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Permeation of Pb^{2+} through calcium channels: fura-2 measurements of voltage- and dihydropyridine-sensitive Pb^{2+} entry in isolated bovine chromaffin cells

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Fura-2 was used to monitor Pb^{2+} entry into isolated bovine chromaffin cells exposed to micromolar concentrations of Pb^{2+} in media containing basal or high concentrations of K^+. The entry of Pb^{2+} consists of voltage-independent and voltage-dependent (K^+-stimulated) components. The voltage-dependent Pb^{2+} entry is enhanced by Ca^{2+} channel agonist BAY K 8644 and blocked by the channel antagonist nifedipine, suggesting the involvement of the L-type Ca^{2+} channels. In contrast to the transient, K^+-depolarization-dependent increase in $[Ca^{2+}]_i$, the increase in $[Pb^{2+}]_i$ is sustained over a period of several minutes, suggesting the absence of channel inactivation and/or the saturation of Pb^{2+}-buffering capacity of the cell cytosol.

Introduction

Much evidence indicates that neurotoxic properties of ionized lead (Pb^{2+}) are related to modification of cellular, calcium-dependent processes [1,2]. We have recently employed the fluorescent, divalent-cation indicator fura-2 to measure $[Pb^{2+}]_i$ and demonstrated that Pb^{2+} readily permeates into isolated chromaffin cells and, at intracellular concentrations in the picomolar range, triggers the secretion of norepinephrine [3]. The mechanism of Pb^{2+}-induced secretion involves, at least in part, a direct 'Ca^{2+}-surrogate' action of Pb^{2+} ions on the secretory mechanism. Although the entry of Pb^{2+} into chromaffin cells occurs readily under nondepolarizing conditions, it has been reported that uptake of Pb^{2+} is stimulated further by K^+-depolarization. The K^+-depolarization-stimulated uptake of Pb^{2+} is enhanced by BAY K 8644 and inhibited by D-600, suggesting that the voltage-dependent entry may be mediated by voltage-gated Ca^{2+} channels [4]. That Pb^{2+} can enter cells through depolarization-activated calcium channels is also suggested by the observation that Pb^{2+}-induced release of acetylcholine from rat cortical synaptosomes is enhanced by K^+-depolarization [5]. In the present work we have studied the entry of Pb^{2+} into K^+-stimulated bovine chromaffin cells using fura-2. We provide real-time measurements of $[Pb^{2+}]_i$ showing a voltage- and a dihydropyridine-sensitive influx of Pb^{2+} and demonstrate that the depolarization-induced changes in $[Ca^{2+}]_i$ and $[Pb^{2+}]_i$ are remarkably different.

Materials and Methods

Isolation of bovine chromaffin cells, preparation of Pb^{2+} buffers and fura-2 measurements were carried out as described previously [3]. Briefly, the cells were dissociated by collagenase treatment of adrenal medullary tissue and allowed to recover for 2–3 h at 37°C in aerated (95% air/5% CO_2) saline solution containing 115 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1

Abbreviations: $[Ca^{2+}]_i$, intracellular free Ca^{2+} concentration; $[Pb^{2+}]_i$, intracellular free Pb^{2+} concentration; BSA, bovine serum albumin; K_d(Pb), dissociation constant of the Pb^{2+}-fura-2 complex; R_b, fura-2 ratio of fluorescence before exposure of cells to Pb^{2+}; R_a, fura-2 ratio of fluorescence after exposure of cells to Pb^{2+}; R_{min}, fura-2 minimum ratio of fluorescence; Sf_2/Sb_{2Pb}, ratio between proportionality coefficients at 380 nm of free fura-2 and the complex Pb^{2+}-fura-2; R_{maxCa} and R_{maxPb}, fura-2 maximum ratio of fluorescence in the presence of saturating concentrations of Ca^{2+} or Pb^{2+}; E_{Pb}, equilibrium potential for Pb^{2+}; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

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mM $MgCl_2$, 0.5 mM NaH_2PO_4, 20 mM $NaHCO_3$, 0.5% BSA, and 10 mM Hepes-NaOH buffer (pH 7.4). Prior to experiments, the cells were pelleted and resuspended in 2 ml of a continuously oxygenated solution consisting of 140 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 5.5 mM glucose, 10 mM Hepes-NaOH buffer (pH 7.4), and 1 μM fura-2/AM in 0.1% DMSO (final concentration). Fura-2 loading was carried out for 45 min at 32°C. After loading incubations, the cells were washed twice by diluting the suspension in 50 ml of the above buffer without Ca^{2+}, and pelleting at $500 \times g$ for 5 min. The cells were then resuspended in the same washing solution, counted in a hemocytometer and their concentration adjusted at about $2.5 \cdot 10^6$ cells/ml for fluorometric experiments. The experiments were carried out at 37°C in a dual excitation, dual emission Spex spectrofluorometer. Fura-2 emission was measured at 505 nm in the ratio mode with excitation at 340 and 380 nm. The fura-2 signals were calibrated by lysing the cells in Triton X-100 and determining R_{min} in presence of EGTA and R_{maxCa} and R_{maxPb} at saturating concentrations of Ca^{2+} and Pb^{2+}, respectively. Cytosolic concentrations of Ca^{2+} were calculated according to Grynkiewicz et al. [6]. Cytosolic concentrations of Pb^{2+} were calculated as described previously [3] from the ratios of fluorescence of fura-2 according to the formula:

$$[Pb^{2+}]_i = K_d(Pb) \cdot \frac{(R_a - R_b)(R_{maxCa} - R_{min})}{(R_{maxPb} - R_a)(R_{maxCa} - R_b)} \cdot \frac{Sf_2}{Sb_{2Pb}}$$

Results and Discussion

We have shown previously that in the absence of Ca^{2+} or Pb^{2+} in the basal medium, the 340/380 nm ratio of fluorescence recorded from the fura-2 loaded chromaffin cells remains stable, indicating average $[Ca^{2+}]_i$ at 60–80 nM. Upon introduction of Pb^{2+} into the medium, the fluorescence ratio increases linearly as a function of time and extracellular $[Pb^{2+}]$ due to accumulation of $Pb^{2+}$and formation of Pb^{2+}-fura-2 complex in the cells [3]. The present results illustrated in Fig. 1A show that the basal rate of Pb^{2+} entry is enhanced as function of cell depolarization by high K^+. Fig 1B. shows that $[Pb^{2+}]_i$ in K^+-stimulated cells increases as an approximately linear function of voltage. This is consistent with the very large Pb^{2+} concentration gradient across the cell membrane ($[Pb^{2+}]_o/[Pb^{2+}]_i \approx 10^6$), and a calculated $E_{Pb} \approx +350$ mV.

The depolarization-stimulated component of Pb^{2+} entry is enhanced by inclusion in the medium of 1 μM BAY K 8644, and reduced by 10 μM nifedipine to the value observed in basal conditions (Fig. 2). Although the foregoing results support the notion that under depolarizing conditions Pb^{2+} entry into the chromaffin cells occurs through the L-type Ca^{2+} channels, the kinetics of the voltage-dependent Ca^{2+} and Pb^{2+}entry into the cells are quite different. This is illustrated in Fig. 3 where fura-2 responses to K^+-depolarization-activated entry of Ca^{2+} and Pb^{2+} are compared. It is evident that while the response to Ca^{2+} is characteris-

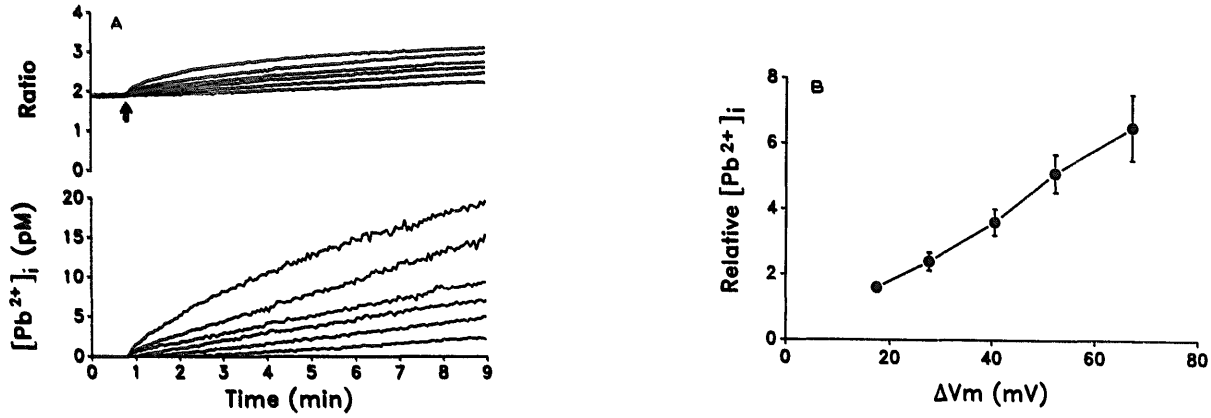

Fig. 1. (A) Effect of the extracellular K^+ concentration on the fluorescence of fura-2 loaded cells treated with Pb^{2+}. Approximately $5 \cdot 10^6$ cells suspended in 2 ml of a solution containing (in mM) 140 NaCl, 5 KCl, 1 $MgCl_2$, 5.5 glucose and 10 Hepes-NaOH buffer (pH 7.4) were mixed with 2 ml of different solutions containing 2 mM citric acid and $Pb(ClO_4)_2$ to yield 0.5 μM free Pb^{2+}. Cell suspensions were mixed with the same buffer or the same buffer modified to contain different concentrations of KCl (15, 25, 45, 75 and 139 mM) and appropriately reduced concentrations of NaCl (130, 120, 100, 70 and 6 mM). Final concentrations of KCl after mixing were (in mM) 5, 10, 15, 20, 40 and 72. Upper panel: 340/380 nm ratios of fluorescence in a representative experiment are shown. The arrow indicates the time of mixing. Higher ratios correspond to higher K^+ concentrations. This response was observed in three different preparations. Lower panel: $[Pb^{2+}]_i$ calculated using ratios of fluorescence from data shown in upper panel. $[Ca^{2+}]_i$ measured before exposure of cells to Pb^{2+} was 110 nM. (B) Voltage-dependence of intracellular Pb^{2+} concentration. Cells were treated as described in (A). Differences in membrane potential between the resting state (5 mM KCl) and depolarizing conditions (x mM KCl) were calculated as 58 mV·log(x/5). Intracellular Pb^{2+} concentrations, after 8 min of Pb^{2+} exposure, were calculated using ratios of fluorescence and expressed relative to the accumulation of Pb^{2+} in resting conditions (5 mM KCl). Results are means ± S.D. of three values obtained in three different preparations.

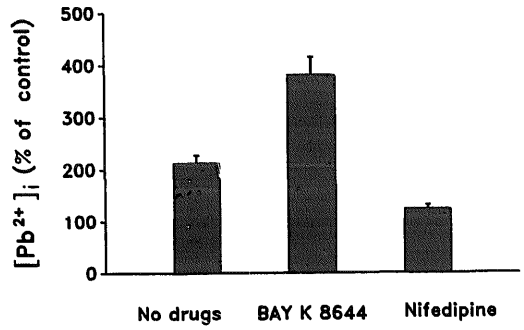

Fig. 2. Effect of BAY K 8644 and nifedipine on the intracellular Pb^{2+} concentrations of fura-2 loaded cells treated with Pb^{2+} in the presence of high K^+. Cells suspended in 2 ml of buffer solution as described in Fig. 1A were mixed with 2 ml of different solutions containing 2 mM citric acid and $Pb(NO_3)_2$ to yield 0.5 μM free Pb^{2+}. Cell suspensions were mixed with the same solution (control) or the same solution modified to contain 45 mM KCl (25 mM final concentration), 100 mM NaCl (120 mM final concentration) and either 2 μM BAY K 8644 (1 μM final concentration) or 20 μM nifedipine (10 μM final concentration) or no drugs. Intracellular Pb^{2+} concentrations, after 3 min of Pb^{2+} exposure, were calculated using ratios of fluorescence and expressed as a percentage of control. Results are means ± S.D. of three experiments carried out in three different preparations. $[Pb^{2+}]_i$ in control cells was 2.0 ± 0.5 pM.

tically transient [7], the fura-2 monitored accumulation of Pb^{2+} is sustained over a period of several minutes. The sustained elevation of $[Pb^{2+}]_i$ is in agreement with the earlier report by Simons and Pocock [4] that K^+-stimulated uptake of Pb^{2+} into chromaffin cells does not inactivate.

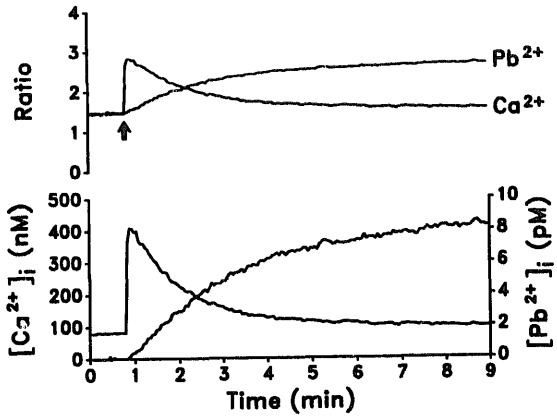

Fig. 3. Fluorescence of fura-2 loaded cells challenged with high K^+ medium containing Ca^{2+} or Pb^{2+}. Cells, suspended in 2 ml of buffer solution as described in Fig. 1A, were mixed with 2 ml of the same solution modified to contain 139 mM KCl (72 mM final concentration), 6 mM NaCl (73 mM final concentration) and either 200 μM $CaCl_2$ or 200 μM $Pb(ClO_4)_2$ (100 μM final concentrations). $CaCl_2$ and $Pb(ClO_4)_2$ were omitted in control experiments. Upper panel: 340/380 nm ratios of fluorescence in a representative experiment are shown. The arrow indicates the time of mixing. The ratio was stable in the absence of Ca^{2+} and Pb^{2+} (control not shown). This paired experiment was carried out two times in two different preparations. Lower panel: Intracellular Pb^{2+} concentrations calculated using ratios of fluorescence from data shown in upper panel.

The present communication provides further evidence that fura-2 can be utilized to monitor cellular accumulation of Pb^{2+}. Although we cannot completely exclude a Pb^{2+}-induced change in $[Ca^{2+}]_i$ our previous work in chromaffin cells indicate that the fluorescent signals from fura-2 loaded cells exposed to Pb^{2+} are associated with Pb^{2+} rather than Ca^{2+} complexation with fura-2 [3]. Furthermore, previous work indicates that Pb^{2+} does not displace Ca^{2+} from subcellular organelles such as mitochondria or endoplasmic reticulum [8]. This procedure provides a temporal resolution that is better than measurement of Pb^{2+} uptake by radiotracer or atomic absorption techniques, and allows measurements of Pb^{2+} permeation in real-time. The results of this study agree with the original observation made using atomic absorption spectrophotometry by Simons and Pocock [4] that Pb^{2+} can permeate through the voltage-gated L-type Ca^{2+} channels in chromaffin cells, and are consistent with the observation that Pb^{2+}-induced release of acetylcholine is enhanced by depolarization of synaptosomes in high K^+ media in a fashion analogous to that observed in the case of Ca^{2+}-evoked transmitter release [5]. Since synaptosomal Ca^{2+} channels are not dihydropyridine-sensitive [9], this suggests that Pb^{2+} probably can permeate readily through various types of Ca^{2+} channels found in neuronal preparations. The channel-mediated entry of Pb^{2+} into neurons could be an important factor in lead neurotoxicity and suggests that highly active neurons could be particularly vulnerable.

Perhaps the most interesting aspect of Pb^{2+} permeation through the voltage-gated Ca^{2+} channels is the persistent elevation of $[Pb^{2+}]_i$. This could reflect continued entry of Pb^{2+} due to a lack of channels inactivation. In this respect Pb^{2+} resembles Ba^{2+} which also permeates readily through the calcium channels with minimal channel inactivation. There are several possible explanations why in contrast to Ca^{2+}, the influx of Pb^{2+} might not cause channel inactivation. For example, the Ca^{2+}-dependent inactivation of L-type channels has been suggested to involve activation of protein phosphatase calcineurin and channel dephosphorylation [10]. It is possible that Pb^{2+} maintains channels in phosphorylated state either by failing to activate protein phosphatases, and/or directly promoting channel phosphorylation. However, an alternative explanation for the persistence of free Pb^{2+} in the cell cytosol might be that a large entry of Pb^{2+} saturates the intracellular divalent-cation sequestration systems more rapidly than in the case of Ca^{2+}. The present data do not allow us to distinguish between these two possibilities.

In conclusion, although lead ions have been long known to act as potent competitive inhibitors of voltage-gated calcium channels [11,12], it is now evident that Pb^{2+} does not merely compete for the divalent

metal cation binding site in the channel but can in fact permeate through the channel. In effect then, Pb^{2+} can disrupt normal spatial and temporal control of the second-messenger functions of Ca^{2+} by on the one hand blocking Ca^{2+} influx and on the other, acting intracellularly as potent Ca^{2+}-surrogate.

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