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

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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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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.

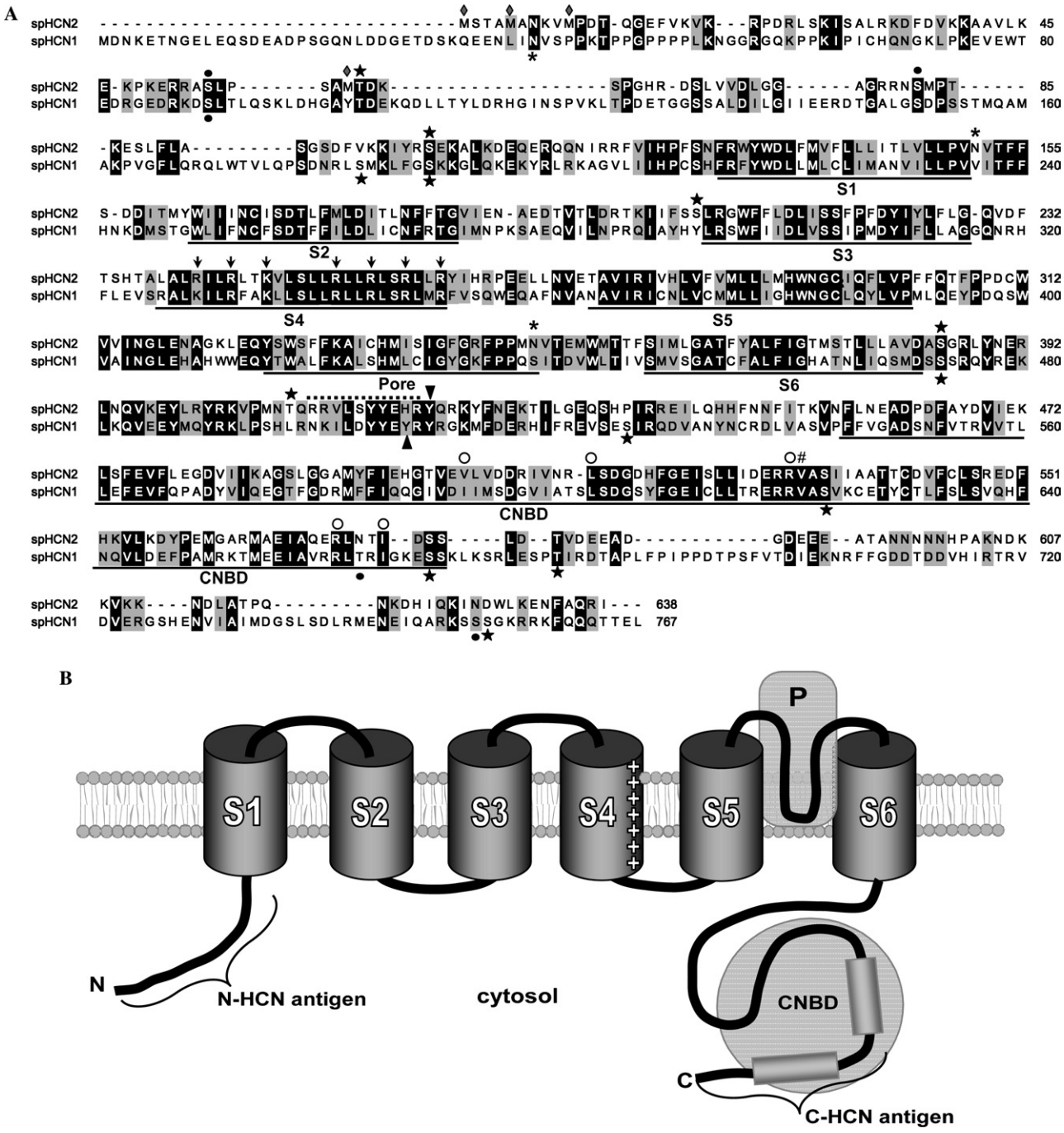

Fig. 1. (A) Alignment of SpHCN1 and SpHCN2. Diamonds show the four 5′ Met residues that could be translation starts. Large dots are potential protein kinase-A phosphorylation sites. Stars indicate potential protein kinase-C phosphorylation sites. Triangles show potential tyrosine phosphorylation sites. Asterisks mark potential N-linked glycosylation sites. The six transmembrane segments are labeled S1–S6. Seven positively charged residues in the S4 voltage sensor are shown by arrows. The ion pore and cyclic nucleotide-binding domain (CNBD) are underlined. Residues important for the cAMP selectivity are marked with empty circles. Dots over SpHCN2 show the peptide obtained by proteomics. Dashes in the sequences are inserted to optimize the alignment. (B) Hypothetical two-dimensional model of SpHCN2. S1–S6, α-helical transmembrane segments; P, ion pore; CNBD, cyclic nucleotide-binding domain. The regions used as antigens (N-HCN and C-HCN) are shown at the two ends.

Phylogenetic analysis

A neighbor-joining tree (Fig. 2) including 16 other HCN channels from a variety of organisms was made using the two closest related cyclic nucleotide-gated channels as out groups. SpHCN2 is at the base of the tree of all HCN channels of all organisms. Because the greatest differences in alignments of these channels are upstream of S1 and downstream of the cyclic nucleotide-binding domain, the tree was remade with-

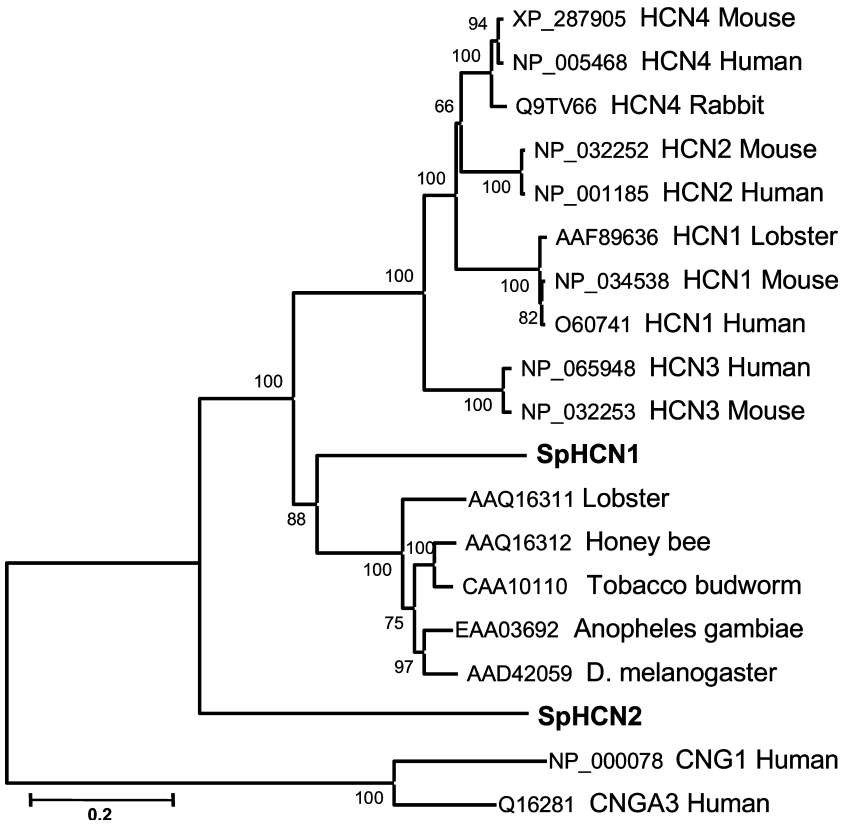

Fig. 2. A neighbor-joining tree of selected HCN channels and the two sea urchin sperm HCN channels made with full-length sequences. SpHCN2 is basal to all other HCN channels. A tree made with the complete sequences of the 30 known eukaryote HCN channels yields the same topology. A tree made with just the core sequences, from the start of S1 to the end of the CNBD, yields the same topology.

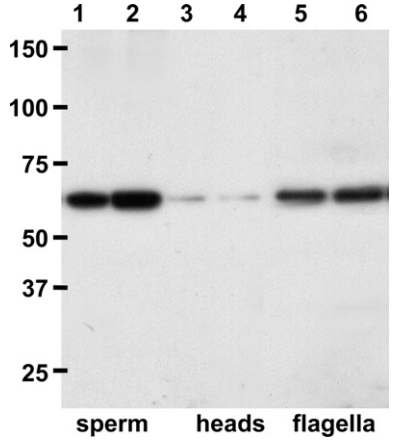

Fig. 3. Western blot of sperm proteins reacted with antibody to residues 1–264 of SpHCN2. A single reacting band is found at 64 kDa. Lanes 1, 3, and 5 are 3 μg protein per lane and lanes 2, 4, and 6 are 6 μg protein per lane. Lanes 1 and 2 are a 1% NP40 extract of whole sperm (10,000*g* supernatant). Lanes 3 and 4 are membranes from sperm heads. Lanes 5 and 6 are membranes from sperm flagella. Antibody to SpHCN1 reacts with a single protein band at 92 kDa [4].

out these two end regions. The "core sequence region," from S1 through the cyclic nucleotide-binding domain, yields a tree with the exact branching order as does the full-length sequences. These data suggest that the SpHCN2 is the most basal HCN channel yet discovered.

Immunoblot and immunofluorescence

Immunoblots using either antibody to the NH_2 or COOH end of SpHCN2 yield the same blot when reacted with NP40 extracts of whole sperm (Fig. 3, lanes 1 and 2), isolated sperm head membranes (lanes 3 and 4), and isolated flagella membranes (lanes 5 and 6). At equal protein loads there is a slight reaction with sperm head membranes, but the greatest reaction is with the flagellar membrane extract at a relative molecular mass of 64 kDa. Antibodies to SpHCN1 react with a single protein band of 92 kDa [3].

Immunofluorescence microscopy shows that SpHCN2 is localized almost exclusively to sperm flagella at all concentrations of antibody tested (Fig. 4). This is the same location of SpHCN1 [4].

Discussion

HCN channels generally have a low selectivity for K^+. For example, SpHCN1 conducts about five times more K^+ than Na^+ [4]. The pore region of HCN

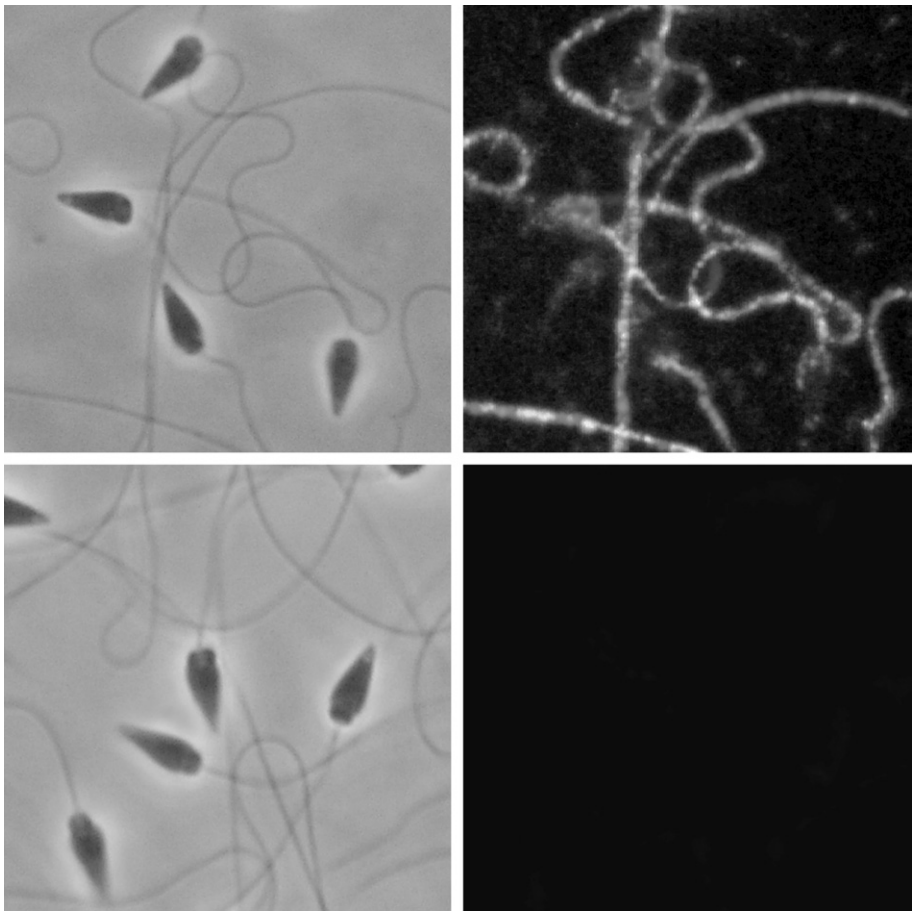

Fig. 4. Immunofluorescence with antibody to SpHCN2. Left panels are phase contrast and right panels are fluorescence. Upper two panels show that the anti-SpHCN2 reacts mainly with flagella. Lower two panels show the negative control without the primary antibody. The widest part of the sperm head is ~1 μm in diameter.

channels is related to K^+ selective channels, which have the triplet GYG motif, considered as the core sequence of the ion selectivity filter [1–3,19]. However, SpHCN2 has a different motif, GFG, indicating that the ion permeability of this channel may differ from that of its homologues.

The voltage sensor (S4) of HCN channels has a particular structure consisting of a string of positively charged residues found in every third position. This series is interrupted by a serine in the middle of the string [3,20]. This characteristic is what differentiates the HCN voltage sensor from the voltage sensor of voltage-activated K^+ channels. The high number of positively charged residues (7–9) in S4 of HCN channels makes their S4 more similar to that of voltage-dependent K^+ channels than to cyclic nucleotide-gated channels (CNG), which have fewer charged amino acids in S4 [2]. The S4 sequence of SpHCN2 is typical of an HCN channel.

HCN channels, like CNG channels, have a cyclic nucleotide-binding domain (CNBD) in the COOH-terminus that confers channel modulation by the direct binding of cAMP or cGMP [2,21]. The crystal structure of this domain in mouse HCN2 is known [22] and it shows similarity to the CNBD in the catabolite gene activator protein (CAP) of *E. coli* [23]. The crystal structure of the CNBD in mouse HCN2 shows that it consists of four α-helices and a β-roll composed of eight β-strands [22]. The cyclic nucleotide interacts with the β-roll and the C-helix of the COOH-terminus. One conserved arginine (in SpHCN1, Arg^{620}; in SpHCN2, Arg^{531}) in the β-roll binds directly to the cyclic phosphodiester moiety and with oxygens in the ribose moiety of the cyclic nucleotide [22]. In the C-helix, a conserved isoleucine of mouse HCN2 (in SpHCN1 Ile^{665}; in SpHCN2, Ile^{576}) has a hydrophobic interaction with the purine ring of cAMP. This Ile is predicted to provide cAMP-binding selectivity together with a conserved arginine (SpHCN1 Arg^{661}; SpHCN2, Arg^{572}) [22]. Other residues in the β-roll that are important for the interaction with the ribose moiety of cAMP are a conserved valine (in SpHCN1 this is substituted as Ile^{592}; in SpHCN2 it is Val^{504}) and a leucine (in SpHCN1 Leu^{603}; in SpHCN2, Leu^{514}). A conserved threonine in the C-helix is partially responsible for the cGMP selectivity in the CNBD of cyclic GMP-dependent protein kinases and

CNG channels [24,25]. Although this Thr is present in all known mammalian HCN channels, SpHCN1 and 2 have a valine (in SpHCN1, Val^{621}; in SpHCN2, Val^{532}, marked with # in Fig. 1A) instead of Thr at this position. HCN channels in general have more selectivity for cAMP compared to cGMP. In addition, SpHCN1 has much higher sensitivity for cAMP over cGMP [4]. Considering these structural facts, it is more likely that SpHCN2 is modulated by cAMP than by cGMP.

Conclusion

A second hyperpolarization-activated and cyclic nucleotide-gated channel has been discovered in sea urchin sperm flagella. The channel is evolutionarily basal to other HCN channels. Its electrical properties, ion selectivity, and cyclic nucleotide dependence will be important to study.

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