KCNK10C

PDB Code: 4BW5

Material and Methods

Entry Clone Source: 4BW5

Entry Clone Accession: IMAGE: 30915621

SGC Construct ID: KCNK10C-c011

GI number: 54207

Vector: pFB-CT10HF-LIC

Amplified construct sequence:

ATGGGCTTGCAGACCGTCATGAAGTG
GAAGACGGTGGTTGCCATCTTTGTGG
TTGTGGTGGTCTACCTTGTCACTGGC
GGTCTTGTCTTCCGGGCATTGGAGCA
GCCCTTTGAGAGCAGCCAGAAGAATA
CCATCGCCTTGGAGAAGGCGGAATTC
CTGCGGGATCATGTCTGTGTGAGCCC
CCAGGAGCTGGAGACGTTGATCCAGC
ATGCTCTTGATGCTGACAATGCGGGA
GTCAGTCCAATAGGAAACTCTTCCAA
CAACAGCAGCCACTGGGACCTCGGCA
GTGCCTTTTTCTTTGCTGGAACTGTC
ATTACGACCATAGGGTATGGGAATAT
TGCTCCGAGCACTGAAGGAGGCAAAA
TCTTTTGTATTTTATATGCCATCTTT
GGAATTCCACTCTTTGGTTTCTTATT
GGCTGGAATTGGAGACCAACTTGGAA
CCATCTTTGGGAAAAGCATTGCAAGA
GTGGAGAAGGTCTTTCGAAAAAAGCA
AGTGAGTCAGACCAAGATCCGGGTCA
TCTCAACCATCCTGTTCATCTTGGCC
GGCTGCATTGTGTTTGTGACGATCCC
TGCTGTCATCTTTAAGTACATCGAGG
GCTGGACGGCCTTGGAGTCCATTTAC
TTTGTGGTGGTCACTCTGACCACGGT
GGGCTTTGGTGATTTTGTGGCAGGGG
GAAACGCTGGCATCAATTATCGGGAG
TGGTATAAGCCCCTAGTGTGGTTTTG
GATCCTTGTTGGCCTTGCCTACTTTG
CAGCTGTCCTCAGTATGATCGGAGAT
TGGCTACGGGTTCTGTCCAAAAAGAC
AAAAGAAGAGGTGGGTGAAGCAGAGA
ACCTCTACTTCCAATCGCACCATCAT
CACCATCACCATCACCACCATGATTA
CAAGGATGACGACGATAAGTGA

Expressed sequence (small letters refer to tag sequence):

MGLQTVMKWKTVVAIFVVVVVYLVTG
GLVFRALEQPFESSQKNTIALEKAEF
LRDHVCVSPQELETLIQHALDADNAG
VSPIGNSSNNSSHWDLGSAFFFAGTV
ITTIGYGNIAPSTEGGKIFCILYAIF
GIPLFGFLLAGIGDQLGTIFGKSIAR
VEKVFRKKQVSQTKIRVISTILFILA
GCIVFVTIPAVIFKYIEGWTALESIY
FVVVTLTTVGFGDFVAGGNAGINYRE
WYKPLVWFWILVGLAYFAAVLSMIGD
WLRVLSKKTKEEVGEAENLYFQ^SHH
HHHHHHHHDYKDDDDK ^ TEV cleavage site

Tags and additions: C-terminal, TEV cleavable decahistidine / FLAG tag.

Host: Spodoptera frugiperda (SF9) insect cells

Growth medium, induction protocol:

Insect cells with a density of 2x106 per litre of cell culture in SF900 medium (Invitrogen) were infected with recombinant baculovirus (5ml P2 virus per litre cell culture) and incubated for 65 hours. Cells were harvested by centrifugation and the pellet was flash frozen in liquid N2.

Extraction method: Frozen pellets were thawed and re-suspended in extraction buffer supplemented with 1% OGNG/ 0.1% CHS and incubated at 4°C on a tube rotator. The extracted protein was separated from insoluble membranes by centrifugation and collected for purification.

Extraction buffer:

50 mM HEPES, pH 7.5; 200 mM KCl;

Column 1: Co-affinity. Cobalt Talon (Clontech), 1 ml of 50 % slurry/ 1L of cells in 1.5 x 10 cm column, washed with extraction/ wash buffer.

Buffers:

Wash buffer: 50 mM HEPES, pH 7.5; 200 mM KCl; 20 mM imidazole; detergent at 3x CMC.
Elution buffer: 50 mM HEPES, pH 7.5; 200 mM KCl; 250 mM imidazole; detergent at 3xCMC

Procedure:

The solubilised membrane protein was batch bound to Cobalt Talon resin equilibrated with extraction buffer at 4°C for one hour and subsequently poured into a gravity flow glass column. The column was then washed with 30CV of wash buffer followed by elution in 1CV fractions until all the protein was eluted.

Desalting:

IMAC purified protein was loaded onto PD10 columns (GE healthcare) pre- equilibrated in extraction buffer supplemented with detergent at 3x CMC, and eluted with the same buffer.

Enzymatic treatment: TEV protease (1:10, TEV:protein) and PNGaseF (1:20, PNGaseF:protein) was added overnight at 4°C to desalted protein. The enzymes were removed and tag cleaved protein was collected by binding to Cobalt talon and collecting the flow-through.

Column 2: Size Exclusion Chromatography. Superose 6 (GE healthcare).

SEC Buffer: 20 mM HEPES pH 7.5, 200 mM KCl, detergent at 2xCMC

Procedure: The protein was concentrated and applied to a Superose 6 gel filtration column equilibrated with gel filtration buffer using an AKTA purifier system.
Protein concentration: Protein was concentrated to ~20mg/ml using a 30kDa cut-off concentrator and back diluted to 9-11mg/ml.
Crystallization: A 50 mM stock of Br-fluoxetine was dissolved in SEC buffer without detergent. This was added to the protein to give a final compound concentration of 5 mM and protein concentration of 9-11 mg/ml. Protein was incubated with compound for 3 h at 4 °C prior to crystallization. The Br-fluoxetine co-crystals grew from a reservoir solution containing 0.1 M MES buffer, pH 6.5, 0.05 M magnesium chloride, 1mM CdCl2 and 14-30 % PEG500DME. Both plate (P212121) and prism (P21) morphologies were observed in identical drops, however the plate morphology diffracted to higher resolution. The plate morphology was enriched using seeding.
Cryocooling: Crystals were cryo-cooled by stepwise transfer into artificial mother liquor containing increasing concentrations of PEG500DME (0.2 M potassium chloride, 0.2 % OGNG/0.02% CHS, 0.1 M MES, pH 6.5, 1 mM CdCl2, 14-30% PEG500DME). The PEG500DME concentration was increased in 5 % steps up to 30 %. 5 mM Br-fluoxetine was added to the cryo-solution in the final 30 % PEG500DME soak solution. Crystals were rapidly cryo-cooled in liquid nitrogen.
Data Collection: Data were collected at 100K from a single crystal on the microfocus beamline I24 at Diamond Light Source using helical / straight line scans with a beamsize of 50?m x 50?m (wxh). Data were acquired at a wavelength close to the Br K edge to maximize the anomalous signal.
Structure Solution: The coordinates of the 'M4 down' conformational state of KCNK10C (4XDJ) were used as a search model in PHASER to provide initial phase estimates for this structure. The crystals belong to spacegroup P212121 with two channel homodimers per asymmetric unit. Structure refined with BUSTER using NCS and TLS restraints and all data to 3.5Å. Ligand binding was verified by clear anomalous difference density for the bromine substituent and allowed placing of the brominated ring. The remainder of the molecule was poorly ordered and has not been modelled. All data to 3.5Å was used in refinement although, due to anisotropy, nominal resolution quoted below is based on Mn I/sI>2 criteria.
Resolution: 3.64Å