor and cholinergic receptor-linked changes in cytosolic Ca^{2+} and membrane potential in primary rat astrocytes. *Brain Res.* **500:** 46-54.

Enkvist, M. O. K., and McCarthy, K. D. (1992). Activation of protein kinase C blocks astroglial gap junction communication and inhibits the spread of calcium waves. *J. Neurochem.* **59:** 519-526.

Evans, T., Helper, J. R., Masters, S. B., Brown, J. H., and Harden, T. K. (1985). Guanine nucleotide regulation of agonist binding to muscarinic cholinergic receptors. Relation to efficacy of agonists for stimulation of phosphoinositide breakdown and Ca^{2+} mobilization. *Biochem. J.* **232:** 751-757.

Fano, G., Belia, S., Mariggio, M. A., Antonica, A., Agea, E., and Spinozzi, F. (1993). Alteration of membrane transductive mechanisms induced by ethanol in human lymphocyte cultures. *Cell. Signal.* **5:** 139-143.

Fatatis, A., and Russell, J. T. (1992). Spontaneous changes in intracellular calcium concentration in type 1 astrocytes from rat cerebral cortex in primary culture. *Glia.* **5:** 95-104.

Fiekers, J. F., and Konopka, L. M. (1996). Spontaneous transients of $[Ca^{2+}]_i$ depend on external calcium and the activation of L-type voltage-gated calcium channels in a clonal pituitary cell line (AtT-20) of cultured mouse corticotropes. *Cell Calcium.* **19:** 327-36.

Finch, E. A., and Goldin, S. M. (1994). Comment. *Science.* **265:** 813-815.

Finch, E. A., Turner, T. J., and Goldin, S. M. (1991). Calcium as a coagonist of inositol 1,4,5-trisphosphate-induced calcium release. *Science.* **252:** 443-446.

Finkbeiner, S. (1992). Calcium waves in astrocytes-filling in the gaps. *Neuron.* **8:** 1101-1108.

Finkbeiner, S. M. (1993). Glial calcium. *Glia.* **9:** 83-104.

Fischer, G., and Kettenmann, H. (1985). Cultured astrocytes form a syncytium after maturation. *Exp. Cell Res.* **159:** 273-279.

Gafni, J., Munsch, J. A., Lam, T. H., Catlin, M. C., Costa, L. G., Molinski, T. F., and Pessah, I. N. (1997). Xestospongins: potent membrane permeable blockers of the inositol 1,4,5-trisphosphate receptor. *Neuron.* **19:** 723-733.

Galione, A. (1994). Cyclic ADP-ribose, the ADP-ribosyl cyclase pathway and calcium signalling. *Mol Cell Endocrinol.* **98:** 125-31.

Gallo, V., Giovannini, C., Suergiu, R., and Levi, G. (1989). Expression of excitatory amino acid receptors by cerebellar cells of the type-2 astrocyte cell lineage. *J. Neurochem.* **52:** 1-9.

Gallo, V., Patrizio, M., and Levi, G. (1991). GABA release triggered by the activation of neuron-like non-NMDA receptors in cultured type 2 astrocytes is carrier mediated. *Glia.* **4:** 245-255.

Gambetti, P., Erulkar, S. E., Somlyo, A. P., and Gonatas, N. K. (1975). Calcium-containing structures in vertebrate glial cells. *J. Cell Biol.* **64:** 322-330.

Garcia, R. L., and Schilling, W. P. (1997). Differential expression of mammalian TRP homologues across tissues and cell lines. *Biochem. Biophys. Res. Commun.* **239:** 279-83.

Genazzani, A. A., and Galione, A. (1996). Nicotinic acid-adenine dinucleotide phosphate mobilizes Ca^{2+} from a thapsigargin-insensitive pool. *Biochem. J.* **315:** 721-5.

Genazzani, A. A., and Galione, A. (1997). A Ca^{2+} release mechanism gated by the novel pyridine nucleotide, NAADP. *Trends Pharmacol. Sci.* **18:** 108-10.

Ghosh, A., and Greenberg, M. E. (1995). Calcium signaling in neurons: molecular mechanisms and cellular consequences. *Science.* **268:** 239-247.

Glaum, S. R., Holzwarth, J. A., and Miller, R. J. (1990). Glutamate receptors activate Ca^{2+} mobilization and Ca^{2+} influx into astrocytes. *Proc. Natl. Acad. Sci.* **87:** 3454-3458.

Goldman, R. S., Finkbeiner, S. M., and Smith, S. J. (1991). Endothelin induces a sustained rise in intracellular calcium in hippocampal astrocytes. *Neurocsi. Lett.* **123:** 4-8.

Golovina, V. A., Bambrick, L. L., Yarowsky, P. J., Krueger, B. K., and Blaustein, M. P. (1996). Modulation of two functionally distinct Ca^{2+} stores in astrocytes: role of the plasmalemmal Na/Ca exchanger. *Glia.* **16:** 296-305.

Grant, A. J., Koski, G., and Treistman, S. N. (1993). Effect of chronic ethanol on calcium currents and calcium uptake in undifferentiated PC12 cells. *Brain Res.* **600:** 280-284.

Greenberg, D. A., Carpenter, C. L., and Messing, R. O. (1987). Ethanol-induced component of 45 Ca^{2+} uptake in PC12 cells is sensitive to Ca^{2+} channel modulating drugs. *Brain Res.* **410:** 143-146.

Grover, A. K., and Khan, I. (1992). Calcium pump isoforms: diversity, selectivity and plasticity. *Cell Calcium.* **13:** 9-17.

Gruol, D. L., and Curry, J. G. (1995). Calcium signals elicited by quisqualate in cultured Purkinje neurons show developmental changes in sensitivity to acute alcohol. *Brain Res.* **673:** 1-12.

Gruol, D. L., and Parsons, K. L. (1994). Chronic exposure to alcohol during development alters the calcium currents of cultured cerebellar Purkinje neurons. *Brain Res.* **634:** 283-290.

Gruol, D. L., and Parsons, K. L. (1996). Chronic alcohol reduces calcium signaling elicited by glutamate receptor stimulation in developing cerebellar neurons. *Brain Res.* **728:** 166-174.

Gu, X., and Spitzer, N. C. (1995). Distinct aspects of neuronal differentiation encoded by frequency of spontaneous Ca^{2+} transients. *Nature.* **375:** 784-787.

Guizzetti, M., Catlin, M., and Costa, L. G. (1997). Effects of ethanol on glial cell proliferation: relevance to the fetal alcohol syndrome. *Front. Biosci.* **2:** e93-98.

Guizzetti, M., and Costa, L. G. (1996). Inhibition of muscarinic receptor-stimulated glial cell proliferation by ethanol. *J. Neurochem.* **67:** 2236-2245.

Guizzetti, M., Costa, P., Peters, J., and Costa, L. G. (1996). Acetylcholine as a mitogen: muscarinic receptor-mediated proliferation of rat astrocytes and human astrocytoma cells. *Eur. J. Pharmacol.* **297:** 265-273.

Guizzetti, M., Wei, M., and Costa, L. G. (1998). Role of protein kinase C α and ε isozymes in DNA synthesis induced by muscarinic receptors in a glial cell line. *Eur. J. Pharmacol.* **359:** 223-233.

Guppy, L. J., Crabbe, J. C., and Littleton, J. M. (1995). Time course and genetic variation in the regulation of calcium channel antagonist binding sites in rodent tissues during the induction of ethanol physical dependence and withdrawal. *Alcohol Alcohol.* **30:** 607-615.

Guppy, L. J., and Littleton, J. M. (1994). Binding characteristics of the calcium channel antagonist [^{3}H]-nitrendipine in tissues from ethanol-dependent rats. *Alcohol Alcohol.* **29:** 283-293.

Hansen, J. W., Streissguth, A. P., and Smith, D. W. (1978). The effects of moderate alcohol consumption during pregnancy on fetal growth and morphogenesis. *J. Ped.* **92:** 457-460.

Hardie, R. C., and Minke, B. (1992). The *trp* gene is essential for a light-activated Ca^{2+} channel in *Drosophila* photoreceptors. *Neuron.* **8:** 643-651.

Hardie, R. C., and Minke, B. (1995). Phosphoinositide-mediated phototransduction in *Drosophila* photoreceptors: the role of Ca^{2+} and *trp*. *Cell Calcium.* **18:** 256-274.

Harootunian, A. T., Kao, J. P. Y., Paranjape, S., Adams, S. R., Potter, B. V. L., and Tsien, R. Y. (1991). Cytosolic Ca^{2+} oscillations in REF52 fibroblasts: Ca^{2+}-stimulated IP_3 production or voltage-dependent Ca^{2+} channels as key positive feedback elements. *Cell Calcium.* **12:** 153-164.

Harris, R. A., and Hood, W. F. (1980). Inhibition of synaptosomal calcium uptake by ethanol. *J. Pharmacol. Exp. Ther.* **213:** 562-568.

Hartwig, A. (1998). Carcinogenicity of metal compounds: possible role of DNA repair inhibition. *Toxicol Lett.* **102-103:** 235-239.

Herschman, H. R. (1991). Primary response genes induced by growth factors and tumor promoters. *Annu. Rev. Biochem.* **60:** 281-319.

Higashi, K., and Hoek, J. B. (1991). Ethanol causes desensitization of receptor-mediated phospholipase C activation in isolated hepatocytes. *J. Biol. Chem.* **266:** 2178-2190.

Higashi, K., Tsukada, K., Hoshino, M., Nomura, T., Takeuchi, T., and Hoek, J. B. (1994). Inhibition of ethanol-induced inositol phosphate formation and Ca^{2+} mobilization by okadaic acid in rat hepatocytes: evidence for a role of protein phosphatases in the modulation of phospholipase C by ethanol. *Alcohol Alcohol.* **29:** 53-59.

Hoek, J. B., Thomas, A. P., Rooney, T. A., Higashi, K., and Rubin, E. (1992). Ethanol and signal transduction in the liver. *FASEB J.* **6:** 2386-2396.

Hoffman, P. L., and Tabakoff, B. (1996). Alcohol dependence: a commentary on mechanisms. *Alcohol Alcohol.* **31:** 333-340.

Hofmann, F., Biel, M., and Flockerzi, V. (1994). Molecular basis for Ca^{2+} channel diversity. *Annu. Rev. Neurosci.* **17:** 399-418.

Holownia, A., Ledig, M., and Menez, J. F. (1997). Ethanol-induced cell death in cultured rat astroglia. *Neurotox. Teratol.* **19:** 141-146.

Holzwarth, J. A., Gibbons, S. J., Broroson, J. R., Philipson, L. H., and Miller, R. J. (1994). Glutamate receptor agonists stimulate diverse calcium responses in different types of cultured rat cortical glial cells. *J. Neurosci.* **14:** 1879-1891.

Hosli, E., and Hosli, L. (1993). Receptors for neurotransmitters on astrocytes in the mammalian central nervous system. *Prog. Neurobiol.* **40:** 477-506.

Hosli, L., Hosli, E., and Kaser, H. (1993). Colocalization of cholinergic, adrenergic and peptidergic receptors on astrocytes. *Neuropharm. Neurotox.* **4:** 679-682.

Hosli, L., Hosli, E., Maelicke, A., and Schroder, H. (1992). Peptidergic and cholinergic receptors on cultured astrocytes of different regions of the rat CNS. *Prog. Brain Res.* **94:** 317-329.

Hosoya, T., Takizawa, K., Nitta, K., and Hotta, Y. (1995). *Glial cells missing*: a binary switch between neuronal and glial determination in *Drosophila*. *Cell.* **82:** 1025-1036.

Huang, G.-J., and McArdle, J. J. (1994). Role of the GTP-binding protein G_O in the suppressant effect of ethanol on voltage-activated calcium channels of murine sensory neurons. *Alcohol. Clin. Exp. Res.* **18:** 608-615.

Huber, A., Sander, P., Bahner, M., and Paulsen, R. (1998). The TRP Ca2+ channel assembled in a signaling complex by the PDZ domain protein INAD is phosphorylated through the interaction with protein kinase C (ePKC). *FEBS Lett.* **425:** 317-22.

Huwiler, A., Briner, V. A., Fabbro, D., and Pfeilschifter, J. (1997). Feedback regulation of extracellular ATP-stimulated phosphoinositide hydrolysis by protein kinase C-alpha in bovine glomerular endothelial cells. *Kidney Int.* **52:** 329-37.

Ilyin, V., and Parker, I. (1992). Effects of alcohols on responses evoked by inositol trisphosphate in *Xenopus* oocytes. *J. Physiol.* **448:** 339-354.

Inagaki, N., Fukue, H., Ito, S., and Wada, H. (1991). Type-2 astrocytes show intracellular Ca^{2+} elevation in response to various neuroactive substances. *Neurosci. Lett.* **128:** 257-260.

Irvine, R. F. (1992). Is inositol tetrakisphosphate the second messenger that controls Ca^{2+} entry into cells? *Adv. Sec. Mess. Phospo. Res.* **26:** 161-185.

Ishiyama, H., Sato, M., Matsumura, K., Sento, M., Ogino, K., and Hobara, T. (1995). Proliferation of hepatocytes and attenuation from carbon tetrachloride hepatotoxicity by gadolinium chloride in rats. *Pharmacol Toxicol.* **77:** 293-8.

Jacobson, J. L., Jacobson, S. W., Sokol, R. J., Martier, S. S., Ager, J. W., and Kaplan-Estrin, G. K. (1993). Teratogenic effects of alcohol on infant development. *Alcoh. Clin. Exp. Res.* **17:** 174-183.

Jeftinija, S. D., Jeftinija, K. V., Stefanovic, G., and Liu, F. (1996). Neuroligand-evoked calcium-dependent release of excitatory amino acids from cultured astrocytes. *J. Neurochem.* **66:** 676-684.

Jensen, A. M., and Chiu, S. Y. (1990). Fluorescence measurement of changes in intracellular calcium induced by excitatory amino acids in cultured cortical astrocytes. *J. Neurosci.* **10:** 1165-1175.

Jensen, A. M., and Chiu, S. Y. (1993). Astrocyte networks. In *ASTROCYTES: Pharmacology and Function*, Murphy, S. (ed). Academic Press, New York. pp. 309-330.

Johnson, R. A., Noll, E. C., and Rodney, W. M. (1982). Survival after a serum ethanol concentration of 1 1/2%. *Lancet.* **2:** 1394.

Jones, A. W., and Sternebring, B. (1992). Kinetics of ethanol and methanol in alcoholics during detoxification. *Alcohol Alcohol.* **27:** 641-647.

Jones, B. W., Fetter, R. D., Tear, G., and Goodman, C. S. (1995). *Glial cells missing*: a genetic switch that controls glial versus neuronal fate. *Cell.* **82:** 1013-1023.

Jones, K. L., Smith, D. W., Ulleland, C. N., and Streissguth, A. (1973). Pattern of malformation in offspring of chronic alcoholic mothers. *Lancet.* **7815:** 1267-1271.

Jouishomme, H., and Rigal, D. (1991). Proliferation and activation of human mononuclear cells induced by ionomycin in a serum-free medium. *Thymus.* **17:** 137-146.

Kataoka, S., Alam, R., Dash, P. K., and Yatsu, F. M. (1997). Inhibition of PDGF-mediated proliferation of vascular smooth muscle cells by calcium antagonists. *Stroke.* **28:** 364-9.

Kawanishi, T., Kawanishi, M., Ohata, H., Toyoda, K., Takahashi, M., Momose, K., and Hayashi, Y. (1994). The relationship between spontaneous calcium oscillations and cell proliferation in cultured smooth muscle cells. *Jpn. J. Pharmacol.* **65:** 59-62.

Kelly, S. J., Pierce, D. R., and West, J. R. (1987). Microencephaly and hyperactivity in adult rats can be induced by neonatal exposure to high blood alcohol concentrations. *Exp Neurol.* **96:** 580-93.

Kettenmann, H., Orkand, R. K., and Schachner, M. (1983). Coupling among identified cells in mammalian nervous system cultures. *J. Neurosci.* **3:** 506-516.

Keyser, D. O., and Pellmar, T. C. (1994). Synaptic transmission in the hippocampus: critical role for glial cells. *Glia.* **10:** 237-243.

Kim, W. T., Rioult, M. G., and Cornell-Bell, A. H. (1994). Glutamate-induced calcium signaling in astrocytes. *Glia.* **11:** 173-184.

Kimelberg, H. K., Jalonen, T., and Walz, W. (1993). Regulation of the brain microenvironment: transmitters and ions. In *ASTROCYTES: Pharmacology and Function*, Murphy, S. (ed). Academic Press, New York. pp. 193-223.

Kligman, D., and Marshak, D. R. (1985). Purification and characterization of a neurite extension factor from bovine brain. *Proc. Natl. Acad. Sci. USA.* **82:** 7136-7139.

Koski, G., Lawrence, K., and Righi, D. (1991). Ethanol-induced inhibition of carbachol-stimulated uptake of calcium in PC12 pheochromocytoma cells. *Neuropharmacology.* **30:** 267-274.

Kovacs, K. A., Kavanagh, T. J., and Costa, L. G. (1995). Ethanol inhibits muscarinic receptor-stimulated phosphoinositide metabolism and calcium mobilization in rat primary cortical cultures. *Neurochem. Res.* **20:** 939-949.

Kovary, K., and Bravo, R. (1991a). Expression of different Jun and Fos proteins during the G0-to-G1 transition in mouse fibroblasts: *in vitro* and *in vivo* associations. *Mol. Cell Biol.* **11:** 2451-2459.

Kovary, K., and Bravo, R. (1991b). The Jun and Fos protein families are both required for cell cycle progression in fibroblasts. *Mol. Cell Biol.* **11:** 4466-4472.

Kuhar, M. J., Birdsall, N. J. M., Burgen, A. S. V., and Hulme, E. C. (1980). Ontogeny of muscarinic receptors in rat brain. *Brain Res.* **184:** 375-383.

Langley, D., and Pearce, B. (1994). Ryanodine-induced intracellular calcium mobilization in cultured astrocytes. *Glia.* **12:** 128-134.

Larsson, C., Simonsson, P., Hoek, J. B., and Alling, C. (1995). Ethanol inhibits the peak of muscarinic receptor-stimulated formation of inositol 1,4,5-trisphosphate in neuroblastoma SH-SY5Y Cells. *Biochem. Pharmacol.* **50:** 647-654.

Larsson, C., Thomas, A. P., and Hoek, J. B. (1998). Carbachol-stimulated Ca^{2+} increase in single neuroblastoma SH-SY5Y cells: effects of ethanol. *Alcohol Clin Exp Res.* **22:** 637-45.

Lee, H. C. (1997). Mechanisms of calcium signaling by cyclic ADP-ribose and NAADP. *Physiol Rev.* **77:** 1133-64.

Lee, H. C. (1998). Calcium signaling by cyclic ADP-ribose and NAADP. A decade of exploration. *Cell Biochem Biophys.* **28:** 1-17.

Lee, H. C., and Aarhus, R. (1995). A derivative of NADP mobilizes calcium stores insensitive to inositol trisphosphate and cyclic ADP-ribose. *J. Biol. Chem.* **270:** 2152-7.

Lee, N. M., and Becker, C. E. (1992). The alcohols. In *Basic and Clinical Pharmacology*, Katzung, B. G. (ed). Appleton & Lange, East Norwalk, Conneticut. pp. 278-286.

Lee, Y. H., Spuhler-Phillips, K., Randall, P. K., and Leslie, S. W. (1996). Effects of prenatal ethanol exposure on voltage-dependent calcium entry into neonatal whole brain-dissociated neurons. *Alcohol. Clin. Exp. Res.* **20:** 921-928.

Lee, Y. S., Sayeed, M. M., and Wurster, R. D. (1993a). Inhibition of cell growth by K^+ channel modulators is due to interference with agonist-induced Ca^{2+} release. *Cell Signal.* **5:** 803-809.

Lee, Y. S., Sayeed, M. M., and Wurster, R. D. (1993b). Inhibition of human brain tumor cell growth by a receptor-operated Ca^{2+} channel blocker. *Cancer Lett.* **72:** 77-81.

Lee, Y. S., Sayeed, M. M., and Wurster, R. D. (1994). Inhibition of cell growth and intracellular Ca^{2+} mobilization in human brain tumor cells by Ca^{2+} channel antagonists. *Mol. Chem. Neuropathol.* **22:** 81-95.

Lemoine, P., and Lemoine, P. (1992). Avenir des enfants de meres alcooliques (etude de 105 cas retrouves a l'age adulte) et quelques constatations d'interet prophylactique. *Ann. Pediatr. (Paris).* **39:** 226-235.

Leslie, S. W., Barr, E., Chandler, J., and Farrar, R. P. (1983). Inhibition of fast- and slow-phase depolarization-dependent synaptosomal calcium uptake by ethanol. *J. Pharmacol. Exp. Ther.* **225:** 571-575.

Leslie, S. W., Brown, L. M., Dildy, J. E., and Sims, J. S. (1990). Ethanol and neuronal calcium channels. *Alcohol.* **7:** 233-236.

Levi, G., Gallo, V., and Patrizio, M. (1992). Release of exogenous and endogenous neurotransmitter amino acids from cultured astrocytes. *Prog. Brain Res.* **94:** 243-250.

Levison, S. W., and Goldman, J. E. (1993). Astrocyte origins. In *Astrocytes: Pharmacology and Function*, Murphy, S. (ed). Academic Press, Inc., New York. pp. 1-22.

Li, W., Llopis, J., Whitney, M., Zlokarnik, G., and Tsien, R. Y. (1998). Cell-permeant caged $InsP_3$ ester shows that Ca^{2+} spike frequency can optimize gene expression [see comments]. *Nature.* **392:** 936-41.

Lilien, L. E., and Raff, M. C. (1990). Analysis of the cell-cell interactions that control type 2 astrocyte development *in vitro*. *Neuron.* **4:** 525-534.

Little, H. J. (1991). Mechanisms the may underlie the behavioural effects of ethanol. *Prog. Neurobiol.* **36:** 171-194.

Loza, J., Carpio, L., Lawless, G., Marzec, N., and Dziak, R. (1995). Role of extracellular calcium influx in EGF-induced osteoblastic cell proliferation. *Bone.* **16:** 341S-347S.

Lynch, M. A., and Littleton, J. M. (1983). Possible association of alcohol tolerance with increased synaptic Ca^{2+} sensitivity. *Nature.* **303:** 175-176.

MacVicar, B. A. (1984). Voltage-dependent calcium channels in glial cells. *Science.* **226:** 1345-1347.

MacVicar, B. A. (1987). Morphological differentiation of cultured astrocytes in blocked by cadmium or cobalt. *Brain Res.* **420:** 175-177.

MacVicar, B. A., Hochman, D., Delay, M. J., and Weiss, S. (1991). Modulation of intracellular Ca^{++} in cultured astrocytes by influx through voltage-activated Ca^{++} channels. *Glia.* **4:** 448-455.

Maier, S. E., Chen, W. J., Miller, J. A., and West, J. R. (1997). Fetal alcohol exposure and temporal vulnerability regional differences in alcohol-induced microencephaly as a function of the timing of binge- like alcohol exposure during rat brain development. *Alcohol Clin Exp Res.* **21:** 1418-28.

Malcolm, C., Grieve, A., Ritchie, L., Schousboe, A., and Griffiths, R. (1996). NMDA receptor-mediated cGMP synthesis in primary cultures of mouse cerebellar granule cells appears to involve neuron-astrocyte communication with NO operating as the intercellular messenger. *J. Neurosci. Res.* **45:** 129-142.

Mangel, A. W., Scott, L., and Liddle, R. A. (1996). Depolarization-stimulated cholecystokinin secretion is mediated by L-type calcium channels in STC-1 cells. *Am. J. Physiol.* **270:** G287-290.

Mangoura, D., Sogos, V., Pelletiere, C., and Dawson, G. (1995). Differential regulation of phospholipases C and D by phorbol esters and the physiological activators carbachol and glutamate in astrocytes from chicken embryo cerebrum and cerebellum. *Brain Res Dev Brain Res.* **87:** 12-21.

Marks, S. S., Watson, D. L., Carpenter, C. L., Messing, R. O., and Greenberg, D. A. (1989). Comparative effects of chronic exposure to ethanol and calcium channel antagonists on calcium channel antagonist receptors in cultured neural PC12 cells. *J. Neurochem.* **53:** 168-172.

Marshak, D. R. (1990). S100ß as a neurotrophic factor. *Prog. Brain Res.* **86:** 169-181.

Martin, F. C., Charles, A. C., Sanderson, M. J., and Merrill, J. E. (1992). Substance P stimulates IL-1 production by astrocytes via intracellular calcium. *Brain Res.* **599:** 13-18.

Masters, S. B., Harden, T. K., and Brown, J. H. (1984). Relationships between phosphoinositide and calcium responses to muscarinic agonists in astrocytoma cells. *Mol. Pharmacol.* **26:** 149-155.

Masters, S. B., Martin, M. W., Harden, T. K., and Brown, J. H. (1985a). Pertussis toxin does not inhibit muscarinic-receptor-mediated phosphoinositide hydrolysis or calcium mobilization. *Biochem. J.* **227:** 933-937.

Masters, S. B., Quinn, M. T., and Brown, J. H. (1985b). Agonist-induced desensitization of muscarinic receptor-mediated calcium efflux without concomitant desensitization of phosphoinositide hydrolysis. *Mol. Pharmacol.* **27:** 325-332.

McCarthy, K. D., and DeVellis, J. (1980). Preparation of separate astroglial and oligodendroglial cell cultures from rat cerebral tissue. *J. Cell Biol.* **85:** 890-902.

McCarthy, K. D., and Salm, A. K. (1991). Pharmacologically-distinct subsets of astroglia can be identified by their calcium response to neuroligands. *Neurosci.* **41:** 325-333.

McDonough, P. M., Eubanks, J. H., and Brown, J. H. (1988). Desensitization and recovery of muscarinic and histaminergic Ca^{2+} mobilization in 1321N1 astrocytoma cells. *Biochem. J.* **249:** 135-141.

Mennerick, S., Benz, A., and Zorumski, C. F. (1996). Components of glial responses to exogenous and synaptic glutamate in rat hippocampal microcultures. *J. Neurosci.* **16:** 55-64.

Messing, R. O., Carpenter, C. L., Diamond, I., and Greenberg, D. A. (1986). Ethanol regulates calcium channels in clonal neural cells. *Proc. Natl. Acad. Sci. USA.* **83:** 6213-6215.

Messing, R. O., Sneade, A. B., and Savidge, B. (1990). Protein kinase C participates in up-regulation of dihydropyridine-sensitive calcium channels by ethanol. *J. Neurochem.* **55:** 1383-1389.

Meyer, T. (1991). Cell signaling by second messenger waves. *Cell.* **64:** 675-678.

Mezna, M., Patchick, T., Tovey, S., and Michelangeli, F. (1996). Inhibition of the cerebellar inositol 1,4,5-trisphosphate-sensitive Ca^{2+} channel by ethanol and other aliphatic alcohols. *Biochem. J.* **314:** 175-179.

Miller, M. W. (1993). Migration of cortical neurons is altered by gestational exposure to ethanol. *Alcohol Clin Exp Res.* **17:** 304-14.

Miller, M. W. (1996). Effect of early exposure to ethanol on the protein and DNA contents of specific brain regions in the rat. *Brain Res.* **734:** 286-94.

Minke, B., and Selinger, Z. (1996). Role of *Drosophila* TRP in inositide-mediated Ca^{2+} entry. *Mol. Neurobiol.* **12:** 163-180.

Minocherhomjee, A. E., and Roufogalis, B. D. (1984). Antagonism of calmodulin and phosphodiesterase by nifedipine and related calcium entry blockers. *Cell Calcium.* **5:** 57-63.

Mironov, S. L., and Hermann, A. (1996). Ethanol actions on the mechanisms of Ca^{2+} mobilization in rat hippocampal cells are mediated by protein kinase C. *Brain Res.* **714:** 27-37.

Mishra, S. K., and Hermsmeyer, K. (1994). Selective inhibition of T-type Ca^{2+} channels by Ro 40-5967. *Circ. Res.* **75:** 144-8.

Missiaen, L., De Smedt, H., Droogmans, G., and Casteels, R. (1992). Luminal Ca^{2+} controls the activation of the inositol 1,4,5-trisphosphate receptor by cytosolic Ca^{2+}. *J. Biol. Chem.* **267:** 22961-22966.

Moloney, B., and Leonard, B. E. (1984). Pre-natal and post-natal effects of alcohol in the rat: I. changes in body weight and exploratory activity. *Alcohol Alcohol.* **19:** 131-136.

Morgan, D. L., Durso, M. H., Rich, B. K., and Kurt, T. L. (1995). Severe ethanol intoxication in an adolescent. *Am. J. Emerg. Med.* **13:** 416-418.

Mullikin-Kilpatrick, D., Mehta, N. D., Hildebrandt, J. D., and Treistman, S. N. (1995). G_i is involved in ethanol inhibition of L-type calcium channels in undifferentiated but not differentiated PC-12 cells. *Mol. Pharmacol.* **47:** 997-1005.

Mullikin-Kilpatrick, D., and Treistman, S. N. (1994). Ethanol inhibition of L-type Ca^{2+} channels in PC12 cells: role of permeant Ions. *Eur. J. Pharmacol.* **270:** 17-25.

Mullikin-Kilpatrick, D., and Treistman, S. N. (1995). Inhibition of dihydropyridine-sensitive Ca^{++} channels by ethanol in undifferentiated and nerve growth factor-treated PC12 cells: interaction with the inactivated state. *J. Pharmacol. Exp. Ther.* **272:** 489-497.

Murphy, T. H., Blatter, L. A., Wier, W. G., and Baraban, J. M. (1993). Rapid communication between neurons and astrocytes in primary cortical cultures. *J. Neurosci.* **13:** 2672-2679.

Nakahara, K., Okada, M., and Nakanishi, S. (1997). The metabotropic glutamate receptor mGluR5 induces calcium oscillations in cultured astrocytes via protein kinase C phosphorylation. *J. Neurochem.* **46:** 1467-1475.

Nakanishi, K., Okouchi, Y., Ueki, T., Asai, K., Isobe, I., Eksioglu, Y. Z., Kato, T., Hasegawa, Y., and Kuroda, Y. (1994). Astrocytic contribution to functioning synapse formation estimated by spontaneous neuronal intracellular Ca^{2+} oscillations. *Brain Res.* **659:** 169-178.

Nedergaard, M. (1994). Direct signaling from astrocytes to neurons in cultures of mammalian brain cells. *Science.* **263:** 1768-1771.

Neer, E. J. (1995). Heterotrimeric G proteins: organizers of transmembrane signals. *Cell.* **80:** 249-257.

Nicoletti, F., Magri, G., Ingrao, F., Bruno, V., Catania, M. V., Dell'Albani, P., Condorelli, D. F., and Avola, R. (1990). Excitatory amino acids stimulate inositol

phospholipid hydrolysis and reduce proliferation in cultured astrocytes. *J. Neurochem.* **54:** 771-777.

Nicotera, P., Zhivotovsky, B., and Orrenius, S. (1994). Nuclear calcium transport and the role of calcium in apoptosis. *Cell Calcium.* **16:** 279-288.

Nishizuka, Y. (1995). Protein kinase C and lipid signalling for sustained cellular response. *FASEB.* **9:** 484-496.

Noronha-Blob, L., Richard, C., and U'Prichard, D. C. (1987). Calcium mobilization by muscarinic receptors in human astrocytoma cells: measurements with quin 2. *Biochem. Biophys. Res. Comm.* **147:** 182-188.

Oakes, S. G., and Pozos, R. S. (1982). Electrophysiologic effects of acute ethanol exposure. II. Alterations in the calcium component of action potentials from sensory neurons in dissociated culture. *Dev. Brain Res.* **5:** 251-255.

Okada, T., Shimizu, S., Wakamori, M., Maeda, A., Kurosaki, T., Takada, N., Imoto, K., and Mori, Y. (1998). Molecular cloning and functional characterization of a novel receptor- activated TRP Ca^{2+} channel from mouse brain. *J. Biol. Chem.* **273:** 10279-87.

Oliva, A. M., Bas, N., and Garcia, A. (1997). Differences in the stimulation of the phosphoinositide cycle by amine neurotransmitters in cultured rat forebrain neurones and astrocytes. *Biochem. Pharmacol.* **54:** 1243-1251.

Orellana, S., Solski, P. A., and Brown, J. G. (1998). Guanosine 5'-O-(thiotriphosphate)-dependent inositol trisphosphate formation in membranes is inhibited by phorbol ester and protein kinase C. *J. Biol. Chem.* **262:** 1638-1643.

Orellana, S., Solski, P. A., and Brown, J. H. (1985). Phorbol ester inhibits phosphoinositide hydrolysis and calcium mobilization in cultured astrocytoma cells. *J. Biol. Chem.* **260:** 5236-5239.

Ouyang, Y. L., Azcona-Olivera, J. I., Murtha, J., and Pestka, J. J. (1996). Vomitoxin-mediated IL-2, IL-4, and IL-5 superinduction in murine $CD4^+$ T cells stimulated with phorbol ester and calcium ionophore: relation to kinetics of proliferation. *Toxicol. Appl. Pharmacol.* **138:** 324-334.

Palfrey, H. C., and Nairn, A. C. (1995). Calcium-dependent regulation of protein synthesis. *Ad. Second Mess. Phosphop. Res.* **30:** 191-223.

Pandey, S. C. (1996). Acute and chronic ethanol consumption effects on the immunolabeling of $G_{q/11\alpha}$ subunit protein and phospholipase C isozymes in the rat brain. *J. Neurochem.* **67:** 2355-2361.

Pandey, S. C., Pandey, G. N., and Smith, T. L. (1996). Chronic ethanol effects on the expression of phospholipase C isozymes and $G_{q/11}$-protein in primary cultures of astrocytes. *Alcohol.* **13:** 487-492.

Parker, E. M., Kaneyama, L., Higashijima, T., and Ross, E. M. (1991). Reconstitutively active G protein-coupled receptors purified from Baculovirus-infected insect cells. *J. Biol. Chem.* **266:** 519-527.

Parpura, V., Basarsky, T. A., Liu, F., Jeftinija, K., Jeftinija, S., and Haydon, P. G. (1994). Glutamate-mediated astrocyte-neuron signalling. *Nature.* **369:** 707-708.

Paulsen, R. E., Raulli, R., Grayson, D. R., and Wroblewski, J. T. (1994). Acute and long-term inhibition of agonist-stimulated phosphoinositide hydrolysis by pulse treatment of cerebellar granule cells with TPA. *Mol Chem Neuropathol.* **22:** 67-79.

Pearce, B., Albrecht, J., Morrow, C., and Murphy, S. (1986a). Astrocyte glutamate receptor activation promotes inositol phospholipid turnover and calcium flux. *Neurosci. Lett.* **72:** 335-340.

Pearce, B., Morrow, C., and Murphy, S. (1986b). Receptor-mediated inositol phospholipid hydrolysis in astrocytes. *Eur. J. Pharmacol.* **121:** 231-243.

Pearce, B., Morrow, C., and Murphy, S. (1988). Characteristics of phorbol ester- and agonist-induced down-regulation of astrocyte receptors coupled to inositol phospholipid metabolism. *J. Neurochem.* **50:** 936-944.

Pellegrino, S. M., Woods, J. M., and Druse, M. J. (1993). Effects of chronic ethanol consumption on G proteins in brain areas associated with the nigrostriatal and mesolimbic dopamine systems. *Alcohol. Clin. Exp. Res.* **17:** 1247-1253.

Pende, M., Fisher, T. L., Simpson, P. B., Russell, J. T., Blenis, J., and Gallo, V. (1997). Neurotransmitter- and growth factor-induced cAMP response element binding protein phosphorylation in glial cell progenitors: role of calcium ions, protein kinase C, and mitogen-activated protein kinase/ribosomal S6 kinase pathway. *J. Neurosci.* **17:** 1291-1301.

Pende, M., Holtzclaw, L. A., Curtis, J. L., Russell, J. T., and Gallo, V. (1994). Glutamate regulates intracellular calcium and gene expression in oligodendrocyte progenitors through the activation of DL-alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors. *Proc. Natl. Acad. Sci. U.S.A.* **91:** 3215-3219.

Perez-Terzic, C. M., Chini, E. N., Shen, S. S., Dousa, T. P., and Clapham, D. E. (1995). Ca^{2+} release triggered by nicotinate adenine dinucleotide phosphate in intact sea urchin eggs. *Biochem J.* **312:** 955-9.

Philipp, S., Cavalie, A., Freichel, M., Wissenbach, U., Zimmer, S., Trost, C., Marquart, A., Murakami, M., and Flockerzi, V. (1996). A mammalian capacitative calcium entry channel homologous to *Drosophila* TRP and TRPL. *EMBO J.* **15:** 6166-6171.

Philipp, S., Hambrecht, J., Braslavski, L., Schroth, G., Freichel, M., Murakami, M., Cavalie, A., and Flockerzi, V. (1998). A novel capacitative calcium entry channel expressed in excitable cells. *EMBO J.* **17:** 4274-82.

Poenie, M. (1990). Alteration of intracellular Fura-2 fluorescence by viscosity: a simple correction. *Cell Calcium.* **11:** 85-91.

Porter, J. T., and McCarthy, K. D. (1997). Astrocytic neurotransmitter receptors *in situ* and *in vivo*. *Prog. Neurobiol.* **51:** 439-455.

Post, G. R., Collins, L. R., Kennedy, E. D., Moskowitz, S. A., Aragay, A. M., Goldstein, D., and Brown, J. H. (1996). Coupling of the thrombin receptor to G12 may account for selective effects of thrombin on gene expression and DNA synthesis in 132 1N1 astrocytoma cell. *Mol. Bio. Cell.* **7:** 1679-1690.

Pozzan, T., Rizzuto, R., Volpe, P., and Meldolesi, J. (1994). Molecular and cellular physiology of intracellular stores. *Physiol. Rev.* **74:** 595-636.

Predki, P. F., and Sarkar, B. (1994). Metal replacement in "zinc finger" and its effect on DNA binding. *Environ Health Perspect.* **102 Suppl 3:** 195-8.

Predki, P. F., Zamble, D., Sarkar, B., and Giguere, V. (1994). Ordered binding of retinoic acid and retinoid-X receptors to asymmetric response elements involves determinants adjacent to the DNA-binding domain. *Mol Endocrinol.* **8:** 31-9.

Privat, A., Gimenez-Ribotta, M., and Ridet, J. L. (1995). Morphology of astrocytes. In *Neuroglia*, Kettenmann, H. and Ransom, B. R. (ed). Oxford University Press, New York. pp. 3-22.

Rabe, C. S., and Weight, F. F. (1988). Effects of ethanol on neurotransmitter release and intracellular free calcium in PC12 Cells. *J. Pharmacol. Exp. Ther.* **244:** 417-422.

Rajasekar, R., and Augustin, A. (1992). Selective proliferation of gamma delta T lymphocytes exposed to high doses of ionomycin. *J. Immunol.* **149:** 818-824.

Reeves, R. H., Yao, J., Crowley, M. R., Buck, S., Zhang, X., Yarowsky, P., Gearhart, J. D., and Hilt, D. C. (1994). Astrocytosis and axonal proliferation in the hippocampus of S100ß transgenic mice. *Proc. Natl. Acad. Sci. USA.* **91:** 5359-5363.

Resnicoff, M., Cui, S., Coppola, D., Hoek, J. B., and Rubin, R. (1996). Ethanol-induced inhibition of cell proliferation is modulated by insulin-like growth factor-I receptor levels. *Alcohol. Clin. Exp. Res.* **20:** 961-966.

Rezazadeh, S. M., Woodward, J. J., and Leslie, S. W. (1989). Fura-2 measurement of cytosolic free calcium in rat brain cortical synaptosomes and the influence of ethanol. *Alcohol.* **6:** 341-345.

Richardson, A., and Taylor, C. W. (1993). Effects of Ca^{2+} chelators on purified inositol 1,4,5-trisphosphate ($InsP_3$) receptors and $InsP_3$-stimulated Ca^{2+} mobilization. *J. Biol. Chem.* **268:** 11528-11533.

Ritchie, T., Kim, H.-S., Cole, R., DeVellis, J., and Noble, E. P. (1988). Alcohol-induced alterations in phosphoinositide hydrolysis in astrocytes. *Alcohol.* **5:** 183-187.

Rodriguez, F. D., Lundqvist, C., Alling, C., and Gustavsson, L. (1996). Ethanol and phosphatidylethanol reduce the binding of [^{3}H]inositol 1,4,5-trisphosphate to rat cerebellar membranes. *Alcohol Alcohol.* **31:** 453-461.

Roifman, C. M., Benedict, S. H., Cheung, R. K., and Gelfand, E. W. (1987). Induction of human B cell proliferation and differentiation by the combination of phorbol ester and ionomycin. *Eur. J. Immunol.* **17:** 701-706.

Rosen, L. B., Ginty, D. D., and Greenberg, M. E. (1995). Calcium regulation of gene expression. *Ad. Sec. Mess. Phosphop. Res.* **30:** 225-253.

Rossett, H. L., Weiner, L., and Edelin, K. C. (1981). Strategies for prevention of fetal alcohol effects. *Obstet. Gynecol.* **51:** 1-7.

Rudge, J. S., Alderson, R. F., Pasnikowski, E., McClain, J., Ip, N. Y., and Lindsay, R. M. (1992). Expression of ciliary neurotrophic factor and the neurotrophins - nerve growth factor, brain-derived neurotrophic factor and neurotrophin 3 - in cultured rat hippocampal astrocytes. *Eur. J. Neurosci.* **4:** 459-471.

Ruis, R. A., Bergamaschi, S., DiFonso, F., Govoni, S., Trabucchi, M., and Rossi, F. (1987). Acute ethanol effect on calcium antagonist binding in rat brain. *Brain Res.* **402:** 359-361.

Ryu, S. H., Kim, U. H., Wahl, M. I., Brown, A. B., Carpenter, G., Huang, K. P., and Rhee, S. G. (1990). Feedback regulation of phospholipase C-beta by protein kinase C. *J. Biol.Chem.* **265:** 17941-5.

Sahai, A., Mei, C., Pattison, T. A., and Tannen, R. L. (1997). Chronic hypoxia induces proliferation of cultured mesangial cells: role of calcium and protein kinase C. *Am J Physiol.* **273:** F954-60.

Saso, K., Higashi, K., Nomura, T., Hoshino, M., Ito, M., Moehren, G., and Hoek, J. B. (1996). Inhibitory effect of ethanol on hepatocyte growth factor-induced DNA synthesis and Ca^{2+} mobilization in rat hepatocytes. *Alcohol. Clin. Exp. Res.* **20:** 330A-334A.

Schmid, C., Keller, C., Schlapfer, I., Veldman, C., and Zapf, J. (1998). Calcium and insulin-like growth factor I stimulation of sodium- dependent phosphate transport and proliferation of cultured rat osteoblasts. *Biochem Biophys Res Commun.* **245:** 220-5.

Schmitt, R., Clozel, J. P., Iberg, N., and Buhler, F. R. (1995). Mibefradil prevents neointima formation after vascular injury in rats. Possible role of the blockade of the T-type voltage-operated calcium channel. *Arterioscler. Thromb. Vasc. Biol.* **15:** 1161-5.

Selinfreund, R. H., Barger, S. W., Pledger, W. J., and Van Eldik, L. J. (1991). Neurotrophic protein S100ß stimulates glial cell proliferation. *Proc. Natl. Acad. Sci. USA.* **88:** 3554-3558.

Selinfreund, R. H., Barger, S. W., Welsh, M. J., and Van Eldik, L. J. (1990). Antisense inhibition of glial S100ß production results in alterations in cell morphology, cytoskeletal organization, and cell proliferation. *J. Cell Biol.* **111:** 2021-2028.

Shao, Y., and McCarthy, K. D. (1993). Regulation of astroglial responsiveness to neuroligands in primary culture. *Neuroscience.* **55:** 991-1001.

Shao, Y., and McCarthy, K. D. (1995). Receptor-mediated calcium signals in astroglia: multiple receptors, common stores and all-or-nothing responses. *Cell Calcium.* **17:** 187-196.

Shears, S. B. (1992). Metabolism of inositol phosphates. *Adv. Second Mess. Phosphop. Res.* **26:** 63-92.

Sheng, M., and Greenberg, M. E. (1990). The regulation and function of *c-fos* and other immediate early genes in the nervous system. *Neuron.* **4:** 477-485.

Short, A. D., Bian, J., Ghosh, T. K., Waldron, R. T., Rybak, S. L., and Gill, D. L. (1993). Intracellular Ca^{2+} pool content is linked to control of cell growth. *Proc. Natl. Acad. Sci.* **90:** 4986-4990.

Simonsson, P., Rodriguez, F. D., Loman, N., and Alling, C. (1991). G proteins coupled to phospholipase C: molecular targets of long-term ethanol exposure. *J. Neurochem.* **56:** 2018-2026.

Simonsson, P., Sun, G. Y., Vecsei, L., and Alling, C. (1989). Ethanol effects on bradykinin-stimulated phosphoinositide hydrolysis in NG 108-15 neuroblastoma-glioma cells. *Alcohol.* **6:** 475-479.

Simonyi, A., Zhang, J.-P., Sun, A. Y., and Sun, G. Y. (1996). Chronic ethanol on mRNA levels of IP_3R1, IP_3 3-kinase and mGluR1 in mouse Purkinje neurons. *NeuroReport.* **7:** 2115-2118.

Singh, S. P., Handa, R. K., Depala, V., Gao, Y., McIlroy, P. J., and Ravindra, R. (1997). The effect of ethanol on muscarinic receptor-G protein coupling in the rat cortex. *Pharmacol. Toxicol.* **81:** 294-9.

Sitsapesan, R., McGarry, S. J., and Williams, A. J. (1995). Cyclic ADP-ribose, the ryanodine receptor and Ca^{2+} release. *Trends Pharmacol. Sci.* **16:** 386-91.

Skattebol, A., and Rabin, R. A. (1987). Effects of ethanol on $^{45}Ca^{2+}$ uptake in synaptosomes and in PC12 cells. *Biochem. Pharmacol.* **36:** 2227-2229.

Smith, S. J. (1992). Do astrocytes process neural information? *Prog. Brain Res.* **94:** 119-136.

Smith, T. L. (1994). Selective effects of ethanol exposure on metabotropic glutamate receptor and guanine nucleotide stimulated phospholipase C activity in primary cultures of astrocytes. *Alcohol.* **11:** 405-409.

Smith, T. L., and Bitrick, M. S. (1995). Lack of involvement of protein kinase C in ethanol-induced inhibition of metabotropic-glutamate receptor function in primary cultures of astrocytes. *Life Sci.* **56:** PL485-489.

Smith, T. L., Yamamura, H. I., and Lee, L. (1986). Effect of ethanol on receptor-stimulated phosphatidic acid and polyphosphoinositide metabolism in mouse brain. *Life Sci.* **39:** 1675-1684.

Smith-Thomas, L., Haycock, J. W., Metcalfe, R., Boulton, M., Ellis, S., Rennie, I. G., Richardson, P. S., Palmer, I., Parsons, M. A., and Mac Neil, S. (1998). Involvement of calcium in retinal pigment epithelial cell proliferation and pigmentation. *Curr. Eye Res.* **17:** 813-22.

Smrcka, A. V., Helper, J. R., Brown, K. O., and Sternweis, P. C. (1991). Regulation of polyphosphoinositide-specific phospholipase C activity by purified G_q. *Science.* **251:** 804-807.

Snell, L. D., Tabakoff, B., and Hoffman, P. L. (1994). Involvement of protein kinase C in ethanol-induced Inhibition of NMDA receptor function in cerebellar granule cells. *Alcohol. Clin. Exp. Res.* **18:** 81-85.

Sneyd, J., Charles, A. C., and Sanderson, M. J. (1994). A model for the propagation of intercellular calcium waves. *Am. J. Physiol.* **266:** C293-C302.

Snyder, R., and Andrews, L. S. (1996). Toxic effects of solvents and vapours. In *Casarett & Doull's Toxicology. The Basic Science of Poisons, 5th Ed.*, Klassen, C. D., Amdur, M. O. and Doull, J. (ed). McGraw-Hill, New York. pp. 737-771.

Sontheimer, H. (1992). Astrocytes, as well as neurons, express a diversity of ion channels. *Can. J. Physiol. Pharmacol.* **70:** S223-S238.

Sontheimer, H. (1994). Voltage-dependent ion channels in glial cells. *Glia.* **11:** 156-172.

Stanimirovic, D. B., Ball, R., Mealing, G., Morley, P., and Durkin, J. P. (1995). The role of intracellular calcium and protein kinase C in endothelin-stimulated proliferation of rat type I astrocytes. *Glia.* **15:** 119-130.

Stiene-Martin, A., Mattson, M. P., and Hauser, K. F. (1993). Opiates selectively increase intracellular calcium in developing type-1 astrocytes: role of calcium in morphine-induced morphologic differentiation. *Dev. Brain Res.* **76:** 189-196.

Streissguth, A. P., Aase, J. M., Clarren, S. M., Randels, S. P., LaDue, R. A., and Smith, D. F. (1991). Fetal alcohol syndrome in adolescents and adults. *J.A.M.A.* **265:** 1961-1967.

Streissguth, A. P., Barr, H. M., and Sampson, P. D. (1990). Moderate prenatal alcohol exposure: effects on child IQ and learning problems at age 71/2 years. *Alcohol. Clin. Exp. Res.* **14:** 662-669.

Sutko, J. L., Airey, J. A., Welch, W., and Ruest, L. (1997). The pharmacology of ryanodine and related compounds. *Pharmacol. Rev.* **49:** 53-98.

Tabakoff, B., Hoffman, P. L., and McLaughlin, A. (1988). Is ethanol a discriminating substance? *Sem. Liver Dis.* **8:** 26-35.

Takuwa, N., Zhou, W., Kumada, M., and Takuwa, Y. (1995). Involvement of intact inositol-1,4,5-trisphosphate-sensitive Ca^{2+} stores in cell cycle progression at the G1/S boundary in serum-stimulated human fibroblasts. *FEBS.* **360:** 173-176.

Tanabe, S., Heesen, M., Yoshizawa, I., Berman, M. A., Luo, Y., Bleul, C. C., Springer, T. A., Okuda, K., Gerard, N., and Dorf, M. E. (1997). Functional expression of the CXC-chemokine receptor-4/fusin on mouse microglial cells and astrocytes. *J. Immunol.* **159:** 905-911.

Taylor, and Marshall. (1992). Calcium and inositol 1,4,5-trisphosphate receptors: a complex relationship. *Trends Biochem. Science.* **17:** 403-407.

Toner, C. C., and Stamford, J. A. (1997). Involvement of N-and P/Q- but not L- or T-type voltage-gated calcium channels in ischaemia-induced striatal dopamine release in vitro. *Brain Res.* **748:** 85-92.

Travis, J. (1994). Glia: the brain's other cells. *Science.* **266:** 970-972.

Trilivas, I., and Brown, J. H. (1989). Increases in intracellular Ca^{2+} regulate the binding of [^{3}H]phorbol 12,13-dibutyrate to intact 1321N1 astrocytoma cells. *J. Biol. Chem.* **264:** 3102-3107.

Tropea, M., Johnson, M. I., and Higgins, D. (1988). Glial cells promote dendritic development in rat sympathetic neurons *in vitro*. *Glia.* **1:** 380-392.

Twombly, D. A., Herman, M. D., Kye, C. H., and Narahashi, T. (1990). Ethanol effects on two types of voltage-activated calcium channels. *J. Pharmacol. Exp. Ther.* **254:** 1029-1037.

Ubl, J. J., and Reiser, G. (1997). Characteristics of thrombin-induced calcium signals in rat astrocytes. *Glia.* **21:** 361-369.

US Public Health Service. (1990). Seventh special report to the US Congress on alcohol and health. In Secretary of Health and Human Services, Public Health Service, Alcohol, Drug Abuse, and Mental Health Administration, National Institute on Alcohol Abuse and Alcoholism, Rockville, MD.

Van Der Zee, E. A., DeJong, G. I., Strosberg, A. D., and Luiten, P. G. M. (1993). Muscarinic acetylcholine receptor-expression in astrocytes in the cortex of young and aged rats. *Glia.* **8:** 42-50.

Verkhratsky, A., and Kettenmann, H. (1996). Calcium signaling in glial cells. *Trends Neurosci.* **19:** 346-352.

Verkhratsky, A., Orkand, R. K., and Kettenmann, H. (1998). Glial calcium: homeostasis and signaling function. *Physiol. Rev.* **78:** 99-141.

Wakui, M., Osipchuk, Y. V., and Petersen, O. H. (1990). Receptor-activated cytoplasmic Ca^{2+} spiking mediated by inositol trisphosphate is due to Ca^{2+}-induced Ca^{2+} release. *Cell.* **63:** 1025-1032.

Wakui, M., and Petersen, O. H. (1990). Cytoplasmic Ca^{2+} oscillations evoked by acetylcholine or intracellular infusion of inositol trisphosphate or Ca^{2+} can be inhibited by internal Ca^{2+}. *FEBS.* **263:** 206-208.

Wayman, C. P., McFadzean, I., Gibson, A., and Tucker, J. F. (1996). Two distinct membrane currents activated by cyclopiazonic acid-induced calcium store depletion in single smooth muscle cells of the mouse anococcygeus. *Br. J. Pharmacol.* **117:** 566-572.

Webb, B., Suarez, S. S., Heaton, M. B., and Walker, D. W. (1995). Ethanol and nerve growth factor effects on calcium homeostasis in cultured embryonic rat medial septal neurons before and during depolarization. *Brain Res.* **701:** 61-74.

Webb, B., Suarez, S. S., Heaton, M. B., and Walker, D. W. (1996a). Calcium homeostasis in cultured embryonic rat septohippocampal neurons is altered by ethanol and nerve growth factor before and during depolarization. *Brain Res.* **729:** 176-189.

Webb, B., Suarez, S. S., Heaton, M. B., and Walker, D. W. (1996b). Cultured postnatal rat medial septal neurons respond to acute ethanol treatment and nerve growth factor by changing intracellular calcium levels. *Alcohol. Clin. Exp. Res.* **20:** 1385-1394.

Weight, F. F. (1992). Cellular and molecular physiology of alcohol actions in the nervous system. *Int. Rev. Neurobiol.* **33:** 289-348.

West, J. R., Hamre, K. M., and Cassell, M. D. (1986). Effects of ethanol exposure during the third trimester equivalent on neuron number in rat hippocampus and dentate gyrus. *Alcohol. Clin. Exp. Res.* **10:** 190-197.

Williams, R. J., and Kelly, E. (1993). Chronic ethanol reduces immunologically detectable $G_{q\ \alpha/11\alpha}$ in NG108-15 cells. *J. Neurochem.* **61:** 1163-1166.

Xiong, W.-C., and Montell, C. (1995). Defective glia induce neuronal apoptosis in the *repo* visual system of Drosophila. *Neuron.* **14:** 581-590.

Yagle, K., and Costa, L. G. (1999). Effects of alcohol on immediate early gene expression in primary cultured rat cortical astrocytes. *Alcohol. Clin. Exp. Res.* **In Press:**

Yao, Y., Choi, J., and Parker, I. (1995). Quantal puffs of intracellular Ca^{2+} evoked by inositol trisphosphate in *Xenopus* oocytes. *J. Phys.* `**482:** 533-553.

Yusa, K., and Tsuruo, T. (1989). Reversal mechanism of multidrug resistance by verapamil: direct binding of verapamil to P-glycoprotein on specific sites and transport of verapamil outward across the plasma membrane of K562/ADM cells. *Cancer Res.* **49:** 5002-6.

Zakour, R. A., Tkeshelashvili, L. K., Shearman, C. W., Koplitz, R. M., and Loeb, L. A. (1981). Metal-induced infidelity of DNA synthesis. *J Cancer Res Clin Oncol.* **99:** 187-96.

Zeitler, H., Ko, Y., Glodny, B., Totzke, G., Appenheimer, M., Sachinidis, A., and Vetter, H. (1997). Cell-cycle arrest in G0/G1 phase of growth factor-induced endothelial cell proliferation by various calcium channel blockers. *Cancer Detect Prev.* **21:** 332-9.

Zhu, X., Jiang, M., and Birnbaumer, L. (1998). Receptor-activated Ca^{2+} influx via human Trp3 stably expressed in human embryonic kidney (HEK)293 cells. Evidence for a non-capacitative Ca^{2+} entry. *J Biol Chem.* **273:** 133-42.

Zhu, X., Jiang, M., Peyton, M., Boulay, G., Hurst, R., Stefani, E., and Birnbaumer, L. (1996). *trp*, A novel mammalian gene family essential for agonist-activated capacitative Ca^{2+} entry. *Cell.* **85:** 661-671.

Zimmermann, A., Sutter, A., and Stephani, U. (1994). Evidence for an NGF-induced autocrine neurotrophic potential of glial cells in nervous system development. *Glia.* **12:** 81-85.

Zou, J. Y., Cohan, C., Rabin, R. A., and Pentney, R. J. (1995). Continuous exposure of cultured rat cerebellar macroneurons to ethanol-depressed NMDA and KCl-stimulated elevations of intracellular calcium. *Alcohol. Clin. Exp. Res.* **19:** 840-845.

APPENDIX A: CALCIUM CALIBRATION CURVE

Absolute calcium concentrations were determined from the ratio of fluorescence of bound (measured at 405 nm) over unbound (measured at 480 nm) indo-1 by generation of a standard curve. Figure 23 shows a representative standard curve. The difference in viscosity between a buffer solution and the intracellular matrix has been reported to confound calcium standard curves, however, it has also been reported that ethanol can correct for this problem (Poenie, 1990). Standard curves, therefore, were generated in the presence of 22% (v/v) ethanol. A calcium buffer calibration kit, containing a zero mM calcium standard and a 'high' (10 mM CaEGTA) calcium standard, were purchased from Molecular Probes (Eugene, OR). These solutions were mixed to give the appropriate concentration, as described in the protocol for the kit.

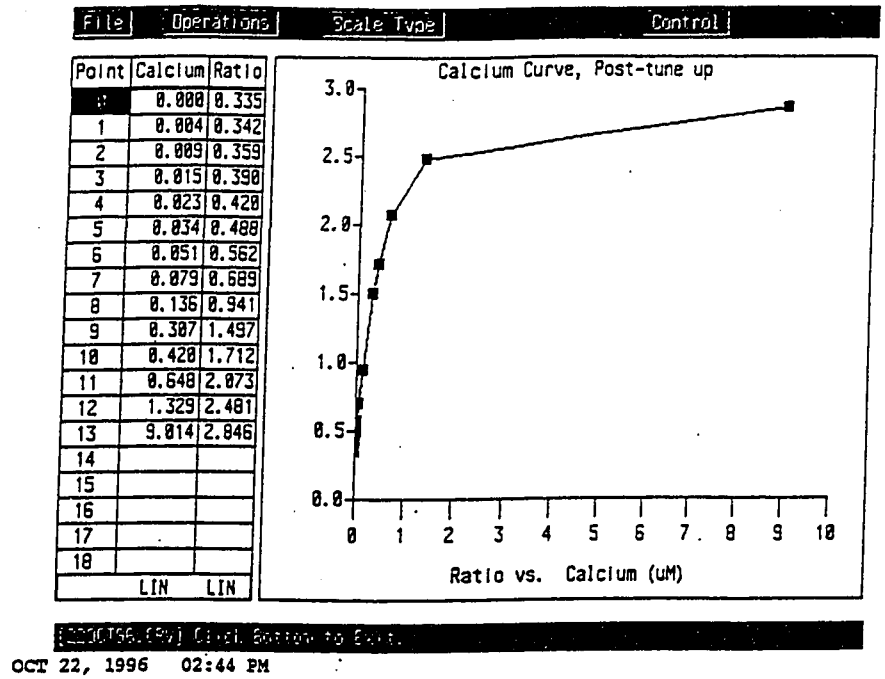


Figure 24. Representative calcium calibration curve.

Representative calibration curve for the ratio of indo-1 fluorescence to calcium concentration (μM), generated in 22% ethanol.

APPENDIX B: ETHANOL AND INDO-1 FLUORESCENCE

The effect of ethanol on indo-1 was investigated to ensure that there were no effects of ethanol on the fluorescence of this dye. The fluorescence of free indo-1 in the presence and absence of 100 mM ethanol was measured using a Perkin Elmer Luminescence Spectrometer LS50 (Norwalk, CT), with an excitation wavelength of 356 nm (Fig. 24). Ethanol did not interfere with the fluorescence of indo-1, and therefore, the effect of ethanol measured on calcium concentrations in astroglia was not an artifact due to interference of ethanol with the fluorescence.

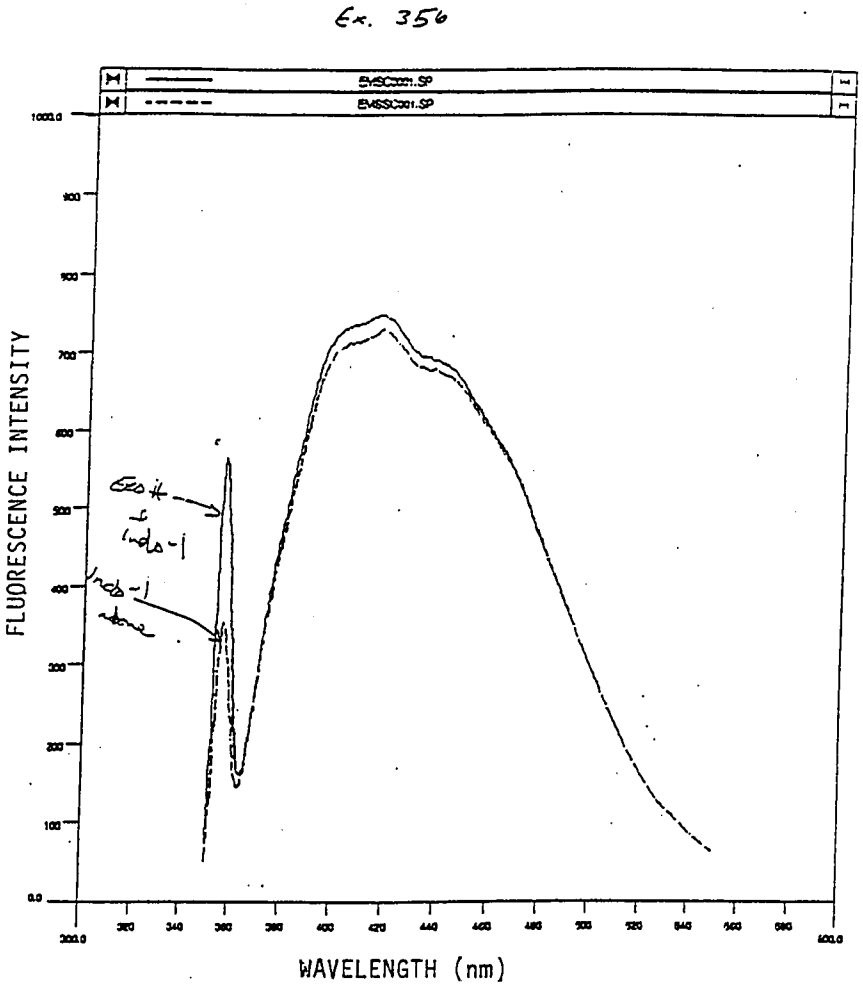


Figure 25. The effect of ethanol on indo-1 fluorescence.

The fluorescence intensity of free indo-1 was measured in calcium free buffer in the range of 350 to 550 nm in the absence (broken line) and presence (solid line) of 100 mM ethanol.

APPENDIX C: PERMEABILIZATION OF CELLS WITH SAPONIN

Stimulation of intracellular calcium release directly using IP_3 was attempted. Cell permeable IP_3 is not available commercially, therefore cells must be permeabilized to allow entry of this compound into cells. Preliminary experiments looking at trypan blue entry after permeabilizing cells for various times, at various temperatures, and with various concentrations of saponin, indicated that incubation with 0.005% saponin for 2.5 min at 37°C was optimal for cell permeabilization. In some instances, a response to IP_3 was seen, indicating the presence of IP_3 stores in these cells, however, this response was not consistent. This can be seen in Fig. 25, in which the top and bottom panels show the calcium response (Fig. 25 a,d) to 100 μM IP_3 following the same permeabilization protocol on the same day. In one chamber cells responded strongly to IP_3, whereas in the other chamber no response was evident. The fluorescence intensity decreased across time in both detectors, with a greater decrease in the fluorescence from detector 2, which measures fluorescence at the wavelength of calcium-bound indo-1 (Fig. 25 c,f). Similar problems occurred following permeabilization with streptolysin. This decrease could be due to photobleaching or loss of dye. Since photobleaching was not a problem in our other experiments, this decrease in fluorescence was most likely due to the loss of dye from the cells. Due to the inconsistent response to IP_3 caused by this leakage, the effects of ethanol on this response could not be studied. The recent synthesis of cell permeant forms of IP_3 (Li *et al.*, 1998) should facilitate this type of experiment and allow the effects of ethanol on direct stimulation of calcium release to be investigated.

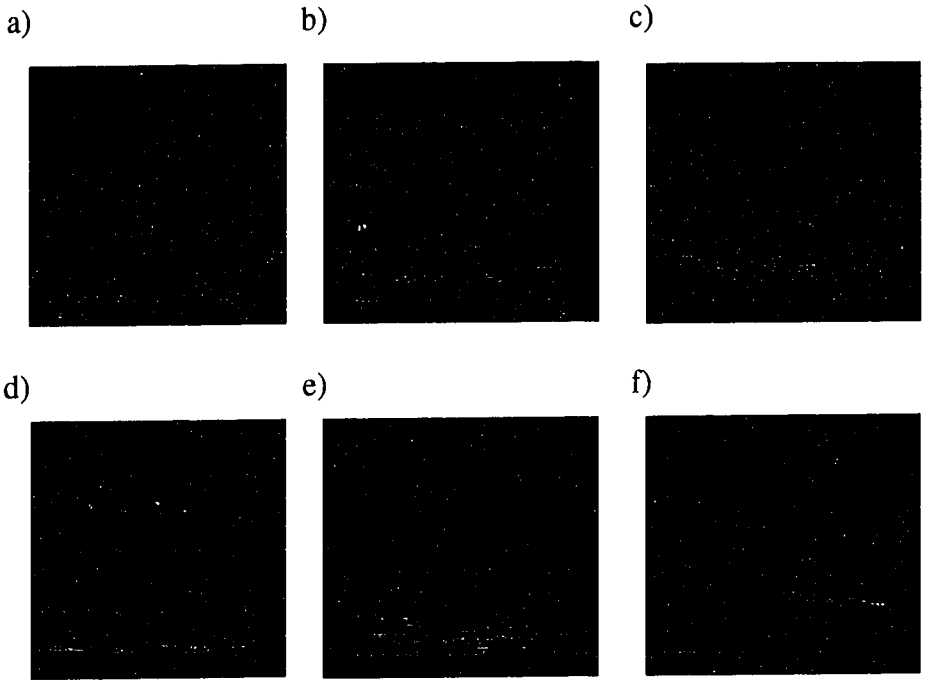


Figure 24. Response of human 132 1N1 astrocytoma to IP_3 following cell permeabilization.

Concentration across time (a,d) and fluorescence levels measured in detector 1 (unbound indo-1, 480 nm, b,e) and detector 2 (bound indo-1, 405 nm, c,f). Each of the 50 lines indicates the calcium concentration (μM) (a,d) or fluorescence intensity (b,c,e,f) in an individual cell, with the vertical line indicating the time of addition of 100 μM IP_3 following permeabilization with 0.005% saponin for 2.5 min at 37°C. Experiments were carried out in an intracellular buffer.

APPENDIX D: RECEPTOR-BINDING SATURATION CURVE

A saturation curve for ^{3}H-N-methylscopolamine binding was generated to determine the k_d and B_{max} for the binding of this compound in human 132 1N1 astrocytoma cells. The total and specific binding of 30 to 3000 pM ^{3}H-N-methylscopolamine was measured in the absence and presence of 10 μM atropine, respectively. A Scatchard analysis of this data was done and the Scatchard plot of the saturation curve is shown in Fig. 26. Based on this analysis, the k_d was 0.15 nM and the B_{max} was 97.03 fmol/mg protein.

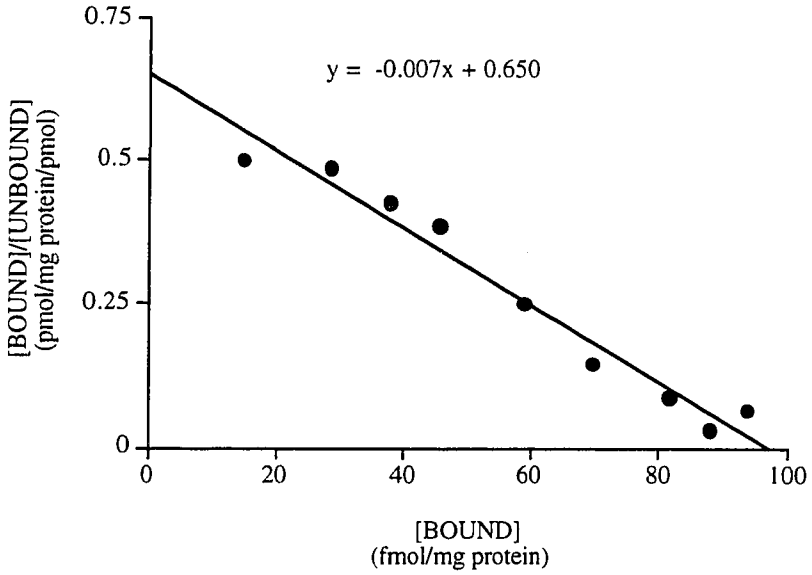


Figure 27. Scatchard plot of binding saturation curve.

Scatchard plot of ^{3}H-N-methylscopolamine binding in human 132 1N1 astrocytoma cells. Specific binding was determined as the difference in binding in the presence and absence of 10 μM atropine. r^2=0.965.

APPENDIX E: XESTOSPONGINS

The effect of xestospongins on primary rat astrocytes was investigated. Researchers at the University of California at Davis found these compounds to be specific, cell permeable blockers of IP_3-mediated intracellular calcium release. Our experiments with these compounds demonstrated that they block muscarinic-receptor-induced calcium release in primary rat cortical astrocytes. These data were published in Neuron (Gafni *et al.*, 1997), which is included as Appendix E.

Neuron, Vol. 19, 723–733, September, 1997. Copyright ©1997 by Cell Press

Xestospongins: Potent Membrane Permeable Blockers of the Inositol 1,4,5-Trisphosphate Receptor

Juliette Gafni,* Julia A. Munsch,† Tien H. Lam,* Michelle C. Catlin,‡ Lucio G. Costa,‡ Tadeusz F. Molinski,† and Isaac N. Pessah*§
*Department of Molecular Biosciences
School of Veterinary Medicine
and Graduate Program in Neurosciences
†Department of Chemistry
University of California
Davis, California 95616
‡Department of Environmental Health
University of Washington
Seattle, Washington 98195

Summary

Xestospongins (Xe's) A, C, D, araguspongine B, and demethylxestospongin B, a group of macrocyclic bis-1-oxaquinolizidines isolated from the Australian sponge, Xestospongia species, are shown to be potent blockers of IP_3-mediated Ca^{2+} release from endoplasmic reticulum vesicles of rabbit cerebellum. XeC blocks IP_3-induced Ca^{2+} release (IC_{50} = 358 nM) without interacting with the IP_3-binding site, suggesting a mechanism that is independent of the IP_3 effector site. Analysis of Pheochormocytoma cells and primary astrocytes loaded with Ca^{2+}-sensitive dye reveals that XeC selectively blocks bradykinin- and carbamylcholine-induced Ca^{2+} efflux from endoplasmic reticulum stores. Xe's represent a new class of potent, membrane permeable IP_3 receptor blockers exhibiting a high selectivity over ryanodine receptors. Xe's are a valuable tool for investigating the structure and function of IP_3 receptors and Ca^{2+} signaling in neuronal and nonneuronal cells.

Introduction

The phosphoinositide signaling cascade plays a prominent role in neuronal signaling. High levels of the inositol 1,4,5-trisphosphate receptor (IP_3R) are expressed in the cerebellum, hippocampus, cerebral cortex, corpus striatum, and olfactory tubercule (Verma et al., 1990). Stimulation of the IP_3R results in Ca^{2+} mobilization from intracellular stores, which in turn activates many cellular processes, including neuromodulation, synaptic plasticity, and sensory perception (Berridge, 1993). Stimulation of G protein–linked or tyrosine kinase–linked cell surface receptors leads not only to production of IP_3 and subsequent Ca^{2+} release from endoplasmic reticulum (ER) stores, but also to coordinated activation of intracellular enzymes such as protein kinase C, phosphatidylinositol 3-OH kinase, and GTPase-activating protein.

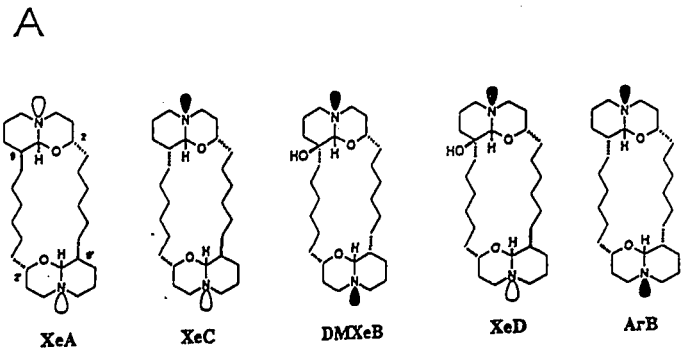
In order to define the importance of the IP_3R in neuronal processes, selective, membrane permeable blockers of the IP_3R are needed. Whereas selective antagonists of the large variety of cell surface receptors are known (Brann et al., 1993; Arrang, 1994; Pin and Duvoisin, 1995), chemical probes that selectively target the IP_3R are currently limited to heparin and IP_3R antibodies. The usefulness of heparin is limited due to its low affinity, lack of selectivity, membrane impermeability, and targeting of the IP_3-binding site (Ghosh et al., 1988; Kobayashi et al., 1988). Evidence that heparin inhibits the synthesis of IP_3 (Berridge, 1993), and stimulates ryanodine-sensitive Ca^{2+} release (Bezprozvanny et al., 1993) have further complicated interpretation of results aimed at understanding the role of the IP_3R in cellular processes. Three IP_3R antibodies have been shown to block IP_3-induced Ca^{2+} release. The 18A10 monoclonal antibody binds to the proposed Ca^{2+} channel region in the carboxy-terminus of the receptor protein (amino acid residues 2736–2747), inhibiting IP_3-induced Ca^{2+} release in cerebellar microsomes (Nakade et al., 1991). Two other IP_3R inhibitory serums have been developed, one targeting the 420 residues within the "coupling" domain (amino acid residues 1379–1798) and the other binding to the 95 residues near the C-terminus (amino acid residues 2604–2698) (Sullivan et al., 1995). Both of these antibodies block IP_3-stimulated Ca^{2+} release and nuclear envelope assembly in Xenopus egg extracts (Sullivan et al., 1995). The main disadvantage of IP_3R inhibitory antibodies is that they are membrane impermeable and thus require specialized techniques (i.e., injection, patching onto cells) and/or disruption of cellular integrity (i.e., patching or permeabilization of cells) to access the IP_3R.

Since a large number of biologically active compounds isolated from marine organisms have been shown to interact with cellular receptors (Crews et al., 1994), we screened 120 species of marine sponges for IP_3R inhibitory activity. We found one sponge, Xestospongia species, to contain five potent blockers of IP_3-mediated Ca^{2+} release (IC_{50}s ranging from 358 nM to 5.9 μM). Structure elucidation revealed the active principles to be the bis-1-oxaquinolizidines xestospongin A (XeA), xestospongin C (XeC), xestospongin D (XeD), araguspongine B (ArB), and demethylxestospongin B (DMXeB). Interestingly, XeA, XeC, and XeD were isolated by Nakagawa et al. (1984) and recognized for their vasodilative activity.

In the present study, we demonstrate that XeA, XeC, XeD, ArB, and DMXeB are potent blockers of IP_3-induced Ca^{2+} release from isolated cerebellar microsomes, and display a high selectivity over the skeletal isoform of the ryanodine receptor (type 1; Ry_1R). The most potent of these blockers, XeC, is a membrane permeable blocker of IP_3-mediated Ca^{2+} release in intact cells. A simple model is presented that relates structural aspects of the xestospongin/araguspongine family of 1-oxaquinolizidine alkaloids to their potency at the IP_3R. XeC provides an ideal pharmacological tool for investigating the structure of the IP_3R, as well as IP_3-mediated Ca^{2+} signaling in neuronal and nonneuronal cells.

§To whom correspondence should be addressed.

Neuron
724



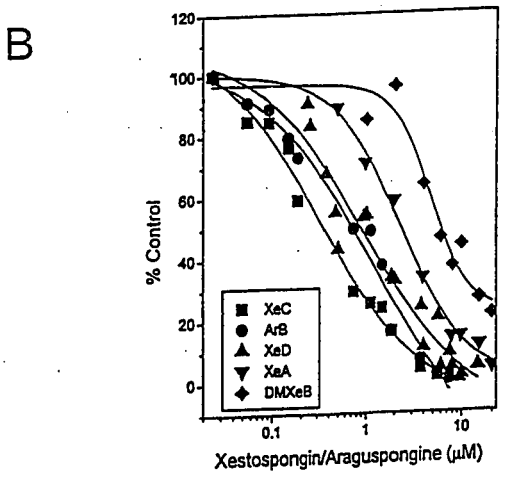


Figure 1. Bis-1-Oxaquinolizidines Isolated from Xestospongia Species Are Potent IP_3R Blockers

(A) Xe and araguspongine structures were extracted and purified from the Australian sponge, Xestospongia species, and tested for their ability to block IP_3-induced Ca^{2+} release from cerebellar microsomes.

(B) Inhibition of IP_3-induced Ca^{2+} release from actively loaded cerebellar microsomes was used to determine the potency of the Xs/araguspongine congeners tested. The IC_{50} values for the five congeners ranged from 358 nM to 5.9 μM (see text for details). Data from each compound are fit to a single site model, and curves for XeC, ArB, XeD, and XeA represent the average of two experiments performed on two different cerebellar microsomal preparations. The DMXeB curve is an average of three experiments. The mean rate (±SD) of Ca^{2+} release for control was 7.0 ± 2.0 nmol Ca^{2+} s^{-1} mg^{-1} with 5 μM IP_3.

Results and Discussion

Subcellular Calcium Transport and Binding Studies

IP_3-induced Ca^{2+} transport across actively loaded rabbit cerebellar microsomes was initially used to screen 120 crude MeOH extracts from 120 species of sponges for IP_3R inhibitory activity. Five potent IP_3R inhibitors were then isolated from Xestospongia species, the only sponge containing potent IP_3R inhibitors, using the mechanism-based assay (Figure 1A). The specificity of the assay for detecting IP_3-mediated Ca^{2+} changes was

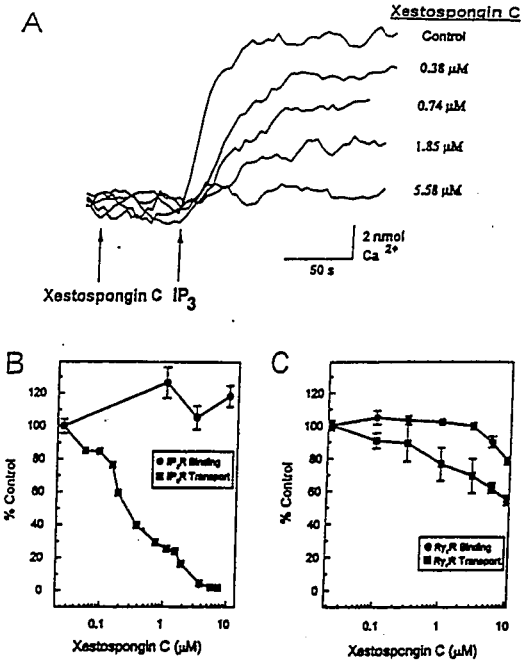


Figure 2. XeC Blocks IP_3-Induced Ca^{2+} Release by a Mechanism Independent of the IP_3-Binding Site

(A) Raw traces showing XeC blocks IP_3 (5 μM)-induced Ca^{2+} release from cerebellar microsomes in a dose-dependent manner.

(B) The dose–response curve for XeC is fit to a multisite model to illustrate the multiphasic nature of inhibition, whereas XeC (1–10 μM) does not affect the ability of [^{3}H]IP_3 (1 nM) to bind to cerebellar microsomes (200 μg protein).

(C) XeC is >30-fold less potent toward blocking [^{3}H]ryanodine binding and caffeine-induced Ca^{2+} release in skeletal SR. XeC (0.1–10 μM) blocked [^{3}H]ryanodine (1 nM) binding and caffeine (20 mM)-induced Ca^{2+} release to a much lesser extent in Ca^{2+}-loaded skeletal vesicles (50 μg protein) enriched in Ry_1R. The control rate (±SD) of caffeine-induced Ca^{2+} release averaged 28.4 ± 6.0 nmol Ca^{2+} s^{-1} mg^{-1}.

initially tested. IP_3-induced Ca^{2+} release was stereoselective, since Ca^{2+} efflux from cerebellar microsomes occurred only following addition of the D- (not L-) IP_3 enantiomer. Heparin (≥20 μM), a known IP_3R inhibitor, also blocked IP_3-induced Ca^{2+} release from cerebellar microsomes, further verifying that IP_3-mediated Ca^{2+} release was being selectively measured. In addition, saturating concentrations (7.5–20 μM) of XeA, XeC, XeD, ArB, or DMXeB did not alter the absorbance of antipyrylazo III or the Ca^{2+} calibration of the dye, verifying that Xe's did not interfere with the ability of antipyrylazo III to detect changes in extramicrosomal Ca^{2+}.

Inhibition curves for XeA, XeC, XeD, ArB, and DMXeB were obtained by measuring Ca^{2+} transport in cerebellar microsomes. For each compound, duplicate experiments using two different cerebellar microsomal preparations were performed to construct the inhibition curves shown in Figure 1B. All Xe's blocked IP_3-mediated Ca^{2+} release in a dose-dependent manner. XeC, which contains one *cis*-fused and one *trans*-fused oxaquinolizidine, proved to be the most potent IP_3R inhibitor with an IC_{50} of 358 nM. The *cis/trans* combination exhibited superior inhibition of IP_3-induced Ca^{2+} release, when compared to the *trans/trans* system in XeA, which has a nearly 10-fold larger IC_{50} of 2535 nM. It is not clear whether the *cis/trans* system is truly a more potent inhibitor than the *cis/cis* system in ArB, which has an IC_{50} of 648 nM, since ArB was not purified to homogeneity (~75 mol % purity). The addition of a hydroxyl group to C9 of XeC (XeD) diminished the potency of the *cis/trans* system and elevated the IC_{50} to 844 nM. Substituting the *cis*-fused oxaquinolizidine of XeD for a *trans*-fused oxaquinolizidine (DMXeB) reduced inhibition of IP_3-induced Ca^{2+} release by nearly an order of magnitude, raising the IC_{50} to 5865 nM.

While the more potent Xe's (XeC, ArB, XeD) tended to produce a multiphasic inhibition of IP_3-induced Ca^{2+} release from cerebellar microsomes, equivalent concentrations of XeC had no effect on the specific binding of [^{3}H]IP_3 to the same membrane vesicles (Figures 2A and 2B). For example, 10 μM XeC did not produce a decrease in specific [^{3}H]IP_3 binding, even though 7.5 μM of XeC completely blocked IP_3-mediated Ca^{2+} release, indicating a noncompetitive mechanism with respect to the IP_3-binding site. The apparent multiphasic nature of Xe inhibition curves (Figure 2B [XeC]) could be explained by the existence of multiple IP_3R isoforms in the microsomal preparations from whole cerebella, which may have different affinities for Xe's. At least three isoforms of the IP_3R have been identified and cloned (Furuichi et al., 1989; Mignery et al., 1990; Südhof et al., 1991; Ross et al., 1992; Maranto, 1994; Yamada et al., 1994; Hamick et al., 1995), and there is evidence that the cerebellum contains more than one of these isoforms (Nakanishi

et al., 1991; Ross et al., 1992). [^{3}H]Ryanodine binding studies and caffeine-mediated Ca^{2+} transport assays using rabbit skeletal sarcoplasmic reticulum (SR) preparations showed that XeC also dose dependently interacts with the Ry_1R (Figure 2C). XeC (10 μM) decreased [^{3}H]ryanodine binding by 22% and caffeine-induced Ca^{2+} release by 46%, indicating that XeC can interact with the Ry_1R, but in a significantly less potent manner. The IC_{20} for blocking caffeine-induced Ca^{2+} release with XeC was (greater than 30-fold its IC_{20} for blocking IP_3-mediated Ca^{2+} release from the IP_3R. Differences in Xe potency toward IP_3R and Ry_1R blockade may also reflect variations in amino acid sequences, especially around the putative pore-forming region (Furuichi et al., 1989; Mignery et al., 1989, 1990; Takeshima et al., 1989).

The mechanism by which Xe's block IP_3-induced Ca^{2+} release could be the result of either: (1) block of the Ca^{2+} channel pore, or (2) an allosteric mechanism that uncouples IP_3 binding from Ca^{2+} release. Although the present study does not discriminate between the two possible mechanisms, the chemical structure of Xe's, a lipophilic elongated core with two partially charged N groups at either end, provide an ideal lipophilic/hydrophilic moiety to fit into the IP_3R channel pore. Tinker and Williams (1995) used a series of monovalent and divalent trimethylammonium derivatives, which bear structural resemblance to Xe's, to define the length of the pore of Ry_2R (cardiac isoform) reconstituted in bilayer lipid membranes. Based on the relative potency for pore blockade, they concluded a pore distance of 10.4 Å. Interestingly, their estimate is consistent with the molecular distance between the partially charged tertiary nitrogens of XeC (11.6 Å). Despite the very similar molecular structures of Xe's, some useful empirical correlations can be drawn regarding how structure relates to potency toward inhibition of IP_3-induced Ca^{2+} release. Two important considerations in this respect are net charge and molecular dimensions. The free bases of Xe are relatively lipophilic molecules and readily soluble in chloroform or methanol. The bridgehead nitrogen of the oxaquinolizidine ring system is moderately basic having a pKa of ~11 for the protonated form. At pH 7.0, the average charge on the bis-oxaquinolizidine molecule is between +1.5 and +2.0, distributed between the two tertiary nitrogens. The molecular structures of XeA, XeC, and ArB differ only with respect to stereochemistry at the oxaquinolizidine ring junction and configurations at C2,2′ and C9,9′, the points of attachment of the methylene side chains. Therefore, XeA, XeC, and ArB are diastereomers. XeA has the more stable *trans* ring junction in both oxaquinolizidine rings and diequatorial substituents at C2 and C9, which are disposed *trans* to each other. By contrast, XeC differs from XeA in possessing the energetically less favorable *cis* ring junction stereochemistry in one of the oxaquinolizidine rings with diequatorial substituents at C2 and C9 that are *cis* to each other. Clearly the *cis*, *trans* orientation of XeC is favored for the IP_3R since it exhibits a 7-fold higher potency compared to XeA. The 2-fold rotationally (C_2) symmetric ArB (Kitagawa et al., 1989; Hoye et al., 1994, 1995) has *cis* ring fusions and *cis* C2,9 substituents (axial at C9 to the piperidine rings) in both heterobicycles. Unfortunately, interpretations about how the structure of ArB relates to IP_3R channel activity are thus far precluded since ArB could not be purified to homogeneity, and the structures of impurities remain to be elucidated. XeD and DMXeB are 9-hydroxy derivatives of XeC and ArB, respectively. Although addition of an axial OH group at C9 in XeD does not increase the molecular cross section (see below), the added polarity and/or introduction of an H-bond donor decreases potency ~2.4-fold. The 16-fold decrease in potency seen with DMXeB is likely to be mainly due to the *cis* ring fusions and *cis* C2,9 substituents in both heterobicycles, with a smaller contribution from the OH group at C9. In this respect, the potent activity seen with the ArB fraction may be attributed to a yet to be identified Xe. XeC has been shown by X-ray crystallography to have a long narrow rod-like shape, and *this feature is preserved in the calculated MM2* structure. The calculated van der Waals profile was defined by a rectangle of width of 8.22 Å, height of 5.78 Å, and molecular cross section of 47.6 Å^2. The molecular cross section (Å^2) is defined here as the area of a rectangular whose sides are the minimum van der Waals height and width of the narrowest projection or profile of the MM2 minimized structure. In this respect, the OH moiety of XeD does not contribute to the molecular cross section (47.6 Å^2) and only produces a small (2.4-fold) decrease in receptor potency. A major structural consequence of stereochemical differences between the alkaloids related to XeC is a significant change in molecular cross section. Inverting the configurations at both the bridgehead nitrogen and C9 of the flexible *cis*, *trans* C2,9 diequatorial (bent) configuration of XeC to the flattened, more rigid *trans*, *trans* C2,9 diequatorial configuration of XeA widens the molecule in order to accommodate bowing of the methylene chains between the two oxaquinolizidine moieties and reduces potency more significantly (7-fold). The van der Waals profile changes accordingly to a width of 9.38 Å, a height of 5.34 Å, and an overall larger molecular cross section (49.8 Å^2). Thus, the molecular dimensions of Xe's appear to be more important in defining potency for IP_3R channel blockade than the presence of an OH at C9, although the presence of the latter disrupts some yet undefined aspect of the interaction with IP_3 receptors. Despite the importance of the cross-sectional area in determining the structure–activity relationship, there are clearly other factors contributing to this relationship. For example, the >16-fold decrease in potency of DMXeB (relative to XeC) could be attributed to the fact that the two *cis*-fused oxaquinolizide rings of DMXeB are more conformationally flexible than the mixed junctions of XeC. Further experiments are needed to determine the XeC binding site and refine the current model.

Cytosolic Calcium Measurements in Intact PC12 Cells and Astrocytes

Pheochromocytoma (PC12) cells have been shown to respond to bradykinin with increased production of IP_3 and transient elevations in cytosolic Ca^{2+} (Fasolato et al., 1991; Grohovaz et al., 1992). Xe's were shown to be effective IP_3R blockers in fura 2–loaded PC12 cells by measuring cytosolic changes in Ca^{2+} following bradykinin addition. Measurements performed in Ca^{2+} replete

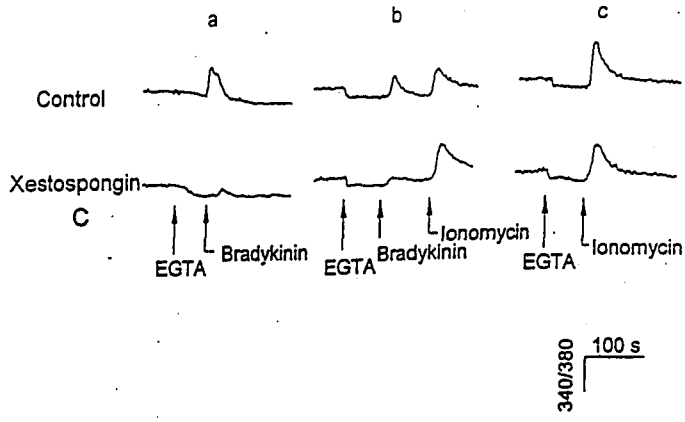


Figure 3. XeC Blocks Bradykinin Responsiveness of PC12 Cells in Ca^{2+}-Depleted Media without Altering the Levels of Ca^{2+} Remaining in the Stores

Fluorometric measurements in PC12 cells using fura-2 AM (5 μg/ml) and Ca^{2+}-depleted media (120 nM and 40 nM Ca^{2+}) show that XeC (20 μM) blocks the peak response to bradykinin (300 nM)-induced Ca^{2+} release. In the presence of 40 nM intracellular Ca^{2+}, addition of ionomycin (5 μM) in place of bradykinin shows that Ca^{2+} stores are equal at the time of bradykinin addition. Each trace is a typical response from triplicate readings. Traces are reported as arbitrary fluorescence units (340 nm/380 nm). The vertical bar represents 1.0 arbitrary fluorescence ratio units. Column a, $[Ca^{2+}]_i$ = 120 nM; column b and c, $[Ca^{2+}]_i$ = 40 nM.

medium (2 mM) revealed that a 10 min application of 20 μM XeC reduced the peak of the bradykinin (300 nM)-induced Ca^{2+} transient by 66 ± 5% of control (±SD; n = 4 determinations). Studies using Ca^{2+}-depleted media (120 and 40 nM free Ca^{2+}) indicated that addition of 20 μM XeC for 10 min blocked bradykinin-induced Ca^{2+} release in PC12 cells to a similar extent as PC12 cells bathed in Ca^{2+} replete buffer (Figure 3). Addition of ionomycin (5 μM) in place of bradykinin revealed that treatment of PC12 cells with 20 μM XeC for 10 min did not significantly affect the level of Ca^{2+} in the stores (Figure 3). Fluorometric measurements of PC12 cells without bovine serum albumin (BSA) and/or a lengthened incubation time of 30 min revealed that XeC more effectively targeted the IP_3R in the absence of BSA and with extended incubation times (data not shown). XeC appears to be maximally effective at blocking bradykinin-induced Ca^{2+} release at bath concentrations of XeC as low as 10 μM, whereas 5 μM XeC results in a bradykinin-induced Ca^{2+} mobilization, which is comparable to control levels (Figure 4). Reintroduction of Ca^{2+} to the extracellular medium ($[Ca^{2+}]_o$ = 2 mM) following bradykinin stimulation showed that capacitative Ca^{2+} influx is reduced proportionally in the presence of 10 and 20 μM XeC (Figure 4). Interestingly, subsequent thapsigargin (500 nM) addition revealed that the Ca^{2+} leak from the ER is significantly reduced in the presence of 10 or 20 μM XeC (Figure 4).

Experiments aimed at determining whether XeC could discriminate between IP_3- and ryanodine-sensitive stores were conducted using ratiofluorescence imaging of fura 2–loaded PC12 cells, a neuronal cell model, which has both ryanodine- and IP_3-sensitive stores (Zacchetti et al., 1991). Addition of caffeine (30 mM) to the extracellular medium resulted in a transient rise in intracellular Ca^{2+} (Figure 5A). Pretreatment of cells with 20 μM XeC for 10 min prior to addition of caffeine did not significantly alter the magnitude of the response to the ryanodine receptor (RyR) ligand (Figure 5B; Table 1). PC12 cells also vigorously responded to 50 μM ryanodine with a sustained rise in cytosolic Ca^{2+} (Figure 5C). Subsequent addition of bradykinin (100 nM) induced an additional but transient rise in cytosolic Ca^{2+}. Addition of XeC (20 μM) 10 min prior to ryanodine did not quantitatively alter the peak response from the ryanodine-sensitive store (peak response [±SD] = 0.16 ± 0.03 and 0.18 ± 0.02 ratio units for control and XeC treatment, respectively; Figure 5D; Table 1). Subsequent addition of bradykinin produced a negligible response from the IP_3-sensitive store. In PC12 cells, ryanodine (50 μM) induced ER Ca^{2+} release from the same store affected by caffeine, as demonstrated by the sequential addition of these compounds (Figure 5E). Addition of caffeine followed by ryanodine also demonstrated a common ryanodine/caffeine-sensitive store (data not shown). Taken together, the results in PC12 cells demonstrate that XeC can discriminate between ryanodine- and IP_3-sensitive Ca^{2+} efflux pathways in intact PC12 cells. PC12 cells acutely treated with Xe's displayed no overt signs of cytotoxicity. Application of 20 μM XeC for 30 min did not induce trypan blue dye leakage into the cytosol of PC12 cells (zero cells stained/two dishes [35 mm^2]), indicating a viable cell population.

Xe's were also shown to block muscarinic-mediated

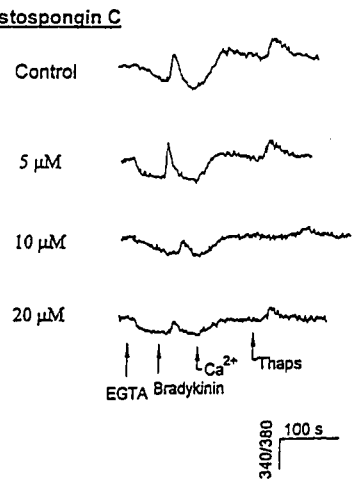


Figure 4. XeC Dose Dependently Blocks Bradykinin-Induced Ca^{2+} Release in PC12 Cells

Fluorometric measurements using fura-2 AM (5 μg/ml) and Ca^{2+}-depleted media (120 nM) show that 20 and 10 μM XeC maximally block the peak response to bradykinin (300 nM)-induced Ca^{2+} release, while 5 μM XeC has no significant effect. Following the reintroduction of Ca^{2+} ($[Ca^{2+}]_o$ = 2 mM), thapsigargin (500 nM) was added to quantitate the ER Ca^{2+} leak. Each trace is a typical response from duplicate experiments. The vertical bar represents 0.5 arbitrary fluorescence ratio units.

IP_3-induced Ca^{2+} release in primary astrocytes, following addition of carbachol. Using confocal fluorescent microscopy, XeC (20 μM) was shown to block IP_3-induced Ca^{2+} release and oscillations in primary astrocytes (Figures 6A and 6B). In addition, XeA was shown to block IP_3-induced Ca^{2+} release in a concentration-dependent manner, with 20 μM XeA completely blocking Ca^{2+} release and oscillations in most cells (Figures 6C–6F). Treatment of astrocyte cultures with 20 μM XeC for 30 min did not cause leakage of lactate dehydrogenase (LDH) into the media (untreated = 3.34 U/l; treated = 2.55 U/l).

Data provided from PC12 cells and primary astrocytes demonstrate that Xe's block phosphoinositide signaling regardless of the agonist used (bradykinin and carbamylcholine, respectively), revealing the general usefulness of these compounds. Further characterization of XeC's effects in PC12 cells provides insight into the Ca^{2+} dynamics of intact cells. Application of ionomycin in place of bradykinin shows that blockade of the IP_3R with XeC does not alter Ca^{2+} store levels in PC12 cells (Figure 3). Reintroduction of Ca^{2+} following bradykinin addition shows that blockade of IP_3-mediated Ca^{2+} release with XeC causes a decrease in Ca^{2+} entry that is proportional to the reduction in Ca^{2+} release from intracellular stores (Figure 4), which supports current theory on capacitative Ca^{2+} entry. A potentially important new finding with XeC is the substantial reduction in the ER Ca^{2+} leak unmasked by thapsigargin, a smooth ER Ca^{2+} pump inhibitor (Figure 4). The XeC-mediated reduction in the ER Ca^{2+} leak may be attributed to blockade of the conformationally distinct leak states of the Ry and/or IP_3 receptor. In this respect, ryanodine-sensitive and insensitive ("leak") Ca^{2+} efflux pathways in skeletal SR (Pessah et al., 1997) and ER Ca^{2+} "leakage" via the IP_3R in brain (Cameron et al., 1995) have been demonstrated. Considering the structural and functional similarity of Ry and IP_3 receptors, the present results support a dual role (Ca^{2+} channel and Ca^{2+} leak) for this class of ER/SR proteins (Cameron et al., 1995; Marks, 1996; Pessah et al., 1997). Xe's not only represent an optimal probe to study IP_3-mediated signaling processes and Ca^{2+} dynamics in intact cells, but provide a unique pharmacological tool to further investigate the structure and functional properties of isolated IP_3-gated channels.

In conclusion, the present paper demonstrates that Xe's represent a new class of blockers of IP_3-induced Ca^{2+} release from isolated ER membrane vesicles having nanomolar to micromolar affinity. The membrane permeant properties of Xe's also make them very useful tools for studying IP_3-mediated signaling in preparations of intact cells. Their ability to discriminate between ryanodine- and IP_3-sensitive stores in intact cells provides a distinct advantage to currently available inhibitors. Although the lipophilic elongated hydrocarbon core undoubtedly contributes to the cell permeant properties of Xe's, it may also promote appreciable partitioning of Xe's into cellular membrane lipids. The latter could account for the need to use 10–20 μM in the bath to attain sufficient concentrations of Xe at the ER surface. However, the physicochemical properties of XeC, which requires higher concentrations in cell culture, do not limit the selectivity of XeC for IP_3R over RyR in PC12 cells (Figure 5), nor do they induce acute cytotoxicity. The actual concentrations of XeC reaching the ER surface are probably significantly lower than those applied to the extracellular medium.

Xe's can be used to further our understanding of the large variety of IP_3-mediated signaling pathways currently identified in neuronal and nonneuronal cells. In this respect, previous research has already shown that Xe's disrupt IP_3R-mediated cellular processes. Three Xe's (XeA, XeC, and XeD), originally isolated by Nakagawa et al. (1984), were identified as vasodilative compounds since they increased blood flow in the coronary, vertebral, and femoral arteries of anesthetized dogs (Nakagawa et al., 1984; Endo et al., 1986). Kitagawa et al. (1989) subsequently reported the vasodilative properties of XeA when the compound was infused into an isolated mesenteric artery preparation from the Sprague-Dawley rat, corroborating the findings of Nakagawa et al. XeD and DMXeB have also been shown to display growth-inhibitory activity in tumor cell lines and/or Micrococcus luteus (Quirion et al., 1992; Pettit et al., 1996). XeD and DMXeB were found to inhibit murine leukemia (L1210, ED_{50} = 0.2, 0.8 μg/ml, respectively) and human epidermoid carcinoma (KB, ED_{50} = 2.0, 2.5 μg/ml, respectively) cell growth activity (Quirion et al., 1992). XeD has also shown growth-inhibitory activity

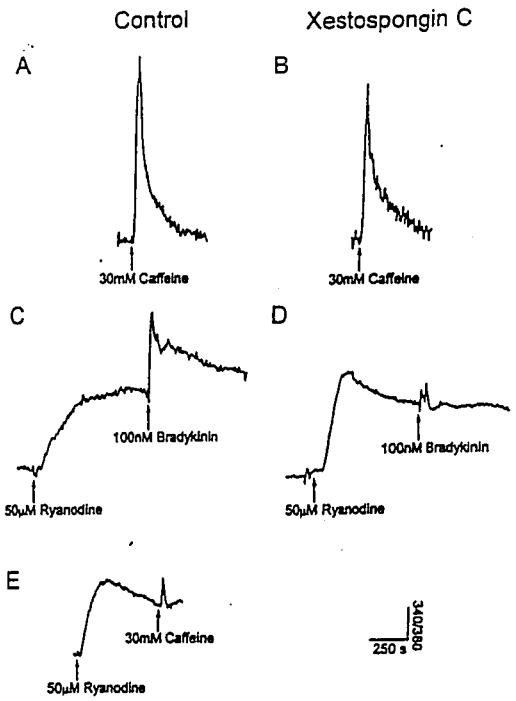


Figure 5. XeC Discriminates between Ryanodine- and IP_3-Sensitive Stores in PC12 Cells

Fluorometric measurements using fura-2 AM (5 μg/ml) and Ca^{2+} (2 mM)-replete media show that PC12 cells possess ryanodine- and IP_3-sensitive Ca^{2+} stores. Traces under control were treated with 4 μl MeOH 10 min prior to caffeine (A) or ryanodine ([C] and [E]) addition. Traces under XeC were treated with 20 μM XeC (in 4 μl MeOH) 10 min prior to addition of caffeine (B) or ryanodine (D). The vertical bar represents 0.05 arbitrary fluorescence ratio units. Table 1 summarizes the peak responses to RyR ligands in the presence and absence of XeC.

in a variety of leukemia (CCRF-CEM, HL-60TB, K-562, MOLT-4, and SR) and breast tumor (MCF7, HS 578T, MDA-MB-435, and MDA-N) cell lines (averaged GI_{50} = 3.62 and 4.53 μM, respectively) and in the bacterium Micrococcus luteus (12.5–25 μg/6 mm disk) (Pettit et al., 1996). The vasodilative and growth-inhibitory activity of Xe's are consistent with their newly recognized IP_3R channel-blocking activity, since vasoconstriction and cell division require IP_3-mediated Ca^{2+} release (Berridge, 1993; Briner et al., 1993; Dauphin et al., 1994; Bretschneider et al., 1995a, 1995b; Sullivan et al., 1995).

Table 1. XeC Inhibits IP_3-Mediated Ca^{2+} Release without Significantly Influencing Ryanodine-Sensitive Stores in PC12 Cells

Treatment	Peak Response[a] (340/380 ± SD)
Bradykinin	
Control (n = 10)	0.12 ± 0.03
20 μM XeC (n = 15)	0.04 ± 0.01
Caffeine	
Control (n = 18)	0.40 ± 0.16
20 μM XeC (n = 20)	0.47 ± 0.28
Ryanodine	
Control (n = 10)	0.16 ± 0.03
20 μM XeC (n = 15)	0.18 ± 0.02

[a]Peak response is the average maximal change in arbitrary fluorescence units (340 nm–380 nm) to addition of 300 nM bradykinin following or 50 μM ryanodine treatment, 30 mM caffeine. The data represent the mean ± SD of the number of determinations indicated in parentheses in column 1. Representative traces are shown in Figure 5.

Experimental Procedures

Materials

D-*myo*-inositol 1,4,5-trisphosphate and L-*myo*-inositol 1,4,5-trisphosphate were obtained from LC Laboratories (Woburn, MA). Sodium heparin (MW 6000) was purchased from Sigma (St. Louis). [^{3}H]D-*myo*-inositol 1,4,5-trisphosphate (specific activity, 21 Ci/mmol) and [^{3}H]ryanodine (specific activity, 81.5 Ci/mmol; purity, >99%) were obtained from New England Nuclear (Wilmington, DE). High-purity ryanodine (>99%; used in radioligand receptor binding assays) and ryanodine mixture (1:3 ryanodine–dehydroryanodine; used in whole cell experiments) were purchased from Calbiochem (San Diego, CA). Bradykinin was obtained from Peptides International (Louisville, KY) and carbachol from Sigma (St. Louis). All other chemicals used were of the highest grade possible.

Collection of Xe's

Two samples (A and B) of the marine sponge Xestospongia species (Phylum Porifera) were collected by hand using SCUBA at two sites

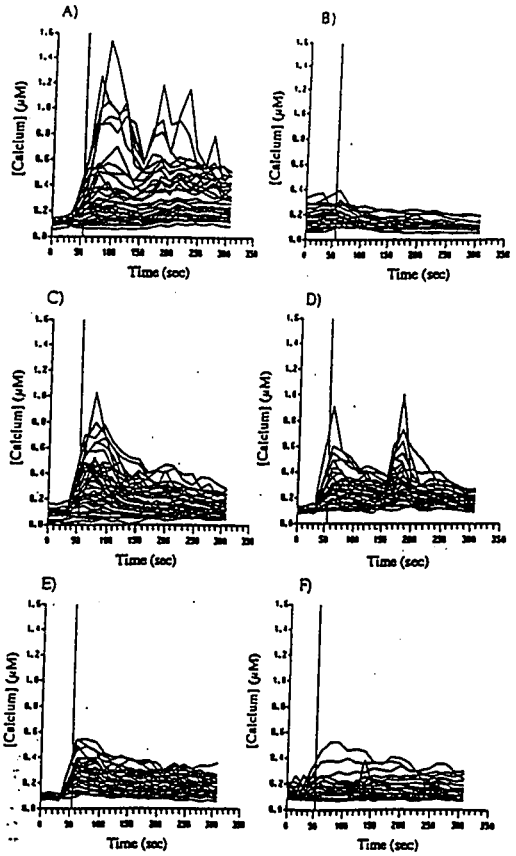


Figure 6. XeC and XaA Effectively Block IP_3-Mediated Ca^{2+} Release and Oscillations in Primary Astrocytes

Each line indicates the Ca^{2+} concentration over time in a single cell. Cells were loaded with Indo-1 AM for 30 min prior to experimentation, and fluorescence was monitored using a scanning confocal fluorescent microscope, as described in Experimental Procedures. Carbachol (1 mM) was added 55 s (indicator line) after a 30 min incubation with control buffer, XeC, or XaA.

(A) Paired control (XeC).

(B) XeC (20 μM).

(C) Paired control (XaA).

(D) XaA, 1 μM.

(E) XaA, 10 μM.

(F) XaA, 20 μM.

from Exmouth Gulf, Western Australia, in 1993. Whole animals were immediately frozen and stored at −20°C for 2 years. The sponge was identified by Mary Kay Harper, Scripps Institution of Oceanography (La Jolla, CA). XaA, XeC, and ArB were isolated from Sample A (collected at a depth of −2 m), and XeD and DMXeB were isolated from sample B (collected at −10 m).

Isolation and Characterization of Xe's

Each sponge sample was processed in a similar manner, and purification of active fractions was assessed for their ability to inhibit IP_3-induced Ca^{2+} release from vesicles as described below. Lyophilized sponge tissue was exhaustively extracted with methanol at 25°C (three times), and the combined methanol extract was concentrated to ~500 ml. The methanol extract was adjusted to ~10% v/v H_2O and extracted with *n*-hexane. The aqueous methanol layer was adjusted to 20% H_2O and extracted with chloroform. Finally, the methanol was removed from the aqueous fraction under reduced pressure, and the residue was extracted with *n*-butanol. IP_3R inhibitory activity was located in the chloroform soluble fraction, which was further purified by column chromatography over silica gel (230–400 mesh, elution with a stepped gradient of 100:0 chloroform–methanol to 0:100 chloroform–methanol). Final purification of the active fractions was achieved by high-pressure liquid chromatography using two different conditions and detection by differential refractometry. The active fraction from sample A was separated on a normal phase silica high-pressure liquid chromotography column (Rainin Dynamax SI, 25 × 300 mm, 1:4 hexane: 2-propanol, containing 0.5% triethylamine, 3 ml/min) to give XaA (0.02% of dry weight) (Nakagawa et al., 1984), XeC (0.03% of dry weight) (Nakagawa et al., 1984), and ArB (0.017% of dry weight) (Otagawa et al., 1989; Hoye et al., 1994, 1995). The active fraction from B was purified on an amino-bonded column (Rainin Dynamax NH_2, 25 × 300 mm, 98:2 hexane: 2-propanol containing 0.05% triethylamine, 3 ml/min) to provide pure XeD (0.0069% of dry weight) (Nakagawa et al., 1984) and DMXeB (0.0084% of dry weight) (Quirion et al., 1992). The structures were identified by comparison of ^1H and ^{13}C NMR data of purified compounds with those of literature values (Nakagawa et al., 1984; Kitagawa et al., 1989; Quirion et al., 1992; Hoye et al., 1994, 1995).

In order to examine how the three-dimensional molecular structure of Xe/araguspongine alkaloids might relate to their ability to

block the IP_3-activated pore, solution structures of XeA, XeC, and ArB were calculated using force field molecular mechanics (MM2) (Chem3D Plus, Cambridge Scientific Computing, Cambridge, MA). The atomic coordinates X-ray crystal structure of XeC (Cambridge Crystallographic Data Center, Cambridge, England) were used as the starting point for MM2 minimization to obtain quantitative structural information for XeC. The MM2 structure of XeA was obtained by force field minimization of a hybrid set of atomic coordinates for XeA. The hybrid data set, in turn, was generated by bond bisection of the dimeric XeC crystal structure, discarding the *cis*-ring heterobicycle and religating two copies of the *trans*-monomer. Both structures preserve the chair–chair conformations of the oxaquinolizidine ring. Distance-dependent dielectric factors were not used in the calculations, and the minimized structures do not take into account possible water of solvation.

Membrane Preparations

Microsomes enriched with the IP_3R were isolated from the cerebellum of 2.5–3 kg male New Zealand white rabbits. Tissue was homogenized with a Potter-Elvehjem homogenizer in 10× (w/v) ice-cold buffer containing 5 mM HEPES (pH 7.4), 320 mM sucrose, 250 μM phenylmethylsulfonyl fluoride, and 5 μg/ml leupeptin. Microsomes were subsequently isolated by differential centrifugation (8,000–100,000 × g pellet). The final microsomal pellet was resuspended with a Dounce homogenizer in buffer lacking protease inhibitors at a protein concentration of 5–10 mg/ml. Microsomes were aliquoted into vials, quickly frozen in liquid nitrogen, and stored at −80°C.

Membrane vesicles enriched in Ry_1R were isolated from the fast-twitch (white) skeletal muscle of the hind limbs and back of 3–4 kg male New Zealand white rabbits. Freshly ground muscle was homogenized in a Waring blender with 4 v of ice-cold homogenization buffer composed of 5 mM imidazole–HCl (pH 7.4), 0.3 M sucrose, 10 μg/ml leupeptin, and 100 μM phenylmethylsulfonyl fluoride. The junctional SR fraction was purified from a discontinuous sucrose gradient as previously described by Saito et al. (1984). The junctional SR fraction was collected from the 38%–45% sucrose interface, pelleted, and then resuspended in ice-cold homogenization buffer at a protein concentration of 3–5 mg/ml. Membranes were aliquoted into vials, quickly frozen in liquid nitrogen, and stored at −80°C. Protein concentrations were determined using the Lowry method (Lowry et al., 1951), with BSA as the standard.

Cell Culture

PC12 cells were obtained from American Type Culture Collection (Rockville, MD) and cultured at 37°C, 5% CO_2 in RPMI 1640 media supplemented with 2.05 mM L-glutamine, 10% heat-inactivated horse serum (at 56°C for 30 min), 5% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin on 75 cm^2 Primaria tissue culture flasks (Falcon 3824, Becton Dickinson, Lincoln Park, NJ). Media was changed every 2 days, and cells were passaged bi-weekly. PC12 cells were plated on laminin-coated glass rectangular (9 × 22 cm) or round (d = 2.5 cm) coverslips at a density of 1 × 10^4 cells/cm^2. Cells were used for experiments 2–3 days after plating.

Primary rat cortical astrocyte cultures were prepared by a modification of the method of McCarthy and De Vellis (1980), as described previously (Gutzzetti et al., 1996). Briefly, the cortices from day 21 fetuses (B&K Universal, Kent, WA) were minced, trypsinized, and washed three times by centrifugation with Dulbecco's Modified Eagle Medium supplemented with 10% fetal bovine serum. The tissue was triturated, filtered, and plated in 75 cm^2 flasks, previously coated with poly-*d*-lysine, at 1.5 × 10^4 cells/cm^2. Within 24 hr of plating, the flasks were shaken, and fresh media was added. Flasks were maintained at 37°C with 5% CO_2 for 9 days and fed every 3–4 days. On day 9 in culture, the flasks were shaken overnight, trypsinized, and reseeded in two-well cover glass chamber slides (Nunc 178656, Intermountain Scientific, Bountiful, UT), previously coated with poly-*d*-lysine, at 2.4 × 10^4 cells/cm^2. Following reseeding, astrocytes were grown for 2 days, then rinsed with phosphate-buffered saline, and serum-deprived for 2 days in Dulbecco's Modified Eagle Medium supplemented with 0.1% Fraction V fatty acid–free BSA. Astrocyte cultures were determined to be at least 95% pure by indirect immunofluorescence with antibodies against glial fibrillary acidic protein and neuron-specific enolase (Accurate Chemical, Westbury, NY).

Calcium Transport Measurements

Release of Ca^{2+} from cerebellar and skeletal membrane vesicles was measured with the metallochromic dye antipyrylazo III in a diode array spectrophotometer (model 8542, Hewlett Packard, Palo Alto, CA).

Cerebellar microsomes were batch-loaded with Ca^{2+} overnight (20 hr) at 4°C in Ca^{2+} transport buffer (8 mM K-MOPS [pH 7.0], 40 mM KCl, 62.5 mM KH_2PO_4, and 250 μM antipyrylazo III) containing 80–160 μg/ml creatine phosphokinase, 20–40 mM phosphocreatine, 4–8 mM Mg–ATP, 4 mM NaN_3, 40 μg/ml leupeptin, 100 μM $CaCl_2$, and 333–667 μg/ml cerebellar microsomal protein. Calcium-loaded microsomes were then diluted 1:3 in Ca^{2+} transport buffer lacking microsomes (final volume 1.2 ml). Upon warming the solution to 37°C (500 s), the ability of Xe'a/aragusponginea to block IP_3 (5 μM)-induced Ca^{2+} release was assessed in temperature-controlled cuvettes with constant stirring.

Skeletal vesicles (50 μg protein/cuvette) were loaded with Ca^{2+} in Ca^{2+} transport buffer containing 18.5 mM K-MOPS (pH 7.0), 92.5 mM KCl, 7.5 mM sodium pyrophosphate, 250 μM antipyrylazo III, 1 mM Mg–ATP, 20 μg/ml creatine phosphokinase, and 5 mM phosphocreatine, with a final volume of 1.2 ml. Immediately prior to measuring caffeine-induced Ca^{2+} release, vesicles were loaded to near capacity with serial additions of 24 nmol $CaCl_2$ in temperature-controlled cuvettes (37°C) with constant stirring. The ability of XeC (0.1–10 μM) to block caffeine (20 mM)-induced Ca^{2+} release via Ry_1R was determined after the dye signal returned to baseline following loading.

Calcium transport was measured using a multisample transporter to sequentially measure two samples. Changes in extravesicular free Ca^{2+} were determined by subtracting the antipyrylazo III absorbance at 790 nm from the absorbance at 710 nm. For each curve, methanol controls were acquired simultaneously to normalize the degree of inhibition found in the samples containing Xe. In each experiment, absorbance signals were calibrated by the addition of 1.0 μg of the Ca^{2+} ionophore, A23187, followed by 20 or 24 nmol additions of $CaCl_2$ from a National Bureau of Standards stock solution. Initial Ca^{2+} release rates were determined by linear regression analysis of the first 15–50 s of Ca^{2+} release.

[^{3}H]IP_3 Binding Assay

Specific binding of [^{3}H]IP_3 to cerebellar microsomes was determined according the methods of Mohr et al. (1993) with minor modifications. The ability of XeC to block the binding of 1 nM [^{3}H]IP_3 to high-affinity sites on cerebellar microsomes (200 μg protein) dose dependently was assayed in a buffer containing 100 mM KCl, 20 mM NaCl, 1 mM EDTA, 0.1% BSA, and 25 mM Na_2HPO_4 (pH 8.0). XeC (1–10 μM) was added to cerebellar microsomes, and the binding reaction was initiated with addition of [^{3}H]IP_3 assay buffer (1 ml final volume). Tubes were incubated at 4°C with constant shaking. After 30 min, assays were terminated by rapid, single manifold filtration through Whatman GF/B filters presoaked with ice-cold assay buffer, followed by immediate washing of filters with 2.5 ml ice-cold assay buffer. Filters were soaked overnight in 5 ml scintillation cocktail, and the radioactivity on the filters was measured using a liquid scintillation counter. Nonspecific binding was measured in the presence of a 500-fold excess of unlabeled IP_3.

[^{3}H]Ryanodine Binding Assay

Specific binding of [^{3}H]ryanodine to skeletal membrane vesicles was determined according to the methods of Pessah et al. (1987). The ability of XeC to dose dependently block the binding of 1 nM [^{3}H]ryanodine to high-affinity sites on skeletal SR (50 μg protein) was assayed in a buffer consisting of 20 mM HEPES (pH 7.1), 250 mM KCl, 15 mM NaCl, 10% sucrose, and 50 μM $CaCl_2$. XeC (0.1–10 μM) was added to skeletal microsomes, and the binding reaction was initiated with addition of [^{3}H]ryanodine assay buffer (500 μl final volume). Following incubation for 3 hr at 37°C, assays were terminated by rapid filtration, with a Brandel (Gaithersburg, MD) cell harvester, through Whatman GF/B glass fiber filters. Filters were rinsed three times with 2 ml ice-cold harvest buffer (20 mM Tris–HCl [pH

7.1], 250 mM KCl, 15 mM NaCl, and 50 μM $CaCl_2$) and soaked overnight in 5 ml scintillation cocktail. Radioactivity on the filters was measured with a liquid scintillation counter. Nonspecific binding of [^{3}H]ryanodine was determined by the addition of 1000-fold excess of cold ryanodine.

Fluorometric Measurements of Cytosolic Calcium in Intact Cells

Two to three days following plating, intracellular Ca^{2+} was measured from PC12 cell populations either using a fluorometer (model F-2000, Hitachi, Japan) or ratiofluorescence video imaging (Photon Technology International, Princeton, NJ). Cells were loaded with a 5 μg/ml solution of cell permeant fura-2 acetoxymethyl ester (fura-2 AM, Molecular Probes, Eugene, OR) in imaging buffer (125 mM NaCl, 5 mM KCl, 2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 2 mM $CaCl_2$, 25 mM HEPES, 6 mM glucose, 0.05% BSA [fraction 5], and 250 μM sulfinpyrazone [pH 7.4]) for 30 min at 25°C. PC12 cells were then rinsed twice and stored in imaging buffer until needed (0 or 30 min). Coverslips were left to equilibrate for 2–3 min in imaging buffer before measurements began. In initial experiments, MeOH (10 μl) or Xe's (20 μM) were added 2 min after recording began, and bradykinin (300 nM) or ionomycin (3 μM) was added 10 min later. Measurements performed in Ca^{2+}-depleted media were made by addition of EGTA (3 and 5 mM final concentrations) 1 min prior to bradykinin/ionomycin addition, to obtain free Ca^{2+} levels of 120 and 40 nM, respectively. In the experiments examining the XeC dose–response relationship and effect of Xe's on the RyR, PC12 cells were incubated with MeOH (4 or 10 μl) or XeC (5–20 μM) for 30 min and 20 min, respectively, in buffer lacking 0.05% BSA, before bradykinin addition. Measurements were performed at a constant 37°C in continually stirred cuvettes (photometry) or a clamp chamber (imaging). The arbitrary fluorescence units of 340 nm/380 nm were used to compare the Xe-treated and control cell populations.

Calcium measurements on primary rat cortical astrocyte cultures were carried out by a modification of the method of Kovacs et al. (1995). At the time of the experiment, media was removed, and cells were rinsed twice with Krebs bicarbonate buffer and loaded with a 2 μM solution of cell permeant indo-1 acetoxymethyl ester (indo-1 AM, Molecular Probes, Eugene, OR) in Krebs buffer, containing 1% Fraction V BSA, 0.001% lipids–cholesterol-rich solution, and 1 mM probenecid, for 30 min at 37°C. Following loading, cells were rinsed with Krebs buffer, without BSA and lipids, and incubated for at least 15 min at 37°C to allow for cleavage of the indo-1 AM ester. Indo-1 was excited at 351–363 nm, and emission was detected simultaneously at 405 and 530 nm to measure the fluorescence intensity of the dye bound and not bound to Ca^{2+}, respectively. Fluorescence measurements were made using an attached-cell analysis and sorter Ultima instrument (Meridian Instruments, Okemos, MI). The ratio of the fluorescence at the two wavelengths was determined and was compared to the ratio for Ca^{2+} standard in solution (Molecular Probes, Eugene, OR) to determine the absolute Ca^{2+} concentration. The fluorescence within 50 cells was quantified over time for each treatment. For Xe experiments, cells were preincubated with the inhibitor for 30 min. Prior to carbachol addition, baseline Ca^{2+} measurements were determined by three scans conducted at 15 s intervals. After the third scan, a 30 s lapse in scanning allowed for the addition of carbachol (1 mM), and then Ca^{2+} changes were monitored every 15 s for 17 scans.

Measurements of Acute Cytotoxicity

In order to assess the acute toxicity of Xe's, trypan blue staining and LDH measurements were performed on PC12 cells and primary astrocyte cultures, respectively. Two days after plating, 4 μl MeOH or 20 μM XeC was added to PC12 cells, and cells were left to incubate at 37°C, 5% CO_2 for 10 or 30 min. Following incubation, media was removed, 0.4% trypan blue in culture media (1:3 w/v) was added, and 10 fields of view (100×) were assessed for the presence of dead cells. Triton X-100 (0.1%)-treated cells were used as a positive control. In astrocyte cultures, the levels of LDH in the media were measured following treatment of cells with 20 μM XeC for 30 min. Incubation for 30 min with buffer alone or a cytotoxic concentration of Triton X-100 (0.1%) were used as controls. LDH levels were measured using the Sigma Diagnostics LDH optimized lactate-dehydrogenase EC 1.1.1.27 UV-test kit (catalog No. DG1340-K).

Acknowledgments

The authors would like to thank Helen Kim for her assistance in the transport assays and Tom Chavez for his contribution to the ryanodine binding studies. We thank Marilyn Olmstead for assistance with conversion of the fraction crystal coordinates of XeC. This work was supported by National Institutes of Health Training Grant ES07059 (J. G.), RO1 ES05002 (L. N. P.), RO1 AI39987 and RO1 AI31680 (T. F. M.), RO1 AA08154 and P30 ES07033 (L. G. C.), and by a Grant-in-Aid of Research from the National Academy of Sciences through Sigma Xi, The Scientific Research Society (J. A. M.).

Received April 4, 1997; revised July 14, 1997.

References

Arrang, J.M. (1994). Pharmacological properties of histamine receptor subtypes. Cell. Mol. Biol. 40, 275–281.

Berridge, M.J. (1993). Inositol trisphosphate and calcium signaling. Nature 361, 315–325.

Bezprozvanny, I.B., Ondrias K., Kaftan E., Stoyanovsky D.A., and Ehrlich, B.E. (1993). Activation of the calcium release channel (ryanodine receptor) by heparin and other polyanions is calcium dependent. Mol. Biol. Cell 4, 347–352.

Brann, M.R., Klimkowski, V.J., and Ellis, J. (1993). Structure/function relationships of muscarinic acetylcholine receptors. Life Sci. 52, 405–412.

Bretschneider, E., Paintz, M., and Glusa, E. (1995a). Inositol 1, 4, 5-trisphosphate and protein kinase C are involved in thrombin- and trap-induced vascular smooth muscle contraction. Agents Actions Suppl. 45, 309–313.

Bretschneider, E., Paintz, M., and Glusa, E. (1995b). Involvement of inositol 1, 4, 5-trisphosphate and protein kinase C in thrombin-induced contraction of porcine ulmonary artery. Biochem. Pharmacol. 49, 33–38.

Briner, V.A., Tasi, P., and Schrier, R.W. (1993). Bradykinin: potential for vascular constriction in the presence of endothelial injury. Am. J. Physiol. 264, F323–F327.

Cameron, A.M., Steiner, J.P., Sabatini, D.M., Kaplin, A.I., Walensky, L.D., and Snyder, S.H. (1995). Immunophilin FK506-binding protein associated with inositol 1,4,5-trisphosphate receptor modulates calcium flux. Proc. Natl. Acad. Sci. USA 92, 1784–1788.

Crews, P., Slate, D.L., Gerwick, W.H., Schmitz, F.J., Scatzman, R., Strulovici, B., Cannon, P., and Hunter, L.M. (1994). Screening for anticancer leads from marine organisms in a mechanism-based drug delivery program. Dev. Oncol. 74, 384–403.

Dauphin, F., Linville, D.G., and Hamel, E. (1994). Cholinergic dilatation and constriction of feline cerebral blood vessels are mediated by stimulation of phosphoinositide metabolism via two different muscarinic receptor subtypes. J. Neurochem. 63, 544–551.

Endo, M., Nakagawa, M., Hamamoto, Y., and Ishihama, M. (1988). Pharmacologically active substances from southern Pacific marine invertebrates. Pure Appl. Chem. 58, 387–394.

Fasolato, C., Zottini, M., Clementi, E., Zacchetti, D., Meldolesi, J., and Pozzan, T. (1991). Intracellular Ca^{2+} pools in PC12 cells. J. Biol. Chem. 266, 20159–20167.

Furuichi, T., Yoshikawa, S., Miyawaki, A., Wada, K., Maeda, N., and Mikoshiba, K. (1989). Primary structure and functional expression of the inositol 1,4,5-trisphosphate-binding protein P400. Nature 342, 32–38.

Ghosh, T.K., Eis, P.S., Mullaney, J.M., Ebert, C.L., and Gill, D.L. (1988). Competitive, reversible, and potent antagonism of inositol 1,4,5-trisphosphate-activated calcium release by heparin. J. Biol. Chem. 263, 11075–11079.

Grohovaz, F., Zacchetti, D., D'Andrea, P., Lorenzon, P., and Meldolesi, J. (1992). $[Ca^{2+}]_i$ effects of bradykinin B2 receptor activation in PC12 cells. Agents Actions. Suppl. *38*, 9–15.

Guizzetti, M., Costa, M., Peters, J., and Costa, L.G. (1996). Acetylcholine as a mitogen: muscarinic receptor-mediated proliferation of rat astrocytes and human astrocytoma cells. Eur. J. Pharmacol. *297*, 265–273.

Hamick, D.J., Jayaraman, T., Ma, Y., Mullet, P., Go, L.O., and Marks, A.R. (1995). The human type 1 inositol 1,4,5-trisphosphate receptor from T lymphocytes. Structure, localization, and tyrosine phosphorylation. J. Biol. Chem. *270*, 2833–2840.

Hoye, T.R., North, J.T., and Yao, L.J. (1994). Conformational considerations in 1-oxaquinolizidines related to the xestospongin/araguspongine family: reassignment of stereostructures for araguspongines B and E. J. Org. Chem. *59*, 6904–6910.

Hoye, T.R., North, J.T., and Yao, L.J. (1995). Conformational considerations in 1-oxaquinolizidines related to the xestospongin/araguspongine family: reassignment of stereostructures for araguspongines B and E. J. Org. Chem. *60*, 4958.

Kitagawa, I., Kobayashi, M., and Kawazoe, K. (1989). Araguspongines B, C, D, E, F, G, H, and J, new vasodilative bis-1-oxaquinolizidine alkaloids from an okinawan marine sponge, *Xestospongia sp.* Chem. Pharm. Bull. (Tokyo) *37*, 1678–1678.

Kobayashi, S., Somlyo, A.V., and Somlyo, A.P. (1988). Heparin inhibits the inositol 1,4,5-trisphosphate-dependent, but not the independent, calcium release induced by guanine nucleotide in vascular smooth muscle. Biochem. Biophys. Res. Commun. *153*, 625–631.

Kovacs, K.A., Kavanagh, T.J., and Costa, L.G. (1995). Ethanol inhibits muscarinic receptor-stimulated phosphoinositide metabolism and calcium mobilization in rat primary cortical cultures. Neurochem. Res. *20*, 939–948.

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

Maranto, A.R. (1994). Primary structure, ligand binding, and localization of the human type 3 inositol 1,4,5-trisphosphate receptor expressed in intestinal epithelium. J. Biol. Chem. *269*, 1222–1230.

Marks, A.R. (1996). Cellular functions of immunophilins. Physiol. Rev. *76*, 631–649.

McCarthy, K.D., and De Vellis, J. (1980). Preparation of separate astroglial and oligodendroglial cell cultures from rat cerebral tissue. J. Biol. Chem. *85*, 890–902.

Mignery, G.A., Südhof, T.C., Takei, K., and De Camilli, P. (1989). Putative receptor for inositol 1,4,5-trisphosphate similar to ryanodine receptor. Nature *342*, 192–195.

Mignery, G.A., Newton, C.L., Archer, B.T.d., and Südhof, T.C. (1990). Structure and expression of the rat inositol 1,4,5-trisphosphate receptor. J. Biol. Chem. *265*, 12679–12685.

Mohr, F.C., Hershey, P.E., Zimanyi, L., and Pessah, I.N. (1993). Regulation of inositol 1,4,5-trisphosphate receptors in rat basophilic leukemia cells. I. Multiple conformational states of the receptor in a microsomal preparation. Biochem. Biophys. Acta *1147*, 105–114.

Nakada, S., Maeda, N., and Mikoshiba, K. (1991). Involvement of the C-terminus of the inositol 1,4,5-trisphosphate receptor in Ca^{2+} release analysed using region-specific monoclonal antibodies. Biochem. J. *277*, 125–131.

Nakagawa, M., Endo, M., Tanaka, N., and Gen-Pei, L. (1984). Structures of xestospongin A, B, C and D, novel vasodilative compounds from marine sponge, *Xestospongia axigua*. Tetrahedron Lett. *25*, 3227–3230.

Nakanishi, S., Maeda, N., and Mikoshiba, K. (1991). Immunohistochemical localization of an inositol 1,4,5-trisphosphate receptor, P400, in neural tissue: studies in developing and adult mouse brain. J. Neurosci. *11*, 2075–2086.

Pessah, I.N., Stambuk, R.A., and Casida, J.E. (1987). Ca^{2+}-activated ryanodine binding: mechanisms of sensitivity and intensity modulation by Mg^{2+}, caffeine, and adenine nucleotides. Mol. Pharmacol. *31*, 232–238.

Pessah, I.N., Molinski, T.F., Meloy, T.D., Wong, P., Buck, E.D., Allen, P.D., Mohr, F.C., and Mack, M.M. (1997). Bastadins relate ryanodine-sensitive and -insensitive Ca^{2+} efflux pathways in skeletal SR and BC_3H1 cells. Am. J. Physiol. *272*, C601–C614.

Petit, G.R., Orr, B., Herald, D.L., Doubek, D.L., Tackett, L., Schmidt, J.M., Boyd, M.R., Pettit, R.K., and Hooper, J.N.A. (1996). Isolation and x-ray crystal structure of racemic xestospongin D from the Singapore marine sponge *Midnates sp.* Bioorg. Med. Chem. Lett. *6*, 1313–1318.

Pin, J.-P., and Duvoisin, R. (1995). Review: neurotransmitter receptors I—the metabotropic glutamate receptors: structure and functions. Neuropharmacology *34*, 1–26.

Quirion, J.C., Sevenet, T., Husson, H.P., Weniger, B., and Debitus, C. (1992). Two new alkaloids from *Xestospongia sp.*, a new Caledonian sponge. J. Nat. Prod. *55*, 1505–1508.

Ross, C.A., Danoff, S.K., Schell, M.J., Snyder, S.H., and Ullrich, A. (1992). Three additional inositol 1,4,5-trisphosphate receptors: molecular cloning and differential localization in brain and peripheral tissues. Proc. Natl. Acad. Sci. USA *89*, 4265–4269.

Saito, A., Seiler, S., Chu, A., and Fleischer, S. (1984). Preparation and morphology of sarcoplasmic reticulum terminal cisternae from rabbit skeletal muscle. J. Cell Biol. *99*, 875–885.

Südhof, T.C., Newton, C.L., Archer, B.T.d., Ushkaryov, Y.A., and Mignery, G.A. (1991). Structure of a novel $InsP_3$ receptor. EMBO J. *10*, 3199–3206.

Sullivan, K.M., Lin, D.D., Agnew, W., and Wilson, K.L. (1995). Inhibition of nuclear vesicle fusion by antibodies that block activation of inositol 1,4,5-trisphosphate receptors. Proc. Natl. Acad. Sci. USA *92*, 8611–8615.

Takeshima, H., Nishimura, S., Matsumoto, T., Ishida, H., Kangawa, K., Minamino, N., Matsuo, H., Ueda, M., Hanaoka, M., Hirose, T., et al. (1989). Primary structure and expression from complementary DNA of skeletal muscle ryanodine receptor. Nature *339*, 439–445.

Tinker, A., and Williams, A.J. (1995). Measuring the length of the pore of the sheep cardiac reticulum calcium-release channel using related molecular calipers. Biophys. J. *68*, 111–120.

Verma, A., Ross, C.A., Verma, D., Supattapone, S., and Snyder, S.H. (1990). Rat brain endoplasmic reticulum calcium pools are functionally segregated. Cell Regul. *1*, 781–790.

Yamada, N., Makino, Y., Clark, R.A., Pearson, D.W., Mattei, M.G., Guenet, J.L., Ohama, E., Fujino, I., Miyawaki, A., Furuichi, T., et al. (1994). Human inositol 1,4,5-trisphosphate type-1 receptor, $InsP_3R1$: structure, function, regulation of expression and chromosomal localization. Biochem. J. *302*, 781–790.

Zacchetti, D., Clement, E., Fasolato, C., Lorenzon, P., Zottini, M., Grohovaz, F., Fumagalli, G., Pozzan, T., and Meldolesi, J. (1991). Intracellular Ca^{2+} pools in PC12 cells. J. Biol. Chem. *266*, 20152–20158.

VITA

Michelle Catherine Catlin

University of Washington

1999

Education

Doctoral Candidate, 1992-Present (Graduation: March 1999)
School of Public Health & Community Medicine
Department of Environmental Health (Toxicology Program)
University of Washington at Seattle, WA, USA

Dissertation: Effects of Ethanol on Muscarinic-Induced Responses in Astroglia
Dissertation Advisor: L. G. Costa, Ph.D.
Courses Include: Molecular Toxicology, Reproductive Toxicology, Biostatistics, Risk Assessment, Neurotoxicology, Epidemiology, Environmental Chemistry, Conservation Biology and Public Policy

Master of Science, 1990-1992
Department of Pharmacology and Toxicology
Queen's University at Kingston, ON, Canada

Thesis: Effects of *in vitro* Exposure to Ethanol and Hypoxia on the Release of γ-Aminobutyric Acid and L-Glutamate in the Hippocampus of the Fetal and Adult Guinea Pig
Thesis Advisor: J. F. Brien, Ph.D.
Courses Include: Biochemical Basis of Pharmacology and Toxicology, Methods in Pharmacology and Toxicology, Environmental Health Hazards

Bachelor of Science (Honours), 1984-1990
Life Science
Queen's University at Kingston, ON, Canada

Relevant Experience

Research Assistant, 1992-Present
Department of Environmental Health, University of Washington at Seattle, WA
Predoctoral dissertation research:

- characterized muscarinic-mediated calcium responses in astroglia and quantified the effect of ethanol on these responses

- determined muscarinic receptor levels by radioactive binding assay
- quantitated IP_3 levels using a radioreceptor assay
- assessed muscarinic-induced cellular proliferation using ^{3}H-thymidine incorporation
- collaborated with researchers at UC Davis to test the efficacy of an inhibitor of the IP_3-linked calcium channel in primary rat astrocytes
- assisted other laboratories with calcium measurements in rat kidney cells, and cultured mouse hippocampal slices
- involved in hiring and training of laboratory assistants
- preparing two manuscripts for publication based on this research

Toxicology Outreach Coordinator, Summer 1998-Present
Health and Environmental Resources for Educators Program,
Department of Environmental Health, University of Washington at Seattle, WA

- assisted in design and preparation of toxicology and environmental health curriculum and resource kit for Kindergarten - Grade 12 education
- trained graduate students for classroom presentations
- presented lecture and led discussion session on toxicology with secondary school students

Toxicology Consultant, Fall, 1998-Present

- identified scientific literature relevant in assessing human health impacts of herbicides
- summarized herbicide research and assessed its implications on previous Bureau of Land Management policy decisions

Teaching Assistant, Spring 1994, 1996, 1997
Department of Environmental Health, University of Washington at Seattle, WA

- prepared and graded assignments and tests for a graduate course in environmental and occupational toxicology
- tutored students on an individual basis
- held review sessions for the class
- designed and presented lectures on plant and animal toxins

Research Assistant, 1990-1992
Department of Pharmacology and Toxicology, Queen's University at Kingston, ON
Master's thesis research:

- characterized a high performance liquid chromatography method to quantify amino acids
- determined the effect of ethanol on the release of γ-aminobutyric acid and L-glutamic acid from guinea pig hippocampal slices using a static-interface superfusion system and high performance liquid chromatography

Teaching Assistant, 1990-1992
Department of Pharmacology and Toxicology, Queen's University at Kingston, ON

- supervised an undergraduate research student
- acted as a demonstrator in undergraduate, graduate and medical pharmacology laboratories

Undergraduate Project/Summer Research Assistant, 1989-1990
Department of Pharmacology and Toxicology, Queen's University at Kingston, ON
Undergraduate research:

- determined behavioural impairment due to *in utero* ethanol exposure using a two-way T-maze and a hyperactivity box; statistical analysis using SAS
- assessed morphological changes by light microscopy

Awards

Best Graduate Student Poster Presentation
Pacific Northwest Association of Toxicologists, 1998
International Union of Toxicology Junior Fellowship, 1998
Sheldon D. Murphy Travel Award
U. of Washington & Pacific Northwest Association of Toxicologists, 1996
Eldon Boyd Fellowship
Dept. of Pharmacology & Toxicology, Queen's, 1991-1992
Dean's Award
Dept. of Pharmacology and Toxicology, Queen's, 1991-1992
Pharmaceutical Manufacturers' of Canada Summer Studentship, 1990

Professional Organizations

Pacific Northwest Association of Toxicologists (PANWAT)
Society of Toxicology (SOT)
Society of Toxicology of Canada (STC)

Committee Activities

Graduate & Professional Student Senator
Department of Environmental Health, U. of Washington, 1993-1997
Curriculum Committee Student Representative
Department of Environmental Health, U. of Washington, 1993-1996
Lead Teaching Assistant
Department of Environmental Health, U. of Washington, 1994

Publications

Catlin, M.C., Guizzetti, M., Ponce, R.A., Costa, L.G., and Kavanagh, T.J. Analytical Cytology: Applications to Neurotoxicology. Submitted for publication in: *Current Protocols in Toxicology.* (M. Maines, G. Sipes, L.G. Costa, D. Reed, and S. Shassa eds) John Wiley and Sons, New York.

Catlin, M.C., Guizzetti, M., and Costa, L.G. (1999). Effects of ethanol on calcium homeostasis in the nervous system: implications for astrocytes. *Mol. Neurobiol.*, In press.

Guizzetti, M., **Catlin, M.**, and Costa, L.G. (1997). Effects of ethanol on glial cell proliferation: relevance to the Fetal Alcohol Syndrome. *Front. Biosci.*, 2: e93-98.

Gafni, J., Munsch, J.A., Lam, T.H., **Catlin, M.C.**, Costa, L.G., Molinski, T.F., and Pessah, I.N. (1997). Xestospongins: Potent membrane permeable blockers of the inositol 1,4,5-trisphosphate receptor. *Neuron*, 19: 723-733.

Catlin, M.C., Penning, D.H., and Brien, J.F. (1995). Effect of exposure *in vitro* to ethanol and hypoxia on g-aminobutyric acid efflux in the hippocampus of the fetal and adult guinea pig. *Reprod. Fertil. Devel.*, 7: 1339-1344.

Catlin, M.C. Effects of *in vitro* exposure to ethanol and hypoxia on the release of g-aminobutyric acid and L-glutamate in the hippocampus of the fetal and adult guinea pig. Master's thesis, Queen's University at Kingston, September, 1992.

Abdollah, S., **Catlin, M.C.**, and Brien, J.F. (1993). Ethanol neuro-behavioural teratogenesis in the guinea pig: behavioural dysfunction and hippocampal morphologic changes. *Can. J. Physiol. Pharmacol.*, 71(10-11): 776-782.

Catlin, M.C., Abdollah, S., and Brien, J.F. (1993). Dose-dependent effects of prenatal ethanol exposure in the guinea pig. *Alcohol*, 10(2): 109-115.

Posters & Presentations

Catlin, M.C., and Costa, L.G. Inhibition of muscarinic-mediated calcium responses in astrocytic cells by ethanol. Pacific Northwest Association of Toxicologists 15th Annual Meeting, Leavenworth, WA, U.S.A. (1998). Poster presentation by M. Catlin.

Catlin, M.C., and Costa, L.G. Ethanol inhibits carbachol-induced calcium responses in glial cells in a concentration- and time-dependent manner. International Congress of Toxicology, Paris, France (1998). Poster presentation by M. Catlin.

Catlin, M.C., and Costa, L.G. Ethanol and muscarinic receptor-induced calcium responses in glial cells. Society of Toxicology 37th Annual Meeting, Seattle, WA, U.S.A. (1998). Poster presentation by M. Catlin.

Catlin, M.C., and Costa, L.G. The effect of ethanol on carbachol-induced calcium responses in glial cells. Pacific Northwest Association of Toxicologists 14th Annual Meeting, Ocean Shores, WA, U.S.A. (1997). Poster presentation by M. Catlin.

Catlin, M.C., and Costa, L.G. The effect of alcohol on intracellular calcium mobilization in glial cells. Society of Toxicology 35th Annual Meeting, Anaheim, CA, U.S.A. (1996). Poster presentation by M. Catlin.

Catlin, M.C., Penning, D.H., and Brien, J.F. Effects of ethanol on the release of g-aminobutyric acid and glutamate in the hippocampus of the fetal and adult guinea pig. 19th Annual Meeting of the Society for the Study of Fetal Physiology, Niagara-on-the-Lake, ON, Canada (1992). Poster presentation by J. Brien.

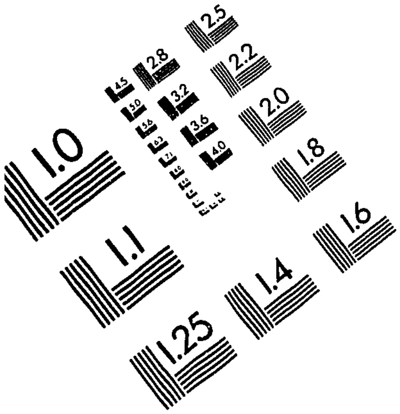
Catlin, M.C., Penning, D.H. and Brien, J.B. Release of glutamate and g-aminobutyric acid in the hippocampus of the fetal, neonatal and adult guinea pig. Society of Toxicology of Canada 24th Annual Symposium, Montreal, QE, Canada (1991). Poster presentation by M. Catlin.

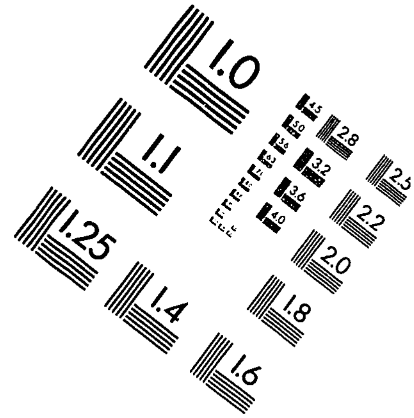
Abdollah, S., **Catlin, M.C.**, and Brien, J.F. Chronic prenatal ethanol exposure in the guinea pig: behavioural dysfunction and hippocampal morphologic changes. Society of Toxicology of Canada 24th Annual Symposium, Montreal, QE, Canada (1991). Poster presentation by S. Abdollah.

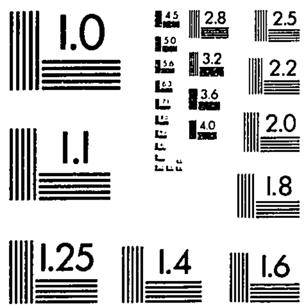
Abdollah, S., **Catlin, M.**, and Brien, J.F. Ethanol CNS teratogenesis in the guinea pig. *Proc. Can. Fed. Biol. Soc.*, **34**, 158 (1991). Platform session presentation by M. Catlin.

Catlin, M.C., Penning, D.H., and Brien, J.B. Glutamate and g-aminobutyric acid efflux in the hippocampus of the fetal and adult guinea pig. 15th Annual Ontario-Quebec Perinatal Investigators' Meeting, Kingston, ON, Canada (1991). Poster session presentation by M. Catlin.

IMAGE EVALUATION TEST TARGET (QA-3)

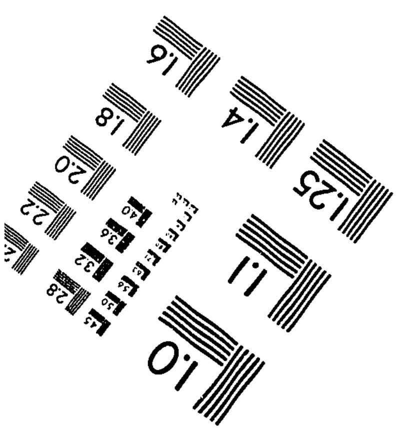


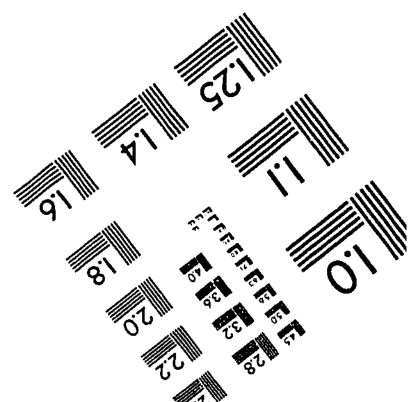




150mm

6"





APPLIED IMAGE, Inc
1653 East Main Street
Rochester, NY 14609 USA
Phone: 716/482-0300
Fax: 716/288-5989