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A novel homology model of TRPC3 reveals allosteric coupling between gate and selectivity filter☆

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ABSTRACT

Utilizing a novel molecular model of TRPC3, based on the voltage-gated sodium channel from *Arcobacter butzleri* (Na_VAB) as template, we performed structure-guided mutagenesis experiments to identify amino acid residues involved in divalent permeation and gating. Substituted cysteine accessibility screening within the predicted selectivity filter uncovered amino acids 629–631 as the narrowest part of the permeation pathway with an estimated pore diameter of <5.8 Å. E630 was found to govern not only divalent permeability but also sensitivity of the channel to block by ruthenium red. Mutations in a hydrophobic cluster at the cytosolic termini of transmembrane segment 6, corresponding to the S6 bundle crossing structure in Na_VAB, distorted channel gating. Removal of a large hydrophobic residue (I667A or I667E) generated channels with approximately 60% constitutive activity, suggesting I667 as part of the dynamic structure occluding the permeation path. Destabilization of the gate was associated with reduced Ca^{2+} permeability, altered cysteine cross-linking in the selectivity filter and promoted channel block by ruthenium red. Collectively, we present a structural model of the TRPC3 permeation pathway and localize the channel's selectivity filter and the occluding gate. Moreover, we provide evidence for allosteric coupling between the gate and the selectivity filter in TRPC3.

1. Introduction

TRPC proteins contribute to spatial control of cytoplasmic Ca^{2+}- and Na^+-gradients as well as membrane potential by formation of non-selective cation channels that are functionally coupled to other plasma membrane ion transport systems [1–3]. Thereby, these channels control global as well as local Ca^{2+}-homeostasis of excitable and non-excitable cells in a rather complex manner. The capability to introduce simultaneous Ca^{2+}- and Na^+-entry into cells has been recognized as a potentially important mechanism for signal diversification [4]. Our understanding of the molecular basis of this feature and our picture of the architecture of the permeation pathway in TRPC channels is still incomplete. So far, structural information from crystallography is lacking and only a few functional domains have been identified, based on homology modeling and site-directed mutagenesis [5–8]. A conserved LFW motif was previously discovered as a potential key element of the permeation pathway. Mutations in the LFW motif completely disrupt channel function in a dominant negative manner, conferring permeation deficiency also to heteromers with wild type proteins [5,6]. This feature is consistent with a central position of the LFW sequence within the channel's pore complex. Nonetheless, a role of the LFW motif in specific channel functions such as selectivity or gating has not been demonstrated. Analyzing the putative pore domain in TRPC channels by mutational neutralization of charged residues identified structural features essential for Ca^{2+}-permeation. For TRPC1, negative charges close to the putative pore helix have been found to determine cation selectivity [6]. Neutralization of these charges causes a clear reduction of the TRPC1-dependent Ca^{2+}-permeability measured in human salivary glands upon heterologous expression of the mutants. Similarly, mutational analysis has uncovered a glutamate residue, that is conserved within the

Abbreviations: BAPTA, 1,2-bis(o-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid; GPCR, G protein coupled receptor; PLC, phospholipase C; DAG, diacylglycerol; SAG, 1-stearoyl-2-arachidonoyl-sn-glycerol.

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pore domain of TRPC3/6/7 and *Drosophila* TRP, as a critical element in the selectivity filter in the *Drosophila* TRP [8]. Based on molecular modeling using KcsA and $K_V1.2$ as template structures, this acidic residue within the central part of the permeation pathway has been identified as key determinant of divalent permeation in TRPC3 [7]. Unfortunately, TRPC molecular models using KcsA or $K_V1.2$ as template suffer essentially from insufficient homology and lack definition. It appears important to consider that permeability properties may be affected by amino acid exchanges in domains distant to but structurally associated with the selectivity filter. Specifically, gating properties may impact on ion selectivity as recently documented for the Orai1 channel [9]. To obtain a more complete picture of the permeation pathway in TRPC3, we set out to explore structural elements in the pore domain by a structure-guided mutagenesis approach, adopting recently published structural information of the bacterial voltage-gated sodium channel Na_VAB [10] for molecular modeling. Our functional analysis of TRPC3 pore mutants clearly demonstrates suitability of the Na_VAB template and confirms E630 as a key residue in the channel's selectivity filter. Furthermore we identify a region forming the occluding gate and suggest a close structural relation and functional coupling between TRPC3 gate and selectivity filter.

2. Methods

2.1. Molecular biology

Site directed mutagenesis was performed using the QuickChange II Site Directed Mutagenesis Kit (Stratagene) according to the manufacturers instructions. Mutagenic primer pairs were designed by the QuickChange Primer Design Program® and synthesized by genXpress® (VWR). Human TRPC3 (UniProtKB ID: Q13507-3) cloned into pEYFP-C1 vector served as a template, resulting in a fusion protein N-terminally tagged to YFP. All mutations were confirmed by sequencing the corresponding cDNAs.

2.2. Cell culture and transfection

Human embryonic kidney 293 (HEK293) cells were cultured at 37 °C and 5% CO_2 in Dulbeco's Modified Eagle Medium (DMEM high glucose with pyruvate, Invitrogen) supplemented with streptomycin (100 μg/ml), penicillin (100 U/ml), L-glutamine (2 mM), HEPES (10 mM) and 10% fetal calf serum. Cells were transiently transfected using FuGENE® HD Transfection Reagent (Promega) as recommended by the vendor. In brief, 200 μl Opti-MEM® (Invitrogen) and 5 μg DNA was mixed thoroughly and then 6 μl transfection reagent was added. After mixing, samples were incubated for 15 min at room temperature and subsequently added to cells grown in 35 mm-dishes. Cells were harvested and plated to cover slips 24 h after transfection and finally subjected to whole-cell patch clamp experiments 48 h after transfection.

2.3. Homology modeling

Phylogenetic analysis [11] revealed that TRP channels are evolutionary related to the calcium and the sodium channels, diverging early in evolution from the very diverse class of potassium channels. We selected the Na_VAB channel as template because evolutionary this is the most related channel crystallized so far. Sequence alignment between Na_VAB and TRPC channels was carried out using muscle 3.7 [12]. The asparagine residue in helix S6, conserved in all calcium and the sodium channels allowed for an unambiguous alignment of the innermost transmembrane helix. Hydrophathy analysis, biophysical properties of membrane exposure and protein interior orientation were as much taken into account as helix capping or termination signals [13] for a manual adjustment of the full sequence alignment, necessary because of the low sequence homology between Na_VAB and TRPC channels. The model of the human TRPC3 ion channel (UniProtKB ID: Q13507-3) was based on the Na_VAB crystal structure (PDB ID: 3RVY) [10] with a resolution of 2.7 Å. Modeling was carried out with MODELLER (version 9v8) [14] applying the automodel protocol. Fifty models were generated. Model quality was assessed using the DOPE score [15] and ProCheck [16]. The best TRPC3 model had a DOPE score of −35341 (Na_VAB template's DOPE score is −41631). The Ramachandran plot of TPRC3 showed 90.9% of residues in the most favorable region, 5.6% in the allowed region, 1.4% in the generously allowed region and 2.0% in the disallowed region, while the Na_VAB template had values of 89.1%, 9.4%, 0.5% and 1.0%, respectively. The model with the best DOPE score was selected for visualization and analysis.

2.4. Electrophysiology

Patch pipettes were pulled from thin-wall filament glass capillaries GC 150TF-7.5 (Havard Apparatus) to a resistance of 3–4 MΩ. A Diaphot Inverted Tissue Culture Microscope (Nikon) was used to identify positively transfected cells by their green fluorescence when illuminated at 514 nm. Whole-cell voltage-clamp experiments were performed at room temperature using a L/A-EPC7-Amplifier (List Medical Electronic) connected with a Digidata-1322A Digitizer (Axon Instruments). Currents were filtered at 3 kHz by a 3-pole Bessel filter and digitized with 8 kHz. Application of linear voltage-ramp protocols ranging from −130 to +80 mV (holding potential 0 mV) was controlled by Clampex 9.2 (Axon Instruments) software.

2.5. Solutions and materials

The standard extracellular solution contained (in mM): 140 NaCl, 10 Hepes, 10 Glucose, 2 Mg_2Cl, 2 Ca_2Cl titrated to pH 7.4 with NMDG. In order to determine permeability of organic cations, experiments were carried out in standard extracellular solution with sodium (Na^+, diameter $d = 1.90$ Å) equimolarly replaced by di- (DMA^+, $d = 4.6$ Å), tri- ($TriMA^+$, $d = 5.2$ Å) or tetra-methyl ammonium ($TetMA^+$, $d = 5.8$ Å) [17]. In order to ensure integrity of channel function, solutions furthermore exhibited physiological concentrations of Ca^{2+} and Mg^{2+} (2 mM each). For measuring currents carried by various monovalent cations, NaCl was equimolarly replaced by LiCl, KCl, CsCl, RbCl or NMDG. The standard intracellular solution contained (in mM): 120 cesium methanesulfonate, 20 CsCl, 15 HEPES, 5 $MgCl_2$, 3 EGTA titrated to pH 7.3 with CsOH. For Ca^{2+}-permeability measurements, solutions were modified as follows: 140 mM NMDG and 10 mM Ca^{2+} extracellularly, 10 mM BAPTA intracellularly and 10 mM Cl^- symmetrically in order to eliminate chloride currents. ECS was pH-adjusted to 7.4 with methanesulfonic acid. Standard chemicals used for solutions as well as carbachol and dithiothreitol were purchased from Sigma Aldrich. Ruthenium red was purchased from Tocris Bioscience.

2.6. Epifluorescence and TIRF microscopy

Fluorescence microscopy was performed using a Zeiss Axiovert Observer D microscope, equipped with a 488 nm and 445 nm laser excitation device and a Hamamatsu Orca D2 camera system. The system is equipped with a 100× objective and condenser/beam deflector (Visitron Systems) for total internal reflection (TIRF) microscopy.

2.7. Data analysis and statistics

Data analysis and graphical display was done using Clampfit 9.2 (Axon Instruments), OriginPro8 (OriginLab Corporation) and