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INVITED REVIEW

Contribution of calcium-conducting channels to the transport of zinc ions

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Abstract Zinc (Zn) is a vital nutrient participating in a myriad of biological processes. The mechanisms controlling its transport through the plasma membrane are far from being completely understood. Two families of eukaryotic zinc transporters are known to date: the Zip (SLC39) and ZnT (SLC30) proteins. In addition, some types of plasmalemmal calcium (Ca)-conducting channels are implied in the cellular uptake of zinc. These ion channels are currently described as systems dedicated to the transport of Ca (and, to some extent, sodium (Na) ions). However, a growing body of evidence supports the view that some of them can also function as pathways for Zn transport. For instance, voltage-gated Ca channels and some types of glutamate-gated receptors have long been known to allow the entry of Zn. More recently, members of the TRP superfamily, another type of Ca-conducting channels, have been shown to permit the uptake of Zn into eukaryotic cells. The aim of this review article is to present the current knowledge supporting the notion that Ca-conducting channels take part in the plasmalemmal transport of Zn.

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Introduction

The metal ion zinc (Zn) is essential to all forms of living cells. Present in extracellular fluids and intracellularly, it is, with iron (Fe), the most abundant trace element of the human body [20, 68]. Proteomic analysis indicated that nearly 10 % of human proteins have the ability to bind Zn [3]. At the cellular level, where its total concentration is estimated to be about 100–200 μM [43], Zn fulfills two main functions: structural and catalytic. Indeed, Zn is a cofactor for many enzymes and is involved in forming and maintaining the correct three-dimensional structure of proteins such as zinc finger proteins [43]. At rest, the cytosolic concentration of free Zn ($[Zn^{2+}]$) in eukaryotic cells depends on its buffering capacities and values ranging from several hundred of picomolar to nanomolar concentrations have been reported [43]. This basal Zn level is however subject to regulation since various physiological factors like the cellular redox status or the electrical activity of excitable cells have been shown to influence $[Zn^{2+}]$ [38, 75]. Changes in $[Zn^{2+}]$ seem to play essential roles in healthy and diseased states. On one hand, dietary Zn deficiency, which is associated with an increased oxidative stress [19], is a nutritional problem affecting human health [9, 42, 44]. On the other hand, Zn excess can also be harmful. This has been illustrated by in vitro studies showing that the culture medium used to maintain cell lines like PC-12, HeLa, or HT-29 and primary cell cultures (cardiac cells and neurons) contains about 5 nM free Zn. This value is close to the baseline extracellular concentration of Zn found in dialysates of rat and human cerebrospinal fluids which has been reported to be ~19 nM [21]. In vitro experiments showed that diminishing or increasing this free Zn ion concentration for instance up to 50 nM is toxic [6]. Another example of the pathophysiological

relevance of Zn is its role in neuronal injury caused by stroke and excitotoxicity during epilepsy. Accumulating evidence suggests that, together with calcium (Ca), the intracellular accumulation of Zn ions participates in ischemic and excitotoxic injury [22, 61, 64].

In addition to its structural and catalytic properties, Zn is also viewed as a signaling ion exerting regulatory functions. It is now regarded as a second messenger [77] able to influence the activity of various enzymes and to control intracellular signaling pathways [64]. Its extracellular concentration is also not constant. Several types of cells have the property to release Zn into the extracellular space [22] like some neuronal [53] and pancreatic cell types [53, 57] where Zn ions are present in vesicles undergoing cycles of exocytosis. In the brain, the concentration of Zn in the synaptic cleft is not known with certainty. Depending on the method and the biological models used, values ranging from 1 to up to 100 μM extracellular Zn have been found [53] (but this view has been questioned, see for instance [50]). Extracellularly, Zn ions positively or negatively modulate the activity of a wide range of ion channels and receptors [46]. For instance, a submicromolar concentration of extracellular Zn is required to influence NMDA receptor activity under physiological conditions [49]. The fact that cells release Zn ions indicates that they need powerful mechanisms for taking up Zn in the first place. However, the mechanisms by which Zn ions enter into cells are still poorly understood. To date, two large families of eukaryotic Zn transporters have been identified: the Zip (SLC39) and ZnT (SLC30) proteins [13, 18]. Besides these actors, other Zn entry routes are likely to exist. Among them, Ca-conducting channels are attractive candidates. They are found in a great diversity of living organisms ranging from prokaryotes to eukaryotes. These Ca-conducting channels form a diverse group of proteins comprising multiple subfamilies with distinct molecular, biophysical, pharmacological, and functional properties. They also differ in their site of expression. Although some of them are located intracellularly (e.g., in the endoplasmic reticulum), most types of Ca-conducting channels described so far are plasma membrane proteins. The aim of this article is to review the current experimental evidence showing that plasmalemmal Ca-conducting channels of several distinct protein families can constitute important Zn entry routes (Fig. 1).

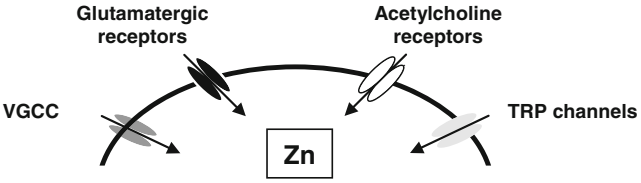

Fig. 1 Ion channel families that have been established to subserve Zn entry. A growing body of experimental data supports the notion that some Ca-conducting channels could participate in the uptake of Zn. So far, members of four distinct superfamilies of Ca-conducting channels (voltage-gated Ca channels (VGCC), glutamatergic receptors, nicotinic acetylcholine receptors, and TRP channels) have been shown to transport Zn. See text and Table 1 for further details and references

Voltage-gated Ca channels

Voltage-gated Ca channels (VGCC) are highly selective Ca channels. They are however capable of permeating other types of cations such as barium (Ba) and strontium (Sr) [29, 55, 67]. Although Zn ions exert inhibitory actions on VGCC [46], they can nevertheless permeate through them. One of the first indications that Ca-conducting channels can transport Zn ions came from electrophysiological recordings carried out on muscle fibers of beetle larvae and showing the existence of Zn-dependent action potentials [24]. But in this study, it was not completely clear whether Zn was permeating through Na or Ca channels. A subsequent work using mature molluscan neurons from subesophageal ganglions of snails showed that channels sensitive to lanthanum (La), cobalt (Co) (nonspecific blockers of Ca-conducting channels), and verapamil (a blocker of L-type VGCC) mediate Zn-dependent action potentials [33]. Single channel analysis of Ca currents on mouse muscle cells also indicated that VGCC could be permeable to Zn [76]. After these pioneering studies on muscle cells, most of the data available so far on the contribution of VGCC to the transport of Zn came from experiments carried out on brain cells and particularly on cultured cortical neurons. Indirect arguments in favor of VGCC as Zn entry channels were provided by studies investigating the neurotoxic properties of extracellular Zn. For instance, a transient bath application of Zn with KCl (or AMPA) to cultured cortical neurons caused a clear cell loss that was partially prevented by Co ions and by other blockers of L-type VGCC (diltiazem, verapamil, nimodipine) [74]. The co-application of KCl (or AMPA) and Zn resulted in a strong elevation of the fluorescence of TSQ, a probe mainly interacting with membrane-bound Zn. This TSQ signal was attenuated by diltiazem. It was subsequently shown that the activation of VGCC that occurs downstream to the depolarization caused by the activation of AMPA channels (see below) can mediate the entry of Zn [23]. This influx, sensitive to blockers of L-type Ca channels, was described as a crucial event leading to the death of rat cortical neurons in culture. This report therefore reinforced the idea that Zn ions can permeate Ca channels. A direct demonstration of the contribution of VGCC in the uptake of Zn came from whole-cell patch clamp experiments conducted on bovine chromaffin cells showing that step-depolarizing pulses elicit Zn inward currents [69]. Chromaffin cells were loaded with Fura-2 and excited at 360 nm. At this wavelength, Fura-2 signals are insensitive to Ca ions. Under these conditions, it was possible to detect a

clear Zn entry occurring at rest. This Zn pathway was blocked by 3 mM nickel (Ni) [69]. Depolarizing the membrane potential of these cells by means of a KCl challenge strongly augmented the entry of Zn, further indicating that VGCC may serve as a Zn entry route [69]. Live cell imaging experiments conducted with the fluorescent probes TSQ [78] and mag-Fura-5 [60] further illustrated the role of neuronal VGCC. In cultured cortical neurons, a KCl-induced depolarization stimulated the entry of Zn that was strongly blocked by gadolinium (Gd), verapamil, and nimodipine and also, but to a smaller degree, by ω-conotoxin GVIA (which inhibits N-type Ca channels) [60]. This entry of Zn through VGCC caused the degeneration of cultured neurons [78]. A more direct demonstration of the ability of neuronal VGCC to transport Zn was obtained by means of electrophysiological experiments performed on cultured murine cortical neurons [34]. In this work, it was shown that Zn ions entered into neuronal cells even in the presence of extracellular Ca. Zn produced a non-inactivating inward current through high-threshold VGCC. This current was sensitive to Gd and nimodipine but weakly blocked by ω-conotoxin GVIA. Under depolarizing conditions, Zn enters neuronal cells mainly via L-type Ca channels but also via ω-conotoxin GVIA-sensitive N-type Ca channels. The involvement of VGCC in the uptake of Zn into neuronal cells has now been well documented by various methodological approaches using electrophysiological recordings, specific Zn probes like FluoZin-3 [14] or the use of ^{65}Zn [63]. This neuronal Zn uptake has been observed in cell cultures and brain slices. While in neuronal cells L- and N-type Ca channels appear as the predominant VGCC transporting Zn, it has been suggested on grounds of pharmacology that in glia cells T-type Ca channels play an important role, since the Zn entry could be attenuated with Ni and mifebradil [62].

In addition to the brain, organs like the heart and the pancreas have Zn-permeant VGCC. In pancreatic β-cells kept in low glucose (basal condition), Zn entry occurred through a non-L-type Ca channel pathway which was hypothesized to be mediated by plasma membrane Zn transporters. Possibly, however, other ion channels, like transient receptor potential (TRP) channels (see below), may also contribute to the basal Zn entry in pancreatic β-cells. However, under stimulatory conditions (high glucose), L-type VGCC mediated the entry of Zn [27, 56]. Pancreatic β-cells thus seem to possess more than one type of Zn uptake system and, depending on their metabolic status, cells switch from one type to another [27]. Several Zn routes have been identified in pancreatic α-cells. Based on their FluoZin-3 experiments, Gyulkhandanyan et al. concluded that Zn is, in part, transported via VGCC insensitive to dihydropyridines (inhibitors of L-type Ca channels) [28]. Gd, a nonspecific but potent blocker of Ca channels, and nitrendipine (a L-type VGCC blocker) were tested. Although applied at a high concentration (50 μM), these agents weakly reduced the Zn uptake under basal and high glucose conditions. This indicates that in pancreatic α-cells, VGCC only poorly contributed to the uptake of Zn. So far, the identity of the plasma membrane channels responsible for Zn entry remains unclear.

In the heart, Fura-2 recordings and whole-cell patch-clamp experiments showed that Zn ions can enter into rat cardiac myocytes (and GH3 cells) via dihydropyridine-sensitive (L-type) VGCC. Furthermore, this influx of Zn can control the expression of a set of genes [4], suggesting that Zn ions can function as a second messenger. L-type VGCC from rat cardiomyocytes transport Zn ions and generate Zn currents. These dihydropyridine-sensitive channels have a higher affinity for Zn (K_d ~0.043 mM) than for Ca (K_d ~4.8 mM) [2].

Glutamate receptors

Studies addressing the question of the neurotoxic properties of Zn identified ionotropic glutamatergic receptors as likely Zn entry gates. As already noted [12], cultures of cortical neurons were the major biological model used to understand the contribution of these receptors.

Several routes are implied in the uptake of Zn under resting (non-depolarizing) conditions. Fura-2 and TSQ recordings showed that in the absence of glutamatergic (or KCl) stimulation Zn uptake seemed to occur in part via channels sensitive to MK-801 or APV (two NMDA channel blockers) and insensitive to CNQX (an AMPA channel blocker) as well as to nifedipine (a L-type Ca channels blocker) [10, 36, 45]. The contribution of NMDA receptors to the import of Zn may seem paradoxical since Zn ions have long been regarded as potent blockers of NMDA receptors [53, 61]. Nevertheless, Zn seems to permeate through them when it is co-applied with NMDA, a maneuver reported to induce a strong intracellular Zn rise sensitive to antagonists of NMDA receptors [60]. The non-NMDA Zn pathway observed at rest was shown to be affected by blockers of the Na/Ca exchanger like benzamil–amiloride, D-methyl-benzamil-amiloride [60], and KB-R7943 [10]. This indicates that the non-NMDA-dependent Zn entry seen at rest involves a route where the influx of Zn is coupled to an efflux of Na [10, 60] (but the contribution of this exchanger in the entry of Zn is controversial [45]). In addition to these pathways (channels and the Na/Ca exchanger), some transporters seem to be implicated also in the entry of Zn into neurons at rest [12].

When Zn is co-applied with AMPA (or kainate), it caused a marked and time-dependent elevation of the TSQ fluorescence, indicating an elevation of [Zn^{2+}] [74]. This Zn signal

recorded under depolarized conditions had two sources: an uptake of Zn via Ca-conducting AMPA/kainate channels and a second pathway involving VGCC (activated by the depolarization that develops after the opening of the AMPA channels) [60, 78]. The recording of Zn currents in cultured hippocampal neurons confirmed that Ca-conducting AMPA/kainate channels have the ability to transport Zn. They have however a greater permeability for Ca than for Zn (P_{Ca}/P_{Zn} ~1.8) [32]. Furthermore, data collected from fluorescence imaging experiments suggest that extracellular Ca ions do not seem to interfere with the permeation of Zn through these channels [32]. Ca-conducting AMPA channels lacking the GluR2 subunit are now regarded as an important Zn entry pathway in the brain [61]. By showing that prion proteins (PrP^C) control the uptake of Zn via an AMPA-dependent process, Watt et al. [73] highlighted the contribution of these glutamatergic receptors in the transport of the metal. Indeed, they found that PrP^C interacts through its N-terminal polybasic region with the AMPA receptor subunits GluA1 and GluA2. Furthermore, it increases the surface expression of the GluA1 subunit. AMPA receptors are mediating the PrP^C-dependent Zn uptake observed in neuronal cells [73].

Acetylcholine receptors

Measurements of reversal potentials indicated that ionotropic cholinergic channels present at the endplate of frog muscles are permeable to Zn ions [1]. This was confirmed by Ragozzino et al. [58] who studied the properties of the mouse muscle nicotinic acetylcholine receptors containing either γ or ε subunits expressed in the human cell line BOSC 23. Although Zn potently inhibited currents through channels containing either (γ and ε) subunit, fluorescence measurements achieved with the Zn probe Newport Green indicated that Zn permeates through these channels. Of note, Ca and magnesium (Mg) did not alter the Zn uptake through these nicotinic acetylcholine receptors. However, the fractional Zn current was higher in channels containing the ε subunit when compared to channels containing the γ subunit [58].

Transient receptor potential channels

The superfamily of mammalian TRP channels comprises 28 members which are organized into six subfamilies named TRPA, TRPC, TRPM, TRPML, TRPP, and TRPV [70]. They form tetrameric structures participating in the transport of Ca with the exception of TRPM4 and TRPM5 which are the only monovalent selective cation channels of the TRP superfamily [30, 40]. Widely expressed, functional TRP channels are predominantly found at the plasma membrane but also, in some instances, on intracellular membranes [16]. The transport of Zn through TRP channels has been documented in five TRP subfamilies and involves the following TRP members: TRPA1, TRPC6, TRPM3, TRPM6, TRPM7, TRPML1, and TRPV6.

TRPM7 was the first TRP protein identified as a Zn-conducting TRP channel [47]. Electrophysiological recordings revealed that it is highly permeable to Zn even in the presence of physiological concentrations of Ca and Mg, indicating that TRPM7 could constitute a physiological pathway for Zn [47]. TRPM6, another TRP channel sharing nearly 50 % of sequence homology with TRPM7, was also shown to be Zn-permeant [41, 66]. Of note, heteromultimeric TRPM6/TRPM7 channels have also a high Zn permeability [41]. In addition to TRPM6 and TRPM7, TRPM3 is another Zn-conducting TRPM channel. The *trpm3* gene encodes for a large number of different protein isoforms, due to a high degree of alternative splicing and alternative exon usage [51, 52]. Importantly, one site of alternative splicing directly and dramatically affects the pore properties of TRPM3 channels. For instance, the splice variant TRPM3α2, which is endogenously expressed in pancreatic β-cells, is permeable to Zn ions, even in the presence of physiological concentrations of Ca and Mg [71, 72]. Since β-cells co-release substantial amounts of Zn together with insulin [7, 59], the influx of Zn through TRPM3 channels might be physiologically relevant, although abundant alternative Zn influx pathways exist in these cells [27]. Interestingly, TRPM1 is a channel having a pore region that has an intermediate length when compared to the two pore variants of TRPM3, by having an insert of seven amino acids (LYAMEIN motif [39]). TRPM1 proteins expressed in a heterologous overexpression system (HEK293 cells) form channels that are Ca- but not Zn-permeable [39]. Instead, TRPM1 channels are inhibited by this metal. This characteristic feature of TRPM1 proteins appears to be dominant because heteromultimeric channels containing TRPM3 and TRPM1 also do not conduct Zn ions. The data on TRPM1 therefore show that not all Ca-permeable TRP channels are Zn-permeable. They rather indicate that permeability to Zn (and possibly other trace metal ions) can be tuned selectively and independently from permeability to Ca. In the genome of *Drosophila melanogaster*, only a single TRPM gene (dTRPM) is present encoding for a protein that is strongly homologous to mammalian TRPM1, TRPM3, TRPM6, and TRPM7 channels. Since the pore region of this protein seems to be rather short, it is, therefore, not surprising that the resulting proteins form Zn-permeant channels [25]. Loss-of-function mutations in dTRPM diminished cellular Zn content, reduced cell size, and led to larval lethality [25]. The cellular size phenotype could be partially rescued by growing the larvae on a medium with high Zn content, but interestingly not with larval food containing high levels of Mg. These data indicate that dTRPM is involved in the cellular Zn homeostasis of fly larvae [25].

The subfamily of TRPML channels (named mucolipins) gathers three isoforms: TRPML1, TRPML2, and TRPML3 (or MCOLN3) [11, 70]. Mutations in the human *trpml1* gene result in a lysosomal storage disorder, mucolipidosis type IV (MLIV) [5, 65]. TRPML channels can function as a lysosomal metal transport system [35] notably TRPML1 which is Zn-permeant [15]. The involvement of TRPML1 channels in Zn homeostasis has been underlined in a recent study showing for instance that TRPML1−/− mice and fibroblasts from MLIV patients have higher levels of chelatable Zn, notably in lysosomes and vacuolar structures [17].

The subfamily of TRPA channels comprises only one member, TRPA1. It is expressed by a subgroup of dorsal root ganglia and trigeminal neurons that respond to noxious stimuli [11, 54, 70]. Extracellular Zn ions are potent agonists of TRPA1 channels triggering their opening with an EC_{50} of ~2 μM, whereas, when present intracellularly, they activated TRPA1 channels with an EC_{50} of ~50 nM [31]. TRPA1 channels heterogeneously expressed in HEK cells as well as native channels from dorsal root ganglia neurons were able to mediate an influx of Zn ions. Mutating a single amino acid (D915A) in the selectivity filter prevented Zn entry through TRPA1 [31]. The authors propose that Zn must gain access to intracellular sites in order to activate the channels.

TRPV6 is a member of the vanilloid family of TRP channels. The overexpression of human TRPV6 channels in HEK cells can give rise to an influx of Zn ions [37]. However, this latter effect was observed in the presence of 1 mM external Zn. Whether a TRPV6-dependent Zn entry could be observed at more physiological extracellular concentrations of Zn has to be shown.

The only member of the TRPC subfamily known to date to mediate Zn currents is TRPC6 [26]. This TRPC isoform was previously described as involved in the uptake of Fe in HEK and PC12 cells [48]. It was however later observed that its overexpression in HEK cells caused a clear augmentation of their total Zn content as well as an enhancement of the size of their mobilizable or labile pool of Zn (without causing any alteration in their iron content). The application of molecules known to activate TRPC6 channels such as SAG and hyperforin gave rise to an uptake of Zn which was sensitive to the extracellular concentration of Ca. This TRPC6-dependent Zn entry upregulated the size of the intracellular non-mitochondrial pool of mobilizable Zn in cultured cortical neurons. None of the effects described above were seen in HEK cells overexpressing TRPC3 channels [26]. Besides TRPC3 and TRPC6, no other TRPC have been tested. Considering all the available literature, it can be seen that Zn permeation has only been studied in a few members of the TRP family (Table 1). Evidently, it would be desirable to have data on the divalent permeation profile of all TRP channels, including the functionally identified heteromultimeric channels that can be formed by various TRP proteins.

Table 1 Listing of ion channels that have been implicated in Zn transport. These channels originate from four distinct ion channel families

Channel family	Channel family members	Key references
Voltage-gated Ca channels (1)	L-type	[4, 23, 27, 34, 56, 60, 74]
	N-type	[34, 60]
	T-type	[62]
Nicotinic acetylcholine receptors	γ/ε-containing subunits	[58]
Glutamate receptors	NMDA	[60]
	AMPA/Kainate	[32, 74, 78]
TRP channels	TRPA1	[31]
	TRPC6	[26]
	dTRPM	[25]
	TRPM3	[71]
	TRPM6	[41, 66]
	TRPM7	[47]
	TRPML1	[15]
	TRPV6	[37]

Conclusions

As illustrated above, Zn can permeate through Ca-conducting channels belonging to various families: glutamatergic receptors of AMPA/kainate and NMDA types; nicotinic acetylcholine receptors; L-, N-, and T-type VGCCs; and TRP channels (Table 1). The only TRP subfamily for which no data are available is the TRPP subfamily. TRPP2 (or polycystin-2) has been shown to transport the monovalent metal lithium (Li) [8] but, clearly, data are missing regarding the biologically relevant metal Zn and this TRP channel subfamily. Also for most of the other families of Ca-permeable channels, the characterization of divalent permeability is far from complete. As many isoforms exist and multimeric channels typically diversify further by forming heteromultimeric channels, completing the picture of Zn permeation of Ca-permeable channels is a daunting task. Nevertheless, given the pathophysiological importance of intracellular Zn, the pursuit of this endeavor appears to be highly worthwhile.

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