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

Examining human voltage-gated cation channels in physiological and pathophysiological conditions

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1. PREFACE

Ion channels are transmembrane proteins which can be categorized by selectivity (i.e. what kind of charged particles they transport) and gating (i.e. what kind of stimuli activates them). Voltage-gated cation channels have been in the focus of ion channels research for decades as they have a lot of vital functions. In excitable cells such as neurons and muscle cells they propagate action potentials (AP), but they contribute to the osmo- and pH regulation, maintenance of intracellular Ca^{2+} levels, cell migration and proliferation in non-excitable cells too. Thus, it is evident, that malfunctioning of these channels could cause pathological conditions, or indirectly influence the prognosis of certain diseases. These include ion channel diseases (i.e. 'channelopathies'), such as epilepsy, Brugada, long-QT and Lambert-Eaton syndromes; and pathological conditions associated with abnormal ion channel function, such as cancer, immune and autoimmune diseases. It follows that these ion channels may be pharmacological targets for the treatment of a number of diseases. In order to minimize the side effects of a given treatment, selective inhibitors must be found so that the therapy is cell- or tissue-specific. To this end, it is essential to understand the ion channel profile of human cells, in particular the presence of auxiliary subunits.

My thesis covers two topics, mapping the expression patterns of different cation channels in physiological and pathological conditions. The physiological condition was represented by B cells obtained from healthy human peripheral blood. Our collaborative project with the Department of Transplantation Medicine (University of Debrecen) investigated the changes in the ion channel profile of B cells during antibody-mediated renal graft rejection. During the study, we noticed the presence of inward ion currents characteristic of voltage-gated sodium (Na_V) channels in some of the B cells obtained from control (healthy) samples, and performed biophysical and expression studies of these ion channels. To describe the pathological condition, a malignant tumour, glioblastoma, was chosen. The activity of $K_{Ca}1.1$ ion channels contributes to the invasiveness and radioresistance of these cells. Since the pore-forming (α) subunit is expressed in many cells, we investigated the functional expression of the auxiliary (β) subunits, which is a good starting point for tissue-specific inhibition of ion channel expression.

2. INTRODUCTION

2.1 Ion channels of the human B cells

B cells, or B lymphocytes, are part of the adaptive immune system and play an important role in the humoral immune response by producing antibodies. They also influence T cell differentiation and antigen presentation by secreting cytokines. The function of ion channels expressed in the cell membrane contributes greatly to this complex behaviour. Most studies on ion channels in human lymphocytes have either focused on T cells or used lymphocyte stocks from peripheral blood, in which the proportion of B cells is low. Thus, the ion channel profile of human B lymphocytes is less defined, paving the way for many new studies.

Human lymphocytes express several ion channels in their cell membrane. These transmembrane proteins are responsible for cell development, cell proliferation, signal transduction and cell activation. Among them are voltage-gated, Ca^{2+}-activated and background potassium channels; voltage-gated and store-operated calcium channels; voltage-gated proton channel and transient receptor potential (TRP) channels.

The best characterized of these ion channels is $K_V1.3$, a member of the voltage-gated potassium channel family, which is found in both B and T cells. The channel is activated by membrane depolarization and its main function is to regulate membrane potential. $K_{Ca}3.1$ is a calcium-activated potassium channel that, upon B cell receptor (BCR) activation, allows calcium influx, essential for signal transduction and cell activation, by regulating the resting membrane potential. There have been several publications on the presence of L-type calcium channels (Ca_V1) in the T and B cell membranes, whose activation is associated with T and B cell receptor (TCR and BCR) stimulation, but there is currently no accepted electrophysiological evidence for their expression. Another calcium channel, the store-operated ORAI1, is also activated upon TCR/BCR stimulation and has a role in cell proliferation and cytokine production. Sodium permeable ion channels in the membrane of human lymphocytes are not well documented in the literature, although a few studies suggest that sodium influx may play a role in the cellular response of lymphocytes by reducing the chance of elevated intracellular calcium concentrations, thus preventing cell death. Among these channels, amiloride-sensitive sodium channels, TRP channels and purinergic receptors have been described.

In recent decades, there have been many publications on lymphocyte ion channels and there is evidence for the presence of voltage-gated sodium channels in the lymphocyte

membrane, but no study has yet investigated and characterized in detail Na_V1 ion channels in human B cells using both electrophysiological and molecular biological methods.

2.2 The voltage-gated sodium channel family (structure and function)

The best-known function of voltage-gated sodium channels (Na_V1) is to generate an action potential through depolarization of the membrane of excitable cells. The Na_V1 ion channel family consists of 9 members ($Na_V1.1$ to 1.9), of which $Na_V1.1$, 1.2, 1.3 and 1.6 are mainly found in the central nervous system, while $Na_V1.7$ to 1.9 are expressed in the peripheral nervous system. Besides neurons, Na_V1 channels are also expressed in muscle cells, another group of excitable cells. $Na_V1.4$ is dominant in skeletal muscle, whereas $Na_V1.5$ is dominant in cardiac muscle. In addition to their main function of inducing AP, voltage-gated sodium channels are also found in the membrane of non-excitable cells such as astrocytes, pancreatic beta cells, keratinocytes, dendritic cells, macrophages, red blood cells, T cells and neutrophil granulocytes. In addition to the maintenance of resting membrane potential in these cells, they have a role in cell migration and differentiation.

They are similar in structure, in that a polypeptide chain of four domains of approximately 230 kDa in size forms the alpha, pore-forming subunit of these ion channels. Each of the four domains (DI-DIV) consists of six transmembrane helices and the intra- and extracellular loops that connect them. The transmembrane helices are labelled S1-S6. The S1-S4 segments make up the voltage sensor (VSD), of which the S4 helix has positively charged amino acids (arginine or lysine every third position), which therefore shifts towards the extracellular space upon membrane depolarization. The S4 helix of the voltage sensor is connected (S4-S5 loop) to the pore (PD), which is formed by the S5-S6 helices and the extracellular loop (P-loop) connecting them.

Na_V1 channels can be distinguished on the basis of biophysical parameters and pharmacology. Biophysical parameters include the kinetics of activation and inactivation, their voltage dependence and the kinetics of recovery from inactivation.

One of the most commonly used Na_V1 inhibitors is tetrodotoxin (TTX), a neurotoxin isolated from pufferfish, which binds reversibly to the pore of voltage-gated sodium channels from the extracellular side. Based on TTX sensitivity, a distinction can be made between TTX-sensitive ($Na_V1.1$-1.4, 1.6 and 1.7; IC_{50} in nanomolar range) and -resistant channels ($Na_V1.5$, 1.8 and 1.9; IC_{50} in micromolar range). Selective Na_V1 inhibitors include GIIIA and GIIIC μ-conotoxins isolated from cone snails, which are selective inhibitors of skeletal muscle-specific

$Na_V1.4$. In addition, a peptide inhibitor, protoxin-II, isolated from tarantula, is often used to selectively inhibit $Na_V1.7$.

2.3 Glioblastoma and the U-87 cell line

Gliomas are tumours that originate from the glial cells of the brain. There are several known groups of gliomas. Multiforme glioblastoma (GBM) is the most malignant with a significant loss of quality of life. Its prognosis is very poor, with an average survival time of less than 15 months despite the best treatments. The treatment consists of classical chemotherapeutic agents (temozolomide, cisplatin, carmustine) combined with radiotherapy and surgery. The primary aim and result of these treatments are still just to reduce the tumor volume (debulking). There is some initial research into a definitive combination therapy to eradicate the tumor, but the results are still limited. Thus, there is a need for new therapeutic agents that effectively address the clinical challenges of treating GBM.

The most commonly used cell line in glioblastoma research is U-87 MG, which stands for Uppsala-87 Malignant Glioma. The tissue sample used as the basis for the cell line was taken from a 44-year-old male patient in 1966. U-87 MG cells are characterised by an epithelial morphology and a hypodiploid genotype close to diploid. Over a period of more than 50 years, genetic mutations in the cell line are likely to have occurred, but there is no literature data on whether there is a difference in invasiveness between U-87 MG cells and glioblastoma.

A novel therapeutic target may be the $K_{Ca}1.1$ (also known as BK, Slo1 or MaxiK) ion channel, which contributes to GBM invasiveness and is expressed dominantly in the membrane of these tumor cells with a distinct splice variant (gBK i.e. glioma BK).

2.4 Role of the KCa1.1 ion channel in the invasiveness of GBM

The invasiveness of GBM has been associated with increased activity of several ion channels. Among these are $Na_V1.7$, ClC-3 (an H^+/Cl^- antiporter), a member of the voltage-gated chloride channel family, and $K_{Ca}1.1$. The latter two share a common theory of the mechanism of invasiveness, based on the fact that cells must shrink to infiltrate brain tissue. According to the cellular osmoregulatory model, water loss is associated with ionic currents, and hence outward currents of potassium and chloride. The former is mostly provided by $K_{Ca}1.1$, while the latter is provided by ClC-3. It is hypothesized that the potassium current of $K_{Ca}1.1$ is accompanied by an increase in intracellular calcium concentration through the activation of calcium store-operated channels (ORAI), so that calcium/calmodulin-dependent protein kinase II (CAMKII) becomes active, causing cytoskeletal changes that activate the ClC-

3 transporter through stretch-gating. In addition, CAMKII directly phosphorylates ClC-3, thus also directly activating the chloride current.

K_{Ca}1.1, likely via the aforementioned mechanism, is also involved in radiation and chemotherapy resistance in GBM.

2.5 Structure and function of K_{Ca}1.1

The K_{Ca}1.1 ion channel is also known as BK, MaxiK and Slo1, in addition to its name in the combined nomenclature. The first two refer to the high K^+ conductivity, which is reported in the literature to be in the range of 200-300 pS. The latter indicates similarity to Drosophila slowpoke.

K_{Ca}1.1, unlike other K_{Ca} ion channels, is voltage-gated in addition to being calcium-dependent; and stretch-gating of the channel has also been described. As most voltage-gated ion channels, K_{Ca}1.1 is tetrameric, i.e. four alpha subunits form the pore of the channel. These alpha monomers are products of the KCNMA1 gene. Similarly, to the Na_V ion channels mentioned above, an alpha subunit consists of a VSD and a PD. However, K_{Ca}1.1 shows differences compared to the structure of classical voltage-gated ion channels. These include the S0 helix in the VSD, which, in addition to voltage sensing, is required for calcium sensitivity and for the modulation by beta subunits. On the C terminal side are the cytosolic RCK1 and RCK2 domains, which have a calcium ion binding site. In addition, RCK1 contains a magnesium ion binding site.

This ion channel is expressed in a variety of mammalian cells/tissues with different biophysical and pharmacological properties. Modulating auxiliary subunits underlie this diverse behaviour. Two types of regulatory subunits are currently known: beta (β) and gamma (γ) subunits. Four subtypes of the former can be distinguished (β1-β4), which are products of the KCNMB1-4 genes and can associate with α subunits in a 1:1 ratio. They are similar in structure, being small proteins (between 20-30 kDa in size) consisting of two transmembrane helices (TM1 and TM2) and a large extracellular loop connecting them. The beta subunits form a complex with the pore-forming alpha subunits.

The beta subunits affect the biophysical parameters of the K_{Ca}1.1 channel as follows: β1; expressed in smooth muscle and kidney, among others; increases the calcium sensitivity of the channel, so that with 10 μM intracellular free Ca^{2+}, the activation V_{50} value is shifted to a more negative range. β2; expressed in the pancreas, spleen, kidney and ovary, like β1, shifts the voltage dependence of channel activation into the negative range. In addition, β2 has an inactivation ball at the N-terminal site, which is responsible for the rapid (20-30 ms time

constant) and complete inactivation of the channel, which also occurs at low $[Ca^{2+}]_i$. β3 has four splice variants (β3a-d), which are mostly expressed in the spleen, pancreas and testis. The β3 subunits do not affect channel activation, however, when a β3a-c subunit is associated with $K_{Ca}1.1$, the ion channel undergoes a very rapid (~1 ms time constant) but incomplete inactivation at more positive test potentials (above +100 mV) when intracellular free Ca^{2+} levels are high (at least 10 μM). Finally, when β4, which is dominantly expressed in the brain, associates with the alpha subunit, the voltage sensitivity of the channel is reduced and the activation V_{50} value is shifted to more positive membrane potential values.

The gamma subunits (γ1-γ4) are ~35 kDa transmembrane proteins encoded by the LRRC26, -52, -55 and -38 genes. They have a similar structure, as they consist of a transmembrane domain (TM), an extracellular leucine-rich domain (LRRD) at the N terminal and a short intracellular C-terminal tail. Expression of LRRC26 (γ1) in a heterologous expression system using HEK-293 cells shifted the channel conductance-voltage dependence (G-V curve) by 120-140 mV into the negative (hyperpolarizing) voltage range. Even in the absence of free intracellular Ca^{2+}, the expression of gamma promotes $K_{Ca}1.1$ activation at membrane potentials below zero. Thus, the association of γ subunits with $K_{Ca}1.1$ greatly alters the voltage sensing of the channel, creating a completely new ion channel phenotype.

2.6 Modulators of $K_{Ca}1.1$

$K_{Ca}1.1$ has a number of modulators. These include agents that act only via the alpha subunits and those that affect the channel via the auxiliary β subunits. The former includes a common K_V inhibitor, tetraethylammonium (TEA), and paxilline (Pax), which inhibit the ion channel at millimolar and nanomolar concentrations, respectively. The latter group includes a number of pharmacons, including scorpion toxins (iberiotoxin, charybdotoxin, slotoxin, etc.), fatty acids (arachidonic acid, ocosahexaenoic acid, eicosapentaenoic acid, etc.), sterane-based compounds (17β-oestradiol, tamoxifen, lithocholic acid, etc.) and ethanol. In the following, I will describe in detail the mechanism of action of the compounds that I used in my experiments.

Lithocholic acid (LCA): a member of bile acids and its main function is the solubilization of fats. It also activates the bile acid-sensitive ion channels (BASIC) and $K_{Ca}1.1$ in a subunit-dependent manner, such that when β1 associates with the ion channel, the peak current increases.

Arachidonic acid (AA): polyunsaturated fatty acid of the ω6 family, which has an important signaling role in inflammatory processes as a secondary messenger. In addition, it acts on several ion channels, such as inhibiting Na_V channels, activating H_V1 and TRP channels.

The effect on $K_{Ca}1.1$ is β subunit-dependent: the application of AA slows down the inactivation of the channel by β2 or β3 subunits and increases the peak current in the case of β1/β2/β3 subunit expression.

Iberiotoxin (IbTx): a 37-amino acid peptide isolated from the venom of the Indian red scorpion, which selectively and reversibly inhibits the $K_{Ca}1.1$ channel at nM concentrations by binding to the extracellular side of the ion channel pore. Inhibition of IbTx also has a β subunit dependence, in that, even at micromolar concentrations, the α/β4 channel complex is not inhibited, and when β1 is associated with the α subunit, the dissociation constant (Kd) is shifted from the ~nM range to the 0.1 μM-1μM range.

Paxilline (Pax): a toxic indole alkaloid produced by the fungus Penicillium paxilli, which selectively and reversibly inhibits $K_{Ca}1.1$ at nanomolar concentrations (Kd~10-50 nM, depending on $[Ca^{2+}]_{ic}$). Pax binds with higher affinity to the closed state of the channel, so that channel block is inversely proportional to P_O (Kd=10 nM if $P_O \approx 0$; Kd=10 μM if $P_O \approx 1$).

Dithiothreitol (DTT): A reducing agent used in biochemical reactions to break disulfide bonds. When β2 or β3 are expressed, it cleaves disulfide bridges formed between extracellular loops responsible for the outward rectification of the tail current, thereby abolishing rectification. Thus, when applied extracellularly at a concentration of mM, DTT causes prominent inward tail currents at negative membrane potentials.

3. AIMS OF THE STUDY

In my PhD thesis, I investigated the expression of voltage-gated cation channels in physiological and pathological conditions. I used two models:

1) B lymphocytes isolated from human peripheral blood were used for the physiological condition. Here, we aimed to investigate the Na_V ion channel expression of B cells, using molecular biology and detailed biophysical characterization using the patch-clamp technique.

2) The pathological condition was represented by glioblastoma cells. Based on the REMBRANDT ("REpository of Molecular BRAin Neoplasia DaTa") database, the KCNMA1 gene is overexpressed in only ~10% of GBM patients, and overexpression of the channel does not correlate with patient survival. On the contrary, expression of the KCNMB3 gene at the mRNA level is increased in gliomas with higher grade and worse prognosis. However, there is no data on which β subunits are functionally expressed and form a complex with the $K_{Ca}1.1$ ion channel in the GBM membrane. Thus, our short-term goal was to determine the phenotype of the $K_{Ca}1.1$ ion channel contributing to invasiveness and radioresistance using primary GBM cells and a glioblastoma cell line.

4. MATERIALS AND METHODS

4.1 Materials

All consumables were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise noted.

4.2 Cell Isolation

4.2.1 Primary GBM cells

Experimentation with GBM tissue samples from patients was approved by the Medical Research Council (ETT-TUKEB,IV/186-1/2022/ECU). The diagnosis was made by a neuropathologist according to WHO criteria. Written informed consent was obtained from all patients. After surgical removal, tumour samples were placed in HBSS (Hank's Balanced Salt Solution) solution and stored on ice during transport. Tissue samples were digested with type I. collagenase for 30 min. They were then homogenized using a 15 ml Dounce homogenizer followed by Pasteur pipettes. This cell suspension was then passed through a 70 µM diameter cell strainer (Corning®, Corning, NY, USA) and plated in a T75 cell culture flask for 2 hours using DMEM medium containing 10% FBS (fetal bovine serum) (37 ˚C and 5% CO_2) and washed twice in 1x PBS (phosphate buffer) before resuspension in cell medium. (Cell purity was verified by labeling with glial fibrillary acidic protein and only cells that showed positivity above 90% were used for the experiment.)

4.2.2 Human peripheral B cells

Peripheral venous blood samples were taken in the morning before electrophysiological measurements each day, using healthy adult donors (no chronic disease or medication, N=8). Between 25-30 ml of blood per donor per day was drawn and collected in sodium heparin tubes. These donors were staff members of the Department of Biophysics and Cell Biology, Faculty of Medicine, University of Debrecen and the Laboratory Medicine.

First, whole blood was diluted twofold in HBSS and then mononuclear cells were separated by density using Histopaque-1077 separating solution and SepMate™ (STEMCELL™ Technologies, Vancouver, Canada) separating tubes. Then, B lymphocytes were isolated from mononuclear cells by magnetic bead separation (human Pan B cell Isolation Kit, Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's instructions. The cell number was determined using a Bürker chamber and the pellet was

resuspended in 40 µl/10^7 cells in degassed buffer (PBS, 0.5% albumin and 2 mM EDTA). Then biotin-conjugated antibodies (antibodies against CD2, CD3, CD4, CD14, CD15, CD16, CD34, CD56, CD61, CD235a and FceRIa; 10 µl/10^7 cells) were added and incubated for 5 min at 4°C. After the incubation period of 10^7 cells, 30 µl of buffer and 20 µl of anti-biotin magnetic beads were added to the cells. After 10 min incubation at 4 °C, B cells were separated using MACS columns (Miltenyi Biotec) (negative selection). B cells that were not used for patch-clamp measurements were plated in TRIzol™ reagent and stored at -80 °C for RT-qPCR.

4.3 Primary GBM and U-87 MG cell cultures

Primary and cell line GBM cells were cultured in T75 flasks in DMEM medium supplemented with 10% FBS, 1% glutamate, 1% penicillin-streptomycin and 1% non-essential amino acids. Primary GBM cells were used for 3 passages, while U-87 MG cells were used for 10 passages. Cells were maintained in a cell incubator at 37 °C and 5% CO_2. Cell passaging was performed in a sterile chamber as follows: GBM cells were removed using a Pasteur or 2ml serology pipette (VWR International, Radnor, PA, USA), then after washing with 1x PBS, 1x trypsin-EDTA solution (0.05% trypsin, 0.02% EDTA) was pipetted into the flask and incubated for 2 min at 37 °C. After the cells were separated, they were transferred to 15 ml centrifuge tubes and centrifuged at 1200 RPM for 5 minutes. After aspiration of the supernatant, the cells were resuspended in the medium and placed in a new cell culture flask.

4.4 Gene silencing with siRNA

U-87 MG cells were transfected in Gibco® Opti-MEM™ (Thermo Fisher Scientific, Waltham, MA, USA) medium containing 2 µl/ml DharmaFECT™ (Horizon Discovery, Lafayette, CO, USA) and 5 µg/ml negative control (AccuTarget™ Negative Control siRNA, Bioneer, Daejeon, South Korea) or siRNAs against KCNMA1, KCNMB1-3. Cells were incubated at 37 °C for 24 hours. The efficiency of silencing was verified by Western blot.

4.5 Electrophysiology

4.5.1 Patch-clamp setup

For electrophysiological measurements, we used a conventional patch-clamp technique in whole-cell configuration. The system is based on an Axopatch 200B (Molecular Devices, CA, USA) amplifier connected to a computer via a Digidata 1550B (Molecular Devices) digitizer, on which data acquisition was performed using the Clampex 10.7 (Molecular Devices) program. Cell selection and pipette positioning were performed using a Nikon Eclipse TS100 (Nikon, Tokyo, Japan) microscope placed on a vibration-free stage and surrounded by a Faraday cage. Cells were plated in a CELLSTAR® (Greiner Bio-One, Frickenhausen, Germany) Petri dish and after plated, the Petri dish was filled with 2 ml of control extracellular solution. Solution exchange was performed via a manual gravity perfusion system with a flow rate of ~0.2 ml/min. A vacuum aspiration system was used to maintain a constant fluid level. To eliminate voltage error due to junction potential during perfusion, the reference electrode was stored in a separate vessel connected to the measuring chamber by an agar bridge (3 M KCl). GC 150F-15 borosilicate glass capillaries (Harvard Apparatus, MA, USA) and P-87 Flaming/Brown micropipette puller (Sutter Instruments, CA, USA) were used to prepare the pipettes. The resistance of the pipettes ranged from 3-8 MΩ depending on the pipette solution.

4.5.2 Solutions

<u>The following solutions were used to measure B lymphocytes:</u>

Control (extracellular) solution: 145 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 2.5 mM $CaCl_2$, 5.5 mM glucose and 10 mM HEPES; pH=7.35 (titrated with NaOH)

Pipette (internal) solution: 145 mM KF, 5 mM Na-aspartate, 11 mM EGTA, 10 mM HEPES, 2 mM $MgCl_2$ and 1 mM $CaCl_2$; pH=7.22 (titrated with KOH)

Na^+ free solution (positive control): 145 mM choline chloride, 5 mM KCl, 1 mM $MgCl_2$, 2.5 mM $CaCl_2$, 5.5 mM glucose and 10 mM HEPES; pH=7.35 (titrated with choline base)

Extracellular solution with high K^+ concentration (HK, for membrane potential measurements): 150 mM KCl, 10 mM HEPES, 5.5 mM glucose, 1 mM $CaCl_2$ and 1 mM $MgCl_2$; pH=7.35 (titrated with KOH)

TTX was diluted in the control solution at the appropriate concentrations (5, 15, 50, 150 nM).

Composition of the solutions used to measure glioblastoma cells:

Control (extracellular) solution: 145 mM Na-aspartate, 5 mM KCl, 1 mM $MgCl_2$, 2.5 mM $CaCl_2$, 5.5 mM glucose and 10 mM HEPES; pH=7.4 (titrated with NaOH)

[Ca^{2+}]-free pipette (internal) solution: 145 mM KF, 11 mM EGTA, 10 mM HEPES, 2 mM $MgCl_2$ and 1 mM $CaCl_2$; pH=7.22 (titrated with KOH)

1 µM free [Ca^{2+}] pipette (internal) solution: 145 mM K-aspartate, 10 mM EGTA, 10 mM HEPES, 2 mM $MgCl_2$, 8.5 mM $CaCl_2$; pH=7.2 (titrated with Tris)

High (~10 µM) free [Ca^{2+}] pipette (internal) solution: 145 mM K-aspartate, 10 mM EGTA, 10 mM HEPES, 2 mM $MgCl_2$, 9.8 mM $CaCl_2$; pH=7.2 (titrated with Tris)

K_{Ca}1.1 modulators were diluted in the control solution on each measurement day at the following concentrations: AA (30 µM), LCA (75 µM), IbTx (~20 nM), Pax (1 µM), DTT (20 mM).

The solvents in which the stock solutions were prepared (dimethyl sulfoxide or ethanol) were added at 1 V/V% to the control solution. Unless otherwise stated, measurements were made using a 1 µM free [Ca^{2+}] pipette (internal) solution.

4.5.3 Voltage and current protocols

B cell measurements:

A holding potential of -100 mV was generally used for voltage-clamp measurements, while 0 nA current was injected for membrane potential measurements (I=0). The sampling and noise filtering (four-pole low-pass Bessel filter built into the amplifier) were chosen according to the Nyquist rule of sampling at 20 kHz and setting the Bessel filter at 5 kHz. The measurements were performed at room temperature. Different voltage protocols were used to map the biophysical parameters, such as step protocol, I-V (voltage dependence of activation), SSI ("steady-state inactivation", voltage dependence of inactivation) and recovery from inactivation.

Glioblastoma measurements:

A holding potential of -100 mV was generally used. Sampling was performed at 20 and 50 kHz. Measurements were performed at room temperature. Step and I-V protocols were used to map biophysical parameters.

4.5.4 Evaluation of patch-clamp data

Patch-clamp data evaluation, statistical analysis and representation were performed using Microsoft Excel (Microsoft, WA, USA), Clampfit 10.7 (Molecular Devices, CA, USA) and Graphpad Prism7 (Graphpad, CA, USA) software. Raw data were first digitally filtered "offline" in Clampfit using a 5-point boxcar function.

To analyze the effect of ion channel modulators, the remaining current fraction (RCF) was calculated for each case using the equation: $= I_{modulator}/I_{control}$, where $I_{modulator}$ is the saturated ion current during modulator application and $I_{control}$ is the peak current before modulator application.

The I-V data were first used to construct G-V curves, for which the conductances were obtained from the equation $G = I/(V_{command} - V_{reverse})$, where G is the conductance, I is the peak current, $V_{command}$ is the command voltage and $V_{reverse}$ is the equilibrium (reverse) potential. The value of $V_{reverse}$ was determined by the Nernst equation $V_{reverse} = \frac{RT}{zF} \ln\left(\frac{[X]_{EC}}{[X]_{IC}}\right)$ based on the composition of the measurement solutions used; where R is the universal gas constant, T is the temperature in Kelvin, z is the ionic charge, F is the Faraday constant, and $[X]_{EC}$ and $[X]_{IC}$ are the extra- and intracellular concentrations of the respective ions). To plot the G-V curves, the conductance data obtained at all command voltages were normalized to the maximum conductance (G/G_{max} = probability of channel opening). To plot the SSI data, I/I_{max} (i.e., the activated channel ratio), was calculated, where I is the peak current at the given pulse and I_{max} is the peak current at the first pulse. The G-V and SSI curves were fitted with Boltzmann's equation $Y = 1/(1 + \exp((V_{50} - V_{command})/k))$; where k is a constant characterizing the slope of the curve) to determine the V_{50} values.

The time constant for the return from inactivation (τv) was obtained as follows: the second pulse (I) of the pulse pair was normalized to the first one (I_{max}) for each pulse pair (I/I_{max}=recovered channel fraction), and the resulting curve was fitted with a single-phase exponential equation ($Y = A\left(1 - \exp\left(-\frac{t}{\tau_v}\right)\right) + Y_0$; where A is the amplitude of the curve, t is the time between pulses, τv is the recovery time constant and Y_0 is I/I_{max} at t=0). The time constant (τi) characterizing the inactivation of the $K_{Ca}1.1$ channel is obtained by the equation; $Y = A\left(1 - \exp\left(-\frac{t}{\tau_v}\right)\right) + Y_0$where C is the saturation value.

4.6 Quantitative polymerase chain reaction (qPCR)

Whole-cell RNA, including messenger RNA, was isolated from cells using TRIzol™ reagent. The purity of the RNA samples was confirmed by UV spectroscopy (NanoDrop 2000 spectrophotometer), where only samples with A260/A280 ratios between 1.8 and 2.1 were accepted. RNA samples were then stored at -80 ˚C until qPCR measurements were performed.

4.6.1 GBM cells

Complementary DNA (cDNA) was prepared using Superscript III™ Reverse Transcriptase kit (Invitrogen, Waltham, MA, USA). PCR was performed using PowerUp™ SYBR™ Green Master Mix (Thermo Fisher Scientific, Waltham, MA, USA) and gene-specific primers. GAPDH was used as a positive control. The reaction was run on a QuantStudio 3 as follows: 40 cycles of denaturation (30 sec/cycle) at 95 ˚C, primer annealing at 55 ˚C for 45 seconds and extension at 72 ˚C for 60 seconds. QuantStudio Design and Analysis software was used to evaluate the data.

4.6.2 B cells

To quantify the expression of Na_V ion channels at the mRNA level, we first prepared cDNA from RNA samples using a kit containing a reverse transcriptase enzyme (High-Capacity cDNA Reverse Transcription Kit, Applied Biosystems, Vilnius, Lithuania). RT-qPCR was performed using LightCycler 480 SyBR Green I Master mix (Roche Diagnostics, Mannheim, Germany) and gene-specific primers (10 µM, Integrated DNA Technologies, Leuven, Belgium). The reaction was run on a LightCycler 96 RT-qPCR (Roche Diagnostics). The PCR cycles were as follows: 10 min incubation at 95 ˚C, followed by 40 cycles of 10 s at 95 ˚C, and finally 1 min at 60 ˚C. The acidic ribosomal phosphoprotein P0 (RPLP0) encoded by the 36B4 gene was used as a positive control. All measurements were performed N=2 times. The threshold cycle (Ct) was determined by the instrument and all values above 35 were considered noise.

4.7 Western blot (WB)

Protein was isolated from U-87 MG cells using Tris lysis buffer (25 mM mercaptoethanol, 1 µl/ml Tween® 20, 10 µl/ml protease inibitor and 50 mM Tris; pH=7.5) and cell sonicator. During isolation, the cell/samples were kept on ice and then the portion not used for WB was stored at -80 ˚C for 1 week. For WB, the protein sample to be used was first centrifuged at 13200 RPM and 4 ˚C for 20 minutes, then after aspiration of the supernatant, they were taken up in loading buffer (6x LB) and the sample was denatured at 98 ˚C for 10 minutes. 120-120 µg of denatured protein was loaded into 12% ProSieve 50 (Lonza, ME, USA) acrylamide gel and electrophoresed (80 mV for 20 min, then 120 mV for 90 min). The gel was then transferred to a PVDF (polyvinylidene fluoride) membrane (100 mV, 90 min) and blocked in 5% milk powder TBST (TBST = Tris-buffered saline + 0.1% Tween® 20) for 1 h. After blocking and washing three times with TBST, overnight incubation with primary antibodies at a dilution of 1:1000 at 4 ˚C was performed. The following day, again after a triplicate TBST wash, incubation with the secondary antibody was performed at room temperature for 2 h. Prior to detecting, the membrane was treated with SuperSignal® West Pico chemiluminescent substrate according to the manufacturer's protocol. Alpha Innotech FluorChem Q MultiImage III was used for chemiluminescence detection.

4.8 Cell synchronization and flow cytometry

U-87 MG cells were synchronized with 4 µg/ml (10 µM) colchicine in M phase metaphase and serum-free DMEM in G0/G1 phase. Treatments were carried out for 24 hours at 37 ˚C and 5% CO_2. The success of the synchronization was verified by propidium iodide (PI) staining, for which treated and untreated cells were first permeabilized with 80% ethanol for 20 min at room temperature, followed by the addition of 2 µg/ml PI to the cells and incubation for 10 min, also at room temperature, until flow cytometry measurements. Data were extracted with a BD FacsAria III (BD Biosciences, NJ, USA) flow cytometer using a 561 nm excitation and 610/20 nm emission filter. Flowjo V10 (BD Biosciences) was used to evaluate the data.

4.9 Statistical analysis

In general, data are expressed as mean ± SEM. Statistical analysis was performed using Graphad Prism 7. Statistical evaluation was conducted by first performing the normality test (D'Agostino-Pearson), then using a two-sample two-tailed t-test or one-way analysis of variance with Tukey's post-hoc test for samples with a normal distribution, and Mann-Whitney or Kruskal-Wallis tests for samples with a non-normal distribution. The effect of glioblastoma modulators was analysed using Wilcoxon test. The significance level (α) was 5%.

5. RESULTS AND DISCUSSION

5.1 Functional Na_V channels are present in the human B cell membrane

5.1.1 Electrophysiological and molecular biology data indicate the expression of *Na_V in human B cells*

Although several studies have suggested the presence of voltage-gated sodium channels in the membrane of human B cells, direct electrophysiological data have not yet demonstrated this. In this study, we have used electrophysiological and molecular biological methods to identify the functional expression of Na_V ion channels in the membrane of human B cells. Approximately 40% of the cells measured with patch-clamp showed an inward current that disappeared in Na^+-free solution and a voltage dependence characteristic of Na_V channels. In contrast, TTX-sensitive sodium currents were found in only 3% of T cells (although protocols to directly detect Na_V channels were not used in the referenced study, so the actual rate may be higher).

Based on the biophysical parameters studied, we cannot clearly identify the Na_V channels present in the cell membrane. The gating of voltage-gated ion channels, including Na_V channels, depends on the expression system, as these parameters are influenced by the lipid composition of the cell membrane and the expression of auxiliary subunits. Thus, the values of most of the biophysical parameters we have investigated show a large overlap between different Na_V ion channels. For example, our data showed an activation V_{50} value of -20.8 ± 1.9 mV, which falls within the published range for most Na_V channels. Only $Na_V1.9$ has been reported to have a more negative activation V_{50} value, which was approximately -40 mV. The values of the time constant (τ_V) for the recovery from inactivation also showed a large variation (7.5-42 ms), suggesting the co-expression of several Na_V channels in the membrane. Among the biophysical parameters, the latter value may better distinguish Na_V channels, since channels with fast recovery (e.g. $Na_V1.3$ and 1.4; τ_V=8-20 ms) and slow recovery ($Na_V1.7$; $\tau_V \approx 150$ ms) are known.

With RT-qPCR we detected the expression of genes encoding TTX-sensitive sodium channels (SCN2A, SCN3A, SCN4A, SCN8A and SCN9A) and TTX-resistant channel (SCN11A). Among these, TTX-sensitive $Na_V1.3$, 1.4 and 1.7 ion channels showed dominant expression, while TTX-resistant $Na_V1.9$ channel was present at lower mRNA levels. The co-expression of TTX-sensitive and resistant Na_V channels in the membrane may explain our pharmacological results, that TTX at a concentration of 150 nM did not cause as much inhibition as would have been expected, if only TTX-sensitive voltage-gated sodium channels

had been present in the B cell membrane. The Hill equation, based on the RCF with 150 nM TTX, gave an IC_{50} value of 84 nM, which is significantly higher than the published IC_{50} values for TTX-sensitive Na_V channels. This value hence supports the lower membrane expression of TTX-resistant Na_V channels. The expression of ion channels is known to change during cell differentiation. For example, the expression pattern of K^+ channels changes in T and B cells, and an increase in the expression of K_V channels have been observed in dendritic cells during maturation, concomitant with a decrease in Na_V channel expression. In a subsequent publication, it was shown that the high expression of Na_V is restricted to a small subpopulation of dendritic cells (CD1a+), further demonstrating the close relationship between ion channel expression and cell differentiation. In addition to cell differentiation, cell injury can also induce expression pattern changes. During mechanical stimulation-induced reactive gliosis in astrocytes, TTX-sensitive Na_V channels were replaced by TTX-resistant channels. As we did not separate B cells to subpopulations in our study, the cell population was highly heterogeneous, which may explain the high variability in TTX sensitivity. This heterogeneity may account for the fact that we only saw Na_V currents in a subset of cells. The sodium current detectable by patch-clamp may be present only in a subpopulation of B cells. The elucidation of the subpopulation(s) functionally expressing voltage-gated sodium channels is an important task for the future to understand the function of Na_V channels in B cells.

5.1.2 Na_V channels influence the resting membrane potential

We measured the membrane potential of B cells in the I=0 current-clamp mode, examining the potential canonical function of Na_V channels, and found that perfusion of sodium-free solution caused reversible hyperpolarization in B cells, which had a more positive resting membrane potential. Based on literature data, the resting membrane potential of lymphocytes falls within a wide range: negative (E_m=(-)70 - (-)60 mV), transient ($E_m \approx$ -40 mV) and more positive (E_m =(-)12 - (-)7 mV) membrane potential values have been reported in various publications. This variability may be due to the different measurement methods used. In studies of an undifferentiated peripheral lymphocyte population, more positive resting membrane potential values are less common due to the low proportion of B cells. Although the SSI curves at our resting membrane potential (Em= -6.8 ± 1.5 mV) show that most Na_V channels are inactivated (at 0 mV Na_V channel availability was 0.05 ± 0.02), the high membrane impedance and low cytoplasmic volume may cause significant membrane potential changes due to activation of few ion channels. Theoretically, Na^+-free extracellular solution could also indirectly induce membrane potential changes through modification of

electrotransport mechanisms (e.g. Na^+/Ca^{2+} exchanger). This possibility has been investigated in dendritic cells, where silencing $Na_V1.7$ caused the resting membrane potential to hyperpolarize and become insensitive to extracellular Na^+ concentrations, which suggests a direct role for Na_V channels, and we therefore suggest that our claim is likely.

The physiological role of voltage-gated Na^+ channels in lymphocytes is not yet fully understood. Previous studies have already suggested a potential canonical function of these channels. In Jurkat T cells, the presence of Na_V channels may be associated with cell migration, as in Matrigel invasion assay, TTX application significantly reduced migration. Furthermore, $Na_V1.5$ plays a role in the positive selection of CD4+ T cell maturation, because inhibition of the function and expression of these ion channels significantly affected the selection processes. The Na_V isoforms that showed high expression in B cells by RT-qPCR in our experiments have not yet been subjected to functional studies in lymphocytes.

Immune cells show many similarities in their ion channel profiles during cell differentiation. For example, the $K_V1.3$ channel, which is expressed in macrophages, dendritic cells, T cells and B cells, has an increased expression during cell activation in most subpopulations. The function of K^+ channels, by hyperpolarizing the membrane and thus maintaining electrochemical gradient, is important for Ca^{2+} influx required for cell activation. It is conceivable that, as in CD1a+ dendritic cells, Na_V channel function in B cells prevents unnecessary cell activation through membrane depolarization.

Considering the low average Na_V peak current (-57.0 ± 12.6 pA), it is possible that these ion channels have non-canonical functions in B cells that are unrelated to ion flux and membrane potential regulation. Such functions have been described for neuronal $K_V2.1$ and 2.2 channels, which form a node between the cell membrane and the endoplasmic reticulum and play a role in membrane trafficking. Similarly, the non-canonical function of the $K_V1.3$ channel was described in vascular smooth muscle cells, in which the operation of the gating mechanism was sufficient for proliferation without the conductance of K^+ ions. Although non-canonical functions cannot be excluded on the basis of our study, the strong hyperpolarization during perfusion of Na^+ -free solution suggests that canonical function of Na_V channels is more likely in B cells.

5.2 K_{Ca}1.1/β3 complexes are functionally expressed in the GBM membrane

In this study, we showed that K_{Ca}1.1 is the dominant K^+ channel in the glioma cell membrane, whose expression is cell cycle dependent, but the expression of the associated β3 subunit is not. Although K_{Ca}1.1 is expressed in many tissues, the expression of the associated beta/gamma subunits have tissue dependence. β3 is less frequently expressed in healthy tissue and has only been described to be expressed in the testis, pancreas and spleen in physiological conditions. Thus, β3 expression may be relevant in the diagnosis and therapy of various diseases. For example, overexpression of the gene encoding the β3 protein (KCNMB3) is associated with a worse prognosis in patients with GBM, and elevated β3 expression has been reported in fibroblast-like synoviocytes (FLS) in rheumatoid arthritis. Furthermore, the functional expression of β3 subunits in the GBM membrane may even serve as a cell surface marker for high-grade gliomas in the future.

5.2.1 β3 is the dominant auxiliary subunit

The presence of K_{Ca}1.1 α/β3 complexes in the GBM cell membrane is supported by the following points:

- **(I) the KCNMB3 transcript was expressed in both GBM and U-87 MG cells, and in the latter, we saw a high copy number by PCR, similar to KCNMA1**
- **(II) macroscopic current increased upon 30 µM AA perfusion, and upon KCNMB3 silencing this current increase was significantly reduced**
- **(III) A rapid and incomplete inactivation occurred upon application of 10 µM free intracellular calcium at +180 mV**
- **(IV) Appearance of tail current at -100 mV upon treatment with 20 mM DTT**
- **(V) Ion current sensitivity to ~20 nM IbTx is consistent with the literature of the K_{Ca}1.1/β3 complex**

These five points suggest functional expression of K_{Ca}1.1/β3 complexes in the GBM cell membrane. Since our results may also be separately related to the expression of other β subunits, they are discussed below.

The β1 subunit, which has been described mainly in smooth muscle, makes the ion channels ready to be activated by bile acids. In our experiment, 75 µM LCA activated the ion channel in U-87 MG cells, but several data argue against its expression. First, we could not detect a KCNMB1 product by qPCR in either primary cells or U-87 MG cells. In addition, no

anti-β1 antibody was detected by WB, although we demonstrated the efficacy of the antibody with control CHO cells, and silencing of KCNMB1 with siRNA failed to significantly alter the LCA effect. Given that bile acids can also act through other ion channels (for example, bile acid-sensitive "BASIC" channels), it is more likely that lithocholic acid did not increase peak current through $K_{Ca}1.1$ in our study. Furthermore, the $K_{Ca}1.1/\beta1$ complex is quite resistant to IbTx (published IC50 values range from 65-370 nM, which contradicts our RCF values), further reducing the likelihood of β1 expression. Finally, β1-associated $K_{Ca}1.1$ channels have a tail current at negative membrane potential, which contradicts our data.

The β2 subunit is expressed at the mRNA level in glioblastoma cells based on qPCR results, which was confirmed by WB as well. KCNMB2 silencing showed a decreasing trend of AA current enhancing effect. Based on the above mentioned observations, β2 might be expressed in the GBM membrane, however, β2 expression leads to complete inactivation at low cytosolic Ca^{2+} concentrations and at 100 mV depolarization, which was not detected in our patch-clamp measurements. Complete inactivation was not seen even using 10 µM intracellular free calcium. The absence of this complete inactivation does not necessarily exclude the presence of β2 membrane, since four β subunits can associate with one α subunit, so the ratio of different β subunits may also affect the biophysical parameters we detect. Stoichiometry will be worth considering in the future, since higher β2 subunit expression (due to rapid and complete inactivation) would imply a reduced driving force for calcium influx, whereas more β3 subunit expression (due to incomplete inactivation) would be associated with prolonged calcium influx.

The β4 subunit is dominant in the CNS and its expression in glioblastoma was expected. In transfected cells, the β4 subunit renders the $K_{Ca}1.1$ channel resistant to IbTx, and our data questions the expression as IbTx inhibited the channel. Nevertheless, there are some publications that suggest that IbTx-sensitive cells can express this subunit, which may be due to membrane processing of β4 subunits in addition to the stoichiometry, but no factual data are available yet.

Expression of gamma subunits is unlikely in the GBM membrane, as KCa1.1/γ complexes open at very negative membrane potentials (as low as -150 mV) even at low intracellular Ca^{2+} concentrations, whereas $K_{Ca}1.1$ ion channels in GBM and U-87 MG started to open at more positive test potentials.

Taken together, our results strongly support a dominant expression of $K_{Ca}1.1/\beta3$ complexes in glioblastomas, in addition to the possibility of β2 subunits in lower numbers.

5.2.2 Cell-cycle analysis reveals that $K_{Ca}1.1$ expression is increased in the M phase

It is known that the expression of certain ion channels changes during the cell cycle. K^+ channels play a major role in cell cycle progression by regulating membrane potential, as changes in K^+ permeability (due to the negative equilibrium potential of K^+) hyperpolarize the membrane when it increases (i.e. channel excitability) and depolarize it when it decreases. In general, high K^+ channel activity has been described at the boundary of the G1/S phase, while low membrane activity of potassium channels is observed in the G2/M phase. In parallel, Na_V channel expression was elevated in the M phase. In a previous study, the cell cycle dependence of $K_{Ca}1.1$ ion channels in human mesenchymal stem cells was investigated and the data showed a decreased proportion of $K_{Ca}1.1$ positive cells in G2/M phase compared to the starvation synchronized population in G0 phase. This is contradicted by our data, as both peak current and current density were significantly higher in the colchicine-treated (M phase enriched) U-87 MG population compared to the unsynchronized population, and this increase in current was found to be Pax-sensitive $K_{Ca}1.1$ current. Similar to our data, another study also found an elevated expression of a high conductance K^+ channel in unfertilized mouse oocytes in the M phase.

Several possible explanations have been raised for the importance of potassium channels in cell cycle progression. Firstly, mitosis requires a transient decrease in cell volume (hence pressure), in which K^+ channels play a big role. It has been described that in medulloblastoma cells, the $K_V10.2$ channel plays a role in the reduction of cell volume required for cell division. The relationship between cell size and proliferation has also been examined in glioma cells, where inhibition of ion channels and changes in the osmolarity of the culture medium (and thus cell volume) also altered the proliferation rate, such that the cell size-distribution curve took on a bell shape. One explanation for the formation of the bell-shaped curve is the macromolecular crowding theory, which suggests that the conformation and activity of macromolecules required to regulate the cell cycle is dependent on their hydration state. According to the model, the activity of molecules follows a Boltzmann distribution, and since this activity can represent both an increase and a decrease in proliferation rate, a volume interval is necessarily formed where cell division is optimal.

In addition, K^+ channels play an important role in calcium signaling by providing the driving force. Thus, by specific inhibition of certain potassium channels, cell cycle blockade can be achieved, leading to reduced cell proliferation. These ion channels include $K_V1.3$, $K_V10.1$, $K_V11.1$ and $K_{Ca}3.1$. Furthermore, it was found that K^+ conductance is not always

required for cell cycle progression, assuming non-canonical functions of potassium channels. Such non-canonical functions were found in the Drosophila EAG channel, which is an ortholog of human $K_V10.1$. By mutating an amino acid in the pore of the channel, a non-conducting ion channel was created, the expression of which increased the proliferation rate in a manner similar to the wild-type EAG channel.

Among the mentioned roles, glioblastoma $K_{Ca}1.1$ is known to have an osmoregulatory role, which may explain the higher $K_{Ca}1.1$ current seen in M phase.

Based on our pharmacological results, in contrast to the alpha subunit, the expression of the beta subunit did not change as a function of cell cycle, as the current-increasing effect of 30 μM AA did not differ significantly between synchronized and unsynchronized populations. Since β3 is equally present in the M phase, tissue-specific inhibition of glioblastoma $K_{Ca}1.1$ channels in a beta subunit-dependent manner could be a potential therapeutic approach in the future.

6. SUMMARY

In my dissertation, I investigated the membrane expression of voltage-gated cation channels in physiological and pathological conditions. The physiological condition was represented by B cells from healthy subjects without chronic disease, while the pathological condition was represented by samples from GBM patients and the U-87 MG cell line.

We were the first to characterize the functional expression of voltage-gated sodium channels in B cells. The molecular biology and electrophysiology data suggest that TTX-sensitive and resistant Na_V channels are simultaneously present and functional in the B cell membrane. Based on our study, these channels perform their canonical function contributing to the regulation the resting membrane potential. Since little is still known about the role of sodium channels in immune cell function, the functional presence of Na_V channels may be worth considering in future pharmacological and functional studies of these cells. Further investigation of which subpopulations of B cells express Na_V channels may provide a basis for selective pharmacological modulation of immune functions.

In glioblastoma cells, we found that the $K_{Ca}1.1$ ion channel forms functional complexes with the β3 subunit in the cell membrane. The expression of β3 in GBM cells could be a future chemotherapeutic target, as in the central nervous system the β4 subunit dominates, so inhibition of the $K_{Ca}1.1$/β3 complexes would be tissue specific for glioblastoma. Also, this expression could be a marker for malignant glioblastoma cells in the future, which would not only aid in therapy but also in diagnostics.

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List of publications related to the dissertation

1. **Fehér, Á.**, Pócsi, M., Papp, F., Szántó, G. T., Csóti, Á., Fejes, Z., Nagy, B. J., Nemes, B. Á., Varga, Z.: Functional Voltage-Gated Sodium Channels Are Present in the Human B Cell Membrane.
   Cells. 11 (7), 1-15, 2022.
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2. **Fehér, Á.**, Pethő, Z., Szántó, G. T., Klekner, Á., Tajti, G., Batta, G., Hortobágyi, T., Varga, Z., Schwab, A., Panyi, G.: Mapping the functional expression of auxiliary subunits of KCa1.1 in glioblastoma.
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   IF: 4.6

List of other publications

3. Szántó, G. T., **Fehér, Á.**, Korpos, É., Gyöngyösi, A., Kállai, J., Mészáros, B., Óvári, K., Lányi, Á., Panyi, G., Varga, Z.: 5-Chloro-2-Guanidinobenzimidazole (ClGBI) Is a Non-Selective Inhibitor of the Human HV1 Channel.
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4. Kovács, Z. M., Óvári, J., Dienes, C., Magyar, J., Bányász, T., Nánási, P. P., Horváth, B., **Fehér, Á.**, Varga, Z., Szentandrássy, N.: ABT-333 (Dasabuvir) Increases Action Potential Duration and Provokes Early Afterdepolarizations in Canine Left Ventricular Cells via Inhibition of IKr.
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