Published in final edited form as:

J Pharmacol Toxicol Methods. 2012 ; 65(3): 122–125. doi:10.1016/j.vascn.2012.03.005.

Novel Approach for Generation of Low Calcium Reagents for Investigations of Heavy Metal Effects on Calcium Signaling

Katelyn Y. Niu^{1,2}, Nathaniel Noyes^{1,3}, and Thomas W. Abrams^{1,2,3,a}

¹Department of Pharmacology, University of Maryland School of Medicine, 655 West Baltimore Street, Baltimore, MD 21201-1559

²Program in Neuroscience, University of Maryland School of Medicine, 655 West Baltimore Street, Baltimore, MD 21201-1559

³Program in Molecular Medicine, University of Maryland School of Medicine, 655 West Baltimore Street, Baltimore, MD 21201-1559

Abstract

Lead exposure can cause learning disabilities, memory loss and severe damage to the nervous system. However, the exact mechanism by which lead causes learning disabilities is not fully understood. The effects of lead on calcium-regulated signaling pathways are difficult to study biochemically; with the traditional method of controlling the free calcium concentration with EGTA, the exact concentrations of free lead and calcium ions in solution are interdependent and prone to error because EGTA also buffers lead. In our approach, we first reduced the free calcium concentration in the solution using calcium-binding resins before adding lead to buffers. The solution was sequentially treated with Chelex-100 ion exchange resin, followed by immobilized BAPTA resin. The final concentration of free calcium in the solution was measured with Fluo-3 indicator. Our protocol successfully produced buffers with free calcium levels below 15 nM, which is substantially below threshold for activation of calcium-dependent enzymes in signaling pathways (which is typically a few hundred nanomolar calcium, when determined *in vitro*). This method provides an improved approach to study the effect of heavy metals on calcium-stimulated signaling pathways.

Keywords

lead; calcium; calmodulin; adenylyl cyclase; synaptic plasticity; calmodulin-dependent kinase II

© 2012 Elsevier Inc. All rights reserved.

^acorresponding author, Department of Pharmacology, University of Maryland School of Medicine, BRB 4-002, 655 West Baltimore Street, Baltimore, MD 21201-1559, Phone: 410-706-5837, Fax: 410-706-0032, tabrams@umaryland.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Introduction

Lead (Pb^{2+}) exposure can have severe effects on the central nervous system and on a number of peripheral systems in the body including the heart, bones, intestines, kidneys and reproductive systems [1-4]. Because Pb^{2+} poisoning often occurs with no obvious immediate symptoms, it frequently goes unrecognized until the consequences are serious. During development, Pb^{2+} exposure can cause learning disabilities, behavioral problems, and, at very high levels, seizures, coma, and even death. More than 400,000 children aged 1 to 5 years in the United States have Pb^{2+} levels in blood greater than the CDC warning level of 10 micrograms of Pb^{2+} per deciliter (which is approximately equivalent to 0.5 μM) [5]. The major source of Pb^{2+} exposure among children in the United States is Pb^{2+} -based paint and Pb^{2+} -contaminated dust found in deteriorating buildings.

There are direct neurotoxic effects of Pb^{2+} on cerebrovascular endothelial cells, and both astroglia and oligodendroglia [6]. Pb^{2+} also causes apoptosis and excitotoxicity in neurons [6]. Pb^{2+} affects storage and release of neurotransmitter, mitochondrial function and second messengers [6]. Pb^{2+} passes through the blood-brain barrier through back-transport via Ca^{2+} -ATPase pumps [7]. Lead is then accumulated intracellularly by passing through Ca^{2+} channels [8]. Some of the toxic effects of Pb^{2+} may result because Pb^{2+} interacts with Ca^{2+} -dependent signaling proteins, such as the Ca^{2+} -dependent regulatory protein calmodulin (CaM), which regulates many Ca^{2+} -dependent pathways. In 1994, Sandhir and Gill [9] determined that Pb^{2+} binds to CaM.

One of the most prominent features of lead poisoning is the presence of lifelong learning disabilities. However, the exact mechanism by which Pb^{2+} causes learning disabilities is not fully understood. Learning and memory depend on a variety of intracellular signaling cascades that utilize Ca^{2+} as a second messenger. Many of these cascades involve CaM. For example, the cAMP response element-binding protein (CREB) is a transcription factor regulated by stimuli that increase cAMP or activate Ca^{2+} /CaM-dependent kinase (CaMKII and CaMKIV) [10-12]. Ca^{2+} /CaM-sensitive adenylyl cyclase (AC) plays a central role in synaptic plasticity during learning [13]. During learning, CaMKII modulates trafficking of postsynaptic receptors for the neurotransmitter glutamate [14].

The effects of Pb^{2+} on Ca^{2+} -regulated pathways are difficult to study biochemically. Ca^{2+} is present in all laboratory solutions and also in animal tissues. The traditional method of controlling the free Ca^{2+} concentration by using EGTA to buffer Ca^{2+} [15, 16] presents a problem in biochemical studies of Pb^{2+} effects because EGTA chelates Pb^{2+} with even higher affinity than Ca^{2+} [17, 18]. The binding of free Pb^{2+} to EGTA displaces bound Ca^{2+} , making the released Ca^{2+} available to stimulate Ca^{2+} -dependent pathways. Thus, when using EGTA in experiments with Pb^{2+} , it is impossible to know the exact concentration of free Pb^{2+} and Ca^{2+} ions in the solution. Although one can calculate the predicted free Pb^{2+} and Ca^{2+} in any EGTA solution, there are actually large discrepancies between the predicted free ion concentrations and the actual concentrations. As Yovell et al. [19] discussed, at concentrations of free Ca^{2+} sufficient to optimally activate Ca^{2+} -dependent signaling pathways (i.e. $>1 \mu\text{M}$), EGTA is close to saturation; small errors in EGTA purity, variability in contaminating Ca^{2+} levels in reagents or shifts in pH produce large changes in free Ca^{2+}

concentrations; these same discrepancies would affect the concentration of free Pb^{2+} . Because of these problems, it has been difficult to accurately determine the role of Pb^{2+} in altering signaling in Ca^{2+} /CaM-dependent pathways. Indeed, various published results have been inconsistent; in some studies, Pb^{2+} stimulated CaM-dependent enzymes, while other studies reported inhibitory effects of Pb^{2+} [20, 21]. Haberman et al. (1983) used Chelex-100 resin to control Ca^{2+} ions in their assay solutions without the use of BAPTA and measured the final Ca^{2+} concentration by atomic absorption [22]. This technique allowed them to reduce free Ca^{2+} concentration to approximately 10^{-7} M, which was the limit of detection. However, based on our measurements, the free Ca^{2+} concentration remaining in solutions after Chelex-100 resin treatment is sufficient to bind to CaM and stimulate CaM-dependent enzymes [28]. Our goal was to develop a new approach for doing biochemical assays under low Ca^{2+} conditions, without the use of EGTA or other traditional Ca^{2+} chelators. To reduce contaminating Ca^{2+} , in this approach, we treated solutions sequentially with two Ca^{2+} -binding resins: Chelex-100 ion exchange resin, which has a low Ca^{2+} affinity, followed by immobilized BAPTA resin, which has a much higher Ca^{2+} affinity.

2. Methods

2.1 Resins

Free Ca^{2+} was reduced with analytical (Biotechnology) Grade Chelex-100 resin (Bio-Rad, CA, USA), used without pretreatment, and Calcium Sponge™ S (BAPTA polystyrene, Invitrogen, USA). Regenerated Calcium Sponge™ S and Chelex-100 resin were never used due to the reduced Ca^{2+} binding capacity after regeneration.

2.2 Reagents

Buffer A, containing 10 mM K-HEPES (pH 7.4) with 95 mM KCl, was used throughout the present study. HEPES was obtained from Affymetrix, OH, USA. KCl and CaCl_2 were purchased from J.T.Baker, NJ, USA. EGTA was obtained from Fluka Biochemika, Switzerland. Fluo-3 calcium indicator (Invitrogen, OR, USA) was used to measure the free Ca^{2+} concentration in buffer. For pilot experiments with Pb^{2+} , high purity Ca^{2+} -free $\text{Pb}(\text{CH}_3\text{CO}_2)_2$ (Alfa Aesar, MA, USA) was used in all experiments to minimize the amount of Ca^{2+} introduced.

2.3 Protocol

Buffer A was incubated with Chelex-100 resin (Bio-Rad, CA, USA) in a batch protocol. For every 1 ml of buffer, 0.05 g of resin was used. The solutions were incubated on a rotator at 4°C overnight. After each incubation, the mixture was centrifuged at 1500 rpm for 5 minutes and the supernatant was collected. This process was repeated three times. The solution became extremely alkaline (pH~10.4) after prolonged incubation with Chelex-100 resin. After three incubations, the pH of buffer A was adjusted to 7.4 with concentrated (37%) hydrochloric acid (HCl, Fisher, USA) to minimize the amount of additional Ca^{2+} introduced.

After Chelex-100 resin treatment and pH adjustment, to further reduce the concentration of free Ca^{2+} , buffer A was applied to a Ca^{2+} Sponge™ S column. Ca^{2+} Sponge™ S resin (0.5

g) was suspended in 5 ml of pH-adjusted, Chelex-100-treated buffer A and packed in a column for a final bed volume of 1 ml. The Ca²⁺ Sponge™ S column was further equilibrated with 2 ml of pH adjusted Chelex-100-treated buffer A. With each 0.5 g of Ca²⁺ Sponge™ S resin, 50 ml of low Ca²⁺ buffer A can be generated. The entire procedure was done at 4°C to minimize the degradation of reagents. The flow speed was kept at 0.6 ml/min to maximize the interaction between free Ca²⁺ in the solution and the Ca²⁺ Sponge™ S resin. Chelex-100 resin-treated buffer A was reloaded onto the same BAPTA-resin column for a total of three times.

The final concentration of free Ca²⁺ in buffer A was measured by Fluo-3 calcium indicator (Molecular Probe, OR, USA) using a Jasco FP-6500 spectrofluorometer. The absorption and emission wavelength for Fluo-3 were 506 nm and 524 nm respectively. A stock solution of 1 mM Fluo-3 was prepared in pH adjusted Chelex-100-treated buffer A. The stock solution was first diluted 1:50 in pH adjusted chelex-100-treated buffer A. Diluted Fluo-3 (1 µl) was added directly to 600 µl of the sample of buffer A to be tested, for a final concentration of 33.3 nM. [With other spectrofluorometers, we increased the Fluo-3 concentration (e.g. 4-fold) to increase the fluorescence signal]. For each sample, after reading the Fluo-3 fluorescence signal, the Fluo-3 was calibrated as follows: The minimum Fluo-3 signal, without Ca²⁺ bound, was first obtained by adding 2.4 µl of 0.5 M EGTA to the sample (after reading each sample). The maximal Fluo-3 signal, with saturating Ca²⁺, was then measured after addition 2.4 µl of 1 M CaCl₂. The dissociation constant (K_d) of Fluo-3 is 400 nM (Invitrogen); therefore, the free Ca²⁺ concentration in a solution is given by the following equation:

$$[Ca^{2+}]_{free} = 400nM[F - F_{min}]/[F_{max} - F]$$

All buffers were measured in three replicates with separate additions of Fluo-3.

2.4 AC activity assay

Membrane preparation—AC activity was evaluated by measuring the production of ³²P-cAMP by AC [23] in rat cerebellar membranes using a method adapted from Lin et al. 1998 [24]. Three-month old male rats were anesthetized with halothane and sacrificed by decapitation. To minimize Ca²⁺ contamination in membrane preparations, cerebella from two rats were homogenized in 5 ml homogenization buffer (50 mM K-HEPES, pH 7.6, 75 mM KCl, 3 mM EGTA, 1 mM DTT), protease inhibitor cocktail (10 µg/ml leupeptin/10 µg/ml aprotinin, 25 µM p-nitrophenyl-p'-guanidinobenzoate hydrochloride and 1 mM benzamidine) and 0.03% Lubrol PX. The Lubrol PX was added to facilitate removal of trapped Ca²⁺. The homogenate was centrifuged at 1,000 × g for 2 min to remove nuclei and large membrane fragments. The supernatant was centrifuged at 30,000 × g for 20 min. The pellet was re-homogenized in 10 ml membrane resuspend buffer (50 mM K-HEPES, pH 7.6, 75 mM KCl, 1 mM DTT and protease inhibitor cocktail) with 0.005% Lubrol PX and re-centrifuged. (This Lubrol PX concentration is only several fold above the CMC to facilitate washout.). The pellet was suspended in 10 ml membrane resuspend buffer without Lubrol

PX and re-centrifuged. The final pellet was suspended in 4 ml Chelex-100 resin-treated buffer A. Membrane aliquots were stored in liquid nitrogen.

Assay conditions—To reduce Ca^{2+} contamination, the AC activity assays contained no ATP-regenerating system. AC assay solution included 10 μM $\alpha^{32}\text{P}$ -ATP (80 $\mu\text{Ci/mL}$), 50 μM ^3H -cAMP ($\sim 3 \times 10^5$ cpm/mL), 0.5 mM IBMX, 3 mM MgCl_2 , 1 mM DTT, and protease inhibitor cocktail, as described above, in Buffer A. Assays were conducted at 30 °C for 8 min and terminated by addition of 100 μl stop solution containing unlabeled ATP and cAMP, plus sodium lauryl sulfate [23] and the reaction mixture was heated at 95° C for 3 min to inactivate the enzyme and solubilize the membrane. Samples were chromatographed on Dowex AG 50W-X4 columns (200-400 mesh, Biorad, Hercules, CA) to separate $\alpha^{32}\text{P}$ -cAMP product from substrate $\alpha^{32}\text{P}$ -ATP. Nucleotide impurities were removed by chromatography on alumina columns (Sigma, St. Louis). $\alpha^{32}\text{P}$ -cAMP was quantified with a scintillation counter. Data were normalized based on ^3H -cAMP recovery.

3. Results

Our objective was to develop an approach for producing buffers in which the concentration of contaminating Ca^{2+} was reduced below the threshold for activating CaM-dependent enzymes, i.e. <100 nM. We initially treated buffers with a series of batches of Chelex-100 resin alone. Despite incubation of buffers with successive Chelex-100 resin for prolonged periods (>1 week) in as many as seven incubation steps, the free Ca^{2+} concentrations did not drop below 80-100 nM. We therefore supplemented the Chelex-100 resin treatment with exposure of the buffer to immobilized BAPTA resin.

The initial concentration of Ca^{2+} in the untreated buffer was 740 nM (Table 1). After a single treatment with Chelex-100 resin, the concentration of Ca^{2+} in the buffer was only reduced to 505 nM. However after the third Chelex-100 resin treatment, the free Ca^{2+} concentration in the solution was reduced to 130 nM. After three passages through an immobilized BAPTA (Ca^{2+} Sponge™ S) column, the concentration of Ca^{2+} decreased to 13 nM. Thus, this method brought down the free Ca^{2+} concentration in our buffer well below 100 nM, which is just below the threshold level for activating CaM and Ca^{2+} -dependent enzymes [25] (see Discussion). Note, we did not use regenerated Ca^{2+} Sponge™ S resin or regenerated Chelex-100 resin because we found the regenerated resin was less effective, presumably due to reduced Ca^{2+} binding capacity.

In order to confirm the efficacy of this two step protocol, we treated a series of samples in parallel through the four Chelex-100 resin incubation steps and serial perfusion (three times) through the Ca^{2+} Sponge™ S column. Volumes were scaled down: 4 ml Chelex-100-treated buffer were applied to 50 mg of Ca^{2+} Sponge™ S, at a flow rate of 0.25 ml/min. The two-step protocol once again resulted in reduced free Ca^{2+} . We observed that with these scaled-down volumes of the immobilized BAPTA columns, the reduction in Ca^{2+} was less effective, and the final free Ca^{2+} was several fold higher than with the larger volume preparations. Nevertheless, the final free Ca^{2+} (~ 60 nM) should be sufficiently low to permit robust stimulation of Ca^{2+} -dependent enzymes. We have also observed variability in batches of Ca^{2+} Sponge™ S resin. We therefore find it critical to monitor the free Ca^{2+} for

each batch of buffer using the Fluo-3 method, particularly before and after the Ca^{2+} Sponge step.

Depending on the purity of the HEPES, KOH and KCl reagents and the quality of the deionized water, we sometimes found the initial buffer had relatively lower initial levels of Ca^{2+} contamination, eliminating the necessity for the series of Chelex-100 incubations prior to using Ca^{2+} Sponge.

In order to test the adequacy of the resulting buffer for making experimental measurements of Ca^{2+} stimulation of CaM-dependent processes, we conducted AC assays on membrane preparations from rat cerebellum. We observed 3-fold stimulation of AC activity by Ca^{2+} in the presence of CaM as compared with in low Ca^{2+} treated buffer. As compared with EGTA, the low Ca^{2+} treated buffer stimulated AC activity two fold. This is much greater activation than would be expected if the final free Ca^{2+} in the assay were in the 10-60 nM range. The higher level of “basal” activity can be accounted for by the addition of cerebellar membranes and reagents including ATP, ^{32}P -ATP, MgCl_2 and DTT, all of which contain contaminating Ca^{2+} .

4. Discussion

The method described above will allow experiments to be conducted in low Ca^{2+} buffers in the absence of a chelator. This method also allows for more accurate determination and control of the concentrations of free Ca^{2+} and free Pb^{2+} in buffers. The sequential use of Chelex-100 resin and Ca^{2+} Sponge™ S was advantageous because Chelex-100 resin is relatively inexpensive (~150 times less expensive, by weight, than Ca^{2+} Sponge™ S) and can reduce the concentration of free Ca^{2+} gradually with a large capacity. However, Ca^{2+} Sponge™ S is required to lower the concentration of free Ca^{2+} below range of threshold Ca^{2+} concentrations that activate Ca^{2+} -dependent enzymes in *in vitro* assays. The threshold Ca^{2+} concentration for activating CaM-sensitive AC is approximately 200 nM [19]. For activating calcineurin and CaMKII, the threshold concentrations of Ca^{2+} are 150 nM [26] and approximately 400 nM [27], respectively. Our protocol successfully produced buffers with free Ca^{2+} levels below <15 nM, which is substantially below these levels. The precise amount of Ca^{2+} and/or Pb^{2+} desired can then be added back to these buffers to test the effects on the activity of enzymes of interest. This method should provide an improved approach to study the effect of heavy metals on enzymes in Ca^{2+} stimulated signaling pathways.

Acknowledgments

This research was supported by National Institutes of Health grant MH-55880. We thank Dr. Joseph Kao for help with the measurements of free Ca^{2+} .

References

1. Navas-Acien A, Guallar E, Silbergeld EK, Rothenberg SJ. Lead exposure and cardiovascular disease--a systematic review. *Environmental health perspectives*. 2007; 115:472–482. [PubMed: 17431501]

2. Pounds JG, Long GJ, Rosen JF. Cellular and molecular toxicity of lead in bone. *Environmental health perspectives*. 1991; 91:17–32. [PubMed: 2040247]
3. Ekong EB, Jaar BG, Weaver VM. Lead-related nephrotoxicity: a review of the epidemiologic evidence. *Kidney international*. 2006; 70:2074–2084. [PubMed: 17063179]
4. Cleveland LM, Minter ML, Cobb KA, Scott AA, German VF. Lead hazards for pregnant women and children: part 1: immigrants and the poor shoulder most of the burden of lead exposure in this country. Part 1 of a two-part article details how exposure happens, whom it affects, and the harm it can do. *The American Journal of Nursing*. 2008; 108:40–9. quiz 50.
5. Centers for Disease Control and Prevention. Preventing Lead Poisoning in Young Children. 2005
6. Lidsky TI, Schneider JS. Lead neurotoxicity in children: basic mechanisms and clinical correlates. *Brain: a journal of neurology*. 2003; 126:5–19. [PubMed: 12477693]
7. Bradbury MW, Deane R. Permeability of the blood-brain barrier to lead. *Neurotoxicology*. 1993; 14:131–136. [PubMed: 8247388]
8. Simons TJ, Pocock G. Lead enters bovine adrenal medullary cells through calcium channels. *Journal of neurochemistry*. 1987; 48:383–389. [PubMed: 2432178]
9. Sandhir R, Gill KD. Effect of lead on the biological activity of calmodulin in rat brain. *Experimental and molecular pathology*. 1994; 61:69–75. [PubMed: 7995380]
10. Gonzalez GA, Montminy MR. Cyclic AMP stimulates somatostatin gene transcription by phosphorylation of CREB at serine 133. *Cell*. 1989; 59:675–680. [PubMed: 2573431]
11. Dash PK, Karl KA, Colicos MA, Prywes R, Kandel ER. cAMP response element-binding protein is activated by Ca²⁺/calmodulin- as well as cAMP-dependent protein kinase. *Proceedings of the National Academy of Sciences of the United States of America*. 1991; 88:5061–5065. [PubMed: 1647024]
12. See V, Boutillier AL, Bito H, Loeffler JP. Calcium/calmodulin-dependent protein kinase type IV (CaMKIV) inhibits apoptosis induced by potassium deprivation in cerebellar granule neurons. *The FASEB journal: official publication of the Federation of American Societies for Experimental Biology*. 2001; 15:134–144.
13. Wong ST, Athos J, Figueroa XA, Pineda VV, Schaefer ML, Chavkin CC, Muglia LJ, Storm DR. Calcium-stimulated adenylyl cyclase activity is critical for hippocampus-dependent long-term memory and late phase LTP. *Neuron*. 1999; 23:787–798. [PubMed: 10482244]
14. Lisman J, Schulman H, Cline H. The molecular basis of CaMKII function in synaptic and behavioural memory. *Nature reviews. Neuroscience*. 2002; 3:175–190. [PubMed: 11994750]
15. MacDonald E, Hellevuo K, Komulainen H. Lead does not affect calmodulin-induced activation of calcium-dependent adenosine triphosphatase in human red blood cell membranes. *Archives of toxicology Supplement= Archiv fur Toxikologie Supplement*. 1986; 9:397–400. [PubMed: 2949723]
16. Oortgiesen M, van Kleef RG, Vijverberg HP. Novel type of ion channel activated by Pb²⁺, Cd²⁺, and Al³⁺ in cultured mouse neuroblastoma cells. *The Journal of membrane biology*. 1990; 113:261–268. [PubMed: 1692342]
17. Schanne FA, Dowd TL, Gupta RK, Rosen JF. Development of ¹⁹F NMR for measurement of [Ca²⁺]_i and [Pb²⁺]_i in cultured osteoblastic bone cells. *Environmental health perspectives*. 1990; 84:99–106. [PubMed: 2112459]
18. Martell AE, Smith RM. Critical stability constants. 1974; 1
19. Yovell Y, Kandel ER, Dudai Y, Abrams TW. A quantitative study of the Ca²⁺/calmodulin sensitivity of adenylyl cyclase in *Aplysia*, *Drosophila*, and rat. *Journal of neurochemistry*. 1992; 59:1736–1744. [PubMed: 1402918]
20. Kern M, Audesirk G. Inorganic lead may inhibit neurite development in cultured rat hippocampal neurons through hyperphosphorylation. *Toxicology and applied pharmacology*. 1995; 134:111–123. [PubMed: 7676445]
21. Vig PJ, Nath R, Desai D. Metal inhibition of calmodulin activity in monkey brain. *Journal of applied toxicology: JAT*. 1989; 9:313–316. [PubMed: 2531770]
22. Habermann E, Crowell K, Janicki P. Lead and other metals can substitute for Ca²⁺ in calmodulin. *Archives of Toxicology*. 1983; 54:61–70. [PubMed: 6314931]

23. Salomon Y. Adenylate cyclase assay. *Advances in Cyclic Nucleotide Research*. 1979; 10:35–55. [PubMed: 222124]
24. Lin AH, Onyike CU, Abrams TW. Sequence-dependent interactions between transient calcium and transmitter stimuli in activation of mammalian brain adenylyl cyclase. *Brain research*. 1998; 800:300–307. [PubMed: 9685686]
25. James-Kracke MR. Calmodulin activation of the Ca²⁺ pump revealed by fluorescent chelator dyes in human red blood cell ghosts. *The Journal of general physiology*. 1992; 99:41–62. [PubMed: 1371307]
26. Kubis HP, Hanke N, Scheibe RJ, Meissner JD, Gros G. Ca²⁺ transients activate calcineurin/NFATc1 and initiate fast-to-slow transformation in a primary skeletal muscle culture. *American journal of physiology Cell physiology*. 2003; 285:C56–63. [PubMed: 12606309]
27. Cohen JE, Fields RD. CaMKII inactivation by extracellular Ca(2+) depletion in dorsal root ganglion neurons. *Cell calcium*. 2006; 39:445–454. [PubMed: 16519936]
28. Noyes N, Niu KY, Abrams TW. Bidirectional effects of lead on calmodulin-dependent enzymes involved in synaptic plasticity and learning. In preparation.

Figure 1. Free Ca²⁺ in replicate batches of buffer treated with Chelex-100 resin followed by Ca²⁺ SpongeTM S resin. Ca²⁺ was measured by Fluo-3 fluorescence as in Methods, except that all samples were in a 96 well plate, Ca²⁺ and EGTA standards were in separate wells from samples, and measurements were made with a Tecan Infinite M1000 Pro plate reader. Each buffer sample was measured in triplicate. Data are means $\pm$ SEM; n = 4. Coefficient of variation = 0.20 for Chelex-100 treated buffer and 0.24 for buffer after Ca²⁺ SpongeTM S. Note, higher levels of final free Ca²⁺ as compared with those in Table 1 may result from the small Ca²⁺ SpongeTM column volume, although the column volume were scaled proportionately to buffer volume.

Figure 2.

Reduction of contaminating Ca^{2+} by Chelex/ Ca^{2+} SpongeTM treatment enables measurement of Ca^{2+} stimulation of adenylyl cyclase without use of EGTA. Adenylyl cyclase activity in cerebellar membranes was assayed with either treated buffer with 3 mM EGTA (without exogenous calmodulin), treated buffer alone + 1 μM calmodulin or treated buffer with 10 μM Ca^{2+} + 1 μM calmodulin. Normally contaminating Ca^{2+} levels would result in near maximal calmodulin stimulation of adenylyl cyclase in the absence of added Ca^{2+} .

Contaminating Ca^{2+} remaining in both cerebellar membranes and untreated assay reagents in buffer alone with calmodulin condition still stimulated adenylyl cyclase activity. Addition of 10 μM Ca^{2+} resulted in three-fold greater adenylyl cyclase stimulation, indicating that the buffer alone stimulation was relatively modest.

Table 1

Free Ca²⁺ concentrations in treated buffers, measured with Fluo-3.

Buffer Treatment	Free Ca ²⁺ concentration (nM)
Buffer before Clielex-100 resin treatment	740
Buffer after 1X Clielex-100 resin treatment	505
Buffer after 2X Clielex-100 resin treatment	214
Buffer after 3X Clielex-100 resin treatment	130
Buffer after 3X Clielex-100 resin treatment and Ca ²⁺ Sponge™ S column	13