INTRACELLULAR CALCIUM CHANGES IN A HYBRID MOUSE DORSAL ROOT GANGLION (DRG) NEURONAL CELL LINE MD3 FOLLOWING γ-AMINOBUTYRIC ACID (GABA) RECEPTOR ACTIVATION

by

Tomonori Yokogawa
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Department of Medicine / Division of Neurology

The University of British Columbia
Vancouver, Canada

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Abstract

Application of γ-aminobutyric acid (GABA) to dorsal root ganglion (DRG) neurons *in vivo* increases their membrane conductance for Cl^-. I studied a mouse DRG neuronal cell line MD3, produced from hybridization between mouse (Balb/C) DRG neurons and N18TG2 neuroblastoma cells, for sensory and pharmacological properties. Immunostaining with markers for neurofilaments demonstrated the cells had neuronal characteristics. In addition, the hybrid cells reacted to CAP and ATP, similar to sensory neurons. Then, I investigated the mechanism and effects of externally applied agonists and antagonists of GABA on MD3 cells *in vitro*. I used fura-2 to monitor GABA-evoked depolarizations in terms of intracellular Ca^{2+}, $[Ca^{2+}]_i$. Application of GABA to hybrid cells caused $[Ca^{2+}]_i$ increases that implied $GABA_A$ receptor ($GABA_AR$) activation. Applications of $GABA_AR$ antagonists bicuculline (BIC) and picrotoxinin (PTX) confirmed the $GABA_AR$ involvement in the intracellular response. BIC, but not PTX, blocked the GABA-mediated elevations in $[Ca^{2+}]_i$. ZAPA, a potent agonist of low affinity $GABA_ARs$, also increased $[Ca^{2+}]_i$ in hybrid cells. The GABA-evoked increases in $[Ca^{2+}]_i$ were not apparent during the absence of Ca^{2+} in the extracellular environment and exposure to dihydropyridines (DHP) nimodipine and nifedipine. The results imply that depolarization-activated Ca^{2+} influx increased $[Ca^{2+}]_i$ during GABA application. I also studied the effects of $GABA_B$ receptor ($GABA_BR$) agonist, β-parachlorophenyl-γ-aminobutyric acid (baclofen), on the hybrid cells. Detectable changes in the $[Ca^{2+}]_i$ were not apparent during $GABA_BR$ activation alone. 2-hydroxy saclofen blocked the inhibitory effect of baclofen and antagonized the $GABA_BR$.

The MD3 hybrid cell line possesses $GABA_A$ and $GABA_BRs$. The augmentation in $[Ca^{2+}]_i$ occurred when GABA activated the $GABA_AR$, increasing Cl^- conductance and depolarizing the membrane potential, thus resulting Ca^{2+} influx through voltage-dependent Ca^{2+}

channels that increase intracellular Ca^{2+}. The source of the elevations in $[Ca^{2+}]_i$ was the extracellular environment. Nifedipine and nimodipine inhibited the GABA-induced transmembrane influx of Ca^{2+} through VGCCs. Activation of $GABA_BRs$ with baclofen attenuated the depolarization-induced increases in $[Ca^{2+}]_i$. In addition, baclofen influenced L-type channels since DHPs inhibited the K^+-mediated $[Ca^{2+}]_i$ elevations. The MD3 cells exhibited similarities to its sensory parent. Nevertheless, it is a useful model system for the examination of GABA and other drug effects.

Table of Contents

List of Figures

List of Tables

List of Abbreviations

AP	action potential
ATP	adenosine triphosphate
Baclofen	β-parachlorophenyl-γ-aminobutyric acid
BIC	bicuculline
BSA	bovine serum albumin
CAP	capsaicin
$[Ca^{2+}]_i$	intracellular free calcium concentration
CNS	central nervous system
DAB	3,3'-diaminobenzidine
DHP	dihydropyridine
DMEM	Dulbecco's Modified Eagle Medium
DMSO	dimethyl sulfoxide
DRG	dorsal root ganglion
EGTA	ethylene glycol-bis(β-aminoethyl ether)- N,N,N',N'-tetraacetic acid
FBS	fetal bovine serum
Fura-2/AM	fura-2 acetoxymethyl ester
GABA	γ-aminobutyric acid
GABAR	γ-aminobutyric acid receptor
$GABA_AR$	γ-aminobutyric acid subtype A receptor
$GABA_BR$	γ-aminobutyric acid subtype B receptor
$GABA_CR$	γ-aminobutyric acid subtype C receptor
GDP	guanine diphoshate
HEPES	N-2-hydroxyethylpiperazine-N'-2-etheansulfonic acid
HHSS	HEPES-buffered Hank's balanced salt solution
HPRT	hypoxanthine phosphoribosyltransferase
HS	horse serum
K_d	dissociation constant
MAb	monoclonal antibody
min	minute(s)
msec	millisecond(s)
nm	nanometre
PAD	primary afferent depolarization
PBS	phosphate-buffered saline
PNS	peripheral nervous system
PTX	picrotoxinin
R_{min}	minimum fluorescence intensity ratio
R_{max}	maximum fluorescence intensity ratio
rpm	revolutions per minute
sec	second(s)
SIT	silicone intensified target
VGCC	voltage-gated calcium channel
ZAPA	(Z)-3-[aminoiminomethul) thio] prop-2-enoic acid

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CHAPTER I. INTRODUCTION

1.1 General Introduction

Certain aspects of the peripheral nervous system are relevant to the study of the dorsal root ganglion (DRG) neuron. For example, the general organization, electrophysiological properties, processes and structure of the sensory ganglia. In this thesis, I focused on GABA effects of the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) increase in a mouse hybrid sensory neuronal cell line, MD3. Since primary cell cultures of neurons are heterogeneous and labour intensive to prepare, I used clonal cells that exhibit neuronal features and immortalization. Every experimental approach has its limitations; however, cell lines represent an unlimited source of material for analyses. I will review the anatomical organization of the central and peripheral nervous systems, especially with a focus on the DRG neuron, and the pharmacological aspects of γ-aminobutyric acid (GABA).

1.2. Synopsis of the Central and Peripheral Nervous Systems

The nervous system depends on input from the external world and the body to maintain homeostasis in the organism. Two major subdivisions of the nervous system include: the central (CNS) and peripheral (PNS) nervous systems. Both systems function synergistically to accomplish necessary actions and have similar anatomical organization and neurochemistry (Sterini, 1997). The CNS consists of the brain and spinal cord while the PNS includes peripheral nerves (spinal and cranial), the autonomic nervous system, and the sensory ganglia. The PNS mediates communication between the CNS, the body of the organism, and the external environment. The CNS monitors the external environment via sight, sound, taste, smell, and touch. It also regulates the internal homeostasis of the organism through other modalities such as pH level, O_2 concentration, distention of internal vessels and viscera, and osmotic pressure of fluids. The CNS interacts with the PNS through the primary afferent fibers in the dorsal root

and the outgoing efferent motor fibers in the ventral horns of the spinal cord (Terzis & Smith, 1990).

1.2.1. Anatomical Organization of the PNS

The PNS is a complex entity composed of cell bodies motor, sensory and autonomic neurons as well as their supportive connective tissue, cellular elements, and associated end-organs. The microscopic structural elements of the PNS include the axons, sensory receptors, neuromuscular junctions, synapses, and ganglia.

Situated near the ventrolateral surface of the spinal cord, the ventral root joins with the dorsal root to form the spinal root. Located inside the ventral root are the somatic motor axons and visceral motor axons. The dorsal root extends from the dorsolateral surface of the spinal cord and has both central and peripheral processes. At the vertebral level, enclosed within the spinal canal, the majority of dorsal roots have both autonomic (visceral) and somatic sensory axons.

The autonomic nervous system innervates the periphery using two neurons. Located in the CNS, the preganglionic neuron sends its axon peripherally to synapse on the postganglionic neuron in the wall of a target tissue or in a peripheral ganglion. The sympathetic system influences activities that expend energy such as increasing the heart rate and the force of each beat, raising arterial pressure, elevating the blood sugar level, and directing blood flow to the skeletal muscles (Barr & Kiernan, 1988). Sympathetic responses occur also during times of stress and emergency (flight or fight response). The parasympathetic system conserves and restores energy by decreasing the rate and force of the heart, lowering blood pressure, and increasing the activity of the digestive system (Barr & Kiernan, 1988).

Peripheral ganglia are collections of sensory, sympathetic or parasympathetic neurons outside the CNS. Both the DRG and cranial ganglia are sensory ganglia. The location of DRGs, also known as posterior root or spinal ganglia, is on the posterior roots of the spinal

nerves. Cranial sensory ganglia such as the trigeminal root ganglia have cell bodies located near the attachment of the nerve roots to the brainstem. The trigeminal root ganglia have functional equivalence to the DRG, innervating the head, neck, and oral structures. Motor ganglia are part of the autonomic nervous system, terminating in the ear, glands, or smooth muscle. The DRG contains the soma of a variable number of ganglia cells including sensory neurons, proximal portions of axons, associated satellite and Schwann cell sheaths, and connective and vascular tissue elements of the PNS (Lieberman, 1976).

1.2.2. Classification of Nerve Fibers

Connective tissue supports afferent and efferent nerve fibers that travel in bundles called fascicles. Spinal nerves have both a dorsal root and a ventral root. The three major categories of afferent fibers, depending upon where the impulse originates, are somatic, visceral, and proprioceptive. Nerve fibers of the dorsal roots are important for sensory information to reach the spinal cord and the rest of the CNS. A whole range of nerve fibers constitutes the peripheral nerves and spinal roots. Erlanger and Gasser originally defined the classification of fibres in a mixed peripheral nerve trunk as one of three groups: *A, B,* or *C* (Perl, 1992).

The *A* fibers, the myelinated somatic afferent and efferent nerve fibers, transmit impulses from afferent endings for pain, temperature, touch, pressure, and vibration and send impulses to motor end plates. The four subgroups of the *A* group are α, β, δ, and γ neurons. α neurons have the most heavily myelinated axons and the highest conduction speeds while the δ neurons have lightly myelinated fibers with the slowest conduction rates of the *A* group (Bessou & Perl, 1969; Lynn & Carpenter, 1982). The *B* fibers consist of myelinated efferent preganglionic fibers of the autonomic system. Their diameter reaches approximately 3 μm in diameter with impulse rates ranging from 3-15 m/s (Bessou & Perl, 1964). The *C* fibers are unmyelinated fibers from the postganglionic sympathetic axons of the autonomic nervous system, and the unmyelinated

afferent fibers of the peripheral nerves and dorsal roots. As with *Aδ* fibres, *C* fibres have an entire range of sensory modalities such as pain, touch, and temperature (Fitzgerald, 1979; Lynn & Carpenter, 1982). The postganglionic sympathetic *C* fibers have diameters ranging from 0.3 to 1.3 μm with impulse speeds from 0.7 to 2.3 m/s. The afferent *C* fibers range from 0.4 to 1.2 μm with conduction speeds ranging from 0.6 to 2.0 m/s (Daniel & Terzis, 1977). Nerve fibres of the *Aδ* and *C* classes transmit pain information (Burgess & Perl, 1967; Kumazawa & Perl, 1977; Fyffe, 1983).

1.3. Anatomical Organization of DRG Neurons

DRG neurons are sensory neurons, located within the dorsal root ganglion of the PNS. They and their cranial nerve equivalents are the primary sensory cells for general somatic sensation. The DRG neuron is a functional unit of the nervous system, capable of receiving, conducting, processing, transmitting stimuli, and making functional contact with other neurons. The vertebrate DRG neuron is a mononucleate cell that possesses a cell body, also known as the soma or perikaryon, and a population of fine cytoplasmic processes (neurites). These extensions of the soma surround tissues, possessing an excitable plasma membrane. The peripheral dendrites of DRG neurons convey pain, pressure, temperature, and stretch impulses from either free endings in skin or a number of specialized end-organs in skin, muscle, tendons, joints, connective tissue, and bone to the somata (Lieberman, 1976).

The DRG neuron is spheroidal, with round or oval profiles (Bunge *et al.*, 1967). They vary enormously in size, ranging from diameters of 10 μm for the smallest cells of small vertebrates to 115 μm for the largest spinal ganglion cells of man (Ohta *et al.*, 1974). The DRG neurons are pseudounipolar with a stem process or the initial tract of the axon. On leaving the cell body, the stem process often pursues a complex course near the cell body. The central

process enters the spinal cord through the dorsal root while the peripheral process extends to the periphery in a peripheral nerve, to receive sensation and terminate in a sensory ending (Taylor & Pierau, 1991). The T- or Y-shaped bifurcation occurs at a variable distance from the cell body, commonly close to or within the axial fibre region (Lieberman, 1976).

1.3.1. Embryology and Morphology of Neurons in Sensory Ganglia

The nervous system develops from ectoderm, the outermost of the three germ layers in the embryo. The derivation of the CNS is primarily from this germ layer; in the PNS, the ectodermal and mesodermal structures combine to produce the final structures. During the third week of gestation, when the neural groove appears on the dorsal surface of the embryo, the longitudinal groove deepens where the lateral margins meet to close the neural groove to form the neural tube. The neural crest is the portion of the ectodermal tissue that separates from the tube, forming a pair of elongated tissue masses along the dorsolateral margins of the neural tube and eventually developing into sensory ganglia (Pannese, 1974). The cells are first bipolar, but the two processes fuse to form the single process of this pseudounipolar type of neuron (Tennyson, 1965, 1970). The stem process that arises from smaller cell bodies is short and unconvoluted, whereas the stem process of larger neurons often at first wind around the parent cell body (Barr & Kiernan, 1988). During development, DRG neuronal processes begin to associate with the surrounding tissues that are forming in the periphery (Pannese, 1974).

Two morphological subtypes of DRG neurons are: "large light" ("light" or L) and "small dark" (SD) neurons (Jacobs *et al.*, 1975, Rambourg *et al.*, 1983). From the statistical analysis of mice and rats, both L and SD neurons have a normal distribution of cell size and overlap each other. The L neuron distribution extends over the entire size range of the ganglion while SD neurons are limited to the lower end of the distribution (Lawson, 1979).

1.3.2. Processes of DRG Sensory Neurons

Situated on structures near the surface of the body and in deeper tissues, somatic sensory axons are the peripheral part of DRG neurons in spinal nerves, and cranial sensory ganglia in cranial nerves. Somatic afferents carry information concerning changes in the external environment. Receptors detect three types of sensations: pain; temperature, warm and cold; and touch, light and deep. Light touch requires only surface stimulation while deep touch requires firmer stimulation that provokes the receptors that lie deeper in the tissues.

DRG neurons are afferent units with dendritic processes, its peripheral afferent fiber and receptive terminals in peripheral tissue, and its central axon's branches and synaptic contacts in central tissue (Perl, 1992). The primary function of the spinal dorsal roots and their cranial equivalents is to serve as afferent conduits to the CNS (Perl, 1992). The axons vary in diameter from a maximum of 20 μm to a minimum of 0.2 μm depending on the destination of the nerve and the sensory modality conveyed (Iggo, 1974). Both the peripherally and centrally directed processes, and stem process that connects them with the cell body, are structurally axons and all three may be myelinated. At its origin, the stem process is small and generally less than 3 μm in diameter in small mammals and leaves the perikaryon rather abruptly, commonly at an acute angle. The diameter of the stem process may increase at some distance from the cell body and the myelinated part of the stem process is usually considerably larger than unmyelinated axons of peripheral nervous tissue.

1.3.3. Electrophysiological Properties of DRG Neurons

Under physiological conditions, the initiation of action potentials (AP) in sensory ganglion neurons develops at, or close to the receptor terminals. Although the position of the soma is off-stream in relation to the impulse traffic 'through-route', ganglion cell perikarya are believed to play a far less dominant role in the electrophysiological activities than is the case in

multi-polar neurons. The terminal portions of the peripheral process are vital in the sensory ganglion cell; however, one should not consider the soma and stem process as silent partners in the process of electrical signalling. The anatomy and the physiology of the spinal cord and the brain stem nuclei, which the sensory ganglion cells terminate, also play a major role because these areas generate activity and control reflex activity. In neurons, the observed resting membrane potential is typically –50 to –60 mV, meaning that the internal environment is more negative than the extracellular component (Gallego & Eyzaguirre, 1978; Mayer & Westbrook, 1983). The calculation of the potential difference (PD) developed across a membrane using the Nernst equation is:

$$E_v = (RT/zF) \log 10\ [K^+]_o/[K^+]_i$$

Determining membrane potentials requires R, the universal gas constant (8.31 J/mol), T, the absolute temperature, F, the Faraday constant (96500 C/mol), z, the charge constant, $[K^+]_o$, the extracellular potassium ion concentration, and $[K^+]_i$, the intracellular potassium ion concentration.

Although initiation is not normally at the level of the cell body, action potentials always invade the soma passing along its processes (Scott *et al.*, 1969; Varon & Raiborn, 1971). Conduction along the initial axon is slow, however, the central terminals of cells with large diameter myelinated axons are depolarized before the soma (Darian-Smith, 1973).

1.3.4. Presynaptic Inhibition

Many hypotheses exist concerning presynaptic inhibition mechanisms. The Cl^- equilibrium potential relative to the membrane potential influences the Cl^- conductance that leads to hyperpolarization, depolarization, or no potential change depending on the Cl^- gradient across the membrane. An increased Cl^- conductance shunts the presynaptic spike, reducing transmitter output (Eccles, 1964; Takeuchi & Takeuchi, 1966). A high extracellular K^+

environment near the presynaptic terminals may induce depolarization (Rudomin *et al.*, 1981). In addition, the decrease in presynaptic Ca^{2+} current may reduce the transmitter release from the presynaptic cell (Dunlap & Fischbach, 1978; Kretz *et al.*, 1986). Previous research provided evidence for primary afferent depolarizations (PAD) produced from GABAergic pathways (Eccles *et al.*, 1963a,b; Curtis *et al.*, 1977). The most accepted hypothesis is the shunting effect of the chloride conductance, lowering the amplitude of the action potential in the nerve terminal (Dunlap & Fischbach, 1978; Kretz *et al.*, 1986), reducing Ca^{2+} influx through voltage-activated channels and transmitter release. However, the exact ionic mechanisms involved in presynaptic inhibition in primary afferents by GABA agonists remain unknown because the nerve terminals are not readily accessible to investigation.

1.4. Pharmacological Responses

1.4.1. γ-Aminobutyric Acid (GABA) Receptors

GABA has many functions that include inhibitory transmitter, seizures, and trophic factor in synaptogenesis (Wolff *et al.*, 1978; Roberts, 1991). It has also been implicated in the pathogenesis of Huntington's disease, Parkinsonism, epilepsy, schizophrenia, tardive dyskinesia, and senile dementia, as well as other behavioral disorders (Mountjoy *et al.*, 1984; Gunne & Andren, 1993; Reynolds *et al.*, 1997; Volpi *et al.*, 1997; Wassef *et al.*, 1999; Brailowsky *et al.*, 1999; Casamenti *et al.*, 1999). The term GABA receptor (GABAR) refers to a GABA recognition site on pre-and postsynaptic membranes. When an appropriate agonist binds to this receptor, a change in membrane permeability permits inorganic ions to cross. For example, a change in permeability to Cl^-, for example, results in hyperpolarization of the receptive neuron for postsynaptic inhibition or depolarization in the case of presynaptic inhibition.

In vertebrates, three major types of GABARs are: type A ($GABA_AR$), B ($GABA_BR$), and C ($GABA_CR$). The GABARs have differences based on their physiology, pharmacology, and anatomical location as well as their second messenger mechanisms. The subunit combination influenced the pharmacological properties of a particular GABAR (MacDonald & Olsen, 1994; Costa, 1998).

1.4.2. $GABA_A$ Receptors

GABA and structural analogues of GABA like muscimol, a substance from the hallucinogenic mushroom *Amanita muscaria*, and synthetic analogues such as 4, 5, 6, 7-tetrahydroisoxazolopyridin-3-ol (THIP), 3-aminopropanesulfonate, peiperidin-4-sulfonate, and isoguvacine activate $GABA_ARs$, controlling the opening and closing of a Cl^- channel conductance (Macdonald & Olsen, 1994).

Astroglia, central and peripheral neurons have $GABA_ARs$ (Grobin *et al.*, 1999). This major inhibitory neurotransmitter receptor is a multi-subunit receptor-channel complex that belongs to a large superfamily of ligand-gated ion channels that includes the nicotinic-cholinergic, ionotropic glutamate, and glycine receptors. The $GABA_AR$-ion channel complex is a heteropentameric glycoprotein of approximately 250 kD, combining multiple polypeptide subunits (Delaney & Sah, 1999). The five distinct classes of polypeptide subunits (α, β, δ, γ, and ρ) have multiple isoforms. Therefore, a structural diversity exists for the $GABA_AR$ with a family of at least 17 genes coding for diverse subunits (α1-6, β1-4, γ1-3, δ, ρ1-2, π, and ε) (Schofield *et al.*, 1987; Olsen & Tobin, 1990; Sieghart, 1995; Hedblom & Kirkness, 1997).

Many pharmacological agents, including anesthetics (for review, see Mody *et al.*, 1991; Weight *et al.*, 1992), ethanol (Sudzak *et al.*, 1986) and endogenous neurosteroids (Majewska *et al.*, 1986), affect GABA-mediated Cl^- conductance. Evidence for the biochemical and structural relationships between GABA and benzodiazepine showed that they share the same receptor

complex and GABA enhances the binding of benzodiazepines (Sigel *et al.*, 1983; Schofield *et al.*, 1987; Stauber *et al.*, 1987). While GABA agonists activate the $GABA_AR$, benzodiazepines increase the frequency of the channel opening without altering the channel conductance or duration of opening (Choi *et al.*, 1977; Macdonald & Barker, 1978a; Macdonald & Barker, 1979). However, barbiturates slightly decrease the opening frequency but prolong the duration of opening (Study and Barker, 1981; Olsen, 1981, 1987; Twyman *et al.*, 1989a,b; Macdonald and Twyman, 1992). The steroids that may include anesthetic steroids and progesterone metabolites also augment GABAR current (Majewska *et al.*, 1986; Callachan *et al.*, 1987a,b). GABA antagonists bicuculline (BIC), picrotoxinin (PTX), methyl 6,7-dimethoxy-4-ethyl-β-carboline-3-carboxylate (DMCM), and penicillin (Raichle *et al.*, 1971; Macdonald and Barker, 1978b) diminish GABA-mediated Cl^- conductance. Therefore, distinct compounds affect the $GABA_AR$ function through several different sites: GABA, PTX / barbiturate, benzodiazepine, and steroid (Olsen, 1981; Sigel *et al.*, 1983; Stauber *et al.*, 1987). Compounds that increase protein phosphorylation may also influence $GABA_AR$ channel function (Whiting *et al.*, 1990). However, the significance of phosphorylation of $GABA_ARs$ remains uncertain but may have an important role for the regulation of the receptor function and expression (Browning *et al.*, 1990, 1993).

GABA antagonists compete with GABA directly at the $GABA_AR$ or non-competitively through modification of the receptor or inhibition of the GABA-activated ionophore. Two classic GABA antagonists are plant convulsants BIC and PTX, each acting differently on the $GABA_AR$ with BIC competitively antagonizing GABA at the receptor level whereas PTX non-competitively blocking GABA-activated ionophores (Johnston, 1991). Other antagonists include petrazepin, the amidine steroid RU5135, and a series of pyridazinyl-GABA derivatives like SR-95531 (Johnston, 1991).

GABA is also an important neurodevelopmental factor and signal molecule during early ontogenesis (Meier *et al.*, 1991; Belhage *et al.*, 1998). The elucidation of the exact mechanism will involve $GABA_AR$ activation since studies discovered that GABA agonists replicate GABA effects and antagonists inhibit neurodifferentiation (Meier *et al.*, 1985; Belhage *et al.*, 1986).

1.4.3. $GABA_B$ Receptors

In mammals, $GABA_BR$ binding sites occur inside and outside the CNS, and are present in lower species. For example, at the neuromuscular synpase of lobsters, both inhibitory and excitatory axon terminals possess $GABA_BRs$ (Miwa *et al.*, 1990). $GABA_BRs$ are BIC- and PTX-insensitive, activated by β-para-chlorophenyl-GABA (baclofen), antagonized by 2-hydroxy-saclofen, and unassociated with Cl^- conductance (Sivilotti & Nistri, 1991; Bowery, 1993). Coupling of $GABA_BRs$ to G-proteins activates intracellular second-messenger pathways. $GABA_BR$ activation also mediates the release of amines, excitatory amino acids, neuropeptides, and hormones. While the $GABA_AR$ is linked to Cl^- channels, the $GABA_BR$ is associated with Ca^{2+} or K^+ channels via second messenger systems (Newberry & Nicoll, 1984; Asano *et al.*, 1985; Inoue *et al.*, 1985; Holz *et al.*, 1986; Ogata *et al.*, 1987; Dolphin & Scott, 1987; Dutar & Nicoll, 1988). Therefore, $GABA_BRs$ activate an inhibitory effect associated with a K^+ conductance increase, a Ca^{2+} conductance decrease, or an adenylyl cyclase functional decrease. Metabotropic $GABA_BR$ require coupling between two distinct gene products: $GABA_BR1$ and $GABA_BR2$ (Kaupmann *et al.*, 1997,1998; Martin *et al.*, 1999; Bowery & Enna, 2000).

In terms of pharmacology, the $GABA_BR$ is different from the $GABA_AR$ by its selective affinity for baclofen and lack of affinity for BIC and muscimol (Bowery *et al.*, 1980,1981; Bowery, 1993). Only a limited number of $GABA_BR$ agonists are active, presumably because they cannot differ significantly in structure from GABA. Baclofen activation of the $GABA_B$ presynaptic receptors decreases Ca^{2+} conductance and hence, transmitter release (Scholz &

Miller, 1991; Diverse-Pierluissi & Dunlap, 1995). Baclofen, (–)-baclofen being the more active form, is virtually inactive at $GABA_A$ sites but is stereospecifically active at $GABA_B$ sites. The most potent agonists are the phosphonic analogues, 3-aminopropyl phosphinic acid and its methyl analogue (Pratt *et al.*, 1989). Unlike the $GABA_AR$, the $GABA_BR$ does not respond to benzodiazepines or barbiturates. The first selective but relatively weak $GABA_B$ antagonist was phaclofen (Kerr *et al.*, 1987). In other brain regions, Lambert *et al.* (1989) observed similar results using the more potent derivatives, saclofen and 2-hydroxy-saclofen. The antagonistic compounds that affect the $GABA_AR$ have no effect on the $GABA_BR$ mechanism. $GABA_BR$ antagonists include CGP 35348, the first phosphinic derivative to be reported (Olpe *et al.*, 1990; Seabrook *et al.*, 1990), and CGP 36742, a brain-penetrating antagonist, competitively antagonizes responses to baclofen when applied to rat cerebrocortical neurons, (Bowery, 1993). Many thioether analogues of saclofen and phaclofen are pharmacologically less active than the original compounds (Allan *et al.*, 1990).

1.4.4. $GABA_C$ Receptors

Recent pharmacological and molecular biological experiments discovered a third class of GABARs, named $GABA_CRs$. In the retina, many types of species, bipolar and horizontal cells had $GABA_CRs$ (Qian & Dowling, 1994; Bormann & Feigenspan, 1995; Enz *et al.*, 1995). Coupled to BIC-insensitive, PTX-sensitive Cl^- channels, $GABA_CRs$ are activated by CACA and TACA (Shimada *et al.*, 1992; Qian & Dowling, 1993).

Although $GABA_CRs$ also gate a Cl^- conductance, they are not blocked by BIC or SR95531 and are markedly less sensitive to PTX. They are also insensitive to modulation by benzodiazepines and barbiturates (Qian & Dowling, 1993; Bormann & Feigenspan, 1995; Johnston, 1996). Assembled from ρ subunits (ρ1, ρ2, ρ3), $GABA_CRs$ share some homology with $GABA_AR$ subunits, but do not appear to coassemble with them (Cutting *et al.*, 1991;

Shimada *et al.*, 1992). However, the significance of these receptors outside of the retina is unknown.

1.5. GABA in DRG Neurons

Past experiments demonstrated that GABA depolarizes the somata and terminals of primary afferent neurons, causing presynaptic inhibition in the spinal cord (Eccles *et al.*, 1963a,b; Schmidt, 1963; Tebecis & Phillis, 1969; Davidson & Southwick, 1971; Barker & Nicoll, 1973; Krnjevic, 1974; Feltz and Rasminsky, 1974; Nishi *et al.*, 1974; Deschenes *et al.*, 1976; Deschenes & Feltz, 1976; Gallagher *et al.*, 1978). Nishi and colleagues (1974) observed in bullfrog DRG neurons that GABA was the most potent depolarizing substance among its analogues, resulting in increased Cl^- conductance. GABA is a major neurotransmitter that generates presynaptic inhibition at central terminals of primary afferents in the dorsal horn, where released GABA simultaneously activates both $GABA_A$ and $GABA_B$Rs.

GABARs in human DRG neurons behaves differently compared to other vertebrate neurons that hyperpolarize during GABA application. Valeyev *et al.* (1996) determined that BIC and PTX did not influence GABA currents in human DRG neurons. However, in rat DRG neurons, both antagonists inhibited GABA-mediated responses (Nowak *et al.*, 1982; Twyman *et al.*, 1989b). These differences may be due to the particular receptor subunit composition in human DRG cells or the experimental design. In the membrane of DRG cells, $GABA_A$Rs and $GABA_B$Rs may exist together or separately. A recent study found 50 % of each kind of receptor in the primary sensory terminals (Si *et al*, 1997).

1.5.1. $GABA_A$ Receptors of DRG Neurons

In mature DRG neurons, BIC competitively and PTX non-competitively inhibit $GABA_A$R subunit proteins that have Cl^- conductance (De Groat, 1972; Nishi *et al.*, 1974;

Gallagher *et al.*, 1978; Choi & Fischbach, 1981; Dunlap, 1984; Inoue & Akaike, 1988). As mentioned above, activation of $GABA_AR$ channel with an appropriate agonist conducts Cl^- ions predominantly. Since the Cl^- equilibrium potential is near resting potential in most neurons, increasing the Cl^- conductance depresses excitability by causing a shunt of the depolarizing effects of an excitatory input. In DRG neurons, the concentration of Cl^- is higher inside compared to the extracellular environment (Feltz & Rasminsky, 1974; Nishi *et al.*, 1974; Gallagher *et al.*, 1978). The activation of $GABA_ARs$ results in the opening of Cl^- channels and a concomitant Cl^- efflux out of DRG neurons, depolarizing the membrane (Nishi *et al.*, 1974; Gallagher *et al.*, 1978; Bormann, 1988). The depolarization activates voltage-gated Ca^{2+} channels (VGCC), allowing Ca^{2+} from the external environment to enter the cells. GABARs affect a variety of VGCCs in DRG neurons.

1.5.2. $GABA_B$ Receptors of DRG Neurons

Initial investigations concerning $GABA_BR$ in DRG neurons found that stimulation or activation of the receptor decreased membrane Ca^{2+} conductance. This depression was attributed to the decrease in the duration of APs in C and Aδ neurons (Dunlap, 1981; Desarmenien *et al.*, 1984) and in DRG neurons of cat, mouse, and rat from voltage clamp studies (Robertson & Taylor, 1986; Green & Cottrell, 1988; Formenti & Sansone, 1991). The action of baclofen on the $GABA_BR$ also produces an inhibition of VGCCs, decreasing the amount of Ca^{2+} influx or shortening the AP duration in the membrane of DRG neurons (Dunlap and Fischbach, 1978; Llinas *et al.*, 1981; Desarmenien *et al.*, 1984; Deisz & Lux, 1985; Dolphin & Scott, 1986,1987). A diminution of Ca^{2+} influx through VGCCs may account for the ability of baclofen and GABA to reduce the evoked-release of neurotransmitters (Bowery *et al.*, 1980,1981; Price *et al.*, 1984).

1.6. Objectives

Previously, our laboratory created the MD3 cell line according to the protocol described by Hammond and colleagues (1986). Immortal cell lines have advantages over primary cultures, owing to ease of maintenance of cells in simple media and acquisition of an infinite lifetime. Neuronal cell lines are also an unlimited source of a pure neuron population, allowing investigations of receptor properties. Fusion of mouse DRG sensory neurons with a mouse neuroblastoma N18TG2, deficient in hypoxanthine phosphoribosyltransferase (HPRT), generated the clonal cell line MD3. The reasons for choosing the neuroblastoma parent include its embryological similarity to neurons (Augusti-Tocco & Sato, 1969); therefore, the chances of eliminating some neuronal gene expression are reduced (Killary & Fournier, 1984). In Platika's study (1985), the hybrid sensory neuronal cells expressed a neuron-like morphology, cell-surface gangliosides and excitable membranes. Successful fusion occurs when these neuronal properties represent the expression of neuronal genes. Suburo and colleagues (1992) also found specific markers and neuropeptides, and cell depolarization in response to capsaicin and bradykinin in their hybrid sensory neuronal cells.

This study investigated the GABA pharmacology of the mouse hybrid sensory neuronal cell line created from hybridization termed MD3. The MD3 cell line may possess GABARs and be a model of GABAR interactions. Therefore, a central hypothesis of this thesis is that GABA binds to the GABA subtype A receptor, opening the Cl^- chloride channels that depolarizes the cell; this activates voltage-gated Ca^{2+} channels, increasing $[Ca^{2+}]_i$ as a consequence of Ca^{2+} influx. GABA subtype B receptor activation may attenuate the evoked elevations in $[Ca^{2+}]_i$. Various GABAR antagonists and Ca^{2+} channel-blockers should inhibit both types of responses. Fura-2 was used to measure the $[Ca^{2+}]_i$ of MD3 cells to determine the presence of GABAR and its responses to different compounds. To determine the mechanisms and effects of GABA-

evoked depolarizations, bath and local applications of GABA-related compounds influenced $[Ca^{2+}]_i$, exhibiting GABAR pharmacology. Determining the GABAR subtype involved in the generation of intracellular transients involved applying antagonists that distinguished between $GABA_A$ and $GABA_BR$ subtypes. A principal mechanism proposed to explain GABA-evoked depolarizations in the mammalian system is that GABA depolarizes the cell through activation of the $GABA_AR$ coupled to anion-selective channels, resulting in efflux of Cl^-.

Chapter II. METHODS

2.1. Cell Culture

We previously produced the hybrid sensory neuronal cell line MD3 by somatic fusion of hypoxanthine phophoribosyl transferase (HPRT)-deficient mouse N18TG2 neuroblastoma cells and Balb/c mouse DRG sensory neurons using methods described by Hammond *et al.* (1986). In brief, DRGs were removed from 3 week old Balb/C mice and incubated in phosphate-buffered saline (PBS) containing 0.25 % collagenase (type I; Worthington Biochemical, Freehold, NJ) and 20 μg/ml DNase (Sigma, St. Louis, MO) for 40 min at 37 °C and dissociated into single cells by repeated pipetting. Single DRG neurons were re-suspended in Dulbecco's modified Eagle's medium (DMEM) containing phytohemagglutinin (40 μg/ml) that facilitates the adherence of DRG neurons to the N18TG2 cells. Then, the N18TG2 neuroblastoma cells were added to the DRG neurons. After a 15 min incubation, the medium was aspirated and the cells were fused with 50 % (v/v) polyethylene glycol (PEG 4000, Sigma). The fusion products were plated in DMEM containing 5 % fetal calf serum, 5 % horse serum (HS), 100 μM hypoxanthine, 0.4 μM aminopterin, and 16 μM thymidine (HAT medium) which selects against HPRT-deficient cells. After 7 days of treatment in HAT medium, cultures were fed with DMEM containing 5 % fetal bovine serum (FBS) and 5 % HS. Individual colonies were isolated and expanded further in DMEM containing 5 % fetal bovine serum (FBS), 5 % HS, 20 μg/ml gentamicin, and 2.5 μg/ml fungizone. One of the expanded colonies was named MD3 to be used in the present study.

Cells frozen in a nitrogen tank were quickly thawed, washed, and resuspended in media. Stock cultures of mouse hybrid sensory neuronal cell line MD3 were grown in tissue culture flasks with growth medium consisting of DMEM with 5 % FBS, 5 % HS, 20 μg/ml gentamicin,

and 2.5 μg/ml fungizone. MD3 cells were maintained in a humidified atmosphere of 5 % CO_2 and 95 % air at 37 °C and fed twice a week. Experiments were performed on MD3 cells, plated onto poly-D-lysine-coated 22 mm glass coverslips (#1 thickness, Carolina Biological Supply, Burlington, NC).

2.2. Immunocytochemistry

MD3 cells on aclar plastic coverslips (9 mm, round) were cultured for 2-4 days. The cells were rinsed thoroughly in PBS, fixed in methanol at –20 °C for 10 min, and incubated with a mouse monoclonal antibody (Ab) against microtubule-associated protein-2 (MAP-2, 1: 100; Sigma Chemical) and a rat monoclonal Ab against high molecular weight neurofilament protein (NF-H, 1:10, Ta51; gift from Dr. V. Lee) at 4 °C for 48 h. Cells treated with the primary antibodies were washed 3 X 2 min in PBS. Appropriate biotinylated anti-mouse or anti-rat IgG Ab was diluted in PBS (1: 200) and applied to the coverslips for 1 h at room temperature (RT). Hybrid sensory neuronal cells were extensively washed in PBS and then incubated in adivin-biotin complex solution (ABC, Vector Laboratories, Mississauga, ON) that binds to the biotinylated secondary antibody for 1 h at RT. The chromogen diaminobenzadine (DAB) revealed this antibody staining. The cells were washed extensively in PBS and incubated for 2 min in 0.1 M Tris-HCl buffer containing 0.5 mM 3,3'-DAB and 0.02 % hydrogen peroxide. Coverslips were dehydrated in 70-100 % ethanol, cleaned in xylene, and embedded on microscopic slides with Permount.

2.3. Preparation of Test Solutions

HEPES-buffered Hank's balanced salt solution (HHBSS, adjusted to pH 7.4 with NaOH) consisted of (in mM): 145 NaCl, 2.5 KCl, 1.0 $MgCl_2$, 20 HEPES, 10 glucose, and 1.8 $CaCl_2$. In

Ca^{2+}-free HHBSS buffer, 50 μM ethylene glycol bis (β-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA) replaced the $CaCl_2$ to chelate any excess Ca^{2+} ions present. GABA, αβ-methylene ATP, capsaicin (CAP), (±)-baclofen, diazepam, 2-hydroxy-saclofen, nifedipine, nimodipine, (Z)-3-[aminoiminomethyl) thio] prop-2-enoic acid (ZAPA), BIC methobromide, and PTX were prepared from frozen aliquots of stock solution (1-100 mM) and diluted in HHBSS buffer. Since CAP, diazepam, nimodipine, and nifedipine are water-insoluble, they were first dissolved in 100 % alcohol and diluted in HHBSS with a final alcohol concentration of 0.01 %.

2.4. Application of Drugs

The experimental protocol involved recording the changes in $[Ca^{2+}]_i$ of the MD3 cells in a series of different solutions (for example, normal saline, Ca^{2+}-free saline, and normal saline with agonist or antagonist, recovery in normal saline). MD3 cells were placed in a recording chamber, with a bath volume of 0.4 ml, on the stage of a Nikon inverted microscope (Nikon Diaphot TMD). This chamber was superfused with the different types of HHBSS buffer using a system of multiple rubber tubes connected to reservoirs containing the various solutions. A three-way solenoid valve allowed switching between the different types of recording solutions. Trials with a marker dye showed that complete replacement of the solution occurred within 30 sec at a flow rate of 2 ml/min. This relatively slow speed of solution exchange does not affect the results because the kinetics of the responses were not measured. An ejection technique with a Picospritzer was used where pressurized nitrogen gas ejected drugs from pipettes. This "puffer" technique allowed rapid and repeated applications of drugs of known concentrations (Fischbach & Choi, 1981). Small quantities of α,β-methylene ATP (100 μM), CAP (30 μM), GABA (10-100 μM), ZAPA (100 μM), or KCl (25-100 mM) were applied to individual cells by pressure microejection (5-20 psi, 10-150 ms) from a two-channel Picospritzer (General Valve,

Fairfield, NJ). A micromanipulator was used to position the single-barrel micropipette over the cells near the recording site. At the start of an experiment, GABA agonists or KCl were applied by pressure to initiate a response. If no response resulted, then the ejection time was increased, or the pipette was moved closer to the cells. Care was taken to optimize the pipette tip-to-cell distance, such that a maximal response was obtained at a given pressure.

Using a double pull protocol, thin-wall glass tubings were drawn under low heat on using an instrument (Narashigi Instrument, Tokyo, Japan) that produced a sharp tip from thin-walled glass (diameter of 1.0 mm o.d., World Precision Instruments). The tips were broken to give a tip diameter of 10-20 μm. The micropipettes were backfilled with a long, thin needle. If the solution could not be expressed from the tip, the electrode was assumed clogged and discarded. Experiments with trypan-blue dye solutions showed the ejection pulses covered the cell area during drug application. In addition, trypan blue allowed visualization of the perfusion flow inside the chamber. BIC (100 nM-100 μM), baclofen (50 nM-100 μM), nimodipine (1-20 μM), nifedipine (1-20 μM), 2-hydroxy-saclofen (500 nM-10 μM), PTX (10-25 μM), and diazepam (1-20 μM) were mixed directly to HHBSS buffer and applied to cells in the recording chamber. At the end of each experimental day, the recording chamber, apparatus and rubber tubings were washed first with 10 % alcohol, followed by distilled water.

2.5. Chemicals and Drugs

GABA, N-2-hydroxyethylpiperazine-N'-2-etheansulfonic acid (HEPES), nifedipine, nimodipine, diazepam, potassium chloride (KCl), bovine serum albumin (BSA), CAP (8-methyl-N-vanillyl-6-nonenamide), EGTA were all purchased from Sigma; ZAPA sulfate from Tocris Cookson (Ballwin, MO); (±)-baclofen, (–)-BIC methobromide, PTX and 2-hydroxy-saclofen from Precision Biochemicals (Vancouver, BC); fura-2/AM, pentapotassium salt ((2-[6-

(bis(carboxymethyl)-amino)-5-methylphenoxy)ethoxy-2-benzofuranyl]5-oxazole carboxylic acid), was purchased from Molecular Probes (Eugene, OR).

2.6. Measurement of Intracellular Free [Ca^{2+}]

2.6.1. Preparation of Cells for Ca^{2+} Recording

Cell cultures grew on poly-D-lysine-coated 22 mm glass coverslips for at least 12 h before initiation of any experiments. Intracellular Ca^{2+} levels were measured using the Ca^{2+}-sensitive fluorescent chelator fura-2 (Grynkiewicz *et al.*, 1985). An ampule containing 50 μg of fura-2/AM (acetoxy methyl ester) was mixed with 50 μL of dimethyl sulphoxide (DMSO) to make a 1 μM stock solution. The stock solution was aliquoted into Eppendorf tubes at 3 μL each and stored in –20 °C. Fura-2/AM was mixed with 0.2 % pluronic F-127 acid (Molecular Probes) and 0.02 % BSA in the recording buffer (HHBSS). The addition of pluronic acid increases the solubility of the highly hydrophobic dye, facilitating the fura-2 loading of the cells. From earlier work (Yoo *et al.*, 1999), adding glucose to HHBSS sustained cell viability during loading, washing, and testing. Without glucose, the cells would also lose their fura-2 fluorescence signal at a much faster rate.

2.6.2. Calcium Imaging

All cells examined were incubated with fura-2/AM (5 μM) in HHBSS for 45 min at 37 °C. Fura-2/AM permeates cell membranes into the cytosol where it is hydrolyzed into fura-2, a non-esteric form that is membrane-impermeable, remaining trapped inside the cell. After loading, the cells were washed twice in recording buffer to remove any excess fura-2 that may be present on the exterior. The cells were incubated a further 15 min at 37 °C to completely de-esterify fura-2/AM to fura-2. Finally, the glass coverslip with the fura-2 loaded cells was mounted on a perfusion chamber and placed onto the stage of the Nikon inverted microscope.

Figure 1 illustrates the overall experimental setup. Cells on glass coverslips were used within a week after plating and passaged between 2-20 times before taking a new vial of cells from the liquid nitrogen storage.

Excitation light from a 100 W xenon arc lamp (Osram, Germany) passed through 340 or 380 nm filters mounted in a plate, reflected by a dichroic mirror and focused onto the cells via a 40X-fluorite epifluorescence objective. The fura-2 fluorescence signal was detected by a silicone intensified target (SIT) video camera (Hamamatsu C2400-08). The ratios of the fluorescence intensity at 340 and 380nm wavelengths were stored on a desktop computer. Before each experiment, cells regions were marked and chosen based on their appearance with an even distribution of fura-2. Regions of high cell density were avoided because of the difficulty in discriminating signals between cells. Ratios were obtained from 8-frame averages of pixel intensities (ranging from 0 to 255) at each of the excitation frequencies.

An appropriate software program, Image-1/FL Quantitative Fluorescence System (Universal Imaging, West Chester, PA), was used on the desktop computer to control optic equipment and the parameters of fluorescence data acquisition (background subtraction, filter wheel/ shutter switch, sampling rate and camera sensitivity). Using ratios should, to a first approximation, normalize for differences in preparation geometry, as well as for the effects of light scattering, illumination nonuniformity, and indicator concentration differences (Grynkiewicz *et al.*,1985). Then, the ratios were converted to a direct measurement of $[Ca^{2+}]_i$ using the following equation:

$$[Ca^{2+}]_i \text{ (in nM)} = K_D\beta(R-R_{min})/(R_{max}-R)$$

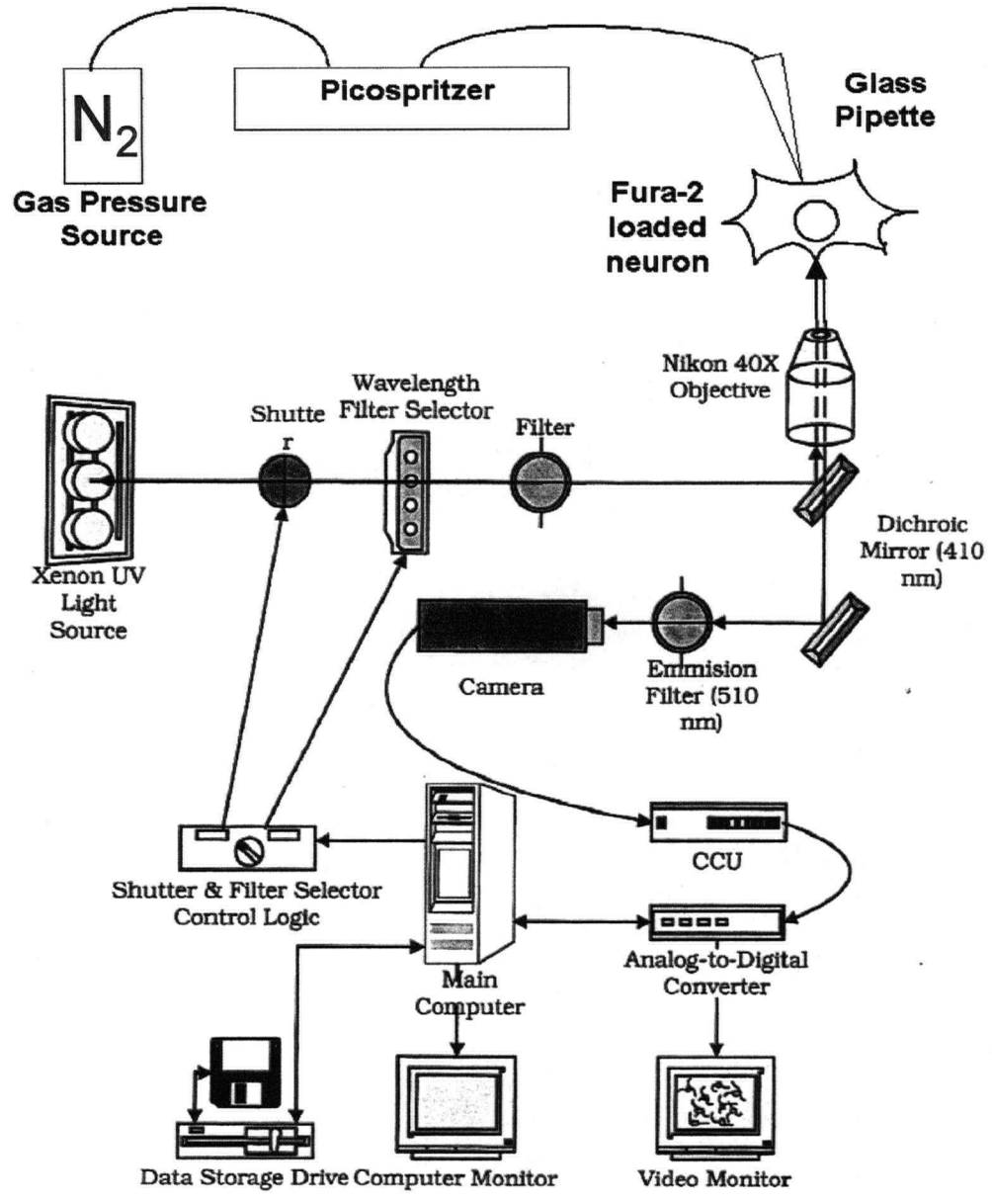

Figure 1 Experimental Setup of Fura-2 Imaging System. Fura-2 loaded cells were mounted in a perfusion chamber and viewed through an inverted microscope. Excitation light passed through 340 or 380 nm filters and reflected off a dichroic mirror. A video camera recorded the fluorescence signal from fura-2 loaded cells and the signals were converted from analog to digital and analyzed by a computer. A picosprizter that was connected to a N_2 pressure source locally applied drugs to the MD3 cells and ejected known drug concentrations rapidly and repeatedly from a single-barrel glass micropipette.

Converting the ratio values to $[Ca^{2+}]_i$ required the values $R_{max} = 0.12$, $R_{min} = 5.5$, $K_D = 221$ (constant), $\beta = 5.4$ nM. R_{min} is the minimum value of the fluorescence ratio (340 nm/380 nm) observed with 0 mM Ca^{2+} HHBSS buffer with 50 μM EGTA. R_{max} is the maximum ratio value when 4-bromo-A23582 (10 μM, Molecular Probes), a Ca^{2+} ionophore, was added to increase the $[Ca^{2+}]_i$.

The imaging system was calibrated using fura-2 pentapotassium salt (fura-2/K5, 5 μM, Molecular Probes) with a series of buffers containing 100 mM KCl, 10 mM MOPS, and Ca^{2+}-EGTA/EGTA in ratios that yielded Ca^{2+} concentrations between 0 and 39.8 μM (pH 7.2) on the current system previously (Yoo *et al.*, 1999). The dissociation constant for fura-2 binding (K_d) and the ratio of fluorescence intensities at 380 nm under zero and saturating Ca^{2+} concentrations (β) were determined to be 221 and 5.4 nM, respectively. The value for K_d in our fura-2 imaging system was similar to values reported by Grynkiewicz *et al.* (224 nM, 1985). Experiments were completed in a dark room at RT (22-24 °C) where full-field images of fura-2 fluorescence at 340 nm and 380 nm were recorded every 10 s. Temperature was previously shown not to influence the K_D value (Uto *et al.*, 1991). Although the individual levels of fluorescence were different in normal saline, the ratio signal was nearly uniform over the population of cells. The resting fluorescence ratio corresponded to Ca^{2+} level of approximately ranging from 38 to 142 nM. Baseline level of cytoplasmic free Ca^{2+} in individual cells was constant during perfusion of HHBSS buffer for up to 2 h. The isobestic point is the wavelength at which the excitation spectra for the Ca^{2+} bound and unbound forms of the dye intersect and not influenced by ion binding. Therefore, in the present system, the fluorescence measured at the excitation wavelength of 340 nm will rise while excitation from 380 nm will produce a fluorescence signal that will decrease when fura-2 and Ca^{2+} form a complex (Thayer *et al.*, 1988). The ratio of these two fluorescent intensities is related to Ca^{2+} concentration in a manner that is independent

of dye concentration, optical path length, or dye bleaching (Thayer *et al.*, 1988). Data are presented as mean ± SEM unless otherwise stated. Most of the data were graphed as measurements of $[Ca^{2+}]_i$ over time. Statistical comparison between control and experimental groups was performed by Student's t-test. The data were analyzed using StatView version 4.02 (Abacus Concepts, Berkeley, CA), Prism version 2.0 software (GraphPad, San Diego, CA), and Microsoft Excel version 8.0 software (Microsoft Corporation, Redmond, WA).

Chapter III. RESULTS

3.1. Identifying the MD3 cell line

From cultures of MD3 cells plated on glass coverslips, I quantified the $[Ca^{2+}]_i$ using microfluorimetry imaging. Recording the fura-2 fluorescent ratio values of mouse hybrid sensory neuronal cells enabled the calculation of $[Ca^{2+}]_i$ from the methods described by Grynkiewicz *et al.* (1985).

3.1.1. Immunocytochemistry

First, the immunocytochemistry technique showed that the MD3 cell line possesses neuronal characteristics. Anti-MAP-2 and anti-NF-H (clone Ta51) Abs labelled the hybrid sensory neuronal cells for neuronal properties (Lee *et al.*, 1982). After growing in culture for at least 24 h, cells were fixed and immunostained with cell type-specific Abs, staining all the cells in culture (Figure 2). The cells appeared to be a morphologically heterogeneous population containing cells of different sizes and different number of processes with cell body diameters ranging between 10 and 25 μm. The majority of the cells had two or more short- or medium-sized processes (89.6 %; n = 1012). Positive immunoreactivity to MAP-2 and NF-H Abs indicate that the MD3 cells express neuronal phenotypes.

3.1.2. Capsaicin-evoked Response

After determining cytoskeletal immunoreactivity, I examined the mouse hybrid sensory neuronal cells for sensitivity to capsaicin (CAP), a pungent tasting ingredient in peppers of the Capsicum family. Sensitivity to CAP helps to identify nociceptive sensory neurons (Bevan & Szolcsanyi, 1990) because of its ability to elicit pain and burning sensations (Simone *et al.*, 1987,1989). It selectively damages sensory neurons, depleting them of neurotransmitter substances like substance P and other neuropeptides (for review see Jancso *et al.*, 1977; Nagy & Hunt, 1983; Holzer, 1988, 1991). Fura-2 imaging showed the effects of CAP on the MD3

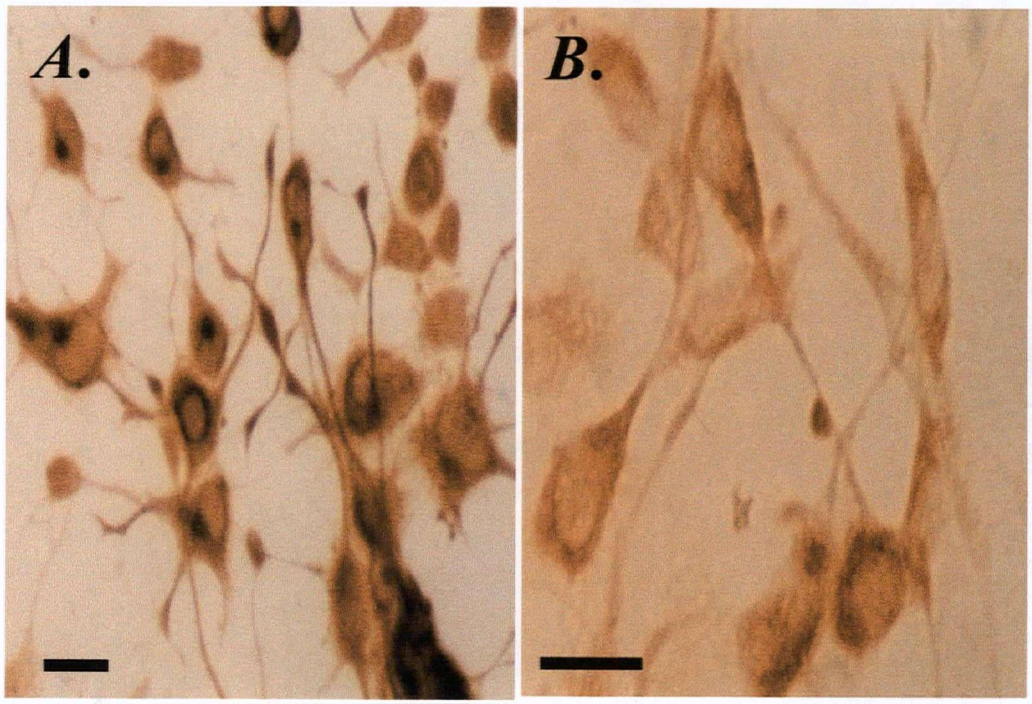

Figure 2*A*,*B* Immunocytochemistry. *A*. Antibodies against NF-H (Ta51) and *B*. MAP-2 specifically labelled the MD3 cells. Diaminobenzidine revealed the antibody staining. The selected antibodies showed that the hybrid sensory neuronal cells possess neuronal characteristics. Scale bars = 10 μm.

cells. The average baseline level of $[Ca^{2+}]_i$ was 89.6 ± 7.4 nM (n = 27). The concentration for CAP was chosen on the results of a previous report that found CAP had an EC_{50} = 1.1 μM on rat DRG neurons (Oh *et al.*, 1996). After observing a stable baseline $[Ca^{2+}]_i$, I applied 3 μM CAP for less than 15 sec increased $[Ca^{2+}]_i$ (14 independent experiments), as illustrated in Figure 3*A*. The rise in cytoplasmic Ca^{2+} from a second application of CAP was ~80 % smaller than the original exposure, or disappeared completely, presumably due to acute desensitization with rapid onset. Subsequent CAP applications were similarly ineffective for observation periods of up to 30 min. In summary, the CAP-evoked response exhibited pronounced acute desensitization and tachyphylaxis when bath applied. However, to circumvent desensitization and evoke repeatable responses, I applied CAP (30 μM) onto MD3 cells with a Picospritzer, resulting in reproducible responses with increased $[Ca^{2+}]_i$ responses with repeated agonist exposures. Consecutive applications of CAP resulted in increases of $[Ca^{2+}]_i$, ranging from 191 to 630 nM (mean increase in $[Ca^{2+}]_i$ = 404 ± 56 nM; n = 14; Figure 3B). In 5 separate experiments, I observed little or no increase in $[Ca^{2+}]_i$ on applying capsaicin after a 10 min pretreatment with capsazepine (5 μM), a CAP receptor antagonist (Walpole *et al.*, 1994). The responsiveness of the MD3 cells to CAP is consistent with the characteristics of sensory neurons *in vitro* and *in vivo* (Bevan & Szolcsanyi, 1990; Simone *et al.*, 1991).

3.1.3. Effects of α,β-methylene adenosine triphosphate

I examined the MD3 cells for responsiveness to α,β-methylene adenosine triphosphate (ATP) because sensory neurons express an ATP-gated cation conductance (Krishtal *et al.*, 1988a,b; Bouvier *et al.*, 1991). The MD3 cells responded to applications of 100 μM α,β-methylene ATP, increasing $[Ca^{2+}]_i$ (mean increase in $[Ca^{2+}]_i$ = 317 ± 20 nM; n = 58; Figure 4). After a 5 min wash with normal HHBSS, a second response to 100 μM α,β-methylene ATP was observed. The responses were similar in reproducibility in terms of their amplitude and time-

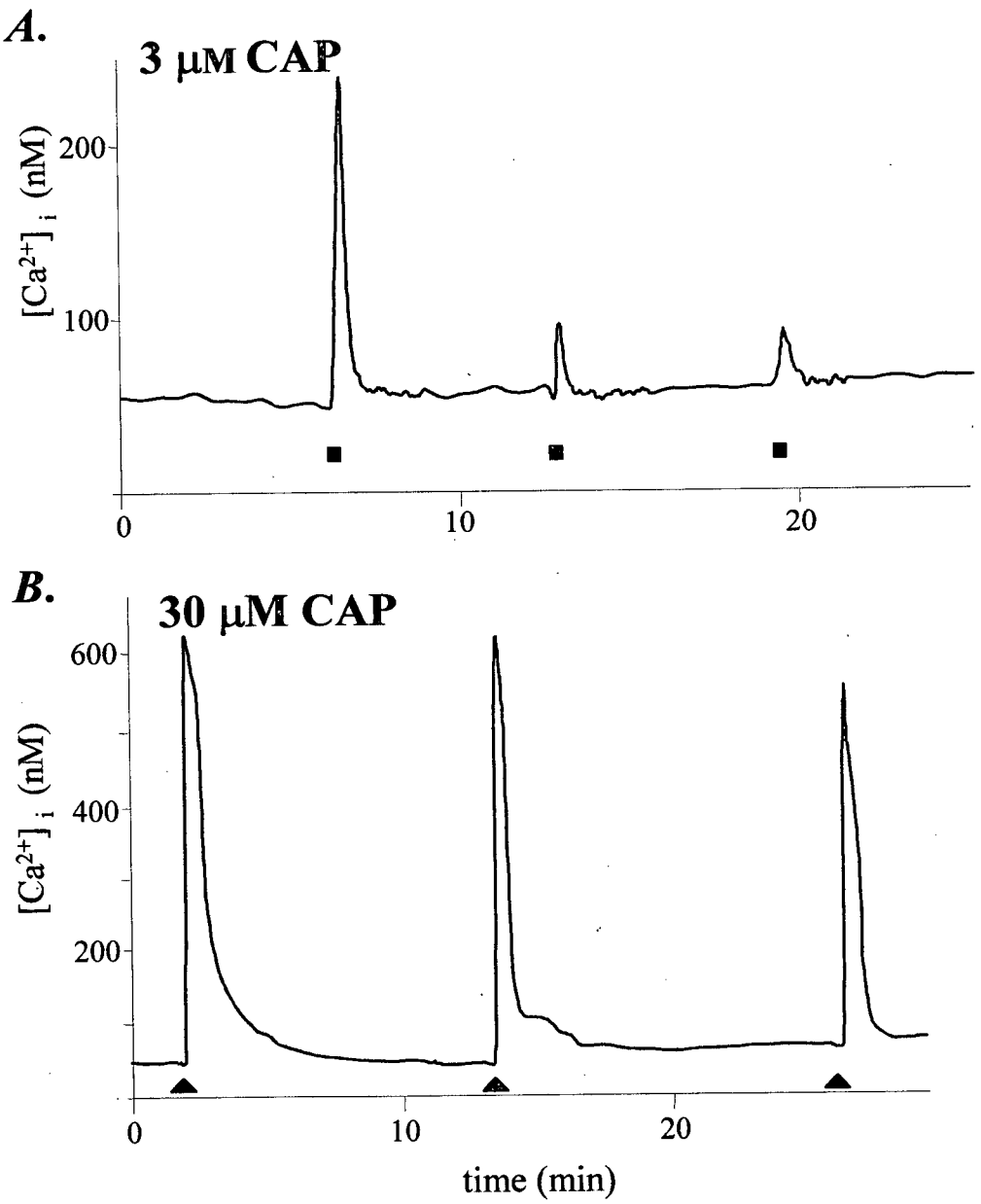

Figure 3*A,B* Capsaicin (CAP)-mediated responses in the MD3 cells. *A*. Application of 3 μM CAP increased $[Ca^{2+}]_i$; however, repeated bath applications led to acute desensitization and tachyphylaxis. *B*. Local applications of 30 μM CAP increased $[Ca^{2+}]_i$ while reducing desensitization. Squares indicate bath applications while arrowheads indicate local applications of CAP.

course. I examined the effects of varying the extracellular Ca^{2+} on the α,β-methylene ATP-mediated response. Application of 100 μM α,β-methylene ATP did not increase $[Ca^{2+}]_i$ in MD3 cells bathing in Ca^{2+}-free recording solution containing EGTA (Figure 4). This figure shows first two responses to α,β-methylene ATP application in Ca^{2+} containing HHBSS buffer, then a no response to α,β-methylene ATP during perfusion of Ca^{2+}-free HHBSS, and finally another response to α,β-methylene ATP in a 1.8 mM Ca^{2+} recording solution. None of the MD3 cells bathed in this manner showed any Ca^{2+} elevation in response to α,β-methylene ATP application during 0 mM Ca^{2+} HHBSS perfusion. Therefore, the rise in $[Ca^{2+}]_i$ depended on extracellular Ca^{2+}. Extracellular EGTA had no effect on the indicator and did not produce obvious morphological deterioration in the cells. Cells contain either purinergic receptors P2X1 or P2X3 that react to α,β-methylene ATP (Collo *et al.*, 1996). P2X3 receptors are present in only CAP-sensitive small-diameter sensory neurons (Chen *et al.*, 1995). Therefore, the sensitivity of MD3 neurons to α,β-methylene ATP is consistent with the presence of purinergic receptors.

3.2. MD3 Cells Express GABA Receptors

I recorded the $[Ca^{2+}]_i$ to determine the mechanisms and effects of GABA-evoked responses in the mouse neuronal MD3 cells. The $[Ca^{2+}]_i$ for MD3 neurons was recorded during and following application of GABA alone and in the presence of various drugs. I monitored the intracellular Ca^{2+} levels to establish baseline levels, then studied $[Ca^{2+}]_i$ following GABA application, or GABA together with GABA antagonists, followed by washout, and finally GABA re-stimulation. The response was biphasic at times, with a rapid, transient rise, declining to a plateau, and finally a gradual decrease to basal levels. Construction of a dose-response curve was not possible because of desensitization to longer applications of GABA. MD3 cells

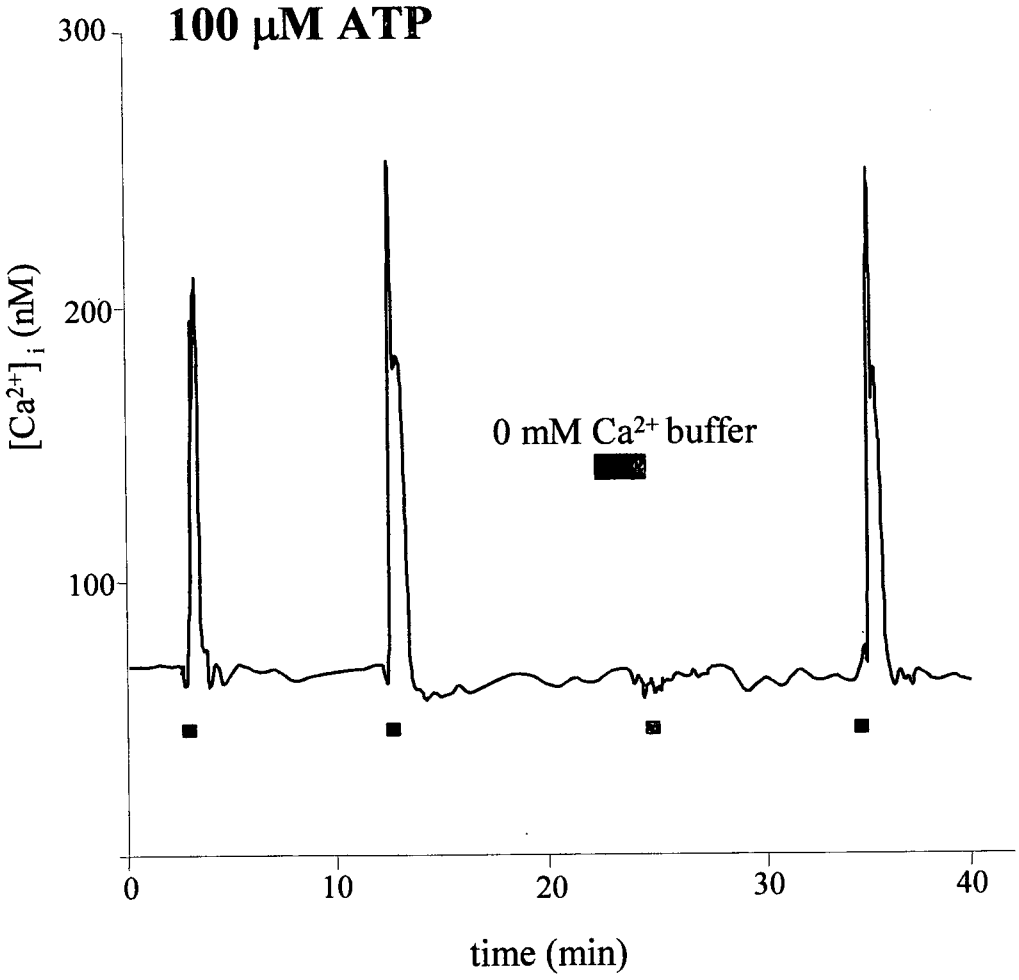

Figure 4 Effect of ATP on hybrid sensory neuronal cells. 100 μM α,β-methylene ATP applications increased $[Ca^{2+}]_i$, implying the presence of purinergic receptors in the MD3 cells. The lack of extracellular Ca^{2+} prevented increases in $[Ca^{2+}]_i$, implying that this rise in $[Ca^{2+}]_i$ depended on the presence of extracellular Ca^{2+}. Filled squares indicate applications of α,β-methylene ATP.

presumably had a high internal [Cl^-] resulting in an efflux of Cl^- on opening of $GABA_AR$-activated channels.

3.2.1. GABA-evoked $[Ca^{2+}]_i$ Elevation

In this study, I identified GABA chemosensitivity with brief pulses of GABA using a HHBSS perfusion system. Applications of GABA (100 μM) evoked repeatable, transient elevations in $[Ca^{2+}]_i$ that recovered to the pre-drug baseline (mean increase in $[Ca^{2+}]_i = 347 \pm 42$ nM; n = 54; Figure 5A). The observed GABA-induced Ca^{2+} increases have similar amplitudes and time courses. MD3 neurons recovered from locally applied GABA within a few minutes, minimizing desensitization. The GABA-evoked changes in intracellular Ca^{2+} had either a simple or complex recovery phase. The complex recovery had an initial fast phase of $[Ca^{2+}]_i$ decline that ended at a level to form a plateau from which $[Ca^{2+}]_i$ recovered at a much slower rate. I, then, investigated the effects of specific $GABA_AR$ agonists to clarify the role of $GABA_ARs$ in the transient increase of $[Ca^{2+}]_i$ in MD3 cells. Similar to GABA, ZAPA (100 μM), a selective and potent agonist of low affinity $GABA_ARs$, also increased $[Ca^{2+}]_i$ of the mouse hybrid DRG neuronal cells (mean increase in $[Ca^{2+}]_i = 393 \pm 44$ nM; n = 24; Figure 6). From these results, I suggest that the MD3 cell line possesses GABARs, more specifically $GABA_ARs$ that were activated, thus increasing $[Ca^{2+}]_i$ caused by the application of GABA or a related agonist.

3.2.2. Effect of Bicuculline on GABA-mediated Response

Application of antagonists of GABAergic transmission were used to examine the nature of the receptors responsible for mediating the response in MD3 cells to GABA stimulation. Applying different compounds that were selective between the $GABA_A$ and $GABA_BR$, I determined the GABAR subtype responsible for the production of the $[Ca^{2+}]_i$ transients. As mentioned in the Introduction, the $GABA_AR$ is part of a superfamily of ligand-gated ion

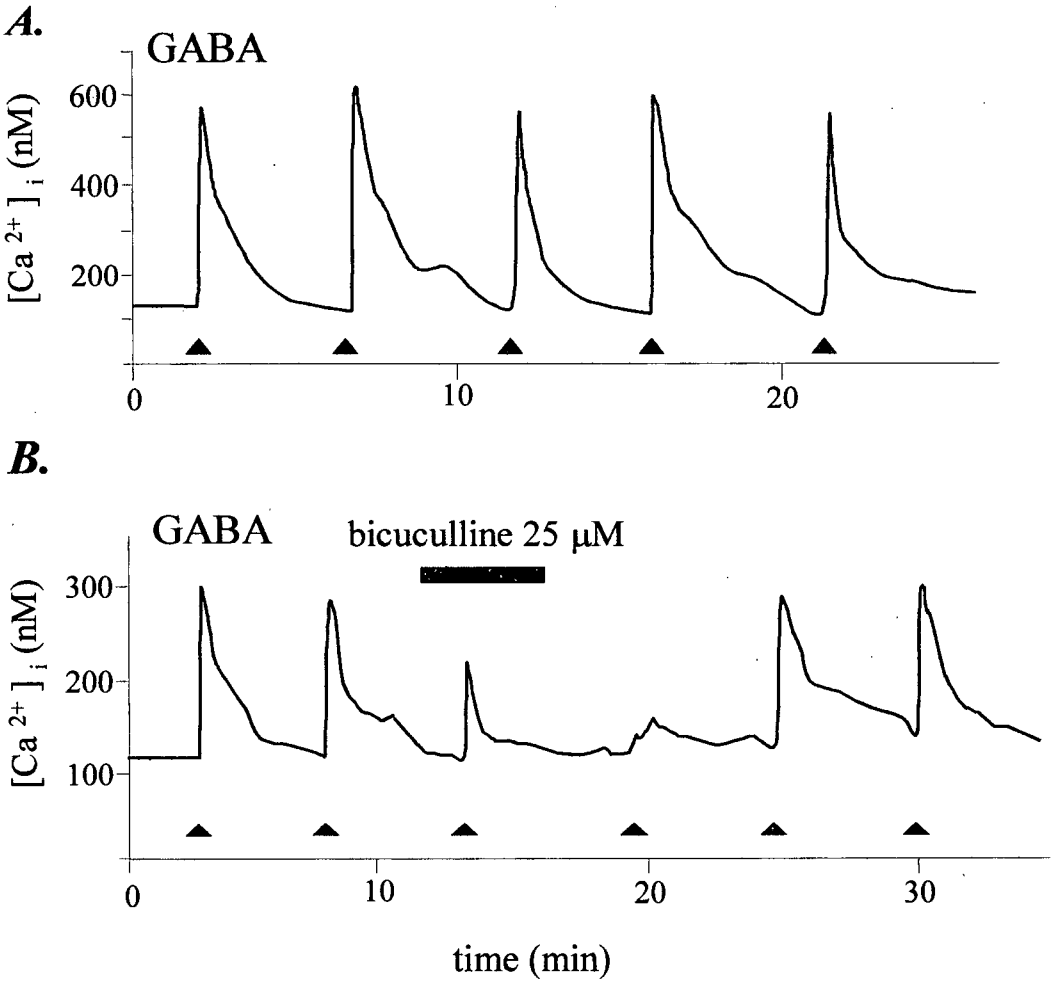

Figure 5*A,B* GABA-induced $[Ca^{2+}]_i$ increases and antagonism of $GABA_A$ receptor. *A.* Repeated, control responses to 100 μM GABA in MD3 cells led to rises in intracellular free Ca^{2+}, $[Ca^{2+}]_i$. *B.* 25 μM bicuculline antagonized the $GABA_A$ receptor and decreased in the rise of intracellular Ca^{2+}. The arrowheads represent local application of GABA. The solid, black bar represents the bath application of bicuculline for 5 min.

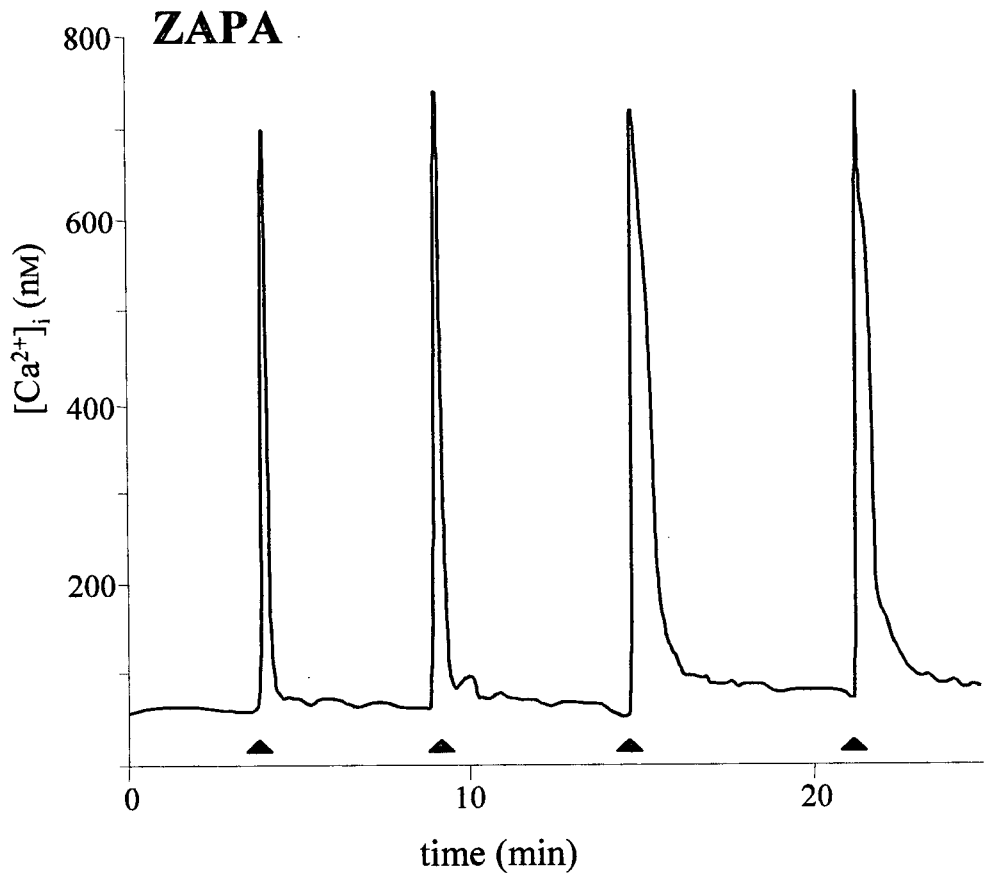

Figure 6 Effects of ZAPA. Local applications of ZAPA (100 μM), a GABA agonist, increased the cytoplasmic free Ca^{2+}, similar to GABA-evoked responses. Arrowheads indicate applications of ZAPA.

channels that on activation increases Cl^- conductance. Applications of $GABA_AR$ antagonists confirmed the involvement of $GABA_ARs$ in increasing internal Ca^{2+} for the hybrid DRG neuronal cells. In the recording chamber, bath application of BIC to the MD3 cells, at concentrations ranging from 100 nM-100 μM, reversibly blocked the increase in $[Ca^{2+}]_i$, resulting from local applications of GABA. BIC reversibly reduced the amplitude of the GABA-mediated response in MD3 neurons but did not alter the time course of the responses (n = 13). Figure 5*B* illustrates the time courses of GABA-activated responses under control conditions and during bath application of BIC. This figure shows first GABA-mediated control responses, followed by pre-treatment of BIC for a period of 4 min, followed by a GABA stimulation of the neurons with BIC present. The concentration-response curve for BIC-mediated inhibition of GABA-induced increase in $[Ca^{2+}]_i$ showed an ED_{50} = 9.3 μM (Figure 7, cf. with Table 1). I suggest from the BIC and ZAPA experiments provide evidence that $GABA_ARs$ influence increases in $[Ca^{2+}]_i$. With BIC present, the amplitude of GABA-evoked rise in intracellular Ca^{2+} was reversibly depressed. However, BIC did not affect the baseline level of $[Ca^{2+}]_i$ during pre-treatment of cells prior to co-application with GABA at the concentrations studied.

3.2.3. Effect of Picrotoxinin and Diazepam on MD3 Neuronal Cells

As with BIC, PTX application did not affect the resting $[Ca^{2+}]_i$ levels in the MD3 cells. When PTX was bath applied for 5 min, as shown in Figure 8, it did not reduce the amplitude of $[Ca^{2+}]_i$ transients evoked by 100 μM GABA (n = 9). Simultaneous application of GABA with 10 μM PTX potentiated the GABA-activated response by an average of 18 ± 4 % over control values. PTX is known to depress GABA-stimulated responses in a non- competitive manner (Gallagher *et al.*, 1978; Akaike *et al.*, 1985); however, in this experiment, PTX did not inhibit the GABA-mediated response. In addition, I applied diazepam to determine if the

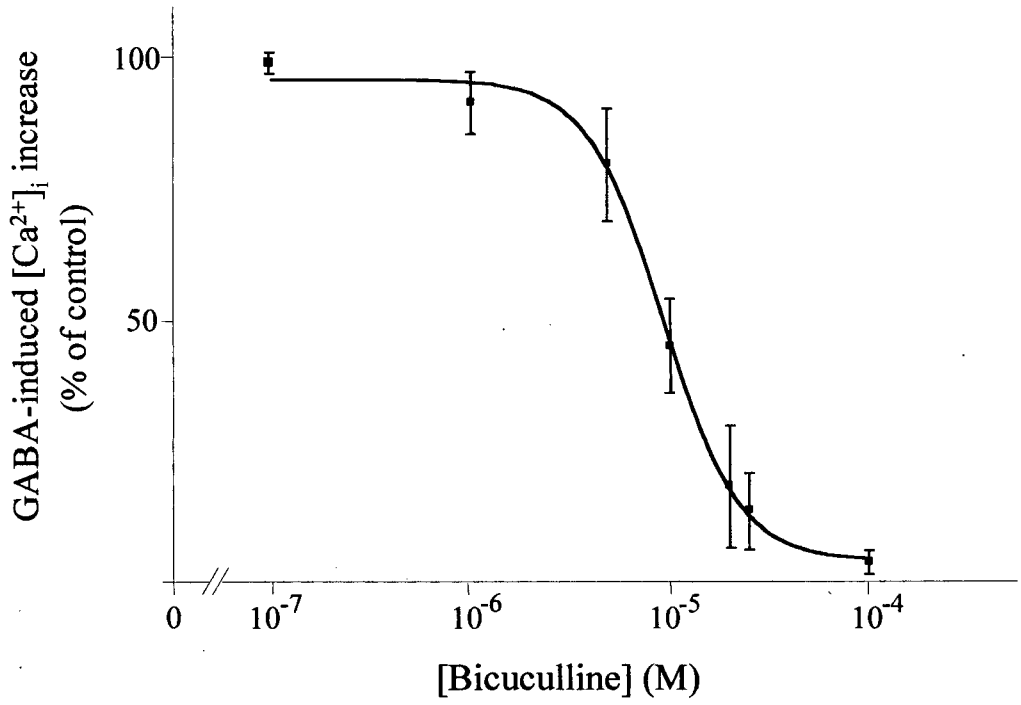

Figure 7 Concentration-response relationship for bicuculline-induced blockade of GABA-mediated increase in $[Ca^{2+}]_i$, (cf. Table 1). GABA was locally applied to MD3 cells to elicit the control responses. Then, bath application of bicuculline (100 nM-100 μM) blocked the GABA-evoked increase in $[Ca^{2+}]_i$. The inhibition was calculated as a percentage of the averaged controls. The ED_{50} for bicuculline was 9.3 μM. The relationship between the mean % inhibition and the logarithm of the bicuculline concentration was fitted with a sigmoidal function using GraphPad software. Data represent mean ± SEM (%) (n = 3-5).

Table 1 Effects of bicuculline on GABA-induced increase in $[Ca^{2+}]_i$. The control responses were averaged elevation in $[Ca^{2+}]_i$ due to two local applications of GABA (100 μM). Bath application of bicuculline (100 nM-100 μM) blocked the responses due to local application of GABA. The percent inhibition of the GABA-evoked response was calculated as a fraction of the averaged control values. The values are expressed as mean ± SEM (%). The p values indicate statistical differences from control values (Student's t-test).

[Bicuculline][a] (μM)	$[Ca^{2+}]_i$ (% of control)[b]	P value	n
0.1	99.3 ± 7.0	0.6845	4
1	91.8 ± 5.9	0.1541	4
5	80.0 ± 10.8	0.0731	4
10	45.2 ± 8.9	0.0005	5
20	18.3 ± 11.7	0.0001	5
25	13.6 ± 7.3	< 0.0001	3
100	3.8 ± 2.4	< 0.0001	4

[a] Bicuculline was applied for 5 min.
[b] Average of 2 responses to GABA applied from micropipette

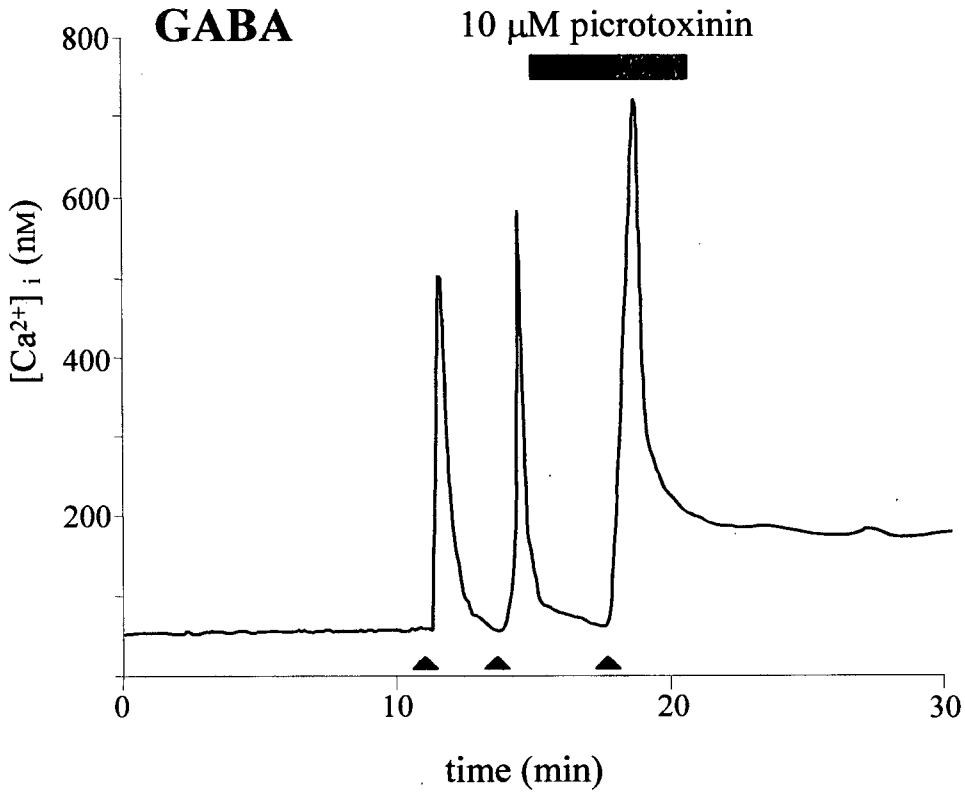

Figure 8 Effects of picrotoxinin. PTX (10 μM) was bath applied to MD3 cells for 5 min prior to GABA (100 μM) stimulation. PTX did not inhibit the GABA response. The arrowheads indicate local applications of GABA. The black, solid bar represents applications of PTX.

benzodiazepine receptor is an integral part of the $GABA_AR$s in MD3 neurons, as described in DRG neurons (Akaike *et al.*, 1989). Bath applications of diazepam (1-50 μM) for 20 min did not significantly affect the $[Ca^{2+}]_i$ responses evoked by local applications of GABA (n = 11; data not shown). The failure of diazepam applications to potentiate the GABA-stimulated response implies that some differences in $GABA_AR$s may exist between MD3 cells and DRG neurons (see Discussion).

3.3. Mechanism of Increase in $[Ca^{2+}]_i$

3.3.1. Absence of Ca^{2+} in the Extracellular Environment

I examined the possible ionic mechanisms involved in the GABA-induced response leading to an increase in $[Ca^{2+}]_i$. To determine the route involved in raising internal Ca^{2+} subsequent to $GABA_AR$ activation, I observed the effect of changes in extracellular Ca^{2+} on the GABA-evoked response. During the perfusion of Ca^{2+}-free extracellular solution (0 mM Ca^{2+}), GABA (100 μM) did not raise the $[Ca^{2+}]_i$, as illustrated in Figure 9*A*. First, control GABA-evoked responses were established in regular recording buffer with 1.8 mM Ca^{2+}, and then the hybrid neurons were perfused in Ca^{2+}-free media with local applications of GABA. The MD3 cells, in the absence of extracellular Ca^{2+}, failed to respond to GABA. Previous studies have reported that incubation of cells in Ca^{2+}-free buffer depletes internal Ca^{2+} stores (Matsuda *et al.*, 1997; Kerper & Hinkle, 1997). The perfusion time was about one minute to avoid depleting intracellular Ca^{2+} stores while exposing MD3 cells to Ca^{2+}-free media. The rate of perfusion enabled complete solution exchange within 30 s. During the replacement of Ca^{2+}-free solution back to normal buffer, applications of GABA were effective again in increasing $[Ca^{2+}]_i$. Also, during the absence of extracellular Ca^{2+}, I applied thapsigargin (1-10 μM) and caffeine (1-10 mM) in the bath for 5 min prior to local application of GABA in 15 cells. Pre-treatment with

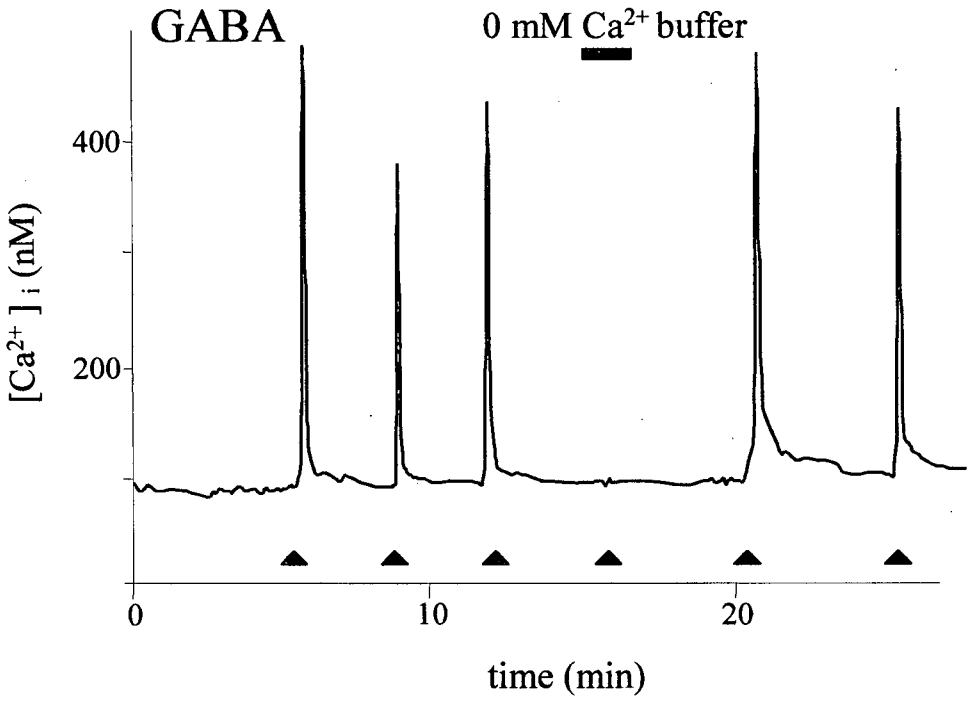

Figure 9 GABA-mediated response with changes in extracellular Ca^{2+}. GABA (100 μM) application during perfusion of Ca^{2+}-free buffer resulted in the disappearance of the increase in $[Ca^{2+}]_i$. The presence of extracellular Ca^{2+} was required to initiate the GABA-mediated response. The arrowheads represent GABA stimulation of MD3 cells. The solid bar represents a 1 min bath application of Ca^{2+}-free HHBSS buffer.

either thapsigargin or caffeine produced little or no change in the baseline $[Ca^{2+}]_i$. The application of either thapsigargin or caffeine also produced no difference in the amplitude of the GABA-induced increase in $[Ca^{2+}]_i$ (data not shown). The inability of the hybrid sensory neuronal cells to respond to GABA in Ca^{2+}-free solution and the return of the GABA-mediated response following the return of Ca^{2+}-containing buffer imply that the increase in $[Ca^{2+}]_i$ following GABA application was a result of transmembrane influx of Ca^{2+}, requiring a Ca^{2+} environment to observe intracellular Ca^{2+} transients. The rise in $[Ca^{2+}]_i$ resulted from a Ca^{2+} influx and not release from internal stores. No indicator signal decrease or cell deterioration occurred from the EGTA in the Ca^{2+}-free HHBSS.

3.3.2 GABA-induced Ca^{2+} Transient Inhibition with Dihydropyridines

My hypothesis was that GABA stimulation of receptors in MD3 cells increased intracellular free Ca^{2+} through VGCCs. Therefore, to determine the consistency of this hypothesis, I assessed the effects of Ca^{2+} channel blockers on VGCC-mediated increase in $[Ca^{2+}]_i$. Neuronal *L-type* Ca^{2+} channels are sensitive to DHP antagonists (Fox *et al.*, 1987a,b; Aosaki & Kasai, 1989). GABA was applied to the hybrid DRG neuronal cells in the presence of nimodipine and nifedipine. I studied the changes in $[Ca^{2+}]_i$ in MD3 cells during and after applications of GABA alone as well as in the presence of a dihydropyridine (DHP). Intracellular Ca^{2+} levels were first monitored for at least 2 min to establish basal levels, then during GABA application, followed by application of GABA and a DHP, and finally GABA alone after washout of the Ca^{2+} channel blocker. Nifedipine and nimodipine, at concentrations of 1-20 μM, attenuated elevations in $[Ca^{2+}]_i$ (n = 27; Figures 10,11). The VGCC blockers were both bath applied, inhibiting the GABA-induced increase in cytoplasmic free Ca^{2+} in the hybrid neuronal cells. Therefore, I suggest the GABA-induced response results from a depolarization because of a transmembrane influx of Ca^{2+} through voltage-gated *L-type* Ca^{2+} channels.

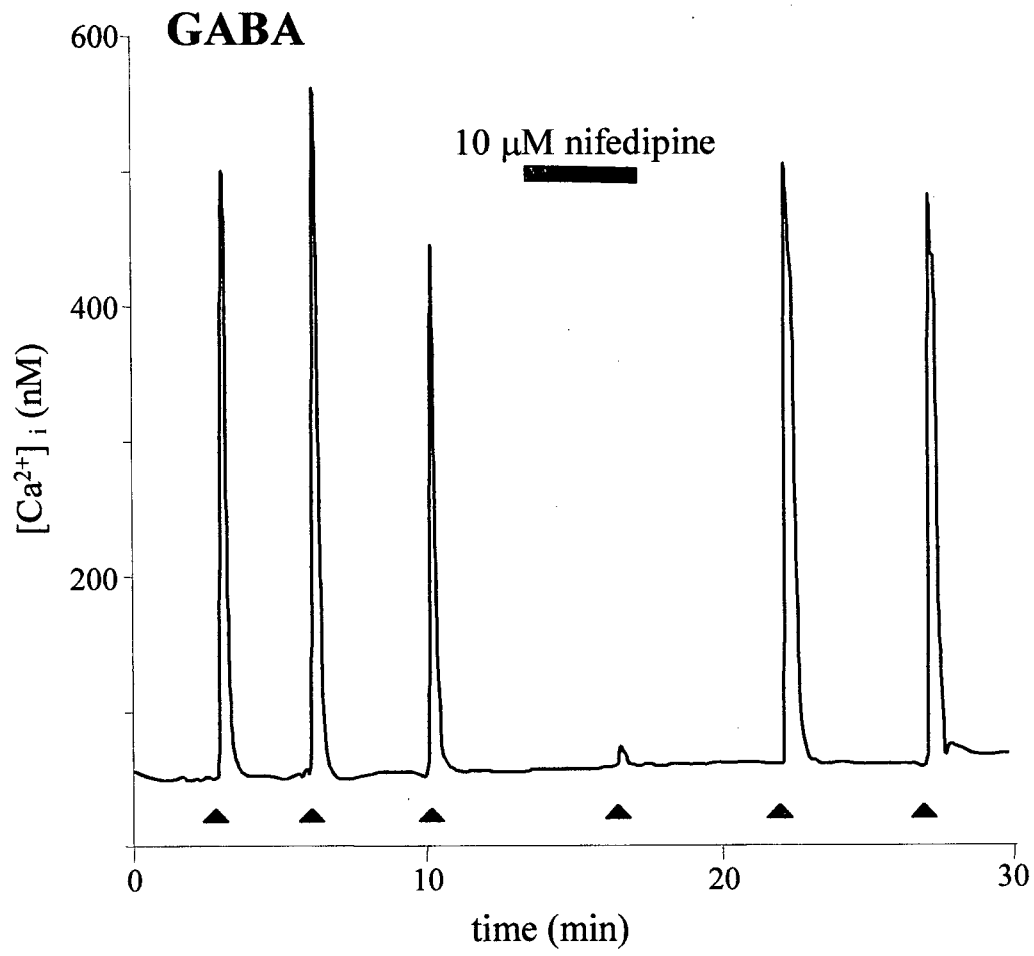

Figure 10 Nifedipine-induced inhibition of GABA-evoked response. Nifedipine (10 μM), an L-type Ca^{2+} channel blocker, inhibited the transient increases in $[Ca^{2+}]_i$. Nifedipine was bath applied for 5 min while GABA was locally applied simultaneously. The arrowheads represent local applications of GABA (100 μM). The solid, black bar represents bath application of nifedipine.

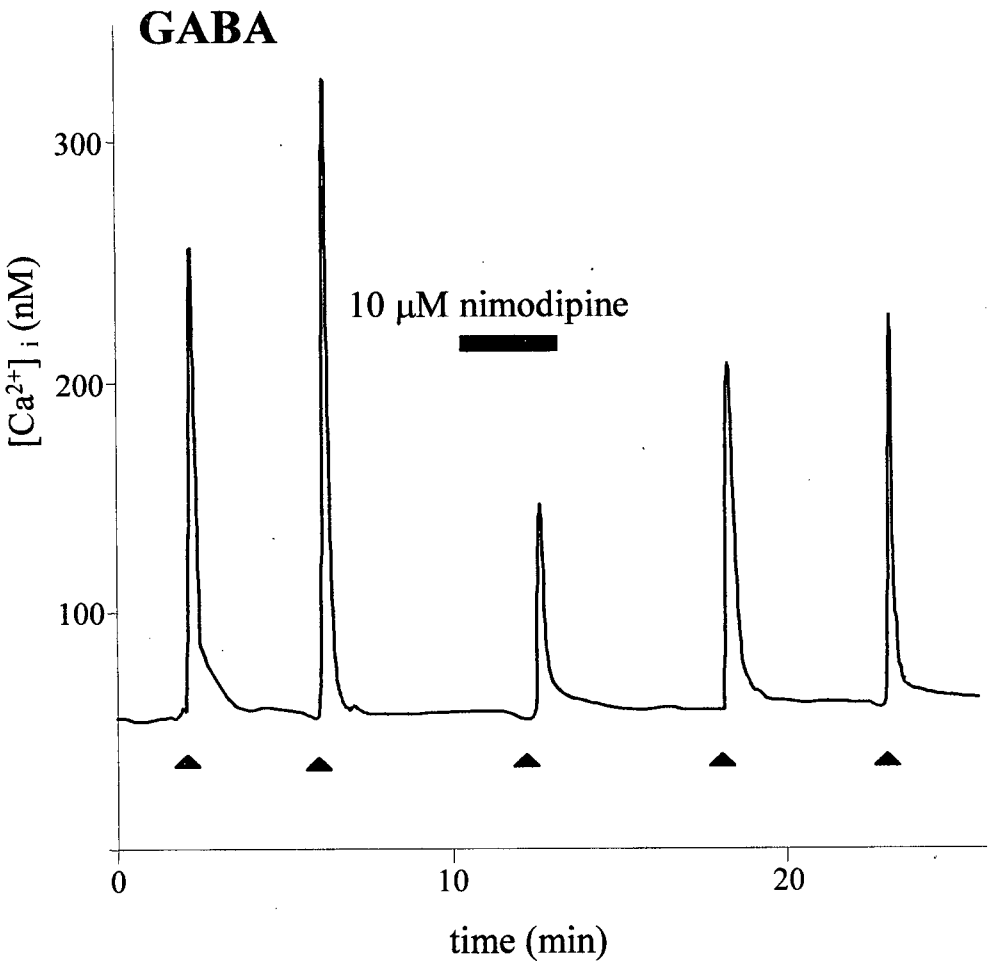

Figure 11 Nimodipine-induced inhibition of GABA-mediated response. Nimodipine (10 μM), an *L-type* Ca^{2+} channel blocker, attenuated the increase in $[Ca^{2+}]_i$ from GABA application. Nimodipine was bath applied for 5 min, followed by a picospritzer application of GABA. The arrowheads represent local applications of GABA (100 μM). The solid, black bar represents bath application of nimodipine.

Extended washing of 10–15 min failed to reverse the effects of nifedipine and nimodipine completely in 12 out of 27 neurons. Both *L-type* Ca^{2+} channel blockers were first dissolved in alcohol and then diluted in HHBSS to a final concentration of 0.01 % alcohol. However, the vehicle, alcohol, had no effect on the basal $[Ca^{2+}]_i$ when cells were applied with GABA in the presence of 0.01 % alcohol-containing HHBSS (data not shown).

3.4. Ionic Mechanism of Depolarization

3.4.1. Depolarization-induced $[Ca^{2+}]_i$ Increase

I also studied K^+-evoked Ca^{2+} entry through voltage-sensitive Ca^{2+} channels in the sensory neuron-derived hybrid cells. High external K^+ depolarizes excitable cells and, consequently, induces Ca^{2+} influx via VGCCs (Benham *et al.*, 1992). Increasing K^+ from its normal level of 2.5 mM to 25 mM elevated $[Ca^{2+}]_i$ in MD3 cells (mean increase in $[Ca^{2+}]_i$ = 538 ± 60 nM; n = 58). Figure 12 illustrates the effect of local applications of K^+ (25 mM) and the subsequent depolarization-induced elevations in $[Ca^{2+}]_i$. When the extracellular Ca^{2+} was replaced with EGTA, no change in $[Ca^{2+}]_i$ resulted from the depolarization with high K^+. Similar to the GABA-mediated response in the absence of external Ca^{2+}, I did not observe an elevation of $[Ca^{2+}]_i$ following K^+-evoked depolarizations when the external solution contained no Ca^{2+}. The response returned following a 3-5 min wash in Ca^{2+}-containing HHBSS buffer. I suggest that the MD3 neurons possess VGCCs that mediate Ca^{2+} when depolarized with K^+. As with GABA-mediated responses, one type of recovery was smooth while the other pattern, observed at times, was more complicated. The initial fast decline of $[Ca^{2+}]_i$ decline ended at a level from which $[Ca^{2+}]_i$ recovered at a much slower rate. Adverse effects of high K^+ solutions

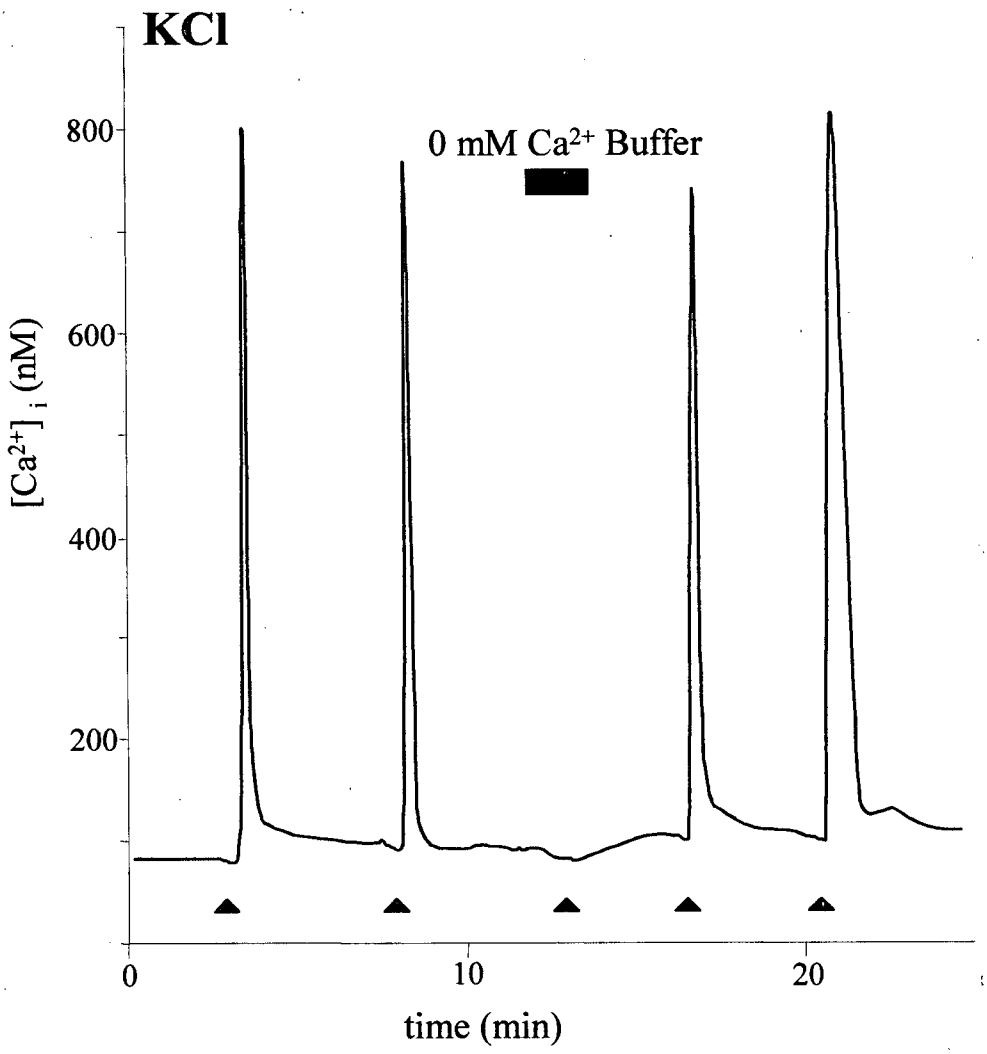

Figure 12 K^+-evoked $[Ca^{2+}]_i$ increase. Local applications of K^+ (100 μM) depolarized hybrid cells and increased their cytoplasmic free Ca^{2+}. When Ca^{2+} was removed from the external solution, no increase in $[Ca^{2+}]_i$ occurred with high K^+ application. However, on return of extracellular Ca^{2+} perfusion, the depolarization-evoked $[Ca]_i$ increases returned. Bar indicates perfusion of zero Ca^{2+} buffer. Arrowheads represent applications of KCl.

were not apparent on the cells because the increase in $[Ca^{2+}]_i$ returned to baseline levels.

3.4.2. Inhibition of K^+-evoked Increase in $[Ca^{2+}]_i$

As with GABA-induced responses, I investigated the effects of Ca^{2+} channel blockers on the K^+-stimulated Ca^{2+} increase. I observed the effects of nifedipine and nimodipine on voltage-dependent Ca^{2+} influx in mouse hybrid DRG neuronal cells in culture. K^+ evoked depolarizations that activate VGCCs, resulting in $[Ca^{2+}]_i$ increases as a consequence of Ca^{2+} influx. To confirm this mechanism, I applied nifedipine and nimodipine to inhibit Ca^{2+} influx through VGCCs. When 25 mM K^+ stimulated the MD3 cells, control responses elevated cytosolic Ca^{2+}. However, pre-treatment of hybrid sensory neuronal cells with nimodipine (1-20 μM) and nifedipine (1-20 μM) inhibited the increase in $[Ca^{2+}]_i$ due to K^+ application (n = 20; Figures 13,14). Full recovery from dihydropyridine application occurred in 10 out of 20 MD3 cells. In some cells (n = 10), however, extended washing for about 10-15 min only partially reversed the total blockade produced by either nimodipine or nifedipine. From the above data, I suggest that similar to GABA, K^+-mediated responses involved Ca^{2+} influx through *L-type* VGCCs in the MD3 cells.

3.5. Activation of $GABA_B$ Receptor

3.5.1. $GABA_B$ Receptor Agonism

Using baclofen, an agonist at $GABA_BRs$, I investigated the presence of $GABA_BRs$ on the MD3 cells. Therefore, in order to observe the effects of $GABA_BR$ stimulation, I first used K^+ to depolarize the cells while applying baclofen. Baclofen application (50 nM-100 μM) for 5

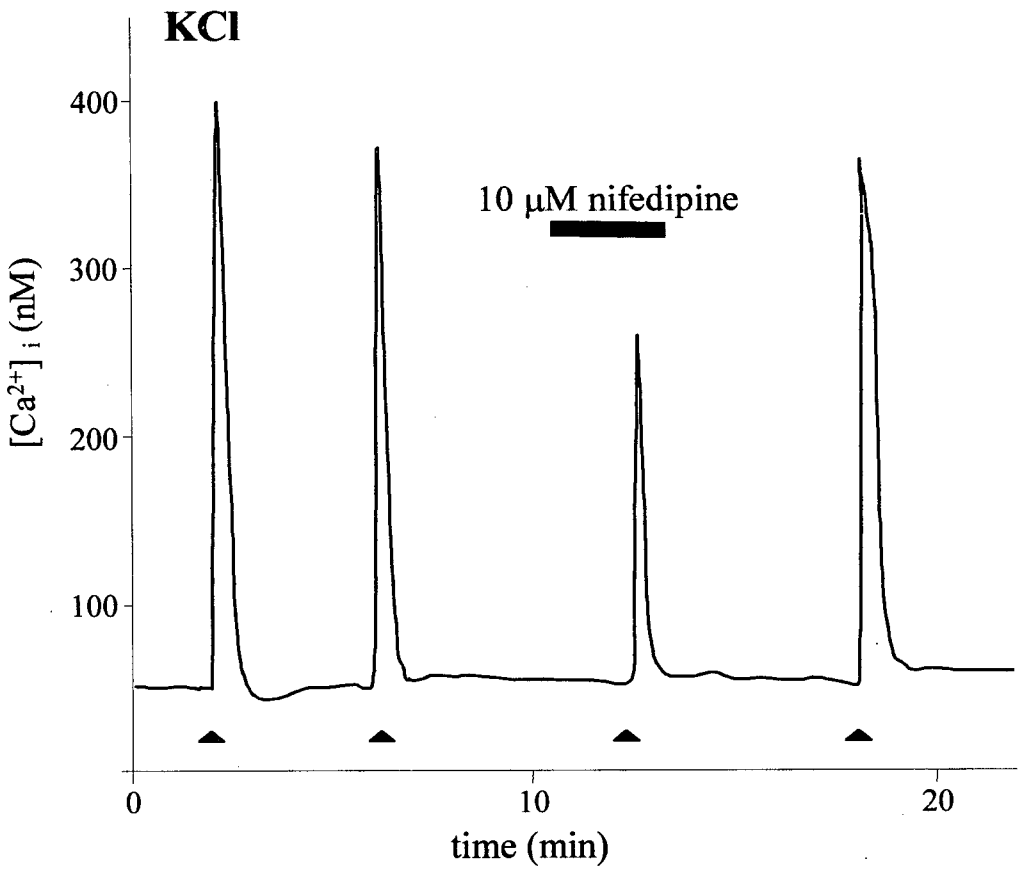

Figure 13 Nifedipine-induced inhibition of K^+-mediated $[Ca^{2+}]_i$ increases. Nifedipine (10 μM), an L-type Ca^{2+} channel blocker, inhibited the transient increases in $[Ca^{2+}]_i$. Nifedipine was bath applied for 5 min while KCl (25 mM) was locally applied intermittently. The arrowheads represent local applications of KCl. The solid, black bar represents bath application of nifedipine.

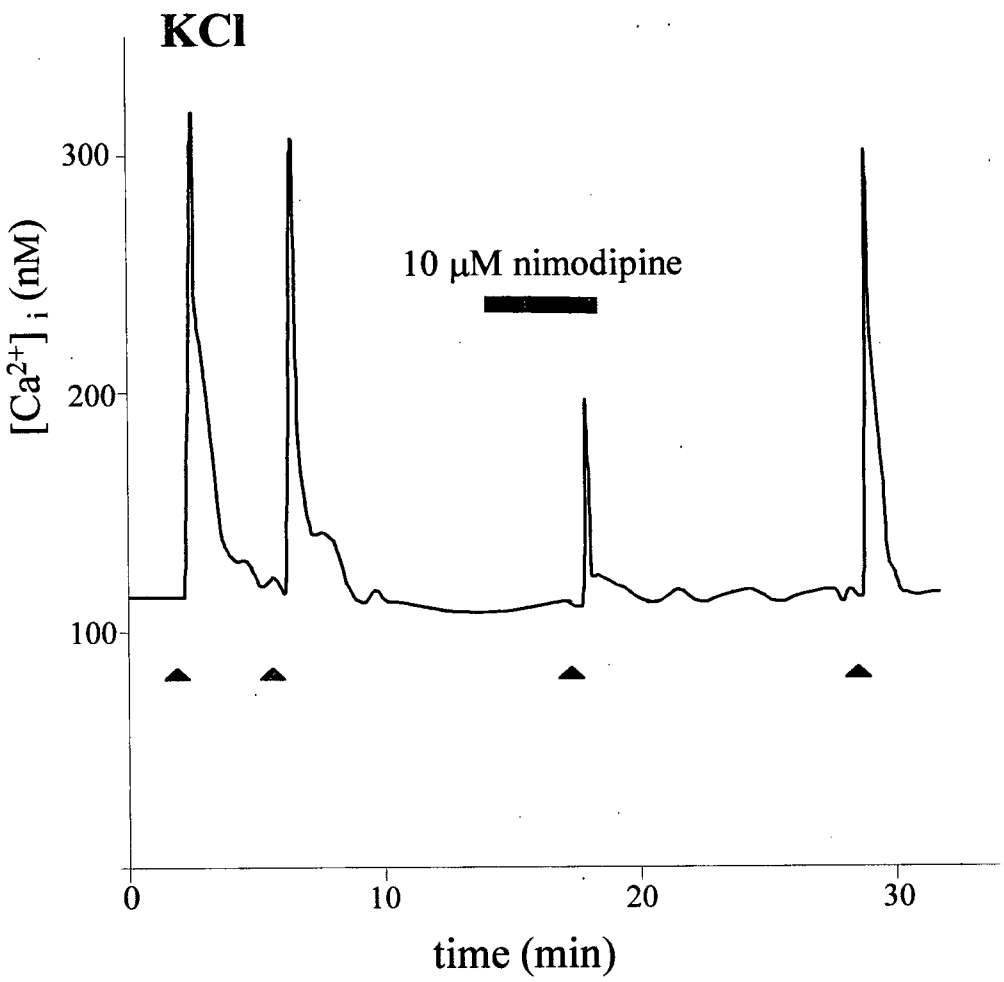

Figure 14 Nimodipine-induced inhibition of K^+-mediated depolarization. Nimodipine (10 μM), an L-type Ca^{2+} channel blocker, attenuated the transient increases in $[Ca^{2+}]_i$. Nimodipine was bath applied for 5 min while K^+ was applied locally. The arrowheads represent local applications of KCl (25 mM). The solid, black bar represents bath application of nifedipine.

min did not produce significant effects on the baseline values of $[Ca^{2+}]_i$ (n = 87). However, during the application of K^+ (25 mM), (±)-baclofen (500 nM-100 μM) significantly reduced the amplitude of the K^+-induced $[Ca^{2+}]_i$ transients by 18–100 % (n = 51; Figure 15*A*). Baclofen (50 nM-100 μM) did not cause any perturbations in baseline $[Ca^{2+}]_i$, implying that there was no constitutive activity involving $GABA_BRs$. The graph in Figure 16 shows the concentration-response curve for baclofen-induced inhibition of K^+-evoked increase in $[Ca^{2+}]_i$ which revealed an ED_{50} = 1.8 μM (cf. with Table 2). In these experiments, the K^+ applications presumably depolarized the MD3 cells, activating VGCCs that permitted Ca^{2+} influx that increased $[Ca^{2+}]_i$.

3.5.2. $GABA_B$ Receptor Antagonism

Once I determined that the MD3 cells possessed $GABA_BRs$, I investigated the ability of 2-hydroxy-saclofen (1-20 μM) to antagonize the inhibitory effect of baclofen (500 nM-10 μM) on the K^+-induced increase in $[Ca^{2+}]_i$ (n = 18). 2-hydroxy-saclofen (1 μM) was bath applied for the first 4 min, followed by a 4 min co-application with baclofen (1 μM). Firstly, bath application of 2-hydroxy-saclofen for 4-8 min had little effect on the baseline $[Ca^{2+}]_i$. However, I observed that 2-hydroxy-saclofen antagonized the effects of baclofen on the K^+-mediated increase in $[Ca^{2+}]_i$. Before 2-hydroxy-saclofen application, baclofen application decreased the rise in $[Ca^{2+}]_i$ by 25-85 % but 2-hydroxy-saclofen prevented any further effects of baclofen-mediated blockade of the K^+-evoked increase in $[Ca^{2+}]_i$ (n = 11; Figure 15*B*). Application of 2-hydroxy-saclofen (1-20 μM) also antagonized the subsequent responses to baclofen. 2-hydroxy-saclofen blocked the effects of baclofen-mediated inhibition of KCl-evoked elevation in $[Ca^{2+}]_i$ (n = 7 ; Figure 17). Therefore, the results show that 2-hydroxy-saclofen antagonized baclofen and blocked the inhibitory effect of baclofen on K^+-mediated increase in $[Ca^{2+}]_i$ through VGCCs. When K^+ presumably depolarized the MD3 cells during co-applications of 2-hydroxy-saclofen and baclofen, the response to K^+ did not decrease as observed during baclofen

application alone (see Figure 15*A*). The above results with 2-hydroxy-saclofen imply that MD3 cells have functional $GABA_BRs$ in addition to $GABA_ARs$.

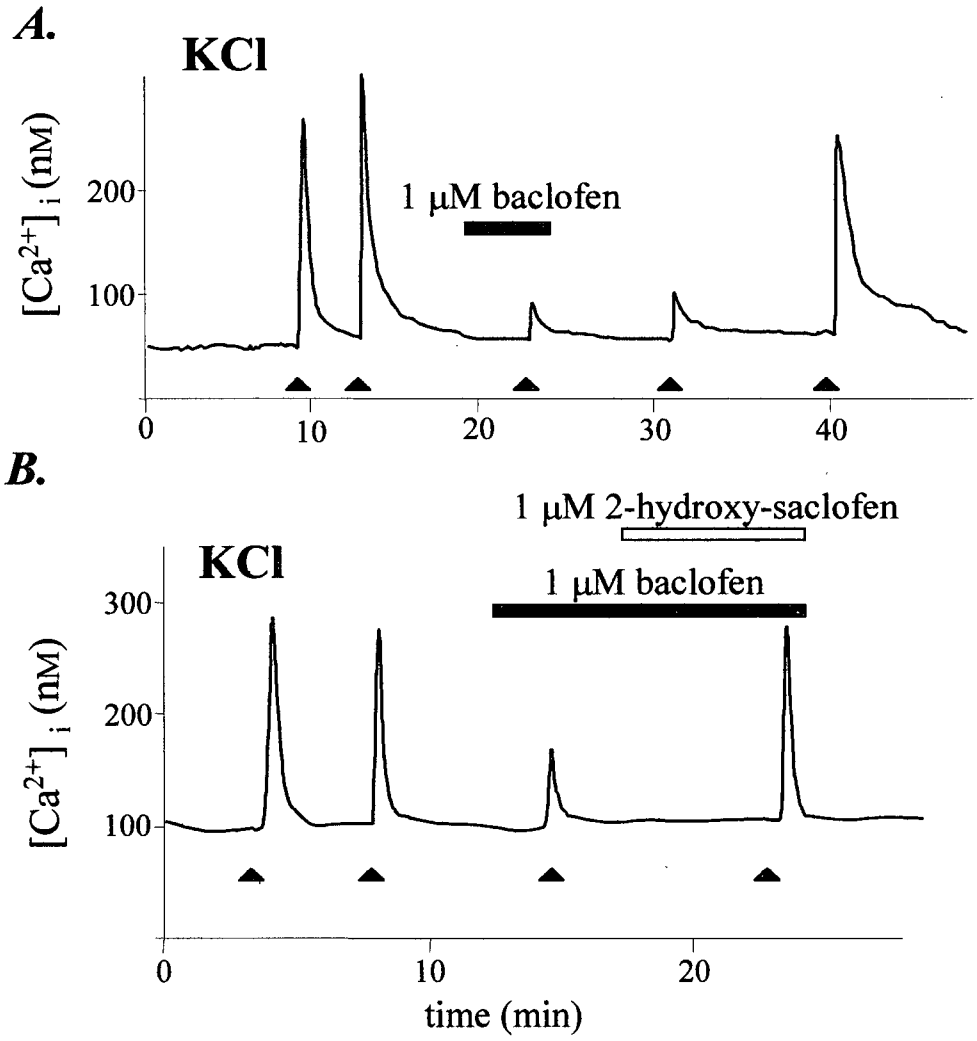

Figure 15*A,B* $GABA_B$ receptor activation and antagonism. *A*. Using KCl to depolarize cells, baclofen (1 μM) decreased the rise in $[Ca^{2+}]_i$. *B*. 2-hydroxy saclofen (1 μM) antagonized the baclofen-induced inhibitory response. The arrowheads represent local applications of KCl (25 mM). The solid, black and white lines represent bath applications of baclofen (10 min) and 2-hydroxy saclofen (4 min), respectively.

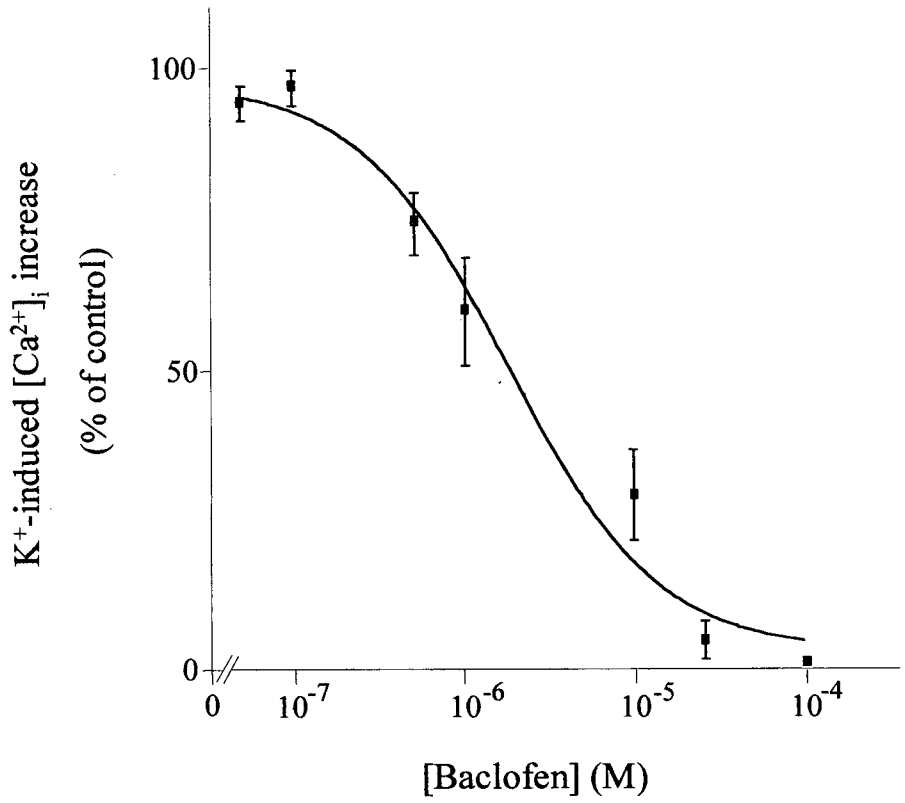

Figure 16 Concentration-response relationship for baclofen-mediated inhibition of the KCl-induced increase in $[Ca^{2+}]_i$, (cf. Table 2). First, KCl was locally applied to MD3 cells for control responses. Then, during bath application of baclofen (50 nM-100 μM) blocked the KCl-evoked increases in $[Ca^{2+}]_i$. The inhibition was calculated as a percentage of two averaged controls. The ED_{50} for baclofen was 1.8 μM. The relationship between the mean % inhibition and the logarithm of the baclofen concentration was fitted with a sigmoidal function using GraphPad software. Data represent mean ± SEM (%) (n = 4-19).

Table 2 Effects of baclofen on the KCl-induced increase in $[Ca^{2+}]_i$. The control responses were an averaged elevation in $[Ca^{2+}]_i$ due to two local applications of KCl (25 mM). Then, bath application of baclofen (50 nM-100 μM) blocked the increase in $[Ca^{2+}]_i$ due to KCl. The percent inhibition of the KCl-mediated response was calculated as a fraction of the averaged control values and expressed as mean ± SEM (%). The p values indicate statistical differences from control values (Student's t-test).

[Baclofen][a] (μM)	$[Ca^{2+}]_i$ (% of control)[b]	P value	n
0.05	94.0 ± 2.8	0.1975	19
0.1	96.7 ± 2.8	0.4595	18
0.5	74.3 ± 5.2	0.0080	15
1	59.6 ± 8.8	0.0107	14
10	28.8 ± 7.6	< 0.0001	13
25	4.6 ± 3.0	< 0.0001	6
100	0.8 ± 0.5	< 0.0001	4

[a] Baclofen was applied for 4 min
[b] Average of 2 responses to KCl applied from micropipette

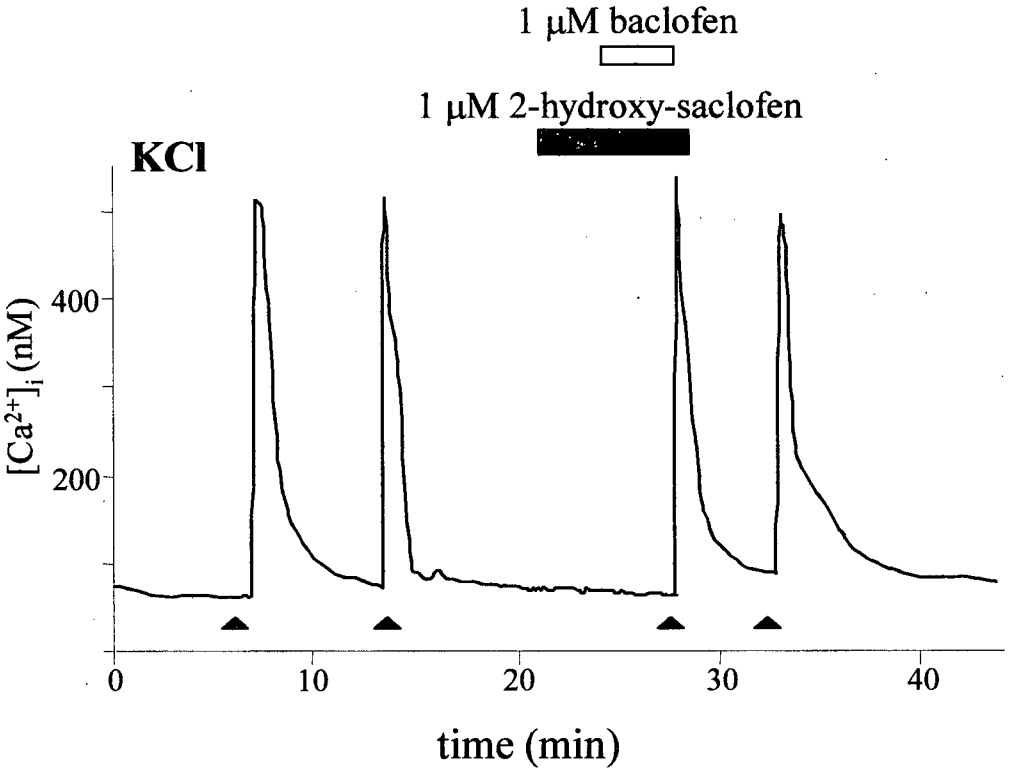

Figure 17 Reverse application of 2-hydroxy-saclofen and baclofen. The hybrid cells were first treated with 2-hydroxy-saclofen, followed by baclofen. 2-hydroxy-saclofen prevented subsequent application of baclofen from influencing the K^+-mediated increase in $[Ca^{2+}]_i$. Arrowheads indicate K^+ (25 mM) application. The solid, black and white bars represent bath applications of baclofen (1 μM) and 2-hydroxy saclofen (1 μM), respectively.

Chapter IV. DISCUSSION

I studied the $[Ca^{2+}]_i$ changes in a mouse hybrid sensory neuronal cell line MD3, generated from mouse DRG sensory neurons and N18TG2 neuroblastoma cells in culture, using Ca^{2+} imaging with fluorescent Ca^{2+}-chelator fura-2. Fura-2 was trapped inside the cells and visualized using digital imaging of the fluorescence (Williams *et al.*, 1985; Connor, 1985, 1986). Fura-2 was found to be effective in competing with the endogenous Ca^{2+} buffers, to capture virtually all incoming Ca^{2+} (Bollman *et al.*, 1998). Upon binding Ca^{2+} ions, the excitation maxima of fura-2 are shifted (Thayer *et al.*, 1988). The indicator method does not give a quantitative measure of the Ca^{2+} influx. However, this procedure directly provides a measure of the $[Ca^{2+}]_i$ perturbations. The technique is very powerful, because Ca^{2+} currents or channel activity is extremely sensitive to the internal dialysis that accompanies methods such as suction-electrode or whole-cell patch recording (Fedulova *et al.*, 1981; Byerly & Hagiwara, 1982; Fenwick *et al.*, 1982).

4.1. Identifying the MD3 Cell Line

4.1.1. Immunocytochemistry

The immunocytochemical findings show that the MD3 cells were neuronal in nature. Immunocytochemical detection of cytoskeletal proteins indicates that MD3 cells have a neuronal phenotype (Lee *et al.*, 1982). Reactivity to monoclonal Abs NF-H and MAP-2 provided an identification of MD3 cells as shown in Figure 2. MAP-2 and neurofilament immunoreactivity also exists in DRG neurons (Naves *et al.*, 1996).

4.1.2. Capsaicin-evoked Response

CAP causes intense burning pain because of its excitatory action on vanilloid receptors of sensory neurons (Geppetti *et al.*, 1988; Simone *et al.*, 1989). The CAP-activated channels are permeable to cations, including Ca^{2+} (Cholewinski *et al.*, 1993; Koplas *et al.*, 1997).

Applications of CAP to the MD3 cells provided a tentative identification that they possessed nociceptive properties similar to sensory neurons *in vivo*. From Figure 3*A*, for example, the hybrid cells responded to 3 μM CAP, raising the $[Ca^{2+}]_i$. The cells showed acute desensitization, a diminished response during constant CAP application, as well as tachyphylaxis, a decreased response to successive applications to CAP. In Figure 3*A*, consecutive bath applications of CAP decreased the increases in cytoplasmic Ca^{2+} with successive stimulation, illustrating both acute desensitization and tachyphylaxis. Acute desensitization was observed as an inactivation of the response during prolonged application of CAP whereas tachyphylaxis is a decrease in responsiveness of the receptor during successive applications (Koplas *et al.*, 1997). CAP stimulates sensory neurons associated with the C- and Aδ-fibres (Bevan & Szolcsanyi, 1990). Therefore, the similar sensitivity to CAP as DRG sensory neurons (Gschossman *et al.*, 2000) implies the hybrid sensory neuronal cells have vanilloid receptors and nociceptive properties.

4.1.3. α,β-methylene ATP-induced $[Ca^{2+}]_i$ Increase

Sensory neurons are known to have an ATP-gated ion conductances (Krishtal *et al.*, 1983, 1988a,b). Once ATP binds to its receptor, a depolarization results from Na^+ influx. With fura-2 microfluoremetry, I measured the accumulation of cytoplasmic Ca^{2+}, most likely through Ca^{2+} influx through ATP-gated cation channels. In general, cells that express P2X1 or P2X3 receptors respond to αβ-methylene ATP (Collo *et al.*, 1996). While P2X1 receptors are expressed in sensory ganglion neurons and spinal cord motoneurons (Valera *et al.*, 1994), the P2X3 receptors are found only in CAP-sensitive sensory neurons (Chen *et al.*, 1995). From Figure 4, 100 μM α,β-methylene ATP applications to MD3 cells elevated $[Ca^{2+}]_i$. However, during perfusion of zero Ca^{2+} HHBSS buffer, α,β-methylene ATP had no effect. Therefore, I suggest that the rise in $[Ca^{2+}]_i$ depended on extracellular Ca^{2+}, resulting from Ca^{2+} influx through the ATP-gated channels as reported in other studies (Krishtal *et al.*, 1988a,b; Bean *et*

al., 1990). The results imply that these channels allow sufficient Ca^{2+} entry in a normal Ca^{2+} gradient to elevate somatic $[Ca^{2+}]_i$. The MD3 cells express receptors that respond to α,β-methylene ATP as well as CAP, corresponding to a nociceptive phenotype. The CAP- and α,β-methylene ATP-mediated responses imply that the hybrid sensory neuronal cell line has nociceptive properties. Also, combining the results from immunocytochemistry and the responses to α,β-methylene ATP and CAP imply the hybrid sensory neuronal cells possess neuronal characteristics.

4.2. GABA Experiments

I investigated the GABA-evoked responses as well as agonists and antagonists in MD3 cells. In the present investigation, GABA produced a transient rise in $[Ca^{2+}]_i$, presumably a consequence of changes in membrane potential. Previous research has revealed that a GABA-induced depolarization in many types of excitable tissue including crustacean stretch receptors of crayfish muscle (McGeer *et al.*, 1961) and frog spinal ganglion (Nishi *et al.*, 1974). GABA enhance $[Ca^{2+}]_i$ in explanted, early postnatal cerebellar granule neurons, mammalian dorsal horn neurons in culture, and early postnatal neocortical neurons of slices (Connor *et al.*, 1987; Yuste & Katz, 1991; Reichling *et al.*, 1994). The data from the current study imply that the $[Ca^{2+}]_i$ transients in this cell line may be due to the VGCC's activation from Cl^- conductance-induced membrane depolarization, resulting from an unusually high internal $[Cl^-]$ and an efflux of Cl^-. In the present study, the time courses of GABA-, KCl-, and ZAPA-evoked increases in $[Ca^{2+}]_i$ varied from one experiment to another. The differences in time course of these drugs may arise from the distance between the ejection pipette and the mouse hybrid sensory neuronal cells.

4.2.1. GABA-induced $[Ca^{2+}]_i$ Increase

After having identified the responses of sensory neurons to CAP and α,β-methylene-ATP sensitivity, and performed immunocytochemistry, I examined the hybrid sensory neuronal cells for GABARs because GABA modulates transmission in primary afferent neurons (Rudomin and Schmidt, 1999). When stimulated with GABA, fura-2 fluorescent imaging methods revealed $[Ca^{2+}]_i$ perturbations in the MD3 cells. GABA evoked reproducible, transient increases in $[Ca^{2+}]_i$ in the MD3 cells, implying that they have receptors for GABA. The data from the experiments are consistent with the hypothesis that GABA activates its receptor, resulting in a Cl^- conductance-induced membrane depolarization that produces Ca^{2+} influx through nimodipine- and nifedipine-sensitive VGCCs, thereby elevating $[Ca^{2+}]_i$. This mechanism has been observed in acute preparations of dorsal spinal cord and the trigeminal complex of the brain stem from 1- and 2-week old rat pups that have both high- and low-threshold Ca^{2+} channels (Huang, 1989; Mintz *et al.*, 1992). Of interest is the fact that $GABA_A$Rs and DHP-sensitive VGCCs form tight spatial coupling in neuronal somata (Hansen *et al.*, 1992).

4.2.2. GABA-evoked Depolarization Mechanisms

Several different mechanisms may explain the role that GABA has in evoking an increase in $[Ca^{2+}]_i$ in the MD3 cells. Current through the Cl^--permeable channel may depolarize the cell when the sustained concentration for the internal Cl^- environment is greater than that in the extracellular space. This process of GABA-induced depolarization occurs in peripheral ganglion neurons (Nishi *et al.*, 1974; Adams & Brown, 1975). However, in other neuronal preparations such as hippocampal pyramidal cells, this mechanism is insufficient to explain both hyperpolarizing and depolarizing effects within a single neuron (Obata *et al.*, 1978; Alger & Nicoll, 1979; Lambert *et al.*, 1991). Nevertheless, the sensory neuron-derived cells presumably

contain a high Cl^- internal environment as demonstrated by their responses to GABA agonists and K^+.

A DRG neuron may generate a high Cl^- internal environment through a variety of mechanisms. To maintain a stable depolarizing Cl^- reversal potential, a neuron may employ an inward pump such as a Cl^--HCO_3-exchanger with the production of CO_2/HCO_3 as the driving force or a Cl^- cation transporter driven by the Na^+ gradient (Kopito, 1990). Evidence for this comes from research on mouse oligodendrocytes and guinea-pig hippocampal neurons where the Cl^- transporter inhibitor, furosemide, reduces GABA-evoked depolarizations (Misgeld *et al.*, 1986; Hoppe & Kettleman, 1989).

Depolarization and subsequent activation of VGCCs would result when the intracellular Cl^- concentration is sufficiently high for a net efflux of Cl^- during activation of GABARs. The electrochemical Cl^- diffusion gradient across the afferent neuron membrane is directed outward, the result of an inwardly directed Cl^- pump (Nishi *et al.*, 1974; Gallagher *et al.*, 1978). Also, a major difference has been substantiated when comparing neurons in terms of their recovery time following intracellular Cl^-injection. Motoneurons recovered in about 30 seconds (Eccles, 1964), 90 seconds for cortical neurons (Kelly *et al.*, 1969), but 30 minutes for the spinal ganglion. One reason for the slow recovery in DRG neurons may be the lower resting Cl^- conductance. In addition, the primary afferent neuron may have effective pumps that prevent outward leakage of Cl^-, not found in motor and cortical neurons (Gallagher *et al.*, 1978).

In previous experiments, removal of Cl^- from the external solution was possible by replacing NaCl with an equimolar solution of sodium isethionate. However, there are conflicting reports. Barker & Nicoll (1973) observed in the amphibian that Cl^--free media produced a small increase in the membrane potential while others found a decrease in the GABA potential (Nishi *et al.*, 1974; Kudo *et al.*, 1975) or no change (Constanti & Nistri, 1976)

in GABA's depolarizing capacity following Cl^- removal. A removal of external Cl^- failed to increase the Cl^- gradient across the nerve terminal membrane during a drug-induced reduction in activity of concentration-dependent Cl^- pump that maintains the gradient.

4.2.3. GABA-evoked Ca^{2+} Influx

As explained earlier, GABA-induced a transient increase in $[Ca^{2+}]_i$ through membrane depolarization, presumably due to an increased Cl^- conductance. Previous studies also have demonstrated that stimulation of $GABA_A$Rs results in Ca^{2+} influx in cultured dorsal horn neurons, as well as neocortical and hippocampal neurons (Reichling *et al.*, 1994; Owens *et al.*, 1996; Canepari *et al.*, 2000). What happens to the hybrid sensory neuron following depolarization? The data show that the GABA-evoked depolarization activated VGCCs since DHPs inhibited the Ca^{2+} transients. The depolarization would activate other voltage-gated channels such as voltage-gated Na^+ and K^+ channels. However, depolarization from GABA-induced Cl^- conductance may inhibit neuronal activity such as APs because it could act as a current shunt, or indirectly activate Ca^{2+}-dependent K^+ currents (Staley & Mody, 1992). The afferent activity along the sensory nerves would then depend on both the excitatory and inhibitory influences as well as their electrophysiological propertiesof the somata.

The GABA-mediated $[Ca^{2+}]_i$ increases may also serve a developmental role, since small tonic elevations of $[Ca^{2+}]_i$ promote neuronal survival and development in embryonic rat spinal cord cultures (Franklin & Johnson, 1992). Also, GABA stimulates early stages of synaptogenesis in cultured neuroblastoma cells, influences the differentiation of cultured cerebellar granule neurons, and disrupts developing cerebellum *in vivo* (Belhage *et al.*, 1998). One study observed the responses to GABA in the cultures diminished over time; hence, the depolarizing response to GABA may result in developmental regulation *in vivo* (Reichling *et al.*, 1994).

The regulation of cytoplasmic free Ca^{2+} concentration is vital to controlling neuronal function (Mironov *et al.*, 1993). The time course of $[Ca^{2+}]_i$ accumulation and the rate of $[Ca^{2+}]_i$ recovery following Ca^{2+} influx may be important factors in the activation of different messenger systems. Many studies have observed transient rises of $[Ca^{2+}]_i$ induced from membrane depolarization in many neuronal types (Tsien & Tsien, 1990; Miller, 1991; Henzi & MacDermott, 1992).

4.2.4. Bicuculline Antagonism of GABA-response

I examined further the GABA-mediated response in the DRG neuronal cell line MD3 to determine the nature of the receptors implicated in enhancing the $[Ca^{2+}]_i$. The results from this section provide evidence that BIC selectively and reversibly blocked the effects of GABA. As shown in Figure 5*B*, BIC antagonized the GABA-evoked response in the mouse hybrid DRG neuronal cells in culture. In invertebrate muscle (Smith & Costanti, 1981) and cat DRG neurons (Gallagher *et al.*, 1978), BIC was a noncompetitive or 'mixed' noncompetitive antagonist. However, on most vertebrate central and peripheral neurons, BIC was a competitive inhibitor of the GABA-evoked response (Curtis *et al.*, 1971; Bowery & Brown, 1974; Levy, 1977; Simmonds, 1980). The BIC effect on the GABA-mediated response in MD3 cells was either by direct competition with GABA at its receptor (competitive inhibition) or decreased Cl^- channel conductance (non-competitive inhibition). However, determining the exact mechanism of antagonism was not possible using the fura-2 microfluoremetry system. Nevertheless, ZAPA applications that activate $GABA_A$Rs increased $[Ca^{2+}]_i$, consistent with the presence of $GABA_A$Rs in MD3 cells (Allan *et al.*, 1997). The observed antagonism by BIC with an ED_{50} = 9.3 μM is consistent with GABA and GABAR interactions (MacDonald & Olsen, 1994).

4.2.5. Effects of Picrotoxinin and Diazepam

The mechanism of PTX inhibition of GABA-activated Cl^- conductance is different in a variety of tissues. PTX inhibits the GABA-evoked response competitively and noncompetitively, or a mixture of both effects on lobster muscle (Takeuchi & Takeuchi, 1969; Shank *et al.*, 1974; Constanti, 1978). PTX had similar effects on neuronal membranes (Gallagher *et al.*, 1978; Homma & Rovainen, 1978; Hori *et al.*, 1978; Akaike *et al.*, 1985). However, no PTX inhibition of the GABA-stimulated response was observed in the MD3 cells, as in hippocampal (Segal, 1993) and human dorsal root ganglion neurons (Valeyev *et al.*, 1996). This inconsistency may be due to GABAR subtypes with different channel properties. Differential manifestation of individual GABAR subunits and the combination of various distinct subunits influences channel properties (Shivers *et al.*, 1989; Verdoorn *et al.*, 1990; Zhang *et al.*, 1991). Picotoxinin-insensitivity may result from unique subunit combinations, such as mutations in the γ2 subunit of the $GABA_AR$ (Shen *et al.*, 1999). In MD3 cells, analogous variations in the benzodiazepine receptor may result in lack of the potentiation of GABA-mediated action by diazepam (Macdonald & Olsen, 1994; Knoflack *et al.*, 1996; Malgrange *et al.*, 1997; Williamson *et al.*, 1998).

4.3. Mechanism of GABA-mediated Increase in $[Ca^{2+}]_i$

I investigated the mechanisms concerning responses in MD3 cells to GABA applications. GABA binds to its receptor, opening the chloride channel to increase Cl^- conductance and depolarizing the cell. However, what is the process that produced the heightened $[Ca^{2+}]_i$ in the hybrid neurons?

4.3.1. Absence of Ca^{2+} in the Extracellular Environment

The treatment of the MD3 cells with 0 mM Ca^{2+} recording buffer during GABA stimulation established that the $[Ca^{2+}]_i$ increase depended on extracellular Ca^{2+}. Perfusion of Ca^{2+}-free buffer through the recording chamber eliminated the GABA-induced response, resulting in no change in the in $[Ca^{2+}]_i$ (Figure 9). This removed the possibility of the Ca^{2+} concentration increase occurring through release of internal stores such as the endoplasmic reticulum. Therefore, $[Ca^{2+}]_i$ increased because of Ca^{2+} influx from the exterior to the interior of the MD3 cell. To minimize depletion of the internal stores during eradication of extracellular Ca^{2+}, the exposure time to EGTA-containing Ca^{2+}-free HHBSS buffer was about a minute. The $[Ca^{2+}]_i$ elevations following GABA applications returned with the perfusion of Ca^{2+}-containing HHBSS buffer. Also, I did not detect a change in baseline $[Ca^{2+}]_i$ on thapsigargin or caffeine application, observed in some cells (cf. Guerini & Carafoli, 1999). However, thapsigargin and caffeine had no effects on GABA-evoked increase in $[Ca^{2+}]_i$, implying that the GABA-induced response did not involve Ca^{2+} release from internal stores. Therefore, Ca^{2+} influx, not Ca^{2+} release from internal stores, and extracellular Ca^{2+} influenced the augmentation in $[Ca^{2+}]_i$ in MD3 neurons.

4.3.2. GABA-response Inhibition with DHPs

The results imply that the GABA-induced response involves $GABA_AR$ activation and extracellular Ca^{2+} influx. The application of DHP antagonists inhibited GABA-mediated rise in cytoplasmic Ca^{2+}, verifying that the transmembrane influx of Ca^{2+} occurred through VGCCs, as in cultured dorsal horn neurons (Reichling *et al.*, 1994) and gonadotropes (Williams *et al.*, 2000). The present study used nimodipine and nifedipine to define the functional presence of *L-type* channels. From Figures 10 and 11, the application of DHPs inhibited the rise in cytoplasmic Ca^{2+} from GABA induction. This provides evidence that the GABA-mediated response initiates entry of Ca^{2+} through *L-type* channels that DHPs potently inhibit (Fox *et al.*,

1987a; Aosaki & Kasai, 1989; Plummer *et al.*, 1989). These channels exhibited a slowly inactivating, voltage-dependent Ca^{2+} current in primary sensory neurons as in chick DRG (Nowycky *et al.*, 1985). In addition, *L-type* Ca^{2+} channels controlled the release of substance P from DRG neurons in chick and rat (Pernery *et al.*, 1986; Holz *et al.*, 1988).

Ca^{2+} channels play a significant functional role, though poorly defined, in neurons (Nowycky, 1992). In addition, direct electrical excitability as well as simultaneous conversion of electrical activity into other functional activities such as transmitter release, regulation of gene expression, or further control of membrane excitability involve Ca^{2+} channels (Nowycky, 1992). VGCCs have an important role in a number of neuronal functions that depend on the intracellular Ca^{2+} concentration. Fox *et al.* (1987a) discovered the co-existence of three types of Ca^{2+} channels in DRG neurons. In addition, various neurotransmitters modulate Ca^{2+} channel currents in many neuronal cell types (Menon-Johansson *et al.*, 1993), representing one of the mechanisms whereby presynaptic inhibition may occur in both the central and peripheral nervous systems (Dunlap & Fischbach, 1978).

In mammalian neurons, cadmium and manganese block Ca^{2+} currents; however, these divalent ions were unsatisfactory as blockers in experiments with fura-2 (Connor *et al.*, 1987). Conner and associates (1987) discovered that cadmium produced a steady increase in the fluorescence ratio of fura-2 while manganese rapidly decreased the fluorescence ratio values at about 10 % of initial values. Therefore, I applied DHPs to investigate its effects on GABA-stimulated the cytoplasmic Ca^{2+} increase. Nimodipine and nifedipine, at concentrations ranging from 1-20 μM, inhibited the GABA-induced depolarization that increase $[Ca^{2+}]_i$ (Figures 10 and 11). Therefore, $GABA_AR$ activation may have depolarized MD3 cells through a Cl^- efflux, initiating Ca^{2+} influx through DHP-sensitive Ca^{2+} channels, leading to an increase in $[Ca^{2+}]_i$.

4.4. K^+-induced Increase in $[Ca^{2+}]_i$

K^+-induced $[Ca^{2+}]_i$ transients produced through VGCCs increased $[Ca^{2+}]_i$ (Figure 12). Nimodipine and nifedipine blocked the rapid depolarization from K^+-applications and mimicked the DHP inhibition of the GABA-activated response (Figures 10 and 11). Thus, these results demonstrate the influx of extracellular Ca^{2+} to be important in the depolarization-induced changes in $[Ca^{2+}]_i$. Previous studies on neurons and neuronal cell lines showed DHP inhibition of Ca^{2+} influx and transmitter release in response to K^+ depolarization (Toll, 1982; Takahashi & Ogura, 1983; Freedman *et al.*, 1984; Sasakawa *et al.*, 1984; Carboni *et al.*, 1985; Enyeart *et al.*, 1985; Perney *et al.*, 1986). DHP Ca^{2+} channel antagonists bind to high-affinity sites and block the depolarization-induced entry of Ca^{2+} (Miller & Freedman, 1984). The inhibition of Ca^{2+} influx is due to the selective interaction of the DHPs with VGCCs (Rane *et al.*, 1987).

Mnay investigations have reported that DHPs do not affect Ca^{2+} influx or Ca^{2+}-dependent transmitter release in intact neurons or synaptosomes (Nachshen & Blaustein, 1979; Ebstein & Daly, 1982; Daniell *et al.*, 1983; Rampe *et al.*, 1984; Ogura & Takahashi, 1984; Shalaby *et al.*, 1984; Boll & Lux, 1985; Perney *et al.*, 1986; Suszkiw *et al.*, 1986). However, other investigations observed the involvement of DHP-sensitive Ca^{2+} channels for both Ca^{2+} entry into and transmitter release from synaptosomes, neurons or neuronal cell lines (Toll, 1982; Takahashi & Ogura, 1983; Freedman *et al.*, 1984; Sasakawa *et al.*, 1984; Turner & Goldin, 1985; Enyeart *et al.*, 1985; Middlemiss & Spedding, 1985; Carboni *et al.*, 1985; Perney *et al.*, 1986). In the present study, the Ca^{2+} channels of the MD3 cell line accounted for Ca^{2+} entry and were sensitive to nimodipine and nifedipine during K^+- and GABA-induced responses.

4.5. $GABA_B$ Receptor Activation.

The results presented in this section show evidence for $GABA_BRs$ in MD3 cells. Baclofen inhibited neurotransmission at peripheral and central synapses such as primary afferent terminals within the spinal cord (Fox *et al.*, 1978). Without affecting the baseline $[Ca^{2+}]_i$, baclofen reduced the K^+-evoked rises in $[Ca^{2+}]_i$ in MD3 cells (Figure 15*A*), agreeing with the results from other studies on rat and chick DRG neurons. $GABA_BRs$ mediated this effect because 2-hydroxy-saclofen did not significantly change the baseline $[Ca^{2+}]_i$ and completely blocked the baclofen-inhibition of KCl-induced responses. Baclofen directly depressed Ca^{2+} currents and modulated $GABA_BR$'s influence on neuronal excitability (Dunlap, 1984; Deisz & Lux, 1985). Another study showed that bath application of baclofen for 5-10 min suppressed the inward current produced by DRG neurons from exposure to GABA (Xi *et al.*, 1997). Baclofen-induced inhibition in the MD3 neurons may involve G proteins. Stimulation of $GABA_BRs$ with baclofen or GABA at the binding site activated G proteins and proceeded through different biochemical pathways (Holz *et al.*, 1986), resulting in the closure of Cl^- channels and a decrease in Cl^- efflux in DRG neurons by phosphorylation of $GABA_ARs$. In chick DRG cultures, pertussis toxin reduced the effect of GABA on AP duration, revealing a G-protein involvement in the process (Holz *et al.*, 1986). $GABA_BR$ activation may mediate a decrease in Ca^{2+} conductance through a direct link with G-proteins. Holz *et al.* (1986) discovered that by incubating DRG neurons with either GDP-β-S, a non-hydrolyzeable analogue of GDP, or pertussis toxin, removed GABA-induced inhibition of Ca^{2+} currents, implying the participation of a G_i-like protein membrane protein. $GABA_BRs$ of embryonic chick sensory neurons *in vitro* decreased Ca^{2+}-dependent APs with no change in the resting conductance. Activation of $GABA_BR$ during an AP inhibits VGCCs in the membrane, decreases extracellular Ca^{2+} flux, and results in low neurotransmitter release from presynaptic terminals. Activation of

$GABA_BRs$ in the MD3 neurons did not lead to an increase in cytoplasmic Ca^{2+}. The inhibitory effects of baclofen persisted for the duration of its presence. The ability of the $GABA_BR$ to modulate second-messenger systems may explain the effect of baclofen in depressing $[Ca^{2+}]_i$ elevations. For example, the $GABA_BR$ decreased cAMP production (Desrues *et al.*, 1995; Hashimoto & Kuriyama, 1997). In the previous section, nimodipine and nifedipine inhibited the KCl-mediated increase in $[Ca^{2+}]_i$. Since high K^+ depolarized the MD3 neurons during baclofen application, then the $GABA_BR$ activation that decreased the influx of Ca^{2+} presumably affected *L-type* Ca^{2+} channels. The observed effects of baclofen at $GABA_BR$, with an ED_{50} = 1.8 μM, may have resulted from a decrease in voltage-activated Ca^{2+} currents, as observed in spinal neurons (Batueva *et al.*, 1999). Given that MD3 cells are similar to DRG neurons, the effects of baclofen and the antagonism by a $GABA_BR$ antagonist imply that $GABA_BR$ activation can influence the voltage-dependent activities in sensory ganglia.

The co-administration of 2-hydroxy-saclofen with (–)-baclofen provided evidence that the effects of baclofen-mediated inhibition of K^+-induced Ca^{2+} transients were specific to the $GABA_BR$ (Curtis *et al.*, 1988; Lambert *et al.*, 1989; Harrison *et al.*, 1990). The results show that bath application of 2-hydroxy-saclofen antagonizes the depressant actions of baclofen in the MD3 neurons. As illustrated from Figure 17, 2-hydroxy-saclofen blocked the inhibitory effects of baclofen during K^+-induced enhancement of $[Ca^{2+}]_i$. The effective treatment with 2-hydroxy-saclofen abolished the effect of (–)-baclofen. From these results, I suggest that the mouse hybrid sensory neuronal cells posses $GABA_BRs$ that respond to baclofen and 2-hydroxy-saclofen, influencing K^+-evoked Ca^{2+} transients.

4.6. Ca^{2+} Sequestration and Efflux Mechanisms

The regulation of cytoplasmic free Ca^{2+} is central to the control of neuronal function. When Ca^{2+} enters through VGCCs, the heightened $[Ca^{2+}]_i$ integrates with and influences other intracellular signals, controlling neurotransmitter release, activation of Ca^{2+}-dependent enzymes, and modulation of membrane ion channels (Benham *et al.*, 1992). In fact, extended increases in cytosolic Ca^{2+} were acutely cytotoxic (Schanne *et al.*, 1979; Choi, 1988, 1995). This present study showed heightened internal Ca^{2+} levels in hybrid DRG neuronal cells from GABAR activation and K^+-evoked depolarizations. Then, how do these cells recover from such acute increases in Ca^{2+} load? One source of help is the mitochondria. In addition to their ubiquitous role as the major synthesizer of ATP in aerobic cells (Nicholls, 1985; Thayer & Miller, 1990), mitochondria have the ability to transport and store Ca^{2+} (Nicholls & Akerman, 1982; Akerman & Nicholls, 1983). The mitochondria have a high rate of Ca^{2+} accumulation. When containing high free Ca^{2+} of 10 μM or more, mitochondria concentrated on the accumulation of the cation rather than ATP synthesis (Nicholls & Akerman, 1982). Two papers identified a role for mitochondrial uptake of Ca^{2+} following large Ca^{2+} loads in DRG neurons (Thayer & Miller, 1990; Duchen *et al.*, 1990). In neuronal tissue, the plasma membrane, inner mitochondrial membrane, and endoplasmic reticulum have a role in the regulation of the cytosolic free Ca^{2+} concentration (Nicholls & Akerman, 1983).

From this current study, GABA may contributes to excitotoxicity as observed in cortical neuron cultures from heightened $[Ca^{2+}]_i$ (Erdo *et al.*, 1991). However, once Ca^{2+} enters the cell through Ca^{2+} permeable channels, Ca^{2+} encounters many obstacles that prevent it from influencing its physiochemical properties. Ca^{2+} binding proteins buffered most of the Ca^{2+} (> 99 %) that enters the cytoplasm (McBurney & Neering, 1987; Neher & Augustine, 1992; Zhou &

Neher, 1993) and taken up into intracellular organelles. However, the two main extrusion mechanisms, sodium-Ca^{2+} exchange and the ATP-driven Ca^{2+} pump, send all the Ca^{2+} back across the plasmalemma.

4.7. Sensory Ganglion Soma

The arbitrary assumption that the effects of GABA on the cell body membranes giving rise to terminals should be similar to the terminals comes from the following evidence: GABA depolarized DRG neurons grown in culture (Nishi *et al.*, 1974; Feltz & Rasminsky, 1974; Deschenes *et al.*, 1976; Obata *et al.*, 1978; Gallagher *et al.*, 1978). Studies observed evidence for synapses on the soma of DRG in chick embryo tissue cultures (Miller *et al.*, 1970; Lodin *et al.*, 1973). Kayahara and colleagues (1981,1984,1986) discovered synapses between the ganglion cell soma and the terminals of cell processes of unknown origin in their ultrastructural study of the cat spinal ganglion *in vivo*.

The DRG has been viewed as a model for GABA interactions at presynaptic sites within the spinal cord (Feltz & Rasminsky, 1974; Valeyev *et al.*, 1999; Rudomin & Schmidt, 1999). The effects of transmitters at sensory ganglion somata may have significance for the mechanisms of action of drugs for potential therapy of sensory nerve injury and allodynia (Gordon *et al.*, 1995; Baccei & Kocsis, 2000; van Hilten *et al.*, 2000). The MD3 cells have many properties of DRG neurons *in vitro* and *in vivo*. They possess functional $GABA_A$ and $GABA_BRs$, providing a simple system for studying drug effects of DRG neurons.

Chapter V. SUMMARY AND CONCLUSIONS

1. I studied the properties of a cell line (MD3) produced from hybridization between mouse DRG sensory neurons and N18TG2 neuroblastoma cells. Immunostaining with MAP-2 and NF-H Abs demonstrated that MD3 cells had neuronal characteristics. In addition, the hybrid sensory neuronal cells reacted to CAP and α,β-methylene ATP, similar to sensory neurons.

2. I investigated the mechanisms and effects of GABA-evoked depolarizations on the mouse hybrid sensory neuronal cells in culture using fura-2 imaging of $[Ca^{2+}]_i$.

3. The MD3 cell line possessed $GABA_A$ and $GABA_B$Rs. $GABA_A$R activation increased the $[Ca^{2+}]_i$ of these sensory neuronal cells. This augmentation of $[Ca^{2+}]_i$ may have resulted from GABA-mediated increase in Cl^- conductance that depolarized the membrane potential, resulting in Ca^{2+} influx through voltage-dependent Ca^{2+} channel activation. BIC, but not PTX, prevented transient rises in $[Ca^{2+}]_i$.

4. Nifedipine and nimodipine inhibited the GABA- and K^+-mediated transmembrane influx of Ca^{2+} through VGCCs, presumably through blockade of Ca^{2+} entry through *L-type* channels. In addition, thapsigargin and caffeine did not affect the GABA-mediated response and no elevation in $[Ca^{2+}]_i$ occurred from application of GABA and K^+ during Ca^{2+}-free HHBSS buffer perfusion. Therefore, GABA- and K^+-mediated responses depended on extracellular Ca^{2+}.

5. Activation of $GABA_BRs$ with baclofen attenuated the K^+-induced increase in $[Ca^{2+}]_i$. 2-hydroxy-saclofen antagonized the inhibitory effect of baclofen on the increase in $[Ca^{2+}]_i$. In addition, baclofen presumably influenced *L-type* channels since DHPs inhibited the K^+-mediated $[Ca^{2+}]_i$ elevation.

6. The MD3 neurons exhibited many properties of DRG neurons *in vivo* and *in vitro*. It provides a model for the examination of drug applications that modulate the GABAR and, thus, is likely to be a useful model system.

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