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LEAD ACTIVATION OF A DEVELOPMENTALLY

REGULATED CALCIUM CHANNEL IN RAT

HIPPOCAMPAL NERVE TERMINALS

by

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B.S. May 1992, Old Dominion University

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ABSTRACT

LEAD ACTIVATION OF A DEVELOPMENTALLY REGULATED CALCIUM CHANNEL IN RAT HIPPOCAMPAL NERVE TERMINALS.

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Old Dominion University and Eastern Virginia Medical School, 1996
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Low level lead (Pb^{2+}) exposure may produce lasting deficits in learning and memory by altering calcium (Ca^{2+}) dependent processes. Isolated presynaptic nerve terminals from rat hippocampus were loaded with the intracellular Ca^{2+} indicator Fura-2. The changes in cytoplasmic free calcium ($[Ca^{2+}]_i$) were measured by stopped-flow fluorescence spectroscopy following depolarization with elevated potassium on a millisecond time scale (Lentzner et al., 1992). Depolarization promoted a rapid increase in Ca^{2+}_i which occured in two kinetically distinguishable phases: a fast component, representing the activity of rapidly inactivating Ca^{2+} channels ($\tau \sim$ 60 msec), and a slow component, which is comprised of slowly inactivating Ca^{2+} channels ($\tau \sim$ 1 sec) and Na^+/Ca^{2+} exchange operating in the "reverse" mode. Low concentrations of Pb^{2+} (0.1 - 0.5 μM) blocked competitively both the rapidly and slowly inactivating channels. At higher concentrations ($\geq$ 1 μM), Pb^{2+} permeated the rapidly inactivating channels. Pb^{2+} permeation was accompanied by a subsequent rise in intracellular Ca^{2+} even in the absence of extracellular Ca^{2+}. The rise in Ca^{2+} was reduced by thapsigargin, suggesting Pb^{2+} activates the release of Ca^{2+} from intracellular stores, possibly an IP_3 sensitive store. The Ca^{2+} release was greatest in younger animals and gradually declined during postnatal development.

Endoplasmic reticulum (ER) vesicles from rat cerebellum were isolated and loaded with chlortetracycline (CTC), a fluorescent Ca^{2+} indicator, and the changes in intravesicular Ca^{2+} were monitored with a cuvette based fluorometer. The addition of Pb^{2+} induced a rapid release of one third to one half of the accumulated Ca^{2+} in the absence of IP_3. Addition of 1 μM IP_3 after the Ca^{2+} release by Pb^{2+} resulted in no further release. Furthermore, the addition of Pb^{2+} after a release by IP_3 also resulted in no further release. These results demonstrated that Pb^{2+} releases Ca^{2+} from an IP_3 sensitive store. Single IP_3 gated Ca^{2+} channels from rat cerebellar ER were reconstituted into artificial planar bilayers to demonstrate that Pb^{2+} directly interacts with the channel molecule. The addition of 1 μM Pb^{2+} to the cytoplasmic side increased channel openings at all membrane potentials. However, the addition of 10 μM Pb^{2+} to the cytoplasmic side decreased channel openings. Thus, Pb^{2+} mimics the effects of Ca^{2+} possibly by interacting at the Ca^{2+} binding/modulatory site(s). These results suggest the inappropriate activation of IP_3 gated Ca^{2+} channels may underlie certain aspects of Pb^{2+} neurotoxicity.

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ATP	Adenosine Triphosphate
BHQ	2,5-Di-(*t*-butyl)-1,4-hydroquinone
BSA	Bovine Serum Albumin
$[Ca^{2+}]_i$	Intracellular Free Calcium Concentration
CTC	Chlortetracycline
DAG	Diacylglycerol
DMSO	Dimethyl Sulfoxide
2,4-DNP	2,4-Dinitrophenol
EGTA	Ethylene glycol-bis (β-aminoethylether)-N,N,N',N'-tetra-acetic acid
ER	Endoplasmic Reticulum
GTP	Gaunosine Triphosphate
HEPES	(N-[2-Hydroxyethyl] piperazine-n'-[2-ethanesulfonic acid])
IP_3	D-(*myo*)-Inositol 1,4,5-trisphosphate, hexapotassium salt
IP_3R	IP_3 receptor
k	Rate Constant
K_{mapp}	Apparent Binding Affinity
LTP	Long term potentiation
MOPS	(3-[N-Morpholino] Propane Sulfonic Acid)
NMDG	N-methyl-D-glucamine
PKC	Protein Kinase C
PIP_2	Phosphatidyl inositol 4,5-bisphosphate

PLC	Phospholipase C
PMSF	Phenylmethylsulfonyl Flouride
PSS	Physiological Saline
VDCCs	Voltage Dependent Calcium Channels
V_{max}	Maximum Magnitude

CHAPTER I

BACKGROUND AND INTRODUCTION

Lead (Pb^{2+}) toxicity is a major health hazard in the pediatric population in the United States. A recent U.S. Dept. of Health study (1988) reported that nearly 5 million children are at risk from the toxic effects of environmental Pb^{2+} exposure from sources including food, drinking water, industrial by-products, and close proximity to industrial sources. Furthermore, at least 30 million homes in this country still contain potentially dangerous levels of Pb^{2+} based paint. Chronic exposure adversely affects the renal, reproductive, hematopoietic, and hepatic systems, but the most sensitive tissue is the nervous system. For many years, it was assumed that after children recovered from the acute phase of Pb^{2+} intoxication, there were no significant residual effects. However, in 1943, Byers and Lord examined 20 children who had recovered from Pb^{2+} poisoning and reported that 19 out of 20 were failing in school or exhibiting behavioral disorders, suggesting that Pb^{2+} exposure may produce lasting cognitive and behavioral deficits. In addition, children are more susceptible than adults to chronic Pb^{2+} exposure (Cantwell, 1975) and deficits are observed in children at levels previously thought to be non-toxic (Needleman, 1983). Hyperactivity, attention deficits, and memory impairment are commonly seen, but in many cases the relationship between low level, developmental Pb^{2+} exposure and such non-specific symptoms as irritability, insomnia, depression, and clumsiness is often overlooked (Jason and Kellog, 1980). Despite extensive investigation, the mechanisms of Pb^{2+} induced toxicity and the higher sensitivity of children are still unknown.

Research in animals has also shown that developmental Pb^{2+} exposure produces deficits in learning, memory, and attention. In monkeys, low level, developmental exposure impairs the ability to perform discrimination reversal tasks and more severe deficits are observed when distracting stimuli are introduced. These animals also displayed learning and memory impairments on tasks that required them to recall previously observed stimuli. Thus, lead treated monkeys exhibit many of the same cognitive and behavioral deficits as children: learning and memory impairments, attention deficits, and the inability to inhibit inappropriate responses (Rice, 1993). Similar impairments are also observed in young rats but not those exposed as adults (Cory-Slechta et al., 1991).

Despite extensive investigation, the neurochemical mechanisms of Pb^{2+} toxicity are still unknown. Recently, much research has focused on the ability of Pb^{2+} to interfere with Ca^{2+} dependent processes. In a number of tissues, Pb^{2+} has been shown to substitute for Ca^{2+} in some Ca^{2+} dependent reactions while inhibiting others. The first reported observation of a Pb^{2+}-Ca^{2+} interaction was made by Kostial and Vouk (1957) who reported that the ability of Pb^{2+} to inhibit cholinergic transmission in the cat nicitating membrane could be overcome by increasing Ca^{2+} concentrations.

Under resting conditions, the Ca^{2+}_i concentration is precisely maintained at a level of about 100 nM, despite extracellular and intracellular Ca^{2+} concentrations in the mM range. Upon depolarization, Ca^{2+}_i increases rapidly by influx through voltage-activated Ca^{2+} channels. Neuronal stimulation by neurotransmitters can lead to increased Ca^{2+}_i by causing second messenger generation which induces Ca^{2+} release from intracellular stores.

Elevated Ca^{2+}_i is buffered by high affinity Ca^{2+} binding proteins in the cytosol and extruded by membrane Ca^{2+}-ATPases and Na^+/Ca^{2+} exchange, or sequestered by Ca^{2+}-ATPases on the endoplasmic reticulum. Much research has focused on the ability of Pb^{2+} to interfere with Ca^{2+}_i homeostasis. Ca^{2+} transport and buffering in neurons and the ability of Pb^{2+} to interfere with these processes will be discussed below.

Calcium Transport Across the Plasma Membrane

Voltage-Dependent Ca^{2+} Channels

Upon membrane depolarization, VDCCs open and allow Ca^{2+} influx which carries depolarizing charge that contributes to excitability. Ca^{2+} influx also causes elevated Ca^{2+}_i which can affect neurotransmitter release, ion channel gating, enzyme regulation, metabolism, gene expression, and neurite extension (Tsien et al., 1988). Multiple types of VDCCs have been described in neurons: large, long-lasting, L-type; transient T-type, neuronal N-type, P-type, and Q-type. Each channel type has different ionic conductances, gating, pharmacology, and activation range. L-type are blocked by dihydropyridines while N-type are sensitive to ω-conotoxin, and P-type are blocked by Ftx. N- and P-type channels are involved in acetylcholine release (Fossier et al., 1994) while N- and Q-type contribute to synaptic transmission in the hippocampal slice preparation (Wheeler et al., 1994).

Pb^{2+} blocks Ca^{2+} permeation through VDCCs in a number of tissues including invertebrate neurons, adrenal chromaffin cells, neuroblastoma cells, dorsal ganglion cells, rat hippocampal neurons, and mammalian forebrain synaptosomes (reviewed in Audesirk,

1993). The most sensitive tissue appears to be rat hippocampal neurons where Pb^{2+} blocks Ca^{2+} channels with a IC_{50} of 80 nM (Audesirk, 1993).

Numerous studies have shown that the activity of intracellular second messengers can modify Ca^{2+} channel activity (Tsien et al., 1988; Dolphin, 1990). In the hippocampal slice preparation, phorbol ester activation of protein kinase C (PKC) increases neurotransmitter release (Parfitt and Madison, 1993). Pb^{2+} has been shown to activate calcium/phospholipid dependent protein kinase C (PKC) at subnanomolar concentrations (Markovac and Goldstein, 1988; Long et al., 1994).

Pb^{2+} permeates Ca^{2+} channels in bovine chromaffin cells (Tomsig and Suszkiw, 1991) and directly triggers catecholamine release at picomolar levels (Tomsig and Suszkiw, 1990). Normal synaptic transmission is dependent at least in part on the appropriate activation and inactivation of neuronal VDCCs. Thus, the ability of Pb^{2+} to interact with presynaptic Ca^{2+} channels is important for determining the neurochemical mechanisms of Pb^{2+} exposure in the hippocampus.

Calcium buffering by cytosolic proteins

Ca^{2+}_i is rapidly buffered by cytosolic proteins that bind Ca^{2+} with high affinity. Calmodulin, parvalbumin, and vitamin D dependent Ca^{2+} binding proteins are present in neurons (Blaustein, 1988). While parvalbumin functions primarily to buffer cytoplasmic Ca^{2+}, calmodulin also functions in second messenger systems (DeLorenzo, 1983). The binding of Ca^{2+} to calmodulin induces conformational changes that lead to the activated form which can regulate intracellular events by activating other enzymes such as protein kinases, adenylate cyclases, Ca^{2+}-ATPases, and phosphodiesterases (Goering, 1993). Pb^{2+}

blocks Ca^{2+} binding to calmodulin, binds with higher affinity, and directly activates calmodulin (Haberman et al., 1983). Thus, Pb^{2+}_i will also interact with Ca^{2+} binding proteins and potentially induce second messenger effects. Furthermore, the buffering of Pb^{2+}_i by Ca^{2+} binding proteins may lead to elevated Ca^{2+}_i which may also disrupt Ca^{2+}_i homeostasis.

Calcium buffering by intracellular Ca^{2+} stores

The ability of cytosolic proteins to buffer Ca^{2+} is rapid but has limited capacity. Therefore, intracellular Ca^{2+} stores are needed to sequester Ca^{2+} until the Ca^{2+} load can be exported across the plasma membrane. In neurons, several organelles function as intracellular Ca^{2+} stores: smooth endoplasmic reticulum, mitochondria, and synaptic vesicles (Blaustein, 1988).

Endoplasmic reticulum

In nerve terminals, the ER plays an important role in Ca^{2+} storage, especially after neuronal activity. The maximum rate of Ca^{2+} uptake by the Ca^{2+}-ATPase on the ER is 0.1-0.2 pM per mg protein per ms (Rasgado-Flores and Blaustein, 1987). However, this is too slow to buffer Ca^{2+} to terminate neurotransmitter release immediately after depolarization. Thus, Ca^{2+} binding proteins initially buffer elevated Ca^{2+}_i so that the ER is able to sequester Ca^{2+} (Blaustein, 1988). Currently, it is not known if the ER sequesters Pb^{2+}.

The ER also functions as a source of Ca^{2+}_i when activated by a second messenger such as inositol 1,4,5-trisphosphate (IP_3). Many neurotransmitters interact with G-protein linked receptors or tyrosine kinase receptors which activate phospholipase C (PLC) to

hydrolyze the lipid precursor phosphatidyl inositol 4,5-bisphosphate (PIP$_2$) to form IP$_3$ and diacylglycerol (DAG) (Berridge, 1993). IP$_3$ binds to an IP$_3$ receptor (IP$_3$R) on the ER to activate Ca^{2+} release. Three subtypes of the IP$_3$R have been characterized with Type-I receptors being the most prevalent subtype in the central nervous system. The Purkinje neuron in the cerebellum expresses high levels of Type-I IP$_3$R (Furuichi et al., 1994). The activity of IP$_3$R-channels is dependent on the presence of cytoplasmic Ca^{2+} in a bell shaped manner with maximal activity occuring ~ 300 nM (Bezprozvanny and Ehrlich, 1991). The activation of IP$_3$ gated channels at low Ca^{2+} levels and inactivation at high Ca^{2+} levels may contribute to regenerative Ca^{2+} oscillations and waves (Berridge, 1993; Pozzan et al., 1994). IP$_3$ binding is also inhibited in a Ca^{2+} dependent manner by calmedin. The IP$_3$R-channel is also modulated by ATP and protein kinases A and C (Pozzan et al., 1994). Finch et al. (1991) used a rapid superfusion system to show that IP$_3$ induced Ca^{2+} release consisted of two kinetically distinguishable components and the rate reached a maximum within 140 ms after the addition of IP$_3$.

Ryanodine sensitive Ca^{2+} stores are present in neurons and mediate Ca^{2+} induced Ca^{2+} release and depolarization activated Ca^{2+} release. These stores are activated by the plant alkaloid ryanodine and caffeine. Three ryanodine receptors have been reported: Type I in skeletal muscle, Type II in cardiac muscle, and Type III in brain (Simpson et al., 1995).

Pb^{2+} may affect the ability of intracellular Ca^{2+} stores to buffer Ca^{2+}. Incubation of osteoblastic bone cells (Schane, 1989b), neuroblastoma-glioma cells (Schane, 1989a), and human platelets (Dowd and Gupta, 1991) in solutions containing Pb^{2+} leads to a sustained

elevation of Ca^{2+}_i. This effect may be the result of Ca^{2+} release from intracellular stores. Although this effect has not been reported for Pb^{2+}, silver (Palade 1987; Brunder et al., 1988; Tatsumi et al., 1988) and mercury (Prabhu and Salama, 1990) can release Ca^{2+} from the sarcoplasmic reticulum.

Recently, Singh (1993) chronically exposed prenatal, neonatal, and adult rats to low level (1 mg/kg/day) Pb^{2+} ingestion, and examined Ca^{2+} mobilization in permeabilized cortical neurons and ^3H-IP_3 binding to endoplasmic reticulum (ER) vesicles. Exposure beginning prenatally reduced the capacity of IP_3 to increase intracellular Ca^{2+} while GTP mediated release was not reduced. The binding of ^3H-IP_3 to ER vesicles was also reduced suggesting that chronic, prenatal Pb^{2+} exposure reduces the number of IP_3 receptors on the ER. However, Pb^{2+} exposure in adult rats did not produce these changes. Chronic exposure in young rats may lead to a down regulation of IP_3 sensitive channels on the ER but this store may be insensitive or absent in adult animals. Thus, a normal developmental transition in intracellular Ca^{2+} storage may correlate with the increased sensitivity of younger animals to Pb^{2+} toxicity.

Mitochondria

Mitochondria have the capacity to sequester large amounts of Ca^{2+}. However, under normal resting conditions, mitochondria do not accumulate Ca^{2+} but may store small amounts when Ca^{2+}_i rises during depolarization (Blaustein, 1988). Under pathological conditions when Ca^{2+}_i is above 5 μM, the mitochondria will sequester Ca^{2+} to the point of crystallization (Rasgado-Flores and Blaustein, 1987).

Pb^{2+} may elevate Ca^{2+}_i by altering mitochondrial function. For example, Pb^{2+} stimulates Ca^{2+} release from mitochondria (Kapoor and Van Rossum, 1984; Chavez et al., 1987) and inhibits Ca^{2+} uptake into mitochondria (Parr and Harris, 1976; Goldstein, 1977).

Synaptic Vesicles

Synaptic vesicles also accumulate Ca^{2+} by a low affinity Ca^{2+}-ATPase (Michaelson et al., 1980; Rephaeli and Parsons, 1982) but most likely do not contribute to the maintenance of Ca^{2+}_i homeostasis.

Calcium extrusion

A very large concentration gradient exists between the extracellular and intracellular space. Neurons must expend metabolic energy to extrude Ca^{2+} in order to maintain normal resting Ca^{2+}_i levels. Neurons have two parallel, independent transport mechanisms on the plasma membrane to export Ca^{2+}: a Ca^{2+}-ATPase and Na^+/Ca^{2+} exchange (Blaustein, 1988).

Ca^{2+}-ATPase

Plasma membrane Ca^{2+}-ATPases have molecular weights of approximately 140 kDa, utilize one ATP molecule to transport one Ca^{2+} ion, and are modulated by calmodulin (Carafoli, 1987).

In erythrocytes, low micromolar concentrations of Pb^{2+} reduces Ca^{2+} transport by the Ca^{2+}-ATPase by 50% (Mas-Oliva, 1989). However, with the concentrations necessary for this effect, it is unlikely that it occurs *in vivo* because Pb^{2+} does not exceed picomolar

levels in experiments with platelets (Dowd and Gupta, 1991) and adrenal chromaffin cells (Tomsig and Suszkiw, 1991).

Na^+/Ca^{2+} Exchange

Na^+/Ca^{2+} exchange is a bidirectional counter transport system on the plasma membrane that transports 3 Na^+ in exchange for 1 Ca^{2+}. Na^+/Ca^{2+} uses the Na^+ electrochemical gradient generated by the Na^+/K^+ ATPase to remove Ca^{2+}. Na^+/Ca^{2+} exchange has a low affinity for Ca^{2+} but a large transport capacity (Blaustein, 1988). The direction of the exchanger can be altered by manipulating the ionic concentrations. Inhibition of the Na^+-K^+ ATPase elevates Na^+_i and promotes Na^+/Ca^{2+} exchange. During an action potential, the electrochemical gradient will cause the exchanger to reverse and promote Ca^{2+} entry. The effects of Pb^{2+} on Na^+/Ca^{2+} exchange have not been determined.

Second Messenger Interactions

Pb^{2+} may also exert its toxic effects by altering second messenger systems (reviewed in Goldstein, 1993). In astrocytes, Pb^{2+} increases inositol 1,4,5-triphosphate levels but does not alter IP_3 mediated Ca^{2+} transients (Dave et al., 1993). Pb^{2+} activates PKC at subnanomolar concentrations (Markovac and Goldstein, 1988; Long et al., 1994). In hippocampal neurons, Pb^{2+} inhibits neurite initiation (Audesirk et al., 1991; Kern et al., 1993) possibly by inappropriately activating calmodulin which stimulates cyclic AMP or CAM kinase (Kern and Audesirk, 1995).

Specific Aims Of The Study

In mammals, the hippocampus is involved with various types of learning and memory, the appropriate maintenance of attention and arousal, and cognitive processing.

Chronic Pb^{2+} exposure impairs the induction of long term potentiation (LTP), a synaptic model of learning and memory, in the hippocampus (Lasley et al., 1993). Since Pb^{2+} exposure adversely affects these processes, it is important to investigate the effect of Pb^{2+} on Ca^{2+}_i homeostasis in presynaptic terminals of the hippocampus. Numerous studies have investigated the effects of Pb^{2+} on the soma; however, less attention has been made to the effects of Pb^{2+} on Ca^{2+} events in the terminal region.

Low level Pb^{2+} exposure may produce long lasting deficits in learning and short term memory by altering Ca^{2+} dependent processes. A series of experiments were begun to examine the effect of acute Pb^{2+} exposure on presynaptic calcium dynamics in nerve terminals ("synaptosomes") from the hippocampus, a structure known to play a role in learning and memory and a site of Pb^{2+} accumulation (Widzowski and Cory-Slechta, 1994; Collins et al., 1982; Grandjean, 1978). The results from these initial experiments served as the foundation for this study and are presented here briefly and in more detail in Chapters 3 and 4. During depolarization, Pb^{2+} permeates one class of voltage activated Ca^{2+} channels and activates the release of Ca^{2+} from a thapsigargin sensitive intracellular Ca^{2+} store. As the animals age, the magnitude of the Pb^{2+} mediated Ca^{2+} release decreases. When the synaptosomes were exposed to nominally Ca^{2+} free solutions for 30 minutes prior to depolarization in the presence of Pb^{2+}, the Pb^{2+} activated Ca^{2+} rise was reduced suggesting that the store is labile. Furthermore, the ability to load the Pb^{2+} sensitive store also diminishes during development (Rhodes and Bartschat, 1995). Thus, a normal developmental transition may correlate with the increased sensitivity of younger animals to Pb^{2+} neurotoxicity.

The release of intracellular Ca^{2+} occurs within 100 ms suggesting a direct interaction between Pb^{2+} and the Ca^{2+} release channels on the endoplasmic reticulum (ER). The Pb^{2+} mediated release of Ca^{2+} is unaffected by pretreatment with ryanodine and caffeine. However, it is reduced in synaptosomes treated with thapsigargin, which depletes the inositol 1,4,5-trisphosphate (IP_3) store. In rats, low level Pb^{2+} exposure beginning prenatally reduces the number of IP_3 receptors on the ER. However, Pb^{2+} exposure in adult animals did not produce this reduction (Singh, 1993). Therefore, it is possible that intracellular Pb^{2+} may activate the IP_3 receptor. The possibility that intracellular Pb^{2+} also causes alterations in second messenger systems that may potentiate the release of Ca^{2+} has not been eliminated, however this study focused on a direct interaction between Pb^{2+} and the IP_3 activated Ca^{2+} channels. Developmental changes in Ca^{2+} storage that may correlate with Pb^{2+} sensitivity were also examined. The initial experiments that lead to this hypothesis are presented in Chapters 3 and 4.

The experiments reported in this study were designed to investigate the following questions:

1.   *Does Pb^{2+} activate the release of Ca^{2+} from an IP_3 sensitive Ca^{2+} store?* The Pb^{2+} activated Ca^{2+} rise is unaffected by pretreatment with ryanodine and caffeine, but is reduced by thapsigargin, which depletes IP_3 sensitive stores. In rats, prenatal Pb^{2+} exposure reduces the number of IP_3 receptors on the ER (Singh, 1993). These results all suggest that Pb^{2+} may be activating the IP_3 receptor. Therefore, ER vesicles were isolated from rat cerebellum, a preparation rich in Type I IP_3 receptors (Furuichi et al., 1994), to

determine if Pb^{2+} directly activates the release of Ca^{2+} from an IP_3 sensitive store. The results from these studies are presented in Chapter 5.

2. *Does Pb^{2+} interact directly with IP_3 activated Ca^{2+} channels?* A more direct way to determine if Pb^{2+} directly activates IP_3 regulated Ca^{2+} channels is to reconstitute single Ca^{2+} channels in an artificial lipid bilayer, where the aqueous and lipid composition is well defined. Channel gating (open and closing) was examined under control conditions and in the presence of Pb^{2+}. The results of these studies are presented in Chapter 6.

3. *Does Pb^{2+} interact at a Ca^{2+} binding site on the IP_3 receptor?* The activity of IP_3 activated Ca^{2+} channels is dependent on the presence of cytoplasmic Ca^{2+} and its' effect on channel activity is a bell-shaped dose response curve with maximal activity occuring ~300 nM (Bezprozvanny et al., 1991). In a number of tissues, Pb^{2+} has been shown to substitute for Ca^{2+} in some Ca^{2+} dependent reactions. The ability of Pb^{2+} to interact at a Ca^{2+} binding site was assessed by controlling the Ca^{2+} levels with EGTA and then adding Pb^{2+}. Simply, if Pb^{2+} is interacting at a Ca^{2+} binding site, the addition of Pb^{2+} should have similar effects on channel gating as the addition of Ca^{2+}. Channel activity in the presence of IP_3 and low Ca^{2+} should increase when Pb^{2+} is added and eventually decrease as more Pb^{2+} is added. The results of these studies are presented in Chapter 7.

4. *Does this Pb^{2+} sensitive store change during development?* Children are more susceptible to low level Pb^{2+} exposure than adults (Cantwell, 1975). Preliminary evidence in rat hippocampal synaptosomes suggests that the ability of Pb^{2+} to release Ca^{2+}_i was greatest in juvenile animals and apparently lost in adult animals. A series of experiments were conducted to quantitate the ability of Pb^{2+} to release Ca^{2+} over development. The

results of these studies are presented in Chapter 8. Preliminary experiments also suggested that the most drastic reduction in Pb^{2+} activated Ca^{2+} release occured between 7 and 9 weeks of age. Interestingly, male rats enter puberty promptly at 5 weeks of age. Thus, neural and hormonal alterations that accompany puberty may induce changes in Ca^{2+}_i storage that limit the ability of Pb^{2+} release Ca^{2+}. To investigate this possibility, a series of pilot experiments was conducted in which male rats were castrated shortly after birth and the ability of Pb^{2+} to release Ca^{2+} was compared at 3 and 10 weeks of age (juvenile vs. adult). The results from these studies are also presented in Chapter 8.

An overall discussion of this study and future research directions are presented in Chapter 9. The outcome of these experiments has contributed to our understanding of neurochemical mechanisms of Pb^{2+} neurotoxicity and possibly the greater sensitivity of young children to Pb^{2+} exposure.

CHAPTER II

MATERIALS AND METHODS

Animals

Male Sprague-Dawley rats between 3 and 12 weeks of age, weighing 35 - 350 g were used for these studies. The rats were housed in a temperature and humidity controlled facility in accordance with USDA regulations and the NIH guide for the Care and Use of Laboratory Animals (NIH Publication No. 85-23, 1985). The animals were allowed free access to drinking water and standard rat chow. The animal experiments presented in this study were reviewed and approved by the Animal Care and Use Committee at Eastern Virginia Medical School.

Synaptosomes

Introduction

The aim of this series of experiments was to study intracellular changes in Pb^{2+} and Ca^{2+} in isolated nerve terminals from the hippocampus, using fluorescent Ca^{2+} indicators and stopped-flow spectroscopy. The biological material used for these studies was the pinched off presynaptic nerve terminal ("synaptosome") from rat brain. The preparative procedure used a modification of Dunkley et al., 1986. This technique is superior to other methods in that it requires less time to prepare synaptosomes (2-3 hours), it has better yield, and it is less contaminated with myelin or glial debris. The synaptosome preparation is limited by several disadvantages: i) the preparation is heterogeneous with respect to cell type and neurotransmitter content; ii) depolarization can only be accomplished by non-physiological means, i.e., elevated potassium; and iii) the intracellular compartment cannot

be manipulated easily. However, these disadvantages are offset by the following: i) it is the only preparation available that allows direct study of most central nerve terminals; ii) current isolation schemes indicate that some enrichment of nerve terminal subtype, according to neurotransmitter content, can be achieved (Dunkley et al., 1986); iii) stopped flow spectroscopy allows the continuous recording of changes in intraterminal calcium on a time scale that approaches that of the neuronal action potential (Bartschat, 1990; Lentzner et al., 1992; Tareilus and Breer, 1992; Tareilus et al., 1993; Thomas et al., 1994); iv) synaptosomes retain essentially all the normal metabolic activity of nerve terminals in situ (Blaustein, 1988), including the ability to maintain appropriate ionic gradients and normal membrane potentials (Nachshen and Blaustein, 1982), the ability to release neurotransmitters by exocytosis, and recycle synaptic vesicle membranes (Drapeau and Blaustein, 1983); and v) freeze-thaw cycles allow the introduction of impermeant molecules into the cytosol with virtually full recovery of synaptosomal activity (Nichols et al. 1989).

<u>Isolation of Nerve Terminals</u>

Isolated nerve terminals were prepared from freshly dissected rat hippocampus as described previously (Bartschat and Rhodes, 1995; Lentzner et al. 1992). In brief, rats weighing 50-350 g were killed by decapitation in accordance with institutional Animal Care and Use Committee guidelines, the forebrains were quickly removed, the hippocampi quickly and carefully dissected free of surrounding structures, and placed in ice-cold 0.32 M sucrose. This preparation was homogenized in a loose-fitting Teflon/glass mortar and pestle at 900 rpm for 6-8 strokes in ice-cold 0.32 M sucrose and 1 mM $MgCl_2$ (adjusted

to pH 7.4 with HEPES-NaOH). The supernatant resulting from a 10 min 1085xg centrifugation was centrifuged for 20 min at 12,100xg, and this pellet was resuspended in the same sucrose solution with the addition 6.5% (vol/vol) Percoll. This suspension was layered atop a 9.1 and 17.4% Percoll solution (in 0.32 M sucrose), and centrifuged at 16000xg for 30 min in a Sorvall SS-34 rotor (total accumulated centripetal force, $\omega^2 t$, was 2.61 X 10^9 rad^2/s). The band settling at the 9.1:17.4% interface was harvested and washed twice with physiological saline (PSS) containing 146 mM NaCl, 2 mM MgCl$_2$, 10 mM dextrose, 0.5 mM Na$_2$HPO$_4$, 0.5 mM sodium pyruvate, and 10 mM HEPES buffer (adjusted to pH 7.4 with NaOH). In some cases, some or all of the Na$^+$ was replaced with N-methyl-D-glucamine (NMDG) mole for mole. The synaptosomes were suspended in a final volume of 10 ml.

Loading with fura-2

The acetoxymethyl (AM) ester form of fura-2 was freshly dissolved in dimethyl sulfoxide (DMSO) and then added to the 10 ml of synaptosomes in PSS [containing bovine serum albumin (BSA; fatty acid free; 1 mg/ml)] to a final concentration of 5 µM at 30°C. The AM ester form of fura-2 passively enters the synaptosome where it is cleaved into the cell impermeant form by cytosolic esterases (as depicted in Figure 2-1). The synaptosomes were allowed to accumulate fura-2 for 30 min, diluted to 50 ml, and incubated for an additional 20 min at 30°C. The synaptosomes were washed with ice-cold PSS without BSA and then divided into 5 ml aliquots corresponding to the number of conditions in that experiment. Typically, each 5 ml aliquot is equivalent to one-half to one rat hippocampus. To assess the contribution of cellular autofluorescence to the total

fluorescence signal preliminary experiments were conducted where a parallel aliquot of synaptosomes was treated identically but without the addition of fura-2. It was determined that autofluorescence caused <10% overestimation of the cytoplasmic free Ca^{2+} concentration; however, this correction was not routinely made in most of the experiments presented herein to minimize the complexity of the protocols.

Stopped-flow spectroscopy

Before each assay, an aliquot of the fura-2 loaded synaptosomes was washed with ice-cold PSS containing no added Ca^{2+} (contaminating free Ca^{2+} was estimated to be between 5 and 10 μM) and resuspended in 5 ml of the same PSS. This suspension was placed in one syringe of the stopped-flow apparatus (syringe A; volume, 1 ml), while the other syringe is filled with the desired reaction solution, typically containing 1 mM Ca^{2+} or 10 μM Pb^{2+} and 4-150 mM K^+ (syringe B; volume, 2.5 ml). In solutions containing an elevated K^+ concentration, Na^+ is replaced with K^+ mole for mole. The solutions were allowed to equilibrate to 30°C for at least 10 min prior to mixing.

Stopped-flow experiments were performed with a Hi-Tech (Salisbury, U.K.) PQ/SF-53 fluorescence spectrophotometer equipped with a dual-grating monochromotor (set at 340 or 380 nm) between the 75-W xenon light source and the reaction cuvette excitation window. The light emitted by the sample was high pass (>465 nm) filtered and measured with a photomultiplier tube (as depicted in Fig. 2-2). The quartz sample cuvette and the syringes containing the reactants were maintained at 30°C by a circulating water bath. The pneumatic rams were driven by a nitrogen pressure of 4-5 bar, which results in
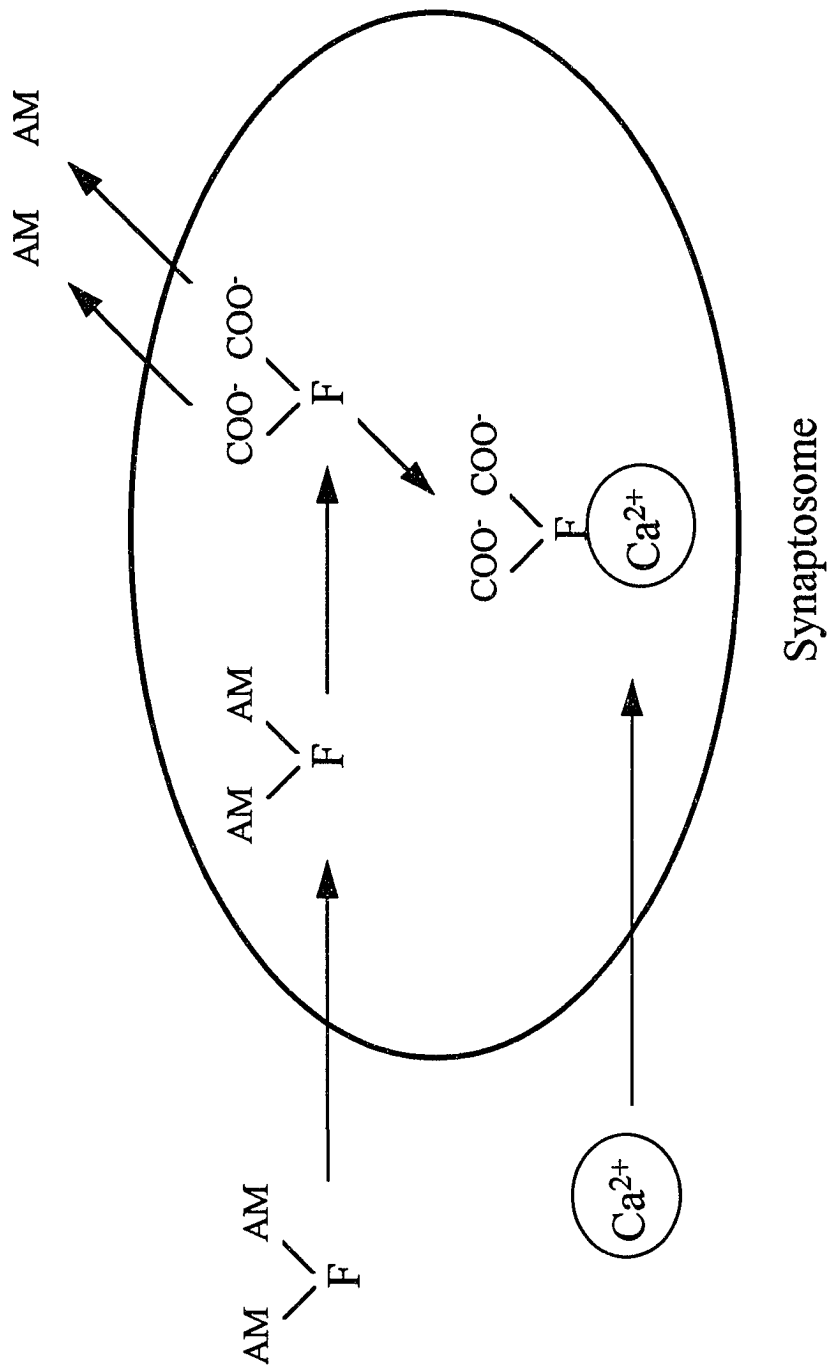
Figure 2-1. Schematic of fura-2 loading in synaptosomes.

an instrument dead time of 1-2 ms. This instrument uses Berger Mk. II Ball mixers. The reacted synaptosomes were collected and stored on ice after each condition.

The photomultiplier output voltage was continuously monitored in real-time and stored on VCR tape for later analysis. To determine reaction rate constants, the data was replayed and digitized at 1 ms/point, and nine "shots" (each "shot" is defined as one injection of synaptosomes) under identical conditions at either 340 or 380 nM were digitally averaged. For each "run" (defined as nine identical "shots" at 340 nm and nine "shots" at 380 nm), the ratio of the averaged 340 and 380 nm data were calculated. At the end of the experiment (consisting of from six to nine "runs," or one synaptosome preparation), the reacted synaptosomes were permeabilized with 25 μM digitonin and divided into two aliquots for conversion of the fluorescence signal to intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$). To one aliquot, 10 mM $CaCl_2$ was added while 10 mM EGTA (pH > 12) was added to the other. These samples were reintroduced into the stopped-flow cuvette without dilution, to determine the 340/380 nm fluorescence ratio of the dye at saturation (R_{max}) and the ratio with $[Ca^{2+}] < 10^{-8}$ M (R_{min}).

At the end of each experiment, the ratio of 340/380 nm fluorescence (R) was converted to $[Ca^{2+}]_i$ by using the following equation (Grynkiewicz et al., 1985):

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

where β refers to the quotient of the Ca^{2+}-free and Ca^{2+}-saturated forms of the dye, determined at 380 nm. The K_D for the fura-2:Ca^{2+} complex was determined to be 240 nM. We have found a small day-to-day variation in the R_{min} and R_{max} values for various
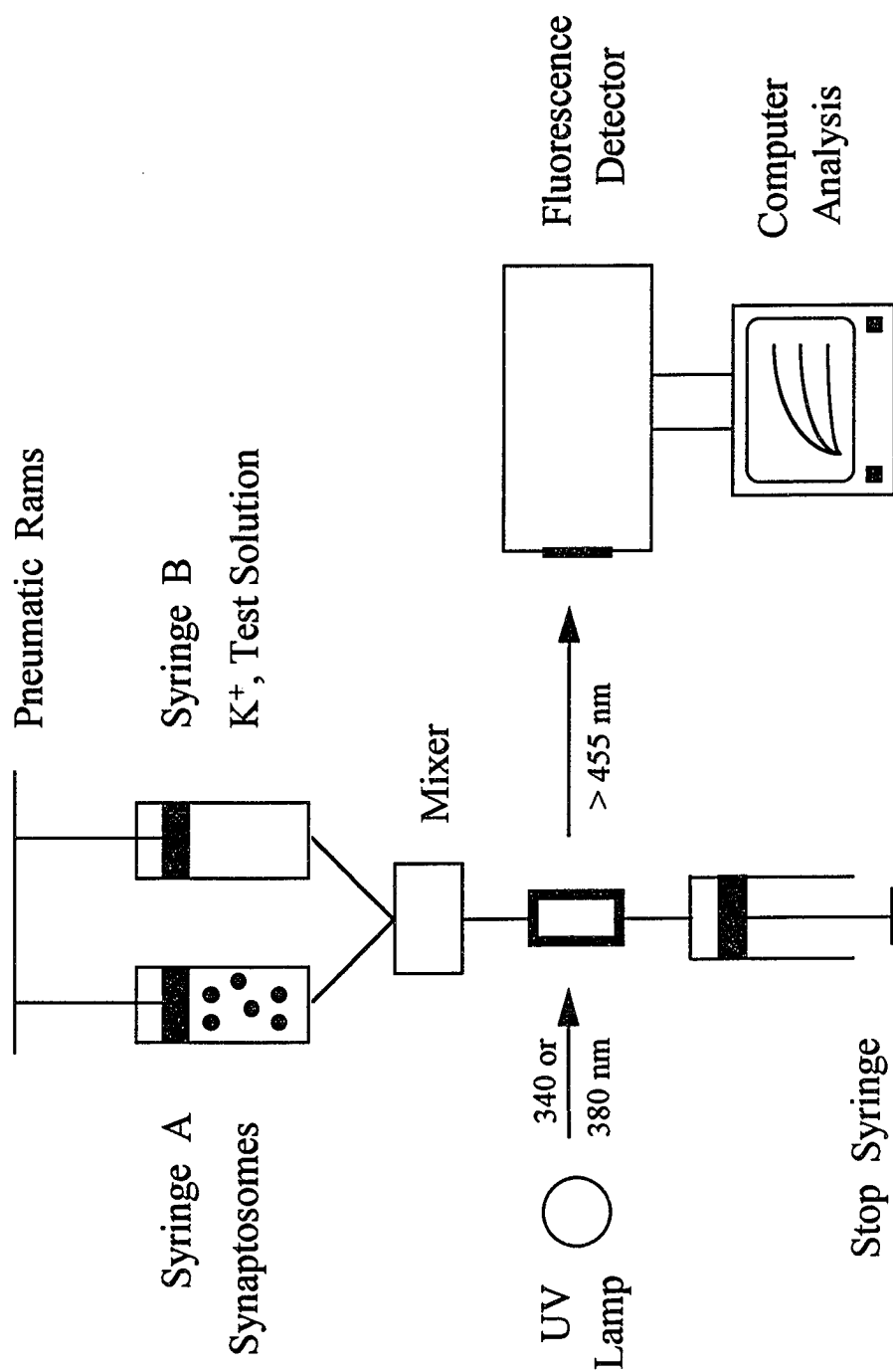
Figure 2-2. Schematic of stopped-flow spectroscopy.

synaptosome preparations, so independent calibrations were performed for each experiment.

The converted data were then computer fitted to a two-component exponential decay equation using either sequential integration or Levenburg-Marquardt algorithms (See Chapter 3, Fig. 1). The Ca^{2+} signal was expressed by five parameters: the calculated $[Ca^{2+}]_i$ at time = 0, the rate constant for influx, k (s^{-1}), and the final change in $[Ca^{2+}]_i$, also referred to as the amplitude or magnitude of influx $[Ca^{2+}]_i$ (nM) for both fast and slow components of Ca^{2+} influx.

Endoplasmic Reticulum Vesicles

Introduction

Certain experiments require unimpeded access to the cytosolic compartment. Unfortunately, the cytosolic compartment within nerve terminals cannot be manipulated easily. Therefore, endoplasmic reticulum (ER) vesicles or microsomes were isolated and the Ca^{2+} flux was monitored with a cuvette based fluorescence system. Unlike stopped-flow spectroscopy, this system allows sequential additions to be made. Ca^{2+} uptake and release were monitored by loading the intracellular stores with chlortetracyine (CTC), a tetracycline antibiotic that preferentially associates with a membrane bordering an area with micromolar or higher levels of Ca^{2+}, such as within intracellular stores (Marcotte et al., 1990). These experiments were performed to determine if Pb^{2+} directly induces Ca^{2+} release from IP_3 sensitive stores.

Microsome preparation

Microsomal vesicles were prepared from freshly dissected cerebelli as previously described (Alderson and Volpe, 1990). Briefly, rats weighing 50-350 g were killed by decapitation in accordance with institutional Animal Care and Use Committee guidelines, the cerebelli were quickly removed, and placed in ice-cold 0.32 M sucrose. In certain instances, cerebelli were removed, placed in ice-cold 0.32 M sucrose and stored in a -20°C freezer until needed. The preparation was minced with scissors and homogenized in a loose-fitting Teflon/glass mortar and pestle at 1300 rpm for 6-8 strokes in ice-cold 0.32 M sucrose, 10 mM HEPES, and 0.1 mM PMSF (adjusted to pH 7.0 with HEPES-NaOH). Following the first spin at 900xg , the pellets were resuspended in 5 vol and homogenized again for 3 strokes at 1300 rpm. The supernatants from both centrifugations were combined and centrifuged at 17,000xg. The resulting supernatant was centrifuged at 100,000xg to obtain the microsomal fraction. This pellet was resuspended in 40 mM KCl, 62.5 mM KH_2PO_4, and 8 mM MOPS (adjusted to pH 7.0 with KOH) and stored at -80°C until needed. This procedure yields membrane vesicles enriched in ER enzymatic markers, with little or no nuclear or mitochondrial contamination (Alderson and Volpe, 1989).

Cuvette based fluorescent spectroscopy

A 200 µl aliquot (~0.5 mg protein) was thawed, added to 2.8 ml of "uptake buffer" containing 40 mM KCl, 62.5 mM KH_2PO_4, 100 µM CTC, and 8 mM MOPS (adjusted to pH 7.0 with KOH) and placed in a continuously stirred cuvette maintained at 37°C. After baseline fluorescence was obtained, Ca^{2+} uptake was initiated by the addition of 1mM Na-ATP. After Ca^{2+} uptake had ceased, Pb^{2+}, IP_3, and other agonists were added

to elicit release. The change in fluorescence was obtained by exciting at 410 nm high pass filtering (>465 nm) the emission. Since chlortetracycline does not allow direct calibration, the baseline fluorescence, peak fluorescence following maximal Ca^{2+} uptake, and the fluorescence after agonist induced release were quantified and the elicited release was expressed as a percentage of the total uptake. Numerous preparations were used for these studies. In preliminary experiments, each preparation was treated with the calmodulin blocker W-7 and the mitochondrial inhibitor 2,4-dinitrophenol to determine the contribution of inverted plasma membrane vesicles or mitochondrial contamination to overall Ca^{2+} uptake. In all preparations, contamination was minimal, and the use of W-7 and 2,4-DNP was unnecessary.

Single calcium channels in artificial lipid bilayers

<u>Introduction</u>

The purpose of this series of experiments was to reconstitute microsomal vesicles into artificial planar bilayers to examine single Ca^{2+} release channels in a membrane of defined lipid composition, separate from the normal cytosolic milieu. This technique was used to determine if Pb^{2+} directly interacts with the channel molecule to elicit release. The voltage sensitivity, ion selectivity, and pharmacology of these channels were examined under control conditions and in the presence of Pb^{2+}.

<u>Lipid preparation</u>

Planar phospholipid bilayers were made by following traditional protocols (Mueller et al., 1962). Briefly, a decane solution of 20 mg/ml phospholipids (phosphatidyethanolamine:phosphatidylserine,75:25) was spread across a 0.15 mm

diameter hole in a polystyrene partition separating two electrolyte solutions. The experimental apparatus was situated on a high mass, pneumatically supported table to reduce noise due to building vibrations. Spontaneous thinning was monitored visually with a dissecting microscope, by observing the disappearance of diffraction colors, and electrically by observing the characteristic increase in capacitance. Thinned membranes were approximately 0.1 mm in diameter, and had capacitances of about 200 pfarad, and had resistances of over 10^8 ohm/cm^2. Under these conditions, bilayer noise was under 1 pA. These experiments were conducted at ambient temperatures.

Bilayer Apparatus

Electrical measurements were made with agar bridges connected to Ag/AgCl electrodes which were connected to a patch clamp amplifier to apply command potentials at the bilayer, and to measure current across the bilayer. The electrodes were prepared by immersion in Chlorox® bleach. Both solutions were stirred with magnetic stirring bars. Membrane vesicles were added the the *cis* chamber. The *trans* chamber was held at virtual ground, and depolarizing steps were applied repeatedly. The traces were filtered at 1000 Hz before being displayed on a digital oscilloscope and stored on a Neurocorder VCR based recording system. Data was analyzed with pClamp software, which is also used to drive the command generator. The patch clamp amplifier currently being used was an Axopatch 1C with integrating headstage configured in the "bilayer" mode. Most of the membrane capacitance was manually nulled; any uncompensated transients were substracted from identical test pulses containing no channel openings (Alvarez, 1986).
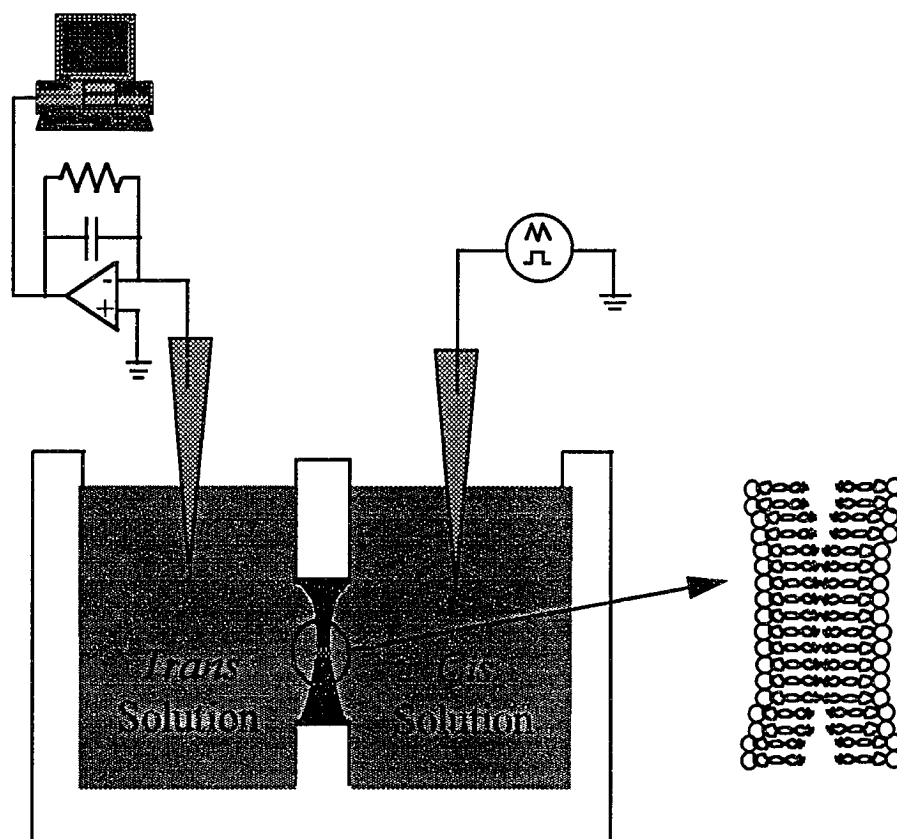
Bilayer Apparatus with
Biconcave Planar Phospholipid
Bilayer Spanning the Aperture
between Cis and Trans
Solutions

Figure 2-3. Schematic of bilayer apparatus.

Channel incorporation

The bilayer was cast in symmetrical 50 mM CsOH, and after bilayer thinning, 350 mM CsOH was added to the *cis* chamber to create an osmotic gradient that promotes membrane incorporation. While maintaining the membrane potential at 0 mV, 5-25 μg of vesicles were added. Incorporation of a Ca^{2+} channel was represented by a positive current fluctuation while chloride channel incorporation was represented by a negative fluctuation. Thus, a positive potential means the *cis* side is positive with respect to the *trans* and a positive current means that the net positive charge is flowing from the *cis* to the *trans* chamber. Cl^- currents were eliminated by using OH^- and NO_3^- solutions. After incorporation, it was necessary to characterize each channel. IP_3 sensitive Ca^{2+} channels were identified by activation by IP_3, inhibition by heparin, and their bell shaped dependence on Ca^{2+}. Furthermore, caffeine, ryanodine and ruthenium red should have no effect on IP_3 sensitive channels. Due to the sensitivity of the channel to Ca^{2+}, Ca^{2+}-EGTA buffer solutions were used to precisely control the free $[Ca^{2+}]$.
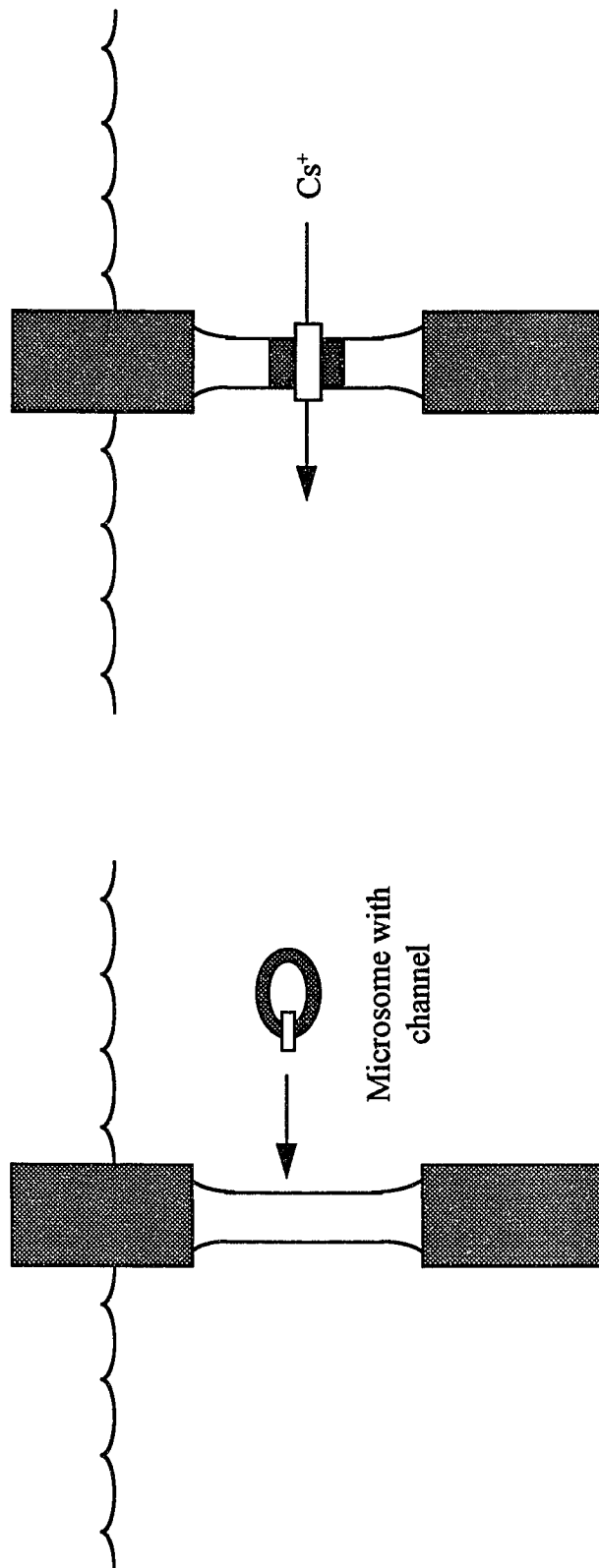
Figure 2-4. Schematic of channel incorporation. From Hille, 1992.

Developmental Aspects In Synaptosomes

<u>Introduction</u>

Preliminary evidence in rat hippocampal synaptosomes suggests that the ability of Pb^{2+} to release Ca^{2+}_i was greatest in juvenile animals and apparently lost in adult animals. The purpose of this series of experiments was to examine the effect of aging on the ability of Pb^{2+} to release Ca^{2+} under control conditions and after pretreatment with thapsigargin. Preliminary experiments also suggested that the most drastic reduction in Pb^{2+} activated Ca^{2+} release occured between 7 and 9 weeks of age. Interestingly, male rats enter puberty promptly at 5 weeks of age. Thus, neural and hormonal alterations that accompany puberty may induce changes in Ca^{2+}_i storage that limit the ability of Pb^{2+} to release Ca^{2+}. Therefore, a series of pilot experiments was conducted in which male rats were castrated shortly after birth and the ability of Pb^{2+} to release Ca^{2+} was compared at 3 and 10 weeks of age (juvenile vs. adult).

<u>Effect of Aging on the Ability of Pb^{2+} to Elicit Ca^{2+} Release</u>

Male Sprague-Dawley rats between 3 and 12 weeks of age, weighing 35-350g were used for these studies. Animals were maintained as previously described, and sacrificed by decapitation at various points over development. Hippocampal synaptosomes were isolated, loaded with fura-2, and mixed with 100 mM K^+ and 10 μM Pb^{2+}, as described previously.

<u>Effect of Castration on the Ability of Pb^{2+} to Elicit Ca^{2+} Release</u>

Twenty four male rats from the same lot and with identical date of birth were divided into 4 groups of 6 rats. Two of these groups were castrated 10 days after birth by the vendor, Hague Sprague-Dawley. The other two groups were used as controls and

were treated identically except for the surgical procedure. The rats became available immediately after weaning, approximately 21 days after birth. At day 21 and day 80, each group of castrated rats was sacrificed by decapitation, in accordance with Animal Care and Use Committee guidelines. The corresponding control groups were sacrificed the following day (day 22, 81 respectively) due to methodological constraints. Following decapitation, the forebrains were quickly removed, the hippocampi quickly and carefully dissected free of surrounding structures, and placed in ice-cold 0.32 M Sucrose. For each group, the hippocampi from 2 rats were combined and treated as one sample. Thus, each condition had 3 replicates. Synaptosomes were isolated, loaded with fura-2, and mixed in the stopped-flow spectrophotometer with 100 mM K^+ and 10 μM Pb^{2+} to determine the ability of Pb^{2+} to induce the release of Ca^{2+}, as described previously.

CHAPTER III

LEAD BLOCKS COMPETITIVELY TWO TYPES OF VOLTAGE-ACTIVATED

CALCIUM CHANNELS IN ISOLATED HIPPOCAMPAL PRESYNAPTIC

NERVE TERMINALS

Introduction

Low level Pb^{2+} exposure produces long lasting deficits in learning and memory in animals and man (Rice, 1993). The hippocampus, an area of the brain known to be involved in learning and memory, may be an important target for the actions of Pb^{2+}. Normal synaptic transmission is dependent at least in part on the appropriate activation and inactivation of neuronal voltage-dependent Ca^{2+} channels (VDCCs), which are blocked by Pb^{2+} ions in a number of tissues (Audesirk, 1993), so it is possible that blockade of VDCCs in the hippocampus may be a contributing factor to Pb^{2+} -induced neurotoxicity.

Pb^{2+} has been shown to block Ca^{2+} uptake in mammalian forebrain synaptosomes (Nachshen, 1984; Suszkiw, 1984) and adrenal chromaffin cells (Pocock, 1987), and blocks Ca^{2+} currents in invertebrate neurons (Busselberg, 1991), cultured neuroblastoma cells (Oortgiesen et al., 1990; Reuveny and Narahashi, 1991), rat dorsal root ganglion cells (Evan et al., 1991), and rat hippocampal neurons. Indeed, the VDCCs in hippocampal neurons appear to be one of the most Pb^{2+} sensitive ion channel types (Audesirk, 1993). However, information on the ability of Pb^{2+} to block hippocampal *nerve terminal* Ca^{2+} channels has been lacking. Accordingly, the experiments presented in this chapter are designed to determine the sensitivity of hippocampal presynaptic Ca^{2+} channels to the

blocking effects of Pb^{2+}, using isolated presynaptic nerve terminals ("synaptosomes") as a model system. Depolarization-dependent changes in presynaptic Ca^{2+} permeability, reflecting the activation of at least two classes of VDCCs, was monitored on a millisecond time scale by following changes in the fluorescence of the intracellular Ca^{2+} indicator fura-2 in a stopped-flow spectrophotometer (Bartschat and Rhodes, 1995; Lentzner et al., 1992).

Results

The membrane potential of synaptosomes is controlled mainly by the K^+ diffusion gradient (Blaustein and Goldring, 1975), so depolarization of the nerve terminals can be elicited by raising the extracellular K^+ concentration. When synaptosomes, previously loaded with the Ca^{2+} indicator fura-2, were mixed in the stopped-flow apparatus with 50 mM K^+ (final $[K^+]$ after mixing = 37 mM) and 1 mM Ca^{2+} (final $[Ca^{2+}]$ after mixing = 0.7 mM), the intracellular Ca^{2+} activity, as reported by calibrated fura-2 signals, promptly rose from a resting level of 268 ± 24 nM (mean ± SEM of 12 experiments) to ~ 0.7 μM by 2 s after depolarization (Fig. 3-1). Computer fit of the Ca^{2+}_i rise with a two- exponential-decay equation revealed the presence of at least two components of Ca^{2+} influx: a fast component with a rate constant, k_1, of 15 ± 3 s^{-1} and an amplitude , A_1, of 205 ± 35 nM; and a slower component of influx with a with a rate constant, k_2, of 1.5 ± 0.4 s^{-1} and an amplitude , A_2, of 247 ± 56 nM (Bartschat and Rhodes, 1995).

The fast component of Ca^{2+} influx represents a class of rapidly inactivating Ca^{2+} channels ($\tau \cong$ 60 msec) that are sensitive to block by La^{3+}, Cd^{2+}, and Co^{2+} (given in decreasing order of potency) but not to the organic Ca^{2+} channel blockers verapamil or

nifedipine and are only weakly susceptible to block by the peptide toxin ω-conotoxin GVIA (Bartschat and Rhodes, 1995). These channels also display voltage-dependent inactivation characteristic of N_T type Ca^{2+} channels in the posterior pituitary (Lemos and Nowycky, 1989).

About 50% of the slow component of Ca^{2+} influx can be blocked when the synaptosomes are depleted of intracellular Na^+, indicating that half of this component reflects Na^+/Ca^{2+} exchange operating in reverse. The remainder represents another class of voltage activated Ca^{2+} channels which inactivate slower ($\tau \cong 1$ sec) than the fast component and have a sensitivity to block by the inorganic channel blockers La^{3+}, Co^{2+}, and Cd^{2+} (given in decreasing order of potency) (Bartschat and Rhodes, 1995). A classification of the precise sub-types of the Ca^{2+} channels present in this preparation remains unfinished.

When the synaptosomes were depolarized with 100 mM K^+ in the presence of 10 μM Ca^{2+}_i, little Ca^{2+}_i rise was observed (Fig. 3-2A). When the Ca^{2+}_o was progressively increased to 1 mM, a concentration-dependent augmentation of Ca^{2+} influx was observed. Kinetic analysis revealed that the effect of Ca^{2+}_o was to increase the magnitude of both components of Ca^{2+} influx, with little effect on the rate constant for inactivation of the fast component, and a small increase in the rate constant for inactivation of the slow component (Lentzner et al., 1992).

When the same experiment was carried out in the presence of 0.5 μM Pb^{2+}, the Ca^{2+} signals obtained at the same Ca^{2+}_o and K^+_o were substantially reduced (Fig. 3-2B). Almost no evoked Ca^{2+}_i rise was seen until the extracellular Ca^{2+} was raised to at least 0.2
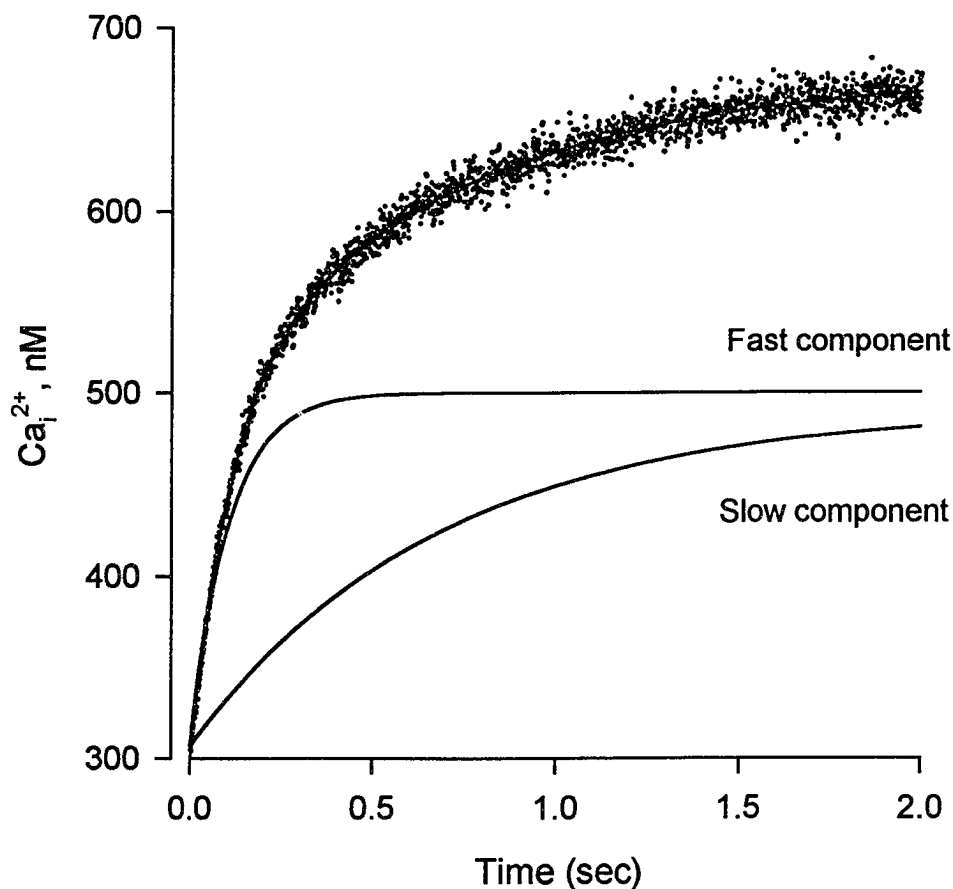
Figure 3-1. Depolarization-activated Ca^{2+} influx into presynaptic nerve terminals is composed of two kinetically distinguishable components. Synaptosomes, previously loaded with Fura-2, were mixed in a stopped flow apparatus (See Figure 2-2) to yield a final $[K^+]_o$ of 37 mM and a final $[Ca^{2+}]_o$ of 0.7 mM. The dots represent the digitized data points for the calibrated $[Ca^{2+}]_i$ signal at time (t), sampled every millisecond, and the solid line passing through the points represents the computer fit of the data to a two exponential equation:

$$Ca^{2+}_i = A_1[1\text{-exp}(-k_1t)] + A_2[1\text{-exp}(k_2t)] + b$$

where A_1 and A_2 represent the amplitudes of the fast and slow components of Ca^{2+} influx, respectively, and k_1 and k_2 represent the rate constants for Ca^{2+} influx through each component. The two labelled lines represent the computer derived time courses for Ca^{2+} influx through the fast and slow components of influx. In this experiment from one synaptosome preparation, $A_1 = 189$ nm Ca^{2+}, $k_2 = 13.5$ sec^{-1}, $A_2 = 217$ nm Ca^{2+}, and $k_2 = 1.35$ sec^{-1}. [Reproduced with permission from Bartschat, D.K., and Rhodes, T.E. (1995) *J Neurochem.* 64, 2064-2072]

mM, and even in solutions containing 1 mM Ca^{2+}, the Ca^{2+}_i signal was substantially reduced.

When control experiments and those experiments containing various Pb^{2+} concentrations were subjected to kinetic analysis, it was determined that the inhibitory effect of 0.1 to 0.5 μM Pb^{2+} was confined to the magnitude of the Ca^{2+} rise mediated by both the fast and slow components, with no effect on the rate constants of inactivation of either component (data not shown). This is consistent with the interpretation that that Pb^{2+} has no effect on the gating of either the fast or slowly inactivating Ca^{2+} channels, and that extracellular Pb^{2+} reduces the single channel conductance of individual Ca^{2+} channels, or fully blocks a fraction of the available Ca^{2+} channels. Differentiation of these two possibilities will require the use of electrophysiological techniques.

Extracellular Pb^{2+} had little effect on Na^+/Ca^{2+} exchange mediated Ca^{2+} uptake under the conditions employed here.

The Ca^{2+}_o dependency of the magnitude of the Ca^{2+}_i rise mediated by the fast component (Panel A) and that for the slow component (Panel B) are presented in Fig. 3-3, for control depolarizations and in the presence of 0.1, 0.3, and 0.5 μM Pb^{2+} . The solid lines represent fit of the data to the Michaelis-Menton equation. The results indicate that, in the absence of Pb^{2+}, Ca^{2+} influx through the fast component is a monotonic function of Ca^{2+}_o with an apparent affinity constant K_{mapp}, of 169 $\pm$ 38 μM and a V_{max} (i.e. the 'maximum magnitude') of 154 $\pm$ 8 nM Ca^{2+} rise. Ca^{2+} influx through the slow component was also monotonic with a K_{mapp} of 41 $\pm$ 10 μM and a V_{max} of 133 $\pm$ 23 nM Ca^{2+} rise. Inclusion of Pb^{2+} in the depolarization medium caused a rightward shift in the Ca^{2+}-influx

Figure 3-2. Effect of increasing $[Ca^{2+}]_o$ and $[Pb^{2+}]_o$ on depolarization-activated Ca^{2+} influx. The traces in Panel A represent experiments performed in the presence of increasing $[Ca^{2+}]_o$ while the traces in Panel B represent identical experiments performed in the presence of 0.5 μM Pb^{2+}. When the the $[Ca^{2+}]_o$ was maintained at 10 μM or less, depolarization of the synaptosomes with 100 mM K^+ elicited little Ca^{2+}_i rise (Panel A). When the $[Ca^{2+}]_o$ was progessively increased to 1 mM, a concentration-dependent augmentation of Ca^{2+} influx was observed. Kinetic analysis revealed that the effect of Ca^{2+} was to increase the magnitude of both components of Ca^{2+} influx, with little effect on the rate constant for inactivation of the fast component, and a small increase in the rate constant for inactivation of the slow component. When the same experiment was performed in the presence of 0.5 μM Pb^{2+}, the Ca^{2+} influx at the same $[Ca^{2+}]_o$ and $[K^+]_o$ were substantially reduced (Panel B). Almost no Ca^{2+} influx was observed until the $[Ca^{2+}]_o$ was increased to 200 μM, and in solutions containing 1 mM Ca^{2+}, the influx was substantially reduced.
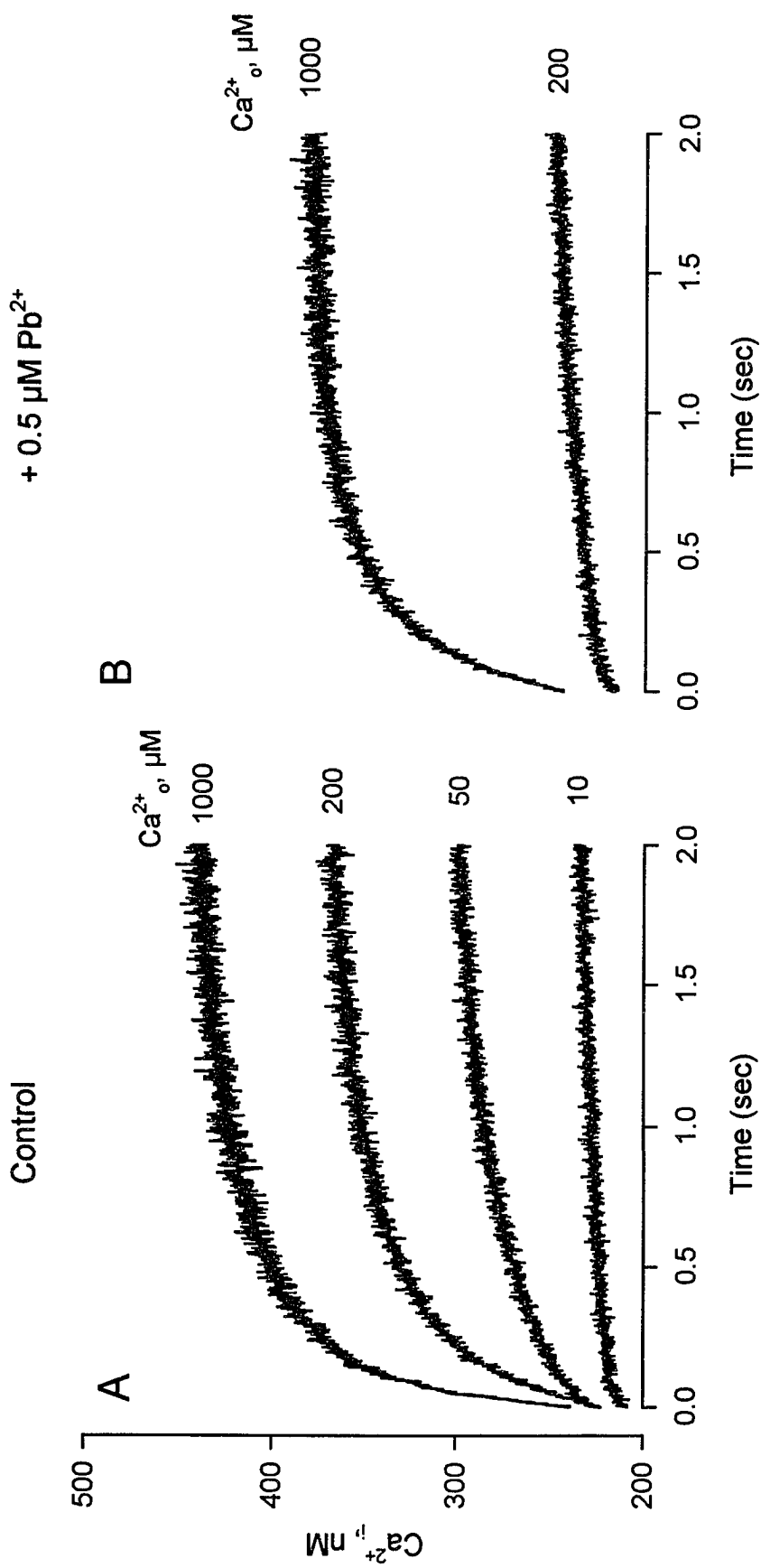
Figure 3-3. Concentration dependence of Pb^{2+} on depolarization-activated Ca^{2+} influx. The magnitude of the fast component (Panel A) and the slow component (Panel B) are plotted versus the $[Ca^{2+}]_o$ from control synaptosomes (open circles), and synaptosomes depolarized in the presence of 0.1 (solid squares), 0.3 (inverted triangles), or 0.5 μM Pb^{2+} (solid triangles). The solid lines are the computer derived best fit to the Michaelis-Menton equation. In the absence of Pb^{2+}, Ca^{2+} influx through the fast component is a monotonic function of $[Ca^{2+}]_o$ with an apparent affinity constant (Km_{app}) of 169 ± 38 μM and V_{max} (maximum magnitude) of 154 ± 8 nM Ca^{2+} rise. Ca^{2+} influx through the slow component was also monotonic with a Km_{app} of 41 ± 10 μM and a V_{max} of 133 ± 23 nM Ca^{2+} rise. Depolarization in the presence of Pb^{2+} caused a rightward shift in the Ca^{2+} influx relationship, consistent with an increase in the Km_{app} for Ca^{2+} permeation through both components of influx.

A

B
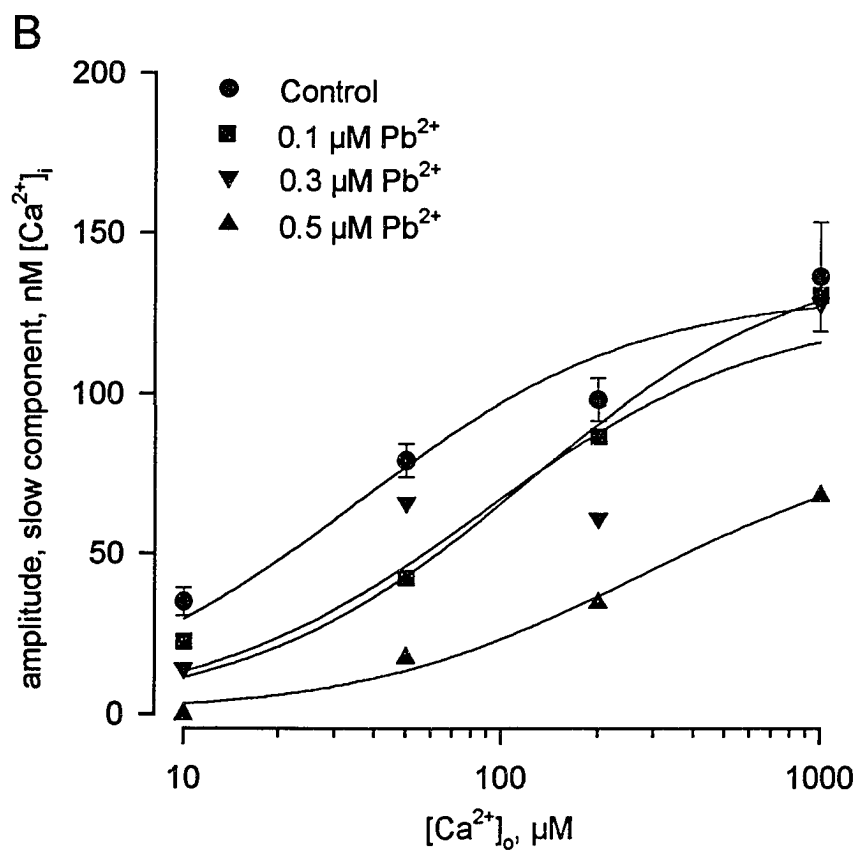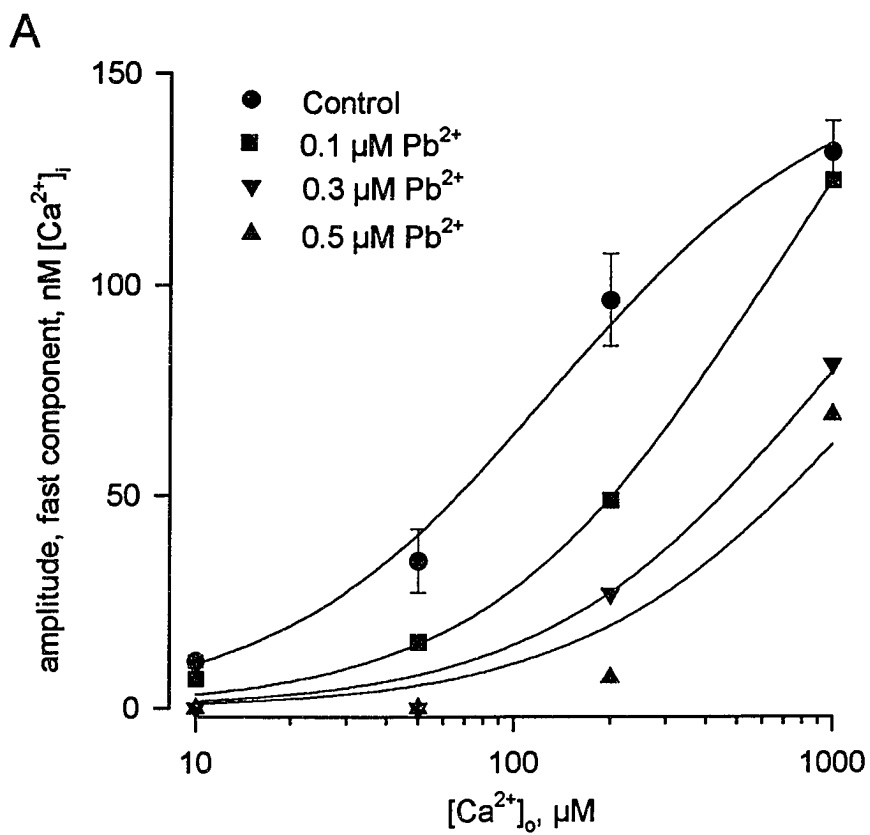
relationship, consistent with an increase in the K_{mapp} for Ca^{2+} permeation for both the fast and slow components of influx.

The data presented in Figs. 3-4 and 3-5 represent a plot of the computer-derived V_{max} (Panel A) or the K_{mapp} (Panel B) for the fast component (open circles) and slow component (closed circles) of Ca^{2+} uptake vs. the extracellular Pb^{2+} concentration. The V_{max} for Ca^{2+} uptake was essentially unaffected by Pb^{2+} over the concentration range studied (i.e. 0.1 to 0.5 μM). In contrast, the K_{mapp} for both the fast and slow components of Ca^{2+} influx was increased as the extracellular Pb^{2+} was raised, consistent with competitive blockade of the presynaptic Ca^{2+} channels by Pb^{2+}. Using these latter data (Figure 3-5) the apparent IC_{50} for block of the fast inactivating Ca^{2+} channels was determined to be 140 nM, and that for the slowly inactivating channels was determined to be 195 nM.

Discussion

Pb^{2+} ions have been shown to interfere with a number of neuronal Ca^{2+} dependent processes, which has lead to the suggestion that some of the neurotoxic effects of this ion may be related to disruption of neuronal Ca^{2+} homeostasis. In addition to the block of Ca^{2+} channels as discussed above, Pb^{2+} permeates Ca^{2+} channels and may act as an intracellular 'Ca^{2+} surrogate' by activating neurotransmitter release directly (Shao and Suszkiw, 1991). However, because of the large range of potencies reported for the blocking ability of Pb^{2+} on various Ca^{2+} channel subtypes (Audesirk, 1993), and in view of the possibility that Pb^{2+} produces behavioral deficits that may be related, in part, to disruption of synaptic transmission in the hippocampus (Rice, 1993), it is important to
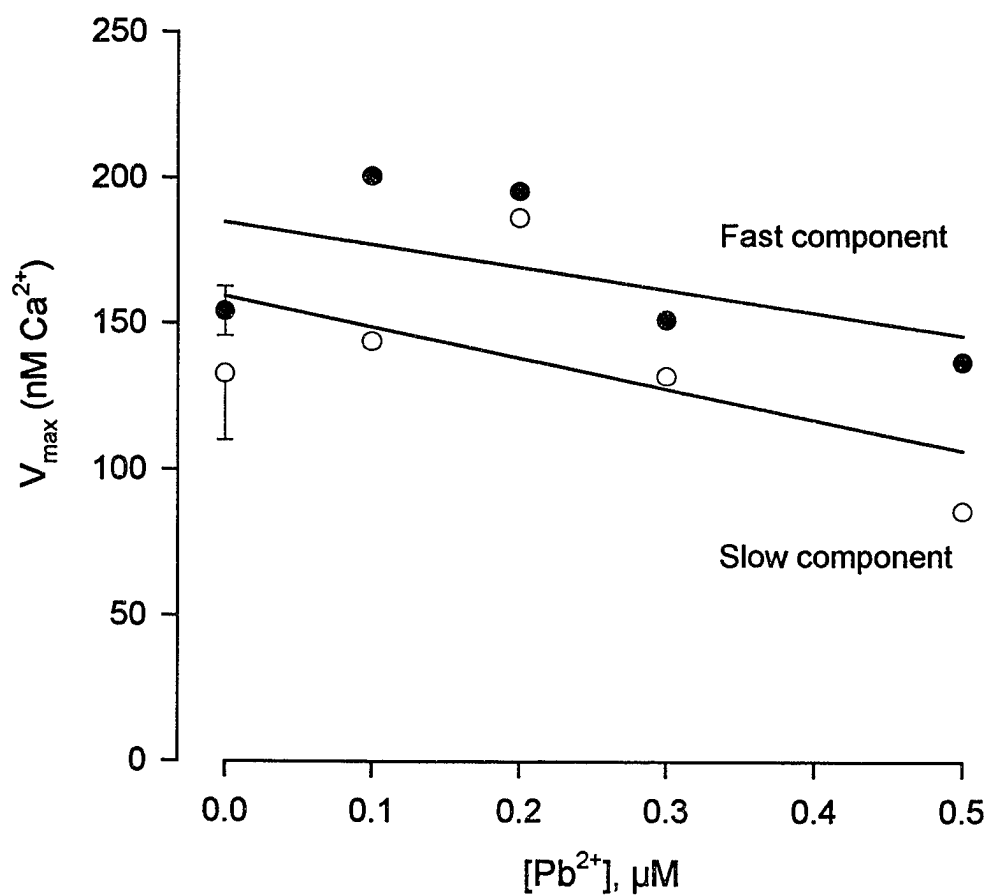
Figure 3-4. V_{max} **for the fast and slow component of Ca^{2+} influx.** The V_{max} was plotted versus the $[Pb^{2+}]_o$ for the fast (closed circles) and slow components (open circles) of Ca^{2+} influx following depolarization with 100 mM K^+_o. The solid lines are the linear regression fit to the data. The V_{max} for Ca^{2+} uptake was essentially unaffected by Pb^{2+} over the concentration range studied (0.1 - 0.5 μM).
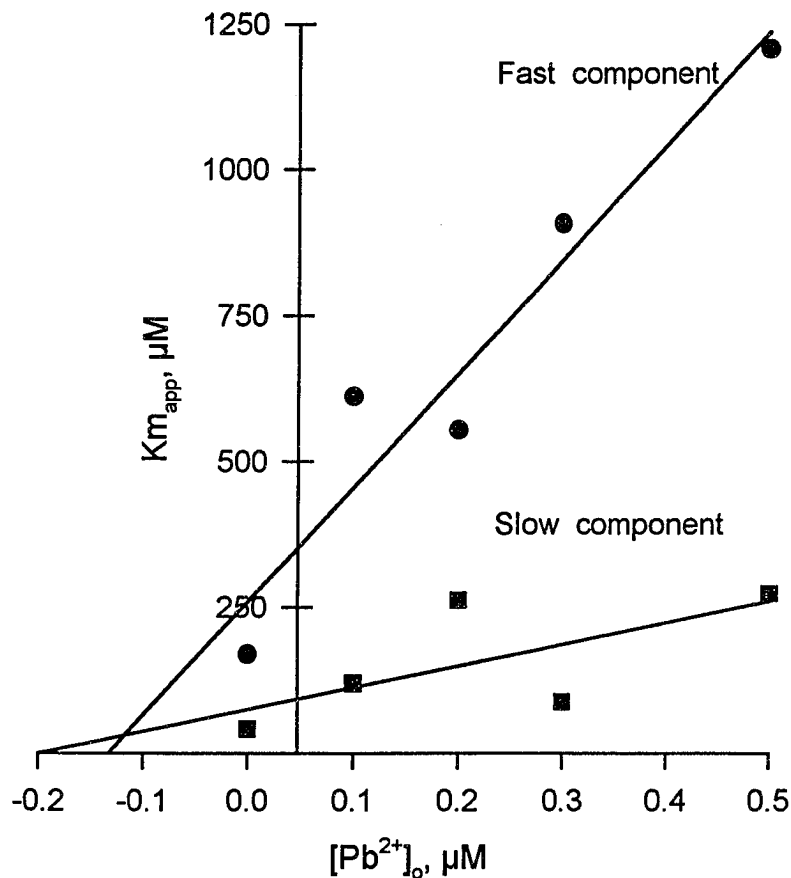
Figure 3-5. Km_{app} for the fast and slow component of Ca^{2+} influx. The Km_{app} (Panel B) are plotted versus the $[Pb^{2+}]_o$ for the fast (closed circles) and slow components (open circles) of Ca^{2+} influx following depolarization with 100 mM K^+_o. The Km_{app} for both the fast and slow components of Ca^{2+} influx increased as the $[Pb^{2+}]_o$ was raised, consistent with competitive blockade of the presynaptic Ca^{2+} channels by Pb^{2+}. The apparent IC_{50} of Pb^{2+} for the fast inactivating channels was determined to be 140 nM and that for the slowly inactivating channels was determined to be 195 nM.

study directly the susceptibility of hippocampal nerve terminal Ca^{2+} channels to the effects of Pb^{2+}, as for the experiments reported herein.

The results presented in this section demonstrate that nerve terminals isolated from rat hippocampus possess at least two classes of voltage-dependent Ca^{2+} channels, and that both of these channels are susceptible to block by Pb^{2+} ions. The ability of Pb^{2+} to block the evoked Ca^{2+} transient in this preparation can be antagonized by elevating extracellular Ca^{2+}, which suggests that Pb^{2+} and Ca^{2+} compete for a binding site within the channel molecule, probably within the ion permeation pathway. Pb^{2+} blocks L- and N-type Ca^{2+} channels in hippocampal neurons with IC_{50}'s of 30 and 80 nM, respectively (Audesirk, 1993), which is similar to the IC_{50} values obtained in the present study (140 nM and 195 nM for rapidly inactivating and slowly inactivating Ca^{2+} channels, respectively). Our results are likely to be an overestimation of the true IC_{50} values, as the actual free Pb^{2+} concentration may be substantially less than that added to the solutions, due to complexion with anions and binding to synaptosomal membranes. It has been estimated that the toxic effects of Pb^{2+} can be seen with plasma concentrations in the pM range (Hernberg, 1980), but insofar as the relationship between the plasma Pb^{2+} concentration and the cerebrospinal fluid Pb^{2+} concentration is unknown , combined with the observation that Pb^{2+} may accumulate in the hippocampus (Widzowski and Cory-Slechta, 1994; Collins et al., 1982; Grandjean, 1978), it is unclear at this time whether Ca^{2+} channel blockade is important in Pb^{2+} neurotoxicity. It does appear that at close to physiological Ca^{2+} concentrations and Pb^{2+} concentrations found in the CSF, Pb^{2+}_{o} probably does not inhibit Ca^{2+} influx into presynaptic terminals to any significant extent which suggests that channel blockade may not contribute to the neurotoxic effect of Pb^{2+}.

CHAPTER IV

LEAD MEDIATES THE RELEASE OF CALCIUM FROM INTRACELLULAR STORES IN ISOLATED HIPPOCAMPAL PRESYNAPTIC NERVE TERMINALS

Introduction

The previous section demonstrated that at physiological Ca^{2+} concentrations and Pb^{2+} concentrations found in the CSF after Pb^{2+} exposure, extracellular Pb^{2+} does not inhibit Ca^{2+} influx into presynaptic nerve terminals to any significant extent. Thus, Ca^{2+} channel blockade may not contribute to Pb^{2+} neurotoxicity. However, Pb^{2+} may exert its' toxic effects by disrupting Ca^{2+} homeostasis within the terminal region. The incubation of osteoblastic bone cells (Schane 1989b), neuroblastoma glioma cells (Schane, 1989a), and human platelets (Dowd and Gupta, 1991) in solutions containing Pb^{2+} leads to a sustained elevation of intracellular Ca^{2+}. Pb^{2+} exposure also increases the total Ca^{2+} content of platelets (Dowd and Gupta, 1991), mouse osteoclasts (Rosen and Pounds, 1989), and rat hepatocytes (Pounds et al., 1982). Elevated Ca^{2+}_i is also observed in Pb^{2+} intoxicated cells (Schane et al., 1989a,b; Dowd and Gupta, 1991). Pb^{2+} may induce this effect by altering second messenger regulation of ion channels which would stimulate Ca^{2+} influx into cells. For example, Ca^{2+} influx may be increased by PKC stimulation, which Pb^{2+} can activate at subnanomolar concentrations (Markovac and Goldstein, 1988; Long et al., 1994). Pb^{2+} may inhibit Ca^{2+} extrusion which would also elevate Ca^{2+}_i. In erythrocytes, low micromolar concentrations of Pb^{2+} reduce Ca^{2+} transport by the Ca^{2+}-ATPase by 50% (Mas-Oliva, 1989). However, with the concentrations necessary for this effect, it is unlikely that it occurs *in vivo* because Pb^{2+} does not exceed picomolar levels in

experiments with platelets (Dowd and Gupta, 1991) and adrenal chromaffin cells (Tomsig and Suszkiw, 1991). The results from the preceding chapter suggest that Pb^{2+} has no effect on Na^{+}/Ca^{2+} exchange. Pb^{2+} may elevate Ca^{2+}_i by altering mitochondrial function. For example, Pb^{2+} stimulates Ca^{2+} release from mitochondria (Kapoor and Van Rossum, 1984; Chavez et al., 1987) and inhibits Ca^{2+} uptake into mitochondria (Parr and Harris, 1976; Goldstein, 1977). Furthermore, Pb^{2+} may effect Ca^{2+} buffering by the ER, the main Ca^{2+}_i store within neurons. In astrocytes, Pb^{2+} increases inositol 1,4,5-triphosphate levels but does not alter IP_3 mediated Ca^{2+} transients (Dave et al., 1993). In rats, low level, developmental Pb^{2+} exposure beginning prenatally, down-regulates IP_3 receptors on the ER but this effect was not observed following exposure in adult rats (Singh, 1993). Thus, alterations in Ca^{2+}_i metabolism may underlie some aspects of Pb^{2+} neurotoxicity.

The aim of the experiments in this section was to monitor intracellular changes in Ca^{2+} and Pb^{2+} on a millisecond time scale by combining fura-2 fluorescence and stopped-flow spectroscopy after depolarization in the presence of Pb^{2+}_o.

<u>Results</u>

As shown in Figure 4-1, when hippocampal synaptosomes were mixed in the stopped-flow device with 100 mM K^{+}, 100 μM Ca^{2+}, and 0.1 μM Pb^{2+}, the overall Ca^{2+} influx was reduced (middle trace) due to competitive blockade of both classes of VDCCs in this preparation by Pb^{2+}. When the same experiment was performed in the presence of 10 μM Pb^{2+}, an initial decrease in the fura-2 signal was observed, characteristic of an interaction between Pb^{2+} and intracellular fura-2. Although Pb^{2+} provokes a shift in the excitation wavelength similar to Ca^{2+}, the quantum yield is less. Thus, when Pb^{2+} displaces

Ca^{2+} on fura-2, a decrease in the fura-2 signal and apparent $[Ca^{2+}]_i$ is observed. This decrease in the apparent $[Ca^{2+}]_i$ was followed by a slow rise in fluorescence that occured even in the absence of Ca^{2+}_o and routinely rose above the initial Ca^{2+}_i measured at the beginning of depolarization.

The traces in Figure 4-2 are from synaptosomes mixed with either 4 (non-depolarizing buffer) or 100 mM K^+ with 10 μM Pb^{2+}. The slow rise in Ca^{2+}_i was only observed when the nerve terminals were depolarized, indicating presynaptic Ca^{2+} channels must be activated. A small drop in fluorescence was observed when the synaptosomes were mixed under basal conditions indicating that some extracellular fura-2 remained despite extensive washing. However, the subsequent rise in Ca^{2+}_i was only observed when the nerve terminals were depolarized.

To further determine if Pb^{2+} was interacting with intracellular fura-2, the nerve terminals were depolarized in the presence of La^{3+}, which blocks presynaptic Ca^{2+} channels. When the synaptosomes were mixed in the stopped-flow apparatus with 100 mM K^+ and 10 μM Pb^{2+}, an initial drop in fluorescence was observed that was followed by the subsequent rise in fluorescence (Fig.4-3, bottom trace) . However, when the same experiment was conducted in the presence of 10 μM La^{3+}, the rapid drop in fluorescence was eliminated. Similar results were obtained with higher La^{3+} concentrations but an increase in the apparent initial Ca^{2+}_i was observed. A linear rise in fluorescence was also observed after depolarization, which was kinetically different from the rise that followed depolarization in the presence of Pb^{2+}. This effect was presumably due to La^{3+} influx,
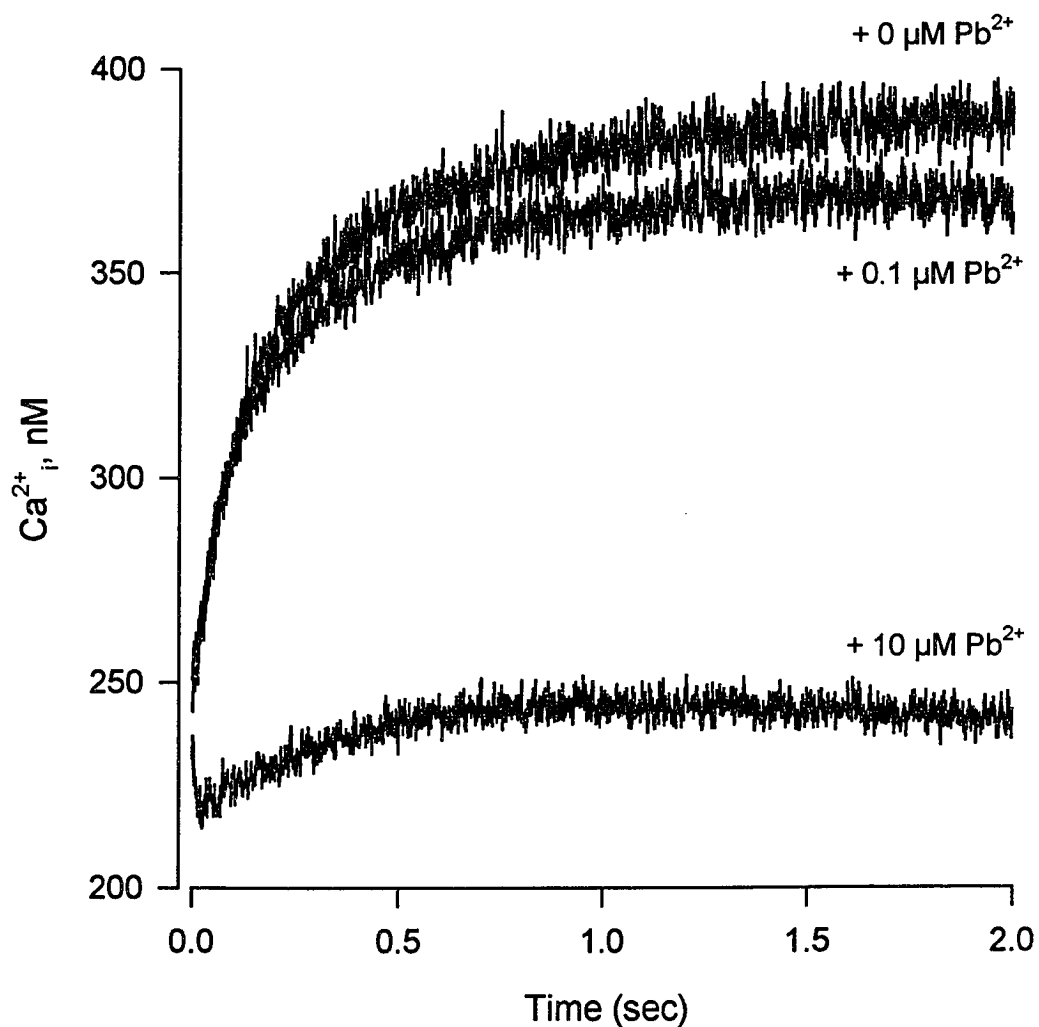
Figure 4-1. **Effect of Pb^{2+}_o on depolarization-activated Ca^{2+} influx.** The synaptosomes were depolarized with 100 mM K^+ in the presence of 100 μM Ca^{2+} and increasing $[Pb^{2+}]_o$ as indicated. These results are from one experiment and similar results were obtained from one other experiment. Note: for clarity, the bottom trace was offset by -25 nM.
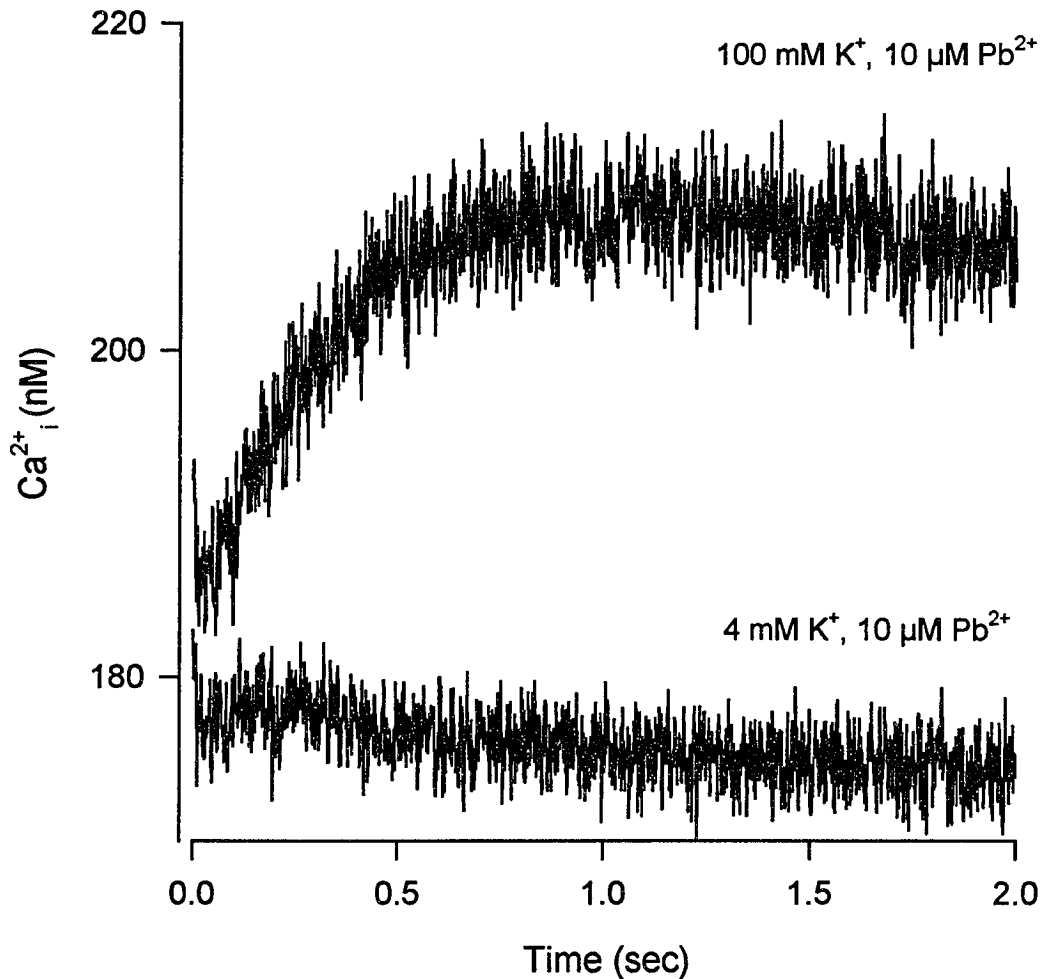
Figure 4-2. Effect of depolarization on the rise in intracellular Ca^{2+} induced by Pb^{2+} influx in depolarized synaptosomes. The synaptosomes were mixed in the stopped-flow apparatus to yield a final concentration of 10 μM Pb^{2+} and either 4 or 100 mM K^+ as indicated. These data were obtained from one synaptosome preparation, and similar results were obtained from one other experiment.

since Na^+/Ca^{2+} exchange readily transports La^{3+} in bovine chromaffin cells (Powis et al., 1994). Furthermore, La^{3+} binds fura-2 with a higher affinity and higher quantal yield than Ca^{2+} does, which possibly explains the apparent increase in Ca^{2+}_i that was observed. As shown in Fig. 4-3, the rate of the rise in fluorescence also increased with increasing La^{3+} concentrations which could also be explained by the ability of Na^+/Ca^{2+} exchange to transport La^{3+} into the terminals. A more relevant result of this experiment was that depolarization in the presence of La^{3+} eliminated the Pb^{2+} dependent signal and the subsequent rise in Ca^{2+}_i, further supporting the observation that Pb^{2+} was interacting with intracellular fura-2.

Combined, the preceding results suggest that following depolarization, Pb^{2+} permeates presynaptic Ca^{2+} channels and induces a rise in Ca^{2+}_i, even in the absence of Ca^{2+}_o.

To determine if Na^+/Ca^{2+} exchange was promoting Pb^{2+} uptake, synaptosomes were loaded with fura-2 and then divided into 2 equal aliquots. One aliquot was washed three times with 4 mM K^+, 146 Na^+ PSS (control) while the remaining aliquot was washed three times with PSS in which N-methyl-D-glucamine (NMDG) replaced Na^+ mole for mole. Synaptosomes that have been partially depleted of intracellular Na^+ by replacement with NMDG showed an unaltered fast component of Ca^{2+} influx, but the amplitude of the slow component was reduced ~50% (Lentzner et al., 1992). This indicates that a portion of the slow component of Ca^{2+} influx represents Na^+/Ca^{2+} exchange operating in the reverse mode, because the prolonged depolarization alters the Na^+ electrochemical gradient such that net Ca^{2+} influx is favored through the exchanger. In this experiment,
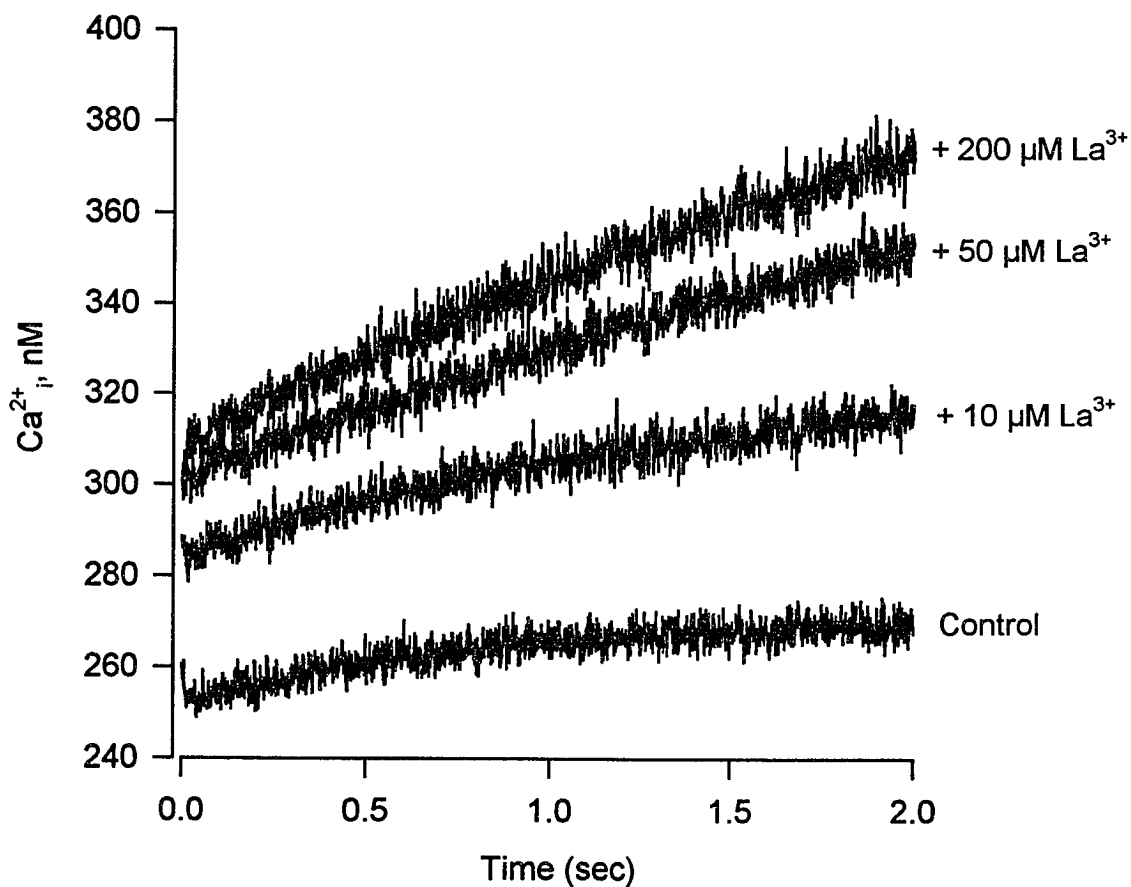
Figure 4-3. Effect of La^{3+} on Pb^{2+} influx in depolarized synaptosomes. The synaptosomes were mixed in the stopped-flow apparatus to yield a final concentration of 100 mM K$^+$, 10 µM Pb^{2+}, and La^{3+} as indicated. These data were obtained from one synaptosome preparation, and similar results were obtained from three other experiments.

the synaptosomes were depolarized with 100 mM K^+ with increasing Pb^{2+}_o. As shown in Fig. 4-4, no difference in Pb^{2+} uptake was observed between control and Na^+ depleted synaptosomes. Furthermore, the amplitudes of the Pb^{2+} induced Ca^{2+} rise were nearly identical for both conditions, further suggesting that Na^+/Ca^{2+} exchange does not transport Pb^{2+} into the terminals.

The use of fura-2 as an indicator of Pb^{2+} was limited by its concomitant interaction with Ca^{2+}. We are currently unable to calibrate both the Pb^{2+} and Ca^{2+} signals. In order to calibrate the Pb^{2+} signal, we would have to assume that no changes in Ca^{2+}_i occur, but that is not the case in this system. This problem can be eliminated by measuring the fluorescence at 359 nm, the isobestic point of the Ca^{2+}-fura-2 complex, thereby eliminating the contribution of changes in Ca^{2+}, allowing the kinetics of Pb^{2+} influx to be examined exclusively. At this excitation wavelength, the observed change in fluorescence should be representative of changes in Pb^{2+}_i only which would ensure that the slow rise in fluorescence that followed Pb^{2+} influx was due to a change in Ca^{2+}_i. Unfortunately, ion selectivity was gained at the expense of the signal:noise ratio and the ratio capability of the indicator.

In Figure 4-5, synaptosomes previously loaded with fura-2, were mixed in the stopped-flow spectrophotometer with 100 mM K^+ and 10 μM Pb^{2+} and excited at 359 nm. Under these conditions, a biphasic decrease in fluorescence was observed. The computer derived rate constant of inactivation for Pb^{2+} influx was determined to be 14.8 s^{-1} which corresponds to the rate constant of the fast component (in this experiment, 15.1 s^{-1}) observed when the nerve terminals were mixed with under control conditions (100 mM
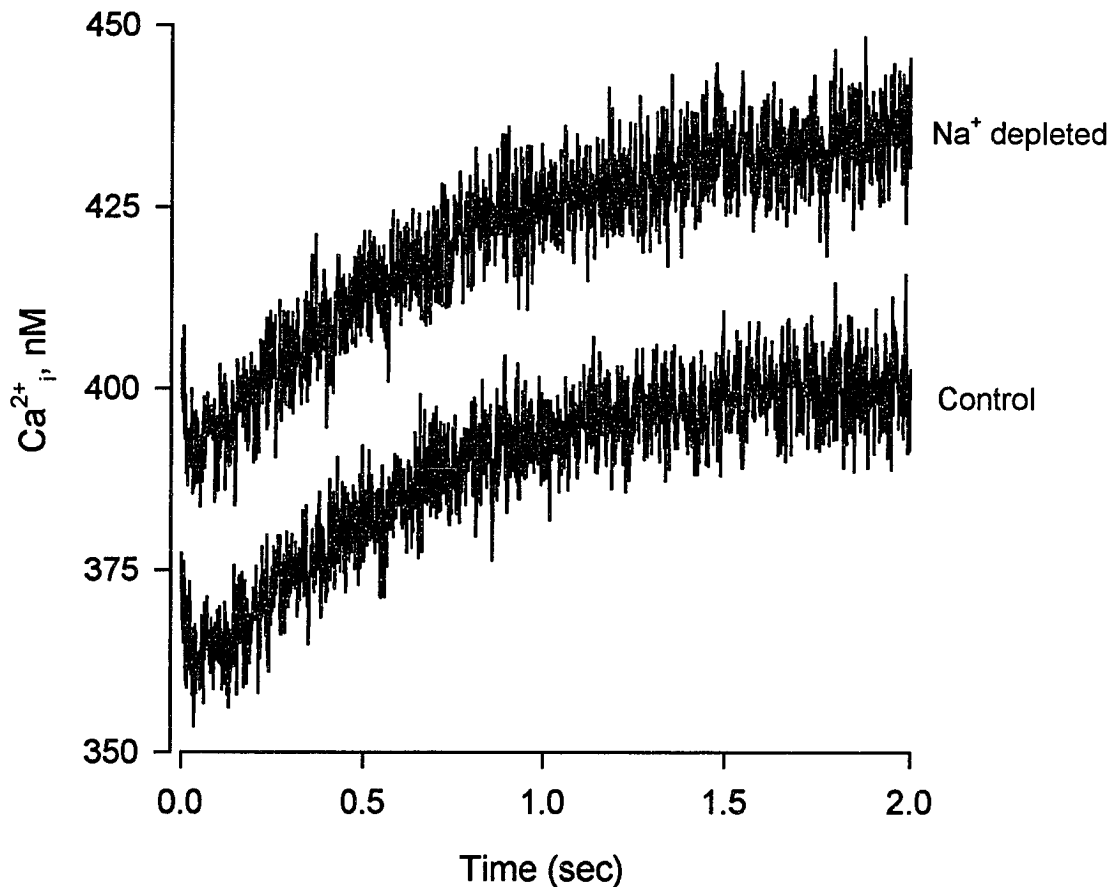
Figure 4-4. Effect of Na$^+$ depletion on Pb^{2+} uptake and the Ca$^{2+}_i$ rise in depolarized synaptosomes. Control and Na$^+$ depleted synaptosomes were mixed in the stopped-flow apparatus with 100 mM K$^+$ and 10 μM Pb^{2+}. These data were obtained from one synaptosome preparation (control, 49.9 nM Ca^{2+}; Na$^+$ depleted, 46.4 nM Ca^{2+}).

K^+, 1 mM Ca^{2+}). Furthermore, this initial decrease in fluorescence was only observed when the terminals were depolarized in the presence of Pb^{2+}. The second and slower decrease in fluorescence that followed Pb^{2+} influx was also observed under control conditions (100 mM K^+, 1 mM Ca^{2+}) and was considered to be artifactual, possibly due to photolysis of the dye. Such an effect would not be observed when the fura-2 signal was ratioed. These results suggest that Pb^{2+} permeation was limited to the rapidly inactivating Ca^{2+} channels in this preparation and that the channels inactivate normally after Pb^{2+} permeation.

Unfortunately, attempts at conducting experiments at an excitation wavelength of 370 nm, the Pb^{2+}-fura-2 isobestic point, were unsuccessful.

The approach of Tomsig and Suszkiw (1992) to calibrate the apparent Pb^{2+}_i signal is problematic because in this system, both Ca^{2+}_i and Pb^{2+}_i were increasing simultaneously. One approach at quantitating the free Pb^{2+}_i concentration following depolarization was to use BTC-5N, a heavy metal indicator whose fluorescence increases in the presence of micromolar levels of Pb^{2+}, but does not respond to high micromolar (100 μM) levels of Ca^{2+} (Molecular Probes, 1995).

BTC-5N is also available in an acetoxymethyl (AM) ester form which will passively diffuse into the presynaptic terminals where the ester groups are cleaved by cytosolic esterases to generate the cell impermeant product. When synaptosomes, previously loaded with BTC-5N, were mixed in the stopped-flow apparatus with 100 mM K^+ and up to 100 μM Pb^{2+}, no change in flourescence was observed (data not shown). A rapid increase in flourescence was observed when the synaptosomes were mixed with 100 mM K^+ and 1 mM Cd^{2+} (data not shown). Upon depolarization, Cd^{2+} readily permeates
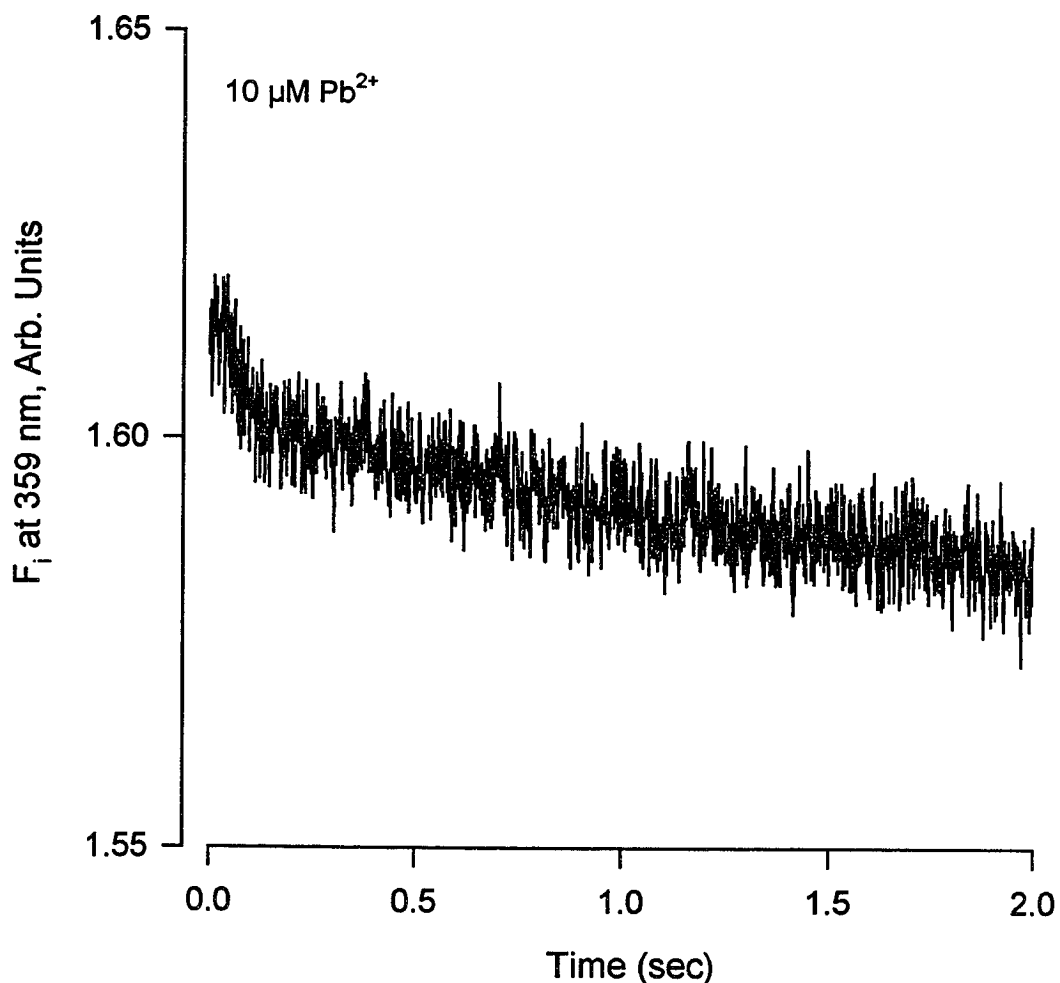
Figure 4-5. Effect of Pb^{2+} influx on fura-2 fluorescence in depolarized synaptosomes. The synaptosomes were mixed in the stopped-flow apparatus to yield a final concentration of 100 mM K$^+$ and 10 μM Pb^{2+}. The monochromator was set at 359 nm, the isobestic point for the Ca^{2+}-fura-2 complex. These data were obtained from one synaptosome preparation, and similar results were obtained from three other experiments.

the rapidly inactivating Ca^{2+} channels in this preparation (D.K. Bartschat, unpublished observations) which confirmed that the indicator had loaded properly. No change in fluorescence was observed when the terminals were depolarized with 100 μM Pb^{2+}. The K_d of Pb^{2+} for BTC-5N is 6.3 μM, which suggests that the Pb^{2+}_i levels after depolarization did not exceed 100 nM and that value is most likely an overestimation.

The results presented in Fig. 4-6 illustrate the concentration dependence of Pb^{2+}_o on the computer derived rate of the Ca^{2+}_i rise. As indicated, a linear increase in the rate of Ca^{2+}_i rise was observed.

The slow rise in fluorescence that followed Pb^{2+} influx also occured in the absence of Ca^{2+}_o, suggesting that the Ca^{2+}_i rise was originating from an intracellular source. To test this hypothesis, an aliquot of synaptosomes was preincubated with 1 μM thapsigargin for 30 min at 30°C prior to depolarization with 100 mM K^+ in the presence of 10 μM Pb^{2+} (final concentrations). Thapsigargin is a cell permeable tumor promoter that releases Ca^{2+} by inhibiting ER Ca^{2+}-ATPases without generating IP_3 (Thasrup et al., 1990). Ca^{2+} uptake by the Ca^{2+}-ATPase is inhibited, allowing the Ca^{2+} leak to go uncountered which causes the store to empty.

As shown by the traces in Fig. 4-7, pretreatment with thapsigargin decreased the Pb^{2+} induced rise by approximately 30% compared with the control conditions. The initial $[Ca^{2+}]_i$ recorded at time 0 was routinely increased following incubation with thapsigargin, confirming elevated cytosolic Ca^{2+} presumably due to intracellular Ca^{2+} store depletion. These results suggest that intracellular Pb^{2+} activated the release of Ca^{2+} from intracellular stores.
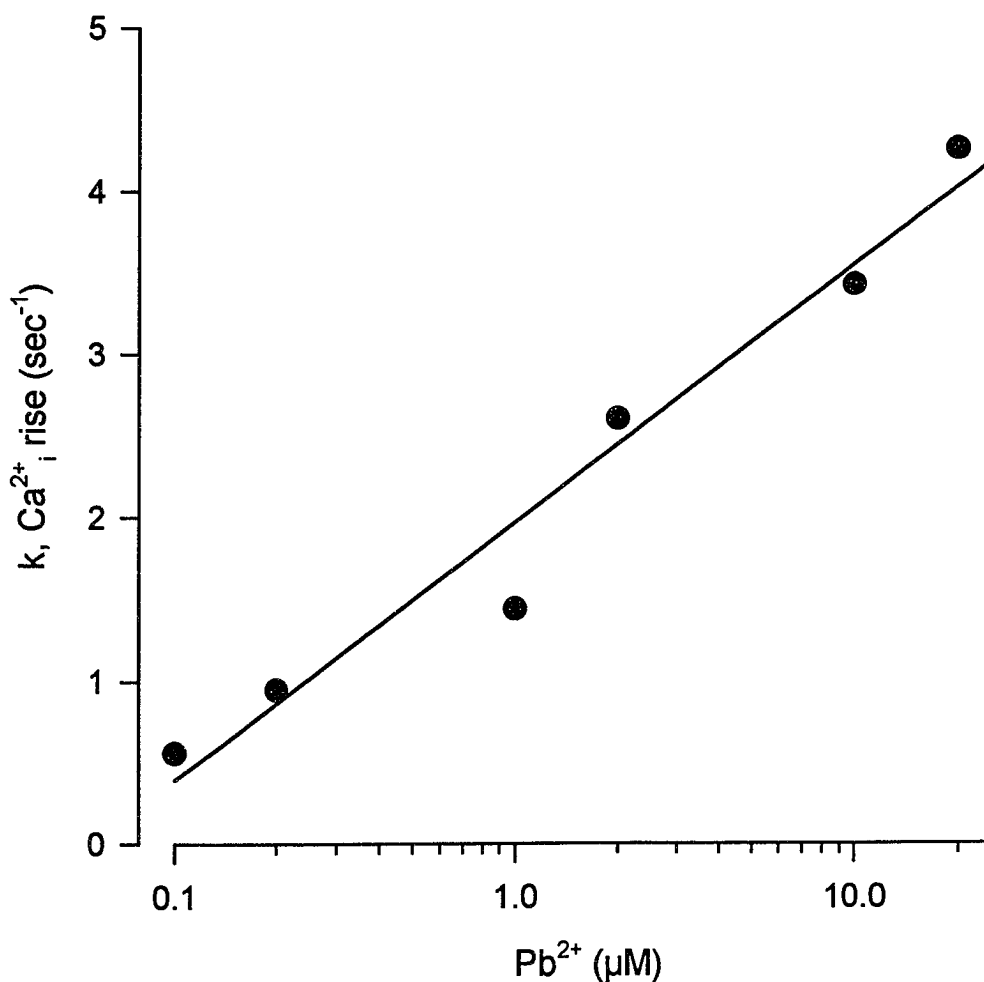
Figure 4-6. Concentration dependence of $[Pb^{2+}]_o$ on the Pb^{2+} activated Ca^{2+} rise. The synaptosomes were depolarized in the presence of various $[Pb^{2+}]_o$ and the increase in Ca^{2+}_i was subjected to a computer fit with a one exponential equation. The derived rate constants were plotted versus the $[Pb^{2+}]_o$. The results are means (where appropriate) for 1 experiment $(0.1, 0.2 \mu M \, Pb^{2)+}$, 2 experiments $(2.0, 20 \mu M \, Pb^{2+})$, 3 experiments $(1.0 \mu M \, Pb^{2+})$, and 6 experiments $(10 \mu M \, Pb^{2+})$.

Neurons possess diverse Ca^{2+} stores that are regulated by various agonists: IP_3, GTP, ADP-ribose, ryanodine, and caffeine (Simpson et al., 1995). Thapsigargin did not provide information on which specific store Pb^{2+} activated because it is a non-specific inhibitor of ER Ca^{2+}-ATPases. To determine if Pb^{2+} was acting upon a ryanodine sensitive store, synaptosomes were preincubated with the plant alkaloid ryanodine for 30 min at 30°C prior to depolarization in the presence of 10 μM Pb^{2+}. In one experiment, the nerve terminals were pretreated with 1 μM ryanodine which activates the channel and causes store depletion. The traces in Fig. 4-8 illustrate that pretreatment with 1 μM ryanodine had a slight effect on the amplitude of the Pb^{2+} activated Ca^{2+} rise. However, in three other experiments, this treatment had no effect on the magnitude of the Ca^{2+}_i rise. In another experiment, the terminals were pretreated with 100 μM ryanodine which blocks the channel. As illustrated in Fig. 4-9, pretreatment with 100 μM ryanodine had no effect on the amplitude of the Ca^{2+}_i rise that followed Pb^{2+} influx. In both cases, the initial Ca^{2+}_i obtained at the onset of depolarization was increased, presumably due to activation of the store. The ryanodine receptor is also activated by caffeine. In Fig. 4-10, the nerve terminals were incubated with 5 mM caffeine for 30 minutes at 30°C prior to depolarization with 100 mM K^+ and 10 μM Pb^{2+}. Furthermore, no effect was observed when the nerve terminals were preincubated with up to 10 mM caffeine (data not shown). These results suggest that Pb^{2+}_i was not activating a ryanodine sensitive store.

To determine if a portion of the Pb^{2+} induced Ca^{2+} rise was originating from the mitochondria, the nerve terminals were pretreated with the mitochondrial inhibitor, 2,4-dinitrophenol for 30 min at 30°C prior to depolarization. As shown by the traces in Fig. 4-

Figure 4-7. Effect of thapsigargin on Pb^{2+} induced Ca^{2+} release in depolarized synaptosomes. The synaptosomes were mixed in the stopped-flow apparatus to yield a final concentration of 100 mM K^+ and 10 μM Pb^{2+} after being preincubated with 1 μM thapsigargin for 30 min at 30°C (control, 46.0 nM Ca^{2+}; thapsigargin, 32.9 nM Ca^{2+}). These traces are from one experiment, and each experiment represents at least 18 replicates per experiment.

Figure 4-8. Effect of 1 µM ryanodine on Pb^{2+} induced Ca^{2+} release in depolarized synaptosomes. The synaptosomes were mixed in the stopped-flow apparatus to yield a final concentration of 100 mM K$^+$ and 10 µM Pb^{2+} after being preincubated with 1 µM ryanodine for 30 min at 30°C. These results are from one experiment (control, 22.7 nM Ca^{2+}; ryanodine, 16.0 nM Ca^{2+}). Similar results were observed in three other experiments.
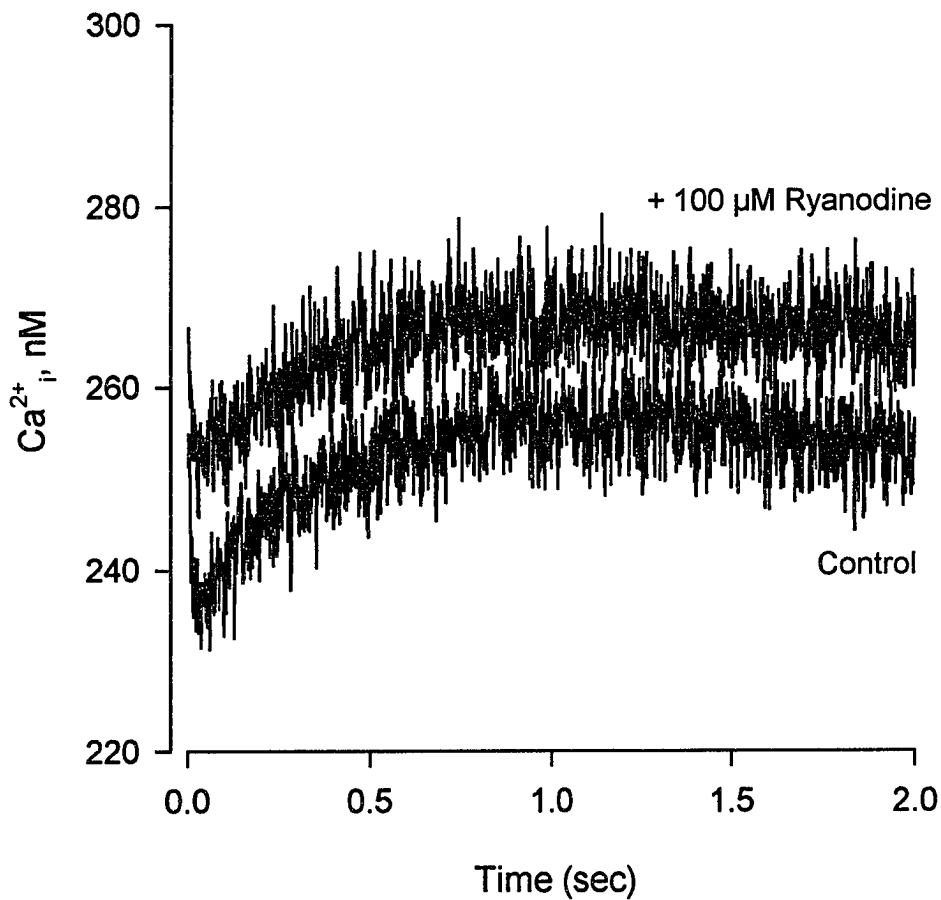
Figure 4-9. Effect of 100 µM ryanodine on Pb^{2+} induced Ca^{2+} release in depolarized synaptosomes. The synaptosomes were mixed in the stopped-flow apparatus to yield a final concentration of 100 mM K$^+$ and 10 µM Pb^{2+} after being preincubated with 100 µM ryanodine for 30 min at 30°C. These results are from one experiment (control, 22.7 nM Ca^{2+}; ryanodine, 22.0 nM Ca^{2+}). Similar results were observed in three other experiments.
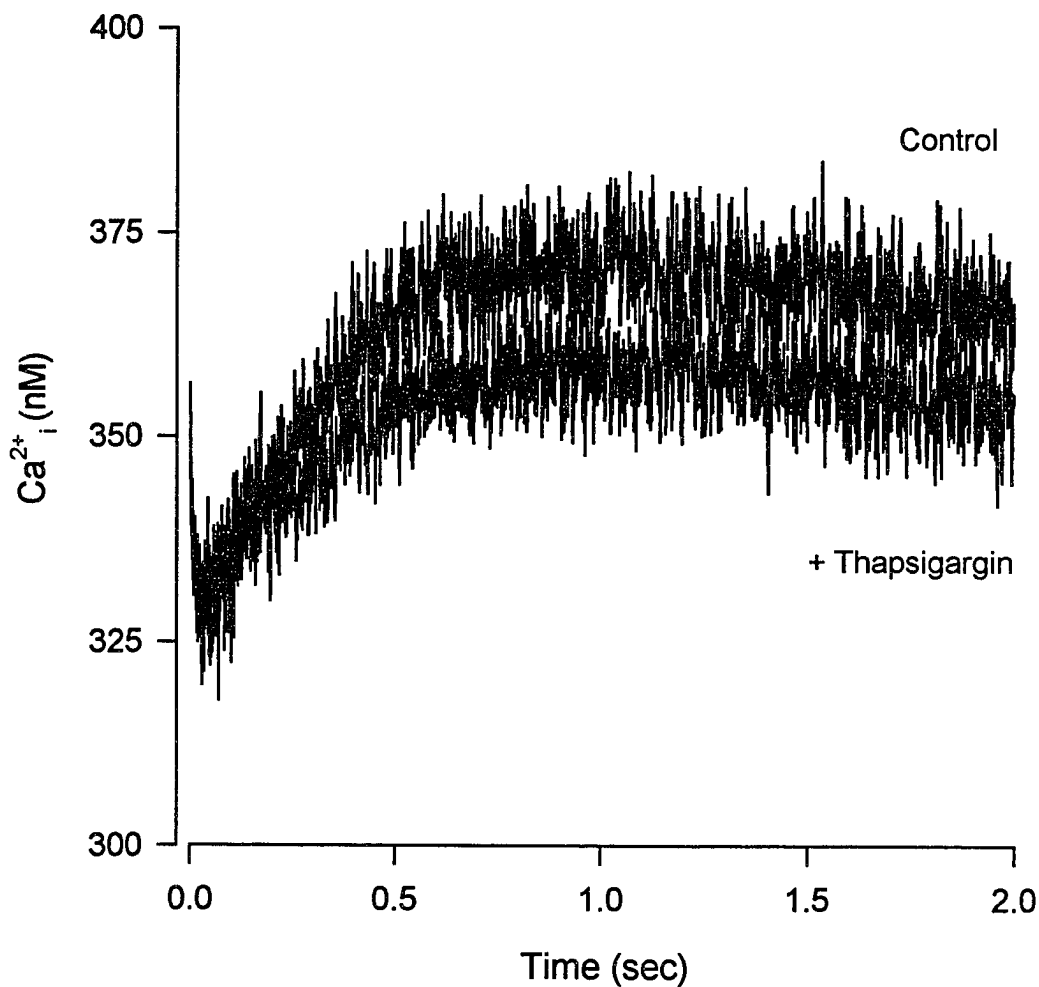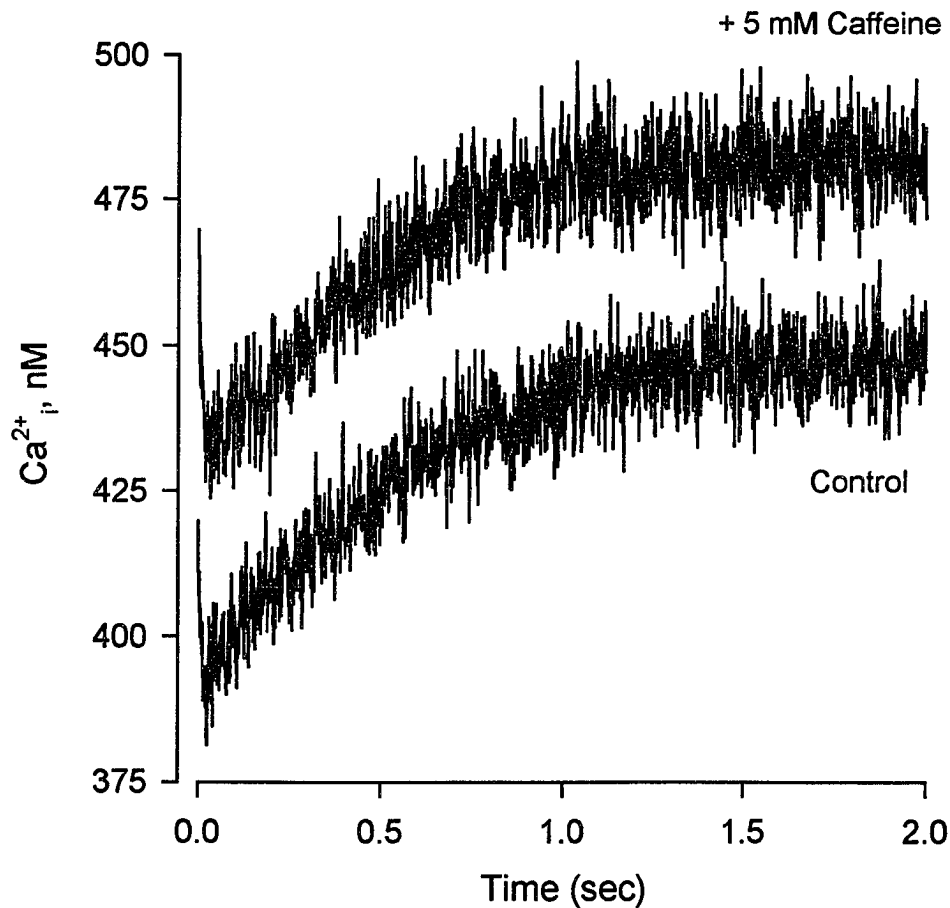
Figure 4-10. Effect of caffeine on Pb^{2+} induced Ca^{2+} release in depolarized synaptosomes. The synaptosomes were mixed in the stopped-flow apparatus to yield a final concentration of 100 mM K$^+$ and 10 µM Pb^{2+} after being preincubated with 5 mM caffeine for 30 min at 30°C. These results are from one experiment (control, 59.4 nM Ca^{2+}; caffeine, 56.3 nM Ca^{2+}).

11, the $Ca^{2+}{}_i$ rise was not affected. An increase in $Ca^{2+}{}_i$ was observed at the onset of depolarization, presumably due to mitochondrial uncoupling, causing mitochondrial Ca^{2+} to be released. The slow decrease in $Ca^{2+}{}_i$ that occured after the Ca^{2+} rise had peaked was most likely due to Na^+/Ca^{2+} exchange transporting Ca^{2+} out of the terminals to lower the $Ca^{2+}{}_i$. This effect was routinely observed when the resting $Ca^{2+}{}_i$ was greater than 500 nM at the onset of depolarization. The highest concentration of 2,4-DNP that was employed was 10 μM, which was the maximal concentration that did not interfere with the fluorescent measurements.

These experiments were routinely conducted at 30°C since incubation at 36°C decreased synaptosomal viability as reflected by a reduction in overall Ca^{2+} influx, due to Ca^{2+} channel rundown, and elevated $Ca^{2+}{}_i$, due to the inability of the terminals to maintain low $Ca^{2+}{}_i$. However, in duplicate experiments, synaptosomes were also incubated with each agonist for 30 min at 36°C but no effect on Pb^{2+} induced Ca^{2+} rise was observed.

Discussion

The results presented in the preceding chapter suggest that Ca^{2+} channel blockade may not contribute to Pb^{2+} neurotoxicity since $Pb^{2+}{}_o$ does not inhibit Ca^{2+} influx into the presynaptic terminals to any significant extent at physiological Ca^{2+} levels and Pb^{2+} concentrations that produce toxicity *in vivo*. However, Pb^{2+} may exert its' toxic effects by altering $Ca^{2+}{}_i$ metabolism. For example, a sustained increase in $Ca^{2+}{}_i$ is observed after incubating osteoblastic bone cells (Schane, 1989b), neuroblastoma glioma cells (Schane, 1989a) and human platelets (Dowd and Gupta, 1991) in solutions containing Pb^{2+}. Pb^{2+} also stimulates Ca^{2+} release from mitochondria (Kapoor and Van Rossum, 1984; Chavez
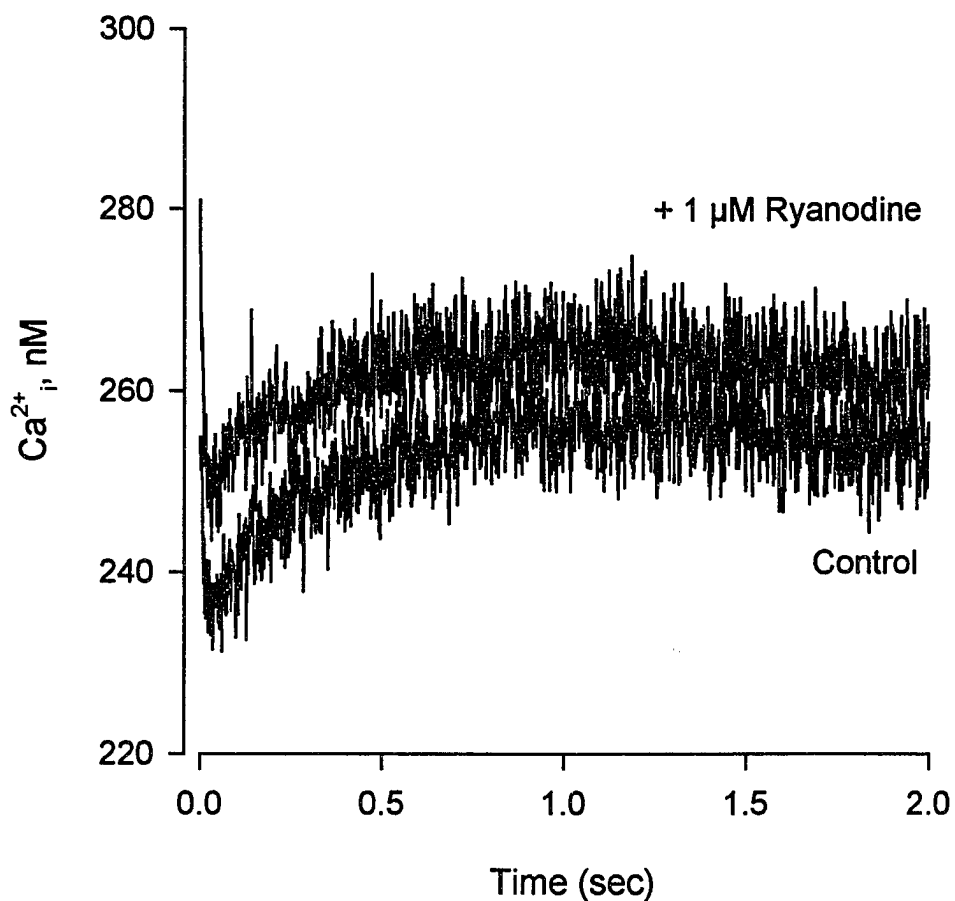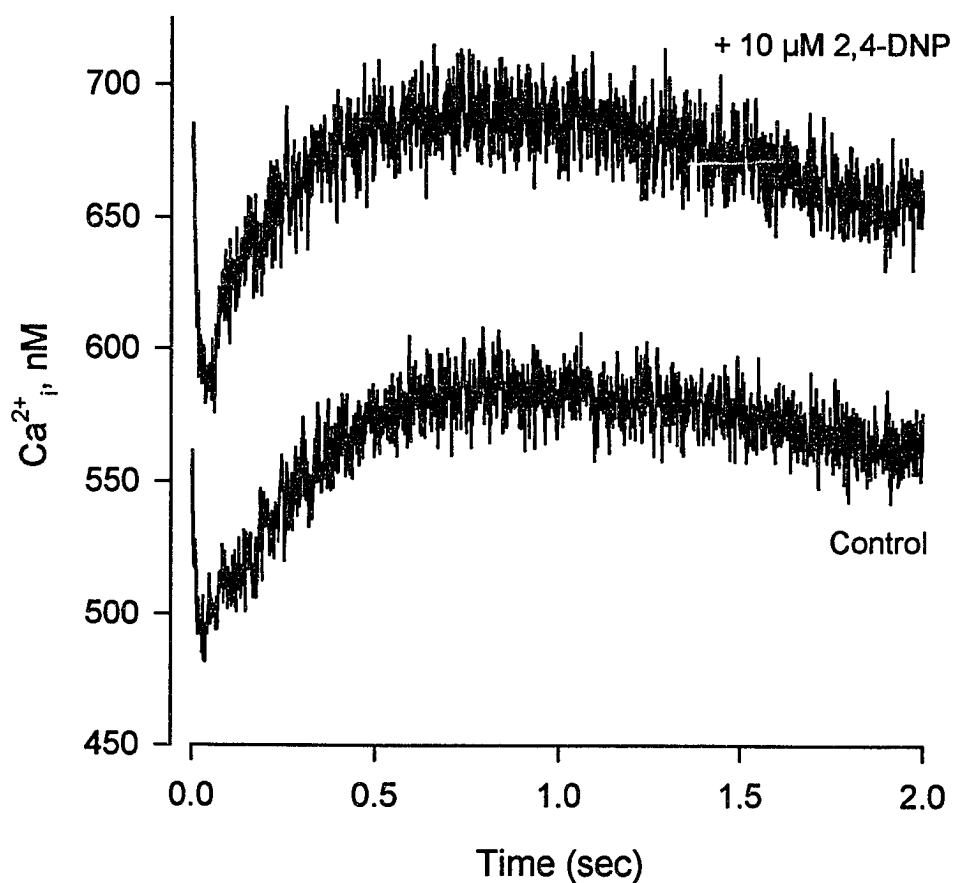
Figure 4-11. Effect of 2,4-dinitrophenol on Pb^{2+} induced Ca^{2+} release in depolarized synaptosomes. The synaptosomes were mixed in the stopped-flow apparatus to yield a final concentration of 100 mM K$^+$ and 10 μM Pb^{2+} after being preincubated with 10 μM 2,4-dinitrophenol for 30 min at 30°C. These results are from one experiment (control, 86.7 nM Ca^{2+}; 2,4-dinitrophenol, 94.9 nM Ca^{2+}). Similar results were observed in two other experiments.

et al., 1987) and inhibits Ca^{2+} uptake into the mitochondria (Parr and Harris, 1976; Goldstein 1977). Pb^{2+} exposure may affect receptor expression on the ER, the main Ca^{2+}_i store in neurons. In rats, low level, developmental exposure beginning prenatally, down regulates IP_3 receptors on the ER but this effect was not observed following Pb^{2+} exposure in adults (Singh, 1993). Thus, alterations in Ca^{2+}_i metabolism may underlie some aspects of Pb^{2+} neurotoxcity.

The results presented in this chapter demonstrated that following depolarization Pb^{2+} permeated VDCCs in hippocampal synaptosomes. This observation was based on several lines of evidence that indicate that the initial decrease in fluorescence was representative of Pb^{2+} influx. For example, the drop in fluorescence was only observed when the nerve terminals were depolarized with elevated K^+. Furthermore, depolarization in the presence of La^{3+}, which blocks VDCCs, also eliminated the Pb^{2+} dependent signal. Experiments conducted at the isobestic point of the Ca^{2+} fura-2 complex demonstrated that Pb^{2+} influx was confined to the class of rapidly inactivating channels. Pb^{2+} was not measurably permeable through the slowly inactivating Ca^{2+} channels and Na^+/Ca^{2+} exchange did not appear to promote Pb^{2+} transport.

The observation that Pb^{2+} permeated VDCCs in this preparation was in agreement with results obtained in other cell types. Simons and Pocock (1987) used atomic absorption spectrophotometry to show that Pb^{2+} permeated a L-type VDCC in adrenal medullary cells. These channels did not inactivate properly after Pb^{2+} influx because Pb^{2+} uptake was still observed after 10 minutes while $^{45}Ca^{2+}$ uptake was complete within 2 minutes. In bovine chromaffin cells, Tomsig and Suszkiw (1991) have shown that Pb^{2+}

also permeates a L-type VDCC that does not inactivate after Pb^{2+} permeation. However, the results presented in this chapter demonstrated that VDCCs inactivated normally after Pb^{2+} permeation. The techniques employed here report changes in presynaptic Ca^{2+} permeability that can be monitored on a millisecond scale, allowing the degree of activation and rate of inactivation of presynaptic Ca^{2+} channels to be determined. These earlier studies utilized techniques with resolutions of seconds (Tomsig and Suszkiw, 1991) and minutes (Simons and Pocock, 1987), and both investigators were unable to eliminate the possibility of Pb^{2+} uptake through other channel types or exchange mechanisms. The Pb^{2+} permeable channel in this preparation is possibly a N-type Ca^{2+} channel (Lentzner et al., 1992) while the channels from these earlier studies were L-type, which inactivate slower than N-type Ca^{2+} channels. Another possible explanation is that the channels have different mechanisms of inactivation such that L-type channels are slowed by Pb^{2+} permeation.

Tomsig and Suszkiw (1992) have shown that fura-2 can also be used to report changes in Pb^{2+}_i because Pb^{2+} causes a similar fluorescence change to that induced by Ca^{2+}. Fura-2 binds Pb^{2+} with a higher affinity than Ca^{2+} ($K_d \cong 4 \times 10^{-12}$ M vs 2×10^{-7} M, respectively). If a large amount of intracellular fura-2 is free (ie., not bound to free Ca^{2+}_i), then Pb^{2+} influx would cause a rise in fluorescence that would be misinterpreted as a rise in Ca^{2+}_i. In the hippocamapal synaptosomes utilized here, the resting $[Ca^{2+}]_i$ is normally ~ 300 nM which suggests that a majority of the fura-2 is bound to Ca^{2+} and upon depolarization, Pb^{2+}_i replaces Ca^{2+} on fura-2 which causes a decrease in flourescence and apparent Ca^{2+}_i. These results are consistent with Pb^{2+}_i levels in the picomolar range after

depolarization. The results obtained with the heavy metal indicator BTC-5N also suggest similar Pb^{2+}_i levels following depolarization.

After Pb^{2+} permeation, a slow rise in flourescence was routinely observed in the absence of Ca^{2+}_o which consistently rose above the initial Ca^{2+}_i levels observed at the onset of depolarization. Incubation of the nerve terminals with thapsigargin, which depletes non-mitochondrial intracellular Ca^{2+} stores, reduced the Pb^{2+} induced Ca^{2+} rise, indicating Pb^{2+} activated the release of Ca^{2+} from intracellular Ca^{2+} stores. The rise in fluorescence was affected by the state of the intracellular stores. For example, the rise was reduced when the synaptosomes were exposed to Ca^{2+} free solutions for prolonged periods of time prior to depolarization in the presence of Pb^2. Furthermore, when the terminals were incubated with Ca^{2+} prior to depolarization, the Pb^{2+} activated rise was increased. These results further demonstrated that Pb^{2+} activated the release of Ca^{2+} from a thapsigargin sensitive, labile store.

Pretreatment with 1 µM ryanodine and 10 mM caffeine to activate ryanodine sensitive Ca^{2+} channels and 100 µM ryanodine to block the channels had no effect on the Ca^{2+}_i rise. These results all suggest that the Ca^{2+} rise did not originate from ryanodine sensitive intracellular Ca^{2+} stores.

Pretreatment of the terminals with 2,4-dinitrophenol, a mitochondrial uncoupler, also had no effect on the Pb^{2+} induced Ca^{2+} rise. While Pb^{2+} has been shown to release Ca^{2+} from mitochondria (Kapoor and Van Rossum, 1984; Chavez et al., 1987), it did not appear that Pb^{2+} affected the mitochondria under the conditions employed here.

Thus, the experiments presented here demonstrate that following depolarization with elevated K^+, Pb^{2+} permeates one class of rapidly inactivating Ca^{2+} channels and activates the release of Ca^{2+} from a thapsigargin sensitive, ryanodine and caffeine insensitive, non-mitochondrial intracelluar store (as depicted in Fig. 4-12).

Pretreatment with thapsigargin did not completely abolish the Pb^{2+} activated Ca^{2+} rise. A significant portion remained that did originate from mitochondria or ryanodine sensitive stores. One possibility is the store may be a previously identified store that is regulated by GTP or cyclic ADP-ribose. Another possibility is that the terminal region contains a store that is insensitive to thapsigargin. IP_3 sensitive stores that are insensitive to thapsigargin have been described in RINm5F cells (Blondel et al., 1993) and BTC-3 cells, insulin secreting cells that have been transfected with type-3 IP_3 receptors (Blondel et al., 1994; Islam and Berggren, 1993). In these cells, IP_3 stimulated Ca^{2+} release is observed even after thapsigargin sensitive stores have been depleted. Such a store may be tight or less leaky and would retain Ca^{2+} even after thapsigargin application. Thapsigargin inhibits the Ca^{2+}-ATPase allowing the normal Ca^{2+} leak to go uncountered which causes the store to deplete, but if a store is tight, thapsigargin would only inhibit re-uptake after an agonist induced release. The terminal region may contain a Ca^{2+} store that is "tight" and would retain Ca^{2+} even after thapsigargin application. In some experiments, the synaptosomes were maintained in Ca^{2+} free solutions for prolonged periods of time but Pb^{2+} induced Ca^{2+} release was only slightly reduced, suggesting the presence of a "tight" store in the terminal region. Type-3 IP_3 receptors have been shown in brain (Furuichi et
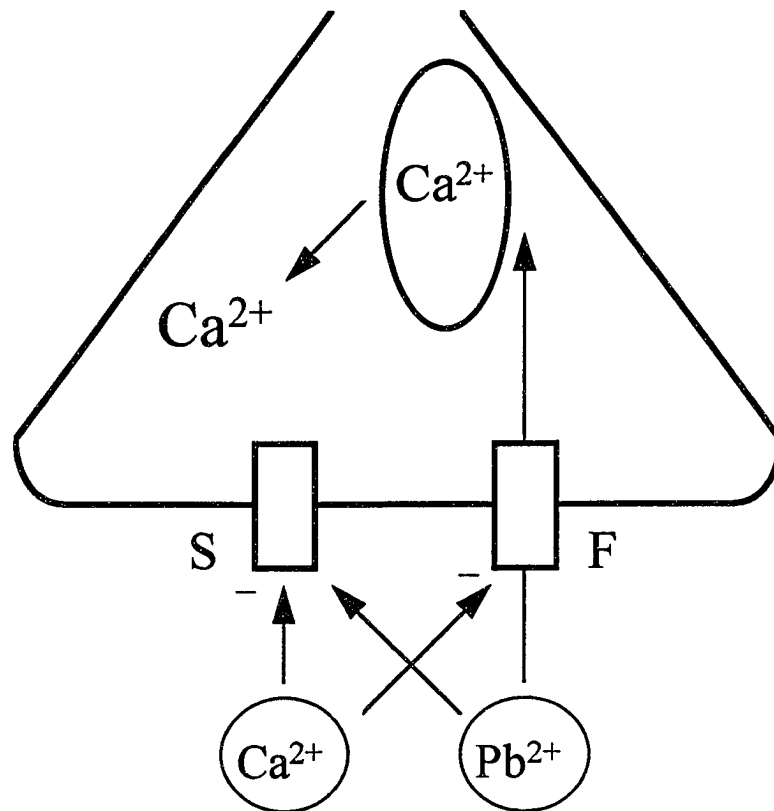
Figure 4-12. Schematic representation of Pb^{2+}-Ca^{2+} interactions in depolarized synaptosomes.

al., 1994). Thus, hippocampal nerve terminals may contain a type-3 IP_3 store as well as the classical IP_3 sensitive, thapsigargin sensitive store.

The rise in intracellular Ca^{2+} occurs within 100 msec suggesting that second messenger production does not mediate this effect but that intracellular Pb^{2+} directly interacts with Ca^{2+} release channels on the ER. The Pb^{2+} mediated release of Ca^{2+} is reduced in synaptosomes treated with thapsigargin, which depletes the inositol 1,4,5-trisphosphate (IP_3) store. Therefore, intracellular Pb^{2+} may activate the IP_3 receptor causing the store to release its contents into the cytosol.

Other investigators have reported elevated Ca^{2+}_i following exposure to Pb^{2+} (Schane, 1989a,b; Simons,1993; Dowd and Gupta, 1991; Rosen and Pounds, 1989; Pounds et al., 1989) but it has not been determined that the effect was the result of the release of Ca^{2+} from intracellular stores. Suszkiw and colleagues (1984) concluded that Pb^{2+} did not release Ca^{2+} from mitochondria or the ER. However, the results presented in this chapter suggest that the elevated Ca^{2+}_i observed after Pb^{2+} exposure may have originated from intracellular Ca^{2+} stores. Although this effect has not been reported for Pb^{2+}, silver (Palade 1987; Brunder et al., 1988; Tatsumi et al., 1988) and mercury (Prabhu and Salama, 1990) can release Ca^{2+} from the sarcoplasmic reticulum.

Another result, which will be fully discussed in Chapter 8, was that the amplitude of the Ca^{2+} rise was highest in younger animals and gradually declined as the animals aged. Chronic exposure in prenatal rats leads to a down regulation of IP_3 sensitive channels on the ER (Singh, 1993) but this store may be insensitive or absent in adult animals. Thus, a

normal developmental transition in intracellular Ca^{2+} storage may correlate with the increased sensitivity of younger animals to Pb^{2+} toxicity.

Following depolarization, Co^{2+} also permeates presynaptic Ca^{2+} channels in hippocampal synaptosomes but Co^{2+} quenches fura-2 fluorescence. When synaptosomes were depolarized in the presence of 0.5 mM Co^{2+}, an initial decrease in the 340 signal was observed which was followed by a slow rise in fluorescence above the initial fluorescence obtained at the onset of depolarization. This effect occured in the absence of Ca^{2+}_o which suggests that Co^{2+} also activates the release of Ca^{2+} from intracellular stores. An interesting observation was that the rise in fluorescence that followed Co^{2+} influx was only seen in juvenile animals (D.K. Bartschat, unpublished observations). Whether this Co^{2+} sensitive store is thapsigargin sensitive or related to the effects of Pb^{2+} is not presently known.

In conclusion, these results demonstrate that following depolarization with elevated K^+, Pb^{2+} permeates one class of rapidly inactivating Ca^{2+} channels and activated the release of Ca^{2+} from a thapsigargin sensitive, ryanodine and caffeine insensitive, non-mitochondrial intracellular store. A large portion of the Ca^{2+} rise was insensitive to thapsigargin, which suggests that the store is tight or less leaky than other thapsigargin sensitive stores. This store may be regulated by IP_3 and inappropriately activated Pb^{2+}. Thus, alterations in Ca^{2+}_i metabolism in the terminal region may contribute to some aspects of Pb^{2+} toxicity. Finally, the store may be insensitive or absent in adult animals which may correlate with the higher sensitivity of younger animals to Pb^{2+} neurotoxicity.

CHAPTER V

LEAD ACTIVATES THE RELEASE OF CALCIUM FROM ISOLATED

ENDOPLASMIC RETICULUM VESICLES FROM RAT CEREBELLUM

Introduction

The previous chapter demonstrated that following depolarization with elevated K^+,

Pb^{2+} permeates one class of rapidly inactivating Ca^{2+} channels and activates the release of

Ca^{2+} from a thapsigargin sensitive, ryanodine and caffeine insensitive, non-mitochondrial

intracelluar store. The rise in Ca^{2+}_i occurs within 100 msec which suggests that Pb^{2+}_i

directly activates Ca^{2+} release channels on the ER. The Pb^{2+} mediated release of Ca^{2+} was

reduced in synaptosomes treated with thapsigargin, which depletes certain IP_3 sensitive

stores. In rats, low level, developmental Pb^{2+} exposure beginning prenatally, down-

regulates IP_3 receptors on the ER, an effect which was not observed following Pb^{2+}

exposure in adult rats (Singh, 1993). Therefore, intracellular Pb^{2+} may activate the IP_3

receptor causing the Ca^{2+} store to release its contents into the cytosol. The inappropriate

activation of IP_3 sensitive Ca^{2+} stores may contribute to certain aspects of Pb^{2+}

neurotoxicity. To determine if Pb^{2+} activates the release of Ca^{2+} from IP_3 sensitive stores,

ER vesicles were isolated from rat cerebellum, a preparation rich in ER vesicles containing

Type I IP_3 receptors (Furuichi et al., 1994). Changes in intravesicular Ca^{2+} were monitored

with a cuvette based fluorometer by loading the microsomes with chlortetracycline (CTC).

Results

In Fig. 5-1, isolated ER vesicles were placed in an "uptake buffer" containing 40

mM KCl, 62.5 mM KH_2PO_4, 8 mM MOPS (adjusted to pH 7.0 with KOH), 2 mM Mg-

ATP, 0.2 mM creatine phosphate, and 0.04 mg/ml creatine phosphokinase. In this experiment, 0.5 mg of vesicle preparation was added to a warm (37°C) cuvette containing 3 ml of uptake medium and 0.25 μM fluo-3. The addition of 10 μM Ca^{2+} caused an initial increase in fluorescence (excitation 488 nm, emission 525 nm), but as the vesicles accumulated Ca^{2+} by an ATP dependent mechanism, the fluorescence decreased towards baseline levels. The addition of 0.1 μM IP_3 resulted in an immediate rise in fluorescence, consistent with a Ca^{2+} release from the vesicles. The decrease in fluorescence that followed indicated that the vesicles were re-accumulating the released Ca^{2+}. The subsequent addition of 0.1 μM IP_3 caused a decrease in fluorescence consistent with the amount of Ca^{2+} that had accummulated. The addition of heparin, which competitively and reversibly inhibits IP_3 binding (Ghosh et al., 1988), and L-myo-IP_3 prevented the release of Ca^{2+} following the addition of IP_3 (data not shown). When the vesicles were pretreated with 1 μM thapsigargin, Ca^{2+} uptake was drastically reduced and the addition of IP_3 did not elicit Ca^{2+} release (data not shown). These results demonstrate that isolated ER vesicles were able to sequester Ca^{2+} and then release it in response to an appropriate agonist (IP_3).

Unfortunately, the use of fluo-3 is not suitable for measuring Pb^{2+} induced Ca^{2+} release since Pb^{2+} also interacts with the indicator. For these experiments, it would be best to utilize an indicator that reported changes in Ca^{2+} from within the store itself. This would eliminate the possibility that the observed change in fluorescence was due to an interaction between Pb^{2+} and the indicator. Mag-fura-2 (Molecular Probes) possesses these characteristics but attempts at loading the vesicles by incubating at 30-36°C for 45 minutes in a circulating H_2O bath, freeze thawing, and passive loading overnight on ice

Figure 5-1. Effect of repeated IP$_3$ additions on fluo-3 fluorescence and isolated ER vesicles from rat cerebellum. Changes in fluo-3 fluorescence are plotted over time. Isolated ER vesicles were placed in an "uptake buffer" containing 40 mM KCl, 62.5 mM KH$_2$PO$_4$, 8 mM MOPS (pH 7.0 with KOH), 2 mM Mg-ATP, 0.2 mM creatine phosphate, and 0.04 mg/ml creatine phosphokinase. In this experiment, 0.5 mg of vesicle preparation was added to a warm (37°C) cuvette containing 3 ml of uptake medium and 0.25 μM fluo-3 (excitation 488 nm, emission 525 nm). The addition of 0.1 μM IP$_3$ resulted in an immediate rise in fluorescence which was followed by a decrease in fluorescence as the vesicle re-accumulated the release Ca^{2+}.
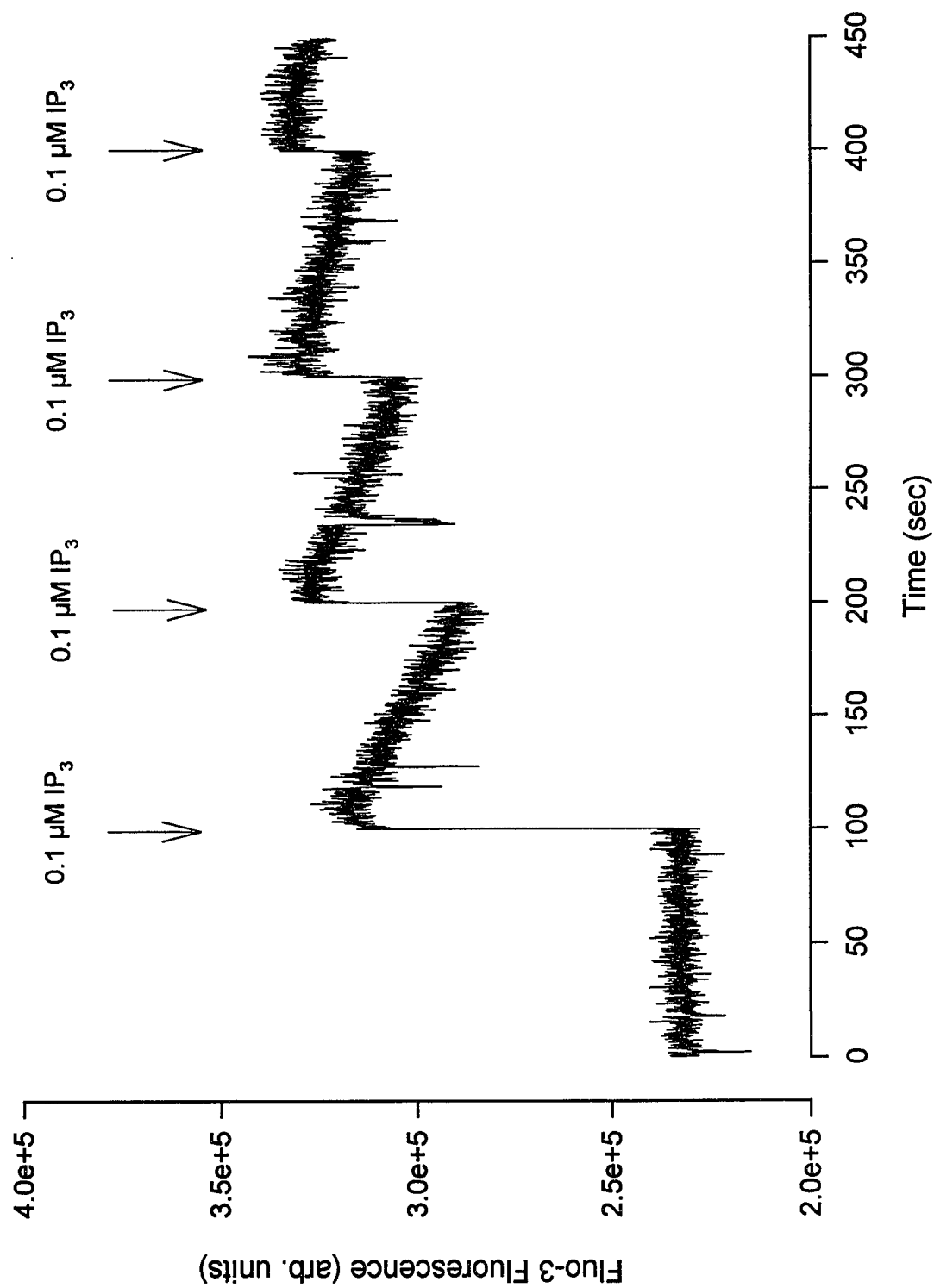
were all unsuccessful. An alternate method used chlortetracycline, a tetracycline antibiotic that has the ability to bind Ca^{2+} and fluoresce. In an aqueous environment, its dissociation constant is approximately 400 μM (Caswell and Hutchinson, 1971) which means the signal must originate from a location in the cell with a high concentration of Ca^{2+}. Chlortetracycline is only slightly lipophilic in an uncomplexed state but when it binds Ca^{2+}, it preferentially associates with a membrane bordering an area with micromolar or higher levels of Ca^{2+}, such as within intracellular stores (Marcotte et al., 1990).

One of the disadvantages of using CTC is that the signal cannot be calibrated directly. Therefore, some experiments were performed where fluo-3 and CTC were used simultaneously with a dual monochromator. In Fig. 5-2, isolated ER vesicles were placed in uptake buffer containing 5-10 μM Ca^{2+}, 100 μM CTC, 0.2 μM fluo-3, and 1mM Na-ATP. As the vesicles accumulated Ca^{2+}, a rise in CTC fluorescence and a decrease in fluo-3 fluorescence was observed. The addition of 1 μM IP_3 resulted in a rise in fluo-3 fluorescence and a decrease in CTC fluorescence, consistent with a release of Ca^{2+} from the vesicles. The decrease in extracellular Ca^{2+} measured by fluo-3 could be used to "calibrate" the rise in intravesicular Ca^{2+} measured by CTC. For example, if the volume of vesicles is approximately 2 μL/mg and approximately 0.5 mg of vesicle preparation was added to the cuvette, the approximate total content of the vesicles in solution was 1 μL. In these experiments, the cuvette volume was 3 ml. If the Ca^{2+} uptake, as determined by the fluo-3 calibration, was 1 μM, this would translate into a Ca^{2+} rise of approximately 3 mM within the vesicles. This technique is limited by the assumption that Ca^{2+} uptake and release is uniform between vesicles.
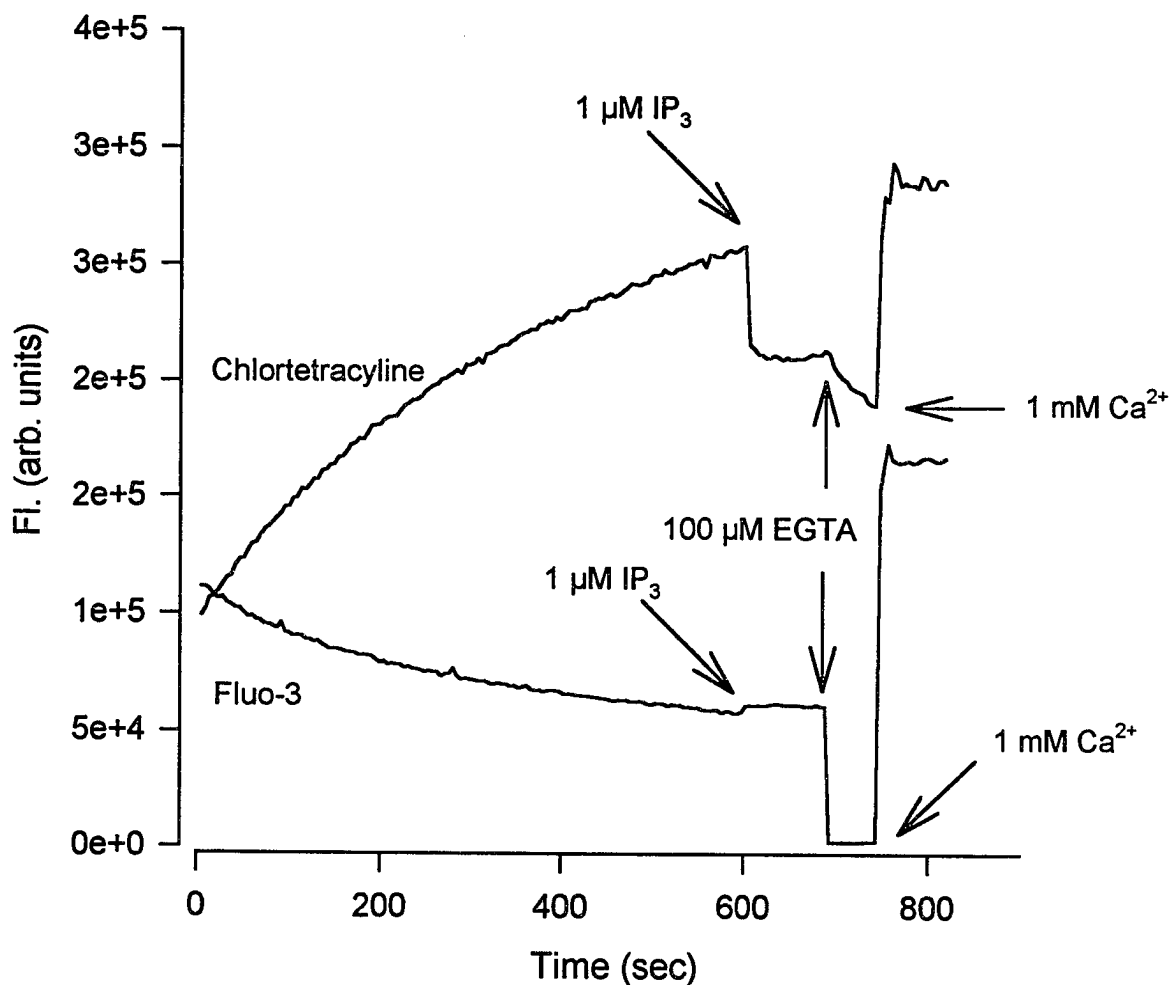
Figure 5-2. **Simultaneous measurement of intravesicular and extravesicular Ca^{2+} by chlortetracycline (CTC) and fluo-3.** Changes in CTC and fluo-3 fluorescence are plotted over time. Isolated ER vesicles were placed in uptake buffer containing 5-10 μM Ca^{2+}, 100 μM CTC, 0.2 μM fluo-3, and 1mM Na-ATP. As the vesicles accumulated Ca^{2+}, a rise in CTC fluorescence and a decrease in fluo-3 fluorescence was observed. The addition of 1 μM IP_3 resulted in a rise in fluo-3 fluorescence and a decrease in CTC fluorescence.

Unfortunately, this is probably not the case. However, this technique does allow us to "quantify" agonist induced Ca^{2+} release measured by CTC.

In experiments where CTC was used to monitor Ca^{2+}, Na-ATP was employed instead of Mg-ATP and an ATP regenerating system because Mg^{2+} can affect the fluorescence of CTC (Marcotte et al., 1990) and in preliminary experments, Mg-ATP caused a significant increase in the background fluorescence which complicated the experiments.

When ~0.5 mg of vesicle preparation was added to a warm (37°C) cuvette containing uptake buffer and 100 µM CTC, an initial rise in fluorescence was observed as the indicator diffused into the vesicles (Fig. 5-3). After baseline fluoresence was obtained, 1 mM ATP was added which resulted in a much larger rise in fluorescence (excitation 410 nm, emission 510 nm) as the vesicles accumulated Ca^{2+}. Ca^{2+} uptake was abolished by the addition of thapsigargin (1 µM), ionomycin (1 µM), or ATP depletion. The subsequent addition of IP_3 resulted in an immediate and concentration dependent decrease in fluoresence, consistent with the release of Ca^{2+} from the vesicles. IP_3 induced Ca^{2+} release was blocked by the addition of heparin which competitively and reversibly inhibits IP_3 binding (Ghosh et al., 1988). No effect on the CTC signal was observed when IP_3 induced release was performed in the presence of 1 µM EGTA. The baseline fluorescence, peak fluorescence after Ca^{2+} uptake, and the fluorescence after release were quantified and the elicited release was expressed as a percentage of total uptake. The results from these experiments are illustrated in Figure 5-4. The solid line through the data points represents the computer fit to the Michaelis-Menton equation with a K_m of

77.5 nM and a V_{max} of 35.6%. When the ER vesicles were pre-incubated with heparin, no release was observed after the addition of 1 μM IP$_3$.

To determine the kinetics of IP$_3$ induced Ca^{2+} release, ER vesicles previously loaded with CTC, were mixed in the stopped flow apparatus with IP$_3$. Prior to mixing, the vesicles were incubated with 10 μM Ca^{2+} and 1 mM Na-ATP for 20 minutes at 36°C. The results presented in Fig. 5-5 illustrate the change in fluorescence observed after mixing the vesicles with 1 μM IP$_3$. Computer fit of the signal revealed the presence of at least two components of Ca^{2+} release: an intermediate component of release with a rate constant, k, of 10.2 s^{-1}; and a slower component with a rate constant, k, of 0.92 s^{-1}. In this experiment, a third and faster component of release was observed but was not kinetically discernable. This most rapid component of release became more appararent when the vesicles were mixed with higher levels of IP$_3$ ($\geq$ 1μM).

A rapid and concentration dependent release of Ca^{2+} was observed when ER vesicles were mixed with increasing concentrations of IP$_3$ (Fig. 5-6). When the vesicles were mixed with 100 nM IP$_3$ (top trace), one component of release was observed. When the vesicles were mixed with IP$_3$ concentrations of 200 nM or greater, two kinetically different components of Ca^{2+} release were observed. Finally, when the vesicles were mixed in the stopped-flow device with concentrations of 1μM or greater, three kinetically distinguishable components of release were observed. Table 5-1 summarizes the rate constant and amplitude (change in CTC fluorescence) for the slow and intermediate components of release.
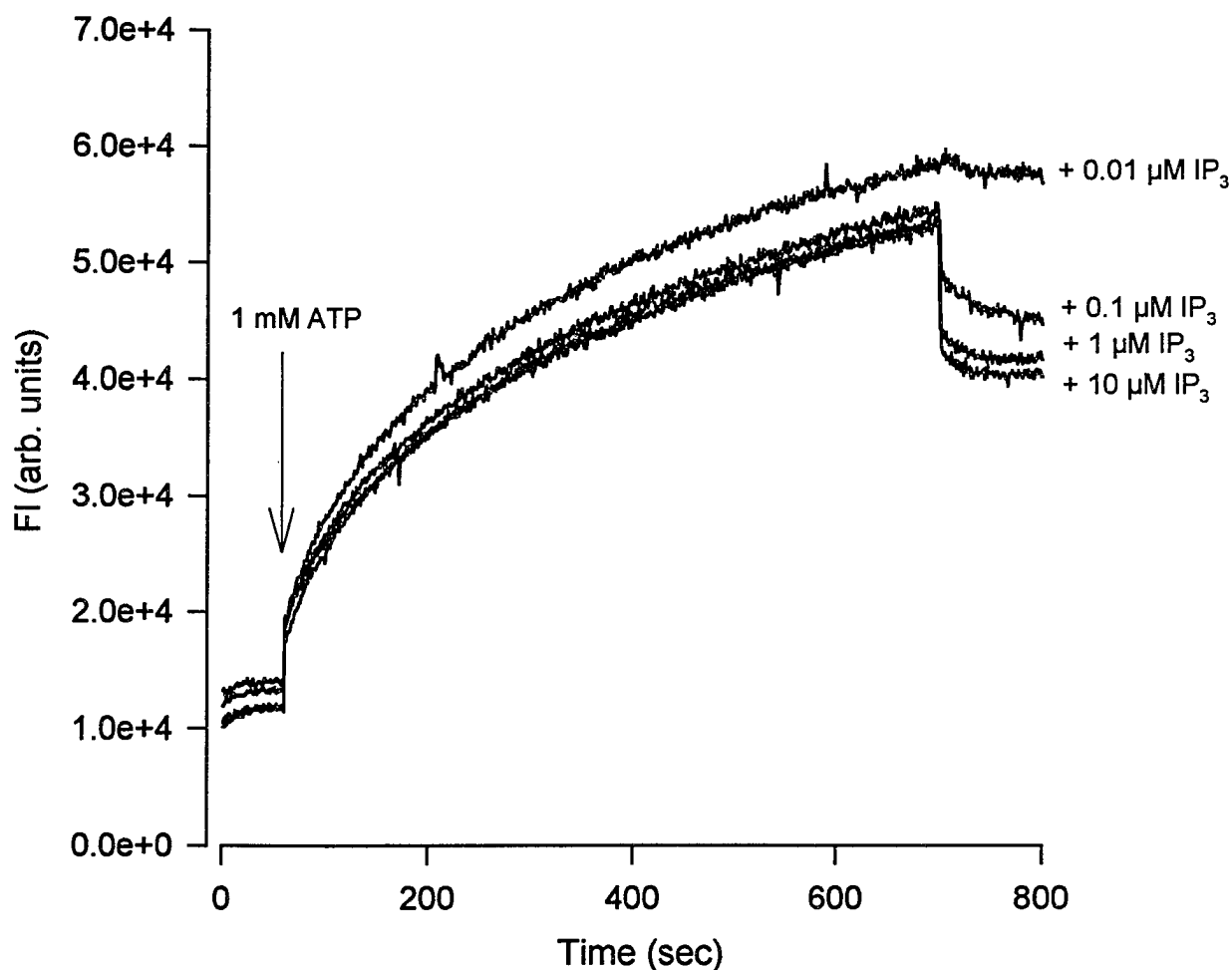
Figure 5-3. Effect of IP₃ on CTC fluorescence in isolated ER vesicles. Changes in CTC fluorescence are plotted over time. Isolated ER vesicles were placed in uptake buffer containing 100 µM CTC. For each experiment, 0.5 mg of vesicle preparation was added to a warm (37°C) cuvette based fluorescent spectrophotometer. A rise in fluorescence (excitation 410 nm, emission 525 nm) was observed following the addition of 1 mM Na-ATP. The addition of IP₃ resulted in an immediate and concentration dependent decrease in fluorescence.
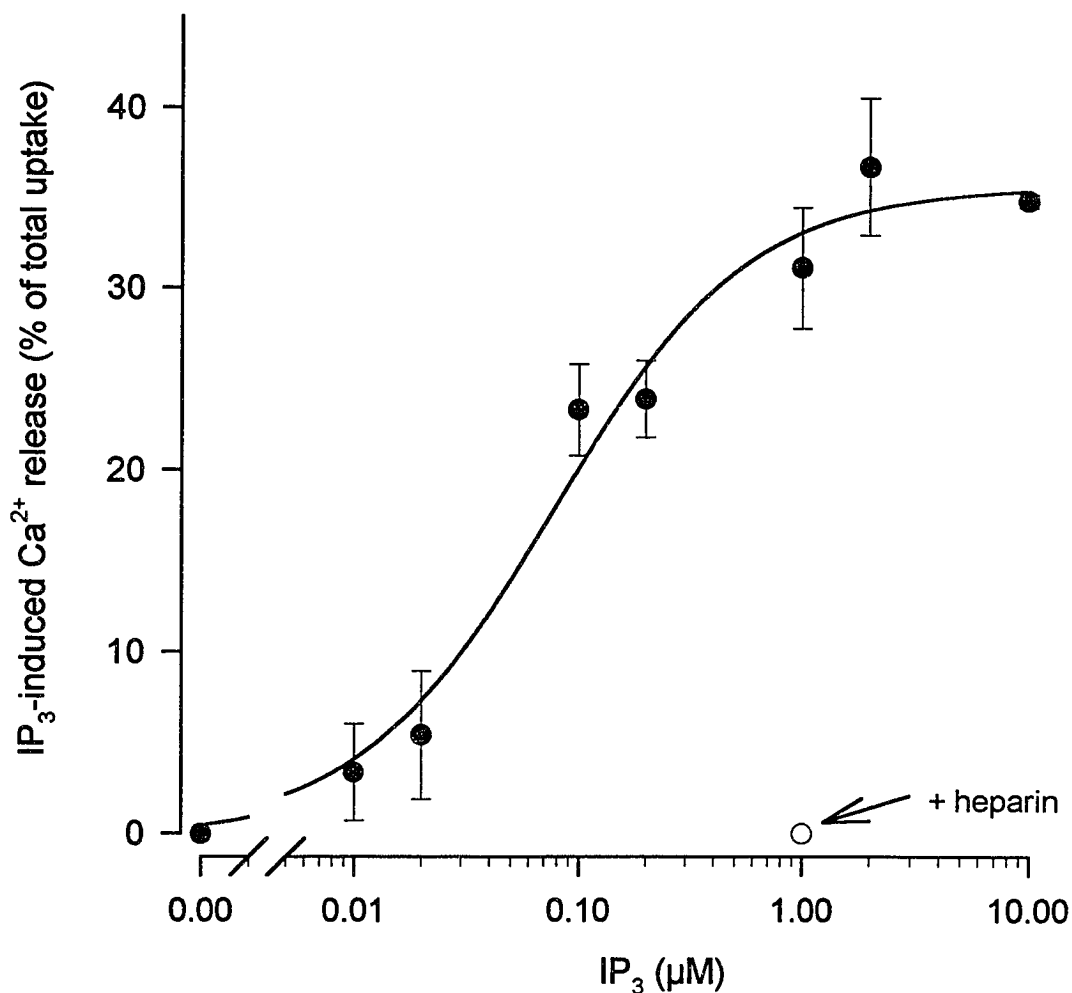
Figure 5-4. Concentration dependence of IP$_3$-induced Ca^{2+} release from ER vesicles. The IP$_3$-induced Ca^{2+} release (% of total uptake) are plotted versus the IP$_3$ concentration (µM). The solid line represents the computer fit to the Michaelis-Menton equation with a K$_m$ of 77.5 nM and a V$_{max}$ of 35.6%. When the ER vesicles were pre-incubated with heparin (o), which competitively and reversibly inhibits IP$_3$ binding, the addition of 1 µM IP$_3$ did not induce Ca^{2+} release.
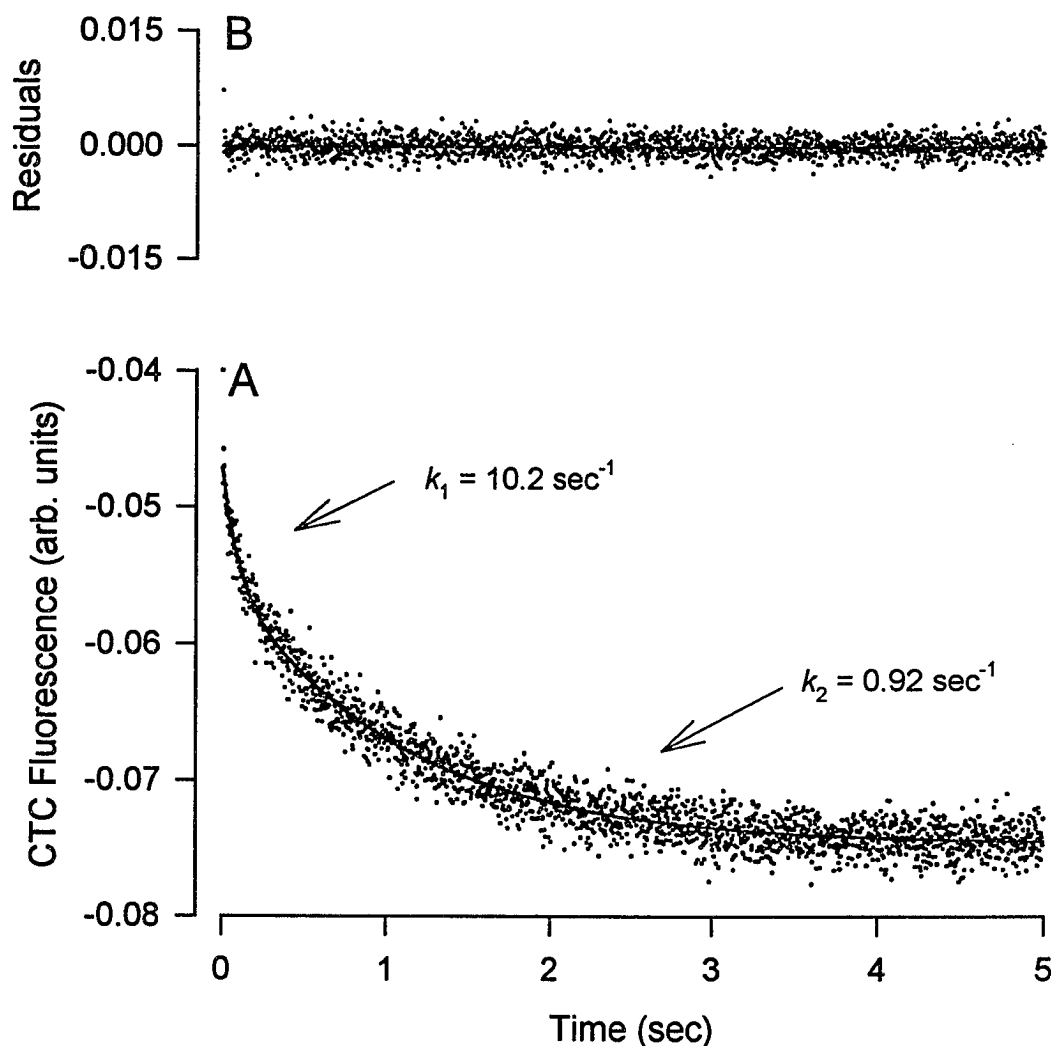
Figure 5-5. IP$_3$ induced Ca^{2+} release from isolated ER vesicles is composed of two kinetically distinguishable components. ER vesicles, previously loaded with CTC, were mixed in the stopped flow apparatus with 1 μM IP$_3$. The dots, sampled every millisecond, represent the digitized Ca^{2+}$_i$ signal and are plotted over time (Panel A). The solid line passing through the points represents the computer fit of the data to a two exponential equation: $\qquad$ Ca^{2+}$_i$ = A$_1$[1-exp(-k$_1$t)] + A$_2$[1-exp(k$_2$t)] + b where A$_1$ and A$_2$ represent the amplitudes of the slow and intermediate components of Ca^{2+} release, respectively, and k$_1$ and k$_2$ represent the rate constants for Ca^{2+} release through each component. In Panel B, the residual errors are plotted over time. These results were obtained from one microsome preparation and similar results were obtained from seven other experiments. Each experiment represents at least 9 replicates per experiment.
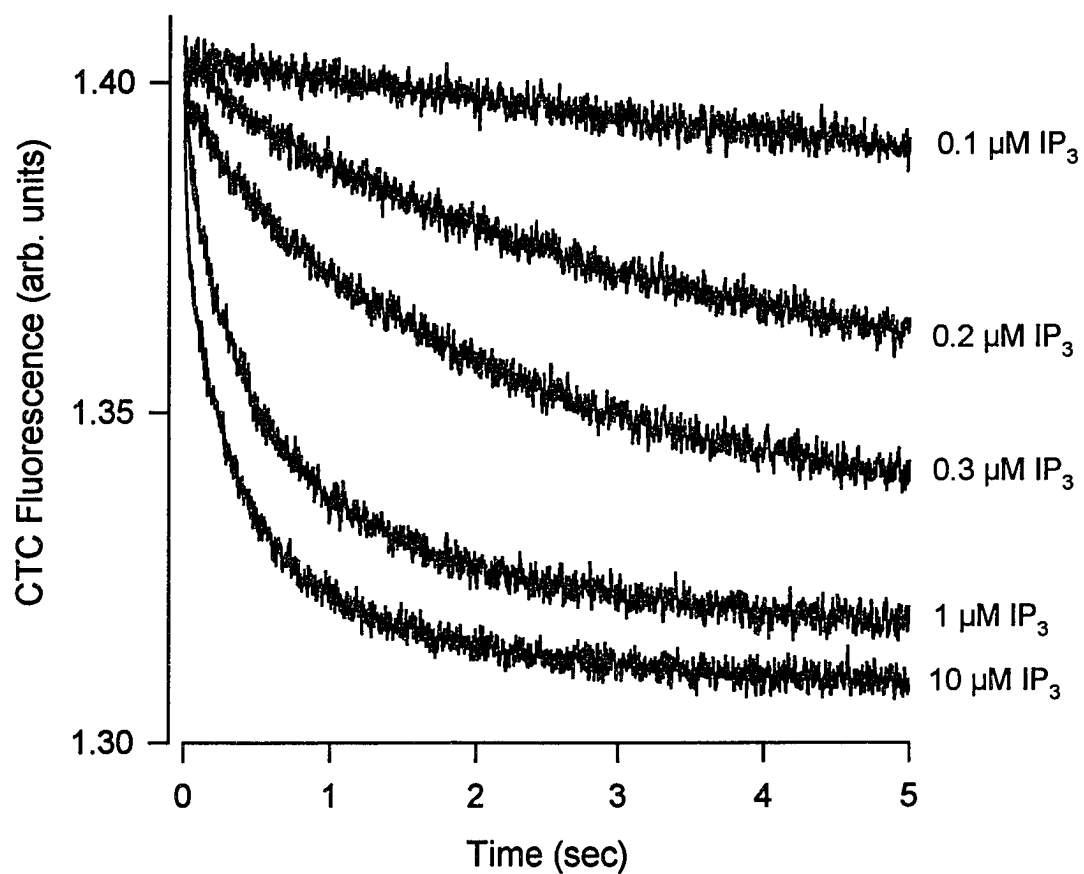
Figure 5-6. Effect of increasing IP₃ concentrations on Ca²⁺ release from isolated ER vesicles. ER vesicles, previously loaded with CTC, were mixed in the stopped flow apparatus with increasing IP₃ as indicated.

To further investigate the kinetics of the most rapid phase of Ca^{2+} release, the vesicles were mixed with IP_3 and the change in CTC fluorescence was monitored for 1 sec (1 data point per 0.5 ms). Under these conditions, this component of release became more apparent. The results from this experiment are shown in Fig. 5-7. Table 5-2 summarizes the rate constant and amplitude (change in CTC fluorescence) for the fast component of release. When the vesicles were mixed with IP_3 concentrations of 1 M or greater, the fast and intermediate components of release were observed. The rate constant of the fast component of release increased as the IP_3 concentration increased (Fig. 5-8). Computer fit of the data to the Michaelis-Menton equation yielded a K_{mapp} of 3.62 µM.

In Fig.5-9, the relationship between the IP_3 concentration and the rate constant of the intermediate component was computer fit with the Hill equation, which yielded a K_{mapp} of 0.29 µM and a Hill coefficient of 1.17. The rate constant of the slow component also increased with increasing IP_3 levels (Fig. 5-10). Computer fit of the data to the Michaelis-Menton equation yielded a K_{mapp} of 0.43 µM.

In the stopped-flow device, half-maximal release was observed with approximately 300 nM IP_3 but when similar conditions were utilized in the cuvette based system, 80 nM evoked half maximal release. The main difference between these two techniques was the rate of mixing, which suggests that the rate of IP_3 exposure may affect the kinetics of Ca^{2+} release.

To determine if Pb^{2+} could activate the release of Ca^{2+} from isolated vesicles, an aliquot (~ 0.5 mg) of ER vesicles was placed in a continuously stirred cuvette containing uptake buffer with 10 µM Ca^{2+} (Fig. 5-11). The addition of 1 mM Na-ATP resulted in a

Table 5-1. Comparison of the kinetics of the slow and intermediate components of IP_3 induced Ca^{2+} release during 5 sec.

IP_3 (µM)	Slow Component			Intermediate Component		
	k	a	n	k	a	n
0.1	0.189	0.0200	3	--	--	--
0.2	0.325	0.0654	2	2.32	0.017	1
0.3	0.418	0.0548	1	2.50	0.0099	1
0.7	0.625	0.0494	1	2.89	0.0397	1
1.0	0.747	0.0497	4	4.01	0.0439	4
2.0	0.893	0.0429	1	4.29	0.0497	1
3.0	1.171	0.0390	1	6.4	0.0325	1
10	0.930	0.0514	3	4.75	0.0826	3
30	1.000	0.0265	1	4.17	0.0403	1

k represents the rate constant of each component in s^{-1}. a represents the change in CTC fluorescence achieved by each component in 5 s.
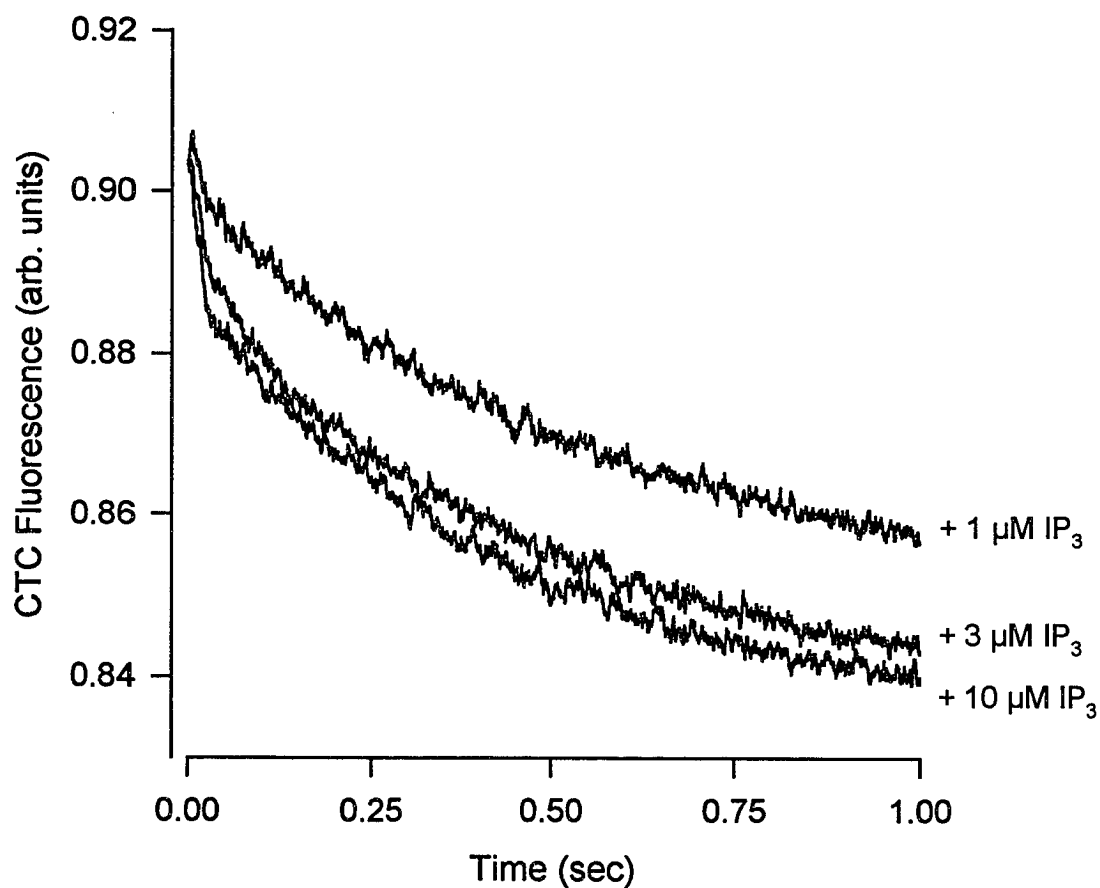
Figure 5-7. IP₃ induced Ca²⁺ release from isolated ER vesicles during 1 second. ER vesicles, previously loaded with CTC, were mixed in the stopped flow apparatus with increasing IP₃ as indicated, and the change in CTC fluorescence was monitored for 1 s (1 data point per 0.5 ms).

Table 5-2. Kinetics of the fast component of IP_3 induced Ca^{2+} release in 1 sec.

IP_3 (μM)	Fast Component	
	k	a
0.7	7.0	0.0118
1.0	31.7	0.0165
3.0	43.0	0.0079
10	73.4	0.0195
30	89.0	0.147

k represents the rate constant of each component in s^{-1}. a represents the change in CTC fluorescence achieved by each component in 1 s. These are the results of one experiment.

rise in fluorescence as the vesicles accumulated Ca^{2+}. The subsequent addition of 10 μM Pb^{2+} resulted in an immediate and concentration dependent release of Ca^{2+}. These results suggest that Pb^{2+} directly activates Type I IP_3 receptors in the absence of IP_3. The addition of 100 μM Pb^{2+} resulted in a slow release of Ca^{2+} which was kinetically similar to the application of thapsigargin and presumably due to the uncoupling of the Ca^{2+}-ATPase on the vesicles.

To determine if Pb^{2+} was activating an IP_3 sensitive store, an aliquot (~0.5 mg) of ER vesicles was placed in uptake buffer containing 1 mM Na-ATP (Fig. 5-12). After the addition of 10 μM Ca^{2+}, a rise in fluorescence was observed as the vesicles accumulated.
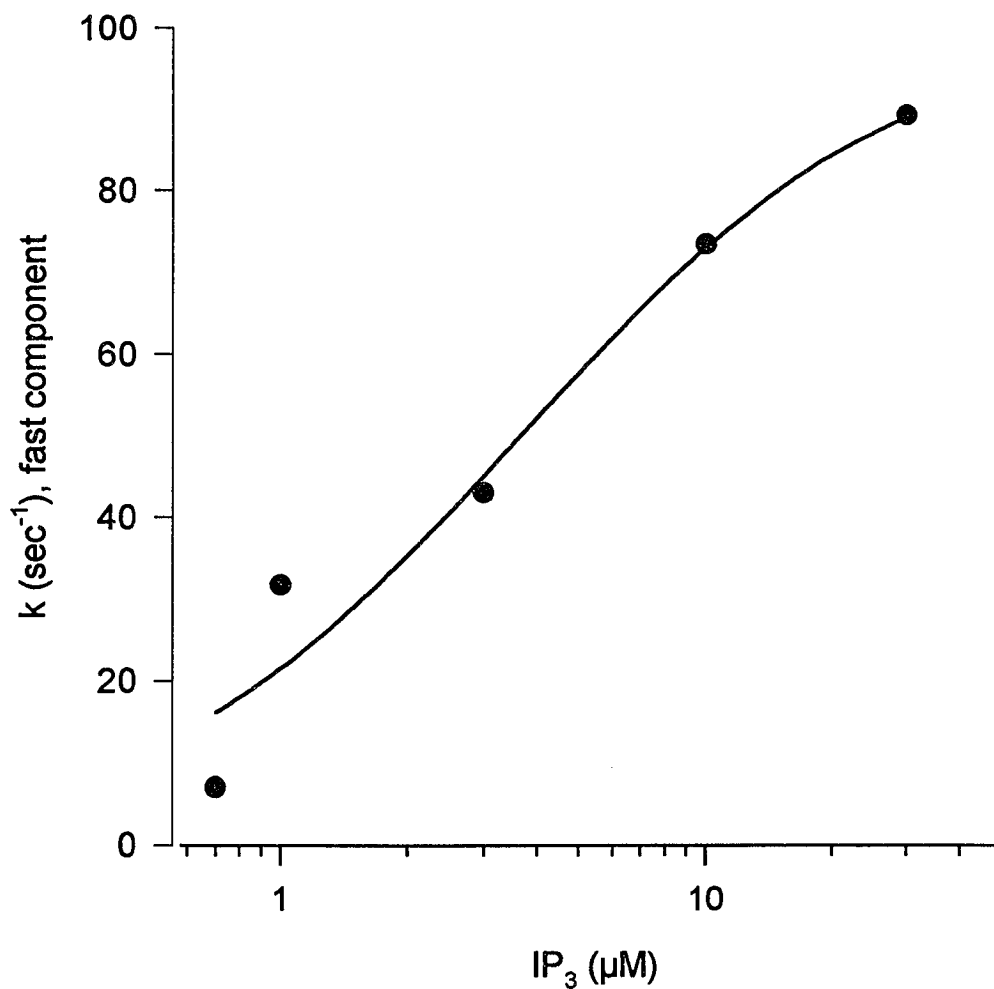
Figure 5-8. Effect of IP$_3$ on the rate constant of the fast component of IP$_3$ induced Ca^{2+} release. The computer derived rate constants are plotted versus the IP$_3$ concentration. The solid line represents the computer fit with the Michaelis-Menton equation which yielded a K$_{mapp}$ of 3.62 µM.
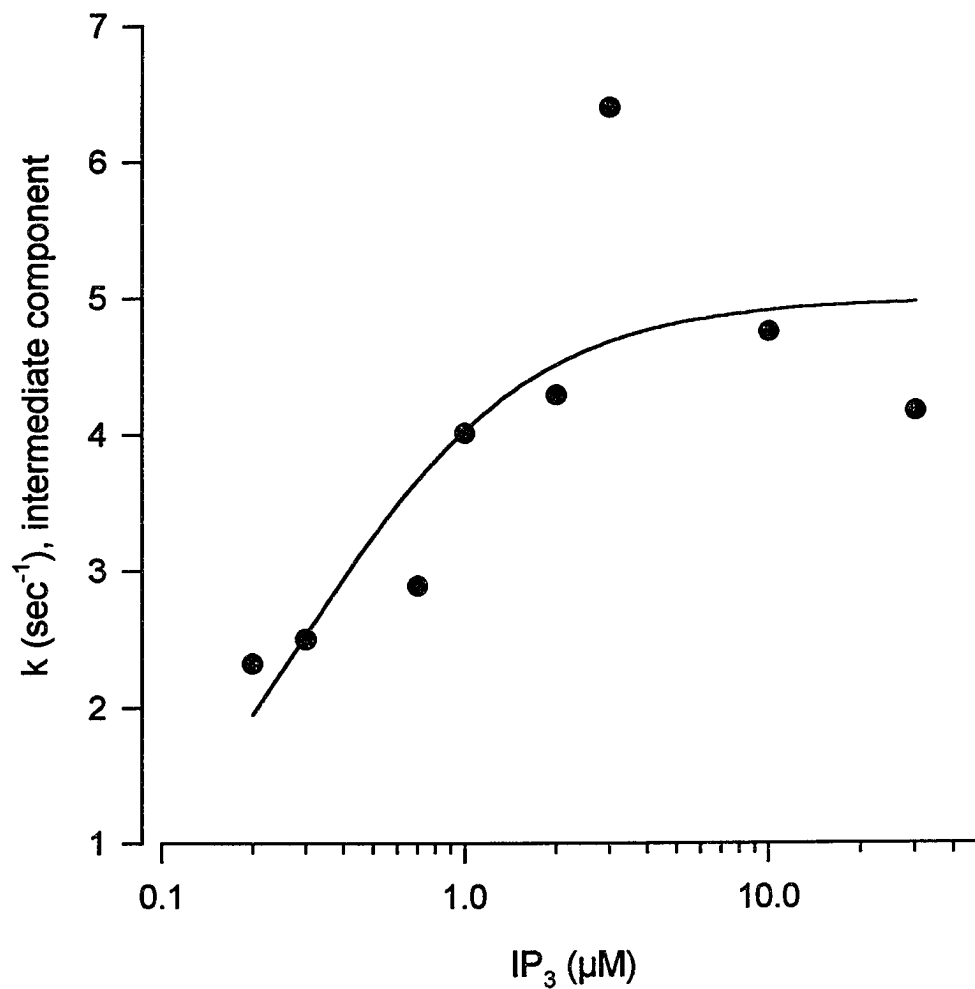
Figure 5-9. Effect of IP₃ on the rate constant of the intermediate component of IP₃ induced Ca²⁺ release. The computer derived rate constants are plotted versus the IP₃ concentration. The solid line represents the computer fit with Hill equation which yielded a K_{mapp} of 0.29 μM and a Hill coefficient of 1.17.
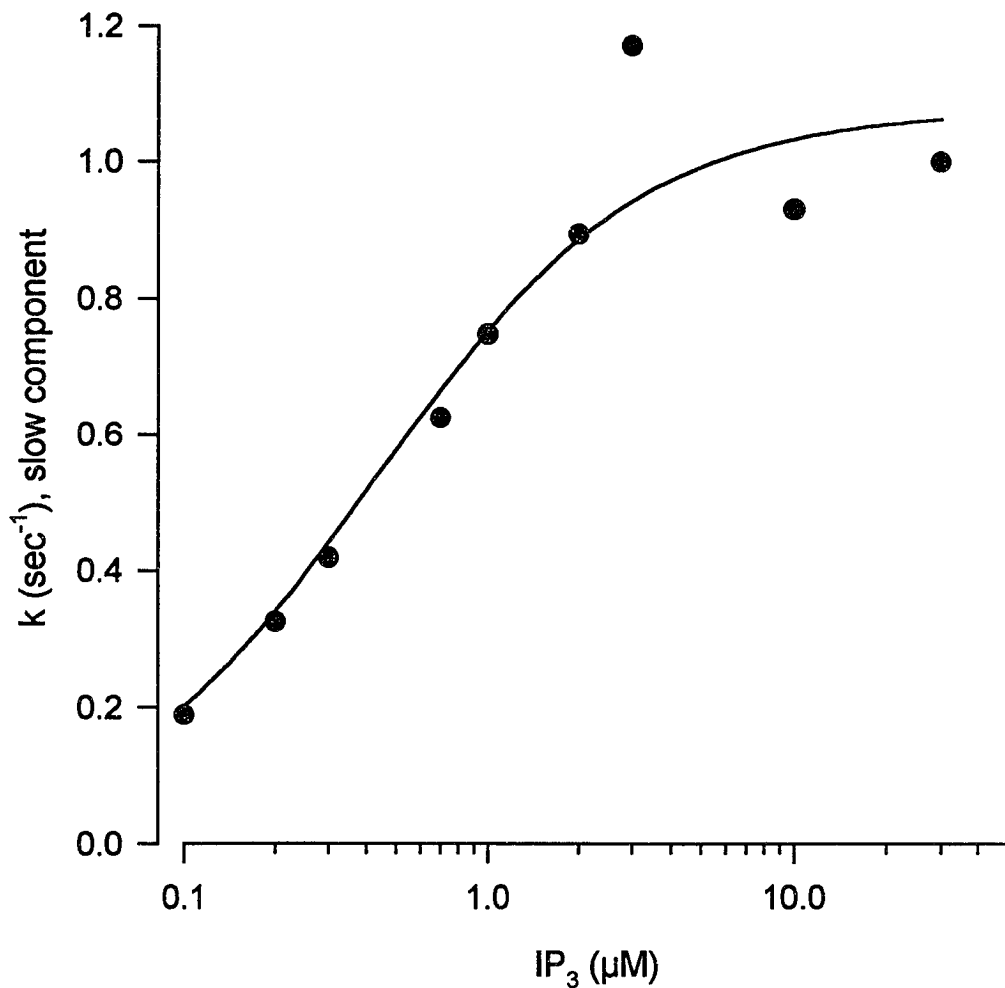
Figure 5-10. Effect of IP₃ on the rate constant of the slow component of IP₃ induced Ca²⁺ release. The computer derived rate constants are plotted versus the IP₃ concentration. The solid line represents the computer fit with Michaelis-Menton equation which yielded a K_{mapp} of 0.43 μM.

Ca^{2+}. The subsequent additon of 10 μM Pb^{2+} induced a rapid release of one third to one half of the accumulated Ca^{2+} (upper trace) in the absence of IP_3. The addition of 1 μM IP_3 after the Ca^{2+} release by Pb^{2+} resulted in no further release. Furthermore, the addtion of Pb^{2+} after a release by IP_3 also resulted in no further release (lower trace). These results demonstrated that Pb^{2+} releases Ca^{2+} from an IP_3 sensitive store. In this experiment, a slower drop in fluorescence was observed after the addition of Pb^{2+}. This effect was not routinely observed and in all other experiments, the fluorescence stabilized after release of by $\leq$ 10 μM Pb^{2+} (as shown in Fig. 5-12). This slower release of Ca^{2+} was kinetically similar to the application of thapsigargin and was presumably due to Pb^{2+} uncoupling the Ca^{2+}-ATPase. The actual free Pb^{2+} concentration may be substantially lower than that added to the solutions, due to complexion with anions, and binding to glassware and microsomal membranes. A Pb^{2+} sensitive electrode is needed to determine the free Pb^{2+} concentration after the addition of Pb^{2+} (Kivalo et al., 1976).

Discussion

Following Pb^{2+} influx into depolarized synaptosomes, the release of intracellular Ca^{2+} occurs within 100 ms suggesting Pb^{2+} directly interacts with the Ca^{2+} release channels on the ER. The Pb^{2+} mediated release of Ca^{2+} occurs in synaptosomes treated with ryanodine and caffeine. However, it does not occur in synaptosomes treated with thapsigargin, which depletes certain IP_3 stores. Therefore, it is possible that intracellular Pb^{2+} elicits Ca^{2+} release by activating IP_3 gated Ca^{2+} channels. To determine if Pb^{2+} activates Ca^{2+} release from IP_3 sensitive stores, ER vesicles were isolated from rat cerebellum, a preparation rich in Type I IP_3 receptors (Furuichi et al., 1994).

The preparation was characterized initially by using fluo-3 and a cuvette based fluorometer to monitor Ca^{2+} flux. The use of fluo-3 was not suitable to monitor Pb^{2+} induced release because Pb^{2+} binds fluo-3 with high affinity and quenches its' fluorescence. Therefore, an alternate technique using CTC was employed. CTC is virtually non-fluorescent in aqueous media in the presence or absence of Ca^{2+} but preferentially accumulates in compartments with micromolar or higher levels of Ca^{2+}, such as within the intracellular stores. When CTC is placed in contact with biological membranes and high levels of Ca^{2+}, its' fluorescence increases as it preferentially associates with the membrane bordering the high Ca^{2+} environment. Some CTC will be bound to the extravesicular membrane but its' contribution to the total fluorescence should be minimal because the extravesicular Ca^{2+} concentration is below the detection limits of CTC (< 10 μM). CTC should have a low rate of efflux from the vesicles because it prefers an apolar, high Ca^{2+} environment to a polar, low Ca^{2+} environment (Marcotte et al., 1990). The results presented in this section demonstrate that CTC can be used to monitor Ca^{2+} fluxes from within the store and is especially useful when the agonist interacts with traditional Ca^{2+} indicators. Furthermore, CTC can be used in conjunction with other Ca^{2+} indicators to "calibrate" the intravesicular signal.

The results presented in this chapter demonstrate that ER vesicles from rat cerebellum are able to sequester Ca^{2+} and release it after the addition of IP_3. The addition of IP_3 resulted in a rapid and concentration dependent Ca^{2+} release which was blocked by preincubation with heparin, which completely and reversibly inhibits IP_3 binding. ATP depletion abolished Ca^{2+} uptake as did the addition of thapsigargin and ionomycin, a pore
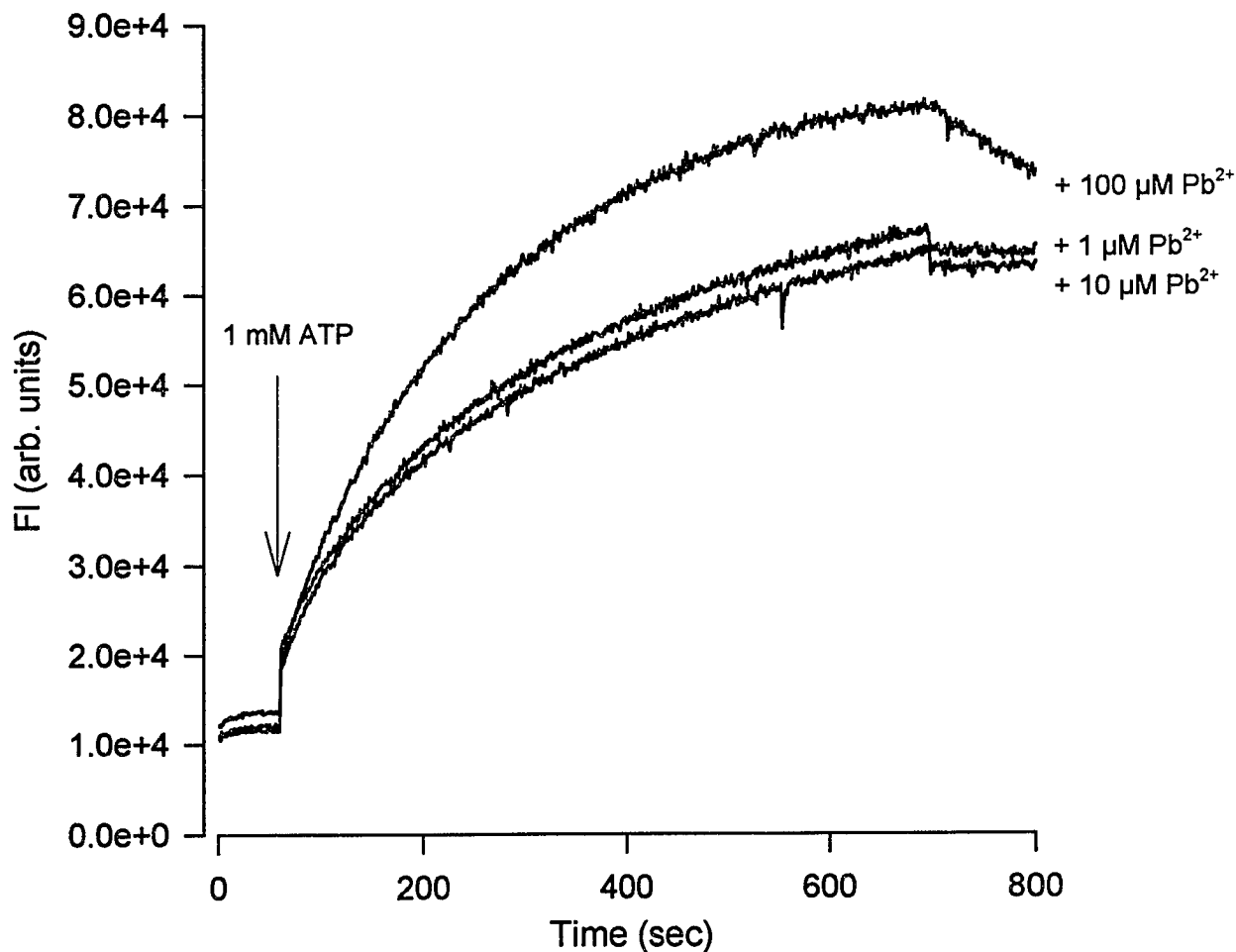
Figure 5-11. Effect of Pb^{2+} on CTC fluorescence in isolated ER vesicles. Changes in CTC fluorescence are plotted over time. Isolated ER vesicles were placed in uptake buffer containing 100 μM CTC. For each experiment, 0.5 mg of vesicle preparation was added to a warm (37°C) cuvette based fluorescent spectrophotometer. A rise in fluorescence (excitation 410 nm, emission 510 nm) was observed following the addition of 1 mM Na-ATP. The addition of Pb^{2+} resulted in an immediate and concentration dependent decrease in fluorescence.
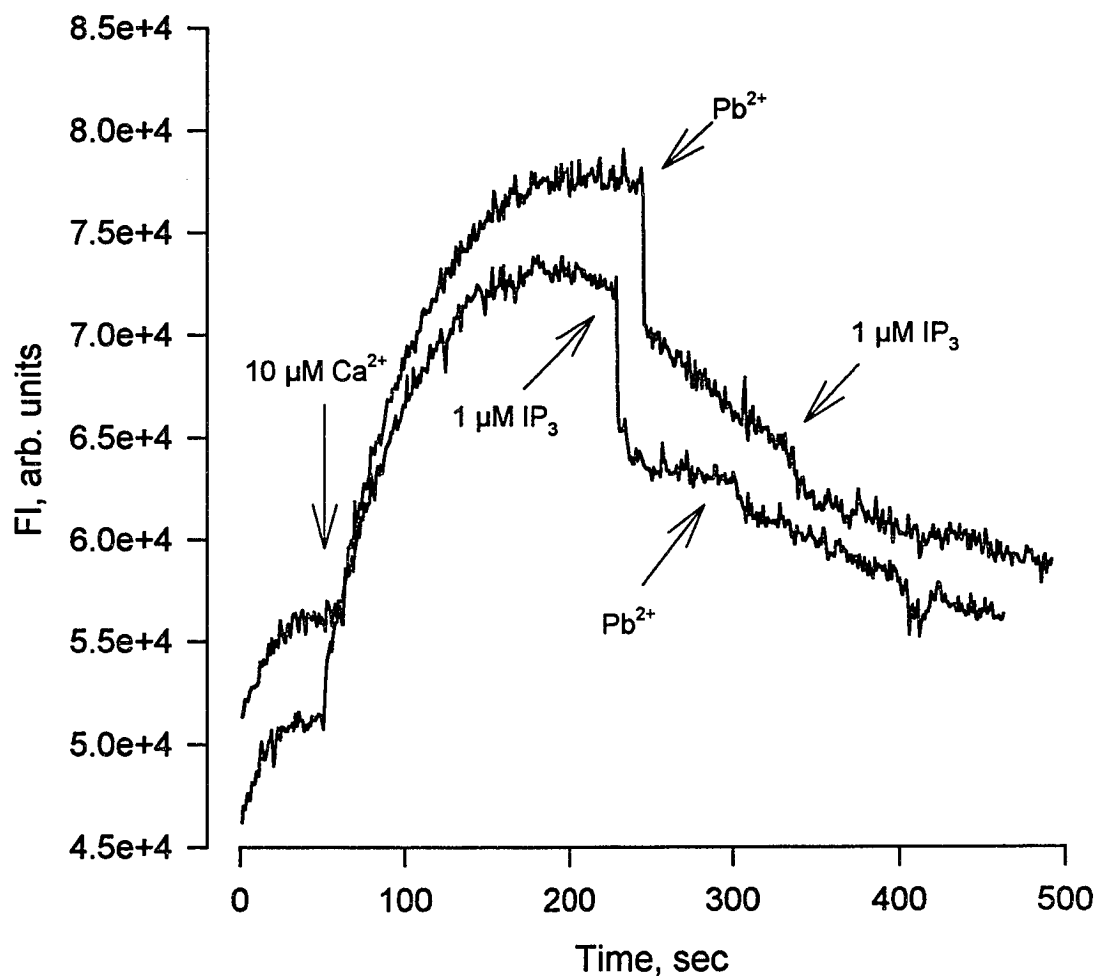
Figure 5-12. Effect of IP$_3$ and Pb^{2+} on CTC fluorescence in ER vesicles. Changes in CTC fluorescence are plotted over time. The ER vesicles were placed in uptake buffer containing 1 mM Na-ATP. The addition of 10 μM Ca^{2+} resulted in a rise in fluorescence as the vesicles accumulated Ca^{2+}. The subsequent additon of 10 μM Pb^{2+} induced a rapid release of one third to one half of the accumulated Ca^{2+} (upper trace) in the absence of IP$_3$. The addition of 1 μM IP$_3$ after the Ca^{2+} release by Pb^{2+} resulted in no further release. Furthermore, the addition of Pb^{2+} after a release by IP$_3$ also resulted in no further release (lower trace).

forming antibiotic. IP$_3$ induced Ca^{2+} release was complete within 20 sec which was consistent with results from other studies (Burnett et al., 1990; Joseph and Rice, 1989; Stauderman et al., 1988). In the experiments utilizing the cuvette based fluorometer presented here, the K$_m$ was determined to be approximately 80 nM, which was consistent with the results of other studies (Stauderman et al., 1988; Supattatone et al., 1988). The larger K$_m$ values reported by other studies (Gandhi and Ross, 1987; Delfert et al., 1986; Joseph et al., 1984) may be due to varying amounts of 3-kinase and 5-phosphatase activities in different preparations (Burnett et al., 1990) and the presence of IP$_3$ receptor subtypes with different binding affinities. Maximal IP$_3$ release was approximately 36%, which was consistent with the results of other studies (Burnett et al., 1990; Joseph and Rice, 1989; Delfert et al., 1986). One possible explanation for the ability of IP$_3$ to release only one third of the accumulated Ca^{2+} is the presence of other Ca^{2+} stores. Ryanodine sensitive stores have been described in this preparation (Volpe et al., 1991).

Stopped-flow spectroscopy and CTC fluorescence were combined to examine the kinetics of IP$_3$ induced Ca^{2+} release on a millisecond time scale. The results from these experiments demonstrated that IP$_3$ activated release consists of three kinetically different components of release that were concentration dependent. At low IP$_3$ concentrations (100 nM), a single, slow component of release was observed. As the IP$_3$ concentration was increased to 200 nM, two kinetically discernable phases of release were observed, a slow and intermediate component of release. At IP$_3$ concentrations of 1 μM IP$_3$ or higher, a third and more rapid phase of release was observed. The computer derived rate constant for each component increased with increasing IP$_3$ levels.

While appearing kinetically different, the intermediate and slow components of release have similar affinities for IP_3 which suggests they may be the same component. These experiments assume uniform receptor density between vesicles. However, this may not be the case. If one population of vesicles has a lower receptor density, then release from those vesicles would appear slower than that from a population with a higher density of the same receptor. Thus, multiple kinetically different phases of release would be observed from a preparation that contains vesicles with the same receptor but at different densities. Heterogenous distribution of IP_3 gated channels has been described in cerebellar Purkinje neurons (Walton et al., 1991; Martone et al., 1993). In pancreatic acinar cells, intracellular injections of IP_3 result in Ca^{2+} release from specific cellular sites suggesting specialized ER sites with a higher density of channels or more sensitive channels (Thorn et al., 1993; Kasai et al., 1993).

The rate constant and IP_3 binding affinity of the fast component were much different from the other two components of Ca^{2+} release. With increasing IP_3 levels, the rate constants of each component increases, most likely due to the activation of more channels. It is less likely that IP_3 was affecting the rate of activation of the channels.

The CTC signal in these experiments was not calibrated so Ca^{2+} release was expressed as small changes in CTC fluorescence. Furthermore, the experimental conditions also approached the saturation point of CTC. Fluo-3 can be used in the stopped-flow apparatus simultaneously with CTC to calibrate the Ca^{2+} signal. By calibrating the fluo-3 signal, the rise in Ca^{2+} outside the vesicles can be converted to the decrease in Ca^{2+} from within the vesicles. The intravesicular Ca^{2+} concentration prior to

mixing can be determined by replicating the Ca^{2+} uptake conditions in the cuvette system and calibrating the loss of Ca^{2+} from the media measure with fluo-3 and converting that to the change in intravesicular Ca^{2+}. Experiments have not be conducted to determine if a linear relationship exists between the change in CTC fluorescence and the change in Ca^{2+}. A calibrated fluo-3 signal could also be used to determine the rate of Ca^{2+} release.

An interesting observation was that the K_{mapp} for these experiments was approximately 300 nM while in the cuvette based system, the K_{mapp} was appoximately 80 nM. The main difference between these two techniques was the mixing rate of IP_3 exposure, which suggests that the rate of the IP_3 rise may affect the kinetics of Ca^{2+} release. Furthermore, the rate of phosphorylation may affect channel gating. The results presented here are from preliminary studies; extensive investigation remains unfinished.

A significant result of these experiments was that Pb^{2+} can activate the release of Ca^{2+} from isolated ER vesicles. Furthermore, Pb^{2+} and IP_3 appear to act on the same store because the addition of IP_3 after a Pb^{2+} induced release resulted in no further release. Thus, Pb^{2+} can activate Type I IP_3 receptors even in the absence of IP_3, possibly through an interaction at a calcium binding site. In these experiments, the addition of 10 μM Pb^{2+} induced a complete release IP_3 sensitive stores. The actual free Pb^{2+} concentration may be substantially less that that added to the solutions, due to complexation of Pb^{2+} with phosphate ions and ATP, and binding to glassware and vesicle membranes. A Pb^{2+} sensitive electrode is needed to determine the true free Pb^{2+} concentration in all experimental solutions (Kivalo et al., .1976).

The effect of Ca^{2+} on the activity of IP_3 gated Ca^{2+} channels is represented as a bell shaped curve. In the presence of a fixed IP_3 concentration, IP_3 gated channels may act as Ca^{2+} activated channels if the Ca^{2+} concentration is below 300 nM (Bezprozvanny and Ehrlich, 1994). Thus, these channels could also be activated by Pb^{2+} under similar conditions. One experiment that remains to be performed is to preincubate the ER vesicles with heparin and then attempt to induce Ca^{2+} release with Pb^{2+}.

Attempts at examining the kinetics of Pb^{2+} induced Ca^{2+} release were unsuccessful. To prevent changes in fluorescence during mixing, it was necessary to mix with a solution that contained an equivalent CTC concentration. This maneuver succeeded in maintaining a stable baseline fluorescence, but also decreased the free Pb^{2+} concentration.

A slow release of Ca^{2+} was observed following the addition of 100 μM Pb^{2+} which suggested that Pb^{2+} may also inhibit the Ca^{2+}-ATPase. However, this effect may not be relevant _in vivo_ because Pb^{2+} does not exceed picomolar levels in experiments with platelets (Dowd and Gupta, 1991) and bovine chrommafin cells (Tomsig and Suszkiw, 1991).

In conclusion, Pb^{2+} directly activates the release of Ca^{2+} from isolated ER vesicles. Furthermore, Pb^{2+} activates the release of Ca^{2+} from IP_3 sensitive stores. Thus, Pb^{2+} directly activates Type I IP_3 receptors in the absence of IP_3, possibly through an interaction at a Ca^{2+} binding/modulatory site. In cultured neuroblastoma cells, small injections of IP_3 or Ca^{2+} causes growing neurites to retract (Bolsover et al., 1992). Thus, increased Ca^{2+}_i disrupts the functional organization of the actin-microfilament network within the growth cone. During developmental Pb^{2+} exposure, Pb^{2+} may inappropriately

activate IP$_3$ sensitive Ca^{2+} channels and elevate Ca^{2+}$_i$, causing growth cone retraction and reduced innervation of target tissues. These effects may contribute to the cognitive and behavioral deficits commonly observed in Pb^{2+} exposed children. Thus, the inappropriate release of Ca^{2+} from from IP$_3$ sensitive stores may contribute to some aspects of Pb^{2+} neurotoxicity.

CHAPTER VI

LEAD DIRECTLY ACTIVATES INOSITOL 1,4,5-TRISPHOSPHATE GATED

CALCIUM CHANNELS FROM RAT CEREBELLUM

<u>Introduction</u>

A more direct way to determine if Pb^{2+} directly activates IP_3 regulated Ca^{2+}

channels is to reconstitute Ca^{2+} channels from cerebellar ER vesicles into artificial planar

bilayers. This technique allows the examination of single Ca^{2+} channels in a membrane of

defined lipid composition, separate from the normal cytosolic milieu. The kinetics of

channel gating (opening and closing) can be examined under control conditions and in the

presence of Pb^{2+}.

The activity of IP_3 gated Ca^{2+} channels is dependent on the presence of cytosolic

Ca^{2+}. The effect of Ca^{2+} on the activity of IP_3 gated channels is a bell shaped curve with

maximal activity occuring around 300 nM (Bezprozvanny et al., 1991). In the presence of

a fixed IP_3 concentration, IP_3 gated channels may act as Ca^{2+} activated channels if the

cytosolic Ca^{2+} is below 300 nM. Under similar conditions, IP_3 gated channels may also

become Pb^{2+} activated channels. Thus, the inappropriate activation of these channels may

contribute to certain aspects of Pb^{2+} neurotoxicity.

<u>Results</u>

At a holding potential of 0 mV, single step changes in conductance of 10 - 12 pA

were observed after channel incorporation. Since IP_3 activatd Ca^{2+} channels have a large

cross sectional permeation pathway and are readily permeable to small cations

(Bezprozvanny and Ehrlich, 1994). Cesium was used as the charge carrier instead of Ca^{2+}

because Cs^+ provides a large single channel current (Ashley, 1989; Ashley and Williams, 1990; Martin and Ashley, 1993). Channel activity was dependent on the presence of IP_3 in the *cis* chamber, indicating these channels incorporate with their cytoplasmic side oriented toward the cis chamber. In a preliminary experiment where 5 channels of identical unitary conductance incorporated into the bilayer, excess heparin, which completely and reversibly inhibits IP_3 binding (Ghosh et al., 1987), eliminated channel activity when added to the *cis* chamber. No effect on channel activity was observed when IP_3 or heparin was added to the *trans* chamber. Channel activity was affected by Ca^{2+} concentrations in the *cis* chamber. The addition of EGTA to reduce free Ca^{2+} levels to less than 10 nM reduced channel gating by more than 99% as did the addition of 100 μM. Both effects were reversible and confined to the *cis* chamber. The addition of 1 - 100 μM ryanodine had no effect on channel gating.

The traces in Fig. 6-1 were from a channel that was reconstituted in 400 mM CsOH *cis* and 50 mM CsOH *trans*. The channel was activated by 20 nM IP_3 in the *cis* chamber indicating that the cytoplasmic side of the channel was oriented toward the *cis* side. Channel activity was also dependent on the presence of Ca^{2+} on the cytoplasmic side. Channel activity was inhibited by reducing the $[Ca^{2+}]$ to nominally zero with EGTA and by increasing the $[Ca^{2+}]$ to 100 μM. In the presence of 1 μM Pb^{2+} (middle trace), channel activity increased at all potentials demonstrating that Pb^{2+} directly activates the channel or a tightly associated subunit. The addition of 1 mM EGTA reduced channel activity to control levels (bottom trace) demonstrating that the effect of Pb^{2+} was reversible.

The traces in Fig. 6-2 are from a channel that was reconstituted in 400 mM CsOH *cis* and 50 mM CsOH *trans*. The channel was activated by 20 nM IP_3 in the *cis* chamber. Under control conditions, an increase in channel activity was observed at all membrane potentials. But the addition of 10 μM Pb^{2+} reduced channel activity at all membrane potentials. These results demonstrated that Pb^{2+} interacts at the Ca^{2+} binding site(s). At low concentrations (Figure 6-1), Pb^{2+} activates IP_3 gated Ca^{2+} channels but at higher concentrations, Pb^{2+} inhibits the channel.

Discussion

The experiments presented in this section were designed to determine if Pb^{2+} activates the release of Ca^{2+} by interacting directly with IP_3 gated Ca^{2+} channels. The activity of IP_3 gated Ca^{2+} channels is dependent on the presence of cytosolic Ca^{2+}. (Bezprozvanny et al., 1991). The effect of Ca^{2+} on the activity of IP_3 gated channels is a bell shaped curve with maximal activity occuring around 300 nM. In the presence of a fixed IP_3 concentration, IP_3 gated channels may act as Ca^{2+} activated channels if the cytosolic Ca^{2+} is below 300 nM. Under similar conditions, IP_3 gated channels may also become Pb^{2+} activated channels.

The results presented here suggest Pb^{2+} directly activates IP_3 sensitive Ca^{2+} channels or a tightly associated subunit. In the presence of IP_3, Pb^{2+} interacts at the Ca^{2+} binding site(s). At low concentrations, Pb^{2+} activates IP_3 gated Ca^{2+} channels but at higher concentrations, Pb^{2+} inhibits channel activity. Furthermore, the effect is reversible because channel activity returned to baseline levels after the addition of EGTA. Thus, the

inappropriate activation of these channels may contribute to certain aspects of Pb^{2+} neurotoxicity.

Figure 6-1. Effect of Pb^{2+} and EGTA on the gating of a single IP_3 activated Ca^{2+} channel from rat cerebellar ER. The channel was reconstituted in 400 mM CsOH *cis* and 50 mM CsOH *trans*. The traces are from a channel that was activated by 20 nM IP_3 in the *cis* chamber indicating that the cytoplasmic side of the channel was oriented toward the *cis* side. Channel activity was also dependent on the presence of Ca^{2+} on the cytoplasmic side. Channel activity was inhibited by reducing the $[Ca^{2+}]$ to nominally 0 with EGTA and by increasing the $[Ca^{2+}]$ to 100 μM. In the presence of 1 μM Pb^{2+} (middle trace), channel activity increased at all potentials. The addition of 1 mM EGTA reduced channel activity to control levels (bottom trace). Channel closing is indicated by the dotted lines.

20 nM IP$_3$, control
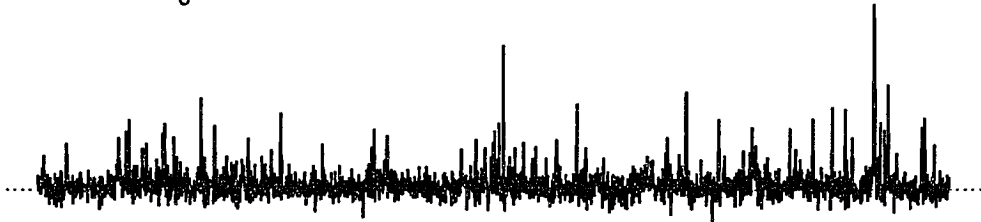
20 nM IP$_3$, + 1 µM Pb^{2+}
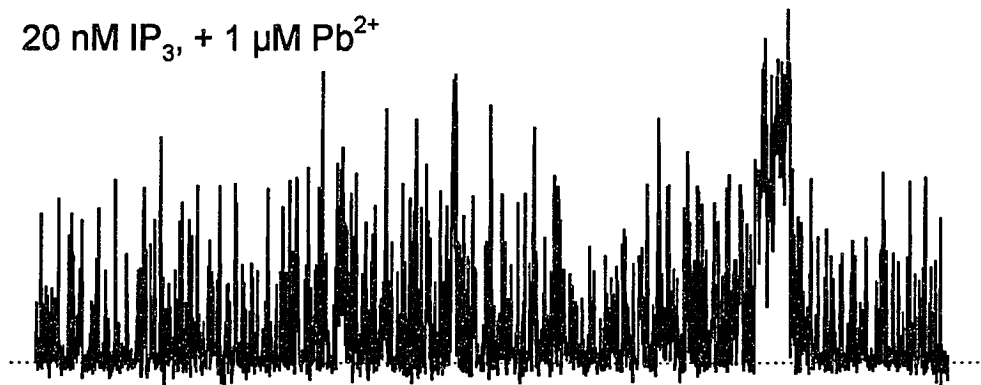
20 nM IP$_3$, + 1µM Pb^{2+}, + 1 mM EGTA

4 pA

0.2 sec

Figure 6-2. Gating of a single IP$_3$ activated Ca^{2+} channel from rat cerebellum under control conditions and after the addition of Pb^{2+}. The channel was reconstituted in 400 mM CsOH *cis* and 50 mM CsOH *trans*. The traces are from a channel that was activated by 20 nM IP$_3$ in the *cis* chamber. Under control conditions, an increase in channel activity was observed at all membrane potentials. But the addition of 10 μM Pb^{2+} reduced channel activity at all membrane potentials. Channel closing is indicated by the dotted lines.
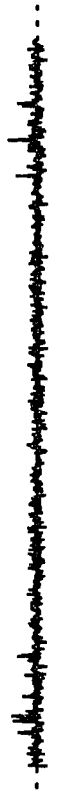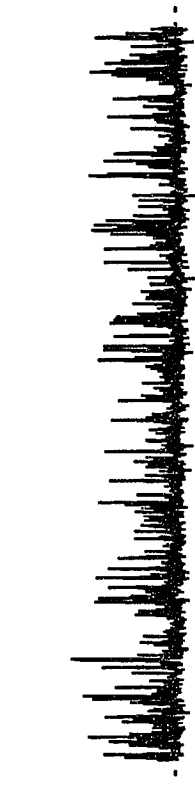
CHAPTER VII

DEVELOPMENTAL ASPECTS OF LEAD NEUROTOXCITY IN RATS

Introduction

Children are more susceptible to low level Pb^{2+} exposure than adults (Cantwell,

1975). Preliminary experiments in rat hippocampal synaptosomes suggests that the ability

of Pb^{2+} to release Ca^{2+}_i was greatest in juvenile animals and apparently lost in adult

animals. A series of experiments was conducted to quantitate the ability of Pb^{2+} to release

Ca^{2+} over development. Preliminary experiments also suggested that the most drastic

reduction in Pb^{2+} activated Ca^{2+} release occured between 7 and 9 weeks of age.

Interestingly, male rats enter puberty promptly at 5 weeks of age. Thus, neural and

hormonal alterations that accompany puberty may induce changes in Ca^{2+}_i storage that

limit the ability of Pb^{2+} release Ca^{2+}. Therefore, a series of pilot experiments was

conducted in which male rats were castrated shortly after birth and the ability of Pb^{2+} to

release Ca^{2+} was compared at 3 and 10 weeks of age (juvenile vs. adult).

Results

In each experiment, the ability of Pb^{2+} to elicit the Ca^{2+} release was assessed four

ways: i) a control load in which the synaptosomes were maintained in solutions with only

contaminating Ca^{2+} levels (< 5 μM); ii) a control load in which the terminals were

pretreated with 1 μM thapsigargin; iii) a Ca^{2+} load in which the synaptosomes were

incubated with 100 μM Ca^{2+}; and iv) a Ca^{2+} load in which the synaptosomes were

pretreated with 1 μM thapsigargin. Each condition was conducted at 30°C for 30 min

prior to depolarization with 100 mM K^+ in the presence of 10 μM Pb^{2+}. Incubation of the

terminals with 100 μM Ca^{2+} reduced the effect of daily Ca^{2+} contamination on the intracellular stores and served as an indicator of the stores' ability to sequester Ca^{2+}.

In Fig. 7-1, synaptosomes from 32 and 74 day old rats were isolated, loaded with the Ca^{2+} indicator fura-2, and mixed in the stopped-flow apparatus with 100 mM K^+ and 10 μM Pb^{2+}. While Pb^{2+} influx was unaffected, the Pb^{2+} activated Ca^{2+} rise was approximately 42% lower in the terminals isolated from the 74 day old rat.

The results in Fig. 7-2 are from synaptosomes incubated with 100 μM Ca^{2+} prior to being mixed in the stopped-flow device with 100 mM K^+ and 10 μM Pb^{2+}. The computer derived amplitudes for the Ca^{2+} rise were pooled among animals in 5 day increments, averaged, and plotted versus age. As depicted, the Pb^{2+} elicited release of Ca^{2+} was greatest in young animals and gradually declined with aging.

Fig. 7-3 illustrates the results from a similar set of experiments where the synaptosomes were maintained in control solutions (contaminating $Ca^{2+} > 1$ μM) for 30 minutes at 30°C. The synaptosomes were mixed with 100 mM K^+ and 10 μM Pb^{2+}. Under these conditions, the overall amplitudes of the Pb^{2+} induced Ca^{2+} rise was decreased compared to those observed when the terminals were exposed to Ca^{2+} prior to depolarization. However, the same trend was present, the evoked Ca^{2+} release was greatest in young animals and lowest in older animals.

The preceding results were from experiments in which the terminals were mixed with 100 mM K^+ and 10 μM Pb^{2+}. This Pb^{2+} concentration became the standard concentration when quanitating the evoked Ca^{2+} rise because it was the lowest concentration that elicited release in older synaptosomes.
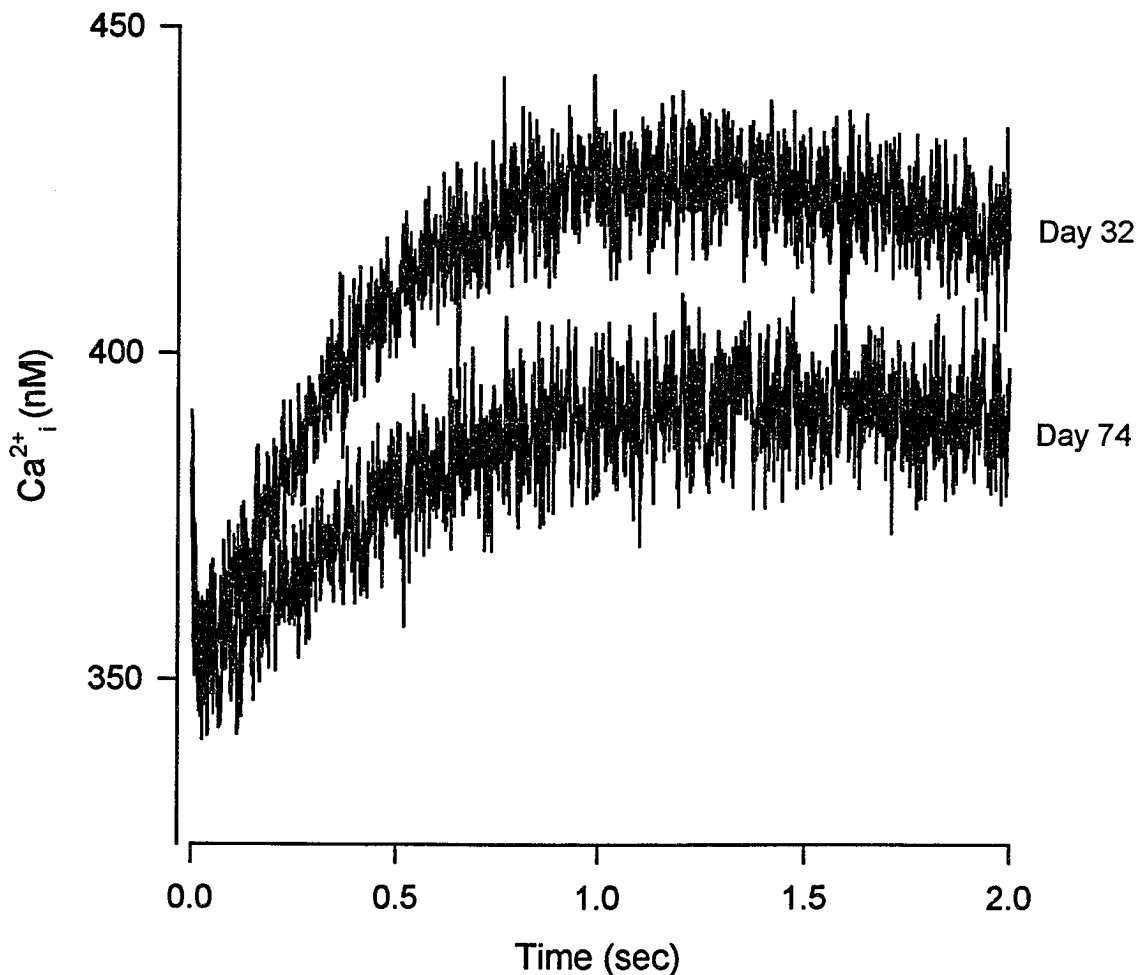
Figure 7-1. Pb^{2+} induced Ca^{2+} release in depolarized synaptosomes from 32 and 74 day old rats. The intracellular Ca^{2+} store appears to be labile so the synaptosomes were exposed to 100 μM Ca^{2+} for thirty minutes prior to depolarization with 100 mM K$^+$ in the presence of 10 μM Pb^{2+}. While the Pb^{2+} influx was unaffected, the Pb^{2+} induced Ca^{2+} release was reduced in synaptosomes from older rats (Day 32, 81.1 nm; Day 74, 46.8 nm). Preincubation with Ca^{2+} prior to depolarization also helped eliminate the effect of daily variations in Ca^{2+} contamination on intracellular store filling during the isolation procedure.
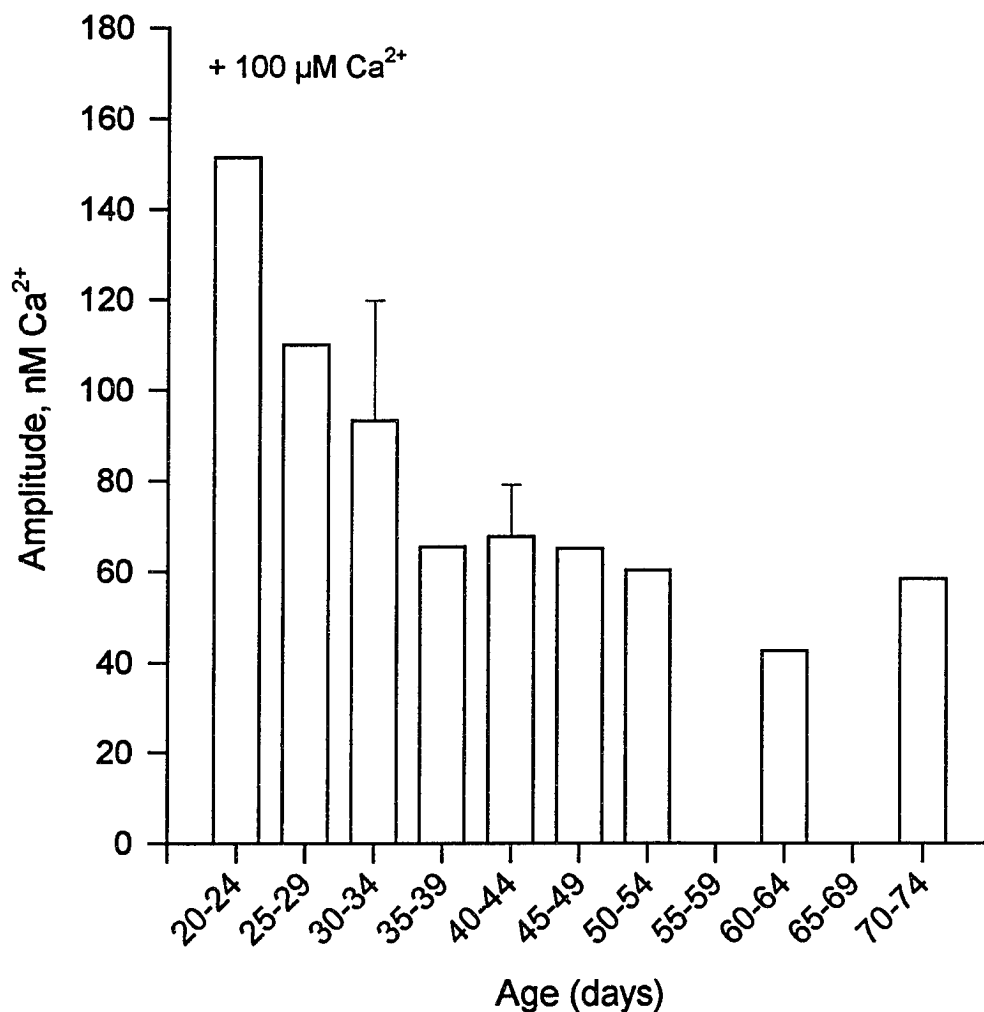
Figure 7-2. Effect of age on the Pb^{2+} induced Ca^{2+} release in depolarized synaptosomes after incubation with Ca^{2+}. The synaptosomes were exposed to 100 μM Ca^{2+} for thirty minutes prior to depolarization with 100 mM K^+ in the presence of 10 μM Pb^{2+}. The amplitude of released Ca^{2+} is plotted versus age. The results are means ± S.E.M (where appropriate) for 6 experiments (30-34 days), 4 experiments (40-44 days), 3 experiments (25-29; 35-39; 45-49; 70-74), 2 experiments (20-24; 50-54), or 1 experiment (60-64). Each experiment represents at least 18 replicates per experiment.
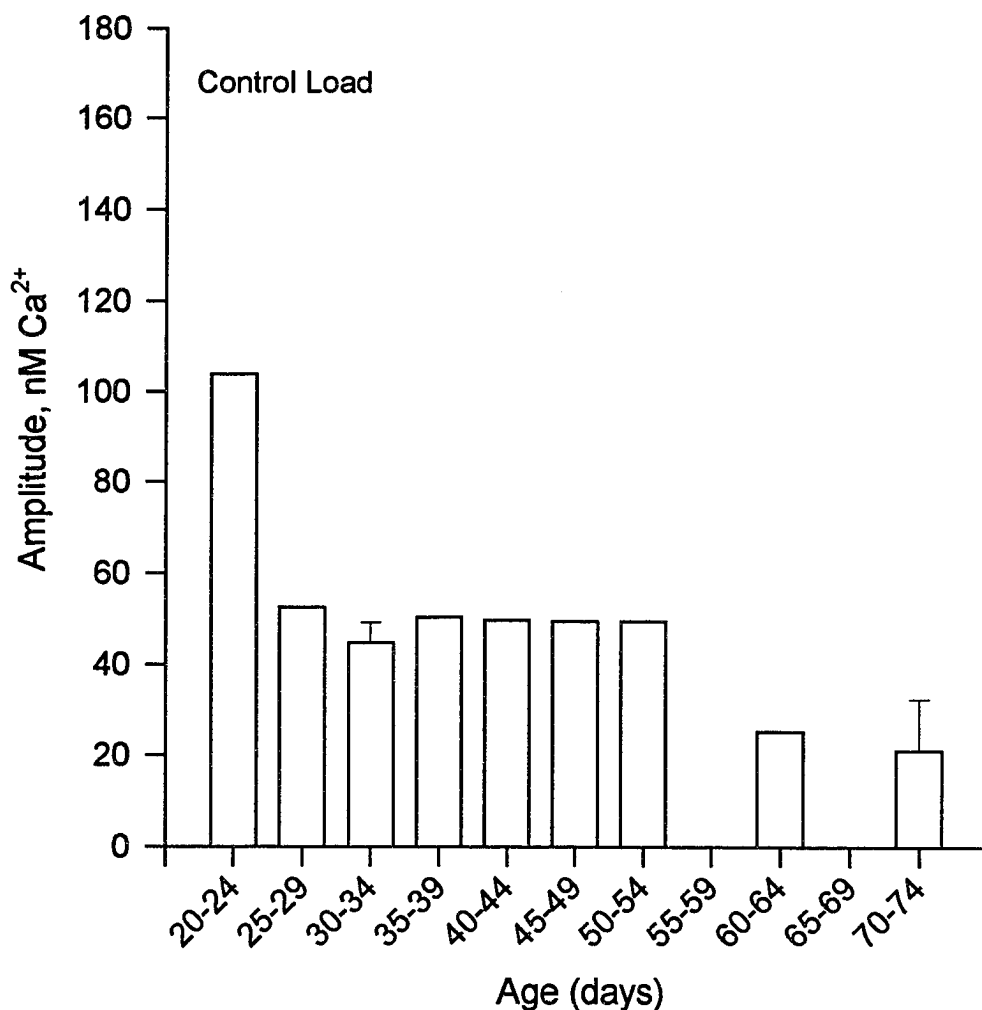
Figure 7-3. Effect of age on the Pb^{2+} induced Ca^{2+} release in depolarized synaptosomes after control load. The synaptosomes were exposed to nominally Ca^{2+} free solutions for thirty minutes prior to depolarization with 100 mM K$^+$ in the presence of 10 μM Pb^{2+}. The amplitude of released Ca^{2+} is plotted versus age. The results are means ± S.E.M (where appropriate) for 6 experiments (30-34 days), 4 experiments (40-44 days), 3 experiments (25-29; 35-39; 45-49; 70-74), 2 experiments (20-24; 50-54), or 1 experiment (60-64). Each experiment represents at least 18 replicates per experiment.

Preliminary experiments also suggested that the most drastic reduction in Pb^{2+} activated Ca^{2+} release occured between 7 and 9 weeks of age. Interestingly, male rats enter puberty promptly at 5 weeks of age. To determine if hormonal alterations that accompany puberty induce changes in Ca^{2+}_i storage and Pb^{2+} sensitivity, a series of pilot experiments was conducted in which male rats were castrated shortly after birth and the ability of Pb^{2+} to release Ca^{2+} was compared at 3 and 10 weeks of age (juvenile vs. adult). The results of these experiments are presented in the Table 8-1.

It was difficult to draw any conclusions from these studies because of possible methodological design problems. The main problem encountered with these studies was a difference in tissue volume between the juvenile and adult conditions. The original design of these experiments was to have an equivalent number of animals between conditions. However, when this experiment was performed, it became apparent that the harvested tissue volume was less for the juvenile rats than for the older rats. Thus, while the same number of hippocampi were used for each condition, a greater synaptosomal yield was obtained from the older animals. The data also confirms this problem. For example, the amplitude of the Pb^{2+} induced Ca^{2+} rise was greater in the adult animals, which was the reverse of what was routinely observed in all other experiments. Furthermore, incubation of juvenile terminals with Ca^{2+} prior to depolarization did not result in a substantially larger Ca^{2+} rise. While no comparisons can be made between the juvenile and adult conditons, it did appear that the Pb^{2+} activated release of Ca^{2+} was slightly greater in the castrated adult rats, possibly suggesting that hormonal alterations may affect Ca^{2+}_i storage which affects the ability of Pb^{2+} to evoke release. At this point, no direct conclusions can

be made until this experiment is repeated with design modifications that correct for differences in tissue volume due to age.

TABLE 8-1: Effect of Castration on the Ca^{2+} Rise Induced by Depolarization in the Presence of 10 μM Pb^{2+}.

	Juvenile		Adult	
Treatment	Control	Castrated	Control	Castrated
Control Load	15.9	23.8	28.8	37.4
Thapsigargin	12.4	19.0	28.7	49.0
Calcium Load	16.0	19.5	49.5	64.6
Thapsigargin	7.8	16.1	45.0	57.7

The results represent the evoked Ca^{2+} rise (nM) after mixing with 100 mM K^+ and 10 μM Pb^{2+}. Each result is the average of 3 replicates.

Discussion

The observation that children are more sensitive to low level Pb^{2+} exposure has been recognized for decades, yet despite extensive investigation the neurochemical mechanisms are still unknown. The results of this chapter provide a novel hypothesis regarding the higher sensitivity of children. In this model, age affects the ability of Pb^{2+} to release Ca^{2+}. The amplitude of the Ca^{2+} rise was greatest in younger animals and gradually

declined over development. Furthermore, depolarization in the presence of higher Pb^{2+} concentrations was necessary to mobilize Ca^{2+} from older synaptosomes.

As presented in Chapter 4, a portion of the Pb^{2+} induced Ca^{2+} rise was sensitive to thapsigargin while the remaining portion appeared to be labile. When the synaptosomes were exposed to nominally Ca^{2+} free solutions for 30 min prior to depolarization in the presence of Pb^{2+}, the Ca^{2+} rise was substantially reduced. Furthermore, thapsigargin had little effect on the Ca^{2+} rise observed in store depleted nerve terminals. It appeared that both stores decreased uniformly with aging. The Pb^{2+} sensitive store may be lost or replaced by some other store during development. Experiments with synaptosomal ER vesicles are needed to determine if the overall storage capacity decreases or if specific stores change with aging.

Alterations in Ca^{2+}_i homeostasis have been reported with aging. In aged cerebral cortical microsomes, the maximal effectiveness of IP_3 to release Ca^{2+} is reduced by 50% while the binding affinity of IP_3 is unaffected. No age related changes in IP_3 potency were observed in the hippocampus, thalamus, or cerebellum. Furthermore, aging had no effect on the ability of microsomes to accumulate Ca^{2+} in the presence of ATP (Burnett et al., 1990), which suggests that the IP_3 sensitive store may be replaced by another store. Likewise, a 50% reduction in the density of IP_3 receptors in the cerebral cortex was observed in aged cortical microsomes while ryanodine receptor density was unaffected (Martini et al., 1994). The reduction in IP_3 receptors may coincide with the decreased ability of Pb^{2+} to mobilize Ca^{2+}. Thus, a normal developmental change in Ca^{2+}_i regulation

may correlate with the greater sensitivity of children to the neurotoxic effects of Pb^{2+} exposure.

The youngest animals used in the Burnett et al. study (1990) were 3 months old which was 2 weeks older than the oldest rats used in the experiments presented here. The results presented here suggest that certain age dependent changes in Ca^{2+}_i storage may be completed by 3 months of age. This possibility may explain why aging had no effect on the potency of IP_3 in hippocampal microsomes. A reduced effect may be observed if microsomes from 1 month old rats were compared to those from 3 month old rats.

In acutely dissociated mouse neurons, basal Ca^{2+}_i concentration was reduced in aged cells and depolarization induced Ca^{2+} influx was reduced. However, depolarization induced IP_3 generation was increased in aged neurons. Thus, IP_3 hydrolysis is increased and activated by lower Ca^{2+}_i due to decreased Ca^{2+} availability (Hartman et al., 1993; 1994). If the IP_3 sensitive store is lost or replaced during development, then increased IP_3 and Pb^{2+} would be needed to evoke the Ca^{2+}_i rise.

The amplitude of the Pb^{2+} induced Ca^{2+} rise began to decline around 4 weeks of age which is one week prior to the onset of puberty in male rats. A group of rats were castrated shortly after birth and the ability of Pb^{2+} to mobilize Ca^{2+} was examined at 3 and 10 weeks of age to determine if neural and hormonal alterations that accompany puberty had any effect on Pb^{2+} sensitivity. Unfortunately, known methodological problems prevent conclusions from being made. This experiment needs to be repeated so that equivalent tissue volumes is obtained from each group.

The youngest rats utilized in these experiments were 21 days old. Additional experiments are needed on younger and older animals to fully determine the time course of this effect.

Thus, a developmental transition in Ca^{2+}_i storage may explain the higher sensitivity of children to Pb^{2+} exposure.

CHAPTER VIII

CONCLUSIONS AND FUTURE DIRECTIONS

The results from these studies provide substantial evidence that Pb^{2+} alters Ca^{2+} homeostasis in the presynaptic terminal. These studies demonstrated that Pb^{2+} blocks presynaptic Ca^{2+} channels. However, at physiological Ca^{2+} concentrations and Pb^{2+} levels observed in Pb^{2+} exposed people, Pb^{2+} does not block Ca^{2+} influx to any significant extent. Upon depolarization, Pb^{2+} permeates one class of rapidly inactivating Ca^{2+} channels and activates the release of Ca^{2+} from intracellular stores. The Pb^{2+} induced Ca^{2+} rise was greatest in young animals and gradually declined over development. Studies performed with isolated cerebellar ER vesicles demonstrated that Pb^{2+} activates the release of Ca^{2+} from IP_3 sensitive stores. Additional evidence with reconstituted IP_3 activated Ca^{2+} channels indicated that Pb^{2+} directly increased channel activity in the presence of IP_3. These conclusions and future research directions are discussed below.

Pb^{2+} blocks Ca^{2+} channels competitively in isolated hippocampal nerve terminals

Depolarization of isolated presynaptic nerve terminals with elevated K^+ promoted a rapid increase in Ca^{2+}_i which occured in two kinetically distinguishable phases: a fast component, representing the activity of rapidly inactivating Ca^{2+} channels ($\tau \sim$ 60 msec), and a slow component, which is comprised of slowly inactivating Ca^{2+} channels ($\tau \sim$ 1 sec) and Na^+/Ca^{2+} exchange operating in the "reverse" mode. Low concentrations of Pb^{2+} (0.1 - 0.5 μM) blocked competitively the rapidly and slowly inactivating channels ($IC_{50} \cong$ 140 nM, 195 nM respectively). At higher concentrations ($\geq$ 1 μM), Pb^{2+} permeated the

rapidly inactivating channels. However, Pb^{2+} did not permeate the slowly inactivating channels and Na^+/Ca^{2+} exchange did not promote Pb^{2+} transport.

Pb^{2+} activates the release of Ca^{2+} from intracellular stores in rat hippocampal synaptosomes

Pb^{2+} permeation was followed by a subsequent rise in Ca^{2+}_i even in the absence of extracellular Ca^{2+}. The rise in Ca^{2+}_i was reduced by thapsigargin, which depletes non-mitochondrial Ca^{2+} stores, demonstrating Pb^{2+} activates the release of Ca^{2+} from intracellular stores. Pretreatment of the terminals with ryanodine (1 μM, 100 μM) and caffeine (10 mM) had no effect on the Ca^{2+} rise induced by Pb^{2+} influx. The Pb^{2+} induced Ca^{2+} rise was unaffected by mitochondrial inhibitors. Thus, Pb^{2+} activates the release of Ca^{2+} from a thapsigargin sensitive, caffeine and ryanodine insensitive, non-mitochondrial store. A substantial portion of the mobilized Ca^{2+} remained after pretreatment with thapsigargin and incubation in Ca^{2+} free solutions, suggesting the presence of a tight or less leaky store in the terminal region. The rise in Ca^{2+}_i occurred within 100 ms suggesting that Pb^{2+} directly activates Ca^{2+} channels on the ER. The Ca^{2+} rise was reduced in terminals treated with thapsigargin, which depletes certain IP_3 sensitive stores. Thus, Pb^{2+} may release Ca^{2+} by activating IP_3 receptors. Furthermore, IP_3 sensitive but thapsigargin insensitive stores have been described in neurons and various other cells. The hippocampal presynaptic terminal may contain a leaky, thapsigargin sensitive store and a tight, thapsigargin insensitive store that are both activated by IP_3 and inappropriately by Pb^{2+}. Thus, the neurotoxic effects of Pb^{2+} may be due in part to interference with Ca^{2+}_i metabolism in the presynaptic terminal.

Developmental aspects of Pb^{2+}- Ca^{2+} interactions in hippocampal synaptosomes

The Pb^{2+} induced release of Ca^{2+} was greatest in younger animals and gradually declined during postnatal development suggesting age dependent changes in $Ca^{2+}{}_i$ storage and Pb^{2+} sensitivity.

Pb^{2+} activates the release of Ca^{2+} from an IP_3 sensitive store

The results of this study clearly demonstrate that Pb^{2+} activates the release of Ca^{2+} from rat cerebellar ER vesicles. Pb^{2+} and IP_3 appear to act on the same store because the addition of IP_3 after Pb^{2+} induced release resulted in no further release. Furthermore, the addition of Pb^{2+} after Ca^{2+} release by IP_3 resulted in no additional release. Thus, Pb^{2+} activates the release of Ca^{2+} from IP_3 sensitive stores.

Pb^{2+} directly activates IP_3 regulated Ca^{2+} Channels

Single IP_3 gated Ca^{2+} channels from rat cerebellar ER were reconstituted into artificial planar bilayers to demonstrate that Pb^{2+} directly interacts with the channel molecule. The addition of 1 µM Pb^{2+} to the cytoplasmic side increased channel openings at all membrane potentials. However, the addition of 10 µM Pb^{2+} decreased channel openings. Thus, the activity of IP_3 gated Ca^{2+} channels appears "bell shaped" for both Pb^{2+} and Ca^{2+}, and Pb^{2+} mimics the effects of Ca^{2+} possibly by interacting at the Ca^{2+} binding/modulatory site(s).

The experiments utilizing cerebellar ER vesicles have demonstrated that Pb^{2+} directly activates the release of Ca^{2+} from IP_3 sensitive stores, in the absence of IP_3. Pb^{2+} also modulates the activity of IP_3 activated Ca^{2+} channels by possibly interacting at a Ca^{2+} binding site. These results sugest that Pb^{2+} may regulate the activity of IP_3 sensitive Ca^{2+}

channels at a Ca^{2+} binding site and promote channel opening in the absence of IP_3 by a possible allosteric interaction. These results demonstrate that Pb^{2+} activates IP_3 sensitive stores but only suggest that Pb^{2+} releases Ca^{2+} in hippocampal synaptosomes by activating an IP_3 sensitive store. These results suggest that inappropriate activation of these channels may underlie some aspects of Pb^{2+} neurotoxicity.

Future research directions are discussed below.

Does Pb^{2+} activate the release of Ca^{2+} by generating IP_3?

This study focused on a direct interaction between Pb^{2+} and IP_3 activated Ca^{2+} channels. The possibility remains that Pb^{2+}_i may also induce alterations in second messenger systems to release Ca^{2+}. For example, Pb^{2+} may generate IP_3 by activating phospholipase C. This possiblity would be tested by loading the synaptosomes with heparin, which inhibits IP_3 binding. The terminals would be subjected to rapid freeze-thaw cycles in the presence of excess heparin, which allows the introduction of impermeant molecules into the cytoplasm without permanently disrupting synaptosomal function (Nichols et al., 1989). If the presence of cytsolic heparin abolishes the Pb^{2+} activated release of Ca^{2+}, this would suggest that Pb^{2+} induces the Ca^{2+} rise by generating IP_3.

Another approach would be to measure IP_3 production in synaptosomes that have been depolarized in the presence of Pb^{2+} on a millisecond time scale in a quench flow device. With this technique, the synaptosomes will be depolarized with elevated K^+ in the presence of Pb^{2+} and 10-1000 msec later the reaction is quenched by a stop solution that contains high concentrations of EGTA and digitonin. The reacted synaptosomes will be collected, the inositol phospholipids extracted, and the IP_3 concentration determined by a

radioreceptor assay (Bredt et al., 1989) or by incorporating ^{3}H-IP$_3$ into intact terminals, and separating the inositol lipids by column chromatography (Chein and Cambier, 1989). If IP$_3$ production precedes or parallels the rise in Ca^{2+}, this would also suggest that Pb^{2+} induces the release of Ca^{2+} by generating IP$_3$. Further experiments would then be necessary to determine the ablity of Pb^{2+} to activate phospholipase C.

What is the relationship between age and the ability of Pb^{2+} to mobilize Ca^{2+}?

The results presented here demonstrated that the ability of Pb^{2+} to release Ca^{2+} was greatest in juvenile animals and gradually declined over development. The youngest animals utilized in these experiments were 3 weeks of age while the oldest animals were 10 weeks of age. Further studies are needed with younger and older animals to examine the time course of this effect.

One possibility for the loss of the Pb^{2+} effect is that the IP$_3$ sensitive store is lost or replaced by some other store. ER vesicles from synaptosomes of various ages will be isolated and the number of IP$_3$ binding sites will be measured by a [^{3}H]-IP$_3$ binding assay (Palmer et al., 1988; Ross et al., 1989). Another approach is to isolate ER vesicles from synaptosomes at various ages and examine the ability of IP$_3$ and Pb^{2+} to release Ca^{2+}. These results will be important in identifying possible transitional periods in IP$_3$ sensitive Ca^{2+}$_i$ storage that could be examined by incorporating Ca^{2+} channels into artificial bilayers.

The amplitude of the Pb^{2+} induced Ca^{2+} rise began to decline around 4 weeks of age which is one week prior to the onset of puberty in male rats. Thus, neural and homonal alterations that accompany puberty may induce changes in Ca^{2+}$_i$ storage that limit

the ability of Pb^{2+} to release Ca^{2+}. The series of pilot experiments presented here need to be repeated with equivalent tissue volumes between the juvenile and adult groups.

The experiments in this study were performed exclusively with tissue from male rats. Experiments with female rats are necessary to determine the ability of Pb^{2+} to mobilize Ca^{2+} and the time course of this effect.

Does Pb^{2+} activate a BHQ sensitive store?

Pretreatment with thapsigargin did not completely abolish the Pb^{2+} activated Ca^{2+} rise. A significant portion remained that did originate from mitochondrial or ryanodine sensitive stores. One possibility is that the terminal region contains a store that is sensitive to 2,5-Di-(t-butyl)-1,4-hydroquinone (BHQ) and weakly sensitive to thapsigargin. BHQ inhibits intracellular Ca^{2+}-ATPases but is structurally unrelated to thapsigargin. The co-existence of multiple isoforms of intracellular Ca^{2+}-ATPases have been described in various preparations (Pozzan et al., 1994). For example, weakly thapsigargin sensitive but BHQ sensitive Ca^{2+}-ATPases have been described in platelets (Papp et al., 1991, 1993). Similar experiments to those presented here will be perfomed to determine if the Pb^{2+} sensitive store is also sensitive to BHQ.

Does low level, developmental Pb^{2+} exposure down-regulate the Pb^{2+} sensitive store?

Singh (1993) reported that low level Pb^{2+} exposure beginning prenatally reduced the number of IP_3 receptors on the ER of isolated cortical neurons. However, Pb^{2+} exposure in adult rats did not produce these changes. A necessary experiment is to subject prenatal and adult rats to low level Pb^{2+} exposure (1 mg/kg/day) by gastric intubation as described by Singh (1993). Synaptosomes will be isolated at various stages

of development and the ability of Pb^{2+} to release Ca^{2+} will be determined. If the Pb^{2+} sensitive store is also down regulated, then experiments will be performed to determine the length of time that is required for this effect to occur.

Does Pb^{2+} induce Ca^{2+} release in other nerve terminal types?

Similar experiments to the ones described here will be performed with synaptosomes from other brain regions (ie., corpus striatum) or in a more defined hippocampal preparation, mossy fiber terminals, to determine if this effect is confined to the hippocampus and whether the results described here are applicable to other areas of the brain.

Do other heavy metals replicate the effect of Pb^{2+}?

Preliminary evidence suggests that Co^{2+} also activates the release of Ca^{2+} from a developmentally regulated intracellular store. Similar experiments will be performed to determine if other heavy metals (methyl mercury, silver, or zinc) also induce Ca^{2+} release from intracellular stores and promote channel activation by interacting at the same sites as Pb^{2+}.

Does Pb^{2+} modulate IP_3 activated Ca^{2+} channels at Ca^{2+} binding site?

Lectin affinity purification of IP_3 receptors yields channels whose activity is insensitive to cytoplasmic Ca^{2+} (Hingorani and Agnew, 1992). A necessary experiment is to isolate IP_3 receptors in this fashion and reconstitute the channels in artificial lipid bilayers to determine if Pb^{2+} is able to activate these channnels.

Does Pb²⁺ interact with other intracellular Ca²⁺ stores?

In the unlikely event that Pb^{2+} does not interact with IP_3 sensitive stores in hippocampal nerve terminals, experiments will be performed to examine the possibility that Pb^{2+} activates another intracellular store. Both GTP (Mullaney et al., 1988; Chueh et al., 1987; Gill et al., 1987) and cyclic ADP-ribose (Meszaros et al., 1993; Morrissette et al., 1993; Lee et al., 1994) activate Ca^{2+} release from intracellular stores in neurons and other cells.

The consequences of Pb^{2+} exposure have been known for centuries, but only recently have the severe and long-lasting effects of low level developmental exposure in children been recognized. Improved epidemiological studies have shown that low level Pb^{2+} exposure can produce lasting cognitive and behavioral deficits and such nonspecific symptoms as irritability, insomnia, depression, and clumsiness (Jason and Kellog, 1980). Despite extensive investigation, the neurochemical mechanisms that underlie these effects are still unknown.

A research model for Pb^{2+} neurotoxicity should incorporate effects that are demonstratable in developing animals. The results presented here demonstrate a novel interaction between Pb^{2+} and intracellular sites important in maintaining Ca^{2+} homeostasis. A significant result was the loss of this effect with aging. This study provides new information regarding the greater sensitivity of children to the neurotoxic effects of Pb^{2+}.

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APPENDIX

REQUEST FOR PERMISSION

August 7, 1996

To: B. Collier
Chief, Editor, Journal of Neurochemistry
Department of Pharmacology and Therapeutics
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I hereby request permission to reprint the following material from your publication:

Bartschat, DK. and Rhodes, TE. (1995) Protein Kinase Modulates Calcium Channels In Isolated Presynaptic Nerve Terminals of Rat Hippocampus. J Neurochem. 64, 2064-2072.

The precise details of the material desired are given below:

Figure 1, Page 2066

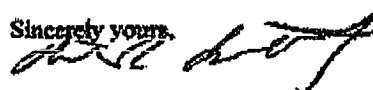The material will appear in the following volume:

Rhodes, TE, Lead Activation of a Developmentally Regulated Calcium Channel in Rat Hippocampal Nerve Terminals

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Approx. no. of pages   175

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Sincerely yours,

Dieter K. Bartschat, M.D., Ph.D.

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_____
(Date)

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Bartschat, D.K. and Rhodes, T.E. (1995) Protein kinase c modulates calcium channels in isolated presynaptic nerve terminals of rat hippocampus. **J. Neurochem.** 64, 2064-2072.

Rhodes, T.E. and Bartschat, D.K. Lead blocks competitively two types of voltage activated calcium channels in isolated hippocampal presynaptic nerve terminals. **Brain Research**, submitted March 1, 1995.

Awards:

Eastern Virgnia Chapter, Society for Neuroscience, Award for Excellence in Research, "Alterations in hippocampal voltage activated calcium channels following traumatic brain injury in the rat"

Selected oral presentation and best poster presentation, EVMS Research Day, "Neurotoxic effects of lead on presynaptic calcium dynamics in rat hippocampal nerve terminals"

Best poster presentation, EVMS Research Day, "Lead activation of inositol 1,4,5-trisphosphate gated calcium channels from rat cerebellum"

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