Lead increases free Ca^{2+} concentration in cultured osteoblastic bone cells: Simultaneous detection of intracellular free Pb^{2+} by ^{19}F NMR

(environmental health/lead toxicity/rat osteosarcoma cells)

FRANCIS A. X. SCHANNE*^{†‡}, TERRY L. DOWD[§], RAJ K. GUPTA^{§¶||}, AND JOHN F. ROSEN*[‡]

Departments of *Pediatrics, [†]Pathology, [§]Physiology and Biophysics, and [¶]Biochemistry, Albert Einstein College of Medicine, Bronx, NY 10461; and [‡]Montefiore Medical Center, Bronx, NY 10467

Communicated by Alfred G. Redfield, April 6, 1989 (received for review October 19, 1988)

ABSTRACT Lead (Pb) has been shown to perturb Ca-mediated cellular processes. However, to date, a direct effect of Pb on intracellular free Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) has not been demonstrated. ^{19}F NMR in combination with 1,2-bis(2-amino-5-fluorophenoxy)ethane-*N,N,N',N'*-tetraacetic acid (5F-BAPTA) was used to simultaneously measure $[\text{Ca}^{2+}]_i$ and intracellular free Pb^{2+} concentration ($[\text{Pb}^{2+}]_i$) in the rat osteoblastic bone cell line ROS 17/2.8. The basal concentration of $[\text{Ca}^{2+}]_i$ in ROS 17/2.8 cells was measured to be 128 ± 24 nM. Treatment with Pb^{2+} at 5 and 25 μM produced sustained 50% and 120% increases in $[\text{Ca}^{2+}]_i$, respectively, over a time course of 5 hr. At a medium Pb^{2+} concentration of 25 μM , the entry of Pb^{2+} into ROS 17/2.8 cells yielded measurable $[\text{Pb}^{2+}]_i$ in cultured cells. Collectively, these findings advance the hypothesis that Pb toxicity is mediated, in part, through perturbations in $[\text{Ca}^{2+}]_i$.

Lead (Pb) toxicity continues to be a major health issue in the United States, involving 9.1% of all preschool children (1). The U.S. Public Health Service estimates that 17.2% of all preschool children are at risk for Pb toxicity, when other sources of Pb from dirt, dust, water, and gasoline are considered, in addition to leaded paint in substandard housing (2). "Low-level" Pb toxicity (blood Pb level of 25–55 $\mu\text{g}/\text{dl}$) includes multiple metabolic (3), neurologic (4, 5), and behavioral (5, 6) disorders. Many recent studies have indicated that even lower blood Pb levels (<25 $\mu\text{g}/\text{dl}$) are associated with impairment in mental development (7), decreased skeletal growth (8), and disturbances in cardiovascular function (9, 10).

Interactions between Pb toxicity and Ca metabolism have been inferred from studies both at the level of the organism as a whole and at the cellular level (11). Based on indirect evidence from a variety of cells and tissues (12–18), the hypothesis has been proposed that Pb^{2+} causes an increase in intracellular pools of Ca and a decrease in the ability of cells to extrude Ca^{2+} to lower the effective intracellular free Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$). The appropriate temporal and spatial resolution of $[\text{Ca}^{2+}]_i$ is critical to the modulation of cellular structure, function, and activity (19). Given the central role of $[\text{Ca}^{2+}]_i$ in the regulation of cellular processes, we and others have proposed that an early, discrete, yet common event in the mechanism of low-level lead toxicity is the inappropriate elevation and regulation of $[\text{Ca}^{2+}]_i$, leading to an important subset of disorders associated with Pb toxicity.

Bone is the major reservoir in the body for both the essential metal ion Ca^{2+} and the toxic metal ion Pb^{2+} ; therefore, the handling of Ca^{2+} and Pb^{2+} by bone cells is of special importance. The rat osteosarcoma cell line ROS 17/2.8, which is

highly differentiated and expresses an osteoblastic phenotype (20), was chosen as a useful model to study the effects of Pb on $[\text{Ca}^{2+}]_i$ in bone cells. ROS 17/2.8 cells synthesize osteocalcin, have high alkaline phosphatase activity, possess $1\alpha,25$ -dihydroxyvitamin D_3 receptors, and respond to parathyroid hormone by increasing adenylate cyclase activity (20) and $[\text{Ca}^{2+}]_i$ (21). An osteoblast-specific function, the production of osteocalcin, the vitamin K-dependent Ca^{2+} -binding protein in bone, is impaired by Pb^{2+} (22).

We previously demonstrated (23) that ^{19}F NMR in combination with 1,2-bis(2-amino-5-fluorophenoxy)ethane-*N,N,N',N'*-tetraacetic acid (5F-BAPTA) distinguishes and measures concentrations of Pb^{2+} and Ca^{2+} in aqueous solution, and this method, originally described by Smith *et al.* (24) to measure $[\text{Ca}^{2+}]_i$, was extended to monolayer/microcarrier cultures of ROS 17/2.8 cells. Using this methodology, we report herein a dose-related increase in $[\text{Ca}^{2+}]_i$ in response to Pb treatment and the measurement of intracellular free Pb^{2+} concentration ($[\text{Pb}^{2+}]_i$) in cultured osteoblastic bone cells.

METHODS

Cell Culture. ROS 17/2.8 cells were maintained and grown to confluence in 75-cm² flasks with Ham's F-12 nutrient mixture supplemented with 5% fetal bovine serum and 28 mM Hepes buffer, pH 7.4 (F-12 medium). ROS 17/2.8 cells were subsequently attached to collagen-coated microcarriers (Cytodex 3, Pharmacia) (23). Cells on beads were maintained for 24–48 hr in 500 ml of F-12 medium in a 1-liter siliconized glass Techne stir vessel at 35 rpm and 37°C in a humidified 95% air/5% CO_2 atmosphere.

Cells were loaded with 5F-BAPTA by incubation with the acetoxymethyl ester of 5F-BAPTA at a concentration of 20 μM for 45 min. Cells on beads were transferred to a 10-mm-diameter NMR tube and continuously superfused with F-12 medium saturated with 95% O_2 /5% CO_2 . The NMR samples were maintained at 30°C throughout the signal accumulation.

NMR. Measurements of 5F-BAPTA-loaded cells were performed with a Varian VXR500 instrument at a Larmor frequency of 470 MHz. Acquisition time was 0.8 sec; pulse angle, 90°; and spectral width, 10 kHz. Each spectrum represented the Fourier transformation of the sum of 2500 free induction decays over a time interval of 33.3 min. Each experiment was begun by acquiring three consecutive spectra in order to establish the baseline $[\text{Ca}^{2+}]_i$ in untreated cells. Treatments were initiated by addition of Pb^{2+} to the superfusion medium.

Abbreviations: $[\text{Ca}^{2+}]_i$, intracellular free Ca^{2+} concentration; $[\text{Pb}^{2+}]_i$, intracellular free Pb^{2+} concentration; 5F-BAPTA, 1,2-bis(2-amino-5-fluorophenoxy)ethane-*N,N,N',N'*-tetraacetic acid.

^{||}To whom reprint requests should be sent at the § address.

The identity of the various spectral peaks was established by a comparison of resonance positions in the cell sample with appropriate standards and by addition of the divalent cation ionophore A23187. Ca-5F-BAPTA and Pb-5F-BAPTA resonances occur at 5.8 and 4.9 ppm, respectively, from the resonance of uncomplexed 5F-BAPTA (23). The concentration ratios for the free and bound species were obtained as ratios of the integrated intensities of the respective resonance peaks by using Varian version 6.1 software. The relative intensities of the complexed and uncomplexed species were unchanged when the pulse recycle time was varied over the range 0.8–3.2 sec in 5F-BAPTA-loaded ROS 17/2.8 cells. This is consistent with short and identical ^{19}F T_1 (relaxation time) values of the complexed and uncomplexed forms, as has been found to be the case in other systems (24–26). Values of $[\text{Ca}^{2+}]_i$ and $[\text{Pb}^{2+}]_i$ are given as the mean $\pm$ SEM.

RESULTS

Fig. 1 represents typical ^{19}F NMR spectra obtained during 100 min without treatment and the subsequent 300 min of Pb^{2+} treatment. The $[\text{Ca}^{2+}]_i$ of untreated cells was 128 ± 24

nM, calculated by using a dissociation constant (K_d) of 5.0×10^{-7} M for the Ca-5F-BAPTA complex (23). With $25 \mu\text{M}$ Pb^{2+} treatment, $[\text{Ca}^{2+}]_i$ increased to 170 ± 28 nM within the first 100 min, with subsequent increments to 220 ± 41 and 284 ± 51 nM within 200 and 300 min, respectively. Overall, the Pb^{2+} -induced rise in $[\text{Ca}^{2+}]_i$ was 120% above control values. Between 200 and 300 min of $25 \mu\text{M}$ Pb^{2+} treatment, a clearly definable intracellular Pb-5F-BAPTA complex was also observed (Fig. 1, bottom spectrum). The intensity of this signal indicated a $[\text{Pb}^{2+}]_i$ of 29 ± 8 pM, calculated with a K_d of 2.1×10^{-10} M for the Pb-5F-BAPTA complex (23). Treatment with $5 \mu\text{M}$ Pb^{2+} produced a 50% increase in $[\text{Ca}^{2+}]_i$ over the same time course; $[\text{Pb}^{2+}]_i$ was not measurable at this medium concentration of Pb^{2+} .

In contrast, treatment of ROS 17/2.8 cells with parathyroid hormone produced a similar but more rapid elevation in $[\text{Ca}^{2+}]_i$ and a prompt return to pretreatment baseline levels within 30 min (23). Treatment of these cells with Pb^{2+} at concentrations up to $100 \mu\text{M}$ for 24 hr did not cause release of lactate dehydrogenase, and incorporation of $[^{14}\text{C}]$ leucine and $[^3\text{H}]$ thymidine was not statistically different from that in untreated cells (27). These data exclude the possibility that the observed increase in $[\text{Ca}^{2+}]_i$ was a reflection of cell death.

FIG. 1. ^{19}F NMR spectra of 5F-BAPTA-loaded ROS 17/2.8 cells treated with $25 \mu\text{M}$ $\text{Pb}(\text{C}_2\text{H}_3\text{O}_2)_2$. Top spectrum, before treatment; middle spectrum, after 1.5 hr of treatment; bottom spectrum, after 3.5 hr of treatment. The frequency scale is given in parts per million (ppm), and the resonance frequencies of free 5F-BAPTA and the Ca-5F-BAPTA and Pb-5F-BAPTA complexes are indicated. The spectral signal-to-noise ratio was enhanced by exponential filtering with a time constant of 6.4 msec, which caused an additional line-broadening of 50 Hz. The spectral baseline was flattened by using standard Varian software to achieve multiple first-order curve-fitting of baseline regions containing no resonances; this eliminated a broad background absorption from fluorine nuclei in the NMR coil-support material.

DISCUSSION

We have demonstrated dose-related increases in $[Ca^{2+}]_i$ in osteoblastic bone cells in response to acute Pb^{2+} treatment. Although the calculated absolute values depend on the K_d of Ca-5F-BAPTA, the main conclusion concerning the increase in $[Ca^{2+}]_i$ with Pb^{2+} treatment is independent of the actual magnitude of the K_d . Our results demonstrate that the increase in $[Ca^{2+}]_i$ in ROS 17/2.8 cells in response to Pb^{2+} represents a prolonged elevation in $[Ca^{2+}]_i$. This inappropriate elevation in $[Ca^{2+}]_i$ may mediate a variety of the toxic consequences of Pb^{2+} by perturbing the temporal and spatial resolution of $[Ca^{2+}]_i$. As a result, one or several loci within the diverse and complex Ca^{2+} messenger system may be impaired, thereby explaining toxic effects of Pb^{2+} on multiple cellular processes.

These NMR measurements represent time-averaged changes in $[Ca^{2+}]_i$, which may occur in response to hormonal stimulation (23) or toxic insult, distinct from the rapid transient changes more readily measured by using the fluorescent and chemoluminescent Ca^{2+} indicators. Furthermore, the demonstration that 5F-BAPTA and ^{19}F NMR can selectively and simultaneously identify and measure both $[Ca^{2+}]_i$ and $[Pb^{2+}]_i$ indicates the potential for this method to be used in toxicological studies of the effects of Pb^{2+} and other heavy metals on $[Ca^{2+}]_i$.

The underlying mechanisms of Pb toxicity are unclear. However, Pb^{2+} has been shown to alter Ca^{2+} -mediated processes in several biological systems (11, 28), and Pb^{2+} can replace Ca^{2+} in binding to and activating intracellular regulatory proteins, including calmodulin (29, 30) and protein kinase C (31, 32). Desaturation kinetic studies have shown that Pb^{2+} both mimics the intracellular distribution of Ca^{2+} and alters cellular Ca^{2+} distribution (17, 33, 34). These earlier reports provide evidence in support of an interaction among Pb^{2+} , Ca^{2+} , and Ca^{2+} -binding regulatory proteins in mediating Pb^{2+} toxicity. Our present data provide direct and simultaneous measurements of $[Ca^{2+}]_i$ and $[Pb^{2+}]_i$. Based upon these measurements, it now appears to be possible to further localize Pb^{2+} - Ca^{2+} interactions to one or several specific sensitive loci within the Ca^{2+} messenger system, thereby providing a mechanistic understanding of Pb^{2+} -induced perturbations of multiple cellular processes.

Direct measurements of $[Ca^{2+}]_i$ and $[Pb^{2+}]_i$ are critical to the understanding of Pb^{2+} toxicity at the cellular level. This understanding could provide a rationale for the development of more effective therapeutic approaches for the treatment of Pb^{2+} toxicity. Our current study has focused on the actions of Pb^{2+} on osteoblastic bone cells. Our methodology, however, is readily adaptable to the study of Pb^{2+} toxicity in other types of cells.

This work was supported in part by U.S. Public Health Service Grants ES01060, ES04006, DK07110, and DK32030 from the National Institutes of Health.

- Centers for Disease Control (1985) *Prevention of Lead Poisoning in Young Children* (U.S. Department of Health and Human Services, Atlanta), CDC 99-2230.
- Agency For Toxic Substances And Disease Registry (1988) *The Nature and Extent of Lead Poisoning In Children In The United*

States: A Report To Congress (U.S. Department of Health and Human Services, Washington, DC).

- Rosen, J. F. (1985) in *Dietary and Environmental Exposure to Lead*, ed. Mahaffey, K. R. (Elsevier/North Holland, Amsterdam), pp. 157-185.
- Needleman, H. L., ed. (1980) in *Low Level Lead Exposure: The Clinical Implications of Current Research* (Raven, New York).
- Davis, J. M. & Svendsgaard, D. J. (1987) *Nature (London)* **329**, 297-300.
- Winneke, G., Kramer, U., Brockhaus, A., Ewers, U., Kaganek, G., Lechner, H. & Janke, W. (1983) *Int. Arch. Occup. Environ. Health* **51**, 232.
- Bellinger, D., Leviton, A., Waternaux, C., Needleman, H. & Rabinowitz, M. (1987) *N. Engl. J. Med.* **316**, 1037-1043.
- Schwartz, J., Angle, C. & Pitcher, H. (1986) *Pediatrics* **77**, 281-288.
- Harlan, W. R., Landis, R., Schmouder, R. L., Goldstein, N. G. & Harlan, L. C. (1985) *J. Am. Med. Assoc.* **253**, 530-534.
- Pirkle, J. L., Schwartz, J., Landis, J. R. & Harlan, W. R. (1985) *Am. J. Epidemiol.* **121**, 246-258.
- Pounds, J. G. (1984) *Neurotoxicology* **5**, 295-332.
- Rosen, J. F. (1983) *Toxicol. Appl. Pharmacol.* **71**, 101-112.
- Cooper, G. P., Suszkiw, J. B. & Manalis, R. S. (1984) *Neurotoxicology* **5**, 247-266.
- Atchison, W. D. & Narahashi, T. (1984) *Neurotoxicology* **5**, 267-282.
- Webb, R. C., Winkist, R. J., Victory, W. & Vander, A. J. (1981) *Am. J. Physiol.* **241**, 211-216.
- Kopp, S. J., Glonek, T., Erlanger, M., Perry, E. F., Perry, H. M. & Barany, M. (1980) *J. Environ. Pathol. Toxicol.* **4**, 205-227.
- Pounds, J. G., Wright, R., Morrison, D. & Casciano, D. A. (1982) *Toxicol. Appl. Pharmacol.* **63**, 389-401.
- Pounds, J. G., Morrison, D., Wright, R., Casciano, D. A. & Shaddock, J. G. (1982) *Toxicol. Appl. Pharmacol.* **63**, 402-408.
- Alkon, D. L. & Rasmussen, H. (1988) *Science* **239**, 998-2004.
- Rodan, G. A. & Rodan, S. B. (1983) in *Bone and Mineral Research*, ed. Peck, W. A. (Elsevier, Amsterdam), Annu. **2**, pp. 244-285.
- Donahue, H. J., Fryer, M. J., Eriksen, E. F. & Heath, H., III (1988) *J. Biol. Chem.* **263**, 13522-13527.
- Markowitz, M. E., Gundberg, G. M. & Rosen, J. F. (1988) *Pediatr. Res.* **23**, 393A.
- Schanne, F. A. X., Dowd, T. L., Gupta, R. K. & Rosen, J. F. (1989) *Environ. Health Perspect.*, in press.
- Smith, G. A., Hesketh, R. T., Metcalfe, J. C., Feeney, J. & Morris, P. G. (1983) *Proc. Natl. Acad. Sci. USA* **80**, 7178-7182.
- Levy, L. A., Murphy, E. & London, R. E. (1987) *Am. J. Physiol.* **252**, C441-449.
- Marban, E., Kitakaze, M., Kusuoka, H., Porterfield, J. K., Yue, D. T. & Chack, V. P. (1987) *Proc. Natl. Acad. Sci. USA* **84**, 6005-6009.
- Long, G. J., Rosen, J. F. & Pounds, J. G. (1988) *Toxicologist* **8**, 23A.
- Simons, T. J. B. (1986) *Br. Med. Bull.* **42**, 431-434.
- Habermann, E., Crowell, K. & Janicki, P. (1983) *Arch. Toxicol.* **54**, 61-70.
- Fullmer, C. S., Edelstein, S. & Wasserman, R. H., (1985) *J. Biol. Chem.* **260**, 6816-6819.
- Markovac, J. & Goldstein, G. W. (1988) *Nature (London)* **343**, 71-73.
- Markovac, J. & Goldstein, G. W. (1988) *Toxicol. Appl. Pharmacol.* **96**, 14-23.
- Pounds, J. G., Wright, R. & Kodell, R. L. (1982) *Toxicol. Appl. Pharmacol.* **66**, 88-101.
- Rosen, J. F. & Pounds, J. G. (1989) *Toxicol. Appl. Pharmacol.*, in press.