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COMMUNICATION

Altered Lipid Composition of Secretory Cells following Exposure to Zinc can be Correlated to Changes in Exocytosis

Lin Ren[a,+], Masoumeh Dowlatshahi Pour[a,b,+], Per Malmberg[a,b], and Andrew G. Ewing[b,c,*]

Abstract: *Micromolar concentration of zinc has been shown to significantly change the dynamics of exocytosis as well as the vesicle contents in a model cell line, providing direct evidence that zinc regulates neurotransmitter release. To provide insight about how zinc modulates these exocytotic processes, neurotransmitter release and vesicle content are now compared with single cell amperometry and intracellular impact vesicle cytometry at a range of zinc concentrations. Additionally, ToF-SIMS images of lipid distributions in the cell membrane after zinc treatment correlate to changes in exocytosis. By combining electrochemical techniques and mass spectrometry imaging, we propose a mechanism by which zinc changes the fusion pore and the rate of neurotransmitter release by changing lipid distributions and results in the modulation of synaptic strength and plasticity.*

Zinc has been found to play an important role in synaptic plasticity and memory formation.[1] It is believed that during synaptic transmission zinc diffuses into the synaptic cleft and then binds to receptors on the pre- or postsynaptic membranes, resulting in regulating pre- or postsynaptic functions. [2] Although a lot of studies have shown zinc is involved in learning and memory, the chemistry of how zinc changes the synaptic strength and further effects on the complex neuron system has been difficult to establish. It is proposed that zinc affects exocytosis, the cellular process critical for a wide range of cellular communications in neurons.[3] We previously found that micromolar zinc not only reduces the amount of catecholamines stored in vesicles but also slows down the exocytotic process, [4] and proposed that zinc-related proteins play important roles in these changes. However, membrane phospholipids are also a likely candidate to be involved in changing the exocytosis process after zinc treatment. This opens a fascinating door to use our chemical measurement methods to investigate the link between zinc and its function in learning and memory as well as zinc deficits in associated diseases.

Mass spectrometry imaging (MSI) can provide simultaneous and parallel detection of many major classes of biological substances.[5] One such technique, time-of-flight secondary ion mass spectrometry (ToF-SIMS) has been successfully utilized to monitor, map and determine the distribution of endogenous biological molecules such as lipids and metabolites directly from the surface of biological materials with high lateral resolution (400 nm to 1-2 μm).[6]

In this work, we used single cell amperometry and intracellular impact vesicle cytometry to study the effect of a range of zinc concentrations on exocytosis and vesicle content of PC12 cells. Simultaneously, ToF-SIMS was used to investigate changes in cell membrane phospholipid content in sibling cultures (Supporting information, Figure S1). By correlating the results of electrochemical methods to ToF-SIMS analysis, we provide a potential mechanism for how zinc influences phospholipid composition and mediates plasticity in exocytosis.

Single cell amperometry, first introduced by Wightman et al.,[7] employs a micrometer-sized electrode placed on top of a cell to detect individual release events (Figure S2-a). Intracellular vesicle impact electrochemical cytometry (Figure S2-b) is used to probe single vesicle content with the insertion of a nanotip microelectrode inside of a living cell.[8] In both techniques, vesicular neurotransmitter is oxidized at the electrode surface resulting in a peak transient, used to count the number of neurotransmitter molecules by applying Faraday's law (see SI, Fig S2). The adrenal phaeochromocytoma (PC12) cell line is a functional model of neurotransmitter release, which can synthesize and store dopamine and sometimes noradrenaline released upon depolarization in a Ca^{2+}-dependent way. After stimulation with high K^{+} solution from a stimulation pipette, the vesicle membrane fuses with the cell membrane and releases part of the vesicle content. Dopamine oxidization at the electrode is recorded as a current transient for each vesicle release event. For intracellular vesicle impact electrochemical cytometry, a nano-tip electrode is inserted through the membrane of the PC12 cell. Without stimulation, the vesicles burst on the electrode and dopamine is oxidized, also recorded as a current transient. We measured the number of molecules released and in vesicles by these two techniques (Figure 1). The average total vesicle content is shown in Figure 1a (black). The vesicle content has a slight increase when the cells are incubated with 0.1 μM zinc. However, at higher concentrations, the vesicle content is significantly decreased, leading to a depletion of the dopamine stored in zinc-treated cells. We also calculated the molecules released from single cell amperometry, however, as shown in Figure 1a (blue), zinc incubation causes no significant difference in the number of molecules released during exocytosis.

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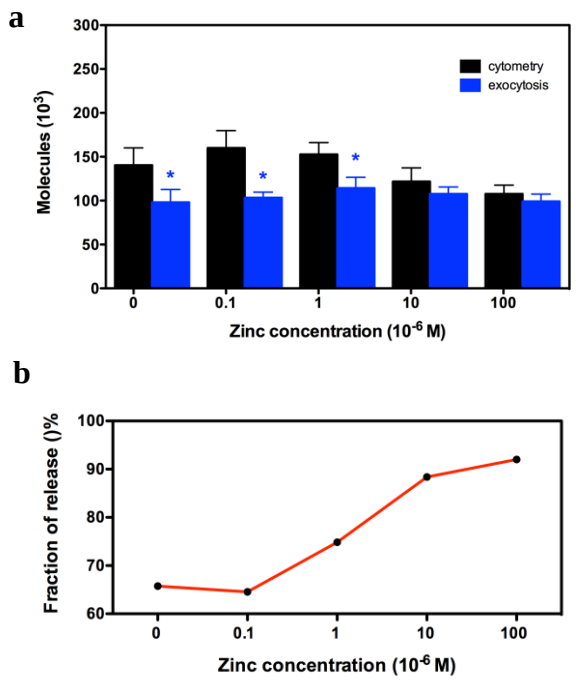

Figure 1. a) Average number of catecholamine molecules quantified per vesicle from intracellular impact cytometry [9] and for exocytotic release from single cell amperometry (blue) after different zinc concentrations (cytometry: controls, 16 cells; zinc treated 0.1 µM, 16 cells; 1.0 µM, 17 cells; 10.0 µM, 19 cells; 100.0 µM, 17 cells and single cell amperometry: controls, 17 cells; zinc treated 0.1 µM, 21 cells; 1.0 µM, 17 cells; 10.0 µM, 14 cells; 100.0 µM,18 cells); b) The fraction of release from cells treated with different zinc concentrations.

When we further compare the total vesicular dopamine content (as measured from intracellular cytometry) with the amount released (as measured from single cell amperometry), the data reveal that, on average, approximately 66 % of the dopamine in each vesicle is released during exocytosis in control cells, whereas the fraction of release increases after 0.1 µM zinc and appears to saturate at about 90% released at 10 and 100 µM zinc (Figure 1b). The hypothesis that exocytosis does not result in complete expulsion of the transmitter in a vesicle has been under investigation for decades. It appears that during exocytosis from a large dense core vesicle, the event does not result in full distention of the fused vesicle and thus rapid closure takes place prior to full release. This is consistent with studies where evidence suggests that in PC12 cells 97% of exocytosis events are followed by rapid endocytosis.[9] In partial release, only a fraction of transmitter molecules is released during exocytosis and vesicle content might, therefore, play an important role in regulating exocytosis and further contribute to synaptic strength.[10] The effect of zinc is very interesting as increased zinc concentrations lead to a much higher fraction of release. This is the first observation that the fraction of release can been dramatically increased from clear partial release to almost full release by a simple chemical treatment. For more insight, we investigated the dynamics of the exocytosis process both in control and zinc-treated cells (Figure S3). Both the width, t_{half}, and declining portion, t_{fall}, of the release peak are significantly changed with zinc treatment, leading to a slower exocytotic process. When an exocytotic event happens, a pore is formed between the vesicle and the plasma membrane through which the vesicle contents are released. The duration of pore opening and closing are apparently affected by zinc leading to a pore that is open longer and closes slower leading to the higher fraction released. What is so significant here is that the extent is so large, changing the events from clearly partial to nearly full.

To understand if zinc affects exocytosis in a reversible way, we employed a zinc chelator to post-treat the zinc-treated PC12 cells. A membrane permeable metal ion chelator, N, N, N' N',-tetrakis (2-pyridylmethyl) ethylenediamine (TPEN), chelates Zn^{2+} in the cytoplasm and other cellular compartments and can strip Zn^{2+} from intracellular proteins. [11] It has been reported that TPEN can prevent overloaded zinc induced neurotoxicity while long time (usually more than 24 hours) exposure to TPEN might be also toxic to cells and cause cell apoptosis. Single cell amperometry and intracellular impact vesicle cytometry were carried out (data traces not shown) and the numbers of catecholamine molecules measured from both techniques are plotted as normalized frequency histograms in Figure 2a. The vesicle content measured from cytometry is again significantly depleted after zinc treatment (blue). Interestingly, the change in vesicle content caused by zinc is reversed by TPEN (red). It has been reported that zinc can affect the gene and protein expression of the vesicular monoamine transporter (VMAT) and results in reduction of dopamine levels, while TPEN can reversibly bind to zinc to inhibit the effect. This contrasts with reserpine which irreversibly blocks the uptake and stock of dopamine by inhibiting VMAT. As shown in Figure S4, after treatment of the drug reserpine, the vesicle content from intracellular cytometry is markedly smaller than untreated cells, approximately 50% of the number, and exposure of the cells to medium with no drug for up to 12 h does not lead to a recovery of dopamine vesicular levels. Exocytotic release, in contrast, as measured from single cell amperometry, shows no significant difference between control, zinc treatment (blue) and TPEN post-treatment (red) (Figure 2a). This result seems to indicate that zinc changes both the vesicle content and exocytotic release in a reversible way which can be drawn back by a specific chelator such as TPEN.

When we investigated the dynamics of exocytosis we found that after TPEN post-treatment, the exocytosis times remain slower (Figure 2b), and the peak current is also smaller (Figure S5) compared to control. Hence, although vesicle content is reversibly changed by zinc, its effect on the exocytotic process is not. It is possible that zinc binds to proteins on the cell membrane to adjust the pore size and duration. Many exocytosis events have a pre-spike foot that represents the fusion pore opening and stopping before opening some more. By studying these pre-spike "feet" we found that after TPEN post-treatment, the dynamics of these feet are similar to those observed after zinc treatment alone (Figure S6). Zinc clearly causes an irreversible change in the cell membrane to change the membrane plasticity and pore size, and this change is not affected by post-treatment of TPEN.

The cell membrane has a complicated composition primarily of lipids and proteins. Although many of the proteins involved in exocytosis have been characterized and the role each plays in regulating exocytosis is becoming clearer, it is possible that membrane phospholipids also play a role in changing the exocytosis process. Understanding the effect of zinc on the lipid composition of the cell membrane might give us more insight into the chemical mechanism of how zinc regulate exocytosis and cell plasticity.

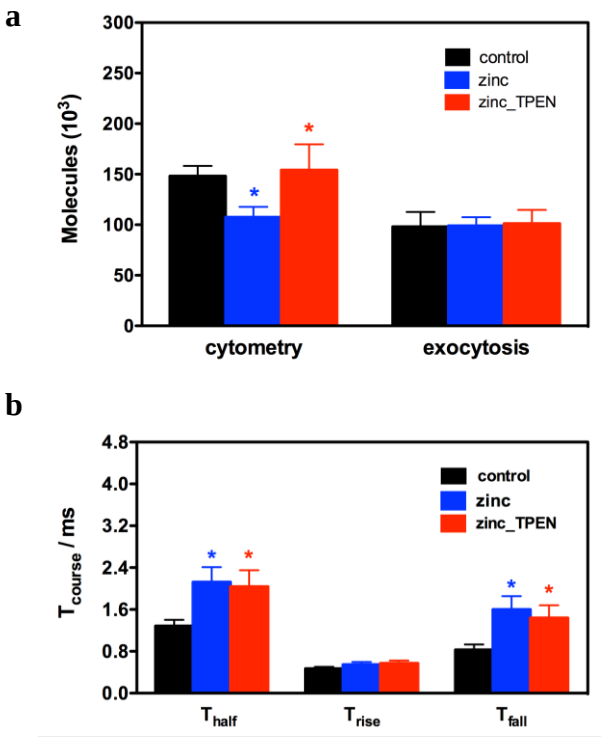

Figure 2. a) Number of molecules per event from single cell exocytosis and intracellular impact cytometry for control, with 100 µM zinc treatment for 3.5 hours and zinc treatment followed by 100 µM TPEN for 1.5 hours; b) Summary of plots for (a) T_{half}, (b) T_{rise} and (c) T_{fall} during exocytotic release measured from single cell amperometry for control, zinc treatment (blue) and TPEN post-treatment (red).

To investigate the distribution of lipid components of cell membrane and to determine how specific lipids are affected by zinc and also zinc-TPEN treatment, ToF-SIMS analysis was applied to PC12 cells treated similarly to those in the electrochemical experiments. The phospholipid signal for the phosphatidylcholine (PC) head group at *m/z* 184 was imaged across cell samples to identify cells in groups of control, zinc-treated and zinc-TPEN treated cells (Figure S7a, b and c, respectively). The phospholipid signals are clearly detected on the cell surface and the cells are healthy following this simple protocol.

To compare and to evaluate individual lipid changes in the lipid content of cell membranes, PCA analysis was performed on all normalized spectra obtained from ToF-SIMS analysis on 5 bunches of cells collected from different silicon wafers for all the samples. The PCA scores plot of the first principle component (t[1]) vs. the second principle component (t[2]) from the positive spectra (Figure S8a) clearly shows that control and zinc treated groups are well separated from each other. Most of this separation is obviously caused by fragments of phospholipids observed at *m/z* 166, *m/z* 184 and *m/z* 224 (Figure S8b). These PC species are increased in association with zinc treatment. Similarly, the scores plot of PCA analysis on negative spectra (Figure S8c) clearly indicates an apparent discrimination between the two groups of control and zinc treated cells. We find that the peaks primarily responsible for the differentiation of the groups (Figure S8d) are fragments at *m/z* 140 and 180 (PE: phosphatidylethanolamine), *m/z* 153 (PI: phosphatidylinositol), *m/z* 255 (fatty acid: palmitic acid) and *m/z* 281 (fatty acid: oleic acid). Interestingly, these fragments are associated with changes between zinc-treated and control. Whereas, there is no clear separation observed between zinc-treated and zinc-TPEN-treated cells (Figure S8e-f). There is also no apparent difference between the control and zinc-TPEN treated cells (Figure S8g-h).

The lipid changes between the three groups of cells were compared with a Welch t-test. The highest loading mass peaks causing separation of the related PCA scores of ToF-SIMS data are found from the corresponding loading plots in both positive and negative modes (Figure S8b and d). The average values of these peaks were then analyzed semiquantitatively with a t-test ($p \leq 0.05$) (Figure 3). The fragments of lamellar-shaped PC lipids significantly increase in the cell membrane, whereas the fragments from high curvature lipid such as PE, PI are reduced in the cell membrane after zinc treatment. Also, fatty acid species (palmitic acid) signals decrease, whereas cholesterol species significantly increase in zinc treated cells. In contrast, there are no significant changes in any lipid species when comparing zinc and zinc-TPEN treated cells. We also checked the lipid distribution between untreated cells and TPEN treated cells, finding no significant difference between these two groups (Figure S9). Most of the lipid species are significantly changed between control and zinc-TPEN treated cells (Table S1), but not TPEN alone. The lipid composition of the cellular membrane is changed by zinc, and these effects are not reversibly changed by chelating zinc after incubation.

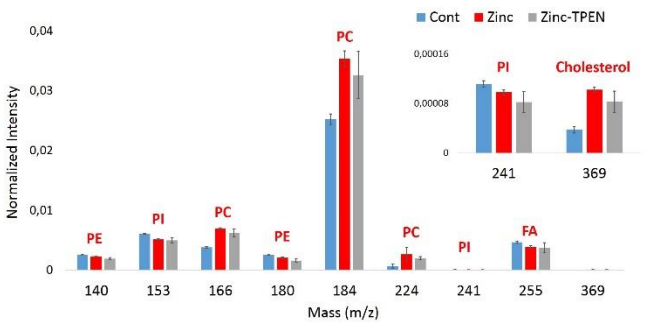

Figure 3. Normalized intensity for negative ions at *m/z* 140 and 180 (PE: phosphatidylethanolamine), *m/z* 153 and 241 (PI: phosphatidylinositol), *m/z* 255 (FA: palmitic acid) and for positive ions at *m/z* 166 (PC fragment), *m/z* 184 (PC: phosphatidylcholine), *m/z* 224 (PC fragment) and *m/z* 369 (cholesterol) in control (blue), zinc treated (red) and zinc-TPEN treated cells (gray). The bar heights show the average value and the error bars show 95% confidence level for each lipid peak in 5 different bunches of the cells in each control, zinc incubated and zinc-TPEN treated samples.

For exocytosis, a simple view of the molecular rearrangements within the two bilayers during membrane fusion is based on the shape model originally proposed in the 1970s.[12] At sites of fusion, the bilayer arrangement of lipids must undergo distortion, and nonbilayer intermediates, facilitated by lipids with noncylindrical shapes. This requires structural reorganization of the membrane lipids to accommodate the high curvature region of the fusion pore. The most abundant of the membrane lipids are the phospholipids, which are polar, ionic, amphipathic. Cholesterol is another major component of cell membranes. Lipid molecules of different kinds can be seen as the

elements of a mosaic, with various shapes similar to cylinders, cones or inverted cones, to contribute to the curvature and bending for membrane dynamics. Membrane composition is an important point of regulation for exocytosis and such observations lend credence to the view that lipids, the major component of membranes, might play some kind of regulatory role in exocytosis.

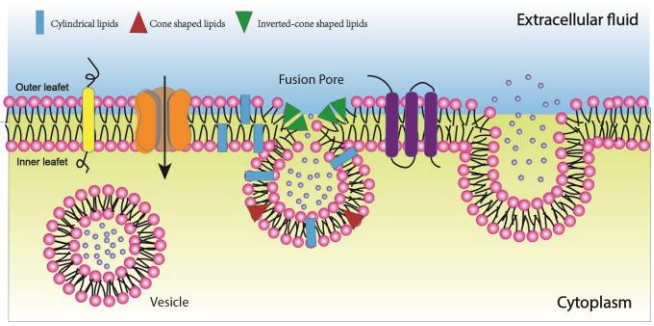

Figure 4. Mechanistic illustration of a model of the fusion pore formation and stabilization during exocytosis.

One hypothesis of how phospholipid species influence exocytosis is that lipid composition contributes to membrane curvature and dynamic cell membrane remodeling during the formation of the exocytotic fusion pore (Figure 4). The chemical properties of different lipid acyl chains or headgroups (length and saturation) can favor different membrane curvatures: for example phosphatidylcholine (PC) and phosphatidylserine are cylindrical lipids that form a flat monolayer; lipids such as phosphatidylethanolamine (PE), phosphatic acid, diacylglycerol or cardiolipin, which have a smaller polar headgroup than that of PC, have a roughly conical shape and thus impose a negative curvature. Conversely, a large headgroup to acyl chain ratio, such as in lysophosphatidylcholine (LPC) or the large headgroups in phosphatidylinositol phosphates (PI) confers an inverted conical shape to favor the bending of the membrane into a positive curvature where the monolayer bends away from the headgroups. It has been reported that addition of different lipids; phosphatidylcholine (PC), phosphatidylethanolamine (PE), sphingomyelin, phosphatidylinositol (PI) and phosphatidylserine can alter exocytosis.[13] Amperometry data in those experiments revealed PC decreases quantal size while increasing vesicular volume and slowing the dynamics of exocytosis; PE led to faster individual release events; PS increased exocytosis frequency; SM slowed vesicle opening and PI had no effect on exocytosis. In our present work, amperometry shows that zinc changes the dynamics of exocytosis and vesicle contents suggesting it affects the formation and stability of fusion pore. We also find that PE and PI are significantly decreased in the membrane after zinc treatment while PC is increased. The mechanism by which zinc causes lipid composition and distribution changes is beyond the scope of this paper, and we focus on the lipidic mechanism (Figure 4). Based on the effects of lipid shape on membrane curvature, when the amount of PE and PI are decreased in the inner leaflet, it is more likely to form a smaller initial fusion pore by inducing less inward curvature during the merging between plasma membrane and vesicle membrane, which is consistent with our amperometric data. After formation of the initial fusion pore, lipid composition can further determine whether the pore expands or closes. Nanavati et al. found stable or irreversibly expanding pores require high spontaneous curvature which result in cylinders in artificial bilayer systems. Here, the increased level of membrane PC in the outer leaflet could support both irreversibly expanding pores and stably fluctuating pores without destabilizing the entire cell membrane. In addition, changes in cholesterol might play an important role by accumulation in high curvature regions to help to maintain the curvature bending and fusion pore stability, again consistent with the increase we observe with ToF SIMS after zinc. Thus, a mechanism of zinc affecting exocytosis is by changing lipid composition leading to a smaller but more stable fusion pore slowing the dynamics of exocytosis and neurotransmitter release.

Lipid composition changes following zinc might result in different protein-lipid interactions and phosphoinositide metabolism in membrane traffic. The SNARE proteins, identified as fusion proteins, have been well characterized in terms of their association with cholesterol-enriched microdomains in a range of cell types and regulate protein-lipid interactions in the early stage of exocytosis, docking and fusion.[14] Interestingly, cholesterol depletion inhibits fusion pore formation and membrane fusion by reorganizing the SNARE protein machinery.[15] We show that increased zinc elevates the level of cholesterol in the cell membrane possibly enhancing the concentration of SNARE proteins required for efficient fusion and thus leading to pores that are open longer and a greater fraction of transmitter released.

Previous studies have suggested that zinc might bind to proteins on the cell membrane to effect exocytosis.[16] Our evidence, comparing structural changes in the cell membrane observed with ToF-SIMS to dynamic observations with single cell amperometry and intracellular vesicle impact electrochemical cytometry, suggests that phospholipids and cholesterol play an important role in regulating fusion pore formation and stabilization. We suggest that zinc affects the chemical structure of the fusion pore and thus transmitter dynamics as part of its modulating synaptic strength and plasticity, or the initial stage of memory.

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Keywords: zinc • regulated exocytosis • vesicle content • amperometry • cytometry

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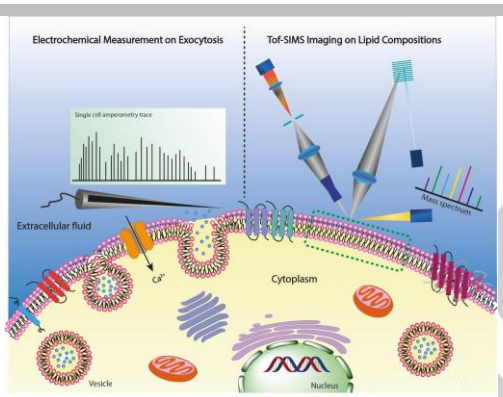
COMMUNICATION

We have employed single cell amperometry and intracellular impact vesicle cytometry to measure the effects of zinc on exocytosis and vesicle content in PC12 cells. We also used mass spectrometry imaging to see the changes in lipid distribution of cell membrane after zinc treatment. Our results show zinc alters lipid compositions which can be correlated to the changes in exocytosis.



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