

SHORT THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (Ph.D.)

**ION CHANNELS IN NATIVE ENVIRONMENT:
CHARACTERIZATION OF ION CHANNELS IN
DENDRITIC AND ENDOTHELIAL CELLS**

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The Examination takes place at the Department of Physiology, Medical and Health Science Center, University of Debrecen at 10 am on April 28th 2011.

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The Ph.D. Defense takes place on April 28th at 12:00 pm at the at the Lecture Hall of the 1st Department of Medicine, Institute for Internal Medicine, Medical and Health Science Center, University of Debrecen

I. Scientific background

A. *Dendritic cells and their role in the immune response*

Dendritic cells are located between the boundaries of inner and outside world and are created to bridge the innate and adaptive immunity. These cells are able to induce, sustain and regulate immune responses by the translocation of antigens (Ag) from periphery to lymphoid niches. They originate from CD34⁺ hematopoietic stem cells and differentiate into multiple different circulating subsets, such as myeloid, lymphoid or plasmacytoid cells. DC subsets can be found in both lymphoid organs (thymus, bone marrow, lymph nodes and spleen) as well as in non-lymphoid organs (such as blood, skin and other peripheral tissues). They can be further characterized by their surface phenotype or by their function – such as migratory -, tissue-resident -, inflammatory -, or tolerogenic DC.

DC are the transporters of pathogen-related information within the immune system as they continuously uptake particles and soluble compounds from their tissue environment. They are the only type of antigen presenting cells that are capable of priming naïve and resting memory T cells as they can migrate from the periphery to the lymph nodes providing them Ag-specific, co-stimulatory and polarizing signals. Pathogen-induced tissue damage as well as multiple environmental factors in the periphery are able to induce DC maturation, which will result in transient increase of Ag uptake, shortly afterwards the loss of endocytic/phagocytic receptors, followed by the presentation major histocompatibility complex (MHC) molecules. DC maturation also includes the production of immunostimulatory cytokines, which are essential for the differentiation and induction of effectors functions of CD4⁺ (T helper - Th) and CD8⁺ (cytotoxic – CTL) T lymphocytes, as well as for their protection from apoptosis

DC not only play a vital role in adaptive immunity, but they are also crucial in the innate immunity as they play role in the activation of major innate effector cells, the natural killer cells. DC are also essential in initiating T cell immunity, numerous pieces of evidence suggest that DC play a role in tolerance, thus avoiding autoimmune responses in the body.

DC were first described in 1973 by Ralph M. Steinman and Zanvil A. Cohn as a novel cell type found in peripheral lymphoid organs. However until the discovery of GM-CSF, which acts as the major factor for the growth and differentiation for DC precursors along with

TNF- α , it was very difficult to purify and characterize a large number of these cell types. In 1992 it was presented, that myeloid DC can be differentiated from CD34⁺ progenitors of the human cord blood and bone marrow by GM-CSF and TNF- α mediated stimulation, which will result in a heterogeneous population of DC. Further on it was shown, that blood monocytes also possess the potential to differentiate into DC in the presence of GM-CSF and anti-inflammatory cytokines such as IL-4 or IL-13 and could be used in DC research. The ability to generate a large number of DC made it possible to study DC biology and to use them in clinical trials and tumor therapy. Among numerous factors that control immune cells' function, ion channels are one of them that play an important role in the regulation of different functions of these cells.

1. The characteristics of DC model cell lines and their role in DC research

KG-1 cells are described to be one of the closest models to human myeloid DC. These cells can be activated by various stimuli and the differentiated cells resemble mature myeloid DC. PMA and ionomycin induces a maturation process in KG-1 cells which is accompanied by changes of morphology, internalizing capacity, and cell surface expression of MHC class I molecules. Based on the phenotypic and functional similarities of IDC, MDC and KG-1 cells we initiated experiments to characterize the ion channels expressed in unstimulated and activated KG-1 cells and compare the results with those obtained for IDC and MDC. We aimed to determine if the KG-1 cell line could be used to understand the function of ion channels in dendritic cells and utilize as a model cell line in electrophysiological studies as well.

2. Ion channels in the immune system

Several studies demonstrated that the expression of ion channels in different immune cells varies during their activation and differentiation. These electrically non-excitable cells possess both voltage-dependent and secondary messenger-gated ion channels that are crucial for their antigen-dependent activation, proliferation and migration. Voltage-gated potassium channels (VGPCs) have been described to be the main ion channels controlling the resting membrane potential and tuning the intracellular Ca²⁺ signaling in monocytes, lymphocytes, macrophages and mouse DC. The dynamic change in the expression of ion channels,

including VGPCs upon distinct extracellular stimuli is important in specific immune responses during the terminal differentiation and activation of these cells. A member of the *Shaker* family, Kv1.3 channel is extensively studied for its potential role in lymphocyte and macrophage activation, and the blockade of these channels is associated with selective inhibition of T cell activation and proliferation. Inwardly rectifying K⁺ channels (K_{ir}2.1), Ca²⁺-activated K⁺ channels (IK_{Ca1} or K_{Ca}3.1) and Kv1.5/Kv1.3 heterotetramer channels have also been described in T cells and in cells of the mononuclear phagocytic system.

The hypothesized role of most of these channels is to maintain a hyperpolarized membrane potential, which enhances and sustains the Ca²⁺ signal upon activation. The intracellular Ca²⁺ signal is also influenced by the presence of Ca²⁺ release-activated Ca²⁺ channels (CRAC) in lymphocytes, macrophages and DC. These channels are responsible for the sustained Ca²⁺ signal required for the expression of numerous functionally important genes that regulate immune responses.

Based on the preliminary evidence regarding the role of ion channels in monocytes and DC we designed experiments to identify and characterize the expression and functional activity of voltage-gated ion channels in DC generated *in vitro* from human peripheral blood monocytes and the DC model cell KG-1. We hypothesized that there's a switch of ion channel expression during DC maturation, which could contribute to the distinct functional activities of these cells associated with their different maturation states.

B. Physiological functions of endothelial cells

Vascular endothelium is a multifunctional and highly specialized cell layer, lining the luminal surface of the entire vascular system. They provide a structural and metabolic barrier between the blood and the underlying tissues as they regulate a variety of vascular functions. They control vessel tone and adjust vascular diameter in response to hemodynamic needs and forces, such as changes in flow rate or in blood pressure. Because of their complex function and pivotal role in numerous pathophysiologic conditions these cells have become the major focus of modern biomedical research.

1. Calcium signals and ion channels in vascular response

The complex function of EC requires a rapid analysis and transduction of various signals that originate from the blood, from the adhering cells, or from the luminal surface of the vessel.

These extracellular signals activate various second messenger systems that are integrated in the EC membrane. Our knowledge about the intracellular signal transduction is still very limited, but among the second messengers Ca^{2+} signaling is known to be the most important. The control of the intracellular free Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) is the main regulating factor in the production and release of various vasoactive factors, e.g. nitric oxide (NO), prostaglandin I_2 , endothelium-derived hyperpolarization factor (EDHF), angiotensin II, endothelin, superoxide anions and thromboxane A_2 , and factors involved in blood clotting. In addition, Ca^{2+} entry is also involved in controlling intercellular permeability, EC proliferation and angiogenesis.

Besides Ca^{2+} channels, channels that control the membrane potential are also important regulators for transmembrane Ca^{2+} fluxes. The membrane potential of vascular endothelial cells is negative compared to the blood and tissue compartments. In general EC membrane potential is more negative in macrovascular than in microvascular EC. According to our current knowledge the resting membrane potential is mainly controlled by K^+ , Cl^- and Na^+ conductances with the relative contribution to the resting membrane potential is $\text{K}^+ : \text{Cl}^- : \text{Na}^+ = 27-95\% : 9-35\% : 3-30\%$.

Endothelial K^+ channels are said to be responsible for setting the membrane potential and their role in endothelium-dependent vasodilatation has been widely implicated. Different types of K^+ channels have been described in EC using various culturing and recording conditions: inwardly rectifying K^+ channel (K_{ir}), large conductance Ca^{2+} activated K^+ channel (BK_{Ca}), small- and intermediate-conductance Ca^{2+} activated K^+ channel (SK_{Ca} and IK_{Ca1} respectively), ATP-sensitive K^+ channels (K_{ATP}). K_{ir} channels are the major class of ion channels setting the resting membrane potential, and quite heterogeneously expressed in different EC types. The gating of K_{ir} channels is also regulated by the $[\text{K}^+]_{ec}$, the increase of extracellular K^+ activates these channels, which leads to hyperpolarization, thus relaxation of the vascular smooth muscle (VSM). In addition to serving as extracellular K^+ sensors, these channels act as amplifiers of hyperpolarization initiated by the opening of other K^+ channels. K_{ir} channels also play an important role in shear-stress induced hyperpolarization of EC, whereas vasoconstrictors (angiotensin II, vasopressin, endothelin and histamine) have been reported to inhibit K_{ir} channels by G-protein dependent mechanism.

Ca^{2+} activated K^+ channels (BK_{Ca} , IK_{Ca1} and SK_{Ca}) are activated by the increase of $[\text{Ca}^{2+}]_i$ and have been described in freshly isolated EC and as well as EC in primary culture. The inhibition of these channels interferes with NO release, but understanding their physiological roles requires further studies.

2. Gap junctions in the vascular response

Endothelium gap junctions are frequently observed between EC of small and large arteries. Gap junctions are cell-to-cell high conductance low resistance channel that allow direct electrical and metabolic communication between EC, EC and smooth muscle cells (SMC) - myoendothelial junction-, and also between EC and lymphocytes/monocytes. Gap junctions form clusters of various hundreds of units in the EC membranes. These cluster formation facilitates interactions and enhances intercellular signaling. Signals for vasoconstriction and vasodilatation travel rapidly along the vessel network through gap junctions, thus EC as well as vascular SMC behave as an electrical syncytium.

3. Polarity of the vascular bed

Most of the described channels have only been detected in cultured or in freshly isolated EC, but very few measurements were done *in vivo* in blood vessels. It is known that ion channels in EC adapt their appearance to external conditions, and gene expression can vary with the cell isolation, culture and growth condition. Therefore the *in vivo* functional impact of the described channels is still a matter of controversy.

Recently several studies focused on the importance of membrane polarity of endothelial and epithelial cells. The exchange of substances between higher organism and the environment takes place across these cells. To perform this function EC have to form tight junctions that seal the extracellular space meanwhile they are polarized into an apical and a basolateral domain, with entirely different structural, biochemical and physiological properties. The functional properties of these cells depend on the differentiation and the appropriately polarized cell organization, which is reliant upon extracellular signals generated from cell-cell or cell-matrix interactions. To retain the ultrastructure of the original tissue, the ion transport properties or the gene expression, optimal conditions have to be provided. Hence the use of non-polarized, poorly differentiated primary cultures or immortalized cell lines is suboptimal for endothelial/epithelial cell research. Summarizing all the difficulties of vascular research and the complexity of EC's physiology, the aim of our study was to set up a method that allows identifying and characterizing the activity of ion channels present on EC '*in situ*', thereby avoid the alterations induced by cell isolation methods or cell culture.

II. Aims

A. *General aims*

In our study we focused on the characterization of ion channels on two different cell types, the human DC and the EC of the *arteria mesenterica superior* in rats. In both cases our goal was to characterize the ion channels under the most physiologic circumstances. Further, we aimed to compare the ion channel expression pattern on IDC, MDC and the DC model cell line KG-1 in unstimulated and stimulated state.

B. *Specific aim 1: Characterizing the ion channels expressed on IDC and MDC*

We aimed to identify and compare the expression and functional activity of voltage-gated ion channels in DC generated *in vitro* from human peripheral blood monocytes using patch-clamp technique. We hypothesized that differentiation/maturation of monocyte-derived DC is accompanied by a change in the ion channel repertoire, which could contribute to the distinct functional activities of these cells associated with their different maturation states. To test this hypothesis we carried out the following experiments:

1. **Characterization of ion channels on IDC and MDC:**

- i. performing whole-cell measurements on IDC and MDC and comparing the obtained currents
- ii. biophysical and pharmacological characterization of the obtained currents on IDC and MDC using whole cell patch clamp technique and high affinity toxins
- iii. confirmation of the presence of characterized channels on IDC and MDC using molecular biology techniques (real time Q-RT-PCR, Western blott)

2. **Characterizing the ion channels in a DC model KG-1:**

- i. performing whole-cell measurements on unstimulated and stimulated KG-1 cells and comparing the obtained currents
- ii. biophysical and pharmacological characterization of the currents on KG-1 cells

C. *Specific aim 2: In situ characterization of the ion channels expressed in EC*

We aimed to set up a method that allows identifying and characterizing the activity of ion channels present on EC '*in situ*' and thereby avoiding the alterations induced by cell isolation and culturing. We used single microvascular myograph to adjust the vessel diameter combined with patch clamp technique to measure the whole cell currents. The development of the method consisted of the following steps:

1. Vessel preparation and identification of EC for optimal electrophysiological measurements

2. Electrophysiological studies on EC

- i. to perform whole cell blind patch clamp experiments on EC layer and characterize the obtained currents (biophysical and pharmacological properties)
- ii. to study the effect of Ach and gap junction blockers on obtained current

III. Materials and Methods

A. *Dendritic cells*

1. **Dendritic cell preparation**

Human myeloid DC were generated from blood monocytes isolated from Buffy Coats. Monocytes were separated by positive selection with anti-CD14-coated magnetic beads from peripheral blood mononuclear cells. Purified monocytes cultured in the presence of IL-4, GM-CSF. Activation of IDC was induced by an inflammatory cocktail containing TNF- α , IL-1 β , IL-6, GM-CSF and prostaglandin E2. MDC were identified by flow cytometry using anti-CD83 mAb.

2. **Dendritic cell line KG-1 cell culture**

KG-1 cells were cultured under standard cell culture conditions and were stimulated with PMA and ionomycin for 4 days before the electrophysiological experiments.

3. **Protein Extracts and Western Blotting**

Dendritic cells lysates were prepared according to standard protocols in the presence of Pepstatin A, leupeptin, Aprotinin and phenylmethylsulfonyl fluoride protease inhibitors. The protein content of the supernatant was determined using Bradford protein assay. Samples were boiled in Laemmli SDS loading buffer and separated on 10% SDS-PAGE. Antibodies against Kv1.5 were used with anti- β -actin antibody as a loading and transfer control. The specificity of the Kv1.5 antibody was tested with a control antigen peptide provided by the manufacturer. Raw 264.7 macrophages and HEK-293 transiently transfected with Kv1.5 cDNA were used as positive controls. Protein biochemistry was done in Prof. Felipe's laboratory (Molecular Physiology Laboratory, Department of Biochemistry and Molecular Biology, Institute of Biomedicine, University of Barcelona, Barcelona, Spain).

4. **Electrophysiology**

Patch-clamping. Standard whole-cell patch-clamp techniques were used using Axopatch-200 and Axopatch-200A amplifiers connected to personal computers. Pipettes were pulled from GC 150 F-15 borosilicate glass capillaries in five stages and fire-polished to gain electrodes

of 2-3 M Ω resistance in the bath. Series resistance compensation up to 85% was used to minimize voltage errors and achieve good voltage clamp conditions ($V_{err} < 5mV$).

Solutions for DC. The normal bath or EC solution was (in mM): 145 NaCl, 5 KCl, 1 MgCl₂, 2.5 CaCl₂, 5.5 glucose, 10 HEPES (pH 7.35, 305 mOsm/kg). 145 mM choline-Cl substituted for NaCl in the choline-EC bath solution (pH 7.35, 305 mOsm/kg). NaCl was replaced by equimolar tetraethylammonium chloride (TEA) in the 10 mM TEA-EC solution. The composition of the pipette solution for current recording in MDC was (in mM): 140 KF, 11 K₂EGTA, 1 CaCl₂, 2 MgCl₂, and 10 HEPES (pH 7.20, ~295 mOsm/kg). This pipette solution was supplemented with 5 mM NaCl for recording currents in IDC.

Solutions for the KG-1 cells. The normal bath or EC solution and the KF-based pipette filling solution were the same as for MDC. The composition of the 1 μ M [Ca²⁺]-containing pipette solution was (in mM): 130 K-aspartate, 10 K₂EGTA, 8.7 CaCl₂, 2 MgCl₂, and 5HEPES (pH 7.20, 295mOsm).

Test substances for DC or KG-1 recording. Toxins (tetrodotoxin, charybdotoxin and margatoxin) were dissolved in the standard EC solution supplemented with 0.1 mg/ml BSA

5. Electrophysiological protocols and data analysis

Voltage protocols with appropriate amplitude and duration were constructed to allow the determination of the kinetic and equilibrium parameters of gating of the voltage-gated K⁺ and Na⁺ channels. The whole-cell current traces or the peak currents obtained using these protocols were analyzed by fitting appropriate model functions to the data points and the fitted parameters of the model equations were used to characterize the biophysical process of gating as follows. A non-linear least square algorithm (Levenberg Marquard) was used for the fitting procedures. Successful fit was confirmed by visual comparison of the data and the fitted function and the analysis of the sum of the squared errors.

The inactivation kinetics of the Na⁺ and the K⁺ current was characterized by $\tau_{in,x}$, the inactivation time constant for either the Na⁺ ($\tau_{in,Na}$) or the K⁺ ($\tau_{in,K}$) current, determined by fitting a single exponential function to the decaying part of the currents.

Recovery from inactivation was characterized by τ_r , the time constant for recovery from inactivation, as determined from fitting a single exponential rising function to the peak currents obtained from a conventional two-pulse protocol.

Voltage-dependence of steady-state activation the conductances were characterized using the membrane potentials at which 50% of the channels are activated. These membrane potentials were determined either from the current-voltage relationships ($V_{m,a}$ for the Na^+ current) or from the conductance-voltage relationships ($V_{1/2}$ for the K^+ current). Voltage-dependence of steady-state inactivation was characterized by the membrane potential ($V_{m,i}$) at which 50% of the channels is not inactivated at equilibrium. To obtain the values of $V_{1/2}$ and $V_{m,i}$ the Boltzmann function was fitted to the data points.

Whole-cell IK_{Ca1} currents in KG-1 cells were studied using voltage steps and voltage ramps with a pipette solution to result in $1\mu\text{M}$ free Ca^{2+} concentration.

Dose-response curves of current inhibition by various toxins were analyzed using the Hill equation assuming 1:1 channel-toxin stoichiometry.

6. Molecular Biology

The DNA, encoding a conserved region of the Na^+ channels was amplified from IDC and MDC cDNA with polymerase chain reaction (PCR), using high fidelity *Pfu* polymerase (Promega) and degenerate primers:

5'-GATTTCCAGGGAGATAAGACAAGCAG-3' and
5'-GAAGCAGAGGCTGAACCTATGAA TT-3'.

Real time Q-RT-PCR was used to quantify the expression of voltage-gated ion channel genes in IDC and MDC. The relative amounts of individual channel transcripts are given in relation to those of 36B4. A "no-RT control" with untranscribed RNA and a "no-template control" with water were performed parallel in all experiments. Each series of experiments were performed in triplicates. The relative expression of the target gene was calculated using the comparative method ($2^{-\Delta\Delta\text{Ct}}$). Cloning and RT PCR experiments were done in Prof. Rajnavölgyi's laboratory in the Dept. of Immunology, University of Debrecen.

B. Endothelial cells

1. Mesenteric artery preparation

The mesenteric vascular bed of adult male Wistar rats (250-300g) were used. Animals were handled according to the rules of the European Union. Vascular segments were mounted as ring preparations (*ca.* 2 mm in length) on a single wire myograph onto two 40 μm wires, by fixing one wire to an isometric force transducer and the second wire to a micrometer screw. Vessel segments were continuously superfused with PSS (37°C). The PSS was equilibrated

with a mixture of 95% O₂ and 5% CO₂, resulting a pH of 7.4. A U-shape was cut out on the dorsal wall of the arterial segment at one end in order to allow direct access to the EC layer underneath. The the internal circumference of the vessel was adjusted to give optimal wall tension for maximum response of the vessel. .

2. “In situ” patch-clamp recording

Macroscopic K⁺ currents were recorded ‘*in situ*’ from EC under whole-cell configuration of the patch-clamp technique. A mechanical was attached to the myograph stage, allowing ‘*in situ*’ recording of the EC currents with patch clamp microelectrodes via the U-shaped cut. The electrode was connected to an Axon Multiclamp 700A amplifier. Pipette resistance was in the range of 4-8 MΩ. Seal resistance was ≈5 GΩ. We corrected routinely for the liquid junction potential. Cell capacitance and series resistance ranging from 10 MΩ to 20 MΩ were automatically compensated through the amplifier’s circuitry.

Macroscopic K⁺ currents were evoked by either families of hyper- or depolarizing steps, over 400 ms in 20 mV increments, either from -60 mV to -200 mV or from -60 to 140 mV, respectively. Pulses were separated by 10 s intervals at the holding potential of -60 mV..

3. Intracellular injection of neurobiotin

In order to ensure that we were recording from EC and for identification purposes, EC were iontophoretically filled with the marker neurobiotin Neurobiotin was iontophoresed into the cell by injecting positive square current pulses in whole-cell clamped cells. After labeling the cell for approximately 15 min, the patch electrode was detached from the cell, the arterial segment was slit open, fixed with paraformaldehyde, subsequently incubated in avidin-biotin complex solution andwith 0.05% diaminobenzidine in the dark. Neurobiotin was visualized after adding H₂O₂ to the solution.

4. Statistics

We obtained electrophysiological data from 61 EC *in situ*. Unsuccessful experiments and cells with incomplete data are not included in the analysis. Depolarizing and hyperpolarizing currents were measured and normalized for the maximal currents obtained in control conditions (I/I_{max}) using GraphPad software..The Remaining Fraction of the current (RF) was calculated as $RF=I/I_0$ where I and I_0 are the peak currents in the presence and absence of a blocker, respectively, at a given membrane potential. The results are expressed as means ± SEM of at least four independent measurements done under identical conditions, unless otherwise indicated. Differences between means were analyzed using Student’s *t*-test for

paired and unpaired observations as appropriate with differences considered statistically significant at $p < 0.05$.

5. Solutions and drugs for EC electrophysiology

The composition of the extracellular PSS was (in mM): 119 NaCl, 4.6 KCl, 1.2 MgCl₂, 24.9 NaHCO₃, 11 glucose, 1.5 CaCl₂, 1.2 KH₂PO₄ and 0.027 EDTA (ethylenediaminetetraacetic acid). The PSS was continuously superfused at a rate of 3ml min⁻¹. In order to investigate the expression of the different K⁺ currents the composition of the IC pipette solution was (in mM): 141 KCl, 5 Na₂ATP, 0.6 CaCl₂, 3 MgCl₂, 10 HEPES (N-2-hydroxyethylpiperadine-*N*-2-ethanesulphonic acid) and 0.1 EGTA (ethyleneglycol-*bis*-(β-aminoether) *N*, *N*, *N'*, *N'*-tetraacetic acid), titrated to pH 7.4 with Tris base.

Drugs used. Acetylcholine HCl (ACh), apamin, barium chloride, 18β-glycyrrhetic acid (18β-gly), iberiotoxin (IbTx), tetraethylammonium (TEA), TRAM34 ([1-[(2-chlorophenyl)diphenylmethyl]-1H-pyrazole) and TRIS-Cl, stock solutions were prepared in distilled water except TRAM34, which was first dissolved in dimethyl sulfoxide and further diluted in distilled water. The final concentration of dimethyl sulfoxide was <0.1%, a concentration which did not affect the electrophysiological activity of EC.

IV. Results

A. *Dendritic cells*

1. **TTX-sensitive Na⁺ current in immature dendritic cells**

Whole-cell currents were measured in IDC in response to a voltage-ramp from -100 mV to $+50$ mV. Marked inward current at membrane potentials more depolarized than -30 mV were registered. To clarify the nature of the charge carrier, ion substitution experiments were performed by changing the normal extracellular bath solution for Na⁺-free choline extracellular solution. The inward current recorded at 0 mV test potential in the Na⁺-containing solution completely disappeared in the Na⁺-free choline based solution. The loss of the Na⁺ current in choline-extracellular solution was quickly reversed by perfusing the bath with normal extracellular bath solution. These experiments demonstrated that the decrease of the inward current is due to the replacement of Na⁺ with the impermeable monovalent choline ion, and thus, strongly argue for the presence of Na⁺ channels in the membrane of IDC. The peak Na⁺ current density at 0 mV test potential was quite variable ranging between -15.1 pA/pF and -110 pA/pF with median and mean current densities of -56.8 pA/pF and -61.9 ± 7.3 pA/pF ($n=20$), respectively.

To characterize the Na⁺ current in IDC we utilized TTX. By adding 100 nM TTX to the normal EC bath the peak current was reduced to $\sim 37\%$ of the control and recovered to the control value within a few pulses following a switch to the toxin-free normal-EC bath. The effect of TTX was concentration dependent and the dose-response data were fit with the Hill equation resulting in a K_d value of 55 nM, thus confirming that inward current detected in IDC is mediated by TTX-sensitive voltage-gated Na⁺ channels.

2. **Biophysical characterization of the Na⁺ current in immature dendritic cells**

Na⁺ channel expressed in IDC were further characterized by determining the biophysical characteristics. Families of depolarizing currents were recorded in IDC from the holding potential of -120 mV to various test potentials. The peak current at each test potential was determined and plotted as a function of the test potential. The peak current-voltage relationship showed that the activation threshold of the current is around -50 mV, and the current reverses close to $+60$ mV. The current-voltage relationship was used to characterize the voltage-dependence of steady-state activation of the Na⁺ channels. Parameters describing

the voltage-dependence of steady-state activation were the test potential at which 50% of the Na^+ channels are activated (midpoint, $V_{m,a}$) and the slope of voltage-dependent gating (s_a). Characteristic values were obtained by fitting individual I-V relationships cell-by-cell and averaging the obtained values. This resulted in $G=4.1\pm 1.2$ nS, $E_{rev}=+64.4\pm 4.2$ mV, $V_{m,a}= -19.8 \pm 3.0$ mV and $s_a= 10.0\pm 0.7$ mV ($n=5$).

The voltage dependence of steady-state inactivation was studied using a conventional pre-pulse protocol. The cell was held at different pre-pulse potentials (V_p) for 5s and then depolarized to 0 mV to evoke whole-cell Na^+ currents with magnitudes proportional to the fraction of Na^+ channels not inactivated by the preceding pre-pulse. The peak currents normalized to the maximal values were plotted vs. V_p cell-by-cell, and the Boltzmann function was fitted to the data points. The characteristic values of the voltage-dependence of steady-state inactivation, i.e., the membrane potential at which 50% of the channels are inactivated was $V_{m,i}= -87.6\pm 3.3$ mV ($n=3$), and the slope was $s_i= -5.1\pm 0.1$ mV ($n=3$).

The inactivation kinetics of the Na^+ current was characterized by the inactivation time constant ($\tau_{i,\text{Na}}$). The $\tau_{i,\text{Na}}$ was determined by fitting a single exponential function to the decaying part of the currents, which demonstrated that inactivation kinetics of the current is getting faster with increasing depolarization.

The kinetics of recovery from inactivation was studied using a conventional two-pulse protocol, where the time between two consecutive depolarizing pulses (IPI) was gradually increased thereby resulting in larger and larger currents during the second pulse. The recovered fraction (RF) of the current as a function IPI was plotted for individual cells and a single exponential function was fitted to the individual datasets, resulting in the time constant for recovery from inactivation of $\tau_r=4.9\pm 0.9$ ms ($n=3$).

Depolarization up to +100 mV and 50 ms in duration failed to elicit any other type of inward or outward current under our experimental conditions ($n>20$).

3. Outward K^+ current in mature dendritic cells

The activation of IDC by an inflammatory cytokine cocktail induced a dramatic change in the electrophysiological properties of the cells. The voltage ramp experiment showed that the inward current characteristic of IDC disappeared in MDC, furthermore, a voltage-gated outward current is activated at membrane potentials more depolarized than -30 mV.

To characterize the outward current 800-ms-long step depolarization pulses to different test potentials from a holding potential of -120 mV in every 90 s were used. This demonstrated that the activation threshold of the obtained current is between -40 and -30 mV, the current quickly activates and almost completely inactivates with a relatively slow kinetics. The leak-

corrected peak current at each test potential was determined and plotted as a function of the test potential. The I_p -V relationship showed that extrapolated reversal potential of the current is more negative than -60 mV, and that the voltage-dependence of the activation of the channels is very steep above -40 mV. Based on these characteristics the expression of voltage-gated K^+ channels was predicted in MDC. The peak K^+ current density at $+50$ mV test potential varied between 6.7 pA/pF and 111.9 pA/pF with median and mean current densities of 38.6 pA/pF and 48.1 ± 9.4 pA/pF ($n=13$), respectively.

The I_p -V relationships were used to calculate the K^+ conductance of the membrane at each test potential and the normalized conductance-test potential (G_{norm} -V) relationships were generated for each cell individually and the Boltzmann function was fitted to the data points. The fits resulted in -25.0 ± 0.8 mV for the midpoint and 5.2 ± 0.4 mV for the slope of the voltage-dependence of steady state activation ($n=4$).

The whole-cell K^+ current in MDC displays inactivation, the current decay was the best fit with a single exponential function. The resulting time constants ($\tau_{i,K}$) were used to characterize inactivation kinetics of the K^+ current. $\tau_{i,K}$ at $+50$ mV was 127.4 ± 9 ms ($n=3$), the inactivation kinetics showed negligible voltage-dependence at membrane potentials more positive than -10 mV.

Based on these and the predominant expression of Kv1.3 channels in various cell types of the immune system we hypothesized that Kv1.3 channels may be expressed in MDC. To test this hypothesis we applied peptide and non-peptide ion channel blockers.

4. Pharmacological characterization of the outward K^+ current

Kv1.3 channels inactivate by the slow (P/C-type) inactivation mechanism which is characterized by the ability of extracellularly applied TEA to inhibit the current and to slow the inactivation process simultaneously by the foot-in-the-door mechanism. This feature is demonstrated as the whole-cell K^+ current is reduced to $\sim 55\%$ of the control in the presence of 10 mM TEA, the single-point estimate of the equilibrium dissociation constant is 12.4 ± 0.7 mM ($n=3$). The inactivation kinetics of the current was slower in the presence of TEA, the time constants were 189 ms for control and 282 ms in the presence of 10 mM TEA.

Two peptide toxins with different affinities and selectivities for Kv1.3, ChTx and MgTx, were used in subsequent pharmacological experiments. The effect of the of 2 nM ChTx was reversible, the peak current was reduced to $\sim 50\%$ of the control upon perfusion with ChTx-containing normal-EC bath and recovered to the control value following a switch to the toxin-free normal-EC bath. ChTx reduced the K^+ current in a dose-dependent manner with an equilibrium dissociation constant of 3.4 nM. The inhibition of the whole-cell K^+ current by MgTx, a Kv1.3 specific toxin, were recorded 300 s after the start of the perfusion with toxin-

containing (50 pM) extracellular solution. The dose-response of the current inhibition by MgTx was then fitted with the Hill equation. The resulting equilibrium dissociation constant was 39.8 pM, which is characteristic of the inhibition of Kv1.3 channels. The wash-out of the toxin was extremely slow requiring 20-30 min for significant recovery from block.

Based on the biophysical and pharmacological data we propose that the channel responsible for the outward K⁺ currents in MDC is Kv1.3.

5. Identification of the electrophysically characterized voltage-gated Na⁺ channel of immature dendritic cells

A PCR-based strategy was used to identify the TTX-sensitive VGSC responsible for the Na⁺ current detected in the membrane of IDC. Prior to this study, no Na⁺ channel-related DNA sequences of DC were known, thus we probed a cDNA library using degenerate primers based on a conserved sequence common to all VGSCs. Using these degenerate primers, a 387-bp portion of the gene was amplified, cloned and sequenced. The nucleotide sequence of five out of twenty cloned constructs fitted perfectly to the human gene SCN9A (GenBank accession number NM_002977), which encodes for the voltage-gated Na⁺ channel 1.7 α subunit. Sequences corresponding to Nav channels other than Nav1.7 were not found in either of the clones. These findings indicate that the molecular identity of the channels responsible for the inward Na⁺ currents in of IDC is Nav1.7.

6. Expression of mRNAs for voltage-gated ion channels in differentiating monocyte-derived dendritic cells

In order to quantify the relative expression of the identified voltage-gated ion channels we measured mRNA expression levels by real time RT-PCR in IDC and MDC activated by an inflammatory cocktail. As several cell types of myeloid origin also express Kv1.5 channel subunits the mRNA expression level for Kv1.5 was also determined along with that of the Nav1.7 and the Kv1.3 channel. To compare the relative expression of the RNA transcripts in IDC and MDC the data were normalized to the expression of the housekeeping gene 36B4. In line with the electrophysiological results the expression of Nav1.7 mRNA was the highest in IDC, whereas the MDC expressed Kv1.3 transcripts. Kv1.5 mRNA transcripts were also isolated from both IDC and MDC, however, at a lower level, with no significant change in the expression during differentiation. The presence of Kv1.5 mRNA and the functional absence of Kv1.5 subunits in the current records indicated Western blot experiments to assess the expression of this subunit in DC of various differentiation states. Western blot experiment

showed that the expression of the Kv1.5 subunit relative to that of β -actin is similar in both IDC and MDC.

7. Electrophysiological characterization of KG-1 cells

Whole-cell currents were recorded in response to depolarizing pulses from a holding potential of -80 mV to different test potential ranging from -70 to $+50$ mV in 10 mV increment in unstimulated and stimulated KG1-cells. The currents recorded in unstimulated and stimulated cells were qualitatively similar; they did not exhibit time-dependent gating. The reversal potential of the current was determined using KF-based solution and was approximately 0 mV in both cell types which is characteristic to a non-specific leak current.

Using KF-containing internal solution did not allow the recording of K_{Ca} currents since the free Ca^{2+} concentration was in the nanomolar range in this solution. Therefore, we substituted fluoride ions with aspartate, and adjusted the EGTA and Ca^{2+} concentration in the pipette solution to result in 1μ M free Ca^{2+} concentration during the measurement of K_{Ca} currents. The whole-cell currents evoked by voltage ramps reversed between -50 and -60 mV when K-aspartate internal solution was used in both cells types. This value was close to the equilibrium potential for K^+ (-83 mV, calculated using the Nernst equation), which indicates that the whole-cell current is dominated by a K^+ selective conductance in this case. The deviation of the reversal potential from the theoretical value is due to the leak current: the reversal potential for leak current is 0 mV, which shifts the reversal potential of the whole-cell current to depolarized potentials.

Whole-cell currents of unstimulated and stimulated KG-1 cells were then reversibly blocked by 10 nM ChTx, a peptide inhibitor of several voltage-gated and K_{Ca} channels. The block of K_{Ca} channels resulted in a diminished slope, but the current returned close to the control level after washing the recording chamber with toxin-free extracellular solution. The magnitude of the K_{Ca} conductance calculated from the slope of the currents was relatively small in both cell types' ~ 1.9 nS. In summary, the time- and voltage-independent but Ca^{2+} -dependent activation of the current and its sensitivity to ChTx point to the presence of K_{Ca} channels in both unstimulated and stimulated KG-1 cells.

B. Endothelial cells

1. Identification of EC with the diffusion of neurobiotin

In order to assure that the recorded currents were from EC and that these cells maintained their connection with their adjacent cells via gap junctions, the marker neurobiotin as a tracer was used. Injection of positive current (10 pA during 500 ms) was used to facilitate the diffusion of neurobiotin from the patch pipette into the cell under current clamp.

By using neurobiotin we showed that EC were homogeneously stained, indicating that they are electrically coupled to each other in both circumferential and longitudinal directions, thus creating a functional unit. Diffusion of neurobiotin from EC to VSMC was not observed, which is consistent with previous studies, that there is no heterocellular dye coupling between EC and VSMC in arterioles.

2. Identification of K_{ir} currents in EC

We tested whether K_{ir} channels are expressed on intact EC. Families of hyperpolarizing voltage pulses from -60 mV to -200 mV ($V_h = -60$ mV) induced rectifying inward currents in intact EC. Hyperpolarizing voltage pulses induced rectifying non-inactivating inward currents in mesenteric EC. The pharmacological properties of the current were tested using a well-known blocker of K_{ir} , Ba^{2+} . Incubation with $30 \mu\text{M}$ Ba^{2+} inhibited the inward currents from -1148 ± 189 to -573 ± 211 pA at -200 mV, ($p=0.008$, $n=8$) and decreased current density from -34 ± 7 to -20 ± 3 pA/pF at -200 mV, ($p=0.018$, $n=8$) indicating that K_{ir} channels are expressed on intact rat mesenteric EC.

3. Calcium activated potassium channels

Endothelial K_{Ca} have been reported in the rat superiormesenteric artery, thus we tested whether outward K_{Ca} currents are expressed in intact EC as well. Cells were held at -60 mV and were subjected to families of depolarizing voltage pulses from -60 mV to 140 mV for 400 ms in 20 mV increments. Depolarizing pulses induced outwardly directed currents in mesenteric intact EC recorded '*in situ*'. The voltage steps elicited non-inactivating outward currents up to test potentials of 140 mV. In order to investigate the nature of the currents we used TEA (10 mM), which significantly decreased the amplitude of these outward currents from 1011 ± 168 to 559 ± 123 pA at 140 mV ($p=0.028$, $n=5$) and the current density from 35 ± 7 to 16 ± 4 pA/pF at 140 mV ($p=0.004$, $n=5$) indicating that K_{Ca} channels are expressed on intact EC.

We further characterize the different types of K_{Ca} channels using IbTx, TRAM34 and apamin, specific blockers of BK_{Ca} , IK_{Ca1} and SK_{Ca} currents, respectively. Blockade of SK_{Ca} channels with apamin ($0.5 \mu\text{M}$) decreased the amplitude of the currents from 453 ± 51 to 206 ± 74 pA at 140 mV ($p=0.004$, $n=6$) and current density from 29 ± 6 to 12 ± 5 pA/pF at 140

mV ($p=0.004$, $n=6$). Blockade of IK_{Ca1} channels with $0.1 \mu\text{M}$ TRAM34 reduced the outward currents from 1129 ± 238 to 479 ± 178 pA at 140 mV ($p=0.002$, $n=5$) and current density from 29 ± 7 to 9 ± 3 pA/pF at 140 mV ($p=0.027$, $n=5$). The blockade of BK_{Ca} currents with IbTx ($0.1 \mu\text{M}$) also had an inhibitory effect on the currents induced by depolarizing steps. IbTx decreased the amplitude of the outward currents from 1040 ± 93 to 639 ± 75 pA at 140 mV pA ($p=0.009$, $n=4$) and current density from 36 ± 4 to 23 ± 5 pA/pF at 140 mV pA/pF ($p=0.004$, $n=4$).

4. Effect of ACh on outward currents

It has been shown previously that ACh increases $[\text{Ca}^{2+}]_i$ by triggering Ca^{2+} release from the intracellular stores, evokes EC hyperpolarization by activation of membrane K_{Ca} channels, which latter promotes Ca^{2+} influx through increasing the electrochemical driving force for Ca^{2+} . The rise of $[\text{Ca}^{2+}]_i$ will result in NO synthesis and release in EC, which will promote relaxation in the underlying VSMC.

We tested whether ACh has an effect on the whole cell currents described above. Cells were held at -60 mV and were subjected to either depolarizing voltage pulses from -60 mV to 140 mV over 400 ms in 20 mV increments or to depolarizing ramps from -60 to 140 mV over 100 ms. Under control conditions, depolarizing voltage pulses or ramps induced non-inactivating outward current. ACh ($10 \mu\text{M}$) induced a significant increase in the amplitude of the outward currents from 689 ± 122 to 995 ± 170 pA at 140 mV ($p=0.007$, $n=14$) and in current density from 25 ± 2 to 35 ± 3 pA/pF at 140 mV ($p=0.001$, $n=14$). Incubation of the artery with 10 mM TEA significantly inhibited the ACh induced current: it reduced the amplitude from 995 ± 170 to 399 ± 137 pA at 140 mV ($p=0.039$, $n=5$), and decreased current density (from 35 ± 3 to 14 ± 6 pA/pF at 140 mV, $p=0.001$, $n=14$, $n=5$, unpaired t-test). This indicates that K^+ channels are responsible for the ACh-mediated increase in outward currents, which may lead to NO release and thus endothelial cell hyperpolarization.

5. Effect of 18β -gly on inward and outward K^+ currents

Neurobiotin staining demonstrated that EC are electrically coupled to each other through gap junctions, which may facilitate electrical coupling between these cells. Thus, we tested whether homocellular gap junctions facilitate electrical spreading of the inward and outward K^+ currents. To block gap junction communication between EC we used 18β -gly. Cells were held at -60 mV and subjected to either hyperpolarizing pulses from -60 mV to -200 mV over 400 ms in -20 mV increments or depolarizing voltage pulses from -60 to 140 mV over 400 ms in 20 mV increments ($V_h=-60$ mV). 18β -gly ($25 \mu\text{M}$) decreased the inward and outward current amplitude (from -1032 ± 122 to -622 ± 126 pA at -200 mV, $p=0.001$, $n=7$;

and from 1078 ± 176 to 526 ± 199 pA at 140 mV, $p=0.007$, $n=6$; respectively). Moreover, when data were expressed as current density, 18 β -gly had not any significant effect (-29 ± 3 vs -25 ± 3 pA/pF at -200 mV, $p=0.369$, n.s., $n=7$; and 31 ± 3 vs 33 ± 6 pA/pF at 140 mV, $p=0.409$, n.s., $n=6$, respectively)

V. Discussion

A. Dendritic cells

Differentiation and maturation of DC from monocyte is linked to marked phenotypic, gene expression and functional changes. Here we describe a dramatic change in the plasma membrane expression of two specific ion channels i.e. Nav1.7 and Kv1.3 in the course of monocyte-derived DC maturation. First time in the literature we demonstrated the presence and activity of voltage-gated Na⁺ channels in the plasma membrane of IDC. The identification of the channel being responsible for the whole-cell current required both electrophysiological and molecular biological approach. Using PCR-based cloning we identified the Na⁺ channels expressed in IDC as Nav1.7 encoded by the SCN9A gene, the cloned sequence did not match the sequence of any other VGSC. The Nav1.7 channel is classified as TTX sensitive; it is inhibited by nanomolar concentrations of TTX. Our results are consistent with this, however, the affinity of the channels for TTX is somewhat lower ($K_d=55$ nM) than reported by Klugbauer et al for human Nav1.7 expressed in oocytes ($K_d=25$ nM) and much lower than for rat Nav1.7 ($K_d=4$ nM). Although we do not know the explanation for these differences it must be noted that the affinities of toxins for ion channels obtained in different expression systems vary significantly.

Regarding the biophysical parameters of gating, the previously published values for the voltage-dependence of steady-state activation of Nav1.7 are between -20 and -25 mV for the $V_{m,a}$, regardless of the expression system used, and our results in IDC are compatible with those ($V_{m,a}=-19.8 \pm 3.0$ mV). Fast inactivation kinetics of Nav1.7 is characterized by time constants in the order of 1 ms at voltages where the current is maximal (-10 to 0 mV), however, biphasic current decay has also been reported. This fast inactivation kinetics has only been achieved by co-expressing the Nav1.7 subunits with the auxiliary β_1 subunit, which accelerates the inactivation kinetics as much as 10-fold. The fast inactivation kinetics reported in this study (0.70 ± 0.09 ms at -10 mV) indicated that Nav1.7 might be in complex with a β_1 subunit in IDC, furthermore, the small size of IDC allow better voltage-clamp conditions as compared to oocytes, thereby resulting in apparently faster kinetics. The

midpoint for the voltage-dependence of steady-state inactivation ($V_{m,i}$) obtained in this study is slightly more negative than reported by Cummins et al. (-73.6 mV), but significantly more negative than -60.5 mV reported by Klugbauer et al. for the human Nav1.7 expressed in HEK cells in both studies.

The α subunits of the Nav1.7 channel are expressed in the dorsal root ganglion neurons, sympathetic neurons, Schwann cells and neuroendocrine cells. Their physiological functions include action potential initiation and transmission in peripheral neurons in response to nociceptive stimuli. These channels are mainly responsible for mechanosensation and for the development of inflammatory pain. IDC reside primarily within or beneath of epithelial surfaces such as the skin, bronchial and gastrointestinal mucosal layers and are also concentrated at peripheral nerve endings and endoneurium, which may suggest a possible relationship between nociceptive stimuli and DC functions.

We also demonstrated that IDC challenged by inflammatory stimuli changed the cell surface expression of ion channels, and thus the MDC are characterized by another set of specific channels. Real time RT-PCR demonstrated the expression of transcripts for Kv1.3 and Kv1.5 in MDC. This raises the possibility that a mixture of Kv1.3 and Kv1.5 homotetramers and Kv1.3/Kv1.5 heterotetramers might be responsible for the whole cell currents observed in MDC, similarly to bone marrow-derived macrophage and MDC of the central nervous system. Based on the following considerations we argue that Kv1.3 determines the properties of the whole-cell currents in our study. The whole-cell current displays single-exponential inactivation kinetics with a time constant of ~ 127 ms at $+50$ mV, which is characteristic of Kv1.3. Kv1.5 homotetrameric channels, on the contrary, have very slow and biphasic inactivation kinetics with time constants of ~ 450 ms and 5s, respectively. Kv1.3/Kv1.5 heterotetramers should have intermediate inactivation kinetics depending on the subunit stoichiometry and calculated from the cooperative interaction between subunits. Thus, the whole-cell current carried by five different species of channels (homotetrameric Kv1.3 and Kv1.5 and three types of heterotetramers) should have complex decay kinetics with slow components in it, which was clearly not the case for whole-cell currents in MDC. In addition, homotetrameric Kv1.5 channels are ~ 30 -fold less sensitive to TEA than Kv1.3 homotetramers. As all four subunits contribute equally to the TEA binding site Kv1.3/Kv1.5 heterotetramers should have lower affinity for TEA than Kv1.3 homotetramers. Again, our results show the contrary, i.e., $\sim 50\%$ of the whole cell current is inhibited by 10 mM TEA, which is characteristic of Kv1.3 channels.

Kv1.5 channels are resistant to inhibition by scorpion toxins used in this study (ChTx, MgTx). Kv1.3/Kv1.5 heterotetramers are less sensitive to MgTx inhibition, depending on the subunit stoichiometry 3 to 100- fold decreases in the affinity was reported by Felipe and colleagues. Thus, a significant proportion of Kv1.5 homo- or heterotetramers would result in

a residual toxin-insensitive current even at a high toxin concentration, which otherwise blocks almost completely Kv1.3 channels. In contrast, we measured higher than 90% blockage of the whole cell current at 50 nM ChTx concentration and a full block at 500 pM MgTx concentration. The presence of the mixture of homo- and heterotetrameric channels would result in a significant deviation of the dose-response relationship from a model in which a single species of channels interacts with a single inhibitory peptide. On the contrary, our dose-response relationships showed K_d values of 3.4 nM and 39.8 nM, for ChTx and MgTx, respectively, which values are characteristic for inhibition of Kv1.3, and fits perfectly to the dose-response relationships, thereby arguing against the existence of Kv1.5 homotetramers and Kv1.3/Kv1.5 heterotetramers in MDC. In conclusion, although mRNA specific for Kv1.5 can be found in both IDC and MDC and Western blot analysis confirmed the expression of Kv1.5 subunits in these cells the protein expression level is too small to affect the properties of whole cell current.

We can exclude the contribution of IK_{Ca1} channels (the Ca^{2+} -activated K^+ channel, which is expressed in many lymphoid cells) to the whole-cell current by the lack of the elevated cytosolic free Ca^{2+} concentration required for the activation of these channels (i.e., the pipette filling solution contained 11 mM EGTA and 140 mM F^-).

Based on these arguments we conclude that Kv1.3 channels are responsible for the whole-cell outward current in MDC, and the properties of the current agree with those reported in the literature for Kv1.3 in human T cells. The expression of Kv1.3 channels is important in the activation of macrophages, where their blockade inhibits the secretion of TNF- α and IL-8 cytokines. Brain macrophages express another type of voltage-gated channel, the Kv1.5, which was proven not to be functional in bone marrow-derived macrophages. The oligomeric composition of functional VGPCs could have a crucial effect on intracellular signaling events, determining the macrophage-specific response upon different physiological stimuli. The presence an outward rectifying K^+ conductance was described in dendritic cells isolated from murine spleen, but the ion channel responsible for that current was not identified. A recent study showed the existence of Kv1.3-like and Kv1.5 currents in MDC of the central nervous system. The lack of a Kv1.5 current in monocyte-derived MDC and its presence in MDC in the central nervous system raises the question whether Kv1.5 expression could be linked to a special function and distribution of these latter cells.

The CD34⁺ erythroleukemia cell line KG-1 has been intensively studied as a potential model of IDC, which can be stimulated with various cytokine cocktails that result in DC-like phenotype and function. Even though there are many phenotypic and functional similarities described between DC and KG-1 cell line, we demonstrated several similarities and

differences between these cells. Although stimulated KG-1 cells were able to induce T cell proliferation and TCR-mediated apoptosis and secrete inflammatory cytokines nearly as efficiently as monocyte derived DC unstimulated KG-1 cells were less efficient to internalize both soluble and particulate materials compared to IDC. This latter was attributed to decreased phagocytic capacity and more restricted expression of receptors mediating internalization of special ligands such as immune complexes or apoptotic bodies. Our electrophysiological assay also revealed striking differences in the ion channel expression of DC and KG-1. We showed biophysically and pharmacologically that the $I_{K_{Ca1}}$ type Ca^{2+} -activated K^+ channel is expressed in both unstimulated and stimulated KG-1 cells. On the contrary, the voltage-gated Nav 1.7 and Kv1.3 channels which were the characteristic channel-type of IDC and MDC, respectively, were not present on the KG-1 model cell line. These data altogether suggest that KG-1 cell line may be used as a model of professional myeloid antigen presenting cells owing to its overlapping phenotypic and functional characteristics with DC, however their ion channel expression profile was markedly different from DC. This sets limitations to the interpretation of the data obtained using KG-1 regarding the factors influencing IDC→MDC maturation and physiological functions of IDC.

In summary, we demonstrated that the inflammation-induced maturation of monocyte-derived DC is accompanied by a substantial change in the cell surface expression of VGSCs. Such alterations are common in the course of the terminal differentiation of immune cells, and the identification of two DC-related ion channels with restricted expression and linked but opposing regulation in MDC may have an impact on the targeted modulation of the cross-talk of immune cells. The Nav1.7 channels are known to be abundantly expressed in the peripheral nervous system, but their presence in the plasma membrane of IDC is unique in the immune system. As VGSCs of non-excitabile immune cells are considered to play a role in phagocytosis and migration, that are principal functions of IDC, the selective blockade of VGSCs can be a useful tool to modulate MDC functions. The presence of Kv1.3 channels in MDC is not surprising as Kv1.3 is probably responsible for adjusting membrane potential, and through this may control Ca^{2+} signaling pathways during the activation of these cells. Further investigation of the specific function of these channels, as well as the regulation of intracellular Ca^{2+} signaling during the maturation process may open up new avenues for targeting DC-directed T lymphocyte activation, polarization and differentiation for immunomodulation.

B. Endothelial cells

Apart from the existence of numerous different studies describing ion-channels in EC, nowadays, the question about the role of these channels in vascular biology is still open. Ion channels can serve as molecular targets for new medications, however to be able to use molecules acting on ion channels properly, we need to define clearly how these channels function under physiological circumstances. Knowing the fact how EC changes its ion channel and surface molecule expression in response to a slight change in the surrounding environment, in the future we need to focus on the development of new experimental setups, that would take us closer to physiological conditions. Physiological conditions do not simply mean the culture EC with VSMC or with extracellular matrix, but there are several other factors that have to be considered during measurements. For instance mechanical forces associated with blood flow play an important role not just in the regulation of vascular tone, but also in vascular remodeling, and in the development of atherosclerosis.

Our experiments showed the expression and characteristics of different K^+ currents recorded '*in situ*' from the EC layer of the rat superior mesenteric artery. Many electrophysiological studies have been conducted on EC, but our goal was to provide an experimental approach that would lead to a better understanding of vascular electrophysiology by creating far more physiological circumstances. We demonstrated that using the patch clamp technique we were able to record ionic currents in a tissue, rather than in isolated EC. We set up and optimized the microvascularmyograph for the studied vessels, where EC maintained their connections with each other as well as with the underlying VSMC. The use of this set up might have overcome the potential alterations of ion channel expression induced by cell isolation or culturing.

Neurobiotin staining proved that we are recording currents from EC and that EC remained electrically coupled through gap junctions to form a functional syncytium. Diffusion of neurobiotin from EC to VSMC was not observed, however the absence of neurobiotin dye transfer to VSMC does not preclude electrical coupling between both cell types. Due to the low optical resolution of the experimental setup we could not accurately measure the size of single EC, but in previous studies it was measured to be around 20-40 μm .

Inhibition of gap junction channels with 18 β -gly resulted in a significant decrease in the amplitude of inwardly and outwardly directed currents, which argues for a network of electrically coupled EC. Since gap junction functions as a low resistance - high conductance pathway, the EC layer form a functional syncytium, coordinating membrane potential changes along a segment of a vessel, thus producing uniform cellular responses. Longitudinal signaling may be complemented by radial movement of currents between EC and VSMC cells via MEGJ located at the EC-VSMC interface and coordinates vasomotor tone.

By using the '*in situ*' patch-clamp technique we identified electrophysiologically and pharmacologically different K^+ channels, such as K_{ir} , SK_{Ca} , IK_{Ca1} and BK_{Ca} in the rat superior mesenteric artery EC.

It is known from the literature that the expression of K^+ channels varies between different EC types, however one of the most widely described ion channel on EC is K_{ir} , which controls the resting membrane potential in non-stimulated cells. In our study we also demonstrated the presence of K_{ir} , which was evoked by voltage steps to hyperpolarizing potentials from the holding potential of -60 mV and was demonstrated to be sensitive to Ba^{2+} . The activation of the inward currents could be evoked by voltage steps to hyperpolarizing potentials from the holding potential of -60 mV. This is virtually in contrast to the behavior of K_{ir} channels which are conducting current at membrane potentials more negative than the equilibrium potential of K^+ (-87 mV in this study). The most important explanation for this inconsistency is that compensation for ohmic leak was not implemented in the records. Several non-voltage gated channels are expressed in EC which do not allow a clear reference voltage for leak subtraction. The presence of leak, in general, shifts the reversal potential of the whole cell currents to more positive values than the equilibrium potential for K^+ calculated from the Nernst equation. Another hallmark of K_{ir} is the lack of significant K^+ currents above the equilibrium potential of K^+ . This feature of K_{ir} could not be demonstrated in this study due to the presence of other non-voltage gated K^+ channels, e.g. IK_{Ca1} . channels are characterized a quasi linear I-V relationship, i.e., outward K^+ current is conducted by these channels above the equilibrium potential of K^+ thereby preventing the recording of an I-V relationship typical for K_{ir} .

In freshly isolated EC and VSMC of the rat mesenteric artery, K_{ir} currents are described to be exclusively expressed in EC, where they have a maximal amplitude of -190 ± 16 pA at -150 mV. There have been few studies demonstrating the presence of K_{ir} channels on isolated VSMC as well (especially on small arteries or arterioles), however K_{ir} currents have been mostly reported on isolated EC. Crane et al demonstrated that in the rat mesenteric artery the removal of endothelium eliminates the sensitivity to Ba^{2+} suggesting that in this vascular bed K_{ir} currents are expressed in the EC layer.

The presence of SK_{Ca} and IK_{Ca1} channels on EC were recorded in our study, and they have also been widely reported on both cultured and freshly isolated small arteries in the past. In freshly isolated mouse aorta EC, the application of apamin plus ChTx inhibited a substantial fraction (44%) of the K^+ currents at $+80$ mV. In EC of the rat carotid artery, the selective IK_{Ca1} blocker TRAM34 reduced the current to 60-70% of the maximal current amplitude

under control conditions. These values are similar to those obtained in the present study, where the blockade with either apamin or TRAM34 inhibited the current to 55-60% and 60-70%, respectively, of the maximal current amplitude under control conditions, suggesting that a significant portion of the overall membrane K^+ conductance in intact ECs carried by SK_{Ca} and IK_{Ca1} channels. Activation of these channels and/or EC hyperpolarization were demonstrated to elicit EDHF mediated responses in the past. Moreover, the ACh-mediated increase of TEA-sensitive K^+ currents suggests the contribution and the activation of KCa currents significantly contributes to the overall membrane K^+ conductance.

In the rat mesenteric artery, ACh increases EC $[Ca^{2+}]_i$ by the release of Ca^{2+} from intracellular stores as well as by Ca^{2+} influx through plasma membrane leading to the activation of SK_{Ca} and IK_{Ca1} channels, which is followed by increased NOS activity and formation of NO. In rat mesenteric artery, the expression of BK_{Ca} channels has been described to be restricted to VSMC, thereby the sensitivity of our outward current to IbTx could potentially imply either the presence of BK_{Ca} on EC or could imply that electrical coupling between EC and VSMC exist through MEGJ. This latter could indicate that membrane currents recorded from intact EC might be modulated by BK_{Ca} currents expressed in VSMC via MEGJ, which is in agreement with other previous reports that demonstrated bi-directionally coupling between the two cell layers. This communication could occur via direct spreading of depolarization and hyperpolarization or with the movement of other signaling molecules such as Ca^{2+} and IP_3 , that can diffuse from one cell type to another.

In conclusion, intact EC are coupled to each other through homocellular gap junctions to form a functional syncytium. K_{ir} as well as SK_{Ca} , and IK_{Ca1} channels coordinate membrane potential changes along the vessel wall and control VSM tone through MEGJ. Furthermore, our recorded BK_{Ca} currents on rat mesenteric artery could potentially arise from VSMC and can modulate EC membrane currents through MEGJ.

In summary the advantages of the experimental approach are the minimal disturbance of EC, which retain their fine relationship with their neighboring cells. Ion channels expression in EC make an important contribution to control the vascular tone *in vivo*; thus impaired function of these channels may play an important role in a range of diseases related to endothelial dysfunction and thus could blunt vasodilatation. Our results show that in the rat mesenteric artery, EC express K_{ir} currents that maintain the membrane potential as well as SK_{Ca} and IK_{Ca1} currents, which could mediate the EC hyperpolarization. ACh increases TEA-sensitive K^+ currents, which are also involved in EC hyperpolarization. High conductance BK_{Ca} currents were recorded from the EC layer, which could indicate that the VSMC hyperpolarization is transmitted to the EC layer through MEGJ. Further, EC and

VSMC are in a sense both detectors and effectors, and the activation of each cell type leads to coordinated responses in the other.

Our future goal is to further investigate the exact role of these channels in Ca^{2+} signaling and vasodilation. Although the complex function of EC along with the network of these cells creates challenges in circulatory research, with these *in situ* results we are confident that our work contributes significantly to the current understanding of EC physiology and the method described here will facilitate further electrophysiological and functional studies done on EC.

VI. Summary

Cells rapidly adjust their gene and ion channel expression upon the change of extracellular environment. To obtain valid measurements it is important to use models closest to the *in vivo* systems. In this work we focused on the characterization of ion channels on two different cell types, the human dendritic cells (DC)c and the endothelial cells (EC) of the arteria mesenterica superior in rats in the most physiologic circumstances. We also used a DC model cell line (KG-1) during our electrophysiological studies to compare the obtained currents to that of DC. In the immune system VGPC, K_{ir} , and K_{Ca} channels have been described to play a major role in controlling the membrane potential and regulating intracellular Ca^{2+} signaling pathways required for proliferation and differentiation. In this study for the first time we described that immature monocyte-derived DC express voltage-gated Na^+ channels (Nav1.7). Transition from the immature to a mature state in DC however was accompanied by the down-regulation of Nav1.7 expression and the up-regulation of voltage-gated $Kv1.3 K^+$ channels. The presence of $Kv1.3$ is common for immune cells; hence, selective $Kv1.3$ blockers may emerge as candidates for inhibiting various functions of mature DCs that involve their migratory, cytokine-secreting, and T cell-activating potential. Both unstimulated and stimulated KG-1 cells expressed K_{Ca} only, which makes them not an ideal model for electrophysiological studies on DC.

EC function could be considerably altered during the process of isolation and cell culture. Previous electrophysiological studies on EC were conducted on isolated or cultured cells, ignoring the complex and fine network of EC and vascular smooth muscle. We developed a method that allows identifying and characterizing the ion channels of EC in their native environment. Rat mesenteric arteries mounted as ring preparations in a microvascular myograph for recording whole cell currents under ‘*blind*’ patch clamp technique. Neurobiotin staining demonstrated that intact EC are electrically coupled through gap junctions and 18β -gly gap junction blocker decreased the outward and inward currents registered. We observed K_{ir} currents sensitive to $BaCl_2$, K_{Ca} currents of small (SK_{Ca}), intermediate (IK_{Ca1}) and high conductance (BK_{Ca}) that were sensitive to apamin, TRAM 34 and IbTx, respectively. Moreover, Ach increased outwardly directed K^+ currents that were sensitive to TEA. Under physiological circumstances K_{ir} current is involved in maintaining the resting membrane potential, where SK_{Ca} and IK_{Ca1} are mainly responsible for membrane hyperpolarization. The BK_{Ca} current reported in this study may arise from the vascular smooth muscle layer and potentially influence EC membrane potential via myoendothelial transfer of current.

VII. List of publications



DEBRECENI EGYETEM EGYETEMI ÉS NEMZETI KÖNYVTÁR
KENÉZY ÉLETTUDOMÁNYI KÖNYVTÁRA

Iktatószám: DEENKÉTK /28/2011.
Tételszám:
Tárgy: Ph.D. publikációs lista

Jelölt: Zsíros Emese

Neptun kód: E6PUPE

Doktori Iskola: Molekuláris Orvostudomány Doktori Iskola

A PhD értekezés alapjául szolgáló közlemények

1. **Zsíros, E.**, Kis-Tóth, K., Hajdú, P., Gáspár, R., Bielansk, J., Felipe, A., Rajnavölgyi, É., Panyi, G.: Developmental switch of the expression of ion channels in human dendritic cells.
J. Immunol. 183 (7), 4483-4492, 2009.
IF:5.646
DOI: <http://dx.doi.org/10.4049/jimmunol.0803003>
2. Hajas, G., **Zsíros, E.**, László, T., Hajdú, P., Somodi, S., Réthi, B., Gogolák, P., Ludányi, K., Panyi, G., Rajnavölgyi, É.: New phenotypic, functional and electrophysiological characteristics of KG-1 cells.
Immunol. Lett. 92 (1-2), 97-106, 2004.
IF:2.136
DOI: <http://dx.doi.org/10.1016/j.imlet.2003.11.021>

További Közlemények

3. Balogh, Z., Fóris, G., Kosztáczky, B., Paragh, G., Seres, I., **Zsíros, E.**, Kónya, G.: The concentration dependent biphasic effect of leptin on endogenous cholesterol synthesis in human monocytes.
Peptides. 28 (10), 2081-2083, 2007.
IF:2.368
DOI: <http://dx.doi.org/10.1016/j.peptides.2007.07.004>
4. Kosztáczky, B., Fóris, G., Paragh, G., Seres, I., **Zsíros, E.**, Koncsos, P., Balogh, Z.: Leptin stimulates endogenous cholesterol synthesis in human monocytes: New role of an old player in atherosclerotic plaque formation.

DOI: <http://dx.doi.org/10.1016/j.biocel.2007.04.012>
IF:4.009

5. Petrohai, Á., Nagy, G., Bősze, S., Hudecz, F., **Zsíros, E.**, Paragh, G., Nyárády, Z., Németh, P., Berki, T.: Detection of citrate synthase-reacting autoantibodies after heart transplantation: An epitope mapping study.
Transpl. Int. 17 (12), 834-840, 2005.
DOI: <http://dx.doi.org/10.1007/~00147-004-0794-4>
IF:1.797

The Candidate's publication data submitted to the Publication Database of the University of Debrecen have been validated by Kenezy Life Sciences Library on the basis of Web of Science, Scopus and Journal Citation Report (Impact Factor) databases.

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