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Defining Potential Roles of Pb^{2+} in Neurotoxicity from a Calciomics Approach

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Abstract

Metal ions play crucial roles in numerous biological processes, facilitating biochemical reactions by binding to various proteins. An increasing body of evidence suggests that neurotoxicity associated with exposure to nonessential metals (e.g., Pb^{2+}) involves disruption of synaptic activity, and these observed effects are associated with the ability of Pb^{2+} to interfere with Zn^{2+} and Ca^{2+} -dependent functions. However, the molecular mechanism behind Pb^{2+} toxicity remains a topic of debate. In this review, we first discuss potential neuronal Ca^{2+} binding protein (CaBP) targets for Pb^{2+} such as calmodulin (CaM), synaptotagmin, neuronal calcium sensor-1 (NCS-1), N-methyl-D-aspartate receptor (NMDAR) and family C of G-protein coupled receptors (cGPCRs), and their involvement in Ca^{2+} -signalling pathways. We then compare metal binding properties between Ca^{2+} and Pb^{2+} to understand the structural implications of Pb^{2+} binding to CaBPs. Statistical and biophysical studies (e.g., NMR and fluorescence spectroscopy) of Pb^{2+} binding are discussed to investigate the molecular mechanism behind Pb^{2+} toxicity. These studies identify an opportunistic, allosteric binding of Pb^{2+} with CaM that is distinct from ionic displacement. Together, this data suggests three potential modes of Pb^{2+} activity related to molecular and/or neural toxicity: (i) Pb^{2+} can occupy Ca^{2+} -binding sites, inhibiting the activity of the protein by structural modulation, (ii) Pb^{2+} can mimic Ca^{2+} in the binding sites, falsely activating the protein and perturbing downstream activities, or (iii) Pb^{2+} binds outside of the Ca^{2+} -binding sites, resulting in allosteric modulation of the proteins activity. Moreover, the data further suggest that even low concentrations of Pb^{2+} can interfere at multiple points within the neuronal Ca^{2+} signalling pathways to cause neurotoxicity.

Introduction

Given that approximately 40% of all proteins have some interaction with physiologically relevant metal cations, the role of non-essential metals on cellular toxicity and neurotoxicity remains a relevant area of research. Of all the known toxic metals, Pb^{2+} has likely received the most attention, as it has been a persistent anthropogenic toxicant for much of recorded human history, and continues to be a health concern (1, 2) in urban areas and countries with emerging industrial production (3, 4). Toxicity associated with Pb^{2+} occurs even at very low

concentrations. The current exposure threshold, as recommended by the CDC, is $<5 \mu\text{g/dL}$ (http://www.cdc.gov/nceh/lead/acclpp/blood_lead_levels.htm). About 20% of Pb^{2+} absorbed by the body, primarily through inhalation and consumption, enters the blood stream where it is redistributed to different tissues and bone (5). The toxicity associated with Pb^{2+} and its relationship to anaemia was documented well over a century ago (6), although it was only in the latter half of the 20th century that Pb^{2+} toxicity was investigated at the molecular level. Exposure to Pb^{2+} produces a variety of systemic effects including anaemia, hypertension, kidney damage, and decrease in male fertility (7–9). Studies have reported that Pb^{2+} ions can pass cross through the blood-brain barrier (BBB) and the placenta in pregnant women (10, 11). More significantly, exposure to Pb^{2+} at even low concentrations produces a number of neurotoxic effects that induce severe cognitive deficiencies such as irreversible IQ loss in children (12), which can in turn lead to subsequent behavioral disorders (13) that persist through adolescence and adulthood.

An increasing body of evidence suggests that the neurotoxicity associated with Pb^{2+} exposure involves disruption of synaptic activity (14, 15), and these observed neurotoxic effects are associated with the ability of Pb^{2+} to interfere with Zn^{2+} and Ca^{2+} -dependent functions, as recently summarized by Neal and Guilarte (16). However, the molecular mechanism for Pb^{2+} has yet to be clearly defined and is a topic of some controversy. In this review, cellular Pb^{2+} toxicity will be discussed in the context of calcium binding proteins (CaBPs) and the relationship between Ca^{2+} , Pb^{2+} , and Ca^{2+} -regulated pathways. The metal binding properties of CaBPs with respect to both Ca^{2+} and Pb^{2+} will be examined, and structural analysis of CaBPs and proteins known to bind Pb^{2+} will be discussed with possible proposed mechanisms for molecular toxicity.

Calcium Signalling and CaBPs

Calcium, one of the most universal and versatile signal indicators, acts as an extracellular first messenger, and as an intracellular second messenger within mammalian cells to regulate almost all aspects of cellular processes. This begins at the inception of life during fertilization, and ends with cell death or apoptosis. Outside of the cell, available Ca^{2+} can reach mM concentrations, whereas cytosolic Ca^{2+} concentration ($[\text{Ca}^{2+}]_{\text{CYT}}$) at a resting state is maintained at about 100 nM, with the endoplasmic reticulum (ER) Ca^{2+} concentration ($[\text{Ca}^{2+}]_{\text{ER}}$) at several hundred μM (Fig. 1). Thus, cells have to maintain a $>10,000$ -fold Ca^{2+} gradient across the plasma membrane and maintain intracellular Ca^{2+} homeostasis to avoid acute, massive fluctuations of Ca^{2+} within cells (17). A specific spatiotemporal pattern of cytosolic Ca^{2+} signalling is made possible by Ca^{2+} from two major sources: the internal Ca^{2+} store (mainly ER or sarcoplasmic reticulum) and the extracellular medium (Fig. 1). The entry of Ca^{2+} across the plasma membrane as mediated by specific receptors and Ca^{2+} channels is usually triggered by stimuli, including membrane depolarization, mechanical stretch, external agonists, depletion of internal stores and intracellular messengers. The mobilization of Ca^{2+} from the internal stores in response to Ca^{2+} itself or an intracellular messenger is primarily mediated by the IP_3 receptors (IP_3R) (18, 19) and the ryanodine receptors (RyR) (20). Several distinct mechanisms such as the plasma membrane Ca^{2+} -ATPase (PMCA), the sarcoplasmic/endoplasmic reticulum Ca^{2+} -ATPase (SERCA), the secretory pathway Ca^{2+} -ATPase (SPCA), the $\text{Na}^+/\text{Ca}^{2+}$ exchanger

(NCX), and the mitochondrial uniporter, are responsible for sequestering Ca^{2+} from the cytosol by transporting Ca^{2+} either to an external medium or into different cellular compartments (17, 21, 22). The temporal and spatial changes in Ca^{2+} concentration, and the interaction between Ca^{2+} and different classes of CaBPs in different cellular environments subsequently stabilizes the CaBPs and regulates numerous biological processes.

Potential Molecular Pb^{2+} Targets in Neurons

Neurons, like other cells, contain numerous proteins that interact either directly or indirectly with Ca^{2+} as part of a broader Ca^{2+} -dependent signalling system (23). Changes in the free Ca^{2+} concentration regulates signalling in neural pathways along the CNS by triggering neurotransmitter release in synapses. Therefore, the well-established relationship between Ca^{2+} and Pb^{2+} makes the intracellular compartment a target-rich environment where Pb^{2+} may compete with Ca^{2+} to gain entrance to the cell and further compete with Ca^{2+} to erroneously activate or inhibit Ca^{2+} -regulated processes via interactions with CaBPs (Fig. 1). This section will focus on several classes of neuronal CaBPs as potential Pb^{2+} targets in the intracellular environment, including CaM (Fig. 2A and 2B), NCS (Fig. 2C), the transmembrane protein synaptotagmin, and extracellular proteins such as family C of GPCR.

Calmodulin

In the neural cell, the intracellular protein CaM, upon being activated by binding with Ca^{2+} (Fig. 2A), regulates a variety of processes (Fig. 1). These processes include: gene expression and modifications to synaptic plasticity (25, 26); inhibition of Ca^{2+} release from intracellular stores through binding with inositol 1,4,5-trisphosphate receptor type 1 (IP₃R1) (27, 28); binding with calcineurin (which also binds Ca^{2+} directly), which activates T cells and may play a role in cognitive function (29, 30); and activation of short transient receptor potential channel 5 (TrpC5) proteins, which participate in the formation of Ca^{2+} -permeable ion channels, and can either inhibit or potentiate neuron growth cone formation (31). There is a preponderance of evidence indicating that Pb^{2+} interacts with CaM (Fig. 2B), and these interactions likely play a prominent role in Pb^{2+} neurotoxicity. Consequently, the ability of CaM to be activated by low concentration of Pb^{2+} suggests that each of these downstream protein interactions represents a potential avenue for neurotoxicity (Fig. 1), and research has already demonstrated that CaM activated at low concentrations of Pb^{2+} can subsequently activate phosphodiesterase (PDE) (32), and it can release norepinephrine (NE) in bovine neuroendocrine chromaffin cells (33).

Additionally, studies have reported that Trcp5 may be directly activated by low micromolar concentrations of Pb^{2+} through interaction with a glutamate residue (E543) (34), and another neurotoxic metal, Hg^{2+} , through interaction with extracellular cysteine residues (C553 and C558) (35). As these interactions are not dependent on CaM, they represent an allosteric effect where toxic metals are binding opportunistically on the protein surface. These results also suggest that blocking of Trcp5 may present an approach to preventing neurotoxic effects of these metals (35).

Synaptotagmin

Synaptotagmin I, one of at least 15 isoforms of synaptotagmin, is a C2 domain-containing protein that is localized to synaptic vesicles (Fig. 1) (36) and plays an important regulatory role in neurotransmitter exocytosis by responding to changes in Ca^{2+} concentration (37). Synaptotagmin I contains a transmembrane domain and a short intravesicular N-terminus domain. The two C2 domains (C2A and C2B), homologous to the C2 domain of protein kinase C (PKC) are a part of their cytoplasmic domain (36), which is also known to bind Pb^{2+} . Synaptotagmin I interacts with the plasma membrane protein syntaxin (38) and negatively charged phospholipids such as phosphatidylserine and phosphatidylcholine (39). These interactions occur in a Ca^{2+} dependent manner via the C2A domain of synaptotagmin I (36). A study by Bouton *et al* in 2001 reported that synaptotagmin binds Pb^{2+} with higher affinity than Ca^{2+} , and that Pb^{2+} at nanomolar concentrations can mimic the effects of Ca^{2+} on synaptotagmin I, producing some of the same effects observed with Ca^{2+} (40). For example, Pb^{2+} was found to protect synaptotagmin I from proteolytic cleavage like Ca^{2+} , but the Pb^{2+} -synaptotagmin complex could not interact with syntaxin. This suggests a direct competitive interaction of Pb^{2+} Vs Ca^{2+} at low Pb^{2+} concentration, and that Pb^{2+} -binding may stabilize the protein by displacing Ca^{2+} , while simultaneously inhibiting the proteins function through the direct interaction with the Ca^{2+} -binding sites, thus interfering with Ca^{2+} -mediated release of neurotransmitters.

Additionally, a study by Garcia and Godwin reported that synaptotagmin II, which shares high sequence identity with synaptotagmin I, will self-associate to form a dimer in the presence of low concentrations of Pb^{2+} (~10 μM), and a multimer at 1.1 mM, while self-association due to Ca^{2+} occurs only at a higher concentration (5 mM) (41). Thus, the availability of Pb^{2+} may lead to inactivation of synaptotagmin through a self-association mechanism.

Family C of GPCRs

The family C of G-protein-coupled receptors (GPCRs) represents one of the largest families of cell surface receptors, consisting of 14 members including the calcium sensing receptor (CaSR), metabotropic glutamate receptors (mGluR), γ -aminobutyric acid (GABA)_B receptors, amino acid taste receptors, pheromone receptors, odorant receptors in fish and several orphan receptor (42). GPCRs have been implicated in neuronal and glial signalling, and function to protect neurons from pathological stresses (43). The cGPCRs are potential targets for Pb^{2+} due to their direct activation by binding to Ca^{2+} , or indirect modulation by interactions with other CABPs, as seen in the case of CaM. Consequently, Pb^{2+} can interfere with downstream Ca^{2+} signalling pathways through displacement of Ca^{2+} , or through opportunistic binding of Pb^{2+} to regions besides the Ca^{2+} binding sites.

γ -aminobutyric acid (GABA)_B receptors

Glutamatergic and GABAergic neurotransmitters within the amygdala are linked with neurons and are related to processes associated with emotion, fear regulation, anxiety, learning and memory (44, 45). GABA is the primary inhibitory neurotransmitter in the adult

central nervous system (CNS). GABA functions as an excitatory neurotrophic factor (46) during early CNS development, and therefore plays a crucial role in cell proliferation, neuronal migration, neurite growth, axonal growth, and synaptogenesis (46).

Rats chronically exposed to low levels of Pb^{2+} have been observed to exhibit reduced Ca^{2+} -dependent glutamate and γ -aminobutyric acid release in the hippocampus (47) indicating presynaptic neuron dysfunction during Pb^{2+} exposure mediated through glutamatergic and GABAergic systems. Studies of hippocampal neurons (48) and brain slices (47) exhibited a deficit in neurotransmission in both the glutamatergic and GABAergic systems, following exposure to Pb^{2+} , as indicated by the impaired excitatory postsynaptic currents (EPSCs) and inhibitory postsynaptic currents (IPSCs). Similarly, Neal *et al* reported that Pb^{2+} exposure resulted in reduced expression of the presynaptic proteins synaptophysin (Syn) and synaptobrevin (Syb), and deficits in vesicular neurotransmitter release which were associated with both glutamatergic and GABAergic synapses (49). Additionally, a study by Wirbisky *et al* analysing tissue up-take, gross morphological alterations, gene expression, and neurotransmitter levels in zebrafish, indicated that Pb^{2+} exposure at lower doses affected the GABAergic system of the developing CNS in a manner that was dependent both on the dose and development stage (50). For example, the GABAergic system exhibited reduced expression of GABA genes *gad2*, *gabral*, *gabbr1a*, and *vgat* at 48 hours post-fertilization (hpf) in the presence of 50 and 100 ppb doses of Pb, while conversely, increased expression of these genes was observed at 60 hpf, but only following treatment with 50 ppb. This may have been due to the lower concentration of Pb^{2+} mimicking the presence of Ca^{2+} in the protein Ca^{2+} -binding site, thereby falsely activating the protein.

Metabotropic glutamate receptor 1 (mGluR1)

Metabotropic glutamate receptor 1 (mGluR1) modulates excitatory neurotransmission, neurotransmitter release, and synaptic plasticity, and is emerging as a potential drug target for various disorders, including a range of psychiatric and chronic neuronal degenerative diseases (51). Structural studies have shown that the receptors are activated when the endogenous agonist L-glutamate (L-Glu), the major excitatory neurotransmitter in the central nervous system, binds at the hinge region of the extracellular domain (ECD) of the receptor. This subsequently stimulates phospholipase C, resulting in accumulation of inositol trisphosphate (ITP) and an increase of intracellular calcium concentration ($[Ca^{2+}]_i$) (52–54), and activation of PKC (55). In addition to being activated by glutamate, mGluR1 is also modulated by extracellular Ca^{2+} , and Jiang *et al* recently identified a Ca^{2+} -binding site within the hinge region of the ECD of mGluR1 comprised of residues D318, E325 and D322 (51), adjacent to the reported L-Glu-binding site (56, 57).

Several studies have identified a crucial relationship between Pb^{2+} neurotoxicity, CaM, mGluRs, and their signalling pathways. The proteins mGluR1, mGluR5, and mGluR7 are known to interact with CaM, which plays a role in mGluR regulation. Picomolar concentrations of Pb^{2+} have been shown to replace micromolar concentrations of Ca^{2+} in a PKC enzyme assay (58), and a relationship between Pb^{2+} and increased PKC activity was reported in a study of lead workers who were observed to exhibit higher tibia bone Pb (BPb) levels and an apparent associated loss of cognitive skills (59). Studies have also reported that

PKC phosphorylation of mGluR5 (60) and mGluR7 (61) inhibits CaM binding and vice-versa. Additionally, mRNA expression of both PKC and CaM was observed to decrease in the hippocampus as a result of chronic lead exposure (62), while conversely, a study by Xu *et al* reported that Pb²⁺ exposure produced no obvious effect on hippocampal *mGluR3* and *mGluR7* mRNA expression, suggesting that these isoforms might not be associated with Pb²⁺-induced neurotoxicity (63). Moreover, a study by Nakajima reported that CaBPI (also known to bind Pb²⁺ (64)) competes with CaM for binding with presynaptic group III mGluRs 4, 7, and 8, in a Ca²⁺-dependent manner, and this binding can be blocked by PKC (61). These results suggest that Pb²⁺ may act on mGluR indirectly, either through binding with PKC or CaM, or by interfering with the expression levels of the two proteins, which may be indicative of another route of neurotoxicity not reported with the GABAergic system and other cited proteins.

N-methyl-D-aspartate receptor (NMDAR)

The N-methyl-D-aspartate receptor (NMDAR) is important for presynaptic plasticity through the generation and/or release of trans-synaptic retrograde signalling molecules, such as brain-derived neurotrophic factor (BDNF) (65–68). Pb²⁺ is a potent NMDAR antagonist (69, 70). Both exposure to Pb²⁺, and inhibition of NMDAR through its antagonist aminophosphonovaleric acid (APV), exhibit similar effects, including the disruption of developing synapses by reducing selective synaptic proteins, Syn and Syb, and this activity can be rescued by BDNF (71). This indicated that the effect is mediated via NMDAR inhibition and disruption of BDNF signalling (14). NMDAR consists of an obligatory NR1 subunit along with one or more accessory subunits from the NR2 and NR3 families (16). Pb²⁺ has been shown to bind the Zn²⁺ regulatory site in NMDAR in a voltage-independent manner with greater binding affinity to its NR2A subunit (72). This result is supported by electrophysiological studies where NR2A-NMDARs are inhibited by Pb²⁺ more than its other forms (73, 74). Excessive Pb²⁺ exposure has also been shown to correlate with reduced NR2A in the hippocampus (75–78), altered expression of NR1 splice variants (79), and slightly increased or unchanged NR2B mRNA levels (49, 75–78, 80). Altered expressions of NR2 family members consequently could interfere with NR2 related downstream pathways such as MAPK signalling (81), calcium/calmodulin kinase II (CaMKII) activity (82) and cyclic AMP response element binding protein (CREB) phosphorylation and binding affinity (80, 83).

Neuronal calcium sensor protein-1 (NCS-1)

Neuronal calcium sensor protein 1 (NCS-1) is a Ca²⁺-sensing EF-Hand protein involved in the regulation of neurotransmission (84), neuron development (85, 86), and cognitive functions (87). NCS-1, when activated by Ca²⁺, interacts with a wide variety of downstream proteins (23), including dopamine receptor D2R (88), Ca²⁺-dependent activator protein for secretion (CAPS), phosphatidylinositol-4 kinase-IIIβ (PI4KIIIβ) (89, 90), ARFI (23, 90), AP1, AP2, TGFBR1, and calcineurin, among others. Despite having a low sequence identity to CaM (20%), NCS-1 possess four EF-Hand motifs similar to CaM (23). Likewise, NCS-1 is very sensitive to small increases in intracellular Ca²⁺ concentrations. As seen in the structure of NCS-1 (Fig. 2C), Ca²⁺ binds in only three of the four EF-Hand sites, while the

fourth site can accommodate Na^+ . Although a review of the literature failed to identify a direct link between Pb^{2+} neurotoxicity and NCS-1, the fact that NCS-1 is an EF-Hand protein with high affinity for Ca^{2+} strongly suggests that it could bind Pb^{2+} in one or more of the EF-Hand sites, including the site that is unable to bind Ca^{2+} , and that interaction with Pb^{2+} could alter downstream activity when NCS-1 interacts with other proteins.

Moreover, while NCS-1 interacts with various proteins that are not directly involved in neurotransmission, these interactions can involve other cellular regulatory and assembly activities that are critical support functions that may be indirectly affected by Pb^{2+} . For example, ADP-ribosylation factor 1 (ARF1) is a GTPase that interacts with NCS-1 and $\text{PI4KIII}\beta$, and regulates vesicular trafficking in cells (23, 91). ARF1 also performs several important functions at the Golgi complex related to vesicle coat assembly and trafficking from the trans-Golgi-network to the plasma membrane and endosomes (92). Although no evidence to date suggests that ARF1 interacts directly with Pb^{2+} , it does interact with Ca^{2+} -binding NCS-1 and may have other functions regulated by Ca^{2+} availability.

Conversely, CAPS does play an important role in neural signalling. CAPS exists in two primary isoforms in neuroendocrine cells and the brain, CAPS1 and CAPS2. CAPS1 is a cytosolic protein that plays a role in large dense-core vesicle (LDCV) exocytosis and neurotransmitter release, while CAPS2 is also expressed in the lung, liver, and testis (93). The two isoforms are expressed differently during development, with low expression of CAPS1 prior to birth, followed by increased expression until postnatal day 21, while expression of CAPS2 remains constant from embryonic day 10 until postnatal day 60. Due to differences in the permeability of the BBB during fetal development as compared with adults, the developing brain may be subject to increased accumulation of Pb^{2+} (94), and Pb^{2+} may act either directly or indirectly (through Pb^{2+} -modulated NCS-1) with CAPS proteins during embryonic or early childhood development. This would represent another potential route for neurotoxicity that could contribute to the observed problems associated with neural development and cognitive deficiencies observed in children.

Stim and Orai

A recent study by Moccia *et al*/reported that Stim1-2 and Orai1-2 are expressed in neurons (95). Stim1 is a Ca^{2+} -sensing EF-Hand protein that responds to decreasing Ca^{2+} concentration in the ER by activating ORAI1 to refill the ER store of Ca^{2+} . Results of this study suggested that store operated Ca^{2+} entry (SOCE) may play a role in the excitation of neuron and the regulation of synaptic activity, and may further contribute to neurological disorders (95). As these are all Ca^{2+} -modulated activities, they also represent potential targets for disruption by Pb^{2+} .

Pb^{2+} effects on functional activity of proteins associated with Ca^{2+}

The effects of Pb^{2+} on the functional activities associated with Ca^{2+} vary in a concentration dependent manner, with protein activation observed at low concentrations and inhibition observed with increasing Pb^{2+} concentration (Fig. 1). At initially low concentrations, CABPs may not be activated by competing essential metals, but instead may be activated by

toxic metals mimicking their native metals. For example, the intracellular protein CaM may be activated by either Pb^{2+} (32) or Cd^{2+} (96), and Richardt *et al* reported that Pb^{2+} will bind with CaM, Parvalbumin, TnC, CaBPI and CaBP II, with the highest Pb^{2+} affinities being observed with CaM and CaBPI (64). Several other studies have reported that Pb^{2+} may initially activate and then deactivate CABPs with increasing concentration. Habermann *et al* (32) found that Pb^{2+} could replace Ca^{2+} in CaM, and the Pb^{2+} -CaM complex subsequently activated cyclic nucleotide phosphodiesterase (PDE) to phosphorylate several membrane proteins. This behavior was observed up to a Pb^{2+} concentration of near 10^{-4} M, which corresponds to the minimum Ca^{2+} concentration required for activation. Phosphorylation was subsequently inhibited with increasing concentration of Pb^{2+} . This suggests that Pb^{2+} may activate CaM at lower concentrations by mimicking Ca^{2+} in the EF-Hand binding sites (Fig. 2D and Fig. 2F), with the reported loss of activity at increasing Pb^{2+} concentration suggesting that Pb^{2+} can bind to another region of the protein, resulting in allosteric inhibition. Additionally, Chao *et al* reported that Pb^{2+} produces a biphasic activation and inactivation of MLCK with increasing Pb^{2+} concentration, but only in the presence of CaM, indicating that Pb^{2+} interacts directly with CaM, not MLCK (97). Chao *et al* reported in a later study that Pb^{2+} activated TnC, which subsequently stimulated myofibrillar ATPase (98). Therefore, the effects of Pb^{2+} on different processes may occur indirectly via interactions with upstream proteins.

Additionally, there is little evidence indicating that Pb^{2+} occupancy of the Ca^{2+} sites inhibits the function of the protein. Instead, the evidence suggests that it impersonates Ca^{2+} in these sites, thereby falsely activating the protein and altering downstream activities at inappropriate times. However, the subsequent inhibition of these proteins at higher concentrations suggests a second mode of toxicity where inactivation or inhibition of the protein is induced as result of Pb^{2+} binding to the proteins in regions outside of the normal Ca^{2+} -binding sites (Fig. 3).

Pb^{2+} toxicity and ionic displacement in proteins

Under normal cellular conditions, proteins are generally not activated by competing essential metal ions (e.g., Ca^{2+} , Zn^{2+} , Mn^{2+} or Fe^{2+}). As example, Mg^{2+} ions can occupy intracellular Ca^{2+} -binding sites in CaM, when the concentration of Ca^{2+} is low, but doing so occurs without activation of CaM. The Mg^{2+} ions are then displaced with an influx of the higher affinity intracellular Ca^{2+} ions to activate the protein. A study by Ouyang *et al* reported that Mg^{2+} binding in the EF-Hand sites of CaM did not produce conformational changes (99), explaining its inability to activate the protein. However, it is possible that Mg^{2+} (and/or Zn^{2+}) allosterically regulates binding of Ca^{2+} to some extent, via binding at auxiliary sites on the protein (100).

The ability of ions to occupy metal binding sites and interact with ligands is related to a number of variables, including: the ionic radius (101); charge and electronegativity of the metal ion; the types of ligands in the binding sites, as well as the structure and coordination geometry of the site; affinity; and ion concentration. Despite the differences in binding geometries and ligand types between Ca^{2+} (6–8 oxygen ligands) and Zn^{2+} (4–6 sulfur and other ligands), Pb^{2+} can also replace Zn^{2+} in proteins (102–107), which points at the

versatility of Pb^{2+} binding and interestingly, several studies have identified this as one route through which Pb^{2+} induces neurotoxicity by disrupting the Zn^{2+} -dependent modulation of NMDAR activation as mentioned above (16, 72). For example, while both Ca^{2+} and Zn^{2+} have identical charges, the ionic radius of Zn^{2+} is about 0.75 Å compared with approximately 1.0 Å for Ca^{2+} , and Zn^{2+} utilizes different binding ligands than Ca^{2+} (more sulfur than oxygen) with a tetrahedral coordination geometry (108), so that the two ions are generally not competitive for their respective target proteins. The stronger binding of Zn^{2+} (10^{18} – 10^{10} M^{-1}) (109, 110) compared to Ca^{2+} (10^7 M^{-1}) (109) to the binding sites in proteins, along with the smaller off rate of Zn^{2+} (10^{-9} s^{-1}) (110) as compared to that of Ca^{2+} (10^3 s^{-1}) (109, 111) should also be considered. Therefore, the exchange of Zn^{2+} with Pb^{2+} is likely much slower and less probable than the exchange of Ca^{2+} with Pb^{2+} , even when the binding constant of Pb^{2+} to Zn^{2+} sites are higher in magnitude than the binding of Pb^{2+} to Ca^{2+} .

Classification of an ion as being a hard, soft, or borderline Lewis acid also appears to play a role in the interaction affinity between ions and proteins. This is particularly relevant with respect to toxic metal ions, which can occupy metal binding sites in proteins, thereby replacing or displacing the targeted essential metal ions. The divalent Ca^{2+} and Pb^{2+} ions are identical in charge and can be similar in size (0.99 – 1.12 Å vs. 1.12 – 1.19 Å, respectively) depending on the coordination geometry (112). However, Pb^{2+} is approximately 2.3 times as electronegative as Ca^{2+} , and is classified as a borderline Lewis acid, whereas Ca^{2+} is classified as a hard Lewis acid. The Pb^{2+} ion therefore tends to be more polarizable with lower energy requirements to achieve its highest occupied molecular orbitals (HOMOs), likely determining its ability to occupy metal binding sites with higher affinities (Fig. 2E).

Structural analysis of Pb^{2+} vs. Ca^{2+} binding sites

Previous work in our laboratory has included a comprehensive statistical analysis of CaBP structural data from the Protein Data Bank (PDB) (113). This study evaluated 1,605 Ca^{2+} -binding sites from 558 PDB files with higher resolutions ($R > 2.0$ Å). The Ca^{2+} -binding sites were further sub-divided into EF-Hand (EF) sites with well-defined pentagonal bipyramidal geometries, and a more general group of structurally-diverse non-EF-Hand (NEF) sites. Structural parameters used to evaluate the sites included binding ligand type (atom type and sidechain), coordination number, and ligand/ion distances and angles. Subsequently, a similar analysis was conducted for proteins reported to bind Pb^{2+} . To better understand how Pb^{2+} displaces Ca^{2+} in proteins, data from both data sets were then compared (112), and the results of these studies (Table 1) indicated that Pb^{2+} sites typically utilized fewer total ligands (CN = 2 – 5) and less mean negative charge by site (charge = –2) than canonical EF-Hand Ca^{2+} sites (CN = 4 – 8, charge = –3). Differences in ligand selections were also observed. While Ca^{2+} utilizes oxygen atoms almost exclusively in the EF-Hand sites, the Pb^{2+} sites were more likely to contain either nitrogen or sulfur atoms in close enough proximity to represent potential ligands in addition to oxygen, making its behavior similar to both Ca^{2+} and Zn^{2+} . Additionally, while the EF sites utilized approximately the same ratio of oxygen from Glu and Asp residues (26.6% and 29.7%), Pb^{2+} was observed to favor Glu over Asp (38.4% vs. 20.3%), presumably due to the longer sidechain of Glu providing more flexibility to accommodate the larger ionic radius of the Pb^{2+} ion (Fig. 2E). This is

consistent with observations that when Pb^{2+} occupies EF-Hand sites, the ion is observed to sit slightly higher above the pentagonal plane than Ca^{2+} (Fig. 2E).

From a structural perspective, the NEF sites exhibited much greater diversity with respect to coordination number than EF sites, and generally could be classified as having a spherical (holo-directed), semi-spherical (hemi-directed), or planar geometry surrounding the Ca^{2+} ion (114), which was also previously reported for binding of Pb^{2+} (108). The holo-directed geometry is consistent with higher CN values observed with EF-Hand Ca^{2+} -binding sites, while the hemi-directed geometry was more prevalent with Non-EF-Hand Ca^{2+} -binding sites, and with Pb^{2+} -binding sites (Table 1). Based on the similarity of these binding sites, and despite variances in the number of coordinating ligands, it is possible that the NEF sites may represent incomplete pentagonal-bipyramidal structures that evolved in response to different needs within the Ca^{2+} -signalling pathways. Comparatively, the hemispheric geometry observed with C2 domain Ca^{2+} -binding sites, as seen with PKC (114), is also observed with Pb^{2+} -binding, and from the perspective of our previous analyses (Table 1), when EF-hand sites were excluded, the differences between binding of Pb^{2+} and Ca^{2+} diminished significantly (112).

Additionally, a detailed comparison of CaM structures bound with Ca^{2+} (PDB ID 1EXR) and Pb^{2+} (PDB ID 1N0Y) revealed only minor deviations in coordination geometry, distances between ligands and ions, bond angles, and RMSD values, when comparing the two protein backbones for the four EF-Hand sites (112). These data therefore suggested only minimal disruption to the structure based on ionic displacement, which was consistent with results reported by Kursula and Majava (115). However, the crystal structure of the Pb^{2+} -bound CaM (Fig. 2B) exhibits binding of several ions outside of the EF-Hand sites, including at the central helix connecting the N- and C-terminal domains of the protein, where significant folding of the protein was observed. While this may potentially represent an artefact of crystallization, the region in question frequently appears as an extended helix in crystalized structures of the Ca^{2+} -loaded protein (116, 117), and as a partially unwound coil in solution (118, 119), and these conformational changes may differentiate the Ca^{2+} -free and Ca^{2+} -loaded states. The flexibility of this region is believed to be necessary for conformational changes associated with peptide binding (Fig. 3), and a recent study has provided evidence that disruption within this region contributes to folding of a more compact structure associated with the Ca^{2+} -bound state (120), as has been reported for osteocalcin (121), while binding of Ca^{2+} in the four EF-Hand sites may result in less flexibility (122). The compact folding observed in the crystalline structure of Pb^{2+} -bound CaM suggested a potential allosteric binding site for Pb^{2+} in this region, and results of structural analyses identified the carboxyl-rich region from residues 78–84 as a strong candidate for binding of Pb^{2+} (Fig. 2A) (123).

Analyses of structural data from the PDB indicate that Pb^{2+} can occupy the binding sites of various metals with a broad range of coordination values, and may also bind opportunistically outside of known binding sites, suggesting multiple binding modes and a promiscuous interaction with proteins, which would be consistent with the systemic effects produced as a result of elevated Pb^{2+} concentrations, including neurotoxicity.

NMR/Binding studies

Although various studies have demonstrated that Pb^{2+} can physically occupy Ca^{2+} -binding sites, displacement of Ca^{2+} by Pb^{2+} would depend on other factors, including concentrations of the ions and their relative binding affinities for a given binding site. Unfortunately, determining accurate binding affinities for Ca^{2+} and Pb^{2+} remains a challenge for several reasons. A general problem is that available analytical instrumentation often lacks sufficient sensitivity to detect very low concentrations of analytes that would correspond to high affinity binding (124). Additionally, for many EF-Hand Ca^{2+} -binding proteins, where binding sites are frequently paired and appear to bind cooperatively, methods to determine K_d values for individual sites, such as deactivating one of the paired sites through site-directed mutagenesis (125), or grafting an individual EF-site into a scaffold protein (126), may not provide reliable affinity data as the process eliminates the effects of cooperativity. Moreover, cooperativity may occur both within and between domains. For example, each of the paired EF-hands in the respective N- and C-terminal domains of CaM appear to bind Ca^{2+} ions cooperatively (127), but further evidence suggests that the domains bind cooperatively, as well, so that Ca^{2+} occupies both EF-hand sites in the higher affinity C-terminal domain sites first, which produces minor conformational changes that enhance binding in the EF-hand sites in the N-terminal domain (128). These cooperative effects appear to be of critical importance for producing conformational changes in responses to fluctuations in Ca^{2+} concentrations (129), which would directly affect Ca^{2+} signal transduction.

As these approaches have provided very limited or incomplete data on affinity, various alternative methods of approximation have been employed to investigate Ca^{2+} -binding affinity at the molecular or domain level, rather than the individual sites. These approaches may involve calculated upper and lower limits (130), relative affinities determined by the order in which the domains bind Ca^{2+} , or spectrofluorometric approaches that monitor fluorescence changes in Phe and Tyr side chains that result from conformational changes associated with metal binding. This latter approach has been used to determine domain-level binding affinities for Ca^{2+} with CaM, as Phe produces strong signals in the N-terminal domain, while Tyr produces similar results in the C-terminal domain (131, 132). In another approach, Richardt *et al* utilized flow dialysis methods to evaluate competitive binding between Ca^{2+} and Pb^{2+} or other toxic metals, with a variety of intracellular Ca^{2+} -binding proteins, and calculations of IC_{50} values for the various protein/metal complexes (i.e., interaction at the molecular level), indicated that the relative binding affinities were: for CaM, $\text{Pb}^{2+} > \text{Ca}^{2+} > \text{Cd}^{2+}$; for troponin C, $\text{Ca}^{2+} > \text{Cd}^{2+} > \text{Pb}^{2+}$; for CaBP I, $\text{Ca}^{2+} \sim \text{Pb}^{2+}$; for CaBP II, $\text{Ca}^{2+} > \text{Pb}^{2+} > \text{Cd}^{2+}$; and for parvalbumin, $\text{Cd}^{2+} \sim \text{Ca}^{2+} > \text{Pb}^{2+}$ (64). Thus, while different CABPs may bind Pb^{2+} , the affinities can vary significantly, indicating that ionic displacement is not universal, and this study verified that CaM represents an important target for Pb^{2+} toxicity at the molecular level.

Nuclear magnetic resonance (NMR) is another powerful tool that can be used to study protein structure and monitor dynamic properties, conformational changes and binding affinity trends. A recent study used NMR and fluorescence spectroscopy to study different aspects of Pb^{2+} binding in CaM, including calculation of dissociation constants (K_d) for

binding of Pb^{2+} with CaM, and to determine whether Pb^{2+} displaces Ca^{2+} in the EF-hand binding sites (123). In the NMR study, data were collected using 2D ^{15}N -HSQC. A baseline of chemical shift data for Ca^{2+} was collected at different concentrations of Ca^{2+} , starting with a Ca^{2+} -free structure. The magnitude of the differences in chemical shifts for residues in the EF-Hand sites was used to monitor binding of Ca^{2+} . Results of this part of the study indicated that Ca^{2+} was first bound to the two EF-Hand sites (III and IV) in the higher affinity C-terminal domain, and these events further altered the structure of the N-terminal domain prior to binding of Ca^{2+} in EF-Hand sites I and II, which would be consistent with interdomain cooperativity. Repeating the experiment, but substituting Pb^{2+} for Ca^{2+} , failed to identify an order of domain occupancy, as was observed with Ca^{2+} , suggesting that the affinities of the four EF-Hand sites for Pb^{2+} were similar, which was consistent with results of other studies (99). However, the addition of Pb^{2+} to Ca^{2+} -saturated CaM produced interesting results which suggested that Pb^{2+} displaced Ca^{2+} only in the N-terminal domain, where sites EF-I and EF-II bind Ca^{2+} with lower affinity, but not the C-terminal domain sites. Moreover, further addition of Pb^{2+} produced conformational changes that were consistent with binding of Pb^{2+} outside of the EF-Hand sites, which led us to hypothesize that increasing concentrations of Pb^{2+} result in opportunistic binding of Pb^{2+} in one or more auxiliary sites on the protein, resulting in significant conformational changes to the protein capable of inhibiting its activity, which would be consistent with the biphasic results observed with the interaction between Pb^{2+} -CaM and PDE (32).

In the same study, K_d values for binding of Ca^{2+} and Pb^{2+} were calculated by monitoring fluorescence changes from Phe in the N-terminal domain and Tyr in the C-terminal domain. While this approach does not allow for separation of the individual binding sites, affinity values for the domains were determined, indicating that K_d values for Ca^{2+} for both the N-terminal ($11.50 \pm 0.68 \mu\text{M}$) and C-terminal ($2.04 \pm 0.02 \mu\text{M}$) were consistent with known intracellular concentrations and previously reported values (133). For Pb^{2+} , the K_d values reported for the N- and C-terminal domains were $1.40 \pm 0.30 \mu\text{M}$ and $0.73 \pm 0.10 \mu\text{M}$, respectively, which indicated that Pb^{2+} had slightly higher affinity for the EF-Hand sites than Ca^{2+} . Interestingly, the tyrosine fluorescence exhibited a biphasic response: an increase in fluorescence similar to the Ca^{2+} response followed by a decrease in intensity with increasing Pb^{2+} concentration. This response was interpreted as either the presence of a single higher affinity binding site (Fig. 3), or a conformational change associated with opportunistic binding of Pb^{2+} in an auxiliary site, and the associated K_d was calculated to be $1.93 \pm 0.32 \mu\text{M}$, which would indicate greater affinity than that observed for Pb^{2+} in the N-terminal domain. If this latter hypothesis were correct, it would help to explain the biphasic response of CaM activation to Pb^{2+} , where trace concentrations can activate the protein, which is subsequently deactivated with increasing concentration. The study further noted that Pb^{2+} did not produce conformational changes when Pb^{2+} was introduced to Ca^{2+} -loaded CaM, despite differences in binding affinity values, which suggests that Pb^{2+} either displaces Ca^{2+} without disrupting the structure, or Pb^{2+} fails to displace Ca^{2+} , but can deactivate the protein through opportunistic binding in a secondary site (123). In the latter case, it is possible that Pb^{2+} may bind opportunistically to regions of the protein outside of the normal metal binding sites, such as the carboxyl-rich central helix of CaM (Fig. 3), thereby allosterically inhibiting protein function through structural alteration. As previously noted, studies have

identified potential secondary sites in CaM that may bind other physiologically relevant ions. Consistent with this, Kursula and Majava identified a 5th Ca²⁺-binding site in CaM, located in the central helix (115), and Bertini *et al* reported that Yb³⁺ may bind in this region as well (134). Results from these studies suggest that this region might represent a potential target for binding of Pb²⁺, and structural studies of Pb²⁺-CaM complexes in our lab further support this hypothesis (123).

A new perspective for the molecular basis of Pb²⁺ toxicity

Historically, molecular toxicity was believed to be caused by the displacement of Zn²⁺ or Ca²⁺ by Pb²⁺ ions, and various studies have since demonstrated that Pb²⁺ can occupy binding sites for many physiologically essential metals, including: Mg²⁺ in pyrimidine 5'-nucleotidase type 1 (135); Zn²⁺ in 5-aminolevulinic acid dehydratase (ALAD) (136, 137), resulting in iron deficiency associated with anaemia; and Fe²⁺ in divalent metal transporter-1 (DMT1), which appears to be involved in transport and uptake of Pb²⁺ (138, 139). The Pb²⁺ ion can also occupy Ca²⁺-binding sites, and/or displace Ca²⁺ in many proteins, including voltage-gated calcium channels (VGCCs) (140), troponin C (98), CaM (32), PKC (58), parvalbumin (64), and synaptotagmin (40). Moreover, CABPs have also been observed to bind a number of different non-essential metals in addition to Pb²⁺, including Sr²⁺, Hg²⁺, Cd²⁺ and many lanthanides (141–145). Similar results have been reported in studies involving other toxic metals (e.g., Hg²⁺ and Cd²⁺) and in different proteins, and these data were generally consistent with a displacement model where toxic metals replace essential metals in binding sites (146).

However, studies have also reported that CABPs may be activated by binding of toxic metals in the primary metal binding sites. As previously noted, the intracellular signalling protein CaM may be activated by either Pb²⁺ (32, 64, 101, 147, 148) or Cd²⁺ (96). Moreover, evidence suggests that Pb²⁺ may initially activate and then deactivate CABPs with increasing concentration, as seen with CaM (147). This in turn affects CaM's subsequent interaction with phosphodiesterase (PDE) (32) and myosin light chain kinase (MLCK) (97). A biphasic response of this nature suggests that toxic metal ions like Pb²⁺ may effectively mimic the Ca²⁺ ion in the Ca²⁺-binding site in certain proteins by inappropriately activating the protein, rather than directly inhibiting the protein function, and increasing concentration subsequently inactivates the protein via a different mechanism or site. Research on CaM suggested that Pb²⁺ may alter the functional activity of the protein by binding opportunistically in regions of the protein outside of the EF-hand Ca²⁺ binding sites in a concentration-dependent manner, and in the presence of Ca²⁺ (112, 123), which would indicate an allosteric inhibitory effect. Similar results were reported by Shirran and Barran (149), and Mills and Johnson (142). Additionally, other studies have identified secondary sites in CaM capable of binding Ni²⁺ (in the Ca²⁺-bound state) (150), or Zn²⁺ (100), which, in the latter study, appears to inhibit binding of Ca²⁺ in the EF-Hand sites. The ability of Pb²⁺ to mimic Ca²⁺ may also produce neurotoxic effects indirectly. Exposure to Pb²⁺ has been observed to inhibit Ca²⁺-dependent glutamate and γ -aminobutyric acid (GABA) release in the hippocampus (47, 151, 152), while results of a study by Ferguson *et al* reported that Pb²⁺ produced an increase in Ca²⁺ efflux from the neural cell by activating CaM, which then stimulates Ca²⁺ ATPase, an enzyme that regulates Ca²⁺ concentration in

the cell (153). Similarly, Pb^{2+} ions may inhibit voltage-gated calcium channels (VGCCs) that regulate synaptic transmission, and subsequently utilize the channels for entry into the cell where they presumably can interact with other proteins (140, 154). Studies also suggest that Pb^{2+} interferes with Ca^{2+} in PKC, a protein that plays a significant role in cognitive functions (155). Additionally, Pb^{2+} may disrupt the Zn^{2+} -dependent modulation of *N*-methyl-D-aspartate receptor (NMDAR) activation (16, 72), which regulates the flow of Ca^{2+} and Na^{+} into the cell.

Collectively, these data indicate that Pb^{2+} may interact with multiple proteins involved in neural pathways, and while Pb^{2+} can interact with proteins that are not specifically CaBPs, the information presented in this review has only focused on the interactions between Pb^{2+} and CaBPs or proteins involved in Ca^{2+} -signalling pathways, and how they relate to neurotoxicity, based on three potential modes of Pb^{2+} activity as follows (Fig. 3):

1. Pb^{2+} may occupy Ca^{2+} -binding sites, altering the structure of the protein and inhibiting the activity of the protein.
2. Pb^{2+} may occupy Ca^{2+} -binding sites and mimic Ca^{2+} , thereby falsely activating the protein and perturbing downstream activities.
3. Pb^{2+} may bind with proteins in sites other than Ca^{2+} -binding sites, thereby altering the conformation and function of the protein.

The picture that is emerging from this research suggests that there are multiple points within the Ca^{2+} signalling pathways in neurons and other cells that may be disrupted by the presence of even low concentrations of Pb^{2+} . All of the available evidence strongly indicates that Pb^{2+} interacts with CaM, either through ionic displacement, or through opportunistic binding, which likely disrupts a wide range of downstream activities, and it is possible that Pb^{2+} could interact with NCS-1 in a similar manner, given the functions of NCS-1 summarized in this review, and their relationships with known effects of neurotoxicity.

The mechanisms discussed in this review can further provide insights into other systemic physiological problems associated with Pb^{2+} exposure. For example, Pb^{2+} may induce nephrotoxicity through high-affinity interaction at low concentrations with CaSR, (156–159), and long term activation of the CaSR can lead to renal injury by inhibiting growth of epithelial cells and stimulating growth of fibroblasts (160–162). Similarly, Pb^{2+} exposure may be a contributing factor in cardiotoxicity, producing structural changes in cardiac tissues (163, 164), while a direct correlation between hypertension and Pb^{2+} exposure has been reported (165).

The above associations however, do not have firmly-established mechanistic explanations, and while Pb^{2+} ions may occupy the Ca^{2+} -binding sites in different CaBPs, it cannot be assumed that they will replace Ca^{2+} ions, as the affinity for Pb^{2+} may vary differently than the affinities for Ca^{2+} . In the case of the neuron, variations in affinities for Ca^{2+} and Pb^{2+} could influence the activation or inhibition of different CaBPs and alter the Ca^{2+} -mediated pathways. For example, while both Ca^{2+} and Pb^{2+} promote exocytosis of vesicular catecholamine, the process is regulated by PKC and calcineurin in the presence of Ca^{2+} , and by CaM kinase II in the presence of Pb^{2+} (166). Because Ca^{2+} -regulated activity within the

neuron is complex due to different types of Ca^{2+} signals that can translate into subtle physiological differences, or produce opposing effects depending on their timing and magnitude (23), it is reasonable to assume that extensive research would be required to decipher the particular variations that might occur as a result of Pb^{2+} introduction into the cell.

Finally, the seemingly incongruent reported dose-response on the loss of IQ of children, where low concentrations of Pb^{2+} appear to produce greater IQ loss than higher exposure to Pb^{2+} (12, 167, 168), may be better understood in the context of a biphasic response of CABPs such as CaM to Pb^{2+} , where the protein is falsely activated at lower Pb^{2+} concentrations, thus initiating deleterious Ca^{2+} -modulated responses, which are subsequently inhibited by further allosteric interaction with Pb^{2+} at higher concentrations. Because anthropogenic Pb^{2+} in the environment represents a continuing problem, as seen recently with the contaminated water supply in Flint, Michigan (<http://www.motherjones.com/environment/2016/02/flint-lead-poisoning-america-toxic-crisis>), understanding the mechanisms associated with Pb^{2+} neurotoxicity is an important step towards understanding the impact of exposure on human health, and as a basis for environmental efforts and the enactment of policies directed towards elimination of this threat.

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Fig. 1.

Ca²⁺ and Pb²⁺ targeted proteins involved directly and indirectly in neurotoxicity and calcium homeostasis are illustrated. The Ca²⁺ gradient is maintained at nM range intracellularly and at a mM range extracellularly in neurons. The black arrows represent the influx and the yellow arrows represent the outflux of divalent metal ions in the system. A general activity profile of receptor proteins is shown by a sigmoid and a bell curve as a result of Ca²⁺ and Pb²⁺ ions, respectively. Pb²⁺ is known to activate Ca²⁺ associated neuronal proteins at low concentrations and deactivate at higher concentrations, as characterized by a bell curve.

Fig. 2.

(A) Proposed allosteric binding site for Pb^{2+} in human Ca^{2+} -bound CaM (PDB ID 4bw8) in carboxyl-rich region of central helix comprising residues 78-84 (DTDSEEE). (B) Pb^{2+} -bound human CaM (PDB ID 4bw8) (C) Human Ca^{2+} -bound NCS-1 (PDB ID 4guk) with four EF-Hand sites similar to those observed in CaM (D) Pb^{2+} bound in site EF-IV of CaM (PDB ID 2v01). (E) Comparison of Ca^{2+} - and Pb^{2+} -binding characteristics with respect to the pentagonal plane. $D_{\text{Lig-Ca}}$ and $D_{\text{Lig-Pb}}$ indicate distances between oxygen ligands and ions. C-Lig-Ca and C-Lig-Pb represent angles ϕ formed between the carbon atoms, ligands and the ions. D_{Ca} and D_{Pb} indicate ion distance above the pentagonal plane formed by C^γ , $\text{O}^{\delta 1}$ and $\text{O}^{\delta 2}$. (F) Ca^{2+} bound in site EF-IV of CaM (PDB ID 3cln). Figs. 2A, B, C, D, and F rendered with Chimera (24).

Fig. 3.

Proposed mechanism for Pb^{2+} toxicity in CaM. Path A illustrates normal activity associated with binding of Ca^{2+} . Ca^{2+} first cooperatively binds the two higher affinity sites EF-III and EF-IV, followed by cooperative binding in sites EF-I and EF-II. The Ca^{2+} loaded protein then interacts with ligand molecules. In Path B, Pb^{2+} binds in a single higher affinity site in the C-terminal domain, while the remaining sites may bind Pb^{2+} with equivalent affinity. Binding may not occur cooperatively. At lower concentration of Pb^{2+} , CaM is activated and functions as if bound with Ca^{2+} . In either the Ca^{2+} or Pb^{2+} loaded states, an increase in the concentration of Pb^{2+} results in Pb^{2+} binding to a secondary site in the central helix, altering the conformation of the protein and allosterically inhibiting the activity of the protein.

Table 1Summary of statistics comparing Pb²⁺ and Ca²⁺ binding sites in proteins.

	Pb		Ca	
	DS HR	DS Final	NEF	EF
Total PDB proteins in study	7	21	525	33
Total binding sites evaluated	27	48	1468	137
Total target ligand atoms	105	177	8549	958
Total O _{aa} ligands	86	118	5626	829
Total O _{HOH} ligands	16	36	2788	127
Total N ligands	3	10	135	2
Total S ligands	0	13	0	0
Total sites with N ligands	3	9	106	2
Mean CN, PLW	4 ± 2	4 ± 2	6 ± 2	7 ± 1
% CN 2–5	78	77	31	4
% CN 6–9	22	17	47	96
Mean CN, PL	3 ± 2	3 ± 2	4 ± 2	6 ± 1
% CN 2–5	70	73	58	9
% CN 6–9	15	8	27	91
Mean charge by site	–2	–2	–1	–3
Total identified bidentate pairs	24	36	777	129
Total sites with bidentate ligands	21	30	664	125
% Sites with bidentate ligands	78	63	45	91
Mean distance, Pb–O _{aa} (Å)	2.7 ± 0.4	2.7 ± 0.4	---	---
Mean distance, Pb–N (Å)	2.7 ± 0.3	2.6 ± 0.4	---	---
Mean distance, Pb–S (Å)	---	3.2 ± 0.3	---	---
Mean distance, Ion–O _{HOH} (Å)	2.8 ± 0.3	2.8 ± 0.4	2.5 ± 0.3	2.1 ± 0.1
Mean distance, Ca–O _{carbonyl} (Å)	---	---	2.4 ± 0.2	2.3 ± 0.1
Mean distance, Ca–O _{SC} (Å)	---	---	2.4 ± 0.2	2.4 ± 0.2
Mean distance, Ca–O _{Bidentate} (Å)	---	---	2.6 ± 0.3	2.5 ± 0.2

CN=Coordination Number. PL=Protein Ligands only. PLW=Protein Ligands and Water. O_{aa}=Amino acid oxygen ligands. O_{HOH}=Water oxygen ligands. O_{carbonyl}=Carbonyl oxygen. O_{SC}=Sidechain oxygen. O_{bidentate}=Bidentate oxygen ligands. DS HR=High resolution dataset (R less than 1.76 Å). DS Final=Total dataset from PDB. NEF=Non-EF-Hand calcium binding sites. EF=EF-Hand calcium binding sites.