Sterol regulation of voltage-gated K+ channels

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1 Abstract

Cholesterol is an essential lipid building block of the cellular plasma membrane. In addition to its structural role, it regulates the fluidity and raft structure of the membrane and influences the course of numerous membrane-linked signaling pathways and the function of transmembrane proteins, including ion channels. This is supported by a vast body of scientific data, which demonstrates the modulation of ion channels with a great variety of ion selectivity, gating and tissue distribution by changes in membrane cholesterol. Here we review what is currently known about the modulation of voltage-gated K+ (Kv) channels by changes in membrane cholesterol content, considering raft association of the channels, the roles of cholesterol recognition sites and those of adaptor proteins in cholesterol – Kv channel interactions. We specifically focus on Kv1.3, the dominant K+ channel of human T cells. Effects of cholesterol depletion and enrichment and 7-dehydrocholesterol enrichment on Kv1.3 gating are discussed in the context of the immunological synapse and the comparison of the in vitro effects of sterol modifications on Kv1.3 function with ex vivo effects on cells from hypercholesterolemic and Smith-Lemli-Opitz patients.

2 Introduction

Cholesterol is a prominent component of the plasma membrane and provides a structural stability for the lipid bilayer of mammalian cells. Cholesterol has an influence on the biophysical properties of the plasma membrane as well as on membrane proteins. Cholesterol, along with the sphyngolipids serves as a molecular glue to attach the lipid rafts together, and it classifies proteins as raft-associated and raft-excluded ones. The cholesterol homeostasis of the human body is rigorously controlled and any unbalances can lead to diseases. Dietary hyperlipidemia/hypercholesteremia is a major health issue, generally leading to atherosclerosis, which nowadays is mainly regarded as an inflammatory immune response (1). Furthermore, inherited disorders of cholesterol synthesis such as SLOS (Smith-Lemli-Opitz Syndrome) that is characterized by several birth defects (microcephaly, cardiac and neuronal deficiencies, behavior problems) (2). While the former is associated with an elevated cholesterol level, in the latter the precursors of cholesterol (7-dehydrocholesterol, 8-dehydrocholesterol) are present at high concentration in the plasma and the tissues.

Ion channels of the plasma membrane are transmembrane proteins that form ion conducting pores to transport ions through the cell membrane. The most ancient and largest group of ion channels are the potassium (K^+) channels, and of these the voltage-gated (depolarization activated) K^+ (Kv) channels are the best studied subgroup. Kv channels consist of 4 subunits, they can be either heteromers or homomers, and each subunit contains six alpha-helical segments (S1-S6) connected by intra or extracellular loops (3). The N and C terminal ends of Kv channels reside intracellularly, and play role in association of individual subunits, binding various auxiliary and adaptor proteins (see chapter 6.2). Though ion channels and neurons/muscle cells are classically related, many non-excitable cells such as immune cells express Kv channels (reviewed in chapter 4), where they regulate various functions such as migration, cytokine production, Ca^{2+} signaling, proliferation etc. During the past two-three decades a large body of information was collected on the interaction of cholesterol/lipids and transmembrane proteins, including ion channels (4, 5). Alanine-scanning mutational analysis revealed that the S1-S4 segments are in contact with the lipid molecules, and S5-S6 and the pore region are surrounded by the remaining segments and isolated from the lipids (6-8). Proper functioning of ion channels was found to require the presence of an annular lipid ring firmly attached to the channel protein (9). Furthermore, many Kv channels were shown to have non-

random distribution in the membrane, they are localized to non-caveolin lipid rafts or caveolea, while others are excluded from the rafts by mechanisms currently not yet known (10-13).

There exist two main theories on how cholesterol modification can modify operation of membrane proteins and ion channels. According to the first, the change in the lipid composition alters the physical-chemical parameters of the membrane (fluidity, stiffness), that generates an increased friction at the interface of the lipids and proteins, and retards the conformational changes (5, 14, 15). The other model explains the changes in the operation of the channel protein with specific binding sites: the membrane proteins have specific hydrophobic pockets, which can modulate the transition between different states upon cholesterol binding (16, 17). However, these two interactions cannot be separated, usually both influence the biophysical features of the ion channel, only their relative weight is different from a channel to another.

In this chapter first we will review the biophysical properties and physiological role of voltage-gated potassium channels, especially those expressed in non-excitable cells. Then we summarize the present knowledge obtained with an electrophysiological method (patch-clamp technique) on Kv channels upon in vitro cholesterol level modification. We report in detail about the localization of Kv channels to rafts, and how the ion-channel associated adaptor proteins (with PDZ domain) can interact with cholesterol and lipids. Finally, a review onion channel related consequences of sterol metabolism (hypercholesteremia, and SLOS: 7DHC accumulation) will be presented based on our latest results.

3 General structure of voltage-gated K^+ (Kv) channels

The product of the genes encoding Kv channels is a channel subunit (Kv1.x-Kv12.x), four of which assemble to form a functional channel. These proteins can be divided into two large classes: the conducting and the non-conducting (silent) channels. Conducting tetrameric channels are formed by the Kv1.x, Kv2.x, Kv3.x, Kv4.x, Kv7.x, Kv10.x, Kv11.x and Kv12.x subunits, while the Kv5.x, Kv6.x, Kv8.x and Kv9.x proteins are not capable of this (18). These latter subunits form heterotetramers with Kv2.x subunits to create functional channels of various properties. Different types of Kv channels can be expressed by a particular cell, which may be formed by homo- or heterotetramers of subunits belonging to the same family (e.g. Kv1.x), which results in the variability of the biophysical parameters. Variability in Kv channel function

is further enhanced by other factors. Auxiliary subunits may attach to the pore-forming α subunits such as Kvβ, KCHIP (K^+ channel interacting protein), KCNE or calmodulin proteins (19) . In addition, the aforementioned silent α-subunits, alternative splicing and posttranslational modifications also contribute to Kv channel function diversity (20).

The opening probability of Kv channels depends on the membrane potential. Increasing depolarization opens an increasing fraction of the channels, and the resultant K^+ efflux causes hyperpolarization establishing a negative feedback loop, which leads to channel closing. During prolonged depolarization some Kv channels enter a non-conducting inactivated state, whose extent and rate of development shows great variability. Classically Kv channels are divided into two groups based on the kinetic properties of the current they pass: A-type (fast inactivating) and delayed rectifier (slowly or non-inactivating) channels. Channels passing typical A-type currents include Kv1.4, Kv3.3, Kv3.4, Kv4.1, Kv4.2 and Kv4.3, which are usually expressed by excitable cells: neurons, muscle and cardiac muscle cells.

The delayed rectifier channels do not inactivate in the millisecond time range and as a result their active period is delayed compared to the voltage-gated Na^+ channels. As such, their main role in excitable cells is the shaping of the repolarization phase of action potentials (21, 22). The typical delayed rectifier channels are Kv1.1, Kv1.2, Kv1.3, Kv1.5, Kv1.6, Kv1.7, Kv1.8, Kv2.x and the Kv7.x.

3.1 Structural features and gating of Kv channels

The voltage-gated K^+ channel of *Drosophyla melanogaster*, dubbed *Shaker*, has supplied a vast amount of information about the structure and function of Kv channels. *Shaker* is a close relative of mammalian Kv1.x channels (accordingly named *Shaker* family) and is the most often applied model system, since it is very easy to work with and expresses very well in various expression systems (23). Its name originates from the fact that the legs of the *Drosophyla* shake uncontrollably during ether anesthesia if the animal carries certain mutant versions of the channel, which modify action potential properties.

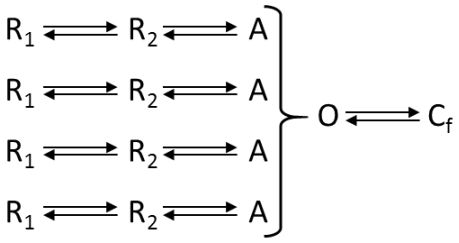
The determination of the X-ray crystal structure of the Kv1.2 channel in 2005 by the MacKinnon group (24) was a great advancement in understanding Kv channel structure, based on which a very detailed model of Kv channels has developed by today. The tetrameric channel is held together by non-covalent bonds among the subunits (25). Each subunit is composed of six transmembrane α-helical segments and the intra- and extracellular loops connecting these

segments. The pore domain that is responsible for ion conduction is formed by the fifth and sixth transmembrane segments (S5 and S6) and the extracellular loop between them (26). This is the region of the channel where most peptide and small molecule blockers of Kv channels bind to prevent ion conduction. The pore region contains the selectivity filter that confers Kv channels their high K^+ selectivity. The filter sequence (TVGYG) is highly conserved across K^+ channels with only a few exceptions (e.g. the SVGFG sequence of Kv11.1 and minor differences in two-pore-domain potassium channels). The selectivity filter is the narrowest part of the pore where K^+ ions pass in a single file and where the strong selectivity over other ions is achieved. The electronegative carbonyl oxygens of the filter residues line the pore and replace the water molecules lost during the dehydration of K^+ ions leaving the solution. The carbonyl oxygens arranged in a well-defined geometry create binding sites for K^+ ions whose entry into the filter will thus be energetically favorable despite losing their hydrate shell. This explains why Na^+ ions that are smaller than K^+ ions do not significantly permeate through the filter: the carbonyl oxygens "designed" for the size of K^+ ions are too far to coordinate Na^+ ions energetically efficiently. Based on the crystal structures of voltage-gated and the pH-gated bacterial KcsA K^+ channels the pore contains four sequential K^+ binding sites, and the existence of an extracellular rehydration / dehydration site is also suggested (27, 28). During ion permeation two of the four sites are likely to be occupied and the electrostatic repulsion between the dehydrated ions accomplishes the very high passage rate (10^7-10^8 ions/s) while preserving high selectivity.

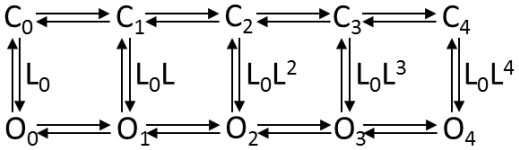
At its intracellular end the selectivity filter widens into a water-filled cavity where K^+ ions are still hydrated. The cavity is closed off by the activation gate at the intracellular side. The activation gate is formed by the crossing of the S6 segments lining the intracellular end of the pore. A highly conserved Pro-Val-Pro motif creates a flexible region, a hinge, where the opening and closing of the activation gate occurs as a kink is created in the S6 segments during gating (29).

Kv channels open in response to membrane depolarization, and since many of them are involved in shaping action potentials, their voltage-dependence must be steep to enable operation within a narrow membrane potential range and fast gating. Changes in membrane potential are detected by the voltage-sensor domains (VSD) of the channel, which are made up of the S1-S4 helices in each subunit. Within the VSDs the S4 segments have a special role, as they carry several positively charged amino acid side chains, whose movement is induced by membrane

depolarization. The moving charges of S4 generate the measurable “gating current”, whose kinetics characterizes the speed of the conformational change associated with gating, and whose integral, the “gating charge” is proportional to the amount of charge moved and thus with the number of gating channels. The conformational change of the VSDs induced by depolarization is transmitted to the pore domain (PD) via a coupling mechanism resulting in the opening of the gate (30). Based on the measured ionic and gating currents with various voltage-protocols different gating schemes had been suggested over the years to describe the steps of activation, which were comprehensively compared by Zagotta et al. (1994) on the *Shaker* channel. Comparing their recorded data to simulations based on the gating schemes, they found that the model in which all VSDs must complete two sequential conformational changes to reach the activated state gave the best match. In the model, pore opening was only allowed after all four VSDs had reached the activated state. The model contains a relatively unstable closed state, which can be reached from the open state. Due to the negligible voltage-dependence of this transition it is likely to cause the fast flipping of the channel between the two states during the activated state of the VSDs.



The model based on the Kv1.2 crystal structure suggests that the VSD-PD coupling is a direct and rigid link via the continuous polypeptide chain of the S4-S5 linker region (28). However, more recent results indicate that this coupling may be much looser and opening of the pore can occur even before the activation of all four VSDs (31). According to this allosteric model, the activation of each VSD increases the probability of pore opening (by a factor of L in the model below), but the activated state of all four is not an absolute requirement



Zagotta et al. (1994) examined the suitability of this model to describe *Shaker* gating, but found it inappropriate. This suggests that Kv channels of different families may have different coupling mechanisms: while *Shaker* follows a linear or deterministic scheme, others, such as Kv7.1 is activated by an allosteric mechanism. Rather than the direct rigid VSD-PD link, coupling may require the presence of lipids, or it may not even require the continuity of the polypeptide chain to accomplish gating (32, 33), so the great diversity of gating models is not surprising.

Long depolarizations drive many Kv channels into a non-conducting inactivated state, which occurs on the intracellular side by fast N-type inactivation in channels passing A-type currents. This mechanism is similar to the inactivation of the fast-inactivating voltage-gated Na^+ channels that is accomplished by the „ball and chain" mechanism. In this, residues at the N-terminal of Kv channels form a ball-like structure tethered to the rest of the channel by a stretch of other residues ("chain"). During depolarization and following the opening of the activation gate the ball binds into the pore via electrostatic interactions thereby blocking the flow of K^+ ions (34).

Another inactivation mechanism, called the C-type, exists in many Kv channels, which blocks ion flow in the selectivity filter at the extracellular end of the pore (35). The movement of the VSDs induced by depolarization rearranges the hydrogen-bond network of the residues in the selectivity filter, which stabilizes the filter in a state wherein the outermost K^+ binding site is widened. The site widened this way is no longer able to complex the K^+ ion and consequently conduction ceases, and the C-inactivated state is formed. In the absence of K^+ ions the entire filter region widens due to the repulsion of the carbonyl oxygens and the channel is able to conduct Na^+ or other larger cations (36). Since the rate of C-type inactivation is determined by the occupancy of the outermost K^+ binding site, any factor influencing this occupancy can modify inactivation kinetics. Such factors include the amino acid residues near the pore entrance, the composition of the solutions (primarily the K^+ concentration) and their pH, and the presence of channel blockers (37).

4 Function of Kv1.3 - an example for the role of voltage-gated ion channels in non-excitable cells

The activity of voltage-gated K^+ channels regulates the permeability of the membrane for K^+ ions, thereby influencing the resting potential of cells, the frequency and duration of action potentials in excitable tissue (38) or, membrane potential driven processes of non-excitable cells such as proliferation and progression of the cell cycle (39). Generally, the physiological function of voltage-gated K^+ channels is determined by several parameters including the biophysics of channel gating, the single-channel conductance, the specific expression level in a given cell and the regulation of all these parameters by interacting partners such as auxiliary subunits, ion channel modulation by transmembrane and intracellular signaling events (phosphorylation/dephosphorylation) and by the physical-chemical environment in the membrane.

This paper focuses on one specific ion channel, the Shaker-related voltage-gated Kv1.3 channel. This channel is expressed in many tissues, including neurons (40), cancer cells (39) and dendritic cells (41), however, its most significant physiological role was described in the regulation of antigen-dependent activation of T cells (recently reviewed in (42, 43).

Kv1.3 is the dominant voltage-gated K^+ channel in human T-cells, its expression in these cells was described in 1984 by Matteson and Deutsch (44) and DeCoursey an co-workers (45). This channel is encoded by the KCNA3 gene (46) and belongs to the Shaker-family of voltage-gated K^+ channels. Accordingly, the structure and gating of this channel conforms to the properties of Shaker-related Kv channels described in chapter 3. It is generally accepted that the main physiological function of Kv1.3 in T cells is controlling the membrane potential of the T cells during the initial steps of T cell activation following presentation of the antigens (reviewed in (47, 48)).

The following properties of this channel and the T-cell membrane are the most important regarding membrane potential control (Fig.1):

1. Voltage-dependence of steady-state activation and inactivation: The channel is activated by depolarization of the membrane, the activation threshold of the channels is close to the resting potential of T cells and the open probability increases very steeply with depolarizations exceeding the activation threshold (49). At the same time, the voltage-dependence of steady-state inactivation of the channels, i.e., the fraction of channels not

accessible for opening at a given membrane potential, is not complete at the resting potential of intact T-cells (50, 51). The combination of these results in a window of membrane potentials at which voltage-gated K^+ channels can be open at steady-state, and therefore, K^+ fluxes through these channels can contribute to the maintenance of the resting membrane potential.

2. Inactivation kinetics: The process of inactivation limits the amount of K^+ current available for membrane potential control. Of the two inactivation processes described in chapter 3.1, Kv1.3 inactivates exclusively by the C-type inactivation mechanism (52) as the channel lacks the N-terminal inactivation particle. Therefore, the kinetics of Kv1.3 inactivation is relatively slow (time constant for inactivation, τ_i=200 ms as opposed to the few milliseconds characteristic for N-type inactivation), allowing significant K^+ efflux through activated channels before entering into the non-conducting state. This also contributes to an efficient control (clamp) of the T cell membrane potential.
3. Input resistance: The lymphocyte membrane has extremely high electrical resistance (10-20 GΩ). Therefore, miniature currents carried by a few open ion channels influence significantly the membrane potential of such cells. Indeed, fluctuation of the resting membrane potential in response to opening of individual ion channels was reported by Maltsev using current-clamp (53), and by Verheugen et al. who determined resting potential fluctuation from the changes in the reversal potential of the current in cell-attached patch configuration (51).

In summary, the biophysical properties of Kv1.3 channels and those of the lymphocyte membrane provide the basis for effective clamping of the membrane potential close to the activation threshold of the channels. Accordingly, transfection of the Kv1.3 channel gene alone into CHO cells was sufficient to shift the resting membrane potential to ~–50mV, as compared to the –5 - –20mV membrane potential measured in non-transfected cells (54). One main topic of this paper is how the biophysical parameters of Kv1.3 gating are affected by modulation of the sterol content of the membrane and how this modulation may result in altered physiological functions of the channel. Therefore, other factors influencing the activity of Kv1.3 (phosphorylation, etc, see above) are not discussed here but have been extensively reviewed elsewhere (48).

4.1 Kv1.3 and T cell activation

A recent review has discussed 14 ion channels and cation transporters that mediate transmembrane ion fluxes in T cells (43). Of these, three ion channels are considered to be the main determinants of the membrane potential regulation of T cells at rest as well as upon antigen stimulation. These include the voltage-gated Kv1.3 and the intermediate conductance Ca^{2+}-activated K^+ channels (KCa3.1) as well as the Calcium Release Activated Ca^{2+} channels (CRAC). To understand the physiological role of these channels we present here a simplified model of T cell activation during an immune response (reviewed extensively in (42, 47, 48, 55). Professional antigen presenting cells (APCs) present processed peptides to T cells via MHC molecules in their plasma membrane. The binding of the peptide-loaded MHC molecules to the T cell receptor (TCR)/CD3 complex and the co-receptors CD4 or CD8) induces the clustering of signaling molecules: this brings kinases and their substrates into molecular proximity allowing for their efficient operation. The enzyme phospholipase C-γ (PLC-γ1) is recruited in this complex, and following its activation cleaves the membrane-associated phosphatidylinositol 4,5-biphosphate (PiP_2) into 1,2-diacylglycerol and inositol 1,4,5-trisphosphate (IP_3), which diffuses freely in the cytosol. IP_3 then binds to its receptors in the endoplasmic reticulum (ER), which results in the release of Ca^{2+} into the cytosol and a significant rise in $[Ca^{2+}]_i$. This transient rise in $[Ca^{2+}]_i$ is followed by a sustained Ca^{2+} signal which is necessary for initiation of T cell proliferation. The sustained Ca^{2+} signal is caused by influx of extracellular Ca^{2+} through CRAC channels located in the plasma membrane. Functional CRAC channels are assembled from the ER calcium sensor STIM1/STIM2, which is translocated to the plasma membrane-ER junctions upon emptying of the stores, and the plasma membrane channel ORAI1. Once the channels are opened the magnitude of Ca^{2+} influx is determined by the electrochemical driving force for Ca^{2+}. It is evident that the depolarizing effect of Ca^{2+} influx needs to be compensated if the driving force for further Ca^{2+} entry is to be kept high. This counterbalancing K^+ efflux is mediated by the voltage-gated Kv1.3 and the Ca^{2+}-activated KCa3.1 K^+ channels. These two channels are activated by Ca^{2+} influx via feed-forward mechanism: Ca^{2+} entry depolarizes T cells to potentials more positive than the opening threshold of Kv1.3 channels, while the increased $[Ca^{2+}]_i$ directly opens the Ca^{2+}-activated KCa3.1 channels. The concomitant K^+ efflux restores and stabilizes the sufficiently hyperpolarized membrane potential providing optimal conditions for further Ca^{2+} entry and driving the proliferation of the T cells specific for a given antigen.

If this compensating K^+ efflux is down-regulated by ion channel blockers or modulation of the biophysical parameters of channel gating by the sterol content of the membrane, the activation of T cells can be inhibited, as we will show it later in this paper.

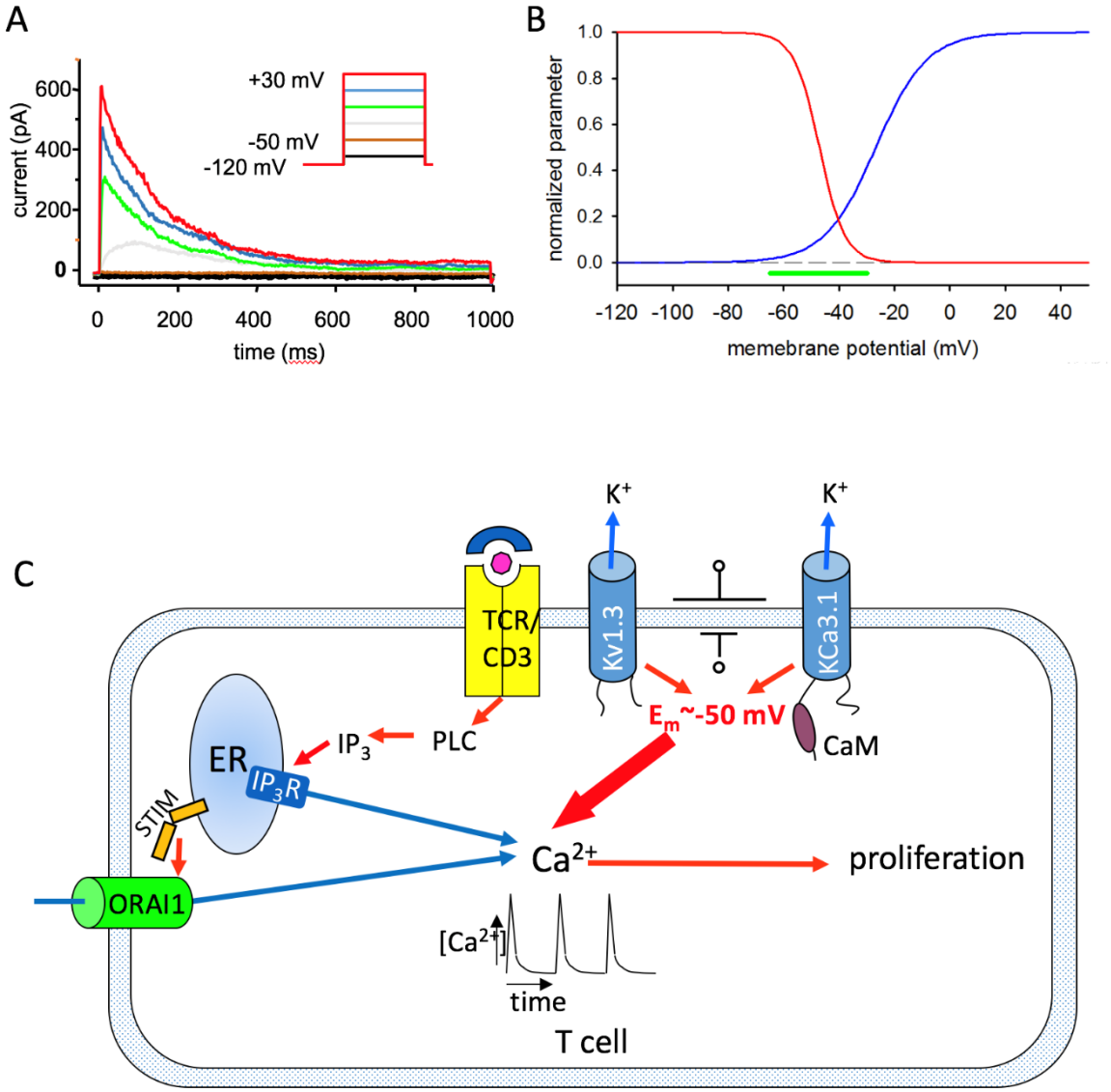


Fig. 1 Properties of Kv1.3 and its role in lymphocyte activation. Panel A: Whole cell Kv1.3 currents recorded in a human T cell. The cell was depolarized from a holding potential of -120 mV to different test potentials ranging from -70 mV to +30 mV in 20 mV steps every 60 s, as

indicated in the inset. Colors of the inset test potentials match the currents recorded at that membrane potential. The activation kinetics of the current progressively becomes faster with increasing depolarizations. The slow decay following the peak current is the consequence of C-type inactivation. Panel B: Voltage-dependence of steady-state activation (blue) and steady-state inactivation (red) of Kv1.3. See text for details. The continuous lines were drawn bases on the data by Hajdu et al. (56). The green bar indicates the membrane potential range where Kv1.3 channels can be open at steady-state. Panel C: Role of cation channels in T cell activation. Stimulation of the T cell receptor complex (TCR/CD3) with antigenic peptide (purple) results in the activation of phospholipase Cγ1 (PLC) and the generation of inositol 1,4,5 triphosphate (IP_3). IP_3 binds to the IP_3 receptor (IP_3R) on the surface of the endoplasmic reticulum (ER) and releases Ca^{2+}. Emptying the ER Ca^{2+} store induces a conformational change in STIM which subsequently activates the ORAI channel located in the plasma membrane which in turn leads to Ca^{2+} influx. Ca^{2+} influx requires a concerted interplay between Ca^{2+} and K^+ channels (Kv1.3 and KCa3.1), the former providing the influx pathway for Ca^{2+} and the latter maintaining a negative membrane potential, which generates the driving force for Ca^{2+} entry. The resulting Ca^{2+} signal is an oscillation of the cytosolic free Ca^{2+} concentration of variable amplitude and frequency (57) which drives T cell proliferation Red arrows show regulatory interactions, blue arrows show ion fluxes, CaM: calmodulin, E_m: membrane potential.

5 Lipid rafts and ion channels

Lipid rafts are specialized membrane domains with a lipid composition different from the bulk of the membrane. While the definition constantly evolves, some features are generally accepted for lipid rafts: enrichment of cholesterol, sphingolipids and specific proteins, increased resistance to detergents and a liquid ordered state of the lipids, in which the acyl chains are stretched out much like in the gel phase while still conserving a fluidity similar to the liquid phase (58). A wide size range has been reported for these microdomains, typically citing 10-200 nm (59), however, their highly dynamic nature precludes precise size determination. Larger platforms can be formed from these nanoscale fluctuating domains, which assemble molecular participants required for specific cellular functions (60). Thus, the main proposed function of lipid rafts is to provide a means for the lateral organization of signaling molecules by controlling

their mobility, proximity and trafficking. A special type of lipid rafts are the caveolae, invaginations of the plasma membrane shaped by caveolin proteins, which are also involved in signal transduction and endocytosis.

Ion channels, as membrane proteins and participants in signal transduction pathways can also interact with lipid rafts, which may consequently influence their function. There have been several reports about Kv channels being associated with or excluded from rafts. Cyclodextrins, which are cyclic oligosaccharides having a hydrophilic external surface and a hydrophobic internal cavity, are routinely used to manipulate the cholesterol content of the plasma membrane. Most often methyl-β-cyclodextrin (MβCD) is used, which can extract cholesterol selectively over other lipids from the plasma membrane (61-63). However, when employed as preloaded with cholesterol or its analogs, it can also specifically enrich the sterol content of the membrane. This provides a tool to disrupt or rearrange the raft structure of membranes and observe its effect on ion channel function. However, it must be noted that cholesterol extraction by cyclodextrins is not selective for lipid rafts (64), so altered channel function cannot be strictly regarded as a consequence of a change in raft structure.

The earliest reports on raft association of Kv channels demonstrated that Kv2.1 is targeted to non-caveolar lipid rafts while Kv1.5 targeted to caveolar lipid rafts. In addition, cholesterol depletion altered raft composition and modified the gating of Kv2.1(10, 11). Later a role for caveolin in regulating Kv1.5 trafficking to cholesterol-rich rafts was demonstrated (65). Other studies found that Kv1.5 behaved differently when overexpressed in heterologous systems compared to that in native cells. In the former case Kv1.5 tended to localize to rafts while in the latter it appeared not to target to these microdomains (66). In leukocytes the tendency of Kv1.3/1.5 heteromers to localize to rafts depended on the stoichiometry of the subunits with higher Kv1.3 ratios favoring raft targeting while higher Kv1.5 ratios favoring exclusion of the channels from rafts. The variable behavior of Kv1.5 is likely to be affected by the association of auxiliary subunits.

Although reports on Kv1.3 found contradicting effects of cholesterol depletion, all studies agreed that Kv1.3 localizes to rafts and that disruption of rafts alters Kv1.3 gating (67-69). Interestingly, recently a domain interacting with caveolin 1 was identified on Kv1.3 suggesting its targeting to caveolar rafts, which appeared very sensitive to various factors and thus regulate the surface localization of the channel (13).

Originally, Kv4.2 was found to be excluded from rafts (10), but another study showed a fraction of Kv4.2 channels to be raft-associated, similar to Kv1.4 (70). The scaffolding protein PSD-95 aided the raft association of Kv1.4, but not that of Kv4.2. Confirming this finding, more recently a partial distribution of Kv4.2 and its auxiliary subunit KChiP2 to rafts was described. MBCD treatment caused a redistribution of channels out of rafts and reduced the current carried by the channels (71). Examination of raft association of Kv channels in pancreatic cells revealed that in α-cells Kv4.1/4.3 channels, while in β-cells Kv2.1, but not Kv1.4 channels target to rafts, and in both cases depletion of cholesterol by MBCD decreased raft association of the channels(72, 73).

Association of the members of the eag family to lipid rafts was also demonstrated. Kv10.1 was found to be distributed between raft and non-raft membrane regions at various ratios, which was influenced by cholesterol and the cytoskeleton. Cholesterol depletion increased current density of Kv10.1 channels without affecting other aspects of gating (74). Both Kv11.1 and its regulatory subunit KCNE2 were also detected in rafts and channel gating was modified by both cholesterol depletion and loading of the membrane (75).

Although certain channels appear more or less prone to target to rafts, these highly variable and sometimes conflicting data indicate that the raft association of a particular Kv channel is likely to be cell specific. Various factors, such as the precise lipid composition, including cholesterol content, the presence of scaffolding proteins and auxiliary subunits influence the affinity of the channel for rafts. For example, the distribution and clustering of Kv2.1 and Kv1.4 channels was significantly different even between atrial and ventricular myocytes (76). In addition to these factors, depletion and loading of cholesterol may have a direct effect on Kv channels via cholesterol binding sites, not only through the disruption or assembly of lipid rafts.

5.1 Ion channels in the immunological synapse

A special lipid microenvironment for Kv1.3 and other channels involved in the regulation of T cell activation can be the immunological synapse. This signaling platform is dynamically assembled upon presentation of the antigen to the T cell by professional antigen presenting cells. The IS signaling platform contains T cell receptor CD3 complex along with the auxiliary, signal transducer and scaffolding proteins (extensively reviewed in (77-79)). Lipid rafts are found to accumulate at the IS, as was reported by staining the cells with cholera toxin B subunit (CTB),

which binds to Ganglioside M1 (GM1), a marker of raft membranes (80-82). High plasma membrane lipid order in the IS was also found using a phase-sensitive membrane dye together with a variety of spectrally-resolved microscopy techniques (83). We have shown that Kv1.3 is co-localized with the T-cell receptor CD3 complex (84) and recruited into the IS formed between cytotxic T cells and target cells (85). Later the KCa3.1 and CRAC channels (STIM/ORAI) were also shown to be localized in the IS (86, 87). Thus, it seems that the most important ion channels regulating T cell activation are dynamically associated with the IS. Furthermore the co-localization of Kv1.3 was shown with CTB-labeled lipid rafts using confocal microscopy and fluorescence cross-correlation (85). Considering the very unique microenvironment in the IS (both protein and lipid) one might imagine that the function of the channels would be dramatically altered upon recruitment into the IS. Unfortunately, very limited information is available about this in the literature due to the difficulty inherent with the experimental system using conventional electrophysiology: one cannot separately study the function of the channels in the IS since the antigen presenting cell impedes access of the pipette to the IS and use of the cell-attached patch recording. One indication that the function of Kv1.3 might be altered upon recruitment to the IS, however, came from our laboratory (88). We whole-cell patch-clamped mouse cytotoxic T cells either alone or following the formation of the IS (conjugated cell). We found that the activation kinetics of the Kv1.3 current was slower whereas the inactivation kinetics was faster in cells conjugated in IS as compared to the standalone cells. In addition, the voltage-dependence of steady-state activation was shifted toward the depolarizing potentials in T cells engaged in IS. As the IS is also a signaling platform we separated the postsynthetic modification of the channel by phosphorylation using kinase inhibitors. We concluded that modulation of the inactivation kinetics is rather related to regulation of the channel by phosphorylation whereas the change in the activation kinetics upon IS formation may be attributed to redistribution of the channels into distinct membrane domains.

6 Electrophysiology of Kv1.x channels upon membrane cholesterol manipulation

We described in chapter 4 that Kv1.3 channels are critical for the activation and proliferation of T lymphocytes. The change in the function of Kv1.3 can lead to inadequate Ca^{2+}-signaling and prevention of the myriad subsequent steps required for an adaptive immune

response. On the other hand, the relatively easy access to the human peripheral T cells make them an attractive model for studying the effect of various factors on the function of voltage-gated ion channels. Also via monitoring the downstream steps, the physiological consequences of the Kv1.3 modulation can be traced.

In the beginning of the investigation of the role of cholesterol in signaling the use of various cyclodextrins that make it possible to manipulate the membrane cholesterol level were applied *in vitro*. First Hajdu et al. applied methyl-beta-cyclodextrin (MβCD) and its cholesterol complexed form (MβCD/CHOL) to modify the cholesterol content of the T lymphocyte membrane (67). One-hour incubation of activated T cells with MβCD/CHOL at concentrations 0.72 and 1 mM could modify the biophysical parameters of the membrane as revealed by fluorescent polarization anisotropy: the increase in the membrane viscosity was observed upon cholesterol loading with MβCD/CHOL, while MβCD treatment had a negligible effect on the membrane fluidity (application of higher MβCD/CHOL or MβCD prevented the stable gigaseal formation during patch-clamp recording). Upon loading the cells with cholesterol (0.72 and 1 mM MβCD/CHOL) a remarkable change in the kinetic parameters was detected: the activation of the Kv1.3 current slowed app. 1.5-fold or more, and the inactivation rate significantly decreased (ca. 50% slower). Furthermore, cholesterol loading shifted the steady-state activation of the Kv1.3 channels toward positive membrane potentials (ca. 7 mV) but the equilibrium inactivation was not changed. It was also reported that cholesterol loading led to a bi-exponential activation of the Kv1.3 current, which could be explained with presence of an activated-closed state and not with co-existence of two channel populations in different lipid environments. This hypothesis is supported by several facts: 1) the ratio of slow and fast current components are not dependent on the depolarizing potential (shift in steady-state activation is not due to two populations in different lipid milieu), 2) inactivation kinetics did not exhibit biphasic decay. The cholesterol removal had no influence on the gating properties of Kv1.3 except a slight speed-up of the inactivation kinetics. The MβCD-treatment did not influence equilibrium parameters of activation and inactivation. The activity of Kv1.3 channels that was characterized by current density (peak current at +50 mV divided by the whole cell capacitance) increased upon cholesterol depletion (only for 1 mM MβCD), whereas cholesterol loading suppressed channel activity hallmarked by the reduced current density, similar to the reports on the Volume Regulated Anion Channel and the Kir2.1 channel (89, 90). Pottosin et al. studied the effect of

MβCD treatment on the Kv1.3 current in Jurkat T lymphoma cell line (69). They incubated the cells with 1 or 2 mM MβCD (for one hour as in Hajdu et al), and observed a vast reduction in cellular cholesterol level (63 and 75 % at 1 and 2 mM, respectively). Cholesterol removal also led to the decrease in the proliferative capacity of Jurkat cells, which partially can be attributed to the collapse of signaling pathways due to the collapse of the lipid raft system. However, they did not report the increase or decrease in the current density of Kv1.3 channels, which could reflect the change in the channel activity. Unlike in T lymphocytes, Kv1.3 channels of Jurkat T cells were affected by the drastic decrease in the cholesterol level: the activation time constant of the whole-cell current increased ca. 2.5-fold after 2 mM but not 1 mM MβCD incubation. Also a right-shift in steady-state activation (11 mV) and inactivation (6 mV) curves was reported upon the higher concentration MβCD treatment. Interestingly, they found that after cholesterol depletion with MβCD the kinetic and equilibrium parameters of activation returned to the value equal to that measured prior to treatment. Bock et al reported that formation of large membrane platforms induced by ceramide addition or stimulating CD95 receptors of Jurkat T cells, inhibited Kv1.3 activity (68). They also disrupted rafts with two cyclodextrins, β-cyclodextrin and β-methyl-cyclodextrin at 2 mM (60 min incubation) and 5 mM (10 min incubation), and could observe the Kv1.3 channel activity drop to 60-75%, as determined from the whole cell peak current at +70 mV. However, no changes on the biophysical parameters were analyzed or studied.

These three studies clearly demonstrate that the raft-associated Kv1.3 channels are in intimate relationship with the lipid environment. Only one paper reported about the effect of excess cholesterol in the membrane on Kv1.3 channels, and in parallel with previous findings on different ion channels (VRAC, BKCa, Kir) the activity of Kv1.3 was suppressed upon cholesterol elevation, which may be assigned to the altered protein-lipid interaction or the restructuring/reorganization of raft system upon cholesterol treatment (67). Moreover, the change in the raft structure itself - fusion of small rafts into large platform - can impair the channel activity, which emphasizes the importance of membrane integrity in Kv1.3 channel operation. The removal of cholesterol in Jurkat cells caused more prominent impact on Kv1.3 current than in primary/native T cells (practically no effect), however, we should note that cholesterol removal with MβCD concentration higher than 1 mM resulted in modification of the Kv1.3 current, which vetoed electrophysiological recording in T lymphocytes. The cholesterol

depletion in Jurkat E6-1 cells manifested in the change of kinetic and equilibrium parameters of activation and inactivation but not in the current density/amplitude, while the other Jurkat cell line responded with reduced peak current upon cholesterol extraction. This latter discrepancy emphasizes that there could be very distinct outcomes of the same treatments probably due to differences in passage number, cell cycle, adaptor protein expression etc. On the other hand, differences of Kv1.3 operation obtained upon cholesterol manipulation in primary and cultured cells promotes the use of primary cells over cell lines so that no ambiguous conclusions about the physiological effect are drawn.

Besides Kv1.3 many more Kv channels were shown to target to lipid rafts and their function is modified by cholesterol removal/loading. Martens et al showed for the first time that Kv2.1 channels expressed in the mouse Ltk^- cell line are localized to non-caveolin lipid rafts (10). When cholesterol was depleted with 2-hydroxypropyl-β-cyclodextrin, which destroyed the raft system, they obtained that Kv2.1 steady-state inactivation curve was largely shifted toward positive membrane potentials (ca. 35 mV increase in the half maximal voltage). Surprisingly, the test potential dependence of equilibrium activation was not affected by cholesterol removal. They also reported that Kv1.5 channels are co-localized with caveolin on the cell surface, in domains distinct from Kv2.1 (11). Treatment of the cell membrane with 2-hydroxypropyl-β-cyclodextrin or fumonisin (inhibitor of sphingomyelin biosynthesis) shifted half maximal voltage of equilibrium activation and inactivation by 10 mV to negative potentials. However, in both studies the biophysical parameters of the Kv4.2 channel, which is not associated with lipid rafts, were not influenced by cholesterol depletion. In contrast, in rat left ventricular myocytes the Kv4.2 channels with their auxiliary subunit KChIP2 are partially localized to lipid rafts. MβCD-treatment induced decrease in whole-cell current and redistribution of Kv4.2 channels to non-raft domains. These two latter cases clearly prove that membrane embedded ion channels can respond to cholesterol modification differently/oppositely in native or heterologous expression systems.

The neuronal Kv10.1 or Eag1 was also shown to partition into two subpopulations in mouse neurons and HEK cells: one localized to and the other was excluded from detergent insoluble membranes (74). Cholesterol extraction had multiple effects on Kv10.1: 1) reduced the fraction of raft-targeted Eag1 channels, 2) increased the whole-cell peak current but did not change any other biophysical parameters.

The cardiac Kv11.1 or hERG channels together with Kv7.1 (KvLQT1 or KCNQ1) turned out to be localized to cholesterol and sphingolipid enriched membrane regions in both canine ventricular myocytes and HEK293 cells (75). Interestingly, a long QT-related mutant of hERG (G601S) is not present in rafts. Furthermore, use of MβCD resulted in a positive shift in the test potential dependence of steady-state activation, and deactivation of the current was also accelerated in HEKs. However, in mouse myocytes only the faster deactivation kinetics was detected upon cholesterol removal. Chun et al. described that increase in the membrane cholesterol of HEK cells decreased the activation rate of hERG channels but had no effect on the deactivation and inactivation kinetics (91). However, cholesterol did not directly modify hERG channel function: activation of PLCβ1 reduces the level of PIP_2 (phosphatidylinositol-4,5-bisphosphate), which uncovers a crosstalk between two lipids, cholesterol and PIP_2 in the regulation of hERG.

6.1 Cholesterol recognition sites may mediate the sterol-protein interactions of Kv1.3

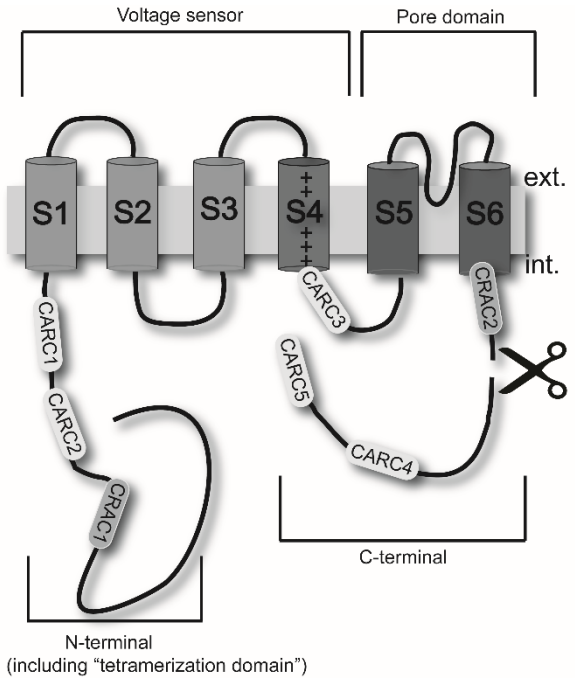


Fig.2 Schematic representation of the cholesterol recognition motifs identified along the Kv1.3 sequence. Each subunit contains at least seven putative cholesterol recognition sites. Two CARC sequence located on the distal end of the C-terminus. As we previously demonstrated removal of the last 84 amino acid residues (including the CARC4 and CARC5 sequences) abolish cholesterol sensitivity of Kv1.3 (4).

Cholesterol can regulate the operation of ion channels indirectly, either via modifying the physical properties of the cell membrane (fluidity, thickness, viscosity, etc.) or modulating the composition and assembly of lipid rafts (92). In recent years much evidence has been gathered supporting an alternative regulatory mechanism where specific and direct cholesterol-protein interactions are responsible for the sterol sensitivity of the ion channels (93). In line with this, several ion channels have been reported to contain one or more of the most widely studied cholesterol recognition sites, the CRAC and CARC motifs (cholesterol recognition amino acid consensus, CRAC: **L/V**-X_{1-5}-**Y**-X_{1-5}-**R/K** and CARC: **K/R**-X_{1-5}-**Y/F**-X_{1-5}-**L/V**, where X is any amino acid) (94-96). These sequences follow a very simple linear algorithm where well defined amino acid residues linked with theoretically unconcerned regions responsible for the cholesterol binding (16). Care must be taken when considering the significance of these motifs due to the flexible requirements of the algorithms (97). However it is notable, that a simple point mutation or deletion of these selected motifs could totally abolish CHOL sensitivity of ion channels (16, 95). Surprisingly, not just the transmembrane segments but the intracellular C-terminal end of the channels also carries functional cholesterol

recognition motifs (16, 95). Screening the amino acid sequence of Kv1.3 we found multiple CRAC and CARC sequences (Fig. 2). Certain motifs overlapped with the conservative parts of the channels like the activation gate or the voltage sensor. Others were mapped on the N- or C-terminal as Singh and his colleagues predicted in a former study (94). Mutational analysis of the voltage sensor or activation gate would be hardly interpretable since these regions are very sensitive to any modifications including single point mutations (98, 99). In contrast, Kv1.3 tolerates the mutations or removal of the entire C-terminus well. We published that the removal of the last 84 amino acid residues (including CARC 4 (K474-Y477-V478) and 5 sequences (K517-K518-F572-V575)) of the C-terminus have no significant effect on the time and voltage dependent properties of Kv1.3 gating (100). Therefore, we aimed to investigate the biological relevance of the two CARC sequences located on the distal C-terminus using the previously characterized ΔC-Kv1.3 construct. Transiently transfected CHO cells were loaded with 420 μM CHOL. Our results showed that the ΔC-Kv1.3 channels were resistant to the CHOL loading of the cell membrane. In contrary to the wild type Kv1.3 neither the $V_{½}$ nor the activation kinetics of ΔC-Kv1.3 changed after the sterol loading of the membrane. We proposed that the lack of CARC4 and CARC5 motifs are responsible for the abolished sterol sensitivity. To prove this concept, we designed specific point mutations along the CARC4 and CARC5 sequences where the key amino acid residues were changed to alanine (mCARC4: K526A-Y529A-V531A, mCARC5: K517A-K518A-F572A-V575A). As expected, the cholesterol sensitivity of the mutated channels was different from the wild type Kv1.3. CHOL loading was ineffective on the activation kinetics of the $mKv1.3_{CARC4}$ and just slightly modified the $V_{1/2}$. In case of the $mKv1.3_{CARC5}$ CHOL loading had modest effect on the $V_{1/2}$ and had negligible influence on the activation kinetics. We propose that CARC motifs of the C-terminal interact with membrane cholesterol of the inner leaflet via their hydrophobic residues and mediate sterol sensitivity of Kv1.3. A similar conclusion was reached by Robinson et al. who envisioned that the C-terminal of the P2X7 ion channel dips back into the plasma membrane and contacts the membrane via palmitoyl groups and hydrophobic residues. Furthermore, in the absence of a well-defined structure of the C-terminus we cannot exclude the possibility that mutations of the putative cholesterol binding pockets indirectly affect alternative regulation of the channel activity by cholesterol.

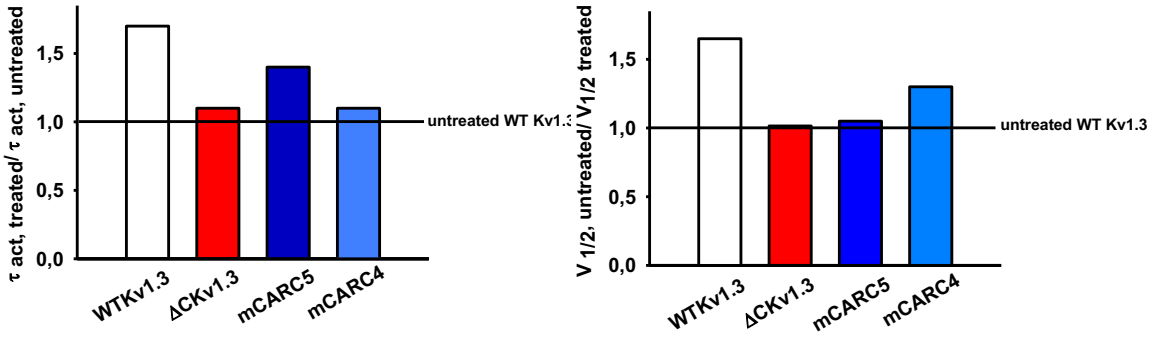


Fig. 3. The effect of 420 μM CHOL loading on the gating properties of various Kv1.3 construct. CHO cells were transiently transfected with the following constructs: wild-type Kv1.3, ΔCKv1.3 (which lacks the last 84 amino acid residues of the C-terminus), a CARC4 mutant (mCARC4, K526A-Y529A-V531A) and a CARC5 mutant (mCARC5, K517A-K518A-F572A-V575A).) and loaded with 420 μM CHOL. To determine the activation time constant and the midpoint of the steady-state activation ($V_{1/2}$) we applied the same protocols as we discussed in Fig. 5. Fig X demonstrates the relative change in the activation time constant and $V_{1/2}$ due to the cholesterol loading in case of a particular construct.

6.2 Adaptor proteins and cholesterol, a possible communication through cholesterol binding

Voltage-gated potassium channels often associate with various adaptor proteins, which can influence their function and localization. These intracellular 'anchoring' proteins, first described in neurons, play roles in trafficking to the plasma membrane, facilitating/hindering lateral membrane diffusion, promoting clusterization or coupling to various signaling proteins (101-111). It was unveiled that Kv1.3 coupled to synapse associated protein 97 (SAP97 or hDLG (human homologue of Drosophila disc large tumor suppressor protein) and postsynaptic density protein (PSD-95 or SAP90) - two members of MAGUK (membrane associated guanylate kinase) proteins - in Jurkat T cells (100). Both PSD-95 and SAP97 possess multiple PDZ domains, which guarantees their interaction with PDZ-binding domain of the C-terminal region. SAP97 is an essential part of Kv1.3-SAP97-Lck signaling complex in the T cell activation, while PSD-95 regulates the localization of Kv1.3 channels to the immunological synapse (IS) between a T cell and an APC (antigen presenting cell) (100, 102). Cortactin and its orthologue HS1 in T cells,

which are actin-binding proteins, can also bind to various ion channels including Kv1.3 where it associates to the SH3 binding domain (PXXP) on the C-terminus. Cortactin hinders the lateral mobility of Kv1.3 upon F-actin polimerization that is a distinctive event occurring in the contact area of a T cell with an APC (101). HS-1, which also accumulates in the IS on the T cell side, can facilitate the residency of Kv1.3 in the IS, which also includes their immobilization due to binding to membrane lipids. These proteins can also attract other signaling elements such as PKC, PKA to the channel, which can modify their operation (106, 110). As PSD-95, SAP-97 and HS1 redistribute to the IS, they probably have a role in attaching Kv1.3 channels to membrane domains/rafts, probably via specific lipid-protein interactions.

In recent years several studies were published that uncovered or predicted the interaction of adaptor proteins and membrane lipids (112) (97, 113-118). Cholesterol binding motifs (cholesterol recognition amino acid consensus, CRAC: **L/V**-X_{1-5}-**Y**-X_{1-5}-**R/K** and CARC: **K/R**-X_{1-5}-**Y/F**-X_{1-5}-**L/V**, where X is any amino acid) can be found in these adaptors proteins, especially within their PDZ domain (114). An intensive computational research project revealed that app. 2000 PDZ domains have membrane binding properties, and many of these have an affinity in or below the micromolar range (112). The PDZ domain of PSD95, SAP-97, PICK1 (protein interacting with C kinase 1) ZO-1 (Zonular occludens 1 or tight junction protein-1 (TJP1)) NHERF1 (sodium-hydrogen antiporter 3 regulator 1) and many others were shown to bind model plasma membranes (113). This interaction was selective for cholesterol, i.e. it was weakened by the replacement of cholesterol with other derivatives or upon its depletion from the membrane. Interestingly, it was described that the protein-binding pocket, which is in charge of the interaction with the PDZ binding domain, and the lipid interacting sequence are separated. A loss-of-function mutation in the lipid-binding motif of the PICK1 PDZ domain, which associates with the GluR2 subunit of the AMPA receptor, prevented its trafficking to the synapse (116). Moreover, the lack of PICK1 interaction with the cell membrane led to the mistargeting of AMPA receptors in neurons.

All the information collected about the interaction between ion channels and adaptor proteins and the cholesterol binding ability of PDZ-domains of adaptor proteins may open a new approach to the understanding of the protein-cholesterol/lipid interaction. To our best knowledge there is no study, which describes the consequence of adaptor protein-ion channel interactions on

cholesterol sensitivity. We suppose these adaptor proteins may participate in anchoring the ion channels to rafts or other cell surface structures, which influence their biophysical parameters.

7 Biological relevance of the cholesterol sensitivity of Kv1.3 in T cells

As we discussed above, *in vitro* manipulation of membrane cholesterol altered the operation of various ion channels including the Kv1.3 channels of T cells (67). These results raised the question whether the in vitro studies have any physiological relevance? Theoretically, human diseases (type 2 diabetes, inborn errors of cholesterol biosynthesis) or pathological conditions (diet induced plasma hypercholesterolemia), which alter the cholesterol content of the cells may result in altered ion channel function and a consequent dysfunction of the ion channel regulated physiological processes. One of the widely accepted methods to study cholesterol related abnormalities is to induce plasma hypercholesterolemia by feeding rodents or primates on high-cholesterol diet and then isolate the cells of interest, whose sterol profile changed due to the diet. The advantage of this approach is that it mimics the high-cholesterol food intake, which is a frequent cause of the human dyslipidemias. Several animal models were investigated by electrophysiological methods using diet induced hypercholesterolemia (reviewed by Bukiya and Rosenhouse-Dantsker (119)). However useful tools animal models are to study human related medical conditions/problems, they cannot substitute the examination of primary cells/tissues. In

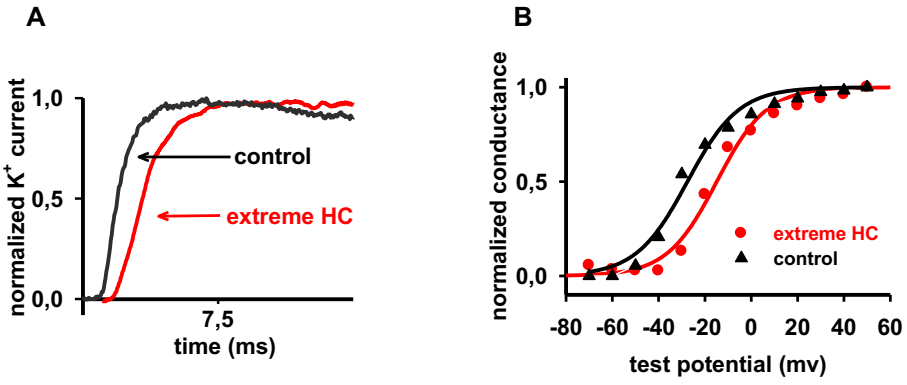


Fig. 4 Effects of the extreme HC on the operation Kv1.3 Fig **A** shows representative activation curves of a control and an extreme HC cell after 15ms long depolarization impulse. As the XA figure demonstrate, extreme HC decelerates the activation kinetics of Kv1.3. Voltage-dependence of equilibrium activation of the Kv1.3 channels in an extreme HC (circle) and a healthy (triangles) T cell along with the best-fit Boltzmann functions is illustrated (**B**). The midpoint of the steady-state activation shifted towards to the positive membrane potentials in extreme HC.

contrast to animal models, only limited information is available about the relationship between K^+ channels and cholesterol related diseases. To gain insight into the relevance of in vitro experiments and animal models we aimed to determine the function of Kv1.3 channels isolated from patients with hypercholesterolemia or Smith-Lemli-Opitz syndrome. It has been published, that in hypercholesterolemia the total cholesterol content of the lymphocytes increased (120). Our hypothesis was that the increased cholesterol content of the HC cells alters the Kv1.3 function similarly to in vitro cholesterol loading (121). Hypercholesterolemic lymphocytes were isolated from two patient groups: the total cholesterol level of group I. patients was between 5.2 and 10 mmol/l (n = 11), patients with total cholesterol more than 10 mmol/l were categorized into group II. (n = 9). Using patch-clamp technique we studied the expression level, the kinetic and equilibrium parameters of the gating of Kv1.3. Our experiments with primary HC cells had a different outcome compared to in vitro loading. The activation kinetics of the current at 0 mV was significantly faster for group I and II, while at +50 mV only for group II than in control cells. We suppose that the slightly accelerated activation kinetics have no significant effect on the ion channel regulated physiological processes. We found that neither the whole-cell peak current of Kv1.3 channels nor the current density changed in hypercholesterolemia. The midpoint and slope of the voltage-dependence of steady-state activation were the same for all groups (1.5 mg/ml in vitro CHOL loading significantly decreased the peak current and current density and shifted the midpoint of the voltage-dependence of steady-state activation to positive potentials(67)). We hypothesized that the discrepancy between the effect of HC and cholesterol loading on Kv1.3 operation could be the consequence of the different membrane cholesterol levels of the studied samples. Therefore we compared the cholesterol content of the control, HC and cholesterol loaded cells. We found that the cholesterol content of group II HC cells was approximately 1.5-fold higher than in the normocholesterolemic cells but it was far below that of the 1.5 mg/ml MbCD/C treated T cells. It seems that the serum cholesterol level has a moderate effect on the plasma membrane cholesterol level. Therefore the in vitro cholesterol loading at the applied concentration (1.5 mg/ml) mimics an extreme situation and has no real biological relevance. During the study we had the opportunity to investigate a patient with extremely high serum cholesterol levels. Analysis of these extreme hypercholesterolemic cells confirmed our conclusion. Similarly to the in vitro cholesterol loading, extreme HC cells had decelerated activation kinetics while the $V_{1/2}$ of steady state activation shifted to the right (Fig X).

Smith-Lemli-Opitz syndrome is a rare inherited disease due to the decreased or abolished activity of the 7-dehydrocholesterol reductase enzyme which catalyzes the last step of the endogenous cholesterol biosynthesis (122, 123). The leading clinical symptoms are neurological and anatomical abnormalities but almost every organ and tissue can be involved (122, 124). As a result of the enzyme block, the precursor 7-dehydrocholesterol and its derivatives accumulate in the cell membrane (2). The increased 7DHC/CHOL ratio may alter the physical properties of the cell membrane and/or modulate the specific sterol-protein interactions. Investigation of the ion channels of SLOS cells can be a useful tool to better understand the effects of sterols on the operation of ion channels. Even so, only limited information is available about the ion channels and transporters in SLOS (125-128). To demonstrate the significance of optimal sterol composition for proper ion channel operation we studied the Kv1.3 channels of lymphocytes isolated from 8 Hungarian SLOS patients. Our results showed that SLOS significantly decelerates the activation kinetics and shifts the midpoint of voltage-dependence of steady-state activation to the right as compared to the control. Tulenko's lab published similar results using skin fibroblasts from SLOS patients. They found a +50 mV shift in the voltage-activation of BK channels using single-channel, inside-out patch-clamp recordings (126). We suppose that the increased 7DHC concentration of the cell membrane is the primary cause for the altered channel function. Our model system where MβCD/7DHC complex was used to increase artificially the 7DHC levels clearly prove this theory, namely in vitro 7DHC loading of healthy lymphocytes adequately mimics the effect of SLOS on Kv1.3 operation (128). Inherited mutations of ion channels often affect the kinetic and equilibrium parameters of ion channel gating causing different channelopathies (129-131). A simple right-shift in the voltage-dependence of steady-state activation reduces the number of functionally available ion channels at a given membrane potential and can have pathophysiological consequences similar to the loss-of-function mutations. In line with this, we found reduced activation and proliferation capacity in SLOS T cells which can be the consequence of the diminished function of Kv1.3. The proliferation of SLOS skin fibroblasts also decreased, which may correlate with decreased activity of BK channels and the Na/K ATPase (125, 126). Because the exact mechanism of the altered cellular functions is not clear we cannot exclude that the nonspecific, toxic effects of 7DHC and its derivatives are responsible for it (132-134). Taken together, results from our lab and from others indicate that cholesterol-related diseases can have pathological consequences via modifying ion

channel operation. Nevertheless, it is important to interpret the results of in vitro studies with caution to avoid overrating their biological relevance.

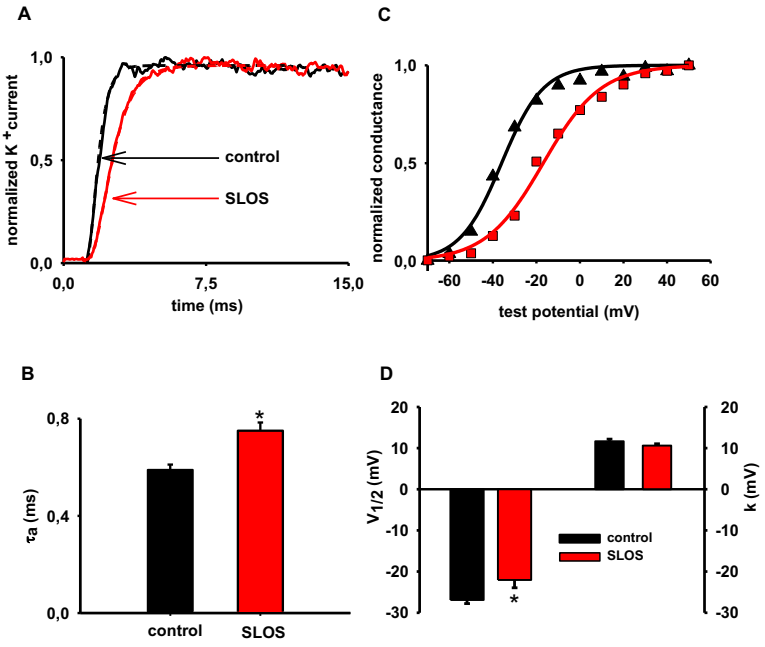


Fig. 5 The effect of SLOS on the gating of Kv1.3 channels. We applied a 15 ms long depolarization impulse to +50mV from the holding potential -120mV to study the kinetic properties of activation of Kv1.3. Current traces evoked in an SLOS and a control T cells were fitted using Hodgkin-Huxley n^{4-} model, and the activation time constant (t_a) was used to describe the activation kinetics (**A**). Our results clearly demonstrate that the SLOS significantly increase the activation time constant of Kv1.3 channels (**B**). Membrane potential dependence of steady-state activation of the K^+ current was determined as follows: cells were held at −120 mV holding potential and depolarized to the test potentials from -70 mV to +50 mV in steps of 10 mV. Peak whole-cell conductance (G(V)) at each test potential was calculated from the peak current (I_{peak}) at test potential V and the K^+ reversal potential (E_K) using $G(V)=I_{peak}/(V_{test}-E_K)$. The G(V) values were normalized for the maximum conductance (G_{norm}) and plotted as a function of test potential along with the best fit Boltzmann function (**C**). Our results shows that there is no difference between the parameters of steady-state activation for the Kv1.3 channels in control and SLOS T cells (slope (k) and midpoint ($V_{1/2}$) (**D**).

8 Conclusions

The regulation of voltage-gated K^+ channels by cholesterol and the cholesterol-dependent membrane microdomains seems to be evident from the review of the literature. The very complex structure of the voltage-gated K^+ channels allows the gating of the channels to be regulated by a variety of mechanisms, among these, the possibility of sterol regulation of the channels was the discussed in this paper. We believe, that the regulation of Kv channels by binding of cholesterol or cholesterol derivatives to the protein will be directly demonstrated in the near future, and this may allow specific targeting of the ion channels for efficient modulation of their physiological functions. The altered biophysical functions of Kv1.3 of T cells in the SLO disease might also predict similar effects of 7DHC on the ion channels of the central nervous system. This may contribute to the very severe neurological symptoms of these patients, but further experiments are needed to prove this hypothesis.

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