
Session III: Cell receptors, cell membranes and intracellular transport

Lectures

L3.1

Intracellular trafficking of diphtheria toxin as an approach to investigation of vesicular transport

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Keywords: endosome maturation; intracellular transport; fluorescent proteins; diphtheria toxin

Diphtheria toxin (DT) is the main pathogenicity factor of *Corynebacterium diphtheria*, the diphtheria causative agent bacteria. The DT tertiary structure is formed by three domains. The catalytic C-domain corresponds to subunit A, and subunit B (SubB) consists of the transmembrane T-domain (Td) and receptor-binding R-domain (Rd). T-domain possesses pH-triggered hydrophobic properties. It is responsible for DT-endosomal membrane interaction and translocation of subunit A into the cytosol.

To examine the effect of Td on endosomal trafficking, we created two types of fusion proteins that were based on SubB (i.e. Rd + Td) or on Rd alone. SubB and Rd were simultaneously labeled with different fluorescent labels and added to Vero cells. They had a low initial colocalization, which increased linearly to almost 70% at 75 min after adding to the Vero cells. Rd showed faster colocalization with early and late endosome and lysosomal markers compared to SubB. Using the live cell imaging technique, it was demonstrated that SubB inhibited acidification within endosomes and maintained pH at the level about 6.5 from 10 to 50 min after addition. pH values in the endosomes with Rd decreased gradually from about 6.3 to about 5.5 during the same time period. Because pH 6.5 corresponds to early endosomes, SubB was in the early endosome compartment during 10 to 50 min after addition. Our data suggests that the T-domain can regulate endosomal pH. This ability may inhibit endosomal proteinases, and thus increase the probability of successfully transporting the DT C-domain to the cytosol.

L3.2

Plasma membrane Ca²⁺-pump in smooth muscle cells: its role, regulation, and new artificial inhibitor

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Keywords: Ca²⁺ pump; plasma membrane; smooth muscle; inhibitor; calixarene

Tight control of cytoplasmic Ca²⁺ concentration is essential for proper cell function. Changes in Ca²⁺ concentration are crucial in smooth muscle cells, because Ca²⁺ determines the state of relaxation/contraction. One key protein that controls cytoplasmic Ca²⁺ concentration is the Mg²⁺,ATP-dependent plasma membrane calcium pump. In this presentation, special attention is given to the kinetic and enzymatic peculiarities of the Ca²⁺ pump in smooth muscles, the molecular coupling of active Ca²⁺-transport, and enzyme hydrolysis. The molecular biology, structure, cell location, and connection with the physiological role of this pump in smooth muscle cells are also considered. Then, changes in plasma membrane Ca²⁺ pump activity under different physical (pH, organic solvents, transmembrane potential, active oxygen species), chemical (heavy metals), and biological (calmodulin, phospholipids, phosphorylation, proteolysis) factors are covered.

Today, there are no selective affine and non-toxic inhibitors or activators of the plasma membrane Ca²⁺ pump; however, in cooperation with the Institute of Organic Chemistry of NASU (Prof. V.I. Kalchenko), a new specific non-toxic affine inhibitor (calixarene C-90, 5,11,17,23-tetra(trifluoro)methyl(phenylsulfonylimino)-methylamino-25,26,27,28-tetrapropoxycalix[4]arene) is proposed to investigate the properties and role of the plasma membrane Ca²⁺ pump in smooth muscle cells. The kinetic mechanism of inhibition, connection between C-90's structure and effectiveness of its inhibitory action on the Ca²⁺ pump and transmembrane potential influence were analyzed. Some consequences of specific Ca²⁺ pump inhibition for smooth muscle cells and smooth muscles are observed.

We are thankful to Prof. S. Kosterin for scientific cooperation, valuable advice and discussions.

L3.3

Activation of ionotropic presynaptic glutamate receptors stimulates a fast synchronous vesicle fusion and a delayed asynchronous exocytosis

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Keywords: NMDA receptors; AMPA/kainate receptors; nerve terminals; GABA; exocytosis

The main goal of the present research was to elucidate the mechanisms that underlie the modulatory effects of presynaptic glutamate receptors on the presynaptic release machinery. We characterize the events induced by glutamate receptor agonists and antagonists in isolated hippocampal and cortical nerve terminals by analyzing the following parameters: I) evoked secretion of [³H]GABA from nerve terminals; II) the involvement of synaptic vesicles in the release process; and III) the plasma membrane potential. The results demonstrate that glutamate receptor-induced modulation of synaptic response strength was due to increasing the release probability of synaptic vesicles. Our results show that the vesicular, but not cytosolic, pool of [³H]GABA is preferentially involved in the response to glutamate receptor activation. We have revealed that glutamate application to nerve terminals induced a complex biphasic process, in which the fast transient phase was followed by a slow prolonged phase. The first phase, a fluorescence transient, was highly sensitive to extracellular Ca²⁺ and was similar to the response to high K⁺; it was decreased in the presence of the NMDA receptor antagonist MK-801. The second phase, a long-lasting process, was absolutely dependent on extracellular Na⁺ and was attenuated in the presence of CNQX, the kainate receptor antagonist. Our data allow us to consider that activation of ionotropic presynaptic glutamate receptors stimulates not only a fast synchronous vesicle fusion, but also a delayed asynchronous exocytosis as a result of inducing the spontaneous fusion of synaptic vesicles with the presynaptic membrane.

L3.4

Evolutionary changes on the way to clathrin-mediated endocytosis in Animals

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Keywords: evolution of endocytic system; FEI; AP2; DNM

The endocytic pathway is an essential system providing internalization of receptors, regulation of signaling, maintenance of plasma membrane homeostasis. Clathrin-mediated endocytosis (CME) is a universal pathway typical for all eukaryotes indicating its evolutionary conservativity. The aim of the work was to analyze the CME in eukaryotes to find out how this system evolved and to identify its specific features.

Comparative proteomic studies were used to trace the emergence of 35 crucial endocytic proteins. Their homologs were searched for in 151 reference eukaryotic proteomes using Hidden Markov Models (HMMs) based on the animal proteins. Sequence similarities and domain architectures were analyzed to identify potential homologs. Among analyzed endocytic components, 22 proteins were identified in the major eukaryotic branches, while 13 proteins were gradually acquired during evolution. The first crucial “wave of emergence” of endocytic components was the acquisition of FCHO, Eps15, and ITSN in opisthokonts. Analysis of the protein-interaction network demonstrated that these proteins constituting the FEI functional complex emerged as an additional interaction hub. Furthermore, the FEI complex became an additional positive factor for the activation of AP2, which is consistent with the structural changes in AP2 homologs. Another opisthokont-specific acquisition corresponds to late endocytic stages and is characterized by the recruitment of the dynamin/SNX9 complex and involvement of the actin polymerization machinery. The emergence of multiple sorting proteins in animals elaborated the cargo recruitment and its specificity in animal cells.

The evolutionary reconstruction demonstrated the basis of the CME process and its progressive changes facilitating CME regulation and specialization in metazoans.

Posters

P3.1

Plasmin affects platelet aggregation and degranulation

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Keywords: plasmin; platelet aggregation; α -granule secretion

Plasmin and plasminogen can specifically bind to platelets, regulating their functions. As we previously showed, Lys-plasminogen, but not its native Glu-form, inhibits platelet aggregation and secretion, and impedes actin cytoskeleton reorganization during platelet activation. Although plasminogen binding to the platelet surface is mediated by its kringle domains, involvement of its catalytic domain in the realization of the observed effects cannot be neglected. The aim of this study was to evaluate the effects of plasmin on human platelet aggregation and α -granule secretion.

Plasmin (1 CU/ml) was found to induce low-amplitude platelet aggregation. Pre-incubation of gel-filtered platelets with plasmin (3 min) in the range of nanomolar concentrations decreased the amplitude of thrombin- and collagen-induced aggregation. Plasmin exerted weak effects on exocytosis of platelet alpha-granules. Using flow cytometry it was shown that platelet activation with strong agonists was accompanied with a 2.3 times increase in P-selectin positive platelets compared to the control. Platelet fluorescence intensity increased by 30 times during thrombin activation and by 20 times in case of collagen. Pre-treatment of platelets with plasmin decreased amount of P-selectin positive cells after stimulation and resulted in a decrease of P-selectin level on the surface of activated platelets. So, it was shown that plasmin facilitates partial release of alpha-granules, but disturbs thrombin- and collagen-induced platelet exocytosis. The similar effect was observed in the case of platelet aggregation. It is possible that plasmin induction of both platelet aggregation and secretion is the result of enzymatic activation of PAR-4.

In conclusion, plasmin can affect platelet activity through modifying platelets' sensitivity to endogenous agonists. The plasmin effects on platelet functioning should be considered when patients undergo fibrinolytic therapy.

P3.2

Deep vs. profound hypothermia: Discrimination of changes in non-pathological and pathological mechanisms of glutamate transport in brain nerve terminals

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Keywords: glutamate; uptake and tonic release; permanent glutamate turnover; glutamate transporter reversal; deep and profound hypothermia; brain nerve terminals

Background

A brain temperature decrease by cardiac arrest is often successfully applied for the prevention of the neurological consequences of ischemia. A study to discriminate between the effects of deep and profound hypothermia (i.e. 27 °C, 17 °C, respectively) on the pathological and non-pathological mechanisms of presynaptic glutamate transport was conducted using rat brain nerve terminals (synaptosomes).

Results

Transporter-mediated uptake and tonic release of L -[¹⁴C] glutamate are oppositely directed processes, the dynamic balance of which determines the physiological ambient level of the neurotransmitter. It was revealed that these processes decreased in a different range in deep/profound hypothermia. Importantly, tonic L -[¹⁴C]glutamate release was almost completely absent in deep hypothermia. As a result, hypothermia-induced changes in ambient L -[¹⁴C]glutamate are not evident in nerve terminals unaffected by ischemia. The ambient level and non-transporter tonic release of L -[¹⁴C]glutamate in nerve terminals, measured in the presence of transporter inhibitor DL -threo- β -benzyloxyaspartate, were decreased with similar efficiency in both hypothermia conditions. A progressive decrease from deep to profound hypothermia was shown in pathological transporter-mediated L -[¹⁴C]glutamate release: 1) stimulated by depolarization of the plasma membrane; 2) during dissipation of the proton gradient of synaptic vesicles by the protonophore FCCP; 3) by means of heteroexchange with competitive transportable inhibitor of the glutamate transporter DL -threo- β -hydroxyaspartate.

Conclusions

Therefore, the direction of hypothermia-induced changes in ambient glutamate is unpredictable in "healthy" nerve terminals and depends on the sensitivity to hypothermia of uptake vs. tonic release. In affected nerve terminals (e.g., in the core and penumbra zones of the insult), pathological transporter-mediated L -[¹⁴C]glutamate release from nerve terminals decreases with progressive significance from deep to profound hypothermia, thereby underlying its potent neuroprotective action.

P3.3

HA – friend or foe? Evidence for an interaction between hyaluronic acid and native/model membranes

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Keywords: hyaluronic acid; human cells; Langmuir monolayers

Hyaluronic acid (HA), an important component of the extracellular matrix, is now commonly used in medicine and cosmetology. HA's importance is related to its involvement in inflammation and tumor progression.

The aim of the study was to examine the effect of exogenous application of HA (of various molecular weights) on human monocytic cells (cell line U-937, not differentiated cells and cells differentiated to macrophages). HA was cytotoxic and decreased cell viability (determined by the MTT assay); however, a HA concentration higher than 200 mg/L was crucial for cell survival. The interaction of HA with native membranes was determined by measuring the binding of biotin-labeled HA to the cell surface. Physicochemical parameter changes (determined using the Langmuir technique) of model membranes composed of specially selected phospholipids allowed us to describe the impact of HA on the polar and hydrophobic parts of membranes and to determine the HA-induced modification of the membrane structure. Non-differentiated and differentiated immune cells responded differently to HA application, suggesting that HA toxicity depends on cell membrane assembly, which was further confirmed by HA's effects on model lipid membranes of appropriate composition; a relatively smaller effect was found for HA of diverse molecular weights.

Reference:

Misra S, Hascall VC, Markwald RR, Ghatak S (2015) Interactions between hyaluronan and its receptors (CD44, RHAMM) regulate the activities of inflammation and cancer, *Front Immunol* 6:201 <http://journal.frontiersin.org/article/10.3389/fimmu.2015.00201/full>

P3.4

The effects of an allosteric modulator of the GABA_B receptor, rac-BHFF, on E_m, synaptic vesicle acidification, and transport of GABA and glutamate in nerve terminals

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Keywords: GABA_B receptor allosteric modulator rac-BHFF; GABA; glutamate; rat brain nerve terminals; membrane potential; synaptic vesicle acidification

Positive allosteric modulators of GABA_B receptors have great therapeutic potential as medications for anxiety, depression, and other disorders. The effects of a recently-discovered positive allosteric modulator of GABA_B receptors, rac-BHFF, on the key characteristics of GABA-ergic and glutamatergic neurotransmission were investigated in cortical and hippocampal presynaptic nerve terminals (synaptosomes).

The ambient level of [³H]GABA, that is a balance between release and uptake of the neurotransmitter, increased significantly in the presence of 10-30 μM rac-BHFF. The initial velocity of synaptosomal [³H]GABA uptake was suppressed by the modulator. In the presence of GABA transporter blocker NO-711, it was shown that rac-BHFF increased the tonic release of [³H]GABA from synaptosomes (at concentrations 3-30 μM). Rac-BHFF within the concentration range of 0.3–30 μM did not enhance the inhibitory effect of (±)-baclofen on depolarization-induced release of [³H]GABA. Rac-BHFF (0.3-30 μM) caused a dose-dependent depolarization of the plasma membrane and dissipation of the proton gradient of synaptic vesicles in synaptosomes, which was shown in the absence/presence of the GABA_B receptor antagonist saclofen using the fluorescent dyes rhodamine 6G and acridine orange, respectively. The described effects of rac-BHFF were not associated with any modulation of presynaptic GABA_B receptors. These features of the modulator also affected synaptosomal L-[¹⁴C]glutamate transport, that is, uptake, release, and the ambient glutamate level in nerve terminals. Therefore, the drug development strategy of using positive allosteric modulation of GABA_B receptors can eliminate the side effects of rac-BHFF in the presynaptic nerve terminals, and *vice versa*, these new properties of rac-BHFF may be exploited appropriately.

P3.5

Effect of cholecalciferol on diabetes-induced impairment of vitamin D₃ signaling in the skeletal system

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Keywords: vitamin D; diabetes type 1; VDR; CYP27B1
Type 1 diabetes mellitus (DM1) is known to be associated with development of severe side effects, including secondary osteoporosis. Furthermore, DM1 leads to disturbances of vitamin D₃ (D₃, cholecalciferol) metabolism and signaling. In this regard, our study was performed to characterize diabetes-related impairments of D₃ bioavailability and the state of the D₃-hormone system in bones and bone marrow by evaluating expressions of D₃ receptor (VDR) and 25-hydroxyvitamin D₃ hydroxylase (CYP27B1). DM1 was induced in male Wistar rats by a single streptozotocin injection (55 mg/kg b.w.). After four weeks of diabetes, the animals were treated with and without D₃ (100 IU, 30 days, per os). The changes in serum 25OHD₃ were measured by ELISA, mRNAs levels of VDR and CYP27B1 in bone tissue and bone marrow were assessed by RT-qPCR. Protein expression of VDR in bone tissue was analyzed by Western blot. It showed that, at a transcriptional level, diabetes caused a marked lowering of VDR in bone and bone marrow, while CYP27B1 expression significantly increased in bone tissue, and extremely decreased in bone marrow. Diabetes was also found to down-regulate the protein level of VDR in bone tissue. These changes were accompanied by a 50% decrease in the content of serum 25OHD₃, indicative of D₃ insufficiency. After D₃ administration, diabetes-induced changes returned to control values. In conclusion, DM1 causes a disturbance in D₃ metabolism and signaling that may result in secondary osteoporosis. D₃ was shown to be effective in correction of diabetes-related failures.

P3.6

Vitamin D₃-deficiency affects the synaptic vesicles docking and fusion steps of exocytosis

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Keywords: vitamin D deficiency; synaptic vesicles; brain dysfunction
Vitamin D₃ may affect brain function by multiple mechanisms. To elucidate the precise mechanism of vitamin D deficiency (VDD) in brain dysfunction we analyzed the aggregation and Ca²⁺-dependent fusion of synaptic vesicles (SVs) in cell-free system. VDD in rats was assessed by 25OH-D₃ level in blood serum. Fusion was performed using fluorescent dye octadecyl rhodamine B. The potential of PMs was assayed with rhodamine 123. Na⁺,K⁺-ATPase activity of synaptosomes was determined spectrophotometrically. The particle diameter was measured by photon correlation spectroscopy.
The size of SV aggregates decreased from 700 nm in control to 450 nm in VDD and their amount was 30% lower under VDD. VDD was accompanied by the reduction of the rate of SV fusion with PMs in the presence of cytosolic proteins (20.5% vs 27% in control, p<0.05). This was likely associated with the alteration of PMs cholesterol. The level of PMs cholesterol after enrichment with mixture of methyl-β-cyclodextrin:cholesterol (1 :8) was 0.78±0.08 as compared to 0.67±0.06 μM/mg of protein in control, p<0.05. The fusion of cholesterol-enriched PMs in control was decreased to the same extent as in VDD. The Na⁺,K⁺-ATPase activity and the potential of synaptosomal PMs decreased by 39.5% and 29.8±0.23%, respectively, under VDD. These alterations may be associated with increased cholesterol content in PMs that was previously shown under type 1 diabetes and suggest that the exocytotic process may be corrected by vitamin D supplementation.

P3.7

The lipids distribution and their dynamics in plasma membranes of living and apoptotic cells

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Keywords: apoptosis; plasma membrane; fluorescent probe

Local changes in the properties of the plasma membrane lipid phases play an essential role in cell biology. Most of the recent lipid raft observations were performed at conditions that are far from the physiological environment. This influences the distribution of the lipid phases in the native plasma membrane and provides only limited information. The aim of the current research was to study the properties, composition and morphology of plasma membranes during apoptosis.

Novel fluorescent probes NR12S, bNR10S, F2N12SM and PA (Kreder, Oncul et al., 2015; Kreder, Pyshev et al., 2015; Niko and Pascal, 2016) were used for lipid order detection. The results suggest a strong lateral heterogeneity of the outer plasma membrane leaflet and a significant difference between living and apoptotic cells. Living cells do not demonstrate a visually distinguishable lateral demixing of the lipid phases in the outer leaflet of the plasma membrane. In contrast, apoptotic cells show the formation of separate domains of the Ld-like phase. Further studies with the recently developed PA probe allowed the direct observation of the lipid phases in both plasma membranes and the membranes of cellular organelles during all stages of programmed cell death. It appeared that plasma membranes have the highest concentration of Lo-like phases, while internal membranes mostly consist of the Ld-like phase. Thus, we observed a significant influence of the externalization of subcellular membranes on the lipid order of the plasma membrane, which, together with scrambling, contributed to strong plasma membrane lipid reorganization upon apoptosis.

P3.8

Requirements for the internalization of antibodies directed against FGFR1

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Keywords: FGFR1; endocytosis; antibody; ADC
Fibroblast growth factors (FGFs) and their receptors (FGFRs) constitute tightly regulated signaling networks that govern developmental processes and metabolism. Numerous tumors are characterized by elevated levels of FGFR, which are correlated with poor patient prognoses. One of the most promising therapeutic approaches to treat FGFR-dependent cancers is based on the concept of Antibody Drug Conjugates (ADC). In this strategy, antibodies coupled to a cytotoxic drug specifically recognize the receptor overproduced by the cancer cells. Receptor binding is followed by the internalization of the ADC-receptor complex to the endosomes via receptor-mediated endocytosis. Next, ADC is degraded in lysosomes and the cytotoxic drug is released, leading to the cell death.

Here, we analyzed the internalization of various antibody fragments that target FGFR1. We show that antibodies in the ScFv or diabody format bind to FGFR1 but do not undergo receptor-mediated endocytosis. In contrast, the same ScFv proteins fused to the Fc fragment are efficiently internalized into the FGFR1-overproducing cells. Binding of Fc-containing antibodies to FGFR1 induces receptor degradation, suggesting that the endocytosed FGFR1-antibody complex is directed towards the lysosomes. The internalization of the antibody-FGFR1 complex is dependent on the activity of clathrin and dynamin. Interestingly, the internalizing antibodies do not trigger the activation of FGFR-dependent signaling cascades, suggesting that receptor kinase function is not essential for FGFR endocytosis.

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P3.9

Adhesion of neutrophils to endothelial cells is regulated by interactions between bradykinin B2 and dopamine D2 receptors

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Keywords: bradykinin B2 receptor; dopamine D2 receptor; neutrophil-endothelial cell adhesion

The bradykinin B2 (B2R) and dopamine D2 (D2R) receptors belong to a large family of G protein-coupled receptors (GPCRs). The direct cooperation and functional modulation of B2R and D2R with other GPCRs has been widely proven. Nevertheless, the interaction between B2R and D2R has not yet been studied. There is some evidence indicating high expression of B2 and D2 receptors in endothelial cells, where they participate in the control of neutrophil-endothelial cell adhesion. Therefore, the aim of this study was to investigate the interaction between B2R and D2R and its consequence in the mediation of neutrophil adhesion. The co-localization of both receptors in the membrane of B2R- and D2R-transfected HEK293 cells was confirmed using confocal microscopy. Alteration of intracellular Ca²⁺ concentration was also observed in co-transfected cells. Moreover, treatment with a B2R agonist changed the level of receptor co-localization. The B2R-D2R interaction was reflected in the adhesion of neutrophils to human umbilical vein endothelial cells (HUV-EC-C). Stimulation of these cells with receptor agonists changed the expression of adhesive proteins as well as the production of interleukin-8 and cAMP. In addition, B2R and D2R agonists modified receptor expression rate. These results suggest that there is cooperation between the B2R and D2R receptors, which is altered when they are stimulated with their respective agonists. It seems that the interaction between these receptors plays an essential role in the processes controlling neutrophil-endothelial cell adhesion.

P3.10

Vitamin D₃-dependent OPG/RANKL system in regulation of bone homeostasis at glucocorticoid-induced osteoporosis

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Keywords: vitamin D₃; 25OHD₃; OPG/RANKL; osteoporosis; glucocorticoid

Long-term glucocorticoid therapy can lead to alterations of bone cell function through an imbalance in the OPG/RANKL (osteoprotegerin/receptor activator of nuclear factor NF- κ B ligand) cytokine system and inhibition of vitamin D₃ turnover.

Wistar female rats received prednisolone (5 g), with or without 100 IU of D₃, daily for 30 days. Serum 25OHD₃, OPG and RANKL levels were measured using ELISA. OPG, RANKL and osteocalcin expression in bone tissue were assessed using Western blot. Ca²⁺ and Pi, alkaline phosphatase (ALP) activity levels were determined using spectrophotometry.

Prednisolone administration reduced serum 25OHD₃ levels by 70%. This led to hypocalcemia and hypophosphatemia, which was accompanied by increased activity of ALP and its bone isoenzyme. Similar changes in mineral metabolism were also observed in bones. Prednisolone reduced OPG levels by 34% and 38%, and increased RANKL levels by 27% and 10% in serum and bone tissue, respectively. The OPG/RANKL ratio was reduced 1.7 times. These changes were accompanied by a significant decrease in osteocalcin expression. Vitamin D₃ co-administration with prednisolone normalized the levels of mineral components and the balance of the OPG/RANKL system by enhancing vitamin D₃ bioavailability.

Our results indicate that glucocorticoid administration altered remodelling by activating bone tissue resorption that correlated with an impairment of vitamin D₃ turnover. Normalization of vitamin D₃ availability might be a potential strategy to reduce the negative effects of glucocorticoids on bone homeostasis.

P3.11

Molecular mechanisms of biological activity of human heparin-binding EGF-like growth factor

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Keywords: growth factors; nuclear trafficking; HB-EGF; EGFR

This work is dedicated to the investigation of the ability of sHB-EGF to induce EGFR nuclear transportation. In order to create the molecular instruments for studying molecular mechanisms of the sHB-EGF/EGFR ligand-receptor complex intracellular traffic, the recombinant analogue of soluble HB-EGF and fluorescent derivatives of sHB-EGF were obtained in both the eukaryotic and prokaryotic expression system. The recombinant sHB-EGF could bind diphtheria toxin and heparin and also stimulate 3T3 mouse fibroblast's proliferation *in vitro*. The recombinant proteins obtained were used in further studies to determine and visualize an intracellular localization of EGFR and its complex with sHB-EGF.

It was shown that nuclear translocation of EGFR can be induced by the soluble form of HB-EGF in human cervical cancer A431 cells. Treatment of A431 cells with sHB-EGF resulted in nuclear localization of EGFR, and such translocation occurs via a retrograde pathway. It was shown by confocal microscopy and co-immunoprecipitation assay that the translocation complex consisted of both ligand and receptor. The chromatin immunoprecipitation assay showed the association of the sHB-EGF/EGFR complex with the promoter region of cyclin D1 in the cell nucleus, and this association was prevented by application of EGFR kinase inhibitor AG1478. The data obtained suggest that sHB-EGF acts similarly to other EGFR ligands and is capable of inducing EGFR nuclear translocation as a part of a ligand-receptor complex in a tyrosine phosphorylation-dependent manner.

P3.12

Detection of new brain proteins that exhibit affinity for thiamine

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Keywords: thiamine; affinity chromatography

The data obtained by affinity chromatography of extracts of different samples of brain tissue under various conditions, consistently repeat and show that thiamine is associated with a group of proteins. Besides the proteins of already known ThDP-dependent enzymes, the most frequent are cytoskeletal proteins, such as actin and tubulin, several signaling proteins, in particular chaperones and proteins from the family 14-3-3. Of particular interest is the binding of th-AS with certain proteins of energy metabolism, in particular, glutamate dehydrogenase (GDH) and malate dehydrogenase (MDH). It is shown that the TPP acts as an inhibitor on the GDH activity of the whole homogenate and as an activator on the GDH activity in the eluates with the AS. It seems that thiamine on the AS is bound with one of the isoforms of GDH, and that it may interact with thiamine. Similarly, affinity chromatography on th-AS with covalently bound thiamine allows to split different isoforms of MDH that may have undergone different post-translational modifications, and have various functions in a cell. In order to confirm the results and decipher the molecular mechanisms and physiological significance of the interaction of these proteins with biologically active derivatives of thiamine, further research is needed. Thiamine in high doses is now used in the treatment of neurodegenerative diseases, and this direction of research is promising in the search for therapeutic intervention and the development of new approaches to the treatment of such diseases as Korsakoff-Wernicke syndrome, Alzheimer's disease and others.

P3.13

Interaction of new piroxicam derivatives with model phospholipid membranes

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Keywords: piroxicam; chemoprevention; phospholipid bilayers

Piroxicam is a non-steroidal anti-inflammatory drug (NSAID). One of molecular targets of NSAIDs is cyclooxygenase-2 (COX-2), which is overexpressed by most solid tumours. Therefore, NSAIDs are utilized as cancer preventive agents. An alternative mechanism by which NSAIDs can be effective is by interacting with cellular membranes and altering their biophysical properties. NSAIDs can induce changes in the fluidity, permeability, and biophysical properties of cell membranes. NSAIDs-induced perturbations of the phospholipid membrane, may also affect the activity of membrane proteins (e.g., COX-2).

In the present work, we describe the effects of six newly synthesized piroxicam derivatives on model lipid membranes using calorimetric, fluorescence, and electron paramagnetic resonance (EPR) spectroscopic experiments. We examined the influence of these new compounds on the phase behaviour of phospholipid bilayers and their membrane location by studying the incorporation of different fluorescent probes (laurdan and prodan). EPR spectroscopy was applied to better understand the local environment and dynamic organization of the studied compounds in the model membranes and confirm their membrane location. The results suggest that the piroxicam derivatives penetrate the model membranes. Further, the experiments revealed that the carbonyl group and the fluorine substituent in the newly synthesized compounds increased their ability to interact with the model membranes when compared to piroxicam.

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P3.14

ATR-FTIR studies of 5-*n*-alkylresorcinols effect on dry DPPC film

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Keywords: ATR-FTIR; alkylresorcinol; DPPC

The 5-*n*-alk(en)ylresorcinols (ARs) are naturally-occurring phenolic lipids. Previous studies have shown that ARs can be incorporated into biological and artificial membranes, thereby affecting their properties and function. If added before liposomal vesicles are formed, ARs decrease the permeability of the membrane. In this sense, we seek to understand the influence of ARs homologs (from C15 to C25) on both the structure and physicochemical properties of dry dipalmitoylphosphatidylcholine (DPPC) films, using Attenuated Total Reflectance Fourier Transfer Infrared Spectroscopy (ATR-FTIR). Chain-length dependent changes in dry AR:DPPC multilayer films were investigated as a function of temperature. Given the analysis of the bands assigned to CH₂ stretching and scissoring, C=O, PO₂⁻, and OH stretching vibrations showed that AR influences the hydrophobic and hydrophilic parts of DPPC layers. In dry AR-doped DPPC films, we observed the appearance of a phase transition associated with an increased amount of gauche conformers of the aliphatic chains. Additionally, we have shown that OH groups of AR form hydrogen bonds with the phosphate groups of DPPC lipids, which may in turn explain the stabilizing effect of ARs on phospholipid membranes.

P3.15

Characterization of recombinant protein CRM197 expressed in *E. coli* for studying the diphtheria toxin

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Keywords: black lipid membranes; CRM197; diphtheria toxin; membrane channels; toxoid

A non-toxic recombinant derivative of diphtheria toxin (DT), CRM197, was obtained by site-directed mutagenesis to replace the Gly at position 52 with Glu. This protein is widely used to study the interaction of DT with target cells and has broad medical and bioengineering applications. CRM197 was expressed in *E. coli* and purified by Ni-NTA chromatography. DT-sensitive Vero and DT-resistant L929 cells were shown to bind and internalize recombinant CRM197. Because the same order of magnitude K_i values were obtained for Vero and L929 cells, this suggests similar K_d values for CRM197. Like DT, CRM197 (2 nM–20 nM) binds to a phosphatidylethanolamine bilayer membrane in saline (pH of 6.0) under negative membrane potential (-40 mV) and creates open channels in the membrane in saline (pH of 4.8) under positive membrane potential (60 mV), which is applied from the side of insertion (Donovan et al., 1981). Conductance of separate DT and CRM197 channels in 1M KCl or 1M NaCl was also the same (20 pS). The relative permeability ratio (P_{Cl⁻}/P_{K⁺}) at 10-fold KCl gradient was estimated from the shift of the reversal potential by Goldman–Hodgkin–Katz equation and amounted to 8.9 ± 0.45 for the total CRM197-induced steady-state current. This coincides favourably with the preferential, though not ideal, anion selectivity of DT channels. Thus, CRM197 reproduced the main properties of DT. In the future, CRM197 can be used as a tool to study the modes of DT action, particularly those involving membrane binding and pore-formation.

P3.16

Sonoporation enhanced sensitizer chlorin e₆ cellular uptake

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Keywords: sonoporation; sonodynamic therapy; chlorin e₆; ultrasound contrast agent microbubbles; fluorescence

In vitro and *in vivo* experimental evidence suggest that porphyrins activated by ultrasound (US) and microbubble (MB) cavitation produce the cell killing effects referred to as sonodynamic therapy. However, during insonication, the cells may undergo sonoporation, which facilitates the intracellular delivery of tetrapyrrolic sensitizers. The question of *human* malignant melanoma Me-45 cell sonoporation significance after US (1MHz; 20% or 100% DC; 0.5W/cm²; 25s) irradiation was addressed in this study by employing chlorin e₆ (Ce₆, 17 μM) sensitizer and BG7573 contrast agent MB at 4.7×10^7 MB/ml concentration and 390:1 MB/cell ratio. Fluorescence microscopy (Leica DMI8; exciter 540–580nm; dichroic 585nm; emission 592–668nm; exposure 3s) and fluorescence spectroscopy (Avantes AvaSpec ULS2048L; excitation 473nm DPSS laser, <100 mW, coupled to Y-shaped fiber bundle; emission filter: LP 535nm) have been used to reveal and quantify the *in vitro* accumulation of Ce₆. The application of US alone was not cytotoxic (>90% viable cells) and MB cavitation induced only a slight decrease of Me-45 cell viability at 72 h after treatment, as measured by MTT assay. Under these experimental conditions, the co-application of US, MB and Ce₆ increased the fluorescence of Ce₆ in the cytoplasm but produced no significant enhancement of Ce₆ cytotoxicity. These data highlight the necessity of defining the roles of sonoporation and sonodynamic therapy when combining US, MB cavitation and sensitizer treatment.

P3.17

Pseudomonas fluorescens SBW25 capability to sense a quorum

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Keywords: quorum sensing; biofilms; pseudomonas
The regulation of biofilm-formation in many pseudomonads is controlled by quorum sensing (QS) and is key to their ability to colonize a wide variety of environments. However, in the soil and plant-associated *P. fluorescens* SBW25, mutations in diguanylate cyclase (DGC) or DGC-associated genes increase c-di-GMP levels to induce bacterial attachment and the expression of cellulose, resulting in the formation of biofilms at the air-liquid interface of static microcosms, with no apparent involvement of QS-dependent behaviour. Nonetheless, a bioinformatics analysis of the SBW25 genome has identified putative N-acyl homoserine lactone (AHL) and α -hydroxy ketone (AHK)-dependent QS pathways, including an AHL/AHK synthase-like protein belonging to the HdtS family (PFLU0050) and a CqsA-like protein identified as 8-amino-7-oxononanoate synthase (PFLU5614). Furthermore, a putative link can be established between QS and the regulation of c-di-GMP levels in SBW25 based on the TpbA/TpbB system of *P. aeruginosa*. In this preliminary work, we have used bioassays with AHL/AHK reporter strains overlaid onto thin layer chromatographs of liquid culture extracts to demonstrate that SBW25 produces detectable levels of AHL and AHKs. Although these quorum compounds have yet to be identified, tests using exogenous dodecanoyl homoserine lactone suggest that in SBW25, biofilm structure, eDNA, and possibly siderophore production, may all be regulated by QS pathways. This work is the first to provide experimental proof that SBW25 is capable of responding to AHL/AHK quorum signals, like many other pseudomonads, and to suggest that biofilm-formation may also be AHL-regulated under different environmental conditions.

P3.18

An efficient approach to purification of antibodies specific to cell surface marker CD34

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Keywords: antibodies purification; CD34; protein renaturation

CD34 is a transmembrane phosphoglycoprotein expressed in human progenitor and stem cells. Antibodies specific to CD34 have broad application for diagnostic, biomedical research, detection and separation of human hematopoietic stem/progenitor cells. Usually, protein A or protein G chromatography is used for purification of antibodies. However, in the case of polyclonal antiserum and scFv, these approaches are not effective.

The research objective was to develop an efficient and inexpensive laboratory approach for the generation and application of an affinity medium based on direct recombinant antigen immobilization for the purification of high-specific antibodies against human antigen CD34.

An extracellular fragment of CD34 antigen that retains immunogenic determinants of cell-surface CD34 was cloned and expressed in *Escherichia coli*. To enhance the protein expression, a modified method of auto-induction in high-density shaking cultures was used. As the result, rCD34 was accumulated in the bacterial inclusion bodies. The matrix-assisted refolding method was utilized to obtain active rCD34. Solubilized under denaturing conditions, rCD34 was immobilized, via His-tag, on the metal affinity column and refolded by decreasing urea gradient. The density of rCD34 on Ni-NTA-agarose after renaturation was around 1.8 mg/ml. The obtained affinity medium was used for one-step purification of antibodies against antigen CD34. The purity of antibodies in eluted fractions was more than 95%.

The antibodies generated are applicable for phenotyping of CD34+ cells using immunocytochemistry and flow cytometry assays. Ni-NTA-agarose with immobilized rCD34 can be useful for selective isolation of highly specific polyclonal, monoclonal and single-chain antibodies against CD34.

P3.19

The effect of DHEA and pregnenolon on GABA metabolism under impaired calcium homeostasis conditions

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Keywords: GABA; glutamate; DHEA; pregnenolone; calcium pump PMCA; neurosteroids

Pregnenolon and dehydroepiandrosteron (DHEA) belong to neurosteroids. Both molecules are known to influence release of γ -aminobutyric acid and glutamate by interaction with their receptors. Therefore, it is crucial to examine impact of neurosteroids on metabolism of neurotransmitters. GABA-shunt is the metabolic pathway involved in synthesis of γ -aminobutyric acid (GABA) from glutamate and conversion of GABA to succinate. It consists of three enzymes: glutamate decarboxylase (present in two isoforms GAD65 and GAD67), GABA-aminotransferase (GABA-T) and succinate semialdehyde dehydrogenase (SSADH). In our earlier work we established that activity of GABA-shunt is dependent on the presence of neuron-specific plasma membrane calcium ATPases (PMCA2 and PMCA3). In present study we examined the effect of DHEA and pregnenolon on the regulation of GABA-shunt under normal and impaired calcium homeostasis.

We used stably transfected pseudoneuronal PC12 cells with suppressed expression of neuron-specific isoforms of PMCA (PMCA2 or PMCA3). Cells were incubated with DHEA or pregnenolon for 30 minutes. We examined activities of GAD, GABA-T and SSADH with fluorimetric and spectrophotometric methods. To determine protein levels of all enzymes, western blot method was used. Our results indicate that DHEA and pregnenolon participate in regulation of GABA-shunt. Changes are dependent on the presence of neuron-specific plasma membrane calcium ATPases. However, not on a specific isoform.

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P3.20

The thiamine metabolism proteins and rat brain astroglial state at alimentary B₁ deficit and its correction

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Keywords: thiamine; GFAP; thiamine deficiency; astroglial cells

The accumulated literature data strongly suggests that almost all of the known neurodegenerative diseases are accompanied by more or less pronounced thiamine deficiency (TD). This fact does not exclude the fact that thiamine deficiency may be the initiating factor for development of some of these pathologies. That is why the study of changes in structural and functional organization of the nerve cells with TD is an important step in determining the mechanisms of neurodegenerative processes. The aim of this study was to determine changes in levels of key proteins of thiamine metabolism and astroglial state at alimentary TD and its correction in rats' brain. The key enzymes of thiamine metabolism are thiamine transporter (THTR-1) and thiamine kinase (TPK). According to modern concepts, astrocytes are considered as the most sensitive central nervous system cells to thiamine deficit. Reduced metabolic activity of the cells is observed in decreased ability to synthesize glial fibrillary acidic protein (GFAP), which is the main component of cytoskeletal intermediate filaments, and is used as astrocytes functional activity marker. The results show the possibility of thiamine-dependent regulation of the TPK and the THTR-1 synthesis in nerve cells. There is also the probability that the normalization of thiamine-dependent processes with thiamine administered to TD animals helps restore metabolic activity of astroglial cells. That might contribute to realization of its neuroprotective function.

P3.21

Ca²⁺-induced changes of normalized fluorescence instead [Ca²⁺]_m measuring – test for mitochondria survival

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Keywords: mitochondria survival testing; intramitochondrial free calcium

Introduction. It is now widely assumed that a value of intramitochondrial free calcium ([Ca²⁺]_m) plays critical role in both normal physiological and pathological conditions. The question of this study is – what parameter can be used as the test of mitochondrial survival?

Methods. In our experiments three models were used: suspension of myometrium mitochondria, suspension of liver mitochondria and digitonin permeabilized myometrium cells. Several parameters were determined: the concentration of ionized Ca in a mitochondria matrix by means of fluorescent probes, total Ca²⁺ accumulation in a mitochondria matrix by means of isotopic method and liver mitochondria respiration by means of a polarographic method.

Results. It was shown that preliminary myometrium mitochondria incubation in the medium containing 3 mM Mg provided the low endogenous level of ionized Ca, however addition of exogenous Ca²⁺ was accompanied by the considerable increase of [Ca²⁺]_m and low level of total cation accumulation, that can be caused by depolarization of mitochondria membranes in this medium. The endogenous Ca²⁺ level was considerably higher in the medium with 3 mM ATP and 3 mM Mg, but the subsequent addition of exogenous Ca was accompanied by small increase of free Ca concentration in the mitochondria matrix.

Conclusion. Thus, a low value of ionized Ca concentration in the mitochondria matrix is not the index of high functional activity of mitochondria. The suggestion has been made that Δ [Ca²⁺]_m in response to the exogenous Ca²⁺ addition (in the units of normalized fluorescence) can be used as test of the mitochondria survival.