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A new hyperpolarization-activated, cyclic nucleotide-gated channel from sea urchin sperm flagella ☆

Blanca E. Galindo, Anna T. Neill, Victor D. Vacquier *

Center for Marine Biotechnology and Biomedicine, Scripps Institution of Oceanography, University of California, San Diego, La Jolla, CA 92093-0202, USA

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Abstract

A sea urchin sperm flagellar hyperpolarization-activated, cyclic nucleotide-gated (HCN) channel is known (SpHCN1) that is modulated by cAMP. Here, we describe a second flagellar HCN channel (SpHCN2) cloned from the same sea urchin species. SpHCN2 is 638 amino acids compared to 767 for SpHCN1. SpHCN2 has all the domains of an HCN channel, including six transmembrane segments (S1–S6), the ion pore, and the cyclic nucleotide-binding domain. The two full-length proteins are 33% identical and 51% similar. The six transmembrane segments vary from 46–79% identity. S4, which is the voltage sensor, is 79% identical between the two proteins. The ion selectivity filter sequence is GYG in the ion pore of SpHCN1 and GFG in SpHCN2. By sequence, SpHCN2 is 73.5 kDa, but it migrates on SDS–PAGE at 64 kDa. Western immunoblots show localization to flagella, which is confirmed by immunofluorescence. A neighbor-joining tree shows that SpHCN2 is basal to all known HCN channels. SpHCN2 might be the simplest pacemaker channel yet discovered.

Keywords: Invertebrate fertilization; Acrosome reaction; Chemotaxis; Cyclic nucleotides; Flagellar motility; Ion channels

Hyperpolarization-activated and cyclic nucleotide-gated (HCN) channels control the rhythmic beating of cardiac cells and the spontaneous firing of neurons [1–3]. One HCN channel, here termed SpHCN1 (but also referred to as SPIH [4] or SpHCN [5]), has been cloned from sea urchin sperm. SpHCN1 is weakly K^+ selective, voltage and cAMP-modulated, and is related to I_h channels [4]. SpHCN1 is phosphorylated in sperm and conducts little Na^+ in the absence of K^+. This channel could be activated by the hyperpolarization induced by binding of the egg peptide speract to sperm receptors [6,7], and could also be responsible for the rhythmic oscillations in free Ca^{2+} that occur spontaneously in the sperm flagellum, which are increased by speract [8]. Speract receptors and SpHCN1 are both in the sperm flagellar membrane [4,5]. Studies of the electrical properties of site-directed mutants of SpHCN1 have added significantly to knowledge of the ion pore gating mechanism of pacemaker channels [9–12].

The sea urchin genome is currently being sequenced and assembled. A tandem mass spectrometry proteomic study of sea urchin sperm flagellar membranes identified a peptide derived from a novel HCN channel [13]. The full-length cDNA sequence encoding this novel channel, here named SpHCN2, was obtained by standard methods and antibodies raised. This paper compares the primary structures of SpHCN1 and 2.

Materials and methods

Sperm and isolation of membranes. Sperm of the sea urchin *Strongylocentrotus purpuratus* were spawned by injection of 0.5 M KCl.

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* Corresponding author. Fax: +1 858 534 7313.
E-mail address: vvacquier@ucsd.edu (V.D. Vacquier).

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Undiluted sperm were stored on ice for <12 h before use. Coelomocytes were removed and the sperm were sedimented [14]. Sperm were homogenized to break the flagellum from the sperm head and these two parts of the cell were separated by differential sedimentation [14]. The membranes of isolated heads and flagella were obtained by the pH 9.1 method [14]. Except where noted, all chemicals were from Sigma.

Tandem mass spectroscopy and cloning. Flagellar membranes were fragmented by CNBr cleavage, Endo-Lys-C, and trypsin digestion, and the peptides were subjected to tandem mass spectroscopy as described [15,16]. A spectrum was correlated with the peptide RRVLSYYEHR found in a translated open reading frame from the sea urchin genome project (http://www.hgsc.bcm.tmc.edu/projects/seaurchin/) [13]. This open reading frame was then used in a BLASTp search against the non-redundant protein database at NCBI (NR; http://www.ncbi.nlm.nih.gov/blast/). *Canis familiaris* K^+/Na^+ hyperpolarization-activated cyclic nucleotide-gated channel 4 was the highest scoring hit. A ~15 kb contig was assembled around the identified open reading frame using trace sequences from the sea urchin genome project database and that was searched against NR to identify the most probable exons. Specific primers were then designed from these candidate sequences.

The full-length cDNA sequence of this novel HCN channel was then obtained by PCR amplification using a Lambda Zap II (Stratagene) testis cDNA library as template. Testis total RNA was also isolated by Trizol (Invitrogen) extraction and cDNA was synthesized by standard procedures. 5′ RACE was performed to obtain the 5′ end, and the 3′ end was obtained by a PCR using specific primers in combination with vector primers.

Sequence analysis. Sites and motifs were found using ProfileScan (http://hits.isb-sib.ch/cgi-bin/PFSCAN) and transmembrane segments and domains were predicted with SMART (http://smart.embl-heidelberg.de/). CLUSTALW in the BioEdit program and MacVector were used for alignments and hydropathy plots. The GenBank accession number for SpHCN2 is DQ079999.

Phylogenetic analysis. Complete sequences were used to generate a neighbor-joining phylogenetic tree of HCN channels using MEGA3 and 3000 replications [17]. The tree was also constructed using the aligned sequences of each HCN channel from the 5′ end of S1 to the 3′ end of the cyclic nucleotide-binding domain. GenBank Accession Nos. were: NP_999729, sea urchin SpHCN1; XP_287905, mouse HCN4; NP_005468, human HCN4; Q9TV66, rabbit HCN4; NP_032252, mouse HCN2; NP_001185, human HCN2; AAF89636, lobster HCN1; NP_034538, mouse HCN1; O60741, human HCN1; NP_065948, human HCN3; NP_032253, mouse HCN3; AAQ16311, lobster; AAQ16312, honey bee; CAA10110, tobacco budworm; EAA03692, *Anopheles gambiae*, and AAD42059, *Drosophila melanogaster*. Cone photoreceptor cGMP-gated channel α subunit (CNGA3), Q16281, and cyclic nucleotide-gated channel α 1, NP_000078, from human were used as out group sequences.

Antibody, immunoblotting, and immunofluorescence. Two sequences, from codons 1 to 264 of the SpHCN2 NH_2-terminus and codons 1609–1917 of the COOH-terminus, were expressed in *Escherichia coli* Rosetta cells using the His-tag pET15b vector (Novagen). Expressed proteins were purified on Ni–NTA–agarose (Qiagen) and sent out for commercial polyclonal antibody production (Orbigen).

For immunoblots, the 10,000*g* supernatant of a 1% NP40 non-ionic detergent extracts of whole sperm, and sperm head and flagellar membrane preparations were dissolved in Laemmli sample buffer and separated by SDS–PAGE. Proteins were transferred to PVDF membranes, blocked with non-fat dry milk in 150 mM NaCl/10 mM Hepes, pH 7.5, and the blots were probed with various dilutions of antibody. Washes were in NaCl/Hepes containing 0.1% Tween 20. Detection utilized an HRP-conjugated goat anti-rabbit antibody and SuperSignal West Dura Extended Duration Substrate (Pierce) following the supplier's directions.

For immunofluorescence, sperm were electrostatically bound to protamine sulfate coated coverslips and fixed in 3% paraformaldehyde/0.1% glutaraldehyde in seawater. Coverslips were blocked in 150 mM NaCl/10 mM Hepes (pH 7.5) containing 5% normal goat serum and 5 mg/ml bovine serum albumin, and then incubated in various dilutions of antibody in blocker. The wash buffer was saline/Hepes containing 0.1% Tween 20. Coverslips were incubated for 1 h in a 1:400 dilution of Alexa-Fluor 546 goat anti-rabbit IgG (Molecular Probes) and washed twice in NaCl/Hepes/Tween before viewing with a fluorescence microscope.

Results

Sequence analysis

The SpHCN2 cDNA open reading frame is 1914 bp encoding a protein of 638 residues with a calculated molecular mass of 73.5 kDa (Fig. 1). An in-frame stop codon was found 5′ to the first Met residue shown. However, Western blots with the two different antibodies show a single reacting band at 64 kDa (Fig. 3). This discrepancy in apparent mass might result from which methionine residue is the start of translation. None of the four Met residues indicated with diamonds at the 5′ end of the sequence (Fig. 1) conforms well to a Kozak translation initiation site [18]. If the fourth Met residue is the start of translation, the predicted mass would be 65 kDa, which is close to that observed on gels. The peptide identified by tandem mass spectroscopy, R^{411}–R^{420}, is between S5 and S6 (dots over SpHCN2). SpHCN2 is 129 residues shorter than SpHCN1, the difference in size of the two proteins being upstream of S1 and downstream of the cyclic nucleotide-binding domain. Where they align, the two proteins are 33% identical and 51% similar (Fig. 1A). The NH_2- and COOH-extensions, which are the major differences between these two channel proteins, could be involved in the differential binding of regulatory proteins.

The most similar regions shared by the two sequences are in S1–S6, the pore region and the cyclic nucleotide-binding domain. S1–S6 range from 46–79% identity and 74–92% similarity. S4, which is the voltage sensor, is the most similar, being 76% identical and 92% similar. The 24 ion pore residues between S5 and S6 are 54% identical and 83% similar. SpHCN2 has seven positively charges residues lining the S4, whereas SpHCN1 has eight [4]. The ion selectivity filter in the pore is GFG in SpHCN2 and GYG in SpHCN1. There are two N-linked glycosylation sites in SpHCN2 at positions N^{151} and N^{350}. There are two cAMP-dependent protein kinase phosphorylation sites at S^{54} and S^{82} and five potential protein kinase-C phosphorylation sites at T^{60}, S^{104}, S^{204}, S^{385}, and T^{409}. There is one potential protein tyrosine kinase site in SpHCN2 and two such sites in SpHCN1 (triangles). The cyclic nucleotide-binding domain is 135 residues and 44% identical between the two proteins.