

SHORT THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (PhD)

Investigation of the gating of voltage-gated potassium channels using
modern biophysical methods

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I. Introduction

Ion channels are special transmembrane proteins that have in common to form a hydrophilic pore across the cell membrane to ensure the flow of ions between extracellular and intracellular spaces. Ion channels can be divided into different families based on their ion selectivity, function, and structure, and are involved in a wide range of biological processes with diverse functions. Voltage-gated potassium channels (K_v) stand in our focus, which play essential roles in mediating a great variety of cellular functions in both excitable and classically non-excitable cells. K_v ion channels consist of four subunits, and each subunit is composed of six transmembrane helical segments (S1-S6). Within subunits, helices are held together by intra- and extracellular amino acid loops, while individual subunits are held together by non-covalent interactions. S1-S4 helices form the voltage sensor domain (VSD), while S5-S6 helices build up the pore domain (PD) of the channel. The structural link between the two domains is provided by the S4-S5 linker in some channels and by intramolecular interactions between transmembrane helices in others. The actual magnitude of the membrane potential is sensed by the VSD, while the activation and C-type inactivation gates and the selectivity filter located in the PD are responsible for coordinating the flow of potassium ions (K⁺). The activation and C-type inactivation gates are located at the intracellular and extracellular ends of the pore, respectively. Gating of the channel takes place through the concerted operation of the voltage sensor, and the activation and inactivation gates. Upon depolarization, the activation gate of the closed state channel (C) opens, thereby the channel enters the functionally conductive state (O), and subsequently, if depolarization persists, the inactivation gate closes leading to the formation of the non-conductive state. In the gating scheme, this is referred to as the OI gating state as a structural state, implying that the activation gate is still open while the inactivation gate is closed. After the end of depolarization, the activation gate closes and the structural state of the inactivated channel shown in the gating scheme is CI, indicating that both the activation gate and the inactivation gate are closed. If the channels are kept at a sufficiently negative membrane potential for a sufficient time, the inactivation gate opens, so that the channel returns to the initial closed state (C) through the process of recovery from inactivation.

Gating of ion channels is a very complex process, which can be influenced at several points by various extrinsic and intrinsic factors. Cholesterol of the cell membrane should be highlighted among extrinsic factors, since it is one of the main determinants of the biophysical properties, vertical and lateral heterogeneity of the membrane, which together are responsible for the formation of different functional microdomains. Interactions between cholesterol and ion channels represent a major focus of research interest, since these interactions or their

alterations can be important factors in many physiological and pathological processes. Recently developed techniques (such as cryo-electron microscopy (cryo-EM) or superresolution microscopy) and significant advances in molecular dynamics (MD) simulations have opened new perspectives in the study of protein-cholesterol interactions, leading to a resurgence of interest in the subject. Previous studies have distinguished between two types of interactions between cholesterol and transmembrane proteins, such as ion channels: a direct, ligand-like interaction in which cholesterol binds to different binding sites on the ion channel to modify its function; and an indirect effect through altering the biophysical properties of the membrane (including its rigidity, thickness, lateral pressure, lipid order, or dipole potential) or the distribution of the channels between lipid rafts and non-raft microdomains. Ion channels and voltage-gated potassium channels in particular, are suitable models for studying transmembrane protein-cholesterol interactions, because during gating both the movements of the voltage sensor and the pore-forming helices and their conformational changes occur in the surrounding lipid membrane, therefore, membrane cholesterol can modify them directly. For ion channels, functional mapping of cholesterol effects is straightforward, as measurement of ion currents can be used to determine a number of current and channel parameters that well-describe the functional consequences of cholesterol-induced modifications (e.g., changes in steady-state activation and inactivation, opening probability, single channel conductance, current activation and inactivation kinetics). First studies in the field were solely limited to the description of electrophysiological effects, then, studies focusing on the mechanisms of action emphasized indirect effects mediated through membrane biophysical parameters and lipid rafts. Recently, with the development of various imaging and computational modeling techniques, the emphasis on the importance of direct interactions has come into focus. However, the approaches of both early and recent studies lack the consideration of the primary intramolecular target (VSD, PD or coupling apparatus), through which cholesterol-mediated functional changes in ion channel function are mediated, regardless of direct or indirect mechanism of action.

Among intrinsic interactions within the ion channel protein that affect the gating process, communication between individual gates of ion channels has come into focus recently. Of these, it was highly relevant to reveal that the gating process involving C-type inactivation is determined by the actual position of the activation gate. The activation and C-type inactivation gates are bidirectionally coupled: opening of the activation gate helps closure of the inactivation gate and inhibits its opening, thus promoting the development of C-type inactivation. At the same time, the closed state of the C-type inactivation gate speeds up the opening of the activation gate and slows its closing. Our data on coupling and probabilities of

gating transitions mainly originate from experiments applying positive membrane potentials, as these membrane potential values provide the driving force and opening probabilities that are necessary for the appearance of easily measurable ion currents. On the contrary, the exact mechanisms of gating transitions at negative membrane potentials, at which data must be collected with a structure-function-based approach in the absence of ion current, are not known.

Consequently, this Thesis focuses on two issues. On the one hand, we investigated which functional domain of voltage-gated potassium channels is primarily involved in mediating the electrophysiological effects of cholesterol to the pore. Does cholesterol primarily affect the function of the voltage sensor domain, which is subsequently transmitted to the pore through the coupling apparatus influencing the ion current, or, conversely, is the pore primarily affected and thus the function of the voltage sensor is not affected by cholesterol? Hereby, we examined the interactions between cholesterol and K_v ion channels from a novel perspective allowing us to get closer to understanding the mechanism of action of cholesterol, which may help to further explore the molecular basis of many diseases associated with changes in membrane cholesterol. The other question studied in our work was how the current state of the activation gate affects the movements of the C-type inactivation gate (i.e., its closure and opening) during the gating process at negative membrane potentials. Can inactivation occur directly from the closed state (C→CI transition) without opening of the activation gate? At negative membrane potentials, is the closure of the activation gate necessary for the channel to recover from the inactivated state into the closed state? In both cases, the movement of the inactivation gate would take place along with the static (closed and open, respectively) state of the activation gate, despite the tight allosteric coupling between the two. The physiological relevance of studying the gating transitions at negative membrane potentials stems from the fact that the population of the CI state, i.e. development of the CI state and recovery from inactivation substantially determine the number of potentially activatable channels, which is one of the determinants of cell excitability.

We have used novel and sensitive methods to examine both of these issues, which allowed us to obtain previously unknown results that shed new light on our current knowledge of fine-tuning the gating of ion channels. We applied two-electrode voltage-clamp fluorometry (TEVCF) technique to determine the functional domain (VSD, PD, or the coupling apparatus between the two) of K_v ion channels primarily affected by membrane cholesterol. This method allows the cysteine introduced by point mutation into the extracellular part of the voltage sensor to be selectively labeled with a cysteine-specific fluorescent dye, so that the movement of the VSD can be tracked throughout the whole gating process, while the current state of the pore

can be followed simultaneously with the help of ion current measurements (similar to the conventional patch-clamp technique). As will be seen later, the magnitude and quality of both ion currents and fluorescence signals (good signal-to-background and signal-to-noise ratios) allowed us to gain sensitive and accurate insight into the primary and previously unknown molecular target of cholesterol within the ion channel. During our experiments related to C-type inactivation, we used *Shaker* ion channels with multiple mutations, which allowed us to selectively study the presence of C-type inactivation from the closed state (C→CI transition) and recovery from inactivation with an open activation gate (OI→O transition). The existence of the C→CI transition was investigated under experimental conditions, where the opening of the activation gate could be sensitively monitored at negative membrane potentials with our rapid perfusion system and a state-dependent cysteine-specific modification assay despite the low driving force and the low opening probability. When studying the OI→O transition, the formation of a Cd²⁺ bridge between the mutated cysteine and a native histidine amino acid made it possible to lock the activation gate in the open state. In both cases, the chemical interaction between cysteine and Cd²⁺ provided the specificity of the experiments. Cd²⁺ containing solutions were precisely applied using a rapid perfusion system to ensure the selective application of Cd²⁺ only in the selected gating states of the channel.

II. Objectives

Although the structural basis, main steps, and basic regulation of gating of K_v ion channels are known, several factors that ensure fine-tuning of the gating process have so far remained unexplored due to the absence of appropriate investigation techniques. Therefore, in our work, we examined the fine-tuning of the activation and C-type inactivation gating of K_v channels along two main issues:

1. Which domain of K_v channels is the primary target of sterols in the membrane within the ion channel? Do the sterols in the membrane exert their previously described effects on the gating of K_v channels through the VSD, which are subsequently transmitted to the coupling apparatus and then to the PD regions, or primarily, directly, affect the function of one of the latter?
2. Does the state of the activation gate determine the development of steady-state inactivation and recovery from inactivation? Considering the gating states of the channel, we examined two basic issues: is the channel able to be directly inactivated at negative membrane potentials in the closed state, i.e. is the C → CI transition possible or is the CI state populated through the OI → CI transition even at negative membrane potentials? Second, does the open-inactivated channel (OI state) have to go through the CI state to recover from inactivation, i.e. is it necessary to close the activation gate for the recovery from inactivation or can the process occur through the OI → O transition at the negative membrane potentials?

The issues described in Objective 1 remain unanswered so far, since previous experiments failed to elucidate the primary target of membrane sterols within K_v ion channels as only ion currents can be measured with conventional patch-clamp techniques, and no direct information can be obtained about the movement of VSD during gating. Thus, based on simple ion current measurements, the primary target of sterols cannot be determined within the ion channel. To answer this question, we used the two-electrode voltage-clamp fluorometry (TEVCF) method in the experiments presented in the Dissertation, which, on the one hand, is able to measure ion currents in the same way as conventional patch-clamp techniques, and thus to determine several biophysical parameters characterizing the channel or its gating process (voltage-dependence of steady-state activation and inactivation, opening probability, single channel conductance, current activation and inactivation kinetics). On the other hand, TEVCF

provides the possibility to determine parameters describing the movement of VSD ($F_{\text{norm}}-V$ curve, fluorescent signal activation kinetics) simultaneously with the measurement of ion currents, after labeling a cysteine introduced by a point mutation into the S3-S4 linker of the VSD with a cysteine-specific fluorescent dye. During activation gating, the S4 helix located in the VSD moves outward from the plane of the membrane, thereby changing the quantum efficiency of the fluorophore. Thus, the change in fluorescent intensity of the signal reflects the movement of the VSD. Our experiments were performed on human 309C Kv1.3 and 322C Kv10.1 ion channels expressed in African claw frog oocytes. The oocyte expression system provided us with the high channel expression required for TEVCF measurements. To determine the target of sterol modulation within the ion channel and the channel specificity of sterol effects, we compared voltage-dependent steady-state and kinetic parameters describing VSD activation and pore opening of control cells and those treated with sterols in the cases of Kv1.3 and Kv10.1 channels characterized by distinct gating mechanisms. To examine the specificity of sterol effects, cholesterol-induced changes were compared with those observed in response to 7-dehydrocholesterol (7DHC) treatment. The latter compound was chosen because of its slight structural difference from cholesterol and its pathophysiological significance in Smith-Lemli-Opitz syndrome. Our experiments can bring us closer to understanding of the mechanism of cholesterol action, which could help to understand the molecular basis of many diseases associated with changes in cell membrane cholesterol content (e.g., hypercholesterolemia, SLO syndrome, Neumann-Pick disease, Gaucher disease).

The questions formulated in Objective 2 focus on the role of the activation gate in steady-state inactivation and recovery from inactivation. Both processes take place at negative membrane potentials. The examination of gating transitions involved in these processes ($C \rightarrow CI$ and $O \rightarrow OI \rightarrow CI \rightarrow C$) is hampered by more factors at negative potentials. One is that at such membrane potentials, the probability of channel opening is small, resulting in small amplitudes of the evoked currents. In addition, the driving force for K^+ is small, which also promotes the appearance of small currents, thus, when determining the state of the activation gate by means of current measurements, the error of the measurements is very large. Furthermore, some of these transitions occur between non-conductive states, so the state of the gates cannot be mapped based on current measurements. The state-dependent cysteine modification method helped us to solve these problems, with the help of which the movements of the activation gate can be accurately tracked or the gate can be locked in a given state even at negative membrane potentials. In our experiments, we examined the state of the activation gate during the development of steady-state inactivation in T449A/V474C *Shaker*-IR channels transiently

expressed in HEK-293 human embryonic kidney cells in inside-out configuration using a state-dependent cysteine modification assay and a rapid-perfusion system. The cysteine at position 474 is only accessible to the intracellular Cd^{2+} when the activation gate is open, and the bound Cd^{2+} inhibits the ion current that can be measured at positive test potentials. Thus, opening of the activation gate at negative membrane potentials can also be tracked. The recovery from inactivation with the activation gate locked in the open state (i.e., the direct $\text{OI} \rightarrow \text{O}$ transition) was examined in T449A/V476C *Shaker*-IR channels at negative membrane potentials. Cd^{2+} forms a cross-link between the cysteine on one subunit and a native histidine on the adjacent subunit, so that the activation gate can be locked in the open state throughout the investigation of the possible recovery from inactivation. The specificity of the interaction between Cd^{2+} and cysteines, and the state-dependent and precise application of Cd^{2+} -containing solutions using the fast-perfusion system, in addition to our designed pulse protocols, ensured that the gating transitions described above can be studied at negative membrane potentials. The relevance of studying gating transitions at negative membrane potentials derives from that both steady-state inactivation and recovery from inactivation substantially determine the number of potentially activatable channels, which is one of the major determinants of cell excitability.

III. Materials and methods

3.1 Molecular biology

Human Kv1.3 (KCNA3, Uniprot B2RA23) channels containing cysteine mutations in the S3-S4 linker were generated by site-directed mutagenesis (QuikChange; Agilent, Santa Clara, CA) in a pBSTA vector containing the wild-type channel. The presence of the given point mutation was verified by sequencing for the given construct. The human Kv10.1 (KCNH1, Isoform 1, Uniprot O95259-2) L322C mutant in a pSGEM vector was from L. A. Pardo (Max Planck Institute, Göttingen, Germany). Plasmids were linearized with HindIII (Kv1.3) or NheI (Kv10.1) and transcribed into mRNA using the Invitrogen mMACHINE mMACHINE T7 Transcription Kit (ThermoFisher, Waltham, MA).

The plasmid encoding wild-type Kv1.3_{FLAG} ion channel used for microscopic measurements was provided by Dr. Péter Hajdú (University of Debrecen, Hungary), while the pCMV6 plasmid encoding wild-type Kv10.1_{FLAG} ion channel was purchased from OriGene (reference number: RC215104). Kv1.3_{FLAG} and Kv10.1_{FLAG} channels contained no other mutations except for the presence of the FLAG epitope. The GFP-GPI plasmid was a gift from Jennifer Lippincott-Schwartz (NIH, Bethesda, MD).

The *Shaker*-IR construct used in experiments examining C-type inactivation (*Shaker*-IR in a GW1-CMV plasmid from R. Horn, Thomas Jefferson University, Philadelphia, PA) contained the deletion of amino acids 6-46 resulting in the absence of N-type inactivation and C301S and C308S point mutations to avoid disturbing effects of interactions between Cd²⁺ and endogenous cysteines. Amino acid substitutions at positions 449 (T449A) and 474 (V474C) or 476 (V476C) in the construct according to *Shaker* B numbering were generated with a QuikChange site-directed mutagenesis kit (Stratagene, San Diego, CA) and verified by sequencing.

3.2 Expression systems, transfection and mRNA injection

Xenopus laevis African claw frog oocytes used for two-electrode voltage-clamp fluorometry (TEVCF) experiments were purchased from EcoCyte Bioscience (Dortmund, Germany). Oocytes were injected with 30-50 nl of mRNA at a concentration of ~1 µg/µl and incubated for 1-3 days at 18 °C in ND93 solution containing 93 mM NaCl, 5 mM KCl, 1.8 mM CaCl₂, 1 mM MgCl₂, 5 mM HEPES and 50 mg/l gentamycin (pH=7.4). Chemicals for the

preparation of all solutions mentioned in this Dissertation were obtained from Sigma-Aldrich (St. Louis, MO).

Human embryonic kidney cells (HEK-293) were purchased from American Type Culture Collection (ATCC, Manassas, VA) and cultured according to the manufacturer's instructions. A calcium phosphate-based transfection kit (Invitrogen, Carlsbad, CA) was used for co-transfection of the EGFP plasmid and the mutant *Shaker* constructs at a ratio of 1 μg : 10 μg . Transfected cells were placed in Corning polystyrene culture dishes with a diameter of 35 mm pretreated with poly-L-ornithine (Sigma-Aldrich) for better adhesion of the cells to excise inside-out patches. Channels were expressed in the cells for 12-36 hours after transfection. EGFP-positive transfected cells were identified using a Nikon TE2000U fluorescence microscope (Nikon, Tokyo, Japan) with bandpass filters of 455-495 nm and 515-555 nm for excitation and emission, respectively. In general, more than 60% of EGFP-positive cells expressed co-transfected *Shaker*-IR channels.

For microscopy measurements, HEK-293 cells grown on 8-well chambered coverglass were transfected with 0.25 μg DNA/well $K_v1.3_{\text{FLAG}}$, $K_v10.1_{\text{FLAG}}$, or GPI-anchored green fluorescent protein (GFP-GPI) protein encoding plasmids using Lipofectamine2000 (ThermoFisher) reagent and a lipid to DNA ratio of 2:1 ($\mu\text{l}/\mu\text{g}$). Channels were expressed for 12-36 hours after transfection.

3.3 Cell membrane sterol modulations

Modification of cell membrane sterol content with cholesterol (Sigma-Aldrich), 25-[N-[(7-nitro-2-1,3-benzoxadiazol-4-yl) methyl]amino]-27-norcholesterol (NBD-cholesterol) (Avanti Polar Lipids, Alabaster, AL) or 7-dehydrocholesterol (7DHC) (Sigma-Aldrich) was performed with custom synthesized sterol-methyl-beta-cyclodextrin (M β CD) complexes (CycloLab Cyclodextrin R&D Laboratory, Budapest, Hungary). Sterol-M β CD complexes used for loading contained 195 μM sterol. Both oocytes and HEK-293 cells were treated with the complexes at room temperature for 60 min before electrophysiological or microscopy experiments. For electrophysiology, sterol-M β CD complexes were dissolved in ND93, while for microscopy in a solution containing 150 mM NaCl, 5 mM KCl, 1.5 mM CaCl₂, 1 mM MgCl₂ and 10 mM HEPES, at a pH of 7.36-7.38. After incubation, oocytes and HEK-293 cells were thoroughly washed in both cases with complex-free solutions.

3.4 Electrophysiology

3.4.1 Two-electrode voltage-clamp fluorometry (TEVCF)

For two-electrode voltage-clamp fluorometry (TEVCF) measurements, oocytes were labeled on ice for 30 minutes with 10 μ M 2-((5(6)-tetramethylrhodamine)carboxylamino)ethyl methanethiosulfonate (TAMRA-MTS, Toronto Research Chemicals, Toronto, ON, Canada) diluted in a depolarizing solution containing 110 mM KCl, 1.5 mM MgCl₂, 0.8 mM CaCl₂, 0.2 mM EDTA and 10 mM HEPES at a pH of 7.1. After labeling, oocytes were extensively washed with ND93 solution and then kept in the dark on ice until the start of measurements. For the measurements, ND93 was used as the extracellular solution, while the intracellular solution was a 3 M KCl solution. Pipettes used for the measurements were pulled from GC 150 F-15 borosilicate glass capillaries (Harvard Apparatus Kent, UK) and had a resistance of 2–3 M Ω .

During TEVCF, setting the membrane potential of oocytes and measurements of ion currents were carried out with an Oocyte Clamp OC-725C amplifier (Warner Instruments, Hamden, CT). Fluorescent signal intensities were measured using a Nikon Eclipse FNI microscope (Nikon, Tokyo, Japan), a 40 \times , 0.8-NA CFI Plan Fluor Nikon water immersion objective and a photodiode (PIN-040A; United Detector Technology, OSI Optoelectronics, Hawthorne, CA). The TAMRA-MTS signal was detected with a 545/25 excitation filter, a 565LP dichroic mirror and a 605/70 emission filter. The signal from the photodiode was amplified with an Axopatch 200A amplifier and data were digitized with an Axon Digidata 1550 (Molecular Devices) digitizer controlled by pClamp10 (Molecular Devices, San Jose, CA). Illumination was provided by an M530L2-C1 green (530 nm) LED (ThorLabs, Newton, NJ). In the case of TEVCF measurements, sampling was performed at a frequency of 5 kHz when measuring ion currents and fluorescent signals. In the figures, fluorescence traces show single recordings without averaging filtered with a Gaussian filter. Online filtering was performed with a frequency value less than half of the sampling frequency, the sampling: filtering ratio was typically 2: 1. Additional offline filtering was used during the analysis of fluorescent signals.

3.4.2 Patch-clamp recordings in outside-out configuration

Single channel parameters were determined by patch-clamp measurements on mechanically devitellinized oocytes in outside-out configuration. The standard intracellular solution adjusted to pH 7.36-7.38 contained 105 mM KF, 35 mM KCl, 10 mM EGTA, and 10 mM HEPES. The standard extracellular solution adjusted to pH 7.36-7.38 contained 150 mM NaCl, 5 mM KCl, 1.5 mM CaCl₂, 1 mM MgCl₂, and 10 mM HEPES. Pipettes used for the

measurements were pulled from GC 150 F-15 borosilicate glass capillaries (Harvard Apparatus, Kent, UK) and had a resistance of 8-9 M Ω . During the measurements, Axopatch 200B and Multiclamp 700B amplifiers were used and data were digitized using Axon Digidata 1550.

3.4.3 Patch-clamp recordings in inside-out configuration

Data were acquired with an Axopatch 200B amplifier (Molecular Devices) and digitized with Axon Digidata 1550 (Molecular Devices) adapter. During measurements, the sampling frequency was set to at least twice the filter frequency. Pipettes were pulled from GC 150 F-15 borosilicate glass capillaries (Harvard Apparatus) and had a resistance of 8-9 M Ω . Only patches with a nonspecific leakage current of less than 5% of the peak current were considered in the studies. Measurements were performed in each case at room temperature (20-24 °C).

The intracellular solution used for inside-out measurements contained 105 mM KF, 35 mM KCl, 10 mM EGTA and 10 mM HEPES. The pH of the solution was titrated to 7.36-7.38 with KOH to give a total K⁺ concentration of 160-165 mM and an osmolarity of 285-295 mOsm/L. The internal solution used to characterize the kinetics of the perfusion system had a similar composition, except that it contained 50 mM K⁺ and the corresponding potassium salts were replaced with 100 mM NaF. For Cd²⁺ modification experiments, the intracellular solution was similar to the standard K⁺-based solution, with the modification that it contained 125 mM KF, 35 mM KCl, and 10 mM HEPES without the presence of EGTA. The standard extracellular solution (present in the pipette in the inside-out configuration) contained 150 mM NaCl, 2 mM KCl, 1.5 mM CaCl₂, 1 mM MgCl₂, and 10 mM HEPES, with an osmolarity of 290 mOsm / L and pH of 7.36-7.38.

A Warner Instruments SF-77A Perfusion Fast-Step perfusion system with a three-outlet perfusion head was used for rapid solution exchange during the measurements. The flow of fluids was continuous at the outlets of the microperfusion, and the appropriate outlet was positioned opposite to the intracellular surface of the inside-out patch by a precision motor synchronized with data acquisition. Inside-out patches were perfused with appropriate intracellular solutions at a flow rate of 0.5 ml/min. The principle and method of the test protocol used to determine the rate of solution exchange has been described previously. The solution exchange was complete in 30 ms.

3.5 Confocal laser scanning and stimulated emission depletion (STED) microscopy

For microscopic analysis, lipid rafts of HEK-293 cells were labeled with two different methods, as described previously. First, GM1-enriched membrane microdomains were labeled by 8 µg/ml AlexaFluor647-tagged subunit B of cholera toxin (CTX-B) (ThermoFisher) for 20 min on ice to prevent internalization of CTX-B. Second, for labeling lipid rafts with GFP-GPI, cells were transfected with a GFP-GPI plasmid.

For labeling ion channels, cells expressing Kv1.3_{FLAG} or Kv10.1_{FLAG} proteins were incubated in the presence of 2 µg/ml anti-FLAG M2-Cy3 antibodies (Sigma-Aldrich) for 30 min on ice. Since the FLAG tag on Kv10.1 is localized on the intracellular side of the protein, labeling of these channels was carried out after fixation of the cells in 3.7% formaldehyde in PBS containing 0.1% BSA and 0.1% Triton X-100.

After labeling of sterol-treated or control cells, images were taken from the flat cell membrane region attached to the coverslip with an LSM880 confocal laser scanning microscope (Carl Zeiss AG, Jena, Germany). NBD, GFP, Cy3 and AlexaFluor647 were excited at 458 nm, 488 nm, 543 nm and 633 nm, respectively, and their emitted intensities were detected in the wavelength ranges of 482–696 nm, 493–598 nm, 548–629 nm, and 638–756 nm, respectively.

For stimulated emission depletion (STED) microscopy measurements, cells expressing Kv1.3_{FLAG} were labeled with 2 µg/ml anti-FLAG M2 antibodies (Sigma-Aldrich) for 30 min at room temperature, which was followed by an incubation with 8 µg/ml AlexaFluor594-CTX-B (ThermoFisher) and StarRed-GAMIG (Abberior, Göttingen, Germany) for 30 min on ice. Images were acquired from the flat cell membrane region attached to the coverslip with an Olympus BX53 microscope equipped with a STED module (Abberior) and an Olympus 100×/1.4 objective using excitation at 594 and 640 nm and a STED laser beam at 775 nm. Detection bands were 608–627 nm and 650–700 nm for AlexaFluor594 and StarRed, respectively.

3.6 Data analysis

3.6.1 Analysis of two-electrode voltage-clamp fluorometry (TEVCF) measurements

Electrophysiological data analyses were performed using Clampfit (v10; Molecular Devices), SigmaPlot (v10; Systat Software, San Jose, CA) and Excel (Microsoft, Redmond, WA) softwares.

During TEVCF measurements, currents and fluorescence signals were recorded at a sampling frequency of 5 kHz. The magnitude of the fluorescence signals was expressed as $\Delta F/F$ in percentage, where ΔF is the change in the signal amplitude, and F is the baseline fluorescence

level at the given holding potential. To correct for photobleaching, the fluorescence signal obtained at a test potential of -100 mV was subtracted from fluorescence signals obtained at different membrane potentials. $F_{\text{norm}}-V$ values of Kv1.3 309C were determined from the steady-state components of the fluorescent signals, normalized to the maximum intensity and plotted as a function of test potential. In contrast with Kv1.3, MTS-TAMRA labeled Kv10.1 322C exhibits a complex fluorescent signal that can be divided into components measured at hyperpolarized (from -180 to -90 mV) and depolarized (from -80 to $+60$ mV) membrane potentials. Signals measured at hyperpolarized membrane potentials had two components of the same polarity and were analyzed similarly to Kv1.3 signals. On the contrary, signals obtained at depolarized membrane potentials had two components of opposite polarities. To determine the absolute value of the signal, the second component was added to the first one with an inverted polarity.

I-V curves were constructed by plotting leak-corrected peak currents as a function of test potential. $V_{1/2}$ and k parameters characterizing the Kv1.3 309C and Kv10.1 322C conductance-voltage (G-V) curves were determined by fitting the

$$I = V \times G_{\text{max}} \times \frac{(1 - e^{-(V-E_{\text{ekv}})/25})}{(1 - e^{-V/25})} \times \frac{1}{1 + e^{-(V-V_{1/2})/k}}$$

equation to current-voltage (I-V) curves, which combines Goldman-Hodgkin-Katz rectification with the voltage-dependence described by a Boltzmann function. Here V and I are the voltage and the current, respectively, and the free parameters are G_{max} the maximum conductance, E_{ekv} the equilibrium potential, and $V_{1/2}$ and k are the half-activation voltage and slope factor of the Boltzmann function, respectively. The number 25 in the Goldman-Hodgkin-Katz equation is the magnitude of the ratio of the product of the universal gas constant and absolute temperature and the product of the charge of the ion and the Faraday constant measured in mV. Then normalized G values at given test potentials were calculated as

$$G(V) = \frac{1}{1 + e^{-(V-V_{1/2})/k}}$$

for each cell, where $G(V)$ is the conductance calculated at the given test potential, V is the actual magnitude of membrane potential, $V_{1/2}$ is the half-activation voltage and k is the slope factor. In oocytes, the value of G was determined in a way different from that in conventional patch-clamp measurements because due to the size of oocytes the intracellular potassium concentration and thus the value of equilibrium potential are not known in the presence of a given internal solution.

To determine the activation time constants for ionic currents a single one-component saturating exponential function was fitted to the rising phase of the traces:

$$I(t) = I_0 \times (1 - e^{-t/\tau_{act}}) + C$$

where $I(t)$ is the actual current amplitude at time t , I_0 is the maximal current amplitude, τ_{act} is the activation time constant, and C is constant, time-independent current component.

For fluorescent signals two-component saturating exponential functions were applied to determine fast (τ_f) and slow (τ_s) time constants:

$$F(t) = F_{0f} \times (1 - e^{-t/\tau_f}) + F_{0s} \times (1 - e^{-t/\tau_s}) + C$$

where $F(t)$ is the actual fluorescence intensity measured at time t , F_{0f} is the amplitude of the fast component of the fluorescent signal, τ_f is the time constant of the fast component of the fluorescent signal, F_{0s} is the amplitude of the slow component of the fluorescent signal, τ_s is the time constant of the slow component of the fluorescent signal, and C is the time-independent fluorescence intensity.

To construct curves characterizing the voltage-dependence of steady-state inactivation (SSI) the fraction of non-inactivated channels at each voltage was calculated as I/I_{-120} where I is the peak current evoked by the depolarization of +50 mV from a given prepulse potential, whereas I_{-120} is the peak current evoked by identical depolarization from the holding potential of -120 mV.

For the quantification of relationships describing steady-state parameters (F_{norm-V} , SSI), a Boltzmann function was fitted to data points to determine the values of half-activation voltage ($V_{1/2}$) and slope factor (k):

$$y = \frac{1}{1 + e^{-(V-V_{1/2})/k}}$$

In the function y is the normalized fluorescence intensity (in the case of F_{norm-V}) or normalized current amplitude (in the case of SSI), V is the actual membrane potential, $V_{1/2}$ is the half-activation voltage and k is the slope factor.

To investigate the Cole-Moore shift of Kv10.1 channels a 500 ms long depolarizing pulse was applied to +40 mV following a 10-s-long prepulse to potentials ranging from -160 mV or -60 mV. To determine the activation time constants of the ionic currents a single exponential function was fitted to currents evoked from each prepulse potential. The first sigmoidal phase of the currents was excluded from the fitting interval.

For determining current decreases caused by sterol loadings in TEVCF measurements, leak corrected peak currents evoked by a +40 mV (for Kv1.3) or +60 mV (for Kv10.1) depolarizing pulses were pooled for each day for the control, cholesterol, and 7DHC loaded

oocytes and then normalized to the mean of the control peaks of the same day. Thus, we obtained a relative current amplitude for each day and SEMs originate from the day-to-day variability of the normalized peak amplitudes.

3.6.2 Analysis of patch-clamp measurements performed in outside-out configuration

For non-stationary noise analysis, outside-out patches were excised from oocytes and 200 ms long depolarizing pulses were applied to +50 mV 200 times every 2 seconds from a holding potential of -100 mV. Traces significantly deviating from the mean due to rundown were omitted from the analysis. For each time point along the trace (isochrones) the mean current of the traces ($\langle I \rangle$) and the variance of the current (σ_I^2) were calculated. Then σ_I^2 was plotted as a function of $\langle I \rangle$, yielding a parabola with the equation:

$$\sigma_I^2 = i \langle I \rangle - (\langle I \rangle^2 / N)$$

where i is the single channel current and N is number of channels in the patch. Single channel current was determined from the first derivative of the parabola at the roots, while single channel conductance was calculated as $i / (V - E_{ekv})$ where V is the applied test potential and E_{ekv} is the equilibrium potential of the K^+ current. Open probability was calculated as the maximum of $\langle I \rangle$ divided by the positive root of the parabola, iN , the theoretical maximum of $\langle I \rangle$ occurring at $P_o = 1$.

3.6.3 Analysis of patch-clamp measurements performed in inside-out configuration

Before analysis, inside-out current traces were digitally filtered with a three-point boxcar filter. All the ionic currents applied for determining conductance-voltage (G-V) and SSI curves were leak corrected before the analysis.

To construct G_{norm} -V curves of mutant *Shaker*-IR channels, inside-out patches were held at -120 and depolarized for 100 ms to test potentials ranging from -100 to +70 mV in steps of 10 mV every 60 s. Conductances at given test potentials (V) were obtained from the leak corrected peak currents (I_{peak}) using the K^+ equilibrium potential (E_{ekv}) and the equation $G = I_{peak} / (V - E_{ekv})$. While isochronal tail current calculation is the standard method for determining G-V curves for non-inactivating currents, the *Shaker*-IR/T449A channels in this study are inactivating, which complicates the interpretation of tail current analysis, thus we used a different method for the analysis. The given G values were normalized to the maximum conductance (G_{norm}) and plotted as a function of the test potential. G_{norm} -V curves were quantified by fitting a Boltzmann-function:

$$y = \frac{1}{1 + e^{-(V-V_{1/2})/k}}$$

where y is the normalized conductance at the given membrane potential (V). Half-activation voltage ($V_{1/2}$) and slope factor (k) were determined from the fits.

To determine the inactivation time constants (τ_{inact}) of the currents 2000-ms-long depolarizing pulses to +50 mV were applied. The decaying part of the current traces was fitted by a single one-component exponential function starting from the 90% of the peak:

$$I = I_0 \times e^{-t/\tau_{inact}} + C$$

where I is the actual current amplitude, C is the magnitude of the steady-state current, I_0 is the amplitude of the inactivating component, t is the time measured from the start of fitting, and τ_{inact} is the inactivation time constant.

To define activation time constants (τ_{act}) of the currents 5-ms-long depolarizing pulses were applied from a holding potential of -120 mV to +50 mV, then evoked current traces were fitted using Hodgkin-Huxley n^4 -model:

$$I = I_0 \times (1 - e^{t/\tau_{act}})^4 + C$$

In the function I is the actual magnitude of the current, I_0 is the maximal current amplitude, τ_{act} is the activation time constant, t is the time measured from the start of fitting, and C is the magnitude of the steady-state current.

For measuring the kinetics of recovery from inactivation pairs of depolarizing pulses were delivered from the holding potential of -120 mV to +50 mV for 200 ms or 400 ms depending on the ion channel construct. The interpulse interval (ipi) at -120 mV varied between 0.5 and 60 s. The fractional recovery was calculated as

$$\frac{I_2 - I_{SS1}}{I_1 - I_{SS1}}$$

where I_2 and I_1 are the peak currents during the second and first pulse, respectively, and I_{SS1} is the steady-state current at the end of the first depolarization. To obtain the time constant of recovery from inactivation (τ_{rec}), data points were fitted by a single one-component saturating exponential function according to

$$I = I_0 \times (1 - e^{-t/\tau_{rec}})$$

where I the current amplitude at time t , I_0 is the maximal current amplitude, and τ_{rec} is the time constant of recovery from inactivation.

To describe steady-state inactivation curves the fraction of non-inactivated channels at each voltage was calculated as I/I_{-120} , where I is the peak current evoked by the depolarization from a given prepulse potential, whereas I_{-120} is the peak current evoked by identical

depolarization from the holding potential of -120 mV. $V_{1/2}$ and k were determined by fitting a Boltzmann-function to the data points.

3.6.4 Analysis of confocal laser scanning and STED microscopy measurements

After labeling of HEK-293 cells and image acquisition using confocal laser scanning or STED microscopy, the extent of co-localization between ion channels and lipid rafts was determined as described previously. Briefly, the Pearson correlation coefficients between the intensities of the raft marker, the antibodies against ion channels and in certain cases the fluorescently labeled cholesterol were calculated. During image processing calculations were carried out in cell membrane pixels selected manually with a “cell mask”. The Pearson coefficients were determined from pixelwise intensity data of individual cells with a custom-written algorithm under Matlab (Mathworks, Natick, MA). During the calculation of the coefficient, in principle, a positive value can be obtained accidentally even in the absence of a real positive colocalization. We used Costes' method to rule out the possibility of this accidental false positive correlation. In this method, the pixelwise intensities of the two fluorophores in a given cell are randomly mixed by an algorithm, and then the Pearson coefficient between the intensities is calculated from the data of pixels of the two new images. The value of the coefficient obtained this way should fluctuate around 0 due to random mixing and subsequent lack of correlation. This process is repeated 100 times by the algorithm, and then the 95% confidence interval of the coefficient is calculated from the obtained data, which statistically determines the expected range of the coefficient in the absence of correlation. If the value of the coefficient calculated from the real data is outside this range, the correlation between the signals of the fluorophores is to be considered statistically significant. As a positive control, the correlation coefficient between two known and widely accepted lipid raft markers was also calculated with our method.

3.7 Statistics

All electrophysiological experiments were carried out on cells originating from at least 3 independent injections or transfections. Microscopy data were obtained by analyzing three or more independent experiments.

Data are represented as mean \pm SEM throughout the Dissertation. The numbers of cells (n) involved in the given analysis are shown in the text. P values were calculated based on ANOVA analysis followed by Tukey's HSD test. Differences were considered significant when $p < 0.05$.

IV. Results

4.1 Sterol effects on steady-state and kinetic parameters of voltage-dependent gating of wild-type Kv1.3

Our previous results on the sterol-sensitivity of Kv1.3 channel gating showed that cholesterol or 7DHC enrichment of the cell membrane reduced the magnitude of whole-cell currents, caused right-shifts of the $G_{\text{norm}}-V$ curve characterizing the voltage-dependence of conductance and slowed channel activation kinetics in human lymphocytes and CHO cells. In the starting phase of our experiments, we set out to reproduce all these observations gained in mammalian cells or human samples with wild-type Kv1.3 channels expressed in the *Xenopus laevis* oocyte expression system. Curves characterizing the voltage dependence of normalized conductance derived from obtained ion currents show that loading of the cell membrane with cholesterol and 7DHC significantly modifies this relationship. Statistical analysis of the data obtained during the measurements shows that the value of $V_{1/2}$ was significantly depolarized after cholesterol and 7DHC loading, similar to that observed in mammalian cells. The time constants characterizing current activation kinetics were also significantly increased by loading the cell membrane with cholesterol or 7DHC, i.e. under these conditions the currents were activated with slower kinetics compared to those measured in untreated (control) oocytes. The voltage dependence of steady-state inactivation also shifted towards depolarization upon loading of the cell membrane with sterols, since quantitative analysis of the values of $V_{1/2}$ showed significant differences compared to the control after both cholesterol and 7DHC loadings. Our results indicate that despite the differences in membrane composition, the *Xenopus* system is a valid model for studying such protein-membrane interactions in mammalian cells.

In order to study voltage sensor movements in Kv1.3 channels using the TEVCF technique, a cysteine must be introduced in the external S3-S4 linker of the channel for fluorophore labeling. Since no previous reports on Kv1.3 TEVCF mutants existed, several residues in the linker were substituted to cysteine by site-directed mutagenesis and tested to find the optimal position (a channel with a high amplitude, easy-to-analyze fluorescent signal, and conductivity and main electrophysiological parameters that do not differ from those of the wild-type channel). An Ala (alanine) \rightarrow Cys (cysteine) mutation at position 309 close to the top of S4 proved to be the most suitable choice for TEVCF as it gave large monophasic voltage-dependent fluorescence signals.

The A309C mutant conserved the basic gating properties of Kv1.3, such as the steepness of the voltage-dependence and activation kinetics, although it shifted the $G_{\text{norm}}-V$ curve characterizing voltage-dependence of its conductance similarly to most TEVCF cysteine mutations. We used MTS-TAMRA in our experiments since labeling by this fluorophore yielded fluorescence signals superior to those of AlexaFluor488 and tetramethylrhodamine maleimide (TMRM). The Kv1.3 has no native extracellular cysteines, which precludes non-specific labeling of the channel. Since the majority of the Dissertation is about the cysteine containing channels used for electrophysiological experiments, Kv1.3 and Kv10.1 refer to these channels without indicating the mutations (A309C and L322C in the cases of Kv1.3 and Kv10.1, respectively), and the channels not containing the introduced cysteines are referred to as wild-type (WT) channels. The presented results on cysteine mutant channels were obtained after MTS-TAMRA staining, however, application of the dye did not result in significant changes in any of the examined parameters or sterol induced effects.

Xenopus laevis oocytes expressing Kv1.3 A309C channels were depolarized to test potentials in the -140 to $+40$ mV voltage range in 10 mV steps for 250 ms every 30 s from a holding potential of -100 mV. By simultaneously recording ionic currents and fluorescence signals using TEVCF technique, we obtained current - voltage (I-V) and relative fluorescence change - voltage ($\Delta F/F-V$) curves. Then, normalized conductance - voltage ($G_{\text{norm}}-V$) and normalized fluorescence change - voltage ($F_{\text{norm}}-V$) curves were constructed and fitted with Boltzmann-functions to obtain half-activation voltages ($V_{1/2}$) and slope factors (k), as detailed in Materials and methods. As expected, the half-activation voltage of the $F_{\text{norm}}-V$ curve was hyperpolarized compared to the $G_{\text{norm}}-V$ half-activation voltage ($V_{1/2} = -41.1 \pm 1.8$ mV (n=12) and -15.8 ± 0.5 mV (n=10), respectively), indicating that VSD activation occurs at more negative potentials than pore opening.

Experiments were repeated on cells loaded with cholesterol or 7DHC. Both sterols induced small but significant hyperpolarizing shifts in the $G_{\text{norm}}-V$ curves characterizing pore opening ($V_{1/2} = -15.8 \pm 0.5$ mV (n=10) for control, -21.1 ± 1.5 mV (n=11) for cholesterol; and -29.0 ± 1.9 mV (n=12) for 7DHC; $p = 0.049$ and $p=0.001$ for cholesterol and 7DHC, respectively), while leaving VSD-related $F_{\text{norm}}-V$ half-activation voltages unaffected ($V_{1/2} = -41.1 \pm 1.8$ mV, n=12 for control; -41.5 ± 1.8 mV, n=11 for cholesterol; and -42.1 ± 2.4 mV, n=12 for 7DHC; $p = 0.992$ and $p=0.937$ for cholesterol and 7DHC, respectively). This suggests a direct sterol effect on the pore or the coupling rather than one mediated by VSD activation. Slope factors of $F_{\text{norm}}-V$ curves were slightly affected by the treatments, cholesterol induced a

significant shallowing of VSD voltage-dependence ($k = 15.4 \pm 0.4$, $n=12$ for control; 22.2 ± 1.8 , $n=11$ for cholesterol; and 18.7 ± 0.8 , $n=12$ for 7DHC, $p=0.002$ and $p=0.099$ for cholesterol and 7DHC, respectively).

An important feature of the voltage-dependent gating of Kv1.3 channel is that at negative membrane potentials, some of the channels get into an inactivated state. This phenomenon is called steady-state inactivation (SSI). The effect of cholesterol on steady-state inactivation of Kv1.3 has not been previously studied. Kv1.3 309C SSI curves characterizing steady-state inactivation showed a similar tendency to $G_{\text{norm}}-V$ curves, being left-shifted by sterols, although only the shift by 7DHC was statistically significant.

4.2 Sterol effects on steady-state and kinetic parameters of voltage-dependent gating of wild-type Kv10.1

In wild-type Kv10.1 channels expressed in oocytes, we observed similar sterol effects as in Kv1.3: reduction of current amplitude, slowing of current activation kinetics and a right-shift in the $G_{\text{norm}}-V$ curve. These results are consistent with previous data examining the effects of cholesterol extraction on wild-type Kv10.1.

For TEVCF measurements on Kv10.1, we used the L322C mutant described and characterized earlier, but applied MTS-TAMRA fluorophore for labeling instead of TMRM. This mutation-dye combination yielded robust multi-phasic voltage-dependent fluorescence signals with higher amplitudes and did not alter channel gating significantly. I-V and $\Delta F/F-V$ curves were recorded during depolarizing pulses ranging from -180 to $+60$ mV in 10 mV steps lasting 800 ms. Pulses were applied every 10 s from a holding potential of -100 mV. In agreement with earlier results, VSD activation in Kv10.1 occurs at very negative potentials ($F_{\text{norm}}-V V_{1/2} = -113.6 \pm 1.9$ mV; $n=9$), while the pore opens in a similar voltage range as Kv1.3 ($G_{\text{norm}}-V V_{1/2} = -25.8 \pm 2.1$ mV; $n=8$). Therefore, the voltage gap between VSD activation and pore opening is much wider in Kv10.1 than in Kv1.3 indicating a looser coupling between the two functional domains, as described in the Introduction. The effect of sterols on Kv10.1 was very similar to those on Kv1.3, as both cholesterol and 7DHC induced significant hyperpolarizing shifts in the $G_{\text{norm}}-V$ curve ($V_{1/2} = -25.8 \pm 1.8$ mV ($n=8$) for control, -33.2 ± 1.8 mV ($n=8$) for cholesterol and -38.2 ± 2.2 mV ($n=9$) for 7DHC; $p = 0.043$ and $p=0.002$ for cholesterol and 7DHC, respectively), but neither had any effect on the half-activation voltage of the $F_{\text{norm}}-V$ relationship ($V_{1/2} = -113.6 \pm 1.9$ mV ($n=9$) for control, -113.5 ± 0.6 mV ($n=9$) for cholesterol and -114.0 ± 1.6 mV ($n=8$) for 7DHC; $p = 0.999$ and $p = 0.981$ for cholesterol

and 7DHC, respectively). Thus, just like in Kv1.3, pore opening, but not VSD activation is affected by sterol loading in Kv10.1.

4.3 Sterol effects on VSD and current activation kinetics

Next, the effects of sterol loadings on VSD activation and current activation kinetics were assessed using A309C Kv1.3 and L322C Kv10.1 constructs. To determine Kv1.3 current activation kinetics, ion currents were fitted by single exponential functions and activation kinetics were characterized by the τ_{act} activation time constant. Current activation kinetics were significantly slowed by both cholesterol and 7DHC loading. This was demonstrated by changes in time constants ($p < 0.05$ at all voltages) and normalized current traces. Fluorescence signals characterizing VSD activation kinetics had a fast component accounting for the majority (>85%) of the amplitude and a smaller slow component. Accordingly, the activation kinetics of the fluorescence signal could be well fitted with a function containing the sum of two exponential terms. There was no change in either of the time constants of double exponential fits in response to sterol loading. Furthermore, no significant changes were observed in the ratios of the amplitudes of the time constants due to sterol loading.

Activation of Kv10.1 currents is known to be modulated by the prepulse holding potential and the external Mg^{2+} concentration, which can result in complex sigmoid kinetics, as opposed to the single exponential activation of the Kv1.3 current. For the comparability of sterol effects on activation kinetics in different channels, we omitted the initial lag phase of the current from the fitting and characterized the rise of the current by single exponentials under all conditions as has been described by others. Both cholesterol and 7DHC loading increased current activation time constants, especially at stronger depolarizations. Next, the effect of sterols on VSD activation kinetics was assessed in the -180 to $+60$ mV range. Fluorescence signals representing backward VSD transitions to deeper closed states at hyperpolarized membrane potentials (between -180 and -110 mV) had two strongly voltage-dependent kinetic components, neither of which was significantly affected by sterols. On the contrary, depolarizing pulses (between -100 and $+60$ mV) produced biphasic signals with two components of opposing polarities. The fast component with negative polarity became more prominent with increasing depolarization and is likely to represent a VSD transition strongly linked to pore opening based on its voltage range and kinetics. The time constant of the slow component showed marked voltage-dependence and was unaffected by sterol treatment, while the fast component was very weakly voltage-dependent and was significantly slowed by both

cholesterol and 7DHC. These observations suggest that VSD transitions between deep closed states are unaltered by sterols, but conformational changes associated with pore opening are slowed.

A characteristic feature of Kv10.1 channels is the Cole-Moore shift, which is apparent as altered current activation kinetics when depolarization occurs from different holding potentials. When channels are activated from very negative potentials they must traverse multiple closed states before opening, a process structurally related to the interaction of the VSD and the PAS domain. Consequently, current activation is sigmoid and slow from more negative holding potentials compared to channels being activated from a more positive holding potential. To examine this phenomenon, current activation kinetics during depolarizing pulses to +40 mV were compared from prepulse holding potentials in the -160 to -60 mV range in control and sterol-loaded cells. Time constants in sterol-loaded cells were similar to control at most hyperpolarized holding potentials (at -160 and -140 mV), but current activation kinetics were significantly slower when opening took place from the “pre-open” closed states in cases of holding potentials more positive than -120 mV. This indicates that the opening transition is slowed by sterols, but the rate-limiting transitions among deep closed states are not.

4.4 Examination of the mechanism of sterol-induced current reduction

Although current densities could not be directly compared due to the lack of cell capacitance information, both sterols significantly reduced whole oocyte currents in cells injected by the same amount of RNA and recorded in the same time range following injection for both A309C Kv1.3 and L322C Kv10.1 channels. Amplitudes were reduced to $71.1 \pm 8.2\%$ and $54.0 \pm 10.6\%$ of the control in Kv1.3 and to $71.3 \pm 8.6\%$ and $58.5 \pm 1.3\%$ in Kv10.1 by cholesterol and 7DHC, respectively. To determine the cause of the current reduction, i.e. whether it is due to the reduction of single channel conductance or the open probability, we performed non-stationary noise analysis on ion currents obtained from excised outside-out membrane patches containing Kv10.1 channels using patch-clamp technique and ion currents evoked by voltage protocols described in Section 3.6.2. Current fluctuations detected during patch-clamp measurements depend on open probability and single channel conductance, and plotting current variance as a function of current amplitude yields a parabola, whose parameters are suitable to determine these properties of the channels. Our noise analysis indicates that both sterols significantly reduced the single channel conductance (control: 19.73 ± 1.44 pS, n=8; cholesterol: 13.30 ± 1.11 pS, n=6, p = 0.009; 7DHC: 14.33 ± 1.04 pS, n=4, p=0.046), while the

open probability was not significantly affected (control: 0.678 ± 0.018 n=8; cholesterol: 0.715 ± 0.035 n=6, p= 0.628; 7DHC: 0.573 ± 0.044 n=4, p=0.067).

4.5 Examination of the raft association of Kv1.3 and Kv10.1

Both Kv1.3 and Kv10.1 were previously shown to be preferentially localized in sphingolipid- and cholesterol-enriched lipid raft microdomains of the cell membrane. Since raft localization was found to modulate structural and functional channel properties and cholesterol is an intrinsic major component of rafts, channel and raft reorganization upon sterol loading is likely to play a role in the observed sterol induced electrophysiological effects, as described in the Introduction. To test this hypothesis, we examined the effects of sterol loading on the distribution of Kv1.3 and Kv10.1 between raft and non-raft membrane microdomains by determining the Pearson correlation coefficients between the fluorescence intensities of antibodies against ion channels and the lipid raft marker cholera toxin B subunit (CTX-B) using confocal and stimulated emission depletion (STED) microscopy. The antibodies recognized FLAG epitopes of Kv1.3 and Kv10.1 channels (Kv1.3_{FLAG} és Kv10.1_{FLAG}). Consistent with previous observations, strongly positive Pearson coefficients were found between Kv1.3 or Kv10.1 and CTX-B signals (0.416 ± 0.013 , n=27 and 0.298 ± 0.019 , n=30, respectively). These values were much larger in magnitude than the limits of the 95% confidence intervals of the coefficient assuming no correlation and were of similar magnitude as obtained in case of positive control samples using two well-established lipid markers, GFP-GPI and CTX-B (0.551 ± 0.020 , n=23). The co-localization is also clearly demonstrated by our confocal images and representative contour plots showing the correlation between pixelwise intensities. In the case of both channels the Pearson coefficients significantly increased in both cholesterol and 7DHC-loaded cells (Kv1.3: 0.492 ± 0.013 , n=34, p=0.001 and 0.500 ± 0.015 , n=32, p=0.001, Kv10.1: 0.373 ± 0.017 , n=29, p=0.010, and 0.395 ± 0.018 , n=24, p=0.002, for cholesterol and 7DHC, respectively) when observed with confocal laser scanning microscopy. The increased raft localization of Kv1.3 in response to sterols was also confirmed at improved resolution (46-60 nm) provided by STED microscopy. Correlation coefficients were significantly more positive in sterol-loaded samples (cholesterol: 0.361 ± 0.019 , n=32, p=0.012, 7DHC: 0.366 ± 0.019 , n=28, p=0.010) than in controls (0.274 ± 0.025 , n=25). Furthermore, when examining the loading pattern of NBD-cholesterol by confocal microscopy, it showed inhomogeneity and based on the strong positive Pearson coefficient values, the fluorescent cholesterol analog incorporated preferentially into CTX-B labeled lipid raft microdomains (0.234 ± 0.025 , n=24),

especially into the vicinity of Kv1.3 channels (0.358 ± 0.023 , $n=24$). Based on the previously observed similar behavior of fluorescently labeled and native cholesterol, these results may suggest that exogenously delivered cholesterol is incorporated preferentially into rafts, especially microdomains containing Kv1.3 proteins, which supports the interaction of sterols and ion channels.

4.6 Introduction of the experimental strategy to investigate the pathways leading to inactivation at negative membrane potentials in the T449A/V474C *Shaker*-IR channel

In *Shaker*-IR channels residue 474 is located in the S6 helix, facing the water-filled cavity of the pore when activation gate is open. A cysteine introduced into this position can only be modified by Cd^{2+} or MTS reagents in the case of an open activation gate. The modification of this cysteine by Cd^{2+} (thus opening of the activation gate) can be monitored by measuring ion currents, since Cd^{2+} binding blocks ion conduction. A T449A amino acid substitution was employed in our construct to speed up C-type inactivation to keep the length of our protocols reasonable for the measurements.

To design a pulse sequence to monitor the status of the activation gate during inactivation, we electrophysiologically characterized the T449A/V474C *Shaker*-IR channel using inside-out patch-clamp configuration. Detailed protocols and data analysis can be found in Section 3.6.3. We first recorded currents evoked by different test potentials, then determined the peak conductance (G) at the given membrane potential by knowing the peak currents and the driving force ($V-E_{\text{ckv}}$), which was followed by normalization and determination of the normalized conductance-voltage relationship ($G_{\text{norm}}-V$ curve). Determination of the half-activation voltage ($V_{1/2} = -51.6 \pm 2.5$ mV) and slope factor ($k = 16.9 \pm 2.1$ mV) was based on a Boltzmann function fitted to the data points, which describes the dependence of the normalized conductance on the test potential for each cell (mean \pm SEM, $n = 5$). The normalized $G_{\text{norm}}-V$ curve was obtained from the averaged G_{norm} values at each voltage (\pm SEM), then we determined the values of activation and inactivation time constants at +50 mV. The current activates quickly, and the inactivation kinetics is fast enough to conduct the experiments on a reasonable time scale in our measurements, these parameters were taken into account when planning the time course of the protocols. We also determined the voltage dependence of steady-state inactivation (SSI) (see Section 3.6.3), which was characterized by the dependence of the fraction of non-inactivated channels (I/I_{-120}) on the prepulse holding potential. The Boltzmann function was fitted to data points of individual cells and the half-activation voltage

($V_{1/2} = -81.7 \pm 0.7$ mV, $n=4$) and the slope factor ($k = -5.8 \pm 0.6$ mV, $n=4$) were determined (\pm SEM, $n=4$). The curve was constructed by averaging I/I_{-120} values measured at each holding potential (\pm SEM, $n=4$). Using pulse pairs to +50 mV and varying the interpulse interval (i_{pi}) the kinetics of recovery from inactivation were determined at -120 mV, which served as the holding potential between pulse protocols for further experiments, to give a recovery time constant of 4.4 ± 0.6 s ($n=5$).

Based on these biophysical properties of T449A/V474C channels, we designed an experimental strategy using state-dependent cysteine accessibility to Cd^{2+} applied from the intracellular side to investigate closed-state inactivation (C \rightarrow CI transition). A pulse sequence consisting of three 5-ms-long depolarizations to +50 mV was applied, and these depolarizing steps fully activated potassium currents. During the first pulse (P_1), control solution (150 mM K^+ , 0 μ M Cd^{2+}) was applied to obtain the maximum current (I_1) on the given inside-out patch. This was followed by a 30-ms-long hyperpolarization to -120 mV to ensure quick and full closure of the activation gate of all channels prior to Cd^{2+} application. The membrane potential was stepped then to various negative values, for example to -90 mV, at which the channel shows a small degree of steady-state inactivation but no macroscopic current can be detected. Although the method may underestimate the extent of steady-state inactivation measured at -90 mV due to the relatively short (3s) holding potentials, the graph well defines the range of negative membrane potentials at which state-dependent cysteine modification experiments can be performed. The extent of steady-state inactivation at -90 mV was assessed by the current (I_2) evoked by the second depolarizing pulse (P_2) according to the formula I_2/I_1 , while inactivated current fraction (IF) was calculated as $IF = 1 - (I_2/I_1)$. An intracellular solution containing 20 μ M or 200 μ M Cd^{2+} was perfused onto the cytosolic surface of the patches during the 20 s pulse. Cd^{2+} exposure of patches was initiated immediately after P_1 and terminated with the completion of the -90 mV holding step, before P_2 . P_2 was also preceded by a 30-ms-long step to -120 mV to avoid Cd^{2+} exposure of the open-state channels at the beginning of P_2 (electromechanical delay in the perfusion system). After P_2 the patch was held at -120 mV for 60 s, which was sufficient to ensure full recovery of the inactivated channels in control conditions. This was followed by the third 5-ms-long depolarizing pulse (P_3), which was used to determine the recovered current fraction according to $RCF = (I_3 - I_2)/(I_1 - I_2)$, where I_1 , I_2 and I_3 represent the leak-corrected current amplitudes evoked by the three sequential pulses. Theoretically, if inactivation originates solely from the C \rightarrow CI transition, i.e., there is no opening of the activation gate prior to inactivation then RCF should equal to 1, because the

closed activation gate prevents Cd^{2+} modification of 474C and current loss. In contrast, if activation gate opening precedes inactivation, Cd^{2+} will modify 474C and current loss will be seen. If all channels contributing to steady-state inactivation have open activation gate at -90 mV and Cd^{2+} modifies all of these channels, then RCF is 0. $0 < \text{RCF} < 1$ indicates that the channels during steady-state inactivation are in a Cd^{2+} accessible state.

4.7 Activation gate opening in the T449A/V474C *Shaker*-IR ion channel at negative membrane potentials

To evaluate the ability of our three-pulse protocol to report specifically on the opening of the activation gate, we used three different control experiments. First, application of a -120 mV holding potential for 20 s between P_1 and P_2 in the absence Cd^{2+} did not decrease I_2 compared to I_1 . No inactivation occurred ($\text{IF} \sim 0$) and consequently, RCF was close to 1. Thus, in our three-pulse protocol the development of steady-state inactivation was avoided at -120 mV ($I_2 = I_1$), the duration of the pulses and the time elapsed between them are sufficient for I_1 and I_3 to be the same when there is no inactivation ($\text{RCF} \sim 1$). In our second control measurements, the intracellular surface of channels was perfused with a solution containing $200 \mu\text{M}$ Cd^{2+} during the holding potential between at -120 mV between P_1 and P_2 . Since neither I_2 (i.e. $\text{IF} \sim 0$) nor I_3 ($\text{RCF} \sim 1$) decreased in these experiments, we concluded that Cd^{2+} exposure had no nonspecific effects if the activation gate did not open and Cd^{2+} perfusion was properly controlled. These data demonstrate that the timing of the start/stop of the Cd^{2+} application is optimized to avoid Cd^{2+} exposure of the open channels when they are closing (end of P_1) or when closed channels are opening (during P_2). A third control experiment showed that all channels that were inactivated at -90 mV holding potential between P_1 and P_2 recover from inactivation in the absence of Cd^{2+} .

To assess the dependence of RCF on the negative holding potential, we applied holding potentials of -90 , -80 or -70 mV between P_1 and P_2 in the presence of $20 \mu\text{M}$ or $200 \mu\text{M}$ intracellular Cd^{2+} . Although no macroscopic current can be detected at -90 mV, a small degree of steady-state inactivation occurs. At -80 and -70 mV, whole-cell currents appear and in parallel, inactivation becomes progressively more prominent. Consistently, IF increases with depolarizing holding potentials both in the absence or presence of Cd^{2+} . In contrast, RCF decreased significantly only if Cd^{2+} was present during the holding potential between P_1 and P_2 . This indicates that activation gate openings occur in the voltage range of -90 to -70 mV. Consequently, Cd^{2+} accesses 474C despite the lack of macroscopic current at -90 mV. The

RCF is significantly decreased in the presence of 20 μM and 200 μM Cd^{2+} regardless of the holding potential. In the absence of Cd^{2+} , RCF values are essentially the same at all holding potentials.

Subsequently, we have shown that a similar current loss can be achieved if Cd^{2+} is used repeatedly many times for a short time. To correlate the increase in the current loss with Cd^{2+} exposure, we applied 20 μM Cd^{2+} on inside-out patches for short (200 ms) durations over many cycles at a holding potential of -90 or -80 mV. The pulse sequence started with a 5 ms depolarization to $+50$ mV to obtain the peak current I_1 , then the patch was held at -120 mV for 60 s. This was followed by the unit cycle, which consisted of 200 ms at -90 mV (or -80 mV) and 800 ms at -120 mV. The unit cycle was repeated $n=200$ times (or 50, or 100 times), followed by a 60 s holding at -120 mV in the absence of Cd^{2+} and the sequence was terminated by a 5 ms depolarization to $+50$ mV to obtain I_2 . Cd^{2+} was either absent or present (20 μM intracellular Cd^{2+}) during unit cycles. The cumulative fraction of channels modified by Cd^{2+} was calculated as $\text{IF} = 1 - I_2/I_1$, as above. Modification of 474C by cumulative exposure to Cd^{2+} was time- and voltage-dependent. A step to -80 mV produces more modification and increased IF than -90 mV. The pulse sequence gave cumulative Cd^{2+} exposure at 100 cycles similar to that obtained with a single 20 s application of 20 μM Cd^{2+} . In summary, our results show that at holding potentials where substantial steady-state inactivation occurs (at -90 , -80 , or -70 mV) the activation gate opens despite the lack of observable currents. This finding indicates that at negative membrane potentials the CI state is populated through the $\text{C} \rightarrow \text{O} \rightarrow \text{OI}$ pathway instead of the direct $\text{C} \rightarrow \text{CI}$ transition as suggested by others.

4.8 Locked-open activation gate prevents recovery from C-type inactivation in *Shaker-IR T449A/V476C* channels

The results of experiments above suggest that an open activation gate is important for inactivation to occur at negative holding potentials. The ability of the channels to recover from inactivation may also mechanistically depend on the conformational status and movements of the activation gate. Locking the activation gate in the open state is a suitable method to investigate determinants for recovery from inactivation.

Previous studies have shown that the activation gate of the *Shaker-IR* 476C mutant channel can be trapped in the open state by Cd^{2+} application from the intracellular side. This is caused by formation of a Cd^{2+} bridge between 476C in one subunit and a native histidine (H486) in a neighboring subunit, thus inhibiting activation gate closing even at negative voltages. A

biophysical characterization of the T449A/V476C *Shaker*-IR channel expressed in HEK-293 cells using inside-out patch-clamp configuration was also performed in this case before designing the pulse protocols used in the experiments. The same protocols and evaluation methods were used as for the characterization of the 474C mutant.

Based on the results obtained during the biophysical characterization, we designed an experimental protocol, which was subsequently used to investigate the effect of locking the activation gate in the open state on the recovery of channels from inactivation. The protocol was first tested under control conditions. In T449A/V476C channels the extent of recovery from inactivation was monitored by measuring current amplitudes evoked by 2-s-long repeated depolarizations to +50 mV from a holding potential of -120 mV. The interpulse interval (ipi) was set to 60 s. Currents were normalized to the peak evoked by the first pulse and plotted as a function of time elapsed from the start of the first depolarizing pulse. Repeated depolarizations in the absence of Cd²⁺ resulted in similar current amplitudes for the T449A/V476C channels, thus, the duration of ipi is sufficient for full recovery of the current under control conditions.

A second control experiment demonstrated the lack of peak current reduction (thus a lack of pore block) by intracellular Cd²⁺ in the absence of 476C. During the measurements the pulse protocol was identical to the one used in the first control experiment except that Cd²⁺ (20 μM) was applied to the cytosolic surface (inside-out configuration) for 1 s. Current inactivation is complete at the beginning of Cd²⁺ perfusion, but the activation gate is open at that time due to the depolarized membrane, so Cd²⁺ is applied to channels in the OI gating state. We recorded identical peak currents even after long (up to 8 s) cumulative exposure of T449A/V476 channels to Cd²⁺. Thus, Cd²⁺ does not influence currents in the absence of 476C.

To determine the dependence of recovery from C-type inactivation on the status of the activation gate we locked-open the activation gate of T449A/V476C *Shaker*-IR channels by intracellular Cd²⁺ exposure (20 μM) following the full inactivation of the current (i.e., in the OI state) at +50 mV. To do this, we essentially repeated the control experiment described before except that T449A/V476C *Shaker*-IR channels were used. Cd²⁺ exposure was timed to the final 1000 ms of the first 2-s-long depolarizing pulses. The T449A/V476C *Shaker* construct inactivates completely by the time the Cd²⁺ application starts ($\tau_i = 136$ ms, Cd²⁺ application starts after $\sim >7\times$ the time constant for inactivation). During the remaining time, patches were perfused by standard intracellular solution. This treatment resulted in complete loss of peak current even after a single pulse. Thus, channels with Cd²⁺-locked open activation gates are unable to recover from inactivation (n=5). We suggest that these channels were trapped in the OI state in response to the treatment and therefore remained nonconductive even after a 60-s-

long holding at -120 mV, which proved to be sufficient for the recovery from inactivation under control conditions. The loss of the current was irreversible. The kinetics of current loss was determined by repeated exposure of the OI channels to 20 μ M Cd^{2+} for short, 200 -ms durations. This resulted in a quantized and significant decrement of peak currents upon cumulative exposure. The loss of the current as a function of cumulative modification time followed a single exponential decay, indicating a specific interaction between the cysteine residue at position 476C and Cd^{2+} .

To confirm that this non-conductive OI state is created by a Cd^{2+} bridge between 476C and H486 in our inactivating V449A construct, we tested whether the Cd^{2+} -induced current loss could be prevented by the protonation of H486 at low pH. A de-protonated H486 is critical for the metal-bridge formation by Cd^{2+} with 476C. During the measurements depolarizing pulses to $+50$ mV were applied from a holding of -120 mV every 15 s. Cd^{2+} application started 200 ms before each pulse and ended simultaneously with the depolarization. The pH of Cd^{2+} containing intracellular solutions was set to 7.36 or 5.3 . The development of the locked-open state at pH 7.36 is indicated by the increased relative amplitudes of the tail currents (compared to tail currents at pH 5.3), which was followed by a slow decay corresponding to inactivation. The trace-to-trace decrease in the peak currents at $+50$ mV indicates that locked-open and inactivated channels do not recover from inactivation. This current phenotype and the inactivation process of open channels with locked-open activation gates have been described previously for V476C mutant *Shaker* channels. In contrast, at pH 5.3 , where the metal bridge formation is inhibited, both the shape of the tail currents and peak amplitudes of the currents at $+50$ mV are conserved during repeated depolarizations. Normalized peak currents decrease during cumulative application of Cd^{2+} at pH 7.36 , however, at pH 5.3 , the peaks are maintained at a steady level ($n=4-5$).

V. Discussion

With the help of the experiments presented in the Dissertation, we examined the role of two factors influencing the fine-tuning of the gating of K_v channels. One of them is the cholesterol in the cell membrane, which has previously been shown to affect the activation gating of K_v ion channels, however, the primary intramolecular target of its action within the ion channel has not been clarified so far. The other issue examined in the Dissertation was the investigation of gating transitions occurring at negative membrane potentials. On the one hand, we examined in these experiments whether the opening of the activation gate is a necessary condition for the C-type inactivation to occur, and, on the other hand, whether closure of the activation gate is required for the process of recovery from inactivation.

Determination of the intramolecular target of cholesterol action within K_v ion channels and the direct investigation of C→CI and OI→O transitions at negative membrane potentials are not possible using the patch-clamp technique based on ion current measurements. On the one hand, we do not obtain information about the movements of VSD during gating simultaneously with ion current measurements in the case of conventional patch-clamp measurements. On the other hand, ion current measurements at negative membrane potentials are characterized by large errors due to the low driving force and low channel opening probabilities at such membrane potentials. Thus, in order to give accurate answers to the two questions formulated in the Objectives of the Dissertation, we had to apply new methods, through which we could examine the fine-tuning of the gating of K_v ion channels from a new approach.

The TEVCF technique used in experiments presented in the first half of the Dissertation allowed us to track VSD movements during the entire gating process simultaneously with ion currents measurements. Thus, in addition to effects of cholesterol on classical electrophysiological parameters, we were able to determine its primary target within the channel (VSD, PD, or the coupling apparatus between the two) using this method. Based on our results, the main target of cholesterol is the PD itself, not the VSD or the coupling apparatus, in both the K_v1.3 gated according to the linear gating model and the K_v10.1 whose gating can be described by the complex gating model.

In the second part of the Dissertation, we designed and used a new combination of voltage protocols and cysteine modification strategies to describe the gating scheme characterizing the development of steady-state inactivation and requirements of recovery from inactivation at negative membrane potentials in *Shaker* ion channels. The specific interactions

between Cd^{2+} and cysteines introduced by point mutations allowed us to detect (474C) and lock (476C) the activation gate opening even at negative membrane potentials. Summarizing our results, the most probable pathway leading to steady-state inactivation is the $\text{C} \rightarrow \text{O} \rightarrow \text{OI} \rightleftharpoons \text{CI}$ transition, thus, direct $\text{C} \rightarrow \text{CI}$ transition is not possible in the *Shaker* ion channel. In addition, closure of the activation gate i.e., the $\text{OI} \rightarrow \text{CI}$ transition, is essential for the recovery from inactivation, so direct $\text{OI} \rightarrow \text{O}$ transition is not possible either at negative membrane potentials. Therefore, according to our most relevant conclusion, the actual position of the activation gate is of essential importance in regulating the processes of steady-state inactivation and recovery from inactivation.

Our results may provide motivation for further examination of cholesterol-membrane protein interactions and are promising for future structure-function and pharmacological studies. Our observations emphasize the importance of exploring indirect cholesterol effects and the underlying molecular targets. Understanding these may contribute to a deeper understanding of the pathomechanisms of several diseases in which one important feature is an increase or change in the cholesterol level of the cell membrane. Such pathological conditions associated with alterations in sterol levels include various tumors, metabolic, neurodegenerative and immunological disorders, and the aging process itself. Investigation of the presence of gating transitions at negative membrane potentials is also biologically relevant, since both steady-state inactivation and recovery from inactivation are of essential significance in determining the number of potentially activatable channels, which is one of the major determinants of cell excitability.

VI. Publications



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Subject: PhD Publikációs Lista

Candidate: Florina Zákány
Neptun ID: E1X0PA
Doctoral School: Doctoral School of Molecular Medicine
MTMT ID: 10045513

List of publications related to the dissertation

1. Szántó, G. T., **Zákány, F.**, Papp, F., Varga, Z., Deutsch, C., Panyi, G.: The activation gate controls steady-state inactivation and recovery from inactivation in Shaker.
J. Gen. Physiol. 152 (8), 1-12, 2020.
DOI: <http://dx.doi.org/10.1085/jgp.202012591>
IF: 4.258 (2018)
2. **Zákány, F.**, Pap, P., Papp, F., Kovács, T., Nagy, P., Péter, M., Szente, L., Panyi, G., Varga, Z.:
Determining the target of membrane sterols on voltage-gated potassium channels.
Biochim. Biophys. Acta. Mol. Cell Biol. Lipids. 1864 (3), 312-325, 2019.
DOI: <http://dx.doi.org/10.1016/j.bbalip.2018.12.006>
IF: 4.402 (2018)





List of other publications

3. **Zákány, F.**, Kovács, T., Panyi, G., Varga, Z.: Direct and indirect cholesterol effects on membrane proteins with special focus on potassium channels.
Biochim. Biophys. Acta. Mol. Cell Biol. Lipids. 1865 (8), 1-27, 2020.
DOI: <http://dx.doi.org/10.1016/j.bbalip.2020.158706>
IF: 4.402 (2018)
4. Kovács, T., Batta, G., **Zákány, F.**, Szöllősi, J., Nagy, P.: The dipole potential correlates with lipid raft markers in the plasma membrane of living cells.
J. Lipid Res. 58 (8), 1681-1691, 2017.
DOI: <http://dx.doi.org/10.1194/jlr.M077339>
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5. Kovács, T., Batta, G., Hajdu, T., Nagyné Szabó, Á. T., Váradi, T., **Zákány, F.**, Csomós, I., Szöllősi, J., Nagy, P.: The Dipole Potential Modifies the Clustering and Ligand Binding Affinity of ErbB Proteins and Their Signaling Efficiency.
Sci. Rep. 6, 35850, 2016.
DOI: <http://dx.doi.org/10.1038/srep35850>
IF: 4.259

Total IF of journals (all publications): 21,826

Total IF of journals (publications related to the dissertation): 8,66

The Candidate's publication data submitted to the iDEa Tudóstér have been validated by DEENK on the basis of the Journal Citation Report (Impact Factor) database.

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