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Pb^{2+} modulates ryanodine receptors from the endoplasmic reticulum in rat brain

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ABSTRACT

Although the neurotoxic mechanism of lead (Pb^{2+}) has been extensively studied, it is not well understood. The effects of Pb^{2+} on free cytosolic calcium (Ca^{2+}) concentration and calcium-regulated events have been suggested to be major mechanisms in Pb^{2+} toxicity. Based on our previous findings that Pb^{2+} changes calcium release through ryanodine receptors (RyRs), the modulation of endoplasmic reticulum (ER) vesicular RyRs by Pb^{2+} was investigated further in the present study. The results of [^{3}H]ryanodine binding assays showed that in the presence of a free Ca^{2+} concentration ($[Ca^{2+}]_f$) of 100 μM, Pb^{2+} modulated the equilibrium of [^{3}H]ryanodine binding to brain RyRs, with a U-type dose-response curve, where minimal binding was observed at a free Pb^{2+} concentration ($[Pb^{2+}]_f$) of 0.39 μM. This modulation was also observed over a time course. Scatchard analysis indicated that both an increase in K_d and a possible decrease in B_{max} were responsible for the decrease in binding induced by low $[Pb^{2+}]_f$. Moreover, the effects of Pb^{2+} on the function of ER RyRs in neurons might also be controlled by other RyR modulators. Whole-cell patch-clamp experiments revealed that dynamic calcium oscillations evoked by specific RyR agonists were depressed rapidly and reversibly by exposure to 10 μM Pb^{2+}. Our study indicates that RyRs are molecular targets of Pb^{2+}, and this interaction disturbs Ca^{2+} signals and leads to neurotoxicity.

1. Introduction

Calcium (Ca^{2+}) is indispensable as an intracellular second messenger for the physiology of organisms and the molecular regulation of cells. Cytosolic free Ca^{2+} is a crucial signal for a variety of neuronal processes including neurotransmitter release, control of membrane excitability, synaptic plasticity, and cognition (Qi and Shuai, 2016; Ludwar et al., 2017; Murali et al., 2017). Ca^{2+} homeostasis in neurons is accurately controlled by several types of Ca^{2+} channels and by the activity of Ca^{2+} transporters located on the plasma membrane or on the endoplasmic reticulum (ER)/sarcoplasmic reticulum (SR) (Fernandez-Morales et al., 2012; Clapham, 1995). Disturbed calcium homeostasis is implicated in both the normal process of aging and the brain pathology prevalent in many neurodegenerative and neurotoxic diseases.

Ryanodine receptors (RyRs), the major intracellular Ca^{2+} release channels located in the ER, are crucial for brain function because they mediate Ca^{2+} release (Berridge, 2002; Ross, 2012; Mattson et al., 2000). Moreover, emerging evidence suggests that by locally controlling levels of cytoplasmic free Ca^{2+} in growth cones and synaptic compartments, RyRs regulate functional and structural changes in nerve cell circuits in both the developing and the adult nervous systems (Mattson et al., 2000), and alterations in Ca^{2+} homeostasis by RyRs contribute to neuronal apoptosis and excitotoxicity, which are linked to the pathogenesis of several different neurodegenerative disorders (Clapham, 1995; Stutzmann et al., 2007; Oules et al., 2012).

Lead (Pb^{2+}) is a neurotoxin that continues to be considered a major global environmental health hazard. However, the mechanism underlying Pb^{2+} toxicity is not completely understood. The effects of Pb^{2+}

Abbreviations: B_{max}, the maximal number of ryanodine-binding site; Ca^{2+}, calcium ion; cADPR, cyclic ADP-ribose; ER, endoplasmic reticulum; GSH, reduced glutathione; K_d, the equilibrium binding constant; Mg^{2+}, magnesium ion; Pb^{2+}, lead ion; PKA, cAMP-dependent protein kinase A; RyRs, ryanodine receptors; SR, sarcoplasmic reticulum; $[Ca^{2+}]_f$, free Ca^{2+} concentration; $[Pb^{2+}]_f$, free Pb^{2+} concentration

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on cytosolic free Ca^{2+} concentration and calcium-regulated events have been suggested to be the major mechanisms involved in Pb^{2+} toxicity (Tomsig and Suszkiw, 1991; Audesirk and Audesirk, 1993; Neal and Guilarte, 2010).

The ionic radius of Pb^{2+} (1.19 Å) is similar to that of Ca^{2+} (1.00 Å), allowing Pb^{2+} to replace Ca^{2+} at the site of an active transporter for Ca^{2+} and to be carried into cells (Martinez-Finley et al., 2012; Kerper and Hinkle, 1997). In addition, Pb^{2+} appears to resemble Ca^{2+} sufficiently closely to disrupt calcium homeostasis and affect calcium signaling, which results in neurotoxicity and other health detriments (Silbergeld and Adler, 1978; Simons, 1986; Audesirk and Audesirk, 1991; Zhang et al., 2012). Interestingly, neurodevelopmental disorders and degenerative diseases that have been shown to be caused by increasing expression of RyRs or enhancing RyR-mediated ER Ca^{2+} release (Stutzmann et al., 2007; Oules et al., 2012) have also been shown to be caused by lead exposure (Wu et al., 2008; Basha et al., 2005). Furthermore, previous results in our laboratory indicated that Pb^{2+} exposure affects RyRs leading to an increase the resting $[Ca^{2+}]i$ in cultured cells (Fan et al., 2013) and increases expression of RyRs to impair cognitive function in rat models (unpublished data). However, whether there are interactions between Pb^{2+} and RyRs is not understood, and this information is needed for further studies.

[^{3}H]ryanodine binding assays are a convenient method to investigate the effect of various agents on the function of RyRs (Lanner et al., 2010; Chu et al., 1990). In particular, a close correlation between ryanodine binding and the gating state of RyRs has been established for various ligands of RyRs (Chu et al., 1990; Lai and Meissner, 1989; Duvshani, 2014). Xenobiotics, if they are ligands, should be able to regulate the opening and closing of RyRs by increasing or decreasing ryanodine binding. To better understand the effect of Pb^{2+} on Ca^{2+} release from RyRs in neurons, the effect of Pb^{2+} on ryanodine binding to ER vesicular RyRs in adult rat brains was investigated in the present study.

2. Materials and methods

2.1. Membrane preparations of endoplasmic reticulum (Paiement et al., 2004; Eriksson et al., 1983)

Specific-pathogen-free (SPF) Sprague-Dawley (SD) rats were purchased from Sino-British SIPPR/BK Lab Animal Ltd. (Shanghai, China) and housed separately in a laboratory animal facility at 20–25 °C and 50–60% humidity with 12 h light/dark cycles. When the weight of the rats reached 200–250 g, they were fasted for 12–24 h and then sacrificed via decapitation after 2% pentobarbital sodium anesthesia injected intraperitoneally (25 mg/kg body weight). Brains were excised and placed in a petri dish containing ice-cold 25% sucrose solution (the average brain, when free of connective tissue and vasculature, weighed approximately 1.5 g/300–400 g body weight). The samples were weighed and homogenized in a volume of ice-cold lysis buffer (0.25 M sucrose, 10 mM imidazole, 0.2 mM PMSF and 1 μM leupeptin, pH 7.4) equivalent to 2.5 times the weight of the brain.

For the preparation of total ER, the homogenate was centrifuged at 8500 × *g* for 13 min in a Sigma 3-18K 12111-H angle rotor to sediment cellular debris and nuclei. The supernatant was transferred into polycarbonate screw-top tubes and centrifuged in a Sigma Ti70 angle rotor at 32165 × *g* for 5 min to remove lysosomes and mitochondria. The supernatant and a portion of the flocculent layer were recovered and centrifuged in a Sigma Ti70 fixed-angle rotor at 114584 × *g* for 47 min. The supernatant was discarded, while the precipitate, which represented the ER fraction, was recovered. All precipitates were collected and suspended either in a storage medium (0.25 M sucrose and 5 mM HEPES, pH 7.4) and stored at − 80 °C in hermetically sealed tubes or in a cold fixative solution of glutaraldehyde and stored at 4 °C for further experiments.

Ninety rats were used for this experiment, and each ER extraction procedure took approximately 5 h with 20–25 rats per batch. All samples were stored on ice at all times throughout the protocol, and all centrifugations occurred at 4 °C while the environmental temperature was controlled at 23 °C. Animal sacrifices were carried out according to the principles and guidelines of the Guide for the Care and Use of Laboratory Animals, China Council on Animal Care.

2.2. Morphological analysis

ER membrane fractions from rat brain were fixed in ice-cold glutaraldehyde (1.5% in 0.05 M phosphate buffer, pH 7.4, 4 °C) for morphological analysis. ER membrane fractions were fixed overnight, fully washed with 0.1 M phosphate-buffered saline three times, stained with uranyl acetate for 2 h, and then post-fixed with 1% osmium tetroxide. Epoxy resin was used to embed the ER membrane fractions after they had been dehydrated in a graded series of ethanol. Finally, the ultrathin sections (75 nm) were viewed by transmission electron microscope (JEM-2100, JEOL, Tokyo, Japan).

2.3. Western blotting

The protein concentrations were determined using a bicinchoninic acid (BCA) protein assay kit (Thermo Fisher Scientific) after samples were suspended in a storage medium (0.25 M sucrose and 5 mM HEPES, pH 7.4). Each sample was diluted with SDS-PAGE loading sample buffer (5 ×), which contained 25% (v/v) glycerol, 10% (v/v) β-mercaptoethanol, 0.5% (w/v) bromochlorophenol blue, 10% (w/v) SDS and 0.312 M Tris–HCl at pH 6.8, and then heated in boiling water for 5 min. A 30 μg sample of protein (one sample from each of the four batches) was loaded in each lane of an electrophoresis gel. These diluted samples and the pre-stained protein markers (Thermo Fisher Scientific) were separated by 10% SDS-PAGE (with a 4% concentrating gel) under reducing conditions and electro-transferred to a Hybond-C nitrocellulose membrane (0.45 μm, Millipore). After being blocked for 1 h at room temperature with either 5% (w/v) nonfat milk (for glyceraldehyde-3-phosphate dehydrogenase, GAPDH and glucose regulated protein78, GRP78) or 5% (v/v) normal goat serum (for RyR2 and RyR3) in TBST at pH 7.4, the membranes were incubated overnight at 4 °C with the following primary antibodies: rabbit anti-RyR2 (1:1000, Millipore Chemicon), rabbit anti-RyR3 (1:1000, Millipore Chemicon), rabbit anti-GRP78 (1:3000, Sigma-Aldrich) and mouse anti-GAPDH (1:35000, Sigma-Aldrich). After being washed 3 times in TBST, the membranes were incubated with horseradish peroxide-conjugated goat anti-rabbit/anti-mouse secondary antibody (1:8000 for anti-rabbit secondary antibody, 1:5000 for anti-mouse secondary antibody, Jackson Lab) in 5% (w/v) nonfat milk/TBST for 2 h at room temperature. Immunoreactive proteins were detected using SuperSignal West Pico Chemiluminescent Substrate (Thermo Fisher Scientific) and visualized using BioRad-ChemiDoc™ XRS + Systems with Image Lab™ Software.

2.4. [^{3}H]ryanodine binding assays

Ten nanomoles of [^{3}H]ryanodine (87 Ci/mmol, Perkin Elmer) was incubated for 3 h at 37 °C with 1 μg/μl membrane protein in 0.2 ml of binding buffer containing 140 mM KCl, 15 mM NaCl, 20 mM HEPES, 50 μM EGTA, and 10% sucrose, at pH 7.4 and supplemented with complete EDTA-free protease inhibitor cocktail (cat. no. 04693132001, Roche Applied Science, Mannheim, Germany) following the manufacturer's instructions. At the end of the incubation time, the samples were filtered through Whatman GF/F glass fiber filters (Perkin Elmer), which were pre-soaked with 2% polyethyleneimine to saturate the nonspecific binding sites. The filters were rapidly rinsed with deionized water 20 times and dried under vacuum. The radioactivity of the samples was measured by using a liquid scintillation counter (MicroBeta, PerkinElmer) after soaking in scintillation cocktail (OptiPhase Supermix, NEN/Perkin Elmer) overnight. Specific binding was defined