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Short Sequence-Paper

Cloning of a swelling-induced chloride current related protein from rabbit heart ☆

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Abstract

Recently, pI_{cln} has been reported to be a regulator of a swelling-induced chloride conductance. We have cloned a cDNA RCL-H1 from rabbit heart, of which primary structure is highly homologous to that of pI_{cln}. Outwardly rectifying currents were recorded in oocytes expressing RCL-H1, which is consistent with the result of pI_{cln}. RNA blot analysis revealed the widespread expression of RCL-H1 mRNA in rabbit tissues. RCL-H1 may play an important role in regulating cell volume and give a clue to revealing molecular structure of swelling-induced chloride channel(s).

Keywords: cDNA cloning; Swelling-induced chloride current; Chloride current; (Rabbit heart)

It is essential for every cell to keep cell volume constant [1–3]. The processes of regulating cell volume in response to osmotic swelling or shrinkage are termed regulatory volume decrease (RVD) or regulatory volume increase (RVI), respectively. The mechanisms of volume regulation have been studied extensively and the underlying ion transport systems are being revealed [2–4]. Activation of chloride channel(s) by swelling is one of the mechanisms responsible for RVD and such swelling-induced chloride current(s) have been identified in some cell types including epithelial cells and cardiac cells [5,6]. Recently, it has been reported that pI_{cln}, which was cloned from MDCK (Madin Darby canine kidney) epithelial cells [7], could play an important role in cell volume regulation by acting as a regulatory protein of the swelling-induced chloride current [8]. In this paper, we describe the molecular cloning of the homologue of pI_{cln} from rabbit heart and show its distribution in rabbit tissues.

$Poly(A)^+$ RNA was prepared from adult rabbit heart by a standard procedure [9] and converted to single-stranded cDNA using oligo(dT) primers. Polymerase chain reaction (PCR) was performed using the cDNA as a template to obtain a probe for hybridization. PCR conditions were as follows: 94°C for 1 min, 45°C for 2 min and 72°C for 3 min for 30 cycles. The primers used for PCR were synthesized according to the amino acid sequences of pI_{cln} and a rat kidney homologue, RKCL-1 [10]. The 5′-primer corresponded to amino acid residues 25–31 and the 3′-primer corresponded to residues 101–107 of pI_{cln}. The amplified DNA fragments (about 250 bp) were subcloned into pBluescript II SK(+) vector (Stratagene) and sequenced using BcaBEST dideoxy sequencing kit (Takara). One PCR clone (pRCL-8) homologous to pI_{cln} was used as a hybridization probe to screen a rabbit heart cDNA library in λZAPII (Stratagene). Approximately $1.7 \cdot 10^6$ plaques were screened with a radiolabelled fragment of pRCL-8 and 12 hybridization-positive clones were isolated under low stringency conditions. The cDNA inserts in λZAPII phage were rescued into pBluescript II SK(−), and the longest insert was sequenced on both strands. Comparison of its sequence with pI_{cln} and RKCL-1 sequences suggested that it lacked two amino acids including the initiating methionine. Therefore, the 5′-rapid amplification of cDNA ends (5′-RACE) was used to complete the 5′-region of the cDNA clone (RCL-H1).

The plasmid containing RCL-H1 was linearized with *Bam*HI and capped run-off cRNA was synthesized in vitro with T7 RNA polymerase (Stratagene). Electrophysiological measurements and the injection of cRNA into *Xenopus laevis* oocytes were carried out essentially in the same way

☆ The nucleotide sequence data reported in this study have been submitted to the GSDB/DDBJ/EMBL/NCBI databases under the accession No. D26076.

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as those reported previously [11]. For electrophysiological assay, oocytes were bathed in ND96 solution (96 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM Hepes, pH 7.5).

RNA blot analysis was carried out by using 5 μg of poly(A)$^+$ RNAs. Poly(A)$^+$ RNAs isolated from the cerebellum, cerebrum, atrium, ventricle, kidney, liver, lung, skeletal muscle of rabbit were electrophoresed on 1.0% agarose gel containing formaldehyde and transferred to Gene Screen Plus membrane (NEN). The hybridization was performed using a 1.8 kb RCL-H1 insert labelled with ^{32}P in a solution of 5 × SSPE, 50% formamide, 5 × Denhardt's solution, 0.1% SDS and 200 μg/ml denatured herring testis DNA at 42°C for 16 h. The membrane was briefly washed in 0.2 × SSC/0.1% SDS at room temperature, followed by two 15 min washes with 0.2 × SSC/0.1%

```
-48  CGC TGT GCC GCA GCT GCC TGC CGG TGT CGT TCC GCG TTC GGT GCA GCC ATG AGC
  1  --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
                                                                 Met Ser

  7  TTC CTC CGG AGT TTC CTG CCT CCC GGG CCC ACG GAA GGG CTG CGG CAC CAG CAG
     --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
  3  Phe Leu Arg Ser Phe Leu Pro Pro Gly Pro Thr Glu Gly Leu Arg His Gln Gln

 61  CCG GAT ACC GAG GCA GTC CTG AAC GGG AAG GGC CTC GGC ACC GGC ACC CTC TAC
     --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 21  Pro Asp Thr Glu Ala Val Leu Asn Gly Lys Gly Leu Gly Thr Gly Thr Leu Tyr

115  ATC GCT GAG AGC CGC CTG TCT TGG TTA GAT GGT TCT GGA TTA GGA TTC TCA CTG
     --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 39  Ile Ala Glu Ser Arg Leu Ser Trp Leu Asp Gly Ser Gly Leu Gly Phe Ser Leu

169  GAA TAT CCC ACC ATT AGC TTA CAC GCG GTA TCC AGG GAC CCA AAT GCC TAT CCA
     --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 57  Glu Tyr Pro Thr Ile Ser Leu His Ala Val Ser Arg Asp Pro Asn Ala Tyr Pro

223  CAA GAG CAT TTG TAT GTT ATG GTA AAT GCC AAA TTT GGA GAA GAA TCA AAA GAA
     --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 75  Gln Glu His Leu Tyr Val Met Val Asn Ala Lys Phe Gly Glu Glu Ser Lys Glu

227  CTT GTT GCT GAT GAA GAG GAA GAC AGT GAT GAT GAT GTT GAA CCT ATT TCT GAA
     --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
 93  Leu Val Ala Asp Glu Glu Glu Asp Ser Asp Asp Asp Val Glu Pro Ile Ser Glu

331  TTT AGA TTT GTA CCT GGT GAT AAA TCA GCA TTG GAG GCA ATG TTC ACT GCA ATG
     --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
111  Phe Arg Phe Val Pro Gly Asp Lys Ser Ala Leu Glu Ala Met Phe Thr Ala Met

385  TGT GAA TGT CAG GCC TTG CAT CCA GAC CCT GAG GAT GAA GAT TCA GAT GAT TAT
     --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
129  Cys Glu Cys Gln Ala Leu His Pro Asp Pro Glu Asp Glu Asp Ser Asp Asp Tyr

439  GAT GGA GAA GAA TAT GAT GTG GAA GCA CAT GAA CAA GGA CAG GGG GAT ATC CCT
     --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
147  Asp Gly Glu Glu Tyr Asp Val Glu Ala His Glu Gln Gly Gln Gly Asp Ile Pro

493  ACG TTC TAT ACC TAT GAA GAA GGA TTG TCC CAT TTA ACA GCA GAA GGC CAA GCC
     --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
165  Thr Phe Tyr Thr Tyr Glu Glu Gly Leu Ser His Leu Thr Ala Glu Gly Gln Ala

547  ACA TTG GAG AGA CTA GAA GGA ATG CTT TCT CAG TCT GTG AGC AGC CAA TAT AAC
     --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
183  Thr Leu Glu Arg Leu Glu Gly Met Leu Ser Gln Ser Val Ser Ser Gln Tyr Asn

601  ATG GCT GGA GTT CGG ACG GAA GAT TCA ATA AGA GAT TAT GAA GAT GGC ATG GAG
     --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
201  Met Ala Gly Val Arg Thr Glu Asp Ser Ile Arg Asp Tyr Glu Asp Gly Met Glu

655  GTA GAC ACT ACA CCA ACA GTT GCT GGA CAG TTT GAA GAT GCA GAT GTT GAT CAC
     --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
219  Val Asp Thr Thr Pro Thr Val Ala Gly Gln Phe Glu Asp Ala Asp Val Asp His

709  TGA AAATGATTTATGCGGCTGGAAGATTCTGCTTTATAACTGTAGGAAAGGACTTGGTGCCTCTTCCTGT
     ---
237  ***
CTGGAGTGGGGTTGATGAAAATGTTTTTGCTTCTTCAAAACTCACTTGAGCCAGTTCTTTCTTGAAACAGC
AAGTTGTCACCAGCAGAAGAAACTATGACCTTTATTAACAAAGGTGAATTAACTAGGAAGGTATTTGTAGT
CTATCACCCCTGAATTTTCTCTGTCTGGTTCCCCTTTGGGTGGGTCTGTAATTTCTGCCTTCACATGTATA
TGTCTTCTGTAAGCTAGCAGCCTATGTGGTGAAATGCACAGGAGCTGGGAGGTGTGGGTAGACTGGGTGGG
AGAGGCTGAAGCTAAAATATGCCTCTTTGGGACTAAAGATACCTTTATCACTTAAAAAGAGAGAGATCTCA
GAGGAATCCTACTGCTGCTCATTCACAGAGTCTTTTACTGAAATGGGACCCAGATTAATTCAGCATCACTA
GGTGACCTCTTTGGAAACCTCCCAGGCACTTGTACCACACTTCCACAGATGCTTCTGACTAGCCTTTG
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Fig. 1. The nucleotide and deduced amino acid sequences of RCL-H1. Nucleotides are numbered from the first nucleotide of the initiation ATG and amino acid residues are numbered from the initiating methionine.

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Rabbit heart (RCL-H1)   MSFLRSFLPPGPTEGLRHQQPDTEAVLNGKGLGTGTLYIAESRLSWLDGSGLGFSLEYPT  60
Rat kidney (RKCL)       ----K---P---SAD---L-------------------------------------  60
MDCK                    ----K---P---SA----Q---E---------------------------------  60
Rat atrium          MLSPA----K---P---SAD---L-------------------------------------  65

Rabbit heart (RCL-H1)   ISLHAVSRDPNAYPQEHLYVMVNAKFGEESKELVADEEEDSDDDVEPISEFRFVPGDKSA 120
Rat kidney (RKCL)       ------------------------R--------PFS---D---DN----------S---- 120
MDCK                    ---------L-----R-----V----------S--*----------A------S---- 119
Rat atrium              ------------------------R--------PFS---D---D----------S---- 125

Rabbit heart (RCL-H1)   LEAMFTAMCECQALHPDPEDEDSDDYDGEEYDVEAHEQGQGDIPTFYTYEEGLSHLTAEG 180
Rat kidney (RKCL)       ------------------------------------------------------------ 180
MDCK                    ------------------------------------------------------------ 179
Rat atrium              ------------------------------------------------------------ 185

Rabbit heart (RCL-H1)   QATLERLEGMLSQSVSSQYNMAGVRTEDSIRDYEDGMEVDTTPTVAGQFEDADVDH 236
Rat kidney (RKCL)       ----------------------------V-T-------E---------------- 236
MDCK                    ----------------------------T------------------------- 235
Rat atrium              ----------------------------V-T-------E---------------- 241
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Fig. 2. Alignment of amino acid sequence of RCL-H1 with that of other pI_{cln} homologues of rat kidney, MDCK and rat atrium. A gap (*) is introduced in MDCK pI_{cln} sequence to maintain optimal alignment with RCL-H1. Identical amino acids are shown by dash (–). Potential phosphorylation sites for tyrosine kinase (●) are indicated.

SDS at 55°C and one 20 min wash with 0.1 × SSC/0.1% SDS at 65°C. Autoradiography was performed on Kodak X-AR film for 40 h at −70°C with an intensifying screen.

Nucleotide sequence analysis of RCL-H1 revealed one long open reading frame, predicting a protein of 236 amino acids with a calculated M_r of 26107.8 (Fig. 1). There was no stop codon in the cDNA sequence 5′ to the first methionine codon. Although we analyzed five independent cDNA clones and eight RACE PCR products, we could extend only 48 bases at the 5′ end to the first methionine codon. The extended 5′-region contained no other methionine codons and no stop codons. However, comparison of amino acid sequence of RCL-H1 with that of pI_{cln} and RKCL-1 indicates that the sequence of RCL-H1 includes the entire protein-coding sequence (Fig. 2). The deduced amino acid sequence of RCL-H1 is 94.1% identical to that of MDCK pI_{cln}, 92.8% identical to that of RKCL-1 and 93.2% identical to that of rat cardiac pI_{cln}. RCL-H1 has the clusters of negatively charged residues (residues 96–106, 139–152) and two putative tyrosine kinase phosphorylation sites (residues 146, 151) in the latter cluster. They are highly conserved among RCL-H1, pI_{cln} and RKCL-1. The physiological significance of these acidic regions is not clear. There is no consensus phosphorylation site for protein kinase A or protein kinase C.

In some oocytes injected with RCL-H1 cRNA, depolarizing pulses produced slowly inactivating outward currents in isotonic solution (Fig. 3). The currents reversed at around −20 mV, which is close to the expected value of Cl^- equilibrium potential in *Xenopus* oocytes [12].

RNA blot analysis revealed that the mRNA of RCL-H1 was expressed at the highest level in the cerebrum, cerebellum, kidney, liver and lung, at moderate levels in the atrium, ventricle and skeletal muscle (Fig. 4). This widespread expression of RCL-H1 suggests that it may play an important role in many cells.

pI_{cln} was originally reported as a novel chloride channel cloned from MDCK cell line. Further studies, however, suggested that pI_{cln} was a swelling-induced chloride con-

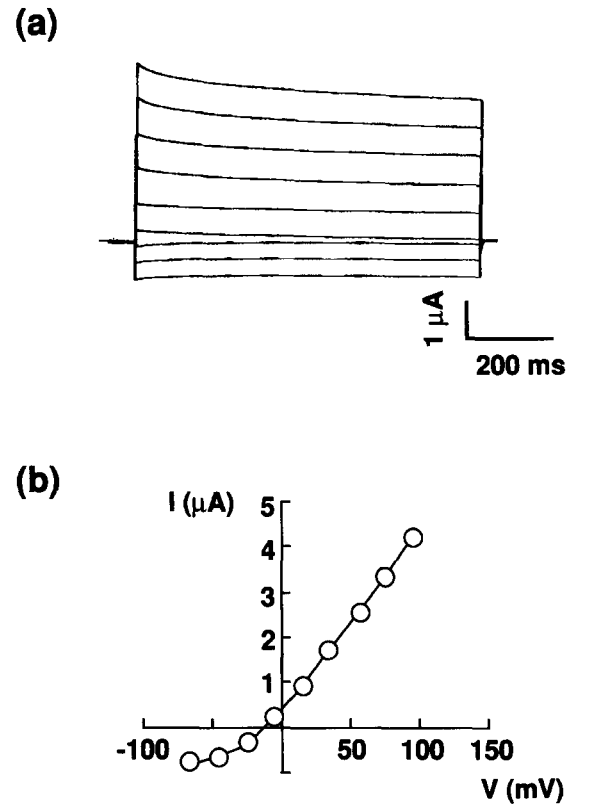

Fig. 3. Currents induced by RCL-H1. (a) Outwardly rectifying currents recorded in an oocyte expressing RCL-H1. The oocyte was bathed in ND96 solution. Current traces elicited by voltage steps to −60, −40, −20, 0, 20, 40, 60, 80 and 100 mV for 800 ms from a holding potential of −20 mV are shown. (b) Current–voltage relationship obtained from the same oocyte of (a). Peak current amplitudes are plotted against membrane potential.

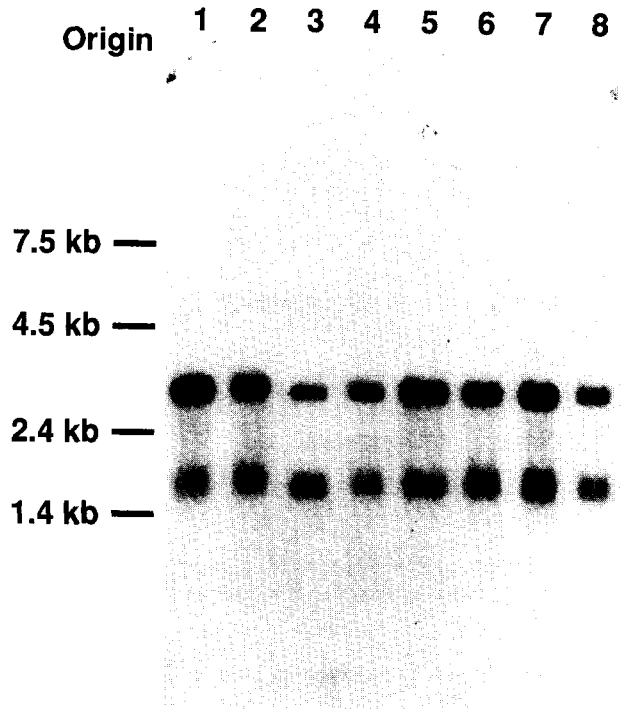

Fig. 4. RNA blot analysis of RCL-H1 mRNA. Poly(A)$^+$ RNAs analyzed are as follows; cerebrum (1), cerebellum (2), atrium (3), ventricle (4), kidney (5), liver (6), lung (7), skeletal muscle (8). The positions of RNA size markers (BRL) are shown on the left. The integrity of each RNA was confirmed by re-probing the same membrane with a β-actin cDNA probe (data not shown).

ductance regulator rather than a channel protein. It was shown that hypotonicity induced a chloride current in uninjected oocytes [13] which was blocked by monoclonal antibody against pI_{cln}. It was also shown that pI_{cln}-associated current was elicited without any osmotic challenge in the oocytes overexpressing pI_{cln}, which is consistent with our results. pI_{cln} and our clone RCL-H1 may participate in cell volume regulation. If RCL-H1 and pI_{cln} are in fact a swelling-induced chloride conductance regulator, they will give a clue to revealing molecular structure of unidentified swelling-induced chloride channel(s).

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