Interactive report

L-type Ca\(^{2+}\) channel-mediated Zn\(^{2+}\) toxicity and modulation by ZnT-1 in PC12 cells\(^1\)

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Abstract

In view of evidence that Zn\(^{2+}\) neurotoxicity contributes to some forms of pathological neuronal death, we developed a model of Zn\(^{2+}\) neurotoxicity in a cell line amenable to genetic manipulations. Exposure to 500 \(\mu\)M ZnCl\(_2\) for 15 min under depolarizing conditions resulted in modest levels of PC12 cell death, that was reduced by the L-type Ca\(^{2+}\) channel antagonist, nimodipine, and increased by the L-type Ca\(^{2+}\) channel opener, \(\text{S(−)}\)-Bay K 8644. At lower insult levels (200 \(\mu\)M Zn\(^{2+}\)+Bay K 8644), Zn\(^{2+}\)-induced death appeared apoptotic under electron microscopy and was sensitive to the caspase inhibitor benzyloxycarbonyl-Ala-Ala-Asp-CH\(_2\)F (Z-VAD); at higher insult levels (1000 \(\mu\)M+Bay K 8644), cells underwent necrosis insensitive to Z-VAD. To test the hypothesis that the plasma membrane transporter, ZnT-1, modulates Zn\(^{2+}\) neurotoxicity, we generated stable PC12 cell lines overexpressing wild type or dominant negative forms of rat ZnT-1 (rZnT-1). Clones T9 and T23 overexpressing wild type rZnT-1 exhibited enhanced Zn\(^{2+}\) eflux and reduced vulnerability to Zn\(^{2+}\)-induced death compared to the parental line, whereas clones D5 and D16 expressing dominant negative rZnT-1 exhibited the opposite characteristics.

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1. Introduction

The transition metal zinc is a normal constituent of transcription factors and metalloenzymes [31,51]. In the central nervous system, an additional pool of chelatable Zn\(^{2+}\) exists in a subpopulation of glutamatergic synaptic vesicles [5,8,13,35] and can be released in a Ca\(^{2+}\)-dependent manner by electrical or chemical stimulation [17], possibly reaching synaptic concentrations in the hundred micromolar range [2]. Zn\(^{2+}\) can modify the behavior of several membrane receptors and channels [6,15,43]; it inhibits GABA and NMDA receptor currents [37,55], blocks voltage-gated Na\(^{+}\) and Ca\(^{2+}\) channels, and potentiates AMPA, glycine, and P\(_{2x}\) receptor currents [16,38].

Given its lack of intrinsic redox activity, Zn\(^{2+}\) has been considered to be relatively nontoxic [3,46]. However, the toxic translocation of presynaptic zinc into postsynaptic neurons has now been implicated in the pathogenesis of selective neuronal death following transient global ischemia, prolonged seizures, and trauma [23,42,45,48]. The cytotoxicity of exogenously applied zinc has been demonstrated in vitro and in vivo. In neuronal as well as nonneuronal cells such as thymocytes, prolonged exposure to even 20–80 \(\mu\)M Zn\(^{2+}\) induces cell death [12,26,41]. Short exposures to 150–600 \(\mu\)M Zn\(^{2+}\) destroys cultured cortical [58] or cerebellar granule neurons [28], and intraparenchymal injection of ZnCl\(_2\) induces neuronal...
necrosis in the rat hippocampus [25]. With concurrent depolarization, the toxicity of extracellular Zn\(^{2+}\) is enhanced such that short exposure to low micromolar concentrations of Zn\(^{2+}\) becomes neurotoxic [54]. This depolarization-induced enhancement likely reflects preferential entry of zinc through L-type voltage-gated Ca\(^{2+}\) channels, leading to toxic elevations of intracellular free Zn\(^{2+}\) ([Zn\(^{2+}\)]\(_{intr}\)) in the range of 300–500 nM [4,40].

Likely opposing such toxic elevations of [Zn\(^{2+}\)], are several mechanisms responsible for maintaining intracellular Zn\(^{2+}\) homeostasis, including binding to metallothioneins [1,10,50,53], and export across the plasma membrane mediated by the ubiquitously expressed zinc transporter, ZnT-1 [34]. Related zinc transporters appear to bear responsibility for transporting Zn\(^{2+}\) into endosomes (ZnT-2) [32] or synaptic vesicles (ZnT-3) [33]. Baby hamster kidney (BHK) cells [34] and N2A cells [49] overexpressing ZnT-1 exhibit resistance to death induced by prolonged Zn\(^{2+}\) exposure. Following transient global ischemia in gerbils, ZnT-1 transcription is upregulated in CA1 pyramidal neurons, consistent with the possibility that ZnT-1 induction may be a cellular strategy to counter ischemia-induced toxic Zn\(^{2+}\) influx [49].

We set out to develop a model of Zn\(^{2+}\) influx-induced cell death in a neuronal cell line. We chose PC12 cells because they have been widely used for investigating multiple aspects of neurobiology, including neuronal differentiation, intracellular signaling pathways, and cell survival [14,56], and specifically express L-type Ca\(^{2+}\) channels [18,47], the primary route of toxic Zn\(^{2+}\) entry into neurons. There is one previous report of Zn\(^{2+}\)-induced death of PC12 cells, utilizing prolonged (24 h) exposure to Zn\(^{2+}\) in the absence of serum, thought to be due to direct inactivation of NGF by Zn\(^{2+}\) [39]. To eliminate this death mechanism and focus on toxic Zn\(^{2+}\) entry, we utilized brief (15 min) exposure to Zn\(^{2+}\), followed by return to serum-containing medium, as the presence of serum abrogated Zn\(^{2+}\)-mediated neurotrophin deprivation-induced death [39].

2. Materials and methods

2.1. Cell culture, drugs, and stable transfection

PC12 cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% heat-inactivated horse serum, 5% fetal bovine serum, and 2 mM L-glutamine (GIBCO BRL) in a humidified incubator at 37°C and 5% CO\(_2\). Twenty-four hours prior to experiments, cells were plated onto poly-L-lysine-coated 24-well (16 mm diameter) plates at a density of 1.25×10⁵ cells/well. ZnCl₂, GdCl₃, and A23187 were purchased from Sigma, and S-(−)-Bay K 8644 and nimodipine from Research Biochemicals International. Benzoyloxycarbonyl-Val-Ala-Asp-CH₂F (Z-VAD) was purchased from Enzyme Systems Products. PC12 cells were transfected using FuGene 6 (Boehringer Mannheim), and stable transfectants were selected in medium containing 500 μg/ml G418.

2.2. Constructs

Wild type rZnT-1 cDNA (kindly provided by Dr. R.D. Palmiter) and dominant negative rZnT-1 cDNA (an 83 bp EagI fragment deletion; [34]) were subcloned into a CMV promoter-driven mammalian expression vector. After mutating the terminal stop codon in both rZnT-1 forms, a fragment encoding a hexameric myc epitope tag (generously provided by Dr. R. Kopan) was inserted in-frame distal to the altered stop codon.

2.3. Toxicity experiments

Immediately before toxic exposures, cells were washed twice using a HEPES-buffered salt solution (HSS) with the following composition: 130 mM Na\(^+\), 5.4 mM K\(^+\), 0.8 mM Mg\(^2+\), 1.8 mM Ca\(^{2+}\), 131 mM Cl\(^-\), 20 mM HEPES (pH 7.4 at 25°C), 15 mM glucose. Exposures of 15 min to toxic solutions were conducted at room temperature in HSS with equimolar substitution of K\(^+\) for Na\(^+\) in solutions with elevated [K\(^+\)]. To terminate toxic exposures, solutions were washed twice with media stock (MS), which consists of Eagle’s minimal essential medium plus 21 mM glucose, and then replaced with MS containing 1% serum (2:1, horse serum:fetal bovine serum) before being returned to a 37°C incubator. A23187 exposures were conducted at 37°C in MS after sham washes. Z-VAD was added to the final replacement solution (MS and 1% serum) following exposures and washes.

2.4. Cell death assays

Cell death was assessed morphologically by phase-contrast microscopy and propidium iodide fluorescence. For propidium iodide staining, cells were incubated for 30 min at 37°C with propidium iodide solution (5 μg/ml) (Molecular Probes), and dead cells (cells with compromised membrane integrity) were visualized by excitation at 488 nm. Cell death was quantified by measuring lactate dehydrogenase (LDH) released by injured cells into the cell medium [21]. LDH values were normalized by subtracting the background LDH released by sham-washed cells from treated cells and scaling to the signal associated with complete cell death (=100), induced by 24 h exposure to 30 μM A23187.

2.5. Electron microscopy

Cells were harvested 12 h following toxic exposures and fixed with 1% paraformaldehyde/2% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) overnight at 4°C. Cultures
were then fixed and embedded. After cutting and staining sections with lead citrate and uranyl acetate, cells were examined and photographed with a JEOL 100 CS electron microscope.

2.6. Immunofluorescence

Plated cells were fixed with cold methanol and immunostained with a monoclonal antibody against the myc epitope (Calbiochem). After incubating cells with a fluorescence-conjugated anti-mouse Alexa 488 secondary antibody (Molecular Probes), myc epitope-positive cells were visualized by fluorescence microscopy (excitation: 495 nm).

2.7. Western blot analysis

To isolate membrane proteins, cells were harvested in lysis buffer (20 mM TrisCl (pH 7.5), 0.32 M sucrose, 0.2 mM EDTA, and 0.5 mM EGTA with protease inhibitors (1 mM dithiothreitol, 1 mM phenyl-methyl-sulfonylfluoride, 20 μg/ml aprotinin, 20 μg/ml leupeptin) and sonicated. After centrifugation of samples at 30,000 g for 30 min at 4°C, membrane pellets were resuspended in a buffer consisting of 20% glycerol, 10 mM TrisCl (pH 8), 1 mM EDTA, and protease inhibitors, and total membrane protein was quantified by BCA measurement (Pierce). Membrane proteins were resolved by SDS-PAGE and transferred onto nitrocellulose. To confirm equal loading, blots were stained with Ponceau-S (Sigma). To detect endogenous rZnT-1 expression, blots were blocked in phosphate buffered-saline (PBS) containing 10% milk, and immunoblotted with anti-myc monoclonal antibody (kindly provided by R. Kopan). Blots were incubated with anti-mouse HRP-conjugated secondary antibody (Vector), and proteins were detected by ECL.

2.8. 65Zn efflux assay

PC12 cultures were washed with MS, allowed to sit for 5 min, and washed with HSS. Then HSS containing 65Zn2+ (2 μCi/ml, 1–5 Ci/g, DuPont NEN Research Products) plus cold Zn2+ ([total Zn2+]=20 μM) was applied to the cells for loading. After the 90-min loading period, the solution was washed three times with HSS, and then cells were returned to HSS plus 5% FBS for efflux. After indicated efflux periods, cells were washed twice with ice-cold quench solution (HSS(–Ca) supplemented with 10 mM CaCl2, 1 mM ZnCl2, 10 mM CaEDTA, and 100 μM GdCl3), and the cultures were placed on ice. After 10 min, the quench solution was aspirated, and the cells were lysed by addition of 100 μl hot 0.2% SDS. The lysate was then dotted onto filter paper (Whatman 3MM, Whatman, Inc.), dried by heating lamp, and counted in a gamma counter (Beckman 4000, Beckman Instruments, Inc.). Other cultures from the same plating were used to determine protein concentrations for each sample.

3. Results

To approximate acute cellular Zn2+ loading followed by later death, we exposed PC12 cells to 500 μM ZnCl2 in the presence of 90 mM K+ for 15 min. Many cells had lost brightness, 12–24 h later, under phase-contrast microscopy and no longer excluded propidium iodide (Fig. 1A); some had detached from the dish. Because of this detachment, we quantified cell death by LDH release to the bathing medium and determined that about a fourth of the total

**Fig. 1.** Bay K 8644 potentiates Zn2+-induced death in depolarized PC12 cells. (A) Phase-contrast (left column) and corresponding propidium iodide fluorescence photomicrographs (right column) of PC12 cells, 24 h following 15 min exposure to 500 μM Zn2+ +90 mM K+, or 500 μM Zn2+ +90 mM K+ +1 μM Bay K 8644. (B) PC12 cell death 24 h after 15 min treatment to the indicated combinations of 90 mM K+, 1 μM Bay K 8644, 500 μM Zn2+, 1 μM nimodipine, 10 μM Gd3+, and 2 mM pyruvate as assessed by LDH efflux to the bathing media, scaled to the near-complete cell death induced by 24 h exposure to 30 μM A23187 (=100). All bars depict mean±S.E.M. from three to four independent experiments (n=12–16 cultures per condition). * indicates significant difference from Zn2+ alone at P<0.05, # indicates significant difference from K+ +Zn2+ at P<0.05. $ indicates significant difference from K+ +Zn2+ +Bay K 8644 at P<0.05. Statistical analysis was performed using a two-way ANOVA followed by a Bonferroni t-test.
cells had died (Fig. 1B). This level of cell death remained stable over the next 24 h (data not shown).

Consistent with earlier studies with primary cortical neurons, blockade of L-type voltage-gated Ca\textsuperscript{2+} channels with 1 µM nimodipine markedly attenuated Zn\textsuperscript{2+}-induced PC12 cell death but only under depolarizing conditions, and enhancement of L-type Ca\textsuperscript{2+} channel opening with 1 µM Bay K 8644 increased cell death such that about half of the total population was killed. This Bay K 8644-enhanced cell death was still sensitive to the broad spectrum voltage-gated Ca\textsuperscript{2+} channel blocker, Gd\textsuperscript{3+} (10 µM), and was attenuated by pyruvate as was shown recently in cortical neuronal cultures [41]. Bay K 8644, K\textsuperscript{+}, Gd\textsuperscript{3+}, or nimodipine by themselves were not toxic in these exposure conditions (data not shown). Death was induced by Zn\textsuperscript{2+} concentrations over the range 30–1000 µM and extracellular K\textsuperscript{+} concentrations over the range 20–90 mM (Fig. 2A, B).

We examined the type of death — apoptosis or necrosis — induced by this protocol. Transmission electron micrographs of PC12 cells 12 h after a 15 min exposure to 200 µM Zn\textsuperscript{2+} in the presence of 1 µM Bay K 8644 and 90 mM K\textsuperscript{+} revealed hallmarks of apoptosis, including apoptotic body formation, preservation of plasma membranes, condensation of chromatin, and mitochondrial integrity (Fig. 3A). In contrast, cells exposed to 1000 µM Zn\textsuperscript{2+} exhibited necrosis, with mitochondrial swelling and disruption of plasma membranes (Fig. 3B). A mixed morphological profile was observed in cells exposed to 500 µM Zn\textsuperscript{2+}, with certain cells appearing necrotic and others apoptotic, and some cells exhibiting a mixture of features (Fig. 3C). Consistent with the morphology, addition of 100 µM Z-VAD attenuated cell death at lower (200–500 µM) but not higher (1000 µM) Zn\textsuperscript{2+} concentrations (Fig. 4). The cell death induced by 500 µM Zn\textsuperscript{2+} in the presence of only K\textsuperscript{+} was also apoptotic in nature, with Z-VAD reducing this death from 24.1 ± 1.5% to 5.1 ± 4.8%, n = 9 from 3 independent experiments. This reduction was the same as that achieved with nimodipine.

To test the hypothesis that PC12 cells exposed to extracellular Zn\textsuperscript{2+} die due to toxic elevations in [Zn\textsuperscript{2+}], we stably expressed the plasma membrane Zn\textsuperscript{2+} transporter, ZnT-1 [34]. We first determined if PC12 cells endogenously express rZnT-1 by immunoblot analysis using a previously characterized polyclonal affinity-purified antibody (Fig. 5A) [29]. To test if rZnT-1 activity can regulate zinc homeostasis in our Zn\textsuperscript{2+} toxicity paradigm, we generated stable PC12 lines expressing wild type and dominant negative rat ZnT-1 constructs. To distinguish transfected from endogenous ZnT-1 protein, we subcloned a hexameric myc epitope tag distal to the 3’ end of the rZnT-1 cDNA.

After transfection, an initial screen using anti-myc immunofluorescence yielded clones expressing the wild-
Fig. 3. Transmission electron micrographs. (A) Neuronal cell apoptosis 12 h after 15 min exposure to 200 μM Zn$^{2+}$+1 μM Bay K 8644+90 mM K$^+$. Bar=2 μm. The arrow points to apoptotic bodies. A portion of a relatively intact cell can also be seen at the right edge of the micrograph. (B) Same as A but with 1000 μM Zn$^{2+}$. Necrosis associated with plasma membrane disruption and swollen mitochondria. (C) Same as A but with 500 μM Zn$^{2+}$. A cell with a mixed death phenotype (arrow) can be seen adjacent to a necrotic cell (arrowhead). Although the former cell demonstrates some features typical of apoptotic death — an intact plasma membrane, a shrunken cell body, and compact chromatin, the cell also exhibits swollen mitochondria.

Fig. 4. Lower levels of Zn$^{2+}$-induced death are sensitive to inhibition by Z-VAD. Cell death 24 h after 15 min exposure to 1 μM Bay K 8644, 90 mM K$^+$, and the indicated concentrations of Zn$^{2+}$, in the presence or absence of 100 μM Z-VAD (mean±S.E.M. from three to four independent experiments, n=12–16 cultures per condition). * indicates a significant difference between Z-VAD treated and untreated cell death at the same Zn$^{2+}$ concentration at P<0.05. Statistical analysis was performed using a two-way ANOVA followed by a Bonferroni t-test.

To confirm the predicted function of overexpressed rZnT-1 proteins, we assessed the ability of lines stably expressing rZnT-1-myc or DN rZnT-1-myc to export preloaded $^{65}$Zn$^{2+}$. Cells were preloaded by 90 min exposure to cold Zn$^{2+}$ plus $^{65}$Zn$^{2+}$ (20 μM total); over the next 2 h, the rZnT-1-myc line, T9, demonstrated enhanced $^{65}$Zn$^{2+}$ extrusion, and the DN rZnT-1-myc cell line, D5, demonstrated reduced $^{65}$Zn$^{2+}$ extrusion, compared to the parental line (Fig. 6). These lines also exhibited altered vulnerability to Zn$^{2+}$-induced death. Over a range of tested Zn$^{2+}$ concentrations between 200 and 1000 μM, the rZnT-1-myc line exhibited reduced vulnerability to Zn$^{2+}$-induced death, and the DN rZnT-1-myc cell line exhibited enhanced vulnerability, compared to the parental line (Fig. 7). To control for potential integration-related effects, we observed similar results with additional lines stably expressing each construct (T23 and D16, data not shown).

4. Discussion

We describe here a model system for investigating Zn$^{2+}$ influx-induced neurotoxicity utilizing PC12 cells. This PC12 cell system exhibited five key features of Zn$^{2+}$ neurotoxicity as previously delineated with primary cortical neurons: (1) cell death was induced by brief Zn$^{2+}$ exposure (15 min); (2) the extent of death was Zn$^{2+}$-concentration dependent; (3) Zn$^{2+}$-induced death was enhanced by concurrent depolarization and largely dependent upon L-type voltage-gated Ca$^{2+}$ channel activation; (4) lower levels of toxic zinc exposure induced apoptosis, whereas higher levels induced necrosis; and (5) death was reduced by the addition of 2 mM pyruvate.

As noted above, Ross et al. have reported that prolonged Zn$^{2+}$ exposure can kill PC12 cells by binding and inac-
Fig. 6. Effects of wild type and dominant negative rZnT-1-myc overexpression on Zn efflux from PC12 cells. Cells were preloaded for 90 min with \(^{65}\)Zn (2 \(\mu\)Ci/ml, total Zn \(^{2+}\) concentration 20 \(\mu\)M). After the efflux period, cells were washed, lysed, and harvested for quantitation of radioactivity by gamma counter (mean\(\pm\)S.E.M. from two to three independent experiments, \(n=8\)–12 cultures per condition). Sister cultures were used to determine the protein concentration of each sample. * indicates significant difference compared to the parental line at \(P<0.05\), using two-way ANOVA followed by a Bonferroni \(t\)-test.

Fig. 7. Effects of wild type and dominant negative rZnT-1-myc expression on Zn\(^{2+}\)-induced death. Cell death 24 h after 15 min exposure to 1 \(\mu\)M Bay K 8644, 90 nM K\(^{+}\), and the indicated concentrations of Zn\(^{2+}\) (mean\(\pm\)S.E.M. from three to four independent experiments, \(n=12\)–16 cultures per condition). * indicates significant difference compared to the parental line at \(P<0.05\), using two-way ANOVA followed by a Bonferroni \(t\)-test.

Fig. 5. Endogenous rZnT-1, and rZnT-1-myc protein expression in PC12 stable lines. (A) Membrane proteins prepared from the parental line were examined for expression of rZnT-1 using a rabbit polyclonal antibody. (B) Membrane proteins prepared from neuroblastoma N2A cells transiently transfected with rZnT-1-myc (lane 1) and DN rZnT-1-myc (lane 2) constructs, the parental PC12 line (lane 3), PC12 clones T9 and T23 expressing rZnT-1-myc (lane 4 and 5), and clones D5 and D16 expressing DN rZnT-1-myc (lanes 6 and 7) were assessed for expression of transfected rZnT-1 constructs using a monoclonal antibody against the myc epitope. (C) Phase-contrast (left column) and corresponding immunofluorescence (right column) photomicrographs of PC12 lines stably transfected with rZnT-1-myc or DN rZnT-1-myc. Control cells were not transfected. In the right column, cells were immunostained with monoclonal antibody against the myc epitope.

since Zn\(^{2+}\) exposure was brief (15 min), and serum-containing media was restored after exposure termination (as noted above, serum prevented death by this neurotrophin inactivation mechanism). Also, while neuronal death in our paradigm was likewise apoptotic, death was markedly reduced by nimodipine (see Fig. 1B) and influenced by the expression of ZnT-1, consistent with mediation by Zn\(^{2+}\)-influx through L-type Ca\(^{2+}\) channels and consequent toxic elevation in intracellular free Zn\(^{2+}\), as occurs in primary neurons.

The vulnerability of PC12 cells to death induced by
exogenously applied extracellular Zn\(^{2+}\) is somewhat less than that of primary central neurons [28,54]. Most likely, this quantitative difference reflects the relative paucity of membrane L-type Ca\(^{2+}\) channels on the former relative to the latter [24] (and unpublished results), although differences in Zn\(^{2+}\) homeostasis mechanisms or downstream toxicity cascades might also contribute. We therefore increased the contribution of the existing L-type channels by increasing extracellular K\(^{+}\) to 90 mM, and adding S(−) Bay K 8644, which acts at the dihydropyridine binding site of transfected ZnT-1 or a dominant negative ZnT-1 to glyceraldehyde-3-phosphate dehydrogenase [41]. These findings extend previous observations regarding the ability of transfected ZnT-1 or a dominant negative ZnT-1 to modify the vulnerability of BHK or N2A cells to prolonged zinc exposure [34,49]. Our results suggest that both endogenous and ectopic ZnT-1 contribute to critical Zn\(^{2+}\) homeostasis after acute Zn\(^{2+}\) exposure under depolarizing conditions. Molecular or pharmacological manipulation of ZnT-1 function may constitute a useful neuroprotective strategy in certain disease settings such as global brain ischemia.

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References


