Activation of the Trk Signaling Pathway by Extracellular Zinc

ROLE OF METALLOPROTEINASES*

In certain brain regions, extracellular zinc concentrations can rise precipitously as intense neuronal activity releases large amounts of zinc from the nerve terminals. Although zinc release has been suggested to play a pathological role, its precise physiological effect is poorly understood. Here, we report that exposure to micromolar quantities of zinc for only a few minutes robustly and specifically activates tropomyosin-related kinase (Trk) receptors, most likely TrkB, in cultured cortical neurons. We further found that Trk activation by zinc is extracellularly mediated by activation of metalloproteinases, which release pro-BDNF from cells and convert pro-BDNF to mature BDNF. These results suggest that activity-dependent release of extracellular zinc leads to metalloproteinase activation, which plays a critically important role in Trk receptor activation at zinc-containing synapses.

Trk neurotrophin receptors are essential for the formation, maturation, and survival of diverse types of neurons during development (1). Recently, Trk receptors were shown to modulate synaptic transmission and plasticity in the adult central nervous system (2, 3), and TrkB activation was shown to be necessary for hippocampal CA1 long term potentiation (4–6). However, the mechanisms underlying activity-dependent activation of synaptic Trk receptors are poorly understood.

The mammalian brain contains a large amount of zinc. Recent work has shown that zinc stored in glutamatergic vesicles is released into the extracellular space (7–9), predominantly during high frequency stimulation (10). Excess zinc release has been implicated as a mechanism of neuronal death in various models of acute brain injury (11, 12). Although synaptic zinc has been suggested to play a role in hippocampal CA3 long term potentiation (4–6), the mechanisms underlying activity-dependent activation of synaptic Trk receptors are poorly understood.

However, the mechanism of these zinc effects has not been well characterized. Since Src kinase is activated as a downstream member of the signaling pathways of several membrane receptors, including Trk (16), we herein examined whether zinc release can lead to Trk activation. This could form a logical pathway for the zinc-based coupling of synaptic activity to neurotrophin signaling.

EXPERIMENTAL PROCEDURES

Cell Cultures—Astrocytic cultures were prepared from newborn mice as previously described (17). After 2 weeks in vitro (DIV14), astrocytic cultures were used for experiments or as feeder cultures for embryonic neuroblastoma. Neurons were obtained from embryonic mouse brain (embryonic day 15–16) and plated either onto astrocytic cultures to generate mixed cultures or onto poly-L-lysine- and laminin-coated culture plates (Nunc) to generate nearly pure neuronal cultures. In the latter, cell division was halted on DIV3 by the addition of 10 μM cytosine arabinoside. More than 95% of cells in the nearly pure neuronal cultures were neurons, as identified by anti-MAP2 antibody immunocytochemistry (18).

Exposure to Zinc and Other Drugs—Brief (≤15 min) exposure of cells to zinc (as ZnCl2), Na3VO4, ionotocin, CaCl2, or aminophosphonic acid (APMA) was performed in Hanks’ balanced salt solution (In Vitrogen) supplemented with 1.8 mM CaCl2 and 0.8 mM MgSO4. In other experiments, K252a, pyridinone, zinc entry blockers, BAPTA-AM, GM6001, or inhibitors of matrix metalloproteinase, MMPI, -II, or -III (Calbiochem), were added in MEM for 30 min prior to zinc exposure and additionally during zinc exposure. After exposure, cultures were washed with MEM and returned to the CO2 incubator. Active MMPs were purchased from Calbiochem.

Immunoprecipitation and immunoblot—For immunoprecipitation, cell lysates or media were centrifuged. Aliquots of supernatant were incubated overnight with the appropriate antibody and then precipitated on Protein A-agarose beads at 4 °C for 1 h. The immunoprecipitates were washed three times with lysis buffer, and proteins were separated by 6–15% SDS-PAGE and transferred to polyvinylidene difluoride membranes (Millipore Corp.) for Western blot analyses. The Supersignal West Dura Extended Duration Substrate (Pierce) and AutoChem System (UVP BioImaging) were used to visualize the immunoreactive bands. To increase the signals for BDNF and pro-BDNF, cultures were incubated for 36 h with 5 μM retinoic acid for 5 days in the same medium.

Antibodies—Anti-BDNF antibody (sc-546; Santa Cruz Biotechnology). Anti-pan-Trk, -TrkA, -TrkB, -TrkC, and -p75NTK antibodies (Santa Cruz Biotechnology); anti-phospho-tyrosine (anti-pY) antibody (Upstate Biotechnology, Inc., Lake Placid, NY); anti-NT-3, -NT-4, and -nerves growth factor (NGF) antibodies (R&D); anti-Src and -p-Src antibodies (BIOSOURCE); and anti-external signal-regulated kinase, -Akt, and -p-Akt antibodies (Cell Signaling Technology).

 Newport Green 2,7-dichlorofluorescein Diacetate-Zinc Staining—Cells were preloaded with 5 μM Newport Green 2,7-dichlorofluorescein diacetate (Molecular Probes, Inc., Eugene, OR) in serum-free MEM for 30 min in the CO2 incubator. After exposure to 10 μM zinc
MMP Zymography—Culture media or cell lysates were centrifuged at 12,000 rpm for 5 min. The resulting supernatants were incubated with 50 μl of gelatin-Sepharose for 60 min and centrifuged at 12,000 rpm for 2 min. Each pellet was washed three times with phenylmethylsulfonfonyl fluoride-containing PBS and electrophoresed using zymography sample buffer and zymogram gels (Novex). The EnzCheck Gelatinase Assay Kit (Molecular Probes) was used for in situ zymography according to the provided protocol. After exposure to zinc, cells were washed with MEM and incubated with 40 μg of DQMM-gelatin-fluorescein isothiocyanate for 1 h at 37°C. Cells were then viewed by fluorescence microscopy and photographed.

In Vitro Treatment of pro-BDNF with MMPs—Ad-BDNF-infected cortical cells were suspended in ice-cold hypotonic lysis medium (1 mM NaHCO₃, 5 mM MgCl₂, 100 μM phenylmethylsulfonfonyl fluoride, 1 μg/ml aprotinin, and 1 μg/ml leupeptin) and lysed by sonication. To remove nuclei and unbroken cells, samples were sedimented at 600 × g for 10 min, and membrane fractions were separated further by centrifugation at 100,000 × g for 1 h. Membrane proteins (25 μg) were incubated with 2 μg/ml of MMP-2, -3, -7, or -9 (Calbiochem) in reaction buffer (50 mM Tris-Cl, pH 5.6, 150 mM NaCl, 5 mM CaCl₂) for 3 h. Immunoblots for BDNF were performed as described above.

RESULTS

Immunoprecipitates with anti-pan-Trk antibody were prepared from protein preparations of mouse cortical cultures that had been exposed to 10 μM zinc for 15 min in serum-free medium. The anti-Trk immunoprecipitates were Western blotted with anti-TrkA, anti-TrkB, anti-TrkC, or anti-pY antibodies. The results revealed that zinc exposure markedly increased the level of phosphorylated Trk (p-Trk) (Fig. 1A). On the other hand, the level of p75NTR was very low in cortical cells as compared with that in PC12 cells (Fig. 1A). Since cortical cells abundantly expressed TrkB but not TrkA or TrkC (barely detectable), the protein band recognized by the anti-pY antibody was most likely that of phosphorylated TrkB. An increase in Trk phosphorylation was first detected at 3 μM zinc and reached a maximum at 30–300 μM zinc (Fig. 1B). Thus, an intermediate concentration of 10 μM was used in subsequent experiments, unless otherwise indicated.

Because our cultures contained both neurons and astrocytes, we examined whether astrocytes were required for the zinc-dependent increase in TrkB phosphorylation. Nearly pure neuronal or astrocytic cultures (>95% of either cell type) were prepared and exposed to 10 μM zinc for 15 min. Western blots showed markedly increased levels of p-Trk in neuronal cultures treated with zinc compared with untreated control cultures (Fig. 1C). In contrast, zinc treatment of astrocytic cultures did not alter the low levels of endogenous TrkB or p-Trk. Since mixed cultures were more resistant to wash injury than nearly pure neuronal cultures, mixed cultures were used in subsequent experiments, unless otherwise indicated.

The time course of zinc-triggered increases in the level of p-Trk was also examined (Fig. 1, D and E). When a 15-min exposure was tested, we observed that as soon as 1 min after the addition of 10 μM zinc, p-Trk levels were higher in treated cultures than in control cultures. Between 5 and 15 min, the level of p-Trk was markedly elevated. The level of p-Trk returned to base line 30 min after washout of zinc, indicating that this effect was completely reversible. Under conditions of continuous exposure, the levels of p-Trk peaked at 15 min and then gradually decreased.

Since 30–100 μM zinc was reported to inhibit the effect of BDNF (19), we examined the effect of zinc on BDNF-triggered Trk phosphorylation. In our cortical culture system, 10 or 300 μM zinc slightly inhibited TrkB phosphorylation induced by a 15-min exposure to 10 ng/ml BDNF (Fig. 1F). However, in the same cells, a 15-min exposure to 10 or 300 μM zinc alone was found to activate Trk.

Since zinc is a known inhibitor of tyrosine phosphatases (20), we examined the possibility that zinc nonspecifically increases the phosphorylation levels of tyrosine kinase receptors. We examined the phosphorylation of epidermal growth factor receptor, insulin receptor β, and insulin-like growth factor-1 receptor β after zinc treatment. After immunoprecipitation with the appropriate antibody, Western blots with anti-pY antibody demonstrated no difference in the phosphorylation of epider-
nal growth factor receptor, insulin receptor β, or insulin-like growth factor-1 receptor β between control and zinc-treated cultures (Fig. 2A). Furthermore, cortical cultures treated with sodium vanadate, a potent inhibitor of tyrosine phosphatases (21), did not alter the levels of p-Trk (Fig. 2B). These data argue against the possibility that zinc-dependent phosphorylation of Trk receptors (most likely TrkB in cortical cultures) is due to nonselective phosphatase inhibition.

Next, we examined the possibility that calcium might also increase the level of p-Trk. Intracellular calcium levels were increased using the calcium ionophore, ionomycin. We found that treatment with ionomycin did not increase the level of p-Trk, even at neurotoxic (22) concentrations (Fig. 2B). These data argue that treatment with ionomycin did not increase the levels of p-Trk (Fig. 2A). Increasing the calcium concentration in the media did not (Ca).

We next addressed whether zinc acts on Trk receptors intracellularly or extracellularly by treating cultures with a zinc ionophore (sodium pyrithione) that greatly facilitates zinc entry into cells (25). Exposure to zinc and pyrithione markedly increased zinc influx over the effect of zinc alone, as visualized by fluorescence staining with the zinc indicator, Newport Green DCF diacetate (Fig. 3). Because the addition of pyrithione slightly decreased zinc-dependent Trk phosphorylation, it seems unlikely that zinc acts intracellularly (Fig. 3B). Conversely, inhibition of zinc entry through known zinc routes with a mixture of MK-801, CNQX, nimodipine, and KB-R7943 (26–29) did not reduce zinc-dependent Trk phosphorylation (Fig. 3B). Furthermore, the addition of an intracellularly acting metal chelator (BAPTA-AM) prior to zinc exposure did not attenuate Trk phosphorylation (Fig. 3B). Together, these results indicate that the site of zinc action on Trk receptors is most likely extracellular.

Next we examined the mechanism for Trk activation by extracellular zinc. Since agonist-independent activation of Trk is possible (30), we examined whether Trk activation by zinc requires the presence of agonists such as brain-derived neurotrophic factor (BDNF) or neurotrophin-4/5 (NT-4/5). Trk-blocking antibodies against BDNF or NT-4/5 attenuated zinc-dependent Trk phosphorylation. The anti-BDNF antibody blocked the effect to a somewhat greater degree than the anti-NT-4/5 antibody (Fig. 4A), whereas a combination of the two antibodies almost completely blocked Trk phosphorylation (Fig. 4A). In contrast, treatment with anti-NT-3 or anti-NGF antibodies at concentrations that block Trk activation in cortical cultures and PC12 cells, respectively, had no effect on zinc-triggered Trk phosphorylation (Fig. 4A).

Because blocking BDNF led to a much greater reduction in Trk phosphorylation as compared with blocking NT-4/5, we focused on BDNF as the representative neurotrophin leading to
Trk phosphorylation in this context and measured changes in the levels of BDNF and pro-BDNF in zinc-exposed cultures. In naive cells, zinc treatment decreased the levels of pro-BDNF in cells, but neither pro-BDNF nor mature BDNF could be detected in media by immunoprecipitation (Fig. 4B). Hence, to increase the signals for BDNF and pro-BDNF, cultures were infected with adenovirus containing the cDNA sequence encoding mouse prepro-BDNF (Ad-BDNF). Immunoblots of anti-BDNF immunoprecipitates from infected cultures showed that the level of pro-BDNF in zinc-treated cells decreased. Concurrent with this decrease, the levels of pro-BDNF and mature BDNF in the media increased compared with control levels (Fig. 4B). Accordingly, we examined the time course of BDNF release by zinc in Ad-BDNF-infected cortical cultures (Fig. 4C). Consistent with the time course of Trk activation by zinc, the levels of pro-BDNF and mature BDNF in the media increased maximally at 15 min and then gradually decreased. Next, we examined whether the BDNF came from neuronal cells. Nearly pure cortical neuronal cultures and SH-SY5Y neuroblastoma cells were infected with Ad-BDNF. As in the mixed cortical cultures infected with Ad-BDNF, the levels of pro-BDNF and mature BDNF in the media increased maximally at 15 min and then gradually decreased.
cultures, exposure to zinc decreased the levels of pro-BDNF in cell lysates and increased the levels of pro-BDNF and mature BDNF in the media (Fig. 4D), indicating that neurons could be a source of BDNF.

Since zinc can injure neurons (12), we examined the possibility that BDNF release is caused by nonspecific release of intracellular proteins from injured cells. Whereas exposure to 500 μM glutamate resulted in widespread neuronal death and glyceraldehyde-3-phosphate dehydrogenase release within 24 h, exposure to 10 or 300 μM zinc for 15 min did not induce the morphological changes indicative of cell death or glyceraldehyde-3-phosphate dehydrogenase release within 1 h (Fig. 4E). In addition, lactate dehydrogenase release into the media, a widely used quantitative indicator of neuronal cell death (18), was no different in cortical cultures 1 h after exposure to 300 μM zinc for 15 min as compared with sham wash controls (24.5 ± 19.4 versus 16.5 ± 20.0 units/ml, respectively; p > 0.5).

In contrast, as neurons lost their membrane integrity after a 24-h exposure to 500 μM glutamate massive lactate dehydrogenase release to the media occurred (277.0 ± 10.5 units/ml). Considering that zinc treatment releases BDNF within 5–30 min (Fig. 4C), it is unlikely that BDNF release was nonspecifically caused by zinc-triggered cell injury.

The zinc-dependent release of pro-BDNF and the concomitant increase of mature BDNF levels in the media raise the possibility that some of the released pro-BDNF is converted to BDNF in the extracellular milieu. Metalloproteinases such as MMPs are candidate proteases for the conversion of pro-BDNF to BDNF (31). Consistent with the activation of metalloproteinase activity by zinc, gelatin zymography of gelatin agarose-precipitated samples showed that the activity of MMP-2 and MMP-9 in the media obtained from zinc-treated cortical cultures was higher than that from control cultures (Fig. 5A). Densitometry showed that 10 μM zinc increased MMP2 activity to 2.99 ± 0.40-fold (S.D.) and MMP9 activity to 2.14 ± 0.59-fold that of the control, whereas 300 μM zinc increased the former to 5.25 ± 1.35-fold and the latter to 3.16 ± 0.40-fold (n = 3 for each, p < 0.05 for all, one-way analysis of variance with post hoc Fisher's LSD test).

In situ zymography using DQ-gelatin-fluorescein isothiocyanate labeled neuron-associated metalloproteinase activity was substantially increased in zinc-treated cultures compared with control cultures (Fig. 5, B–D). A metalloproteinase inhibitor

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**FIG. 5. Involvement of metalloproteinases in zinc-triggered Trk activation.** A, zymography on gelatin gels of samples prepared from media of sham wash control cultures or cultures exposed for 15 min to 10 or 300 μM zinc. Zinc exposure triggered increased activity of MMP-2 and MMP-9 (n = 3). B–D, in situ zymography using DQ-gelatin-fluorescein isothiocyanate of sham wash control cultures (B) and cultures exposed to 10 μM zinc (C) or 300 μM zinc (D) for 15 min. Metalloproteinase activity increased in zinc-treated cells (n = 3). Scale bar, 100 μm. E, anti-Trk immunoprecipitates of samples obtained from sham wash control (CTL) or cultures exposed to zinc alone (Zn) or zinc plus GM6001 (500 μM; +GM) or MMP inhibitor I (50 μM), -II (100 nM), or -III (500 nM) (n = 3) were Western blotted with anti-pY and anti-TrkB antibodies. F, activation of Trk after a 15-min exposure to the indicated concentrations of APMA, an MMP activator (n = 3). G, activation of Trk after a 15-min exposure to active MMP-2, MMP-3, MMP-7, or MMP-9 (0.1 μg/ml) (n = 3). H, anti-BDNF Western blots of membrane fractions (25 μg) obtained from mixed cortical cell cultures without (CTL) or with 3-h incubation with a 2 mg/ml concentration of the indicated MMPs. All tested MMPs increased conversion of pro-BDNF to mature BDNF (n = 3).

**FIG. 6. Metalloproteinases mediate the decrease of pro-BDNF in cells and the increase of BDNF in media.** A, anti-BDNF immunoprecipitates of cell lysates obtained from naive cortical cell cultures (top) and media from Ad-BDNF-infected cortical cultures (bottom) were Western blotted with anti-BDNF antibodies. Cells were exposed to 10 μM zinc or zinc plus GM6001 (500 μM) or MMP inhibitor I (50 μM), -II (100 nM), or -III (500 nM) for 15 min (n = 3). Levels of pro-BDNF were decreased in cell lysates and increased in media following zinc treatment; this effect was reversed by the addition of metalloproteinase inhibitors. B, levels of pro-BDNF in cell lysates obtained from naive cortical cell cultures (top) and levels of pro-BDNF and mature BDNF in media from Ad-BDNF-infected cortical cultures (bottom), after a 15-min exposure to 10 μM APMA (n = 3). APMA decreased pro-BDNF levels in cells but increased levels of pro-BDNF and mature BDNF in media.
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(GM6001) and inhibitors for MMP-I, -II, and -III substantially reduced zinc-dependent Trk phosphorylation (Fig. 5E). Conversely, the addition of the metalloproteinase activator, APMA, to cultures in the absence of zinc increased the levels of p-Trk (Fig. 5F). Furthermore, treatment with active MMP-2 and MMP-9 (the dominant forms in cortical cells) as well as MMP-3 and MMP-7 (known to cleave proneurotrophins) (31) all increased the levels of p-Trk to varying degrees (Fig. 5G), suggesting that all MMPs can mediate the conversion. Consistent with the previous report (31), MMP3 and MMP9 were more effective at converting pro-BDNF to BDNF under our in vitro conditions.

In addition to their effects on Trk phosphorylation by zinc, metalloproteinase inhibitors attenuated both the decrease of pro-BDNF levels in cells and the increase of pro-BDNF and mature BDNF levels in media of zinc-treated cultures infected with Ad-BDNF (Fig. 6A). Conversely, the metalloproteinase activator (APMA) alone decreased the pro-BDNF levels in cells and simultaneously increased the levels of pro-BDNF and mature BDNF in the media (Fig. 6B). Together, these results suggest that MMPs mediate zinc-dependent Trk activation. Hence, it is likely that in addition to pro-BDNF/mature BDNF conversion, MMPs mediate pro-BDNF release to the media from zinc-treated cells.

DISCUSSION

Normally, extracellular zinc concentrations in the brain are well below 1 μM (10, 12). However, following intense neuronal activity, local zinc concentrations can rise as high as several tens to hundreds of μM (7, 8, 10, 12). The present results suggest that such increases in extracellular zinc levels probably lead to activation of Trk receptors, particularly TrkB.

Although further studies may be needed to fully elucidate the mechanism of zinc-triggered Trk activation, our data suggest that the extracellular zinc-mediated activation of MMPs is a crucial event for Trk activation. Zinc treatment releases pro-BDNF from cells in an MMP-dependent manner, leading to increased pro-BDNF levels in the media. In addition, levels of mature BDNF also increased in the media, which may occur via metalloproteinase-mediated conversion (31). Since pro-BDNF can activate TrkB (32) as well as p75NTR, and mature BDNF preferentially activates TrkB (31), both forms may contribute to the TrkB activation seen in the present study. Although p75NTR can be additionally activated by the release of pro-BDNF, our cortical neurons normally express p75NTR at very low levels (33), and hence the main effect of pro-BDNF release may be the activation of TrkB at least in this system.

It is unknown how zinc treatment increases pro-BDNF release from cells. In A431 cells, zinc has been shown to activate MMPs and release heparin-binding epidermal growth factor, a membrane-anchoring epidermal growth factor receptor ligand, probably through MMP-mediated proteolysis (34). However, in cortical cell cultures, simple proteolytic release of pro-BDNF is unlikely, because 1) pro-BDNF is not a membrane protein and 2) no apparent size alteration occurs in released pro-BDNF as compared with cellular pro-BDNF. Further studies may be warranted to precisely elucidate the relevant mechanisms.

Extracellular matrix MMPs and a disintegrin and metalloproteinase (ADAM) family proteases in the cell membrane are two structurally similar classes of metalloproteinases that can act in the extracellular space. Because of their similar structures, activation of MMPs and ADAMs occurs by a similar mechanism, wherein proteolytic cleavage of the inhibitory pro domain by upstream kinases such as furin exposes catalytic zinc sites (35). However, in certain cases, nitration or oxidation of inhibitory cysteine residues unmasks the catalytic site and thus activates MMPs in the absence of cleavage (36). Although the present study did not directly address the mechanism of metalloproteinase activation by zinc, the extracellular action of zinc suggests that it may activate metalloproteinases either directly by binding to the inhibitory cysteine residues or indirectly by activating oxidative pathways (36, 37) or upstream proteases. Further studies will be needed to elucidate the precise activation mechanism. Metalloproteinase species that specifically mediate zinc-dependent Trk activation were not precisely identified in the present study, since most MMP inhibitors inhibit several metalloproteinases including ADAMs. Whereas our data seem to indicate that zinc activates MMP-2 and MMP-9, which are the major forms in mouse cortical cultures, it is possible that yet uncharacterized metalloproteinases localized in synapses play larger roles.

Previously, 30–100 μM zinc has been shown to inhibit neurite outgrowth by BDNF in PC12 cells (19). However, in our cortical cultures, 10–300 μM zinc only slightly inhibited TrkB activation by BDNF. Rather, 10–300 μM zinc alone substantially activated TrkB, indicating that activation of Trk by zinc outweighs its possible inhibitory effect on BDNF/TrkB interaction. The zinc-induced inhibition of the BDNF/TrkB interaction seen in this study was lower than that observed previously (19), perhaps due to differences in cell types. Another possibility is that since free zinc concentration varies depending on the media composition (i.e. the presence or absence of zinc-binding molecules such as amino acids and various proteins), the free zinc concentrations in our experiments may have been substantially lower than the total calculated concentrations.

In sum, synthetically triggered TrkB activation has been proposed to play a critical role in synaptic plasticity such as hippocampal long term potentiation (4–6). The present study shows that zinc release may play an important role in the activity-dependent activation of TrkB or other Trk receptors at zinc-containing glutamatergic synapses. This work will provide an important basis for the study of synaptic zinc in relation to activity-dependent synaptic plasticity.

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