A Novel Class of Ligand-gated Ion Channel Is Activated by Zn\(^{2+}\)*

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In mammals, the superfamily of “Cys loop,” ligand-gated ion channels (LGICs), is assembled from a pool of more than 40 homologous subunits. These subunits have been classified into four families representing channels that are gated by acetylcholine, serotonin, \(\gamma\)-aminobutyric acid, or glycine. By searching anonymous genomic sequence data for exons that encode characteristic motifs of the channel subunits, we have identified a novel LGIC that defines a fifth family member. Putative exons were used to isolate a full-length cDNA that encodes a protein of 411 amino acid residues. This protein (ZAC) contains all of the motifs that are characteristic of Cys loop channel subunits but cannot be assigned to any of the four established families on the basis of sequence similarity. Genes for ZAC are present in human and dog but appear to have been lost in mouse and rat genomes. Transcripts of ZAC subunits were detected in human placenta, trachea, spinal cord, stomach, and fetal brain. Transfection of human embryonic kidney cells with ZAC subunit cDNA caused expression of spontaneous current. By screening with a broad range of potential agonists and antagonists, we determined that tubocurarine inhibits the spontaneous current whereas Zn\(^{2+}\) activates the expressed receptors. The absence of Zn\(^{2+}\)-activated channels in rats and mice may explain why this fifth member of the LGIC superfamily has evaded detection until now.

Ion channels that are gated by acetylcholine, serotonin, \(\gamma\)-aminobutyrate, and glycine are a superfamily of homologous neurotransmitter receptors (nACh,\(^1\) 5-HT\(_3\), GABA\(_{\text{Ac}}\), and glycine receptors, respectively). Each of these four receptor families is composed of multiple receptor subtypes derived from distinctive assemblies of five homologous subunits (1, 2). Although a wide variety of subunit genes has been identified in mammalian genomes (42 in human), each subunit can be assigned to one of the four receptor families on the basis of the sequence similarity and, in most cases, functional activity. Many of these subunits confer unique properties to recombinant receptors in which they assemble, properties that have often been poorly defined in prior studies of native tissues (3, 4). Consequently, the identification of novel receptor subunits continues to reveal unexpected properties of this receptor superfamily.

Most of the known subunits were identified by traditional methods of cross-hybridization or expression cloning. More recently, data bases of ESTs have been used to identify more distantly related homologues through computational searches for characteristic structural motifs (5–7). However, all of these methods have inherent limitations that can hinder the identification of a complete gene family. This is particularly relevant for subunits that are distantly related, that confer unexpected properties, and that are expressed at low levels in discrete locations or for short time periods during development. For such subunits, identification may only be possible through the analysis of a completely sequenced genome. Previously, during the early stages of sequencing the human genome, we used the draft sequence data to identify an elusive subunit of 5-HT\(_3\) receptors (8). Here we have used a similar approach to uncover a very distinctive member of the subunit superfamily. This subunit cannot be assigned to any of the known receptor families on the basis of sequence similarity or functional properties. It appears to represent an old and distinct branch of the receptor superfamily that displays unique functional properties. Moreover, it also appears to be the first example of a subunit from this receptor superfamily that is expressed in human tissues but has been lost from at least some rodent species.

EXPERIMENTAL PROCEDURES

Isolation of the Human ZAC Subunit cDNA—A consensus peptide sequence of subunits for 5HT\(_3\) and nACh receptors was used to search the nr and hGtgs data bases using the TBLASTN algorithm (www.ncbi.nlm.nih.gov/blast/). Homologous peptide fragments were identified within the six-frame translation of a human genomic DNA fragment (GenBank™ accession number AC018665). Oligonucleotide primers were designed from the genomic sequence to amplify the 5’- and 3’-flanking sequences from fetal brain and spinal cord cDNA libraries using the Marathon system (Clontech). Amplification at 95 °C for 45 s, 60 °C for 60 s, and 72 °C for 2 min was performed for 35 cycles using the XL-PCR system (PerkinElmer Life Sciences). Reaction products were purified from agarose gels and sequenced directly. The open reading frame of the new subunit cDNA (termed ZAC) was amplified from a spinal cord cDNA library as described above using primers containing nucleotides 1–21 (sense) and 1266–1289 (antisense) of the ZAC subunit cDNA sequence (GenBank™ accession number AF512521). The cloned product was sequenced over its entire length to ensure that no mutations had been introduced. Sequence alignments were generated by the ClustalW program from the MacVector package of sequence analysis software (Oxford Molecular Group). A cladogram was constructed using the neighbor-joining method with pairwise distances measured by absolute differences and gaps ignored. The bootstrap consensus was generated using 1,000 replications.

Northern Blot Analysis—Samples of ~2 μg of poly(A)\(^+\) mRNA (Clontech) underwent electrophoresis on a 1.2% formaldehyde agarose gel, were transferred to nylon membranes, and were hybridized with an
Figs. 1A and 1B. Comparative sequence analysis and mRNA expression for the human ZAC subunit. A, alignment of human nAChR \( \alpha_7 \), 5-HT \( \alpha_2 \), and ZAC subunit sequences. Conserved residues are boxed and highlighted for identity (heavy shading) or chemical similarity (light shading). The potential signal sequence (S) and four putative transmembrane domains (M1–M4) are indicated by lines over the corresponding sequences. B, a cladogram of a ClustalW alignment of human subunit sequences that are homologous to residues 27–319 of the nACh receptor \( \alpha \) subunit (nAChR \( \alpha \)). The distance matrix employed neighbor joining (12) with bootstrapping (1000 repetitions). The rooting outgroup was the GABA \( \alpha_1 \) subunit (GABA, \( \alpha_1 \)). Branch points predict the order of divergence from a common ancestral gene. C, hybridization of poly(A)\(^+\) mRNA from small intestine (lane 1), placenta (lane 2), lung (lane 3), peripheral blood leukocytes (lane 4), and brain (lane 5) with a \( ^{32}\)P-labeled riboprobe for the ZAC subunit (top panels). The same blots were probed with \( ^{32}\)P-labeled fragments of the human glyceraldehyde phosphate dehydrogenase cDNA (bottom panels).

antisense \( ^{32}\)P-labeled riboprobe that was derived from the ZAC subunit cDNA (nucleotides 1–447 of GenBank™ accession number AF512521). The blots were washed at 60 °C in 0.1× SSC, 0.1% SDS before exposure. The blots were stripped and reprobed with \( ^{32}\)P-labeled fragments of the glyceraldehyde-3-phosphate dehydrogenase cDNA (nucleotides 789–1146; Ref. 9).

Cloning of Orthologous Genomic Fragments from Mouse, Rat, and Dog—Fragments of genomic DNA were amplified using primer pairs that correspond to the 3′-ends of genes encoding the mouse, rat, and dog orthologues of the human galanin receptor 2 and KIAA1067 genes. For mouse and rat, these were 5′-CACCCGACATCCACCTCGACA and 5′-CTCATCTGCCTCTCAGTGTA (nucleotides 1–93 and 5146–5169 of GenBank™ accession number AF512522). For dog, they were 5′-CCAGGGTATTACCCGGGTAAGGA and 5′-GGACGGTGGCCGACATGATCGA (nucleotides 1–24 and 5846–5869 of GenBank™ accession number AF512523). Aliquots of DNA (50 ng) from mouse, rat (Clontech), or dog (Novagen) were amplified using the XL-PCR system (Applied Biosystems). Reaction products were purified from agarose gels and sequenced directly.

Cell Culture and Transfection—HEK cells were grown in Dulbecco’s modified Eagle’s medium, supplemented with 10% calf serum, 100 IU/ml penicillin, and 100 μg/ml streptomycin. When cells approached confluence, they were seeded into 35-mm diameter dishes and transfected with cDNA encoding the human ZAC subunit (in pcDNA1.1/amp) and GFP (in pCDM8). Cells were transfected using calcium phosphate precipitation (6). Cells were used 24–44 h after transfection.

Electrophysiology—The whole-cell patch-clamp technique was used to record currents from HEK cells. The bath was continuously perfused (5 ml/min) with an extracellular solution containing (in mM): NaCl, 140; KCl, 4.7; MgCl\(_2\), 1.2; CaCl\(_2\), 2.5; glucose, 11; and HEPES, 10 (pH 7.4 with NaOH). The electrode solution contained (in mM): KCl, 140; MgCl\(_2\), 2.0; EGTA, 11; and HEPES, 10 (pH 7.4 with KOH). The intracellular solution used to characterize the cation permeability of ZAC channels contained (in mM): KCl, 70; N-methyl-D-glucamine, 70; MgCl\(_2\), 2.0; EGTA, 11; and HEPES, 10 (pH 7.4 with HCl). The intracellular solution used to determine the contribution of Cl\(^−\) to the ZAC currents contained (in mM): KCl, 70; K-glucamine, 70; MgCl\(_2\), 2.0; EGTA, 11; and HEPES, 10 (pH 7.4 with KOH). Junction potentials were nullified prior to each experiment. Recorded currents were filtered (0.1–10 kHz), amplified (Axoclamp 2B, Axon Instruments), low pass-filtered at 1 kHz, and digitized (Digidata 1320, Axon Instruments, Foster City, CA) for acquisition onto the hard drive of a personal computer. Currents were averaged, superimposed, and measured using pCLAMP software (Axon Instruments). Zn\(^{2+}\) concentration-response data were obtained by prolonged (10 s) pressure ejection of randomized agonist concentrations from low resistance pipettes as described previously (10). Zn\(^{2+}\)-activated currents often exhibited run-up. To compensate for this, 1 mM Zn\(^{2+}\) was applied before each concentration of Zn\(^{2+}\). The amplitudes of the Zn\(^{2+}\)-activated currents were subsequently normalized to the cur-
rent elicited by the prior application of 1 mM Zn$^{2+}$. Graphs of concentration-response relationships were fitted using the logistic function as described previously (10). Current-voltage relationships were analyzed by averaging at least two currents recorded at each holding potential. Individual current-voltage relationships were plotted, and a linear fit to points either side of current reversal yielded the equilibrium potential. All data are expressed as the arithmetic mean ± S.E., and statistical comparisons were made using the Student’s t test.

RESULTS

Cloning of the Human ZAC Subunit cDNA—Searches of the draft human genomic sequence with a consensus sequence of conserved residues revealed exons of a novel LGIC subunit within unannotated fragments of a bacterial artificial chromosome clone from chromosome 17q23. The exon sequences were used to amplify flanking exons and a contiguous cDNA containing the complete open reading frame of the subunit. The subunit forms Zn$^{2+}$-activated channels (see below) and was termed ZAC. The ZAC subunit cDNA encodes a polypeptide of 411 amino acid residues (Fig. 1A). It has a signal sequence, a Cys-Cys motif, four predicted transmembrane domains, and several invariant residues that underpin the conserved secondary structure of the Cys loop LGIC superfamily (11). However, the sequence of the ZAC subunit is not closely related to any known subunits of this superfamily (maximum of 15% amino acid identity with 5-HT$_3$A and nAChR$_7$ subunits; Fig. 1A). This is much lower than the level of identity between known subunits of 5-HT$_3$ receptors (30–40%) or nACh receptors (25–

![Image](http://www.jbc.org/)

**FIG. 2.** Spontaneous currents mediated by ZAC are blocked by TC. A, $I_{\text{ont}}$ recorded from an HEK cell expressing recombinant ZAC appeared rapidly upon establishing the whole-cell configuration at -60 mV. Uncompensated capacitive currents appear during whole-cell recording in response to -5 mV steps. Leak currents were negligible in cells expressing GFP alone. B, TC (100 μM) applied with pressure for 3 s reduced $I_{\text{ont}}$ in cells expressing ZAC but had no effect on cells expressing GFP alone. C, the TC-inhibited current exhibits rectification. The TC-inhibited current amplitude was plotted against holding potential. Data points are average current amplitudes from three recordings normalized to the amplitude of currents recorded at -60 mV. Vertical bars represent ± S.E.

![Image](http://www.jbc.org/)

**FIG. 3.** Zn$^{2+}$ activates ZAC. A, leak subtracted Zn$^{2+}$ (1 mM)-activated currents, recorded from a cell expressing ZAC, with intracellular and extracellular solutions containing equal K$^+$ and Na$^+$ concentrations (~140 mM), respectively. Traces are averages of two currents recorded at each potential. The graph illustrates that $I_{\text{ont}}$ exhibited pronounced outward rectification with equilibrium potentials dependent on the concentration of intracellular K$^+$. $I_{\text{ont}}$ were recorded from cells expressing ZAC with electrode solutions containing either 140 mM K$^+$ (open circles) or in which K$^+$ was substituted by equimolar NMDG$^+$ (70 mM; filled circles). B, a graph illustrating the time-dependent run-up of Zn$^{2+}$ (1 mM for 2 s)-activated currents. Data points fitted with a sigmoid function were $I_{\text{ont}}$ amplitudes at each time point normalized to the maximum $I_{\text{ont}}$ recorded from each cell (n = 9). The inset graph illustrates a lack of $I_{\text{ont}}$ run-up recorded from the same cells. Representative current traces recorded from a single cell are shown in the right panel.
of those activated by 1 mM Zn\(^{2+}\) amplitudes are expressed as percentage activation of ZAC by Zn\(^{2+}\)/H9262 presence of the drugs indicated. Each trace represents three currents averaged under control conditions or in the presence of the drugs indicated.

Vertical bars represent ± S.E. B, spontaneous and Zn\(^{2+}\) (1 mM)-evoked currents were unaffected by ondansetron (1 nM), mecamylamine (10 \(\mu M\)), \(\alpha\)-bungarotoxin (10 \(\mu g/ml\)), and ketamine (100 \(\mu M\)). Each trace represents three currents averaged under control conditions or in the presence of the drugs indicated.

A logistics fit to the TC (100 \(\mu M\))-evoked current amplitude recorded from cells expressing ZAC. Exemplar Zn\(^{2+}\) (1 mM)-activated currents recorded from the same cell under control conditions and in the presence of TC are illustrated. Each trace is the average of three currents. A logistics fit to the TC (30 nm–100 \(\mu M\)) concentration-response relationship yielded an IC\(_{50}\) of 6.6 ± 0.8 \(\mu M\) (n = 4). Current amplitudes are expressed as percentage of control. Vertical bars represent ± S.E.
bition of channel activity (13). Surprisingly, the local application of Zn\textsuperscript{2+} (1 mM) caused activation of inward currents in cells expressing ZAC (Figs. 3–5) but not in cells expressing GFP alone (n = 4).

**Activation of ZAC Channels by Zn\textsuperscript{2+}**—Zn\textsuperscript{2+}-activated currents (I\textsubscript{Zn}) had an equilibrium potential (E\textsubscript{Zn}) of −5 ± 1 mV (n = 6) and outward rectification similar to that of I\textsubscript{spont} (Figs. 2C and 3A). We used alternative electrode solutions to examine the ionic selectivity of ZAC. Substitution of half the intracellular K\textsuperscript{+} with N-methyl-D-glucamine (70 mM), caused a right shift in the current-voltage relationship (Fig. 3A), producing a positive Zn\textsuperscript{2+} equilibrium potential (2 ± 3 mV, n = 3). This demonstrates a role for intracellular K\textsuperscript{+} ions in I\textsubscript{Zn}. Substitution of half of the intracellular K\textsuperscript{+} Cl\textsuperscript{−} with K\textsuperscript{+}-gluconate (70 mM) had no significant effect on Zn\textsuperscript{2+} equilibrium potential (n = 4), demonstrating that the channels have negligible Cl\textsuperscript{−} permeability.

There was little correlation between the amplitudes of I\textsubscript{Zn} and I\textsubscript{spont} in cells expressing ZAC. Furthermore, run-up of I\textsubscript{Zn} was frequently observed during an experiment without a corresponding change in the amplitude of I\textsubscript{spont} (Fig. 3B). These observations suggest that Zn\textsuperscript{2+} activates channels that are closed prior to its application. In cells expressing ZAC, Zn\textsuperscript{2+} caused a concentration-dependent activation of currents, with a threshold of −30 μM and an EC\textsubscript{50} of 540 ± 9 μM (Fig. 4A). As with the I\textsubscript{spont}, I\textsubscript{Zn} was insensitive to bath-applied α-bungarotoxin (10 μg/ml), mecamylamine (10 μM), ondansetron (1 nM), and ketamine (100 μM; Fig. 4B). By contrast, TC caused a concentration-dependent inhibition of I\textsubscript{Zn} (Fig. 5) with an IC\textsubscript{50} of 6.6 ± 0.8 μM (n = 4).

**Absence of Functional ZAC Subunit Genes in Rodents**—We were unable to amplify fragments of an orthologous gene from mouse or rat genomic DNA using degenerate primers designed from the ZAC amino acid sequence (data not shown). More surprisingly, searches of the draft mouse and rat genomic sequences (www.celera.com and www.ncbi.nlm.nih.gov) failed to yield fragments of orthologous genes. The human ZAC gene is located between genes encoding the galanin2 receptor and KIAA1067 (Fig. 6A). Consequently, we amplified and sequenced the region between the orthologues of these two genes from mouse, rat, and dog genomic DNA. For the human sequence, exon 5 is in uppercase, inside brackets. Nucleotides that are conserved between human and other species are shaded. C, alignment of the deduced amino acid sequences for the human and dog ZAC subunits (hZAC subunit and dZAC subunit, respectively). Conserved residues are shaded.
in these species, and they are no longer capable of expressing a functional ZAC subunit. In contrast, dog genomic DNA from this region encodes a full-length ZAC subunit that displays 84% amino acid conservation with the human ZAC subunit (Fig. 6C).

**DISCUSSION**

The ZAC subunit displays all of the structural motifs that are common to the superfamily of Cys loop LGICs. However, its distinctive sequence and function prevent its classification within any of the four established receptor families. Sequence analysis indicates that a common ancestral gene gave rise to the present day ZAC, nACh, and 5-HT_2_ receptor subunit genes. Since they diverged, a functional ZAC subunit gene has been retained by some mammals (e.g. human and dog) but has been lost by others (e.g. mouse and rat). Most human genes, including those for all previously identified LGIC subunits, have rodent orthologues. However, there is evidence for a differential loss of a limited number of genes from either rodent or human genomes (e.g. Refs. 14 and 15). With respect to ZAC, this raises the possibility that humans and rodents differ in some fundamental cell signaling mechanisms. To date, the properties of endogenous LGICs have been studied predominantly in rodent cells. An absence of ZAC from mice and rats may explain why its unusual properties have not been described previously.

ZAC exhibits TC-sensitive spontaneous activity. Some nACh and GABA_A receptor subtypes are also spontaneously active, either in their natural environment (16) or when expressed in recombinant systems (17). Persistent GABA_A channel openings attenuate cellular excitability and can contribute to the maintenance of resting membrane potential (18). Spontaneous nACh channel activity may participate in embryonic muscle development (16). The physiological role of spontaneous ZAC remains to be determined. However, the ability of TC to block this activity will be useful for characterizing the properties of native ZAC in tissue preparations.

Zn^{2+} causes a concentration-dependent activation of ZAC. Zn^{2+} can also modulate the activity of related LGICs, although, in most cases, it inhibits channel function (19–22). However, at the glycine receptor, its effect is biphasic, potentiating glycine-evoked responses at low concentrations and inhibiting them at higher concentrations (20). Zn^{2+} may activate ZAC through an action at the putative N-terminal loop B ligand-binding domain. Subunits of each LGIC family display characteristic loop B residues that may reflect a role in agonist selectivity (11). Spontaneous nACh channel activity may participate in embryonic muscle development (16). The physiological role of spontaneous ZAC remains to be determined. However, the ability of TC to block this activity will be useful for characterizing the properties of native ZAC in tissue preparations.

A relatively high concentration of Zn^{2+} (>30 μM) is required for activation of ZAC. Zn^{2+} is concentrated in various tissues, including the forebrain, testes, and neuroendocrine cells (24, 25). In the hippocampus, pituitary, and pancreatic β-cells, Zn^{2+} is concentrated in vesicles at high concentrations (>1 mM; Ref. 25). During vesicular release, it is possible that micromolar concentrations of Zn^{2+} are present within the synaptic cleft. However, high affinity binding to extracellular albumin is likely to reduce free Zn^{2+} concentrations to the low nanomolar range within a short distance from its source. Native ZAC may be located close to a vesicular source of free Zn^{2+}. Indeed, fetal brain is among a variety of human tissues that express transcripts of the ZAC subunit. Expression of ZAC subunit mRNA was detectable in several other human tissues. However, the absence of a functional ZAC subunit gene in rats and mice precludes the use of these rodents for more detailed localization studies. To resolve the distribution of ZAC expression at the cellular level, it will be necessary to perform such studies on tissues from human, dog, or other non-rodent species.

It is possible that, in its native environment, the affinity of ZAC for Zn^{2+} is increased by a cofactor or by post-translational modification. The observed run-up of Zn indicates an increased ability of Zn^{2+} to activate the channel during the course of an experiment. Future experiments will examine whether this phenomenon is caused by post-translational regulation. It is also possible that Zn^{2+} is not the physiological agonist for ZAC. However, with the knowledge that Zn^{2+} behaves as an agonist and TC as an inhibitor of spontaneous gating, these agents will be useful tools for characterizing the properties of native ZAC receptors in tissues from human and other non-rodent species.
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