Published in final edited form as:

Angew Chem Int Ed Engl. 2017 April 24; 56(18): 4970–4975. doi:10.1002/anie.201700095.

Zinc Regulates Chemical Transmitter Storage in Nanometer Vesicles and Exocytosis Dynamics Measured by Amperometry

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Abstract

We applied electrochemical techniques with nano-tip electrodes to show that micromolar concentrations of zinc not only trigger changes in the dynamics of exocytosis, but also vesicle content in a model cell line. The vesicle catecholamine content in PC12 cells is significantly decreased after 100 μ M zinc treatment, but, catecholamine release during exocytosis remains nearly the same. This contrasts the number of molecules stored in the exocytosis vesicles, which decreases, and we find that the amount of catecholamine released from zinc-treated cells reaches

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nearly 100 percent content being expelled. Further investigation shows that zinc slows down exocytotic release allowing time for this to occur. Our results provide the missing link between zinc and the regulation of neurotransmitter release processes, which might be important in memory formation and storage.

Graphical Abstract

We have employed single cell amperometry and intracellular vesicle impact electrochemical cytometry to investigate the effects of zinc on exocytosis and vesicle content in PC12 cells. Our results show that zinc not only changes the transmitter storage, but also the exocytosis dynamics by leading a more stable fusion pore opening and closing process.

Keywords

zinc regulated exocytosis; vesicle content; amperometry; cytometry

Zinc is an essential micronutrient involved in structural and regulatory cellular functions. Changes in zinc concentration participate in modulating fundamental cellular processes such as proliferation, secretion, ion transport and most importantly learning and memory in a mechanism that is not well understood. The level of zinc in the hippocampus probably exceeds 1 mmol/L and is only weakly coordinated with any endogenous ligand. Coupled with the integral involvement of the hippocampus in memory, this implicates a possible role for zinc in the learning and memory process.^[1] Zinc deficiency has also been shown to impair cognitive and motor functioning. Animals who experience severe zinc deficiency early in their fetal development are not only at increased risk for abortion and fetal abnormalities, but also have difficulty in maze-learning tasks, particularly when shock is used in the learning procedure when tested as adults.^[2] Micromolar zinc has been reported to influence many physiological and pathological processes in the neuronal system,^[3] however, whether and how it modulates the response of various ion channels and further alters synaptic strength and plasticity to effect learning and memory is difficult to establish. Exocytosis, the release of chemical transmitters from vesicles to the extracellular environment, is the main mechanism of signal transduction and neuronal communication.^[4] Numerous studies have indicated that both learning and memory are somehow expressed through neurotransmitter release in neurons,^[5] which provides us a pathway to study the

mechanism by which zinc is involved in neurotransmitter release to effect on synaptic strength or learning and memory.

Single cell amperometry is the most used technique to study exocytosis release, in which neurotransmitters excreted during exocytosis are quantified by oxidation at carbon-fiber micro-electrodes.^[6] Recently, we also discovered nanoscale mammalian vesicles can adsorb to carbon-fiber electrodes and subsequently rupture and expel their contents.^[7] Therefore, a nano-tip carbon-fiber electrode has been inserted into a single cell in an approach called intracellular vesicle impact electrochemical cytometry to measure the content of individual nanoscale vesicles directly in the cytoplasm of living cells.^[8] In this work, with the employment of both single cell amperometry and intracellular vesicle impact electrochemical cytometry (Scheme 1), we have been able to investigate the effects of micromolar zinc on exocytotic release and vesicle storage in cultured pheochromocytoma (PC12) cells.

With these two techniques, we have found that the chemical content of vesicles in PC12 cells is significantly decreased after 100 μM zinc treatment. However, the amount of vesicle catecholamine released during exocytosis remains almost the same. When we calculate the number of molecules stored in vesicles and released during stimulated exocytosis, we find that the fraction of catecholamine released during each exocytosis event from zinc-treated cells is almost 100%, in contrast to most release events.^[4] Further investigation shows that zinc slows down exocytotic release with significant increases in half time and fall time compared with control cells, which indicates the formation of a much more stable fusion pore during vesicle opening. Although zinc does not change the amount of molecules released, it does change vesicle catecholamine storage, and it changes the rate of neurotransmitter release to make longer events, which might regulate synaptic strength and plasticity.

Prior to single cell amperometry, PC12 cells were incubated with 100 μM Zn^{2+} in RPMI 1640 media for 3.5 h and control cells were only incubated with RPMI 1640 media for same intervals. Fluorescence images showed obvious zinc increase in the intracellular environment of the PC12 cells after zinc treatment; however, there was very little zinc in control PC12 cells (Figure S1, Supplementary information). To compare the amount of catecholamine released and catecholamine stored in vesicles, we employed nano-tip carbon fiber electrodes in both techniques to obtain a similar signal to noise ratio. For single cell amperometry, the nano-tip electrode was placed on top of a single PC12 cell and held at a +700 mV potential vs. Ag/AgCl reference electrode. After stimulation with high K^+ solution, the vesicle membrane fuses with cell membrane and releases part of the vesicle content (mainly dopamine in our case), which is recorded as a current transient (Scheme 1a, the optical experimental system is shown in Figure S3a).^[9] Figures 1a and 1b show typical amperometric traces for exocytotic release obtained with single cell amperometry for control and zinc-treated cells, respectively, where each current transient corresponds to a single vesicle release event. Both control and zinc data from all the current transients have been plotted as a normalized frequency histogram of vesicle catecholamine released during stimulated exocytosis. The number of catecholamine molecules, N , from individual current transients can be quantified with Faraday's equation ($N=Q/nF$) where n is the number of

electrons exchanged in the oxidation reaction (2 for catecholamines) and F is Faraday's constant (96 485 C/mol). As shown in Figure 1c, there is no significant difference in the number of molecules released during exocytotic release between control with zinc-treated cells (Figure 1d is the normalized frequency histogram describing the distribution of $\log[\text{molecules}]$, which provides a near-Gaussian distribution and little difference for zinc-treated cells).

In contrast, significant differences for zinc-treated cells were obtained when we used intracellular vesicle impact electrochemical cytometry to measure the vesicle catecholamine storage with a nano-tip electrode held at +700 mV potential inserted into a cell. Without stimulation, the vesicles burst and catecholamines are oxidized on the electrode, which are also recorded as current transients. Each transient represents the total catecholamine content of a single vesicle (Scheme 1b, the optical experimental setup is shown in Figure S3b). As shown from the typical traces in Figure 2a and 2b, there are fewer events and lower transient currents for zinc-treated cells compared to control. The numbers of catecholamine molecules in single vesicles were calculated from all the transients, and these values are plotted as normalized frequency histograms in Figure 2c. This reveals a significant depletion of vesicular catecholamine content in zinc-treated cells. The $\log[\text{molecules}]$ plots are well-fit to a Gaussian distribution, where the standard deviation of the Gaussian is 0.338 for zinc treated and 0.367 for control cells. The similarity of the standard deviation implies that zinc has equally decreased the catecholamine content of all vesicles in the cells.

Analysis of typical amperometric traces can provide characteristic information of exocytotic and cytometry events. Figure 3 shows the average peaks and average number of catecholamine molecules from single cell exocytosis and intracellular vesicle impact electrochemical cytometry. As shown in Figures 3a and 3b, treatment with 100 μM zinc (red transients) leads to lower amplitude and broader exocytotic events, as well as smaller and sharper cytometry transients compared to control. The average number of catecholamine molecules per single event in exocytosis is $98\,000 \pm 1\,500$ molecules for control cells (11 cells, error is SEM) versus $99\,000 \pm 8\,500$ molecules for zinc-treated cells (18 cells), thus showing no significant difference (Figure 3c). The average total vesicle content shown in Figure 3d as measured with intracellular vesicle impact electrochemical cytometry is $148\,000 \pm 1\,000$ molecules for control cells (21 cells) versus $107\,000 \pm 1\,000$ for zinc-treated cells (17 cells), a significant drop.

In catecholaminergic cell lines, dopamine is efficiently transported by the vesicular monoamine transporter (VMAT) into monoaminergic vesicles that are then used for exocytosis. Vesicular catecholamine storage can be regulated by several kinds of VMAT inhibitors or stimulators to reduce or increase vesicular content.^[9] It has been reported in previous studies that zinc exposure affects the gene and protein expression of VMAT-2 in rats and results in reduction of dopamine levels.^[10] However, there have been no direct measurements to reveal the effect of zinc on catecholamine levels in single cells and especially in single vesicles. Our results show that the vesicular catecholamine content is altered in zinc-treated PC12 cells with a 27 % reduction compared to control cells.

The total vesicular catecholamine content, as measured in intracellular impact electrochemical cytometry, is decreased after zinc treatment and the amount released as measured in single cell amperometry is approximately the same. With less storage of transmitter, how is exocytotic release enhanced to produce the higher fraction released and what does it mean? To answer this question, we analyzed the peaks from single cell amperometry to get kinetic information of exocytotic release. As described in Figure 4a, the peak current, i_{max} , rise time t_{rise} , defined as the time from 25% to 75% of the maximum on the ascending part of the transient, peak half width t_{half} , defined as the width of the exocytotic transient at half of its magnitude, and the fall time t_{fall} , defined as the time from 75% to 25% of the maximum on the descending part of the transient are all parameters related to the dynamics of the release event. The corresponding exocytosis parameters with and without zinc treatment are summarized in Table S1 (Supplementary information). As shown in Figure 4b, a decrease in peak current, i_{max} , is observed after zinc incubation, which is in agreement with the depletion of single vesicle content caused by zinc. However, the change is not significantly different ($p = 0.411$) owing to variability in peak height data. The peak half time, t_{half} , is increased by 66 % ($p = 0.006$), indicating slower vesicle opening and closing after zinc treatment (Figure 4c). The value of t_{half} is a marker of duration of exocytotic events.^[11] The rise time, t_{rise} , and fall time, t_{fall} , are the characteristics of the vesicular influx and efflux of catecholamine during exocytotic release, which are believed to be temporal indicators of the opening and closing of the fusion pore. As shown in Figure 4d, there is a slight but not significant increase in t_{rise} indicating that the opening process of the fusion pore has not been affected greatly. However, the result from Figure 4e shows a large increase in t_{fall} , +92 % ($p = 0.004$), which suggests that the closing of the fusion pore has been significantly slowed down and the pore stays open for a longer time compared to control cells. This allows the vesicle to release a larger fraction of its load (92 % instead of 66 %) during exocytosis after zinc treatment.

We analyzed the pre-transient feet from single cell amperometry to gain more insight into the opening phase of the exocytosis event when affected by increased zinc. A pre-transient foot is recorded as a small current increase in the beginning of amperometric detection, representing leakage of neurotransmitter through a fusion pore formed as an early stage of vesicle fusion.^[12] Feet were analyzed according to Figure 4a. The values i_{foot} , t_{foot} and Q_{foot} represent the lifetime, current amplitude, and charge of the pre-transient foot^[6b] and are shown in Figure S4 (Supplementary information). With zinc treatment, i_{foot} decreased. As the foot current is thought to be proportional to vesicle content, the reduction of this content by zinc is consistent with the reduced vesicular load observed. In contrast, t_{foot} increased, indicating that the zinc treatment induced a more stable fusion pore and the total number of molecules released during the foot is the same. Both release during the foot and that during main release events are slowed by zinc, but the number of molecules released does not change. This leads to the surprising hypothesis that regulation of exocytosis by zinc might result more from a change in event dynamics rather than a change in the number of molecules, and this appears to be regulated by fusion pore opening and closing.

In last decades, complementary genetic, biochemical and molecular approaches have been used to identify the SNARE (soluble N-ethylmaleimide-sensitive fusion protein attachment protein receptor) complex as the basic membrane fusion machinery to direct vesicular

trafficking and exocytosis, which is a carefully regulated process.^[13] One important characteristic of this regulated exocytosis is its acute regulation by many enzymatic proteins such as protein kinases (protein kinase A and protein kinase C) which are believed to be involved in regulating multiple stages in the exocytosis event, including the fusion pore opening and closing processes.^[14] Protein kinase C has been suggested to induce changes in cell morphology correlating with the reorganization of submembranous actin, resulting in the constriction of fusion pore and changes in the vesicular fraction of neurotransmitter release.^[11a, 15] In most cell types, one or more protein kinase C isoforms influences the morphology of the F-actin cytoskeleton and thereby regulates processes that are affected by re-modelling of the microfilaments.^[16] It has been reported that the activity of MARCKS (myristoylated alanine-rich C-kinase substrate), a filamentous actin cross-linking protein which may be a regulated cross-bridge between actin and the plasma membrane, is inhibited by protein kinase C-mediated phosphorylation.^[17] In previous work, the effect of latrunculin A, an inhibitor of actin cross-linking, has been shown to increase the value of t_{fall} in amperometric measurements of exocytosis and thus lengthens the time of exocytotic events and increases the number of molecules released.^[11a, 15] This was suggested to occur via a decrease in the rate of closing of the fusion pore. Thus, we hypothesize a mechanism for the effect of zinc on exocytosis where zinc enhances the activity of cytosolic protein kinase C.^[18] Zinc has been shown to promote protein kinase C-mediated phosphorylation and induce the binding of protein kinase C to plasma membranes similarly to the reversible binding induced by calcium.^[19] This proposed mechanism is shown in Figure 5 suggesting that the effect of zinc on protein kinase C phosphorylation results in the inhibition of actin cross-linking proteins and binding proteins. The following actin fragmentation causes a more loose actin network which results in a stable fusion pore and longer exocytotic events before the pore closes again. This thus explains the increase in half time and fall time of the exocytotic events in zinc-treated cells compared with control cells. A looser actin network might be expected to cause a larger pore opening for exocytosis; however, we do not see this and so the data suggest this process stabilizes the pore at the expense of a smaller diameter.

To further confirm this hypothesis, we used time-of-flight secondary ion mass spectrometry (ToF-SIMS) analysis to investigate zinc distribution and localization in PC12 cells after zinc treatment. For each experiment, a cell from each control and zinc-treated group was trapped and exposed to depth profiling analysis using Bi_3^+ as the primary ion beam and C_{60}^{+2} as a sputter source for etching. Depth profiling data are shown in Figure 6 for the cells treated with zinc and in supplemental Figure S5 for control cells, respectively. Two zinc species, $[\text{ZnOH}_3]^+$ (green) and $[\text{ZnO}_2\text{H}]^+$ (purple), identified based on their mass and their respective isotopic pattern (data not shown), were selected to be monitored while profiling through the cells. Another fragment, $[\text{C}_5\text{H}_{15}\text{PNO}_4]^+$, which is one characteristic fragment of phosphatidylcholine from the lipid membrane, was used as an indicator of distance from the cell membrane. With continued etching through the cell, the intensity of the $[\text{C}_5\text{H}_{15}\text{PNO}_4]^+$ (blue) decreases as expected. There are no obvious zinc species in control cells, also as expected (Figure S5, Supplementary information). However, the two zinc species are clearly observed and differ in their 3D profiles in the zinc-treated cells (Figure 6). With continued etching from the membrane to inside of the cell, the $[\text{ZnO}_2\text{H}]^+$ fragment is found to have high intensity in the top-most layer of the analyzed cell and much lower intensity through

the cell, similarly to the $[C_5H_{15}PNO_4]^+$ fragment. In contrast, the $[ZnOH_3]^+$ signal rises significantly as we sputter through the cell. These data suggest that $[ZnO_2H]^+$ is mainly located close to the cell membrane surface while $[ZnOH_3]^+$ seems to be present more inside of the cell. This then implies that there are different originating sources for these two zinc related species after zinc treatment.^[20] This suggests that the species localizing near the cell membrane might be related to zinc species binding to protein kinase C present on the cellular membrane to regulate the rate of exocytosis, whereas the other species that localize inside of the cell might have an effect on VMAT to influence vesicular catecholamine transport and storage in vesicles.

In conclusion, we have employed nano-tip electrodes for single cell amperometry and intracellular vesicle impact electrochemical cytometry to investigate the effects of zinc on exocytosis and chemical content of vesicles in PC12 cells. Our data show that although zinc does not change the amount of catecholamine released in each event, it does change the vesicle content, changes the rate of release to induce longer events, and results in a higher fraction of content released in exocytotic release. In contrast to these results, L-DOPA changes the vesicle content and amount released equally, so the fraction released is unchanged.^[8a] Cisplatin on the other hand changes the fraction released, but not the vesicle content.^[22] Thus, it seems apparent that the fraction released during exocytosis and as shown here the dynamics of the event are critical parameters in the exocytosis event. As zinc affects learning, these experiments suggest that the rate of exocytosis, the length of release events, and the fraction of chemical messenger released are likely to be important factors in synaptic strength and plasticity.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We acknowledge support from the European Research Council (ERC Advanced Grant), the Knut and Alice Wallenberg Foundation in Sweden, the Swedish Research Council (VR), and the U.S. National Institutes of Health (NIH).

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Figure 1.

Representative amperometric traces of exocytotic release from cells a) without and b) with 100 μM zinc treatment for 3.5 h; c) Normalized frequency histograms describing the distributions of the molecules and d) $\text{log}[\text{molecules}]$ released from control cells (black, $N=358$ from 11 cells) and zinc treatment cells (red, $N=735$ from 18 cells), bin size: 1.0×10^4 molecules. Fits were obtained from a Gaussian distribution of the data.

Figure 2.

Representative amperometric traces of vesicle content in cells a) without and b) with 100 μ M zinc treatment for 3.5 h; c) Normalized frequency histograms describing the distribution of the molecules d) $\log[\text{molecules}]$ observed in vesicles of control cells (black, $n=1355$ from 21 cells) versus zinc-treated cells (red, $n=1093$ from 17 cells), bin size: 2×10^4 molecules. Fits were obtained from a Gaussian distribution of the data.

Figure 3.

Average peaks obtained from a) single cell amperometry and b) intracellular vesicle impact electrochemical cytometry at control (black) and zinc- treated (red) cells. Average molecules of catecholamine quantified per c) exocytotic release and d) vesicle content from control (blank-filled) and zinc treatment (dot-filled) PC12 cells. Pairs of data sets were compared with a Mann-Whitney rank-sum test; ***, $p < 0.001$; **, $p < 0.01$; *, $p < 0.1$.

Figure 4.

a) Scheme showing the peak analysis. Comparisons of b) peak current, c) half peak width, d) rise time and e) fall time from single cell amperometry with control cells (blank-filled, 11 cells) and zinc treated cells (dot-filled, 18 cells); Pairs of data sets were compared with Mann-Whitney rank-sum test; ***, $p < 0.001$; **, $p < 0.01$; *, $p < 0.1$.

Figure 5. Mechanism showing zinc activates the activity of protein kinase C and promotes the actin fragmentation, resulting in a more stable fusion pore and longer exocytotic event.

Figure 6. ToF-SIMS depth profile analysis for zinc location in zinc-treated cells. Cell depth is approximately 250 nm estimated by the ion fluence.^[21]

Scheme 1.

Schematic diagram of single cell amperometry and intracellular vesicle impact electrochemical cytometry.