TOXICOLOGICAL SCIENCES **130(1)**, 1–3 (2012)
doi:10.1093/toxsci/kfs221
Advance Access publication July 12, 2012

Sense in Pb^{2+} Sensing

Henk P. M. Vijverberg and Remco H. S. Westerink[1]

Institute for Risk Assessment Sciences, Utrecht University, Utrecht, The Netherlands

[1]To whom correspondence should be addressed at Institute for Risk Assessment Sciences, Utrecht University, PO Box 80177, 3508 TD Utrecht, The Netherlands. Fax: +31302535077. E-mail: r.westerink@uu.nl.

Received May 16, 2012; accepted July 3, 2012

It is generally acknowledged that Pb^{2+}, which is sequestered by live cells from their direct environment, affects a large number of cellular processes at picomolar to micromolar concentrations. However, resolving the specific molecular targets and mechanisms responsible for the neurotoxic effects of this xenobiotic metal is hampered by the lack of suitable tools to investigate the intracellular dynamics of Pb^{2+} at low concentrations. Fluorescent Ca^{2+} indicators have been used as Pb^{2+} sensors and have proven useful to detect cellular Pb^{2+} entry and to estimate the overall intracellular free Pb^{2+} concentration associated with adverse cellular effects. Despite the high affinity of these Ca^{2+} indicators for Pb^{2+}, their utility for more advanced studies is limited. This is merely due to their moderate metal selectivity and uncertainties about the subcellular (co)localization of the indicators and the targets. Novel Pb^{2+} sensors, specifically developed for this purpose, still lack affinity to sense toxicologically relevant intracellular concentrations of Pb^{2+}. Nonetheless, the development of genetically encoded protein sensors for Ca^{2+}, Zn^{2+}, and, recently, also for Pb^{2+} opens a new and promising perspective to resolve spatiotemporal changes in intracellular Pb^{2+} in relation to cellular signaling and intracellular divalent metal homeostasis. Such a development is required for enabling more systematic studies of the intracellular dynamics of Pb^{2+}, which are essential for progress in mechanistic knowledge and will ultimately reveal the critical toxic targets of Pb^{2+} at the subcellular and molecular level.

Key Words: **Pb^{2+} toxicity; Live-cell fluorescent imaging; Ca^{2+} imaging; indo-1; fura-2; Met-lead 1.59; Fluorescence Resonance Energy Transfer (FRET); Metal neurotoxicity.**

Pb^{2+} TOXICITY AND CELLULAR EFFECTS

Over the past 50 years, the substantial effort made to study cognitive and neurodevelopmental effects associated with low-level exposure has greatly increased the concern about the neurotoxic potential of Pb^{2+}. This has resulted in a recurrent lowering of the acceptable level of Pb^{2+} in human blood and has founded the notion that a safe level of Pb^{2+} in blood may not exist at all (Grandjean, 2010; Jusko *et al.*, 2008). Despite the steadily increasing concern about health effects of Pb^{2+}, it has not yet been possible to pinpoint a primary molecular target for the neurotoxic effects of Pb^{2+}. Studies into its cellular and molecular mechanism of action have shown that Pb^{2+} enters the cell via multiple pathways (for recent papers, see Chang *et al.*, 2008; Martinez-Finley *et al.*, 2012). Pb^{2+} has been reported to activate and/or inhibit a range of cellular proteins, e.g., voltage- and ligand-gated ion channels, calmodulin, protein kinase C, calcineurin, Ca^{2+}/calmodulin-dependent protein kinase II, and synaptotagmin, presumably by mimicking or antagonizing physiological effects of Ca^{2+} and other divalent metals, e.g., Zn^{2+} (for reviews, see Neal and Guilarte, 2010; Suszkiw, 2004; Vijverberg *et al.*, 1994). Estimates of Pb^{2+} concentrations to induce these cellular effects vary by at least six orders of magnitude, i.e., from the picomolar range for protein kinase C to the micromolar range for some voltage- and ligand-gated ion channels (Kern and Audesirk, 2000; Sun *et al.*, 1999; Vijverberg *et al.*, 1994). Difficulties to reliably measure intracellular Pb^{2+} concentration ($[Pb^{2+}]_i$)—in particular for the lower end of the toxicologically relevant concentration range—complicate the interpretation of observed intracellular effects and hamper the progress of mechanistic research. However, some recent developments in tools available for live-cell imaging of Pb^{2+} and other metals appear to offer a novel perspective.

Ca^{2+} INDICATORS AS INTRACELLULAR METAL SENSORS

Fluorescent dyes, based on Ca^{2+} chelating compounds and originally developed for live-cell Ca^{2+} imaging, e.g., indo-1 and fura-2, show high affinity for several other metal ions and for Pb^{2+} in particular. When titrated with Pb^{2+} in ethylene glycol tetraacetic acid (EGTA)–buffered solution, Pb^{2+} excites fura-2 and quenches the fluorescence of indo-1 with apparent affinities in the picomolar range. Both dyes show selectivity for $Pb^{2+} > Zn^{2+} >> Ca^{2+} > Mg^{2+}$ (Grynkiewicz *et al.*, 1985; Jefferson *et al.*, 1990; Kerper and Hinkle, 1997; Tomsig and Suszkiw, 1990). Pb^{2+} and Ca^{2+} cause similar changes in the excitation spectrum of fura-2. Because the spectrum of fura-2 in the presence of Ca^{2+} differs little from that in the presence of Pb^{2+}, the utility of fura-2 for live-cell Pb^{2+} imaging is limited (Tomsig and Suszkiw,

© The Author 2012. Published by Oxford University Press on behalf of the Society of Toxicology. All rights reserved.
For permissions, please email: journals.permissions@oup.com

Downloaded from https://academic.oup.com/toxsci/article/130/1/1/1666649 by guest on 25 April 2024

1990). Because Pb^{2+} quenches indo-1 fluorescence over the entire emission spectrum and Ca^{2+} alters the intensity ratio at specific wavelengths of the emission spectrum, indo-1 is suited to measure intracellular Pb^{2+} and Ca^{2+} simultaneously. Taking advantage of these favorable properties of indo-1, it has been shown in rat pheochromocytoma (PC12) cells that Pb^{2+} triggers sustained exocytosis of neurotransmitter-containing vesicles once its intracellular concentration reaches a level of 10–30nM in the absence of simultaneous changes in the intracellular Ca^{2+} concentration (Westerink and Vijverberg, 2002). Under intracellular conditions, the apparent affinities of indo-1 for Ca^{2+} and for Pb^{2+} are much lower than in EGTA-containing saline buffers commonly used for dye calibration. In the absence of EGTA, the Ca^{2+}-insensitive (isosbestic) point of the indo-1 emission spectrum shifts 20 nm to shorter wavelengths and the apparent affinities for Ca^{2+} and Pb^{2+} are markedly reduced (Hove-Madsen and Bers, 1992; Kerper and Hinkle, 1997; Owen *et al.*, 1991; Westerink and Vijverberg, 2002). The reduced affinity of indo-1 inside the cell precludes the detection of the lowest effective concentrations of intracellular Pb^{2+}. Apart from the technically complicating factors and limitations inherent in Ca^{2+} indicators (for review, see Takahashi *et al.*, 1999), the metal selectivity order of fura-2 and indo-1 makes the use of these dyes for live-cell imaging prone to errors caused by the intracellular presence of background levels of essential metals and of contaminating levels of xenobiotic metals. In this respect, it is noteworthy that a nominal Ca^{2+}- and Pb^{2+}-free saline solution, even when prepared carefully from pure water and chemicals, will be contaminated with up to 0.4µM Ca^{2+} and ~20nM Pb^{2+} (Westerink and Vijverberg, 2002). In addition, the background, resting concentration of cytosolic Zn^{2+} may amount to 0.4nM (Vinkenborg *et al.*, 2010), which is 2.5 times higher than the reported K_d value of indo-1 for Zn^{2+} (Jefferson *et al.*, 1990). However, consistent information on the affinities of various essential and xenobiotic metals for the commonly used ratiometric Ca^{2+} indicators and a detailed characterization of the spectral effects of these metals are lacking. For single-wavelength fluorescent Ca^{2+} indicators, the situation readily becomes too complex to measure intracellular metal ions in the presence of cytosolic Ca^{2+}, Mg^{2+}, and Zn^{2+}.

SPECIFIC Pb^{2+} INDICATORS

Several indicators have been synthesized with the specific purpose of live-cell Pb^{2+} imaging, e.g., the ratiometric indicators leadfluor-1 (He *et al.*, 2006) and leadglow (Marbella *et al.*, 2009) and the protein sensor Met-lead 1.59 (Chiu and Yang, 2012). The low affinity for Pb^{2+} precludes the use of the fluorescent indicators leadfluor-1 and leadglow for sensing toxicologically relevant $[Pb^{2+}]_i$. Met-lead 1.59, a genetically encoded protein probe sensitive to visible light, is based on the principle of fluorescence resonance energy transfer (FRET). This novel probe consists of a protein from heavy metal–resistant bacteria (*Cupriavidus metallidurans*)—the Pb^{2+} sensor—with a pair of cyan and yellow fluorescent proteins attached at each end. Met-lead 1.59 is excited with blue light, and the cyan/yellow emission ratio is used to measure $[Pb^{2+}]_i$. Titration with Pb^{2+} shows that Met-lead 1.59 has high- and low-affinity sites for Pb^{2+} with K_d values of 69nM and 22µM, respectively, and with a total dynamic range of 1.73. The dye calibration also shows that the major part (~75%) of the dynamic range is associated with low-affinity Pb^{2+} binding. In intact HEK293 cells, transfected with Met-lead 1.59 and exposed to high concentrations (10–50 µM) of Pb^{2+}, it is possible to detect an increase of $[Pb^{2+}]_i$ after a delay of 1–2 h (Chiu and Yang, 2012). Although the presence of low- and high-affinity binding sites widens the concentration range in which Met-lead 1.59 is responsive to Pb^{2+}, both the live-cell imaging and the dye calibration indicate that Met-lead 1.59 is not very sensitive and is not very responsive to low concentrations of Pb^{2+}. In addition, a sensor with more than one metal binding domain is inconvenient in an environment in which multiple metal ion species are present. The selectivity order of Met-lead 1.59 is Zn^{2+}, Pb^{2+} > Cu^{2+}, Cu^{+} >> Ca^{2+}, Mg^{2+}, Mn^{2+}, and Fe^{2+} (Chiu and Yang, 2012). Depending on the affinities and on the concentrations present, multiple metals may bind to high- or low-affinity sites of Met-lead 1.59 in a mutually dependent or independent way, which may complicate the interpretation of changes in fluorescence ratio. It remains to be confirmed how the FRET probe behaves under intracellular conditions, i.e., at intracellular pH and ionic strength, a relatively high concentration of Mg^{2+} and low concentrations of other divalent metals, e.g., Ca^{2+} and Zn^{2+}, and proteins present at the same time. Thus far, the ratiometric UV Ca^{2+} indicators outperform indicators developed with the specific purpose of live-cell Pb^{2+} imaging particularly with respect to their sensitivity to Pb^{2+}.

FUTURE PROSPECTS

Despite the current drawbacks of Met-lead 1.59, the development of genetically encoded FRET sensors holds promise for an exciting future in which it is possible to transfect cells with DNA encoding one or several proteins to sense and monitor real-time changes in the intracellular environment. Extensive expertise has been built up with a variety of Ca^{2+} sensors, which have been used as tools to study intracellular Ca^{2+} dynamics, Ca^{2+} buffering and compartmentalization associated with cellular signaling, and Ca^{2+} homeostasis (for review, see Hires *et al.*, 2008; Rudolf *et al.*, 2003). The sensitivity of genetically encoded Ca^{2+} indicators is steadily being improved (e.g., Muto *et al.*, 2011; Tian *et al.*, 2009). Systematic progress has also been made in the engineering of genetically encoded fluorescent Zn^{2+} indicators and has yielded a set of Zn^{2+} sensor proteins with affinities covering over six orders of magnitude to detect picomolar to micromolar concentrations of Zn^{2+} (for review, see Vinkenborg *et al.*, 2010). However, the sensitivity of the genetically encoded indicators to various essential and xenobiotic metal ions remains largely unknown and needs to be established. Because Pb^{2+} is known to activate calmodulin

Downloaded from https://academic.oup.com/toxsci/article/130/1/1/1666649 by guest on 25 April 2024

comparable to but much more potently than Ca^{2+} (Habermann *et al.*, 1983), calmodulin-derived, single-domain sensors are of particular interest for Pb^{2+} sensing. The modification of protein indicators for subcellular targeting is another step to produce multiple, picomolar- to nanomolar-affinity Pb^{2+} sensors for specific intracellular domains or organelles. Because cellular and subcellular targeting of genetically engineered protein sensors has been shown feasible (for a recent review on subcellular targeting of aequorin, see Webb and Miller, 2012), all components appear available to produce the necessary tools for resolving the intracellular dynamics of Pb^{2+} in a not too distant future. Until such sophisticated tools become available for toxicologists, using indo-1 makes sense in Pb^{2+} sensing.

FUNDING

This research was funded by the Faculty of Veterinary Medicine of Utrecht University.

REFERENCES

Chang, Y. F., Teng, H. C., Cheng, S. Y., Wang, C. T., Chiou, S. H., Kao, L. S., Kao, F. J., Chiou, A., and Yang, D. M. (2008). Orai1-STIM1 formed store-operated Ca^{2+} channels (SOCs) as the molecular components needed for Pb^{2+} entry in living cells. *Toxicol. Appl. Pharmacol.* **227,** 430–439.

Chiu, T. Y., and Yang, D. M. (2012). Intracellular Pb^{2+} content monitoring using a protein-based Pb^{2+} indicator. *Toxicol. Sci.* **126,** 436–445.

Grandjean, P. (2010). Even low-dose lead exposure is hazardous. *Lancet* **376,** 855–856.

Grynkiewicz, G., Poenie, M., and Tsien, R. Y. (1985). A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. *J. Biol. Chem.* **260,** 3440–3450.

Habermann, E., Crowell, K., and Janicki, P. (1983). Lead and other metals can substitute for Ca^{2+} in calmodulin. *Arch. Toxicol.* **54,** 61–70.

He, Q., Miller, E. W., Wong, A. P., and Chang, C. J. (2006). A selective sensor for detecting lead in living cells. *J. Am. Chem. Soc.* **124,** 6246–6247.

Hires, S. A., Tian, L., and Looger, L. L. (2008). Reporting neural activity with genetically encoded calcium indicators. *Brain Cell Biol.* **36,** 69–86.

Hove-Madsen, L., and Bers, D. M. (1992). Indo-1 binding to protein in permeabilized ventricular myocytes alters its spectral and Ca binding properties. *Biophys. J.* **63,** 89–97.

Jefferson, J. R., Hunt, J. B., and Ginsburg, A. (1990). Characterization of indo-1 and quin-2 as spectroscopic probes for Zn^{2+} protein interactions. *Anal. Biochem.* **187,** 328–336.

Jusko, T. A., Henderson, C. R., Jr, Lanphear, B. P., Cory-Slechta, D. A., Parsons, P. J., and Canfield, R. L. (2008). Blood lead concentrations < 10 μg/dL and child intelligence at 6 years of age. *Environ. Health Perspect.* **116,** 243–248.

Kern, M., and Audesirk, G. (2000). Stimulatory and inhibitory effects of inorganic lead on calcineurin. *Toxicology* **150,** 171–178.

Kerper, L. E., and Hinkle, P. M. (1997). Cellular uptake of lead is activated by depletion of intracellular calcium stores. *J. Biol. Chem.* **272,** 8346–8352.

Marbella, L., Serli-Mitasev, B., and Basu, P. (2009). Development of a fluorescent Pb^{2+} sensor. *Angew. Chem. Int. Ed. Engl.* **48,** 3996–3998.

Martinez-Finley, E. J., Chakraborty, S., Fretham, S. J., and Aschner, M. (2012). Cellular transport and homeostasis of essential and nonessential metals. *Metallomics* **4,** 593–605.

Muto, A., Ohkura, M., Kotani, T., Higashijima, S., Nakai, J., and Kawakami, K. (2011). Genetic visualization with an improved GCaMP calcium indicator reveals spatiotemporal activation of the spinal motor neurons in zebrafish. *Proc. Natl. Acad. Sci. U.S.A.* **108,** 5425–5430.

Neal, A. P., and Guilarte, T. R. (2010). Molecular neurobiology of lead (Pb^{2+}): Effects on synaptic function. *Mol. Neurobiol.* **42,** 151–160.

Owen, C. S., Sykes, N. L., Shuler, R. L., and Ost, D. (1991). Non-calcium environmental sensitivity of intracellular Indo-1. *Anal. Biochem.* **192,** 142–148.

Rudolf, R., Mongillo, M., Rizzuto, R., and Pozzan, T. (2003). Looking forward to seeing calcium. *Nat. Rev. Mol. Cell Biol.* **4,** 579–586.

Sun, X., Tian, X., Tomsig, J. L., and Suszkiw, J. B. (1999). Analysis of differential effects of Pb^{2+} on protein kinase C isozymes. *Toxicol. Appl. Pharmacol.* **156,** 40–45.

Suszkiw, J. B. (2004). Presynaptic disruption of transmitter release by lead. *Neurotoxicology* **25,** 599–604.

Takahashi, A., Camacho, P., Lechleiter, J. D., and Herman, B. (1999). Measurement of intracellular calcium. *Physiol. Rev.* **79,** 1089–1125.

Tian, L., Hires, S. A., Mao, T., Huber, D., Chiappe, M. E., Chalasani, S. H., Petreanu, L., Akerboom, J., McKinney, S. A., Schreiter, E. R., *et al.* (2009). Imaging neural activity in worms, flies and mice with improved GCaMP calcium indicators. *Nat. Methods* **6,** 875–881.

Tomsig, J. L., and Suszkiw, J. B. (1990). Permeation of Pb^{2+} through calcium channels: Fura-2 measurements of voltage- and dihydropyridine-sensitive Pb^{2+} entry in isolated bovine chromaffin cells. *Biochim. Biophys. Acta* **1069,** 197–200.

Vijverberg, H. P. M., Oortgiesen, M., Leinders, T., and van Kleef, R. G. D. M. (1994). Metal interactions with voltage- and receptor-activated ion channels. *Environ. Health Perspect.* **102**(Suppl. 3), 153–158.

Vinkenborg, J. L., Koay, M. S., and Merkx, M. (2010). Fluorescent imaging of transition metal homeostasis using genetically encoded sensors. *Curr. Opin. Chem. Biol.* **14,** 231–237.

Webb, S. E., and Miller, A. L. (2012). Aequorin-based genetic approaches to visualize Ca^{2+} signaling in developing animal systems. *Biochim. Biophys. Acta* **1820,** 1160–1168.

Westerink, R. H. S., and Vijverberg, H. P. M. (2002). Ca^{2+} independent vesicular catecholamine release in PC12 cells by nanomolar concentrations of Pb^{2+}. *J. Neurochem.* **80,** 861–873.

Downloaded from https://academic.oup.com/toxsci/article/130/1/1/1666649 by guest on 25 April 2024