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MOLECULAR CLONING OF THE HUMAN VOLUME-SENSITIVE CHLORIDE CONDUCTANCE REGULATORY PROTEIN, pICln, FROM OCULAR CILIARY EPITHELIUM

Juan Anguita[1†], Michael L. Chalfant[4], Mortimer M. Civan[2,3,4], and Miguel Coca-Prados[1*]

[1]Departments of Ophthalmology and Visual Science, Yale University School of Medicine, New Haven, CT

Departments of [2]Physiology and [3]Medicine, and the [4]Graduate Group in Bioengineering, University of Pennsylvania, Philadelphia, PA

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Summary: Chloride channels in the ocular ciliary epithelium are believed to play a key role in aqueous humor formation. We isolated a cDNA clone from a λUni-ZAP cDNA library of human nonpigmented ciliary epithelial (NPE) cells encoding the swelling-induced chloride channel/channel regulator pICln. The human clone contains an open reading frame of 237 amino acids (M_r 26,293). The deduced human amino-acid sequence shows 90.2% and 92.7% identity with counterparts isolated from rat kidney and the canine kidney epithelial cell line MDCK. Human NPE cell lines exhibited significant levels of pICln transcripts. Complementary perforated-patch, whole-cell patch clamping demonstrated that swelling activates Cl^- channels of the NPE cells, as suggested by ruptured-patch measurements. The results document the molecular isolation and identification of a human cDNA clone of a Cl^- conductance regulator from ocular cells displaying volume-activated Cl^-channels.

Chloride channels play a critical role in transepithelial secretion and absorption, and are absolutely required for the regulatory response to swelling of nonpigmented ciliary epithelial (NPE) cells [1]. Recent

†Present address: Dept. of Internal Medicine, Section of Rheumatology, Yale University School of Medicine.

*Corresponding author. FAX: 203-785-6123.

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electrophysiologic measurements support the view that the Cl^- channels underlying the volume regulatory response are also responsible for isosmotic Cl^- secretion by the NPE cells [2]. To understand at the molecular level how Cl^- channels are regulated in the ciliary epithelium, the chloride channels must be identified and characterized at the electrophysiological and gene levels. Recently, we have isolated a partial clone of the human ciliary body homologue of pICln by the polymerase chain reaction (PCR) [2]. The pICln gene is considered a chloride channel by some [3] and a regulatory protein of a chloride channel by others [4]. The electrophysiological properties of the chloride currents activated by hypotonic perfusion of the nonpigmented ciliary epithelial cells [2] are very similar to those described for pICln [4]. We report here the isolation of a cDNA clone containing the entire coding region of pICln. This will allow us to investigate their functional expression, regulation and possible interaction with cytosolic proteins.

MATERIAL AND METHODS

cDNA cloning and sequencing of human pICln: a human nonpigmented ciliary epithelial cell line cDNA library in λUni-ZAP XR (Stratagene) [5] was screened with a previously isolated probe pHCBICln (0.4-kb cDNA clone) generated by PCR [2]. Hybridization was carried out in 50% formamide, 5XSSC (1XSSC, 0.15M NaCl, 15mM sodium citrate), 50 mM phosphate, pH5; 1XDerhardt's; 0.1%SDS; 50 μg/ml salmon sperm DNA; and ^{32}P-labeled pHCBICln probe at 42°C for 18h. The filters were washed at 42°C for 20 min to a final stringency of 0.1X SSCPE (0.15M NaCl, 15 mM sodium citrate, 1.3 mM KH_2PO_4, 100 mM EDTA) containing 0.1% sodium dodecyl sulfate. Five phages hybridizing to the probe were plaque-purified and characterized by standard restriction endonuclease mapping procedures. The largest, pICln-H-2.9, was sequenced by the dideoxynucleotide method [6] using Sequenase (U.S. Biochemicals) and synthetic oligonucleotide primers.

RNA isolation and Northern analysis: RNA was isolated from cell lines established from the human ciliary epithelium by the acid guanidinium thiocyanate-phenol-chloroform method [7]. For Northerns 20 μg of total RNA was subjected to agarose gel electrophoresis and transferred to nitrocellulose. After transfer the membranes were crosslinked with a uv crosslinker apparatus (Stratagene), prehybridized, hybridized with the 1.5-kb EcoRI/ApaI fragment of pICln-H-2.9, and then washed as previously described [5].

Patch clamping: Micropipettes were pulled from low melting-point glass (#PG10165-4, WPI), coated and fire polished to resistances of ≈1-3 MΩ. The filling solution contained (in mM): 15 NaCl, 10 HEPES (half acid and half sodium salt), 5 $CaCl_2$, 35 CsCl, and 60 Cs_2SO_4, at ≈260 mOsm and at pH

≈7.4. To form perforated patches [8,9], 170 μg/ml amphotericin-free solution alone. After seal formation, amphotericin diffused to and partioned in the membrane, producing a slow capacitative transient. The final steady-state access resistance was reached in ≈30 min. The isosmotic Tyrode's perfusate contained (in mM): 77.0 NaCl, 0.8 $MgCl_2$, 3.3 KCl, 1.8 $CaCl_2$, 0.8 KH_2PO4, 21.0 $NaHCO_3$, 10.5 N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES), 5 glucose, and 110 sucrose, at 310-320 mOsm and pH adjusted to 7.4. Cells were also perfused with a hypotonic solution containing the same ionic and glucose concentrations, but free of sucrose, at 200-205 mOsm. Where appropriate, the Cl^- concentration was reduced by isosmotic replacement with methylsulfonate.

Nucleotide sequence accession number: The nucleotide sequence data reported in this work has been submitted to GenBank/EMBL and assigned accession number U17899.

RESULTS AND DISCUSSION

A human ciliary epithelial cDNA library in λUni-ZAP XR [5] was screened with a 0.4-kb probe (pHCBICln) derived by PCR amplification [2] using a set of primers deduced from the rat nucleotide sequence of the cDNA encoding the MDCK-type chloride channel [10]. Five cDNA clones hybridizing to the probe were obtained by screening approximately $1x10^5$ recombinant phage plaques. DNA sequencing revealed that all the clones, which overlapped, encoded the human homologue of pICln [4,10]. The largest of the clones, pICln-H-2.9, was selected and both strands of this clone were sequenced with the sequencing strategy shown in Fig 1. The cDNA sequence and predicted amino acid sequence of pICln-H-2.9 are shown in Fig 2. The ATG beginning at nucleotide position 75 is presumed to be the start codon. The predicted molecular size of pICln-H-2.9 based on this putative start is 26-kDa which is comparable to the molecular sizes of the pICln chloride channels or channel regulators cloned from rat kidney (26-kDa) [4,10] and canine cell line MDCK (25.9-kDa) [11]. Both strands of the human pICln-H-2.9 were sequenced with the sequencing strategy

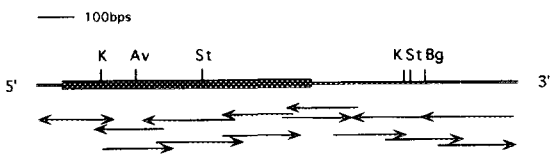

Figure 1. Restriction map analysis and sequencing strategy of the human pICln cDNA. Arrows indicate the origin and direction of nucleotide sequencing and the extent of the sequence obtained from pICln cDNA and subclones. The open reading frame is shown by a black box. Restriction sites: K, *Kpn I*; Av, *Ava II*; St, *Stu I*; Bg, *Bgl II*.

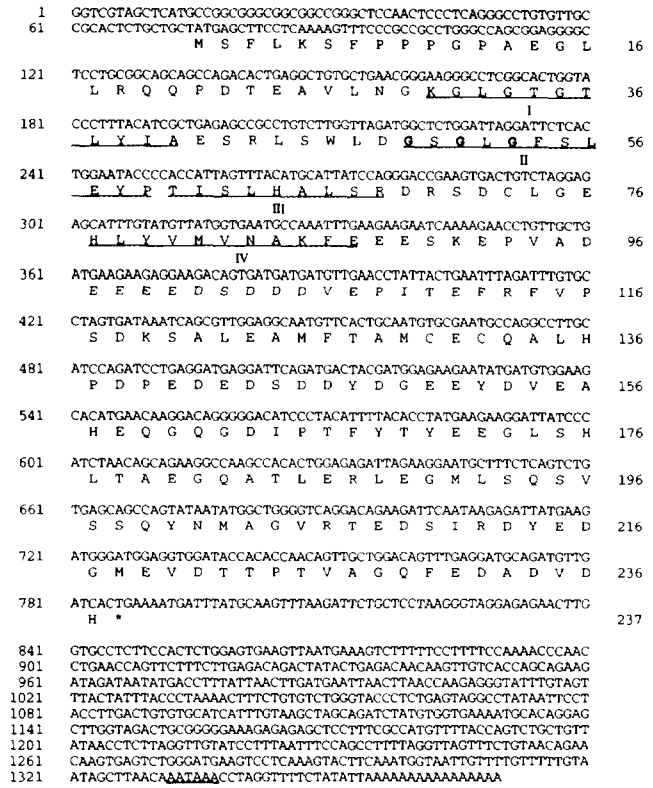

Figure 2. Nucleotide and protein sequence of the human pICln cDNA clone. Nucleotides are numbered on the left; amino acids (single-letter code) are numbered on the right, starting at the initiating methionine as +1. The deduced amino acid sequence is given under the open reading frame. The polyadenylation signal is underlined. The four putative transmembrane β-sheets (I-IV) are also underlined, and the consensus nucleotide-binding site (GXGXG) in the II transmembrane β-sheet is also indicated by bold letters.

shown in Fig 1. The human pICln-H-2.9 is 1.3-kb long has a 74-nucleotide untranslated region at the 5'-end, followed by a 711 nucleotide open reading frame, and a 569-nucleotide 3'-untranslated sequence with a poly(A)$^+$ tail and the polyadenylation signal AATAAA at position 1332. The open reading frame encodes a polypeptide of 237 amino acids with a M_r of 26,293. The nucleotide sequence of the human pICln-H-2.9 cDNA exhibits 90.2% identity with the rat pICln [4,10] and 92.7% identity with the canine pICln [11]. These results indicate that the human, rat and dog cDNA clones encode the same protein with small species differences.

A comparison of the amino acid sequence of the human pICln with the rat and dog pICln is shown in Fig 3. The human pICln consists of 237 amino acid residues, whereas the rat has 236 and the dog has 235 amino

acid residues. The four putative transmembrane β-sheets (I-IV) are highly conserved among the three species, the I (amino acids 30-40) and II (amino acids 49-59) regions are conserved 100% whereas region III (amino acids 60-68) differs in a single amino acid and region IV (amino acids 77-88) differs in two amino acids. The region of largest dissimilarity resides in a stretch of six amino acids which are amino acid substitutions (70-75) between transmembrane β-sheet regions III and IV. The half which includes the C-terminus end of the human pICln protein exhibits marked homology with the rat and dog (>98%) pICln proteins. The putative nucleotide-binding site (GSGLG) is also conserved among the three species (amino acids 49-53).

A Northern blot containing total RNA from human ocular ciliary epithelial cell lines was hybridized with the 1.3-kb pICln-H-2.9 cDNA as probe. The human pICln encodes a single RNA of about 1.6-1.8 -kb in size (Fig 4). This is consistent with previous results observed for the rat and dog pICln clones [4,11].

We also verified that the NPE cells display volume-activated Cl^- channels, using a less invasive mode of patch clamping than previously applied [12]. Perfusion with 50% hypotonic solution reversibly increased whole-cell conductance by a factor of 5-7 in six experiments and by 20 in a sixth. In five experiments, reducing the external Cl^- concentration from 85.5 to 12.6 mM significantly reduced outward current under hypotonic conditions (Fig 5). The Cl^--dependent fraction of the total conductance was four-fold smaller under baseline conditions. In three experiments, 100μm

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          1                                                  50
Human     MSFLKSFPPP GPAEGLLRQQ PDTEAVLNGK GLGTGTLYIA ESRLSWLDGS
Rat       .......... .S.D..RL.. .......... .......... ..........
Dog       .......... .S....RQ.. .E........ .......... ..........

          51                                                 100
Human     GLGFSLEYPT ISLHALSRDR SDCLGEHLYV MVNAKFEEES KEPVADEEEE
Rat       .......... .....V...P NAYPQ..... ....R.G... ...FS.-.D.
Dog       .......... .....V...L NAYPR..... V.....G... ..S..--...

          101                                                150
Human     DSDDDVEPIT EFRFVPSDKS ALEAMFTAMC ECQALHPDPE DEDSDDYDGE
Rat       .DN......S .......... .......... .......... ..........
Dog       .........A .......... .......... .......... ..........

          151                                                200
Human     EYDVEAHEQG QGDIPTFYTY EEGLSHLTAE GQATLERLEG MLSQSVSSQY
Rat       .......... .......... .......... .......... ..........
Dog       .......... .......... .......... .......... ..........

          201
Human     NMAGVRTEDS IRDYEDGMEV DTTPTVAGQF EDADVDH  237
Rat       .......... V.T....... E......... .......  236
Dog       .......... T......... .......... .......  235
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Figure 3. Deduced amino acid sequences of pICln in human, rat and dog. The alignment shows the high level of conservation of amino acids on the four transmembrane β-sheets, positions: I(30-40), II(49-59), III(60-68), IV(77-88) and the half C-terminus in the vertebrate chloride channel-regulator pICln.

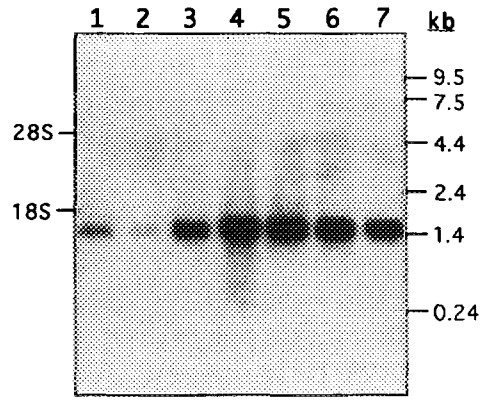

Figure 4. Expression of pICln in human cell lines derived from the ocular ciliary epithelium. Twenty micrograms of total cellular RNA/lane from the following established cell lines: lane 1, ODM-2; lane 2, HOSV; lane 3, ODM-T; lane 4, HNPSV-C19; lane 5, ODM-C4 ; lane 6, ODM-C2; lane 7, HNPSV- SubCl-1 of the human ocular ciliary epithelium were hybridized in a Northern blot with the human pICln probe. RNA molecular weight markers and ribosomal 28S/18S markers are indicated.

of the Cl^--channel blocker 5-nitro-2-(3-phenylpropylamino)-benzoate (NPPB) reduced both the outward and inward currents measured under hypotonic conditions (Fig 5). NPPB abolished the Cl^- dependence of the whole-cell currents. In two experiments, the Cl^--difference currents ($I_{\Delta Cl}$) were measured using a voltage-step protocol over the range from -140 to 100 mV. $I_{\Delta Cl}$ displayed no significant voltage activation or inactivation. All

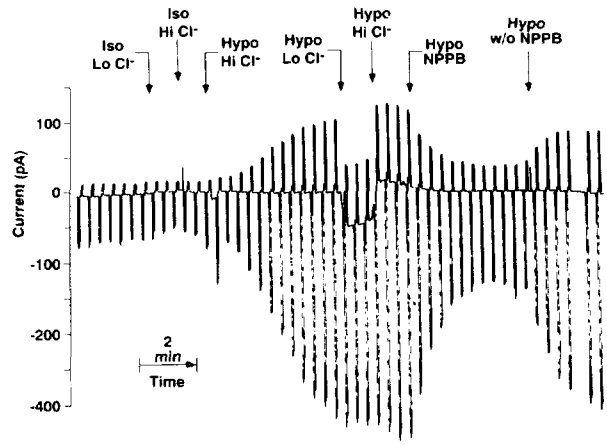

Figure 5. Perforated-patch whole-cell recording of an NPE cell. The cell was sequentially perfused with isotonic solutions containing high (85.5 mM) and low (12.6) Cl^- concentrations, hypotonic high- and low-Cl^- solutions, and hypotonic high- Cl^- solution with (NPPB) or without (w/o NPPB) 100 μM NPPB.

of these findings are similar to those we have previously observed using the more invasive ruptured-patch mode of patch clamping [12]. However, in contrast to the previous approach [12], the perforated-patch technique has permitted us to visually detect cell swelling and shrinkage just preceding the changes in Cl^- conductance triggered by hypo- and isotonic perfusates, respectively. Thus, the present results document the association of volume-activated Cl^- channels with a highly conserved homologue of rat and canine pI_{Cln} in human nonpigmented ciliary epithelial cells.

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