

## Sense in Pb<sup>2+</sup> Sensing

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It is generally acknowledged that Pb<sup>2+</sup>, 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<sup>2+</sup> at low concentrations. Fluorescent Ca<sup>2+</sup> indicators have been used as Pb<sup>2+</sup> sensors and have proven useful to detect cellular Pb<sup>2+</sup> entry and to estimate the overall intracellular free Pb<sup>2+</sup> concentration associated with adverse cellular effects. Despite the high affinity of these Ca<sup>2+</sup> indicators for Pb<sup>2+</sup>, 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<sup>2+</sup> sensors, specifically developed for this purpose, still lack affinity to sense toxicologically relevant intracellular concentrations of Pb<sup>2+</sup>. Nonetheless, the development of genetically encoded protein sensors for Ca<sup>2+</sup>, Zn<sup>2+</sup>, and, recently, also for Pb<sup>2+</sup> opens a new and promising perspective to resolve spatiotemporal changes in intracellular Pb<sup>2+</sup> 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<sup>2+</sup>, which are essential for progress in mechanistic knowledge and will ultimately reveal the critical toxic targets of Pb<sup>2+</sup> at the subcellular and molecular level.

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

### Pb<sup>2+</sup> 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<sup>2+</sup>. This has resulted in a recurrent lowering of the acceptable level of Pb<sup>2+</sup> in human blood and has founded the notion that a safe level of Pb<sup>2+</sup> in blood may not exist at all (Grandjean, 2010; Jusko *et al.*, 2008). Despite the steadily increasing concern about health effects of Pb<sup>2+</sup>, it has not yet been possible to pinpoint a primary molecular target

for the neurotoxic effects of Pb<sup>2+</sup>. Studies into its cellular and molecular mechanism of action have shown that Pb<sup>2+</sup> enters the cell via multiple pathways (for recent papers, see Chang *et al.*, 2008; Martinez-Finley *et al.*, 2012). Pb<sup>2+</sup> 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<sup>2+</sup>/calmodulin-dependent protein kinase II, and synaptotagmin, presumably by mimicking or antagonizing physiological effects of Ca<sup>2+</sup> and other divalent metals, e.g., Zn<sup>2+</sup> (for reviews, see Neal and Guilarte, 2010; Suszkiw, 2004; Vijverberg *et al.*, 1994). Estimates of Pb<sup>2+</sup> 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<sup>2+</sup> concentration ([Pb<sup>2+</sup>]<sub>i</sub>)—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<sup>2+</sup> and other metals appear to offer a novel perspective.

### Ca<sup>2+</sup> INDICATORS AS INTRACELLULAR METAL SENSORS

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

1990). Because  $\text{Pb}^{2+}$  quenches indo-1 fluorescence over the entire emission spectrum and  $\text{Ca}^{2+}$  alters the intensity ratio at specific wavelengths of the emission spectrum, indo-1 is suited to measure intracellular  $\text{Pb}^{2+}$  and  $\text{Ca}^{2+}$  simultaneously. Taking advantage of these favorable properties of indo-1, it has been shown in rat pheochromocytoma (PC12) cells that  $\text{Pb}^{2+}$  triggers sustained exocytosis of neurotransmitter-containing vesicles once its intracellular concentration reaches a level of 10–30 nM in the absence of simultaneous changes in the intracellular  $\text{Ca}^{2+}$  concentration (Westerink and Vijverberg, 2002). Under intracellular conditions, the apparent affinities of indo-1 for  $\text{Ca}^{2+}$  and for  $\text{Pb}^{2+}$  are much lower than in EGTA-containing saline buffers commonly used for dye calibration. In the absence of EGTA, the  $\text{Ca}^{2+}$ -insensitive (isosbestic) point of the indo-1 emission spectrum shifts 20 nm to shorter wavelengths and the apparent affinities for  $\text{Ca}^{2+}$  and  $\text{Pb}^{2+}$  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  $\text{Pb}^{2+}$ . Apart from the technically complicating factors and limitations inherent in  $\text{Ca}^{2+}$  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  $\text{Ca}^{2+}$ - and  $\text{Pb}^{2+}$ -free saline solution, even when prepared carefully from pure water and chemicals, will be contaminated with up to 0.4  $\mu\text{M}$   $\text{Ca}^{2+}$  and ~20 nM  $\text{Pb}^{2+}$  (Westerink and Vijverberg, 2002). In addition, the background, resting concentration of cytosolic  $\text{Zn}^{2+}$  may amount to 0.4 nM (Vinkenburg *et al.*, 2010), which is 2.5 times higher than the reported  $K_d$  value of indo-1 for  $\text{Zn}^{2+}$  (Jefferson *et al.*, 1990). However, consistent information on the affinities of various essential and xenobiotic metals for the commonly used ratiometric  $\text{Ca}^{2+}$  indicators and a detailed characterization of the spectral effects of these metals are lacking. For single-wavelength fluorescent  $\text{Ca}^{2+}$  indicators, the situation readily becomes too complex to measure intracellular metal ions in the presence of cytosolic  $\text{Ca}^{2+}$ ,  $\text{Mg}^{2+}$ , and  $\text{Zn}^{2+}$ .

### SPECIFIC $\text{Pb}^{2+}$ INDICATORS

Several indicators have been synthesized with the specific purpose of live-cell  $\text{Pb}^{2+}$  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  $\text{Pb}^{2+}$  precludes the use of the fluorescent indicators leadfluor-1 and leadglow for sensing toxicologically relevant  $[\text{Pb}^{2+}]_i$ . 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  $\text{Pb}^{2+}$  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  $[\text{Pb}^{2+}]_i$ . Titration with  $\text{Pb}^{2+}$  shows that Met-lead 1.59 has high- and low-affinity sites for  $\text{Pb}^{2+}$  with  $K_d$  values of 69 nM and 22  $\mu\text{M}$ , 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  $\text{Pb}^{2+}$  binding. In intact HEK293 cells, transfected with Met-lead 1.59 and exposed to high concentrations (10–50  $\mu\text{M}$ ) of  $\text{Pb}^{2+}$ , it is possible to detect an increase of  $[\text{Pb}^{2+}]_i$  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  $\text{Pb}^{2+}$ , 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  $\text{Pb}^{2+}$ . 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  $\text{Zn}^{2+}$ ,  $\text{Pb}^{2+}$  >  $\text{Cu}^{2+}$ ,  $\text{Cu}^+$  >>  $\text{Ca}^{2+}$ ,  $\text{Mg}^{2+}$ ,  $\text{Mn}^{2+}$ , and  $\text{Fe}^{2+}$  (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  $\text{Mg}^{2+}$  and low concentrations of other divalent metals, e.g.,  $\text{Ca}^{2+}$  and  $\text{Zn}^{2+}$ , and proteins present at the same time. Thus far, the ratiometric UV  $\text{Ca}^{2+}$  indicators outperform indicators developed with the specific purpose of live-cell  $\text{Pb}^{2+}$  imaging particularly with respect to their sensitivity to  $\text{Pb}^{2+}$ .

### 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  $\text{Ca}^{2+}$  sensors, which have been used as tools to study intracellular  $\text{Ca}^{2+}$  dynamics,  $\text{Ca}^{2+}$  buffering and compartmentalization associated with cellular signaling, and  $\text{Ca}^{2+}$  homeostasis (for review, see Hires *et al.*, 2008; Rudolf *et al.*, 2003). The sensitivity of genetically encoded  $\text{Ca}^{2+}$  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  $\text{Zn}^{2+}$  indicators and has yielded a set of  $\text{Zn}^{2+}$  sensor proteins with affinities covering over six orders of magnitude to detect picomolar to micromolar concentrations of  $\text{Zn}^{2+}$  (for review, see Vinkenburg *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  $\text{Pb}^{2+}$  is known to activate calmodulin

comparable to but much more potently than Ca<sup>2+</sup> (Habermann *et al.*, 1983), calmodulin-derived, single-domain sensors are of particular interest for Pb<sup>2+</sup> sensing. The modification of protein indicators for subcellular targeting is another step to produce multiple, picomolar- to nanomolar-affinity Pb<sup>2+</sup> 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<sup>2+</sup> in a not too distant future. Until such sophisticated tools become available for toxicologists, using indo-1 makes sense in Pb<sup>2+</sup> sensing.

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