PH.D. THESIS

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I. INTRODUCTION

In living systems the cell-cell communication is mediated by extracellular signalling molecules. Among these molecules one can find hormones, neurotransmitters and growth factors. In the process of signal transduction such an external chemical stimulus evokes some kind of cellular response by metabolic changes in the intracellular space. Natural chemicals that bind specifically to receptors are ligands, the structural analogues of them are agonists or antagonists. The first one has an effect similar to that of the natural ligand, while the latter does not induce biological response, but it is able to inhibit the effects of agonists or antagonists via competition. In many cases, the indirect effects of receptors are mediated by G-proteins. These are situated in the inner layer of the cell membrane, and make a connection between receptors and enzymes that catalyze the synthesis of secondary messengers.

Cyclic adenosine monophosphate (cAMP) belongs to the family of secondary messengers. The level of cAMP is determined by the adenylate-cyclase enzyme, which is regulated by two kinds of G-proteins: G_s stimulates, while G_i inhibits the operation of the enzyme. The former is permanently activated by cholera-toxin, the latter is inactivated by pertussis-toxin, so both toxins contribute to an increase in the level of cAMP. The usage of them makes possible to decide if the G_i or G_s mediated signalling pathway was activated by a given stimulus. The phosphorylation events in signal transduction processes can influence the functional properties of ion channels. This kind of modulation of ion channels has already been described by many types of channels. The functional changes can affect the channel- current kinetic parameters (activation, inactivation), the voltage-dependence of activation, the current amplitude, or the combination of these, inducing a complex change in operation.

G-proteins can have a direct effect on the operation of certain channels, this means that signal transduction is mediated by membrane-components, and no cytoplasmic proteins take part in that.

Adenosine is a compound that can be found in any cell of the body. It is released into the extracellular space under certain physiological conditions, when there is an increased oxigen demand/oxigen supply ratio. Adenosine is able to evoke many kinds of effects in several tissues and cells, among them, dilation of coronary arteries, and reducing peripheric vessel resistance. In the guinea pig it decreases atrial myocardial contractile force, while in aorta it produces relaxation. In contrast, in lung arteries adenosine induces contraction, which can influence the rhytm of respiration.

Adenosine exerts it effect by binding to specific cell surface receptors. Adenosine-receptors can be grouped into A_1, A_2 and A_3 receptors, A_2 receptors can be further subdivided into A_{2a} and A_{2b} types. All of these receptors are G-protein-linked. The signal transduction mediated by A_1 receptors is via G_i and G_o proteins, accordingly it inhibits the activity of adenilate-cyclase enzyme (and thus decreases the level of cAMP). The effect evoked through A_1 can be blocked by perussis toxin. However, A_2 receptors are G_s protein-coupled receptors, so they stimulate adenylate cyclase. The A_3 receptors are G_i-coupled. The differences between A_{2a} and A_{2b} are structural and pharmacological.

There are several selective adenosine receptor agonists and antagonists, which bind to different receptor-types with different affinities. This enables the identification of receptors on a pharmacological basis. Relatively high affinity agonists are N6-cyclopentyl-adenosine (CPA) (to A_1), 2-[p-(2-carbonyl-ethyl)-phenylethylamino]-5'-N-ethylcarboxamido-adenosine (CGS) (to A_{2a}), 5'-N-ethyl-carboxamido-adenosine (NECA) (to A_{2b}). 1,3-dipropyl-8-cyclopentylxanthin (DPCPX) is a well-known A_1 antagonist. Widely used nonselective adenosine-receptor antagonists are caffeine, theophylline and 8-phenyl-theophylline (8-PT).

Although the best known signalling pathway of adenosine receptors is the inhibition or activation of adenylate-cyclase activity, several effects of adenosine also includes changes in ionic conductances, especially the modulation of K^+ and Ca^{2+} channels, which, of course, also has as a consequence, changes in the transmembrane potential. It is known that membrane-potential plays a crucial role in signal transduction, however up till now the possible role of the changes in membrane-potential in the response of cells to adenosine-receptor activation has not been investigated in details.

The relaxing effect of adenosine on the vessel smooth muscle is realized by mechanisms that decrease the intracellular Ca^{2+}-level or reduce the calcium-sensitivity of the contractile apparatus. K^+-channels play an important role in the mediation of relaxation: Their increased activity contributes to the hiperpolarization of the smooth muscle cell, thus less amount of Ca^{2+} ions can enter into the cell through the voltage-dependent Ca^{2+}-channels, in this way the smooth muscle contraction decreases.

In this process both Ca^{2+}-activated K^+-channels and ATP-dependent K^+-channels play a role. Blocking of the large conductance Ca^{2+}-activated K^+-channels leads to the inhibition of the adenosine-induced coronary artery dilation, while the activation of ATP-dependent K^+-channels due to adenosine relaxes the artery. Recently voltage-dependent potassium channels have also been shown to influence vascular tone. Because of this, the investigation of the effects of new or already known adenosine analogues on the potassium currents of muscle cells can be a useful method for the functional investigation of the analogue.

Positron-emission tomography (PET) makes possible to investigate the distribution of adenosine-receptors in the central nervous system and heart muscle. In the last decades for such investigations several protocols were developed, using different ligands. This technique requires the application of radioactively labeled ligands, with high activity and selectivity. 18F-NECA is

the radiolabeled derivative of the A_2 adenosine-receptor agonist NECA, where a positron-emitting fluorine atom is bound to the original molecule. F-NECA is the non-emitting derivative of 18F-NECA. 18F-NECA has already been proven to be a suitable radioligand for the visualization of adenosine-receptors by PET.

The sperm cells of both freshwater and seawater are immotile in the male reproductive organ or in a solution that has the same osmolarity as the seminal plasma. The sperm cells become motile immediately when they get into an environment similar to where spawning occurs. Freshwater fish sperm cells become motile when they are taken in hypoosmotic solution, while seawater fish sperm-motility is initiated right after it is diluted in hyperosmotic seawater. It has been demonstrated for both freshwater and seawater fish species, that changes in intracellular K^+ and Ca^{2+}-concentrations is a result of the shift of the environment to the hyper- or hypoosmotic direction.

The osmolality of the seminal plasma of the ascidian *Ciona* is similar to that of seawater, so there is no change in osmolality at spawning. It is the sperm activating and attracting factor (SAAF), a substance from the egg, which is responsible for the activation of the *Ciona* sperm, inducing Ca^{2+}-influx via Ca^{2+}-channels.

It is common for all species that the behaviour of their sperm cells is determined primarily by Ca^{2+}-ions. Sperm cells express several Ca^{2+}-permeable channels and these channels are indispensable for the regulation of sperm motility. Mechanically sensitive, stretch-activated channels can be found in plant and animal cells. These channels play a role in the responses to many kinds of mechanical effects, like regulation of cell volume, increase in intracellular Ca^{2+}-concentration, and cell proliferation. In most cases mechanically sensitive channels are cation-selective, passing through Ca^{2+}-ions and monovalent cations. Gadolinium blocks stretch-activated channels and thus inhibits several physiological processes.

Sperm cells show different sensitivity for the change of environmental osmolality. This raises the question whether osmoregulated, mechanically sensitive Ca^{2+}-permeable channels play a role in the mechanism of the initiation of sperm motility in seawater and freshwater fish species. The Ca^{2+}-influx necessary for the initiation of sperm motility may be provided by these channels.

The existence and possible biological role of cell surface super-structures of immunologically important signalling molecules involving Major Hystocompatibility Complex antigens class I and class II (MHC I and MHC II), the Interleukin-2 cytokine receptor (IL-2R) complex, and the Intercellular Adhesion Molecule-1 (ICAM-1) were recently described in a series of experiments. Nano- and micrometer-scale hetero- and homo-associations of these molecules has been found to show remarkable compositional identity among the different cell lines examined.

Besides their established role in antigen presentation, MHC class I and class II molecules have recently been assigned also an active "non-classical" role in transmembrane signalling: they may induce apoptosis or activate cells in a liniage and differentiation state dependent manner. The same tyrosine and serine kinases involved in the signalling of IL-2 receptor were shown to be also coupled to MHC-elicited pathways.

Based on these observations, the lipid rafts organizing receptor clusters of the above molecules can be regarded as functional units of signalling, the outcome of which being determined by the quality and stochiometry of the protein components.

Beside of the the composition of lipid rafts, however, there is another important physiological factor shaping transmembrane signalling: membrane potential. Voltage- and Ca^{2+}-dependent K^{+} channels are among the main candidates responsible for maintaning a potential window necessary for proper signalling. These ionic channels may directly or indirectly be coupled to the MHC- and IL-2R-containing lipid rafts.

Pi2 toxin, a fraction of molecules extracted from the toxin of *Pandinus imperator* scorpion, is a selective and efficient blocker of Kv1.3 voltage-dependent potassium channels (K_d = 44 pM), that have a high expression number on the surface of immune cells. It is advantageous to use Pi2 toxin in toxin-hindrance experiments because it has much higher molecular mass compared to other potassium channel inhibitors (4-AP, TEA) and thus it occupies much more space.

If the receptors of lipid rafts in question are labeled by primary antibodies, and golden beads conjugated to secondary antibodies are added as secondary antibodies, then a net-like spatial structure can be formed over the ion channels being in possible proximity, because of the homoassociation of the receptors. This network composed of golden beads and antibodies can hinder the channels from the peptide toxin molecules arriving in the perfusion system, which can result in the delaying of the toxin binding to the channel mouth and the delaying of the inhibition of channel current.

II. AIMS

We wished to examine the effect of ligand binding to A_1 and A_2 adenosine receptors on the membrane potential and potassium conductance of smooth muscle cells. We planned our measurements to be performed on DDT1 MF-2 cells, these are smooth muscle cells derived from hamster vas deferens, expressing both A_1 and A_2 types of receptors and voltage-dependent K^+-channels can also be found in their cell membranes, therefore they provide a capable system for the above examinations.

Furthermore, we wished to compare the changes mediated by A_1 and A_2 receptors in the contraction and relaxation of certain tissues, caused by NECA and F-NECA (adenosine receptor agonists), and their effects on the membrane potassium conductance in single cells. The tissues on which we wished to

complete our investigations were: guinea pig atrial myocardium, pulmonary artery and aorta, on which previously A_1 and A_{2b}-mediated changes in contractility had been described. The measurements on single cells were planned on DDT1 MF-2 cells, using the patch-clamp technique.

Extracellular osmolality can have an effect on the physical state of the membrane and thus on the ion channels being there. Regarding this, we aimed to investigate the role of mechanosensitive channels in the activation of the sperm cells of seawater fish *Takifugu niphobles*, freshwater fish *Cyprinus carpio*, and the ascidian *Ciona intestinalis*. With a view to this, we planned the application of the stretch-activated cathion-channel blocking Gd^{3+}, as well as fluorimetric membrane fluidity and light microscopic motility measurements.

Being aware of the modulation of Kv1.3 channels by certain receptors and kinases we tried to decide whether the cell surface receptors playing a role in the immune response and Kv1.3 molecules are in physical proximity. We wished to perform our experiments on Kit 225 K6 human T-lymphoma cell line, while the receptors in question were MHC I, MHC II, IL-2Rα subunit and VLA-4 integrin, which were associated to the same lipid rafts on these cells. As a negative control, we wished to use TrfR, which belongs to a raft different from the previously mentioned one.

III. MATERIALS AND METHODS

Cells The hamster vas deferens smooth cell line (DDT1 MF-2) was obtained from the European Collection of Animal Cell Cultures (Salisbury, UK). DDT1 MF-2 cells were cultured at 37 ^{o}C in humidified atmosphere of 5% CO_2 and 95% O_2. The growth medium was Dulbecco's modified Eagle's medium supplemented with 2mM L-glutamine and 10 % (v/v) fetal calf serum. Cells were collected by vigorous shaking of the flask and washed with PBS. For the flow cytometric measurements we set the concentration of the cell suspension to

$2x10^7$/ml. To remove endogenous adenosine, we added adenosine deaminase to the cells (2 U/ml) 10 minutes before starting the measurement. To check the viability of the cells we used trypan blue labeling, according to which 90 % of the cells was viable. Kit 225 K6 is a human T-lymphoma cell line with helper phenotype. For its proliferation the regular addition of IL-2 ligand is needed. Cells were cultured in RPMI-1640 medium containing 10 % fetal calf serum, penicillin and streptomycin. Every 48 hours 20 units/ml recombinant IL-2 ligand was added to the cells.

Electrophysiology Patch-clamp measurements were performed in whole cell configuration with an Axopatch-200-A patch clamp amplifier in conjunction with a Digidata 1200 computer interface (Axon Instruments, Foster City, CA, USA). Low-pass filtering was applied at half of the sampling frequency. Patch-electrodes of 3-4 MΩ resistance were fabricated from GC150 F-15 borosilicate glass capillaries (Clark Electromedical Instruments, Reading, UK). Pipettes were filled with KF-based intracellular solution containing EGTA. Cells were measured in NaCl-based extracellular solution containing Ca^{2+} and Mg^{2+}. Gigaseals in the order of 1-10 GΩ were formed on the cells by applying negative pressure to the interior of the patch-pipette. Subsequently, whole-cell measuring configurations were established by the application of negative pressure pulses. In most of the experiments the leak was negligible compared to the K^+ currents. Series resitance was in the range of 3-10 Mohm, 80 % correction and 40 % prediction compensation was used for the series resistance. All patch-clamp measurements were performed at 20 ^oC. Current protocols were started 12 min after entering whole-cell configuration or later. K^+ channel properties of the examined cells remained stable for up to several hours as they were kept on ice until the patch-clamp experiments. Adenosine receptor ligands were solved in NR and carried to the cells with a continuous perfusion system, using valves. We evaluated patch-clamp data using the pClamp8 program package (Axon Instruments, Inc.).

Flow cytometry Flow cytometric membrane-potential measurements were performed by using the negatively charged oxonol dye, which is distributed between the two sides of the membrane according to the Nernst equation. For the measurements we used a modified FACSTAR flow cytometer equipped with argon-ion laser (Becton-Dickinson, Parsippany, USA). The fluorescence of the oxonol dye was excited with the 488 nm line with 200-400 mW power. The output optics included a 520 nm highpass filter to filter scattered excitation light, and a 540 nm band filter. We used the small angle forward-scattered light for electronic gating during data collection, which enabled the ignoring of dead cells from the set of cell to be analysed. Cells were run at a concentration of 10^6/ml, in room temperature. We started to measure fluorescence histograms after the formation of the staining equilibrium. To investigate the possible modifying effect of the secondarily given ligand, the ligand was given to the stained cell suspension after the establishment of the equlibrium.

Contractility measurements

(a) atrial myocardium After the killing of guinea pigs by a blow on the head under ether anaesthesia, the chest was opened and the heart was quickly excised and washed in oxygenated Krebs solution at 30 ^oC. Left atria were isolated and set up in 10 ml vertical organ baths containing 37 ^oC Krebs solution. The atria were electrically paced with 3 Hz, applying twice the threshold voltage, by a programmable stimulator. Isometric contractions were measured by means of a transducer, recorded by a polygraph. An initial resting tension of 10 mN was applied on the preparations. Following the stabilization of the contractile parameters, we recorded cumulative NECA and F-NECA dose-response (E/[A]) curves.

(b) pulmonary artery and aorta Circular segments were prepared from the middle part of the main pulmonary arteries and the thoracic aorta of guinea pigs. The tissues were fixed vertically in a 10 ml tissue chamber containing 37 ^oC Krebs solution at 5 % CO_2. Tension of pulmonary and aortic vascular strips was

measured isometrically on transducers and the ouput fed to potentiometric recorders. The specimens were given an initial tension of 10 mN, and after the equilibration period, preparations were incubated and washed with 1 μM noradrenaline.

When the contraction was stable, 10 uM NECA was applied to the organ bath and was allowed to produce a maximal response. The tissues were then washed several times with fresh, noradrenaline-containing (1 μM) Krebs solution and allowed to recover. To examine the effects of NECA and F-NECA on pulmonary arteries, non-cumulative measurements were performed, while on thoracic aortae cumulative measurements were done with these adenosine analogues. 8-PT, an agent modulating the action of purine compounds, was added during the resting state for 30-40 minutes, afterwards the tissues got noradrenaline, as a result of which they contracted to the level of control tone. After the experiments where A_1 adenosine receptor mediated contractions evoked by NECA or 19F-NECA were studied, tissues were lightly blotted, and their length and weight were measured. Their response to adenosine analogues were then calculated by determining the increase in mechanical force (in mN units), and dividing this by the cross-sectional area of the tissue (in mm^2 units).

measurement of membrane fluidity 10 μl TMA-DPH stock-solution was added to 1 ml cell suspension (final concentration of TMA-DPH: 10^{-6} M), and cells were then incubated for 5 minutes in quartz cuvette in the presence or without 20 μM $GdCl_3$. 2 ml FPS (sperm remained immotile) or 2 ml distilled water (sperm became motile) was added to the cuvettes and then the anisotropy was measured. Added FPS and distilled water contained TMA-DPH or $GdCl_3$ in the same concentrations.

immunogold labelling for cell surface proximity measurements In the first labeling step freshly harvested cells were washed twice in 4 ^oC-os PBS (pH 7.4). The cell pellets were suspended in 100 μl of PBS (1 x 10^6 cells/ml) and labeled by incubation with approximately 10 μg of unlabeled whole mAbs (serving as

targets for the immunogold beads of anti-Fc fragment specificity in a second step of labeling) for 40 minutes on ice. To avoid possible aggregation of the mAbs, they were air-fuged (at 9 x 10^4 rpm, for 30 min) before labeling. The labeled cells were washed with excess cold PBS twice. Labeling with the unstained whole mAbs was followed by, as a second labeling step, incubation with polyclonal secondary antibodies conjugated to gold beads of 30-nm diameter (Aurogamig g-30 against the Fc fragment of the unstained whole mAb; from Amersham Pharmacia) on ice for another 40 minutes.

patch-clamp wash-in time measurements with the L243 monoclonal antibody After the formation of stable whole-cell configuration, depolarizing pulses were added to the cell at least 5 times. The pulses started from a holding potential of –120 mV and rose up to +50 mV for a time interval of 20 ms. The pulses were repeated every 15 s. When we made sure that the current level was stable, in equilibrium, 30 μl of L243 antibody solution (the solvent was PBS) was pipetted directly onto the cell. The peak current level was continuously monitored by depolarizing pulses, and a gradual decrease was detected, which reached a lower equilibrium in 3-6 minutes. The concentration of the antibody was 2.5 mg/ml.

IV. RESULTS AND DISCUSSION

adenosine-receptor-mediated signalling events in smooth muscle cells The application of A_1 adenosine receptor agonist CPA (50 nM) caused hyperpolarization of the DDT1 MF-2 smooth muscle cells. DPCPX, an A_1-receptor antagonist (1 μM) significantly decreased the agonist induced hyperpolarizing effect. Preincubation of cells with 100 μM 4-AP, which is a voltage-dependent potassium-channel blocker, or with 1 μM DPCPX completely abolished the effect of CPA on the membrane-potential. Patch-clamp

experiments performed on DDT1 MF-2 cells showed an outward, voltage-dependent current. This current could completely be inhibited by perfusing with the voltage-dependent potassium channel blocker 4-AP (5 mM) or TEA (10 mM). Application of CPA in the range of 10 nM-10 μM gradually increased this current, producing an increase of 30 % at the concentration of 1 μM. Thus, changes in potassium permeability could underly the CPA-induced hyperpolarization.

The A_{2A}-receptor agonist CGS 21680 in the concentration range of 10 nM – 3 μM exerted an effect opposite to that of the A_1 agonist CPA on the membrane potential and potassium conductance of DDT1 MF-2 cells. The addition of 1 μM A_{2A} adenosine receptor antagonist CSC caused repolarization and the membrane-potential returned close to the original value. CPA also abolished CGS 21680 induced depolarization on cells. CPA supposedly has this property because the A_1-receptors have a four times greater expression level on DDT1 MF-2 cells compared to that of A_{2A} receptors. When during the patch-clamp experiments CGS 21680 (10 nM – 10 μM) was perfused to the cells we experienced the immediate decrease of the whole cell potassium current. At higher concentrations CGS 21680 also accelerated the inactivation of the current and the change of inactivation was most expressed at the concentration of 100 nM. To demonstrate that in the changes of the properties of the potassium current, receptors exert their effects in a G-protein-independent manner, the experiment was repeated on pertussis-toxin-treated cells at the most efficient CGS 21680 concentration (100 nM). No significant difference was found between pertussis-toxin treated (200 μg / ml, incubated for 4 hours) and the control cells neither in the blocking of the peak current (73 ± 12 % and 66.9 ± 8.8 %, respectively, n=5), nor in the change of current inactivation kinetics (54 ± 9.7 % and 64.8 ± 12 %, respectively, n=5).

According to our best knowledge this was the first time to successfully demonstrate the modulation of the potassium current on DDT1 MF-2 cells by selective adenosine-receptor-activating agents. Molleman et al. did not find any effect of either high dosage adenosine (1 mM) or ATP on membrane currents on this cell line. However, these findings do not contradict our results, as the 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 potassium current may not be significant. In the present study we examined subtype-specific agonist-induced processes were investigated, so we could separate the different effects.

The response of potassium channels to adenosine-analogues specific for different subtypes can vary from tissue to tissue. Dart and Standen found that although both A_1 and A_2 types of receptors can be found in pig coronary smooth muscle cells, binding to A_1 adenosine-receptor activates current, but this is not the case for A_{2A} receptors.

According to our conception, the stimulation or inhibition of adenylate cyclase following A_{2A} or A_1 receptor-activation occurs by a mechanism in which among the first steps there is a ligand-induced change in transmembrane-potential, as the change in membrane-polarity can induce the voltage-dependent conformational change of the enzyme.

Our results suggest that DDT1 MF-2 cells are unique in a way that they react by increasing their potassium conductance and hyperpolarization to A_1-receptor activation, in contrast, respond with decreased potassium current and depolarization to ligand treatment against the A_2 receptor. This means that these cells can be useful targets of pharmacological research, because they provide the possibility of tracking A_1 and A_{2A}-mediated mechanisms on the same cell line.

examination of contractility changes of tissues evoked by NECA and F-NECA adenosine-derivatives In electrically stimulated atrial myocardium – a tissue expressing A_1 adenosine receptors – both NECA and F-NECA decreased

contractile force in a dose-dependent manner. Recording and analyzing the dose-response curves for NECA and F-NECA we could see that there was no significant difference between the two compounds regarding maximal effect (E_{max}) and Hill-coefficient (n_H), while the apparent affinity for F-NECA (EC_{50}) was less than that of NECA (7.50 ± 0.18 and 8.23 ± 0.12, respectively).

In isolated strips of guinea pig pulmonary arteries precontracted with 1 μM noradrenaline, both NECA and F-NECA exerted a concentration-dependent phasic contraction (a fast initial contraction, which is A_1-mediated according to literature). Dose response curves taken for the two compounds showed that E_{max} and Hill-coefficient values did not differ significantly for NECA and F-NECA. However, EC_{50} values derived from the curves were also different here (F-NECA: 6.20 ± 0.01, NECA: 6.58 ± 0.05).

The above show that the affinity of F-NECA is less for both myocardial and vascular A_1 adenosine-receptors. The similarity in maximal effect (E_{max}) and Hill coefficient (n_H) can be explained by suggesting that NECA and F-NECA act via similar signalling pathways.

In 1 μM noradrenaline-pretreated guinea pig aorta preparations – a tissue expressing A_{2B} adenosine receptors - NECA and F-NECA evoked dose-dependent relaxation. Dose-response curves showed no significant differences in either maximal effect (E_{max}), or Hill-coefficient (n_H), or the EC_{50} values characterizing apparent affinities.

In the next series of experiments guinea pig pulmonary arteries (a tissue containing A_1 and A_{2B} adenosine receptors) were used, where the vascular A_1 adenosine receptors were inhibited by DPCPX, a specific A_1 adenosine receptor antagonist. This way, the A_{2B} adenosine receptor-induced fast relaxation could be reliably examined. To examine this relaxation, dose-response curves were taken on pulmonary arteries concerning NECA and F-NECA. We could not detect any difference between the E_{max}, Hill coefficient (n_H) or EC_{50} values characterizing the effects of NECA and F-NECA, therefore we suggest that

NECA and F-NECA are equivalent agonists on vascular A_{2B} adenosine-receptors.

To check if NECA and F-NECA exert their effect via adenosine-receptors, we took dose-response curves also in the presence of 1 μM 8-PT (a nonselective adenosine-receptor-antagonist). 8-PT shifted both the dose-response curves of NECA and F-NECA to the right, and the maximal relaxation (E_{max}) caused by these compounds decreased as well. To further analyze the effect of 8-PT, we determined the EC_{25} values of the E(A) curves taken for NECA and F-NECA in the presence or absence of 8-PT, and calculated concentration ratios (EC_{25} after 8-PT / EC_{25} before 8-PT) from them. These ratios for NECA and F-NECA were 3.83 ± 0.69 and 4.94 ± 0.54, respectively. The equivalence of NECA and F-NECA on A_{2B} receptors is thus justified also by the measurements with 8-PT.

Whole-cell current measured on DDT1 MF-2 smooth muscle cells had two components: an inwardly rectifying, voltage-dependent Na^+-current and an outwardly rectifying, voltage-dependent K^+-current. As sodium current inactivated earlier than potassium current activated, it was possible to measure the potassium current independently of the sodium current. We also performed the pharmacological analysis of the voltage-dependent, outwardly rectifying potassium conductance with non-peptide (TEA) and peptide (ChTx, MgTx) type blocking agents. The values of half-maximal dose (EC_{50}) for these blockers were: TEA: 2.1 mM, MgTx: 43 pM, ChTx: 910 pM, which is characteristic for the properties of Kv1.3 channels. The treatment of NECA or F-NECA significantly decreased whole cell potassium current of DDT1 MF-2 cells. In response to 5 μM NECA the peak current caused by depolarization from -120 mV to +40 mV decreased to 61.2 ± 8.3 % (n = 3) of the original value, while it fell to 80.6 ± 4.7 %, when F-NECA was used.

Perfusion treatment with NECA or F-NECA significantly accelerated the inactivation of the membrane current as well. Accordingly, we could see a

reduction of the exponentially fitted inactivation time constant to 55.1 ± 18.0 % of the original value due to 5 μM NECA, while 5 μM F-NECA had less effect, diminishing the time-constant to 80.1 ± 15.3 % of the control value. The two agents also differently influenced the activation time-constant of the current: while 5 μM NECA exerted a fall of 73.9 ± 10.0 %, 5 μM F-NECA did not cause any significant change, setting the time-constant to 104.6 ± 1.8 % of the original value.

The differences between the effects of NECA and F-NECA can be a consequence of the differences in their chemical structure, which may cause alterations in the affinity of these compounds to A_1 and A_{2B} adenosine-receptors. As both A_1 and A_{2B} receptors are present on the surface of DDT1 MF-2 cells (Ramkumar et al.), and NECA is an agonist for both receptors (Stinnakre and Renterghem), the functional response to the binding of NECA or F-NECA is the sum of the A_1- and A_{2B}-mediated effects.

examination of the role of mechanical stretch-activated channels in the activation processes of sperm cells Spermatozoa of *Takifigu niphobles* were immotile in SLS or NoCaSLS solutions, which correspond to their original environment. To evoke motility we used ASW or Ca^{2+}-free NoCaASW solutions, which correspond to seawater. When diluted in ASW, sperm cells of *Takifigu niphobles* began to show a motility of 90.6 ± 2.0 %, with a speed of 163.2 ± 5.35 m/s. When sperm cells were taken in NoCaASW, there was no significant difference compared to ASW either regarding the motile fraction (87.6 ± 3.1 %), or in velocity (149.7 ± 7.3 μm/s), indicating that extracellular Ca^{2+} is not needed to initiate motility. This observation of us is in accordance with the results of Oda and Morisawa, who found that the motility of the spermatozoa of *Takifigu niphobles* could be initiated without extracellular Ca^{2+}.

As a result of the depletion of intracellular Ca^{2+} from sperm cells by incubation with NoCaSLS containing 10 μM A231186 calcium-ionophore, the cells did not became motile after diluting them in NoCaASW. After the addition

of 100 μM Ca^{2+}, the cells became motile again, and their speed values got close to those of control values. These results suggest that intracellular calcium is required for the initiation of the motility of *Takifigu niphobles* spermatozoa.

Thus, the role of Ca^{2+} seems to be substantial in this process: Oda and Morisawa measured a 5-fold increase in the intracellular Ca^{2+}-concentration, even in the absence of extracellular Ca^{2+}, when *Takifigu niphobles* sperm motility was initiated by hyperosmotic solution. They also found that sperm cells got motile in physiological solution 10-15 s after loading the cells with Ca^{2+} by the application of extracellular Ca^{2+} and A23187 Ca^{2+}-ionophore. So according to their results, Ca^{2+} stored in intracellular stores, can initiate sperm motility even without a change in external osmolality.

Our results partially contradict this, because our measurements showed that sperm cells of *Takifigu niphobles* remained immotile in solutions of physiological osmolality containing various concentrations of Ca^{2+} (1-100 μM) and 10 μM A23187. To adjust the intracellular Ca^{2+}-level, the extra- and intracellular Ca^{2+}-concentrations were balanced by applying 10 μM A231186, and using external solutions of different Ca^{2+}-concentrations. When cells were kept in isotonic SLS, the increase in intracellular Ca^{2+}-concentration did not induce sperm cell motility. However, when cells were diluted in ASW with the same Ca^{2+}-concentration, they became motile. These findigs of us provide serious evidence that the increase in intracellular Ca^{2+} alone is not enough for the initiation of sperm motility, and that the shift of osmolality towards hypertonic direction is the primary eliciting factor here.

To examine the role of calmodulin we used W-7 calmodulin-antagonist. The use of 100 μM W-7 reduced motility to 8 ± 3 % of control value in 10 minutes. TFP, another calmodulin-antagonist (200 μM) reduced motility to 3 ± 1 % of control value.

We also investigated the effects of several different ion-channel blockers on the motility of *Takifigu niphobles* spermatozoa. Out of 21 potassium channel blockers of Econokit EK-300 (Alomone Labs) only Penitrem A (10 μM, 15 minutes incubation) decreased motility (43 ± 12). Calcium channel blockers of EK-400 did not have any effect on the motility of the cells, as the chloride channel blocker chlorotoxin given at 25 μM concentration.

As calmodulin-inhibitors blocked the motility of the sperm cells of puffer fish, furthermore neither blockers of cell membrane Ca^{2+}-channels, nor K^{+}-channels influenced the motility of puffer fish sperm, it can be concluded that the interaction of calmodulin and Ca^{2+} released from internal stores plays a central role in the activation of puffer fish sperm cell motility elicited by hyperosmotic treatment.

Mechanical stretch-activated ion-channel blocker gadolinium changed the motility of the spermatozoa of both seawater puffer fish and freshwater carp. Gadolinium applied in 5, 10 or 20 μM decreased the motility of puffer fish spermatozoa from the control value of 92 ± 3 % to 17 ± 9, 11 ± 4 and 6 ± 3 %. Gadolinium also decreased carp sperm motility in a dose-dependent manner. Motility fell to fourth of control value when sperm cells were incubated for 1 minute in FPS containing 20 μM gadolinium, and decreased to 3 % when 40 μM gadolinium-concentration was applied. In contrast, gadolinium-treatment (20 or 40 μM) did not influence significantly the motility of the ascidian *Ciona* sperm cells initiated by SAAF or valinomycin.

We also measured the membrane-fluidity of the resting, motile and gadolinium-treated (40 μM $GdCl_3$) carp sperm cells. The fluorescence anisotropy value of resting, TMA-DPH-labelled sperm cell sample was 0.242 ± 0.02. Hypoosmotic treatment, which also induces sperm-motility, increased membrane-fluidity (measured anisotropy value: 0.182 ± 0.02). Treatment with

gadolinium rigidized the membranes of both resting cells and those exposed to hypoosmotic treatment.

Gadolinium binds to the lipid part of the cell membrane (Ermakov et al.), and the changes in the physical properies of the bilayer may also have an effect on the signal transduction processes. High affinity binding of lantanides influences the physical properties of the phospholipid bilayers. These changes can affect the dinamics of the conformational changes of the transmembrane proteins (Cantor). Gadolinium actively removes Ca^{2+} from the surface and causes the reordering of certain fatty acid chains, besides, it produces phase-separation (Li et al.). The condensation of the neighbouring lipids in the presence if Gd^{3+}, can exert pressure on the transmembrane proteins, and this pressure can prevent the conformational transition evoked by the mechanical tension. $GdCl_3$ (40 μM) significantly decreases the membrane fluidity of carp sperm, and it balances the fluidizing effect of hypotonic treatment. According to this observation we can say that gadolinium provokes the structural change of plasma-membrane.

theory of steric partition of toxin-binding To investigate the associations of cell surface proteins (receptors, ion-channels) we developed a method which is based on earlier theoretical biophysical articles dealing with the phenomenon of hindered surface diffusion. In our model we examined the modification of binding of toxin to ion channel, due to proximal spherical particles. Our results suggest that protein-proximity can be detected by golden-bead-mediated delaying of the lateral component of toxin-diffusion, which means in quantitative approach that we measure a change in the k_{on} determined from patch-clamp measurements.

examination of cell surface distribution of the receptors in question The extent of delay is also influenced by the grade of homoassociation. On Kit 225 K6 human T-lymphoma –a cell line that we used– the intermolecular distances of MHC I, MHC II, IL-2Rα, VLA-4 and TrfR, calculated from flow cytometric

energy transfer efficiencies, were 7.0 ± 0.1, 7.8 ± 0.2, 8.8 ± 0.2, 8.1 ± 0.2 and 7.5 ± 0.1 nm, respectively. From our experiments we also determined the relative expression levels: taking MHC I, which had the highest expression level, as a basis (100 %), meaning 1.25 x 10^6 binding places, the values for MHC II, IL-2Rα, VLA-4 and TrfR were 76.6 ± 8.6, 34.2 ± 8.3, <4.0 and 15.3 ± 1.5, respectively.

measurement of molecular associations Our experiments showed that VLA-4 integrin has a significant heteroassociation with MHC II molecule, the energy-transfer efficiency between them was E = 17.6 ± 2.5. We found a somewhat less, still significant co-localization of VLA-4 and MHC I molecules, here E = 13.7 ± 3.6. As a negative control the pair of TrfR and MHC I was used, where E = 3.5 ± 2.0. The energy transfer was the highest between MHC II and MHC I (20.6 ± 1.5).

detection of the hindrance of toxin and antibody-binding We performed patch-clamp experiments on four different samples related to one cell surface receptor, in which we examined the blocking properties of Pi2 scorpion-toxin on Kv1.3 channels regarding the Kit 225 K6 cell line. The τ wash-in time constant derived from the blocking curves on the control (unlabeled) cells was τ_{in} = 27.3 ± 1.7 s. For the sample that had been incubated in the presence of only secondary golden beads (g30:GAMIG) we got τ_{in} = 26.1 ± 1.7 s, which is not a significant difference. For the cells labeled primarily with antibodies, the value of τ_{in} regarding MHC I, MHC II, IL-2Rα, VLA-4 and TrfR was 30.1 ± 1.4, 28.7 ± 1.6, 29.9 ± 1.3, 27.6 ± 1.7 and 27.8 ± 1.6 s, respectively. These values only slightly differ from those of control. However, on cells labeled primarily by antibodies and secondarily by immunogold particles, we found that the values of τ_{in} increased significantly except for VLA-4 and TrfR: 45.9 ± 4.7, 40.0 ± 3.1, 36.3 ± 2.0, 29.6 ± 1.7 and 29.2 ± 1.2. This means that Kv1.3 channels are situated close to the rafts containing MHC-molecules, and far from those

containing TrfRs. The little difference obatined for VLA-4 integrins may be a consequence of their little expression level.

The relative change of the channel-toxin association rate constant, k_{on}, due to secondary labelling, taking primary labelling as a basis, was also calculated. As a result we got that $\Delta k_{on}/k_{on}$ was the highest for MHC I and MHC II (-42.7 ± 9.8 % and -40.0 ± 10.3 %, respectively).

Taking into consideration that there is such a decrease in toxin-binding wash-in time-constant (τ_{on}) we can conclude that Kv1.3 channels are in the lipid rafts containing MHC molecules, or at least, the majority of them is in the distance from the rafts comparable to the size of an immunogold particle. This is, according to Jürgens et al. and Murphy et al., 30-50 nm, the sum of the diameter of the golden bead and the thickness of the covering antibody-layer.

As the expression level of MHC II is 76.6 ± 8.6 % of that of MHC I, MHC II may statistically be closer to Kv1.3 channel. For the IL-2Rα and VLA-4 integrin, $\Delta k_{on}/k_{on}$ was found to be -17.5 ± 10.6 % and -13.0 ± 10.3 %, respectively. The value for VLA-4 can even be significant, if one takes into consideration the relatively low expression level of this protein (< 4 %). $\Delta k_{on}/k_{on}$ was only -2.8 ± 10.4 % for TrfR (no significant delaying).

The fact that we measured less relative change in k_{on} in the case of IL-2Rα subunit, is thought to be because although IL-2Rα forms clusters similar in size to those of MHC I or MHC II proteins, they have a significantly less density, according to the higher receptor-receptor distance (8.8 nm for IL-2Rα and 7.0 nm for MHC I), and the related confocal microscopic images. These results suggest that Kv1.3 channels are in comparable, although not so expressed molecular vicinity of the IL-2Rα subunits than of MHC molecules.

In an experiment analogous to the above, we wished to examine the delaying of cell surface receptor-antibody binding due to a proximal antibody-golden bead complex. In our experiments we found that *in situ* treatment of Kit

225 K6 cells by L243 anti-MHC II antibody in whole-cell configuration, decreased the current of Kv1.3 channels by 53 ± 15 %. Taking this phenomenon as a basis, we planned an experiment, in which we used three samples: an unlabeled, an MHC I-labeled (W6/32 antibody), and an MHC I and golden bead-labeled (W6/32 + g30) cell suspension.

When L243 antibody was given to the cells of the samples, the current of the channels decreased gradually. The exponentially fitted relaxation time constants τ_{in} derived from these measurements were 8.3 ± 2.8, 11.3 ± 4.2 and 37.4 ± 9.5 %. According to this, the bigger ligand we bind to the MHC I, which is in the vicinity of the MHC II, the higher the delaying effect of the MHC II-labeling L243 is on the channel current. According to the literature, we think that the binding of L243 to MHC II evokes an intracellular signalling process, and this leads to the inhibition of Kv1.3 channels (indirect effect). A depolarization of the cell membrane was found by Bene et al. due to the binding of an antibody to MHC II on JY B lymphoblast cells, and Pieri et al. as a response to IL-2 binding to IL-2 receptor on HUT-102B2 T-lymphoblast cells. These observations are in accordance with our view that both receptors are part of the same transmembrane signalling platform, the MHC-containing lipid rafts on this cell line.

V. SUMMARY

As result of our experiments, we could make conclusions on the regulation of cell membrane cation channels by receptors and extracellular osmolality and the physiological background of this.

1. On DDT1 MF-2 smooth muscle cells A_1 adenosine-receptor agonist CPA and A_{2A} adenosine receptor agonist CGS 21680 inversely modulated

membrane potential. With A_1 and A_{2A} antagonists we could prevent these changes.

Application of CPA dose-dependently increased the outward-rectifying voltage-dependent potassium current, in contrast, CGS 21680 decreased it. On the basis of this, the changes in potassium conductance can be responsible for the alterations in membrane potential. CGS 21680 dose-dependently accelerated the inactivation of the potassium channels. The application of pertussis-toxin did not influence the potassium-current blocking effect of CGS 21680, indicating that this A_{2A}-agonist does not modulate the channels via G-proteins.

2. Examining guinea pig tissues, in atrial myocardium and pulmonary artery both adenosine-receptor agonist NECA and its PET radiotracer analogue F-NECA exerted a concentration-dependent reduction in contractile force and phasic contraction (A_1-mediated effect). F-NECA had less effect for both kinds of tissue.

On thoracic aorta preparations and DPCPX-pretreated pulmonary arteries NECA and F-NECA induced relaxation in a dose-dependent manner, however no significant difference was found between the two analogues (A_{2B}-receptor mediated effect).

Both NECA and F-NECA significantly decreased the voltage-dependent potassium current on DDT1 MF-2 cells, and accelerated the inactivation of the current as well. Nevertheless, the effect was less expressed for F-NECA. From the above one can conclude that F-NECA is a functionally less active analogue of NECA.

3. We examined the role of mechanical stretch-activated cation channels in the signalling process leading to the activation of spermatozoa of different species. These channels were found to be inhibited by gadolinium, which dose-dependently decreased, according to our experiments, the sperm motiliy of the seawater puffer fish and the freshwater carp. Gadolinium also inhibited the shift in isoelectric point of a certain protein of puffer fish sperm, a shift which is

related to initiation of motility. We suggest that this protein mediates the effect of the stretch-activated channels. Our fluorimetric anisotropic measurements showed that hypoosmotic treatment, which initiates the motility of carp sperm, increases membrane-fluidity, while gadolinium prevents this. Therefore, stretch-activated cation-channels play a role in the activation of sperm-motility of species, where sperm gets into a medium with different osmolality at spawning.

4. In the next series of experiments we investigated the proximity of ion channels and receptors on a human T-lymphoma cell line. By using the patch-clamp technique, we demonstrated that golden nanobeads of 30 nm diameter bound to MHC I, MHC II glycoproteins, IL-2Rα subunit, VLA-4 integrin and TrfR cell surface receptors differently modulate the binding of Pi2 toxin to Kv1.3 channels. Modulation was seen in the increase of exponential τ_{in} toxin wash-in time constant compared to unlabelled cells. With our data we demonstrated the proximity of Kv1.3 channels to MHC-containing rafts. Taking into consideration the already proven vicinity of MHC I and MHC II we performed a control experiment in which the steric partition of (MHC II receptor-L243 antibody) binding was investigated by antibodies and golden beads bound to MHC I. This way L243-mediated channel modulation was successfully inhibited. We therefore evaluated a new method, with whitch we could detect receptor-channel or receptor-receptor proximities, on the basis of the steric partitioned diffusion, followed by a concentration-jump.

VI. PUBLICATIONS

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