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The zinc sensing receptor, ZnR/GPR39, in health and disease

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1. ABSTRACT

While zinc has had a well-established structural role for many years, it is only during the last two decades that its role as a signaling molecule has been recognized. Ionic zinc, Zn^{2+}, that is endogenously released during physiological activity acts as a first messenger, triggering the activity of a distinct Zn^{2+}-sensing-receptor, ZnR. The ZnR is a member of the Gq-coupled receptor family, and the molecular moiety mediating its activity is GPR39. In this review, we will discuss the role of the ZnR/GPR39 in mediating Zn^{2+}-dependent signaling in epithelial tissues and in neurons, where Zn^{2+} homeostasis plays physiological as well as pathological roles. Importantly, ZnR/GPR39 activates signaling that regulates a remarkably wide range of cell functions, including proliferation, differentiation and survival, as well as modulation of ion transport, and thereby, regulation of Na^+, H^+ and Cl^- homeostasis. Moreover, signaling activated by ZnR/GPR39 plays a key role in mediating effects of Zn^{2+} in health and disease. Thus, ZnR/GPR39 provides a unique target for therapeutically modifying the actions of zinc in a specific and selective manner.

2. INTRODUCTION

Ionic zinc, Zn^{2+}, plays an essential role in the development and function of every system in the body. Its involvement is particularly prominent in the digestive, immune, nervous, secretory and integumentary systems (1-6). The utility of zinc in treatment of airway spasms or skin wounds, was described already in the 19th century (7, 8). A link between Zn^{2+} and carbonic anhydrase was shown 70 years ago (9, 10), and this was followed by the identification of Zn^{2+} as a cofactor of numerous enzymes. Zinc was then found to play an important structural role in various proteins, particularly transcription factors (11-13). Based on its importance in numerous physiological and cellular processes, it is hardly surprising that changes in extracellular as well as intracellular Zn^{2+} content trigger cellular signaling pathways. Moreover, Zn^{2+} transport into or out of organelles or its release from buffering proteins into the cytoplasm trigger subcellular changes in Zn^{2+} that is now considered a second messenger (14, 15). In this way, Zn^{2+} is now recognized as a metal ion that acts as a first or second messenger in physiological processes (16, 17). While most cellular Zn^{2+} is bound to proteins, transient changes in its extracellular or intracellular

concentration occur under both physiological and pathological conditions. Its functional significance is shown definitively by the potentially lethal effects of Zn^{2+} deficiency or increases of cellular Zn^{2+} (18, 19).

The strict maintenance of intracellular Zn^{2+} homeostasis is accomplished by means of a plethora of discrete Zn^{2+} transporters and buffer proteins (16, 20-22). These are largely represented by three families of proteins: 1) the 10 member zinc transporter family (ZnTs, SLC30), which transports this ion out of the cell or into cytoplasmic organelles and vesicles, 2) the 14 member Zip (SLC39) family that moves Zn^{2+} into the cytoplasm, and 3) the Zn^{2+} chelating metallothioneins, loosely binding this ion within the cytoplasm (20, 21, 23-25). Cellular Zn^{2+}- containing vesicles are particularly abundant in the pancreas, brain, mammary gland, salivary gland and digestive system, and exocytosis of these vesicles triggers transient changes in extracellular Zn^{2+} levels (26-31). Extracellular Zn^{2+} levels were also increased following injury of keratinocytic cells that resulted in release of endogenous Zn^{2+} (32). Activity of the released Zn^{2+} as a signaling molecule requires that changes in Zn^{2+} concentration are transient. Several mechanisms could operate to quickly induce reuptake of Zn^{2+} into the cells via the extensive transporter system consisting of Zip and ZnT proteins mentioned above. Buffering of intracellular Zn^{2+} by metallothioneins or extracellular Zn^{2+} by albumin or citrate may also serve to lower the extracellular free Zn^{2+} concentration (13, 33).

Extracellular Zn^{2+} acts as a signaling molecule via the ZnR/GPR39 (34, 35), which has been functionally identified as a Zn^{2+}-dependent, G-protein coupled receptor that senses changes in extracellular Zn^{2+} and, in response, activates downstream signaling pathways. Importantly, ZnR/GPR39 can be activated by endogenous Zn^{2+} released from vesicles or following injury (32, 36-38). Extracellular Zn^{2+} also *indirectly* activates cell signaling via allosteric modulation of ZnR/GPR39 signaling , and hence, dissection of the pathways activated by this ion may reveal specific therapeutic targets. For example, while Zn^{2+} interacts with major neuronal membrane transporters, e.g., the dopamine transporter, NMDA, glycine and GABA and modulates their activity (39-43), the distinct downstream signaling pathways triggered by the ZnR/GPR39 may be essential for regulating neuronal inhibition (37, 38, 44). In epithelial cells, Zn^{2+} regulates the activity of purinergic receptors and the store-operated channel (SOC), representing an important link between Zn^{2+} and intracellular Ca^{2+} (45-47). In fibroblasts, extracellular Zn^{2+} upregulates the PI3 kinase pathway, leading to activation of AKT and increased cell survival (48). Similar effects have also been shown to be mediated by Zn^{2+}-dependent activation of the mitogen-activated protein kinase (MAPK) (49). Although intracellular Zn^{2+} may affect these pathways via regulation protein phosphatases (50, 51), ZnR/GPR39 is the major link between Zn^{2+} and both, the PI3 and MAP kinase pathways (52, 53).

3. ZnR/GPR39 IN HEALTH AND DISEASE

3.1. A distinct Zn^{2+}-sensing Gq-protein coupled Receptor

The ZnR/GPR39 is activated by physiological concentrations of extracellular Zn^{2+}, inducing release of Ca^{2+} from thapsigargin-sensitive intracellular stores via the IP3 pathway (35, 54). Inhibitors of Gαq (55, 56), inositol 1,4,5-trisphosphate (IP3) receptor and the phospholipase C (PLC) attenuate this Zn^{2+}-dependent Ca^{2+} rise, indicating that the Ca^{2+} release is mediated by activation of a Gαq-coupled receptor (35, 52). ZnR/GPR39-dependent Ca^{2+} release has thus far been observed in numerous epithelia, including colonocytes, keratinocytes, pancreatic cells, prostate cancer cells and salivary gland cells (52, 57-59). Interestingly, Zn^{2+} has a well-established role in the normal function of these tissues, and Zn^{2+} dyshomeostasis is associated with diarrhea, growth retardation, skin lesions, impaired salivary secretion and taste disorders (60, 61). The ZnR/GPR39-dependent Ca^{2+} rise induced by Zn^{2+}, enhances activation of the mitogen activated protein kinase, MAPK, and PI3 kinase pathways that are closely linked to enhanced cell survival and proliferation (62). Thus, ZnR/GPR39 may be the mediator of many of the well-established, health-promoting functions of Zn^{2+} (63). Finally, Zn^{2+} dyshomeostasis is also associated with neurological disorders, including Alzheimer's disease, ischemia and epilepsy (64-67). The ZnR/GPR39 has been identified in neurons postsynaptic to vesicular Zn^{2+}-containing synaptic boutons (68). The physiological role of ZnR/GPR39 in its diverse contexts will be discussed further in this review.

It should be emphasized that ZnR/GPR39 is highly specific to Zn^{2+}, as other biologically relevant heavy metal ions (e.g. Mn^{2+}, Cu^{2+} and Fe^{2+}) do not produce a Ca^{2+} response (35). In addition, ZnR/GPR39 is sufficient and necessary to trigger Zn^{2+}-dependent signaling. Nevertheless, it can interact with another, well-described cation receptor, the Ca^{2+} sensing receptor (CaSR (69, 70)). Heterodimerization of GPCRs diversifies the physiological response of these receptors to their ligands and may play an important role in their regulation (71, 72). The ZnR/GPR39 and CaSR exhibit similarities in their signaling pathways, for instance, both are activated via a Gq-dependent mechanism. Although Zn^{2+}-dependent activity does not require the presence of extracellular Ca^{2+}, this ion alters the apparent cooperativity and affinity of ZnR/GPR39 to Zn^{2+} (57). Similarly, spermine, a CaSR ligand, synergistically increases the cellular response when applied with Zn^{2+}, though it does not activate the ZnR/GPR39 itself (53). Finally, silencing

of the CaSR downregulates ZnR/GPR39 response, and direct interaction between the CaSR and ZnR/GPR39 is monitored using co-immunoprecipitation (53). Previous studies have shown that changes in the surface expression of the CaSR occur following exposure to its ligand (73). We posit that CaSR localization, following spermine or Ca^{2+} application, also affects the surface expression of ZnR/GPR39, thereby enhancing the Zn^{2+}-dependent response.

Regulation of GPCRs activity is also achieved by desensitization, occurring after a brief exposure to their ligands. In this process, the desensitized receptor is internalized and may undergo degradation (74, 75). Differences in the degree of functional desensitization among GPCRs reflect the ratio between the recycling versus degradation of the receptor (76). Following its release, decrease of extracellular Zn^{2+} level may be facilitated by its reuptake via the Zip transporters, found in most cells, or chelation by Zn^{2+}-binding proteins. However extracellular Zn^{2+}, in contrast to most GPCRs ligands, is not rapidly degraded. Protection of cells from excessive Ca^{2+} signals triggered by continued activation of ZnR/GPR39 is achieved via desensitization of the ZnR/GPR39. Exposure to subtoxic concentrations of Zn^{2+} leads to profound and prolonged desensitization likely involving ZnR/GPR39 degradation (52, 58, 68). In the normal prostate Zn^{2+} is found in the presence of citrate, an extracellular Zn^{2+} binding protein that is especially abundant in the prostate. While the complex of Zn^{2+} with citrate does not activate ZnR/GPR39 signaling, it does induce desensitization of the ZnR/GPR39 (58). This may suggest a mechanism by which the ZnR/GPR39 is quiescent in the normal prostate when Zn^{2+} is largely complexed with citrate, but nevertheless desensitizes the receptor. However, during carcinogenesis when citrate and Zn^{2+} levels decrease (77-78), ZnR/GPR39 will be re-sensitized and can function to enhance cell proliferation in the prostate (58).

3.2. Crosstalk between Zn^{2+}, pH and ZnR/GPR39

Analysis of the structural basis for constitutive activity of GPR39, known then as an orphan receptor, revealed that application of Zn^{2+} increased the activity of this receptor (79, 80). Though constitutive activity of this receptor was less than half compared to other neurotensin- and ghrelin-receptor family members, Zn^{2+} was considered of little physiological significance and not suggested as an endogenous ligand of GPR39. Instead, obestatin, a short peptide linked to obesity, was suggested to activate GPR39 (81), but activation of GPR39 by this peptide was not reproduced in further studies (82, 83). In contrast, an unbiased study identified Zn^{2+} as the endogenous agonist of GPR39 (84). The Zn^{2+} binding site on GPR39 was found to consist of two histidine residues: His17, His19 (85), and an aspartate residue: Asp313. This Asp313 was suggested to act as a tethered inverse agonist that upon binding of Zn^{2+} is diverted to enable Zn^{2+} binding to the histidines. Binding of Zn^{2+} to histidine occurs via an imidazole group and is pH sensitive, being most efficient between pH 7-8. Hence, when the extracellular pH drops to 6.5, the Zn^{2+} activated, ZnR/GPR39-dependent Ca^{2+} response and subsequent phosphorylation of MAP or PI3 kinase is completely abolished (32, 86, 87). Attenuation of Zn^{2+}-dependent signaling at pH 6.5 is short-lived and reversible (86, 87). By overexpressing GPR39 mutated at what was expected to be the pH sensitive residues, i.e., His17 and His19, we showed that ZnR/GPR39 maintains its pH-dependence and Zn^{2+} signaling is still abolished at pH 6.5. Similarly, other extracellular-facing histidines fail to reverse the pH sensitivity. Eventually, Asp313 was identified as the pH sensing component for ZnR/GPR39. The replacement of this residue with the pH insensitive alanine results in Zn^{2+}-dependent Ca^{2+} responses that are similar at pH 7.4. or 6.5, while its substitution by His or Glu pH-sensitive residues restores the pH sensitivity of the receptor (86). Reducing the activity of the ZnR/GPR39 following changes in extracellular, but not intracellular, pH may result from different protonation states of the involved residues (88). Yet another possible mechanism involves local conformational changes of the binding site as was shown for pH sensitivity of the Ca^{2+}-sensing receptor and the mGluR4 glutamate receptor (89, 90).

Altogether, ZnR/GPR39 mediates Zn^{2+}-dependent signaling while tuned to sense physiologically-relevant changes in extracellular pH (86). Importantly, changes in this range of pH commonly occur under physiological conditions within the digestive system lumen, the epidermis and the brain (91-95). Hence, the pH sensitivity of ZnR/GPR39 may serve as an important regulator of physiological and pathological responses to Zn^{2+}. For example, inflammatory bowel disease may induce local pH changes that render ZnR/GPR39 signaling inefficient for Zn^{2+} enhancement of proliferation. Such mechanism could underlie the erosion of the epithelial layer, occurring in this disease (96).

Interestingly, ZnR/GPR39 itself regulates the pH of the intracellular and extracellular microenvironments via upregulation of Na^+/H^+ exchange (NHE) activity. The activity of NHE exchangers is upregulated following a drop in intracellular pH, serving as an important factor in the recovery from intracellular acid loads in many cell types (97). Activation of ZnR/GPR39 signaling, and its downstream phosphorylation of the MAPK pathway, results in upregulation of NHE activity in colonocytes, keratinocytes and neurons (32, 36, 52, 87, 98). In all of these cells, the effect of Zn^{2+} on regulation of NHE is completely lost in the absence of ZnR/GPR39. Such upregulation of NHE activity may have important effects on the function of neurons

or epithelial cells. In neurons, intracellular acid load, accumulated during repetitive firing, is largely the result of metabolic H^+ generation (99). The intracellular pH changes can affect neuronal excitability by modulating the activity of ion channels, transporters and receptors (100-102). Thus, ZnR/GPR39-dependent regulation of intracellular pH may play a role in neuronal excitability.

By mediating Na^+-dependent H^+ export, NHE exchangers may induce changes in the extracellular pH while accelerating recovery of the intracellular environment. In neurons, acidifying the cell surfaces that abut the synaptic cleft can regulate various functions, including GABA signaling or dendritic spine growth (103, 104). On the other hand, sustained NHE activity contributes to tissue acidosis during ischemic neuronal injury (105). Under these conditions, inhibition of NHE was claimed to be neuroprotective (106, 107). The pH sensitivity of neuronal ZnR/GPR39, which is inactive at acidic pH, suggests a homeostatic mechanism by which NHE-mediated decrease of the extracellular pH following Zn^{2+} activation of ZnR/GPR39, serves to prevent excessive tissue acidification (87). In keratinocytes, ZnR/GPR39 also upregulates NHE activity (32), which may result in an acidic apical surface. In the skin, such acidification is required for formation of an effective permeability barrier (108) and NHE1 is an important regulator of this function (109). Interestingly, Zn^{2+} deficiency is commonly associated with inflammation, possibly reflecting breakdown of this barrier. Elegant *in vivo* experiments have shown an important role for NHE2 activation in gastric epithelial repair (110), interestingly NHE2 activity in this study did not induce changes in surface pH because they may have been masked by HCO_3 buffering. It is not clear if ZnR/GPR39 upregulation of NHE activity in the presence of physiological HCO_3 affects extracellular pH in various tissues, hence further studies comparing the role of ZnR/GPR39 and NHE in the presence of physiological concentrations of HCO_3 are required.

3.3. Zn^{2+} acts as a neurotransmitter via ZnR/GPR39

3.3.1. The Zn^{2+}-containing neuron

Deficiency of Zn^{2+} is associated with developmental malformations and impaired cognitive performance (66, 111). There is an increasing body of evidence suggesting that disturbances of Zn^{2+} homeostasis play an important role in the etiology of various neurological disorders, including Alzheimer's disease (112, 113), amyotrophic lateral sclerosis (114, 115), ischemia (116-118), spreading depression (119-121) and autistic spectrum disorders (122, 123). Zinc deficiency has also been closely associated to epilepsy, with lack of dietary Zn^{2+} leading to enhanced susceptibility to epileptic seizures in mouse models (124, 125) as well as in humans (126-133). Furthermore, Zn^{2+} administrated to mice, in studies using a kindling model of epilepsy, reduced seizure activity (134, 135). In contrast, excessive rise of extracellular as well as intracellular Zn^{2+}, correlates with neurotoxicity and cell death (51, 118, 136-138). Rapid increases in the concentration of intracellular Zn^{2+} may occur following an episode of oxidative or nitrosative stress, inducing liberation of Zn^{2+} bound to intracellular proteins and frequently resulting in cell death (18, 51, 118, 139). For this reason, Zn^{2+} chelation is increasingly contemplated as a potentially viable therapeutic strategy in some neurological conditions such as ischemia/stroke (138, 140-142), though such an approach could be detrimental in others (143). In contrast, in Alzheimer's disease, metal ionophores that may restore intracellular Zn^{2+} levels have been considered for use as therapeutic agents (144).

A unique pool of Zn^{2+}, representing a mere 10% of total brain zinc, is concentrated in synaptic vesicles within a subclass of excitatory cortical neurons, it is often referred to as 'synaptic Zn^{2+}'. It is this Zn^{2+} that is demonstrated by the so-called 'Timm's' staining method (29, 145, 146). Loading of Zn^{2+} into synaptic vesicles is mediated by a specific transporter, ZnT3, which is expressed in discrete regions of the brain, including hippocampus, amygdala, neocortex and auditory brainstem among other regions. Knockout of the ZnT3 gene results in mice lacking synaptic Zn^{2+} (125, 147). Early studies did not show a clear phenotype of these mice except for enhanced susceptibility to seizure, yet later studies indicate that ZnT3 knockout mice may also have impaired learning, memory and fear-conditioning processes and autistic-like behavior (148-154). Synaptic Zn^{2+} is stored together with glutamate, the principle excitatory neurotransmitter of the mammalian CNS, and is co-released with it into the synaptic cleft (136, 155) in a Ca^{2+}- and activity-dependent manner (156-159). Synaptic Zn^{2+} has been suggested to modulate membrane excitability via direct interaction with post synaptic targets, e.g., $GABA_A$, NMDA and glycine receptors (39, 66, 160-165). Binding of synaptic Zn^{2+} to ZnR/GPR39 provides a pathway underlying, for example, the effects of Zn^{2+} during seizure (44).

3.3.2. ZnR/GPR39 in neurons

Metabotropic pathways, triggering slow intracellular second messenger systems, play a critical role in neurotransmission (166). Indeed, metabotropic receptors activate intracellular signaling leading to delayed modulation of ion channels and membrane transporters. Glutamate receptors (mGluRs), for example, mediate changes in synaptic plasticity by activating the mitogen-activated protein kinase, MAPK, pathway (167, 168). Hence, we hypothesized that the metabotropic Zn^{2+} sensing receptor ZnR/GPR39 is a distinct target of synaptic Zn^{2+} that may underlie the

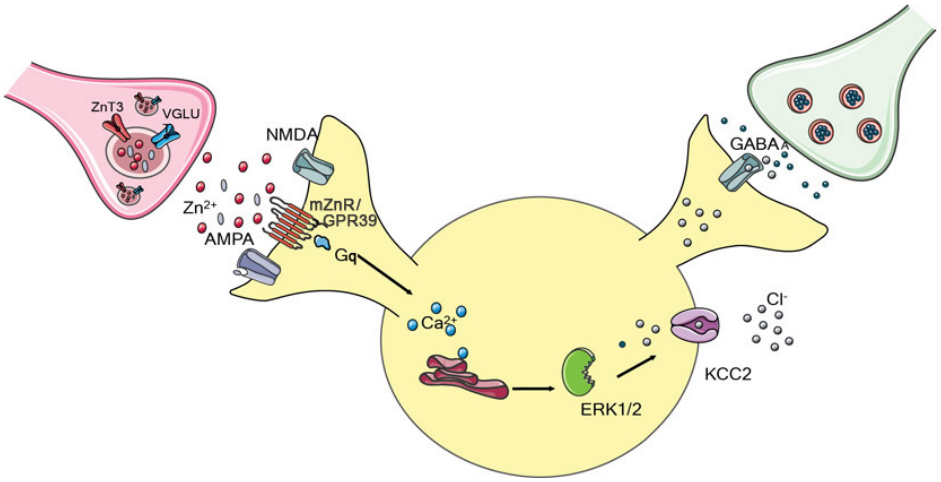

Figure 1. Schematic representations of ZnR/GPR39 signaling in CA3 neurons. Zn^{2+} is transported into synaptic vesicles by ZnT3 and is stored and co-released together with glutamate. Synaptically released Zn^{2+} may then bind ZnR/GPR39, inducing post-synaptic intracellular Ca^{2+} rises and activation of the MAPK pathway. ZnR/GPR39 signaling enhances KCC2 activity and the Cl^- gradient, affecting $GABA_A$ inhibitory responses. Image was created using Servier Medical Art. ZnT3 - Zn^{2+} transporter 3, VGLUT – Vesicular glutamate transporter, ZnR/GPR39 – metabotropic Zn^{2+}-sensing receptor, mGluR – metabotropic glutamate receptor, AMPA and NMDA are ionotropic glutamate receptors, MAPK – mitogen activated kinase.

effects of this ion in the brain. Initial efforts focused on the hippocampus, as this region demonstrates intense Timm's staining, particularly of mossy fiber boutons aligned with their postsynaptic, CA3 neurons (28). Application of exogenous Zn^{2+} resulted in intracellular Ca^{2+} release from the endoplasmic reticulum stores in these neurons, but not in adjacent astroglia (38, 68). Pharmacological dissection of the signaling pathway triggered by Zn^{2+} revealed that intracellular Ca^{2+} rise is PLC and Gq dependent (see Figure 1), in accordance with ZnR/GPR39 activity seen in epithelial cells. Furthermore, activation of MAP kinase by Zn^{2+} in neurons requires ZnR/GPR39 activation and the resulting Ca^{2+} release (68, 87). Importantly, physiological stimulation of the mossy fibers was shown to trigger endogenous synaptic Zn^{2+} release (157, 158) and ZnR/GPR39-dependent Ca^{2+} rises in the post synaptic CA3 neurons (38). The presence of a non-membrane permeable, extracellular, Zn^{2+} chelator reduced the stimulus-induced Ca^{2+} rise suggesting that the endogenous synaptic Zn^{2+} is sufficient for activating ZnR/GPR39. ZnR/GPR39-dependent Ca^{2+}-signaling was monitored when exogenous Zn^{2+} was applied to hippocampal slices from ZnT3 knockout mice, which lack synaptic Zn^{2+}, but stimulation of the mossy fibers in these slices yielded a significantly decreased Ca^{2+} signal in the CA3 neurons that was similar to the signal triggered in WT slices in the presence of the Zn^{2+} chelator (38). Thus, synaptic Zn^{2+} release is essential for a postsynaptic metabotropic response, indicating the physiological relevance of this receptor (68). Importantly, the Zn^{2+}-dependent Ca^{2+} signaling is absent in GPR39 knockout mice supporting the conclusion that the molecular moiety mediating neuronal ZnR responses is GPR39 (38, 169). Similar ZnR/GPR39 activity, triggered by physiologically relevant electrostimulation of synaptic Zn^{2+}-containing fibers, has been described in the dorsal cochlear nucleus, a region of the auditory brainstem (37). In these neurons, activation of ZnR/GPR39 inhibits glutamate release by inducing synthesis of the endocannabinoid, 2-arachidonoylglycerol (2-AG), which reduces synaptic strength (37). A role for ZnR/GPR39 in regulation of the CREB/BDNF/TrkB pathway, and thereby in depression, has also been postulated, though it is not clear at present how Gq signaling activates this pathway or whether these effects are lost in ZnR/GPR39 knockout mice (170, 171).

In the hippocampus, ZnR/GPR39 activation by synaptic Zn^{2+} results in enhancement of inhibitory tone, apparently by means of an increase in the neuronal Cl^- efflux pathway (38, 169). The major neuronal outward transporter of Cl^- is the K^+/Cl^- cotransporter, KCC2, which is necessary and sufficient to create a Cl^- equilibrium potential that is negative with respect to the resting membrane voltage (172, 173). The activity of KCC2 is therefore crucial for rendering Cl^- channels, i.e. $GABA_A$ and glycine receptors, activity as inhibitory (174, 175). This important co-transporter is highly regulated via its phosphorylation, and by changes in its expression during neuronal activity, thereby modulating the inhibitory effect of GABA and glycine (176-178). Interestingly, ZnR/GPR39 activation in neurons and downstream phosphorylation of MAPK results in enhanced K^+-dependent Cl^- transport that is mediated by KCC2. This activity is not reproduced in ZnR/GPR39 knockout mice (38, 169). It has been shown that Gq-dependent signaling enhances KCC2 surface expression and thereby upregulates KCC2-dependent transport (38). These results were the first demonstration of a direct and distinct target

for synaptic Zn^{2+} and suggest that this ion acts as a neurotransmitter (Figure 1). Moreover, in this way, neuronal inhibitory tone is enhanced by Zn^{2+} binding to ZnR/GPR39.

3.3.3. ZnR/GPR39 plays a homeostatic role during seizure activity

Importantly, decreased KCC2 function renders the, normally inhibitory, $GABA_A$ post-synaptic potentials excitatory thereby increasing seizure susceptibility (175, 176, 179). On the other hand, a study in neonatal rats revealed that a single seizure episode *in vivo* or a brief treatment of acute brain slices with the excitotoxin, kainite, result in activation of KCC2 in the hippocampus. This correlated with an increase in the plasmalemal fraction of KCC2, and not with an increase in its overall concentration (180). As suggested above, loss of synaptic Zn^{2+}, thought to act as an inhibitory neuromodulator, is associated with epileptogenesis (181, 182). Moreover, administration of a Zn^{2+}-deficient diet is sufficient to reduce synaptic Zn^{2+} levels, and results in greater susceptibility to kainate-induced seizures (183, 184). Similarly, ZnT3 knockout mice show enhanced sensitivity to seizure-inducing pharmaceuticals, including the glutamatergic excitotoxin, kainite, which induces limbic seizures (124). More recent studies show that ZnT3 knockout mice are also more prone to febrile hyperthermia induced seizures (133). Interestingly, Zn^{2+} supplementation may decrease febrile seizure recurrence in children (185).

It was demonstrated that Zn^{2+}, acting via ZnR/GPR39, upregulates KCC2 activity and may be sufficient to decrease seizure severity. Indeed, ZnR/GPR39 knockout animals exhibit an enhanced susceptibility to kainate-induced seizures, exhibiting significantly higher behavioral seizure severity scores and more seizures over longer periods of time, compared to wildtype controls (44). Treating hippocampal slices with kainate is sufficient to upregulate KCC2 activity in an extracellular Zn^{2+}-dependent manner. Kainate-induced upregulation of KCC2 is also dependent on ZnR/GPR39 signaling, as it is abolished by treatment with Gq, PLC or MAPK inhibitors. Finally, kainate-induced KCC2 upregulation is absent in ZnR/GPR39 knockout mice, suggesting that the receptor itself is necessary for this process. Taken together, these findings support a homeostatic role for ZnR/GPR39 triggered by seizure-induced synaptic Zn^{2+} release (44).

3.4. ZnR/GPR39 signaling in epithelial cells

3.4.1. The digestive system

The intestinal epithelial layer separates the body from the luminal contents, which includes metabolites but also toxins, bacteria and pathogens. Hence, it is hardly surprising that this layer undergoes continuous renewal of its cell population every 3-4 days, a process requiring continuous cell proliferation and differentiation. This layer of epithelium, on one hand, is responsible for the selective uptake of digested metabolites from the lumen, which necessitates multiple transporter proteins. On the other hand, this epithelium requires tight anatomical and physiological barrier to prevent invasion by foreign organisms and substances. Numerous studies link Zn^{2+} to proper function of the digestive system, including absorption and barrier functions (186-189). At the cellular level, Zn^{2+} promotes proliferation, differentiation, survival (17, 98) and barrier formation (190-193) in colon epithelial cell (colonocyte) cultures. The presence of a Zn^{2+}-triggered, Gq-dependent mechanism for activation of Ca^{2+} cellular signaling in colonocytes enabled the demonstration of a functional ZnR/GPR39 in these cells (35).

Initial studies indicated that GPR39 is widely-expressed throughout the digestive system (194, 195). We subsequently showed that luminal application of Zn^{2+} is sufficient to activate ZnR/GPR39, suggesting that the receptor is present on the apical side of colon epithelial cells, facing the colonic lumen (98). Sources of luminal Zn^{2+} that could interact with ZnR/GPR39 include exogenous or dietary Zn^{2+}, as well as endogenous sources, e.g., Zn^{2+} released from pancreatic digestive enzymes, Zn^{2+} from salivary gland vesicles or from Paneth cells in the intestinal epithelium (26-31, 196). In addition, Zn^{2+} is released from all mammalian cells following injury or death (32), and more selectively via a process mediated by Zn^{2+} transporters such as ZnT6 (196). Finally, endogenous Zn^{2+} released into the gastrointestinal lumen via epithelial shedding alone may account for its physiological concentration (98), sufficient to activate the ZnR/GPR39.

As mentioned above, Zn^{2+}- activated, ZnR/GPR39-dependent Ca^{2+} signaling plays a key role in enhancing proliferation of epithelial cells (35, 98). The downstream pathways activated by ZnR/GPR39 include MAPK and AKT, both hallmarks of cell proliferation and survival (36, 52). Both are of major importance to the constant renewal of the epithelium, required to replace those cells constantly shed into the intestinal lumen. It seems noteworthy as well that ZnR/GPR39 has been implicated in accelerating proliferation and differentiation of pre-adipocytes (198). The short chain fatty acid, butyrate, present at high concentrations in the colon (199), imposes an acidic stress and exerts a pro-apoptotic effect on colonocytes (199-202). The acidic load imposed on colonocytes by the presence of butyrate (203) is reduced by activation of ZnR/GPR39 and its downstream activation of NHE (52, 98). Colonocyte cell death, induced by prolonged exposure to

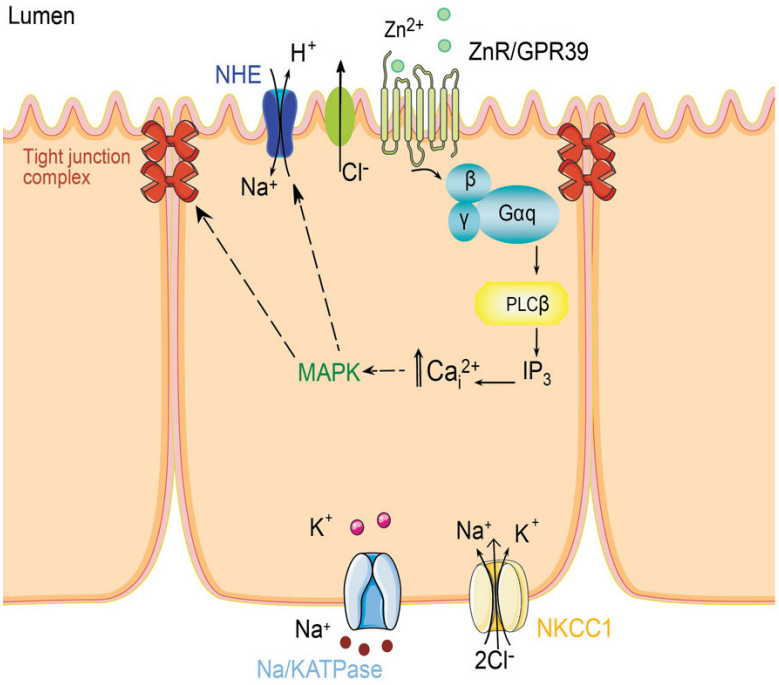

Figure 2. Schematic representations of ZnR/GPR39 signaling in colonocytes. Colonocytes form a permeability barrier via expression of tight junction complexes on the apical side and express ion transporters regulating Na^+, Cl^- and K^+ absorption. Zn^{2+} on the luminal side may activate the ZnR/GPR39 and trigger downstream Ca^{2+} rises and ERK1/2 pathway activation. This results in upregulation of the Na^+/H^+ exchanger and the formation of tight junctions. Image was created using Servier Medical Art. NHE - Na^+/H^+ exchanger, ZnR/GPR39 – metabotropic Zn^{2+}-sensing receptor, MAPK – mitogen activated kinase, Na/KATPase – Na^+/K^+ ATPase, NKCC1 – $Na^+/K^+/Cl^-$ cotransporter 1.

butyrate, is attenuated by activation of ZnR/GPR39-dependent MAP and PI3 kinase pathways (98). The rescue of cells from butyrate-induced cell death, however, requires further activation of the pro-survival glycoprotein, clusterin, also known as apolipoprotein J (204, 205). A role for ZnR/GPR39 in enhancing proliferation and cell survival (see Figure 2) may also underlie the effects of Zn^{2+} in therapies accelerating healing of gastric ulcers (206).

It has been suggested that Zn^{2+} reduces the severity and extends the times between bouts of inflammatory ulcerative disease, such as occur in Crohn's disease and colitis (186, 187, 207-209)). A major factor in these diseases is breakdown of the epithelial barrier that precedes the inflammatory response (210, 211). Under conditions of Zn^{2+} deficiency, occurring due to insufficient dietary Zn^{2+} or genetically-induced by loss of transporters responsible for Zn^{2+} absorption, barrier function is compromised, leading to increased permeability (190, 212, 213). On the other hand, Zn^{2+}-dependent activation of ZnR/GPR39 enhances expression of junctional complex proteins ZO-1 and occludin, (36), critical elements of the specialized junctions between adjacent colonocytes that compose the intestinal barrier (214, 215). Moreover, the role of ZnR/GPR39 in regulating expression of these proteins was elucidated by their significant reduction in the colon epithelium of ZnR/GPR39 knockout mice (98). Interestingly, redistribution of ZO-1 takes place early on during epithelial cell shedding, as it is essential for maintaining the barrier function during the ongoing epithelial renewal in the intestines (216). It would be of interest, then, to examine the effect of Zn^{2+}, released from redundant colon epithelial cells, on the expression and reorganization of ZO-1 during incorporation of new cells into the epithelial layer.

A crucial function of the intestinal epithelium is regulation of ion and solute transport, by which osmotic gradients for water movement are maintained (217). Interestingly, under normal conditions or during experimentally-induced diarrhea, Zn^{2+} was suggested to modulate ion transport in the colon (218, 219). In colon cell lines and native colonocytes, Zn^{2+}, acting via ZnR/GPR39, promotes upregulation of Na^+/H^+ exchanger activity (52, 98). While this reduces the intracellular acid load, it also induces rapid uptake of Na^+ from the lumen. Because of the key role played by the colonocytic apical NHE3 in attenuating diarrhea (217, 220, 221), its upregulation by ZnR/GPR39 in the presence of Zn^{2+} is likely to enhance this protective effect.

3.4.2. Skin

One of the first physiological functions demonstrated for extracellular zinc involves its

enhancement of wound healing. Zinc deficiency has long been linked to skin lesions (222, 223). Topical application of zinc-containing ointments, moreover, stimulates wound healing and re-epithelialization processes (8, 223-225). Importantly, severe skin lesions manifest in both dietary and genetic Zn^{2+} deficiencies and can be reversed by dietary Zn^{2+} supplementation (226, 227). Congenital Zn^{2+} deficiency, accompanied by severe skin lesions, is also characteristic to disorders linked to dysfunction of Zn^{2+} transporters, such as Acrodermatitis Enteropathica (AE), a genetic mutation in the intestinal Zn^{2+} uptake transporter ZIP4, or the Transient Neonatal Zinc Deficiency (TNZD), a symptomatic disorder in breastfed babies that is associated with mutations in the mammary gland Zn^{2+} transporter ZnT2 (60, 228-230). Extracellular Zn^{2+}, at concentrations found in the epidermis (222, 223, 231) triggers Ca^{2+} release from thapsigargin-sensitive stores that is largely mediated by ZnR/GPR39 (32). Moreover, injury of keratinocytes releases Zn^{2+} at concentrations that are sufficient to trigger ZnR/GPR39 response in neighboring keratinocytes (32). Surprisingly, this Zn^{2+}-dependent Ca^{2+} response in a keratinocyte epithelial skin cell-line possesses a dramatically higher affinity to Zn^{2+} compared to other cells (32). This may be explained by the tendency of G-protein coupled receptors to form dimers with other members, thereby affecting their affinity to their endogenous ligands (71, 72). Indeed, ZnR/GPR39 is able to interact with CaSR (53), suggesting interaction between GPCRs may underlie the high affinity of the keratinocytic ZnR/GPR39 to Zn^{2+}. In keratinocytes, Zn^{2+}-dependent upregulation of MAPK activity and NHE ion transport are both mediated via activation of ZnR/GPR39 (32). The importance of ZnR/GPR39 upregulation of NHE activity in keratinocytes is underlined by the role of this transport in induction and maintenance of an acidic local extracellular microenvironment essential to the efficacy of the permeability barrier in the skin (108, 203, 232). It appears likely, therefore, that ZnR/GPR39 activation may regulate the formation of the permeability barrier and enhance anti-inflammatory effects, associated with topical Zn^{2+} application during wound healing. Moreover, accelerated wound closure in the presence of Zn^{2+} indicates that activation of ZnR/GPR39 also directly promotes healing, while silencing the receptor or pharmacologically inhibiting its signaling pathway reverses the effect (32). Interestingly, although in colonocytes ZnR/GPR39 upregulation of NHE does not affect cell survival (98), this pathway clearly enhances keratinocytes proliferation (32). Since the keratinocytic-ZnR/GPR39 undergoes rapid and profound desensitization following exposure to high levels of extracellular Zn^{2+} (32), topical application of Zn^{2+} for extended periods (for example in bandages) may actually hinder wound healing. As such, the discovery of agonists that activate, but that do not desensitize, ZnR/GPR39 could provide better wound healing solutions.

3.5. A paracrine role for ZnR/GPR39

Signaling pathways activated by ZnR/GPR39 are also involved in secretion of first messengers that may trigger paracrine signaling in neighboring cells. Indeed, activation of ZnR/GPR39 in a salivary gland duct epithelial cell line, HSY, induces release of ATP (57). Moreover, application of Zn^{2+} to co-cultures of HSY cells, expressing a functional ZnR/GPR39, and vascular smooth muscle cells (VSMCs) that do not express a functional ZnR (57), induces a Ca^{2+} rise in both cell types. To determine a paracrine effect, we showed that treatment with the non-permeable ATP scavenger apyrase inhibited the Zn^{2+}-dependent Ca^{2+} rise in the VSMC but not in the HSY cells (57). Altogether these experiments suggested that ZnR/GPR39 activity in HSY cells triggers ATP release that activated metabotropic signaling in the neighboring VSMC.

Such paracrine effects influence and could amplify the increased proliferation and migration processes elicited by Zn^{2+}- activated ZnR/GPR39 to neighboring cells, not expressing the receptor. Hence, Zn^{2+}-activated ZnR/GPR39 may enhance the wound healing ability by promoting proliferation and migration of both keratinocytes and fibroblasts (233), although fibroblasts lack a functional ZnR (35). Taken together, ZnR/GPR39 may be capable of augmenting growth of neighboring metastatic cells following increased secretion of ATP or S100A4 (234).

3.6. ZnR/GPR39 in cancer

Since ZnR/GPR39 enhances cell proliferation and migration a role in carcinogenesis must be considered. A role for Zn^{2+} in the development of breast cancer has been explored, and changes in the expression or function of transporters from the ZIP and ZnT families have been linked to the development and progression of the malignant process (235-237). Similarly, butyrate has been shown to induce apoptosis of colon cancer cells. The finding that ZnR/GPR39 rescues colonocytes from butyrate-induced apoptosis (98), suggests a potential role for this receptor in the etiology of colon cancer that has yet to be addressed. In contrast, Zn^{2+} via ZnR/GPR39 signaling-induces release of intracellular Ca^{2+} in androgen-independent, but not in androgen-dependent prostate cancer cells (58). Changes in intracellular Ca^{2+} may also regulate S100A calcium binding proteins that enable cell migration and invasion of cancer cells, and are specifically linked to enhanced prostate cancer growth (238-240). Specifically, S100A4 is present in tumor interstitial fluid and is thought to enhance metastatic cell proliferation and angiogenesis (241). Proliferation and invasiveness of a prostate cancer cell line (PC3) is enhanced by S100A4 via induction of the metalloprotease, MMP-9 (242). Importantly, S100A4

expression is triggered in PC-3 cells by extracellular Zn^{2+} at concentrations that activate ZnR/GPR39 (53). In contrast, S100A4 expression is reduced following silencing of ZnR/GPR39 with siGPR39 (53). It will be interesting to assess the effect of ZnR/GPR39 expression on the level of extracellular S100A4 in prostate tumors and its relation to metastasis.

Robust changes in extracellular Zn^{2+} during prostate tumorigenesis have been suggested to serve as a marker for prostate cancer (243, 244). In PC-3 cells, ZnR/GPR39 activation induces PI3K pathway upregulation, measured via phosphorylation of AKT (53). Surprisingly, ZnR/GPR39 expression is itself associated with higher levels of total AKT expression, which are associated with a more malignant phenotype of adrenal carcinomas (245). In addition, constitutive activation of the PI3K signaling pathway is correlated with severity of prostate tumors (246). Importantly, zinc itself and zinc transporters, most prominent ZIP1, ZIP2, ZIP3 and ZnT7, have been implicated in playing an important role in prostate cancer (5, 77, 243, 247-250), but whether there is a causative or synergistic link between these changes and ZnR/GPR39 activity is not understood. Finally, changes in GPR39 expression levels and patterns were observed in human esophageal squamous cell carcinoma (251) and in gastric adenocarcinomas (252), but a role for Zn^{2+} in activation of ZnR/GPR39 was not determined.

4. CONCLUSIONS AND FUTURE PROSPECTS

It is now established that ZnR/GPR39 is a distinct target for free ionic Zn^{2+} released in response to physiological or pathological activity. The receptor is a functional GPCR that mediates Zn^{2+}-dependent signaling in epithelial cells and in neurons, providing for the first time, a molecular target to explain many of the physiological actions of Zn^{2+}. Described for many years as a ubiquitous structural element of virtually all cells, Zn^{2+} is now identified, in addition, as a signaling molecule in a wide variety of contexts. The ZnR/GPR39 is an important regulator of Zn^{2+}-dependent signaling, and may serve as a handle to modulate physiological processes.

Although Zn^{2+} plays a role in many pathological conditions, Zn^{2+} itself, because of its lack of specificity and the often conflicting intracellular versus extracellular effects, offers a far less attractive address for therapeutic interventions than a distinct GPCR. Hence, ZnR/GPR39 is a potentially promising candidate for therapeutic intervention in diseases as diverse as epilepsy and colitis, which in general are poorly controlled by existing therapies. By focusing on ZnR/GPR39, the molecular pathways responsible for the symptoms of diseases may be directly and effectively targeted. That the Gq protein-coupled receptor family (GPCR) is currently a major focus of the pharmaceutical industry (253, 254), provides reason for optimism that specific and effective agonists and antagonists for ZnR/GPR39 will be forthcoming.

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