Human Hyperpolarization Activated Cyclic Nucleotide Gated Ion Channel 4 (HCN4)

A Target Enabling Package (TEP)

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Target Nominator	SGC Internal Nomination
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Target PI	Liz Carpenter (SGC Oxford)
Therapeutic Area(s)	Cardiovascular, Inflammation (homologue of HCN2, an inflammatory pain target), Neuropsychiatry and neuro genetic disorders
Disease Relevance	Mutations in HCN4 are found in individuals with the cardiac conditions Brugada syndrome 8 (OMIM: 613123) and Sick sinus syndrome 2 (OMIM: 163800). Variants have also been observed in patients with mood disorders and obsessive compulsive disorders (Kelmendi et al., Neurosci Lett, 2011) HCN4 is a close homologue of HCN2, another cyclic nucleotide gated ion channel that is associated with inflammatory pain (1).
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SUMMARY OF PROJECT

HCN4 is one of four hyperpolarisation activated cyclic nucleotide-gated ion channels. It is responsible for the pacemaker or funny (If) current in the heart and is required for maintenance of a stable heartbeat. Mutations in HCN4 lead to a number of arrhythmias. HCN4 is the target for the angina drug ivabradine, which reduces HCN4 activity. However, ivabradine is non-selective, affecting all of the four HCN channels. HCN4 is a close homologue of HCN2, which is a target for neuropathic and inflammatory pain treatment. We have solved the structure of HCN4 both in complex with cyclic AMP (cAMP) and without nucleotide. Comparison of our HCN4 structure with that of the related HCN1 channel (86% identity) allows us to suggest ways to design selectivity for small molecule inhibitors between these closely related channels.

SCIENTIFIC BACKGROUND

Biology Background

HCN4 channels are non-selective potassium and sodium channels that are regulated by voltage and cAMP. They are highly expressed in the heart, thalamus and testes (2). HCN4 has roles in the central and peripheral nervous systems and in the heart. In particular, HCN4 is found in the sinoatrial node (SAN) in the right atrium of the heart, the region that is responsible for the generation of the heartbeat. HCN4 has the unusual characteristics of being activated by hyperpolarisation of the membrane, combined with very slow gating. Hyperpolarisation causes HCN4 to open, allowing the inward flow of sodium and potassium ions, which reduces the membrane potential. This current is known as the pacemaker or funny (If) current and it is required for diastolic depolarisation of the SAN cells. This depolarisation triggers the electrical impulses that spread through the atria and then the ventricles of the heart. This causes the coordinated contraction of the atria, followed by the ventricles, propelling blood through the heart to the lungs and the rest of the body. Heart rate is regulated by the sympathetic and parasympathetic nervous systems, through receptors that activate or inhibit adenylate cyclase. Changes in cAMP levels, regulate HCN4 through its cyclic nucleotide binding domains. HCN4 is therefore central to the formation and regulation of the heartbeat.

Therapeutic control of the heart rate has been facilitated by the development of an HCN4 channel blocker, ivabradine, which is used for treating angina and heart failure. Although ivabradine is effective in treating heart problems, it does have side effects due to the lack of selectivity for the other three HCN channels. One of the aims of this work is to promote the design of more selective HCN channel blockers.

Genetic Linkage to Cardiac Arrhythmias

Given the fundamental role of HCN4 in pacemaking in the heart, it is not surprising that a number of cardiac arrhythmias are associated with mutations in HCN4, including Brugada syndrome 8 (OMIM: 613123) and Sick sinus syndrome 2 (OMIM: 163800). Patients with Brugada syndrome have apparently normal heart structures, abnormal ECGs and a high incidence of adult sudden death syndrome. Patients with sick sinus syndrome 2 (SSS) have syncope, dizziness and fatigue. They have bradycardia (a heart rate below 40 beats per minute), arrhythmias and atrial fibrillation later in life. They often need to have pacemakers fitted to ensure a reliable heart rhythm. Table 1 below lists a series of missense variants in HCN4, found in patients with these diseases, their pathologies and their effects on channel function. In addition there are several mutations that lead to truncation of HCN4. Mutations are heterozygous and for SSS inheritance is dominant.

Disease associated mutations

Point mutations in HCN4 implicated in heart diseases characterised by arrhythmias are tabulated. Many of the mutations were identified in the Online Mendelian Inheritance in Man, OMIM® database, McKusick-Nathans Institute of Genetic Medicine, Johns Hopkins University (Baltimore, MD), 2018. https://omim.org/)

Missense variant	Disease	GoF/ LoF	Defect	Reference
Pro257Ser	Early onset atrial fibrulation	LoF	Trafficking	(3)
Ala414Gly	Sick synus syndrome 2 with cardiac noncompaction	LoF	Large negative shift in voltage dependence	(4)
Gly480Arg	Sick sinus syndrome 2	LoF	Activated at more negative voltages, Trafficking	(5)
Try481His	Bradycardia and left ventricular noncompaction	LoF	N/D	(4)
Gly482Arg	Bradycardia and left ventricular noncompaction	LoF	Large negative shift in voltage dependence of activation	(4)

Gly482Arg	Sinus node disease, left ventricular noncompaction, and MVP	LoF	No channel activity	(6) (7) (8)
Ala485Val	Sinus bradycardia	LoF		(9)
Val492Phe	Brugada syndrome	LoF	Required a more negative voltage for activation	(10)
Arg524Gln	Inappropriate sinus tachycardia	GoF	Increased sensitivity to cAMP	(11)
Lys530Asn	Tachycardia-bradycardia syndrome	LoF	Heteromeric channel gave significant hyperpolarizing shift in the half-maximal activation voltage	(12)
Asp553Asn	Sick sinus syndrome 2	LoF	Trafficking	(13)
574 del, trunc	Sick sinus syndrome 2 - sinus bradycardia and intermittent AF	LoF	No CNBD, no cAMP sensitivity	(14)
Ser672Arg	Sick sinus syndrome 2	LoF		(15)
13 bp ins, 695X	Sick sinus syndrome 2 with cardiac noncompaction	LoF	No sensitivity to cAMP	(16)
Ser841Leu	Brugada syndrome 8	LoF	N/D	(17)
Gly1097Trp	Atrioventricular block	LoF	Reduction in peak currents, negative voltage shifting	(18)

Table 1 Point mutations in HCN4 $^{L201\text{-}S719}$ associated with disease

HCN4 in the nervous system and its potential involvement in mood disorders

HCN channels are widely distributed throughout the central and peripheral nervous systems, where they are responsible for the I_h or hyperpolarisation activated current. They are thought to be involved in a range of functions, including maintenance of the resting membrane potential, cellular excitability and plasticity.

One report of association between mood and anxiety disorders and polymorphisms on HCN4 (19). This report identifies three common single nucleotide polymorphisms in introns in the HCN4 gene that are correlated with mood disorders, but further work will be required to determine the significance of these associations.

The HCN channels interact with PEX5L/TRIP8b, an adaptation factor which is thought to interact with two allosteric sites in the cytoplasmic domains of HCN channels. TRIP8b is thought to affect function, trafficking and turnover of HCN channels. Knockout mice lacking the TRIP8b protein are said to be “depression resistant”, behaving in a similar way to mice treated with antidepressants, so the TRIP8b-HCN interaction is of interest for treatment resistant depression.

A recent study (20) has shown that a family of endoplasmic reticulum (ER) transmembrane proteins interact with and modulate HCN4. Despite being homologous, these regulatory proteins, namely, the Lymphoid-restricted membrane protein (LRMP) and the inositol trisphosphate receptor-associated guanylate kinase substrate (IRAG), have opposite effects. They cause loss and gain of function, respectively. Interestingly, the actions of both regulators are independent of trafficking and cAMP binding.

In addition, HCN4 has been implicated in responses to sour taste (21).

Seizures

Mutations in the related HCN1 and HCN2 channels are associated with epileptic encephalopathies and epilepsy. Gabapentin is used to treat seizures and the mechanism of action of this drug is currently unclear,

but one report has suggested that gabapentin reduces HCN4 activity in neurons, suggesting that one of the modes of action of gabapentin (or potentially one of the causes of side effects) could be through HCN4 interactions.

Inflammation

HCN4 is highly homologous (90% identity in the region studied here) to HCN2, another member of the hyperpolarisation activated cyclic nucleotide gated family. Mutations in HCN2 are associated with febrile seizures and epilepsy. HCN2 is thought to play a central role in inflammatory and neuropathic pain. It has been proposed as a potential target for pain. Understanding the differences between HCN2 and HCN4 is therefore important for design of HCN2 selective tools.

RESULTS – THE TEP

Purification

We used a truncated version of HCN4 ($HCN4^{L201-S719}$) lacking the first 200 and the last 484 residues, which are predicted to be disordered. . A 6-His-tag followed by a TEV protease cleavage site were added to the N-terminus for purification. HCN4 was expressed using the baculovirus/insect cell expression system. The protein was extracted and purified using the dodecyl maltoside (DDM) detergent, supplemented with cholesteryl hemisuccinate (CHS) (10:1 ratio, wt:wt) for extraction. Protein was purified in 0.02% DDM, 0.002% CHS containing buffer, using Talon resin, TEV cleavage, reverse purification and size exclusion chromatography. The final yield was up to 1.5 mg/Litre of cell culture. The construct used was initially optimized for crystallisation, giving crystals that diffracted to 8Å, but the structure was subsequently solved by cryo-EM.

Chemical matter

Cyclic AMP is a native ligand of this hyperpolarisation activated channel. Cyclic AMP binds to the cyclic nucleotide binding domain, and, in tandem with the action potential of the membrane, causes the HCN4 channel to open.

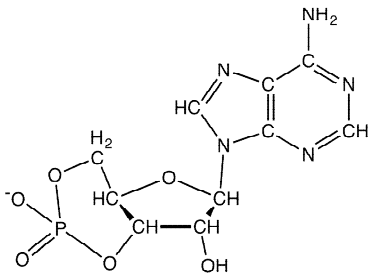

Figure 1: Structure of cyclic AMP

Structures

HCN4 was solved by cryo –EM

- 3.4 Å HCN4 without ligands
- 3.4 Å HCN4 with cyclic AMP

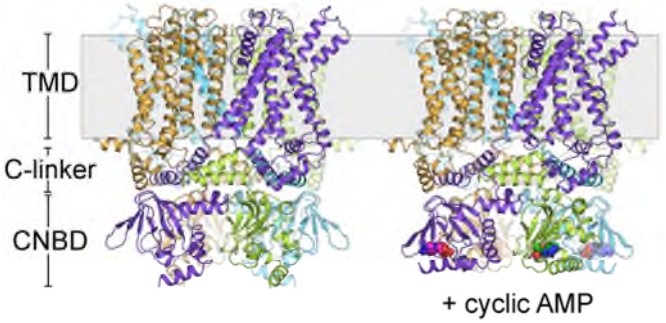

Figure 2 Structures of HCN4 in Apo and cAMP bound form

We solved the structure of HCN4$^{L201-S719}$ by single-particle cryo-EM with and without its native ligand cyclic-AMP, to 3.4Å resolution. The truncated construct was chosen to improve stability and expression levels of the protein in detergent-solubilised form. The tetrameric channel consists of the C-terminal cytoplasmic cyclic nucleotide binding domain (CNBD) which is connected to the transmembrane ion channel and voltage sensor domains via an α-helical C-linker domain which forms a disc-like structure below the membrane (Figure 2). The overall structure of HCN4 resembles that of HCN1 (22),with which is shares 86% sequence identity.

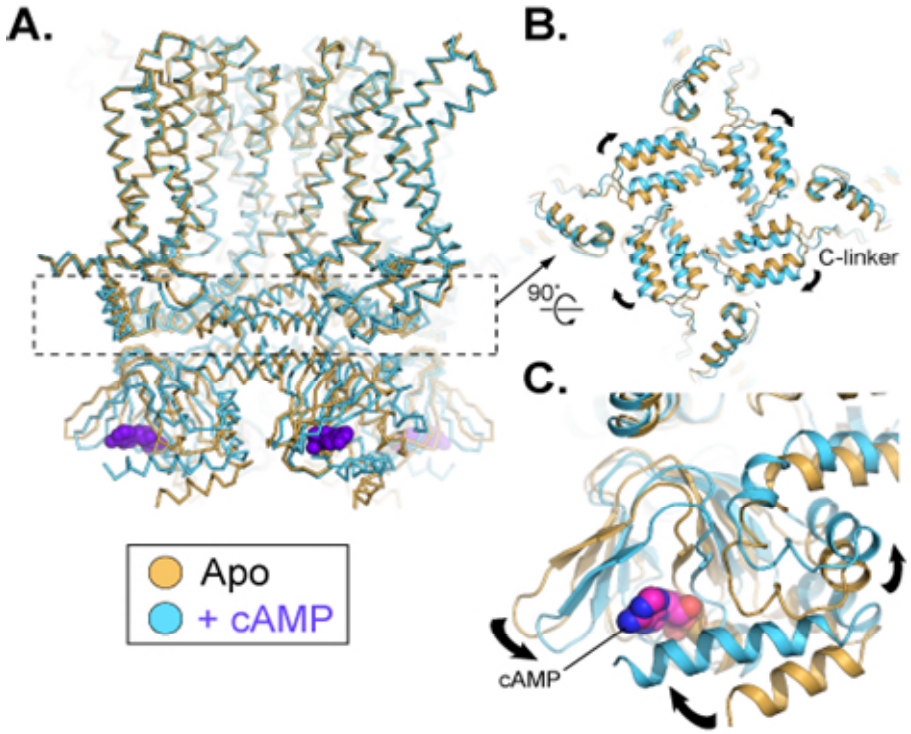

Figure 3 Cyclic AMP binding to HCN4 causes rotation of CNBDs and compaction of structure. (**A**) Superposition of Apo (gold) and cAMP-bound (cyan) structures. (**B**) View of C-linker ring from cytoplasmic face. (**C**) Conformational changes induced on cAMP binding.

IMPORTANT: Please note that the existence of small molecules within this TEP only indicates that chemical matter can bind to the protein in a functionally relevant pocket. As such, these molecules should not be used as tools for functional studies of the protein unless otherwise stated as they are not sufficiently potent or well-characterised to be used in cellular studies. The small molecule ligands are intended to be used as the basis for future chemistry optimisation to increase potency and selectivity and yield a chemical probe or lead series.

Upon binding of cyclic AMP to the CNBDs there is a twisting motion observed in the cytoplasmic half of the protein relative to the transmembrane region. This also results in the opening of the C-linker domain. However, we do not observe an opening of the ion channel itself (Figure 3A and 3B). The binding of cAMP also induces a compaction in the structure of the CNBDs and C-linker towards the TMDs. The C-terminal helix of the CNBDs coordinates the cAMP molecule and the whole domain moves towards the central axis of the channel (Figure 3C). HCN channels are uniquely activated at hyperpolarising membrane potentials therefore the structures are in a closed state with the voltage sensors adopting a depolarised conformation.

Biochemical and biophysical assays

The ion channel properties of HCN4 has been studied extensively using electrophysiology techniques (2).

In addition to these well characterised methods, we have developed a thermostability assay for HCN4, allowing us to detect direct binding of ligands such as cyclic AMP to the pure protein in detergent. This provides an orthogonal assay to confirm direct interaction between the channel and potential ligands.

Thermostability assays using the CPM cysteine modification dye as an indication of protein unfolding

This method uses a membrane protein-specific method for measuring protein stability with increasing temperature (23). The CPM coumarin derivative dye modifies exposed cysteine residues, forming a fluorescent adduct. This leads to an increase in fluorescence as the protein unfolds and cysteine residues are exposed. We used this method to detect binding of cyclic AMP to our detergent-purified HCN4 samples.

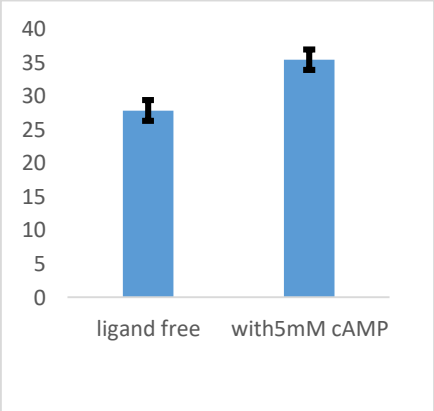

Figure 2 Thermostabilisation of HCN4 in presence of cAMP

- Quadruplicate measurements of two biological samples are shown.
- Equipment: Mx3005P™ Real-Time PCR System with ANS filter set to observe fluorescence emission maximum at 470 nm.
- The $Tm_{1/2}$ for the HCN4 sample used for structure determination was 27.7 °C.
- Addition of cyclic AMP gave a 7.5 °C stabilization.

Future plans

- We are investigating the HCN4-ivabradine interaction.
- We also plan to investigate the interactions between HCN4 and TRIP8b/PEX5L.
- We also intend to obtain structures of HCN2, in order to compare this close relative, for the purposes of finding ways to build in selectivity between HCN channels for design of molecules that specifically target each of the HCN channels.

CONCLUSION

- In this work we report a target enabling package for HCN4, the ion channel responsible for the pacemaker or funny channel activity in the sinoatrial node of the heart and the I_h current in neurons.
- HCN4 is the target for the angina drug ivabradine and it may also play a role in gabapentin activity.
- We have obtained protein, cryo-EM structures and a complex with the natural ligand cyclic AMP for HCN4.
- In addition we have developed a biophysical method for detecting binding of ligands to HCN4, which can be used as an orthogonal assay in conjunction with standard electrophysiology techniques.
- This opens the door to the design of more selective HCN channel blockers, with fewer side effects.

FUNDING INFORMATION

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ADDITIONAL INFORMATION

Structure Files

PDB ID	Structure Details
TBA	Apo HCN4
TBA	HCN4 in complex with its natural ligand cyclic AMP

Materials and Methods

Protein Expression and Purification Procedures

Vector: pFB-LIC-Bse (available from The Addgene Nonprofit Plasmid Repository)
Cell line: DH10Bac, Sf9 cells
Tags and additions: N-terminal, TEV protease cleavable-6-His- tag

Wild-type sequence (with tag):
MGHHHHHHSSGVDLGTENLYFQSMLPEAEVRLGQAGFMQRQFGAMLQPGVNKFSLRMFGSQKAVEREQERVKSAGFW
IIHPYSDFRFYWDLTMLLLMVGNLIIIPVGITFFKDENTTPWIVFNVVSDTFFLIDLVLNFRTGIVVEDNTEIILDPQRIKMKYLKS
WFMVDFISSIPVDYIFLIVETRIDSEVYKTARALRIVRFTKILSLLRLLRLSRLIRYIHQWEEIFHMTYDLASAVVRIVNLIGMMLLL
CHWDGCLQFLVPMLQDFPDDCWVSINNMVNNSWGKQYSYALFKAMSHMLCIGYGRQAPVGMSDVWLTMLSMIVGAT
CYAMFIGHATALIQSLDSSRRQYQEKYKQVEQYMSFHKLPPDTRQRIHDYYEHRYQGKMFDEESILGELSEPLREEIINFNCRK
LVASMPLFANADPNFVTSMLTKLRFEVFQPGDYIIREGTIGKKMYFIQHGVVSVLTKGNKETKLADGSYFGEICLLTRGRRTAS
VRADTYCRLYSLSVDNFNEVLEEYPMMRRAFETVALDRLDRIGKKNS

Expression
The full length gene for human HCN4 was obtained as a gift from GSK. This gene was cloned into the pFB-LIC-Bse vector and baculoviruses were produced by transformation of DH10Bac cells. *Spodoptera frugiperda* (Sf9) insect cells in Sf-900 II SFM medium (Life Technologies) were infected with recombinant baculovirus and incubated for 72 h at 27°C in shaker flasks and then collected by centrifugation at 900Xg and the pellet flash frozen in liquid nitrogen.

Cell Lysis and detergent extraction of membrane protein
Extraction Buffer, (EXB): 50 mM HEPES (pH 7.5), 150 mM NaCl, 5% Glycerol, Roche complete EDTA free protease inhibitors.

The insect cell pellet was resuspended in 40 ml of EXB / 1L pellet in a 50 ml tube and thawed in tepid water, mixing constantly to keep the sample cold. Cells were lysed with a EmulsiFlex-C5 homogenizer (Avestin) (chilled at 4 °C, 2 passes). 5 mL of 10 % : 1 % (w:w) stock of DDM/CHS was added per Litre of cell culture and the volume was adjusted by the addition of EXB to a final volume of 50 mL/L of cell culture. The sample was rotated in the cold room for 1 h, then extracted membrane proteins were separated from insoluble membranes by centrifugation at 35,000 g for 45 min at 4 ^{o}C.

Column 1: Co^{2+} talon resin (0.5 ml volume in a gravity-flow column):
Wash Buffer (WB): 50 mM HEPES (pH 7.5), 20 mM imidazole (pH 8.0), 150 mM NaCl, 5 % Glycerol, 0.02% DDM, and 0.002 % CHS.
Elution Buffer (EB): 50 mM HEPES (pH 7.5), 300 mM imidazole (pH 8.0), 150 mM NaCl, 5 % Glycerol, 0.02% DDM, 0.002 % CHS.
Gel Filtration Buffer (GFB): 20 mM HEPES (pH 7.5), 150 mM NaCl, 0.02 %DDM, 0.002 % CHS.

The detergent-extracted membrane protein fraction from each litre of cells was combined with 1 ml of pre-equilibrated slurry of 50 % Co^{2+} talon resin (pre-equilibrated with EXB after washing in H_2O). The sample was rotated in the cold room for 1 h then collected in an econo column fitted with a filter. The residual talon resin was washed with 5 ml wash buffer and pipetted onto the econo column. The talon resin was washed with 20x talon resin volume of WB and the protein eluted with 2x Talon resin volume of elution buffer in 1 ml fractions.

The peak fractions were combined and the imidazole removed using two PD10 columns per purification (pre-equilibrated with SEC buffer). Protein was eluted from the PD10 columns using SEC buffer.

TEV protease cleavage and reverse purification

TEV protease was added at a ratio of 10 : 1 (HCN4: TEV protease (w:w)). Samples were incubated on ice in the cold room overnight. The 6X His tagged TEV was separated from the tag cleaved HCN4 using cobalt affinity chromatography as before, with the flow through containing pure protein.

Column 2: Superose 6 Size Exclusion Chromatography

The protein sample was concentrated in a 100 kDa PES centrifugal concentrator (pre-equilibrated with SEC buffer), with mixing every 10 mins, to a final volume of 500 µL. After centrifugation at 21, 000 g for 30 min the sample was subjected to size exclusion chromatography on a Superose 6 column in SEC buffer. 3-4 0.5 ml peak fractions containing HCN4 protein were concentrated with a Sartorius 2 ml PES 100 kDa concentrator pre-equilibrated with SEC buffer, at 3,220 g to a final concentration of no more than 15 mg/ml. The protein was centrifuged at 21,000 g for 15 mins, then flash frozen in liquid nitrogen.

Assay protocols

Thermostability assay using CPM dye fluorescence

This method is based on the protocol described in Alexandrov et al., 2008 (23). Concentrated protein was defrosted and diluted to 1 mg/ml before aliquoting into assay plate well with each well containing 2 µg HCN4 in a final volume of 25 µl. The remaining volume was made up with GFB with or without addition of ligand (5 mM) and 5 µl of 0.04 mg/ml CPM (7-Diethylamino-3-(4'-Maleimidylphenyl)-4-Methylcoumarin) dye (diluted from a 4 mg/ml stock made up in DMSO) was added fresh before a 30 s centrifugation at 800 X g of the assay plate. Fluorescence measurements were carried out in an Mx3005P™ Real-Time PCR System with ANS filter with initial temperature set to 25℃ and the plate heating to 95C at a rate of 1℃ per minute.

Cryo-EM grid preparation

Concentrated protein was defrosted and diluted to 5 mg/ml. When ligands were added they were left to incubate with the protein for 1 hour at this stage and then the sample was centrifuged at 21,000 X g for 30 minutes before plunge freezing onto cryo EM grids. Sample was applied to glow discharged holey carbon coated grids (1.2-µm hole size, 1.3 µm spacing, 300 or 400 mesh; Quantifoil) Grids were blotted at 100% humidity for 3- 5 s and plunge-frozen in liquid ethane cooled by liquid nitrogen with a vitrification apparatus (Vitrobot Mark IV, FEI).

Cryo EM data collection and processing

Cryo-EM data collection was carried out at the eBIC national facility on a Titan Krios operated at 300 kV at liquid nitrogen temperature. Data were acquired with EPU software on a direct electron detector (K2 Summit, Gatan) mounted behind an energy filter (GIF Quantum LS, Gatan) and operated in zero-loss mode (0–20 eV). Movies were recorded in electron-counting mode (total dose 48e^{-}/Å^{2}) with underfocus in a range from 1.1 to 2.5 µm. The calibrated magnification used was 37,037× (corresponding to a pixel size of 1.34Å).

All initial processing was carried out in RELION 2.0. Frames in each movie stack were aligned with MOTIONCOR2 and CTF parameters were estimated using CTFFIND 4.0. Dose weighted stacks were subjected to semi-automatic particle picking using Gautomatch.

For both datasets particles were semi automatically picked and put through several rounds of 2D class averaging to remove bad particles. These were then subjected to template based 3D classification with a low pass filtered (60 Å) model of HCN4 based on HCN1 used as a template. An initial 3D classification, run with no symmetry imposed, clearly indicated that HCN4 formed a tetrameric channel. Further 3D classification with *C*4 symmetry imposed identified a subset particles that were subsequently used for a first 3D 'gold-standard' refinement wherein two half sets of the data were refined independently. Both datasets yielded reconstructions to 3.4Å resolution (FSC=0.143). The optimised particle sets from RELION were refined in parallel using cryoSPARC which gave resolutions of 3.3 Å.

Structure building and refinement procedures

The unliganded structure was built manually in COOT based on the coordinates of the unliganded HCN1 channel (PDB: 5U6O). The structure was refined against the *B*-factor sharpened cryoSPARC map to 3.4Å resolution using REFMAC and *phenix.real_space_refine*. This refined model was initially fitted automatically to the 3.4Å cAMP reconstruction using morphed refinement in *phenix.real_space_refine* and subsequently manually corrected in COOT. For the unliganded complex, the final model comprises residues Met214-Leu708. In the cAMP complex, the C-terminus is resolved to Ile714 due to ordering of the C-terminal CNBD helix on nucleotide binding.

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