Two SUR1-specific Histidine Residues Mandatory for Zinc-induced Activation of the Rat $K_{\text{ATP}}$ Channel

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**Running title:** Molecular site of $K_{\text{ATP}}$ channels activation by zinc
SUMMARY

Zinc at micromolar concentrations hyperpolarizes rat pancreatic β-cells and brain nerve terminals by activating ATP-sensitive potassium channels (K\textsubscript{ATP}). The molecular determinants of this effect were analyzed using insulinoma cell lines and cells transfected with either wild type or mutated K\textsubscript{ATP} subunits. Zinc activated K\textsubscript{ATP} in cells co-expressing rat Kir6.2 and SUR1 subunits, as in insulinoma cell lines. In contrast, zinc exerted an inhibitory action on SUR2A-containing cells. Therefore, SUR1 expression is required for the activating action of zinc, which also depended on extracellular pH and was blocked by diethylpyrocarbonate, suggesting histidine involvement. The five SUR1-specific extracellular histidine residues were submitted to site-directed mutagenesis. Out of them, two histidines (H326 and H332) were found to be critical for the activation of K\textsubscript{ATP} by zinc, as confirmed by the double-mutation H\textsubscript{326,332}A. In conclusion, zinc activates K\textsubscript{ATP} by binding itself to extracellular H326 and H332 of the SUR1 subunit. Thereby zinc could exert a negative control on cell excitability and secretion process of pancreatic β-and α-cells. Actually we have recently showed such mechanism is occurring in hippocampal mossy fibers, a brain region characterized, like pancreas, by an important accumulation of zinc and a high density of SUR1-containing K\textsubscript{ATP}.

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INTRODUCTION

$K_{\text{ATP}}$ channels are tetradimeric complexes of two structurally unrelated subunits (1-4): an inwardly rectifying $K^+$ channel subunit ($\text{Kir}6.x$) which serves as an ATP-inhibitable pore (5), and a sulfonylurea receptor subunit (SUR) which belongs to the ATP-binding cassette transporter superfamily and endows the channel with sensitivity to Mg-nucleotides, channel openers and sulfonylureas (6). To date, two Kir6.x genes have been described, Kir6.1 and Kir6.2 (7-9). As for the SUR subunit, two closely related genes, SUR1 and SUR2 have been cloned, with different splice variants such as SUR2A and SUR2B (6; 10-12). Depending on tissues or organs, the different molecular forms of SUR and Kir6.x proteins co-assemble to form $K_{\text{ATP}}$ channels with different functional and pharmacological properties (13; 14). Cardiac and skeletal muscle $K_{\text{ATP}}$ channels comprise of Kir6.2 and SUR2A, whilst vascular smooth muscle $K_{\text{ATP}}$ channels combine the subunit SUR2B with either Kir6.1 or Kir6.2. The pancreatic $\beta$-cell $K_{\text{ATP}}$ channels involved in insulin secretion comprise of Kir6.2 and SUR1. This pattern is also abundant in the mammalian central nervous system, especially in the hippocampus mossy fiber nerve terminals (15). In addition, several other subunit combinations have been described in the central nervous system (14).

Strikingly, several structures possessing $K_{\text{ATP}}$ channels of the Kir6.2 and SUR1 type, such as pancreatic $\beta$-cells and hippocampal mossy fibers, also contain substantial amounts of zinc (16-18). Puzzled by this co-localization, we found that micromolar concentrations of zinc hyperpolarize $\beta$-cells from an insulin-secreting pancreatic line (RINm5F) by activating $K_{\text{ATP}}$ channels (19). Moreover, this mechanism was also found using mossy fiber synaptosomes of the rat hippocampus (20). In both cases $K_{\text{ATP}}$ activation by zinc resulted in a decreased secretion of insulin from $\beta$-cells and of glutamate from mossy fiber terminals. This effect might be
physiologically relevant since it suggests that the transition metal could be involved in a paracrine or autocrine negative feedback regulation of release. However, in contrast to our results, Kwok and Kass (21) observed a block of cardiac $K_{ATP}$ channels by low concentrations of extracellular divalent cations, including zinc. This discrepancy led us to hypothesis that zinc could induce opposite effects on different types of $K_{ATP}$, depending on the subunit composition of the channel. The cloning of cDNAs encoding $K_{ATP}$ channel subunits afforded us the opportunity to determine the molecular basis of zinc-induced activation of recombinant $K_{ATP}$ channels.

Thus, the present study aimed to investigate the mechanisms by which zinc activates or inhibits $K_{ATP}$ channels and more particularly whether definite molecular compositions of SUR and Kir subunits are critical for these effects. Preliminary results (22) suggested that the SUR1 subunit was involved in $K_{ATP}$ activation by extracellular zinc. So far, however, systematic subunit-combination analysis and clear-cut identification of domain and/or amino acid residues involved in zinc binding to $K_{ATP}$ have not been performed. To achieve this, we used electrophysiological techniques to investigate the effects of zinc on recombinant $K_{ATP}$ reconstituted in HEK293T cells by transient transfection of various combinations of rat SURx and Kir6.x subunits. By using pH modifications, pharmacological tools and site-directed mutagenesis, we identified two histidine residues present in the extracellular side of SUR1 as mandatory for activation of $K_{ATP}$ channels by zinc.

**EXPERIMENTAL PROCEDURES**

**Cell culture**

Human embryonic kidney cells (HEK293T) and insulin-producing cell lines (RINm5F and INS-1E) were obtained from the Laboratoire de Biochimie Clinique, CMU, Geneva. Cells were cultured
at 37 °C and in a humidified 5% CO₂/95% air atmosphere, in RPMI 1640 medium (GIBCO BRL, Life Technologies AG, Basel, Switzerland) supplemented with 10% fetal calf serum, 11 mM glucose, 100 U/ml of penicillin and 100 µg/ml of streptomycin. One to five days before experiments were carried out, RINm5F, INS-1E and transfected HEK293T cells were seeded out onto Falcon 3001-type Petri dishes (Becton Dickinson UK Ltd., Plymouth, England) at 100-200,000 cells per dish in RPMI 1640 medium.

**Cloning**

Rat Kir6.1 and Kir6.2 cDNAs (GenBank D42145.1 and X97041.1, respectively) were cloned from rat lung and the rat insulinoma cell line RINm5F, respectively, using a RT-PCR-based strategy with 5'- and 3'- untranslated region-specific primers based upon the published sequence (7; 8). The resulting PCR products (1290 bp and 1180 bp for Kir6.1 and Kir6.2, respectively) were subcloned into pRCII (Invitrogen BV, Groningen, The Netherlands), and the sequences were confirmed on six independently isolated clones. cDNA encoding the rat sulfonylurea receptor SUR1 (GenBank X97279.1), was isolated by RT-PCR from RINm5F cells. Three overlapping PCR products were generated using specific oligonucleotides primer pairs based upon the known sequence (10). The amplified products were subcloned into pBluescript II KS+ (Invitrogen BV, Groningen, The Netherlands). The sequences were confirmed on several independently isolated clones before the reconstitution of the full-length cDNA.

**Site-directed mutagenesis**

Sequence of the SUR1 and SUR2A subunits of K<sub>ATP</sub> were obtained from GenBank, (accession numbers: X97279.1 and D83598.1 respectively). In vitro site-directed mutagenesis of selected histidine residues on rat SUR1 gene were performed each separately on the pcDNA3/SUR1
vector using the QuickChange site-directed mutagenesis kit from Stratagene. Each mutation introduced a single amino acid, changing a histidine to an alanine residue in the corresponding coding region of the cDNA. The double mutation H_{326+332}A was done on the single cDNA mutant histidine_{332} by using mutagenic primers (F/326 and R/326) introducing the H_{326}A mutation. Mutagenesis and amplification reactions were performed with a PCR Mastercycler (Eppendorf) in 50 µl reaction volume, containing 1.5 mM MgCl2, with 50 ng DNA pcDNA3/SUR1, 125 ng of each primer, 1 ml of mix dNTPs 10mM, 3 pfu units and pfu buffer. Incorporation and extension of the mutagenic primers were done with the following cycling program: 1 run at 95°C during 30 sec and 25 runs of 30 sec at 95°C, 1 min at 55°C and 20 min at 68°C. The non-mutated parental DNA template was digested with DpnI during 1 hour at 37°C and 5 µl of the PCR mix was used to transform DH5α competent bacteria by heat shock at 37°C. Sequence of each mutated cDNAs was confirmed by full sequencing.

**Transfections**

The coding regions of wild-type rat Kir6.1, Kir6.2, SUR1, SUR2A or mutated SUR1 cDNAs were subcloned into the expression vector pcDNA3 (Invitrogen BV, Groningen, The Netherlands). The combinations of these plasmids were transfected into HEK-293T cells grown until 70 % confluence in Falcon 3004-type Petri dishes (Becton Dickinson UK Ltd, Plymouth, England). Transfections were performed overnight by CaCl2 precipitation with 4 µg / well of Kir6.x + SURx combinations (ratio 1:3), together with 0.5 µg of vector pEGFP (Invitrogen BV, Groningen, The Netherlands) in fresh DMEM plus 10 % FCS medium. Twelve hours after transfection cells were seeded out onto Falcon 3001-type Petri dishes and cultured in RPMI 1640 medium. The cells expressing GFP were identified by fluorescence microscopy and used for electrophysiological recordings.
Electrophysiology

Whole-cell recordings were performed using fired-polished electrodes, pulled from borosilicate glass, and showing an open resistance of 2-3 MΩ. Signals were amplified using an Axopatch 200-B amplifier and filtered through a 4-pole low-pass Bessel filter at 1 or 2 kHz, before digitisation with a Digidata 1200 interface and analysis with pClamp 8 software (Axon Instruments, Inc., Forster City, CA, USA). Capacitative transients and series resistance were compensated (≥ 70%), using the circuitry incorporated to the amplifier. The external solution contained (in mM): 145 NaCl; 3 KCl; 2 CaCl₂; 2 MgCl₂; 10 Hepes; 10 D-glucose. The pH was adjusted (with NaOH) to 7.2 in standard conditions and from 5.6 to 8.0 for pH-dependence studies. The patch pipette solution contained (in mM): 10 NaCl; 140 KCl; 1 MgCl₂; 10 Hepes; 1 EGTA; 1 Mg-ATP; pH: 7.2 (KOH). All experiments were performed at room temperature (20-22°C), and the equilibrium potential for K+ ions (E_K⁺), calculated after correction of the liquid junction potential, was about -82 mV.

Data expression

In histograms, data are expressed as mean ± S.E.M. of zinc-induced changes in holding current normalized to control level that is, to the current measured before zinc applications. Control current level was determined by subtraction of tolbutamide- or glibenclamide-insensitive currents. n indicates the number of individual cells recorded each conditions.

Chemicals

Diethylpyrocarbonate (DEPC) was obtained from Brunschwing, (Basel, Switzerland), Glibenclamide from RBI (Fluka Chemie AG, Buchs, Switzerland) and zinc chloride and other
inorganic salts (with negligible listed zinc contamination) were from Sigma (Sigma-Aldrich Co, Fluka Chemie AG, Buchs, Switzerland, puriss. p.a. ACS reagent grade). All solutions were prepared in distilled-deionized water to minimize basal zinc levels.

(Figure 1 about here)

RESULTS

Zinc activates native and recombinant Kir6.2/SUR1 K<sub>ATP</sub> channels

Figure 1 (A and B) shows that extracellular zinc at micromolar concentrations activates K<sub>ATP</sub> current in two insulinoma cell lines (RINm5F and INS-1E), confirming our previous observation (19). The current enhanced by zinc reversed itself at approximately -80 mV and was fully blocked by tolbutamide. The rat Kir6.2/SUR1 K<sub>ATP</sub> was reconstituted in HEK293T cells, a human embryonic kidney cell line, which does not express endogenous K<sub>ATP</sub> channels (23). Like native insulinoma cell lines, Kir6.2/SUR1-transfected HEK293T cells displayed an outward current which reversed itself at about -80 mV. This current was activated by zinc (10 µM), and suppressed by tolbutamide (Figure 1C). HEK293T cells transfected with e-GFP alone or with only one K<sub>ATP</sub> subunit, either Kir6.2 or SUR1, did not display K<sub>ATP</sub> or any endogenous current sensitive to zinc (Figure 1D).

(Figure 2 about here)

The effect of zinc on currents carried by K<sub>ATP</sub> channels of different subunit compositions

The effect of zinc was investigated on several clones transfected with different combinations of K<sub>ATP</sub> channel subunits. Cells were held at -40 mV and the effects of drugs were recorded as a function of time. The pattern shown by the Kir6.2/SUR1 clone was very similar to that of
RINm5F cells. Zinc reversibly enhanced a pre-existing current that was also enhanced by diazoxide and completely abolished by tolbutamide. Actually, the effect of zinc was twofold: zinc induced first a rapid activation (“ON effect”), which was followed by a slower inhibiting effect (“OFF effect”). The latter could easily be seen upon zinc washout (Figure 2A and B). In the present work we focused our investigation on the activating ON effect. In cells transfected with Kir6.1/SUR1 subunits, zinc exerted a stronger activation than in Kir6.2/SUR1 clones (Figure 2C). In contrast, cells transfected with either the Kir6.2/SUR2A or the Kir6.1/SUR2A combination presented a glibenclamide-sensitive current, which was not activated but inhibited by zinc application (Figure 2D and E). The inhibitory effect of zinc was particularly visible when pinacidil was used to previously open this type of K<sub>ATP</sub> channels.

A more quantitative evaluation is summarized in Figure 2F, where the effect of zinc is expressed as a function of the pre-existing K<sub>ATP</sub> current amplitude. RINm5F cells and the Kir6.1/SUR1 and Kir6.2/SUR1 clones characteristically displayed an activating effect. In contrast, Kir6.1/SUR2A and Kir6.2/SUR2A clones exhibited an inhibitory effect. Therefore, activation of K<sub>ATP</sub> current by zinc requires SUR1 subunit expression. Also in the presence of Kir6.1, combined with either SUR1 or SUR2A subunits, the amplitude of zinc-induced effects was enhanced.

(Figure 3 about here)

**pH dependency of zinc-induced K<sub>ATP</sub> activation**

It has been shown that zinc ions can interact with proteins by direct binding to either histidine, cysteine, aspartate or glutamate residues (24-26). The binding of zinc to these amino acids is expected to be differently affected by changes in H<sup>+</sup> ion concentration. In the case of K<sub>ATP</sub>, this should modify zinc-induced activation of the channel. To test this, the action of zinc on the K<sub>ATP</sub>
current was examined as a function of extracellular pH on RIN-5F cells. As shown in Figure 3A, zinc provoked a more pronounced activation of the $K_{ATP}$ current at pH 8.0 than at pH 6.8. At pH 5.6, zinc was clearly inhibitory. By titrating the effect of zinc as a function of pH over the range of 5.6 and 8.0, we found a reverse point which was close to pH 6.4 (Figure 3B). This profile of pH-dependency suggested a histidine side chain (pKa ~ 6-7) rather than a cysteine (pKa ~ 8-9) as the relevant target at the zinc binding site (27).

(Figure 4 about here)

**The histidine modifying reagent diethylpyrocarbonate blocks zinc effect**

Diethylpyrocarbonate (DEPC), a substance which reacts with neutral imidazole groups, provided an additional test to confirm the involvement of histidine residues. We pre-treated Kir6.2/SUR1 transfected HEK293T cells with 1 mM DEPC for 1-2 minutes. As a result the activating action of zinc on $K_{ATP}$ current was abolished, but the basic $K_{ATP}$ current was not affected. The blockade by DEPC could be partially reversed by giving hydroxylamine (0.1 M) for a few minutes (Figure 4).

To examine nevertheless a possible role of cysteine residues, we used the sulfhydryl-modifying reagent methanethiosulfonate ethyltrimethylammonium (MTSET$^+$), a compound which is poorly permeable through membranes (28) and thus will act only on extracellular sites under our conditions. At the concentration of 1 mM for 1-2 min, MTSET$^+$ application did not modify the effects of zinc on Kir6.2/SUR1 transfected HEK293T cells (not illustrated). These observations strengthened the hypothesis that histidine residues on the SUR1 subunit were crucial for the activation of $K_{ATP}$ channels by zinc, most probably without implication of extracellular cysteines.
**Binding site for zinc ions on the $K_{ATP}$ channel SUR1 subunit: site directed mutagenesis**

Comparison of the extracellular segments of SUR subunits of the rat $K_{ATP}$ channel revealed that five histidine residues are present in SUR1 but not in SUR2A. These are: H11 and H160, located in the first transmembrane domain (TMD-0); H326 and H332 in TMD-1, and H1273 in TMD-2.

SUR1-specific extracellular histidines were individually mutated and replaced by the neutral amino acid, alanine. Figure 5 illustrates the site of the different mutations (Figure 5A) and the corresponding $K_{ATP}$ currents recorded in the response to voltage ramps before, during and after zinc application (Figure 5B). Averaged zinc-induced changes in wild type and mutated channels are expressed in Figure 5C as a percentage of the control $K_{ATP}$ current measured before zinc application. Mutations H11A; H160A and H1273A had no effect, as zinc enhanced the $K_{ATP}$ current in these clones to the same extent as in the wild type Kir6.2/SUR1 channels. In contrast, both mutations H326A and H332A, and even more so the double mutation H326+332A, converted the activation into inhibition. Thus, the site of zinc activation of rat $K_{ATP}$ channel requires the H326 and H332 residues of SUR1, which are situated on the first extracellular loop of the second transmembrane domain (TMD-1).

*(Figure 5 about here)*

**DISCUSSION**

**SUR1 is the target of $K_{ATP}$ activation by zinc**

Our results should contribute to solve a discrepancy concerning the opposite effects of extracellular zinc ions on $K_{ATP}$ channels of β-pancreatic and neuronal types versus the cardiac type. On the one hand, Kwok and Kass (21) reported that micromolar zinc concentrations inhibited the opening of cardiac $K_{ATP}$ channels, on the other hand, our data indicated that the
same treatment activated $K_{ATP}$ channels in murine pancreatic $\beta$-cells (19) and hippocampal mossy fiber nerve terminals (20). We showed here that zinc effects are actually twofold. Inhibition occurs with all types of channels but activation is prevalent in SUR1-containing $K_{ATP}$. Therefore, the SUR1 subunit is absolutely required for the activating action. Channels containing the SUR1 subunit are particularly expressed in pancreatic $\beta$-cells as well as hippocampus granular cells and their mossy fiber terminals (15), whereas SUR2A and SUR2B subunits are present in $K_{ATP}$ channels of cardiac and skeletal muscles as well as of vascular and other smooth muscles (13; 14). This provides an explanation for the opposite effects of zinc on $K_{ATP}$ channels present in different structures. It is interesting, in this connection, to recall also that pancreatic $\beta$-cells and mossy fiber terminals of hippocampus are not only rich in SUR1 containing channels but contain the highest zinc concentration of the organism (16; 17).

**Identification of zinc binding sites on ion channels**

Zinc can interact with various ligand-gated and voltage-gated ion channels generally causing inhibition. Interestingly, the action of zinc on glycine receptors has been reported to be biphasic, potentiation and inhibition, corresponding to separate binding sites (29). Our present study suggests the same for $K_{ATP}$ channels where activation or inhibition by zinc may occur at different sites of the Kir and SUR subunits.

Histidine residues have been identified as essential for zinc inhibition of several subtypes of GABA receptors, depending on critical localization of histidines on definite subunits (28; 30-32). Also, in recombinant NMDA receptors critical histidines (H42, H44 and H128) are required for high-affinity, voltage-independent, pH-dependent zinc inhibition (33; 34).
Activation of acid-sensing ion channels by zinc implicates two critical histidines (H162 and H339) as well (35). In addition to histidine residues, cysteines have also been shown to participate in zinc binding to ion channels. As an example C546 is critical for zinc blockage of human skeletal muscle chloride channels (27).

The case of AMPA type glutamate receptor is particularly interesting in connection with the present work since it generates currents that are potentiated by micromolar concentrations of zinc (36). Histidine 412 seems to be critical for zinc binding on the AMPAR since it is present on GluR2-4 (sensitive to zinc) but not on GluR1 (non-sensitive) subunits (37).

We also identified two histidines (H326 and H332) as critical amino acid residues for the activation action of zinc on the extracellular side of K\textsubscript{ATP} SUR1 subunit. The site is localised on the short extracellular loop between the first and second segment of TMD1. The zinc binding site seems to be fully independent from the sulfonylurea binding site which is localized in the intracellular side of SUR on TMD2 (38; 39). Indeed, tolbutamide equally blocked K\textsubscript{ATP} current in all mutated clones (Figure 5) and the activation by zinc was additive to the activation induced by diazoxide (Figure 2).

**Physiological significance of K\textsubscript{ATP} channel activation by zinc**

By linking the cell metabolism with the membrane potential, and thereby electrical activity, K\textsubscript{ATP} channels regulate important physiological functions in various tissues. In the pancreatic \(\beta\)-cells, the K\textsubscript{ATP} channels are critically involved in the control of glucose-induced insulin secretion. Increase in glucose metabolism in the vicinity of K\textsubscript{ATP} channels depolarizes the \(\beta\)-cells membrane, leading to opening of voltage-dependent Ca\textsuperscript{2+} channels and thereby to increase in insulin secretion (40). Zinc is co-stored with insulin in pancreatic granules and co-released by
exocytosis (17). We found that micromolar concentrations of zinc hyperpolarized the β-cells by activating $K_{\text{ATP}}$ channels, reducing cell excitability and calcium spike firing (19). As a consequence, further release of insulin is reduced (V. Bancila et al., in preparation). In this way, zinc could ensure a negative feedback on insulin and on its own secretion. However, a more relevant physiological role for activation of pancreatic $K_{\text{ATP}}$ channels by β-cells-secreted zinc can be to regulate glucagon release from α-cells. Indeed, α-cells are also provided with SUR1-containing $K_{\text{ATP}}$ channels (41) and, since intra-islet circulation is expected to carry zinc from β-cells toward α-cells, release of glucagon will be reduced when insulin and zinc co-release is intense. This hypothesis is supported by the work of Ishihara and colleagues (42) who demonstrated that zinc secreted by β-cells is implicated in the suppression of glucagon secretion observed in response to β-cells activation. In this view, zinc would amplify the overall effect of insulin by decreasing that of glucagon. Thereby, the same stimulus may control reversely regulated secretion from the two main pancreatic cellular populations.

Similarly, in mossy fibre/CA3 neuron synapses of the hippocampus, zinc is concentrated in synaptic vesicles of nerve terminals. On stimulation, it is released (43-45) and recent experiments showed that zinc at low concentrations activates presynaptic $K_{\text{ATP}}$ channels, reducing further glutamate release (20). At this level, zinc could play a role of negative feedback on transmission.

**Pathological implications**

Such data may be relevant to pathological states of the pancreas, in view of the importance of β-cell dysfunction in the pathogenesis of type-2 diabetes, or hyper-insulinism. Some studies (46-48), but not all (49-51), have reported disorganized oscillations of insulinemia in patients with type-
2 diabetes and their near relatives with mild glucose intolerance. Insulin resistance of obese patients also accompanied by a decrease in the regularity of pulsatile insulin secretion (52). Because oscillations of insulinemia favor optimal glucose homeostasis, one may speculate that a disturbance of this process in type-2 diabetes contributes to the disease. It would be most interesting to investigate whether the regulation of $K_{ATP}$ channels and/or the feedback action of zinc might be perturbed in these affections.

Zinc and $K_{ATP}$ channels might also be involved in pathological conditions affecting the central nervous system. We recently found that micromolar zinc protects nerve terminal from excessive depolarization, preventing massive transmitter release and neuronal death consecutive to anoxic insult. This neuroprotective effect was antagonized by tolbutamide, indicating $K_{ATP}$ implication (20). Moreover, over-expression of SUR1 or Kir6.2 subunits in mice has been reported to render animals more resistant to ischemic insults (53; 54). Also, Kir6.2 KO mice are more sensitive to hypoxia-induced seizures (55).

In addition, zinc chelation or zinc deficiency is associated with an increased seizure susceptibility (56; 57). Mice knock-out for the vesicular zinc transporter ZnT3 are more vulnerable to chemically-induced seizures and consequent neuronal damage (58). These observations could be explained by the mechanism described herein, i.e. a failure of $K_{ATP}$ activation when synaptic zinc is low or absent.

Conclusions

In pancreatic $\beta$-cells and hippocampal mossy fibers, zinc is concentrated in large amounts and released on activity. These cells are also characterized by a high density of $K_{ATP}$ channels composed of the SUR1/Kir6.2 subunits. Since the extracellular concentration of zinc following release probably reaches the range ($\sim 1 \mu M$) needed for activation of $K_{ATP}$ of this type, zinc can...
act as a negative feedback regulator of secretion of both insulin and glutamate. This effect is mediated by two particular amino acids (H326 and H332) of the rat SUR1 subunit, located in the first extracellular loop of TMD-1.

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ABREVIATION FOOTNOTES

K$_{\text{ATP}}$, ATP-sensitive K$^+$ channel; SUR, sulfonylurea receptor; WT, wild type; Kir, inward rectifier potassium channel
FIGURE LEGENDS

Fig. 1. Zinc activates $K_{ATP}$ current of native insulinoma cells, and cells co-expressing rat Kir6.2 and SUR1 channel subunits. Cells were held at -80 mV in voltage-clamp whole-cell configuration and currents were recorded in response to 3 s voltage ramps from -120 to -40 mV, in the presence or absence of 10 $\mu$M zinc in the extracellular medium. (A and B) In native insulinoma RINm5F or INS-1E cells, zinc enhanced a basic membrane current which reversed itself at approximately -80 mV. Tolbutamide (200 $\mu$M) suppressed both the pre-existing $K_{ATP}$ current and its enhancement by 10 $\mu$M zinc (bottom traces; the two traces in this recording cannot be distinguished). (C) Zinc exerted a clear activating effect on HEK293T cells co-expressing rat Kir6.2 and SUR1 channel subunits. (D) No $K_{ATP}$ current and no zinc effect were recorded under the same experimental conditions in HEK293T cells transfected with the SUR1 subunit alone (control and zinc traces cannot be distinguished).

Fig. 2. The action of zinc on $K_{ATP}$ depends on the channel subunit composition. Cells were voltage-clamped at -40 mV in the whole-cell configuration. Drugs were perfused in the extracellular medium at the indicated concentrations during the periods of time marked by corresponding blocks. Zn, zinc 10 $\mu$M; D, diazoxide 100 $\mu$M; T, tolbutamide 200 $\mu$M; G, glibenclamide 5 $\mu$M; P, pinacidil 5 $\mu$M. Calibration: 10 sec and 100 pA (200 pA in C and E). (A) In native RINm5F cells, zinc enhanced a basic outward current. However, the initial increase in current amplitude (ON effect) was followed by a slower inhibiting effect which was clearly visible on zinc washout (OFF effect). Also diazoxide activated the outward current, an effect which was slightly additive to that of zinc. Tolbutamide fully blocked the current, attesting that it
arose from opening of $K_{\text{ATP}}$ channels. (B) The same pharmacological pattern was obtained by using HEK293T cells co-expressing the rat Kir6.2 and SUR1 subunits. (C) With the Kir6.1/SUR1 composition, zinc and diazoxide exerted a stronger activating action. (D) In contrast, zinc and diazoxide did not activate, but rather inhibited, the $K_{\text{ATP}}$ current of HEK293T cells co-expressing rat Kir6.2 and SUR2A subunits. (E) Finally, when HEK293T cells expressed the rat Kir6.1/SUR2A combination, pinacidil markedly activated the $K_{\text{ATP}}$ current, but zinc inhibited both the basic current and even more so the pinacidil-enhanced current. In mutants containing the SUR2A subunit, glibenclamide was used instead of tolbutamide to suppress $K_{\text{ATP}}$ currents. (F) The rapid action of zinc ($I_{Zn}$), either positive (activation) or negative (inhibition), is expressed as the ratio to the corresponding basic outward current ($I_C$). Mean ± S.E.M. values of the number or determinations indicated above histograms. The activating effect of zinc depended on the presence of SUR1; it was larger when Kir6.1 was co-expressed instead of Kir6.2.

**Fig. 3. The pH dependence of zinc action on $K_{\text{ATP}}$ currents in insulinoma cells.** (A) Outward currents were recorded from RIN-5F cells held at -40 mV at the indicated pH values and tolbutamide was added at the end to identify signals as $K_{\text{ATP}}$ currents. Zinc activated $K_{\text{ATP}}$ current when pH was higher than 6.4, but inhibited it at lower pH values. (B) The effect of zinc on $K_{\text{ATP}}$ current ($I_{Zn}$) was measured as the ratio to basic current ($I_C$), and expressed (mean ± S.E.M) as a function of pH. The number of determinations is indicated.

**Fig. 4. Effects of DEPC and hydroxylamine on the activating action of zinc on $K_{\text{ATP}}$ currents.** HEK293T cells co-transfected with rat Kir6.2 and SUR1 subunits were recorded in whole-cell configuration. (A) Currents elicited by 3 s voltage ramps from -120 to -40 mV (holding potential -80 mV). Application of 10 µM zinc enhanced the outward current reversing at
about -80 mV (traces in the top). After pre-incubation with 1mM DEPC during 1-2 min, the
effect of zinc was practically annihilated. (B) In a series of 6 determinations (± s.e.m.), the
activating effect of zinc was expressed as a ratio to the basic outward current. Pre-incubation with
DEPC reduced zinc effect to 12%. Application of hydroxylamine (0.1 M) to DEPC-blocked cells
for 1-2 min partly re-activated the action of zinc on the \( \text{K}_{\text{ATP}} \) current.

**Fig. 5.** Determination of histidine residues involved in the activating action of zinc on \( \text{K}_{\text{ATP}} \)
channels. The action of zinc on \( \text{K}_{\text{ATP}} \) currents was determined in series of rat SUR1 mutants,
where each extracellular SUR1-specific histidine was replaced by alanine. SUR1 mutants were
co-transfected in HEK293T cells with the rat Kir6.2 subunit. (A) Site of the SUR1 histidine
mutation. TMD-0 to TMD-2, transmembrane domains. (B) Effect of 10 \( \mu \)M zinc in the
corresponding clones. Currents were measured in response to 3 s voltage ramps from -120 to
-40 mV (holding potential: -80 mV). Zinc (10 \( \mu \)M) was applied during the time indicated by the
black bar. Calibration: 1 nA and 20 sec (C) Percent \( \text{K}_{\text{ATP}} \) current increase, or decrease, caused by
zinc in the corresponding clone. Mean ± s.e.m. of the indicated number of determinations.
Mutations H326A and H327A, and the double mutation H326A+H327A suppressed zinc activation of
\( \text{K}_{\text{ATP}} \). Mutations H11A, H160A and H1273A did not affect the zinc activation of \( \text{K}_{\text{ATP}} \) current, whose
amplitude remained equal to that of the wild type rat SUR1/Kir6.2 clone.
Fig. 4

(A) Graph showing the current response with and without Zn²⁺.

(B) Bar graph showing the ratio of I_Zn/I_c for Control, DEPC, and Hydroxylamine.
Two SUR1-specific histidine residues mandatory for zinc-induced activation of the rat KATP channel
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