Identification of a New Outwardly Rectifying Cl⁻ Channel That Belongs to a Subfamily of the CIC Cl⁻ Channels*

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A new outwardly rectifying Cl⁻ channels (ORCC) that belongs to CIC Cl⁻ channel family has been identified from rat kidney and designated as CIC-5. CIC-5 cDNA encodes a polypeptide of 746 amino acids, which is indicated by hydrophobicity analysis to have structural features that are common of the CIC family. However, the amino acid sequence was weakly homologous to those of other CIC Cl⁻ channels except for CIC-3, which we recently identified as a Ca²⁺-sensitive ORCC. Northern blot analysis of rat tissues showed that CIC-5 mRNA was predominantly expressed in the kidney and colon. To characterize the functional properties of CIC-5 by whole cell patch-clamp technique, we established the stably transfected CHO-K1 cell line using intranuclear microinjection technique. The transfected cells induced outwardly rectifying and 4,4'-disothiocyanostilbene-2,2'-disulfonic acid-sensitive Cl⁻ currents on whole cell configuration. Following the identification of two highly homologous ORCCs, CIC-3 and CIC-5, a new subfamily encoding ORCC has emerged in the CIC family. Furthermore, CIC-5 was almost identical to a partial human cDNA, which is recently reported to be that of the ClC-5 gene. The molecular structure and functional properties of CIC-5 will provide an important insight into ORCCs and the pathogenesis of Dent’s disease.

Many physiological studies have demonstrated the presence of outwardly rectifying Cl⁻ channels (ORCCs) in a variety of cells (1–4). Single channel recordings by patch-clamp technique have shown that they have strong outward rectification in a positive membrane voltage and that their conductance is about 40 picoamperes (1–4). Recently, ORCC has attracted special interest in relation to cystic fibrosis transmembrane conductance regulator (CFTR) (5–9). Patch-clamp studies in the epithelial cells of patients with cystic fibrosis have shown that cyclic AMP-dependent ORCC is not properly regulated in these patients (5–9), indicating the possible importance of ORCC in the pathogenesis of cystic fibrosis. When the CFTR gene was cloned in 1989 (10, 11), it soon became clear that CFTR encodes a small linear Cl⁻ channel of ~10 picoamperes and not ORCC (12). This discrepancy has been puzzling investigators, but recent new findings that CFTR acts as an ATP-permeating channel may solve this problem. Schiewietz et al. (13) have shown that CFTR is able to permeate ATP in addition to Cl⁻ and that ATP transported from inside to outside of the cell in turn activates the purinergic receptors on the cell surface. Activation of the purinergic receptor then stimulates ORCC. On the basis of these findings, subsequent attention has been focused on the molecular structure of ORCC.

Recently, we have cloned and characterized a Ca²⁺-sensitive ORCC, CIC-3, an intriguing member of the CIC family (14, 15). Only 20–24% of the amino acid sequence encoded by CIC-3 is identical to those of other cloned CIC Cl⁻ channels, i.e. CIC-0, -1, -2, -K1, and -K2 (16–20). These findings led us to the hypothesis that CIC-3 may belong to a new subfamily encoding ORCCs in the CIC family. In the present study, we examined whether a new member of ORCC exists in the CIC family using a homology-based cloning strategy. Here, we report a new cDNA clone encoding an ORCC, CIC-5, isolated from rat kidney. CIC-5 is highly homologous to CIC-3 and CIC-4 and almost identical to a partial human cDNA, which is recently reported as a strong candidate for Dent’s disease (21). We further established the stably transfected mammalian cells and characterized its channel properties by the patch-clamp technique.

EXPERIMENTAL PROCEDURES

Methodology

Reverse Transcription PCR—First, we made degenerate PCR primers that corresponded to the second and third membrane spanning domains of CIC-3: sense strand, CCGGATCCGGNATCHCNGARHT-NAARAC and antisense strand, CGGAATCTGACNARNGNGC-CYCTYTT (where N = A/C/G/T; H = A/C/T; R = A/G; Y = C/T). Gomerulidi midisodiated by conventional technique (22) were reverse-transcribed using avian myeloblastosis virus reverse transcriptase (Boehringer Mannheim) at 42 °C for 60 min and then heated at 94 °C for 5 min. The synthesized cDNA was used for subsequent PCR in the following profile: 94 °C for 1 min, 55 °C for 1 min, 72 °C for 3 min, 35 cycles. The PCR product was cut with EcoRI and BamHI on both ends, ligated into EcoRI- and BamHI-cut pSPORT1 (Life Technologies, Inc.), and then sequenced. One clone, pH17, had about 74% nucleotide sequence identity with that of the corresponding region of CIC-3.

Isolation of CIC-5 cDNA Clone and DNA Sequencing—An oligo(dT)-primed directional rat kidney cDNA library in XgtIIA (19) was screened under high stringency (6 × saline/sodium/potassium EDTA (SSPE), 50% formamide, 5 × Denhardt’s solution, 1% SDS, 100 μg/ml salmon sperm DNA) at 42 °C with a 160-bp PCR clone (pH17) labeled with [α-32P]dCTP (3000 Ci/mmol, Amersham) (2.5 × 10⁶ cpm/μg). The screening yielded four positive clones from 3 × 10⁶ plaques. The clone pH1 was cut with NotI and SalI, and a 2.5-kb insert was subcloned into NotI- and SalI-cut pSPORT1 and designated as CIC-5. To sequence the isolated cDNA, nested deletion clones were prepared using the Erase-A-Base system (Promega) and sequenced by T₇ DNA polymerase by the
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Primary Structure of ClC-5—PCR cloning strategy with rat microdissected glomeruli as a template was adopted to isolate a new ORCC that is homologous to ClC-3 and is predominately expressed in the kidney. From the PCR products we subcloned and sequenced 32 clones. Sequencing revealed the existence of a PCR clone (ph 17) that was highly homologous to ClC-3 (74% nucleotide identity). Using this PCR clone as a probe, a cDNA clone was obtained with a 2568-bp insert designated as ClC-5 was isolated from rat kidney cDNA library. The nucleotide sequence surrounding the translation initiation codon (5'-GAATCATGG-3') conforms with the Kozak sequences for translation initiation sites (Fig. 1A) (25, 26) and initiates the longest reading frame. The 5'-untranslated sequence does not contain stop codon. The first stop codon occurs at nucleotide 2449, resulting in an open reading frame of 746-amino acid protein (Fig. 1A). The predicted translation product has a calculated molecular mass of 83,089 daltons. A hydropathy plot of the predicted amino acid sequence by the method of Kyte and Doolittle (27) is shown in Fig. 1C. The hydropathy profile shows at least 12 hydrophobic regions, which is similar to those of other members of the ClC Cl− channel family (28). This protein has two potential N-glycosylation sites (amino acid positions at Asn-38 and Asn-408) (29). There are also consensus sequences for phosphorylation by cAMP-dependent protein kinase and protein kinase C (30, 31). The consensus cAMP-dependent protein kinase phosphorylation sites are located at position Thr-349 and Thr-350, and sites for protein kinase C are located at position Ser-397, Ser-628, Thr-37, Thr-291, Thr-409, Thr-544, Thr-676, and Thr-724.

The protein sequence of ClC-5 is highly homologous to that of rat ClC-3 (77%), to those of rat and human ClC-4 (76%) (32) (Fig. 1B), and to that of a partial human cDNA (99%) (21). In contrast, the overall amino acid identity of ClC-5 with other members of the ClC family is very low (29% amino acid sequence identity with Torpedo channel, ClC-0; 31% with ClC-1, 33% with ClC-2, 27% with ClC-K1). To establish possible evolutionary relationships among these ClC Cl− channels, a phylogenetic tree was constructed using DNA/s computer software (Mac version 3.2, Hitachi, Yokohama, Japan) (Fig. 2). According to the phylogenetic analysis, the eight members of the ClC Cl− channel family can be classified into two subfamilies: the ClC-0/CIC-1/CIC-2/CIC-K1/CIC-K2 chloride channels and the ClC-3/CIC-4/CIC-5 chloride channels.

Tissue Distribution—In Northern blot analysis of a variety of rat tissues, the full-length CIC-5 cDNA probe hybridized with a band at approximately 9.5 kb, as shown in Fig. 3, A and B. In a Northern blot where total RNAs were electrophoresed, the expression of CIC-5 was detected only in the kidney and colon (Fig. 3A). In contrast, transcripts of the same size were also noted in lower amounts in the heart, brain, lungs, and testis, in addition to the kidney on a blot prepared with poly(A)− mRNA (Fig. 3B).

Functional Expression—To characterize the function of ClC-5, we transfected the coding sequence of cloned CIC-5 cDNA into CHO-K1 cells using dexamethasone-inducible mammalian expression vector (pMAM-neo) (33). Transcription of the insert is under the control of the Rous sarcoma virus promoter and the dexamethasone-inducible mouse mammary tumor virus long terminal repeat. One stably transfected cell clone (J 2702) selected by resistance to G418 for 3 months showed the induction of ClC-5 mRNA at 3.6 kb in response to DEX (5 μM for 24 h) (Fig. 4). In contrast, no bands were
detected in any of the mock-transfected cells with or without DEX treatment. These findings indicated the isolation of the stably transfected cell line (J2702) expressing ClC-5.

To examine whether ClC-5 actually acts as a Cl$^{-}$ channel, the whole cell patch-clamp technique was applied to J2702 cells. Fig. 5 shows a typical trace of the whole cell currents following the changes in holding membrane potential between $-75$ and $+25$ mV (A) and their I-V relationship (E). The transfected cells generated large, time-dependent and outwardly rectifying currents ($1,562 \pm 128$ pA at $+25$ mV membrane potential, $n = 12$), in contrast to the mock-transfected CHO-K1 cells ($70 \pm 8$ pA at $+25$ mV, $n = 10$; Fig. 5, A and B). The I-V curve based on the currents at the end point of voltage pulses revealed an outwardly rectifying current-voltage relationship (Fig. 5E). The extracellular partial Cl$^{-}$ replacement with gluconate reduced the overall current and resulted in a shift of the reversal potential toward the positive direction, thus indicating that the current was Cl$^{-}$ selective (Fig. 5E).
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DISCUSSION

The molecular structures of ORCCs have not yet been identified because of the lack of their molecular cloning. In this study, we successfully isolated CIC-5, a new ORCC which is highly homologous to CIC-3, by a sequence homology-based strategy. We further established the stably transfected mammalian cultured cell line expressing CIC-5 using the intranuclear microinjection technique.

The predicted amino acid sequence of CIC-5 is highly homologous with not only CIC-3 (77%), but also with CICN4 (78%), and its rat counterpart CIC-4 (78%), which has been recently isolated from Xp22.3 region using positional cloning strategy (28, 32) (Fig. 1B). Phylogenetic analysis indicated the presence of two subfamily in the CIC family (Fig. 2). A partial deletion of CICN4 is known to cause delays in psychomotor functions and mental retardation. Furthermore, the amino acid sequence of CIC-5 is almost identical (99%) to a human sequence predicted from a partial cDNA (780 bp) recently isolated as a candidate molecule of Dent’s disease (an X-linked hereditary renal tubular disorder) from Xp11.22 region using positional cloning strategy (21). It is tempting to speculate that this subfamily of CIC-5 channel is related to human disease. Further clarification of physiological function of these channels is needed to elucidate the pathogenesis of these disease.

In the motif analysis, CIC-5 has two potential N-glycosylation sites. One of the N-glycosylation sites (Asn-408) is located in the segment between D8 and D9, and this glycosylation motif is well conserved among all CIC channels known so far. Kiefer et al. (34) have shown recently that CIC-0, CIC-1, CIC-2, and CIC-K1 channels are glycosylated at this segment in vitro translation experiments. Therefore, this glycosylation site between D8 and D9 should be located outside of the cells.

2 During the submission of this draft the full-length human cDNA, which is implicated in Dent’s disease, has been published as CLCN5 by Fisher et al. (Fisher, S. E., Bakel, I. D., Lloyd, S. E., Pearce, S. H. S., Thakker, R. V., and Craig, I. W. (1995) Genomics 29, 598–606).
In addition, there are two consensus sequences for phosphorylation by cAMP-dependent protein kinase in ClC-5 cDNA (see "Results"). This finding suggest that ClC-5 protein itself might be modulated by cAMP-dependent protein kinase-mediated phosphorylation. However, any change in the whole cell current was not induced in response to cAMP in the present study (Fig. 6). This result revealed that cAMP-mediated signaling does not directly modulate the channel properties. However, it cannot be neglected that cAMP-mediated signaling indirectly modulates the function of ClC-5. A lack of channel regulator(s) in the transfected cells may disrupt the pathway of cAMP-mediated signaling.

Now, eight members of the CIC family have been cloned. Some of these CIC channels have been functionally characterized by transient expression system using Xenopus oocytes. However, the functional expression in Xenopus oocyte may not be ideal for characterization of CIC-3, -4, and -5 channels. For unknown reasons, the expression of these channels has been difficult in the oocytes except for CIC-3 (for review, see Refs. 28 and 35). Also a Ca²⁺-dependent Cl⁻ channel is present in the oocytes (36, 37) and sometimes disturbs the detection of the exogenously expressed Cl⁻ currents. We thought that mammalian cells were more suitable for proper functional expression of ClC-5, which was obtained from the rat. Accordingly, we stably transfected ClC-5 cDNA into a mammalian cultured cell line, CHO cells, using the intranuclear microinjection technique. This transfection system has the following advantages over others: 1) the stable transformed cell line can be obtained more frequently and more easily because the transfection efficiency of intranuclear microinjection is higher than those of other indirect methods (0.2 versus 0.001) (38, 39), 2) the dexamethasone-inducible expression vector (pMAM-neo) is useful to induce the overexpression of the transformed full-length gene, 3) a patch-clamp study has demonstrated that only a small linear Cl⁻ channel is endogenous in CHO cells (40).

In the stably transfected mammalian cells with ClC-5, the whole cell currents showed a typical outward rectification that was time-dependently activated following depolarization of membrane voltage (Fig. 5E). The profile and amplitude of the currents (~1 nA at +25 mV, Fig. 5A) were different from those of the endogenous small linear Cl⁻ channel in the wild-type CHO cells (40). Several lines of evidence suggest that these currents were induced by a Cl⁻-selective channel. First, Cl⁻ was the predominant current-carrying ion under the conditions of patch-clamp experiments. Second, the replacement of Cl⁻ with gluconate in the bath solution reduced the currents and caused a positive shift in the reversal potential (Fig. 5E). Third, the currents were inhibited by Cl⁻ channel blockers, DPC and
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nephrocalcinosis, nephrolithiasis, and eventual renal failure (43–45). Although the primary mechanism responsible for this disease has not been clarified, the feature of low molecular weight proteinuria indicates the dysfunction of proximal tubule. The previous physiological studies have shown that the acidification of endosomal compartment is a limiting factor for the uptake of filtered proteins via active endocytotic pathway in the proximal tubule, in which the Cl⁻ conductance in endosomal membrane is responsible for the acidification of endosomal compartment (46–49). The Cl⁻ channel expressed in endosomes has been isolated from rat kidney cortex and has been shown to be voltage-dependent and DPC- and DIDS-sensitive (50). The further studies on CIC-5 may help to evaluate the hypothesis that the defect of the Cl⁻ channel is responsible for Dent's disease.

In summary, a new molecule of ORCC was isolated from rat kidney and functionally characterized. Following the elucidation of two structurally and functionally homologous ORCCs (CIC-3 and CIC-5), the existence of a subfamily encoding ORCCs in CIC Cl⁻ channel family has emerged. Although CIC-4 has not yet been functionally characterized as a Cl⁻ channel, it could belong to the same subfamily as CIC-3 and CIC-5. The analysis of the structure and function of these ORCCs proteins will provide us with the framework to evaluate their detailed regulatory mechanisms at a molecular level.

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