Pore Helix-S6 Interactions Are Critical in Governing Current Amplitudes of KCNQ3 K^+ Channels

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Supplemental Materials and Methods

cDNA constructs

Human KCNQ3 and KCNQ4 (Genbank accession number AAC96101and AF105202, respectively) were kindly given to us by Thomas Jentsch (Zentrum fur Molekulare Neurobiologie, Hamburg Germany). For TIRF experiments, plasmids were sub-cloned into pEYFP-N1 vectors (Clontech, Mountainview, CA) using standard techniques. Myc-tagged KCNQ4 (wild-type and mutants) were generated by sub-cloning each channel in frame into cytomegalovirus-myc (pCMV) vector (Clontech) after the myc epitope.

Cell culture and Transfection

Chinese hamster ovary (CHO) cells were grown in 100-mm tissue-culture dishes (Falcon, Franklin Lakes, NJ) in DMEM medium with 10% heat-inactivated fetal bovine serum plus 0.1% penicillin/streptomycin in a humidified incubator at 37°C (5% CO_2) and passaged every 4 days. Cells were discarded after ~30 passages. For the total internal reflection fluorescent (TIRF) experiments, CHO cells were first passaged onto 35 mm plastic tissue culture dishes and transfected 24 h later with Polyfect reagent (QIAGEN), according to the manufacturer's instructions and as previously described (1). For experiments with wild-type and mutant KCNQ3, the cDNA composition transfected was 1 μg KCNQ3 (wild-type) or mutant and 0.3 μg of EGFP. In KCNQ2 + KCNQ3 experiments, cells were transfected with 0.8 μg each of KCNQ2 and KCNQ3 and 0.3 μg EGFP. For experiments on wild-type or mutant KCNQ4, cells were transfected with 1.5 μg of wild-type or mutant (C643A) KCNQ4 and 0.3 μg of EGFP. The next day, cells were plated onto cover glass chips, and experiments were performed over the following 1-2 days.

Native PAGE and Immunoblotting

Cells were grown in 100-mm culture dishes and individually transfected with myc-tagged wild-type or mutant KCNQ4 and EGFP. After 48h, cells were subjected to either sham wash, H_2O_2 (500 μM, 15 min) alone, or H_2O_2 followed by DTT (2 mM, 15 min). Whole-cell lysate proteins were separated using non-denaturing native polyacrylamide gel electrophoresis (PAGE). Immunoblots was probed with mouse anti-myc primary antibodies (Clontech) at 1:1000 dilution overnight at 4 ˚C in a blocking solution containing 5% nonfat dry milk (Carnation, Wilkes-Barre, PA) in TBS and Tween-20, and subsequently treated with goat-mouse horseradish peroxidase-conjugated secondary antibodies (1:25,000 dilution, 45 min room temperature; Jackson Immunoresearch, West Grove, PA). Blots were developed by enhanced chemiluminescence (Supersignal, Pierce) and exposed on X-ray film (Biomax, Rockville, MD).

Perforated-patch electrophysiology

Pipettes were pulled from borosilicate glass capillaries (1B150F-4, World Precision Instruments) using a Flaming/Brown micropipette puller P-97 (Sutter Instruments), and had resistances of 2-4 MΩ when filled with internal solution and measured in standard bath solution. Membrane current was measured with pipette and membrane capacitance cancellation, sampled at 5 ms and filtered at 500 Hz by means of an Axopatch 1D amplifier and PULSE software (HEKA/Instrutech). In all experiments, the perforated-patch method of recording was used with amphotericin B (600 ng/ml) in the pipette (2). Amphotericin was prepared as a stock solution as 60 mg/ml in DMSO. In these experiments, the access resistance was typically 7-10 MΩ 5-10 min after seal formation. Cells were placed in a 500 μl perfusion chamber through which solution flowed at 1-2 ml/min. Inflow to the chamber was by gravity from several reservoirs, selectable by activation of solenoid valves (Warner Scientific). Bath solution exchange was essentially complete by < 30 s. Experiments were performed at room temperature.

KCNQ currents were studied by holding the membrane potential at -80 mV, and applying 800 ms depolarizing pulses from 60 mV to -80 mV, every 3 s. KCNQ-current amplitude was measured at 60 mV. To estimate voltage dependence, tail current amplitudes were measured ~20 ms after the repolarization at -60 mV,

normalized, and plotted as a function of test potential. The data were fit with Boltzmann relations of the form: $I/I_{max}=I_{max}/\{1+\exp[(V_{1/2}-V)/k]\}$, where I_{max} is the maximum tail current, $V_{1/2}$ is the voltage that produces half-maximal activation of the conductance and k is the slope factor. Cell populations were compared using two tailed *t*-test. Data are given as the mean ± SE.

The external Ringer's solution used to record KCNQ currents in CHO cells contained (in mM): 160 NaCl, 5 KCl, 2 $CaCl_2$, 1 $MgCl_2$ and 10 HEPES, pH 7.4 with NaOH. The pipette solution contained (in mM): 160 KCl, 5 $MgCl_2$ and 10 HEPES, pH 7.4 with KOH with added amphotericin B (600 ng/ml). Hydrogen peroxide (H_2O_2) and dithiothreitol (DTT) stock solutions were prepared fresh for dilution to a final concentration (500 μM H_2O_2 and 2 mM DTT) in Ringer.

Total Internal Reflection Fluorescence (TIRF) microscopy

Fluorescence emission from enhanced yellow fluorescent protein (YFP)-tagged KCNQ3 WT and KCNQ3 mutants (F344A, F344C, F344W and H646C) were collected at room temperature using TIRF (also called evanescent field) microscopy. TIRF generates an evanescent field that declines exponentially with increasing distance from the interface between the cover glass and the cytoplasm, illuminating only a thin section (300 nm) of the cell very near the cover glass, including the plasma membrane (3). All TIRF experiments were performed in the TIRF microscopy core facility housed within the Department of Physiology. Images were not binned or filtered, with pixel size corresponding to a square of 122 × 122 nm.

Homology modeling

Three-dimensional models of the KCNQ3 pore domain (end of S5-turret domain-pore helix-selectivity filter-S6) were generated using the crystal structure of the pore domain of the bacterial K^+ channel, KcsA (4) as a template (PDB accession number 1K4C) using the program SWISS-MODEL. The initial alignment between WT KCNQ3 and KcsA was generated with ClustalW full-length multiple alignment. Then, the alignment of residues L281-H367 of KCNQ3 to residues L49-H124 of KcsA were submitted for automated comparative protein modeling implemented in the program suite incorporated in SWISS-MODEL (5) using the former sequence as a target protein and the latter as template structure. Then, mutant structural models were individually made by selecting the mutation desired using the program DeepView/Swiss-PdbViewer 4.02 (Swiss Institute of Bioinformatics, Lausanne, Switzerland). The resulting models were subsequently energy-optimized using GROMOS96 in default settings within DeepView/Swiss-PdbViewer and the models with the most likely conformation are represented here.

References

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