A Sodium/Zinc Exchange Mechanism is Mediating Extrusion of Zinc in Mammalian Cells.

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Abstract

Zinc influx, driven by a steep inward electrochemical gradient, plays a fundamental role in zinc signaling and in pathophysiologies linked to intracellular accumulation of toxic zinc. Yet, the cellular transport mechanisms that actively generate or maintain the transmembrane gradients are not well understood. We monitored Na⁺ dependent Zn²⁺ transport in HEK293 cells and cortical neurons, using fluorescent imaging. Treatment of the HEK293 cells with CaPO₄ precipitates induced Na⁺ dependent Zn²⁺ extrusion, against a 500 fold transmembrane zinc gradient, or zinc influx upon reversal of Na⁺ gradient. Thus indicating that Na⁺/Zn²⁺ exchange is catalysing active Zn²⁺ transport. Depletion of intracellular ATP did not inhibit the Na⁺ dependent Zn²⁺ extrusion, consistent with a mechanism involving a secondary active transporter. Inhibitors of the Na⁺/Ca²⁺ exchanger failed to inhibit Na⁺-dependent Zn²⁺ efflux. In addition, zinc transport was unchanged in HEK293 cells heterologously expressing functional cardiac or neuronal Na⁺/Ca²⁺ exchangers. Thus indicating that the Na⁺/Zn²⁺ exchange activity is not mediated by the Na⁺/Ca²⁺ exchanger. Sodium-dependent zinc exchange, facilitating the removal of intracellular zinc, was also monitored in neurons. To our knowledge, the Na⁺/Zn²⁺ exchange activity described here is the first example of a mammalian transport mechanism capable of Na⁺ dependent active extrusion of zinc. Such mechanism is likely to play an important role, not only in generating the transmembrane zinc gradients, but also in protecting cells from the potentially toxic effects of permeation of this ion.
**Introduction**

Extracellular free zinc may reach micromolar concentrations in the brain, pancreatic islets and in seminal fluid (1). In contrast, free intracellular zinc ([Zn$^{2+}$])$_i$ concentrations range from picomolar to nanomolar. Such a steep electrochemical gradient (2,3), that may even exceed the transmembrane calcium gradient, has fundamental physiological and pathophysiological implications. For example, influx of zinc into hippocampal neurons has been suggested to play a role in the induction of LTP in CA3 neurons (4,5). In cardiac myocytes, zinc influx induces gene expression (6). Regulation of extracellular zinc may also be important for modulation of zinc signaling via the recently characterized zinc sensing receptor (7). Epileptic seizures or transient global ischemia typically result in the release of massive amounts of zinc into the synaptic cleft, a phenomenon that leads to increase of [Zn$^{2+}$]$_i$, and is considered to play a key role in neuronal cell death following these episodes (8,9). Similarly, zinc permeation into pancreatic β–cells leads to islet destruction occurring in streptozotocin induced diabetes (10).

The steep transmembrane zinc gradient, characterizing mammalian cells, must be maintained by an active mechanism that extrudes cellular zinc. Such a mechanism is also essential for protecting cells against excessive intracellular zinc. In plants and yeast, a H$^+$/Zn$^{2+}$ exchanger catalyses the active extrusion of zinc into vacuoles (11). Much less is known about such mechanisms in mammalian cells. Although proton/zinc exchange activity has been demonstrated in plasma membrane vesicles derived from brain cells (12), it seems unlikely that the small magnitude of the proton electrochemical gradient is sufficient to catalyze the formation of steep Zn$^{2+}$ gradients across the plasma membrane of mammalian cells. Reversal of Na$^+$/Ca$^{2+}$ activity has been suggested to be involved in the accumulation of zinc in neurons but not in the efflux of neuronal zinc (13). Furthermore, other studies have suggested that zinc...
allosterically inhibits cation transport mediated by the Na⁺/Ca²⁺ exchanger (14). The ZnT-1 is yet another protein suggested as a mediator of zinc efflux, and its expression was shown to confer resistance against zinc toxicity and to lower intracellular zinc (15,16). However, it is not clear if ZnT-1 is a transporter per-se, a regulator of zinc transport, or whether it catalyses zinc extrusion against a zinc transmembrane gradient (17). Thus, the identity of an active cellular zinc extrusion mechanism in mammalian tissues remains to be elucidated.

In the present report, we provide evidence that novel Na⁺-dependent Zn²⁺ exchange activity can be induced. Utilizing the transmembrane electrochemical Na⁺ gradient, this exchanger catalyses active extrusion of cellular zinc against an estimated 500-fold zinc gradient. Such a transporter could play a major role in the transport of zinc in tissues such as the brain, prostate and pancreas.

**Materials and Methods**

**Cell cultures and plasmid transfection**- HEK293 (human embryonic kidney cell line) were cultured in DMEM as described previously (18). Treatment with calcium phosphate (CaPO₄) precipitates was performed using the protocol for cell transfection, without plasmids, on a 20-30% confluent culture (19). The CaPO₄ treatment is identical to the classical transfection protocols and in agreement with pervious studies (18) the procedure did not result in apparent morphological changes indicative of acute stress. Transport experiments were carried out 30-48 hours following CaPO₄ treatment. Neuronal cultures were prepared as described previously (20,21).

**Expression of Na⁺/Ca²⁺ exchanger isoforms**- The role of the Na⁺/Ca²⁺ exchanger in zinc transport was assessed in HEK293 cells, heterologously expressing the Na⁺/Ca²⁺ exchanger.
The cells were transfected with the Na\(^+\)/Ca\(^{2+}\) neuronal or cardiac isoform (22) (kindly provided by Dr. Hannah Rahamimoff) plasmids using CaPO\(_4\) precipitation or Fugene-6 (Roche, Switzerland). Rates of Ca\(^{2+}\) transport in cells transfected by either of the methods were similar.

Semiquantitative RT-PCR Analysis- Total RNA was isolated from CaPO\(_4\) treated and control HEK293 cells using Trizol reagent (Invitrogen). The cDNA was synthesized with 0.125, 0.5 and 2 µg of total RNA using reverse transcriptase and oligo (dT) primers (Ez-First Strand kit, Biological Industries, Beit Haemek, Israel). Fragments of hZnt1, hNCX1 and glyceraldehyde-3-phosphate dehydrogenase were amplified from equal volumes of cDNA templates using 21, 24 and 25 cycles respectively. The hZnt1 amplification primers used were 5'-AATACCA GCAACTCCAACGG-3' and 5'-ATTGCTTCAAACGGCACAAGCTGGG-3', the hNCX1 primers used were 5'- TCTGCGGATAATGGTAAGGG-3' and 5'-CCATAGGAGCACAAAG AGGC-3' and the glyceraldehyde-3-phosphate dehydrogenase primers used were 5'-CCACCATGGAGAA GCTGGGGGCTC-3' and 5'-AGTGATGGCATGGAACACTGTG-TCAT-3'.

Fluorescent Imaging of ion transport- The imaging system consisted of a Zeiss Axiovert 100 (Zeiss, Germany) inverted microscope, Polychrome II monochromator (T.I.L.L. Photonics, Germany) and a cooled CCD camera (PCO, Germany) as previously described (7). Fluorescent imaging measurements were acquired using Axon Imaging Workbench 2 (Axon Instruments, USA). HEK293 cells grown on cover slides were incubated for 30 minutes with 5 µM of Fura-2 AM (TEF - Lab, USA) in 0.1% BSA in sodium Ringer’s solution. Following dye loading, the cells were washed in sodium Ringer’s solution and the cover slides mounted in a chamber that allowed perfusion of cells. Fura-2 was excited at 340 nm and 380 nm wavelength light and imaged with a 510 nm long pass filter (7). [Zn\(^{2+}\)], was calibrated using the zinc ionophore, pyrithione, and the zinc chelator TPEN as previously described (23). For sodium loading HEK293 cells were treated essentially as previously described (24). Briefly, 100 µM ouabain
(Sigma) was added to the cells for 4-5 hours, intracellular Na⁺ reached 30-40 mM following the incubation as calibrated in cells loaded with 10µM SBFI (TefLabs, USA). Zinc imaging in neurons was performed in cultured cortical neurons loaded with Newport Green (5µM, 60 min at 25°C). Coverslips were mounted on the stage of an inverted Nikon microscope as previously described (21). Newport Green, excitation was at 490 nm, emission at 530 nm, and calibrated zinc values were calculated as described (13,21). Similarly to the HEK293 experiments, Na⁺ free solutions were made by iso-osmotically replacing Na⁺ with N-Methyl-Glucamine (NMG). The results shown are the means of 4-6 independent experiments, with averaged responses from 30-50 cells in each experiment.

**Assays for zinc and calcium transport.**

The zinc influx assay aimed to study the reversibility of the exchanger was performed by perfusing the cells with a Na⁺-free (NMG) Ringer’s containing 400 µM zinc (reversal medium). Active zinc efflux was monitored by first loading the cells with zinc by perfusion with the reversal medium reaching a steady state at about 80 nM of [Zn²⁺]ᵢ. Cells were then perfused with Na⁺ Ringer’s containing 50 µM zinc. Zinc transport mediated by the Na⁺/Ca²⁺ exchangers was monitored using the zinc influx assay (using reversal medium) in cells heterologously expressing the cardiac or the neuronal isoform of this exchanger. When [Zn²⁺]ᵢ reached a steady state the perfusing solution was switched to Na⁺ Ringer’s solution containing the intracellular zinc chelator, TPEN (50µM), for 30-60 seconds and then TPEN was removed by washing the cells with Na⁺ Ringer’s solution. Rates of calcium transport were determined while perfusing the cells with Na⁺-free Ringer’s solution. Cells were depleted of ATP, by incubation in Ringer’s solution containing 10mM KCN and 5 mM deoxy-glucose for 10 min as previously
described (25). Zinc transport was monitored immediately following the ATP depletion using Ringer’s solution containing deoxy-glucose instead of glucose.

**Results**

During our studies of zinc transporters heterologously expressed in HEK293 cells, we also monitored zinc transport in mock-transfected cells. Massive zinc influx was observed in cells (Figure 1) perfused with Na\(^+\)-free Ringer’s solution containing 400 \(\mu\)M zinc, that were pre-treated with CaPO\(_4\) precipitate according to a standard transfection protocol. Such zinc influx was not observed in cells treated with Fugene-6, nor did it require the presence of plasmid, indicating that the CaPO\(_4\) treatment induced zinc transport activity. Zinc transport was monitored using Fura-2, which has 100 fold greater affinity to zinc than to calcium (6,26). Nevertheless, to confirm that the increase in Fura-2 fluorescence shown in figure 1 is indeed related to the rise in \([Zn^{2+}]_i\), we performed the following experiment (Fig 2): Fura-2 loaded cells were initially perfused with Na\(^+\)-free Ringer’s. Addition of extracellular zinc (200 \(\mu\)M) resulted in an increase of Fura-2 fluorescence, to about 80 nM \([Zn^{2+}]_i\). Subsequent application of the membrane-permeable heavy metal chelator, TPEN, resulted in reduction of fluorescence to background level, indicating that the observed changes in fluorescence were related to changes in \([Zn^{2+}]_i\). Taken together, our results indicate that treatment of the HEK293 cells with CaPO\(_4\) precipitates, induced the acceleration of zinc influx.

**Active zinc transport is catalysed by a Na\(^+\)-dependent ion exchange mechanism**

The increase in Zn\(^{2+}\) influx in the absence of Na\(^+\) shown in figure 1 may be related to reversal activity of a putative Na\(^+\)/ Zn\(^{2+}\) exchanger triggered by CaPO\(_4\) precipitates. To test this hypothesis, treated cells were perfused with Ringer’s solution supplemented with 400 \(\mu\)M zinc,
containing either Na\(^+\) or NMG. As shown in Figure 3, zinc influx in the absence of Na\(^+\) (0.18±0.03 nM/s) was accelerated by about 6 fold compared to influx rate in the presence of Na\(^+\) (0.03±0.01 nM/s). If a sodium-dependent zinc exchange mechanism is indeed responsible for the zinc influx, then in the presence of extracellular Na\(^+\), acceleration of active zinc efflux would be expected. To address this question, cells were first loaded with zinc using Na\(^+\)-free Ringer’s solution, as described in Fig 1, reaching a steady state of 60-80 nM [Zn]. The rate of zinc efflux against its gradient was then monitored in the absence or presence of Na\(^+\). As shown in Fig 4a, Na\(^+\) accelerated the rate of zinc efflux by about 5 fold compared to the efflux rate from zinc loaded cells that were washed with the Na\(^+\) free Ringer’s solution. Thus, our results indicate that a Na\(^+\) dependent zinc efflux is catalysed in HEK293 cells. Active zinc efflux such as shown above, may involve either a primary or a secondary active mechanism. To distinguish between the two mechanisms, the rate of zinc efflux was determined in ATP depleted HEK293 cells (25). As summarized in Fig 4b, rates of zinc influx catalysed by the reversal of activity of the exchanger were similar in the control and the ATP-depleted cells. Rates of active zinc efflux were even slightly (10%) higher in ATP-depleted cells, indicating that the active zinc efflux is not catalysed by a primary pump. Furthermore, because the sequestration of zinc into the mitochondria is totally dependent on intact trans-mitochondrial membrane gradient (27,28), the insensitivity of the Na\(^+\)/Zn\(^{2+}\) exchange activity to ATP depletion, using cyanide, indicates that the reduction in intracellular zinc does not involve sequestration of zinc into this organelle. Taken together, the Na\(^+\)-dependence of Zn\(^{2+}\) efflux and influx (Fig 3-4), indicates that a secondary, active, reversible Na\(^+\)/Zn\(^{2+}\) exchanger catalyses efflux of zinc against an estimated gradient of 500 fold.
**Sodium and zinc dependence of the Na\(^+\)/Zn\(^{2+}\) exchange activity**

The apparent affinity of the external site for zinc was determined by dose response analysis (Figure 5a), zinc influx was mediated by the reversal of the exchange activity (see fig 1) while changing extracellular zinc concentration. Fitting the experimental data yielded a Hill coefficient for Zn\(^{2+}\) of 1.4±0.5 and an apparent Km for extracellular zinc of 50±8 µM. The dose response for the Na\(^+\)-dependent Zn\(^{2+}\) efflux was determined by monitoring zinc efflux while varying extracellular Na\(^+\), iso-osmotically replaced by NMG. As shown in Fig 5b, the dose response profile of Na\(^+\)-dependent zinc efflux was sigmoidal, yielding an apparent Km for sodium of 96±4 mM, with an estimated Hill coefficient of 3.5±0.3. To further analyze the coupling between zinc and sodium, we analyzed the sodium dependence of zinc influx with the exchanger operating in the reverse mode. To maximize the sodium gradient cells were loaded with Na\(^+\) using the Na\(^+\)/K\(^+\)ATPase inhibitor, ouabain (100 µM) (see methods) and rates of zinc influx were determined in cells perfused with Ringer’s solution containing various concentrations of Na\(^+\) (Fig 5c). Increasing extracellular Na\(^+\) concentrations resulted in a reduction of zinc influx rate (Hill coefficient of 2.5±0.5 and apparent Km of 41±5 mM). These results further indicate that zinc transport is coupled to Na\(^+\) gradients.

**Zinc transport is not mediated by the Na\(^+\)/Ca\(^{2+}\) exchanger.**

The Na\(^+\)/Ca\(^{2+}\) exchanger is abundantly expressed in brain regions where intense zinc homeostasis activity occurs (29,30), and upon reversal of the sodium gradient, has been suggested to accelerate zinc influx into cortical neurons (13). In contrast, other studies indicated that zinc, allosterically inhibits neuronal Na\(^+\)/Ca\(^{2+}\) exchange activity (14). Thus, the ability of the Na\(^+\)/Ca\(^{2+}\) exchanger to catalyse zinc transport remains controversial. We have, therefore, assessed the potential role of the Na\(^+\)/Ca\(^{2+}\) exchanger in catalysing zinc transport.
Brain and cardiac isoforms of the Na\(^+\)/Ca\(^{2+}\) exchanger (kindly provided by Prof. Hannah Rahamimoff from The Hebrew University, Israel) were heterologously expressed in HEK293 cells as described above. Cells expressing the various isoforms were loaded with Fura-2 and the rate of zinc influx was first determined. TPEN (50 µM), was used to verify that the fluorescent signal resulted from changes in \([Zn^{2+}]_i\), and to lower it to resting level. Subsequently, the rate of Ca\(^{2+}\) transport was determined by perfusing the cells with Na\(^+\)-free Ringer’s solution in which zinc was replaced by Ca\(^{2+}\) (see Fig 6a). Figure 6b shows a Na\(^+\)-dependent increase in Ca\(^{2+}\) but not Zn\(^{2+}\) transport in cells expressing either the cardiac or neuronal isoforms of the Na\(^+\)/Ca\(^{2+}\) exchanger. Thus, our results indicate that neither of these Na\(^+\)/Ca\(^{2+}\) exchanger isoforms is involved in zinc transport, suggesting that Na\(^+\)/Zn\(^{2+}\) exchange is catalysed by a distinct exchanger.

We further analyzed changes in the Na\(^+\)/Ca\(^{2+}\) exchanger (NCX1) mRNA levels, using RT-PCR, in HEK293 cells treated with CaPO\(_4\) precipitate versus control. No change in mRNA was demonstrated for NCX1 following the CaPO\(_4\) treatment (Fig 6c). Apart from the Na\(^+\)/Ca\(^{2+}\) exchanger, the ZnT-1 transporter has been suggested to catalyze active zinc efflux in mammalian cells (16). We therefore analyzed changes in ZnT-1 mRNA levels, using RT-PCR, and did not see any changes either (Fig 6c). In another study we found that expression of ZnT-1 does not lead to an increase in active zinc efflux in HEK293 cells (Segal et al. in preparation). Thus, while CaPO\(_4\) induces Na\(^+\)/Zn\(^{2+}\) exchange, it had no effect on ZnT-1 or NCX1 expression, indicating that these proteins are unlikely to mediate Na\(^+\)/Zn\(^{2+}\) exchange.

**Sensitivity of the Na\(^+\)/Zn\(^{2+}\) exchange activity to inhibitors**

The use of inhibitors is an important tool for the functional identification and characterization of an exchanger. We have, therefore, studied the sensitivity of the Na\(^+\)/Zn\(^{2+}\) exchange activity to various inhibitors of cation transporters (Fig. 7a). As shown in Fig 7b, application of KB-
R7943, an inhibitor of the Na⁺/Ca²⁺ exchanger, failed to inhibit Na⁺/Zn²⁺ exchange, further supporting our assertion that it is distinct from the Na⁺/Ca²⁺ exchanger. The Na⁺/Zn²⁺ exchange was inhibited by Ni 4mM (Fig 7a,b) an inhibitor of sodium and calcium channels as well as the Na⁺/Ca²⁺ exchanger, or 150 µM verapamil, a non-selective calcium channel blocker. In contrast, treatment with the dihydropyridine nifedipine (another calcium channel blocker) or EIPA (the amiloride analogue and Na⁺/H⁺ exchanger inhibitor) failed to block Na⁺/Zn²⁺ exchanger activity (data not shown).

**Presence of Na⁺/Zn²⁺ exchange activity in cultured neurons**

In neurons, prolonged rise of intracellular free zinc is injurious (5,31) and it is, therefore, critical for these cells to achieve rapid clearance of toxic cytosolic zinc loads. Previous studies suggested a possible role for the Na⁺/Ca²⁺ exchanger as a pathway for Zn²⁺ extrusion in cortical neuronal cultures (13). However, the above results in HEK293 cells suggest a Na⁺ dependent Zn²⁺ exchanger distinct from the Na⁺/Ca²⁺ exchanger. We therefore studied the role of a Na⁺/Zn²⁺ exchanger as a pathway for transmembrane zinc movement in neuronal cells. Zinc fluxes in cortical neurons were determined using Newport green. This dye, while specific, has a lower affinity for zinc, making it suitable for monitoring the large zinc fluxes encountered in neurons (2). To monitor zinc transport, neurons were depolarized using high K⁺ (60mM) in Na⁺-free buffer (sodium replaced by NMG) containing 50µM zinc. Depolarization of the neurons triggered rapid influx of zinc via L-Type Calcium Channels (LTCC) (2,13). The intracellular zinc level remained high and fairly stable as long as the neurons were perfused with Na⁺-free buffer (Fig 8a). When NMG-containing solution was replaced with Na⁺ a steep, ~10 fold, increase in the rate of zinc efflux was monitored. To determine if this Na⁺/Zn²⁺ exchange could attenuate LTCC-mediated intracellular Zn²⁺ accumulation, we monitored zinc transport, under external physiological sodium and zinc concentrations (32). As shown in Fig
8a, when cells were depolarized in the presence of Na⁺ (instead of NMG Ringer’s solution) LTCC-related zinc accumulation decreased and the subsequent rate of zinc efflux increased (calculated rates are shown in Fig 8c). These results indicate that Na⁺/Zn²⁺ exchange is indeed playing a physiological role in reducing the accumulation of intracellular zinc concentration in neurons. Application of the Na⁺/Ca²⁺ exchanger inhibitor, KB-R7943, did not attenuate the effect of external Na⁺ on intracellular accumulation or the subsequent zinc efflux (Fig 8b-c), indicating that the Na⁺/Zn²⁺ exchange in neurons is not mediated by the Na⁺/Ca²⁺ exchanger.

**Discussion**

Transmembrane zinc gradients play fundamental physiological and pathophysiological roles in mammalian cells (33), but the mechanisms responsible for the formation of these gradients and particularly the transporters involved are unknown. We now provide evidence for Na⁺/Zn²⁺ exchange activity which utilizes the electrochemical Na⁺ gradient, to catalyse the active extrusion of intracellular zinc. To our knowledge, this is the first demonstration of an active zinc extrusion mechanism in mammalian cells. Activity of Na⁺/Zn²⁺ exchange is demonstrated by the Na⁺-dependent extrusion of zinc against a 500-fold zinc gradient. The similar Zn²⁺ efflux observed in cells that were depleted of ATP argues against a mechanism involving an active ion pump. Lowering of intracellular Zn²⁺ may also be catalyzed by sequestration into the main storing organelle for zinc in the cell, the mitochondria (28,34). Such sequestration of zinc should be inhibited following the collapse of the mitochondrial electrochemical gradient. Rate of intracellular Zn²⁺ reduction, however, did not change following the cyanide treatment (at concentrations sufficient to collapse the mitochondrial trans-membrane gradient) thus indicating that there is no zinc sequestration under these conditions. Another important
hallmark of an exchanger is demonstrated by the reversibility of the Na⁺/Zn²⁺ exchange activity upon the reversal of the Na⁺ gradient. The relatively low rates of the Na⁺/Zn²⁺ exchange that we monitor (<nM/s) may preclude direct fluorescent and radioisotopic measurement of Na⁺ transport because of the relatively low affinity and quantum efficiency of Na⁺-sensitive fluorescent dyes (35) and the relatively high activity of endogenous transporters, e.g. Na⁺/K⁺ATPase, that are capable of catalysing cytoplasmic Na⁺ transport at millimolar rates (36). Therefore the present study on the rate of changes of intracellular zinc concentration strongly indicates that in HEK293 cells the reduction in Zn²⁺ is mediated by a secondary trans-membrane transporter but not by sequestration.

We have previously suggested that the Na⁺/Ca²⁺ exchanger might be involved in zinc transport (13). These findings were based on the use of a general cation transport inhibitor and the demonstration of electrogenic Na⁺ dependent zinc transport. We now demonstrate that the Na⁺/Zn²⁺ exchange activity is distinct from the known Na⁺/Ca²⁺ exchangers. This is based on our findings that heterologous expression of the neuronal or cardiac isoforms of the exchanger increased the influx rates of Ca²⁺ but failed to accelerate the rate of Zn²⁺ transport. In addition, the Na⁺/Ca²⁺ exchanger inhibitor, KB-R7943, as well as other inhibitors of this exchanger, failed to reduce Na⁺/Zn²⁺ exchange activity in HEK293 and in cultured neurons, further supporting the notion that they are different proteins. Notwithstanding the distinction between the two exchangers, the Na⁺/Zn²⁺ exchange activity shares several features with the Na⁺/Ca²⁺ exchanger. For example, both catalyze an exchange of divalent ions with Na⁺, utilizing a similar electrogenic mode of transport. Therefore, the Na⁺/Zn²⁺ exchanger may be a novel member of the Na⁺/Ca²⁺ exchanger family. Indeed, the plant H⁺/Mg²⁺/Zn²⁺ exchanger does not catalyze H⁺/Ca²⁺ exchange, although it shares 30% homology with the mammalian Na⁺/Ca²⁺ exchanger (37).
The effect of CaPO$_4$ precipitates on the induction of proteins is well documented in many cell types and numerous papers (38-40). Application of these precipitates has a profound effect, activating the MAP kinase pathway, proto-onconges such as c-fos and c-jun and the nuclear transcription factor CREB (41,42). All these proteins are partially responsible for induction of gene expression and therefore may play a role also in the induction of the gene involved in Na$^+$/Zn$^{2+}$ exchange activity (43). Interestingly, CaPO$_4$ treatment is also known to induce the expression of zinc-dependent metalloproteinases (42,44). Regardless of the induction mechanism, this pre-treatment will be an invaluable tool for both the functional screening and the cloning of the Na$^+$/Zn$^{2+}$ exchanger gene.

The ability of the Na$^+$/Zn$^{2+}$ exchange activity to catalyze zinc efflux against a 500-fold transmembrane gradient may have important physiological implications. The rapid and active extrusion of zinc such as that catalyzed by the exchanger is of particular relevance for neurons due to their exposure to high extracellular zinc concentrations and their susceptibility to zinc toxicity following short exposure (8,9,45). Our results indicate that the Na$^+$/Zn$^{2+}$ exchange in neurons effectively attenuates zinc accumulation triggered following opening of the LTCC, by significantly accelerates the rate of zinc efflux. Hence, this transporter is capable of playing a key role in protecting brain cells against toxic accumulation of zinc, encountered during excitotoxic syndromes such as ischemia and epilepsy. The Na$^+$/Zn$^{2+}$ exchange activity may also play a role in resting intracellular zinc homeostasis, thus in regulating LTP, which in the CA3 region of the hippocampus is linked to intracellular zinc concentrations (4). Ion exchangers participate in the vectorial transport across epithelia (30). It will, therefore, be interesting to determine the activity and expression of the Na$^+$/Zn$^{2+}$ exchanger in tissues such as prostate, where the rate of vectorial zinc transport is particularly high (46). The physiological importance of zinc gradients and the pathophysiological implications of its collapse are well
recognized (5). Hence, future study of this novel, secondary active mammalian zinc transport activity is expected to yield new insight on the homeostasis of this ion.

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References:

1. Vogt K., Mellor J., Tong G., and Nicoll R. (2000) Neuron 26, 187-196.
2. Canzoniero L. M., Sensi S. L., and Choi D. W. (1997) Neurobiol Dis 4, 275-279.
3. Outten C. E., and O'Halloran T. V. (2001) Science 292, 2488-2492.
4. Li Y., Hough C. J., Frederickson C. J., and Sarvey J. M. (2001) J Neurosci 21, 8015-8025.
5. Choi D. W., and Koh J. Y. (1998) Annu Rev Neurosci 21, 347-375.
6. Atar D., Backx P. H., Appel M. M., Gao W. D., and Marban E. (1995) J Biol-Chem 270, 2473-2477.
7. Hershfinkel M., N.Grossman, Moran A., and Sekler I. (2001) PNAS 98, 11749.
8. Suh S. W., Chen J. W., Motamedi M., Bell B., Listiak K., Pons N. F., Danscher G., and Frederickson C. J. (2000) Brain Res 852, 268-273.
9. Kim B. J., Kim Y. H., Kim S., Kim J. W., Koh J. Y., Oh S. H., Lee M. K., Kim K. W., and Lee M. S. (2000) Diabetes 49, 367-372.
10. Palmiter R. D., and Findley S. D. (1995) EMBO-J 14, 639-649.
11. Kasir J., Ren X., Furman I., and Rahamimoff H. (1999) J Biol Chem 274, 24873-24880.
12. Gryniewicz G., Poinie M., and Tsien R. Y. (1985) J. Biol. Chem. 260, 3440-3450.
13. Gutser U. T., and Gleitz J. (1998) Neuropharmacology 37, 1139-1146.
14. Wang Y. H., Li F., Schwartz J. H., Flint P. J., and Borkan S. C. (2001) Am J Physiol Cell Physiol 281, C1667-1675.
34. Sensi S. L., Ton-That D., Weiss J. H., Rothe A., and Gee K. R. (2003) *Cell Calcium* **34**, 281-284.
35. Diarra A., Sheldon C., and Church J. (2001) *Am J Physiol Cell Physiol* **280**, C1623-1633.
36. Despa S., Islam M. A., Pogwizd S. M., and Bers D. M. (2002) *J Physiol* **539**, 133-143.
37. Shaul O., Hilgemann D. W., de-Almeida-Engler J., Van Montagu M., Inz D., and Galili G. (1999) *Embo J* **18**, 3973-3980.
38. McCarthy G. M., Westfall P. R., Masuda I., Christopherson P. A., Cheung H. S., and Mitchell P. G. (2001) *Ann Rheum Dis* **60**, 399-406.
39. McCarthy G. M., Augustine J. A., Baldwin A. S., Christopherson P. A., Cheung H. S., Westfall P. R., and Scheinman R. I. (1998) *J Biol Chem* **273**, 35161-35169.
40. Bai G., Howell D. S., Howard G. A., Roos B. A., and Cheung H. S. (2001) *Osteoarthritis Cartilage* **9**, 416-422.
41. Zeng X. R., Sun Y., Wenger L., and Cheung H. S. (2003) *Cells Tissues Organs* **174**, 63-72.
42. Reuben P. M., Brogley M. A., Sun Y., and Cheung H. S. (2002) *J Biol Chem* **277**, 15190-15198.
43. Cheung H. S., Sallis J. D., and Struve J. A. (1996) *Biochim Biophys Acta* **1315**, 105-111.
44. Sun Y., Wenger L., Brinckerhoff C. E., Misra R. R., and Cheung H. S. (2002) *J Biol Chem* **277**, 1544-1552.
45. Sheline C. T., Behrens M. M., and Choi D. W. (2000) *J Neurosci* **20**, 3139-3146.
46. Costello L. C., Liu Y., Zou J., and Franklin R. B. (1999) *J Biol Chem* **274**, 17499-17504.
Figure legends

**Fig 1: Induction of zinc transport in cells treated with CaPO₄.** HEK293 cells treated with CaPO₄ precipitation or Fugene-6 for 36 hours, then loaded with Fura-2, and perfused with Na⁺-free Ringer’s solution containing 400µM zinc. The results show that CaPO₄ but not Fugene-6, treatment induce zinc transport. Data are the mean±SE of at least four experiments with about 30 cells sampled in each slide.

**Fig 2: Changes in Fura-2 fluorescence are related to intracellular zinc level.** HEK293 cells treated with CaPO₄ precipitates and loaded with Fura-2, were perfused with 200µM zinc in sodium-free solution. Following the fluorescent increase to steady state, TPEN (50µM) a membrane permeable zinc chelator was added. The addition of TPEN lead to a decrease in the fluorescent signal back to a baseline level, indicating that changes in Fura-2 fluorescence reflect [Zn]ᵢ changes.

**Figure 3: Zn²⁺ influx is accelerated in the absence of Na⁺.** HEK293 cells treated with CaPO₄ precipitation were perfused with Ringer’s solution containing zinc (400 µM) with or without sodium (NMG substitute for sodium in the sodium free Ringer's solution). Zinc influx is accelerated by about 6 fold in the absence of Na⁺ (0.18±0.03 nM/s and 0.03±0.01 nM/s for NMG and Na⁺ Ringer's solution, respectively).

**Figure 4: Active Zn²⁺ extrusion in HEK293 cells is Na⁺ dependent, mediated by a secondary active transport.** a. HEK293 cells were loaded with Zn²⁺ as described in fig 1. When [Zn]ᵢ reached a steady state, cells were perfused with Na⁺-containing or Na⁺-free Ringer’s solutions containing 50 µM zinc, and changes in intracellular zinc were monitored. The Na⁺-dependent zinc
efflux, against its electrochemical gradient, indicates that the transport is mediated by Na\(^+\)/Zn\(^{2+}\) exchange. (0.09±0.01 nM/s and 0.44±0.03 nM/s for NMG and Na\(^+\) Ringer's, respectively).

b. The effect of ATP depletion on the initial rate of zinc influx (via the exchanger in reverse mode) and efflux (80 nM intracellular zinc against 50\(\mu\)m extracellular zinc). Initial rates of zinc transport did not decrease following ATP depletion indicating that the transport is not mediated by a primary pump.

**Fig 5: Dose response of the Na\(^+\)/Zn\(^{2+}\) exchanger for Na\(^+\) and Zn\(^{2+}\).** a. Rates of zinc influx, monitored in a Na\(^+\)-free buffer, were plotted versus external [Zn\(^{2+}\)]. Data were fitted to a Michaelis-Menten equation, yielding a Hill coefficient of 1.4±0.3 and an apparent Km of 50±8\(\mu\)M zinc. b. Active efflux of zinc, from zinc loaded cells was plotted versus external sodium concentration. The fitted line yielded a Hill coefficient of 3.5±0.5 and an apparent Km of 96±4 mM sodium. c. Zinc influx, into cells loaded with Na\(^+\) (30-40 mM), was determined at various extracellular Na\(^+\) concentrations. The fitted line yielded a Hill coefficient of 2.5±0.5 and an apparent Km of 41±5 mM sodium.

**Fig 6: Zinc transport is not mediated by the Na\(^+\)/Ca\(^{2+}\) exchanger.** Rates of zinc and calcium influx were monitored in HEK293 cells heterologously expressing the cardiac or neuronal isoforms of the Na\(^+\)/Ca\(^{2+}\) exchanger. a. The experimental paradigm. Cells were perfused with Na\(^+\)-free Ringer’s solution and zinc (400\(\mu\)M) was added, following application of TPEN, cells were then perfused with Ca\(^{2+}\) Ringer’s solution without zinc (see methods). b. The initial rates of Zn\(^{2+}\) and Ca\(^{2+}\) transport in cells expressing the brain and cardiac isoforms vs. control cells (averaged for both isoforms that were not significantly different). The results show that expression of the Na\(^+\)/Ca\(^{2+}\) exchanger increased the rate of Ca\(^{2+}\) transport as expected, but had no significant effect
on rates of zinc transport, thus indicating that the activities of the Na\(^+\)/Zn\(^{2+}\) and the Na\(^+\)/Ca\(^{2+}\) are distinct. c. Semiquantitative RT PCR analysis of ZnT-1 and NCX1 mRNA levels in cells treated with CaPO\(_4\) precipitates versus control cells, Glycerol-3-phosphate dehydrogenase (G3PD) served as a control. No significant change was observed in mRNA levels, indicating that changes in the expression pattern of these putative zinc transporters do not account for the CaPO\(_4\)-dependent changes in Na\(^+\)/Zn\(^{2+}\) exchange.

**Fig 7: Inhibition of Na\(^+\)/Zn\(^{2+}\) exchange in HEK-293 cells.** a. An example of the experimental paradigm used to determine the inhibitor sensitivity on Zn\(^{2+}\) efflux showing the effect of Ni. HEK293 cells were loaded with zinc and the rates of zinc efflux were determined as described in Fig 4. b. The rate of zinc efflux in the presence of the indicated inhibitors (verapamil 150\(\mu\)M, Ni 4mM and KB-R-7943 100\(\mu\)M). Nickel and verapamil but not the Na\(^+\)/Ca\(^{2+}\) inhibitor, KB-R-7943, inhibited zinc influx.

**Fig 8: Na\(^+\)/Zn\(^{2+}\) exchange in cultured cortical neurons.** a. Zinc influx was induced by depolarization of cortical neurons loaded with Newport green in the presence (□) or absence (▼) of Na\(^+\). Cells perfused first with NMG-buffer (●) were then switched to Na\(^+\)-containing buffer (at the time marked) or NMG perfusion was continued (▼). The switch to Na\(^+\)-buffer triggered enhancement of Zn\(^{2+}\) influx rate. b. Cells depolarized in NMG-buffer (the same graph as in a) or in the presence of Na\(^+\) and 10\(\mu\)M KB-R7493, inhibitor of the Na\(^+\)/Ca\(^{2+}\) exchanger. Inclusion of the latter compound did not inhibit the Na\(^+\)-dependent Zn\(^{2+}\) efflux. c. Calculated rates of zinc efflux, as measured in a-b.
Figure 1

[Graph showing experimental results with control, Fugen-6, CaPO_4, and Zn^2+ at 400 µM.

Zn (µM)

CaPO_4

Zn^2+ 400 µM

Fugen-6

control

Time (s)
Figure 2

![Fluorescent Signal over time graph with annotations for Zn²⁺ and TPEN]
Figure 3
Figure 4
Figure 5

(a) Zn influx rate (% of maximal rate) vs. [Zn]_ex (µM)

(b) Zn efflux rate (% of maximal rate) vs. [Na]_ex (mM)

(c) Zn influx rate (% of maximal rate) vs. [Zn]_ex (µM)
Figure 6

(a) Fluorescent Signal (F/F₀) over Time (s) showing the influx rate of Zn²⁺ and Ca²⁺ transport.

(b) Bar graph comparing Zn²⁺ and Ca²⁺ transport rates between Transfected and Control groups.

(c) Western blot analysis of G3PD, hZnt1, and hNCX1 with total RNA concentrations of 2, 0.5, and 0.12 µg. The untreated and CaPO₄ conditions are indicated.
Figure 7

(a) Graph showing the time course of Zn efflux in control and Ni 4mM conditions. The graph indicates a peak in efflux at approximately 200-300 seconds, with a subsequent decrease.

(b) Bar graph showing the rate of Zn efflux for different conditions: Control, Verapamil, Ni, and KB-R7943. The graph indicates a higher rate of efflux in the Ni and KB-R7943 conditions compared to the control and Verapamil conditions.
Figure 8

![Graphs showing zinc efflux with different conditions](http://www.jbc.org/)

- **Panel a**: High K+ and zinc, wash Na, Na added at mark, NMG.
- **Panel b**: High K+ and zinc, NMG, KB-R7943.
- **Panel c**: Bar graph showing rate of Zn efflux (nM/min) with Na, NMG, and KB-R7943.
A sodium zinc exchange mechanism is mediating extrusion of zinc in mammalian cells
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