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Copper and protons directly activate the zinc-activated channel

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ABSTRACT

The zinc-activated channel (ZAC) is a cationic ion channel belonging to the superfamily of Cys-loop receptors, which consists of pentameric ligand-gated ion channels. ZAC is the least understood member of this family so in the present study we sought to characterize the properties of this channel further. We demonstrate that not only zinc (Zn^{2+}) but also copper (Cu^{2+}) and protons (H^+) are agonists of ZAC, displaying potencies and efficacies in the rank orders of $H^+ > Cu^{2+} > Zn^{2+}$ and $H^+ > Zn^{2+} > Cu^{2+}$, respectively. The responses elicited by Zn^{2+}, Cu^{2+} and H^+ through ZAC are all characterized by low degrees of desensitization. In contrast, currents evoked by high concentrations of the three agonists comprise distinctly different activation and decay components, with transitions to and from an open state being significantly faster for H^+ than for the two metal ions. The permeabilities of ZAC for Na^+ and K^+ relative to Cs^+ are indistinguishable, whereas replacing all of extracellular Na^+ and K^+ with the divalent cations Ca^{2+} or Mg^{2+} results in complete elimination of Zn^{2+}-activated currents at both negative and positive holding potentials. This indicates that ZAC is non-selectively permeable to monovalent cations, whereas Ca^{2+} and Mg^{2+} inhibit the channel. In conclusion, this is the first report of a Cys-loop receptor being gated by Zn^{2+}, Cu^{2+} and H^+. ZAC could be an important mediator of some of the wide range of physiological functions regulated by or involving Zn^{2+}, Cu^{2+} and H^+.

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1. Introduction

The zinc-activated channel (ZAC) is a ligand-gated ion channel (LGIC) belonging to the Cys-loop receptor superfamily which also includes the nicotinic acetylcholine (nACh), 5-hydroxytryptamine type-3 ($5\text{-}HT_3$), γ-aminobutyric acid type A ($GABA_A$) and glycine receptors [1]. These classical members of this superfamily play important roles in physiology and pathology and thus constitute interesting drug targets in a wide range of disorders [2–5]. ZAC is the least understood member of this family, partly because the gene encoding for ZAC is a pseudogene in mouse and rat genomes, which has complicated explorations into the physiological function of the receptor [1,6].

ZAC displays very little amino acid sequence similarity with other members in the superfamily, and thus it is classified within its own subgroup, the closest matches to ZAC being the human 5-HT3A, 5-HT3B and nACh α7 subunits that exhibit approximately 15% amino acid sequence identity to ZAC [1,6]. Expression studies have detected ZAC mRNA in human fetal whole brain, spinal cord, pancreas, placenta, prostate, thyroid, trachea and stomach [1,6]. Furthermore, the presence of ZAC mRNA has been demonstrated by RT-PCR in adult human hippocampus, striatum, amygdala and thalamus [6]. The presence of ZAC has also been demonstrated in human hippocampal CA3 pyramidal cells and in the polymorphic layer of the dentate gyrus by immunolocalization techniques [6].

Transition metals are known to be essential for proper functioning of various proteins critical for cellular viability and for numerous cellular processes, and thus the intracellular and extracellular concentrations of metals are tightly regulated by metal binding proteins and metal specific transporters [7,8]. Additionally, metal ions can influence the activity of cells by modulating various ion channels, no more so than within the CNS where zinc (Zn^{2+}) and other metals are recognized as

Abbreviations: ZAC, zinc-activated channel; LGIC, ligand-gated ion channel; nACh, nicotinic acetylcholine; 5-HT, 5-hydroxytryptamine; GFP, green fluorescent protein; MOPS, 10 3-(N-morpholino)propanesulfonic acid; CTR1, high affinity copper uptake protein 1; GLIC, *Gloeobacter violaceus* pentameric ligand-gated ion channel; ATP7A, ATPase, Cu^{2+} transporting, alpha polypeptide; ATP7B, ATPase, Cu^{2+} transporting, beta polypeptide.

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neuromodulators of N-methyl-D-aspartate, $GABA_A$, nACh, 5-HT_3 and glycine receptors [9]. Zn^{2+} is essential for normal prenatal and postnatal development and is especially important for fetal brain function [10]. Levels of Zn^{2+} and other metals also appear to be important in neurodegenerative disorders such as Alzheimer's disease as well as other neurological disorders that result in neuronal loss, including epilepsy and ischemia [11–14]. Within the periphery, metals play important roles in the proper functioning of gastrointestinal, immune and reproductive systems [10]. The pancreas and prostate accumulate and release abundant Zn^{2+} and impairment of Zn^{2+} signaling is associated with diabetes, infertility and cancer [15]. Hence, given the overlapping location of ZAC and the systems affected by imbalances in metal signaling, the receptor may govern important functions for human health.

In our previous study of ZAC, this channel was shown to spontaneously open (open in the absence of agonist) when expressed in human embryonic kidney 293 (HEK293) cells, and application of Zn^{2+} was found to evoke a reversible inward current [1]. However, the small amplitudes of Zn^{2+}-activated currents through ZAC in HEK293 cells hampered the investigation of the properties of the channel. In the present study, we have explored further the functional properties of ZAC expressed in COS-7 cells, another mammalian cell line often employed to study recombinant proteins. We report the discovery of two additional agonists for ZAC (Cu^{2+} and H^+) and present new findings about the biophysical properties of the receptor.

2. Materials and methods

2.1. Cell culture and transfections

COS-7 cells (ATCC CRL-1651) and HEK293 cells (ATCC CRL-1573) were cultured in Dulbecco's Modified Eagle's Medium supplemented with 10% calf serum, 100 i.u./ml penicillin and 100 μg/ml streptomycin at 37 °C in a humidified 5% CO_2, 95% air atmosphere. Cells were electroporated (110 V, 20 ms Biorad Gene Pulser Xcell, Hercules, CA) with cDNA encoding for human ZAC along with GFP cDNAs (in pCDM8). Both cDNAs encoding a myc-tagged and the un-tagged ZAC were used, and since no differences in agonist-evoked current characteristics were observed between cells electroporated with the two constructs, the data were pooled. Cells were used 24–72 h after electroporation.

2.2. Patch-clamp electrophysiology

The whole-cell configuration of the patch-clamp technique was used to record currents from voltage-clamped COS-7 cells. For agonist concentration–response relationship experiments, cells were superfused, at a rate of 2 ml/min, with an extracellular solution containing (in mM): 140 NaCl, 5 KCl, 1.2 $MgCl_2$, 2.5 $CaCl_2$, 10 3-(N-morpholino)propanesulfonic acid (MOPS), 11 glucose and adjusted to pH 7.4 with NaOH. MOPS was used in this study as it is a Good's zwitterionic buffer that does not form complexes with the metals, unlike the typical HEPES buffer [16]. Borosilicate glass patch pipettes (resistance 2–5 MΩ) contained (in mM): 140 KCl, 2 $MgCl_2$, 0.1 $CaCl_2$, 1.1 EGTA, 10 HEPES, and adjusted to pH 7.4 with KOH.

In the experiments on H^+-activated currents, acidic solutions that were rapidly applied to ZAC-expressing cells consisted of extracellular solutions adjusted to a pH of 7.0 with HCl. Solutions with a pH range from 4.5 to 6.5 were made up fresh on the day of experiments from addition of HCl to a solution containing (in mM): 110 NaCl, 5 KCl, 1.2 $MgCl_2$, 2.5 $CaCl_2$, 10 Trisodium citrate dihydrate, 11 glucose. In the ion substitution experiments, Zn^{2+}-activated currents were recorded in extracellular solution (E1) where KCl was replaced with additional NaCl and both $MgCl_2$ and $CaCl_2$ were reduced to lower their influence upon zinc-mediated current amplitude. E1 consisted of (in mM): 145 NaCl, 0.1 $MgCl_2$, 0.1 $CaCl_2$, 10 MOPS, 11 glucose, adjusted to pH 7.4 with measured amounts of NaOH. To determine the permeability of K^+ relative to Cs^+ E1 was exchanged with E2 where all NaCl in E1 was replaced with KCl (pH adjusted with KOH). Extracellular solution E1 was exchanged with solution E3 or E4 to determine the permeability of Ca^{2+} and Mg^{2+} relative to Cs^+ respectively. These solutions consisted of (in mM): 100 $CaCl_2$ ($MgCl_2$ for E4), 5 histidine, 10 glucose (pH 7.4). Intracellular recording solution (I1) contained (in mM) 140 CsCl, 2 $MgCl_2$, 0.1 $CaCl_2$, 1.1 EGTA, 10 HEPES, adjusted to pH 7.4 with CsOH. The free intracellular calcium concentration was estimated to be 10 nM [17].

Experiments examining the inhibitory properties of Ca^{2+} and Mg^{2+} at ZAC were conducted with recording solutions I1 and E1 (as detailed above). The concentration of extracellular Ca^{2+} was then raised from 0.1 mM to between 1 and 75 mM by the addition of $CaCl_2$. Studies examining the block of ZAC by Mg^{2+} were conducted by raising the $MgCl_2$ concentration to 30 mM. Solutions containing the raised concentrations of Ca^{2+} and Mg^{2+} in the presence or absence of Zn^{2+} were focally applied to the cell via a SF-77B fast-step perfusion system (Warner Inst., Hamden, CT).

Agonists were applied to GFP-positive cells via the fast-step perfusion system. All experiments were carried out at 32–33 °C using a recording chamber and in-line perfusion heaters (Warner Inst.). Typically, liquid junction potentials were nulled with an open electrode in the recording chamber prior to experiments. For the ion substitution studies, liquid junction potentials were measured with an open-tipped electrode and corrected post hoc. Cells were voltage-clamped at −60 mV unless otherwise noted.

2.3. Data acquisition and analysis

Currents were recorded with an Axopatch 200B amplifier (Molecular Devices, Sunnyvale, CA), filtered at 2 kHz and digitized at 20 kHz with a Digidata 1320A (Molecular Devices) and analyzed using either Clampfit (pClamp, Molecular Devices) or Graphpad Prism v.4 software (Graphpad Software, Inc., San Diego, CA). All data are expressed as the arithmetic mean ± S.E.M., and, unless stated otherwise, statistical comparisons were made using the student *t*-test with statistical significance set at $p < 0.05$.

ZAC-mediated currents exhibit an increase in current amplitude over the course of the experiment [1]. To compensate for this run-up phenomenon during concentration–response experiments 1 mM Zn^{2+} was applied before the subsequent agonist application. The amplitudes of the agonist-evoked currents were normalized to the current elicited by the prior application of 1 mM Zn^{2+}. Concentration–response relationships were fitted by nonlinear regression to a sigmoidal dose–response with variable slope equation (Graphpad).

Time constants for activation and decay of ZAC-activated currents evoked by maximal concentrations of Zn^{2+} (1 mM), Cu^{2+} (30 μM), or H^+ (10 μM, pH 5) were determined by fitting exponential functions to the corresponding component of the current wave form using the Levenberg–Marquardt algorithm with least squares minimization (Clampfit Ver. 9.2; Molecular Devices). A period of time (approximately 10 min) after the establishment of the whole-cell configuration was allowed before the ZAC-activated currents were used for analysis. This allowed for changes in current waveform that occurred during run-up to stabilize. The activation phase of the macroscopic current (between 10% and 90% of peak current amplitude) was fitted by a single exponential function. The rate of current decay upon removal of Zn^{2+}, Cu^{2+} or H^+ was determined by fitting traces to exponential equations from the start of current decay to a point at which 90% of the current had

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