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Molecular mechanism of Zn^{2+} agonism in the extracellular domain of GPR39

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Abstract **Ala substitution of potential metal-ion binding residues in the main ligand-binding pocket of the Zn^{2+}-activated G protein-coupled receptor 39 (GPR39) receptor did not decrease Zn^{2+} potency. In contrast, Zn^{2+} stimulation was eliminated by combined substitution of His^{17} and His^{19}, located in the N-terminal segment. Surprisingly, substitution of Asp^{313} located in extracellular loop 3 greatly increased ligand-independent signaling and apparently eliminated Zn^{2+}-induced activation. It is proposed that Zn^{2+} acts as an agonist for GPR39, not in the classical manner by directly stabilizing an active conformation of the transmembrane domain, but instead by binding to His^{17} and His^{19} in the extracellular domain and potentially by diverting Asp^{313} from functioning as a tethered inverse agonist through engaging this residue in a tridentate metal-ion binding site.**

Keywords: G protein-coupled receptor; GPR39; Metal ion; Zn^{2+} binding site

1. Introduction

In addition to its structural and catalytic role in a multitude of proteins such as enzymes and transcription factors, Zn^{2+} also acts as a signaling molecule. Thus, many cell types secrete Zn^{2+}, including neurons, pituitary cells, prostate epithelial cells, mast cells, granulocytes, Paneth cells in the intestine, exocrine pancreatic cells as well as β-cells of the endocrine pancreas [1]. Moreover, a number of cell-surface proteins are affected by Zn^{2+}; including ion channels, neurotransmitter transporters and seven transmembrane domain (7TM), G protein-coupled receptors [2,3]. Within the 7TM receptor family, Zn^{2+} inhibits binding and/or activation of the D_4 dopamine receptor, the μ-opioid receptor, and the D_2 dopamine receptor [4–6]; while it stimulates and/or potentiates the NK_3 receptor, the MC_1 and MC_4 melanocortin receptors and the β_2-adrenergic receptor [7–10].

G protein-coupled receptor 39 (GPR39) is a 7TM receptor belonging to the Ghrelin receptor family which is expressed in metabolic, endocrine tissues such as the gastrointestinal tract, the liver, adipose tissue and the endocrine pancreas [11]. Originally, we described that Zn^{2+} acts as a potent and efficacious agonist for GPR39, which has been confirmed by several groups, while a report that a fragment from the ghrelin precursor called obestatin could be a ligand for GPR39 has not been independently reproduced [12–16]. Interestingly, in an attempt to characterize a GPR39-activating component from fetal bovine serum, Zn^{2+} was ultimately isolated as being this factor [17]. In the current study, we map the Zn^{2+}-binding site in GPR39 through mutagenesis and pharmacological characterization of the mutants (Fig. 1).

2. Materials and methods

2.1. Receptor construction

The cDNA of the human GPR39 was provided by K. Hansen (7TM Pharma) and corresponds to GenPept NP_001499. The M2 FLAG epitope was inserted at the N-terminus. Mutants constructed using the PCR overlap extension method [18] were verified by DNA sequencing.

2.2. Cell culture

HEK-293 cells were grown at 37 °C in 5% CO_2, 95% humidity in DMEM with Glutamax, 10% fetal bovine serum, 100 U/mL Penicillin G and 100 μg/mL streptomycin. The doxycycline-inducible GPR39 stable cell line was created using the FLP-In T-Rex system (Invitrogen).

2.3. Inositol phosphate accumulation assay

Twenty thousands cells/well were seeded O/N in 96 well plates then transient transfections were performed using 30 ng receptor DNA/well with Effectene reagent (Qiagen). Fresh medium containing 10 μCi/mL *myo*-[2-^{3}H]inositol was added the next day. Following ~24 h incubation, cells were washed with HBSS, compounds were added to cells in HBSS with 10 mM LiCl and incubated for 45 min at 37 °C. Cells were lysed in 10 mM formic acid on ice for $\geqslant$ 30 min. Ysi-SPA beads were reconstituted (10 mL/g) and then diluted 8-fold in H_2O before use. Eighty microliters were combined with 20 μL cell lysate in white 96 well plates, shaken 5–30 min, centrifuged at 400×*g* for 5 min, incubated at RT $\geqslant$ 8 h and radioactivity (cpm) measured in a TopCount-NXT scintillation counter (Packard). Experiments were performed in triplicate.

2.4. Cell surface ELISA

~48 h post-transfection (as above), cells were washed with PBS, fixed for 10 min in 3.7% formaldehyde, washed (3 × 10 min) with

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Abbreviations: GPR39, G protein-coupled receptor 39; 7TM, seven transmembrane domain; ECL, extracellular loop; IP, inositol phosphate; ELISA, enzyme-linked immunosorbent assay

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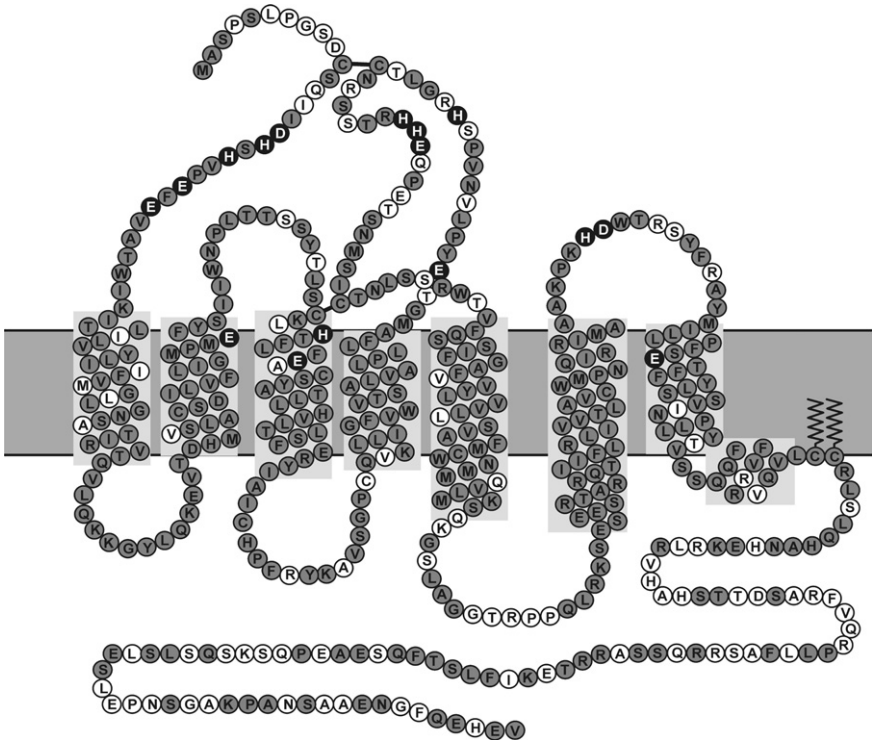

Fig. 1. Serpentine diagram of human GPR39. Amino acid residues conserved in human, mouse and rat GPR39 are shown in gray circles; those analyzed in this study are shown as white letters in black circles.

PBS, blocked for 30 min in PBS with 3% milk powder and 50 mM Tris–HCL pH 7.5, incubated with 1/1000 dilution of anti-FLAG antibody (SIGMA) in blocking buffer for 1–2 h, washed, incubated in 1/1250 dilution of goat anti-mouse horse radish peroxidase-conjugated secondary antibody (Pierce) in blocking buffer for 1 h, washed, visualized by addition of 100 μL TMB Plus substrate (Kem-En-Tec) and reaction was stopped with 100 μL 0.2 M H_2SO_4. Absorbance was measured at 450 nm, 1 s on a Wallac Victor2 (Perkin Elmer). Experiments were performed in quadruplicate.

2.5. Data analysis

Concentration–response curves were generated and EC_{50} values determined using Prism (version 5, GraphPad Software) and fitting normalized data by non-linear regression to a four-component logistic equation [log(agonist) versus response – variable slope].

3. Results

Previously we and others have demonstrated that human GPR39 is activated by Zn^{2+} [15,17,19]. Mouse and rat GPR39 exhibit similar concentration-response curves for Zn^{2+}, as compared to human GPR39, in inositol phosphate (IP) accumulation assays (data not shown) in agreement with a recent report using Zn^{2+}-induced Ca^{2+} mobilization as a functional read out [17]. Thus, Zn^{2+} activation appears to be a conserved characteristic of GPR39. To further support a direct action of Zn^{2+} on GPR39, we generated a doxycycline-inducible HEK-293 cell line in which increasing Zn^{2+} activation of IP accumulation was observed as a function of increasing cell surface expression of GPR39 as determined by enzyme-linked immunosorbent assay (ELISA) (Fig. 2).

In proteins, Zn^{2+} is most efficiently coordinated by His, Cys, Asp and Glu residues [20]. Because the four extracellular Cys

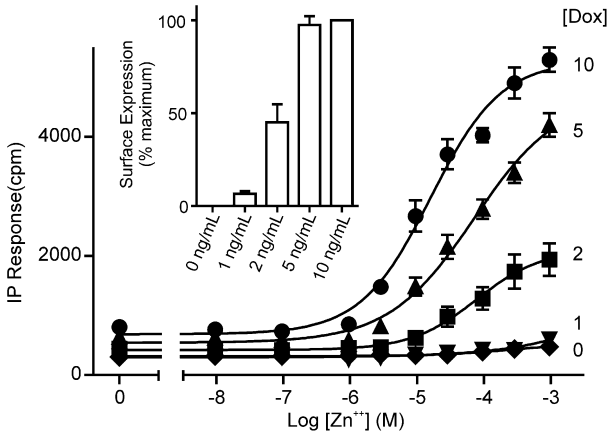

Fig. 2. Functional responses in a stable inducible GPR39 cell line. GPR39 expression was induced by addition of 0 (◆), 1 (▼), 2 (■), 5 (▲), or 10 (●) ng/mL doxycycline for ~24 h prior to inositol phosphate accumulation assays in response to Zn^{2+}. Data are expressed in cpm. The inset shows cell surface expression at each induction level as a percent of the maximal expression as determined by ELISA. Data shown are means ± S.E.M. of at least three separate experiments.

residues in GPR39 all are involved in disulfide bridge formation and therefore not available to bind Zn^{2+}, these were not included in the present study (manuscript in preparation). A total of sixteen residues located either in the main ligand-binding pocket or in the extracellular domains were replaced individually or in combinations with Ala residues and the mutant receptors were transiently expressed in HEK-293 cells and monitored for cell surface expression and spontaneous as well as Zn^{2+}-induced IP accumulation (Figs. 3 and 4, Table 1).

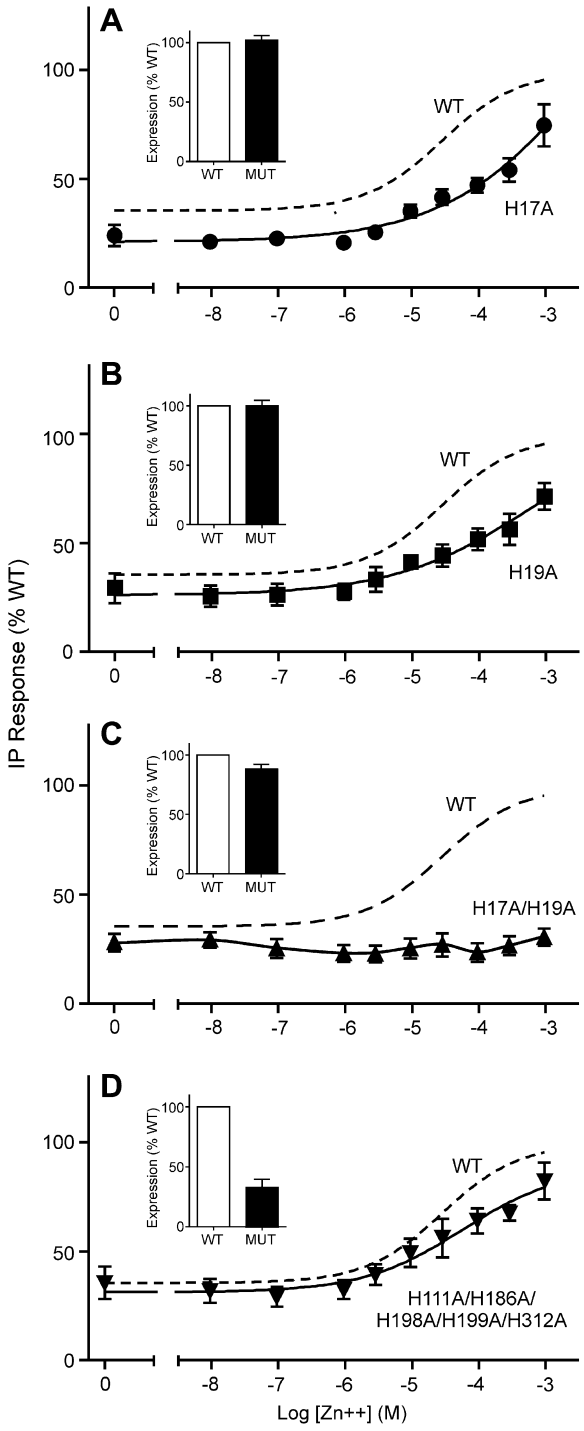

Fig. 3. Inositol phosphate accumulation assays. Zn^{2+} concentration-response curves (CRC) for GPR39 mutants (A) H17A (B) H19A (C) H17A/H19A (D) H111A/H186A/H198A/H199A/H312A. The dashed line shows wild-type GPR39 for comparison. Insets show cell surface expression of each mutant as a percent of wild-type GPR39 expression as determined by ELISA. Data shown are means ± S.E.M. of three or more separate experiments.

Previously, we have built activating metal-ion sites in the β_2-adrenergic receptor through introduction of metal-ion binding residues at key positions on the opposing faces of TM-III and TM-VII [21,22]. In this context, it was surprising that no deleterious effect – rather a 2-fold *increase* – was observed in Zn^{2+} potency upon Ala-substitution of either HisIII:04 (His^{111}) or GluVII:06 (Glu^{330}) in GPR39, because these residues are located on the opposing faces of TM-III and TM-VII. Like these two residues, GluIII:09 (Glu^{116}) is facing the main-ligand-binding pocket of GPR39 and is conserved in the ghrelin receptor, where it is known to be a major anchor point for both peptide and non-peptide agonists [23–25]. However, in GPR39 GluIII:09 (Glu^{116}) is apparently not part of the Zn^{2+}-binding site as Ala substitution did not lead to a decrease but rather to an almost 15-fold increase in the agonist potency of Zn^{2+} (Table 1). This mutant had a somewhat decreased expression level (Table 1); however, as shown in Fig. 2 a decrease in the expression level of GPR39 does not lead to increased potency for Zn^{2+}. Thus, the activating metal-ion binding site of GPR39 does not appear to be located in the main ligand-binding pocket of the receptor as substitutions of potential metal-coordinating residues in this pocket all appear to increase the potency of Zn^{2+}, rather than decreasing it.

In the extracellular domains of GPR39, Ala substitution of most potential metal-ion binding residues had little or no effect on activation by Zn^{2+}, i.e. only decreasing the potency of the metal ion by a maximum of 2- to 3-fold. Importantly, although individual substitutions of either His^{17} or His^{19} located in the N-terminal segment with Ala residues decreased the potency of Zn^{2+} by 3.4-fold and 2.4-fold, respectively (Fig. 3A and B), the combined H17A/H19A double mutant did not respond to Zn^{2+} at all (Fig. 3C). Ala substitution of four other residues in ECL-2 also showed somewhat decreased Zn^{2+} potencies as compared to wild-type GPR39, including His^{186} (2.4-fold), His^{198} (3-fold), His^{199} (3.1-fold) and Glu^{200} (2.7-fold) (Table 1). However, these residues are not essential for the Zn^{2+}-induced activation as, for example, a receptor with a combination of multiple of these His residues replaced with Ala had a Zn^{2+} response similar to wild-type GPR39 (Fig. 3D).

Surprisingly, Ala substitution of Asp^{313}, located in the middle of extracellular loop 3 (ECL-3), resulted in a receptor displaying very high constitutive activity (Fig. 4A). Although difficult to determine due to this high ligand-independent signaling, it was not possible to detect a significant stimulatory effect of Zn^{2+} in the D313A GPR39 mutant. In contrast, Ala substitution of the neighboring residue His^{312} did not significantly affect constitutive or Zn^{2+}-induced activity (Fig. 4B).

4. Discussion

When initiating the mapping of the Zn^{2+}-binding site in GPR39, we expected to find a site where Zn^{2+}, by analogy to other small molecule agonists of 7TM receptors, would act as an agonist by "holding" the receptor in an active conformation through binding between the extracellular ends of TM-III, -VI and -VII [22,26]. However, based on the results of the present study, we propose instead a very different mechanism of action, where Zn^{2+} binds in the extracellular domain to a site involving His^{17} and His^{19}, located in the N-terminal extension. The relatively high Zn^{2+} potency indicates that a third residue is involved in the Zn^{2+} binding, which in principle could be located in the main ligand-binding pocket. However, this notion is not supported by the mutational analysis. Instead we suggest that Asp^{313} located in ECL-3, which appears to normally

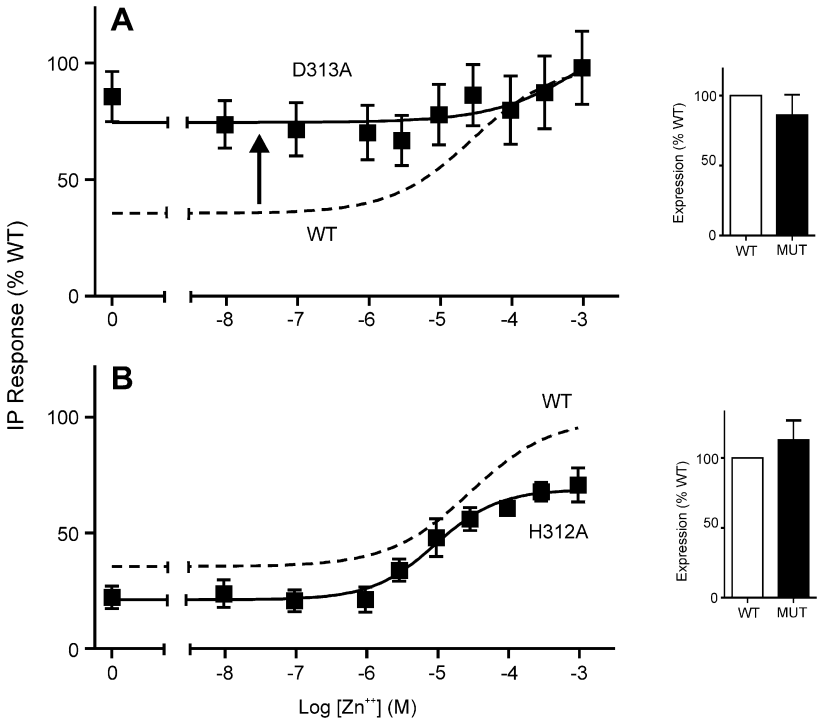

Fig. 4. Inositol phosphate accumulation assays. Zn^{2+} concentration-response curves (CRC) for GPR39 mutants (A) D313A (B) H312A. The dashed line shows wild-type GPR39 for comparison. Insets to the right show cell surface expression of each mutant as a percent of wild-type GPR39 expression as determined by ELISA. Data shown are means ± S.E.M. of three or more separate experiments.

Table 1
Pharmacological characterization of WT and mutant GPR39[a]

	Potency, EC_{50}			N[c]	% C.A.	E_{max}[d]	Expression
	−Log (M)	μM	Fold[b]				
WT	4.5 ± 0.08	28	1.0	30	36 ± 1	100	100
D16A	5.2 ± 0.54	6	0.2	4	39 ± 7	60 ± 8	68 ± 6
H17A	4.0 ± 0.12	94	3.4	4	24 ± 5	75 ± 10	102 ± 4
H19A	4.2 ± 0.16	68	2.4	4	29 ± 7	71 ± 6	100 ± 4
H17A/H19A	> 3	> 1000	> 36	7	28 ± 4	31 ± 4	88 ± 4
E22A/E24A	5.2 ± 0.13	6	0.2	5	38 ± 4	75 ± 5	49 ± 5
E90A	4.9 ± 0.13	14	0.5	4	65 ± 5	120 ± 11	74 ± 4
H111A	4.9 ± 0.20	14	0.5	4	25 ± 5	85 ± 9	38 ± 2
E116A	5.6 ± 0.22	2	0.07	4	37 ± 10	59 ± 6	9 ± 3
E177A	5.2 ± 0.31	6	0.2	5	27 ± 3	38 ± 5	24 ± 4
H186A	4.2 ± 0.18	68	2.4	4	28 ± 6	73 ± 7	88 ± 2
H198A	4.1 ± 0.15	83	3.0	5	42 ± 3	88 ± 8	92 ± 4
H199A	4.1 ± 0.18	86	3.1	4	23 ± 5	48 ± 5	101 ± 6
E200A	4.1 ± 0.11	76	2.7	5	30 ± 3	86 ± 7	96 ± 6
H312A	4.9 ± 0.13	11	0.4	4	22 ± 5	71 ± 7	113 ± 14
D313A	> 3	> 1000	> 36	4	86 ± 11	98 ± 16	86 ± 14
E330A	4.8 ± 0.11	16	0.6	5	23 ± 1	58 ± 3	94 ± 6
H111/186/198/ 199/312A	4.5 ± 0.21	34	1.2	3	32 ± 8	82 ± 12	30 ± 9

[a]Transiently transfected HEK-293 cells were analyzed for function in inositol phosphate accumulation assays in response to 10-point Zn^{2+} concentrations and for cell surface expression by ELISA. Values shown are ±S.E.M., where applicable.
[b]Determined by EC_{50} mutant − EC_{50} WT.
[c]N represents the number of separate experiments.
[d]E_{max} values represent % wild-type efficacy at maximal Zn^{2+} challenge (1 mM).

function as what could be considered a ''tethered inverse agonist'', participates in the metal-ion binding. Thus, Zn^{2+} could act as an agonist by engaging Asp^{313} in a tridentate metal-ion binding site involving also His^{17} and His^{19} in the N-terminal domain, and thereby preventing Asp^{313} from acting as an inverse agonist which would increase receptor signaling.

Thus, removal of the side chain of Asp^{313} through Ala substitution results in a highly constitutively-active receptor, which did not respond normally to Zn^{2+}. Interestingly, in the ghrelin receptor a SNP resulting in introduction of an acidic Glu residue in ECL-2 for an Ala residue selectively eliminates the high constitutive signaling of that receptor, which – in an

opposite manner – is analogous to the situation in GPR39, i.e. the mutation introduces an acidic "tethered inverse agonist" instead of removing it [27,28]. This proposed mode of action of Zn^{2+} in GPR39 is reminiscent of how Cu^{2+} acts in the metal-ion site engineered trypsin of Craik and coworkers, where the metal-ion engages the His residue of the active triad of the enzyme in an engineered, neighboring metal-ion site which prevents the residue from being part of the catalytic process [29].

Most naturally-occurring Zn^{2+} sites that have been mapped in 7TM receptors are located in the upper part of the main binding pocket or in the extracellular domains. For example, in the NK_3 receptor, Zn^{2+} acts as an allosteric modulator through binding to two His residues located *i* and *i*+4 at the extracellular end of TM-V [7]. In the melanocortin MC_1 receptor, a free Cys residue in ECL-3, possibly in combination with a key Asp residue at the extracellular end of TM-III, appears to form the binding site through which Zn^{2+} acts as an ago-allosteric modulator [8]. Zn^{2+} inhibition of ligand binding in the μ-opioid receptor requires a His residue at the extracellular end of TM-VII and may also involve an Asp residue in ECL-2; whereas in the dopamine D_2 receptor, two His residues at the extracellular end of TM-VI/ECL-3 appear to form the binding site for the metal ion [5,6]. In contrast, in the β_2-adrenergic receptor a His residue in *intra*cellular loop 3 negated the Zn^{2+}-enhanced binding [9,10]; however this residue was not responsible for the Zn^{2+}-mediated potentiation of cAMP accumulation. It should be noted that, for example, two His residues located in the N-terminal domain of the β_2-adrenergic receptor were not addressed in that study and that these residues – by analogy to the observations in the present study of GPR39 – could well be responsible for the potentiating effect of Zn^{2+}.

Zn^{2+} is likely the endogenous ligand for GPR39. In the endocrine pancreas Zn^{2+} functions as a chemical messenger co-stored and secreted from the insulin-producing β-cells [11,30]. Zn^{2+} inhibits glucagon secretion from the neighboring α-cells and it has been argued that the main signal initiating glucagon secretion during hypoglycemia is in fact the decrease in Zn^{2+} [31,32]. Zn^{2+} also appears to have an autocrine feedback function on the β-cells. Interestingly, treatment with Zn^{2+} can prevent or ameliorate both streptozotocin-induced and spontaneous diabetes in mice [33–35]. Moreover, a non-synonymous polymorphism in a Zn^{2+} transporter (ZnT-8) was recently reported to be associated with type-2 diabetes and autoantibodies directed against this protein were found in 60–80% of new onset type-1 diabetes [36,37]. Both type-1 and type-2 diabetes are associated with excessive apoptosis of pancreatic β-cells [38]. Zn^{2+} is a potent inhibitor of cell death and Zn^{2+}-depletion can lead to apoptosis [39,40]. Recently, GPR39 was demonstrated to inhibit cell death and it is tempting to speculate that the protective action of Zn^{2+} in diabetes models could be mediated through GPR39 [41].

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