

# Effects of Toxic Environmental Contaminants on Voltage-Gated Calcium Channel Function: From Past to Present

William D. Atchison<sup>1,2,3</sup>

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Voltage-gated  $\text{Ca}^{2+}$  channels are targets of the number of naturally occurring toxins, therapeutic agents as well as environmental toxicants. Because of similarities of their chemical structure to  $\text{Ca}^{2+}$  in terms of hydrated ionic radius, electron orbital configuration, or other chemical properties, polyvalent cations from aluminum to zinc variously interact with multiple types of voltage-gated  $\text{Ca}^{2+}$  channels. These nonphysiological metals have been used to study the structure and function of the  $\text{Ca}^{2+}$  channel, especially its permeability characteristics. Two nonphysiological cations,  $\text{Pb}^{2+}$  and  $\text{Hg}^{2+}$ , as well as their organic derivatives, are environmental neurotoxicants which are highly potent  $\text{Ca}^{2+}$  channel blockers. These metals also apparently gain intracellular access in part by permeating through  $\text{Ca}^{2+}$  channels. In this review the history of  $\text{Ca}^{2+}$  channel block produced by  $\text{Pb}^{2+}$  and  $\text{Hg}^{2+}$  as well as other nonphysiological cations is traced. In particular the characteristics of  $\text{Ca}^{2+}$  channel block induced by these environmental neurotoxic metals and the consequences of this action for neuronal function are discussed.

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**KEY WORDS:** Neurotoxicity; methylmercury; inorganic mercury; lead; polychlorinated biphenyls; solvents; synaptosomes; nerve terminal function; neuromuscular junction; ion channels.

## INTRODUCTION

Because of their portal location, membrane proteins in general are the first line of defense encountered when a cell is exposed to a toxic chemical. Thus they are obligatorily exposed to numerous chemical toxicants. Moreover, because of the number of crucial signaling roles which  $\text{Ca}^{2+}$  plays in cellular function and their role in maintenance of life, i.e., skeletal muscle contractility (respiration), cardiac muscle contractility, and neurotransmitter release,  $\text{Ca}^{2+}$  channel proteins are natural evolutionary targets for the action of a number of toxins used by predatory animals (spiders, fish-hunting cone snails, and certain snakes). However the same properties make  $\text{Ca}^{2+}$  channel

proteins susceptible to interaction with other toxic agents encountered in the environment. When this fact is coupled with the fact that a number of environmentally important metals have chemical properties similar to those of  $\text{Ca}^{2+}$  (ionic radius, electron shell configuration, ionic charge), it becomes clear that these "foreign metals" can dramatically affect  $\text{Ca}^{2+}$  channel function. In this review paper I will examine the effects which several nonphysiological and often toxic metals have on voltage-gated  $\text{Ca}^{2+}$  channel function. I will trace the evolution of our understanding of the actions of these agents on  $\text{Ca}^{2+}$  channel function. In doing so, I will attempt to point out areas in which (a) study of effects of toxic metals has broadened our understanding of the structure and/or function of  $\text{Ca}^{2+}$  channels; (b) the role which  $\text{Ca}^{2+}$  channels may play in the toxicity of certain environmentally relevant toxic metals; and (c) some prominent gaps within our understanding of the effects which these metals have on  $\text{Ca}^{2+}$  channel function. A number of fine reviews have examined portions of this issue in detail over the years, and readers are referred back to these for more information (Atchison, 1988; Atchison

<sup>1</sup> Neuroscience Program, Department of Pharmacology and Toxicology, Michigan State University, East Lansing, Michigan.

<sup>2</sup> Institute for Environmental Toxicology, Michigan State University, East Lansing, Michigan.

<sup>3</sup> To whom correspondence should be addressed at Michigan State University, B331 Life Sciences Building, East Lansing, Michigan 48824-1317; e-mail: atchiso1@msu.edu.

and Hare, 1994; Audesirk, 1985, 1993; Audesirk *et al.*, 2000; Büsselberg, 1995; Cooper *et al.*, 1984; Cooper and Manalis, 1983; Shafer, 2000; Sirois and Atchison, 1996).

As a prelude to this, I think it is first important to define one of the most commonly misused and abused words in this area—*toxin*. A *toxin* is a naturally occurring substance of plant or animal origin. Thus chemicals which block  $\text{Ca}^{2+}$  channel function such as  $\omega$ -conotoxin, calcicludine, and  $\omega$ -agatoxin are all correctly defined as *toxins*. The action of these agents on  $\text{Ca}^{2+}$  channel function is covered in other excellent papers within the series, and will not be considered specifically in this review. A *toxicant*, on the other hand, is a general term which describes any chemical that has a toxic action; thus all toxins are also toxicants. The focus of this review will be on several environmental toxic metals, lead (Pb) and mercury (Hg) which obviously are neither of plant or animal origin, as well as on several other interesting, and environmentally important agents. Thus, while these agents are all clearly neurotoxicants, they are *not* neurotoxins.

While the actions of metals ranging from aluminum to zinc have been studied on membrane ion channels, and  $\text{Ca}^{2+}$  channels in particular, these metals can be divided into three categories: (1) physiologically relevant metals, i.e., those found as normal cellular constituents; (2) non-physiological, but environmentally relevant metals, i.e., those for which exposure in the environment definitely causes clinical evidence of neurotoxicity; and (3) non-physiological, and nonenvironmentally relevant metals, i.e., those for which exposure in the environment is either particularly unlikely, or unlikely to cause neurotoxicity. In the first group are divalent cations such as  $\text{Ca}^{2+}$ ,  $\text{Mg}^{2+}$ ,  $\text{Zn}^{2+}$ ,  $\text{Cu}^{2+}$ , and  $\text{Fe}^{+2/+3}$ . These agents are clearly important in terms of effects on  $\text{Ca}^{2+}$  channel function, because they are encountered by the cell regularly. In some cases, such as for  $\text{Zn}^{2+}$ , they may affect  $\text{Ca}^{2+}$  channel function as a result of being released from a cell during normal metabolic activity, and can subsequently affect the cell adversely. The second group, environmentally relevant metals, include those generally ascribed as being toxic concerns in the environment, and those which clearly cause neurotoxicity. The most obvious metals in this group are  $\text{Pb}^{2+}$  and  $\text{Hg}^{2+}$  (particularly the organic forms) for which well-described neuropathology exists, as well as  $\text{Cd}^{2+}$  for which environmental exposure leads to toxicity, though not necessarily in the nervous system. Virtually all heavy metals inhibit voltage-gated  $\text{Ca}^{2+}$  channels to some extent. The question then becomes whether actions of these metals on  $\text{Ca}^{2+}$  channels contributes to the overall spectrum of clinical toxicity. As will become apparent later, this is likely, though not certainly, the case with these metals. The third group includes metals for which

normal exposure in the environment is rather unlikely, and if it occurs, there is little evidence for neurotoxicity. This includes metals such as  $\text{Ba}^{2+}$ ,  $\text{Sr}^{2+}$ ,  $\text{Co}^{2+}$ ,  $\text{Ni}^{2+}$ , as well as other metals. However, whereas these metals are unlikely to produce  $\text{Ca}^{2+}$  channel toxicity in a clinical setting, they have nonetheless been important agents in helping us understand the structure and/or function of voltage-gated  $\text{Ca}^{2+}$  channels. These metals will not be the focus in this review, but in certain instances, I will discuss them to illustrate specific points.

### EARLY FUNCTIONAL STUDIES DEMONSTRATE $\text{Ca}^{2+}$ CHANNEL BLOCKING ACTION OF METALS AND SUBSEQUENT PERMEATION INTO CELL

Early studies of effects of environmental toxicants on  $\text{Ca}^{2+}$  channels actually predated the conclusive demonstration of existence of voltage-gated  $\text{Ca}^{2+}$  channels in mammalian neurons, and certainly predated identification of subtypes of  $\text{Ca}^{2+}$  channels. These studies depended on indirect measures of neurotransmitter release in response to presynaptic nerve stimulation as a “bioassay” of  $\text{Ca}^{2+}$  channel function based on the well-known role which  $\text{Ca}^{2+}$  entry played in neurotransmitter release (Baker *et al.*, 1971; Katz and Miledi, 1969; Miledi, 1973). Mechanistic studies designed to examine early effects of toxic metals on  $\text{Ca}^{2+}$ -dependent processes focused on the well-described release of acetylcholine (ACh) from motor nerve endings at the neuromuscular junction in response to nerve stimulation. Numerous early studies initially focused on this preparation because of its well-characterized microscopic anatomy, physiology, and biochemistry. However, at least for methylmercury (MeHg), there was some additional rationale, inasmuch as an abnormally increased incidence of neuromuscular weakness resembling myasthenia gravis was reported in the Iraqi episode of MeHg poisoning in the early 1970s (Bakir *et al.*, 1980; Rustam *et al.*, 1975).

### LEAD

Because of the interest in  $\text{Pb}^{2+}$  as an environmental neurotoxicant, it was one of the first agents whose effects on synaptic function were examined, and it remains one of the most widely studied of the heavy metals. The neurotoxic effects of  $\text{Pb}^{2+}$  are not directed clinically at the neuromuscular junction. Thus this is an example of use of this synaptic circuit as a “bioassay” for  $\text{Ca}^{2+}$  channel function. It should be noted that these were some of

the earliest mechanistic studies demonstrating that divalent cations could alter membrane-oriented  $\text{Ca}^{2+}$  signaling. Moreover, observations made in this study that  $\text{Pb}^{2+}$  induces apparent intracellular actions in the nerve terminal were also an important stimulus to seek pathways by which  $\text{Pb}^{2+}$  entered the terminal, and led to the notion that certain types of  $\text{Ca}^{2+}$  channels serve as a permeation pathway for  $\text{Pb}^{2+}$  into the cell (see below). Manalis and Cooper (1973) first examined the effects of  $\text{Pb}^{2+}$  on neuromuscular transmission at frog neuromuscular junction. The effects of  $\text{Pb}^{2+}$  noted in that study are now recognized as being similar to those of other heavy metals, both those which are relevant environmental contaminants, and those which are not recognized environmental neurotoxicants.  $\text{Pb}^{2+}$  first decreased the amplitude of the nerve-evoked end plate potential (EPP) and then paradoxically increased the frequency of occurrence of spontaneously occurring miniature end plate potentials (MEPPs). Each of these effects occurred in a concentration-dependent manner and each was observed in the  $\mu\text{M}$  range of  $\text{Pb}^{2+}$ . Furthermore, both effects could be reversed by washing the  $\text{Pb}^{2+}$  out of the preparation using a  $\text{Pb}^{2+}$ -free bathing solution. As will be apparent, many of the results obtained using these early model systems were extremely prescient of those seen today with contemporary electrophysiological methods. Pronounced differences occurred in the time course of effects of  $\text{Pb}^{2+}$  on the EPP and MEPPs. This led to the conclusion that the effects were mediated by distinct actions. The increase in MEPP frequency was ascribed as being due to intracellular actions of the metal either to release  $\text{Ca}^{2+}$  from intracellular stores, or as is now known to be the case, a direct stimulatory action of  $\text{Pb}^{2+}$  itself on the release mechanism (Toth *et al.*, 1987). Iontophoretic application of ACh to the end plate in the presence of  $100 \mu\text{M}$   $\text{Pb}^{2+}$  elicited the normal depolarizing responses of amplitude similar to that of responses elicited in  $\text{Pb}^{2+}$ -free solutions, implying a lack of contribution of postjunctional effect of  $\text{Pb}^{2+}$  on the muscle nicotinic receptors. Thus the effects observed were limited to the presynaptic terminal. Subsequent studies revealed that  $\text{Pb}^{2+}$  had a similar spectrum of effects on the mammalian neuromuscular junction (Atchison and Narahashi, 1984; Pickett and Bornstein, 1984).

On the basis of these initial observations, subsequent studies were designed to characterize the nature of block of neurotransmission by  $\text{Pb}^{2+}$ . The  $\text{Ca}^{2+}$  dependence of the effect of  $\text{Pb}^{2+}$  on EPP amplitude was examined by varying the extracellular  $\text{Ca}^{2+}$  concentration in the absence and presence of  $\text{Pb}^{2+}$  (Cooper and Manalis 1983, 1984a,b; Manalis *et al.*, 1984). At  $1 \mu\text{M}$ ,  $\text{Pb}^{2+}$  caused a parallel shift to the right of the relationship between EPP amplitude and extracellular  $\text{Ca}^{2+}$  concentration. Thus

$\text{Pb}^{2+}$  acted as a competitive antagonist to  $\text{Ca}^{2+}$  in this process. Combining  $\text{Pb}^{2+}$  with  $\text{Cd}^{2+}$  was additive in blocking the EPP, but reduced the effectiveness of  $\text{Pb}^{2+}$  at increasing MEPP frequency (Cooper and Manalis, 1984a), suggesting that the two metals competed, possibly for entry into the cells. Similarly at the rat neuromuscular junction,  $\text{Pb}^{2+}$  shifted the sensitivity of the release process to  $\text{Ca}^{2+}$  to the right again implying a competitive relationship between  $\text{Ca}^{2+}$  entry and  $\text{Pb}^{2+}$  concentration (Atchison and Narahashi, 1984). However at mammalian neuromuscular junctions the concentrations of  $\text{Pb}^{2+}$  needed to block evoked release of ACh were considerably higher than at amphibian neuromuscular junctions. These observations led to the early conclusion that  $\text{Pb}^{2+}$ -induced block of the EPP resulted from a  $\text{Ca}^{2+}$  channel blocking action of  $\text{Pb}^{2+}$ .

### $\text{Hg}^{2+}$ AND METHYLMERCURY (MeHg)

There was considerable interest in the neurotoxicity of MeHg during the 1970s and 1980s because of several well-publicized and massive episodes of human exposure. During the massive episode of poisoning with MeHg in Iraq, a unique feature noted was the extremely high incidence of a "myasthenia gravis-like" type of neuromuscular weakness (Rustam *et al.*, 1975). Thus the early studies of actions of mercurials in general, and MeHg in particular on neuromuscular function had an obvious clinical rationale. As such, initial studies of effects of mercurials on synaptic transmission were directed primarily at determining what effects acute bath application of  $\text{Hg}^{2+}$  and MeHg had on neuromuscular function. Because MeHg is the most prevalent form of mercury encountered in the environment, and the most neurotoxic of the mercurials, more in-depth studies have been done using this compound. However, ultimately these studies were redirected to examine the effects of mercurials on voltage-gated  $\text{Ca}^{2+}$  channel function.

Intracellular microelectrode recordings at frog sartorius muscle (Cooper and Manalis, 1983; Juang, 1976; Manalis and Cooper, 1975) and rat (Atchison *et al.*, 1984; Atchison and Narahashi, 1982) and mouse diaphragm (Atchison *et al.*, 1984) revealed that the primary effect of both divalent inorganic mercury ( $\text{Hg}^{2+}$ ) and MeHg, like those of  $\text{Pb}^{2+}$ , was to decrease the amplitude of the nerve-evoked EPP and subsequently to increase and then decrease to complete block spontaneous release, measured as MEPP frequency. At the time at which the nerve-evoked EPP was blocked by MeHg, MEPPs of normal amplitude and duration still occurred, thus the effect represented a presynaptic effect rather than postsynaptic receptor block.

In every instance in which it was tested, block of nerve-evoked release of neurotransmitter by inorganic or organic mercury could not be reversed by washing the preparation with  $\text{Hg}^{2+}$ -free solutions (Atchison and Narahashi, 1982; Traxinger and Atchison, 1987). For both inorganic  $\text{Hg}^{2+}$  and MeHg the effects on nerve-evoked release of ACh were time-dependent, but not necessarily concentration-dependent. That is, higher concentrations reduced the time required to block transmitter release. For low concentrations of mercurials (0.1–10  $\mu\text{M}$ ) complete block of the EPP did not occur for 60 min or more (Juang, 1976; Manalis and Cooper, 1975); at higher concentrations of MeHg (2–100  $\mu\text{M}$ ) virtually complete block of nerve-evoked EPPs occurred after 5–30 min of exposure (Atchison *et al.*, 1986; Atchison and Narahashi, 1982; Traxinger and Atchison, 1987). The decrease in EPP amplitude was progressive with time, and proceeded to complete block. This effect did not attain a steady-state short of total block, and hence a strict concentration dependence does not occur. This has been another constant observation across numerous experimental paradigms with MeHg, as will be seen later in his review for studies of isolated  $\text{Ca}^{2+}$  currents in individual cells in culture (Hajela *et al.*, 2003; Peng *et al.*, 2002a; Sirois and Atchison, 2000). There may be a lower threshold concentration of MeHg below which block of the EPP, and hence of  $\text{Ca}^{2+}$  channel function does not occur (Atchison and Narahashi, 1982) but this remains unclear, because the latent period preceding its blocking action is concentration-dependent for MeHg. Thus it may simply be that for extremely low concentrations of MeHg that the latent period of inactivity is longer than the recording session permits.

A unique aspect of block of neurotransmission by mercurials was that both  $\text{Hg}^{2+}$  and MeHg caused a transient increase in EPP amplitude that preceded block of the EPP. This effect occurred after 10–20 min of exposure to the mercurial (Atchison and Narahashi, 1982; Binah *et al.*, 1978; Cooper and Manalis, 1983; Juang, 1976; Manalis and Cooper, 1975; Traxinger and Atchison, 1987) and lasted for between 10 and 35 min.

Unlike the situation for  $\text{Pb}^{2+}$ , block of the EPP by MeHg appears initially to be independent of the extracellular  $\text{Ca}^{2+}$  concentration, because increasing the bath  $\text{Ca}^{2+}$  concentration from 2 to 4 or 8 mM did not prolong the latent period preceding block of the EPP, nor decrease the degree of block caused by MeHg (Atchison *et al.*, 1986). Thus MeHg differs from  $\text{Pb}^{2+}$  and indeed most polyvalent cations for which block of  $\text{Ca}^{2+}$  channel function is reversed by increasing the  $[\text{Ca}^{2+}]_e$ . However MeHg also impairs sodium conductances (Shafer *et al.*, 2002; Shafer and Atchison, 1992; Shrivastav *et al.*, 1976; Traxinger and Atchison, 1987), and thus axonal impulse conduc-

tion, although this effect only occurs at high concentrations. However if membrane excitability is increased, raising the extracellular  $\text{Ca}^{2+}$  concentration will cause a partial reversal of effects of MeHg on EPP amplitude (Traxinger and Atchison, 1987). Thus there are apparently complex effects of MeHg on both neuronal  $\text{Na}^+$  and  $\text{Ca}^{2+}$  channels.

The functional block by MeHg of nerve terminal  $\text{Ca}^{2+}$  and  $\text{Na}^+$  channels has also been examined directly at intact neuromuscular junctions of the mouse *triangularis sterni* motor nerves (Shafer and Atchison, 1992). This was done by examining the effect of MeHg on potential changes arising from  $\text{Na}^+$  and  $\text{Ca}^{2+}$  channel function as measured from the perineurial sheath surrounding motor nerves (see Mallart, 1985; Mallart and Brigant, 1982). When potassium channels are blocked, two distinct components that are dependent on  $\text{Ca}^{2+}$  can be discerned (Penner and Dreyer, 1986; Xu and Atchison, 1996). These two  $\text{Ca}^{2+}$ -dependent voltage changes are presumed to reflect distinct subpopulations of  $\text{Ca}^{2+}$  channels, because they have differential pharmacological sensitivity (Xu and Atchison, 1996). At 100  $\mu\text{M}$ , MeHg rapidly blocked both  $\text{Ca}^{2+}$  components as well as the  $\text{Na}^+$  component of the perineurial currents, however at 50  $\mu\text{M}$ , both  $\text{Ca}^{2+}$ -dependent components were blocked by MeHg prior to block of the  $\text{Na}^+$ -dependent components. Once again, washing the preparation with MeHg-free solution did not reverse the block nor did increasing the intensity or duration of the stimulation of the intercostal nerve innervating that terminal.

#### **BLOCK OF $\text{Ca}^{2+}$ -DEPENDENT RELEASE OF ACh BY OTHER NONPHYSIOLOGICAL POLYVALENT METALS**

The ability of heavy metal ions to block evoked release of ACh by preventing  $\text{Ca}^{2+}$  entry has been examined extensively at both amphibian and rat neuromuscular junction preparations. In addition to  $\text{Pb}^{2+}$  and  $\text{Hg}^{2+}$ , a wide range of polyvalent cations can block ACh release at the neuromuscular junction. This effect has generally been assumed to be due to block of  $\text{Ca}^{2+}$  channel function. It has been described for  $\text{Cd}^{2+}$  (Cooper and Manalis, 1984a; Forshaw, 1977),  $\text{Co}^{2+}$  (Kita and Van Der Kloot, 1973; Weakly, 1973),  $\text{Er}^{3+}$  (Metral *et al.*, 1978),  $\text{Gd}^{3+}$  (Molgo *et al.*, 1991),  $\text{La}^{3+}$  (Kajimoto and Kirperkar, 1972),  $\text{Mg}^{2+}$  (del Castillo and Engbaek, 1954; Jenkinson, 1957),  $\text{Mn}^{2+}$  (Balnave and Gage, 1973),  $\text{Pr}^{3+}$  (Alnaes and Rahamimoff, 1975),  $\text{Tl}^{3+}$  (Wiegand *et al.*, 1984),  $\text{Ni}^{2+}$  (Kita and Van Der Kloot, 1973), triethyl- $\text{Sn}^{4+}$  (Allen *et al.*, 1980), and  $\text{Zn}^{2+}$  (Benoit and Mambrini, 1970). For only  $\text{Tl}^{3+}$  is block

Table I. Ability of Polyvalent Heavy Metals to Block Nerve-Evoked End Plate Potentials (EPPs) at Vertebrate Motor Nerve Terminals<sup>a</sup>

Metal	Species	Approximate minimal effective concentration ( $\mu\text{M}$ )	Comments	Reversibility	Refs.
Be <sup>2+</sup>	Frog	<100	Competitive w/ $\uparrow$ [Ca <sup>2+</sup> ]	Reversible	Bloch <i>et al.</i> (1968)
Mg <sup>2+</sup>	Frog	<1000	Competitive w/ $\uparrow$ [Ca <sup>2+</sup> ]	Reversible	Dodge and Rahamimoff (1967)
	Rat	<1000		Reversible	Hubbard <i>et al.</i> (1968a)
Cr <sup>3+</sup>	Frog	<10		Irreversible	Cooper <i>et al.</i> (1984a)
Mn <sup>2+</sup>	Frog/toad	<50	$K_D = 150 \mu\text{M}^b$	Reversible	Meiri and Rahamimoff (1972)
Co <sup>2+</sup>	Frog	<20	$K_D = 1.8 \mu\text{M}$	Reversible	Weakly (1973)
Ni <sup>2+</sup>	Frog	?	95% $\downarrow$ by 500 $\mu\text{M}$	Reversible	Benoit and Mambrini (1970)
Zn <sup>2+</sup>	Frog	?	56% $\downarrow$ by 100 $\mu\text{M}$	Reversible	Benoit and Mambrini (1970)
Y <sup>3+</sup>	Frog	<12.5	80% $\downarrow$	Partially reversible	Bowen (1971)
Cd <sup>2+</sup>	Frog	<1	$K_D = 1.7 \mu\text{M}$	Reversible	Cooper <i>et al.</i> (1984b)
	Rat	<10		Reversible	Forshaw (1977)
Sn <sup>4+</sup>	Mouse	<0.1		Irreversible	Allen <i>et al.</i> (1980)
La <sup>3+</sup>	Frog	<1	70% $\downarrow$ in "m" by 1 $\mu\text{M}^c$	Reversible	DeBassio <i>et al.</i> (1971)
Pr <sup>3+</sup>	Frog	15	EPP first $\uparrow$ then $\downarrow$	Not reported	Alnaes and Rahamimoff (1974)
Gd <sup>3+</sup>	Frog	5		Reversible, depending on time of exposure	Molgo <i>et al.</i> (1991)
Er <sup>3+</sup>	Frog		$\downarrow$ by $\sim$ 80% at 15 $\mu\text{M}$	Reversible	Miledi (1966)
Hg <sup>2+</sup>	Frog	<0.1	EPP first $\downarrow$ then $\uparrow$	Irreversible	Manalis and Cooper (1975)
	Frog	<10	No initial $\uparrow$ in EPP	Not reported	Juang (1976)
MeHg <sup>+</sup>	Frog	<10	EPP first $\uparrow$ then $\downarrow$	Not reported	Juang (1976)
	Rat/mouse	20	Occasionally an initial $\uparrow$ in EPP	Irreversible	Atchison <i>et al.</i> (1984), Atchison and Narahashi (1982)
Tl <sup>3+</sup>	Rat	5	4-AP caused partial reversal	Irreversible	Wiegand <i>et al.</i> (1984, 1986)
Pb <sup>2+</sup>	Frog		$K_D = 0.99 \mu\text{M}$	Reversible	Manalis and Cooper (1973)
	Rat	20	$\downarrow$ "m" Competitive w/ $\uparrow$ [Ca <sup>2+</sup> ]	Reversible	Atchison and Narahashi (1984)
	Rat	<5	$\downarrow$ "m"	Reversible	Pickett and Bornstein (1984)

<sup>a</sup> Adapted and updated from Cooper *et al.* (1984a).

<sup>b</sup>  $K_D$  = dissociation constant for the metal as compared to Ca<sup>2+</sup>.

<sup>c</sup> "m" = mean quantal content.

of EPP amplitude thought not to be due to Ca<sup>2+</sup> channel block (Wiegand *et al.*, 1984). Table I, modified from the work of Gary Cooper (Cooper *et al.*, 1984), lists relative potencies of various nonphysiological heavy metals for blocking Ca<sup>2+</sup>-dependent release of ACh at various vertebrate neuromuscular preparations. This table demonstrates that polyvalent metals vary widely in their effectiveness at blocking Ca<sup>2+</sup> channel function. However, examination of the table also suggests that the ability of a cation to reduce ACh release appears to be correlated approximately to its atomic mass and valence. Pb<sup>2+</sup> and Hg<sup>2+</sup>, which interestingly are also the only two of these metals with prominent environmental neurotoxicity, are by far the most potent of these cations at blocking ACh release. Conversely, La<sup>3+</sup> and Sn<sup>4+</sup>, which while having a lower atomic mass, and a higher valence (+3 and +4, respectively) are almost equal in potency to Hg<sup>2+</sup> and Pb<sup>2+</sup>. The extremely high potency of some of these metals such as Hg<sup>2+</sup>, Pb<sup>2+</sup>, and La<sup>3+</sup> suggests that they block Ca<sup>2+</sup> channels and hence ACh release by high-affinity interaction perhaps within the Ca<sup>2+</sup> channel pore.

### STIMULATION OF ASYNCHRONOUS, SPONTANEOUS RELEASE (MEPP FREQUENCY) AS AN INDEX OF INTRACELLULAR ACTION OF THE METAL

Because the frequency of occurrence of MEPPs is not highly dependent on the [Ca]<sub>e</sub>, but rather on the [Ca]<sub>i</sub>, the ability of these heavy metals to stimulate MEPP frequency reflects an intracellular effect. Thus it depends upon their mobility across the membrane through Ca<sup>2+</sup> channels or other entry pathways and their subsequent effectiveness at stimulating exocytosis. This latter process can occur either indirectly as by causing Ca<sup>2+</sup> release from internal stores which MeHg does (Levesque and Atchison, 1987) or directly by interacting with the exocytotic process as Pb<sup>2+</sup> apparently does (Shao and Suszkiw, 1991; Tomsig and Suszkiw, 1991). Shown in Table II is a listing of qualitative effects of the numerous multivalent inorganic cations which have been studied on release of ACh at the neuromuscular junction in comparison with their ionic radii and spontaneous free energies of hydration. As

**Table II.** Ability of Polyvalent Heavy Metals to Increase MEPP Frequency at Vertebrate Motor Nerve Terminals<sup>a</sup>

Ion	Ionic radius <sup>b</sup> (pm)	Hydration energy <sup>c</sup> (kcal/mole)	Effect on MEPP frequency	Refs.
Be <sup>2+</sup>	30	563	0, (-)	Blioch <i>et al.</i> (1968)
Cr <sup>3+</sup>	75.5	1105	0, (-)	Cooper <i>et al.</i> (1984)
Ni <sup>2+</sup>	69	507	0	Benoit and Mambrini (1970)
Zn <sup>2+</sup>	74	492	0	Benoit and Mambrini (1970)
Sn <sup>4+</sup>	69	1827	0	Allen <i>et al.</i> (1980)
Mg <sup>2+</sup>	71	464	0, (-)	Hubbard <i>et al.</i> (1968b)
Co <sup>2+</sup>	72	497	↑	Weakly (1973)
Mn <sup>2+</sup>	80	445	↑	Balnave and Gage (1973)
Hg <sup>2+</sup>	83	441	↑↑↑↑	Manalis and Cooper (1975)
Gd <sup>3+</sup>	107.8 <sup>d</sup>	na	↑↑↑↑	Molgo <i>et al.</i> (1991)
Cd <sup>2+</sup>	92	437	↑	Forshaw (1977)
La <sup>3+</sup>	117.2	793	↑↑↑↑	DeBassio <i>et al.</i> (1971)
Ca <sup>2+</sup>	114	382	↑	Hubbard <i>et al.</i> (1968b)
Pb <sup>2+</sup>	112	359	↑↑↑↑	Atchison and Narahashi (1984); Manalis and Cooper (1973)
Sr <sup>2+</sup>	132	350	↑	Mellow (1979)
Ba <sup>2+</sup>	149	316	↑	Silinsky (1977)
MeHg	na	na	↑↑↑↑	Atchison and Narahashi (1982)

<sup>a</sup>Adapted and updated from Cooper *et al.* (1984a).

<sup>b</sup>Goldschmidt radius, from Huheey (1983).

<sup>c</sup>From Basolo and Pearson (1968).

<sup>d</sup>M<sup>3+</sup> hydrated ionic radius.

was the case for blocking nerve-evoked release, the very potent Ca<sup>2+</sup> channel blockers Hg<sup>2+</sup>, Pb<sup>2+</sup>, and La<sup>3+</sup> are similarly the most effective at increasing MEPP frequency. Conversely those relatively weak Ca<sup>2+</sup> channel blockers Co<sup>2+</sup>, Mn<sup>2+</sup>, Mg<sup>2+</sup>, and Ni<sup>2+</sup> are poorly effective at increasing MEPP frequency. Finally other metals including Cd<sup>2+</sup>, Cr<sup>3+</sup>, and Sn<sup>4+</sup> have virtually no effect on MEPP frequency, despite being rather potent blockers of nerve-evoked release. Because of the presence of fixed negative charges residing on glutamate residue within the channel pore (see below) permeability of Ca<sup>2+</sup> over other alkaline earth metals is favored (Silinsky and Mellow, 1981). Thus cations with hydration energies significantly different than those of Ca<sup>2+</sup> should have altered mobility within the channel. As is apparent from Table II, hydration energies for both Hg<sup>2+</sup> and Pb<sup>2+</sup> are fairly close to those of Ca<sup>2+</sup>. Moreover these ions are extremely effective at increasing MEPP frequency. In contrast, Cr<sup>3+</sup> and Sn<sup>4+</sup> have hydration energies dramatically greater than that of Ca<sup>2+</sup>, and have virtually no effect on MEPP frequency despite blocking nerve-evoked release very potently. This may reflect the fact that these metals do not permeate readily through the pore, and hence do not gain significant access to the intracellular space. For other metals such as Mn<sup>2+</sup> and Cd<sup>2+</sup> this simple explanation apparently does not hold, as it is clear from a number of studies that Cd<sup>2+</sup> at least and perhaps Mn<sup>2+</sup> as well, both permeate the Ca<sup>2+</sup> channel pore. Their inability to increase MEPP frequency

may relate more to their lack of effect either to increase intracellular Ca<sup>2+</sup> concentration or to stimulate the release process directly.

The situation is somewhat more complex for organometals such as MeHg, triethyl-Pb<sup>2+</sup>, or trimethyl-Sn. For these metals the complicating variable of potentially bulky organic groups is counteracted somewhat by enhanced lipophilicity, perhaps providing additional entry paths into the cell (see Atchison, 1987; Hewett and Atchison, 1992a; Miyamoto, 1983).

## MEASUREMENT OF Ca<sup>2+</sup> CHANNEL FUNCTION IN SYNAPTOSOMES

As the field of Ca<sup>2+</sup> channel physiology progressed, it became clear that there were fundamental differences among channel subtype phenotypes, and that these differences were related in part to site-specific localization of different Ca<sup>2+</sup> channel subtypes, as well as distinct pharmacological and biophysical characteristics and might also be related to species-dependent differences. Because of the concern that the phenotype of Ca<sup>2+</sup> channels within the nerve terminal was not identical to that in the cell soma and because at the time there was little consensus regarding the number and identity of phenotypes of Ca<sup>2+</sup> channels, most of the subsequent mechanistic studies of effects of metals on Ca<sup>2+</sup> channel function

employed nerve terminal preparations. These were for the most part, not amenable to examination of their function by patch clamp methodology because of their small size. (Most mammalian nerve terminals have a diameter of approximately 1  $\mu\text{m}$  or less, making it impossible to record from them using conventional patch electrode methodology, but see Nelson, 1986.) Furthermore, because neurotoxicity associated with environmental agents such as Pb<sup>2+</sup> and Hg<sup>2+</sup> is primarily directed at the CNS, studies next attempted to focus on cellular actions of these metals on central nerve terminals as opposed to peripheral somatic terminals. As a consequence, alternate models needed to be used to examine function of non-physiological cations on Ca<sup>2+</sup> channel activity. Use of synaptosomes—suspensions which are made up of large numbers of pinched off nerve terminals—permitted a series of rather sophisticated biochemical studies of ion channel function. These spherical particles, which have a diameter of approximately 1–2  $\mu\text{m}$  contain typical intracellular organelles including most prominently synaptic vesicles as well as mitochondria and endoplasmic reticulum (Gray and Whittaker, 1962). Importantly, metabolic pathways and neurotransmitter synthetic pathways are retained intact within synaptosomes. Synaptosomes contain voltage-gated ion channels for sodium, potassium, and calcium (Bartschat and Blaustein, 1985; Bicalho *et al.*, 2002; Hewett and Atchison, 1992b; Weller *et al.*, 1985) and can release neurotransmitters in a Ca<sup>2+</sup>-dependent manner following depolarization (see Suszkiw and O'Leary, 1983; Suszkiw and Toth, 1986; Turner and Dunlap, 1995). They contain a number of different types of Ca<sup>2+</sup> channels including P-, N-, L-, and Q-type channels (Alvarez Maubecin *et al.*, 1995; Turner and Goldin, 1985; Zhang *et al.*, 1993). Moreover they have the advantage of being able to be used simultaneously for measurements of Ca<sup>2+</sup> channel function and neurotransmitter release, which permitted important cause-and-effect-type studies linking distinct Ca<sup>2+</sup> channel phenotypes with release of neurotransmitter (Lemos and Nowycky, 1989; Turner *et al.*, 1992, 1995; Turner and Dunlap, 1995). This preparation has been widely used for studies of nerve terminal ion channel function in the 1980s and still remains a valuable model today (see Holz and Turner, 1998; Leenders *et al.*, 2002; Polzin *et al.*, 2002; Tomizawa *et al.*, 2002).

Ca<sup>2+</sup> channel function in the synaptosomal model is examined most typically using uptake of radiolabeled Ca (<sup>45</sup>Ca<sup>2+</sup>) in response to chemical-induced depolarization, however less direct measures such as measures of fura-2 fluorescence or release of neurotransmitters in response to KCl-induced depolarization have also been used. The most common and most sensitive technique however involves uptake of <sup>45</sup>Ca<sup>2+</sup>, usually during KCl-induced depolariza-

tion. A major problem with these measurements has always been that the time course over which depolarization occurred was dramatically longer than that which occurred physiologically in the nerve terminal, where the length of depolarization was on the order of millisecond. A significant breakthrough in this area came about from work in Mordecai Blaustein's lab (Nachshen and Blaustein, 1982) in which reasonably short (0.5 to 2-s duration) depolarizations could be reliably applied and the uptake of <sup>45</sup>Ca<sup>2+</sup> resolved into distinct kinetic phases. This process has been refined even further by Tim Turner (Turner and Dunlap, 1995) through use of an innovative turntable arrangement to reduce the length of depolarization over which transmitter release occurs even more.

Depolarization-dependent Ca<sup>2+</sup> influx into synaptosomes occurs in two temporally distinct phases (Nachshen, 1985; Nachshen and Blaustein, 1980; Suszkiw and O'Leary, 1983). The fast phase inactivates after 1–2 s of depolarization and is associated with neurotransmitter release (Drapeau and Blaustein, 1983; Suszkiw and O'Leary, 1983). The slow phase, which remains active for 20–90 s after depolarization, may be mediated by the combination of actions of a reverse Na<sup>+</sup>–Ca<sup>2+</sup> exchanger and a slow, perhaps noninactivating Ca<sup>2+</sup> channel (see Suszkiw *et al.*, 1986; Turner and Goldin, 1985). The fast component is subject to inactivation, as it can be eliminated by predepolarization of the synaptosomes suspension in either the presence or absence of Ca<sup>2+</sup> (Nachshen and Blaustein, 1980). Both voltage-dependent and Ca<sup>2+</sup>-dependent inactivations are thought to occur in synaptosomes (Suszkiw *et al.*, 1986, 1989). Moreover permeability of Ca<sup>2+</sup> channels in synaptosomes mirrors that of other systems in that they are permeable to Ca<sup>2+</sup>, Sr<sup>2+</sup>, and Ba<sup>2+</sup> (Nachshen and Blaustein, 1982; Nelson, 1986).

Pharmacologically, the two components of <sup>45</sup>Ca<sup>2+</sup> have different characteristics. The fast component of <sup>45</sup>Ca<sup>2+</sup> influx in synaptosomes is sensitive to block by a myriad of multivalent cations. Nachshen (1985) found La<sup>3+</sup>, Pb<sup>2+</sup>, and Cd<sup>2+</sup> were among the most potent blockers of <sup>45</sup>Ca<sup>2+</sup> influx, with  $K_i$  values  $\leq 10 \mu\text{M}$ . Other metals such as Co<sup>2+</sup>, Ni<sup>2+</sup>, Hg<sup>2+</sup>, and Zn<sup>2+</sup> also blocked this fast component of <sup>45</sup>Ca<sup>2+</sup> influx; higher concentrations were necessary however, and  $K_i$  values ranged from 10 to 100  $\mu\text{M}$ . Nelson (1986) incorporated synaptosomal Ca<sup>2+</sup> channels into the lipid bilayers. Single channel recordings made from the synaptosomal Ca<sup>2+</sup> channels indicated that La<sup>3+</sup> and Cd<sup>2+</sup> as well as verapamil were potent blockers of channel function.

The synaptosomal system has been used extensively to examine the function of nerve terminal Ca<sup>2+</sup> channels in response to environmental metals such as Hg<sup>2+</sup>, Pb<sup>2+</sup>, and Cd<sup>2+</sup> in attempts to correlate actions on Ca<sup>2+</sup>

channels with the previously described block of  $\text{Ca}^{2+}$  dependent nerve-evoked release of ACh and later with other neurotransmitters as well. The early hypothesis that  $\text{Pb}^{2+}$  and Hg (and MeHg) blocked neurotransmitter release by virtue of their block of  $\text{Ca}^{2+}$  channel function was now directly testable, and considerably strengthened by the observations that these metals blocked uptake of  $^{45}\text{Ca}^{2+}$  into synaptosomes (Atchison *et al.*, 1986; Suszkiw *et al.*, 1984). For these studies extremely brief exposure periods (1 s) were used to examine channel function in isolation. This was done by combining the metal of interest with the KCl-depolarizing solution and the radiotracer, and precisely limiting the exposure of this "cocktail" to the synaptosomes using a metronome (Nachshen and Blaustein, 1980). This type of situation minimized the potential for interaction of the metal with other intracellular processes which could confound interpretation of channel-mediated effects.

Cooper's group continued the pioneering work on effects of  $\text{Pb}^{2+}$  on nerve terminal function, using the synaptosomal system (Suszkiw *et al.*, 1984). Simultaneous effects of  $\text{Pb}^{2+}$  on release of  $[^3\text{H}]\text{ACh}$  and  $^{45}\text{Ca}$  uptake were examined in rat forebrain synaptosomes.  $\text{Pb}^{2+}$  blocked the "fast phase" of  $^{45}\text{Ca}$  uptake with a  $K_i$  of  $\sim 1.1 \mu\text{M}$ . In comparison,  $\text{Cd}^{2+}$  had a  $K_i$  of  $\sim 2.2 \mu\text{M}$ . For both of these metals, these inhibitory concentrations were in very close agreement with their inhibitory concentrations at vertebrate neuromuscular junctions ( $K_{\text{Pb}} = 0.99 \mu\text{M}$ ;  $K_{\text{Cd}} = 1.7 \mu\text{M}$ ). Furthermore, for both of these metals, this action was competitive with  $[\text{Ca}^{2+}]_e$ .

Initial studies of effects of mercurials on  $\text{Ca}^{2+}$  channel function were similarly designed to follow up on the initial observations that MeHg depressed the  $\text{Ca}^{2+}$ -dependent release of neurotransmitters. The effects of MeHg and  $\text{Hg}^{2+}$  were compared on the two phases of  $^{45}\text{Ca}$  uptake into rat forebrain synaptosomes during KCl-induced depolarization.  $\text{Hg}^{2+}$  caused a concentration-dependent decrease in total uptake of  $^{45}\text{Ca}$  during KCl-induced depolarization. The maximal inhibition occurred at  $200 \mu\text{M Hg}^{2+}$ , which suppressed  $^{45}\text{Ca}$  uptake to approximately 5% of the mercury-free values. MeHg similarly suppressed total  $^{45}\text{Ca}$  uptake although the maximal inhibition produced by MeHg was less than that produced by  $\text{Hg}^{2+}$  (70% at  $200 \mu\text{M MeHg}$ ). Effects of MeHg occurred both on synaptosomes that were not previously depolarized to examine the "fast uptake" component as well as on the "slow uptake" component.  $^{45}\text{Ca}$  uptake during 1 s of incubation with MeHg was reduced significantly at concentrations greater than  $25 \mu\text{M}$ . MeHg was more potent as a blocker on the slow phase of uptake as compared to the fast phase. The effect of MeHg on  $^{45}\text{Ca}$  uptake was examined as a function of the  $[\text{Ca}^{2+}]_e$ ; increasing  $[\text{Ca}^{2+}]_e$

was only marginally effective at overcoming the block of  $^{45}\text{Ca}$  uptake produced by MeHg. Thus once again, the synaptosomal studies corroborated the earlier neuromuscular junction studies in terms of qualitative characteristics of blocking action, and correlation of concentrations that block nerve-evoked release of ACh at the neuromuscular junction and uptake of  $^{45}\text{Ca}$  into isolated nerve terminals.

Because the actions of MeHg on  $\text{Ca}^{2+}$  channel function did not seem to be as clearcut as were those of divalent inorganic cations such as  $\text{Pb}^{2+}$  and  $\text{Cd}^{2+}$ , the characteristics of actions of MeHg have been studied in considerable detail. Depolarization of nerve terminals caused by increasing the extracellular KCl concentration in the presence of a fixed concentration of MeHg caused a greater percentage of block of synaptosomal  $^{45}\text{Ca}^{2+}$  influx, suggesting the effect to be voltage-dependent. Thus, MeHg may somehow associate with the channel, perhaps in a manner similar to  $\text{Ca}^{2+}$ , as stronger depolarizations increased the percentage of block. Block was not dependent on whether the channel was in the closed, activated, or inactivated state at the time of MeHg exposure (Shafer *et al.*, 1990). These results suggest that MeHg may interact in a unique manner with  $\text{Ca}^{2+}$  channels as compared to other heavy metals, and possibly that the ability of MeHg to block  $\text{Ca}^{2+}$  uptake may be dependent on the strength of the interaction between MeHg and the channel. MeHg also alters the ionic selectivity of the synaptosomal  $\text{Ca}^{2+}$  channels by decreasing the influx of  $^{85}\text{Sr}$  to a greater extent than it did  $^{45}\text{Ca}^{2+}$  or  $^{133}\text{Ba}^{2+}$ .

MeHg also interacts with  $\text{Ca}^{2+}$  channels in synaptosomes isolated from rat cerebellum (Yan and Atchison, 1996). Pretreatment synaptosomes with the N-type peptide blocker  $\omega$ -conotoxin GVIA (1 or  $5 \mu\text{M}$ ) or the N-, P/Q-type blocker  $\omega$ -conotoxin MVIIC (0.14– $0.5 \mu\text{M}$ ), but not  $\omega$ -agatoxin IVA (10 or  $10 \text{ nM}$ ) or nifedipine ( $10 \mu\text{M}$ ), partially inhibited the ability of MeHg to inhibit KCl-depolarization-induced  $^{45}\text{Ca}^{2+}$  uptake. This suggested that in cerebellar nerve terminals, that MeHg interacts with channels sensitive to these toxins.

In contrast to the effects of MeHg in synaptosomes  $^{45}\text{Ca}^{2+}$  uptake block by  $\text{Hg}^{2+}$  occurs in a voltage-dependent and competitive manner (Atchison *et al.*, 1986; Eason and Aronstam, 1984; Hewett and Atchison, 1992a; Nachshen, 1984). While other inorganic metals such as  $\text{Co}^{2+}$ ,  $\text{Cd}^{2+}$ ,  $\text{Ni}^{2+}$ , and  $\text{Mn}^{2+}$  also block  $\text{Ca}^{2+}$  influx into synaptosomes competitively, they typically exhibit low or no voltage-dependence of their blocking action. Moreover these ions do not alter ion selectivity of the channel (Nachshen and Blaustein, 1982) which MeHg does (Shafer *et al.*, 1990). Electrically neutral organic mercurials such as *p*-chloromercuribenzoate (PCMB) and dimethyl mercury, or those which contain



a negative charge, such as PCMBs, failed to block KCl-depolarization-stimulated uptake of <sup>45</sup>Ca<sup>2+</sup> into synaptosomes, even at extremely high concentrations (Hewett and Atchison, 1992a). This suggests that lipophilicity alone cannot account for the unique blocking action of MeHg. However, the ability of MeHg to block Ca<sup>2+</sup> channels in the noncompetitive fashion, even in the absence of prior channel activation, and to alter the ionic selectivity, suggests that its inherent lipophilicity is an important determinant in its ability to block Ca<sup>2+</sup> channel function. Perhaps MeHg interacts with a portion of the channel not accessible to the inorganic divalent ions.

Because they contain multiple subtypes of Ca channels, synaptosomes have also been used for studies of binding of radiolabeled ligands to distinct Ca<sup>2+</sup> channel subtypes. Binding studies of competition among binding ligands specific for certain phenotypes of Ca<sup>2+</sup> channels have also been used to examine the effects of mercurials on voltage-gated Ca<sup>2+</sup> channels. MeHg decreases the specific binding <sup>3</sup>[H]-nitrendipine, a dihydropyridine L-type Ca<sup>2+</sup> channel antagonist in synaptosomes. This occurred at 100 μM MeHg, a concentration which also inhibited <sup>45</sup>Ca<sup>2+</sup> influx. MeHg also inhibited the binding of <sup>125</sup>I-ω-conotoxin GVIA in rat PC12 cells (Shafer *et al.*, 1990). Thus MeHg may interact with L-type and N-type Ca<sup>2+</sup> channels.

### VOLTAGE CLAMP STUDIES OF TOXIC METAL EFFECTS ON Ca<sup>2+</sup> CHANNELS IN NATIVE CELLS

A panoply of metals has been shown by whole-cell voltage clamp measurements of native currents to block the function of voltage-gated Ca<sup>2+</sup> channels. However in the concentration range of low μM–mM, specific blocking effects have been demonstrated for fewer cations including Cd<sup>2+</sup>, Co<sup>2+</sup>, Gd<sup>3+</sup>, Cr<sup>3+</sup>, Hg<sup>2+</sup>, La<sup>3+</sup>, Mg<sup>2+</sup>, Mn<sup>2+</sup>, Ni<sup>2+</sup>, Pb<sup>2+</sup>, and Zn<sup>2+</sup>. Once again, the data from these studies mirrors that of earlier functional studies at the neuromuscular junction and in synaptosomes, in that Pb<sup>2+</sup> and Hg<sup>2+</sup> are among the most potent of the heavy metal inhibitors of Ca<sup>2+</sup> channel function.

### EFFECTS OF Pb AND ITS ORGANIC DERIVATIVES ON NATIVE Ca<sup>2+</sup> CHANNELS

In view of the clear evidence that Pb<sup>2+</sup> blocks Ca<sup>2+</sup> entry and the extensive series of studies using synaptosomes channels, it was natural that eventually the effects of Pb<sup>2+</sup> on voltage-gated Ca<sup>2+</sup> channels would be exam-

ined directly using electrophysiological techniques in cells in culture. As reviewed by Audesirk (1993) the effects of Pb<sup>2+</sup> have been studied using electrophysiological methods in cells from a number of mammalian and gastropod species. In every case reported, current flow through the types of Ca<sup>2+</sup> channels present in that preparation was very sensitive to block by Pb<sup>2+</sup>. However, these studies have revealed some rather interesting aspects associated with effects of Pb<sup>2+</sup> on Ca<sup>2+</sup> channels, some of which were clearly unanticipated.

The effects of acute bath application of Pb<sup>2+</sup> have been studied in transformed (PC12, N1E-115, SH-SY5Y) as well as primary (rat DRG, hippocampal pyramidal neurons, chromaffin) cells in culture and in gastropod neurons. Table III modified from that of Audesirk (1993) summarizes the salient points of these studies—namely that Pb<sup>2+</sup> appears to block voltage-gated Ca<sup>2+</sup> channel currents in every preparation tested, that the ability of Pb<sup>2+</sup> to block current is related in part to the charge carrier concentration as well as the neuron, that in general, similar concentrations of Pb<sup>2+</sup> are needed to block current from multiple subtypes of high-voltage-activated Ca<sup>2+</sup> channels, and this effect is variably reversible. Rigorous comparison is difficult because the studies have been done using a bewildering range of charge carrier concentrations and distinct pulse protocols. Furthermore, comparison of potency of Pb<sup>2+</sup> for block of a given phenotype of Ca<sup>2+</sup> channel subtype is impaired by (1) the lack of pharmacological isolation of distinct Ca<sup>2+</sup> channel subtypes and (2) inconsistency in reporting of [Pb<sup>2+</sup>], i.e., “free Pb<sup>2+</sup>” as opposed to “added Pb<sup>2+</sup>” (see Audesirk, 1993).

The concentration range over which Pb<sup>2+</sup>-induced block of channel function occurs is generally quite low. That is, Pb<sup>2+</sup> is quite potent as a blocker of Ca<sup>2+</sup> channel current. This conclusion was hardly surprising given the earlier results of both neuromuscular junction and synaptosome experiments. In general, effective block is produced by [Pb<sup>2+</sup>] in the low μM range. Values range from an IC<sub>50</sub> of 300 nM free Pb<sup>2+</sup> in bovine adrenal chromaffin cells (Sun and Suszkiw, 1995), ~1 μM in human SH-SY5Y neuroblastoma cells (Reuveny and Narahashi, 1991), 4.8 ± 0.8 μM in N1E-115 cells (Vijverberg *et al.*, 1994), and 10–50 μM in differentiated PC12 cells (Hegg and Miletic, 1996) (see Table III). In crayfish muscle fibers, blocking concentrations range from 50 to 300 μM (Zacharova *et al.*, 1993). Several factors appear to contribute to the varied range of concentrations over which block was observed. First, interestingly, Audesirk and Audesirk (1993) reported that transformed cell lines appeared to be somewhat more resistant to Pb<sup>2+</sup> than were primary cultures of neurons. The reason underlying this observation is unknown. Second, in some cases, efforts were

Table III. Comparison of Inhibition of Voltage-Gated Ca Channels by Pb in Various Cell Types<sup>a</sup>

Cell type	Exposure	[Ca/Ba]	Channel type(s) tested & effect	Reversibility	Refs.
<i>Lymnaea</i> B cells	Acute	2 mM Ba	Unknown, 38% inhib at 5 nM free Pb	Irreversible	Audesirk and Audesirk (1989)
<i>Lymnaea</i> RPeD1 cells	Acute	2 mM Ba	Unknown, 31% inhib at 30 nM free Pb	Irreversible	Audesirk and Audesirk (1989)
<i>Aplysia</i> -various cells	Acute	10–40 mM Ca	Unknown, ~175 <sup>b</sup> nM was inhib at 10 mM Ca; 200 nM <sup>b</sup> at 40 mM Ca	Reversible	Büsselberg <i>et al.</i> (1991b)
Rat, DRG	Acute	10 mM Ba	“L-” <sup>c</sup> , “N-type” – 200 nM <sup>b</sup> ; “T-type” – 500 nM <sup>b</sup>	Partially reversible	Evans <i>et al.</i> (1991)
Human SH-SY5Y neuroblastoma	Acute	100 mM Ba	“L-,” “N-type” – 200 nM <sup>b</sup>	Reversible	Reuveny and Narahashi (1991)
Mouse N1E-115 neuroblastoma	Acute	50 mM Ba/ 2 mM Ba	“L-type” – 200 nM <sup>b</sup> / IC <sub>50</sub> = 5 μM	Reversible	Oortgiesen <i>et al.</i> (1990), Vijverberg <i>et al.</i> (1994)
Mouse N1E-115 neuroblastoma	Acute	50 mM Ba	“L-type” – 700 nM, “T-type” – 1300 nM <sup>b</sup>	Reversible	Audesirk and Audesirk (1991)
Rat hippocampal neurons	Acute	10 mM Ba	“L-type” – 30nM, “N-type – 80 nM	Reversible	Audesirk and Audesirk (1993)
Bovine adrenal chromaffin cells	Acute	5 mM Ca	IC <sub>50</sub> = 300 nM	Reversible	Sun and Suszkiw (1995)
Rat PC12 cells	Acute	10 mM Ca	27% inhib at 1 μM; 38% inhib at 10 μM; 46% inhib at 50 μM	Irreversible	Hegg and Miletic (1996)
Rat PC12 cells	Chronic	10 mM Ca	60-day tx w/ 25, 50 μM ↑ peak & sustained current	na	Hegg and Miletic (1997)
Rat PC12 cells	Acute	5, 10, 20 mM Ca	IC <sub>50</sub> = 1.2 μM; (Ca5), 2.0 μM; (Ca10), 7.5 μM; (Ca20)	Partially reversible, full reversal required chelation	Shafer (1998)
Rabbit recombinant cardiac channels in HEK cells & <i>Xenopus</i> oocytes	Acute	10 mM Ba	IC <sub>50</sub> = 169 nM in HEK cells & IC <sub>50</sub> = 152 nM in <i>Xenopus</i> oocyte	Partially reversible, full reversal required chelation	Bernal <i>et al.</i> (1997)
Human recombinant channels in HEK 293 cells	Acute	20 mM Ba	α <sub>1E</sub> (IC <sub>50</sub> = 0.10 μM), α <sub>1C</sub> (IC <sub>50</sub> = 0.38 μM) α <sub>1B</sub> (IC <sub>50</sub> = 1.31 μM)	α <sub>1E</sub> Partially reversible α <sub>1C</sub> , α <sub>1B</sub> fully reversible	Peng <i>et al.</i> (2002b)

<sup>a</sup>Modified and updated from Audesirk (1993).

<sup>b</sup>Indicates approximately [free Pb] in unbuffered saline similar to that in original paper as measured in Audesirk’s laboratory (see Audesirk, 1993, Table 2).

<sup>c</sup>“ ” indicates that the exact phenotype was not confirmed by pharmacological or molecular biological means.

made to report “[free Pb<sup>2+</sup>]” following either measurement of the actual Pb<sup>2+</sup> concentration in the medium using a Pb-electrode, or rigorous buffering of the Pb<sup>2+</sup> in the medium to attain a specific “[free Pb<sup>2+</sup>],” while for the majority of studies values were simply reported using the applied [Pb<sup>2+</sup>]. Third, the concentration of charge carrier varied widely. Whole-cell recording studies also appeared to confirm, at least indirectly, the competitive relationship between Ca<sup>2+</sup> and Pb<sup>2+</sup>. This is seen as the increased concentrations of Pb<sup>2+</sup> necessary to block Ca<sup>2+</sup> channel function, as the charge carrier (Ca<sup>2+</sup>/Ba<sup>2+</sup>) concentration is increased (Shafer, 1998). Because of this characteristic and the fact that normally, supraphysiological concentrations of charge carrier are used to facilitate recordings of current, the true IC<sub>50</sub> for Pb<sup>2+</sup> may well be markedly overestimated.

The relative sensitivity of various phenotypes of Ca<sup>2+</sup> channels to Pb<sup>2+</sup>-induced block is an important issue that

has been tested only cursorily in studies of native channels. It has typically been addressed, by varying pulse protocols in attempts to isolate “high-voltage-activated” or “low-voltage-activated” current components. Studies in which pharmacological antagonists were applied to block distinct components of current (see, e.g., Mintz and Bean, 1993) have typically not been done. Thus for cells in which multiple components of “high-voltage-activated” current exist (L-, N-, P/Q-types), comparisons of actions of Pb<sup>2+</sup> on distinct channel subtypes are at best approximate. For example in SH-SY5Y cells which appear to contain N- and L-type Ca<sup>2+</sup> current components, block of the two types of current by Pb<sup>2+</sup> was equivalent (Reuveny and Narahashi, 1991). Similarly, Vijverberg *et al.* (1994) reported that in mouse N1E-115 neuroblastoma cells, which contain a fast transient as well as a noninactivating current, sensitivity of the two components to Pb<sup>2+</sup> was also approximately similar, and Hegg and Miletic (1996) reported similar potency

of block by Pb<sup>2+</sup> on peak and sustained current amplitude in PC12 cells. However, using primary hippocampal neurons, Audesirk and Audesirk (1993) reported that putative L-type current was more sensitive to Pb<sup>2+</sup> than was N-type, while in N1E-115 cells L-type current was more sensitive than was T-type current, and Ujihara *et al.* (1995) reported that P-type Ca<sup>2+</sup> channels in hippocampal pyramidal cells were most sensitive to Pb<sup>2+</sup>-induced block. Thus conclusions regarding the relative sensitivity of different subtypes of Ca<sup>2+</sup> channels based on studies of mixed components of current in native cells are inconclusive at this point. This issue is only now being addressed specifically in studies utilizing recombinant expressed channels (see below), for which more conclusive results can be attained.

[Ca<sup>2+</sup>]<sub>e</sub>-dependence of potency of Pb<sup>2+</sup> (and Cd<sup>2+</sup>) was examined by Shafer (1998). As one might expect on the basis of the prior studies at vertebrate neuromuscular junctions and in mammalian brain synaptosomes, the blocking potency of Pb<sup>2+</sup> and Cd<sup>2+</sup> was competitively related to the [Ca<sup>2+</sup>]<sub>e</sub>. As detailed below, this undoubtedly reflects competition between Ca<sup>2+</sup> and the nonphysiological cation for entry into the channel pore.

Effects of Pb<sup>2+</sup> on kinetic properties of Ca<sup>2+</sup> channel currents is another area in which studies are lacking. Kinetic properties are generally best examined in microscopic studies of single channel currents. This has not yet been done for exposure of cells Pb<sup>2+</sup>; however, at least on the basis of whole-cell recordings, it is not obvious that Pb<sup>2+</sup> dramatically or consistently affects the voltage dependence of activation or inactivation of Ca<sup>2+</sup> channels or the kinetics of opening and closing. Büsselberg *et al.* (1991b) reported that Pb<sup>2+</sup> caused a slight shift in the voltage dependence of activation of *Aplysia* neuron Ca<sup>2+</sup> channels, resulting in the need for larger voltage steps to induce maximal current amplitude. Audesirk and Audesirk (1991) found no effect of Pb<sup>2+</sup> on voltage dependence of activation or inactivation of low-voltage-activated, T-type Ca<sup>2+</sup> channels for mouse N1E-115 neuroblastoma cells, but found that the steady-state inactivation of the high-voltage-activated, L-type channels in the same cells was enhanced slightly by Pb<sup>2+</sup>. Hegg and Miletic (1996) reported some voltage dependence of effects of Pb<sup>2+</sup> to increase Ca<sup>2+</sup> channel currents in rat PC12 cells (see below). However, in other instances, effects of Pb<sup>2+</sup> on activation and inactivation were unremarkable (Audesirk and Audesirk, 1993). But, as noted above, there are to date no studies reported of effects of Pb<sup>2+</sup> on microscopic current, hence the effects of Pb<sup>2+</sup> on kinetics of channel opening and closing have not yet been examined rigorously.

Among the numerous studies of macroscopic currents in response to Pb<sup>2+</sup> exposure, some rather surprising results have been obtained. These have generally resulted from studies in which Pb<sup>2+</sup> was given either chronically or applied acutely in the patch pipet to produce intracellular effects. However, in either case, the locus was probably intracellular. In the first report of an action of Pb<sup>2+</sup> to increase Ca<sup>2+</sup> channel current amplitude, Audesirk (1987) treated the pond snail *Lymnaea* with 5 μM Pb<sup>2+</sup> in the artificial "pond" bathing solution for 6–12 weeks. In contrast to the inhibitory effects on Ba<sup>2+</sup> currents which he saw following acute in vitro exposure, Ba<sup>2+</sup> currents in B cells of animals exposed in vivo to Pb<sup>2+</sup> were almost 2× as large those recorded from B cells from unexposed controls. (Both sets of recordings were made in Pb<sup>2+</sup>-free physiological saline.) In more recent studies in bovine adrenal chromaffin cells (Sun and Suszkiw, 1995), extracellular application of Pb<sup>2+</sup> caused a concentration-dependent and reversible inhibition of Ca<sup>2+</sup> currents. The estimated IC<sub>50</sub> for this effect was within the range of concentrations ascribed to block high-voltage-activated current components—300 nM free Pb<sup>2+</sup>. In the absence of Pb<sup>2+</sup>, increasing the intracellular-free Ca<sup>2+</sup> concentration above 10 nM caused the initial amplitude of the Ca<sup>2+</sup> current to be reduced, and the rate of current rundown to increase. Under these conditions when Pb<sup>2+</sup> was applied *intracellularly*, it prevented Ca<sup>2+</sup>-dependent reduction of current amplitude. Moreover kinetics of current rundown changed from exponential to linear. The estimated EC<sub>50</sub> for this effect of Pb<sup>2+</sup> was approximately 0.2 nM. Sun and Suszkiw (1995) interpret this result as a Pb<sup>2+</sup>-induced inhibition of Ca<sup>2+</sup>-dependent inactivation, resulting in an apparent increase in the amplitude of Ca<sup>2+</sup> currents. Thus once Pb<sup>2+</sup> enters the cell, it appears to interact with those regions of the channel responsible for Ca<sup>2+</sup>-dependent inactivation to prevent this effect. This effect occurs despite Pb<sup>2+</sup> also increasing intracellular free [Ca<sup>2+</sup>] (Schanne *et al.*, 1989a,b). However, Shafer (1998) did not see a similar effect in nerve-growth-factor- (NGF) differentiated PC12 cells when 5 μM free Pb<sup>2+</sup> was applied intracellularly and 10 mM Ba<sup>2+</sup> was used as a charge carrier. Under these conditions, intracellular Pb<sup>2+</sup> decreased current amplitude.

Increase in current amplitude was seen in a small percentage of NGF-differentiated PC12 cells following acute extracellular exposure to Pb<sup>2+</sup> at 1, 10, and 50 μM (Hegg and Miletic, 1996), while in most cells, these concentrations of Pb caused a concentration-dependent inhibition of current which was irreversible by wash. The increase in amplitude of Ca<sup>2+</sup> currents was irreversible, was seen in a small percentage of cells at each concentration of Pb<sup>2+</sup>, and was voltage-dependent in that the amplitude decreased

progressively with depolarization from  $-20$  to  $+40$  mV. The current-voltage relationships for these increased amplitude currents in response to  $Pb^{2+}$  were normal. In a subsequent study, Hegg and Miletic (1997) exposed PC12 cultures to  $Pb^{2+}$  (10, 25, and  $50 \mu M$ —"applied"  $Pb^{2+}$ ) chronically. Concentration- and time-dependent increases in current amplitude were seen following  $Pb^{2+}$  exposure. While no effect was seen at any concentration at 1 month of exposure, after 2 months, cultures exposed to 25 or  $50 \mu M Pb^{2+}$  exhibited increased amplitudes of both peak and sustained components of current. Cultures exposed to  $10 \mu M Pb^{2+}$  showed little effect. After 2 months of exposure to the higher concentrations of  $Pb^{2+}$ , there was a shift in the current-voltage relationship such that maximal current amplitude was attained at 0 mV instead of  $+10$  mV. During a 3rd month of exposure to  $Pb^{2+}$ , increased current amplitudes were maintained at the two higher concentrations of  $Pb^{2+}$ .

From the standpoint of  $Pb^{2+}$  neurotoxicity, an interesting question is whether the actions of  $Pb^{2+}$  on voltage-gated  $Ca^{2+}$  channels contribute to the clinical signs seen upon chronic exposure to  $Pb^{2+}$  in vivo. The free concentrations of  $Pb^{2+}$  in the blood, and, by extension, presumably in the interstitial fluids of patients during  $Pb^{2+}$  toxicity, are typically considered to be below  $10^{-8}$  M (Goldstein, 1992; Simons, 1993). Thus it is unclear whether a significant component of  $Ca^{2+}$  channel block by extracellular  $Pb^{2+}$  actually occurs in vivo. However accumulation of  $Pb^{2+}$  during chronic exposure could result in intracellular concentrations of  $Pb^{2+}$  which are sufficiently high to modify  $Ca^{2+}$  channel activity due to intracellular actions. Because  $Ca^{2+}$ -dependent inactivation of  $Ca^{2+}$  channels is thought to serve as a feedback mechanism to prevent  $Ca^{2+}$  overload within the cell, interference with this mechanism by lead could result in sustained elevations of intracellular  $Ca^{2+}$  an effect which is well-known to be cytotoxic, and long implicated in the role of  $Pb^{2+}$ -induced neurotoxicity.

In the only study found in which effects on  $Ca^{2+}$  channel function were examined following *chronic in vivo* exposure to  $Pb^{2+}$ , rats were exposed to a high concentration (500 ppm) of  $Pb^{2+}$  in the drinking water for 64–85 days. This exposure regimen produced blood- $Pb^{2+}$  levels as determined by atomic absorption spectrophotometry, of  $22.7 \mu g/dL$  of blood (Grover *et al.*, 1997).  $Ba^{2+}$  (2 mM) currents were recorded from acutely dissociated medial septal/nucleus diagonal band neurons. No effect was seen on amplitudes of either peak or sustained currents following  $Pb^{2+}$  exposure. However, there was reduced current rundown induced by stimulation at 1/6 Hz, but not at 1/20 Hz in the  $Pb^{2+}$ -exposed group as compared to a control group treated with Na-acetate in the drinking water. This reduced rundown could be due to impeded

$Ca^{2+}$ -dependent inactivation. Interestingly, in these cells treated chronically with  $Pb^{2+}$ , a subsequent acute  $Pb^{2+}$  challenge still produced current inhibition.

There are two reports of effects of organo-lead compounds on voltage-gated  $Ca^{2+}$  channel function. Gawrisch *et al.* (1997) examined the effects of trimethyl- $Pb^{2+}$  on rat DRG neurons using whole-cell recording techniques. Trimethyl- $Pb^{2+}$  was similarly effective as inorganic  $Pb^{2+}$  at blocking  $Ca^{2+}$  channel current; the range of effective inhibitory concentrations was 1–5  $\mu M$  for ~50% inhibition. No voltage dependence was seen, although the inhibitory effect was reportedly dependent upon the channel being in the open state. Büsselberg *et al.* (1991a) used two-microelectrode voltage clamp on *Aplysia* neurons to study the effects of triethyl- $Pb^{2+}$ . Perfusion with triethyl- $Pb^{2+}$  (5–50  $\mu M$ ) caused a delayed reduction of current which was evident after ~2 min of exposure, but then continued even during wash with triethyl- $Pb^{2+}$ -free solution. Thus, organo- $Pb^{2+}$  compounds produced inhibitory effects on  $Ca^{2+}$  channel function which were not remarkably different from those of  $Pb^{2+}$ .

#### EFFECTS OF $Hg^{2+}$ AND MeHg ON NATIVE $Ca^{2+}$ CHANNELS

Perhaps because of the unique nature of its chemical structure (monovalent and methylated) there have been a number of studies specifically of the effects of MeHg on whole-cell currents carried through  $Ca^{2+}$  channels. Similarly, because of chemical differences between MeHg and  $Hg^{2+}$ , a number of studies have sought to compare the  $Ca^{2+}$  channel blocking action of the organomercurial with the inorganic divalent form. Results from a number of these studies are summarized in Table IV. Salient features of these studies include the fact that current inhibition by MeHg, and generally  $Hg^{2+}$ , is not reversible. Moreover, for MeHg, current inhibition is progressive, although as described below, the rate of block is concentration-dependent. Furthermore, in general, MeHg is quite potent as a blocker of voltage-gated  $Ca^{2+}$  channel function; typically block occurs in the range of low  $\mu M$  concentrations, irrespective of the divalent cation charge carrier concentration. Moreover, when cells are exposed chronically to MeHg, extremely low concentrations (10–30 nM) appear to be able to impair channel function (Shafer *et al.*, 2002).

The first study of effects of MeHg on  $Ca^{2+}$  channel currents was meant to follow up on observations made using synaptosomal  $^{45}Ca$  flux measurements and radioligand binding studies in both synaptosomes and PC12 cells (Shafer *et al.*, 1990; Shafer and Atchison, 1989). MeHg

**Table IV.** Inhibitory Effects of Methyl mercury (MeHg) and Hg<sup>2+</sup> on Ca<sup>2+</sup> Channel Currents

Cell type	Hg species	[Ba/Ca]	Channel types examined	Blocking conc. of Hf <sup>2+</sup> / MeHg	Reversibility	Refs.
Rat PC12 cell <i>Aplysia</i>	MeHg Hg <sup>2+</sup>	10/20 mM Ba 10 mM Ca	"N"/"L" <sup>a</sup> "inactivating," "noninactivating"	0.25–5 μM 20 μM	Irreversible Irreversible	Shafer and Atchison (1991) Büsselberg <i>et al.</i> (1991a)
Rat PC12 cell Rat DRG cells	Hg <sup>2+</sup> Hg <sup>2+</sup>	10 mM Ca 10 mM Ba	L-type "N"/"L," "T"	0.3 μM Hg increased I <sub>Ca</sub> N/L channel IC <sub>50</sub> = 1.1 μM; T channels blocked in range of 0.5–2 μM;	na Irreversible	Rossi <i>et al.</i> (1993) Pekel <i>et al.</i> (1993)
Rat DRG cells	MeHg	10 mM Ba	HVA (presumed L-type), LVA (presumed T-type)	IC <sub>50</sub> = 2.6 μM	Irreversible	Leonhardt <i>et al.</i> (1996a,b)
Rat hippocampal pyramidal cell Rat PC12 cell	Hg <sup>2+</sup> /MeHg MeHg	10 mM Ca 10 mM Ca	HVA (presumed L-type), LVA (presumed T-type) "N"/"L"	At 1 μM, T current transiently increased 5, 10 μM cause complete block, 1, 2.5 μM incomplete block	Irreversible Irreversible	Szucs <i>et al.</i> (1997) Shafer (1998)
Rat cerebellar granule cell Rat PC12 cell HEK 293 cell	MeHg MeHg Hg <sup>2+</sup> /MeHg	5 mM Ba 10 mM Ca 10 mM Ba	N/L/P-Q specific blockers used "N"/"L," RecombinantN/R(α <sub>1B</sub> , α <sub>1E</sub> )	0.25, 0.5 μM incomplete block, 1 μM complete 30 nM for 24 h, 29% reduction Hg <sup>2+</sup> IC <sub>50</sub> = 2.2 μM = N 0.7 μM = R, MeHg IC <sub>50</sub> = 1.3 μM = N 1.1 μM = R Incomplete block at 5 μM	Irreversible na Irreversible for MeHg, partially reversible Hg <sup>2+</sup> Irreversible	Sirois and Atchison (2000) Shafer <i>et al.</i> (2002) Hajela <i>et al.</i> (2003) Peng <i>et al.</i> (2002a)
HEK 293 cell	MeHg	10 mM Ba	RecombinantL (α <sub>1C</sub> )	Incomplete block at 5 μM	Irreversible	Peng <i>et al.</i> (2002a)

<sup>a</sup> indicates that the exact phenotype was not confirmed by pharmacological or molecular biological means.

(1–20  $\mu\text{M}$ ) rapidly and completely blocked whole-cell currents carried by  $\text{Ba}^{2+}$  (20 mM) through both N- and L-type  $\text{Ca}^{2+}$  channels in NGF-differentiated PC12 cells (Shafer and Atchison, 1991). These effects occurred in the absence of nonspecific effects on leak or capacitive currents. The rate of onset of current block was concentration-dependent. At low concentrations of MeHg (1, 2, or 5  $\mu\text{M}$ ), block progressed with time, but did not either reach a steady-state, or achieve maximum level during 5 min of exposure. At 5 and 10  $\mu\text{M}$  MeHg, peak and end currents were blocked by approximately equivalent amounts—32 and 26% for peak and end currents respectively, at 5  $\mu\text{M}$  MeHg, and 77 and 70% at 10  $\mu\text{M}$  MeHg. Thus, for these cells, there was no obvious difference in sensitivity of inactivating or noninactivating current components. Following exposure to MeHg for 2 min, a similar degree of inhibitory action occurred both when the channel was activated repeatedly and following a period of channel inactivity, indicating that MeHg had access to the channel by means other than entry to the open pore. As was noted in previous studies with synaptosomes, block by MeHg of  $\text{Ca}^{2+}$  channel function did not depend upon the state of the channel; blocking potency was approximately the same whether the channel was exposed to MeHg during activity, or at rest. Over the range of stimulation frequencies of 0.1, 0.2, and 0.4 Hz, increasing the rate of stimulation enhanced the rate of block of  $\text{Ba}^{2+}$  current by MeHg. Furthermore washing the preparation with MeHg-free physiological saline did not reverse its effect. In this respect the action to MeHg differed from those of metals such as  $\text{Cd}^{2+}$  and in some cases  $\text{Pb}^{2+}$ , for which blocking effect is generally reversed rather readily by washing with metal-free solution (Shafer, 1998). At 10  $\mu\text{M}$ , MeHg altered the apparent ion selectivity of PC12 cell  $\text{Ca}^{2+}$  channel currents. Under these conditions the order of potency was  $\text{Ca}^{2+} > \text{Sr}^{2+} > \text{Ba}^{2+}$  (Shafer and Atchison 1991).

Since this original report, there have been several other studies of effects of MeHg in several types of primary cultures including rat DRG (Pekel *et al.*, 1993), hippocampal pyramidal cells (Szűcs *et al.*, 1997), and most recently in cerebellar granule cells—a primary target of MeHg toxicity in vivo (Sirois and Atchison, 2000), as well as further studies in PC12 cells (Shafer, 1998; Shafer *et al.*, 2002). In primary cultures of hippocampal pyramidal and cerebellar granule cells (Sirois and Atchison, 2000), very low concentrations (0.25, 0.5  $\mu\text{M}$ ) of MeHg block  $\text{Ba}^{2+}$  current. Once again, there is clearly a correlation of needing higher MeHg concentrations to block function at higher charge carrier concentrations, but nevertheless, primary cultures do appear to be more sensitive to MeHg than are transformed cells. For example, at 1  $\mu\text{M}$  MeHg and with 5 mM Ba, current was blocked completely by MeHg in

granule cells whereas in PC12 cells with 10 mM Ca, 5–10  $\mu\text{M}$  MeHg was needed to cause complete block of current. Block of  $I_{\text{Ca}}$  (5 mM) occurred rapidly with 5  $\mu\text{M}$  MeHg (Shafer, 1998). Within 3–4 min of application, block of  $I_{\text{Ca}}$  at 1 and 2.5  $\mu\text{M}$  MeHg was incomplete. In comparison, in granule cells, using 5 mM  $\text{Ba}^{2+}$  as charge carrier, 0.25  $\mu\text{M}$  MeHg for  $\sim 3$  min caused about a 15% reduction in current amplitude, which continued unabated after MeHg was washed out of the bath solution (Sirois and Atchison, 2000).

As shown below, there are a number of issues for which no clear consensus has been attained in studies with MeHg. However, there are several issues on which all studies seem to agree. First, block of  $\text{Ca}^{2+}$  channel function by MeHg is irreversible by washing with metal-free solution. In fact, Sirois and Atchison (2000) reported that block continued even during the “washout” phase. Furthermore, in PC12 cells (Shafer, 1998) block of current by MeHg could not even be reversed using the chelator *d*-penicillamine, and current block was only partially reversible ( $\sim 2/3$  reversible) by the chelator DMPS. Second, block of  $\text{Ca}^{2+}$  channel current is generally progressive with MeHg, proceeding evidently to complete block, if the recording can be held long enough. This response is similar to what was originally observed at the mammalian neuromuscular junction (Atchison and Narahashi, 1982).

As was the case for  $\text{Pb}^{2+}$ , subtype-specific actions of MeHg (and  $\text{Hg}^{2+}$ ) have not been extensively characterized in native currents. Szűcs *et al.* (1997) reported that high-voltage-activated currents were less sensitive to MeHg (block at 5 and 10  $\mu\text{M}$ ) while low-voltage-activated currents were markedly reduced by 2  $\mu\text{M}$  MeHg. Leonhardt *et al.* (1996a,b) examined the effects of MeHg on DRG cells and reported an  $\text{IC}_{50}$  of  $\sim 2.6$   $\mu\text{M}$  in the presence of 10 mM  $\text{Ba}^{2+}$  for both high-voltage- and low-voltage activated-currents. In the only study in which pharmacological interactions between identified pharmacological components of  $I_{\text{Ba}}$  and block by MeHg was examined (Sirois and Atchison, 2000), none of the toxins tested ( $\omega$ -agatoxin IV A,  $\omega$ -conotoxin GVIA,  $\omega$ -conotoxin MVIIC, and calcicludine), nor nimodipine was able to prevent block of  $I_{\text{Ba}}$  by 0.5  $\mu\text{M}$  MeHg. Thus apparently MeHg can block  $\text{Ca}^{2+}$  channel current through all the major subtypes of high-voltage-activated channels (L-, N-, P/Q-, and R-type), and can do so by actions at sites other than those occupied by these ligands. Further, when only a partial degree of block was produced by MeHg, and then  $\omega$ -conotoxin MVIIC was applied, an immediate further block of current occurred. Thus again the sites of action of these toxins were apparently not occluded by MeHg.

As a result, as with  $\text{Pb}^{2+}$  it is not possible on the basis of studies of native currents to ascertain whether or

not certain subtypes of Ca<sup>2+</sup> channels are more or less sensitive to MeHg. Once again, this issue will need to be resolved definitively through studies of recombinant, expressed channels (see below).

Another issue about which results are contradictory is the voltage dependence of effect of MeHg. No voltage dependence of block was seen in granule cells and no change occurred in the current-voltage relationship (Sirois and Atchison, 2000). Upon intracellular application of MeHg to PC12 cells, MeHg still blocked current with approximately the same potency, however there was no obvious effect on the current-voltage relationship either (Shafer, 1998). However Leonhardt *et al.* (1996a,b) reported that for rat DRG neurons, the potential at which maximal current was elicited was shifted in the depolarizing direction in a concentration-dependent manner by MeHg.

Use dependence was seen for MeHg in granule cells, but only at the lowest concentrations (Sirois and Atchison, 2000). At 1  $\mu$ M, channel block appeared to be less specific, and no use-dependence was noted. Szücs *et al.* (1997) noted no use-dependence for MeHg in hippocampal pyramidal cells, using 10 mM Ca<sup>2+</sup> as charge carrier, but the concentration of MeHg that they used for this experiment was not given. However, given that 1  $\mu$ M MeHg appeared to be the lowest concentration that they tested, it is not surprising that they may not have seen use dependence.

In the lone published study of effects of chronic exposure to MeHg on Ca channel function, Shafer *et al.* (2002) found that very low concentrations of MeHg (30 nM) reduced the amplitude of Ca<sup>2+</sup> currents 24 h after exposure in NGF-differentiated PC12 cells. In the same experimental paradigm, the N-type Ca<sup>2+</sup> channel antagonist  $\omega$ -conotoxin GVIA (500 pM) did not affect current amplitude. Exposure of PC12 cells during NGF-induced differentiation to MeHg for 6 days at 3, 10, or 30 nM caused a concentration-dependent reduction of Ca<sup>2+</sup> currents in comparison to control cells. At 30 nM MeHg overt cytotoxicity occurred during the 6 days at exposure, however neither 10 nor 3 nM MeHg caused significant cytotoxicity. While at 3 nM MeHg no effect was evident, at 10 nM current amplitude was reduced significantly across most of the test potential range (-10 to +40 mV). Both the inactivating and noninactivating components of current in these differentiating PC12 cells were reduced equally (~40%) by MeHg. No obvious effects of MeHg were seen on the current-voltage relationship. With the short-term treatment paradigm the action of MeHg on Ca<sup>2+</sup> channels appeared to be selective, as the Na<sup>+</sup> current amplitude was not affected following 24 h of MeHg treatment, but was affected after 6 days. Treatment for 6 days with MeHg did not reduce either the number of radiolabeled  $\omega$ -conotoxin GVIA or saxitoxin binding sites. Thus the reduction in

whole-cell current amplitude was presumably not due to reduced expression of voltage-gated Ca<sup>2+</sup> channels. This was a very important study, in that it demonstrated conclusively that longer exposure paradigms at quite low, and environmentally relevant concentrations of MeHg can affect Ca<sup>2+</sup> channel function.

Overall, the effects of MeHg Ca<sup>2+</sup> channel function have been somewhat perplexing. Whereas single cell recordings of Ca<sup>2+</sup>/Ba<sup>2+</sup> currents in response to acute application of MeHg are obviously readily blocked, indirect measures of Ca<sup>2+</sup> channel function in identical systems suggest that at later times of exposure Ca<sup>2+</sup> channel function may even be facilitated by MeHg. This was shown by studies in isolated cerebellar granular neurons (Marty and Atchison, 1997) in culture and NG108-15 neuroblastoma cells (Hare and Atchison, 1995) in which during fura-2 recordings of increased fluorescence produced by MeHg, several types of Ca<sup>2+</sup> channel blockers including nifedipine,  $\omega$ -conotoxin GVIA, and Ni<sup>2+</sup> were able to slow or abolish part of the MeHg-induced increase in fura-2 fluorescence, suggesting that activation of Ca<sup>2+</sup> channels may occur at later times in exposure to MeHg. Furthermore, this aspect evidently contributes to cytotoxicity with MeHg, because Sakamoto *et al.* (1996) found that treatment of rats with Ca<sup>2+</sup> channel blockers reduced toxicity produced by MeHg. On the basis of the study of Shafer *et al.* (2002) it appears as if at least for PC12 cells that longer term exposure to MeHg does not increase Ca<sup>2+</sup> channel current as longer term exposure to Pb<sup>2+</sup> does (Audesirk, 1987; Hegg and Miletic, 1997). Thus, as detailed below, the data from fura-2 studies may reflect more the entry of MeHg into the cell by Ca<sup>2+</sup> channels than a direct stimulatory action of the metal on the channels. However, this will require studies directed specifically at addressing this question.

Although inorganic mercury (Hg<sup>2+</sup>) is not the environmental concern that MeHg is, it is still neurotoxic, and causes its own spectrum of neurotoxicity. Moreover, it has frequently been used in comparison to organomercurials such as MeHg, hence there are several studies which examine the effects of Hg<sup>2+</sup> on voltage-gated Ca<sup>2+</sup> channel function. These studies have generally been rather descriptive, but have pointed up some interesting differences in action on the channel between the organomercurial and Hg<sup>2+</sup>.

Low  $\mu$ M concentrations of Hg<sup>2+</sup> irreversibly reduce whole-cell Ca<sup>2+</sup> currents in both the abdominal ganglia neurons of *Aplysia* and primary cultures of rat dorsal root ganglion (DRG) neurons (Pekel *et al.*, 1993). The blocking action of Hg<sup>2+</sup> in these two preparations differs in several regards. In DRG neurons Hg<sup>2+</sup> blocks currents carried through presumed T-, L-, and N-type Ca<sup>2+</sup> channels in the concentration range of 0.5–2  $\mu$ M, while in *Aplysia*

neurons,  $\text{Ca}^{2+}$  currents are only blocked at much higher concentrations of  $\text{Hg}^{2+} > 20 \mu\text{M}$ . In DRG neurons, the current-voltage relationship was altered by  $\text{Hg}^{2+}$ ; the voltage at which maximum current was elicited was shifted to more positive membrane potentials. Block of current by  $\text{Hg}^{2+}$  in DRG neurons was rapid in onset. In contrast, in *Aplysia* neurons, block of current with inorganic  $\text{Hg}^{2+}$  was much slower, and never reached steady-state value over a perfusion period of 13 min. Leak current amplitude was increased in DRG neurons at  $\text{Hg}^{2+}$  concentrations  $> 2 \mu\text{M}$ . Conversely in *Aplysia* neurons leak current was not affected even at  $50 \mu\text{M}$   $\text{Hg}^{2+}$ . However studies in the *Aplysia* neurons were conducted *in situ*, whereas those using DRG cells occurred in primary culture. Thus some of the concentration-dependent differences may reflect uptake of  $\text{Hg}^{2+}$  by other tissue. Furthermore, the *Aplysia* preparations were bathed in an artificial seawater which contained approximately  $3 \times$  the chloride concentration as did the solution in which DRG cells were studied. Thus the comparatively much higher chloride concentration might have affected the speciation of mercury in the studies. This makes it difficult to compare results across the two preparations.

In bovine chromaffin cells  $\text{Hg}^{2+}$  blocked  $\text{Ca}^{2+}$  channel current with an  $\text{IC}_{50}$  value of approximately  $3 \mu\text{M}$  and hillslope of 1.46 (Weinsberg *et al.*, 1995). At  $100 \mu\text{M}$ ,  $\text{Hg}^{2+}$  blocked current completely;  $\text{Cd}^{2+}$  had the same effect at that concentration.

Szücs *et al.* (1997) compared the effects of  $\text{Hg}^{2+}$  and MeHg on hippocampal pyramidal neurons in culture. Presumed L-type  $\text{Ca}^{2+}$  channels were inhibited irreversibly by both  $\text{Hg}^{2+}$  and MeHg. Currents were again separated in the cells by altering the holding potential; no pharmacological means was used to separate or identify these currents. No evidence of use dependence was seen for MeHg in the cells, however the effect of  $\text{Hg}^{2+}$  was partially use-dependent. The time to peak current was slowed somewhat by  $\text{Hg}^{2+}$ . Interestingly, low-voltage-activated  $\text{Ca}^{2+}$  currents were transiently increased in amplitude by  $\text{Hg}^{2+}$ . At  $1 \mu\text{M}$  current amplitude was potentiated for  $> 250$  s whereas at  $5 \mu\text{M}$  current amplitude was potentiated for much shorter period of time.

One particularly interesting study which correlates well with the effects of  $\text{Hg}^{2+}$  at the neuromuscular junction is that of Rossi *et al.* (1993). In this study nM concentrations of  $\text{Hg}^{2+}$  increased the amplitude of  $\text{Ca}^{2+}$  currents in PC12 cells. Increased current amplitude occurred at  $300 \text{ nM}$   $\text{Hg}^{2+}$  and at every step potential between  $-60$  and  $+50 \text{ mV}$  when elicited from a holding potential of  $-70 \text{ mV}$ . The enhanced  $\text{Ca}^{2+}$  current in response to  $\text{Hg}^{2+}$  was reduced by application of verapamil ( $10 \mu\text{M}$ ). Shifting the holding potential of electrophysiological studies

from  $-70$  to  $-50 \text{ mV}$  did not significantly alter the effect of  $\text{Hg}^{2+}$ . Thus the increasing  $\text{Ca}^{2+}$  current observed following acute application of  $\text{Hg}^{2+}$  was due primarily to activation of high-voltage-activated  $\text{Ca}^{2+}$  channels, in this model predominantly L-type. At the same concentration of  $\text{Hg}^{2+}$  increases in intracellular  $\text{Ca}^{2+}$  due to bradykinin or ATP (i.e., agonist-mediated  $\text{Ca}^{2+}$  responses) were not potentiated, and in fact were inhibited. Thus this effect was apparently specific for voltage-gated  $\text{Ca}^{2+}$  channels.

To summarize, effects of  $\text{Hg}^{2+}$  on  $\text{Ca}^{2+}$  channel function appear to vary rather distinctly from those of MeHg, and in fact to resemble much more the effects of  $\text{Pb}^{2+}$  aside from the clear problems of reversibility. Moreover, there appear to be some potentially interesting effects of low concentrations of  $\text{Hg}^{2+}$  to stimulate  $\text{Ca}^{2+}$  channel function. Perhaps, like  $\text{Pb}^{2+}$ , this reflects intracellular actions to prevent normal  $\text{Ca}^{2+}$ -induced inactivation. Thus this issue too clearly merits a closer examination.

#### EFFECTS OF ENVIRONMENTAL TOXICANTS ON RECOMBINANT $\text{Ca}^{2+}$ CHANNEL CURRENTS

One area in which toxicologically oriented studies have lagged behind the biophysics and physiology is in the use of recombinant channels. Through use of cloned channels of known phenotype, expressed heterologously in a system in which contributions from contaminating types of current are minimized, the ability of a metal to interact with the channel can be examined most unambiguously.

At present there are only two published reports (Bernal *et al.*, 1997; Peng *et al.*, 2002b) dealing with effects of  $\text{Pb}^{2+}$  on recombinant, heterologously expressed channels. One deals principally with L-type cardiac-type  $\text{Ca}^{2+}$  channels and the other compares three phenotypes of human neuronal  $\text{Ca}^{2+}$  channels.

In the first published report of effects of environmental metals on current carried through recombinant  $\text{Ca}^{2+}$  channels, Bernal *et al.* (1997) reported that  $\text{Pb}^{2+}$  caused a concentration-dependent reduction in amplitude of cloned L-type  $\text{Ca}^{2+}$  channels from rabbit heart stably expressed in the HEK 293 cell line. When only the  $\alpha_{1C}$  subunit was expressed, and  $10 \text{ mM}$   $\text{Ba}^{2+}$  was used as charge carrier, concentrations as low as  $30 \text{ nM}$   $\text{Pb}^{2+}$  caused significant reduction in current amplitude. Subsequently several combinations of the cRNA for the  $\alpha_{1C}$  subunit in combination with several types of  $\beta$  and/or  $\alpha_{2\delta}/\beta$  subunits were used. The resulting control current amplitude varied widely, however  $\text{Pb}^{2+}$  appeared to block



these currents with equivalent potency. An IC<sub>50</sub> for the pooled results was 152 ± 87 nM. Native L-type currents from ventricular myocytes were similarly blocked by Pb<sup>2+</sup>. Current produced by coexpression of  $\alpha_{1C}$  and  $\beta_2$  subunit in *Xenopus laevis* oocytes in the presence of 10 mM Ba<sup>2+</sup> was somewhat less sensitive to block by Pb<sup>2+</sup>; an apparent IC<sub>50</sub> for Pb<sup>2+</sup>-induced antagonism was approximately 1  $\mu$ M. Bernal *et al.* found that Pb<sup>2+</sup>-induced inhibition was not completely reversible by washing with Pb<sup>2+</sup>-free solutions. The extent of irreversibility of Pb<sup>2+</sup> was concentration-dependent. To induce complete reversal of Pb<sup>2+</sup>-induced block, Bernal *et al.* needed to apply a chelator such as EDTA, DMPS, or DMSA, all of which are commonly used to chelate Pb<sup>2+</sup> clinically, to remove the Pb<sup>2+</sup>-induced blocking effect.

In contrast, our recently published study (Peng *et al.*, 2002b) compared how specific subtypes of neuronal high-voltage-activated Ca<sup>2+</sup> channels were affected by acute exposure to Pb<sup>2+</sup>. Expression cDNA clones of human  $\alpha_{1C}$ ,  $\alpha_{1B}$ , or  $\alpha_{1E}$  subunit genes encoding for neuronal L-, N-, and R-subtypes of Ca<sup>2+</sup> channels respectively were transfected into HEK293 cells along with a constant  $\alpha_2\delta$  and  $\beta_3$  subunit. Currents through the respective transiently expressed channels were measured using 20 mM Ba<sup>2+</sup> as charge carrier. As expected, Pb<sup>2+</sup> significantly reduced current amplitude through all three types of Ca<sup>2+</sup> channels in a concentration-dependent manner, however, there was a clear difference in sensitivity among the three channel phenotypes. The order of potency was as follows:  $\alpha_{1E}$  (IC<sub>50</sub> = 0.10  $\mu$ M), followed by  $\alpha_{1C}$  (IC<sub>50</sub> = 0.38  $\mu$ M) and  $\alpha_{1B}$  (IC<sub>50</sub> = 1.31  $\mu$ M). This mirrors to some extent the results of Audesirk and Audesirk (1993) in E18 rat hippocampal pyramids, in which presumed L-type channels were more susceptible to Pb<sup>2+</sup> than were N-type channels. For all three phenotypes of recombinant channels, Pb<sup>2+</sup>-induced inhibition was reversible to some extent, however block of current through  $\alpha_{1C}$ - and  $\alpha_{1B}$ -containing Ca<sup>2+</sup> channels was more readily reversed after washing with Pb<sup>2+</sup>-free solution than were  $\alpha_{1E}$ -containing Ca<sup>2+</sup> channels. While current-voltage relationships were not altered after 3-min exposure to Pb<sup>2+</sup> for any of the three types, the steady-state inactivation relationships were shifted to more negative potentials for  $\alpha_{1B}$ - and  $\alpha_{1E}$ - , but not  $\alpha_{1C}$ -subunit containing channels. Pb<sup>2+</sup> accelerated the inactivation time of current in all three subtypes of Ca<sup>2+</sup> channels in a concentration- and voltage-dependent manner. Furthermore, results suggested that Pb<sup>2+</sup> was more likely to combine with Ca<sup>2+</sup> channels in the closed state. Therefore, there do appear to be differences in susceptibility to Pb<sup>2+</sup> among subtypes of Ca<sup>2+</sup> channels even when they are expressed in the same cell type. Surprisingly  $\alpha_{1E}$ -containing channels are more sensitive to Pb<sup>2+</sup> than are

$\alpha_{1C}$ - or  $\alpha_{1B}$ -containing channels. Moreover all three of these Ca<sup>2+</sup> channel phenotypes exhibited high sensitivity to the inhibitory action of Pb<sup>2+</sup>.

We have recently also examined and compared the actions of MeHg with Hg<sup>2+</sup> on recombinant Ca<sup>2+</sup> channels expressed transiently in the HEK293 cell system (Hajela *et al.*, 2003; Peng *et al.*, 2002a). Transient expression of human neuronal cDNA clones of the  $\alpha_{1C}$  Ca<sup>2+</sup> channel subunit in combination with  $\alpha_2\delta$  the  $\beta_{3a}$  Ca<sup>2+</sup> channel subunits was used to examine the actions of MeHg on a single, defined phenotype (neuronal L-type) of voltage-gated Ca<sup>2+</sup> channels (Peng *et al.*, 2002a). MeHg caused a concentration-dependent (0.125–5.0  $\mu$ M) and time-dependent reduction in the current; onset of block was hastened by increasing the concentration. Surprisingly, block was not complete even at 5  $\mu$ M MeHg, despite the fact that inhibition had reached a plateau level; approximately 20–25% of the current remained. The dihydropyridine-type L-type Ca<sup>2+</sup> channel antagonist nimodipine could cause complete block of current on its own, and could block the residual component of current which remained in the presence of 5  $\mu$ M MeHg. Furthermore in the presence of MeHg the L-type dihydropyridine agonist Bay K 8644 caused an enhancement of current amplitude. As has been shown in every other system tested, the inhibitory action of MeHg on recombinant L-type channels was not reversed by washing with MeHg-free solution (Peng *et al.*, 2002b). We also compared the effects of Hg<sup>2+</sup> and MeHg on recombinant human neuronal N- and R-type Ca<sup>2+</sup> channels again transiently expressed in HEK293 cells (Hajela *et al.*, 2003). Whereas MeHg was unable to block  $\alpha_{1C}$ -mediated current completely, it did cause a concentration-dependent (0.125–5.0  $\mu$ M), and time-dependent block of current carried by  $\alpha_{1B}$ - (N-type) and  $\alpha_{1E}$ - (R-type) containing recombinant channels which was complete at the higher concentrations of MeHg. Inhibitory effects of MeHg were again irreversible following washing with MeHg-free solutions (Hajela *et al.*, 2003). MeHg was approximately equally effective at blocking current through N- and R-type recombinant channels; the apparent IC<sub>50</sub> values taken after 2 min of exposure to MeHg were 1.3 and 1.1  $\mu$ M respectively. In contrast, inorganic Hg<sup>2+</sup> exerted somewhat differential sensitivity for N- and R-type channels. The IC<sub>50</sub> values for Hg<sup>2+</sup> were 2.2 and 0.7  $\mu$ M respectively. Like MeHg, Hg<sup>2+</sup> blocked both types of current completely. However inhibitory effects of Hg<sup>2+</sup> on N-type but not R-type current were reversed in part by washing the cells with Hg<sup>2+</sup>-free physiological saline solution. Thus once again, differences in sensitivity among channel subtypes seem to exist at least for Hg<sup>2+</sup>. Clearly more in-depth studies using cloned channels are warranted, and

the interaction of MeHg with DHP-type  $\text{Ca}^{2+}$  channel blockers appears potentially to be quite interesting.

### EFFECTS OF SOME OTHER ENVIRONMENTAL CHEMICALS ON $\text{Ca}^{2+}$ CHANNEL FUNCTION

#### Solvents

Tillar *et al.* (2002) demonstrated that the volatile organic solvent toluene was able to induce a reversible block of  $\text{Ca}^{2+}$  current in PC12 cells. However in some cases toluene potentiated  $\text{Ca}^{2+}$  current, most notably at test potentials near the threshold for current activation in the cells. The current expressed in differentiated PC12 cells was found to be more sensitive to toluene than that in undifferentiated PC12 cells. The amplitude of current remaining at the end of the voltage pulse was inhibited to a greater extent than was the peak current amplitude, suggesting either that toluene enhanced the inactivation process, or perhaps preferentially interacted with rapidly inactivating components of current in the cells. The solvent trichloroethane (TCE) was similarly able to alter  $\text{Ca}^{2+}$  current amplitude.

Okuda *et al.* (2001) specifically examined the effects of TCE on rat DRG neurons in culture. TCE caused a reversible inhibition of current through both low-voltage-activated and high-voltage-activated  $\text{Ca}^{2+}$  channels. The  $\text{IC}_{50}$  values 5.76 and  $3.99 \times 10$  mM, respectively. The Hill coefficients of the low-voltage-activated and high-voltage-activated  $\text{Ca}^{2+}$  channels were 0.61 and 1.04, respectively. The high-voltage-activated  $\text{Ca}^{2+}$  currents inactivated at more negative potentials in the presence of TCE.

#### Polychlorinated Biphenyls (PCBs)

Arachlor 1254, a commercial mix of a number of PCBs induces recurring  $\text{Ca}^{2+}$  oscillations in neocortical neurons in culture. These effects were blocked by nifedipine (1  $\mu\text{M}$ ) or removal of extracellular  $\text{Ca}^{2+}$ , indicating a role of L-type  $\text{Ca}^{2+}$  channels. However these responses were also blocked by TTX, suggesting that they resulted from synaptic activity among the cells in culture, rather than from a direct effect of the PCBs on L-type  $\text{Ca}^{2+}$  channels (Inglefeld and Shafer, 2000).

#### Tetrandrine

The alkaloid tetrandrine, a bis-benzyl-isoquinoline derivative from the plant *Stefania Tetrandra*, blocked

$\text{Ca}^{2+}$  currents in neurons of spinal cord of fetal mice (Bickmeyer and Wiegand, 1993). The  $\text{IC}_{50}$  value for this was 8  $\mu\text{M}$ . The slowly inactivating components of high-voltage-activated current was affected more readily by tetrandrine than was the low-voltage-activated component. Rubio *et al.* (1993) examined the mechanism of action of tetrandrine on  $\text{Ca}^{2+}$  currents of single bullfrog cardiac cells. Low concentrations (10 nM to 1  $\mu\text{M}$ ) of tetrandrine slightly increased current amplitude through L-type  $\text{Ca}^{2+}$  channels elicited from a holding potential of  $-100$  mV; at higher concentrations, tetrandrine inhibited current. When the holding potential was  $-50$  mV inhibition was enhanced. Tetrandrine-induced block of the L-type  $\text{Ca}^{2+}$  channels was primarily tonic, although use-dependent effects of tetrandrine were evident at high rates of stimulation.

### ION PERMEABILITY IN $\text{Ca}^{2+}$ CHANNELS—A POTENTIAL TARGET FOR HEAVY METALS

Among the areas in which nonphysiological metals have contributed perhaps most significantly to our understanding of  $\text{Ca}^{2+}$  channel function is in their use to examine ion permeability. Most voltage-gated ion channels possess the ability to discriminate for or against movement of certain ions through the channel. This property is extremely important for some types of ion channels, and less so for others. It is dependent upon a number of variables including the radius of the pore at the so-called "selectivity filter;" the nature of the ionic charges within the pore, as well as those in the intracellular and extracellular "vestibules" of the channel. Some channel types are such as voltage-gated  $\text{Ca}^{2+}$  channels are highly discriminating for a particular ion while other channel types such as the nicotinic-acetylcholine-receptor- (nAChR) activated channel are more promiscuous. However, not even the most discriminating channel type is permeable exclusively to only a single type of ion, although as in the case of the potassium channel, which is permeable to ions such as  $\text{Rb}^+$ , these ions may not be physiologically relevant. Moreover, as is the case for monovalent cation permeation of the voltage-gated  $\text{Ca}^{2+}$  channel, it may only occur under artificial conditions, and thus have no normal physiological relevance. Nonetheless, these differences in permeability of an ion channel to "foreign" ions have been extremely useful in elucidating biophysical characteristics of the channel and its pore. This is particularly germane to the  $\text{Ca}^{2+}$  channel field, where the "atypical" and "non-physiological" cations  $\text{Ba}^{2+}$  and  $\text{Sr}^{2+}$  have both been used to characterize function of the channel. Moreover, insofar as these ions have different permeabilities, they can be

used to differentiate among different subtypes of Ca<sup>2+</sup> channels (Tsien *et al.*, 1988).

The mechanism by which an ion channel can discriminate between ions and subsequently select those ions which pass through the channel is of critical importance for our discussion of actions of toxic metals on Ca<sup>2+</sup> channels. As discussed below, data suggest that the intrinsic pore of the Ca<sup>2+</sup> channel contains one or two distinct binding sites for Ca<sup>2+</sup>. On a simplistic basis, Ca<sup>2+</sup> can be thought to move through the open channel in what amounts to a billiard ball type of fashion with binding and subsequent displacement of a Ca<sup>2+</sup> ion from a binding site possibly to another and passing ultimately into the intracellular space. The extracellular concentration gradient driving force for Ca<sup>2+</sup> is huge—on the order of 20,000:1. Hence, when the channels open, Ca<sup>2+</sup> normally moves through it very expeditiously. Naylor (1988) has estimated a rate of influx to be in excess of 1 million Ca<sup>2+</sup> ions *per second per* channel protein. This rapid throughput rate, in turn, implies that binding of Ca<sup>2+</sup> within the pore is readily reversible.

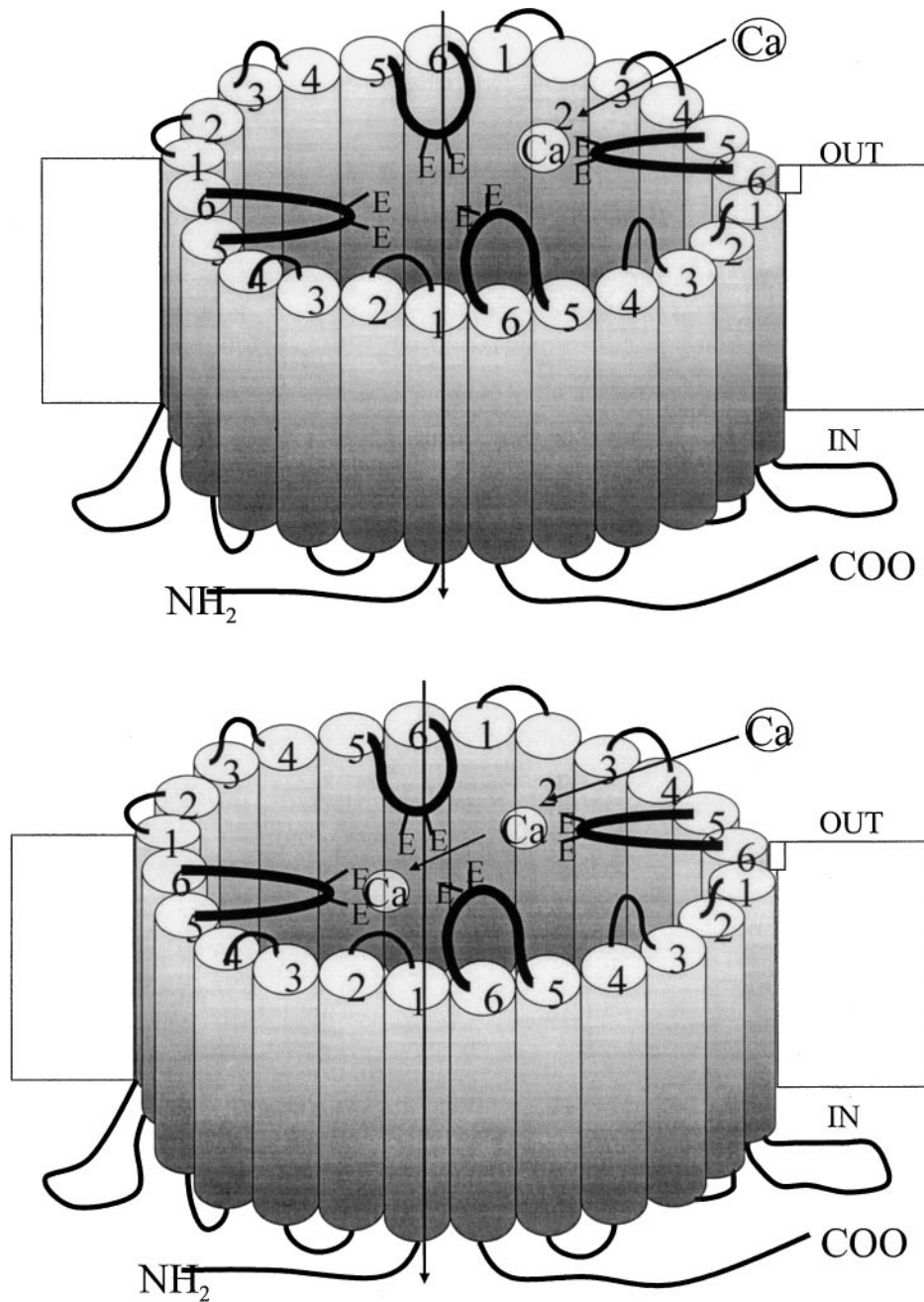
Nonphysiological cations can also enter cells through Ca<sup>2+</sup> channels by virtue of their similarity to Ca<sup>2+</sup> in chemical structure, hydrated radius, and electron orbital configuration (see Table II). However, this is not the only problem that voltage-gated Ca<sup>2+</sup> channels must deal with. Ca<sup>2+</sup> channels must also discriminate between monovalent and divalent cations, despite the fact that Na<sup>+</sup> and K<sup>+</sup> predominate on either side of the membrane. Consequently the Ca<sup>2+</sup> channel selectivity filter must be equipped to select Ca<sup>2+</sup> or other divalent cations with very high affinity. This implies that some sort of distinct chemical binding reaction takes place in the channel pore to discriminate divalent from monovalent cations. However as noted above the downside to this is that if the binding affinity for Ca<sup>2+</sup> is too high, its “off-rate” will be slow and ion throughput from the channel will be impaired. Thus the Ca<sup>2+</sup> channel pore must maintain a delicate “balance” between high-affinity discriminatory interactions and maintaining rapid ion conductance.

There is evidence that the internal membrane pore of the Ca<sup>2+</sup> channel contains just such a feature. The structure of the Ca<sup>2+</sup> channel has been described in excellent detail elsewhere in this series of reviews. The reader should refer to those reviews as well as the original papers for details on the structure. We will however consider this problem briefly here. As shown in Fig. 1, each of the four homologous pore-forming segments of the Ca<sup>2+</sup> channel  $\alpha_1$  subunit consists of a region between segments S5 and S6 which contains an amino acid sequence with glutamate residues. These glutamate residues are highly conserved.

This region appears to be a pore-lining region and the carboxylic acid moieties on the glutamate residues are thought to be critical for binding Ca<sup>2+</sup> ions and other divalent cations (Bahinski *et al.*, 1997; Ellinor *et al.*, 1995; Mikala *et al.*, 1993; Parent and Gopalakrishnan, 1995; Tang *et al.*, 1993; Yang *et al.*, 1993; Yatani *et al.*, 1994). By providing a strategically located series of negative charges which set up a range of negativity to which Ca<sup>2+</sup> ions are attracted, these residues are generally considered to contribute to the high-affinity interactions of the channel with Ca<sup>2+</sup>.

While there is yet no consensus on the exact number of binding sites, or the relative importance of individual glutamate residues to ion permeation, there is general agreement among the relevant studies that the four residues do not contribute equally to divalent cation binding (Kim *et al.*, 1993; Tang *et al.*, 1993; Yang *et al.*, 1993). Several models have been put forth to explain how Ca<sup>2+</sup> channel selectivity and ion permeation occurs. In one model (Almers and McCleskey, 1984; Hess and Tsien, 1984) a high-affinity binding site for Ca<sup>2+</sup> is located at each end of the channel pore. In this model Ca<sup>2+</sup> selectivity resulted from higher affinity of Ca<sup>2+</sup> binding to these sites as compared to other ions. The rapid flux of Ca<sup>2+</sup> associated with the Ca<sup>2+</sup> channel was attributed to electrostatic ion-ion interactions. Another model (Armstrong and Neyton, 1991) is predicated on the pore having a single site to which a single Ca<sup>2+</sup> ion combined with high affinity. Tsien *et al.* (1987) hypothesized the presence of two high-affinity Ca<sup>2+</sup>-binding sites with EC<sub>50</sub> of approximately 1  $\mu$ M located in the middle of the pore. Increasing the extracellular Ca<sup>2+</sup> concentration from  $\mu$ M to the mM range induced electrostatic repulsion between the Ca<sup>2+</sup> ions forcing them to move through the pore with a high throughput rate (Tsien *et al.*, 1987). The alternative model—the one-site model—hypothesized the presence of a single high-affinity binding site which was negatively charged and continuously occupied under normal physiological conditions (Armstrong and Neyton, 1991). In this model, permeation resulted from cationic ion exchange at the site. The Ca<sup>2+</sup>-binding sites could alternate between single and double occupancy, with the rate limiting step being the departure of the ion from the doubly occupied site. However no model has adequately explained all of the observations; thus more recent studies have been directed at trying to understand permeation characteristics using mutants of the normal glutamate residues found at the sites.

An elegant series of studies from several different labs (Ellinor *et al.*, 1995; Parent and Gopalakrishnan, 1995) have involved site-directed mutagenesis against the four glutamate residues, with subsequent heterologous



**Fig. 1.** A schematic representation of cation permeation through the pore-forming  $\alpha_1$  subunit of the  $\text{Ca}^{2+}$  channel. The four sets of grey cylinders represent the six transmembrane segments of each of the four domains of the  $\alpha_1$  subunit. Solid lines represent the extra- and intracellular connection regions. Both the amino- and carboxy-terminals are intracellular. The four extracellular connecting regions between segments 5 and 6 are believed to be the pore-forming assembly. A set of two glutamate residues in each of these four segments line the pore and selectivity filter. The carboxylate groups of the glutamates are believed to interact with divalent cations at higher affinity than monovalent cations. Both the glutamates in each segment are believed to bind the  $\text{Ca}^{2+}$  ions. Displacement of an already bound  $\text{Ca}^{2+}$  by another incoming  $\text{Ca}^{2+}$  ion is thought to move it inward perhaps binding to another pair of glutamates, although the exact mechanism and models describing this are still unresolved. Part 1 of figure shows an initial  $\text{Ca}^{2+}$  bound and part 2 shows its displacement and subsequent movement by an additional  $\text{Ca}^{2+}$ .

transfection of the cloned and mutated channel. These studies were all conducted using L-type Ca<sup>2+</sup> channels, although a more recent study has examined N-type Ca<sup>2+</sup> channel permeation as well (Wakamori *et al.*, 1998). Ellinor *et al.* (1995) constructed quadruple mutants in which the glutamate residues in all four membrane repeats were substituted with either glutamine or alanine. cRNAs encoding for these mutant channels were expressed heterologously in *Xenopus* oocytes. The interaction of Ca<sup>2+</sup> with these quadruple mutant channels was assessed by comparing the ability of low concentrations of Ca<sup>2+</sup> to block current carried through these channels by Li<sup>+</sup> (Lansman *et al.*, 1986; Tang *et al.*, 1993). This monovalent cation inward current is normally blocked by extremely low concentrations of Ca<sup>2+</sup> (Almers and McCleskey, 1984; Hess and Tsien, 1984; Lux *et al.*, 1990). However in quadruple mutants of both the glutamine and alanine phenotypes, the potency of Ca<sup>2+</sup> as an antagonist of Li<sup>+</sup>-mediated current was reduced dramatically. Whereas the IC<sub>50</sub> for Ca<sup>2+</sup> in wild-type cells was slightly less than 1 μM, in the mutants IC<sub>50</sub> values were in the range of 1 mM. Thus the apparent affinity of Ca<sup>2+</sup> for these residues was reduced more than 1000× by replacement of the glutamate residues.

Cd<sup>2+</sup> apparently interacts like Ca<sup>2+</sup> with binding determinants within the Ca<sup>2+</sup> channel pore (Tsien *et al.*, 1987), although apparently with stricter requirements than for Ca<sup>2+</sup> itself (Lippard and Berg, 1994). Cd<sup>2+</sup>-induced block of Li<sup>+</sup>-mediated current through the quadruple mutants was examined; IC<sub>50</sub> values were reduced from 1.4 nM in the wild type (Yang *et al.*, 1993) to approximately 10 μM for glutamine and 250 μM for alanine mutants (Ellinor *et al.*, 1995). Similarly when pairs of glutamate residues were mutated simultaneously using alanine as a substitute, the potency of Ca<sup>2+</sup>-mediated block of Li<sup>+</sup>-mediated current was again reduced dramatically from the wild type. When the glutamate residues were altered individually, the interaction of the channel with Ca<sup>2+</sup> was reduced, but not in a consistent fashion. Thus there was asymmetry in the interaction of Ca<sup>2+</sup> with the various glutamate residues.

Another question tested in the paper by Ellinor *et al.* (1995) was whether the carboxylic acid groups on the glutamate residues were involved in direct interaction with divalent cations by means of specific binding, or whether they simply provided electrostatic interactions. Substitution of glutamate to aspartate, which is also negatively charged, reduced dramatically the potency of Cd<sup>2+</sup> as a blocking ion for each of the four glutamate sites.

In the study of Parent and Gopalakrishnan (1995), mutational analysis of the glutamate residue in transmembrane repeat 3 also showed that the presence of a nega-

tive charge was critical in maintaining affinity for Ca<sup>2+</sup>. Substitution of glutamate with a positively charged lysine also caused a marked decrease in affinity of Ca<sup>2+</sup>. However in their study substitution of aspartate residues for glutamate residues produced equivalent results suggesting that the carboxylic acid groups were critical for high-affinity Ca<sup>2+</sup> binding. Their results also suggest that the glutamates located at positions 1145 and 1446 on the L-type Ca<sup>2+</sup> channel (α<sub>1C</sub>) were critical in maintaining divalent as opposed to monovalent ion selectivity within the pore. Parent and Gopalakrishnan (1995) suggested that these residues contributed to two high-affinity divalent cation binding sites near the external vestibule as suggested in an earlier model (Kuo and Hess, 1993a,b).

### Ca<sup>2+</sup> CHANNELS AS ROUTES OF ENTRY OF TOXIC METALS INTO CELLS

Irrespective of the precise model by which permeation selectivity occurs, it is clear that the Ca<sup>2+</sup> channel has structural features that it will permit it to pass other cations in lieu of Ca<sup>2+</sup>. The extremely high affinity of Cd<sup>2+</sup> for this channel suggests that other metals may similarly exert pronounced effects on permeation through the channel, as well as use the channel for their own purposes. The presence of critical carboxylic acid residues may be especially important for interactions with metals such as Pb<sup>2+</sup> and Hg<sup>2+</sup>, both of which have high affinity for these types of groups. The concept that even when ion channels are highly specific for an individual ion they are not exclusively permeated by that ion also extends to nonphysiological metals of toxicological interest. Ca<sup>2+</sup> channels are considered to be a route of entry into cells for many heavy metals including Cd<sup>2+</sup> (Hinkle *et al.*, 1987; Taylor 1988), Pb<sup>2+</sup> (Cooper and Manalis, 1983; Simons and Pocock, 1987; Tomsig and Suszkiw, 1991), Hg<sup>2+</sup> (Miyamoto, 1983), MeHg (Atchison, 1987), and Zn<sup>2+</sup> (Winegar and Lansman, 1990).

This concept has been demonstrated in several ways. The first of these, as mentioned above, relates to metal-induced increases in MEPP frequency. Because MEPP frequency does not depend so much on the extracellular Ca<sup>2+</sup> concentration as on the intracellular concentration, the ability of a metal to increase MEPP frequency has been taken as an index of an intracellular effect, a "bioassay" as it were, to examine the ability of an ion to traverse the membrane and subsequently elevate [Ca<sup>2+</sup>]<sub>i</sub> following entry of the metal into the cell. For example, at the frog neuromuscular junction, tetrodotoxin (TTX) and Co<sup>2+</sup> prevent the Hg<sup>2+</sup>-induced increases in MEPP frequency

when used together, but not when used alone (Miyamoto, 1983). The ability of  $\text{Co}^{2+}$  and TTX to prevent this action of  $\text{Hg}^{2+}$  was taken as evidence that  $\text{Hg}^{2+}$  could pass through the membrane via these types of channels. Conversely the MeHg-induced increase in MEPP frequency was not blocked by this combination of treatments, suggesting that while inorganic  $\text{Hg}^{2+}$  can pass through  $\text{Na}^+$  and  $\text{Ca}^{2+}$  channels, that MeHg can also enter the cell by routes other than those blocked by  $\text{Na}^+$  and  $\text{Ca}^{2+}$  channel blockers. Along these same lines, Atchison (1987) demonstrated that activation of  $\text{Na}^+$  and  $\text{Ca}^{2+}$  channels in the absence of  $\text{Ca}^{2+}$  and presence of an impermeant organic monovalent cation *could* facilitate the increase in MEPP frequency induced by MeHg. This again suggested that the metal gained access to the intracellular compartment at least in part by means of entry through voltage-gated ion channels.

Another means of demonstrating entry of metals into the cell by means of  $\text{Ca}^{2+}$  channels has involved measures of cell fluorescence using fluorophores such as fura-2 (Tomsig and Suszkiw, 1991). For example, in fura-2 loaded rat cerebellar granule cells, extracellular  $\text{Pb}^{2+}$  (5–50  $\mu\text{M}$ ) induced an increase in fluorescence ratio in the absence of any specific stimulus. This effect was concentration-dependent, but was not affected by mM concentrations of  $\text{Ca}^{2+}$  or  $\text{Mg}^{2+}$ . It was, however, reversed by the membrane permeate heavy-metal chelator TPEN (100  $\mu\text{M}$ ) indicating that it resulted from influx of  $\text{Pb}^{2+}$ . The rate of rise was increased dramatically by depolarization with high KCl concentrations. This latter effect was not antagonized by nimodipine nor was it enhanced by BayK8644. However  $\omega$ -agatoxin IVA (200 nM) reduced the onset of this effect (Mazzolini *et al.*, 2001).

An interesting technique for demonstrating the role of  $\text{Ca}^{2+}$  channels in permeation of toxic metals was reported by Hinkle and Osborne (1994) and made use of the subclone of the rat PC12 cell line—PC18 cells (Tank *et al.*, 1986) which lack voltage-gated  $\text{Ca}^{2+}$  channels. In these cells,  $\text{Cd}^{2+}$ -induced changes in fura-2 fluorescence were not affected by L-type  $\text{Ca}^{2+}$  channel blockers, whereas in normal PC12 cells, they were.

### RELATIONSHIP BETWEEN EFFECTS OF METALS ON $\text{Ca}^{2+}$ CHANNELS AND THEIR NEUROTOXICITY

What role does  $\text{Ca}^{2+}$  channel block play in cell toxicity of heavy metals? In general, studies of the effects of polyvalent heavy metals on  $\text{Ca}^{2+}$  channel function have been more valuable in providing information about the

structure and function of the channel protein itself, than they have at providing information about relevant neurotoxicology. Clearly  $\text{Ca}^{2+}$  channels provide a route for entry into cells of environmentally relevant metals such as  $\text{Pb}^{2+}$ , and MeHg. Also, entry of the metal into the cell and subsequent cytotoxicity can be delayed by use of  $\text{Ca}^{2+}$  channel blockers *in vitro* (Marty and Atchison, 1997) and *in vivo* (Sakamoto *et al.*, 1996). Thus at a minimum,  $\text{Ca}^{2+}$  channels contribute to the early stages of toxic metal accumulation. However, an objective assessment of the comparative importance of altered  $\text{Ca}^{2+}$  channel function to relevant neurotoxicity is harder to make. As noted by Audesirk (1993) there is little information available about the minimal concentrations of a metal that cause significant inhibition of current through various types of  $\text{Ca}^{2+}$  channels. The concentrations used in many studies far exceed those that occur typically during environmental exposure. However differential sensitivity of different types of  $\text{Ca}^{2+}$  channels does occur to the same metal, so while some types of  $\text{Ca}^{2+}$  channel function may not be affected at a particular metal concentration, others may be. This issue is only now being addressed unequivocally through use of recombinant  $\text{Ca}^{2+}$  channels expressed heterologously, so that the phenotype of channel under study is known and studied in isolation (Hajela *et al.*, 2003; Peng *et al.*, 2002a,b). But, for the most part it is unclear as to whether there is a toxic threshold concentration below which certain some types of  $\text{Ca}^{2+}$  channels are unaffected by particular metals. A second major issue, as noted later in the gaps of information regarding effects of metals on  $\text{Ca}^{2+}$  channels, is the role which chronic exposure to the metal plays on the channel. There are several reasons why most of the studies to date utilized acute exposure of the cells in culture to bath-applied metals. Chief among these is the likelihood that chronic exposure to the metal evinces a more complicated response in the cell—consisting of a number of effects, some of which are secondary or even nonspecific. Moreover as noted above, the membrane and its proteins are the first part of the cell to be exposed to a metal. Thus it is logical to consider early-onset membrane limited responses. However, in real life, neurotoxicity does not typically occur in this manner, but rather entails more long-term, lower concentration types of exposure. Thus an important shortcoming of studies to date has been a lack of studies in which cells are exposed chronically to neurotoxic metals. There are some notable exceptions and these have been pointed out in the prior section as in the original experiments by the Audesirks (Audesirk and Audesirk, 1989) using *Lymnaea* and the more recent experiments by Shafer *et al.* (2002) for MeHg and Hegg and Miletic (1997) for  $\text{Pb}^{2+}$  using PC12 cells. A third issue, which again is only now beginning to be examined (Shafer, 1998; Sun

and Suszkiw, 1995), is the role which intracellular effects of metals play on Ca<sup>2+</sup> channel function. Because of the importance of the  $\beta$  subunit in modulating Ca<sup>2+</sup> channel current kinetics, it would be surprising if highly reactive metals such as Pb<sup>2+</sup> and Hg<sup>2+</sup> did not affect the function of this subunit. Furthermore, in this regard, intracellular modulation of Ca<sup>2+</sup> channel function is critical for its optimal utilization. There are well-known sites on various subunits for modulation as by phosphorylation. Both Pb<sup>2+</sup> and Hg<sup>2+</sup> alter intracellular phosphorylation; as such this represents yet another site at which these metals could alter Ca<sup>2+</sup> channel function apart from actions within the pore. Finally, experiments to date have largely ignored the issues of metal speciation and actual concentration. *In vivo* metals are unlikely to exist principally in their free ionized state (e.g., Pb<sup>2+</sup>, Hg<sup>2+</sup>, MeHg<sup>+</sup>), but rather as alternate ionic species (e.g., PbOH<sup>+</sup>, HgOH<sup>+</sup>, or PbCl<sup>+</sup>; HgCl<sup>+</sup>), coordination complexes with various anions, or complexes with cysteine, for example. Studies of effects of metals on Ca<sup>2+</sup> channels have not yet taken into account the existence of these species. Thus the answer to the question—What role to Ca<sup>2+</sup> channels play in neurotoxicity of certain metals?—remains a cautious—“They’re likely to have some role, but the exact extent remains unknown.”

#### GAPS IN OUR UNDERSTANDING OF THE EFFECTS OF ENVIRONMENTAL TOXICANTS ON Ca<sup>2+</sup> CHANNEL FUNCTION

As alluded to in the previous section, despite numerous studies of actions of certain environmental toxicants on Ca<sup>2+</sup> channel function, there are a number of unresolved questions that remain. Several of these have been outlined well in recent reports by Shafer (2000) and Audesirk *et al.* (2000). Among the most important of these deals with whether chemical toxicants interact only with the channel protein itself or also interacts intracellularly to alter the varied signals which modulate Ca<sup>2+</sup> channel function. Because Ca<sup>2+</sup> channel function is modulated on a long-term basis by a number of variables, neurotoxicants could disturb Ca<sup>2+</sup> channel function by effects on pathways that modulate channel activity such as by altering GTP binding protein function, altering phosphorylation of the channel, or intracellular concentration of free Ca<sup>2+</sup>, which is well-known to contribute to the inactivation properties of voltage-gated Ca<sup>2+</sup> channels. Whereas it is clear that both Pb<sup>2+</sup> and MeHg affect protein phosphorylation in isolation, no studies have yet been directed at determining how this may contribute to aberrant function of voltage-gated Ca<sup>2+</sup> channels. Similarly given the complex inter-

actions between voltage-gated Ca<sup>2+</sup> channels and regulation of intracellular Ca<sup>2+</sup>, and the well-known ability of toxicants such as MeHg (Hare *et al.*, 1993; Marty and Atchison, 1997) and polychlorinated biphenyls (PCBs) to increase intracellular Ca<sup>2+</sup> (Mundy *et al.*, 1999) studies of the interaction between these two components would also be valuable. Third although it is clear that subunit subtype composition especially with regards to the  $\alpha_1$  subunit plays a crucial role in determining pharmacological selectivity of antagonists for Ca<sup>2+</sup> channels, studies of effects of environmental toxicants on phenotypically defined Ca<sup>2+</sup> channels, or on specific components of the channel protein, are only now beginning (Bernal *et al.*, 1997; Hajela *et al.*, 2003; Peng *et al.*, 2002a,b). Moreover because a number of these toxicants gain access to the intracellular compartment, it will be important to begin to examine how they interact with intracellularly directed subunits such as the  $\beta$  subunit which nonetheless play critical roles in Ca<sup>2+</sup> channel function. In this regard, because of the important role of the  $\beta$  subunit of the Ca<sup>2+</sup> channel in regulating the expression and localization of Ca<sup>2+</sup> channels, chronic effects of environmental toxicants on this subunit could lead to diminished and/or aberrant expression and placement of the channels in the membrane. Thus studies need to be directed at possible intracellular actions of environmental toxicants on Ca<sup>2+</sup> channels. Moreover, a significant area in which research is lacking deals with the effects of chronic exposure to neurotoxicants on Ca<sup>2+</sup> channel function. Almost all of the studies to date have involved short-term bath application of chemicals directly to the cell. While these have unquestionably been necessary initial studies, and have provided a compendium of important, and in some cases rather surprising results, they do not approach the types of situations experienced *in vivo*. As such their ultimate relevance to true environmental exposure is remains unknown. Thus there remain a number of very important and significant issues in this field which needed addressing.

#### CONCLUSION

Nonphysiological polyvalent cations have played an important role in our understanding of voltage-gated Ca<sup>2+</sup> channel function. Some of these such as Cd<sup>2+</sup>, Ni<sup>2+</sup>, and Mn<sup>2+</sup> have been used as important pharmacological tools to block the function of these channels or to isolate distinct channel phenotypes. Others, such as Pb<sup>2+</sup> and Hg<sup>2+</sup>, are important environmental contaminants whose toxic action may in part be mediated by effects at Ca<sup>2+</sup> channels. Finally, some such as Zn<sup>2+</sup> and Mg<sup>2+</sup> have been shown to exert discrete effects on channel function of their

own effects which may be important in their capacity as normal physiologic divalent cations. While many questions remain to be answered, these chemicals will continue to play an important role in our understanding of the normal physiological functions of voltage-gated Ca<sup>2+</sup> channels as well as their disruption in pathophysiological states.

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