Cellular/Molecular

Zn^{2+} Inhibits Mitochondrial Movement in Neurons by Phosphatidylinositol 3-Kinase Activation

Latha M. Malaiyandi, Anthony S. Honick, Gordon L. Rintoul, Qiming J. Wang, and Ian J. Reynolds
Department of Pharmacology, University of Pittsburgh, Pittsburgh, Pennsylvania 15261

Mitochondria have been identified as targets of the neurotoxic actions of zinc, possibly through decreased mitochondrial energy production and increased reactive oxygen species accumulation. It has been hypothesized that impairment of mitochondrial trafficking may be a mechanism of neuronal injury. Here, we report that elevated intraneuronal zinc impairs mitochondrial trafficking. At concentrations just sufficient to cause injury, zinc rapidly inhibited mitochondrial movement without altering morphology. Zinc chelation initially restored movement, but the actions of zinc became insensitive to chelator in <10 min. A search for downstream signaling events revealed that inhibitors of phosphatidylinositol (PI) 3-kinase prevented this zinc effect on movement. Moreover, transient inhibition of PI 3-kinase afforded neuroprotection against zinc-mediated toxicity. These data illustrate a novel mechanism that regulates mitochondrial trafficking in neurons and also suggest that mitochondrial trafficking may be closely coupled to neuronal viability.

Key words: **green fluorescent protein; organelle transport; signal transduction; wortmannin; mitochondrial membrane potential; oxidative stress**

Introduction

Neural zinc is a highly regulated ion, much of which is tightly coordinated to proteins, leaving a small pool of zinc that remains free and mobile (Frederickson, 1989). Elevated intracellular free zinc ($[Zn^{2+}]_i$) results from entry through several Ca^{2+}-permeable pathways (Weiss et al., 1993; Koh and Choi, 1994; Yin and Weiss, 1995; Sensi et al., 1997; Cheng and Reynolds, 1998) and also from oxidant-mediated release from intracellular stores (Aizenman et al., 2000). Elevated $[Zn^{2+}]_i$ results in neuronal injury *in vitro* (Choi et al., 1988; Koh and Choi, 1994), and it has been suggested that its accumulation may contribute to neurodegeneration associated with ischemia (Koh et al., 1996; Lee et al., 2002), epileptic seizures (Frederickson et al., 1988), and head trauma (Suh et al., 2000).

Although an unambiguous mechanism for Zn^{2+}-mediated neurotoxicity has not been identified, several lines of evidence suggest mitochondria and energy metabolism as subcellular targets for the toxic actions of $[Zn^{2+}]_i$ (Dineley et al., 2003; Sensi and Jeng, 2004). Zinc can inhibit glycolysis (Sheline et al., 2000), the tricarboxylic acid cycle (Brown et al., 2000), and complexes in the electron transport chain (Skulachev et al., 1967; Kleiner, 1972, 1974; Link and von Jagow, 1995; Mills et al., 2002). It has also been shown that Zn^{2+} dissipates mitochondrial membrane potential ($\Delta\Psi_m$), decreases oxygen consumption, and enhances reactive oxygen species (ROS) accumulation (Dineley et al., 2005). However, it remains unclear which, if any, of these processes represent the critical target for the neurotoxic actions of zinc.

It has long been appreciated that mitochondria constantly move, divide, and fuse throughout the cell (Bereiter-Hahn and Voth, 1994). It is reasonable to suggest that mitochondrial trafficking in neurons serves to position mitochondria to deliver ATP to regions of high energy demand (van Blerkom, 1991) and to aid in the regulation of local Ca^{2+} homeostasis (Spira et al., 2001; Yi et al., 2004), although direct evidence that mitochondrial trafficking is driven by energy demand remains sparse. Nevertheless, recent studies suggest that interruption of trafficking is one consequence of excessive activation of NMDA receptors and the subsequent calcium entry (Rintoul et al., 2003). Other potential neurotoxins, such as nitric oxide (Rintoul et al., 2004) and ATP depletion (Rintoul et al., 2003), also inhibit mitochondrial movement, suggesting that impaired delivery of mitochondria could be an important contributor to neuronal injury.

In this report, we investigated the effects of $[Zn^{2+}]_i$ on mitochondrial trafficking. Using a mitochondrially targeted enhanced yellow fluorescent protein (mt-eYFP), we observed that movement is substantially diminished with ionophore-induced elevations of $[Zn^{2+}]_i$ that are just sufficient to cause injury. We further show that these actions of zinc are mediated by a signaling cascade that involves activation of phosphatidylinositol (PI) 3-kinase, and that transient inhibition of PI 3-kinase prevents both the impairment of trafficking and zinc-induced neuronal injury.

Materials and Methods

Materials. All reagents were purchased from Sigma (St. Louis, MO) unless otherwise specified.

Received March 4, 2005; revised Aug. 27, 2005; accepted Aug. 29, 2005.

This work was supported by National Institutes of Health Grant NS34138 (I.J.R.), American Heart Association (AHA) Postdoctoral Fellowship 0325734U (G.L.R.), and AHA Predoctoral Fellowship 0315338U (L.M.M.). We thank Dr. Roger Y. Tsien for providing the mt-eYFP construct and Dr. Elias Aizenman for providing the luciferase reporter construct. We thank David J. Levinthal, Drs. Donald B. DeFranco, Jes K. Klarlund, Yu Jiang, and Daniel Altschuler for providing critical reagents and helpful suggestions, and our colleagues in the lab for assistance in preparing this manuscript. We also thank Anthony J. Filiano for preparation of cell cultures, Xiaoping Hu, Yue Luo, and Ganwei Lu for assistance with Western blots, and Groundskeeper Willie for technical assistance in fluorescence microscopy.

Correspondence should be addressed to Dr. Ian J. Reynolds, Merck Research Laboratories, 770 Sumneytown Pike, Mail Stop WP42-229, West Point, PA 19486. E-mail: ian_reynolds@merck.com.

DOI:10.1523/JNEUROSCI.0868-05.2005

 0270-6474/05/259507-08$15.00/0

Cell culture. All procedures using animals were in accordance with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* and were approved by the Institutional Animal Care and Use Committee at the University of Pittsburgh. Primary cultures of rat forebrain neurons were prepared from embryonic Sprague Dawley rat pups (17 d in gestation) and grown in a 37°C incubator containing 5% CO_2. Cortices were removed, trypsinized at 37°C for 30 min, and plated on poly-D-lysine-coated 31 mm glass coverslips. Cells were plated in medium containing DMEM with 10% FBS and 1% penicillin/streptomycin. Cultures contain <5% glia in the total cell population. Five hours after initial plating, plating medium was completely removed and replaced with N2-NB media (Neurobasal media with 0.5% penicillin/streptomycin and 1% N2 supplement). Cells were refed with N2-NB media 4 d after plating, whereby approximately one-third of the volume was removed and replaced with an equal volume of fresh N2-NB media. Cells were fed 8 and 11 d after plating with B27-NB media (Neurobasal media with 0.5% penicillin/streptomycin and 2% B27 supplement minus antioxidants) using the same feeding protocol described above. For preparation of the insulin-depleted cells, cultures were fed only with B27-NB media beginning at 5 h after initial plating. All experiments were performed on neurons after 13–15 d in culture.

Transfection using DNA constructs. The mt-eYFP construct (Llopis et al., 1998) consists of eYFP fused to the mitochondrial targeting sequence from subunit IV of human cytochrome *c* oxidase. The luciferase reporter plasmid consists of the luciferase gene placed in a mammalian expression vector under the control of a cytomegalovirus promoter (Gossen and Bujard, 1992). After 10–12 d *in vitro*, neurons were transiently transfected using a modified calcium phosphate transfection technique (Xia et al., 1996). This procedure typically generates 1–2% efficiency, with maximal protein expression 24–72 h after transfection.

Fluorescence microscopy. All experiments were performed using a HEPES-buffered salt solution (HEBSS) containing the following (in mM): 137 NaCl, 5 KCl, 10 $NaHCO_3$, 0.6 KH_2PO_4, 0.6 Na_2HPO_4, 0.9 $MgSO_4$, 1.4 $CaCl_2$, 20 HEPES, and 5.5 glucose, pH adjusted to 7.4 with NaOH. Neurons were perfused at 5 ml/min during the course of each experiment, and the chamber temperature was maintained at 37°C with heated buffer. The personal-computer-based system for data acquisition is as described previously (Dineley et al., 2002) using SimplePCI imaging software (Compix, Cranberry, PA). Using a 40× water immersion objective, mitochondrial movement in a field containing a single cell was observed by illuminating at 490 nm and acquiring images every 6 s. After a 3–5 min rinse in HEBSS buffer, cells were perfused with the appropriate stimulus (diluted in buffer from stock concentrations).

For $[Zn^{2+}]_i$ measurements, we used the Zn^{2+}-sensitive fluorophore mag-fura-2 AM (Invitrogen, Eugene, OR). Coverslips were incubated with 5 μM mag-fura-2 for 20 min at 37°C. With constant perfusion at 10 ml/min, fluorescence was measured by illuminating the indicator alternatively at 335 and 375 nm. For $\Delta\Psi_m$ experiments, neurons were incubated with 5 μM rhodamine 123 (Invitrogen) for 10 min at 37°C (Vergun et al., 2003). Cells were excited at 490 nm and fluorescence was measured in 10–20 individual neurons for each coverslip. For nucleic acid staining, coverslips were incubated in HEBSS containing 1.5 μM Hoechst-33342 for 15 min at room temperature (Dineley et al., 2000). Cells were illuminated at 340 nm and fluorescent images were obtained randomly from three to six cell-containing regions per coverslip.

Quantification of mitochondrial movement. Mitochondrial movement was analyzed using a macro-based analysis program as described previously (Rintoul et al., 2003). Briefly, a 255 × 255 pixel subfield containing mitochondria was selected, in which pixel images in successive images are subtracted. A movement event for each pixel was registered if the change in fluorescence between successive fields exceeded 20 fluorescence units and a quantitative measurement of movement was obtained by combining pixel events in a field over 2 min. Movement was represented as average event counts/pixel, which was normalized to prestimulus movement values.

Toxicity. For the simplicity of assaying only cells that express mt-eYFP, we transfected neurons with the luciferase construct to measure neuronal injury. For toxicity experiments, coverslips of luciferase-transfected neurons were washed three times with HEBSS. Reagents were diluted in HBSS at the desired concentration and 1 ml of solution was applied to the coverslips for the desired time at room temperature. Stimuli were terminated by washing at least three times with excess HEBSS. The wash buffer was then replaced with the original media and returned to the incubator. Twenty-four hours later, cells were rinsed and lysed in PBS. Lysates were used to measure luciferase enzyme activity using the BriteLite Ultra-High Sensitivity Luminescence Reporter Gene Assay System (PerkinElmer Life Sciences, Boston, MA) according to the manufacturer's instructions. Arbitrary luminescence units were normalized to control, nontreated cells.

Western blotting. Cells were lysed in lysis buffer containing the following: 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1.5 mM $MgCl_2$, 1% Triton X-100, 5 mM EGTA, 20 μM leupeptin, 1 mM AEBSF [4-(2-aminoethyl) benzenesulfonylfluoride], 1 mM $NaVO_3$, 10 mM NaF, and 1 tablet of protease inhibitor. Protein concentration was determined by a microprotein assay using the BCA protein assay kit according to the manufacturer's instructions (Pierce, Rockford, IL). Approximately 30 μg of lysates were mixed with equal volumes of 2× SDS sample loading buffer (60 mM Tris-HCl, pH 7.5, 2 mM EDTA, 10 mM 2-mercaptoethanol, 20% glycerol, and 2% SDS) and size-fractionated by electrophoresis on 4–15% Tris-HCl Ready Gels (Bio-Rad, Hercules, CA) at 30 mA for 1 h followed by electrotransfer onto a nitrocellulose membrane at 80 V for 2 h. The membrane was preblotted with 5% dry milk in PBS-Tween (1× PBS, 0.1% Tween 20) at room temperature for 1 h. The blots were probed with rabbit antiserum raised against Akt, and phosphosphorylated-S473 Akt (Cell Signaling Technology, Beverly, MA) antibodies. Goat anti-rabbit IgG coupled to horseradish peroxidase (1:1000; Pierce) was used as the secondary antibody. Bands were visualized by the SuperSignal West Dura Extended Duration Substrate (Pierce).

Statistical analyses. Statistical analysis was performed using Prism 4.01 (GraphPad Software, San Diego, CA). All data are presented as mean ± SE consisting of four to six experiments for each condition from at least three different cell culture preparations. Comparisons were made using Student's *t* test and one-way ANOVA with Bonferroni's multiple comparison and Dunnett's post tests to compare all conditions to control, with p values <0.05 regarded as significant.

Results

Concentrations of Zn^{2+} that inhibit mitochondrial movement parallel Zn^{2+} concentrations that are neurotoxic

We fluorescently labeled neuronal mitochondria using an mt-eYFP. The ionophore sodium pyrithione (Pyr) was used to increase $[Zn^{2+}]_i$ (Dineley et al., 2000). As shown in Figure 1*a*, buffer, sodium pyrithione alone (20 μM), or 0.3 μM Zn^{2+}/Pyr did not affect mitochondrial movement. However, higher concentrations of Zn^{2+} significantly inhibited mitochondrial movement, with concentrations as low as 1 μM resulting in a significant decrease in movement (supplemental video 1, available at www.jneurosci.org as supplemental material). Notably, this stimulus consistently stopped movement of all mitochondria in a given field and did not preferentially alter the movement of retrograde- versus anterograde-moving mitochondria. This Zn^{2+}-mediated effect was irreversible up to 20 min after the stimulus was washed out. Interestingly, this concentration-dependent movement inhibition corresponded to the concentration dependence of Zn^{2+} toxicity (Fig. 1*a*, inset).

To establish that this was not a Ca^{2+}-induced phenomenon, we perfused cells with Zn^{2+}/Pyr under Ca^{2+}-free conditions to confirm that the stimulus was still effectively inhibiting movement (Fig. 1*b*). There is evidence that, under depolarizing conditions, cellular Zn^{2+} entry occurs through plasma membrane Ca^{2+}-permeable pathways (Sensi et al., 1999). As a more physiological approach to elevate $[Zn^{2+}]_i$, we observed that mitochondrial movement was significantly inhibited with elevated Zn^{2+} (300 μM) in the presence of high K^+ buffer (Fig. 1*b*). Evidence

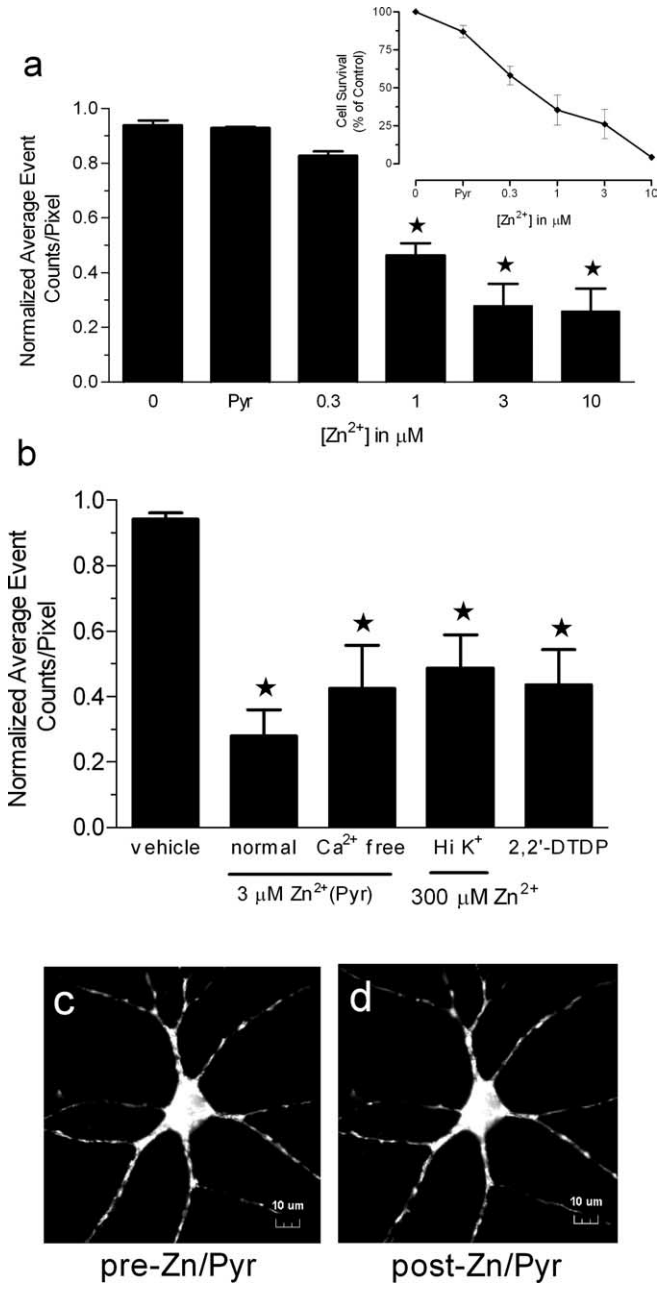

Figure 1. $[Zn^{2+}]_i$ inhibits mitochondrial movement at cytotoxic concentrations. ***a***, Neurons expressing mt-eYFP were perfused with buffer, pyrithione (20 μM) alone, or in the presence of a range of [$ZnCl_2$] (0.3–10 μM) for 10 min. The bars represent mitochondrial movement measured at the end of the stimulus. The inset demonstrates that neurons undergo Zn^{2+}-mediated toxicity at parallel concentrations that affect mitochondrial movement. ***b***, Mitochondrial movement is inhibited when neurons were perfused with (1) 3 μM Zn^{2+}/Pyr in Ca^{2+}-free buffer, (2) 300 μM Zn^{2+} in an elevated K^+ buffer, or (3) 10 μM 2,2′-DTDP. Fluorescent images were taken before (***c***) and after (***d***) 3 μM Zn^{2+}/Pyr to show that no gross morphological changes occur with acute Zn^{2+} treatment. *Treatments were significantly different when compared with control (buffer alone). $p < 0.01$ for one-way ANOVA with Dunnett's post test. Results are representative of four to six different cultures. Error bars indicate SE.

that exposure to strong oxidizing agents can generate $[Zn^{2+}]_i$ fluxes in neurons is indicative of Zn^{2+} release from cytosolic reservoirs, including metallothionein (Aizenman et al., 2000). Application of the sulfhydryl oxidizing agent 2,2′-dithiodipyridine (DTDP) (10 μM) significantly inhibited mitochondrial movement, suggesting that $[Zn^{2+}]_i$ release in addition to Zn^{2+} entry is sufficient to induce the effect (Fig. 1*b*). Our previous study showed that Ca^{2+}-induced cessation of movement was also associated with a substantial change in mitochondrial morphology (Rintoul et al., 2003). However, the representative fluorescent images taken before (Fig. 1*c*) and after (Fig. 1*d*) Zn^{2+} exposure demonstrated that there are no gross morphological changes that occur with Zn^{2+} exposure.

Zn^{2+} inhibition of mitochondrial movement is partially sensitive to chelation

To determine whether chelation can restore Zn^{2+}-mediated movement inhibition, we applied the membrane-permeant heavy metal chelator *N*,*N*,*N*′,*N*′-tetrakis-(2-pyridylmethyl)-ethylenediamine (TPEN) (50 μM) immediately after Zn^{2+}/Pyr exposure (Fig. 2*a*). Notably, TPEN partially restored movement when applied after 1 μM Zn^{2+} but was ineffective at restoring movement when applied 10 min after 3 μM Zn^{2+}. Additionally, Figure 2*b* shows that TPEN restored movement when applied after shorter exposures to 3 μM Zn^{2+} but became ineffective after treatments longer than 6 min. We also observed that TPEN rescued neurons from injury when immediately applied after a 5 min Zn^{2+}/Pyr exposure (Fig. 2*c*) but did not protect against toxicity after a 10 min (Fig. 2*d*) Zn^{2+} treatment. Together, these results indicate that the TPEN effects are concentration and time dependent. The insensitivity to TPEN after longer or larger Zn^{2+} exposures suggested the initiation of a signaling cascade, which is irreversible after simple Zn^{2+} chelation and therefore becomes independent of Zn^{2+}.

As described above, oxidant exposure diminished mitochondrial movement when 2,2′-DTDP (30 μM) was applied at concentrations that inhibit mitochondrial movement to the same extent as 3 μM Zn^{2+}/Pyr. If TPEN was applied after 2,2′-DTDP, movement was not restored (supplemental Fig. 1*a*, available at www.jneurosci.org as supplemental material). However, if 2,2′-DTDP and TPEN were applied simultaneously, movement was inhibited to a lesser degree (supplemental Fig. 1*c*, available at www.jneurosci.org as supplemental material). Likewise, a similar trend was observed when 100 μM H_2O_2 was applied to mimic ROS exposure (supplemental Fig. 1*b*,*d*, available at www.jneurosci.org as supplemental material). Interestingly, the reducing agent dithiothreitol also did not reverse movement inhibition (supplemental Fig. 1*e*, available at www.jneurosci.org as supplemental material), suggesting that although this phenomenon is potentially ROS mediated, movement is not restored when shifted to a more reduced environment. These findings are consistent with ROS-mediated Zn^{2+} release contributing to the inhibition of trafficking but also suggest that there may be an additional ROS-induced Zn^{2+}-independent effect on mitochondrial movement.

Inhibitors of PI 3-kinase prevent Zn^{2+}-induced movement inhibition

To identify the Zn^{2+}-activated signaling cascade involved in the movement effect, we investigated several agents that selectively inhibit protein kinases (supplemental Fig. 2, available at www.jneurosci.org as supplemental material). Of all of the compounds that we tested in our movement paradigm, only the PI 3-kinase inhibitors, wortmannin (1 μM) and 2-(4-morpholinyl)-8-phenyl-4*H*-1-benzopyran-4-one (LY294002) (30 μM), were effective in that (1) they did not affect mitochondrial movement by themselves, and (2) they blocked the Zn^{2+}-mediated effect at the concentration (3 μM Zn^{2+}/Pyr) and time (10 min) that was

TPEN insensitive (Fig. 3*a*). One concern was that wortmannin was preventing ionophore-induced $[Zn^{2+}]_i$ accumulation by blocking its cellular entry or chelating the $[Zn^{2+}]_i$ before it could mediate any effects on movement. We excluded these possibilities by monitoring $[Zn^{2+}]_i$ in mag-fura-2-loaded neurons that were perfused with 3 μM Zn^{2+}/Pyr, either with or without wortmannin (1 μM) pretreatment (Fig. 3*b*). Both conditions produced equal mag-fura-2 responses that were TPEN-reversible, confirming that wortmannin was not preventing entry or binding $[Zn^{2+}]_i$.

Preventing Akt phosphorylation does not prevent Zn^{2+}-induced movement inhibition

As a possible downstream effector of PI 3-kinase, we investigated the role of Akt phosphorylation in Zn^{2+}-induced movement inhibition (Fig. 4). We monitored phosphorylated Akt levels in neurons treated with 3 μM Zn^{2+}/Pyr for 10 min, either in the presence or absence of wortmannin (1 μM) or a putative Akt inhibitor [1L-6-hydroxymethyl-*chiro*-inositol-2-(*R*)-2-*O*-methyl-3-*O*-octadecykcarbonate] (10 μM; Calbiochem, La Jolla, CA) (Fig. 4*a*). This phosphatidylinositol ether analog has been reported to selectively inhibit Akt phosphorylation and prevent growth of HT-29, MCF, HeLa, and PC-3 cancer cell lines. This novel class of Akt inhibitors selectively blocks Akt activation and downstream substrates without affecting upstream kinases or other kinase pathways. We used insulin (100 nM) as a positive control in these experiments. As demonstrated in Figure 4*a*, both insulin and Zn^{2+} rapidly induced Akt phosphorylation within 10 min in a wortmannin-sensitive manner. Interestingly, treatment with Akt inhibitor alone increased phosphorylation from control levels, and this compound did not significantly reduce phosphorylation in neurons compared with either insulin or Zn^{2+} alone.

We also monitored the effects of these treatments on mitochondrial movement. As shown in Figure 4*b*, perfusion with 100 nM insulin for 10 min did not significantly decrease mitochondrial movement compared with untreated cells, although a 10 min stimulus of 100 nM insulin is sufficient for Akt phosphorylation in primary neurons (Schubert et al., 2004). Treatment with the Akt inhibitor itself slightly decreased mitochondrial movement and also did not prevent the Zn^{2+}-induced movement effect. Although previous studies have used this inhibitor to prevent Akt phosphorylation in cell lines (Kozikowski et al., 2003), it appears to be less effective in neurons.

As an alternative to the Akt inhibitor, we transiently cotransfected a hemagglutinin-tagged dominant-negative (DN)-Akt (K179M) plasmid along with the mt-eYFP plasmid. Overexpression of DN-Akt did not prevent the Zn^{2+}-mediated movement inhibition (supplemental Fig. 3, available at www.jneurosci.org as supplemental material), arguing against Akt activation by PI 3-kinase as a downstream event. We additionally tested other targets of PI 3-kinase that are activated in Akt-independent pathways (supplemental Fig. 3, available at www.jneurosci.org as supplemental material). Blocking ADP-ribosylation factor 6 (ARF6) phosphorylation using a dominant-negative construct (Langille et al., 1999) or inhibiting target of rapamycin (TOR) phosphorylation using rapamycin (Lynch et al., 2001) did not

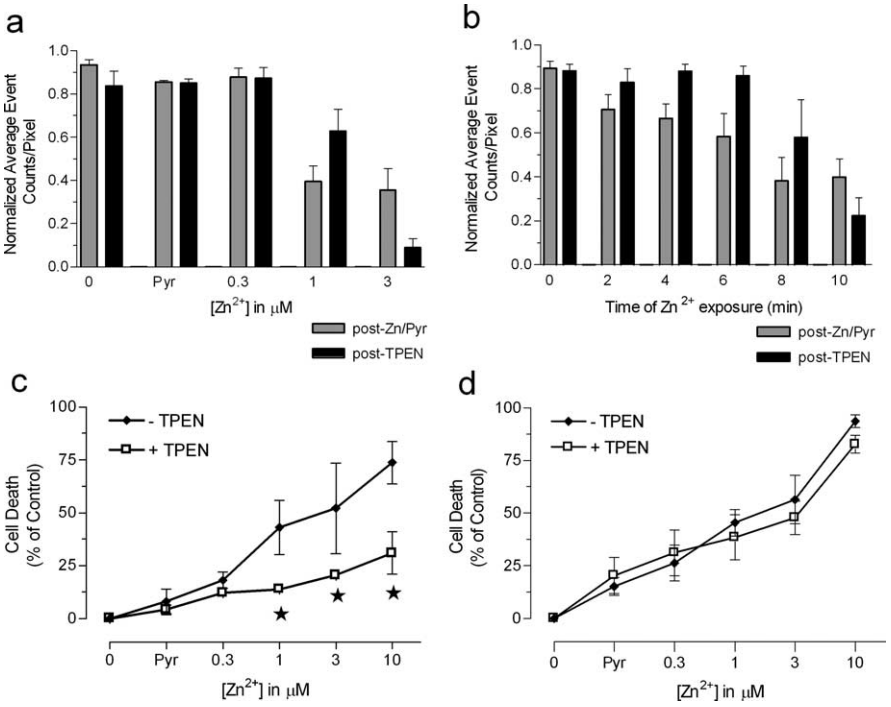

Figure 2. Zn^{2+} removal partially restores movement inhibition in a concentration- and time-sensitive manner. ***a***, Neurons were perfused with Zn^{2+}/Pyr (0–10 μM) for 10 min followed by 50 μM TPEN for 10 min. Mitochondrial movement was measured both after the Zn^{2+} (gray bars) and TPEN stimuli (black bars). ***b***, Neurons were perfused with 3 μM Zn^{2+}/Pyr for varying times (0–10 min) followed immediately by TPEN. Zn^{2+}-induced toxicity was assayed in neurons exposed to 0–10 μM Zn^{2+}/Pyr for 5 min (***c***) or 10 min (***d***) immediately followed by TPEN treatment. ★ $p < 0.05$ by paired Student's *t* test. Results are representative of four to six different cultures. Error bars indicate SE.

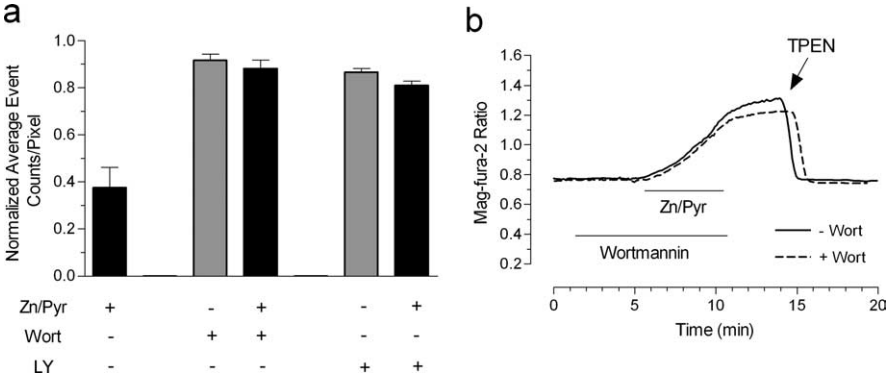

Figure 3. PI 3-kinase inhibitors prevent Zn^{2+}-induced movement inhibition. ***a***, Neurons were perfused with either wortmannin (1 μM) or LY294002 (30 μM) for 10 min before and during 3 μM Zn^{2+}/Pyr treatment, and mitochondrial movement was analyzed after application of inhibitors (gray bars) and Zn^{2+} in the presence of inhibitors (black bars). ***b***, Neurons loaded with mag-fura-2 were exposed to 3 μM Zn^{2+}/Pyr for 5 min either without (solid line) or pretreated with 1 μM wortmannin (dotted line). In both conditions, TPEN (50 μM) was applied after the Zn^{2+} stimulus. Results are representative of four to six different cultures. LY, LY294002; Wort, wortmannin. Error bars indicate SE.

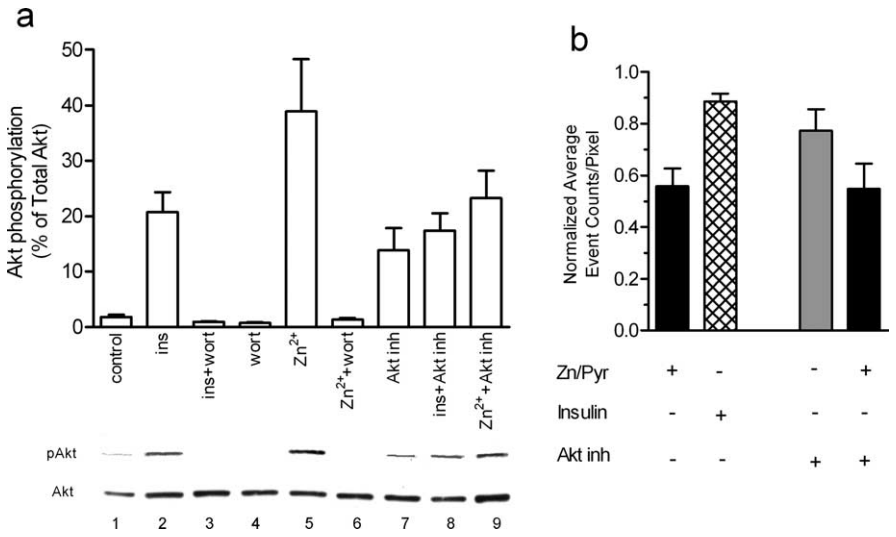

Figure 4. Zn^{2+} induces Akt phosphorylation. ***a***, Representative Western blot for phosphorylated Akt from neurons treated for 10 min with 3 μM Zn^{2+}/Pyr or 15 min with 100 nM insulin, in either the presence or absence of wortmannin (1 μM) or Akt inhibitor (10 μM) pretreatment for 10 min. The lane numbers correspond to the bar conditions depicted in the graph directly above. Relative protein levels were normalized to total Akt protein levels as shown in the graph, which is a compilation of results from at least three different culture preparations. ***b***, Neurons were exposed to 3 μM Zn^{2+}/Pyr, 100 nM insulin, or pretreated for 10 min with Akt inhibitor (10 μM) before Zn^{2+}. Movement was measured after insulin (hatched bar), inhibitor (gray bar), and Zn^{2+} (black bar) treatments. Results are representative of four to six different cultures. inh, Inhibitor; ins, insulin; wort, wortmannin. Error bars indicate SE.

prevent the Zn^{2+}-mediated inhibition, indicating that these protein kinases are not directly involved in the movement paradigm. Thus, although PI 3-kinase activation appears to be an essential component of the effect of zinc, it alone is apparently not sufficient to account for the actions of zinc on mitochondrial movement.

Zn^{2+} effects on mitochondrial movement do not result from compromised mitochondrial function

We have shown previously that acute application of the mitochondrial uncoupler carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP), which rapidly dissipates $\Delta\Psi_m$, decreases mitochondrial movement in primary neurons (Rintoul et al., 2003). Zinc, at some concentrations, can also impair mitochondrial function (Sensi et al., 1999; Dineley et al., 2000). However, an examination of the effects of these zinc treatments on mitochondrial membrane potential show that zinc only produced a significant depolarization at relatively high concentrations (Fig. 5*a*,*b*). In addition, pretreating neurons with wortmannin (1 μM) did not prevent the inhibition of mitochondrial movement produced by FCCP (750 nM) exposure (Fig. 5*c*). Together, these results argue against mitochondrial dysfunction as the key mechanism in Zn^{2+}-mediated movement inhibition.

Wortmannin protects against Zn^{2+}-induced neurotoxicity

As shown in Figure 3*a*, preperfusion with wortmannin prevented Zn^{2+}-mediated inhibition of mitochondrial movement. However, the effectiveness of wortmannin was decreased when Zn^{2+} was increased from 3 to 10 μM (Fig. 6*a*). Interestingly, wortmannin protected against Zn^{2+}-induced neurotoxicity with concentrations at or less than 3 μM Zn^{2+}/Pyr, but not with 10 μM (Fig. 6*b*,*c*), suggesting a correlation between PI 3-kinase activation in Zn^{2+}-mediated movement inhibition and kinase activity during neurotoxicity.

Sustained as opposed to transient activation of PI 3-kinase does not protect against Zn^{2+}-induced toxicity

Although PI 3-kinase is generally considered to be a prosurvival signaling mechanism, a recent study demonstrated that transient PI 3-kinase inhibition protects primary neurons from oxidative stress (Levinthal and DeFranco, 2004). As described above, our data suggest that wortmannin protected against Zn^{2+}-induced neurotoxicity at concentrations that inhibited mitochondrial movement. Although wortmannin is a fast-acting inhibitor (half-life of 90 min), it is also relatively unstable in an aqueous solution. The other selective PI 3-kinase inhibitor, LY294002, has a longer half-life (3.5 h) and produces a more sustained kinase inhibition (Jones et al., 1999). Because we were unable to prevent Zn^{2+}-induced neurotoxicity with LY294002 (Fig. 7*a*), we speculated that the neuroprotection that we observed with wortmannin could be explained by a transient inhibition of the kinase. To explore this possibility, neurons were repeatedly exposed to wortmannin during the course of Zn^{2+} toxicity. Sustained kinase inhibition with wortmannin no longer prevented neurons against elevated $[Zn^{2+}]_i$ (Fig. 7*b*), in agree-

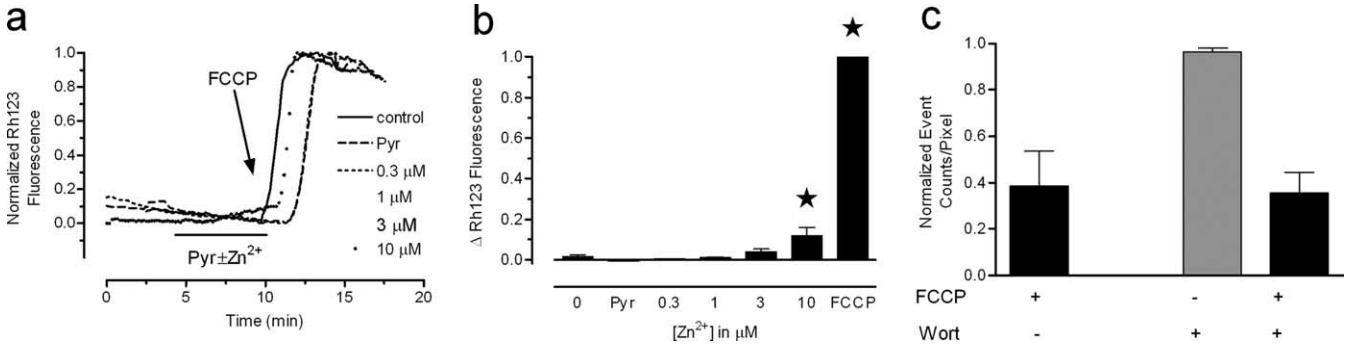

Figure 5. Zn^{2+}-induced movement inhibition does not grossly affect mitochondrial function. ***a***, Representative traces of $\Delta\Psi_m$ changes in neurons using rhodamine 123 exposed to pyrithione (20 μM) alone or in the presence of Zn^{2+} (0.3–10 μM) for 5 min. FCCP (750 nM) was applied at the end of each experiment. ***b***, Summary graph of rhodamine 123 changes in neurons exposed to Zn^{2+}/Pyr. ***c***, Mitochondrial movement in neurons was measured after FCCP (750 nM) exposure for 5 min, either with or without 10 min wortmannin (1 μM) pretreatment. ★ $p < 0.01$ by ANOVA with Dunnett's *post hoc* test. Results are representative of four to six different cultures. Wort, Wortmannin; Rh123, rhodamine 123. Error bars indicate SE.

ment with the hypothesis that only transient inhibition of PI 3-kinase affords protection against Zn^{2+} toxicity.

Discussion

The main finding from this study is that elevated $[Zn^{2+}]_i$ modulates mitochondrial movement through a signaling mechanism that is most commonly portrayed as a prosurvival pathway. In our experiments, acute induction of pathophysiological $[Zn^{2+}]_i$ consistently stopped mitochondrial movement in cortical neurons. This effect was reversible only with early Zn^{2+} chelation, indicating that a Zn^{2+}-induced signaling cascade had been initiated, which subsequently mediated movement inhibition. Interestingly, both Zn^{2+}-induced movement changes and neurotoxicity were prevented with inhibitors of the PI 3-kinase pathway. Thus, we demonstrated a signaling mechanism that regulates both mitochondrial trafficking and neuronal viability in parallel.

Recent studies have identified several modulators of mitochondrial trafficking in central neurons. In particular, elevated intracellular Ca^{2+} is an effective inhibitor of movement, an effect that is likely to be mediated by inhibition of ATP synthesis as well as potential disruption of the cytoskeleton (Rintoul et al., 2003). Nitric oxide also inhibits movement (Rintoul et al., 2004), probably as a result of inhibition of the electron transport chain. However, the mechanism by which Zn^{2+} inhibits movement is distinct in that it appears to occur without clear disruption of mitochondrial function. Our findings of a role for PI 3-kinase are consistent with a recent report by Chada and Hollenbeck (2003), who reported that focal stimulation of NGF receptors in sympathetic neurons causes mitochondria to "dock" or stop in the region of NGF application via a mechanism sensitive to inhibition by wortmannin, and likely by undergoing docking interactions with the actin cytoskeleton (Chada and Hollenbeck, 2004). Interestingly, this phenomenon did not occur with global application of NGF, which would more closely parallel the conditions used here. Nevertheless, the basic concept of PI 3-kinase-mediated control of mitochondrial trafficking is clearly similar between the two model systems. From our experiments, it appears that PI 3-kinase is involved in the actions of zinc described here, but the downstream target of the signaling cascade is less obvious. Although the conditions used here result in Akt phosphorylation, agents like insulin that mimic this action of zinc do not recapitulate the effects on trafficking. Also, neither Akt inhibitors nor dominant-negative Akt prevents the effects of zinc. Likewise, neither TOR nor ARF6 inhibition interrupted the actions of zinc. Thus, the step(s) between PI 3-kinase activation and the mitochondrial target remains unidentified. Moreover, recent studies have highlighted the role of other signaling cascades in the regulation of mitochondrial trafficking, including interactions with phosphatidylinositol 4,5-bisphosphate species (De Vos et al., 2003), activation of protein kinase A (Okada et al., 1995), and tumor necrosis factor-induced p38 activation (De Vos et al., 2000). In addition to mitochondria, others have implicated the regulation of synaptic vesicular transport by kinesin motor proteins (Okada et al., 1995; Zhao et al., 2001). With regard to the present study, we also demonstrated that mitochondrial trafficking is regulated by a signaling mechanism critical for cell survival.

A number of different mechanisms for zinc-mediated toxicity have been suggested. The data shown here illustrate the point that there are multiple effects of Zn^{2+}, which occur over a range of

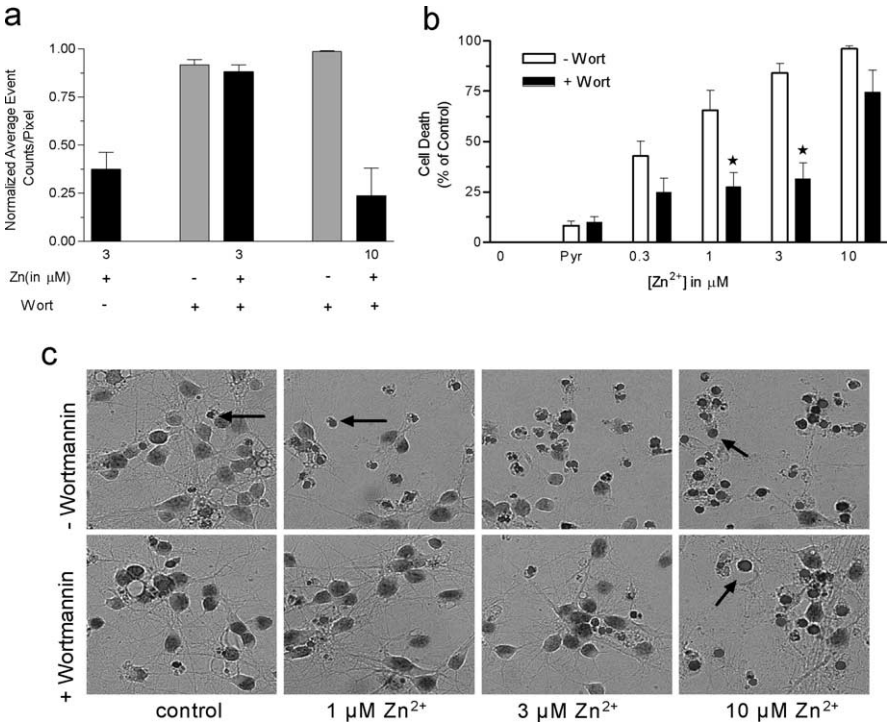

Figure 6. Wortmannin prevents both Zn^{2+}-mediated movement inhibition and neurotoxicity in a concentration-sensitive manner. ***a***, Movement was analyzed in neurons after 3 or 10 μM Zn^{2+}/Pyr stimuli pretreated with 1 μM wortmannin. ***b***, Extent of cell death was measured in neurons pretreated with wortmannin (1 μM) before Zn^{2+} (0–10 μM) exposure. ***c***, Representative Hoechst staining in neurons exposed to varying concentrations of Zn^{2+}/Pyr, either with or without wortmannin, where cell death (as indicated by arrows) is represented by extensive nuclear condensation (concentrated staining in smaller, rounder cell bodies) compared with healthier-looking cells (diffuse staining in larger cell bodies). ★$p < 0.005$ by Student's *t* test. Results are representative of four to six different cultures. Wort, Wortmannin. Error bars indicate SE.

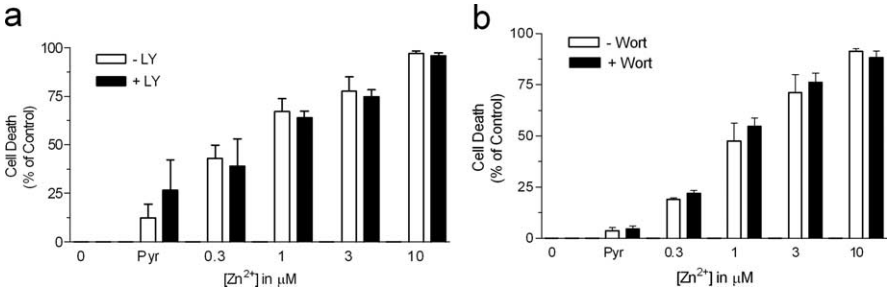

Figure 7. Sustained inhibition of PI 3-kinase does not protect against Zn^{2+}-mediated toxicity. ***a***, Extent of cell death was measured in neurons pretreated with LY294002 (30 μM) before Zn^{2+} (0–10 μM) exposure. ***b***, Toxicity assayed in neurons pretreated with wortmannin (1 μM) followed by 0–10 μM Zn^{2+}/Pyr (in the presence of wortmannin) that were given additional wortmannin pulses 3 and 6 h after Zn^{2+} application. Results are representative of four to six different cultures. LY, LY294002; Wort, wortmannin. Error bars indicate SE.

concentrations. We observed that low concentrations of Zn^{2+} that inhibit movement and produce injury have no effect on $\Delta\Psi_m$. Higher concentrations of zinc produce a form of injury that is not prevented by wortmannin and is also associated with mitochondrial depolarization. Zinc-mediated mitochondrial depolarization has been reported in a number of studies, both in intact cells (Sensi et al., 1999) as well as in isolated mitochondria (Dineley et al., 2005). However, these results suggest that, although mitochondria are clearly a target for zinc action, the most sensitive mechanisms associated with cytotoxicity do not involve gross disruption of mitochondrial function. Evidently, this phenomenon is not strictly dependent on the source of zinc. To mimic oxidant-labile $[Zn^{2+}]_i$, we applied 2,2′-DTDP to neurons and observed that mitochondrial movement significantly decreased with concentrations as low as 10 μM. This was surprisingly low, given that 100 μM 2,2′-DTDP was applied to cells to see mag-fura-2 responses in neurons (Aizenman et al., 2000). In addition, we also observed movement inhibition with 100 μM H_2O_2, which was used to mimic ROS exposure. Interestingly, TPEN did not restore mitochondrial movement when applied after either 2,2′-DTDP or H_2O_2. Together, these results raise the possibility that, apart from a direct Zn^{2+} effect, a secondary ROS-induced mechanism may also modulate mitochondrial movement.

Indirect PI 3-kinase activation by Zn^{2+} has been reported in other studies. Eom et al. (2001) found that Zn^{2+} induced stimulation of the JNK (c-Jun N-terminal protein kinase) through the PI 3-kinase pathway in primary mouse cortical neurons in culture, and Kim et al. (2000) demonstrated that extracellular Zn^{2+} activates p70 S6 kinase through PI 3-kinase signaling in Swiss 3T3 cells. These findings differ from our own in that PI 3-kinase activation is dependent on the activation of other pathways, which may be attributable to the exposure of 10 times higher concentrations of Zn^{2+} (except in the absence of ionophore), longer Zn^{2+} exposures necessary to achieve Akt phosphorylation, or varying sensitivities among cell types. We did not observe that the activation of other protein kinases plays a direct role in the Zn^{2+}-mediated movement effect, because inhibitors of these pathways did not protect against movement inhibition (supplemental Fig. 2, available at www.jneurosci.org as supplemental material). We cannot entirely exclude the possibility that these kinases are activated either downstream of or in parallel with PI 3-kinase; however, their activation did not affect Zn^{2+}-mediated changes in mitochondrial trafficking or neuronal injury. Zn^{2+} activates a number of other protein kinases involved both in pro-cell and anti-cell survival mechanisms. For example, studies have demonstrated that $[Zn^{2+}]_i$ can activate members of the MAP (mitogen-activated protein) kinase family. Zn^{2+} activation of the stress-activated p38 (McLaughlin et al., 2001), as well as its activation of the traditionally prosurvival ERK (extracellular signal-regulated kinase) (Du et al., 2002), is hypothesized to contribute to neuronal injury and subsequent cell death. In addition, activation of PKC (protein kinase C) in neurons plays a role in Zn^{2+}-induced oxidative neuronal injury (Noh, 1999). Because signal transduction pathways are rarely activated in a linear manner, it is more than likely that there is cross talk between these pathways with respect to Zn^{2+}-mediated neuronal injury.

PI 3-kinase activity has traditionally been associated with many key cellular processes, including cell growth and survival, membrane trafficking, neurite outgrowth, and cytoskeletal reorganization (Foster et al., 2003). Indeed, extensive literature demonstrates that growth factor-mediated activation of the PI 3-kinase/Akt pathway by insulin (Patel et al., 1993) or IGF-1 (insulin-like growth factor 1) (Dudek et al., 1997; Schubert et al., 2004), NGF (Chada and Hollenbeck, 2003), and BDNF (Zheng and Quirion, 2004), is neuroprotective against a range of cellular stresses. In addition, Luo et al. (2003) have demonstrated that constitutive activation of Akt is neuroprotective, whereas Akt deactivation promotes multiple models of neuronal cell death, including NMDA excitotoxicity, or NO- and H_2O_2-elicited injury. Including the present study, however, only one other report by Levinthal and DeFranco (2004) recently provided an example of PI 3-kinase activity associated with cell death. The authors demonstrated that glutamate-induced oxidative neuronal injury is mediated by activation of PI 3-kinase. Moreover, a window of transient PI 3-kinase inhibition afforded protection against oxidative glutamate toxicity, which is consistent with our results involving Zn^{2+} toxicity. We also observed that transient PI 3-kinase inhibition was neuroprotective, but this protection was overcome with sustained kinase inhibition. This provides insight about the bipolar nature of PI 3-kinase: short-term activation is detrimental to cell health, whereas long-term activation is essential for cell survival.

References

Aizenman E, Stout AK, Hartnett KA, Dineley KE, McLaughlin B, Reynolds IJ (2000) Induction of neuronal apoptosis by thiol oxidation: putative role of intracellular zinc release. J Neurochem 75:1878–1888.

Bereiter-Hahn J, Voth M (1994) Dynamics of mitochondria in living cells: shape changes, dislocations, fusion, and fission of mitochondria. Microsc Res Tech 27:198–219.

Brown AM, Kristal BS, Effron MS, Shestopalov AI, Ullucci PA, Sheu KF, Blass JP, Cooper AJ (2000) Zn^{2+} inhibits alpha-ketoglutarate-stimulated mitochondrial respiration and the isolated alpha-ketoglutarate dehydrogenase complex. J Biol Chem 275:13441–13447.

Chada SR, Hollenbeck PJ (2003) Mitochondrial movement and positioning in axons: the role of growth factor signaling. J Exp Biol 206:1985–1992.

Chada SR, Hollenbeck PJ (2004) Nerve growth factor signaling regulates motility and docking of axonal mitochondria. Curr Biol 14:1272–1276.

Cheng C, Reynolds IJ (1998) Calcium-sensitive fluorescent dyes can report increases in intracellular free zinc concentration in cultured forebrain neurons. J Neurochem 71:2401–2410.

Choi DW, Yokoyama M, Koh J (1988) Zinc neurotoxicity in cortical cell culture. Neuroscience 24:67–79.

De Vos KJ, Severin F, Van Herreweghe F, Vancompernolle K, Goossens V, Hyman A, Grooten J (2000) Tumor necrosis factor induces hyperphosphorylation of kinesin light chain and inhibits kinesin-mediated transport of mitochondria. J Cell Biol 149:1207–1214.

De Vos KJ, Sable J, Miller KE, Sheetz MP (2003) Expression of phosphatidylinositol (4,5) bisphosphate-specific pleckstrin homology domains alters direction but not the level of axonal transport of mitochondria. Mol Biol Cell 14:3636–3649.

Dineley KE, Scanlon JM, Kress GJ, Stout AK, Reynolds IJ (2000) Astrocytes are more resistant than neurons to the cytotoxic effects of increased $[Zn^{2+}]_i$. Neurobiol Dis 7:310–320.

Dineley KE, Malaiyandi LM, Reynolds IJ (2002) A reevaluation of neuronal zinc measurements: artifacts associated with high intracellular dye concentration. Mol Pharmacol 62:618–627.

Dineley KE, Votyakova TV, Reynolds IJ (2003) Zinc inhibition of cellular energy production: implications for mitochondria and neurodegeneration. J Neurochem 85:563–570.

Dineley KE, Richards LL, Votyakova TV, Reynolds IJ (2005) Zinc causes loss of membrane potential and alters production of reactive oxygen species in isolated brain mitochondria. Mitochondrion 5:55–65.

Du S, McLaughlin B, Pal S, Aizenman E (2002) *In vitro* neurotoxicity of methylisothiazolinone, a commonly used industrial and household biocide, proceeds via a zinc and extracellular signal-regulated kinase mitogen-activated protein kinase-dependent pathway. J Neurosci 22:7408–7416.

Dudek H, Datta SR, Franke TF, Birnbaum MJ, Yao R, Cooper GM, Segal RA, Kaplan DR, Greenberg ME (1997) Regulation of neuronal survival by the serine-threonine protein kinase Akt. Science 275:661–665.

Eom SJ, Kim EY, Lee JE, Kang HJ, Shim J, Kim SU, Gwag BJ, Choi EJ (2001) Zn^{2+} induces stimulation of the c-Jun N-terminal kinase signaling pathway through phosphoinositide 3-kinase. Mol Pharmacol 59:981–986.

Foster FM, Traer CJ, Abraham SM, Fry MJ (2003) The phosphoinositide (PI) 3-kinase family. J Cell Sci 116:3037–3040.

Frederickson CJ (1989) Neurobiology of zinc and zinc-containing neurons. Int Rev Neurobiol 31:145–238.

Frederickson CJ, Hernandez MD, Goik SA, Morton JD, McGinty JF (1988) Loss of zinc staining from hippocampal mossy fibers during kainic acid induced seizures: a histofluorescence study. Brain Res 446:383–386.

Gossen M, Bujard H (1992) Tight control of gene expression in mammalian cells by tetracycline-responsive promoters. Proc Natl Acad Sci USA 89:5547–5551.

Jones SM, Klinghoffer R, Prestwich GD, Toker A, Kazlauskas A (1999) PDGF induces an early and a late wave of PI 3-kinase activity, and only the late wave is required for progression through G_1. Curr Biol 9:512–521.

Kim S, Jung Y, Kim D, Koh H, Chung J (2000) Extracellular zinc activates p70 S6 kinase through the phosphatidylinositol 3-kinase signaling pathway. J Biol Chem 275:25979–25984.

Kleiner D (1972) On the inhibition of mitochondrial electron transport by Zn^{2+} ions. FEBS Lett 20:229–232.

Kleiner D (1974) The effect of Zn^{2+} ions on mitochondrial electron transport. Arch Biochem Biophys 165:121–125.

Koh JY, Choi DW (1994) Zinc toxicity on cultured cortical neurons: involvement of *N*-methyl-D-aspartate receptors. Neuroscience 60:1049–1057.

Koh JY, Suh SW, Gwag BJ, He YY, Hsu CY, Choi DW (1996) The role of zinc in selective neuronal death after transient global cerebral ischemia. Science 272:1013–1016.

Kozikowski AP, Sun H, Brognard J, Dennis PA (2003) Novel PI analogues selectively block activation of the pro-survival serine/threonine kinase Akt. J Am Chem Soc 125:1144–1145.

Langille SE, Patki V, Klarlund JK, Buxton JM, Holik JJ, Chawla A, Corvera S, Czech MP (1999) ADP-ribosylation factor 6 as a target of guanine nucleotide exchange factor GRP1. J Biol Chem 274:27099–27104.

Lee JM, Zipfel GJ, Park KH, He YY, Hsu CY, Choi DW (2002) Zinc translocation accelerates infarction after mild transient focal ischemia. Neuroscience 115:871–878.

Levinthal DJ, DeFranco DB (2004) Transient phosphatidylinositol 3-kinase inhibition protects immature primary cortical neurons from oxidative toxicity via suppression of extracellular signal-regulated kinase activation. J Biol Chem 279:11206–11213.

Link TA, von Jagow G (1995) Zinc ions inhibit the QP center of bovine heart mitochondrial bc1 complex by blocking a protonatable group. J Biol Chem 270:25001–25006.

Llopis J, McCaffery JM, Miyawaki A, Farquhar MG, Tsien RY (1998) Measurement of cytosolic, mitochondrial, and Golgi pH in single living cells with green fluorescent proteins. Proc Natl Acad Sci USA 95:6803–6808.

Luo HR, Hattori H, Hossain MA, Hester L, Huang Y, Lee-Kwon W, Donowitz M, Nagata E, Snyder SH (2003) Akt as a mediator of cell death. Proc Natl Acad Sci USA 100:11712–11717.

Lynch CJ, Patson BJ, Goodman SA, Trapolsi D, Kimball SR (2001) Zinc stimulates the activity of the insulin- and nutrient-regulated protein kinase mTOR. Am J Physiol 281:E25–E34.

McLaughlin B, Pal S, Tran MP, Parsons AA, Barone FC, Erhardt JA, Aizenman E (2001) p38 activation is required upstream of potassium current enhancement and caspase cleavage in thiol oxidant-induced neuronal apoptosis. J Neurosci 21:3303–3311.

Mills DA, Schmidt B, Hiser C, Westley E, Ferguson-Miller S (2002) Membrane potential-controlled inhibition of cytochrome *c* oxidase by zinc. J Biol Chem 277:14894–14901.

Noh K-M (1999) Mediation by membrane protein kinase C of zinc-induced oxidative neuronal injury in mouse cortical cultures. J Neurochem 72:1609–1616.

Okada Y, Sato-Yoshitake R, Hirokawa N (1995) The activation of protein kinase A pathway selectively inhibits anterograde axonal transport of vesicles but not mitochondria transport or retrograde transport *in vivo*. J Neurosci 15:3053–3064.

Patel RA, Kurian P, Raizada MK, Crews FT (1993) Insulin stimulates phosphatidylinositol 3-kinase activity in rat neuronal primary cultures. J Neurochem 61:360–363.

Rintoul GL, Filiano AJ, Brocard JB, Kress GJ, Reynolds IJ (2003) Glutamate decreases mitochondrial size and movement in primary forebrain neurons. J Neurosci 23:7881–7888.

Rintoul GL, Bennett VJ, Papaconstandinou N, Reynolds IJ (2004) Nitric oxide reversibly inhibits mitochondrial movement in primary forebrain neurons. Soc Neurosci Abstr 30:453.5

Schubert M, Gautam D, Surjo D, Ueki K, Baudler S, Schubert D, Kondo T, Alber J, Galldiks N, Kustermann E, Arndt S, Jacobs AH, Krone W, Kahn CR, Bruning JC (2004) Role for neuronal insulin resistance in neurodegenerative diseases. Proc Natl Acad Sci USA 101:3100–3105.

Sensi SL, Jeng JM (2004) Rethinking the excitotoxic ionic milieu: the emerging role of Zn^{2+} in ischemic neuronal injury. Curr Mol Med 4:87–111.

Sensi SL, Canzoniero LM, Yu SP, Ying HS, Koh JY, Kerchner GA, Choi DW (1997) Measurement of intracellular free zinc in living cortical neurons: routes of entry. J Neurosci 17:9554–9564.

Sensi SL, Yin HZ, Carriedo SG, Rao SS, Weiss JH (1999) Preferential Zn^{2+} influx through Ca^{2+}-permeable AMPA/kainate channels triggers prolonged mitochondrial superoxide production. Proc Natl Acad Sci USA 96:2414–2419.

Sheline CT, Behrens MM, Choi DW (2000) Zinc-induced cortical neuronal death: contribution of energy failure attributable to loss of NAD^+ and inhibition of glycolysis. J Neurosci 20:3139–3146.

Skulachev VP, Chistyakov VV, Jasaitis AA, Smirnova EG (1967) Inhibition of the respiratory chain by zinc ions. Biochem Biophys Res Commun 26:1–6.

Spira ME, Oren R, Dormann A, Ilouz N, Lev S (2001) Calcium, protease activation, and cytoskeleton remodeling underlie growth cone formation and neuronal regeneration. Cell Mol Neurobiol 21:591–604.

Suh SW, Chen JW, Motamedi M, Bell B, Listiak K, Pons NF, Danscher G, Frederickson CJ (2000) Evidence that synaptically-released zinc contributes to neuronal injury after traumatic brain injury. Brain Res 852:268–273.

van Blerkom J (1991) Microtubule mediation of cytoplasmic and nuclear maturation during the early stages of resumed meiosis in cultured mouse oocytes. Proc Natl Acad Sci USA 88:5031–5035.

Vergun O, Han YY, Reynolds IJ (2003) Glucose deprivation produces a prolonged increase in sensitivity to glutamate in cultured rat cortical neurons. Exp Neurol 183:682–694.

Weiss JH, Hartley DM, Koh JY, Choi DW (1993) AMPA receptor activation potentiates zinc neurotoxicity. Neuron 10:43–49.

Xia Z, Dudek H, Miranti CK, Greenberg ME (1996) Calcium influx via the NMDA receptor induces immediate early gene transcription by a MAP kinase/ERK-dependent mechanism. J Neurosci 16:5425–5436.

Yi M, Weaver D, Hajnoczky G (2004) Control of mitochondrial motility and distribution by the calcium signal: a homeostatic circuit. J Cell Biol 167:661–672.

Yin HZ, Weiss JH (1995) Zn^{2+} permeates Ca^{2+} permeable AMPA/kainate channels and triggers selective neural injury. NeuroReport 6:2553–2556.

Zhao C, Takita J, Tanaka Y, Setou M, Nakagawa T, Takeda S, Yang HW, Terada S, Nakata T, Takei Y, Saito M, Tsuji S, Hayashi Y, Hirokawa N (2001) Charcot-Marie-Tooth disease type 2A caused by mutation in a microtubule motor KIF1Bβ. Cell 105:587–597.

Zheng WH, Quirion R (2004) Comparative signaling pathways of insulin-like growth factor-1 and brain-derived neurotrophic factor in hippocampal neurons and the role of the PI3 kinase pathway in cell survival. J Neurochem 89:844–852.