Ph.D. THESIS

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ABBREVIATIONS

AA	arachidonic acid
4-AP	4-aminopyridine
BK_{Ca}	Ca^{2+}-activated K^+ channel
cAMP	cyclic adenosine-monophosphate
ChTx	Charybdotoxin
Con A	concanavalin A
CPA	N^6-cyclopentyladenosine
CGS 21680	2-[p-(2-carbonylethyl)phenylethylamino]-5'-N-ethylcarboxamidoadenosine
CPX	1,3-dipropyl-8-cyclopentylxanthine
CSC	8-(3-chlorstyryl)caffeine
DHA	docosahexaenoic acid
DRM	detergent-resistant membrane domain
FBS	foetal bovine serum
FA	fatty acid
F-NECA	5'-N-(2-[^{18}F]fluoroethyl)-carboxamidoadenosine
G_i	inhibitory G protein
G_s	stimulatory G protein
HPL	human peripheral lymphocyte
K_{ATP}	ATP-sensitive K^+ channel
K_{Ca}	Ca^{2+}-activated K^+ channel
K_V channel	voltage-gated potassium channel
LA	linoleic acid
Lck	Src-type phosphotyrosine kinase
MgTx	Margatoxin
MUFA	monounsaturated fatty acid
NECA	5'-N-ethyl-carboxamidoadenosine
NxTx	Noxiustoxin
OA	oleic acid
PA	palmitic acid
PET	positron emission tomography
PTx	pertussis toxin
PUFA	polyunsaturated fatty acid

P	purinergic
SA	stearic acid
SFA	saturated fatty acid
Src	tyrosine kinase identified from sarcoma virus
TEA^+	tetraethylammonium ion
TREK-1	2 pore K^+ channel
TRAAK	arachidonic acid-stimulated K^+ channel
TTx	tetrodotoxin
ZM 241385	4-(2-[7-amino-2-(2-furyl)[1,2,4]triazolo[2,3-a][1,3,5] triazin-5-ylamino]ethyl)phenol

1. INTRODUCTION

The potassium (K^+) channels of human peripheral lymphocytes (HPLs) play significant roles in the signalling processes required for immune responses and in maintaining the membrane in the cell hyperpolarized hereby starting mitogen stimulation. T-cells become active following antigen presentation. This presentation induces a cascade of biochemical processes leading proliferation of the T-cells. Several types of K^+ channels were identified in the membrane of T-lymphocytes, the most considerable of which proved to be the Ca^{2+} activated K^+ channels ($K_{Ca}3.1$ or $IK_{Ca}1$) and the voltage-dependent, depolarization-activated $K_V1.3$ channels. These channels control the intracellular Ca^{2+} signals directly, which are essential for the T-cell proliferation. Blockers of voltage-activated potassium (K_V) channels [such as the tetraethylammonium ion (TEA^+), Charibdotoxin (ChTx), Noxiustoxin (NxTx) and Margatoxin (MgTx)] inhibit the mitogen-induced proliferation of the cells – this is one of the evidences for the key role of the K^+ channels in T-cell activation. The increase in the K^+ conductance of the membrane of resting T-cells during mitogen stimulation and the increase of expression level of $K_{Ca}3.1$ channels during T-cell activation show the role of K^+ channels in T-cell activation and concomitant proliferation. It is probable that K^+ channels create the right conditions for increasing of intracellular Ca^{2+} level necessary for the mitogen response via hyperpolarization of the membrane.

$K_V1.3$ belongs to the Shaker family of voltage-gated K^+ channels. The functional channel in T-lymphocytes consists of four identical subunits. One subunit contains six α-helical transmembrane segments (S1-S6) linked by extra- and intracellular loops. The first four (S1-S4) segments are supposed to form the voltage sensor domains of the channel (four domains per channel) and responsible for the voltage dependent activation of the K_V channels, while the fifth and the sixth (S5-S6) segments with the connected extracellular loop (P) (all the four subunits together) form the inner conducting pore region to allow K^+ ions to flow through the membrane.

The membrane depolarization-induced activation (opening) of voltage-gated K^+ channels is thought to be a conformational change initiated in the first four segments of the channel protein, and after the activation, the channels come into a non-conducting, inactivated state. Inactivation can be carried out by two mechanisms. The fast inactivation, called N-type, lasts for a few milliseconds and appears to be due to a “ball-and-chain” mechanism, in which the N-terminus of the protein behaves like a blocker tethered to the cytoplasmic side of the channel and directly occludes the pore to cause the inactivation. The slow inactivation, called C-type, lasts many seconds and is the result of a complex mechanism sensitive to external cations and the amino acid composition of the channel pore. This process is essentially produced by a conformational change restricted to the

selectivity filter and the extracellular gate of the channel. C-type inactivation does not require intact N-type inactivation but N-type inactivation can accelerate C-type inactivation. Inactivation of $K_V1.3$ channels is mediated solely by the slow, P/C-type mechanism.

Lipid composition of plasma membrane influences membrane properties such as fluidity considerably. Fluidity of membranes fundamentally influences membrane processes and functions, operation of membrane-bound proteins. Kinetic characteristics and liquid crystal state of the membrane can influence membrane-related functions, the conformation and flexibility of membrane-bound proteins, permeability processes, the kinetic parameters of ion channels in cytoplasm membrane and enzyme activities. Under various conditions, the lipid order and composition of the plasma membrane may change. This change may significantly modify membrane fluidity altering the essential physical properties in the affected portions of the membrane and especially disturb the function of ion channels. Since K^+ channels are transmembrane proteins, i.e. they span the membrane, their function can be influenced by lipid–protein interactions. Gating of ion channels involves conformational changes in the protein, and changes in the channel lipid environment can therefore modulate the activation and inactivation kinetics of ion channel.

Effects of plasma membrane cholesterol on membrane fluidity and ion transport are well known. This substance can be found in both leaflet of the lipid bilayer and essentially modifies dynamic parameters of the membrane. It inhibits the adequate operation of receptor proteins in the concentrations different from physiological, and the enrichment of the membrane with cholesterol decreases while cholesterol depletion increases the activity of ion channels. It can be explained by the increase of membrane microviscosity caused by elevation of cholesterol content, and it acts on the intramolecular movements of the channel protein via partly mechanical way partly modification of lipid–protein interactions.

There is appreciable interest in the use of long-chain polyunsaturated fatty acids (PUFAs) as natural agents to control destructive inflammatory responses and autoimmunity. Of most interest are n-3 PUFAs. Several studies indicate that diets rich in n-3 PUFAs are anti-inflammatory and immunosuppressive *in vivo* and *in vitro*. Cellular constituents of both acquired and natural immunity are affected. PUFAs dose-dependently inhibit signal transduction in T-cells while saturated fatty acids (SFAs) and monounsaturated fatty acids (MUFAs) do not change Ca^{2+} response. PUFA enrichment of the cell membrane selectively modifies the cytoplasmic leaflet of detergent-resistant membrane domains (DRMs). Similarly, triacylglycerols containing PUFAs inhibited Concanavalin A (Con A)-stimulated lymphocyte proliferation; tryacylglycerols with an SFA or oleic acid (OA) content are not inhibitory.

Eicosanoids are also potent modulators of lymphocyte functions. The type of fat taken in the diet can influence the amount and type of eicosanoids made. Presumably, the inhibitory actions of fatty acids (FAs) on cell proliferation is not a result of a conversion to other metabolites but direct effects.

Modification of the membrane fatty acids (FAs) influences the functions of several membrane enzymes and ion channels, including K^+ channels [e.g. ATP-sensitive K^+ (K_{ATP}) channel, TREK-1 (2 pore K^+ channel), TRAAK (arachidonic acid stimulated K^+ channel), Ca^{2+} activated K^+ (BK_{Ca}) channel, ATP-activated K_1 background channel and phosphatidylinositol-4,5-bisphosphate (PIP_2) activated K_{ATP} channel). Modification of ion channels by FAs or other charged lipids is carried out probably via their direct interactions with the channel protein itself or with some other channel-associated components.

Dietary FAs pass into the blood circulation, where they can be taken up by the lymphocytes. Once they have been incorporated into the lymphocyte membrane, they may modulate the structure and fluidity of the membrane bilayer and consequently the function of the membrane proteins, including ion channels. Change in the function of the ion channels of the HPLs may cause a change in the cellular immune response. Since dietary FAs can incorporate into the cell membrane, diet can be important in maintaining the adequate lipid composition.

Extracellular signalling molecules (e.g. neurotransmitters, hormones, growth factors) mediate the communication among cells. These molecules behave as external chemical stimuli in signal transduction processes, and evoke cellular response via induction of metabolic changes in the intracellular space. Signalling molecules usually bind to receptors specifically, thus they are the natural ligands of their receptors. In many cases, G proteins located in the inner layer of the membrane mediate the effect of receptors and make a connection between the receptors and enzymes that catalyse the synthesis of secondary messengers.

Adenylyl cyclase enzyme determines the level of the secondary messenger cyclic adenosine monophosphate (cAMP). This enzyme is stimulated by G_s (stimulatory) proteins and is inhibited by G_i (inhibitory) proteins. The former one is activated by cholera toxin permanently, the latter one is inactivated by pertussis toxin (PTx) and so both these toxins contribute to elevation of the level of cAMP. It can be established with the use of these toxins that whether the G_i or the G_s mediated signalling pathway was activated by a given stimulus.

Adenosine is an endogenous modulator of cellular functions in the central nervous system and in the peripheral tissues. It can be produced by almost all cells and can be released into the extracellular space in case of an increased oxygen demand/oxygen supply ratio. A number of effects of adenosine are known, e.g. it has auto- and paracrin regulatory effect and it is a neurotransmitter.

It regulates the extent of coronary perfusion (enlarges the coronary artery and causes bradycardia), while blocks the secretion of some neurotransmitters by activating the presynaptic purinergic (P) receptors. Most of the effects of adenosine can be attributable to interaction with cell surface receptors that belong to the family of purinergic receptors.

The endogenous ligands of purinergic receptors are adenosine, AMP, ADP and ATP. These receptors were classified by Burnstock as adenine nucleoside sensitive P_1 and adenine nucleotide sensitive P_2 purinergic receptors, depending on their preferential interaction with adenosine (P_1) or ATP (P_2). In this way, P_1 receptors are identical with the adenosine receptors. The P_1 sites are further subdivided into A_1, A_{2a}, A_{2b} and A_3 adenosine receptors, on the basis of their differential selectivity for a series of adenosine analogues and amino acid sequence investigation of recombinant adenosine receptors. All of these receptors are seven-transmembrane domain proteins coupled to regulatory G-proteins. The signal transduction mediated by A_1 receptors occurs via G_i and G_0 proteins while A_2 receptors are G_s coupled and A_3 receptors are G_i coupled. A_1 and A_3 receptors inhibit the adenylyl cyclase activity (i.e. formation of cAMP) while A_2 receptors stimulate it. The effect evoked through A_1 can be blocked by PTx.

Tissue adenosine receptors can be identified by specific adenosine analogues (agonists) or selective adenosine receptor antagonist. These are the structural analogues of the natural ligands; agonists have effect similar to that of the natural ligands, antagonists do not induce biological response but can inhibit the effect of ligands and agonists. Adenosine agonists are adenosine derivatives all together while adenosine antagonist can be xanthine- (e.g. caffeine) or non-xanthine derivatives. N^6-cyclopentyl-adenosine (CPA) is an A_1 agonist, 2-[p-(2-carbonyl-ethyl)-phenylethylamino]-5'-N-ethylcarboxamidoadenosine (CGS 21680) is a selective A_{2a} agonist and 5'-N-ethyl-carboxamidoadenosine (NECA) is not selective, it has all the A_1, A_2 and A_3 agonist feature. 4-(2-[7-amino-2-(2-furyl)[1,2,4]triazolo[2,3-a][1,3,5] triazin-5-ylamino]ethyl)phenol (ZM 241385) is a non-xanthine type A_{2a} selective antagonist and 1,3-dipropyl-8-cyclopentylxanthine (CPX or DPCPX) is a highly specific A_1 receptor antagonist.

Positron emitting labelled forms of agonists and antagonists having high receptor specificity are usable for mapping the distribution of adenosine receptors in the body and for studying the kinetic features of ligand binding. 5'-N-(2-[^{18}F]fluoroethyl)-carboxamidoadenosine (^{18}F-NECA), the radiolabelled derivative of NECA has already proved to be a suitable radioligand for positron emission tomography (PET) imaging of adenosine receptors.

Although the best known signalling pathway of adenosine receptors is the activation or inhibition of adenylyl cyclase activity, many of the effects of adenosine are coupled to changes of the conductance of cell membrane, particularly the modification of K^+ and Ca^{2+} channels, involving an alteration also in the membrane potential. It is known that membrane potential plays a crucial

role in signal transduction, therefore examination of the possible role of the change of membrane potential in response of the cells to adenosine receptor activation may be reasonable.

The presence of both A_1 and A_2 type of adenosine receptors in certain smooth muscle cells has been previously demonstrated. Such cells are the DDT_1 MF-2 smooth muscle cells derived from Syrian hamster vas deferens. These cells possess a very responsive adenylyl cyclase system.

In some cases, a correlation was revealed between purinergic receptor activation and the K^+ conductance of cell membrane. For example, P_1 receptors activate an outward rectifying K^+ current in mature follicular oocytes of Xenopus laevis. Since stable analogue compounds of cAMP generate K^+ current, it is presumable that adenosine receptors stimulate the adenylyl cyclase system and K^+ current is modified via cAMP level alterations.

In addition to P_1 receptors, presence of voltage-dependent K^+ channels has been shown in DDT_1 MF-2 cells, so a P_1 mediated K^+ channel activation is also probable in these cells. In this way, these cells can be useful pharmacological targets to analyze the A_1 and A_{2a} receptor mediated mechanisms in the same cell line.

Glibenclamide sensitivity of a voltage-gated K^+ channel (rabK_V1.3) from rabbit brain and liver expressed in *Xenopus* oocytes was described. Thus, studying of the glibenclamide sensitivity of the voltage-gated K^+ current in DDT_1 MF-2 cells can be established.

2. AIMS OF THE STUDY

1. Molecular composition of phospholipids in the cytoplasmic membrane is supposed to modify the transition kinetics of transmembrane proteins (ion channels and transporters). Change in the function of the ion channels of the peripheral blood lymphocytes may cause a change in the cellular immune response.

   We set out to investigate how FAs with different carbon chain lengths and degrees of unsaturation influence the function of the $K_V1.3$ channels of the HPLs, thereby modifying the immune responses of the cells. We studied the effect of palmitic acid (PA), stearic acid (SA), OA, linoleic acid (LA), arachidonic acid (AA) and docosahexaenoic acid (DHA) on the kinetic and steady-state parameters of the $K_V1.3$ channels.

2. DDT_1 MF-2 cells express both the A_1 and A_2 type receptors, and voltage-gated K^+ and Na^+ channels can also be found in their membrane. Stable analogue compounds of cAMP generate K^+ current, therefore it is presumable that the adenylyl cyclase system is stimulated by adenosine receptors and K^+ current is modified via cAMP level alterations. We suppose the presence such a P_1 mediated K^+ channel activation in DDT_1 MF-2 cells. According to our hypothesis, these cells could constitute an ideal modell system for studying the regulation of voltage-gated ion channels by P_1 receptors in the same cell line.

   Therefore we intended to investigate how the interaction of certain adenosine agonists (NECA) and antagonists (ZM 241385) with the P_1 receptors modify the voltage-gated ion currents on DDT_1 MF-2 cells.

   Since the K_{ATP} channel blocker 1-{4-[2-(5-chloro-2-methoxybenzamido)ethyl]phenylsulfonyl}-3-cyclohexylurea (glibenclamide, Gilemal) proved to influence the conductance of certain voltage-gated ion channels, we were about to examine how glibenclamide influences voltage-gated currents studied in our experiments.

   Furthermore, we aimed to compare the effect of NECA and F-NECA on the K^+ conductance of the membrane of DDT_1 MF-2 cells mediated by adenosine A_1 and A_2 receptors, hereby to obtain informations about whether this agonist is applicable for PET diagnostics.

3. MATERIALS AND METHODS

Linking FAs to foetal bovine serum (FBS)

Individual FAs were dissolved in abs. etanol and titrated with saturated ethanolic KOH solution (free from carbonate ion) in the presence of phenolphthalein as indicator. The solvent was evaporated off by bubbling N_2 through the solution under a N_2 atmosphere. FBS (2.0 cm^3 solution to 100 μmol of FA) was added to the residue immediately, and the solution was kept at 37 °C for 1 h. The complex generated was kept at –20 °C until utilization, and was added freshly (5.0 μl) to the medium used for cell culturing (5.0 cm^3).

Cell culturing

HPLs were separated from human peripheral blood obtained from healthy volunteers by centrifugation, using Histopaque-1077 separation solution (density 1.077 g/cm^3). The blood was diluted and the separated mononuclear cells were washed with Hank's balanced salt solution (HBSS) at pH=7.4, supplemented with 25 mM Hepes. In the control medium or in media containing different SFAs and unsaturated fatty acids (UFAs) (50 μM) bound to FBS, the cells were cultured in Petri dishes (d = 3 cm) for 3 days at 37 °C in a humidified atmosphere containing 5% CO_2 at a density of $2 \cdot 10^6$ cells/cm^3. The control medium was RPMI-1640 containing 15 mM Hepes, supplemented with 10% (V/V) FBS, 2 mM L-glutamine and 50 mg/dm^3 gentamycin. 20 $\mu g/cm^3$ phytohaemagglutinin A (PHA) and 100 CU/cm^3 IL-2 were also added to each culture medium to stimulate proliferation. For the gas chromatography measurements, lymphocytes from 4 donors were obtained. Samples from each donor were distributed to 7 portions. 6 were supplemented with the different FAs and 1 was used as control. For patch-clamp measurements, lymphocytes from all together 23 donors were used. Each sample was distributed to two parts. One was supplemented with a given fatty acid and one was used as control.

The DDT_1 MF-2 smooth muscle cell line, derived from a steroid-induced leimyosarcoma tumor of Syrian hamster vas deferens, was obtained from the European Collection of Animal Cell Cultures (Porton Down, Salisbury, UK). The cells were cultured at 37 °C in a humidified atmosphere containing 5 % CO_2 in a closed 25 cm^2 flask on Dulbecco's modified Eagle's medium (DMEM) supplemented with 2 mM L-glutamine and 10 % (V/V) fetal calf serum. Cells were passaged twice a week by removing used medium from the flask and adding 5 cm^3 fresh medium to it. Then the cells were displaced from bottom using a rubber and 0.3–0.5 cm^3 of the suspension was put to a new flask containing 8 cm^3 fresh medium.

Before the experiments, the cultured cells were washed twice in PBS at 250g at 4 °C for 8 min and then suspended in ≈ 1 cm^3 of extracellular solution (see *Electrophysiology* section) and kept on ice until use. For patch-clamp measurements the cells (20-40 µl of the suspension) were adhered to Petri dishes (d = 3 cm) containing 1.5 cm^3 of extracellular solution. For the gas chromatographic measurements, HPLs were kept dry, and cell membrane was preparated.

Analysis of FA composition

Lipids were extracted with chloroform/methanol (2:1, V/V) from individual cultured HPL samples according to the method described by Folch et al (1957). Methyl esters of the total phospholipid FAs were prepared by transesterification in absolute methanol containing 5 % HCl at 80 °C for 2.5 h. The methyl esters were separated on an FFAP column (0.25 mm i.d. capillary column 30 m long from Supelco, Bellefonte, PA) in a Hewlett-Packard Model 6890 gas chromatograph. The measurements were made in duplicates and the averages of the two measurements were used. Peaks were identified with the aid of authentic standards from Sigma. The reported data are means ± standard errors of the means (SEM) of N = 3 independent experiments.

Electrophysiology

Ion currents were measured by the patch-clamp technique in whole-cell configuration, voltage-clamp mode, at room temperature, using an Axopatch-200 amplifier connected to Axon Instruments TL-1-125 and Digidata 1200 computer interfaces with varying sampling rates. Low-pass filtering was applied at half of the sampling frequency or less, using Warner Instruments LPF-8 instrument. Clampex 8.2 software (Axon Instruments, Foster City, Cal., USA) was used for data acquisition.

The extracellular solution contained NaCl, pH = 7.35. Pipettes were pulled from glass capillaries and filled with the internal solution containing KF, pH = 7.23. Adenosine receptor ligands were solved in the extracellular solution and carried to the cells using a gravitational perfusion system.

Measurement of K^+ currens:

The holding potential was kept at –120 mV, cell membranes were depolarized to +50 mV, and the duration of the depolarization was 15 ms on lymphocytes and 150 ms on DDT_1 MF-2 cells for characterization of the activation kinetics whereas it was 1 s on lymphocytes and 2 s on DDT_1 MF-2 cells for characterization of the inactivation kinetics. Depolarizing pulses were applied every 30 s for short depolarizations (i.e. measurement of activation kinetics) and every 60 s for long depoalrizing pulses (during the measurement of the inactivation kinetics) to avoid cumulative inactivation of the current. Online leak-subtraction was applied. Voltage-dependence of steady-state

activation on HPLs was determined by depolarizing the cells from the holding potential of –120 mV to test potentials for 1 s, ranging from –70 to +50 mV, in 10 mV steps. On DDT_1 MF-2 cells, the holding potential was set to –120 mV, depolarizing pulses were applied from –80 to +60 mV in 20 mV steps, lasting for 2 s. Subsequent leak-subtraction was applied. Voltage-dependence of steady-state inactivation was determined by depolarizing the HPLs to +50 mV for 15 ms from the holding potential applied for 2.5 min in the range between –100 mV to –20 mV, in 10 mV steps. Subsequent leak-subtraction was applied.

Measurement of Na^+ currens:

Depolarizing pulses were applied between -80 and +90 mV in 10 mV steps for 7 ms, from the holding potential of –120 mV on DDT_1 MF-2 cells. Online leak-subtraction was applied.

Data analysis

Clampex 8.2 (Axon Instruments, Foster City, Cal., USA), Microsoft Excel and SigmaPlot 8.0 softwares were used for data acquisition.

In cases of subsequent leak-subtraction, current traces were software-corrected to ohmic leak current and digitally filtered (3-point boxcar smoothing) prior to analysis.

For determination of the activation and the inactivation time constants (τ_a and τ_i), according to the Hodgkin–Huxley model, exponential function was fit to the current curves using the Levenberg–Marquardt algorithm.

To determine the parameters (the midpoint of the voltage-dependence, $V_{1/2,a}$ and the slope factor, s_a) characterizing the voltage-dependence of steady-state activation of $K_V1.3$ channels on HPLs, the peak K^+ conductance of the membrane (G_K) at each membrane potential was calculated. The actual G_K values were normalized to the largest value of G_K and the normalized conductance-voltage relationships were fit using the Boltzmann's function. The voltage-dependence of steady-state inactivation ($V_{1/2,i}$ and s_i) was also characterized using the Boltzmann's function.

Dose–response curves were fitted by the Hill's equation in measurements carried out on DDT_1 MF-2 cells.

Statistical analysis

The characteristic values of the activation and inactivation time constants for each HPL were calculated as the averages obtained from depolarizations in a succession of at least 3 pulses.

In case of HPLs, the use of the median, rather than of mean, is indicated by the large donor-to-donor variability of the data (see below) and the non-normal distribution of the time constants.

For each cell preparation (donor) a control (untreated) group and FA-treated group was established. The values of τ_a, τ_i, $V_{1/2,a}$, s_a, $V_{1/2,i}$ and s_i obtained from control cells for a given FA-

treatment of different donors were compared using the Kruskal–Wallis one-way ANOVA on ranks procedure. The analysis showed that there are significant differences between control cells of different donors. Similarly, in some cases cells of different donors treated with the same FA showed statistically significant differences in the measured parameters. Therefore, for statistical comparisons the following normalization procedure was used on a cell-by-cell basis. The measured parameter characteristic to a given cell in response to FA treatment was normalized to the average of the same parameter obtained from control cells of the same donor. The resulting normalized values were then pooled for each treatment, the means and SEMs were calculated, and used in a Student's t-test and tested against a null hypothesis of $\mu=1$. The limit of statistical significance was $P<0.05$.

Statistical comparisons were made in SigmaStat 3.11 program. Dunn's method was used for both multiple comparisons versus control and all pair-wise multiple comparisons.

Experiments carried out on DDT_1 MF-2 cells were self-controlled.

4. RESULTS AND DISCUSSION

4.1. Effect of the phospholipid composition of citoplasm membrane on the function of $K_V1.3$ channels in HPLs

Monitoring of FA incorporation

Gas chromatographic measurements were carried out to determine the incorporation of the FAs into the lymphocyte membrane. In each case, we experienced a statistically significant increase in the relative amount of the given FA after the treatment relative to the total amount of FA in the HPL membrane (t-test, $P < 0.05$).

The relative amount of PA increased from the 29.00 ± 1.51 % measured in the control (C) (N=3, where N is the number of measurements on HPLs from different donors) to 33.66 ± 0.56 % in the treated cells (N=3). Increase in the relative amount of SA in the membrane was from 32.01 ± 2.71 % to 40.56 ± 1.26 % (N=3), of OA from 6.10 ± 3.12 % to 20.15 ± 5.42 % (N=3), of LA from 4.32 ± 2.19 % to 17.75 ± 2.87 % (N=3), of AA from 10.63 ± 1.49 % to 14.54 ± 2.49 % (N=3), and of DHA from 2.50 ± 1.29 % to 16.89 ± 0.65 % (N=3).

PUFAs modify the activation and inactivation kinetics of $K_V1.3$ channels

To compare the effects of the various treatments, we performed a detailed statistical analysis of the measured time constants (see *Statistical analysis* section in the Materials and Methods). In several cases, the all pair-wise comparison (Kruskal–Wallis one-way ANOVA on ranks, $P<0.05$) revealed significant differences in the activation time constant (τ_a), the inactivation time constant (τ_i) and the midpoint of the voltage-dependence of steady-state activation ($V_{1/2,a}$) of the current recorded from control cells isolated from different donors. Other steady-state parameters did not show donor-by-donor variability. As a consequence of this statistically significant donor-by-donor variability, the data obtained on the HPLs isolated from different donors were not pooled into one control group and merged treatment groups, but the values measured on the FA-treated HPLs were normalized to the control values for the same donor (see details in the *Statistical analysis* section).

The incorporation of SA into the HPL membrane did not alter the activation time constant of the $K_V1.3$ channels. The ratio of the activation time constants was 1.05 ± 0.04 relative to the control value. Neither the saturated PA nor the monounsaturated OA yielded significant changes in this parameter, the ratios were 1.07 ± 0.14 and 0.97 ± 0.03, respectively. On the other hand, the PUFA LA, AA and DHA decreased the activation time constant of the current, with ratios of 0.91 ± 0.02, 0.64 ± 0.07 and 0.73 ± 0.11, respectively.

Qualitatively similar FA treatment-induced changes were obtained for the inactivation time constant (τ_i) of the $K_V1.3$ channels. LA, AA and DHA significantly decreased the inactivation time constant of the HPLs, while PA, SA and OA did not affect it. The ratios of the inactivation time constants for LA, AA, DHA, PA, SA and OA were 0.67 ± 0.08, 0.51 ± 0.05, 0.54 ± 0.09, 0.92 ± 0.06, 0.97 ± 0.03 and 1.13 ± 0.13, respectively.

FA incorporation leaves the steady-state parameters of activation and inactivation of the $K_V1.3$ channels unchanged

The voltage dependence of the normalized whole-cell conductance (steady-state activation) characterizes the equilibrium distribution of ion channels between the closed and open states at a given membrane potential. The voltage dependence of the steady-state inactivation describes the proportion of channels that are not inactivated at a given membrane potential.

Neither of the FAs affected the voltage-dependence of steady-state activation of $K_V1.3$ channels. This was verified by the statistical analysis. For considerations detailed in the *Statistical analysis* section the tests were performed on the normalized parameters of the Boltzmann functions. The treated/control ratios of the midpoint of the voltage dependence of the steady-state activation ($V_{1/2,a}$) were: SA: 1.02 ± 0.08, OA: 1.06 ± 0.16, LA: 1.07 ± 0.18, AA: 1.13 ± 0.04, and DHA: 0.97 ± 0.04. The ratios of the slope factors (s_a) were: SA: 1.03 ± 0.05, OA: 1.04 ± 0.05, LA: 0.99 ± 0.16, AA: 1.05 ± 0.10, and DHA: 0.98 ± 0.09.

Similarly, FA treatment of HPLs did not induce significant changes in the voltage dependence of steady-state inactivation of $K_V1.3$ either. The treated/control ratios of the fitted parameters using the best-fit Boltzmann's function were as follows. The ratios for the midpoints of the voltage dependence of the steady-state inactivation ($V_{1/2,i}$) were: SA: 1.08 ± 0.05, OA: 0.95 ± 0.07, LA: 0.98 ± 0.01, AA: 1.06 ± 0.01, and DHA: 1.07 ± 0.12; and for the slope factors (s_i): SA: 0.96 ± 0.09, OA: 1.05 ± 0.12, LA: 1.09 ± 0.08, AA: 1.05 ± 0.04, and DHA: 0.91 ± 0.07. The statistical analysis was performed on the normalized values of $V_{1/2,i}$ and s_i (see above).

The motivation to study the effect of FAs on the activity of $K_V1.3$ channels came from three sources of data in the literature. First, the activity of $K_V1.3$ channels critically determines the lymphocyte activation processes; second, alterations in the lipid composition of the lymphocyte membrane dramatically influence certain immune reactions; and third, lipid environment in the membrane modulates the function of membrane proteins in general, and that of ion channels in particular.

The membrane potential-dependence of antigen activation of T-cells is well documented. Considering the very narrow membrane potential window under which $K_V1.3$ channels operate at

steady-state (i.e. the intercept of the voltage-dependence of steady-state activation and inactivation) any small change in the voltage-dependence of these parameters might have profound influence of the availability of the K^+ current to control the membrane potential of T-cells. Under these conditions changes in the kinetics of $K_V1.3$ gating, especially those that influence the time spent in the conducting (open) state, might very efficiently alter the contribution of the K^+ conductance to the total membrane conductance, and thus, modulate the membrane potential of T-cells. For example, the ≈2-fold increase in the inactivation kinetics following PUFA treatment described in this study reduces the lifetime of the open state very effectively. The effect of the increased inactivation rate is barely compensated by the faster opening kinetics of the current following PUFA treatment since inactivation kinetics is ≈2 orders of magnitude slower than activation kinetics therefore the area under the I(t) curve (i.e. amount of charge transferred) is more sensitive to changes in the inactivation kinetics as compared to changes of the activation kinetics. Thus the combined effect of the faster activation and inactivation kinetics is a reduced K^+ flux which may explain the beneficial effects of PUFA in the suppression of immune and autoimmune processes.

Several studies have indicated that diets rich in n-3 PUFAs are anti-inflammatory and immunosuppressive both *in vivo* and *in vitro*. It is thought that the inhibitory effect of the FAs was not a result of conversion to other metabolites, but was a direct effect. Similarly, triacylglycerols with a PUFA content inhibited Con A-stimulated lymphocyte proliferation in a concentration- and dose-dependent manner in the rat, while tryacylglycerols containing SFAs or OA were not inhibitory.

Regarding the molecular mechanism of the action of PUFAs Stulnig et al. (1998) reported that the enrichment of Jurkat T-cell lipids with PUFAs inhibited very early signalling events. They argued that PUFA-induced inhibition of T-cell activation was due to displacement of the Src family kinase Lck from the cytoplasmic layer of DRMs. Our study indicates that suppression of the activity of $K_V1.3$ may be a mechanism complementary to the displacement of Lck in the inhibition of immune reactions.

The opening and the subsequent slow (P/C-type) inactivation step involves a series of conformational changes in the voltage sensor domain and the pore domain of the channel protein. As K^+ channels are embedded in the cell membrane, their function can be influenced by lipid–protein interactions. This scenario was highlighted recently by the X-ray crystallographic structure of the human $K_V1.2$ K^+ channel, which showed several potentially lipid exposed surfaces of the channel ($K_V1.2$ is closely related to $K_V1.3$.). Transmembrane helices S5 and S6 along with the pore helix and the selectivity filter from each of the four subunits of a functional channel together form a single pore domain whereas helices S1-S4 of each subunit form the voltage sensor domains of the channel (4 per channel). Based on the X-ray structure the following lines of evidence point to the

importance of protein–lipid interactions: 1) the presence of a detergent/lipid mixture was necessary during the crystallization process, 2) the voltage sensor domain is not tightly packed with the pore domain, the space separating the hydrophobic surfaces of the pore and voltage sensors would be filled with lipids in native membranes, 3) key residues for voltage sensing (Arg 1 and 2 counted from the extracellular end of S4) are located on the lipid-facing surface of S4 helix.

Several effects of the lipid composition of the membrane were attributed to the alteration of microviscosity (fluidity) of the membrane, change in the membrane curvature and lateral pressure profile in the membrane and the recruitment into or exclusion from lipid rafts of ion channels. For the following reasons we favour the idea that the effect of PUFAs on $K_V1.3$ channels are related to changes in the physico-chemical environment of the channel, rather than to a specific interaction with the gating machinery of the channels. First, qualitatively similar changes in the activation and inactivation kinetics of $K_V1.3$ were observed for PUFAs with different acyl chain length, degree of saturation and the position of the double bonds. Second, a common feature of all PUFAs is that the acyl chains are bent to an extent depending on the degree of unsaturation and therefore they alter the mechanical properties of the membrane e.g causing an increase in the fluidity (decrease the microviscosity).

Anel et al. (1993) showed that cis-UFAs, but not trans-UFAs or SFAs alter the lipid acyl chain order and inhibit the cell function of cytotoxic T-lymphocytes (CTLs). In our experiments, only all-cis unsaturated derivatives were used and, with the exception of the MUFA OA, they were all effective in modulating the $K_V1.3$ channel kinetics.

Alternatively, treatment of lymphocytes with PUFAs may act by altering the postsynthetic modification of $K_V1.3$ channels by protein kinases and phosphatases and the platform for this modulation could be the lipid rafts of T-cells. It has previously been described that $K_V1.3$ channels are localized in lipid rafts within the T-cell membrane and that the n-3 PUFA EPA selectively incorporates into T-cell rafts and alters their composition. This latter is reflected in the displacement of acylated signalling proteins from the cytoplasmic leaflet of lipid rafts including that of the Src family kinase Lck. As the acivity of $K_V1.3$ and its gating is widely regulated by phosphorylation on tyrosine and serine/threonine residues, the disruption of the signalling complex of lymphocytes by PUFAs is also a feasible alternative to explain our results.

We reported here that voltage dependence of steady-state activation and inactivation is insensitive to the accumulation of PUFAs, MUFA or SFAs in the HPL membrane. This means that the equilibrium distribution of the channels between closed and open as well as non-inactivated and inactivated states at a given membrane potential are the same regardless of the FA treatment (i.e. $\Delta\Delta G$ between the corresponding states is unchanged). On the contrary, the kinetics reaching the open and inactivated states were accelerated by PUFAs which can be envisioned as a decrease in

the height of the energy barrier (ΔG) separating the closed and open, and the open and inactivated states, respectively. Thus, the energetics of channel gating is affected specifically by the PUFAs, which is reflected in the rates of the transitions, but not in the equilibrium distribution between the states. This scenario may be compatible by the decrease in the microviscosity of the membrane upon PUFA treatment.

4.2. Adenosine analogues modify the operation of K^+ channels on DDT_1 MF-2 smooth muscle cells in a dose-dependent manner

Identification of ion currents in DDT_1 MF-2 cells

Patch-clamp experiments carried out on DDT_1 MF-2 cells revealed two voltage-dependent currents: an outward rectifying K^+ current and an inward rectifying Na^+ current. As the Na^+ current activated and inactivated earlier than the K^+ current activated, it was possible to measure them independently.

To verify that we actually measured K^+ current, we performed the pharmacological analysis of the channels. Well-known K^+ channel blockers, 4-AP (5 mM) and TEA^+ (10 mM) reversibly blocked the whole-cell current, the ratios of currents measured in the presence and absence of blockers (I/I_0, remaining fraction) were 0.52 ± 0.10 (N=5) and 0.29 ± 0.11 (N=3), respectively. The pharmacological analysis of the channels using peptide type blocking agents (ChTx, MgTx) has been previously reported in Ph.D. thesis by Bálint Rubovszky. Received parameters correspond to that of $K_V1.3$ channels.

Adenosine agonists and antagonists modify K^+ currents in DDT_1 MF-2 cells

Application of NECA, an effective agonist for both the A_1 and A_2 receptors, in a concentration range between 1 nM – 60 μM caused significant decreasing of the K^+ current of DDT_1 MF-2 cells in a dose-dependent manner. The value of half-maximal dose (EC_{50}) was 165.13 μM, Hill's coefficient (n_H) was 0.38. The inhibition was most expressed at the concentration of 5 μM where the remaining fraction of the current was 0.65 ± 0.02 (N=3). Since NECA is an agonist for both the A_1 and A_2 receptors, a complex, two-phase process can occur during its application. The inhibition of the K^+ current could be observed with the use of lower concentrations of NECA (A_{2a} effect) while the increase of the K^+ current could exceed the inhibition of it with the use of higher concentrations of NECA (A_1 effect). According to our measurements, NECA also altered the kinetics of the current resulting faster activation, e.g. the ratio of the activation time constants (τ/τ_0) was 0.70 ± 0.10 at the concentration of 5 μM (N=5).

Concerted action of NECA and TEA^+ was experienced. First NECA (0.5 μM) then TEA^+ (10 mM) was added to the same cell (N=2). Up to our expectations, the inhibitory effect of these two molecules was not competitive, since these two substances bind to different places: TEA^+ acts on K^+ channel directly while NECA takes its effect through adenosine receptor. NECA blocked current was further reduced by TEA^+, the remaining fraction of the current was 0.27 ± 0.05 (keeping in mind the effect of single TEA: the remaining fraction of the K^+ current was 0.29 ± 0.11).

^{18}F-NECA is a β^+ decaying isotope labelled derivative of NECA. It can be introduced into the body and used for PET imaging (studying the distribution of adenosine receptors and their kinetics). To avoid contact with β^+ decaying substance, non-radioactive form of it, ^{19}F-NECA was used in our patch-clamp experiments.

F-NECA did not exert any influence on either the amplitude of K^+ current (remaining current fraction was: 1.00 ± 0.01; N=2) or the activation time constant (τ/τ_0 was: 0.98 ± 0.04) at the concentration of 1 μM. However, 5 μM F-NECA diminished the current, the remaining fraction was 0.85 ± 0.03 (N=3) but did not produce any significant change in the activation time constant 0.99 ± 0.05 (N=4).

A_2 adenosine receptor antagonist ZM 241385 (1 μM) resulted in a decrease of the K^+ current, the remaining fraction was 0.81 ± 0.07 (N=4). The effect was irreversible, washing the cells with normal extracellular solution did not interrupt the effect of ZM 241385. ZM 241385 did not alter the activation time constant at this concentration, τ/τ_0 was 0.95 ± 0.09 (N=4).

K_{ATP} channel blocker glibenclamide irreversibly decreased the K^+ current in a concentration range of 1–20 μM in a dose-dependent manner, the remaining fraction was 0.77 ± 0.05 at the concentration of 3 μM. The value of half-maximal dose (EC_{50}) was 15.00 μM, Hill's coefficient (n_H) was 1.00. Glibenclamide did not exert any effect on the kinetic parameters of the current, the treated/control ratio for the activation time constants was 1.08 ± 0.18 at the concentration of 3 μM.

Adenosine receptor activation and inactivation does not influence Na^+ currents in DDT_1 MF-2 cells

The fast inward rectifying current was identified as a voltage-gated Na^+ current on the basis of the followings: 1. 100 nM *tetrodotoxin* (TTx) reversibly blocked this current, 2. the channel was possessed of the kinetical parameters and voltage–current characteristics of the voltage-gated fast Na^+ channels of excitable cells, 3. the current was not detectable in Na^+ free extracellular environment.

Studied P_1 agonists and antagonists did not influence the conductance of Na^+ channels of DDT_1 MF-2 cells showing that these ion channels do not take part in the studied process. Measured

remaining fractions were: NECA (1 μM): 0.97 ± 0.04 (N=3), ZM 241385 (1 μM): 1.02 ± 0.11 (N=3), glibenclamide (3 μM): 0.99 ± 0.03 (N=3).

Results demonstrated in the present study are in agreement with our earlier observations presented by Bálint Rubovszky in his Ph.D. thesis. On the basis of all these, we can conclude that receptors take effect on the channels via G protein independent way during changes in the properties of K^+ current.

According to our best knowledge, this was the first time to successfully demonstrate the modulation of the K^+ current in DDT_1 MF-2 cells by selective P_1 adenosine receptor agonists and antagonists. Although Molleman et al. failed to demonstrate any effect of high dosage of adenosine (1 mM) on membrane current and ATP-induced currents in this cell line, these findings do not contradict our results, as adenosine is not a specific ligand. It can also bind to P_2 receptors, and the weak binding of adenosine to either A_1 or A_2 receptors can evoke a wide range of receptor activation processes, as a result of which the effect on the K^+ current may not be significant. In the present study subtype-specific agonist and antagonist induced processes were investigated, therefore different effects could be manifested separately.

The response of K^+ channels to adenosine analogues specific for different subtypes varies from tissue to tissue. Our results suggest that DDT_1 MF-2 cells are unique in their ability to respond with increasing their K^+ conductance and hyperpolarization to A_1 receptor activation and decreased K^+ current and depolarization to the A_2 receptor activation. Therefore, these cells can be useful tools of pharmacological research, as they provide the possibility of tracking A_1 and A_2 mediated mechanisms in the same cell line.

It has been shown that a close relation exists between membrane potential and adenylyl cyclase activity which phenomenon is Ca^{2+} independent. This observation is in harmony with our findings and the reported adenylyl cyclase stimulation by receptor binding of A_{2a} agonists. Our data support the assumption that the adenylyl cyclase inhibition by agonist binding to A_1 adenosine receptors may be transmitted by the induced membrane potential increase. Based on the voltage-dependence of adenylyl cyclase, we suggest that stimulation or inhibition of adenylyl cyclase by A_2 or A_1 receptor influence can occur by a mechanism involving a ligand-induced change in the transmembrane potential among the first elementary steps, as alterations in the membrane polarity may lead to voltage-dependent conformational change of the enzyme.

The two adenosine analogues, NECA and its fluorine-labelled derivative F-NECA, have different effects on the K^+ conductance of DDT_1 MF-2 smooth muscle cells. This can be a consequence of the differences in their chemical structure which may lead to differences in their affinity to A_1 and A_2 adenosine receptors. As both A_1 and A_2 receptors are found to be present on

DDT_1 MF-2 cells, and NECA is an agonist for both receptors, the elicited functional response to the binding of NECA or F-NECA is the sum of the effects mediated via A_1 and A_2 subtypes. Our results suggest that the stimulation of cAMP dependent signaling pathways via receptor binding is different for NECA and F-NECA. Although the alteration in electrophysiological properties caused by F-NECA is less prominent, both compounds reduce K^+ current. Therefore F-NECA can be used as moderately less effective analogue from the electrophysiological point of view. Since F-NECA behaves in a different way from NECA in the studied electrophysiological process, it is needed to be circumspect with the appreciation of PET data.

The voltage-gated K^+ channel (rabK_V1.3) from rabbit brain and liver cloned to Xenopus oocytes proved to be glibenclamide sensitive, in contrast to other Shaker family channels described (glibenclamide is an inhibitor for K_{ATP} channels). The glibenclamide sensitivity of rabK_V1.3 was on the order that of the native K_V channel in vascular smooth muscle cells from rabbit. Glibenclamide sensitivity of other K_V channel than rabK_V1.3 and the K^+ channels in DDT_1 MF-2 smooth muscle cells has not been documented.

5. SUMMARY

Characteristics of voltage-gated cation channels in the cell membrane were studied in this thesis.

1. We examined the effect of certain FAs on the kinetics of $K_V1.3$ channel gating of PHA- and IL-2-stimulated HPLs. Our study indicated that the effect carried out by FAs depended on the degree of unsaturation and/or the chain length of the FAs. All of the studied PUFAs (LA, AA and DHA) influenced the activation and inactivation kinetics of the $K_V1.3$ channels of HPLs (decreased the time constans mentioned above), whereas the MUFA OA and the SFAs (PA and SA) were ineffective. This pattern parallels the efficacy of FAs to interfere with lymphocyte activation processes. However, these FAs did not affect the voltage-dependence of steady-state activation and steady-state inactivation of the channels. The efficacy of the incorporation of FAs into the HPL membrane was confirmed by gas chromatographic measurements, each treatment specifically increased the relative contribution of the corresponding FA to the total amount of FAs in plasma membrane of HPLs.

   We have demonstrated here that enrichment of the membrane with various PUFAs can modulate the kinetics of $K_V1.3$ channels in HPLs probably via alteration of microenvironment in the lipid bilayer. In this way, a diet rich in PUFAs can be important in maintaining the suitable lipid composition of the membrane for proper operation of these channels or induction of lipid correction. T-cell activation is based on the operation of the voltage-gated K^+ channels, the Ca^{2+} activated K^+ channels and the Ca^{2+} release activated Ca^{2+} channels, and $K_V1.3$ channels expressed in T-lymphocytes control the membrane potential and signal transduction. Therefore the PUFA-induced modification of $K_V1.3$ kinetics might have important consequences regarding the inhibition of the activation of T-cells under pathophysiological conditions. This might contribute to the beneficial effects of dietary PUFA supplement in autoimmune reactions and/or during chronic inflammation.

2. We have revealed a relationship between the extent of K^+ currents and adenosine receptor stimulation or inhibition in DDT_1 MF-2 smooth muscle cells. On the basis of both this present study and our earlier measurements presented by Bálint Rubovszky in his Ph.D. thesis, we can conclude that applied adenosine agonists (NECA, F-NECA, CPA, CGS 21680) and antagonists (ZM 241385) take part in the adenosine mediated process and verify the presence of A_1 and A_2 adenosine receptors in the studied cell line. During the changes in the properties of K^+ current,

receptors act on the channels in a G protein independent way. On the other hand, the examined P_1 agonists and antagonists did not influence the conductance of Na^+ channels in this smooth muscle cells.

DDT_1 MF-2 cells have proved to be uniqe to respond to both the A_1 receptor activation with increase in K^+ conductance and hyperpolarization, and the A_2 receptor activation with decreased K^+ current and depolarization. In this way, these cells can be useful pharmacological targets to analyze the A_1 and A_2 receptor mediated mechanisms in the same cell line.

6. PUBLICATIONS

6.1. Publications served as a basis of this thesis

Székely, A., Kitajka, K., Panyi, Gy., Márián, T., Krasznai, Z.: Nutrition and immune system: Certain fatty acids differently modify membrane composition and consequently kinetics of $K_V1.3$ channels of human peripheral lymphocytes. *Immunobiology* **212 (3)**, 213-227. (2007) **IF: 1,812**

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Márián, T., Rubovszky, B., Szentmiklósi J. A., Trón, L., Balkay, L., Boros, I., Gáspár, R., **Székely, A.**, Krasznai, Z.: A_1 and A_2 adenosine receptor activation inversely modulates potassium currents and membrane potential in DDT_1 MF-2 smooth muscle cells. *Jpn. J. Pharmacol.* **89**, 366–372. (2002) **IF: 1,792**

6.2. Other publications

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6.3. Posters, abstracts and summaries of oral presentations related to this thesis

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6.5. Other poster

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