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Molecular and electrophysiological characterization of nucleotide-sensitive chloride current-inducing protein of *Fasciola hepatica*☆

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Abstract

Nucleotide-sensitive chloride current regulating proteins (ICln's) of the chloride channels have been characterized from man and animals. An ICln of *Fasciola hepatica* (ICln-Fh) consisting of 231 amino acids revealed high similarities to both consensus domain of ICln's and two acidic residue-abundant patches in its C-terminus. Native ICln-Fh protein was confirmed present in *F. hepatica* soluble extract by immunoblotting. The recombinant ICln-Fh protein expressed in collagenase-defolliculated *Xenopus* oocytes induced fast rising and outward rectifying Cl^- currents ($I_{Cln\text{-}Fh}$). The recombinant ICln-Fh protein, however, did not trigger cell swelling-induced Cl^- currents ($I_{Cl\text{-}swell}$). The $I_{Cln\text{-}Fh}$ currents were significantly reduced by substituting external Cl^- with gluconic acid and by externally adding cAMP. Collectively, these results suggest that ICln-Fh protein is an inducer of Cl^- currents in *F. hepatica*.

Keywords: *Fasciola hepatica*; Chloride current-inducing protein; Nucleotide-sensitive; Outward rectifier

1. Introduction

Fasciola hepatica is a trematode parasitic of herbivorous mammals and humans [1]. As a member of the platyhelminths, *F. hepatica* is one of the lowest bilateraria that diverged early from the metazoan lineage [2]. Trematodes are the first metazoan group to possess a centralized nervous system [3]. Several neurotransmitters characterized in higher animals were identified from the nervous system of *F. hepatica*.

The normal functions of organs and cells are tightly associated with the cytoarchitecture. The control of cell volume is therefore vital for organisms. Increases in cell volume (cell swelling) can occur after a sudden decrease in the extracellular osmolarity, an increase in the intracellular solute level, or transiently after an increase in the extracellular osmolarity. All living organisms encounter these osmolarity changes. The activation of Cl^- and K^+ channels is a widely established strategy of the cells to counteract swelling, and leads to a net efflux of salt followed by water [4–6]. Chloride channels are categorized into three classes; ligand-gated Cl^- channels, cystic fibrosis transmembrane conductance regulator (CFTR) channel, and CLC channels [7]. Alterations of the chloride permeability of cell membranes result in various

Abbreviations: ICln, nucleotide-sensitive chloride current regulating protein; I_{Cln}, nucleotide-sensitive chloride current; ICln-Fh, ICln of *Fasciola hepatica*; $I_{Cln\text{-}Fh}$, I_{Cln} of *Fasciola hepatica*

☆ *Note:* Nucleotide sequence reported herein has been deposited in GenBank under Accession number AY496957.

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human diseases, such as cystic fibrosis (CFTR) [8,9], myotonia congenita (CLC-1), Dent's disease (CLC-5), and Barter's syndrome (CLC-Kb) [10].

Nucleotide-sensitive chloride current-inducing proteins (ICln) have been suggested to be the ion channels underlying swelling-dependent chloride current [4,5,6,11,12]. ICln was initially proposed to encode a chloride channel since the over-expression of ICln in *Xenopus* oocytes produces the induction of chloride conductance [11]. In cells, the ICln proteins were found associated with the cell membrane [12]. The reconstituted ICln's in lipid bilayers elicited the chloride currents sharing some features of the swelling-dependent anion channel, i.e. rectification and nucleotide sensitivity [4].

Ion channels are an important family of membrane proteins and are involved in the normal functioning of the organs and cells of trematodes, such as *Schistosoma mansoni* and *F. hepatica* [13]. Moreover, several useful anthelmintic drugs are known to act on various ion channels, such as nicotinic acetylcholine receptor channels, GABA-gated chloride channels and glutamate-gated chloride channels [14,15]. In *F. hepatica*, a hyperpolarization-activated cation current was identified in acutely-isolated single cells by the whole-cell patch-clamp technique [16]. Recently, three types of single channel activities were characterized in *F. hepatica* [17]. However, the properties of various ion channels in *F. hepatica* remain largely uncharacterized.

In this study, a cDNA encoding ICln protein was cloned from *F. hepatica* and the electrophysiological properties of its encoded protein were characterized in *Xenopus* oocytes.

2. Materials and methods

2.1. Adult F. hepatica

Metacercariae of *F. hepatica*, purchased from a laboratory (Baldwin Aquatics, Monmouth, OR) were administered to rabbits with a gastric tube. The adult flukes were recovered from the bile ducts of rabbits 6–12 months after infection, and used immediately or stored at −20 °C until use.

2.2. Cloning a ICln-Fh cDNA

In the course of cloning potassium channel cDNAs from *F. hepatica* using degenerate oligonucleotide probes, one cDNA clone was found to encode a putative protein homologous with chloride current regulating proteins. Described hereafter are experimental methods employed in the cloning and characterization of this cDNA clone.

Total RNA was extracted from the adult flukes by CsCl-gradient ultracentrifugation and poly(A)$^+$ mRNA's were selected from the total RNA by oligo(dT) column chromatography. An adult *F. hepatica* cDNA library was constructed in bacteriophage lambda ZAP II vector according to the manufacturer's instruction (Stratagene, La Jolla, CA).

Degenerate oligonucleotides were designed and synthesized (Bioneer Co. Chungju, Korea) based on the conserved pore-forming and calcium-binding domains of Kir2, Kir3, Kir4, Kv1, SKik, and BKca channels [18,19]. The five synthetic degenerate oligonucleotides were pooled and purified using a PCR purification spin column (QIAGEN GmbH, Hilden, Germany). The adult *F. hepatica* cDNA library (independent 2×10^5 pfu) was screened with DIG-labeled oligonucleotide probes and the plaque hybridization technique using the Luminescent Detection kit (Roche). Positive plaques were isolated through three rounds of screening using the same oligonucleotide probes. Recombinant plasmids were in vivo excised and the cDNA inserts were sequenced with BigDye Terminator Cycle Sequencing kit and an automatic sequencer (ABI, Foster City, CA).

cDNA sequences and their putative polypeptide sequences were analyzed using DNASIS (Hitachi Software Engineering Co., Yokohama, Japan) and programs from several institutions (http://www.ncbi.nlm.nih.gov; http://sosui.proteome.bio.tuat.ac.jp; http://bioinf.cs.ucl.ac.uk; http://www.cbs.dtu.dk). Proteins homologous to the putative polypeptide were identified in GenBank using BLAST. A multiple alignment was prepared with CLUSTAL W and refined manually with reference to the predicted secondary structure of the putative ICln-Fh polypeptide.

2.3. Expression and purification of recombinant ICln-Fh protein

The forward primer was designed using the cDNA sequence corresponding to the N-terminus of ICln-Fh with the Kozak sequence (underlined) added to its 5′-end (5′-gccgccgccATGCAGCTGGTTCAATCCAATGGA-3′), and reverse primer corresponding to the C-terminus of ICln-Fh (5′-tctagtttaACAATCCGCAAACT-3′). With these two primers and *AmpliTaq* Gold DNA polymerase (QIAGEN Co.), ICln-Fh cDNA containing the whole coding region was amplified from cDNA clone FhICln4 and subcloned into a prokaryotic expression vector, pCR@T7/NT-TOPO@ (Invitrogen). In this expression construct (pTP-FhCl), the N-terminus of deduced ICln-Fh polypeptide was placed in-frame to a prokaryotic expression tag peptide (6xHis-Xpress epitope). Using this construct, the recombinant ICln-Fh protein can be expressed both in prokaryotic and eukaryotic host cells.

To overexpress the recombinant ICln-Fh protein, *E. coli* BL21[DE3]pLysS was transformed with the expression construct plasmid DNA, pTP-FhCl. Expression of the recombinant protein was induced by adding IPTG to the culture medium to a final concentration of 1 mM. The bacterial cells were harvested and ruptured by sonication. An equilibrated Ni-NTA affinity column (QIAGEN Co.) was charged with the cleared bacterial supernatant containing approximately 10 mg of the fusion protein. The bound protein was eluted at conditions according to manufacturer's instruction. Recombinant protein was subjected to SDS-polyacrylamide gel