XIII International Meeting of the **European Calcium Society** ECS2014

September 13 - 17, 2014

Centre de Congrès, Aix-en-Provence, France

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The organisers of ECS2014 wish to express their gratitude to the following sponsors whose support makes a vital contribution to the success of the meeting.

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Welcome

As Chair of the Scientific Committee, it is my great pleasure to welcome you to Aix-en-Provence and the XIII biennial meeting of the European Calcium Society. Our conference this year represents a landmark in ECS history, coming 25 years after the first meeting in Brussels. During that quarter century our meetings have changed beyond recognition, we have built a substantial membership that extends well beyond European borders, and ECS conferences have become established as a leading international forum for calcium researchers around the globe.

The meeting this year breaks new ground for the ECS in several respects. First, we have moved from a three day to a four day meeting. The slightly extended programme makes long distance travel easier to justify, but also has an impact on the cost of registration and housing – we strive to achieve the right balance and would welcome your views on the longer format. Second, for the first time our meeting is based not at one of the scientific hubs of European calcium research, but in a city selected for its location, beauty and cultural heritage. Instead of having a local organiser we have managed the conference centrally, on which note a special word of thanks is due to our Treasurer, Roland Pochet, and our General Secretary, Jan Parys, for their extraordinary commitment to pulling the meeting together.

In a third break with tradition, we designed the scientific programme by inviting ECS members to propose topics for the scientific sessions. This was clearly a popular move as we received many more suggestions than we could accommodate. My sincere thanks go to the Session Chairs, who nominated speakers and selected short talks from the submitted abstracts, and for their creative input to what promises to be a highly informative and successful scientific meeting.

I also want to recognise the generous support of our sponsors and would invite you to interact with them during the meeting. Their contributions help in many ways, one of the most visible being that they enable the ECS to direct funds to supporting the participation of younger scientists through the ECS Fellowships scheme. For the Aix meeting we are delighted to have awarded 10 Junior Travel Fellowships, and 3 Travel Fellowships for Established Scientists from Emerging Countries.

Finally I would like to wish you all a productive and enjoyable meeting and hope that you take the opportunity during these few days to see something of the beautiful city of Aix.

Stephen E Moss
Chair of the Scientific Committee

General Information

REGISTRATION

The registration desk will open on Saturday 13th September at 10.00 a.m. and remain open throughout the meeting.

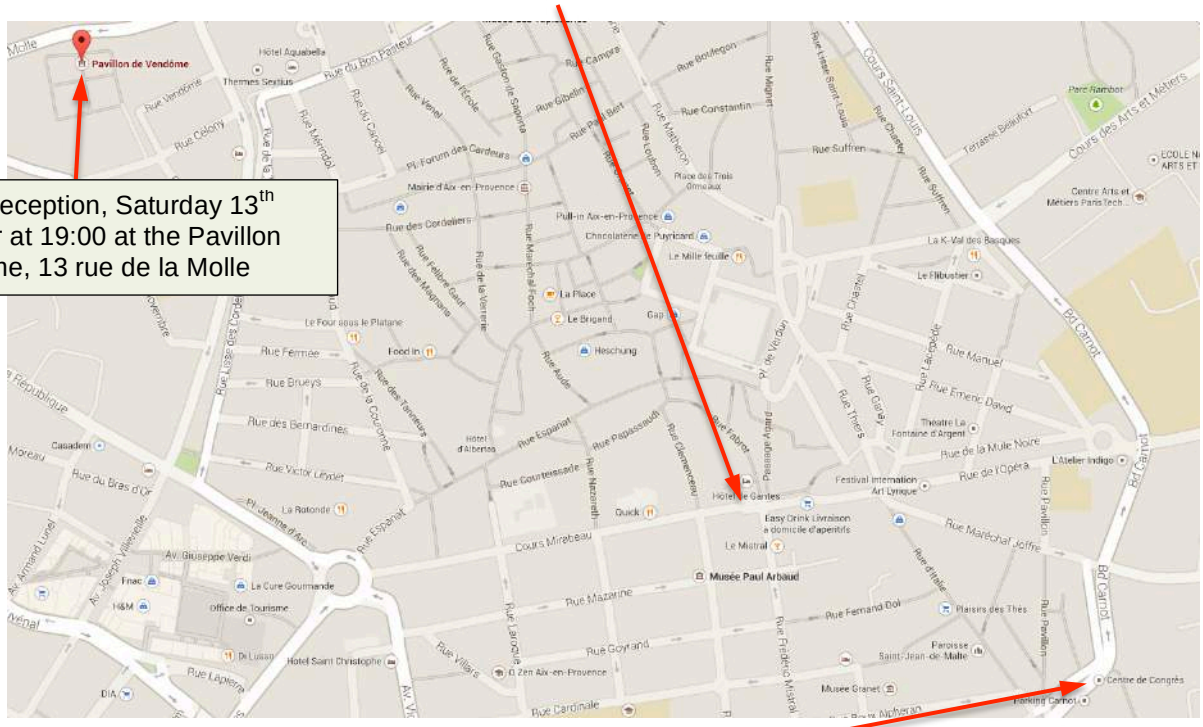
NAME BADGES

Delegates are requested to wear their name badges at all times in the conference centre in order to gain admittance to the auditorium, poster sessions and lunch and coffee breaks.

BANQUET

The cost of the banquet is included in your registration but delegates are required to pay an additional €5 reservation fee. The banquet will take place at Les 2 Garçons, 53 Cours Mirabeau. See information at registration desk.

Welcome reception, Saturday 13th September at 19:00 at the Pavillon de Vendôme, 13 rue de la Molle



LOCATION

The conference centre is located on Bd Carnot.

LANGUAGE

The language of the conference will be English, all talks and posters should be in English, no simultaneous translation will be provided.

POSTERS

Poster boards are 120 cm wide and 180 cm high. Posters should be put up as soon as possible after registration, and remain in place until after the Berridge Lecture on the Tuesday evening. On Sunday evening, presenters of odd numbered posters are requested to attend their posters, and on Monday evening, presenters of even numbered posters are requested to attend their posters. The ECS will award prizes of €500 for the two best posters at the awards ceremony following the Berridge Lecture.

GENERAL ASSEMBLY

All ECS members present are invited and encouraged to attend the general assembly at 19.00 on Sunday September 14th.

SATURDAY TOUR OF AIX

Only a limited number of people may accompany the official tour guides. Places will be allocated on a first come first served basis. Email Roland Pochet (rpochet@ulb.ac.be) to request a place, stating English or French guide.

WEB SITE

Please refer to the ECS web site at http://www.ulb.ac.be/assoc/ecs/aix2014.html for regular updates and programme changes, and keep in touch with the organisers and fellow calcium enthusiasts on our Facebook page: https://www.facebook.com/pages/European-Calcium-Society/137424812975200?fref=photo

Schedule at a glance

Day 1. Saturday 13th September

10.00 Registration opens

16:00 Welcome from the ECS President, Marc Moreau

16:15 Plenary Lecture – Hon Cheung Lee (Hong Kong)
With an introduction from Andreas Güse (Germany)

THE CYCLIC ADP-RIBOSE/NAADP/CD38-SIGNALING PATHWAY: PAST AND PRESENT

17.15 Tour of Aix

19.00 Welcome Reception at the Pavillon de Vendôme

Day 2. Sunday 14th September

Session 1 – Calcium and ER Stress

Chair: Joachim Krebs

08.30 Keynote Lecture: Marek Michalak (Canada)
Calcium signaling and coping with the ER stress

09.00 Feroz Papa (USA)
Small molecule targeting of the terminal UPR in diabetes

09.20 Pico Caroni (Switzerland)
Early disease mechanisms in ALS motoneurons: roles of ER stress and autophagy

09.40 Hibiki Kawamata (USA)
ER proteins oxidative modifications alters calcium signaling in familial ALS

09.55 Thomas Simmen (Canada)
Regulation of ER-Mitochondria Calcium Crosstalk by TMX1

10.10 – 10.40 COFFEE BREAK

Session 2 – Ca^{2+}-Binding Proteins in Ca^{2+}-Signaling

Chair: Jim Putney and Walter Chazin

10.40 Keynote Lecture: Mitsuhiko Ikura (Canada)
Structural basis for key checkpoints in the store-operated calcium entry mechanism

11.10 Michael Overgaard (Denmark)
Cardiac arrhythmia calmodulin mutations and regulation of the RyR2 calcium channel

11.30 Sara Linse-Snogerup (Sweden)
EF-hands as vehicles in biomolecular interaction studies

11.50 Milena Pejic (Germany)
Ca^{2+}-dependent membrane organization mediated by annexin A2

12.05 Marie-Luce Bochaton-Piallat (Switzerland)
S100A4 and calmodulin promote smooth muscle cell phenotypic transition. Implications in atherosclerosis

12.20 LUNCH AND POSTERS

Session 3 – Neuronal Calcium Signaling and Optogenetics

Chair: Jacques Haiech

14.30 Keynote Lecture: Valentina Emiliani (France)
Controlling brain signalling with wave front shaping and optogenetics

15.00 Pau Gorostiza (Spain)
Optical control of calcium-regulated exocytosis

15.20 Jana Hartmann (Germany)
STIM1 required for neuronal Ca^{2+} signaling and mGluR1-dependent synaptic transmission

15.40 Robert Blum (Germany)
Continuous calcium influx compensates fast ER calcium loss in resting neurons

15.55 Khaled Machaca (Qatar)
Dynamic trafficking of the store-operated Ca^{2+} entry channel Orai1

16.10 COFFEE BREAK

Session 4 – Ca^{2+}-Signaling and Cancer Stem Cells

Chair: Catherine Leclerc and Marc Moreau

16.40 Keynote Lecture: Jacques Haiech (France)
Calcium signaling in normal and cancer stem cells: different languages for different fates

17.10 Rodrigo Resende (Brazil)
Spontaneous calcium signaling directs stem cells fate to neuronal cells

17.30 Francesco Moccia (Italy)
Distinct remodelling of the Ca^{2+} signalling toolkit in endothelial progenitor cells under different neoplastic conditions

17.50 Francisco Aulestia (France)
Cancer stem cells: Ca^{2+} influx through store-operated Ca^{2+} channels

18.05 Elena Poser (Italy)
Sorcin links cell cycle, calcium levels in endoplasmic reticulum and multi drug resistance in cancer

18.20 POSTER SESSION AND BAR

19.00 GENERAL ASSEMBLY

Day 3. Monday 15th September

Session 5 – Ca^{2+} in Sensory Cells and Signalling

Chair: Karl-W Koch

08.30 Keynote Lecture: Kathryn Medler (USA)
Calcium signaling in taste cells

09.00 Marc Spehr (Germany)
Calcium signaling in chemical communication

09.20 Daniele Dell'Orco (Italy)
Molecular aspects of the regulation of Ca^{2+}/cGMP homeostasis in rod photoreceptor cells in normal and altered conditions

09.40 Javier Garcia-Sancho (Spain)
GAP, a novel aequorin-based fluorescent ratiometric probe suitable for calcium imaging of intracellular organelles

09.55 Ming-Dong Zhang (Sweden)
Neuronal calcium binding proteins 1/2 localize to dorsal root ganglia and excitatory spinal neurons

10.10 COFFEE BREAK

Session 6 – Calcium and Mechanisms of Plasma Membrane Protection

Chair: Annette Draeger and Volker Gerke

10.40 Keynote Lecture: Paul McNeil (USA)
Calcium and vitamins: signaling and promoting membrane repair

11.10 Alain Brisson (France)
Annexin-A5 participates in membrane repair in the placenta

11.30 Annette Draeger (Switzerland)
Dealing with damage: the annexin survival kit

11.50 Katherine Hajjar (USA)
Annexin A2 modulates inflammasome activation in severe colitis by enabling lysosomal membrane repair

12.05 Bethan Kilpatrick (UK)
Defective calcium signalling in Parkinson Disease; clues from a lysosomal storage disorder

12.20 LUNCH AND POSTERS

Session 7 – Calcium in the Nervous System

Chair: Ula Wojda and Jacek Kuznicki

14.30 Keynote Lecture: Robert Burgoyne (UK)
Sense and specificity in neuronal calcium signaling

15.00 Axel Methner (Germany)
In vivo evidence that Bax inhibitor-1 is a Ca^{2+} channel with a role in immune cell function and survival

15.20 Dominique Debanne (France)
Analog-digital modulation of synaptic transmission in cortical neurons

15.40 Bipan Kumar Deb (India)
Drosophila septin, Pnut is a modulator of neuronal Store operated calcium entry

15.55 Maria Ryazantseva (Russia)
STIM sensors as molecular targets for memory loss treatment connected with calcium homeostasis impairments in Familial Alzheimer's Disease

16.10 COFFEE BREAK

Session 8 – Ca^{2+}-Channels and Pumps and Cancer

Chair: Greg Monteith

16.40 Keynote Lecture: Natalia Prevarskaya (France)
Calcium and calcium channels in initiation and progression of prostate cancer

17.10 Greg Monteith (Australia)
Calcium ATPases and breast cancer

17.30 Albrecht Schwab (Germany)
Calcium regulation of migration pathways important in cancer

17.50 Haidar Akl (Belgium)
Transforming pro-survival IP_3 signaling in B-cell cancers into pro-apoptotic Ca^{2+} signaling upon Bcl-2 antagonism at the endoplasmic reticulum

18.05 Abhishek Aggarwal (Austria)
The Calcium Sensing Receptor is a tumor suppressor in the colon regulating proliferation, differentiation, and epithelial to mesenchymal transition

18.20 POSTER SESSION AND BAR

20.00 CONFERENCE BANQUET at the restaurant **Les 2 Garçons,** 53 Cours Mirabeau, 13100 Aix-en-Provence

Day 4. Tuesday 16th September

FREE MORNING

13.00 POSTER SESSION

Session 9 – Modelling Intracellular Calcium Dynamics

Chair: Geneviève Dupont

14.30 Keynote Lecture: Martin Falcke (Germany)
General properties of IP_3-induced Ca^{2+} spiking

15.00 Rudiger Thul (UK)
Calcium oscillations in the presence of cellular variability

15.20 Alexandra Zahradnikova (Slovakia)
Sparks and waves in cardiac myocytes - insights from an allosteric model of ryanodine receptor gating

15.40 S. Rüdiger (Germany)
Modulation of elementary calcium release and its role in the puff-to-wave transition

15.55 Insa Wolf (Germany)
Fast subcellular Ca^{2+} signals in T cell signaling

16.10 COFFEE BREAK AND POSTERS

18.00 Berridge Lecture: Richard Tsien (USA)
With an introduction from Philippe Lory (France)

A SHUTTLE MECHANISM COMBINING NANODOMAIN AND LONG-DISTANCE CALCIUM SIGNALING

19.00 AWARD CEREMONY

Day 5. Wednesday 17th September

Session 10 – Ca^{2+}-Signalling at Membrane Contact Sites

Chair: Nicolas Demaurex

08.30 Keynote Lecture: Chris Loewen (Canada)
Polarization of the yeast ER through ER-septin tethering

09.00 Paula Nunes (Switzerland)
Dissecting ER-phagosome membrane contact sites

09.20 Emily Eden (UK)
The role of Ca^{2+} in the formation and function of ER contact sites with the endolysosomal pathway

09.40 Donald Gill (USA)
Calcium signal generation through STIM-induced ER-plasma membrane junctions

09.55 Paula Heister (UK)
Glucose and NAADP trigger elementary intracellular Ca^{2+} signals in pancreatic ß-cells

10.10 COFFEE BREAK

Session 11 – New Horizons in Mitochondrial Calcium Signalling

Chair: Michael Duchen and Gyorgy Szabadkai

10.40 Keynote Lecture: Gyorgy Hajnoczky (USA)
Molecular mechanisms of mitochondrial calcium uptake

11.10 Diego De Stefani (Italy)
New insights into the regulation of the mitochondrial calcium uniporter

11.30 Giles Hardingham (UK)
Probing the role and regulation mitochondrial Ca^{2+} uptake in neurons

11.50 Frank Gellerich (Germany)
Tissue specific differences of mitochondrial gas pedal: cytosolic Ca^{2+} regulates the mitochondrial energization with pyruvate

12.05 Yuequan Shen (China)
Structural and mechanistic insights into MICU1 regulation of mitochondrial calcium uptake

12.20 DRABIKOSWKI AWARD and CONCLUDING REMARKS

12.30 MEETING CLOSES

Speaker Abstracts

Plenary Lecture

THE CYCLIC ADP-RIBOSE/NAADP/CD38-SIGNALING PATHWAY: PAST AND PRESENT

Lee, Hon Cheung
Beijing University Shenzhen Graduate School, China

The concept advanced by Berridge and colleagues that intracellular Ca^{2+} stores can be mobilized in an agonist-dependent and messenger (IP3)-mediated manner have put Ca^{2+}-signaling at the center stage of signal transduction mechanisms. During the late '80s, we showed that the Ca^{2+} stores can be mobilized by two other messengers unrelated to IP3 and identified them as cyclic ADP-ribose (cADPR), a novel cyclic nucleotide from NAD, and nicotinic acid adenine dinucleotide phosphate (NAADP), a linear metabolite of NADP. Their messenger functions have now been well documented in a wide range of cellular systems spanning three biological kingdoms. Accumulated evidence indicates that the target of cADPR is the ryanodine receptor in the endoplasmic reticulum, while that of NAADP is the two-pore channel in the lysosomes. As cADPR and NAADP are structurally and functionally distinct, it is remarkable that they are synthesized by the same enzyme. We first identified the Aplysia ADP-ribosyl cyclase as one such enzyme and, through homology, found its mammalian homolog, CD38. Gene knockout studies in mice confirm the important roles of CD38 in a wide range of physiological functions from insulin secretion, susceptibility to bacterial infection, to social behavior of mice through modulating neuronal oxytocin secretion. We have elucidated the catalytic mechanisms of the Aplysia cyclase and CD38 to atomic resolution by X-ray crystallography and site-directed mutagenesis. This presentation will give a historical account of the cADPR/NAADP/CD38-signaling pathway and describes some current efforts in elucidating the regulation and membrane topology of CD38.

CALCIUM SIGNALING AND COPING WITH THE ER STRESS

Marek Michalak

Department of Biochemistry, University of Alberta, Edmonton, Alberta, Canada T6G 2S7

The disruption of the energy or nutrient balance is the fundamental cause of endoplasmic reticulum (ER) stress, a process that mobilizes corrective strategies to reestablish ER and cellular homeostasis known as the unfolded protein response (UPR). The ER stress coping response [e.g. unfolded protein response (UPR)] is composed of discrete pathways that are controlled by a collection of common regulatory components which may function as a single entity involved in reacting to ER stress. These corrective strategies allow the cellular network to reinstate energy/nutrient homeostasis and avoid cell death. For example, the UPR stress sensor IRE1 signals through its endoribonuclease activity, processing the mRNA of the transcription factor XBP-1, which regulates genes related to protein folding and quality control. We performed small interfering RNA (siRNA) arrays and microRNA (miRNA) profiling array analysis to search for coping responses (including signaling pathways) that were modulated by disrupted ER calcium homeostasis such as ER calcium stores depletion. We employed a small interfering RNA (siRNA) library screen combined with deep sequencing analysis of micro RNA (miRNA) to identify factors that mediate UPR modulation. We uncovered a mechanism dependent on an ER oxidoreductase and miR-322 that regulates the function of IRE1 and showed that PDIA6, an ER resident oxidoreductase, interacted with cysteine residues in the luminal domain of IRE1 and was required to maintain IRE1 activity as monitored by phosphorylation of IRE1 and XBP1 mRNA splicing. ER calcium depletion and activation of store operated calcium entry reduced the abundance of different miRNAs including miR-322, which increased PDIA6 mRNA stability and consequently stabilized IRE1 activity during the ER stress response. This work identifies PDIA6 as a unique component of the UPR and demonstrates interplay between ER and cytosolic calcium, PDIA6, IRE1 and miR-322 as a part of a novel pathway (coping mechanisms) activated by disrupted ER calcium homeostasis and activation of SOCE as an adaptive response to coping with ER stress.

ALLOSTERIC INHIBITION OF THE IRE1α RNASE PRESERVES CELL VIABILITY AND FUNCTION DURING ENDOPLASMIC RETICULUM STRESS

Rajarshi Ghosh[1,2,5,6,7,12], Likun Wang[1,5,6,7,12], Eric S. Wang[2,12], B. Gayani K. Perera[8], Aeid Igbaria[1,5,6,7], Shuhei Morita[1,5,6,7], Kris Prado[1,5,6,7], Maike Thamsen[1,5,6,7], Deborah Caswell[2], Hector Macias[1,5], Kurt F. Weiberth[1,5,6,7], Micah J. Gliedt[1,6], Marcel V. Alavi[3], Sanjay B. Hari[8], Arinjay K. Mitra[8], Barun Bhhatarai[10], Stephan C. Schürer[9,10], Erik L. Snapp[11], Douglas B. Gould[3,4], Michael S. German[1,5], Bradley J. Backes[1,6], Dustin J. Maly[8], Scott A. Oakes[2,5], Feroz R. Papa[1,5,6,7]

Department of Medicine[1], Department of Pathology[2], Department of Ophthalmology[3], Department of Anatomy[4], Diabetes Center[5], Lung Biology Center[6], California Institute for Quantitative Biosciences[7], University of California, San Francisco. San Francisco, CA 94143. U.S.A. Department of Chemistry[8], University of Washington, Seattle. Seattle, WA 98195. U.S.A. Center for Computational Science[9], Department of Molecular and Cellular Pharmacology[10], Miller School of Medicine, University of Miami, FL 33136, U.S.A. Department of Anatomy and Structural Biology[11], Albert Einstein College of Medicine, Bronx, NY 10461, U.S.A. Equal contribution[12]

Depending on endoplasmic reticulum (ER) stress levels, the ER transmembrane multi-domain protein IRE1a promotes either adaptation or apoptosis. Unfolded ER proteins cause IRE1a lumenal domain homo-oligomerization, inducing *trans* auto-phosphorylation that further drives homo-oligomerization of its cytosolic kinase/ endoribonuclease (RNase) domains to activate mRNA splicing of adaptive XBP1 transcription factor. However, under high/chronic ER stress, IRE1α surpasses an oligomerization threshold that expands RNase substrate repertoire to many ER-localized mRNAs, leading to apoptosis. To modulate the effects of its kinase-controlled rheostat, we developed ATP-competitive IRE1α *K*inase *I*nhibiting *R*Nase *A*ttenuators—KIRAs—that allosterically inhibit IRE1α's RNase by breaking oligomers. One optimized KIRA, KIRA6, inhibits IRE1α *in vivo* and promotes cell survival under ER stress. Intravitreally, KIRA6 preserves photoreceptor functional viability in rat models of ER stress-induced retinal degeneration. Systemically, KIRA6 preserves pancreatic ß-cells, increases insulin, and reduces hyperglycemia in Akita diabetic mice. Thus, IRE1α powerfully controls cell fate, but can itself be controlled with small molecules to reduce cell degeneration. Further implications of small molecule control over the UPR will be discussed.

EARLY DISEASE MECHANISMS IN ALS MOTONEURONS: ROLES OF ER STRESS AND AUTOPHAGY

Pico Caroni and Francesco Roselli

FMI, Basel, Switzerland

Transgenic mice expressing disease-related mutant human SOD1(G93A) ubiquitously using a human SOD1 promoter construct provide the only mouse model that recapitulates essential features of a neurodegenerative disease in mice, including selective vulnerability of particular neurons (here motoneurons), late onset, relentless progression, neuronal loss and death. A particularly valuable feature of SOD1 mutant mice is that disease progresses in a highly stereotype manner, and that the occurrence of pathological processes in any individual can be predicted within 2-3 days. These unique features of the mouse model have allowed us to investigate disease processes longitudinally, addressing questions of causality. Here we will provide evidence that two very early disease-driving processes in the mutant mice involve gradually increasing ER stress and autophagy overload in vulnerable motoneurons. The latter accounts for major mitochondrial pathology and for gradual subpopulation-selective loss of motoneuron function and behavioral performance long before any loss of motoneurons. We will discuss the cellular mechanisms linking these pathogenic processes to motoneuron excitability, cellular stress pathways and disease progression.

ER PROTEINS OXIDATIVE MODIFICATIONS ALTERS CALCIUM SIGNALING IN FAMILIAL ALS

Hibiki Kawamata[1], Yongjie Yang[2], and Giovanni Manfredi[1]

[1]Brain and Mind Research Institute, Weill Medical College of Cornell University. 407 East 61st Street, RR511 New York, NY 10065; [2]Department of Neuroscience, and Sackler School of Biomedical Sciences, Tufts University,136 Harrison Ave, Boston, MA, 02111, USA

Protein misfolding and the formation of insoluble aggregates containing multiple proteins are pathological disease hallmarks of many neurodegenerative diseases, including amyotrophic lateral sclerosis (ALS). Mutations in superoxide dismutase 1 (SOD1) are associated with autosomal dominant familial ALS. SOD1 is a soluble and ubiquitous antioxidant protein, which, when mutated, forms intracellular aggregates with toxic functions. Through cell fractionation experiments, SOD1 aggregates have been identified in various subcellular compartments of mutant cells, including the endoplasmic reticulum (ER)[1]. Recently, we have discovered the role of mutant SOD1 in enhancing redox protein modification of the ER resident protein STIM1, and resulting alterations in store operated calcium entry (SOCE) and intracellular calcium dynamics. These changes were first found in mutant SOD1 astrocytes from the spinal cord of a mouse model of ALS, which exert non-cell autonomous toxicity on motor neurons[2]. Importantly, abnormal STIM1 redox modifications and intracellular calcium abnormalities in astrocytes were ameliorated by supplementation of the redox agent glutathione. The treatment resulted in attenuation of mutant SOD1 astrocyte toxicity. We identified abnormal ER calcium dynamics also in primary skin fibroblasts of ALS patients, suggesting that this may be a general pathogenic mechanism in ALS. The ER is involved in a multitude of cell functions, including calcium homeostasis, lipid biosynthesis, and protein folding and secretion. The ER protein folding machinery involves a series of coordinated redox relay systems. Production of H_2O_2 and GSSG during protein folding contributes to the oxidized environment of the ER. Misfolding of proteins in the ER is cleared through activation of the ER associated degradation (ERAD). In addition, the unfolded protein response is activated to cope with overburdening of the protein folding system. Despite these clearance mechanisms, aberrant protein folding processes can result in enhanced oxidation and ER oxidative stress. We hypothesized that mutant SOD1 in the ER causes continuous futile cycles of protein folding and unfolding, resulting in ER oxidative stress, which in turn leads to oxidative modification of ER proteins, including STIM1 glutathionylation, and calcium dynamics disruption.

1. Urushitani M et al. FASEB J. 2008, 22(7): 2476-87
2. Kawamata H et al. J Neurosci. 2014, 34(6): 2331-48

REGULATION OF ER-MITOCHONDRIA CALCIUM CROSSTALK BY TMX1

Arun Raturi[1], Emily Lynes[1], Dimitar Ourdev[1], Shairaz Baksh[1,2], Eliana Lucchinetti[1,2,3], Klaus Ballanyi[4], Michael Zaugg[1,2,3] and Thomas Simmen[1]

[1]Department of Cell Biology, [2]Department of Pediatrics, [3]Department of Anesthesiology and Pain Medicine, [4]Department of Physiology, University of Alberta, Edmonton, Alberta, Canada T6G2H7.

Work from our laboratory has demonstrated that endoplasmic reticulum (ER) chaperones regulate the calcium-mediated crosstalk between the ER and mitochondria. They do so dependent on their localization within the ER. For instance, the moiety of calnexin that localizes to the mitochondria-associated membrane (MAM) promotes SERCA2b activity, whereas calnexin has to be associated with the rough ER to promote protein folding. We now describe a novel mechanism based on the ER thioredoxin-containing transmembrane protein TMX1 that critically regulates ER-mitochondria calcium flux. TMX1 interacts with SERCA2b, but not with the IP3R like calnexin. In contrast to calnexin, TMX1 inhibits the SERCA2 calcium pump in a redox-dependent manner. The activity of TMX1 promotes mitochondria-directed calcium transfer, as it reduces ER calcium uptake. TMX1 requires its thioredoxin domain as well as its localization to the MAM for this function. Via this mechanism, TMX1 is critical for mitochondria respiration and ATP production, but also determines the speed of apoptosis during oxidative stress. Conversely, low levels of TMX1 lead to increased ER calcium content, but reduced transfer of calcium towards mitochondria, concomitant with an increased distance between mitochondria and the ER, inhibited mitochondria metabolism and reduced apoptosis. Interestingly, low levels of TMX1 are observed in cancer tissue, in particular in melanoma. Therefore, TMX1 acts as a tumor suppressor and major determinant of the Warburg phenotype.

STRUCTURAL BASIS FOR KEY CHECKPOINTS IN THE STORE-OPERATED CALCIUM ENTRY (SOCE) MECHANISM

Peter B. Stathopulos, Tadateru Nishikawa, Masahiro Enomoto, Min-Duk Seo, Le Zheng, Geneviève M. Gasmi-Seabrook, & Mitsu Ikura

Princess Margaret Cancer Centre and Department of Medical Biophysics, University of Toronto, Toronto, Ontario, Canada, M5G 1L7.

In response to receptor activation, a cascade of membrane signaling events liberate inositol 1,4,5-trisphosphate (IP3) into the cytoplasm, which travels to endoplasmic reticulum (ER) where it activates the IP3 receptor (IP3R). Upon this ligand-induced activation, the gigantic tetrameric Ca2+ channel (M.W. approx. 1.2 GDa) IP3R release ER Ca^{2+} into the cytoplasm and plays a vital role in eukaryotic calcium signaling. We determined the structure of IP3 binding region in IP3R1, enabling to speculate how the ligand binding may activate the IP3R channel (Bosanac et al. Nature 2002; Seo et al. Nature 2012). Depletion of the ER Ca^{2+} store leads to further Ca^{2+} influx from the extracellular space into the cell, a phenomenon first described by Jim Putney in 1986. This store-operated Ca^{2+} entry (SOCE) mechanism is mediated by the ER Ca^{2+} sensor protein STIM and the plasma membrane Ca^{2+}-release activated Ca^{2+} (CRAC) channel Orai as well as TRP channels. The luminal domain of STIM directly binds Ca^{2+} and undergoes a conformational change (Stathopulos et al. Cell 2008), hence transmitting the Ca^{2+} signal to the cytoplasmic domain, which then activates plasma membrane (PM)-localized Orai proteins. Human STIM1 encodes three coiled-coil (CC) motifs in the cytosolic region, named CC1, CC2, and CC3 from the N- to C-terminus. Our NMR structure of CC1-CC2 (Stathopulos et al. Nature Commun. 2014) revealed that two extended alpha-helices form a helical hairpin, and two of these U-shaped domains are symmetrically arranged in an antiparallel homodimer. Mutations in full-length STIM1 which stabilize CC1 within the CC1-CC2 context result in Ca^{2+}-independent, constitutive activation of Orai channels, while mutations that destabilize CC1 can attenuate channel activity. We propose that the CC1-CC2 dimer plays a central role in the CRAC activation, indispensable for both the transduction of the Ca^{2+} sensing signal from the luminal domains to the minimal cytosolic Orai activating regions and the arrangement of Orai subunits into functional channels. By highlighting the three key checkpoints described above, I will review the current status of the structural mechanism of the SOCE/CRAC mechanism. Supported by CIHR, HSFC, and CRC.

CARDIAC ARRHYTHMIA CALMODULIN MUTATIONS AND REGULATION OF THE RYR2 CALCIUM CHANNEL

Michael T Overgaard

Department of Chemistry and Biotechnology, Aalborg University, Aalborg, Denmark

Calmodulin (CaM) is the central mediator of intracellular Ca^{2+} signalling in cardiomyocytes, where it conveys the intricate Ca^{2+} transients to the proteins controlling cardiac contraction. We recently identified two separate mutations in CaM (N53I and N97S) linked to dominantly inherited catecholaminergic polymorphic ventricular tachycardia (CPVT), an arrhythmic disorder in which exercise or acute emotion can lead to syncope and sudden cardiac death. Following studies have identified additional CaM mutations, all in individuals affected by severe cardiac arrhythmias: CPVT, long QT syndrome (LQT), iodopathic ventricular fibrillation (IVF), or a combination of these. Given the ubiquitous presence of CaM in all eukaryote cells, it is particular intriguing that carriers of CaM mutations all present cardiac phenotypes. Here we show that three-day zebrafish embryos, injected with CPVT CaM mRNA (N53I and N97S), display an increase in heart rate specifically under ß-adrenergic stimulation, without any other detectable pathologies or developmental abnormalities. Intriguingly, we find that the biophysical impact of these mutations is small, but distinctly opposing, suggesting that they may impose different molecular disease mechanisms. CaM is a key regulator of the cardiac sarcoplasmic reticulum Ca^{2+} release channel (RyR2), and mutations in RyR2 are known to cause CPVT. We therefore investigated the effect of CPVT CaM on RyR2 interaction and Ca^{2+} release properties, and included some of the LQT CaM variants for comparison. Our results show that the both CPVT and LQT causing CaM mutations lead to aberrant RyR2 channel regulation and excessive Ca^{2+} release. We therefore propose that dysregulation of RyR2 is a highly likely disease mechanism and convergence point for CaM mutations causing CPVT, and a potential component of the clinical manifestation for some CaM mutations causing LQT.

EF-HANDS AS VEHICLES IN BIOMOLECULAR INTERACTION STUDIES AND BIOTECHNOLOGY TOOLS

Sara Linse

Lund University

The widespread phenomenon of protein reconstitution demonstrates that many native folds can tolerate breaks in the polypeptide chain, permitting assembly of the fold from more than one chain segment (e.g. EF-hand fragments) using native contacts in a reaction akin to intramolecular folding. Like reconstitution, the process of 3D domain swapping relies on native contacts, and often uses the same chain segments that are also observed to reconstitute.

The EF-hand is a prototypical subdomain. It often occurs paired or in higher order assembly with a high level of preference for the native pairing/higher order assembly. The high specificity of EF-hand assembly is also manifested in the related process of 3D domain swapping leading to dimers and higher order multimers of EF-hand proteins. We will present examples of reconstitution studies to probe the EF-hand assembly and to assess its molecular driving forces.

The high specificity of EF-hand pairing can be harnessed for development of tools for biotechnology and biomedicine. We will show how the EF-hands of calbindin D9k have been used in development of a general method for protein stabilization. In contrast to most of the conventional methods for protein stabilization, our method is based on simple thermodynamic principles. It relies on the following assumptions: 1) The structure and function of the protein can be reconstituted from two fragments. 2) The affinity between the fragments is correlated to the free energy of unfolding of the intact protein. The split-GFP

system is used as a readout and calbindin D9k was used as a first test case in this system and then as a positive control in the first stabilization project, due to its very high affinity and stability.

Finally, we will also show examples of a novel affinity-tag purification system based on the EF-hands from calbindin D9k - a purification system relying on mild condition and changes in calcium/EDTA concentrations. The same system is also developed for easily regenerated immobilization on sensorchips for surface plasmon resonance studies.

Ca^{2+}-DEPENDENT MEMBRANE ORGANIZATION MEDIATED BY ANNEXIN A2

Milena Pejic[1], Patrick Drücker[2], David Grill[1], Hans-Joachim Galla[2], Volker Gerke[1]

[1]Institute of Medical Biochemistry, ZMBE, University of Muenster, von-Esmarch-Str. 56, D-48149 Muenster, Germany; [2]Institute of Biochemistry, University of Muenster, Wilhelm-Klemm-Str. 2, D- 48149 Muenster, Germany

Annexin A2 (AnxA2), a member of the annexin family of proteins that bind Ca^{2+}-dependently to negatively charged phospholipids, is enriched at phosphatidylinositol-4,5-bisphosphate (PIP_2) and cholesterol containing membrane microdomains. This domain formation and lipid binding is particularly pronounced when AnxA2 resides in a heterotetrameric complex with its intracellular ligand S100A10, a member of the S100 family of EF hand-type proteins. (AnxA2-S100A10)2 or A2t binds with high affinity to PIP_2 and 1-palmitoyl-2-oleoyl-sn-glycero-3 phosphatidylserine (POPS) and is capable of clustering these lipids in the background of a 1-palmitoyl-2-oleoyl-sn-glycero-3 phosphatidylcholine (POPC) bilayer. To study the membrane organizing properties of annexin A2 we analyzed interactions of the monomeric protein, A2t and a mutant comprising the C-terminal core domain of annexin A2 with giant unilamellar vesicles (GUVs) of complex lipid compositions. We show that, in the presence of Ca^{2+}, cholesterol and glycosphingolipids are enriched together with PIP_2 in clusters induced by annexin A2. Further, we identify these clusters as sites for annexin A2 driven inward vesicle budding. To quantify the Ca^{2+}-dependent binding parameters of annexin A2 and derivatives to PIP_2 and PS containing membranes we employed the quartz crystal microbalance technique. Solid supported bilayers of varying lipid composition were used to study the influence of acyl chain saturation and PIP_2 on protein binding. We show for the first time that annexin A2 and its derivatives exhibit a positive cooperative binding that is independent of PIP_2 and acyl chain saturation of the backbone lipids.

S100A4 AND CALMODULIN PROMOTE SMOOTH MUSCLE CELL PHENOTYPIC TRANSITION. IMPLICATIONS IN ATHEROSCLEROSIS

[1]Chiraz Chaabane, [1]Matteo Coen, [2]Claus W. Heizmann, [1]Marie-Luce Bochaton-Piallat

[1]Department of Pathology and Immunology, Faculty of Medicine, University of Geneva, Geneva, Switzerland; [2]Department of Pediatrics, Division of Clinical Chemistry and Biochemistry, University of Zürich, Zürich, Switzerland.

During atherosclerosis, smooth muscle cells (SMCs) accumulate into the intima and undergo phenotypic changes. In pig and human, we isolated SMC subpopulations that exhibit features of the synthetic phenotype, typical of intimal SMCs (high proliferative and migratory activities and low level of differentiation). By means of a proteomic approach, we identified markers typical of the synthetic phenotype and explore their role in the phenotypic SMC modulation. From the porcine coronary artery we isolated 1) spindle-shaped SMCs and 2) rhomboid SMCs. Rhomboid SMCs displayed the features of the synthetic phenotype. We identified S100A4 as being a marker of the rhomboid SMCs in vitro and of intimal SMCs, both in pig and man. S100A4 is an intracellular calcium-binding protein and exhibits extracellular functions via the receptor for advanced glycation end products (RAGE). Treatment of spindle-shaped SMCs (devoid of S100A4) with S100A4-rich conditioned medium collected from S100A4-transfected SMCs induced a transition towards a rhomboid phenotype, which was associated with increased proliferative, migratory and proteolytic activities as well as SMC differentiation marker downregulation. It yielded activation of NF-kappaB in a RAGE-dependent manner. Conversely blockade of extracellular S100A4 in rhomboid SMCs with S100A4 neutralizing antibody induced a transition from rhomboid to spindle-shaped phenotype, decreased proliferative activity and upregulation of SMC differentiation markers. Therefore extracellular S100A4 plays a pivotal role in SMC phenotypic changes. From human carotid endarterectomy specimens, and in particular, from the media, we isolated two distinct SMC subsets: 1) large SMCs and 2) small SMCs. Small SMCs were obtained only after coculture with macrophage-derived foam cells isolated from the atherosclerostic plaque. Small SMCs displayed the features of the synthetic phenotype. We identified calmodulin (CaM) as being predominant in small SMCs. Coculture of large SMCs with macrophage-derived foam cells induced a transition to the small phenotype with increased CaM expression. The CaM inhibitor W-7 prevented the large-to-small phenotypic transition. In vivo, CaM was markedly expressed in SMCs of atherosclerotic plaques and barely detectable in the media. Therefore macrophage-derived foam cells promote the selective migration from the media of synthetic SMCs. S100A4 and/or calmodulin could be new targets to prevent SMC accumulation during atherosclerosis.

CONTROLLING BRAIN SIGNALLING WITH WAVE FRONT SHAPING AND OPTOGENETICS

Valentina Emiliani

Wavefront-Engineering Microscopy Group, Neurophotonics Laboratory, CNRS and University Paris Descartes, UMR8250 Paris, France

Optogenetics enables the control and readout of complex computational events in the brain through the precise stimulation and inhibition of neuronal circuits and activation of functional reporters. Remarkably, the cell-specific expression and light sensitivity of optogenetics tools, have permitted efficient, in-depth and selective control of specific neuronal network with relatively simple optical methods based on wide field illumination. However, the fascinating perspective of optically orchestrating neuronal circuitry in the brain of freely moving animals motivates today the development of more sophisticated illumination methods. An ideal light delivery method should be: efficient, robust to scattering, span multiple spatial scales, and feature high spatial (micron) and temporal (millisecond) resolution. To accomplished these goals, our laboratory has recently demonstrated a series of new methods based on wave front shaping to generate shaped single- and two-photon (1P-2P) excitation volumes into neural tissue, including computer generated holography (CGH), generalized phase contrast (GPC), and temporal focusing (TF). Specifically, we have shown that wave front shaping, accomplished with a liquid crystal matrix, enables dynamic control of the light at the sample plane matching the geometry of structures or circuits of interest with micrometer lateral and axial resolution.

Here I will review some of these methods and will present a new holographic system where multi-scale light illumination with near-cellular resolution in freely behaving mice is achieved by coupling an optical microscope for computer generated holography to a fiber bundle and a micro-objective. To demonstrate stable and precise photoactivation, the fiber bundle is also coupled to a versatile imaging system that permits fluorescence and functional imaging with different illumination modes comprising wide field, structured illumination and confocal imaging. Proof of principle experiments using the fiberscope to photostimulate and monitor functional responses in cerebellar molecular layer interneurons co-expressing GCaMP5-G and ChR2-tdTomato in anesthetized and freely moving mice will be presented. This new method will permit the control and monitor of circuit dynamics in freely behaving animals with unprecedented precision.

OPTICAL CONTROL OF CALCIUM-REGULATED EXOCYTOSIS

Pau Gorostiza

ICREA Research Professor at the Institute for Bioengineering of Catalonia (IBEC) in Barcelona

Neurons signal to each other and to non-neuronal cells as those in muscle or glands, by means of the secretion of neurotransmitters at chemical synapses. In order to dissect the molecular mechanisms of neurotransmission, new methods for directly and reversibly triggering neurosecretion at the presynaptic terminal are necessary. Here we exploit the calcium permeability of the light-gated channel LiGluR in order to reversibly manipulate cytosolic calcium concentration, thus controlling calcium-regulated exocytosis in bovine chromaffin cells. Amperometric recordings reveal that optical stimulation consistently triggers exocytosis, and secretion of catecholamines can be adjusted between zero and several Hz by changing the wavelength of illumination. Using whole-cell patch-clamp to quantify membrane capacitance and calcium influx, differences in secretion efficacy are found between the activation of LiGluR and native voltage-gated calcium channels (VGCCs). Our results show that the distance between sites of calcium influx and vesicle release is longer when calcium influx is triggered by LiGluR than by native VGCCs. Thus, LiGluR activation directly and reversibly increases the intracellular calcium concentration, and allows controlling calcium-regulated exocytosis without the need of applying depolarizing solutions or voltage clamping in chromaffin cells. LiGluR is a useful tool to study the secretory mechanisms and their spatiotemporal patterns in neurotransmission, and enables studying other calcium-dependent processes such as muscular contraction or cell migration.

STIM1 IS REQUIRED FOR NEURONAL Ca^{2+} SIGNALING AND MGLUR1-DEPENDENT SYNAPTIC TRANSMISSION

Jana Hartmann[1]; Rosa M. Karl[1], Ryan P.D. Alexander[1], Helmuth Adelsberger[1], Monika S. Brill[2], Charlotta Rühlmann[1], Anna Ansel[1], Kenji Sakimura[3], Yoshihiro Baba[4], Tomohiro Kurosaki[4], Thomas Misgeld[2] and Arthur Konnerth[1]

[1]Institute of Neuroscience, Technical University Munich, Biedersteiner Str. 29, 80802 Munich, Germany; [2]Chair for Biomolecular Sensors and German Center for Neurodegenerative Diseases (DZNE), Technical University Munich, Biedersteiner Str. 29, 80802 Munich, Germany; [3]Brain Research Institute, Niigata University, Niigata 951-8585, Japan; [4]Immunology Frontier Research Center, Osaka University, 9F Integrated Life Science Building, Osaka University, 3-1 Yamada-oka, Suita, Osaka 565-0871, Japan

In cerebellar Purkinje cells (PCs), the metabotropic glutamate receptor type 1 (mGluR1) is highly expressed and is crucial for cerebellar function. Synaptic activation of mGluR1 is followed by IP_3 receptor-dependent Ca^{2+} release from endoplasmic reticulum (ER) Ca^{2+} stores and a slow excitatory postsynaptic current mediated by the transient receptor potential channel TRPC3. The mechanism of TRPC3 activation is unknown as is the mechanism by which the ER Ca^{2+} content is maintained. In non-excitable cells TRPC channels are known to interact with the stromal interaction molecule 1 (STIM1), which is also critical for Ca^{2+} store replenishment. Therefore, we tested here the hypothesis that STIM1 may be involved in TRPC3 activation in brain neurons. First, using immunostaining and quantitative single cell RT-PCR we found that STIM1 is present in PCs and its expression level is ten times higher than that of its homolog STIM2. When analyzing newly generated PC-specific STIM1-deficient ($STIM1^{pko}$) mice, we observed that the absence of STIM1 in PCs causes distinct alterations in cerebellar motor function. Using whole-cell recordings and confocal Ca^{2+} imaging in acute cerebellar slices we found that both mGluR1-mediated Ca^{2+} release and TRPC3-mediated

inward currents are largely abolished in the absence of STIM1. Focal UV-hydrolysis of caged IP_3 (NPE-IP_3) in PC dendrites evoked a localized dendritic Ca^{2+} transient in control mice but not in $STIM1^{pko}$ mice. Ca^{2+} signals evoked by local dendritic application of caffeine, an agonist of ryanodine receptors, were also strongly attenuated in $STIM1^{pko}$ mice. By monitoring Ca^{2+} release signals from dendritic stores evoked by the mGluR-specific agonist DHPG in normal and Ca^{2+} free external medium we found that STIM1 is required for the replenishment of Ca^{2+} stores in PCs at resting membrane potential. In contrast to that, both DHPG-evoked Ca^{2+} signals and TRPC3-mediated inward currents were transiently restored in $STIM1^{pko}$ mice following depolarization-evoked Ca^{2+} influx through voltage-gated Ca^{2+} channels. Thus, the results identify STIM1 as a powerful regulator of Ca^{2+} homeostasis in a central mammalian neuron. In addition, we demonstrate that STIM1 is a messenger that couples mGluR1 and TRPC3 in cerebellar PCs by controlling intracellular Ca^{2+} levels at resting membrane potential.

CONTINUOUS CALCIUM INFLUX COMPENSATES FAST ER CALCIUM LOSS IN RESTING NEURONS

Samira Samtleben & Robert Blum

Institute for Clinical Neurobiology, Julius-Maximilians-University of Würzburg, 97078 Würzburg, Germany

The endoplasmic reticulum (ER) of neurons acts as a highly dynamic and tightly regulated calcium store. Here we asked how neurons maintain their free ER calcium level at rest. First we developed improved viral vectors for targeted esterase-induced dye loading (TED) enabling us to reliably label the ER lumen with a red-fluorescent carboxylesterase. Upon ER targeting this esterase releases high amounts of the green-fluorescent low-affinity calcium indicator Fluo5N in the ER lumen, thus enabling direct ER calcium imaging in presence of extracellular calcium. The new TED vectors show improved ER targeting and a bright red label in the ER lumen of living neurons. We developed mouse lines expressing the red fluorescent carboxylesterase as well. The first progeny of seven founder lines is available and shows a strong expression of the red TED construct in principal neurons. Here we applied TED to cultured hippocampal neurons and show that neuronal ER calcium is rapidly lost from the somatic and dendritic ER lumen, when neurons are kept under calcium free conditions. This loss of ER calcium was not prevented from cytosolic sites, neither in somatic nor in dendritic areas carrying a high density of ER structures. This suggested that a pronounced continuous calcium influx is needed to maintain the level of free ER calcium in a resting neuron. Next, we performed calcium imaging in the ER and in the cytosol and treated neurons acutely with SKF96365, an inhibitor of store-operated calcium entry (SOCE). Interestingly, in presence of extracellular calcium, the SOCE inhibitor caused a rapid drop in ER calcium, but no obvious changes in cytosolic calcium levels were observed. The fast loss of ER calcium, which could not been monitored in the cytosol, let us assume that leakage of ER calcium via the plasma membrane occurs in locally restricted areas of neurons and bypasses the immediate rescue by activity of the sarcoplasmic/endoplasmic reticulum calcium ATPase (SERCA).

DYNAMIC TRAFFICKING OF THE STORE-OPERATED Ca^{2+} ENTRY CHANNEL ORAI1

Rawad Hodeify[1], Senthil Selvaraj[1], Jennifer Wen[2], Abdelilah Arredouani[1], Satanay Hubrack[1], Sara N. Al-Thani[1], Kiyotaka Toshimori[3], Chizuru Ito[3], Timothy McGraw[2] and Khaled Machaca[1,#]

[1]Department of Physiology & Biophysics, Weill Cornell Medical College in Qatar, Doha, Qatar; [2]Department of Biochemistry, Weill Cornell Medical College, New York, USA; [3]Department of Anatomy & Developmental Biology, Chiba University, Japan.

Store operated Ca^{2+} entry (SOCE) is a ubiquitous Ca^{2+} influx pathway central to many physiological functions, including activation of cells in the immune system, muscle development and secretion. The minimal machinery mediating SOCE include an ER Ca^{2+} sensor, STIM1 and a plasma membrane highly Ca^{2+}-selective channel, Orai1. Although much is known about the molecular regulation of SOCE, the biogenesis and trafficking of Orai1 remain poorly defined. Here we dissect the dynamic trafficking of Orai1 and show that it is enriched intracellularly at steady state and that this distribution shifts following store depletion. We quantitatively define the rates of exocytosis and endocytosis of Orai1, and show that the protein localizes to a novel sub-plasmalemal intracellular compartment at rest. Furthermore we show that the C-terminal cytoplasmic domain of Orai1 is required for its trafficking to the plasma membrane.

Session 4 – Ca^{2+}-Signaling and Cancer Stem Cells

CALCIUM SIGNALING IN NORMAL AND CANCER STEM CELLS: DIFFERENT LANGUAGES FOR DIFFERENT FATES

Jacques Haiech

Laboratory of therapeutic innovations, UMR CNRS 7200, University of Strasbourg, School of Pharmacy, 74, route du Rhin, 60401 ILLKIRCH, France

A new paradigm has appeared in the last decade in the field of cancer. Solid tumors contain cancer-initiating cells that exhibit stemness properties and are able to resume tumor growth. Therefore, a tumor may be described as an organoid, i.e an organ-like with developmental defects. Such new paradigm opens new avenues for therapeutic innovation but also rejuvenates basic questions on tumor growth and plasticity. The calcium signal appears to be specific to a given cell type and a specific physiological state. The calcium signal relies on the expression of a specific subset of genes belonging to a calcium signal toolkit. We consider that this toolkit is composed of three sets of genes coding for proteins allowing i) calcium entry, ii) calcium removal and iii) calcium detection (about 300 genes). By analyzing the expression profile of this toolkit in a set of normal tissues, normal stem cell and cancer stem cells, it becomes possible to describe transcriptional modules and to infer potential calcisomes present in a given type of cell or tissue. Taking into account that the calcium entry and the calcium removal are regulated by calmodulin, we propose the utilization of calmodulin antagonists to differentially perturb calcium signals in a normal or cancer stem cells.

STEM CELLS AND CALCIUM SIGNALING

Rodrigo R. Resende

Cell Signaling and Nanobiotechnology Laboratory, Federal University of Minas Gerais, Belo Horizonte, MG 31270-901, Brazil

The increasing interest in stem cell research is linked to the promise of developing treatments for many lifethreatening, debilitating diseases, and for cell replacement therapies. However, performing these therapeutic innovations with safety will only be possible when an accurate knowledge about the molecular signals that promote the desired cell fate is reached. Among these signals are transient changes in intracellular Ca^{2+} concentration $[Ca^{2+}]_i$. Acting as an intracellular messenger, Ca^{2+} has a key role in cell signaling pathways in various differentiation stages of stem cells. The aim of this presentation is to present a broad overview of various moments in which Ca^{2+}-mediated signaling is essential for the manteinance of stem cells and for promoting their development and differentiation, also focusing on their therapeutic potential.

DISTINCT REMODELLING OF THE Ca^{2+} SIGNALLING TOOLKIT IN ENDOTHELIAL PROGENITOR CELLS UNDER DIFFERENT NEOPLASTIC CONDITIONS

Francesco Moccia

Laboratory of General Physiology, Department of Biology and Biotechnology "Lazzaro Spallanzani", University of Pavia, via Forlanini 6, 27100 Pavia, Italy

Endothelial progenitor cells (EPCs) may be recruited from bone marrow to sustain the metastatic switch in a number of solid cancers, including breast cancer (BC) and renal cellular carcinoma (RCC). Consistently, preventing EPC mobilization has been shown to cause tumour shrinkage. Novel anti-angiogenic treatments have recently been introduced in therapy to inhibit tumour vascularization by interfering with VEGFR-2 signalling; unfortunately, these drugs blocked tumour angiogenesis in pre-clinical murine models, but resulted far less effective in human patients. Understanding the molecular mechanisms driving EPC proliferation and tubulogenesis could outline novel targets for alternative anti-angiogenic treatments. Store-operated Ca^{2+} entry (SOCE), which is activated by a depletion of the intracellular Ca^{2+} pool, regulates the growth of human EPCs, where is mediated by the interaction between the endoplasmic reticulum Ca^{2+}-sensor, Stim1, and the plasmalemmal Ca^{2+} channels, Orai1 and TRPC1. EPCs do not belong to the neoplastic clone: thus, unlike tumour endothelium and neoplastic cells, they should not remodel their Ca^{2+} toolkit in response to tumour microenvironment. However, our recent work demonstrated that EPCs isolated from naïve RCC patients (RCC-EPCs) undergo a dramatic remodelling of their Ca^{2+} toolkit by down-regulating the expression of inositol-1,4,5-receptors (InsP3Rs), as well as their intraluminal Ca^{2+} levels, and up-regulating Stim1, Orai1 and TRPC1, as well as SOCE amplitude. Similar to RCC-EPCs, the Ca^{2+} machinery is re-arranged in EPCs isolated from BC patients (BC-EPCs). However, the molecular nature of the alterations in the Ca^{2+} toolkit is subtly different from that reported in RCC-EPCs, suggesting that tumour microenvironment may differentially impact on circulating EPC depending on the neoplasm. Moreover, EPCs are dramatically less sensitive to VEGF stimulation both in terms of Ca^{2+} signalling and of gene expression when isolated from tumour patients. Conversely, the pharmacological abolition of SOCE suppresses proliferation in both RCC- and BC-EPCs. These results question the suitability of VEGFR-2 as a therapeutically relevant target for anti-angiogenic treatments and hint at Stim1, Orai1, and TRPC1 as more promising alternatives.

CANCER STEM CELLS: Ca^{2+} INFLUX THROUGH STORE-OPERATED Ca^{2+} CHANNELS

Aulestia, F.J., Moreau, M. and Leclerc, C.

Centre de Biologie du Développement UMR 5547 118 route Narbone 31062 Toulouse-France.

Among the adult malignant primary brain tumours, glioblastoma multiforme (GBM) is the most common and deadliest grade IV astrocytoma. GBM contain a small subpopulation of self-renewing cells that have characteristic of embryonic cells, share many properties for adult neural stem cells (NSCs) and are capable of initiating tumour growth. These cells called Glioblastoma initiating Cells (GiCs) follows the cancer stem cell model which suggests that these cells are responsible for the

initiation and the growth of tumours (Galli et al., 2004). We propose to identify new selective signalling elements to specifically target GiCs. In this work we focus in one aspect of the calcium signaling, the store operated calcium entry (SOCE). The store-operated calcium entry is involved in various cellular functions. Increasing evidence suggests that SOCE is an important event in driving the expression of the malignant phenotypes, such as proliferation, migration, invasion, and metastasis (Chen et al., 2013). Understanding the SOCE in Glioblastoma cancer and cancer stem cells may thus shed a light on prognostic biomarkers or the potential therapeutic targets. We have used targeted aequorins to selectively measure calcium fluxes in different organelles. We have investigated the SOCE in OB1-glioblastoma initiating cells-previously isolated from glioblastoma multiforme (Patru et al., 2010). We have emptied the stores of the OB1 with tBHQ the reversible SERCA inhibitors and EGTA treatment. The re-adding of 1mM calcium to OB1 produces a high calcium increase in the nucleus and cytosol and the refilling of the endoplasmic reticulum.

SORCIN LINKS CELL CYCLE, CALCIUM LEVELS IN ENDOPLASMIC RETICULUM AND MULTI DRUG RESISTANCE IN CANCER

Elena Poser[1], Vasiliki Lalioti[2], Andrea Ilari[3], Annarita Fiorillo[3], Ilaria Genovese[3], Gianni Colotti[3]

[1]Department of Biochemistry "A. Rossi Fanelli", Sapienza University of Rome, Piazzale Aldo Moro 5 00185 Roma, [2]Centro de Biologia Molecular Severo Ochoa (Consejo Superior de Investigaciones Cientificas), Madrid; [3]Institute of Biology, Molecular Medicine and NanoBiotechnology (IBMN-Italian National Council of Research), Rome

Sorcin is a 22 kDa calcium binding protein belonging to the family of proteins with 5 EF hand motifs and overexpressed in many multi drug resistant tumors. Structurally, sorcin forms dimer through the unpaired EF5. We have solved the crystal structure of human sorcin in the apo-, in the calcium-bound, and in the magnesium-bound forms. Calcium binding to EF3, EF2 and EF1 entails conformational changes that determine sorcin activation via the exposure of hydrophobic surfaces and the consequent interaction with specific targets. Phosphorylation of residues located in EF3, EF4 and EF5 is another form of sorcin regulation, that may also modulate affinity for calcium of the protein in a sort of cross-talk between calcium binding and non-calcium binding EF hands. Sorcin regulates the activity of calcium channels (ryanodine receptors, SERCA pumps, Na-Ca exchangers) and keeps under control intracellular calcium levels in Endo/Sarcoplasmic Reticulum (ER). Analysis of sorcin interactome shows that sorcin interacts with proteins associated with microtubules, proteins located in the nucleolus, proteins involved in differentiation and cell cycle, many kinases such as Polo-like kinase 1 (PLK1), Aurora A and Aurora B kinases. Our in vitro interactome fits with the in vivo observations. Sorcin shows a characteristic behavior during the cell cycle: after nuclear envelope breakdown, it accumulates at the apical region of mitotic spindle; during late anaphase and early telophase, most sorcin accumulates in the region of the cleavage furrow; in late telophase, sorcin relocates in the reforming nuclei and concentrates in the midbody. We have also demonstrated that knock down of sorcin in fibroblasts blocks the cells in G2/M, provoking accumulation of rounded polynucleate cells and increasing apoptosis and cell death. Sorcin overexpression increases calcium concentration in the ER, may protect cells from ER stress, and prevents apoptosis. The mitotic defects observed upon sorcin silencing, together with the high levels of sorcin expression associated with cancers and with sorcin upregulation in Multi Drug Resistant (MDR) tumor cells, suggest that sorcin might be a regulator of mitotic progression and apoptosis, and a potential target to sensitize resistant tumor cells.

CALCIUM SIGNALING IN TASTE CELLS

Kathryn F Medler

University at Buffalo, Dept of Biological Sciences, Buffalo, NY 14260

Detecting and identifying chemicals in the environment is a common ability shared by all organisms. This is categorized as the oldest sensory system, due to the need to identify potentially harmful versus beneficial chemicals in the environment. In higher organisms, the ability to detect chemicals is shared by two sensory systems, taste and olfaction. While olfaction is important in many social behaviors, taste is used to detect nutrients as well as potentially harmful compounds to avoid. Thus, the sense of taste is critical to survival.

Despite the importance of taste, surprisingly little is known about the cellular mechanisms generating and regulating responses to taste stimuli. In part, this is due to the nature of the peripheral taste receptor cells, which are derived from epithelial cells but have neuronal properties. Like epithelial cells, taste cells routinely turnover, generating a mixed population of cells at different life cycle stages. Taste receptor cells also use different signaling pathways depending on the chemical structure of the stimulus being detected. All of these features add to the complexity of the taste system.

We do know that all taste responses depend on calcium signals to generate appropriate responses in the taste cells which relay information to the central nervous system. Some taste cells have conventional synapses and rely on calcium influx through voltage-gated calcium channels. Other taste cells lack these synapses and depend on calcium release from stores to formulate an output signal through a hemichannel. Beyond establishing these characteristics, few studies have focused on understanding how these calcium signals are formed or regulated. We have identified multiple calcium clearance mechanisms that regulate cytosolic calcium levels in taste cells after an evoked taste signal is generated. We have also identified the presence of a constitutive calcium influx that contributes to maintaining appropriate calcium homeostasis in these cells. We found that multiple factors can contribute to the evoked taste signals but these factors vary across the different taste cell populations. Clearly, calcium signaling is a dynamic process in taste cells and is more complex than has previously been appreciated.

CALCIUM SIGNALING IN CHEMICAL COMMUNICATION

Marc Spehr

Dept. of Chemosensation, Institute for Biology II, RWTH Aachen University, Aachen Germany

In olfactory sensory neurons (OSNs), cytosolic calcium controls the gain and sensitivity of olfactory signaling and, thus, serves as an essential regulator of OSN function. Important components of the molecular machinery that orchestrates the complex spatiotemporal calcium dynamics in OSNs have been described, but key details are still missing. Here, we demonstrate a critical physiological role of mitochondrial calcium mobilization in mouse OSNs. Combining a novel mitochondrial calcium imaging approach with patch-clamp recordings, organelle mobility assays, and ultrastructural analysis of individual OSNs, our study identifies mitochondria as key determinants of olfactory signaling. We show that mitochondrial calcium mobilization during sensory stimulation shapes the cytosolic calcium response profile in OSNs, ensures a broad dynamic response range, and maintains sensitivity of the spike generation machinery. When mitochondrial function is impaired, olfactory neurons function as simple stimulus detectors rather than intensity encoders. Moreover, we describe activity-dependent recruitment of mitochondria to olfactory knobs, a mechanism that provides a context-dependent tool for OSNs to maintain cellular homeostasis and signaling integrity.

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MOLECULAR ASPECTS OF THE REGULATION OF Ca^{2+}/CGMP HOMEOSTASIS IN ROD PHOTORECEPTOR CELLS IN NORMAL AND ALTERED CONDITIONS

Daniele Dell'Orco

Department of Life Sciences and Reproduction, Section of Biological Chemistry, University of Verona, strada le Grazie 8, 37134 Verona, Italy

Light sensitivity in rod and cone photoreceptor cells is finely regulated by negative feedback loops that involve Ca^{2+} as the main second messenger. The homeostasis of Ca^{2+} is strictly connected to that of another second messenger, guanosine 3´,5´-cyclic monophosphate (cGMP). In the dark, cGMP binds to a cyclic nucleotide-gated channel in the plasma membrane, keeping it open and allowing the influx of Na^+ and Ca^{2+} while the Na^+/Ca^{2+}, K^+-exchanger, a second transport system in the plasma membrane, continuously extrudes Ca^{2+}. Illumination of a photoreceptor cell results in a significant decrease of the cytoplasmic Ca^{2+}-concentration, which is detected by at least three Ca^{2+}-sensor proteins of the EF-hand superfamily, among which guanylate cyclase-activating proteins (GCAPs) control the activity of membrane bound guanylate cyclases in a Ca^{2+}-dependent manner. GCAPs contribute to shaping rod and cone photoresponses under diverse light regimes, and the operation of their different forms in rods or cones can be described in a Ca^{2+}-relay model, which allows the signalling cascade to make gradual responses to small changes in Ca^{2+}, thus accounting for two to eight different GCAP isoforms existing in species from fish to human.

Single point mutations in GCAP1 are known to cause severe disturbance of their Ca^{2+}-sensing properties resulting in the onset of cone and cone-rod dystrophies (COD). Biochemical and biophysical approaches are extremely useful to highlight altered structure/function relationships in COD-associated forms of GCAPs. However, in order to fully understand the dynamic changes of the homeostasis of both cGMP and Ca^{2+} in a photoreceptor cell under disease-associated conditions, a more comprehensive kinetic description of the phototransduction cascade is necessary. Such system-level mathematical description of the biochemical cascade based on experimentally determined parameters allows the simulation of the photoresponse in mouse rods under several illumination stimuli. Recent results on the characterization of four COD-related GCAP1 mutants will be presented, which suggest that the contribution of

GCAP1 to the dynamic synthesis of cGMP in rod cells depends on the expression level of the wild-type form, and could be partly compensated by the other present Ca^{2+}-sensor GCAP2, in complete agreement with the Ca^{2+}-relay model.

GAP, A NOVEL AEQUORIN-BASED FLUORESCENT RATIOMETRIC PROBE SUITABLE FOR CALCIUM IMAGING OF INTRACELLULAR ORGANELLES

Jonathan Rojo-Ruiz, Paloma Navas-Navarro, Francisco Javier Aulestia, Maria Teresa Alonso and Javier Garcia-Sancho

Instituto de Biologia y Genetica Molecular (IBGM), Universidad de Valladolid & CSIC, Valladolid, Spain

Genetically encoded calcium indicators (GECI) allow monitoring subcellular Ca^{2+} signals inside organelles. Most GECI contain endogenous calcium-binding proteins whose functionality in vivo may be perturbed by competition with cellular partners. We describe here a novel family of fluorescent Ca^{2+} probes based on the fusion of two Aequorea victoria proteins, GFP and apo-aequorin (GAP). Both proteins have been used extensively with no report of interferences with Ca^{2+} homeostasis or other secondary effects. GAP exhibited green fluorescence whose excitation spectrum was shifted by Ca^{2+}. GAP displayed a unique combination of features: dual-excitation ratiometric imaging, high dynamic range, good signal-to-noise ratio, insensitivity to pH and Mg^{2+}. Ca^{2+} affinity could be tuned by mutations in the aequorin EF hands. Ca^{2+} calibration was uncomplicated with a maximum ratio increase of three to fourfold and a Hill coefficient of 1. We have targeted GAP to five distinct organelles and behaviour was as expected for a selective Ca^{2+} probe. Both virus-induced expression as well as cell lines stably expressing targeted GAPs were successfully achieved. Transgenic mice for endoplasmic reticulum-targeted GAP exhibited a robust long-term expression and reproducible performance in various neural tissues including hippocampus, cerebral cortex, cerebellum, spinal motor neurons or dorsal root sensory neurons. Expression patterns in other tissues will be advanced. This biosensor fills a gap in the actual repertoire of Ca^{2+} indicators for organelles and is a valuable tool for in vivo Ca^{2+} imaging applications.

NEURONAL CALCIUM BINDING PROTEINS 1/2 LOCALIZE TO DORSAL ROOT GANGLIA AND EXCITATORY SPINAL NEURONS

Ming-Dong Zhang[a,b,1], Giuseppe Tortoriello[b], Brian Hsueh[c], Raju Tomer[c], Li Ye[c], Nicholas Mitsios[d], Lotta Borgius[a], Gunnar Grant[a], Ole Kiehn[a], Masahiko Watanabe[e], Mathias Uhlén[f], Jan Mulder[a,d], Karl Deisseroth[c], Tibor Harkany[b,g]* and Tomas Hökfelt[a,1]*

[a]Department of Neuroscience, Karolinska Institutet, Stockholm, Sweden; [b]Division of Molecular Neurobiology, Department of Medical Biochemistry & Biophysics, Karolinska Institutet, Stockholm, Sweden; [c]Departments of Bioengineering and Psychiatry, Howard Hughes Medical Institute, Stanford University, Stanford, CA 94305, USA; [d]Science for Life Laboratory, Department of Neuroscience, Karolinska Institutet, Stockholm, Sweden; [e]Hokkaido University School of Medicine, Sapporo, Japan; [f]Science for Life Laboratory, Albanova University Center, Royal Institute of Technology, Stockholm, Sweden; [g]Department of Molecular Neurosciences, Center for Brain Research, Medical University of Vienna, Spitalgasse 4, A-1090 Vienna, Austria
*THö and THa share senior authorship

Neuronal calcium binding proteins 1 and 2 (NECAB1/2) are members of the phylogenetically-conserved EF-hand Ca^{2+}-binding protein superfamily. NECABs have up till now been explored only to a limited extent and so far not at all at the spinal level. Here, we describe the distribution, phenotype and nerve injury-induced regulation of NECAB1/NECAB2 in mouse dorsal root ganglia (DRGs) and spinal cord. In DRGs, NECAB1 and NECAB2 are expressed in around 70% of, mainly, small and medium-sized, neurons. Many co-localize with calcitonin gene-related peptide and isolectin B4 and thus represent nociceptors. NECAB1/2 neurons are much more abundant in DRGs than the so far studied Ca^{2+}-binding proteins (parvalbumin, calbindin, calretinin and secretagogin). In the spinal cord, the NECAB1/NECAB2 distribution is mainly complementary. NECAB1 labels interneurons and a plexus of processes in superficial layers of the dorsal horn, commissural neurons in the intermediate area, and motor neurons in the ventral horn. Using “CLARITY”, a novel, bilaterally connected neuronal system with dendrites that embrace the dorsal columns like palisades is observed. NECAB2 is present in cell bodies and presynaptic boutons across the spinal cord. In the dorsal horn, most NECAB1/NECAB2 neurons are glutamatergic. Both NECAB1/2 are transported into dorsal roots and peripheral nerves. Peripheral nerve injury reduces NECAB2, but not NECAB1, expression in DRG neurons. Our study identifies NECAB1 and NECAB2 as abundant Ca^{2+}-binding proteins in pain-related DRG neurons and a variety of spinal systems, providing molecular markers for known and unknown neuron populations of mechanosensory and pain circuits in spinal cord.

CALCIUM AND VITAMINS: SIGNALING AND PROMOTING MEMBRANE REPAIR

Paul L. McNeil

Georgia Regents University, Augusta, Georgia, USA

Calcium is the trigger for the key events of plasma membrane repair, an observation made by Heilbrunn almost 70 years ago, and one that preceded his hypothesis that calcium was the trigger for skeletal muscle contraction. We now know that calcium entering cytoplasm from the extracellular environment through large plasma membrane disruptions (> 1 µm) triggers SNARE-, annexin- and dysferlin-mediated homotypic and exocytotic fusion events that add a patch to the defect site. Whereas calcium entering through smaller disruptions (nanometer scale) triggers local assembly of endosomal complex required for transport (ESCRT) proteins, that drive outward bending of the membrane and the formation of a small bud, which rids the cell of the defect. I will propose in my talk that this repair-triggering role is not only a biologically fundamental one, since cell death is the rapid result of repair failure, but also that it is an evolutionarily primitive one. I will also present my recent research on vitamin E. The biological role of vitamin E was also discovered approximately 70 years ago: deficiency results in profound muscle wasting disease. We have found that vitamin E is required for skeletal muscle myocyte plasma membrane repair. This explains why this vitamin is important for muscle health and adds a new concept to our understanding of the repair mechanism.

ANNEXIN-A5 PARTICIPATES IN MEMBRANE REPAIR IN THE PLACENTA

Anthony Bouter[1], Sisareuth Tan[1], Romain Linares[1], Romain Carmeille[1], Alain R. Brisson[1], Séverine Degrelle[2], Guillaume Pidoux[2], Danièle Evain-Brion[2]

[1]Molecular Imaging and NanoBioTechnology, UMR-5248 CBMN CNRS-University of Bordeaux-IPB, Pessac, F-33600, France; [2]UMR-S767 INSERM, University Paris Descartes, Paris, France

Annexin-A5 (AnxA5) is the smallest member of the annexins, a group of soluble proteins that bind to membranes containing negatively-charged phospholipids, principally phosphatidylserine, in a Ca^{2+}-dependent manner (1). AnxA5 presents unique properties of binding and self-assembling on membrane surfaces, forming highly ordered two-dimensional (2D) arrays (2). We showed recently that AnxA5 plays a central role in the machinery of cell membrane repair (3), promoting the repair of membrane damages via the formation of 2D protein arrays at membrane damaged sites and preventing the extension of membrane ruptures (4). As the placenta is the richest source of AnxA5 in humans (5), we decided to investigate whether AnxA5 was involved in membrane repair in the placenta. We addressed this question at the level of human placental BeWo cells, either mononucleated cytotrophoblasts or multinucleated syncytiotrophasts (ST). Using established procedures of laser irradiation and fluorescence microscopy, we investigated the localization of AnxA5 after cell injury and compared the repair capacity of cells expressing AnxA5 and cells rendered deficient in AnxA5 by RNA interference. These studies demonstrated that AnxA5 participates in the membrane repair machinery in placental cells. In parallel, we revisited the question of the exposure of phosphatidylserine and the localization of AnxA5 at the level the ST membranes in human placenta, by electron microscopy and immunogold labeling. We show that most of the ST membrane surfaces do not expose phosphatidylserine, nor Anx5, in marked contrast with previous studies concluding to the presence of an AnxA5 layer covering the surface of syncytiotrophoblasts (6). This ensemble of results highlights the protective role of AnxA5 in the placenta.

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DEALING WITH DAMAGE: THE ANNEXIN SURVIVAL KIT

Heidi Wolfmeier, Roman Schoenauer, Alexander P. Atanassoff, Eduard B. Babiychuk, Annette Draeger

Institute of Anatomy University of Bern, Switzerland

Bacterial pore-forming toxins, such as pneumolysin (PLY), a key virulence factor of S. pneumonia, permeabilize eukaryotic cells by forming large trans-membrane pores. However, membrane repair mechanisms allow the cells to reseal their membrane in order to prevent the uncontrolled influx of calcium and the efflux of cytoplasmic constituents. After injury, the Ca^{2+}-concentration gradient fulfils a dual function: it provides guidance cues for the repair machinery and directly activates the molecules, which have a repair function.

Whereas pore-induced Ca^{2+}-entry from the extracellular milieu is of paramount importance for the initiation of plasmalemmal repair, the active Ca^{2+}-sequestration that prevents excessive Ca^{2+}-elevation during its execution is no less important. The efficacy of plasmalemmal repair does not only define the fate of targeted cells but also intensity, duration and repetitiveness of PLY-induced Ca^{2+}-signals in cells that are able to survive after PLY-attack. Members of the annexin protein family are ubiquitously expressed, Ca^{2+}-dependent, phospholipid-binding proteins with fusogenic properties. Their unique and distinct Ca^{2+}-sensitivities of membrane binding enable them to serve both as sensors of membrane perforation and as executors of plasmalemmal repair.

ANNEXIN A2 MODULATES INFLAMMASOME ACTIVATION IN SEVERE COLITIS BY ENABLING LYSOSOMAL MEMBRANE REPAIR

Elle Flood[1], Min Luo[1], Huigen Chen[1], Brian Scharf[2], Christopher Huber[1], Nadia Hedhli[1], Dena Almeida[1], Ellen Scherl[1], Laura Santambrogio[2], Katherine A. Hajjar[1]

[1]Weill Cornell Medical College, New York, USA; [2]Einstein College of Medicine, Bronx, USA

Inflammasomes are environmental “danger” sensors that are activated in response to production of reactive oxidative species (ROS), opening of the P2X7 potassium channel, release of mitochondrial DNA, or leakage of lysosome contents. In response to stress signals, cells assemble inflammasomes, large multi-protein complexes that activate caspase-1, enable secretion of the pro-inflammatory cytokines IL-1ß and IL-18, and initiate pyroptosis, a pro-inflammatory form of programmed cell death. Annexin A2 (ANXA2) is a phospholipid-binding protein that may prevent inflammasome activity in vitro by translocating, in a calcium-dependent manner, to injured membranes, such as the mechanically-stretched sarcolemma or dendritic cell lysosomal membranes damaged by wear debris microparticles from artificial joints. In the dextran sodium sulfate (DSS) model of inflammatory bowel disease, we found that Anxa2$^{-/-}$ mice displayed markedly increased colonic edema, bleeding, and epithelial cell erosion, in addition to striking leakage of lysosomal cathepsins and H^+ ions into the cytoplasm of colonic epithelial cells. DSS-Anxa2$^{-/-}$ colonic tissue displayed uniquely exaggerated NLRP3 inflammasome activity as demonstrated by marked caspase-1 activation and apoptotic epithelial cell death. Compared to DSS-Anxa2$^{+/+}$ controls, DSS-Anxa2$^{-/-}$ mice also revealed dramatically elevated plasma levels of IL-1ß and TNFα. Interestingly, mice lacking S100A10, an ANXA2 binding partner, showed no increase in susceptibility to DSS colitis. DSS-induced colonic inflammation in the Anxa2$^{-/-}$ mouse was completely reversed upon treatment with the specific cathepsin inhibitor CA074Me or with a blocker of inflammasome activation, glyburide. Adoptive transfer of Anxa2$^{-/-}$ macrophages into Anxa2$^{+/+}$ mice recapitulated the extreme Anxa2$^{-/-}$ phenotype in DSS colitis. Finally, in human patients with moderate to severe ulcerative colitis, ANXA2, normally cytoplasmic, co-localized with lysosomal membranes within mucosal cells in inflamed regions of colon, whereas it remained cytoplasmic in non-involved areas. Together, these data suggest a strong anti-inflammatory role for ANXA2, through its ability to repair the lysosome’s limiting membrane, thus maintaining lysosomal integrity. ANXA2 could serve as a disease-modifying factor in human inflammatory bowel disease.

DEFECTIVE CALCIUM SIGNALLING IN PARKINSON DISEASE; CLUES FROM A LYSOSOMAL STORAGE DISORDER

Bethan S. Kilpatrick[1], Joana Magalhaes[2], Michelle S. Beavan[2], Michael W.J. Cleeter[2], Alisdair McNeill[2], Duncan Bloor-Young[3], Grant C. Churchill[3], Michael R. Duchen[1], Anthony H. Schapira[2] and Sandip Patel[1]

[1]University College London, Department of Cell and Developmental Biology, United Kingdom; [2]Department of Clinical Neurosciences, University College London Institute of Neurology, United Kingdom; [3]University of Oxford, Department of Pharmacology, United Kingdom

Significant advances towards understanding the genetic basis for Parkinson’s disease (PD) have been made, however the cellular mechanisms underlying this common neurodegenerative disorder remain to be fully defined. Notably mutations in GBA1, which underlie the lysosomal storage disorder Gaucher’s disease (GD), have been identified as the most frequent genetic risk factor for PD. Since deviant Ca^{2+} signalling features in many neurodegenerative diseases including GD, we considered a role for intracellular Ca^{2+} storage dysregulation in the pathology of PD. Ca^{2+} signals were compared in GD and PD patients carrying the common N370S mutation in GBA1. We show that ER Ca^{2+} content in primary fibroblasts from healthy individuals was increased in an age dependent manner. In younger GD and PD patients ER Ca^{2+} content was higher relative to aged-matched controls and thus resembled ER Ca^{2+} of older individuals. Enhanced ER Ca^{2+} content was associated with increased responses to the ryanodine receptor activator, cyclic ADP-ribose. Healthy individuals carrying a GBA1 mutation did not show disrupted ER Ca^{2+} signalling, identifying Ca^{2+} homeostasis as a defining feature and potential biomarker for PD development. Overexpression of mutant GBA1 in dopaminergic SHSY5Y cells, but not knock-down, recapitulated defective ER Ca^{2+} responses, implicating mis-folded GBA1 in the pathology of PD. Conversely, lysosomal Ca^{2+} signals were reduced in GD and PD fibroblasts and associated with disrupted lysosome morphology. We conclude that accelerated remodelling of ER-lysosomal Ca^{2+} stores by pathogenic GBA1 might predispose to PD. Therefore, Ca^{2+} signalling proteins could represent new therapeutic targets for both GD and PD.

SENSE AND SPECIFICITY IN NEURONAL CALCIUM SIGNALLING

Robert D Burgoyne

Department of Cellular and Molecular Physiology, Institute of Translational Medicine, University of Liverpool, Crown Street, Liverpool, L69 3BX, UK

Changes in intracellular Ca^{2+} concentration regulate many different aspects of neuronal function on varied time scales from less than milliseconds to days. The specific effects of Ca^{2+} are to some extent determined by the magnitude, localisation and time-course of the intracellular Ca^{2+} signal but are also determined by the nature and characteristics of the particular Ca^{2+} sensor protein(s) that is activated. A further significant factor is the target specificity of the Ca^{2+} sensor. We have been investigating two families of EF-hand containing Ca^{2+} sensor proteins that are expressed in neurons, these are the neuronal calcium sensor (NCS) proteins and the CABPs. Each family is encoded by multiple genes in mammals (14 for NCS proteins and 6 CaBPs) some of which can be expressed as splice isoforms. A key question is why are there such a large number of these Ca^{2+} sensors and how can they have specific non-redundant functions despite being closely related in sequence and structure? An answer to this requires an understanding of the structural basis for their interaction with their specific target proteins. Using a combination of biochemical and structural approaches the nature of their target proteins and their interactions have been characterised. This has provided insights into the amino acids that determine specificity of target binding. For the NCS proteins a crucial aspect of specificity comes from the shape and size of a hydrophobic groove exposed on Ca^{2+} binding, the nature of surrounding residues and differing contributions of the variable C-terminal tail of the proteins. We have extended these studies and confirmed structural predictions by testing key aspects of the features of NCS-1 using a physiological assay in the model organism Caenorhabditis elegans. We are now using this approach to define the Ca^{2+} signalling pathway underlying NCS-1-dependent changes in behaviour.

IN VIVO EVIDENCE THAT BAX INHIBITOR-1 IS A Ca^{2+} CHANNEL WITH A ROLE IN IMMUNE CELL FUNCTION AND SURVIVAL

Dmitrij Lisak[1], Orhan Aktas[2], Ari Waisman[3], Nadine Hoevelmeyer[3], Michael Gliem[2], Teresa Schacht[1] and Axel Methner[1]

[1]University Medical Center Mainz, Dept of Neurology, Mainz, Germany; [2]University of Düsseldorf, Dept of Neurology, Düsseldorf, Germany; [3]University Medical Center Mainz, Institute of Molecular Medicine, Mainz, Germany

The endoplasmic reticulum (ER) plays a key role in the synthesis, folding and sorting of proteins and disturbances of this delicate system cause cell death by the unfolded protein response. The ER however also serves as the major intracellular calcium (Ca^{2+}) store and Ca^{2+} release from this store controls a vast array of cellular functions. At the interface of both functions is Bax Inhibitor-1 (BI-1), an evolutionarily conserved multi-faceted protein for which others and we have proposed various mechanisms by which BI-1 mediates Ca^{2+} flux from the ER and protects from ER stress. Recent structural evidence obtained from a bacterial homologue of BI-1, BsYetJ, demonstrated that BI-1 is indeed a Ca^{2+}-leak channel regulated by pH, supporting earlier conclusions of our groups. Despite its evolutionarily conserved role in such important functions like ER stress and Ca^{2+} regulation, lack of functional BI-1 appears to have no obvious effect in vivo. Mice deficient in functional BI-1 display no gross abnormalities and have no recognizable histopathological abnormalities. We delved deeper into this and found that these mice suffer from leukopenia pointing to a role of BI-1 in the immune system. We then used experimental autoimmune encephalomyelitis (EAE), vaccination, and postischemic inflammation to further clarify this role. This showed a predominant susceptibility of KO B cells which have increased cytosolic and ER Ca^{2+} levels leading to increased translocation of the Ca^{2+} dependent transcription factors NFAT and NFκB; markers of ER stress and XBP-1 splicing were in contrast similar in WT and KO cells. These results suggest that the major role of BI-1 in vivo involves its effects on the intracellular Ca^{2+} homeostasis in immune cells.

ANALOG-DIGITAL MODULATION OF SYNAPTIC TRANSMISSION IN CORTICAL NEURONS

Dominique Debanne, Sylvain Rama, Andrzej Bialowas, Vincenzo Marra, Edmond Carlier and Laure Fronzaroli Molinieres

U1072, INSERM, Aix-Marseille Université, Marseille, France

Synaptic transmission generally depends on action potentials (APs) in an all-or-none (digital) fashion. Recent studies indicate however, that sub-threshold depolarization of the presynaptic neuron facilitates spike-evoked transmission, thus creating an analog-digital (AD) mode of synaptic modification. We show here that transmission at CA3-CA3 synapses can be enhanced by presynaptic voltage in two ways: transmission is facilitated when the presynaptic AP is preceded by either a long (>5sec) depolarization or a brief (0.05-0.2sec) hyperpolarization. The depolarization-induced AD facilitation (d-ADF) results from the inactivation of Kv1 channels whereas the hyperpolarization-induced AD facilitation (h-ADF) is due to the recovery from inactivation of Nav channels. d- and h-ADF occur under physiological conditions and are additive. h-ADF is also observed at L5-L5 synapses, indicating that it can be generalized to many central synapses. In conclusion, our study shows that fast and slow modes of analog-digital facilitation coexist at central synapses, indicating that the informational content of the presynaptic AP is highly adjustable.

DROSOPHILA SEPTIN, PNUT IS A MODULATOR OF NEURONAL STORE OPERATED CALCIUM ENTRY

Bipan Kumar Deb, Trayambak Pathak and Gaiti Hasan

National Centre for Biological Sciences, Tata Institute of Fundamental Research, Bangalore, India

A variety of signals can trigger uptake of extracellular calcium into neurons. Certain signals like the neurotransmitter acetylcholine can bind to one of its cognate receptors, the muscarinic acetylcholine receptor

and activate downstream signaling leading to generation of the second messenger inositol-1,4,5-trisphosphate(IP_3)(1). Binding of IP_3 to the IP_3 receptor on the membrane of the endoplasmic reticulum (ER) leads to depletion of the ER calcium stores. This drop in ER calcium levels is sensed by STIM proteins, which subsequently oligomerize and translocate to ER-PM junctions where they help organize and open the Store operated calcium entry (SOC) channel, Orai, leading to uptake of extracellular calcium, a process called Store operated calcium entry (SOCE)(2). We have demonstrated a requirement for SOCE in neurons of the Drosophila flight circuit(1). Subsequent analysis showed a genetic interaction between IP3 receptor and STIM/Orai, in regulation of neuronal SOCE (3). Septins are GTPases that form heterooligomeric structures in cells, that regulate a variety of cellular phenomenon (4). Recently, septins of the SEPT2 subgroup have been shown to act as coordinators of SOCE in HeLa and Jurkat T cells (5). Using direct measurement of SOCE from primary neuronal cultures obtained from central nervous system of Drosophila, we uncovered a novel function for a Drosphila septin, Pnut (homolog of hSEPT7) in modulation of IP_3 receptor function and SOCE in neurons. Genetic manipulations of Pnut levels show that this protein regulates SOCE in neurons of the Drosophila flight circuit. Flight deficits in the adult organism obtained on abrogating SOCE in neurons of the flight circuit can be rescued by reducing Pnut levels. Our findings show that the mechanism employed by Pnut (Drosophila SEPT7) for regulation of SOCE in neurons is distinct from the role mammalian septins of the SEPT2 subgroup play in modulation of SOCE in HeLa and Jurkat T cells.

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STIM SENSORS AS MOLECULAR TARGETS FOR MEMORY LOSS TREATMENT CONNECTED WITH CALCIUM HOMEOSTASIS IMPAIRMENTS IN FAMILIAL ALZHEIMER'S DISEASE

Maria Ryazantseva[1], Ksenia Skobeleva[1], Anna Panova[2], Liubov Glushankova[1], Nikolai Kamyshev[2], Elena Kaznacheyeva[1]

[1]Institute of Cytology RAS, St. Petersburg, Russia, Tikhoretsky ave 4, 194064; [2]Pavlov Institute of Physiology RAS, St. Petersburg, Russia, Makarova emb.6, 199034

Familial Alzheimer's disease (FAD) which leads to memory impairments is caused by mutations in presenilin-1 (PS1) gene in approximately 50% of cases. PS1 is well known as a component of the gamma-secretase enzyme which cleaves APP to A-beta. To become a catalytic part of enzyme PS1 holoprotein undergoes an endoproteolysis. It was shown that mutations in PS1 gene disrupt endoproteolysis increasing holoprotein level in brain tissue of FAD patients. In our study we found effects of FAD PS1 mutants (PS1DE9, PS1 D247A) on activity of store-operated and voltage-gated calcium channels in neurons and Neuro2a cell line. The PS1 endoproteolysis levels were estimated with SDS PAGE and western-blot of total cell lysates which demonstrated increased PS1 holoproteins levels. Disrupted channels activities were detected with direct single-cell electrophysiological measurements. Intracellular calcium concentrations were measured in calcium imaging experiments with fura2-AM. Experiments with ER calcium sensors STIM knock-downs demonstrated the effects were caused by impaired signal transduction from ER to calcium channels in plasmatic membrane (PM) under control of ER calcium levels, which was demonstrated by experiments with knock-downs. The impaired intracellular signal transduction by STIM sensors was revealed in live confocal imaging experiments. Moreover a feeding of Drosophila melanogaster transgenes expressing human mutated PS1 in cholinergic nervous system with pharmacological inhibitor of STIM sensor signal transduction led to rescue of the memory loss detected by courtship based assay.

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CALCIUM AND CALCIUM CHANNELS IN INITIATION AND PROGRESSION OF PROSTATE CANCER

Charlotte Dubois, Fabien Vanden Abeele, V'yacheslav Lehen'kyi, Maylis Raphael, Natalia Prevarskaya

Inserm U1003, Equipe labellisée par la Ligue Nationale Contre le Cancer, Laboratory of Excellence, Ion Channels Science and Therapeutics; Université Lille I Sciences et Technologies, Villeneuve d'Ascq, France

Normal cell progression to their malignant derivatives is associated with remodeling of the proteins controlling such major cellular functions as apoptosis and proliferation. Here, we show that prostate cancer cells use ORAI and TRP protein redistribution as an oncogenic switch mechanism. In particular, ORAI3 and TRPV6 remodeling results from genomic and microenvironment perturbations that disrupt the equilibrium of channels and favor the formation of novel Ca^{2+} channels activated in a store-independent manner. This remodeling of Ca^{2+} signaling in turn induces cell progression to a more aggressive pro-proliferative phenotype. Our study specifically positions these channels at the center of molecular machinery linking dysregulated arachidonic acid metabolism, calcium homeostasis, and oncogenesis.

CALCIUM PUMPS OF THE PLASMA MEMBRANE AND SECRETORY PATHWAY IN BREAST CANCER CELLS

Amelia Peters, Jane Lee, Eloise Dray, Sarah Roberts-Thomson and Gregory Monteith

The School of Pharmacy, The University of Queensland, Pharmacy Australia Centre of Excellence. Brisbane. Australia

Lactation is the physiological function of the breast and is associated with the transport of Ca^{2+} ions into milk for infant growth and development. The transport of Ca^{2+} into milk occurs via direct efflux across the breast epithelial membrane via plasma membrane Ca^{2+}-ATPases (PMCAs) and secretion via secretory Ca^{2+}-ATPases (SPCAs) that also can transport Mn^{2+} ions. Expression of the PMCA2 isoform of the plasma membrane Ca^{2+} pump and the two secretory pathway Ca^{2+} pumps SPCA1 and SPCA2 are increased in some breast cancers. PMCA2 and SPCA1 but not SPCA2 mRNA are present in MDA-MB-231 breast cancer cells. MDA-MB-231 breast cancer cells are representative of the basal breast cancer subtype, which is associated with poor clinical prognosis. Silencing of PMCA2 and SPCA1 both reduce the proliferation of MDA-MB-231 breast cancer cells. PMCA2 silencing in MDA-MB-231 breast cancer cells augments the effectiveness of the clinically used cytototoxic agent doxorubicin. The silencing of SPCA1 does not appear to increase the effectiveness of clinically used cytotoxics. However, 2D-DIGE analysis has identified a number of proteins sensitive to SPCA1 silencing in breast cancer cells including heat shock protein 60.

CALCIUM REGULATION OF MIGRATION PATHWAYS IMPORTANT IN CANCER

Albrecht Schwab & Nikolaj Nielsen

Institut für Physiologie II, Robert-Koch-Str. 27 b, 48149 Münster

Cancer and host cells within the tumor stroma strongly influence each other and create a tumor microenvironment with distinct physical and chemical properties that are permissive for tumor progression. This is particularly valid in pancreatic ductal adenocarcinoma (PDAC). PDAC is characterized by a pronounced fibrosis (desmoplasia) resulting from the mutual growth factor-dependent stimulation of pancreatic stellate cells (PSCs) and cancer cells. The ability to migrate plays a central role in this mutual interaction which eventually leads to a hypoxic and acidic tumor microenvironment. There is growing evidence that transient receptor potential (TRP) channels and STIM/ORAI proteins affect key calcium-dependent mechanisms of cancer and stroma cell migration. Here we focused on the TRPC family of which several members are expressed in PSCs. We could show that TRPC1, 3 and 6 channels play distinct roles in PSC migration in response to different stimuli such as PDGF or hypoxia. Notably, they are either upregulated in the PDAC stroma or by hypoxia in an in vitro setting. Mechanistically, TRPC channels affect on PSC migration by cooperating with other Ca^{2+} sensitive ion channels (e.g. KCa3.1) or other intracellular Ca^{2+} sensitive effector proteins such as calpain. In summary, our results indicate that TRPC channels are important mediators in the adaptation of PSCs to the PDAC microenvironment.

TRANSFORMING PRO-SURVIVAL IP_3 SIGNALING IN B-CELL CANCERS INTO PRO-APOPTOTIC Ca^{2+} SIGNALING UPON BCL-2 ANTAGONISM AT THE ENDOPLASMIC RETICULUM

Akl H.[1], Vervloessem T.[1], La Rovere R.[1], Janssen A.[2], Monaco G.[1], Vandenberghe P.[3], Distelhorst C.W.[4], Baes M.[5], Erneux C.[6], Mikoshiba K.[7], Parys J.B.[1], De Smedt H.[1], and Bultynck G.[1]

[1]KU Leuven, Cellular and Molecular Medicine, Leuven, Belgium; [2]UZ Leuven, Development and Regeneration, Leuven, Belgium; [3]UZ Leuven, Human Genetics, Leuven, Belgium; [4]Case Western Reserve University, Cleveland, Ohio, USA; [5]KU Leuven, Cell Metabolism, Leuven, Belgium; [6]Université Libre de Bruxelles, Institut de Recherche Interdisciplinaire et Biologie Humaine et Moléculaire, Brussels, Belgium; [7]Riken Brain Science Institute, Wako, Japan

In addition to its mitochondrial role, the anti-apoptotic oncoprotein Bcl-2 acts at the endoplasmic reticulum, directly binding and inhibiting inositol 1,4,5-trisphosphate (IP_3) receptors (IP_3Rs), intracellular Ca^{2+} channels that critically control cell proliferation and death. A peptide (TAT-IDPS) that targets the BH4 domain of Bcl-2, but not that of Bcl-XL, reversed Bcl-2's inhibitory action on IP_3Rs and promoted pro-apoptotic Ca^{2+} signaling in chronic lymphocytic leukemia (CLL) patient cells and in diffuse large B-cell lymphoma (DL-BCL) cell lines. The apoptotic sensitivity of DL-BCL cells to TAT-IDPS was dictated by the levels of IP_3R2, the IP_3R isoform with the highest sensitivity to IP_3. In DL-BCL cells expressing high IP_3R2 levels, TAT-IDPS caused a Ca^{2+} rise in both the cytosol

and in the mitochondria. However, healthy cells displaying high IP_3R2 levels, like primary hepatocytes, were resistant to TAT-IDPS. Hence, an additional factor must be present in B-cell malignancies critical for TAT-IDPS-induced cell death. Since a key feature of B-cell neoplasias is the constitutively active B-cell receptor (BCR) resulting in chronic phospholipase C (PLC)/IP_3 signaling, we explored the relevance of basal IP_3 levels in our models. Remarkably, pharmacological and genetic manipulation of the basal IP_3 signaling was detrimental for CLL and DL-BCL cells, showing its role in cancer cells survival. Inhibition of the on-going IP_3 signaling, using the PLC inhibitor (U-73122) or by transfecting the cells with vectors expressing either a high-affinity IP_3 sponge or an IP_3 5'-phosphatase, suppressed TAT-IDPS-induced Ca^{2+} rise in the cytosol and mitochondria and prevented subsequent apoptotic DL-BCL and CLL cells death. Eventually, primary hepatocytes, displaying high IP_3R2 levels, were sensitized to TAT-IDPS-induced cell death by boosting their basal IP_3 signaling with the PLC activator, m-3M3FBS. The importance of basal IP_3 signaling for TAT-IDPS-induced cell death in CLL was independent of its BCR status (mutated or unmutated). Thus, our data indicate that basal, pro-survival chronic IP_3 signaling renders CLL and DL-BCL cells dependent on high Bcl-2 levels to prevent excessive pro-apoptotic IP_3R-mediated Ca^{2+} signaling.

THE CALCIUM SENSING RECEPTOR IS A TUMOR SUPPRESSOR IN THE COLON REGULATING PROLIFERATION, DIFFERENTIATION, AND EPITHELIAL TO MESENCHYMAL TRANSITION

Abhishek Aggarwal, Maximilian Wohlgenannt, Samawansha Tennakoon, Anastasia Meshcheryakova, Sabina Baumgartner-Parzer, Diana Mechtcheriakova, Enikö Kállay

Medical University of Vienna, Vienna, Austria

The classical role of the calcium sensing receptor (CaSR), a G protein coupled receptor, is regulation of calcium homeostasis. CaSR is also expressed in tissues not directly involved in calcium homeostasis like the colon. In colorectal cancer (CRC) we found that CaSR expression is down-regulated, leading to the hypothesis that loss of CaSR provides growth advantage to transformed cells. We stably transfected HT29 CRC cells with the wild type CaSR ($HT29^{CaSR\text{-}WT}$) or an empty vector as control ($HT29^{EMP}$). Proliferation of $HT29^{CaSR\text{-}WT}$ significantly decreased compared with the $HT29^{EMP}$ cells. $HT29^{CaSR\text{-}WT}$ cells were more differentiated than $HT29^{EMP}$ cells having higher alkaline phosphatase activity. These effects were more pronounced when cells were treated with cinacalcet, an allosteric modulator of the CaSR. On treating the cells with the calcilytic NPS 2143, a negative allosteric modulator of the receptor, we could reverse the anti-proliferative, pro-differentiating effects. $HT29^{CaSR\text{-}WT}$ further showed a more epithelial phenotype than $HT29^{EMP}$ cells. Since cancer cells can undergo epithelial to mesenchymal transition (EMT), we investigated expression of a wide spectrum of EMT markers. Expression of mesenchymal markers (alpha-Smooth Muscle Actin and CD90) and EMT associated transcription factors (Snail, ZEB and FoxC2) were significantly down-regulated in the $HT29^{CaSR\text{-}WT}$ cells, with a parallel upregulation in expression of epithelial markers (E-cadherin and EpCAM). Moreover, we could show a reduction in nuclear to cytoplasmic translocation of β-catenin in $HT29^{CaSR\text{-}WT}$ cells compared with $HT29^{EMP}$ cells and that reintroducing the CaSR led to a decreased invasive behavior of the $HT29^{EMP}$ cells. To translate these findings *in vivo* we investigated expression levels of markers of proliferation, differentiation and EMT markers in the colon of CaSR/PTH double knockout mice. Animals lacking CaSR had significantly increased expression proliferation markers of, as well as mesenchymal and EMT associated transcription factors. On the other hand, markers of differentiation and epithelial markers were down-regulated. *Ex vivo*, in a cohort of CRC patients, we found significant inverse correlations between CaSR expression and markers of proliferation and EMT, and positive correlations with differentiation markers. These data further our understanding of the molecular mechanism by which the CaSR protects colon epithelial cells from malignant transformation.

GENERAL PROPERTIES OF IP_3-INDUCED Ca^{2+} SPIKING

Kevin Thurley[1,2,3], Stephen C. Tovey[2], Gregor Moenke[1], Victoria L. Prince[4], Abha Meena[2], Andrew P. Thomas[4], Alexander Skupin[5,6], Colin W. Taylor[2,*], Martin Falcke[1,*]

[1]Mathematical Cell Physiology, Max Delbrück Center for Molecular Medicine, Robert Rössle Str. 10, 13125 Berlin, Germany. [2]Department of Pharmacology, Tennis Court Road, Cambridge, CB2 1PD, UK. [3]Charité-Universitäts medizin Berlin, Institute for Theoretical Biology, Invalidenstr. 43, 10115 Berlin, Germany. [4]Department of Pharmacology and Physiology, New Jersey Medical School, Rutgers, The State University of New Jersey, 185 S Orange Avenue, Newark, NJ 07103, USA. [5]Luxembourg Centre for Systems Biomedicine, 7 Avenue des Hauts Fourneaux, Esch sur Alzette 4362, Luxembourg. [6]National Center for Microscopy and Imaging Research, University of California San Diego, 9500 Gilman Drive, La Jolla, CA 92093, USA

Ca^{2+} is a ubiquitous intracellular messenger that regulates diverse cellular activities. Extracellular stimuli often evoke sequences of intracellular Ca^{2+} spikes, and spike frequency may encode stimulus intensity. However, the timing of spikes within a cell is random because each interspike interval has a large stochastic component. We found that the average interspike interval also varied considerably between individual cells. To evaluate how individual cells reliably encoded stimuli when Ca^{2+} spikes exhibited such unpredictability, we combined Ca^{2+} imaging of single cells with mathematical analyses of the Ca^{2+} spikes evoked by receptors that stimulate formation of inositol 1,4,5-trisphosphate (IP_3). This analysis revealed that signal-to-noise ratios were improved by slow recovery from feedback inhibition of Ca^{2+} spiking operating at the whole-cell level and that they were robust against perturbations of the signaling pathway. Despite variability in the frequency of Ca^{2+} spikes between cells, steps in stimulus intensity caused the stochastic period of the interspike interval to change by the same factor in all cells. These fold changes reliably encoded changes in stimulus intensity, and they resulted in an exponential dependence of the average interspike interval on stimulation strength. We conclude that Ca^{2+} spikes enable reliable signaling in a cell population despite randomness and cell-to-cell variability, because global feedback reduces noise, and changes in stimulus intensity are represented by fold changes in the stochastic period of the interspike interval.

Within the large cell-to-cell variability, which most likely is everyday experience in all Ca^{2+} signaling labs, we found that all investigated pathways exhibit an exponential concentration response relation and a linear relation between the average ISI and the SD of ISI. The slope of the linear relation (the inverse of the signal to noise ratio) and the constant in the exponent of the concentration response relation (agonist sensitivity) are the same for all cells of the same type and under the same stimulation. Thus, they can serve as quantitative characterization of Ca^{2+} signalling pathways.

CALCIUM OSCILLATIONS IN THE PRESENCE OF CELLULAR VARIABILITY

Rüdiger Thul

School of Mathematical Sciences, University of Nottingham, Nottingham NG7 2RD, UK

Calcium oscillations constitute one of the main pathways by which cells process and transmit information. Given the need for reliable and robust cell signalling it is remarkable that calcium oscillations - often recorded as whole cell calcium spikes - exhibit great variability. A challenge in understanding cellular calcium dynamics is therefore to predict the response of a cell to a given stimulus. I will present two main approaches to address this central question. Firstly, I will introduce a computationally non-expensive framework that allows the study of whole cell calcium signals that are driven by subcellular dynamics in three-dimensional cellular environments. The model makes non-intuitive predications and is ideally suited to probe the dependence of calcium waves on cellular variability such as variations in the position of calcium release sites or the strength of calcium release and resequestration. In the second part I will use tools from statistical inference to predict the time-dependent rate of calcium spikes from measured calcium traces. The two approaches complement each other as the first provides a mechanistic bottom-up view while the latter yields a probabilistic description of whole cell behaviour.

SPARKS AND WAVES IN CARDIAC MYOCYTES - INSIGHTS FROM AN ALLOSTERIC MODEL OF RYANODINE RECEPTOR GATING

Alexandra Zahradnikova

Department of Muscle Cell Research, Institute of Molecular Physiology and Genetics, Slovak Academy of Sciences, Bratislava, Slovak Republic

Diastolic calcium concentration in the cytosol of cardiac myocytes fluctuates due to spontaneous calcium release events through clusters of ryanodine receptors (RyRs). This process is fundamental for proper function of cardiac muscle. In failing heart and in arrhythmias caused by RyR mutations, spontaneous calcium release events proliferate into potentially arrhythmogenic calcium waves. Here we analyze how the characteristics of microscopic calcium release events, that is, calcium sparks, calcium spikes and calcium waves, relate to gating and to organization of RyRs in calcium release sites. Gating of RyR channels was described by the aHTG gating model [1]. Efflux of Ca^{2+} from release sites and diffusion in the cytosol was modeled using stochastic generalization [2] of the fire-diffuse-fire model [3] in MATLAB. We show how RyR2 gating parameters, especially the balance between the binding of activating Ca^{2+} and non-activating Mg^{2+} ions, the allosteric interaction between Ca^{2+} binding and RyR opening, and the rate of Mg^{2+} unbinding determine the amplitude and the frequency of occurrence of calcium release events [4,5] and their propagation into calcium waves. Simulations revealed regulation of the propensity of calcium release events to organize into self-propagating calcium waves by the sensitivity of Ca^{2+} spark activation to cytosolic calcium concentration and by the number of activated RyRs per release site.

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MODULATION OF ELEMENTARY CALCIUM RELEASE AND ITS ROLE IN THE PUFF-TO-WAVE TRANSITION

S. Rüdiger, M. Rückl, Ch. Nagaiah[1], F.W. Johenning[2]

Institut für Physik, Humboldt-Universität zu Berlin, Germany, [1]Johann Radon Institute for Computational and Applied Mathematics, Austrian Academy of Sciences, Linz, Austria, [2]Neuroscience Research Center, Charitè-Universitätsmedizin Berlin, Germany

Localized releases of calcium through clusters of inositol 1,4,5-trisphosphate receptor channels constitute elementary signals called calcium puffs. Diffusion of calcium leads to synchronization of release from clusters, but the exact mechanism of formation of calcium waves from the cluster coupling is unclear. To elucidate the transition from puffs to waves, we here analyze the release dynamics of a cluster of IP_3R channels. We first formulate a model of single channel gating and calcium reaction-diffusion processes. In simulations for a cluster of 16 channels we find that puffs are not stereotyped events of fixed size and duration but are rather sensitive to parameters such as the stimulation strength and the diffusive calcium transport. Upon an increase of stimulation, the local dynamics undergo a qualitative change with more frequent and synchronized IP_3 unbinding and binding. For large IP_3 concentrations there is a broad distribution of lifetimes, with a certain probability of channel states resulting in release events lasting several seconds. This release for higher stimulation is found to be sufficient to trigger channels in adjacent clusters to open. Hence, the local modulation of calcium release controls the transition from puffs to waves.

FAST SUBCELLULAR Ca^{2+} SIGNALS IN T CELL SIGNALING

I.M.A. Wolf[1], B.P. Diercks[1], E. Gattkowski[1], R. Fliegert[1], R. Werner[2], D. Schetelig[2], M .von Osten[3], S. Feske[4], A. Flügel[3], A.H. Guse[1]

[1]The Calcium Signalling Group, Department of Biochemistry and Molecular Cell Biology; [2]Department of Computational Neuroscience, University Medical Center Hamburg-Eppendorf, Martinistrasse 52, 20246 Hamburg, Germany; [3]Institute for Multiple Sclerosis Research, Dept. of Neuroimmunology, University Medical Center Göttingen, Germany; [4]Department of Pathology & Cancer Institute, New York University Medical Center, New York, USA

T-lymphocytes are activated by antigen presenting cells upon immune synapse (IS) formation. Mediating this activation, second messengers like nicotinic acid adenine dinucleotide phosphate (NAADP) initiate elementary, spatially restricted Ca^{2+} release events from Ca^{2+} stores such as the endoplasmic reticulum (ER) or lysosomes. Further, rapid Ca^{2+} depletion of the ER may evoke early Stim/ORAI-dependent Ca^{2+} entry from the extracellular space. The propagation of NAADP mediated Ca^{2+} signals is crucial in effector T cells, since an inhibition of NAADP by BZ194 resulted in decreased effector function (Cordiglieri et al. (2010) Brain, 133: 1930). Since NAADP is formed within seconds upon T cell activation (Gasser et al. (2006) J Biol Chem, 281: 16906) elementary Ca^{2+} signals may be initiated by NAADP. Both ryanodine receptors (RyR) as well as two-pore channels (TPC) have been suggested as target receptors of NAADP. To provide further insights into the concerted activity of Ca^{2+} releasing NAADP target channels, we analyzed elementary Ca^{2+} signals in T-lymphocytes mediated by NAADP (e.g., via microinjection) and upon IS formation. An improved high resolution Ca^{2+} live cell imaging setup was developed, enabling single cell Ca^{2+} measurements of up to 50 fps with a spatial resolution of 153x153 nm/pixel. Main characteristics of NAADP mediated elementary Ca^{2+} signals were an area of 0.21 μm^2, an amplitude of 72 $\pm$ 4 nM and a duration to peak of 1$\pm$ 0.1 s. Elementary Ca^{2+} signals in proximity to the IS were observed within the first second after IS formation. In a RyR knockdown Jurkat T cell clone #10 (Langhorst et al (2004) Cell. Signalling, 16:1283) local Ca^{2+} signals in response to both NAADP microinjection and IS formation were diminished. Further, elementary Ca^{2+} signals in primary T cells from $Ryr^{-/-}$ and $Orai1^{-/-}$ mice are currently analyzed and will be discussed. Taken together, a high resolution Ca^{2+} live cell imaging setup revealed a crucial role of RyR and NAADP in elementary Ca^{2+} signals in T cells during IS formation.

Berridge Lecture

A SHUTTLE MECHANISM COMBINING NANODOMAIN AND LONG-DISTANCE CALCIUM SIGNALING

Huan Ma[1], Rachel D. Groth[2], Samuel M. Cohen[1], John F. Emery[1], Bo-Xing Li[1], Richard W. Tsien[1]

[1]NYU Neuroscience Institute and Department of Neuroscience and Physiology, NYU Langone Medical Center, New York, NY 10016, USA; [2]Centers for Therapeutic Innovation, Pfizer Inc., 1700 Owens Street, San Francisco, CA 94158, USA

The functional importance of Ca^{2+} signaling is both a triumph and a challenge. How can the ubiquitous reliance on this signaling be reconciled with the need for specificity of communication? This question applies to many cell types, be they secretory, contractile or immunological but it is most intensively studied in excitable cells that use Ca^{2+} signals for excitation-response coupling. The answer often invokes events localized to a signaling nanodomain. In excitation-secretion (E-S) coupling, the Ca^{2+}-sensor synaptotagmin is positioned within nanometers of the Ca^{2+} fluxing pore. In excitation-contraction (E-C) coupling, a voltage-sensitive gating protein imparts its conformational change to a Ca^{2+} release channel (RyR) in the neighboring sarcoplasmic reticulum. Excitation-transcription (E-T) coupling, the topic here, is critically important for excitable cells, but much less understood than E-C or E-S coupling. In E-T coupling nanodomain signaling might seem superfluous since transcriptional regulation occurs in the nucleus, micrometers away from even the nearest surface membrane. Yet, it is generally accepted that one subclass of Ca^{2+} channels, CaV1 (L-type channels) have a privileged role in regulating transcription, contrary to expectations if only global Ca^{2+} signals mattered. Our studies on activation of nuclear CREB, a transcription factor critical in development, homeostasis and memory, resolve this tension by demonstrating the importance of both nanodomain and long-distance signaling. We describe how local signaling at CaV1 channels is relayed onward to the nucleus: by a novel shuttle mechanism that transports Ca^{2+}/calmodulin from the surface membrane to the nucleus. We report that the shuttle protein is γCaMKII, that its phosphorylation at Thr287 by ßCaMKII protects the Ca^{2+}/CaM signal, and that calcineurin (CaN) triggers its nuclear translocation. Both ßCaMKII and CaN act in close proximity to CaV1 channels, underlying their dominance; γCaMKII operates as a carrier, not as a kinase. Upon arrival within the nucleus, Ca^{2+}/CaM activates the nuclear CaMK cascade (CaMKK and its substrate CaMKIV, the CREB kinase). This mechanism supports a dedicated line to the nucleus and resolves some longstanding puzzles about rapid CaM/CaMK-dependent signaling. Interestingly, altered regulation of CaV1, γCaMKII, ßCaMKII and CaN crops up in multiple neuropsychiatric disorders, emphasizing the pathophysiological significance of Ca^{2+}/CaM signaling.

POLARIZATION OF THE ENDOPLASMIC RETICULUM IN YEAST BY ENDOPLASMIC RETICULUM - SEPTIN TETHERING

Christopher Loewen

Department of Cellular and Physiological Sciences, Life Sciences Institute, University of British Columbia, Vancouver, British Columbia, V6T 1Z3, Canada

Polarization of the plasma membrane (PM) into domains is an important mechanism to compartmentalize cellular activities and to establish cell polarity. Polarization requires formation of diffusion barriers that prevent mixing of proteins between domains. Recent studies have uncovered that the endoplasmic reticulum (ER) of budding yeast and neurons is polarized by diffusion barriers, which in neurons controls glutamate signaling in dendritic spines. The molecular identity of these barriers is currently unknown. Here we show that a direct interaction between the ER protein Scs2 and the septin Shs1 creates the ER diffusion barrier in yeast. Barrier formation requires Epo1, a novel ER-associated subunit of the polarisome that interacts with Scs2 and Shs1. ER-septin tethering polarizes the ER into separate mother and bud domains, one function of which is to position the spindle in the mother until M phase by confining the spindle capture protein Num1 to the mother ER.

DISSECTING ER-PHAGOSOME MEMBRANE CONTACT SITES: PLATFORMS FOR GENERATING LOCALIZED Ca^{2+} HOTSPOTS

Paula Nunes, Cyril Castelbou, Daniele Guido, Samuel Bouvet, Nicolas Demaurex

University of Geneva, Department of Cellular Physiology and Metabolism, Switzerland

Both global Ca^{2+} elevlations as well as localized Ca^{2+} hotspots have been observed during phagocytosis, a process through which immune cells engulf foreign particles that is essential for an effective immune response. Recently, we showed that the mechanism underlying Ca^{2+} hotspot generation near phagosomes involves the store-operated Ca^{2+} entry master regulator STIM1. Using a combination of fluorescence and electron microscopy in neutrophils from STIM1-deficient mice and in phagocytic fibroblasts lacking STIM1, we showed that STIM1 is recruited to phagosomes and promotes the formation of membrane contact sites (MCS) between the ER and phagosomes. Our findings indicate that these MCS are required for high-efficiency phagocytosis by generating localized intracellular Ca^{2+} hotspots through the opening of phagosomal store-operated Ca^{2+} channels. Our current research efforts are focused on further dissecting the mechanisms that drive ER-phagosome MCS formation as well as their function, through both candidate as well as unbiased proteomic approaches. Our preliminary data identify novel molecules that comprise ER-phagosome contact sites and/or regulate their function as platforms that allow precise spatial localization of signaling events within the cell.

THE ROLE OF Ca^{2+} IN THE FORMATION AND FUNCTION OF ER CONTACT SITES WITH THE ENDOLYSOSOMAL PATHWAY

Emily Eden[1], Sandip Patel[2], Clare Futter[1]

[1]University College London, Institute of Ophthalmolgy, London, UK; [2]University College London, Cell and Developmental Biology, London, UK

The ER forms membrane contact sites (MCSs), microdomains of close membrane apposition (<30nm), with a diverse range of functionally distinct organelles, including mitochondria, lipid droplets and the plasma membrane. MCSs are increasingly being recognized as important platforms for inter-organellar exchange, with key roles in lipid and calcium exchange as well as signaling regulation reported. We have previously identified MCSs between the ER and endosomes that provide sites of interaction for endocytosed EGF receptor (EGFR) and the ER-localised phosphatase, PTP1B. We found that the EGFR:PTP1B interaction at MCSs promotes the EGF-stimulated formation of intraluminal vesicles (ILVs) within the endosome and downregulation of EGFR tyrosine kinase activity. The Ca^{2+} and phospholipid binding protein, AnnexinA1, has been localized to endosomes where it is phosphorylated by EGFR kinase and also promotes EGF-stimulated ILV formation. We now show that the Ca^{2+}-dependent interaction of tyrosine phosphorylated AnnexinA1, with its binding partner S100A11, is central to the regulation of ER MCSs with EGFR-positive endosomes. We also found that antagonism of NAADP, that mobilizes Ca^{2+} from acidic organelles, had profound effects on the endocytic pathway, resulting in reduced formation of both ER-endosome contact sites and EGF-stimulated ILVs. Furthermore lysosomes were enlarged, likely resulting from aberrant Ca^{2+}-dependent fusion events, suggesting a role for ER-endolysosomal Ca^{2+} exchange across MCSs in the maintenance of endosomal morphology. We have additionally identified AnnexinA1-independent ER contact sites with endosomes and lysosomes that are regulated by ER-localised VAPs. We found a striking correlation between the extent of these MCSs with LDL-cholesterol transport to the ER for esterification. Moreover, these MCSs are also Ca^{2+}-sensitive, with BAPTA-mediated Ca^{2+} chelation or NAADP antagonism resulting in reduced MCS formation. We further show that the well-established regulator of cholesterol egress, NPC1, also regulates the formation of AnnexinA1-independent ER-endolysosomal MCSs. Loss of NPC1 function is known to result in reduced acidic organelle Ca^{2+} stores. These findings reveal an important role for Ca^{2+} in MCS formation with downstream effects on egress of LDL-cholesterol predicted, raising the intriguing possibility that endolysosmal/ER Ca^{2+} signal amplification could occur at, and indeed be coupled to, sites of lipid exchange across ER-endolysosomal MCSs.

CALCIUM SIGNAL GENERATION THROUGH STIM-INDUCED ER-PLASMA MEMBRANE JUNCTIONS

Yandong Zhou, Xizhuo Wang, Natalia A. Loktionova, Youjun Wang, and Donald L. Gill

Department of Cellular and Molecular Physiology, The Pennsylvania State University College of Medicine, Milton S. Hershey Medical Center, Hershey, PA 17033, USA

STIM proteins are finely tuned ER Ca^{2+} sensor proteins that mediate the induction of Ca^{2+} signals through the generation of junctions between ER and plasma membrane. Within these intermembrane junctions, STIM proteins tether and activate the highly Ca^{2+} selective family of Orai Ca^{2+} channels in the plasma membrane. The entering Ca^{2+} sustains Ca^{2+} oscillations, maintains Ca^{2+} homeostasis, and provides crucial long-term Ca^{2+} signals in many cell types which control gene expression and cellular growth. Small changes in ER Ca^{2+} induce STIM1 to undergo an intricate self-triggering process and translocation into the ER-PM junctions. Also widely expressed among cells, the little-studied STIM2 protein differs subtly from STIM1 in its N-terminal domain, affecting luminal Ca^{2+}-sensitivity self-activation. The STIM1 cytoplasmic STIM-Orai activating region (SOAR) has been structurally resolved. While the SOAR sequence in STIM2 is highly conserved, we reveal it has a profoundly diminished interaction with and gating of Orai1 channels. We narrowed this distinction in Orai1 activation to a small sequence in SOAR, within which substitution of a single phenylalanine in STIM1 with leucine in STIM2 confers a severe decrease in Orai1 channel-gating efficacy. This residue is strategically positioned at the structural apex of the SOAR domain. Modification of this single residue within the intact STIM1 protein reveals its pivotal role in both interaction with and gating of the Orai1 channel within the membrane junctions. The results not only pinpoint a crucial locus of STIM-Orai coupling but also reveal a physiologically profound distinction between STIM1 and STIM2. Other recent results are pinpointing new areas within the Orai1 channel protein that mediate both binding with and channel gating by STIM proteins. The four transmembrane domains of each of the hexameric Orai channel subunits, include a pore-lining helix which is tightly associated with the other three transmembrane helices. We are defining some novel regions in the tight membrane helical complex that exert profound control over the gating properties of the channel and which are primary sites of control by STIM proteins. The results are hence revealing the molecular architecture of the transmembrane contact sites the result in Ca^{2+} signal generation.

GLUCOSE AND NAADP TRIGGER ELEMENTARY INTRACELLULAR Ca^{2+} SIGNALS IN PANCREATIC ß-CELLS

Paula Maria Heister, Trevor Powell & Antony Galione

Department of Pharmacology, Mansfield Road, Oxford OX1 3QT, UK

Pancreatic beta cells release insulin upon rise in blood glucose. The exact mechanism of this stimulus-secretion coupling, and its failure in Diabetes Mellitus Type 2, remain to be elucidated. The consensus model, as well as currently prescribed anti-diabetic drugs, are based around the observation that glucose-triggered ATP production in the beta cell leads to closure of cell membrane KATP channels, depolarisation, calcium influx, and exocytosis (Ashcroft et al., 1984; Cook and Hales, 1984). However it has been demonstrated by the activation and inactivation of this pathway using genetic and pharmacological means that other mechanisms are likely to be involved (Henquin, 1998; Seghers et al., 2000). Here we show using total internal reflection fluorescence (TIRF) microscopy (Axelrod, 1981) that glucose as well as novel calcium mobilising messenger Nicotinic Acid Adenine Dinucleotide Phosphate (NAADP), known to be present in beta cells (Johnson and Misler, 2002; Masgrau et al., 2003), lead to elementary intracellular calcium signals. Optical quantal analysis of these reveals a unitary event amplitude equivalent to that of known elementary calcium signals IP3 receptor mediated blips (Parker and Ivorra, 1990; Parker et al., 1996) and ryanodine receptor mediated quarks (Cheng et al., 1993). This suggests that an alternative stimulus-secretion mechanism based on intracellular calcium signalling via NAADP may be at work in beta cells when they react to glucose.

MOLECULAR MECHANISMS OF MITOCHONDRIAL Ca^{2+} UPTAKE

György Hajnóczky, Melanie Paillard, György Csordás, Erin Seifert

MitoCare Center, Thomas Jefferson University, USA

Mitochondrial Ca^{2+} uptake is central to cell metabolism, signaling and survival. Recent studies identified MCU as the pore of the mitochondrial Ca^{2+} uniporter and MICU1 as its critical regulator. MICU1 and its paralogs, MICU2 and MICU3 are EF-hand proteins and are the primary candidates to confer Ca^{2+} sensitivity to the Ca^{2+} uniporter. We will present studies of the molecular mechanisms of the MICU-dependent closure of the uniporter at low [Ca^{2+}] levels and its cooperative activation when [Ca^{2+}] increases. Furthermore, we will present clues to the MICU-dependence of the tissue specific mitochondrial Ca^{2+} uptake profiles.

NEW INSIGHTS INTO THE REGULATION OF THE MITOCHONDRIAL CALCIUM UNIPORTER

Diego De Stefani

Department of Biomedical Sciences, University of Padova, Padova, Italy

The Mitochondrial Calcium Uniporter (MCU) is the protein of the inner mitochondrial membrane responsible for Ca^{2+} uptake into the organelle matrix, which represents a critical event for the control of cellular signaling, aerobic metabolism and apoptosis. Despite its recent identification, the list of the MCU modulators is rapidly growing, and now includes MCUb, the MICU family (that includes MICU1, MICU2 and MICU3), MCUR1, EMRE and SLC25A23, thus revealing a unique complexity that highlights the pleiotropic role of mitochondrial calcium signaling. Recent evidences suggest that these different components are intimately related one to the other and each one participates in conferring specific features to MCU-mediated Ca^{2+} channeling properties. As an example, I will show that the MICU family can form homo and/or heterodimers that are responsible for the sigmoidal relationship between MCU opening and extramitochondrial calcium. Indeed, in the proposed model, at low [Ca^{2+}], the dominant effect of MICU2 largely shuts down MCU activity; at higher [Ca^{2+}], the stimulatory effect of MICU1 allows the prompt response of mitochondria to Ca^{2+} signals generated in the cytoplasm. Moreover, new data on the role of MICU3, EMRE, MCUR1 and new regulators will be presented.

PROBING THE ROLE AND REGULATION MITOCHONDRIAL Ca^{2+} UPTAKE IN NEURONS

Giles Hardingham

Centre for Integrative Physiology, University of Edinburgh, UK

Mitochondrial Ca^{2+} plays important roles in cellular physiology and pathophysiology, and the mitochondrial calcium uniporter Mcu is an important contributor to mitochondrial Ca^{2+} uptake. Knockdown of Mcu in forebrain neurons reduces mitochondrial Ca^{2+} uptake following excitotoxic activation of NMDA receptors, induces some perturbations to basal neuronal metabolism, and offers a degree of protection against excitotoxicity. However, Ca^{2+} uptake is not abolished, suggestive of other routes of entry as well, which may play a greater or lesser role dependent on the concentration or source of cytoplasmic Ca^{2+}. Also of note, the coupling of cytoplasmic Ca^{2+} to mitochondrial Ca^{2+} can be subject to dynamic regulation, and appears to be reduced by prolonged periods of elevated cytoplasmic Ca^{2+}, such as those triggered by synaptic activity. Activity-dependent Ca^{2+} signals result in the transcriptional repression of Mcu, and also modify the expression (both up and down) of a range of genes involved in mitochondrial Ca^{2+} homeostasis, including several not associated with the Mcu complex. Expression of certain mitochondrial Ca^{2+} homeostasis genes, including Mcu, also appear to vary according to neuronal type, and work from others suggest that they can also vary between synaptic and non-synaptic mitochondria. Overall, a picture is emerging whereby the control of mitochondrial Ca^{2+} levels, and the precise machinery involved, may depend on wide range of factors.

TISSUE SPECIFIC DIFFERENCES OF MITOCHONDRIAL GAS PEDAL: CYTOSOLIC Ca^{2+} REGULATES THE MITOCHONDRIAL ENERGIZATION WITH PYRUVATE

F.N. Gellerich[1,2], Z. Gizatullina[1,2], T. Gainutdinow[2], J. Seppet[1], K. Mut[1], R. König[2], M. Riek-Burchard[2], G. Debska-Vielhaber[1], S. Vielhaber[1]

[1]Department of Neurology, Otto von Guericke University of Magdeburg and [2]Department for Behavioral Neurology, Leibniz Institute for Neurobiology, Magdeburg, Germany

We have shown that Ca^{2+}_{cyt} activates the glutamate/malate- as well as the glycero-3-phosphate-dependent OXPHOS of isolated brain mitochondria (BM) by more than 300%[1-4]. In contrast the OXPHOS capacity with pyruvate/malate increases under the same conditions by 15 % only[1-4]. The strong activation of state 3Glu/Mal and state 3G3P by Ca^{2+}_{cyt} is realized via aralar, the glutamate-aspartate carrier or the mtG3PDH. Both enzymes have regulatory Ca^{2+} binding sites at the mitochondrial surface making their activity Ca^{2+}_{cyt} sensitive. Since both enzymes are central components of the malate aspartate shuttle (MAS) and glycero-3-phosphate shuttle (G3PS) Ca^{2+}_{cyt} also controls the activities of these shuttles which are metabolically connected with the oxidizing reactions of pyruvate formation via their common substrate couple NADH/NAD^+. We called this mechanism “mitochondrial gas pedal”[1]. We now demonstrate the existence of the gas pedal in rat synaptosomes: The Ca^{2+}-chelating agent BAPTA-AM decreases (i) the Ca^{2+}_{cyt} from 76 ± 11 nM to 15 ± 11 nM, (ii) the glucose/lactate dependent respiration by 47 %, and (iii) the cytosolic pyruvate concentration by 50 %. The inhibition of respiration can be reversed by pyruvate additions although the Ca^{2+}_{cyt} remains low, indicating that Ca^{2+}_{cyt} controls the mitochondrial substrate supply via the rate of pyruvate formation causing variations of cytosolic pyruvate concentrations. Similar results we obtained very recently with human fibroblasts. We found that the gas pedal uses either the MAS (heart, red muscle), the G3PS (astrocytes) or both (neurons, white muscle). These differences are detectable by the substrate specific pattern

of mitochondrial substrate oxidation rates. Based on these findings we are able to assess respirometrically typical neuronal and astrocytic properties in BM isolated from brain regions with heterogenous cellular composition in neurodegenerative models. Our model predicts that at sufficiently low Ca^{2+}_{cyt} the mitochondria (e.g. in neurons and red muscle) are de-energized so preventing dangerous large ROS formation at resting conditions[1].

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STRUCTURAL AND MECHANISTIC INSIGHTS INTO MICU1 REGULATION OF MITOCHONDRIAL CALCIUM UPTAKE

LeLe Wang, Xue Yang, Siwei Li, Zheng Wang, Yu Liu, Jianrong Feng, Yushan Zhu, Yuequan Shen*

**College of Life Sciences, Nankai University, Tianjin, China*

Mitochondrial calcium uptake is a critical event in various cellular activities. Two recently identified proteins, the mitochondrial Ca^{2+} uniporter (MCU), which is the pore-forming subunit of a Ca^{2+} channel, and mitochondrial calcium uptake 1 (MICU1), which is the regulator of MCU, are essential in this event. However, the molecular mechanism by which MICU1 regulates MCU remains elusive. In this study, we report the crystal structures of Ca^{2+}-free and Ca^{2+}-bound human MICU1. Our studies reveal that Ca^{2+}-free MICU1 forms a hexamer that binds and inhibits MCU. Upon Ca^{2+} binding, MICU1 undergoes large conformational changes, resulting in the formation of multiple oligomers to activate MCU. Furthermore, we demonstrate that the affinity of MICU1 for Ca^{2+} is approximately 15–20 lM. Collectively, our results provide valuable details to decipher the molecular mechanism of MICU1 regulation of mitochondrial calcium uptake.

Poster Abstracts

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001

REGULATION OF EPITHELIAL CELL PHENOTYPE BY ANNEXIN A8 AND WNT SIGNALLING

Katharina Lueck, Amanda Carr, John Greenwood, Stephen E Moss

Department of Cell Biology, UCL Institute of Ophthalmology, 11-43 Bath Street, London EC1V 9EL, United Kingdom

The retinoic acid derivative Fenretinide (FR) is capable of transdifferentiating retinal pigment epithelial (RPE) cells towards a neuron-like phenotype in culture. Microarray analysis of FR-treated ARPE-19 cells revealed down-regulation of Annexin (Anx)A8 and specific Wnt signalling proteins in transdifferentiated cells. AnxA8, a calcium-dependent phospholipid-binding protein, is expressed in the RPE cell cytosol, where it may be involved in membrane and cytoskeletal organisation and cell proliferation. The aim here was to analyse the role of AnxA8 and its interaction with Wnt signalling in RPE cell transdifferentiation. Human RPE cells were seeded at a concentration of 2,200/ml and treated with 3% charcoal dextran-treated foetal bovine serum (FBS) for 24h. 3µM FR or vehicle (0.1% dimethylsulfoxide) was added every day for 7 days. As a second approach, AnxA8 was suppressed in RPE cells using short interfering RNA (siRNA). FR and AnxA8 siRNA treatment both induced a decrease in AnxA8 expression and inhibited cell proliferation. It further led to transdifferentiation of ARPE-19 cells into neuron-like cells and a concomitant up-regulation of the neuronal markers Calbindin and Calretinin analysed by qPCR and immunofluorescence. Additionally, expression of Wnt signalling proteins such as ß-Catenin, Frizzled-1, Frizzled-4, Wnt2b and Wnt3a was decreased. The reduction in AnxA8 and cell morphology changes induced by FR, were not reversible by inhibiting Wnt signalling using Dickkopf-1 and DAPT. Wnt signalling activators such as recombinant Wnt3a or SB216763 (glycogen synthase kinase-3ß inhibitor) were able to partially reverse the FR effect. These data imply an important role for AnxA8 in maintaining RPE phenotype. Down-regulation of AnxA8 appears to be sufficient for neuronal transdifferentiation of RPE cells and the expression of neuronal markers. Further, the interdependence of AnxA8 and Wnt proteins suggests that AnxA8 might be an important regulator of Wnt signalling.

002

EXPRESSION AND LOCALIZATION OF THE TRPV1 CATION CHANNEL IN MOUSE CARDIOMYOCYTES AND SKELETAL MUSCLE CELLS

Païta L., Al-Mawla R., Bidault-Ducreux S., Lotteau S., Chouabe C., Dupré-Aucouturier S., Bonvallet R., Durand A., Ovize M., Van Coppenolle F.

INSERM U1060-CarMeN-"Team 5- Cardioprotection"; 8 Avenue Rockefeller 69373 Lyon cedex 08-France

The sarcoplasmic reticulum (SR) calcium homeostasis is due to a dynamic balance between the capture of the cytosolic calcium by the calcium pumps such as SERCA (Sarco Endoplasmic Reticulum Calcium ATPase) and calcium leak. The calcium release occurs under different ways, mostly actively across calcium channels such as IP3 (Inositol Tri-phosphate) and ryanodine receptors, but also passively via calcium leak channels. Few data are available concerning the functional characterization of SR calcium leak channels in cardiomyocytes. These channels are involved in the regulation of the reticular calcium concentration as well as in the exchange of calcium between SR and other intracellular organelles. Therefore, their characterization is important for a better understanding of the physiology of cardiomyocytes. Recently (Lotteau et al., 2013), we have demonstrated that TRPV1 (Transient Receptor Potential Vanilloid 1), a cationic channel, is a functional calcium leak channel of the SR of mouse skeletal muscle cells. TRPV1 is activated by acidosis, high temperature (>42°C), and by molecules such as capsaicin. Currently, we are pursuing our work on C57Bl6 mouse cardiomyocytes. Our preliminary results confirm that: (i) TRPV1 is expressed in cardiac muscle cells (immunoblots); (ii) TRPV1 is present on the longitudinal part of the SR (immunofluorescence); (iii) TRPV1 acts as a functional calcium leak channel (calcium imaging). Since TRPV1 is activated by an acid intracellular pH, it is interesting to investigate further the role that this channel might have in cardiomyocytes during hypoxia-reoxygenation.

Lotteau S., Ducreux S., Romestaing C., Legrand C., Van Coppenolle F. Characterization of functional TRPV1 channels in the sarcoplasmic reticulum of mouse skeletal muscle. PLoS One. 2013; 8(3):e58673

003

FLUOXETINE INDUCES EARLY NECROSIS BY RESPIRATORY CHAIN INHIBITION AND MITOCHONDRIAL Ca^{2+} OVERLOAD IN LYMPHOCYTE CELL LINES

Emilie Charles[1], Mehdi Hammadi[1], Anne-Marie Vacher[1], Thomas Ducret[2], Anne Devin[3], Pierre Vacher[1].

[1]INSERM U916, 229 cours de l'Argonne, 33076 Bordeaux cedex, France; [2]INSERM U1045, 146 rue Léo Saignat, 33076 Bordeaux cedex, France; [3]CNRS UMR5095, 1 Rue Camille Saint-Saens, 33077 Bordeaux cedex, France

Selective Serotonin Reuptake Inhibitor (SSRI) antidepressants, such as fluoxetine (Prozac), have been shown to induce cell death in cancer cells, suggesting a potential use for cancer treatment. SSRIs are also able to increase cytosolic calcium concentration ($[Ca^{2+}]$cyt). However, there is an ongoing controversy on the mechanisms involved in this increase, and its physiological consequences on cells are not understood. Our data show that fluoxetine inhibits the respiratory chain, which is necessary for mitochondrial ATP production. Fluoxetine also induces an increase in $[Ca^{2+}]$cyt by emptying the ER through the translocon, a Ca^{2+} leakage structure. As mitochondrial ATP is particularly needed for the Ca^{2+} reuptake in the ER, we postulated that fluoxetine induces a decrease in mitochondrial ATP production, resulting in the inhibition of the Sarcoplasmic/ER Ca^{2+}-ATPases (SERCAs), the emptying of the ER and an increase in the $[Ca^{2+}]$cyt. Furthermore, once released from the ER, Ca^{2+} quickly accumulates in the mitochondria. It is widely known that a mitochondrial Ca^{2+} overload can lead to the Permeability Transition Pore opening and to cell death by apoptosis/necrosis. Thus, we observed that fluoxetine induces an early necrosis in normal T lymphocytes and

Jurkat cell line and a late apoptosis in Jurkat. These results shed light on a part of SSRIs-induced cell death and will thus help us understand their effects on cancer cells in order to propose an improvement of the chemotherapy treatments.

004

A SECRETAGOGINERGIC LOCUS OF THE MAMMALIAN HYPOTHALAMUS CONTROLS STRESS HORMONE RELEASE

Roman Romanov[1,2], Alan Alpar[1], Ming-Dong Zhang[1,2], Amit Zeisel[1], André Calas[3], Marc Landry[3], Robert Schnell[1], Arpad Dobolyi[4], Mark Olah[5], Lauren Spence[6], Miklos Palkovits[5], Matthias Uhlen[7], Harald H. Sitte[8], Ludwig Wagner[9], Sten Linnarsson[1], Tomas Hökfelt[2] & Tibor Harkany[1,10]

[1]Division of Molecular Neurobiology, Department of Medical Biochemistry & Biophysics, Karolinska Institutet, SE-17177 Stockholm, Sweden; [2]Department of Neuroscience, Karolinska Institutet, SE-17177 Stockholm, Sweden; [3]Laboratory for Central Mechanisms of Pain Sensitization, Interdisciplinary Institute for Neuroscience, CNRS UMR 5297, Université Bordeaux 2, Bordeaux, France; [4]Department of Anatomy, Semmelweis University, H-1094 Budapest, Hungary; [5]Department of Human Morphology and Developmental Biology, Semmelweis University, H-1094 Budapest, Hungary; [6]Institute of Medical Sciences, University of Aberdeen, Aberdeen AB25 2ZD, United Kingdom; [7]Science for Life Laboratory, Albanova University Center, Royal Institute of Technology, SE-10691 Stockholm, Sweden; [8]Center for Physiology and Pharmacology, Institute of Pharmacology, Medical University of Vienna, A-1090 Vienna, Austria; [9]University Clinic for Internal Medicine III, General Hospital Vienna, A-1090 Vienna, Austria and [10]Department of Molecular Neurosciences, Center for Brain Research, Medical University of Vienna, A-1090 Vienna, Austria.

A hierarchical hormonal cascade along the hypothalamo-pituitary-adrenal axis orchestrates bodily responses to stress. Although corticotropin-releasing hormone (CRH), produced by parvocellular neurons of the hypothalamic paraventricular nucleus (PVN) and released into the portal circulation at the median eminence, is known to prime downstream hormone release, the molecular mechanism regulating phasic CRH release remains poorly understood. We found a cohort of parvocellular neurons interspersed with magnocellular PVN neurons expressing recently discovered Ca^{2+}-binding protein secretagogin. Single-cell transcriptome analysis combined with protein interactome profiling identifies secretagoginergic neurons as a distinct CRH-releasing neuron population reliant on secretagogin's Ca^{2+}-sensor properties and protein-interactions with the exocytosis release machinery to liberate this key hypothalamic releasing hormone into the hypophyseal portal circulation. Pharmacological tool combined with RNA-interference demonstrate that secretagogin's loss-of-function occludes adrenocorticotropic hormone release from the pituitary and lowers peripheral corticosterone release in response acute stress. Cumulatively, these data define a novel secretagoginergic neuronal locus and molecular axis underpinning stress responsiveness.

005

NECAB2: CHARACTERIZATION OF A CALCIUM-BINDING PROTEIN

Teresa Schacht[1], Katrin Frauenknecht[2], Armagan Özgür[3], Hatice Yigit[4], Peter Zentis[5], Lothar Gremer[6], Lukasz Majewski, Michael K.E. Schäfer[7], Claus Seidel[5], Jacek Kuznicki, Dieter Willbold[6] and Axel Methner[1]

[1]Department of Neurology, [2]Department of Neuropathology, and University Medical Center and Focus Program Translational Neuroscience (FTN) of the Johannes Gutenberg-University Mainz, Mainz, Germany. [3]University Hospital Düsseldorf, Dept. of Neurology, Düsseldorf, Germany. [4]Department of Molecular Cell Biology, MainzFocus Program Translational Neuroscience (FTN) of the Johannes Gutenberg-University Mainz, Mainz, Germany. [5]Department of Molecular Physical Chemistry, Düsseldorf, Germany. [6]Department of Biophysics, Düsseldorf, Germany. [7]Department of Anesthesiology, and University Medical Center and Focus Program Translational Neuroscience (FTN) of the Johannes Gutenberg-University Mainz, Mainz, Germany

NECAB2 is a Neuronal Ca^{2+}-binding protein characterized by the combination of EF-hand Ca^{2+}-binding domains, two coiled-coil domains and a monooxygenase domain. It is expressed exclusively in the brain with strongest expression in the striatum where it co-localizes and interacts with metabotropic glutamate and adenosine receptors increasing their basal constitutive signaling. High concentrations of Ca^{2+} drastically reduce the ability of these proteins to interact with NECAB2. Prokaryotic homologs of NECAB2 possess monooxygenase activity and are involved in the detoxification of reactive oxygen species, suggesting a similar function in eukaryotes distinct from the interaction with membrane receptors. A structural analysis of prokaryotic NECAB2 homologs also suggests that the active enzyme is a homodimer. We also observed a high expression of NECAB2in the striatum. The strong expression in the neuropil is probably caused by a dendritic rather than axonal localization of NECAB2. No colocalization with the presynaptic protein synaptobrevin, but high enrichment in synaptic microsomes suggests that Necab2 is a postsynaptic protein. Recombinant NECAB2 dimerizes and oligomerizes in the presence of a chemical crosslinker similar to its prokaryotic orthologues. We also showed that mammalian NECAB2 dimerizes in a Ca^{2+}-dependent manner. We then generated mutants of NECAB2 that lack distinguishable domains or have a non-functional EF hand (by mutating Glu residues to Ala) to further address the different functions of NECAB2. We figured out that the CC1 domain is responsible for the interaction with the metabotropic glutamate receptor 5 and homodimerization. Our data suggests that the increase in the cytosolic Ca^{2+} concentration causes homodimerization of NECAB2 in the cytosol, resulting in subsequent receptor desensitization in a negative feedback loop. The dimerization probably activates its monooxygenase activity, which probably serves to process reactive oxygen species and protons generated in active, stimulated neurons.

006

A CARDIAC ARRHYTHMIA CAUSING CAM MUTANT IS UNABLE TO FULLY ACTIVATE CAMKII DUE TO ITS INABILITY TO INDUCE PHOSPHORYLATION OF NOVEL SITES

Martin W. Berchtold[1], Katarzyna Kulej[2], Triantafyllos Zacharias[1], Raffaela F. Torggler[1], Kevin Wang[3], Martin R. Larsen[2], Thomas Jespersen[4], Jau-Nian Chen[3] and Jonas M. la Cour[1]

[1]Department of Biology, University of Copenhagen, August Krogh Building, 2100 Copenhagen, Denmark; [2]Protein Research Group, Department of Biochemistry and Molecular Biology, University of Southern Denmark, 5230 Odense, Denmark; [3]Department of Molecular, Cell and Developmental Biology, University of California, Los Angeles, CA 90095, USA; [4]Danish National Research Foundation Centre for Cardiac Arrhythmia, Department of Biomedical Sciences, University of Copenhagen, 2200 Copenhagen, Denmark

Calmodulin (CaM) is a highly conserved Ca^{2+}-binding protein playing a major role in Ca^{2+} signaling of eukaryotes. CaM modulates multiple target proteins of which several have been associated with cardiac pathophysiology. Recently, a number of CaM mutations have been identified in patients suffering from arrhythmogenic syndromes. We have investigated the effect of six of these mutations on growth and viability of a genetically modified vertebrate cell line specifically designed for studying CaM and found that a LQTS associated CaM mutation (D129G) was incapable of supporting cell viability. Furthermore, this mutation caused severe cardiac arrhythmia in zebrafish. We identified CaMKII as a route possibly being disabled for this specific LQTS mutation since it could only moderately activate CaMKII in vitro. Quantitative mass spec analysis revealed intact CaMKII T286 autophosphorylation. However, the CaM mutant was unable to fully activate CaMKII due to a limited ability to induce phosphorylation of six newly identified sites involved in CaMKII activation. These results introduce a novel regulatory level of CaMKII signaling with possible importance for cardiac physiology.

007

AN EXPLORATION OF Ca^{2+} SIGNALING DURING HEART DEVELOPMENT IN ZEBRAFISH

Sarah E. Webb[1], Lingyu Zhou[1], T. Katherine Tamai[2], David Whitmore[2] and Andrew L. Miller[1,3]

[1]Division of Life Science, HKUST, Clear Water Bay, Kowloon, Hong Kong; [2]Department of Cell and Developmental Biology, University College London, London, UK; [3]Marine Biological Laboratory, Woods Hole, MA 02543, USA.

Ca^{2+} signaling has been extensively studied in the mature, functioning heart. However, in addition to excitation-contraction coupling, it is also becoming apparent that Ca^{2+} signaling also plays a major role in regulating heart development via excitation-transcription coupling. With regards to the latter, Ca^{2+} has been reported to act via CaM kinase and calcineurin, and subsequently HDACII, NFATc, and CREB to activate transcription of the Mef2, Gata4 and Nkx2.5 transcription factors. These, in turn activate multiple downstream targets. It is not clear, however, how Ca^{2+} initiates and/or coordinates these various different cardiac myogenic signaling networks in complex multicellular developing embryos. Our goal is to study Ca^{2+} signaling in the developing zebrafish heart in situ. We have attempted to visualize Ca^{2+} signals in the developing heart using fluorescent Ca^{2+} dyes, such as calcium green-1 dextran and Fura-2, as well as the bioluminescent Ca^{2+}-sensitive reporter, aequorin. We have also generated a line of transgenic zebrafish that express Nano-lantern (Ca^{2+}), a new bioluminescent Ca^{2+} indicator, which is driven by the nkx2.5 promoter, and work is currently underway to identify stable founder fish. This fish line has the potential to allow measurement of Ca^{2+} signaling in the developing heart in situ over the extended time periods required to study organogenesis. To modulate Ca^{2+} levels more directly in the developing heart, work to generate another line of transgenic fish expressing the Ca^{2+}-binding protein, parvalbumin, is also in progress. Preliminary parvalbumin over-expression experiments appear to show defects in the flow of blood through the heart outflow tract, suggesting an attenuation of contractile activity and/or a morphological defect. Our new lines of transgenic fish will provide powerful tools for studying Ca^{2+} signals generated during normal and abnormal heart development, as well as during heart regeneration. Supported by RGC awards: HKUST662211, 662113 and 16101714; ANR/RGC award: A-HKUST601/13; and TRS award: T13-706/11-1.

008

KNOCK-DOWN AND RESCUE OF TPC2-MEDIATED Ca^{2+} SIGNALING REQUIRED FOR MYOFIBRILO-GENESIS IN ZEBRAFISH SLOW MUSCLE CELLS

Jeffrey J. Kelu[1], Hayley L. H. Chan[2], Sarah E. Webb[1], Margarida Ruas[2], John Parrington[2], Antony Galione[2] and Andrew L. Miller[1,3]

[1]Division of Life Science and State Key Laboratory of Molecular Neuroscience, HKUST, Clear Water Bay, Hong Kong, PRC; [2]Department of Pharmacology, University of Oxford, Mansfield Road, Oxford OX1 3QT, UK; [3]Marine Biological Laboratory, Woods Hole, MA 02543, USA.

Ca^{2+} signaling is reported to play a key role in the regulatory networks that control differentiation in many cell types, including vertebrate skeletal muscle. Our recent work has characterized sarcoplasmic reticulum (SR)-based Ca^{2+} signals generated during the differentiation of slow muscle cells (SMCs) in intact zebrafish. We present new data, which suggest that in zebrafish embryos, a novel member of the voltage-gated channel superfamily, the two pore channel 2 (TPC2), which is located in the membranes of acidic organelles and proposed to be activated by NAADP, also plays a crucial role in the differentiation of SMCs. Using a line of transgenic zebrafish that express the Ca^{2+}-sensitive bioluminescent protein aequorin specifically in skeletal muscle cells, morpholino (MO)-based knockdown of TPC2 resulted in a dramatic attenuation of the Ca^{2+} signals and a significant disruption of myofibrilogenesis. Furthermore, treatment of embryos with an inhibitor of NAADP/TPCs (*trans*-Ned-19) or of the vacuolar-type H^+ ATPase that depletes the Ca^{2+} store in acidic organelles (bafilomycin A1), also resulted in a disruption of both Ca^{2+} signaling and myofibrilogenesis. Co-loading embryos with the MO and a TPC2 mRNA resulted in a partial rescue of the Ca^{2+} signaling signature.

Finally, we show that TPC2 and acidic vesicles are both expressed in a striated pattern in the cytoplasmic domain of SMCs. Together these data provide evidence to suggest that TPC2-mediated Ca^{2+} release from acidic organelles triggers the SR-generated Ca^{2+} transients shown to be essential for myofibrilogenesis.

009

CHANGES IN S100 PROTEINS EXPRESSION DURING KERATINOCYTE DIFFERENTIATION: EFFECT OF S100A6 ON EPIDERMAL GROWTH

Wieslawa Lesniak, Agnieszka Graczyk, Barbara Sobiak

Laboratory of Calcium Binding Proteins, Nencki Institute of Experimental Biology, 3 Pasteur St., 02-093 Warsaw, Poland

Most genes encoding S100 calcium binding proteins are located within a gene cluster called Epidermal Differentiation Complex (EDC) on human chromosome 1 (q21), together with genes encoding proteins that form the impermeable skin barrier (1). Such gene location suggests that S100 proteins can be implicated in epidermal growth but their expression and function in the epidermis have not been studied systematically. We examined the mRNA level of 9 (out of 15) S100 genes located in the EDC and, based on the results, established the relative abundance of S100 proteins in primary human keratinocytes (HEKa cells). Furthermore, we monitored changes in expression of S100 genes during keratinocyte differentiation (induced by increased Ca^{2+} concentration in the culture medium) and examined if they correlated with the extent of cytosine methylation within the gene promoters. We found that individual S100 gene exhibited different expression patterns in differentiating keratinocytes - some S100 genes revealed stable expression while some were down- or upregulated. Interestingly the expression pattern seemed to correlate with the position of a given gene within the EDC gene cluster. In subsequent experiments we focused on the role of the S100A6 protein (calcyclin) in the epidermis. To achieve this goal we checked the effect of S100A6 knock-down or overexpression in kertinocytes on the growth of epidermis in a 3-dimensional organotypic culture. Our study has shown that keratinocytes overexpressing S100A6 proliferate more intensively than control ones and form thicker epidermis containing more Ki67 –positive cells. In a subsequent step we performed immunohistofluorescence analysis of protein markers of various stages of epidermal differentiation, namely keratin 14 (K14), keratin 10 (K10) and loricrin. On the basis of this analysis we can conclude that high S100A6 level in keratinocytes induces epidermal growth but substantially delays the process of epidermal differentiation.

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010

SPONTANEOUS Ca^{2+} SIGNALLING AND ULTRASTRUCTURAL CHANGES IN PULMONARY VEIN SLEEVE CELLS DURING AGEING – RISK FACTORS FOR AF?

Rietdorf, K., Masoud, S. and Bootman, M.D.

The Open University, Department of Life, Health and Chemical Sciences, Walton Hall, Milton Keynes, MK7 6AA

Atrial fibrillation (AF) is the most common form of a sustained cardiac arrhythmia, with age and obesity being the most significant risk factors (1). Substantial evidence indicates that cardiomyocytes located in the pulmonary veins (pulmonary vein sleeve cells; PVCs) cause AF by generating ectopic electrical activity (2). Electrical ablation, to isolate PVCs from their left atrial junctions, is a major treatment for AF (3). In small rodents, the sleeve of PVC cardiomyocytes extends along the pulmonary veins deep inside the lungs (4), and is present in lung slices (5).

Since ageing is a major risk factor for AF development, we investigated changes in the ultrastructure of PVCs, and their spontaneous and electrically-paced Ca^{2+} signals in aged mice, comparing slices from mice at 3 months and 24 months of age. Slices were loaded with Ca^{2+} sensitive fluorescent indicators, enabling us to measure both spontaneous Ca^{2+} signals and Ca^{2+} signals resulting from electrical pacing. We found a higher incidence of spontaneous Ca^{2+} signals, and an increased resistance to electrical pacing, in slices from 24 month-old mice. Importantly, increased spontaneous Ca^{2+} signalling and resistance to electrical pacing are known triggers of arrhythmic events in the heart.

Using scanning electron microscopy, we found a striking difference in the density of mitochondria in PVCs from young and old mice: PVCs from 24 month-old mice contained a greater density of mitochondria of heterogeneous shapes, whereas PVCs from 3 month-old mice contain fewer mitochondria with more consistent, circular shapes. Since mitochondria are the most significant buffers of cytosolic Ca^{2+} changes in cardiomyocytes, it is plausible that an increased mitochondrial density will significantly alter the spatial and temporal properties of PVC Ca^{2+} signalling. As well as being a greater Ca^{2+} buffer, the larger number of mitochondria could potentially act as an additional Ca^{2+} source in the cells, thereby contributing to the pro-arrhythmic activity found in PVCs from older mice.

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011

PHOSPHOLAMBAN OVEREXPRESSION CAUSES SKELETAL MUSCLE MYOPATHY AND IS MORE PROMINENT IN FEMALE MICE

Val A. Fajardo, Eric Bombardier, Catherine Bellissimo, Ian C. Smith, Khanh Tran, A. Russell Tupling

Department of Kinesiology, University of Waterloo, Waterloo, Ontario, Canada, N2L 3G1.

The sarco(endo)plasmic reticulum Ca^{2+}-ATPase (SERCA) pump catalyzes the active transport of Ca^{2+} into the sarcoplasmic reticulum and is largely responsible for muscle relaxation as well as regulation of intracellular Ca^{2+} levels. Impaired SERCA activity has been observed in a variety of skeletal muscle myopathies and therapeutic strategies aimed towards improving SERCA function have all resulted in attenuated disease pathology. Phospholamban is a small 52 amino-acid peptide that physically interacts with and inhibits the SERCA pumps. Studies using transgenic rabbits overexpressing phospholamban (PlnOE) in slow-twitch type I fibres report signs of skeletal muscle myopathy including atrophy, fibrosis, mononuclear cell infiltration, and central nucleation. In this study, we assessed the skeletal muscle architecture and function in mice overexpressing phospholamban in their slow-twitch type I fibres. Similar to that in PlnOE rabbits, soleus and gluteus minimus muscles from PlnOE mice displayed atrophy, mononuclear cell infiltration, fibrosis, and central nucleation. Analysis of isometric contractile properties in the soleus muscles from PlnOE mice reveal lower force generating capacity at both sub-maximal and maximal frequencies as well as slower twitch kinetics compared with their wild-type littermates ($p < 0.05$). Furthermore, statistical analysis by sex indicated a significant interaction between sex and genotype ($p<0.05$) whereby female mice were more affected by the phospholamban overexpression transgene than males. Specifically, we observed more pronounced soleus muscle atrophy, lower twitch- and tetanic force-generating capacity and slower twitch kinetics in females compared with males. In conclusion, our results provide novel insight into skeletal muscle myopathy caused by impaired SERCA function, and future studies will seek out mechanisms explaining the newly discovered sex differences.

012

STRUCTURAL PLASTICITY OF CALMODULIN ON THE SURFACE OF CaF2 NANOPARTICLES PRESERVES ITS BIOLOGICAL FUNCTION

Alessandra Astegno[1]*, Elena Maresi[1], Valerio Marino[2], Paola Dominici[1], Marco Pedroni[1], Fabio Piccinelli[1] and Daniele Dell'Orco[2]

[1]Department of Biotechnology, University of Verona, Verona, Italy; [2]Department of Life Sciences and Reproduction, Section of Biological Chemistry, University of Verona, Verona, Italy.

Nanoparticles are increasingly used in biomedical applications and are especially attractive as biocompatible and biodegradable protein delivery systems. Herein, the interaction between biocompatible 20 nm CaF2 nanoparticles and the ubiquitous calcium sensor calmodulin has been investigated in order to assess the potential these particles to serve as suitable surface protein carriers. Calmodulin is a multifunctional messenger protein that activates a wide variety of signaling pathways in eukaryotic cells by changing its conformation in a calcium-dependent manner. Isothermal titration calorimetry and circular dichroism studies have shown that the interaction between calmodulin and CaF2 nanoparticles occurs with physiologically relevant affinity and that the binding process is fully reversible, occurring without significant alterations in protein secondary and tertiary structure. Experiments performed with a mutant form of calmodulin having impaired Ca^{2+}-binding ability in the C-terminal lobe suggest that the EF-hand Ca^{2+}-binding motifs are directly involved in the binding of calmodulin to the CaF2 matrix. The residual capability of nanoparticle-bound calmodulin to function as a calcium sensor protein, binding to and altering the activity of a target protein, was successfully probed by biochemical assays. Even if efficiently carried by CaF2 nanoparticles, calmodulin may dissociate thus retaining the ability to bind the peptide encompassing the putative C-terminal calmodulin-binding domain of glutamate decarboxylase and activate the enzyme. We conclude that the high flexibility and structural plasticity of calmodulin are responsible for preservation of its function when bound in high amounts to a nanoparticle surface.

013

CALCIUM SENSING RECEPTOR SIGNALLING IN COLON CANCER CELLS

Samawansha Tennakoon, Izabella Karpat, Abhishek Aggarwal, Luca Hegedus, Enikö Kallay

Department of Pathophysiology and Allergy Research, Medical University of Vienna, Austria

Background: The calcium-sensing receptor (CaSR), a G protein-coupled receptor, which is able to sense changes in extracellular calcium levels and to regulate various cellular functions such as cell proliferation and survival. During colorectal tumourigenesis, CaSR is down regulated. This led us to hypothesize a deregulation of CaSR–mediated signalling in the colon. Therefore, the aim of this study is to investigate the colon cancer specific signalling pathways regulated by the CaSR.
Methods: Live cell intracellular calcium imaging with Fura-2 fluorescent dye was performed to determine the intracellular calcium levels. Phosphorylation of ERK1/2 and p38 was determined by either traditional or in-cell western blotting in the colon cancer cell line Caco2-15 in the presence and absence of calcium. Involvement of CaSR was confirmed with specific allosteric modulators of the CaSR (agonist NPSR-568 and antagonist NPS2143). Further, the influence of the receptor was investigated on stably transfected Caco2-15 overexpressing the wild type CaSR (CaSR-WT). The cells transfected with the empty vector (CaSR-Emp) was used as the control.
Results: Increasing concentrations of extracellular calcium, increased intracellular calcium levels in Caco2-15 cells. Live cell imaging showed strong intracellular calcium oscillations in CaSR-WT system upon treatment with calcium compared with the CaSR-Emp cells. In Caco2-15 cells calcium treatment showed significant up-regulation of ERK1/2 phosphorylation ($p<0.001$), while phosphorylation of p38 was inhibited. Maximal effect was reached within 5 minutes. The positive allosteric modulator NPSR-568, further enhanced the effect of calcium while the negative

modulator NPS2143 reduced the action of calcium. Moreover, a time dependent treatment of CaSR-WT cells with calcium confirmed the influence of the CaSR. Conclusions: We have shown that the CaSR is, at least in part, responsible for regulating intracellular calcium levels. Whether this is achieved by opening cell-membrane channels or intracellular stores needs to be proven. Moreover, the CaSR-mediated signalling modulates the distinct MAPK pathway in a time and concentration-dependent manner. The involvement in the regulation of these pathways could be linked to the cancer preventive effects of calcium.

014

IMPACT OF MYRISTOYLATION AND Mg^{2+} ON Ca^{2+}-SENSING PROPERTIES OF ZEBRAFISH GCAPS

Sulmann, S., Vocke, F., Scholten, A. and Koch, K.-W.

Biochemistry Group, Department of Neuroscience, University of Oldenburg, 26111 Oldenburg, Germany.

In photoreceptor cells many processes are mediated by proteins, which can sense subtle changes in the intracellular Ca^{2+} concentration. Among these calcium sensor proteins the guanylate cyclase-activating proteins (GCAPs) play a fundamental role in the restoration of the dark adapted state by controlling the membrane bound guanylate cyclase (GC) in retinal rod and cone cells. Bovine photoreceptors express two GCAP isoforms, namely GCAP1 and GCAP2, which have different Ca^{2+} affinities and show different structural implications for the N- terminal attached myristoyl group. These GCAP isoforms activate the bovine GC in a Ca^{2+} relay mode fashion: in their Ca^{2+}-free/Mg^{2+}-bound state they activate the GC, when the Ca^{2+} level raises the GCAP isoforms are sequentially filled up with Ca^{2+} and switch to an inhibitory conformation. The GC-GCAP system is more complex in zebrafish rods and cones, which express six GCAP isoforms (zGCAP1-5, zGCAP7) that differ in their Ca^{2+} affinities, Ca^{2+} sensitive GC activation and in their spatial-temporal expression profiles. In the present study we investigated some unresolved issues that are central to the molecular and cellular function of zGCAPs. We tested, whether all zGCAPs are myristoylated in living cells using an approach of in vivo click chemistry in combination of fluorescence microscopy and further compared zGCAPs with the Ca^{2+} sensor recoverin for performing a putative Ca^{2+} dependent myristoyl switch. The influence of Mg^{2+} on Ca^{2+} binding and on the concerted Ca^{2+}-related conformational changes was monitored by isothermal titration calorimetry (ITC) and a technique based on the surface plasmon resonance phenomenon (SPR). In summary, zGCAPs are myristoylated, but do not undergo a Ca^{2+}-myristoyl switch; all have at least two high affinity binding sites for Ca^{2+} and Mg^{2+} controls the affinity for Ca^{2+} as well as Ca^{2+}-induced conformational changes. Thus, zGCAPs are equipped with properties suitable to operate in sequential step by step order.
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015

STIM1 AND ORAI1 MEDIATE HISTAMINE-EVOKED CALCIUM ENTRY AND NFAT SIGNALING IN HUMAN UMBILICAL VEIN ENDOTHELIAL CELLS

Meng-Hua Zhou[1,#], Hongying Zheng[1,#], Hongjiang Si[1], Yixin Jin[1], Carlos Muñoz-Garay[2], Yubin Zhou[3], David C. Zawieja[1], Lih Kuo[1,4], Xu Peng[1], and Shenyuan L. Zhang[1].

[1]Department of Medical Physiology, College of Medicine, Texas A&M Health Science Center, Temple, TX 76504, USA; [2]Instituto de Biotecnología, Universidad Nacional Autónoma de México, Apdo. Postal 510-3, Cuernavaca 62250, Morelos, Mexico; [3]Center for Translational Cancer Research, Institute of Biosciences & Technology, Texas A&M Health Science Center, Houston, TX 77030, USA; [4]Department of Surgery, Scott & White Memorial Hospital, College of Medicine, Texas A&M Health Science Center, Temple, TX 76504, USA
# Both authors contributed equally to this work.

Histamine is an important immunomodulator involved in allergic reactions and inflammatory responses. In endothelial cells, histamine induces Ca^{2+} mobilization by releasing Ca^{2+} from the endoplasmic reticulum and eliciting Ca^{2+} entry across the plasma membrane. Herein, we show that histamine-evoked Ca^{2+} entry in human umbilical vein endothelial cells (HUVECs) is sensitive to blockers of Ca^{2+} release-activated Ca^{2+} (CRAC) channels. RNA interference against STIM1 and Orai1, the activating subunit and the pore-forming subunit of CRAC channels, respectively, abolishes this histamine-evoked Ca^{2+} entry. Furthermore, overexpression of dominant-negative CRAC channel subunits inhibits while co-expression of both STIM1 and Orai1 enhances histamine-induced Ca^{2+} influx. Interestingly, gene silencing of STIM1 or Orai1 also interrupts the activation of calcineurin/nuclear factor of activated T-cells (NFAT) pathway and the production of interleukin 8 triggered by histamine in HUVECs. Collectively, these results suggest a central role of STIM1 and Orai1 in mediating Ca^{2+} mobilization linked to inflammatory signaling of endothelial cells upon histamine stimulation.

016

TUBULAR AGGREGATE MYOPATHY IS CAUSED BY MUTATIONS IN THE CALCIUM SENSOR STIM1

Johann Böhm[1], Frédéric Chevessier[2], Catherine Koch[1], Karima Ghorab[3], Norma B. Romero[4], Bruno Eymard[4], Marc Bartoli[5], Jocelyn Laporte[1]

[1]IGBMC (Institut de Génétique et de Biologie Moléculaire et Cellulaire), Illkirch, France; [2]University Hospital Erlangen, Erlangen, Germany; [3]Centre Hospitalier Universitaire de Limoges, Limoges, France; [4]Groupe Hospitalier Pitié-Salpêtrière, Paris, France; [5]Faculté de Médecine de Marseille, Marseille, France

Calcium (Ca^{2+}) is a physiological key factor and plays a pivotal role in a plethora of cellular functions. In skeletal muscle, calcium triggers contraction and acts as a second messenger controlling growth and differentiation. In order to ensure normal muscle function, calcium storage in the sarcoplasmic reticulum and release to the cytoplasm need to be tightly regulated. STIM1 (Stromal Interaction Molecule 1) is the main calcium sensor in the sarcoplasmic

reticulum. It contains an intraluminal region with two EF hands and a SAM domain, a single transmembrane domain, and a cytosolic part interacting with other proteins. The EF hands sense and bind calcium within the sarcoplasmic reticulum. Upon calcium store depletion, STIM1 unfolds, oligomerizes and activates calcium release-activated calcium channels (CRAC) as ORAI1 to trigger extracellular calcium entry. This STIM1-dependant mechanism of calcium store refill is known as store-operated calcium entry (SOCE). Using high-throughput sequencing, we have identified STIM1 mutations as the genetic cause of tubular aggregate myopathy (TAM). This progressive muscle disorder can involve muscle weakness, cramps and pain. Biopsies from TAM patients typically show prominent aggregates consisting of densely packed membrane tubules. These aggregates also appear as secondary features in a variety of inherited and acquired muscle disorders and strikingly accumulate in normal muscle with age. All 10 STIM1 mutations identified to date (including 6 unpublished) affect highly conserved amino acids in the EF hands. In order to assess their impact on the biological function of STIM1, we transfected C2C12 myoblasts with STIM1-YFP constructs. In contrast to wild-type STIM1, all mutants were constitutively oligomerized, indicating that calcium sensing was impaired. To further investigate the pathomechanism underlying the disorder, we measured the response to SOCE in myoblasts from TAM patients. Compared to controls, TAM myoblasts displayed increased calcium levels under basal condition. Addition of calcium to the medium induced a massive intracellular calcium increase in TAM myoblasts, while only moderate calcium influx was observed in control myolasts. These results demonstrate that the STIM1 mutations impair calcium sensing and induce constitutive STIM1 clustering and activation of the calcium channels, resulting in aberrant calcium homeostasis in muscle cells from TAM patients.

017

DISTRIBUTION OF Ca^{2+} CHANNEL ISOFORMS IN HACAT KERATINOCYTES

Will Talbot[1], Helen Burrell[2] and Alec Simpson[1]

[1]Cellular and Molecular Physiology, Institute of Translatonal Medicine, University of Liverpool, UK; [2]School of Pharmacy and Biomolecular Sciences, Liverpool John Moores University, U.K.

Intracellular and extracellular Ca^{2+} homeostasis are both important for the normal barrier and protective functions of skin. An extracellular Ca^{2+} gradient within the epidermis has been widely reported, although its' exact form and how it is maintained is unclear. In low extracellular Ca^{2+} keratinocytes proliferate and in high Ca^{2+} they differentiate. In the epidermis, the stratum granulosum contains lamellar bodies, which provide lipid and proteins that make up the permeability barrier of skin. A surprising feature of lamellar body secretion is that it is inhibited by opening of voltage-gated Ca^{2+} channels (VOCs) and the activation of ryanodine receptors (RYRs). Consequently, lowering Ca^{2+} promotes exocytosis. In contrast, activation of phospholipase C and IP_3 receptors (IP_3Rs) is associated with lamellar body secretion. RYRs, IP_3Rs and VOCs show differential expression in epidermis. RYR1 is expressed throughout, but mostly strongly in the upper differentiated layers where RYR2 is also expressed - in and around the stratum granulosum. RYR3 is found evenly throughout the epidermis as are VOCs. HaCaT cells are a human keratinocyte cell line that can be grown into a stratified epithelium (or pseudoskin). We propose that the spatial relationships between RYRs, IP_3Rs, VOCs and possibly other influx channels determines whether Ca^{2+} release or influx promotes or inhibits lamellar body secretion. We are using the HACAT cell line, to investigate the spatial relations between the Ca^{2+} channels and to examine the functionality of VOCs in keratinocytes. Immunofluorescence reveals distinct sub-cellular localisation of Ca^{2+} release channel isoforms. RYR1 and IP_3R1 occurred near the plasma membrane with areas of co-localisation. IP_3R2, IP_3R3, RYR2 and RYR3 isoforms were distributed throughout the cytoplasm, each in a unique pattern. In confluent cells, RYR1 and IP_3R1 could be localised to membrane invaginations along with involucrin and caveolin. We also find that resting Ca^{2+} is largely influenced by VOC. These results are consistent with the potential for tight spatial regulation of distinct Ca^{2+}-dependant processes. Further investigations are needed to determine the spatial relations and functionality of these channels, and in particular, how they shape Ca^{2+} signals in keratinocytes and the differentiated layers of the epidermis.

018

DUAL FUNCTIONS OF S100A8/A9 IN INFLAMMATION: FOCUS ON NADPH OXIDASE REGULATION AND AUTOCRINE SIGNALLING IN NEUTROPHILS

V. Schenten, S. Bréchard, N. Jung, F. Tolle, S. Plançon and E.J. Tschirhart

University of Luxembourg, Life Sciences Research Unit, Calcium Signalling and Inflammation Group, Luxembourg.

S100A8 and S100A9 are members of the S100 sub-family of EF-hand Ca^{2+}-binding proteins and are abundantly expressed in the cytosol of circulating neutrophils. Formation of Ca^{2+}-dependent heterodimers and heterotetramers is likely a pre-requisite for S100A8/A9 biological activity. These molecular mediators are found in high concentrations at sites of inflammation or in the serum of patients with inflammatory diseases (e.g rheumatoid arthritis). S100A8/A9 have diverse effects and multiple functions in the immune response, infection and tissue injury. In this work we focused first on the intracellular role of S100A8/A9 in the regulation of the NADPH oxidase (NOX) activity, the major source of reactive oxygen species in neutrophils. These reactive oxygen species are designed to kill invading pathogens but can also cause tissue damage and perpetuation of inflammation when excessively produced. NOX activation is regulated by the integration of diverse signaling pathways, such as Ca^{2+} signaling and kinase activation. S100A8/A9 could constitute the molecular switch between the Ca^{2+}-dependent signaling cascade and NOX activation. Therefore we characterized S100A8/A9-dependent mechanisms of NOX regulation in neutrophil-like HL-60 cells. The second part of our work intended to study the signalling pathways induced by S100A8/A9. Indeed, S100A8 and S100A9 can be secreted by neutrophils in the extracellular environment where they are considered as damage-associated molecular pattern molecules which can amplify the pro-inflammatory response either by activation of neutrophils (autocrine mode) or other inflammatory cell types (paracrine mode). To investigate these autocrine effects, neutrophil-like HL-

60 cells were stimulated with purified S100A8/A9 and the induction of diverse pro-inflammatory molecules was measured. Our results show that, among other, TNF-α or IL6 mRNA expression is up-regulated in S100A8/A9-treated cells. Clearly, S100A8/A9 can be involved in the pathophysiology of many inflammatory diseases and understanding more precisely their various functions will be a major advance for the development of selective therapy.

019

Ca^{2+}-BINDING CALMYRIN 2 INTERACTS WITH RAB PROTEINS AND REGULATES ENDOCYTOSIS

Potrzebowska K.[a], Błazejczyk M.[a], Jaworski J.[b], Kuźnicki J.[a,e], Hoogenraad C.C.[c], Wojda U.[a,d]

[a]Laboratory of Neurodegeneration, [b]Laboratory of Molecular and Cellular Neurobiology, International Institute of Molecular and Cell Biology, Warsaw, Poland; [c]Cell Biology, Faculty of Science, Utrecht University, Utrecht, the Netherlands; [d]Laboratory of Preclinical Studies, eLaboratory of Calcium Binding Proteins, Nencki Institute of Experimental Biology, Warsaw, Poland

Recent studies indicate importance of Ca^{2+} in the regulation of endosome fusion and vesicular transport, but the identification and elucidation of the roles of particular Ca^{2+} sensor proteins in endocytosis is not complete. Calmyrin 2 (CaMy2, Cib2) belongs to the Ca^{2+}-binding EF-hand calmyrin protein family. Calmyrins are structurally most similar to Neuronal Ca^{2+} Sensors (NCSs). NCS proteins regulate many neuronal functions including neuron development, membrane trafficking and synaptic plasticity. Importance of such NCSs as calcineurin and hippocalcin in the regulation of endocytosis was also indicated. Recently we have found that CaMy2 is preferentially expressed in hippocampus and cortex neurons (Blazejczyk et al., 2009). Endogenous CaMy2 was present in neurites and the Golgi apparatus, and was found in the membranous fraction. Here we report the identification of first neuronal CaMy2 protein targets. In accordance with the subcellular localization in neurons, CaMy2 interacts with Rab proteins involved in early endosome trafficking and fusion. The interaction was identified in extracts of rat brain by pull-down using CaMy2 as bait, followed by mass spectrometry, and confirmed by coimmunoprecipitation. Moreover, we demonstrated colocalization of CaMy2 with Rab proteins on endocytic vesicles of rat hippocampal neurons in primary culture. Knock-down of CaMy2 with shRNA in hippocampal neurons greatly affected the distribution of Rab-positive vesicles. Our data suggest that CaMy2 is a novel Ca^{2+}-sensor that could be involved in endocytosis.

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020

HIGH SPATIO-TEMPORAL RESOLUTION Ca^{2+} IMAGING IN T CELLS DURING IMMUNE SYNAPSE FORMATION

B.-P. Diercks[1], R. Fliegert[1], D. Schetelig[2], R. Werner[2], A.H. Guse[1], I.M.A. Wolf[1]

[1]The Calcium Signalling Group, Department of Biochemistry and Molecular Cell Biology, University Medical Centre Hamburg-Eppendorf, Hamburg, Germany; [2]Department of Computational Neuroscience, University Medical Centre Hamburg-Eppendorf, Hamburg, Germany

Upon immune synapse (IS) formation fast, spatially restricted Ca^{2+} signals, termed elementary Ca^{2+} signals may occur. To analyze these signals, high spatial and temporal resolution Ca^{2+} imaging is necessary. Fura-2 is a frequently used ratiometric Ca^{2+} indicator. However, as excitation shift indicator with two separate excitation wavelengths, Fura-2 is unsuitable for millisecond measurements of spatio-temporal Ca^{2+} events because ratioing of two images acquired at different excitation time points may lead to systematic errors. Using non-ratiometric Ca^{2+} indicators, such as Fluo dyes, and F/F0 ratioing, artifacts may occur, particularly near the plasma membrane of cells undergoing morphological changes. To solve these problems an improved emission shift method using two different Ca^{2+}-dyes was established. The combination of a single wavelength dye (Fluo4) and an emission shift indicator (FuraRed) enables the measurement and characterization of subcellular Ca^{2+} signals despite cellular movement and changes in cell shape. With only one excitation wavelength for both dyes an acquisition rate of 40 to 50 frames/sec at 153x153 nm/pixel spatial resolution is obtained. Baseline noise analysis revealed that at highest acquisition speed Ca^{2+} signals ≥36 nM difference were resolved. In order to mimic IS and thus evoke directed, locally restricted Ca^{2+} signals, Jurkat T cells were stimulated with anti-CD3-coated beads. To detect sites of elementary Ca^{2+} signals upon IS formation the first 100 frames upon cell-bead contact (covering approx. 2.5 sec) were stacked in a maximum intensity projection using ImageJ, thus creating an output image containing the maximum value over all images in the stack at the particular pixel location. Local areas containing the top 10-20% intensity of the output image were used to create regions of interest ≥0.21 μm^2. Elementary Ca^{2+} signals were observed close to the plasma membrane in the first 10 to 15 sec upon IS formation (3.8 counts/per cell), showing the following characteristics: amplitude 72±4 nM, duration to peak 1±0.1 sec. Using our improved high resolution imaging method, the characterization of elementary Ca^{2+} signals in T cells is technically feasible. Elementary Ca^{2+} signals occurring upon IS formation in T cells are very fast, e.g. starting within milliseconds, and are spatially and temporarily restricted.

021

THE CALCIUM-REGULATED PHOSPHATASE CALCINEURIN REGULATES COORDINATED OUTGROWTH OF ALL TISSUE PROGENITOR CELLS OF REGENERATING ZEBRAFISH APPENDAGES

Satu Kujawski[1,5], Weilin Lin[2], Florian Kitte[1], Mandy Börmel[3], Steffen Fuchs[4], Guruschandar Arulmozhivarman[1], Sebastian Vogt[5], Denise Theil[1], Yixin Zhang[2] and Christopher L. Antos[1]*

[1]DFG-Center for Regenerative Therapies Dresden, Technische Universität Dresden, Fetscherstrasse 105, 01307 Dresden, Germany; [2]B CUBE, Center for Molecular Bioengineering, Technische Universität Dresden, Arnoldstrasse 18, 01307 Dresden, Germany; [3]Institute of Biochemistry, Swiss Federal Institute of Technology Zürich, CH-8093 Zürich, Switzerland; [4]Universität Würzburg, 97072 Würzburg, Germany; [5]Max-Planck Institute for Cell and Molecular Biology, Pfotenhauerstrasse 108, 01307 Dresden, Germany.

Animals that regenerate organs and appendages are able to control stem and progenitor cells to reform these structures in a proportionally coordinated manner to the same dimensions as the original structures. Coordinated proportional regeneration involves controlled regulation between allometric and isometric tissue growth. It is unclear what executes this control. We show that inhibition of the calcium-regulated phosphatase calcineurin results in continued allometric regenerative outgrowth of regenerating fish appendages beyond their original dimensions and continued transcription of regeneration-associated genes. Congruent with these results, calcineurin activity is low when the rate of blastema outgrowth is highest, and its activity increases as the regeneration rate decreases. Furthermore, inhibition of calcineurin promotes outgrowth of uninjured fins, which is associated with the activation of several genes involved in growth and regeneration. Growth rate measurements and morphometric analysis of proximodistal asymmetry indicate that calcineurin inhibition shifts fin regeneration from a distal isometric growth program to an allometric proximal program. This shift is associated with the promotion of retinoic acid signaling, a signal transduction mechanism that conveys positional information along the proximodistal axis. In summary, we identified a calcium-calcineurin-mediated mechanism that operates as a molecular switch between distal isometric growth and proximal allometric growth.

022

INHIBITION OF INSP$_3$ RECEPTOR SIGNALLING INDUCES AUTOPHAGY

Tala Chehab[1], Katja Rietdorf[1], Jan B. Parys[2], Geert Bultynck[2] and Martin D.Bootman[1]

[1]Department of Life, Health and Chemical Sciences, The Open University, Walton Hall, Milton Keynes, UK and [2]Dept. Cellular and Molecular Medicine, K.U. Leuven, Leuven, Belgium

Macroautophagy (autophagy) is an important process for cell survival and homeostasis that involves degradation of aggregated or misfolded proteins, dysfunctional organelles and pathogens through a lysosomal pathway. Autophagy occurs as a housekeeping process, and is greatly up-regulated in response to nutrient stress. Recent work has suggested that intracellular Ca^{2+} signals can positively and negatively regulate autophagic flux. Exactly how Ca^{2+} signals switch cells between pro-autophagic and anti-autophagic states is not clear. It is likely that the source and characteristics of Ca^{2+} signals are critical in determining cellular responses. One source of Ca^{2+} signals that regulate autophagic flux is inositol 1,4,5-trisphosphate receptors (IP_3Rs). This study examined the effect of inhibiting Ca^{2+} release from IP_3Rs on autophagic flux, and compared the magnitude of autophagy following IP_3R inhibition with that evoked by starvation and rapamycin (an mTOR inhibitor).

HeLa cells stably expressing GFP-tagged LC3 responded to rapamycin with a significantly enhanced autophagic flux, in a time- and dose-dependent manner. Similarly, nutrient starvation induced autophagy in a time-dependent manner. Expression of an IP_3 5'-phosphatase evoked a substantial increase in autophagic flux that was comparable to rapamycin and nutrient starvation. The expression of IP_3 5'-phosphatase significantly reduced cellular Ca^{2+} signals evoked by application of the IP_3-generating hormone, histamine. These data indicate that the activation of IP_3Rs is critical to prevent induction of autophagy. Loss of IP_3R function triggers autophagy with a similar magnitude to that evoked by pharmacological inhibition of mTOR and nutrient starvation.

023

STRUCTURAL EFFECTS OF Mg^{2+} ON THE REGULATORY STATES OF NEURONAL CALCIUM SENSORS OPERATING IN VERTEBRATE PHOTOTRANSDUCTION

Valerio Marino[1], Stefan Sulmann[2], Karl-Wilhelm Koch[2] and Daniele Dell'Orco[1]

[1]Department of Life Sciences and Reproduction, Section of Biological Chemistry, University of Verona, 37134 Verona, Italy; [2]Department of Neurosciences, Biochemistry Group University of Oldenburg, 26111 Oldenburg, Germany.

Several lines of evidence suggest that free Mg^{2+} plays an important role in phototransduction, as the Neuronal Calcium Sensors (NCS) Recoverin and Guanylate Cyclase Activating Proteins 1 and 2 (GCAP1 and GCAP2) are also capable of binding Mg^{2+} via their EF-hand motifs. Previous studies showed that a Mg^{2+}-bound state is required for GCAP1 in order to activate GC and that Recoverin binds Mg^{2+} without triggering its physiological conformational change. No structural studies were performed so far about GCAP2, for which the effects that Mg^{2+} could exert were only hypothesized. Here we compared the effects of physiological [Mg^{2+}] (1 mM) on the switch states of these three NCS in their myristoylated (myr) and non-myristoylated (nonmyr) form over the extreme conditions of high and low [Ca^{2+}], mimicking respectively dark and light states of the photoreceptor cell. We performed Circular Dichroism spectroscopy measurements to assess the differences in thermal stability, secondary and tertiary structure of all NCS in the aforementioned conditions. Intrinsic fluorescence spectroscopy titrations and Isothermal Titration Calorimetry were performed for monitoring the binding of Mg^{2+} to GCAP2. Molecular dynamics simulations (200 ns, all-atom force field) were performed to assess structural properties of GCAP1 in putatively activator, inhibitor and transitory states. Our

results confirm that Mg^{2+} is unable to trigger the physiological conformational change of Recoverin (myristoyl switch) and that it decreases its thermal stability. Mg^{2+} induces a conformational change in GCAP2 both at high and low [Ca^{2+}], however these variations are more substantial for apo-myrGCAP2. Apo-GCAP1 is responsive to Mg^{2+}, acquiring a different tertiary structure from both apo and Ca^{2+}-bound states, though this difference is lost when Ca^{2+} is saturating. GCAP1 seems to be stabilized by the presence of Mg^{2+} in solution, more notably its Ca^{2+}-bound form. Molecular dynamics simulations point out that myrGCAP1 has a highly flexible loop (125-135) when at least one divalent cation is bound to EF-3. In line with experimental data, this is sufficient to stabilize the entire structure. Moreover all simulated transitory states show very similar dynamic properties, which differ from both apo and Ca^{2+}- or Mg^{2+}- loaded forms.

024

DIVERSITY OF RECOVERINS IN ZEBRAFISH ROD & CONE PHOTORECEPTOR CELLS

[1]Scholten, A., [1]Koch, K.W., [1]Kramer, Ch., [2]Zang, J. & [2]Neuhauss, S.

[1]Department of Neurosciences, Biochemistry Group, University of Oldenburg, D-26111-Oldenburg, Germany; [2]Institute of Molecular Life Sciences, University of Zürich, Switzerland

Two classes of neuronal Ca^{2+} sensor proteins (NCS proteins) - recoverins and guanylate cyclase-activating proteins (GCAPs) - play a crucial role in shaping the light response of photoreceptor cells and in adaptation to different background light intensities. The first step to shut down the light response in rod photoreceptor cells, for example, is the inactivation of the light-receptor rhodopsin via phosphorylation by G-protein coupled receptor kinase 1 (GRK1). The activity of GRK1 is blocked by Ca^{2+}-loaded, membrane-bound recoverin. A drop in intracellular Ca^{2+}-concentration after a light stimulus leads to the release of Ca^{2+} from recoverin and eventually to an activation of GRK1. Cone photoreceptor cells exhibit much faster shutoff kinetics and the Ca^{2+} homeostasis is more complex compared to rods. Cones, for example, express a second, additional GRK-isoform (GRK7). While both GRK-isoforms are regulated by the same recoverin in mammals, four individual members of the recoverin gene family, rcv1a, rcv1b, rcv2a and rcv2b, can be found in the zebrafish genome. Nothing is known so far about the biochemical properties of zebrafish recoverins (zRecs), their Ca^{2+}-affinities or their abilities to regulate four different GRK-isoforms (zGRK1a, zGRK1b, zGRK7a and zGRK7b). To investigate the recoverin/GRK-system in zebrafishes in more detail, we compared the expression profiles of zRecs with those of GRKs. There is at least one putative zRec/zGRK-pair both in rods and all types of cones. We used heterologous expression in E.coli to characterize the biochemical properties of zRecs. Recombinant proteins are myristoylated and all seemed to undergo a Ca^{2+}-myristoyl switch, but they differed in their Ca^{2+}-sensing properties. Because the expression patterns of all zRecs overlapped at least partially with that of zGRK7a we performed interaction studies with the N-terminus of GRK7a. So far, only zRec1a and 2a seemed to interact with the N-terminus of zGRK7a. Our data point to an intricate network of Ca^{2+}-sensing proteins that shape the dynamic range of target regulation in response to fluctuating intracellular Ca^{2+}.

025

TANDEM PORE DOMAIN K^+ CHANNEL (K2P) OPENERS; EFFECTS ON NEURONAL Ca^{2+} RESPONSES AND HVA CHANNELS

Avi Ring, Anders Ziegler, Rita Tanso

Deptmt protection, Norwegian Defence Research Est., Box 25, 2027 Kjeller, Norway

Two-pore-domain potassium (K2P) channels determine the K^+ leak current which underlies the resting membrane potential in most vertebrate cells. Modulation of K2P channels is therefore expected to critically influence excitability, e.g., in the CNS and in heart. A number of different types of membrane soluble drugs have been shown to facilitate opening of K2P channels. It has been suggested that the mechanism of interaction of the drugs with K2P channels is due to their perturbation of the physical properties of the lipid membrane. The membrane-channel interaction then modulates K2P activity due to the effects on the relative stability of different channel conformations.
Methods: In-vitro neural cell cultures were used to examine the effects of K2P modulating drugs on Ca^{2+} signaling. NMDA, kainate or nicotine were used to trigger depolarization induced Ca^{2+} influx. Fura-2 and a fluorescence plate reader were used to investigate the kinetics of the neurotransmitter mediated intracellular Ca^{2+} changes in primary cultures of chick embryo cortical neural cells and the human neuronal cell line SH-SY5Y. Cells were stimulated with low concentrations of agonists in order to enhance assay sensitivity to effects of membrane potential modulation. This sensitivity to membrane potential was further promoted by including, in the experimental buffers, voltage dependent open channel blockers (of NMDA- or nicotine-receptors). We show that the experimental model used is suitable to assay effects of membrane potential on the net Ca^{2+} response.
Results: All except one of the different types of K2P channel openers tested had significant modulating effects on high voltage activated (HVA) Ca^{2+} channels (p/q-, n- or L-type). The exception was the K2P channel opener drug BL-1249 which significantly inhibited the NMDA stimulated Ca^{2+} responses although it had no significant effects on depolarization stimulated HVA-channel Ca^{2+} influx.
In summary, our results suggest that the modulation of Ca^{2+} responses to glutamatergic and nicotinerigic stimulation by poly-unsaturated fatty acids as well as NS-309 and Lysophatidylinositol (LPI) are more significantly influenced by their effects on HVA channels than are their effects on K2P channels.

026

Ca^{2+}-DEPENDENT REPAIR OF PNEUMOLYSIN PORES: A NEW PARADIGM FOR HOST CELLULAR DEFENSE AGAINST BACTERIAL PORE-FORMING TOXINS

Heidi Wolfmeier[1], Roman Schoenauer[1], Alexander P. Atanassoff[1], Daniel R. Neill[2], Aras Kadioglu[2], Annette Draeger[1], Eduard B. Babiychuk[1]

[1]Department of Cell Biology, Institute of Anatomy, University of Bern, Bern 3009, Switzerland; [2]Department of Clinical Infection Microbiology & Immunology, Institute of Infection & Global Health, University of Liverpool, Liverpool L69 7BE, UK.

Pneumolysin (PLY), a key virulence factor of S. pneumonia, permeabilizes eukaryotic cells by forming large trans-membrane pores. PLY imposes a puzzling multitude of diverse, often mutually excluding actions on eukaryotic cells. Whereas cytotoxicity of PLY can be directly attributed to the pore-mediated effects, mechanisms that are responsible for the PLY-induced activation of host cells are poorly understood. We show that PLY-pores can be repaired and thereby PLY-induced cell death can be prevented. Pore-induced Ca^{2+}-entry from the extracellular milieu is of paramount importance for the initiation of plasmalemmal repair. Nevertheless, active Ca^{2+}-sequestration that prevents excessive Ca^{2+}-elevation during the execution phase of plasmalemmal repair is of no less importance. The efficacy of plasmalemmal repair does not only define the fate of targeted cells but also intensity, duration and repetitiveness of PLY-induced Ca^{2+}-signals in cells that were able to survive after PLY-attack. Intracellular Ca^{2+}-dynamics evoked by the combined action of pore formation and their elimination mimic the pattern of receptor-mediated Ca^{2+}-signaling, which is responsible for the activation of host immune responses. Therefore, we postulate that plasmalemmal repair of PLY-pores might provoke cellular responses that are similar to those currently ascribed to the receptor-mediated PLY effects. Our data provide new insights into the understanding of the complexity of cellular non-immune defense responses to a major pneumococcal toxin that plays a critical role in the establishment and the progression of life-threatening diseases. Therapies boosting plasmalemmal repair of host cells and their metabolic fitness might prove beneficial for the treatment of pneumococcal infections.

027

ACID SPHINGOMYELINASE MEDIATES THE REPAIR OF TOXIN-DAMAGED PLASMALEMMA BY REGULATING MICROVESICLE SHEDDING

Schoenauer Roman[1], Wolfmeier Heidi[1], Atanassoff Alexander P[1], Schneider-Schaulies Sibylle[2], Babiychuk Eduard B[1], Draeger Annette[1]

[1]Institute of Anatomy, University of Bern, Switzerland; [2]Institute of Virology and Immunobiology, University of Würzburg, Germany

Bacterial pore-forming toxins compromise plasmalemmal integrity leading to Ca^{2+}-influx, leakage of the cytoplasm and cell death. Cells have developed tools to shed active toxin pores and remove them from the membrane in the form of microparticles. This shedding is mediated by Ca^{2+} sensitive proteins of the annexin family. The activation of sphingomyelinases leads to the formation of ceramide, which condenses into platforms, thereby altering the biophysical properties of the plasmalemma. This in turn might affect the ability of the plasmalemmal repair machinery to shed the toxin pores. Therefore we have analyzed the role of acid (ASM) and neutral (NSM) sphingomyelinases during membrane repair after the treatment with the pore-forming toxin listeriolysin O (LLO) in Jurkat cells in which either enzyme was knocked down. Our experiments revealed that the ASM knock-down cells are significantly more prone to injury by LLO compared to control or NSM knock-down cells. We show that microparticle release was significantly decreased in LLO treated ASM knock-down cells. We suggest that microparticle release is part of a defense mechanism, which is regulated by ceramide formation.

028

KCNIP1 A Ca^{2+}-DEPENDENT TRANSCRIPTIONAL REPRESSOR REGULATES THE SIZE OF THE NEURAL PLATE IN XENOPUS

Isabelle Néant[1,2,3,4], Britt Mellström[4], Paz Gonzales[4], Jose R. Naranjo[3,4], Marc Moreau[1,2,3] and Catherine Leclerc[1,2,3]*

[1]Université Toulouse 3, Centre de Biologie du Développement, 118 routes de Narbonne, F31062 Toulouse, Cedex 04, France, [2]CNRS UMR5547, Toulouse, F31062, France, [3]GDRE CNRS n°731 Toulouse France, [4]Centro Nacional de Biotechnología, CSIC and CIBERNED, Madrid, Spain

In amphibian embryos, our previous work has demonstrated that calcium transients occurring in the dorsal ectoderm at the onset of gastrulation are necessary and sufficient to engage the ectodermal cells into a neural fate by inducing neural specific genes. Some of these genes are direct targets of calcium. We search for a direct transcriptional mechanism by which calcium signals are acting. The only known mechanism responsible for a direct action of calcium on gene transcription involves an EF-hand Ca^{2+} binding protein which belongs to a group of four proteins (kcnip1 to 4). Kcnip protein can act in a Ca^{2+}-dependent manner as a transcriptional repressor by binding to a specific DNA sequence, the Downstream Regulatory Element (DRE) site. The affinity of kcnip to the DRE is modulated by the calcium occupancy of its EF-hand sites. In Xenopus, among the four kcnips, we show that only kcnip1 is timely and spatially present in the presumptive neural territories and is able to bind DRE sites in a Ca^{2+}-dependent manner. The loss of function of kcnip1 results in the proliferation of neural progenitors and in the expansion of the neural plate. Later on this leads to impairment in the development of anterior neural structures. We propose that, in the embryo, at the onset of neurogenesis Kcnip1 is the Ca^{2+}-dependent transcriptional repressor that controls the size of the neural plate.

029

DISTINCT DYNAMICS OF INTRACELLULAR $[Ca^{2+}]_I$ AND $[cAMP]_I$ UPON ADRENERGIC RECEPTOR STIMULATION IN SINGLE RAT ASTROCYTES

Anemari Horvat[1], Robert Zorec[1,2,*], Nina Vardjan[1,2*]

[1]Laboratory of Neuroendocrinology–Molecular Cell Physiology, Institute of Pathophysiology, Faculty of Medicine, University of Ljubljana, Zaloška 4, 1000 Ljubljana, Slovenia; [2]Celica Biomedical, Tehnološki park 24, 1000 Ljubljana, Slovenia
*Correspondence to RZ and NV

Astrocytes are exceptional cells for many reasons; one of them is that they store glycogene. Glycogene can be metabolized to glucose, from which lactate or even the gliotransmitters glutamate and ATP can be synthesized. Both glucose and lactate can be transferred to neurons. An important regulator of glucose formation in astrocytes is cAMP-mediated signaling pathway, however detailed measurements of this second messenger in single astrocytes are lacking. The aim of this study was to introduce a real-time method of monitoring the cytosolic concentration of cAMP ($[cAMP]_i$) by confocal microscopy and the fluorescent FRET-based cAMP nanosensor Epac1-camps and protein kinase A (PKA) nanosensor AKAR2 that were introduced into astrocytes by lipofection. The results have shown that the addition of extracellular adrenergic receptor agonists noradrenaline and adrenaline raised $[cAMP]_i$ with a time-constant of 15 s, likely through Gs-protein activation, which then activates PKA. PKA is able to activate enzymes that induce glycogene degradation and an augmentation of cytosolic free glucose (Prebil et al., 2011). It was reported that cAMP-signaling pathway may interact with changes in cytosolic $[Ca^{2+}]_i$. We asked whether a similar pattern can be recorded in astrocytes. Interestingly, even though the application of adrenaline elicited periodic time-dependent increases in $[Ca^{2+}]$i in more than 85% of astrocytes, adrenaline- or noradrenaline-induced increases in $[cAMP]_i$ reached a steady state value and were devoid of temporal oscillations. These results indicate that cAMP-mediated signaling in astrocytes plays a role in relatively slow, integrating signaling processes.

030

FUNCTIONAL DEFECTS IN CALCIUM SIGNALING AS A DIAGNOSTIC TOOL FOR AUTISM SPECTRUM DISORDER

Galina Schmunk, Ian Smith, Ian Parker, J. Jay Gargus

Center for Autism Research and Translation, University of California, Irvine

Autism spectrum disorder (ASD) is a neurological disorder characterized by signs and symptoms that include lack of social skills, language deficiency, and stereotypic repetitive behaviors. Its presentation is highly variable from patient to patient in expressivity and severity of ASD symptoms. The etiology of ASD is ill-defined, and diagnosis often involves a clinical odyssey as no reliable biomarkers have been identified. However, the high heritability of ASD suggests a strong genetic component, and it is generally understood that ASD can manifest from both monogenic and polygenic disorders. Re-sequencing and genome-wide association studies (GWAS) have identified numerous “risk” alleles for ASD, many of which appear to cluster in a few signaling pathways. This supports a convergence hypothesis, which proposes that key “hubs” within signaling pathways operate as convergence points at which *many* mutated genes exert their additive ASD pathogenic effects, likely in concert with unknown environmental stressors. This complexity and heterogeniety makes it highly unlikely that a diagnostic “gene panel” could be devised; however *functional* assays on diagnostic skin biopsies have proven critical to advancing diagnosis and specific treatments for a host of similarly complex genetic disorders such as lysosomal storage and mitochondrial diseases.

Genetic studies have implicated genetic defects in Ca^{2+} channels and Ca^{2+} signaling-associated proteins with an increased risk for ASD. However, little is understood of the mechanistic involvement of disrupted Ca^{2+} signaling in ASD. We therefore analyzed intracellular Ca^{2+} signaling in cell lines derived from multiple independent patients and matched controls to define core signaling defects shared in common by three distinct genetic lesions strongly associated with ASD. We find that fibroblasts from patients with dominant hamartin or tuberin mutations causing tuberous sclerosis type 1 or type 2, or pathogenic expansion FMRP alleles causing Fragile X syndrome, have significant defects in both global and local inositol trisphosphate-mediated Ca^{2+} signals. These highly reproducible findings have direct implications for the development of objective diagnostic tests, and additionally serve as a gateway to discovery of novel small molecule therapeutics for ASD.

031

BIOACTIVE SPHINGOLIPIDS AND CALCIUM HOMEOSTASIS IN CULTURED RAT ASTROCYTES

Matjaž Stenovec[1,2]*, Saša Trkov[1,2]*, Marko Kreft[1,2,3], Robert Zorec[1,2]

[1]Celica Biomedical Center d.o.o., Tehnološki park 24, 1000 Ljubljana, Slovenia; [2]Laboratory of Neuroendocrinology-Molecular Cell Physiology, Institute of Pathophysiology, Faculty of Medicine, University of Ljubljana, Zaloška 4, 1000 Ljubljana, Slovenia; [3]Department of Biology, CPAE, Biotechnical Faculty, University of Ljubljana, Večna pot 111, 1000 Ljubljana, Slovenia
*Matjaž Stenovec and Saša Trkov contributed equally to this work.

In the brain, alterations in sphingolipid metabolism contribute to several neurologic disorders; however, their effect on astrocytes is largely unknown. Here, we identified bioactive sphingolipids that affect intracellular free calcium concentration ($[Ca^{2+}]$i), mobility of peptidergic secretory vesicles, signaling pathways involved in alterations of calcium homeostasis and explored the relationship between the stimulus-evoked increase in $[Ca^{2+}]$i and attenuation of vesicle mobility. Confocal time-lapse images were acquired to explore $[Ca^{2+}]$i signals, the mobility of fluorescently tagged peptidergic vesicles and the structural integrity of the microtubules and actin filaments before and after the addition of exogenous sphingolipids to astrocytes. The results show that fingolimod (FTY720), a recently introduced therapeutic for multiple sclerosis, and sphingosine, a releasable constituent of membrane sphingolipids, evoked long-lasting increases in $[Ca^{2+}]$i in

the presence and absence of extracellular Ca^{2+}; the evoked responses were diminished in the absence of extracellular Ca^{2+}. Activation of phospholipase C and inositol-1,4,5-triphosphate receptors was necessary and sufficient to evoke increases in $[Ca^{2+}]i$ as revealed by the pharmacologic inhibitors; Ca^{2+} flux from the extracellular space intensified these responses several fold. The lipid-evoked increases in $[Ca^{2+}]i$ coincided with the attenuated vesicle mobility. High and positive correlation between increase in $[Ca^{2+}]i$ and decrease in peptidergic vesicle mobility was confirmed independently in astrocytes exposed to evoked, transient Ca^{2+} signaling triggered by purinergic and glutamatergic stimulation. It is concluded that cell-permeable sphingosine-like lipids exert complex, Ca^{2+}-dependent effects on astrocytes and likely alter their homeostatic function in vivo.
Keywords: Astrocytes; Calcium; FTY720; Phospholipase C; Sphingosine; Vesicle traffic

032

IMPAIRED SECRETION OF SAPPα UNDER HIGH EXTRACELLULAR Ca^{2+} CONDITIONS

Wegierski T, Gazda K., Kuznicki J.

Laboratory of Neurodegeneration, International Institute of Molecular and Cell Biology, Ks. Trojdena 4, 02-109 Warsaw, Poland

Alzheimer's disease (AD) is the most common neurodegenerative disease. The deposition of amyloidß–peptide (Aß) fibers, products of ß–amyloid precursor protein (APP) regulated proteolysis, is widely believed to be a crucial factor involved in the pathogenesis of AD. However, altered calcium homeostasis has been recently proposed as an early event in disease development. There are two major pathways of APP processing. In amyloidogenic pathway, APP is sequentially cleaved by ß–secretase and γ–secretase, leading to formation of toxic Aß peptides. In non–amyloidogenic pathway, α–secretase precludes cleavage by ß–secretase, releasing an extracellular fragment (sAPPα) and a membrane–associated C–terminal fragment (C83). C83 is further cleaved by γ–secretase, which results in formation of P3 and AICD peptides. The soluble, extracellular sAPPα was proposed to have neuroprotective functions, but the exact mechanism of its action is poorly understood. One objective of our research is to understand how disturbances in cellular calcium levels contribute to differences in APP proteolysis. Here we show that elevated extracellular Ca^{2+} concentration ($[Ca^{2+}]e$) leads to a reduction in sAPPα release and concomitant accumulation of APP inside cells. Probing with antibodies raised against different epitopes within APP points to sAPPα as the accumulated species. Ectopically expressed APP with truncation at the alpha site shows the same effect. On the other hand, secretable luciferase neither accumulates nor co-localizes with retained sAPPα. Our data suggest a specific block in secretion of the sAPPα fragment under conditions of high $[Ca^{2+}]e$. The results give insight into the mechanism of APP processing and trafficking, depending on the Ca^{2+} levels, and may help to elucidate the role of APP in different pathophysiological conditions.

033

EFFECT OF ATMB, A PHARMACOLOGICAL MODULATOR OF TRPM8 ON BREAST CANCER CELL PROLIFERATION

Kunsala T.D.S Yapa, Amelia A. Peters, Irina Vetter, Sarah J. Roberts-Thomson, Gregory R. Monteith.

The School of Pharmacy, The University of Queensland, Brisbane, QLD

Introduction: Many studies have now shown that aberrant expression of specific calcium channels is a feature of some cancers, including those of the breast. The calcium permeable ion channel, transient receptor potential cation channel subtype M, member 8 (TRPM8) has been identified as a potential therapeutic target in prostate cancer, however very few studies have assessed the role of TRPM8 in breast cancer cells.
Aims: To characterise the role of TRPM8 in breast cancer cell proliferation.
Methods: MDA-MB-231 and SK-BR-3 breast cancer cells were treated with 0.1 µM - 100 µM of the TRPM8 inhibitor: N-(3-aminopropyl)-2-[(3-methylphenyl)methoxy]-N-(2-thienylmethyl)-benzamide hydrochloride (1:1) hydrate (AMTB) (Sigma-Aldrich). Cells were plated in 96-well plates (24 hours), then incubated with the TRPM8 inhibitor, AMTB (72 hours) and MTT assays (Promega) were performed to determine the effect of AMTB on viable cell number. The effect of AMTB on voltage gated sodium channel (Na_v) activity was assessed using a high-throughput FLIPR membrane potential assay based on depolarisation of HEK293 cells stably expressing hNav isoforms (Scottish BioMedical) by veratridine. Real-time RT-PCR (Life Technologies) was used to determine mRNA levels of TRPM8 and Na_v subtypes 1.1-1.9 in breast cancer cell lines.
Results: The TRPM8 inhibitor, AMTB, decreased viable cell number by greater than 70% in both MDA-MB-231 and SK-BR-3 cell lines at 100 µM. However, no TRPM8 or very little mRNA transcripts were detected in MDA-MB-231 and SKBR-3 cells respectively. Analysis of AMTB, showed its inhibition of some voltage gated sodium channels. Subsequent real time RT-PCR assessment detected the presence of mRNA for Na_v subtypes 1.3, 1.5, 1.7 in MDA-MB-231 and Na_v subtypes 1.4, 1.6, 1.7 in SK-BR-3 breast cancer cells.
Conclusion: The effects of the TRPM8 inhibitor AMTB on breast cancer cells may be predominately mediated by its inhibition of voltage gated sodium channels. Further studies are required to assess the consequences of voltage gated sodium channel inhibition on calcium signalling and proliferation in breast cancer cells.

034

INTESTINAL DELETION OF PMCA1 LEADS TO CALCIUM MALABSORPTION, SECONDARY HYPERPARATHYROIDISM AND REDUCED BONE MINERAL DENSITY

Emanuel E. Strehler*, Zachary C. Ryan#, Adelaida G. Filoteo*, Elizabeth J. Cartwrightˆ, Ludwig Neysesˆ, Rajiv Kumar*#

**Dept. of Biochemistry and Molecular Biology and #Division of Nephrology and Hypertension, Dept. of Medicine, Mayo Clinic College of Medicine, Rochester, MN, USA; ˆInstitute of Cardiovascular Sciences, Manchester Academic Health Sciences Centre, Manchester M139PL, UK*

The plasma membrane calcium pump isoform 1 (PMCA1) is widely distributed throughout the body and is believed to be critical for the trans-cellular movement of calcium and overall calcium balance. The central role of PMCA1 in Ca^{2+} homeostasis in vivo has not been established because homozygous deletion of the Pmca1/Atp2b1 gene in the germ line is associated with embryonic lethality. We generated conditional knockout mice in which the Pmca1 is specifically deleted in intestinal epithelial cells (Pmca1EKO mice) by crossing “floxed” Atp2b1f/f (Pmca1f/f) mice with villin-Cre transgenic mice expressing the Cre-recombinase under the control of the villin promoter. Pmca1EKO mice were fertile but when crossed with Pmca1f/f control mice produced litters in which the number of Pmca1EKO pups born alive was lower than expected on the basis of Mendelian ratios. Pmca1EKO pups were stunted compared to their normal littermates and showed severe growth retardation at weaning and into adulthood. Pmca1EKO mice displayed characteristics of secondary hyperparathyroidism, including elevated serum concentrations of PTH and 1α,25-dihydroxyvitamin D3, an increased urinary phosphorus to creatinine ratio, and reduced bone mineral density of the spine and femur. Active intestinal Ca^{2+} transport in response to the administration of vitamin D was impaired in Pmca1EKO mice as determined by the everted gut sac method, suggesting the importance of hormonally regulated PMCA1-mediated Ca^{2+} absorption in the intestine. We conclude that PMCA1 is required for trans-cellular calcium transport and calcium absorption in the intestine, and thus plays an essential role in the establishment and maintenance of overall body calcium balance.

035

ENDOPLASMIC RETICULUM CALCIUM RELEASE THOUGH ITPR2 CHANNEL LEADS TO MITOCHONDRIAL CALCIUM ACCUMULATION AND SENESCENCE

Clotilde Wiel[1,2,3,4,6], Hélène Lallet-Daher[1,2,3,4,6], Delphine Gitenay[1,2,3,4], Baptiste Gras[1,2,3,4], Benjamin Le Calvé[1,2,3,4], Arnaud Augert[1,2,3,4], Mylène Ferrand[1,2,3,4], Natalia Prevarskaya[5], Hélène Simonnet[1,2,3,4], David Vindrieux[1,2,3,4] and David Bernard[1,2,3,4]

[1]Inserm U1052, Centre de Recherche en Cancérologie de Lyon, Senescence escape mechanisms lab, [2]CNRS UMR5286, F-69000 Lyon, France. [3]Centre Léon Bérard, F-69000 Lyon, France. [4]Université de Lyon, F-69000 Lyon, France. [5] Inserm U1003, Equipe labellisée par la Ligue Nationale contre le cancer, Laboratory of Excellence, Ion Channels Science and Therapeutics; Université Lille I Sciences et Technologies, Villeneuve d’Ascq, F-59655 France.

Senescence is involved in various pathophysiological conditions. Besides loss of Rb and p53 pathways, little is known about other pathways involved in senescence. We have performed a loss-of-function genetic screen and have identified two calcium channels; ITPR2 (IP3R2) and MCU as new senescence regulators. We show that loss of ITPR2, known to mediate endoplasmic reticulum (ER) calcium release, as well as loss of MCU, necessary for mitochondrial calcium uptake, enable escape from oncogene-induced senescence (OIS). During OIS, ITPR2 triggers a calcium release from the ER, followed by mitochondrial calcium accumulation through MCU channels. Mitochondrial calcium accumulation leads to a subsequent decrease in mitochondrial membrane potential, ROS accumulation and senescence. This ER-mitochondria calcium transport is not restricted to OIS, but also involved in replicative senescence. Our results show a functional role of calcium release by the ITPR2 channel and its subsequent accumulation in the mitochondria.

036

PMCA2 IN HUMAN BREAST TISSUE WITH LACTATIONAL CHANGE AND IN HUMAN BREAST CANCER CELLS

Amelia A Peters[1], Wei C Lee[1], Eloise Dray[2], Chanel E Smart[3], Lynne Reid[3], Leonard da Silva[3], Sunil R Lakhani[3], Sarah J Roberts-Thomson[1], Gregory R Monteith[1].

School of Pharmacy, The University of Queensland[1], Brisbane, QLD; Queensland University of Technology[2], Brisbane, QLD; The UQ Centre for Clinical Research (UQCCR), The University of Queensland[3], Brisbane, QLD.

The plasma membrane Ca^{2+} ATPase isoform, PMCA2, pumps Ca^{2+} from the cytosol into the extracellular space. PMCA2 has a restricted tissue expression, and is expressed in cells including the cerebellar Purkinje cells and the cochlear hair cells. PMCA2 expression is increased in mouse mammary gland cells during lactation where it plays a major role in the excretion of Ca^{2+} into milk; however, PMCA2 expression has not been assessed in human breast tissue during lactation. We have previously shown that PMCA2 mRNA levels are elevated in some breast cancer cell lines and that pan-PMCA antisense inhibits the proliferation of MCF-7 breast cancer cells and reduces the percentage of S-phase positive cells. However, little is known about the consequences of PMCA2 silencing on human breast cancer cells. This study aimed to assess PMCA2 expression in breast tissue exhibiting lactational change and in human malignant breast samples. This study also aimed to assess the role of the PMCA2 isoform in breast cancer cell proliferation. Immunohistochemistry using a rabbit anti-PMCA2 ATPase polyclonal antibody showed membranous PMCA2 expression in the luminal epithelium of breast tissue exhibiting lactational change. Tissue microarrays were used to assess PMCA2 expression in human breast tumor samples. Nine of 96 breast tumours (9.4%) showed membranous PMCA2 staining. No significant correlation with estrogen, progesterone or HER2 receptor status was demonstrated. High-content imaging showed that knockdown of PMCA2 in MDA-MB-231 human breast

cancer cells is associated with a reduction in cell number and an inhibition of the percentage of S-phase positive cells. The effect of PMCA2 knockdown combined with various cytotoxics (cisplatin, doxorubicin or mitomycin C) on cell proliferation was assessed in MDA-MB-231 cells using a kinetic imaging system (IncuCyte). The proliferation curves showed that PMCA2 silencing promotes the effects of some cytotoxics. These findings indicate that PMCA2 protein is up regulated in human lactation and is a feature of some breast cancers. Inhibitors of PMCA2 in combination with other agents may represent a therapeutic strategy for women with breast cancers that overexpress this Ca^{2+} pump.

037

SODIUM/CALCIUM EXCHANGER IS UPREGULATED BY SULFIDE SIGNALING, FORMS COMPLEX WITH THE ß1 AND ß3 BUT NOT ß2 ADRENERGIC RECEPTORS

Markova J[1], Hudecova S[1], Soltysova A[1], Sirova M[1], Csaderova L[2], Lencesova L[1], Ondrias K[1], Krizanova O[1]

[1]Institute of Molecular Physiology and Genetics, Slovak Academy of Sciences and [2]Molecular Medicine Center, Slovak Academy of Sciences, Vlárska 5, Bratislava, Slovak republic

Hydrogen sulfide (H_2S) as a novel gasotransmitter regulates variety of processes, including calcium transport systems. Aim of our work was to determine effect of a sulfide signalling on the NCX type 1 (NCX1) expression and function in HeLa cells, to investigate the relationship of ß-adrenergic receptors with the NCX1 in the presence and/or absence of H_2S, and to determine physiological importance of this potential communication. As a H_2S donor, we used morpholin-4-ium-4-methoxyphenyl (morpholino) phosphinodithioate - GYY4137. We observed increased levels of the NCX1 mRNA, protein, and activity after 24 h of GYY4137 treatment. This increase was accompanied by elevated cAMP due to the GYY4137 treatment and was completely abolished, when the NCX1 was silenced. Increased cAMP levels would point to an upregulation of ß-adrenergic receptors. Indeed, GYY4137 increased expression of the ß1 and ß3 (but not ß2) adrenergic receptors. These receptors co-precipitated, co-localized with the NCX1, and induced apoptosis in the presence of H_2S. We assume that H2S potentiates signalling of the NCX1 and its reverse mode, in which there is an accumulation of calcium ions within the cell. H_2S can therefore contribute to altered calcium signalling. Our results suggest that sulfide signalling plays a role in regulation of the NCX1, ß1 and ß3 adrenergic receptors, their co-localization and stimulation of apoptosis, which might be of a potential importance in a cancer treatment.

038

ROLE OF INDIVIDUAL TYPES OF THE IP3 RECEPTORS IN CANCER

Krizanova O.

Institute of Molecular Physiology and Genetics, Slovak Academy of Sciences, Bratislava, Slovakia

IP_3 receptors are intracellular calcium channels localized mainly on the endoplasmic reticulum. Function of these receptors in tumor´s development, growth and metastasis formation is not fully elucidated yet. Although three known types of the IP_3 receptors share relatively high homology, their functions in the tumor cells differ and the question about pro-apoptotic or anti-apoptotic effect is an issue in many publications. It was already shown that the type 1 and 2 IP_3 receptors participate in the activation of inner, mitochondrial pathway of apoptosis, while the type 3 IP_3 receptor has a proliferative effect, thus promoting cancer cell growth and metastasis formation. Thus, activation of the type 1 or type 2 IP_3 receptors and blocking type 3 IP_3 receptors might be a potential challenge in a cancer treatment. Type 1 IP_3 receptors might not only induce apoptosis through the activation of mitochondrial pathway, but also affect transcriptional activity of several genes through variety of transcription elements and transcription factors (e.g. ATF6, OCT4, SOX2, RARE, etc.). This observation would suggest wider modulation of the transcriptional activity by IP_3 receptors of type 1, probably due to changes in the intranuclear calcium. Supported by grants APVV 51/0045/11; VEGA 2/0074/13 and CEMAN

039

IDENTIFICATION AND CHARACTERIZATION OF A NOVEL PROTEIN FAMILY AS A NEW COMPONENT OF TRPM8 REGULATION AND ITS IMPLICATION IN CANCER

Dimitra Gkika[1,9], Loic Lemonnier[1,9], George Shapovalov[1,9], Dmitri Gordienko[1,§], Céline Poux[2], Michela Bernardini[1,6], Alexandre Bokhobza[1], Gabriel Bidaux[3], Cindy Degerny[4], Kathye Verreman[4], Basma Guarmit[5], Mohamed Benahmed[5], Yvan de Launoit[4], Felix Viana[7], Rene J. Bindels[8], Alessandra Fiorio Pla[1,6], Natalia Prevarskaya[1].

[1]Inserm U1003, Equipe labellisée par la Ligue Nationale contre le cancer, Villeneuve d'Ascq, France; Université des Sciences et Technologies de Lille (USTL), 59655 Villeneuve d'Ascq Cedex, France; [2]CNRS UMR 8198, Villeneuve d'Ascq, France; GEPV, Université des Sciences et Technologies de Lille (USTL), 59655 Villeneuve d'Ascq Cedex, France; [3]Laboratoire Biophotonique Cellulaire Fonctionnelle, Institut de Recherche Interdisciplinaire, USR3078 CNRS, Parc scientifique de la Haute Borne, Villeneuve d'Ascq, F-59655 France; [4]CNRS UMR 8161, Institut de Biologie de Lille, Université de Lille—Nord de France, Institut Pasteur de Lille, 59019 Lille Cedex, France; [5]Inserm, INSERM U895, Centre Méditerranéen de Médecine Moléculaire, Hôpitall'Archet, 06204 Nice, France; [6]Department of Life Science and Systems Biology, University of Torino, 10123 Torino, Italy; [7]Instituto de Neurociencias de Alicante, Universidad Miguel Hernandez – CSIC, Apartado 18, San Juan de Alicante, 03550 Alicante, Spain; [8]Department of Physiology, Radboud University Nijmegen Medical Centre, 6500HB Nijmegen, the Netherlands; [9]contributed equally

to this work; [§]Current address: Laboratory of Molecular Pharmacology and Biophysics of Cell Signalling, Bogomoletz Institute of Physiology, State Key Laboratory of Molecular and Cell Biology, 01024 Kiev, Ukraine

TRPM8 is a cold sensor, highly expressed in the prostate among other non-temperature sensing organs, regulated by the downstream receptor-activated signaling pathways. However, little is known about the intracellular proteins necessary for channel function. Here, we identified two previously unknown proteins that we have named “TRPM8 Channel-Associated Factors” (TCAFs) as new TRPM8 partner proteins, and demonstrate that they are necessary for channel function. TCAF1 & 2 both bind to the TRPM8 channel and influence its cell surface trafficking while exerting opposing effects on its activity via the introduction of new kinetic states during gating. The functional interaction of TCAF1/TRPM8 leads to the inhibition of both prostate cancer cell velocity of migration and migratory persistence, as also suggested by the loss of expression of TCAF1 in metastatic human specimens. The identification of the TCAFs introduces a novel mechanism for the modulation of TRPM8 by which the channel activity is stabilized and reveals the pathophysiological role of the TCAF1/TRPM8 complex.

040

TARGETING MITOCHONDRIAL- AND ENDOPLASMIC RETICULUM-BCL-2 TO INDUCE APOPTOSIS IN CANCER CELLS

Akl H.[1], Vervloessem T.[1], La Rovere R.[1], Janssen A.[2], Monaco G.[1], Vandenberghe P.[3], Distelhorst C.W.[4], Welkenhuyzen K.[1], Missiaen L.[1], De Smedt H.[1], Parys J.B.[1], & Bultynck G[1].

[1]KU Leuven, Cellular and Molecular Medicine, Leuven, Belgium; [2]UZ Leuven, Development and Regeneration, Leuven, Belgium; [3]UZ Leuven, Human Genetics, Leuven, Belgium; [4]Case Western Reserve University, Cleveland, Ohio, USA

Bcl-2 is an oncoprotein, which contributes to cancer formation and progression, like in chronic lymphocytic leukemia (CLL) and in diffuse large B-cell lymphoma (DLBCL). Bcl-2 impinges its anti-apoptotic and proliferative roles at two different sites into the cell. At the mitochondria, Bcl-2 interacts via its hydrophobic cleft with pro-apoptotic members of the Bcl-2 family to inhibit apoptosis. Small molecules (like ABT-199) that disrupt this interaction have already entered the cancer therapy arena. At the endoplasmic reticulum (ER), Bcl-2 controls constitutively active Ca^{2+} signals that affect apoptosis and proliferation. The regulation of intracellular Ca^{2+} by Bcl-2 is mediated through its BH4 domain, which interacts with the inositol 1,4,5-trisphosphate receptor (IP_3R), an intracellular Ca^{2+}-release channel. We developed a peptide tool (TAT-IDPS) that targets the BH4 domain of Bcl-2, reverses its inhibitory action on IP_3Rs thereby triggering pro-apoptotic Ca^{2+} signaling in primary cells and in DLBCL cell lines. Based on their apoptotic blocks, DLBCL cell lines are classified into three groups. In one group the activation of Bax and Bak is prevented owing to Bcl-2. These cells are described as being ‘‘primed for death’’ and sensitive to BH3 mimetics, like ABT-199. Five “primed for death” DLBCL cell lines were compared for their apoptotic sensitivity toward TAT-IDPS and ABT-199 treatments. Analysis of the EC50 of the two components elucidates an opposite correlation between TAT-IDPS- and ABT-199-induced apoptosis in the DLBCL cell lines. Moreover, HA14-1 was reported to exert inhibitory properties on sarco/endoplasmic reticulum Ca^{2+}-ATPases (SERCA). Annexin-V/7AAD staining of OCI-LY-1 cells, which are resistant to TAT-IDPS, treated with HA14-1, TAT-IDPS and with both HA14-1 and TAT-IDPS showed a synergistic apoptotic effect of TAT-IDPS together with HA14-1. After chelating extracellular Ca^{2+}, cytosolic [Ca^{2+}] was detected in the different conditions. While neither TAT-IDPS nor HA14-1 did empty the ER Ca^{2+} store, the combination of the two drugs led to ER Ca^{2+} depletion. Hence, our findings indicate that some cancer cells require Bcl-2 at the mitochondria to prevent Bax activation, an action mediated by Bcl-2’s hydrophobic cleft, while others require Bcl-2 at the ER to prevent IP_3R hyperactivity, an action mediated by Bcl-2’s BH4 domain.

041

DIFFERENTIAL EFFECTS OF HISTONE DEACETYLASE INHIBITORS ON THE EXPRESSION OF PMCAS IN BREAST TUMOR CELLS

Karolina Varga[1], Katalin Pászty[2], Rita Padányi[3], Luca Hegedüs[1], Jean-Philippe Brouland[4], Béla Papp[5] and Agnes Enyedi[1,3]

[1]Institute of Enzimology, Research Centre for Natural Sciences, Hungarian Academy of Sciences, Budapest, Hungary; [2]Molecular Biophysics Research Group of the Hungarian Academy of Sciences and Department of Biophysics, Semmelweis University, Budapest, Hungary; [3]Department of Molecular Cell Biology, Hungarian National Blood Transfusion Service, Budapest, Hungary; [4]Service d’Anatomie et Cytologie Pathologiques, Hôpital Lariboisière, Paris, France; [5]Institut National de la Santé et de la Recherche Médicale, U978 and Université Paris-13, PRES Sorbonne Paris-Cité, Bobigny, France

Remodeling of intracellular Ca^{2+} homeostasis is an important step in cancer progression, which is controlled by several components of the Ca^{2+} signaling toolkit. It is reported in the literature that the plasma membrane Ca^{2+} ATPase (PMCA) proteins also contribute in this process because the expression of different PMCA isoforms was altered in several types of cancer cells. Previously we found marked PMCA4b upregulation during short chain fatty acid (SCFA)-induced differentiation of MCF-7 breast cancer cells that was further enhanced by phorbol 12-myristate 13-acetate (PMA), while there were no remarkable changes in the expression levels of the other PMCA isoforms. The altered PMCA4b expression led to changes in the Ca^{2+} signaling pattern, suggesting that the protein plays an important role in the Ca^{2+} clearance mechanisms of differentiated breast cancer cells. Underlying the importance of PMCA4b in the maintenance of Ca^{2+} homeostasis in mammary epithelium, next we examined the protein expression in normal breast tissue samples and found high PMCA4 expression in breast ductal epithelial cells. To gain more insight into the mechanisms of SCFA and PMA treatments, we investigated the effects of the PKC inhibitor GF 109203X hydrochloride on PMCA expression and found that the SCFAs and PMA act through different pathways. Because of the anticancer activity of the histone deacetylase inhibitors (HDACi), some of them are used in clinical trials either alone or in combination with other drugs. Therefore, we examined whether these FDA-approved epigenetic

drugs affected PMCA expression. We treated different types of breast cancer cells (MCF-7, ZR-75-1, BT-474, AU-565, MDA-MB-231) with suberoylanilide hydroxamic acid (SAHA) or valproate; and found a substantial upregulation of PMCA4b expression in MCF-7, ZR-75-1, BT-474 and AU-565 cells while the triple negative MDA-MB-231 cells did not respond in this respect. However, an enhanced expression of the PMCA1 isoform was observed in MDA-MB-231 cells upon valproate treatment. Our results show that PMCA expression is selectively regulated in breast cancer cell lines having different genetic background, and may provide molecular insights into the behavior of distinct breast cancer types.
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042

CALCIUM SIGNALLING IN HYPOXIA-INDUCED EPITHELIAL–MESENCHYMAL TRANSITION IN BREAST CANCER CELLS

[1]Iman Azimi, [2]Felicity M. Davis, [3]Paraic A. Kenny, [4]Erik W Thompson, [5]Sarah J Roberts-Thomson, [6]Gregory R Monteith.

[1,2,5,6]School of Pharmacy, The University of Queensland, Brisbane, QLD, Australia; [3]Department of Developmental and Molecular Biology, Albert Einstein College of Medicine, New York, New York, United States of America; [4]St. Vincent's Institute, Fitzroy, Victoria, Australia; University of Melbourne Department Surgery, St. Vincent's Hospital, Fitzroy, Victoria, Australia; [5]Institute of Health and Biomedical Innovation and School of Biomedical Sciences, Queensland University of Technology, Kelvin Grove, Queensland, Australia

Hypoxia as a hallmark of the cancer microenvironment induces epithelial-mesenchymal transition (EMT) in breast cancer cells, a process whereby cancer cells acquire a more invasive phenotype. We have recently characterised the remodelling of calcium (Ca^{2+}) signalling as a consequence of EMT induced by epidermal growth factor (EGF). In this study we aimed to characterise the remodelling of Ca^{2+} signalling in a model of hypoxia-induced EMT in MDA-MB-468 breast cancer cells. The induction of EMT by hypoxia (1% O_2) was confirmed by assessing the protein level of vimentin (after 48 h hypoxia) and mRNA levels of some of other EMT markers including vimentin, Snail, Twist, N-cadherin and the CD44/CD24 ratio (after 24 h hypoxia). The mRNA levels of fifty Ca^{2+} pumps, Ca^{2+} channels and receptors in the presence and absence of hypoxia (24 h) were assessed using real time RT-PCR. This led to identification of four specific targets which were significantly up-regulated in hypoxia compared to normoxia (21% O_2), including P2Y6. This purinergic receptor, showed a three-fold mRNA induction by hypoxia. Pharmacological inhibition of P2Y6 by its selective inhibitor MRS2578, significantly reduced cellular migration of the mesenchymal like MDA-MB-231 breast cancer cell line. Gene expression profiling of 458 breast tumours revealed elevation of P2Y6 in basal breast cancer subtypes compared to other subtypes. Furthermore, breast cancer patients with high P2Y6 levels showed reduced overall survival rates compared to patients with low levels of P2Y6 (total patient number = 1115, P-value = 0.019). In conclusion, alterations in Ca^{2+} signalling and P2Y6 purinergic receptor expression is a feature of hypoxia-induced EMT. Further studies are required to identify if a specific Ca^{2+} pump, Ca^{2+} channel or receptor may represent a target for the mesenchymal phenotype of breast cancer cells and offer a therapeutic strategy for the control of breast cancer metastasis.

043

THE ROLE OF THE PLASMA MEMBRANE Ca^{2+} PUMP PMCA4B IN Ca^{2+} HOMEOSTASIS AND MIGRATION OF BRAF MUTANT MELANOMA CELLS

Hegedus, L.[1,2], Garay, T.[3], Molnar, E.[3], Varga, K.[1], Grusch, M.[4], Kallay, E.[2], Hegedus, B.[3,5], Enyedi, A.[1,6]

[1]Research Centre For Natural Sciences, Hungarian Academy Of Sciences; [2]Department of Pathophysiology and Allergy Research, Medical University of Vienna; [3]2nd Institute of Pathology, Semmelweis University, Budapest; [4]Institute of Cancer Research, Medical University of Vienna; [5]Department of Thoracic Surgery, Medical University of Vienna; [6]Hungarian National Blood Transfusion Service, Budapest, Hungary

Targeting mutant BRAF fundamentally changed melanoma therapy. Nevertheless, BRAF mutant melanomas often show limited response due to resistance. While a number of resistance mechanisms have been identified, the role of Ca^{2+} signaling is rather unexplored. The Ca^{2+} extrusion proteins in the plasma membrane, the plasma membrane Ca^{2+}ATPases (PMCAs) are critical regulators of intracellular Ca^{2+} homeostasis, and thus regulate vital cellular processes. We treated several BRAF mutant and wild-type melanoma cell lines with the mutant BRAF-specific inhibitor vemurafenib and found that specifically in the cells with BRAF mutation PMCA4b expression was markedly enhanced. Immunocytochemical analysis showed that the upregulated PMCA4b protein was located mainly in the plasma membrane of these cells. In accordance with the increased expression of the PMCA the Ca^{2+} signal also changed characteristically and specifically; in the vemurafenib-treated BRAF mutant cells the intracellular Ca^{2+} concentration declined to basal level faster after the peak than in the untreated BRAF mutant cells. We used time-lapse video microscopy to analyze cell migration of BRAF mutant melanoma cells and found marked inhibition of migration after treating the cells with vemurafenib for 24 to 48 hours. Our experiments suggested that PMCA4b expression was downregulated in mutant BRAF cells; therefore, we stably expressed GFP-tagged PMCA4b in wild type BRAF (MEWO) and mutant BRAF (A375) cells to understand the correlation between altered PMCA4b expression and migratory characteristics of BRAF mutant cells. In both cell lines the newly introduced PMCA4b localized mostly in the plasma membrane that was further enhanced by treating the cells with vemurafenib. Interestingly, the PMCA4b expressing BRAF mutant A375 cells changed their morphology which became more evident after treatment. We analyzed the migratory behavior of these cells and found that PMCA4b overexpression inhibited cell migration specifically in BRAF mutant cells to a similar extent observed in the parental cells upon vemurafenib treatment. Our results suggest that PMCA4b plays an important role in the regulation of intracellular Ca^{2+} homeostasis and migratory characteristics of melanoma cells in a BRAF-specific manner.
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044

THE ROLE OF ORAI1 AND STIM2 IN PRIMARY HUMAN MELANOCYTES AND MELANOMA

Hedwig Stanisz, Stephanie Saul, Thomas Vogt, Markus Hoth, Alexander Roesch, Ivan Bogeski

Department of Biophysics, School of Medicine, Saarland University, 66421 Homburg, Germany; Department of Dermatology, Venerology and Allergology, University Hospital of the Saarland, Homburg, Germany.

UV radiation of the skin triggers secretion of hormones that activate plasma membrane receptors on melanocytes and induce rapid increase in intracellular Ca^{2+} levels, which is essential for proper cellular function. A fraction of these Ca^{2+} ions is released from the endoplasmic reticulum (ER) via the IP3 receptors, while the larger fraction enters the melanocytes through plasma membrane Ca^{2+} channels. In many cell types, this Ca^{2+} entry is mediated by Ca^{2+} release-activated Ca^{2+} channels which are composed of three Orai isoforms at the plasma membrane and two Ca^{2+}-sensing proteins at the ER, STIM1 and STIM2. Once ER Ca^{2+} levels drop, STIM proteins oligomerize and aggregate thereby activating Orai channels. Here, we show that both human melanocytes and melanoma express all Orai and STIM isoforms and that Orai1 and STIM2 are the predominant isoforms. In melanocytes, Orai1 channels control endothelin-1 induced melanogenesis and cell growth. In melanoma, the role of Orai1 and STIM2 is more complex. Our results indicate that silencing of Orai1 and/or STIM2 strongly correlates with enhanced proliferation and increased expression of MITF, a marker for proliferative melanoma phenotype. On the contrary, silencing of Orai1 and/or STIM2 significantly reduced melanoma invasive and migratory potential as well as the expression of JARID1B and BRN2, markers for a non-proliferative, tumor-maintaining melanoma phenotype. Immunohistochemistry of primary melanomas and lymph node metastases revealed an elevated Orai1/STIM2 expression in the invasive tumor rims. Our results imply a role for Orai1/STIM2 in skin pigmentation and their potential involvement in UV-induced stress responses of the human skin. In melanoma, pharmacological tuning of Orai1 and particularly STIM2 might prevent metastasis and render melanomas more susceptible to conventional therapy.

045

LOCALIZED STORE-OPERATED CALCIUM INFLUX REPRESSES CD95-DEPENDENT APOPTOTIC EFFECTS OF RITUXIMAB IN NON-HODGKIN B LYMPHOMAS

Pierre Vacher[1,2,3], Anne-Marie Vacher[1,2,3], Raphael Pineau[4], Isabelle Soubeyran[1,2,3], Celine Pangault[5,6,7], Karin Tarte[5,6,7], Pierre Soubeyran[1,2,3] Thomas Ducret[2,8,9], and Laurence Bresson-Bepoldin[1,2,3].

[1]Institut Bergonié, Comprehensive Cancer Centre, F-33000 Bordeaux, France; [2]Univ. Bordeaux, F-33076 Bordeaux, France; [3]INSERM, U916 VINCO, F-33000 Bordeaux, France; [4]Animalerie mutualisée, Univ. Bordeaux, F33400 Talence, France; [5]INSERM, UMR 917, Rennes, F-35043, France; [6]Univ. Rennes 1, Rennes, F-35043 France; [7]Centre Hospitalier Universitaire (CHU) Pontchaillou, F-35033 Rennes, France; [8]Centre de Recherche Cardio-Thoracique de Bordeaux, F-33000 Bordeaux, France; [9]INSERM, U 1045, F-33000 Bordeaux, France

The anti-CD20 monoclonal antibody Rituximab (RTX) is routinely used to treat B-cell malignancies. However, a majority of patients relapse. An improvement in the complete response was obtained by combining Rituximab with chemotherapy, at the cost of increased toxicity. In SUDHL4 and Raji cells, we observed that RTX provokes an IP3-dependent Ca^{2+} release and a subsequent activation of Store-Operated Ca^{2+} Entry (SOCE) through Orai1/STIM1 Ca^{2+} release-activated Ca^{2+} (CRAC) channels. Using Ca^{2+} imaging and immunofluorescence, we demonstrated that RTX induces the recruitment of CD20, CD95, Orai1 and STIM1 into a cluster (capping), eliciting a polarized Ca^{2+} entry. Inhibition of this Ca^{2+} entry sensitized lymphoma cell lines and primary human follicular lymphoma cells to Rituximab-induced caspase 8-dependent apoptosis in vitro, and improved the anti-tumoral effect of Rituximab in vivo. The activation of caspase 8 and the capping of CD95, hallmarks of the CD95 activation pathway, suggests that RTX-induced apoptosis was at least partly due to activation of the CD95 pathway. These results reveals that Ca^{2+} entry exerts a negative feedback loop on Rituximab-induced apoptosis, suggesting that associating CRAC channel inhibitors or hypocalcemic agents with Rituximab may improve the treatment of patients with B-cell malignancies.

046

TRPC1 AS A REGULATOR OF MIGRATION AND PROLIFERATION OF FOLLICULAR ML-1 THYROID CANCER CELLS

Asghar MY[1], Magnusson M[1], Kemppainen K[1], Blom N[2], Törnquist K[1,2]

[1]Åbo Akademi University, Biosciences, Turku, Finland; [2]The Minerva Foundation Institute for Medical Research, Helsinki, Finland

Human follicular thyroid cancer is a highly differentiated and aneuploid form of thyroid cancer. In aggressive forms of follicular thyroid cancers, as represented by the ML-1 cell line, metastasis to bones and lungs is common. Both the lipid sphingosine 1-phosphate (S1P), and members of the transient receptor potential canonical (TRPC) non-selective cation channels, are involved in maintaining normal cellular processes, but are also important regulators of cancer cell proliferation and migration. In normal thyroid cells, TRPC1, 3, 4 and -5, are expressed. In ML-1 cells, TRPC1, 3, 4, 5, 6 and -7 are expressed. In the present investigation, we examined the role of TRPC1 in cellular migration and proliferation. For this purpose, we created stable ML-1 shNT TRPC1 knock-down cells. Our results showed that the TRPC1 expression in TRPC1 knock-down cells was downregulated by 85% and 50% on mRNA and protein level, respectively. There was a significant decrease in serum-evoked invasion and migration of TRPC1 knock-down cells, as compared to mock-transfected control cells. Furthermore, in mock-transfected control cells, S1P significantly increased invasion and migration while there was no significant effect

of S1P on invasion and migration in TRPC1 knock-down cells. In addition, proliferation assay showed a significant and sustained decrease in the proliferation rate of TRPC1 knock-down cells due to a prolonged G1 phase of the cell cycle, compared to mock-transfected control cells. In knock-down cells, there was no effect on tumor suppressor p-53 expression, but a significant increase in the expression of the cyclin-dependent kinase inhibitors p-21 and p-27. Also, cyclin D2 and D3 were significantly down-regulated in TRPC1 knock-down cells. We conclude that in human follicular thyroid ML-1 cancer cells, TRPC1 is of significant importance in regulating invasion, migration and proliferation of these cells.

047

TRPC1 CHANNEL KNOCK-DOWN ATTENUATES THE EXPRESSION OF RECEPTORS INVOLVED IN MIGRATION OF ML-1 CELLS

Asghar Muhammad Yasir[1], Magnusson Melissa[1], Kemppainen Kati[1], Blom Nina[2], Törnquist Kid[1,2].

[1]Åbo Akademi University, Biosciences, Turku, Finland; [2]The Minerva Foundation Institute for Medical Research, Helsinki, Finland

TRPC1 is widely expressed in human tissues and is involved in regulation of many cellular processes, including cancer cell proliferation and migration. Human follicular thyroid cancer ML-1 cells express several forms of TRPC channels, including TRPC1. To investigate the role of TRPC1 in ML-1 cells, we generated stable TRPC1 knock-down cells. We showed that the proliferation, invasion and migration were significantly decreased in these cells, compared with control ML-1 cells. Previously, we have reported that sphingosine 1-phosphate (S1P), through S1P receptor 1 and -3, and VEGF receptor 2 (VEGFR2) regulate migration in ML-1 cells. Thus, we investigated the expression of S1P receptors in TRPC1 knock-down cells. In these cells S1P3, but not S1P1, and VEGFR2 were significantly downregulated. To corroborate our findings, we transfected wild-type ML-1 cells with a dominant negative, non-conducting TRPC1 mutant. In these cells, both S1P3 and VEGFR2 were downregulated. Furthermore, protein kinase C delta (PKCδ) was downregulated in TRPC1 knock-down cells, and decreasing PKC delta expression using siRNA, decreased the expression of S1P3. Our previous studies have shown that S1P3 regulates the expression of HIF-1 alpha and MMP2 and -9. In TRPC1 knock-down cells, HIF-1 alpha, MMP2 and -9 were downregulated. Furthermore, in TRPC1 knock-down cells, receptor-operated calcium entry was decreased, compared with control cells. To investigate whether the decreased receptor expression was due to attenuated calcium entry in the cells, the cells were incubated with the intracellular calcium chelator BAPTA-AM. In these cells, both S1P3 and VEGFR2 were downregulated. Furthermore, inhibiting calmodulin or calmodulin-dependent kinases with pharmacological inhibitors attenuated the expression of S1P3 and VEGFR2. We conclude that the expression of S1P3 and VEGFR2 is mediated by a calcium-dependent mechanism, and that TRPC1 has a crucial role in this process. This regulation is important for the migration of the thyroid cancer.

048

SERCA EXPRESSION DEFECTS IN CHOROID PLEXUS NEOPLASIA

Lamia Ait Ghezali[1,2], Atousa Arbabian[3], Astrid Jeibmann[4], Martin Hasselblatt[4], Giorgio G. Hallaert[5], Caroline Van den Broecke[6], Françoise Gray[7], Jean-Philippe Brouland[7], Nadine Varin-Blank[1,2], and Bela Papp[1,2]

[1]Institut National de la Santé et de la Recherche Médicale, UMR U978, Bobigny, France; [2]Université Paris-13, PRES Sorbonne Paris-Cité, 74 rue Marcel Cachin 93017 Bobigny, France; [3]UMR 168 CNRS, Institut Curie, 11, rue Pierre et Marie Curie, 75005 Paris, France; [4]Institute of Neuropathology, University Hospital Münster, Münster, Germany; [5]Department of Neurosurgery, Ghent University Hospital, Ghent, Belgium; [6]Department of Pathology, Ghent University Hospital, Ghent, Belgium; [7]AP-HP, Service d'Anatomie et Cytologie Pathologiques, Hôpital Lariboisière, 2, rue Ambroise Paré, 75010 Paris, France

SERCA-type calcium pumps (Sarco/Endoplasmic Reticulum Calcium ATPases) accumulate calcium in the endoplasmic reticulum (ER). Because ER calcium storage is critical for the initiation of second messenger-induced calcium signals, and is also required for intra-ER chaperone functions, SERCA-dependent calcium transport constitutes a key nodal point in the control of several important cell functions such as proliferation, programmed cell death or differentiation, and is also involved in highly specialized effector functions of various fully differentiated cell types. Choroid plexus is a highly specialized organ located in cerebral ventricles that by a complex set of homeostatic solute transport processes elaborates cerebrospinal fluid. Choroid plexus epithelial cells can give rise to benign or malignant tumors, and can also display hyperplastic changes, characterized by non-tumoral epithelial overgrowth. We show that normal mature choroid plexus epithelial cells express simultaneously the SERCA2 and SERCA3-type ER calcium pumps, and that SERCA3 expression is decreased or lost in choroid plexus tumors. In contrast with benign or malignant tumors, epithelial cells in choroid plexus hyperplasia express SERCA3 abundantly. SERCA3 expression in normal primary choroid plexus epithelial cells grown in vitro could be induced by various short chain fatty acid-type cell differentiation-inducing agents, including valproate. These observations show that ER calcium homeostasis is deficient, due to the loss of SERCA3 expression, in choroid plexus tumors when compared to normal epithelium, and suggest that SERCA3 immunohistochemistry may be useful to distinguish hyperplastic lesions from adenomas in this tissue. The loss of SERCA3 expression has been observed also in gastric, colon and breast carcinoma when compared to the corresponding normal tissue. Moreover, it has also been shown that SERCA3 expression is induced during the differentiation of colon and gastric carcinoma, of lung adenocarcinoma, as well as of various myeloid leukemia cell lines and primary malignant cells in vitro. Taken together these observations show that the loss of SERCA3 expression during tumorigenesis is a widespread phenomenon in tissues which express this protein in normal, fully differentiated cells, and that SERCA3 may serve as a useful new phenotypic tumor marker “by loss of expression” for these tissues.

049

SPHINGOSINE KINASE 1 MODULATES CALCIUM SIGNALING IN A COMPARTMENT-SPECIFIC MANNER

Ilari Pulli[1], Christoffer Löf[2], Tomas Blom[3], Kid Törnquist[1,4]

[1]Åbo Akademi University, Tykistökatu 6A, 20520 Turku, Finland; [2]University Of Turku, Kiinamyllynkatu 10, 20520 Turku, Finland; [3]University Of Helsinki, 00014 Helsinki, Finland; [4]Minerva Foundation Institute For Medical Research, Biomedicum Helsinki, 00270 Helsinki, Finland

Sphingosine kinase 1 (SK1) is an oncogenic protein that converts sphingosine to the lipid messenger molecule sphingosine-1-phosphate (S1P). Sphingosine is considered to be a pro-apoptotic lipid whereas S1P promotes cell survival. SK1/S1P signaling is known to act through direct intracellular mechanisms as well as through extracellular stimulation of G-protein coupled S1P receptors (S1PR1-5). While SK1 and S1P are known regulators of cytosolic calcium, their role in compartmentalized calcium signaling is poorly characterized. In the present study, we elucidate the role of SK1 in compartment-specific calcium signaling. For this, we created HeLa cell lines with depleted or overexpressed SK1, respectively, and employed targeted aequorin fusion proteins to measure calcium concentrations in specific organelles such as the mitochondria and the endoplasmic reticulum (ER), along with the plasma membrane (PM) and caveolin-1-enriched PM compartments. Fura-2 AM was used for assessment of cytosolic calcium. We show that SK1 modulates the inositol trisphosphate (IP_3)-induced release of calcium from the ER calcium stores without significantly affecting the initial rise of cytosolic calcium, whilst the pattern of cytosolic calcium oscillations is altered. Interestingly, the altered release of ER calcium is sensed by the mitochondria, whereas SK1 does not affect the intrinsic calcium uptake capacity of mitochondria in permeabilized cells. Furthermore, the SK1-mediated change in IP3-induced ER calcium release is registered at the caveolin-1-enriched compartments of the PM, but not at the overall PM. Also, SK1 induces a change in the caveolar microdomain of calcium during receptor-operated calcium entry (ROCE) without affecting the PM microdomain. The observed SK1-mediated change in ROCE at the caveolae is abolished by inhibition of S1PR1-3. In addition, we demonstrate that SK1 affects cellular respiration and cell migration. As calcium is involved in mitochondrial energy metabolism, and caveolae have been shown to play a role in cell migration, we find it possible that SK1 controls these processes through compartment-specific calcium regulation. Collectively, these data may help to explain some of the oncogenic actions of SK1.

050

CHANNEL COMPLEXE SK3/ORAI1/TRPC1 PROMOTING COLORECTAL CANCER CELL HCT116 MOTILITY: EFFECT OF CETUXIMAB AND AKT/RAC1 PATHWAYS

M. Guéguinou[1], T. Harnois[2], A. Gambade[1], A. Chantôme[1], J.P. Haelters[3], P.A. Jaffrès[3], G. Weber[1], P. Bougnoux[1], N. Deliot[2], N. Bourmeyster[2], B. Constantin[2], T. Lecomte[4], C. Vandier[1], M. Potier-Cartereau[1].

[1]INSERM UMR1069 « Nutrition, Croissance et Cancer », Université de Tours; [2]Equipe ERL 7368 CNRS Université de Poitiers; [3]CNRS UMR 6521-Université de Brest; [4]CNRS UMR 7292 Université de Tours, France

We recently identified in HCT116 colon cancer cells, a new channel complex involving SK3/Orai1/TRPC1 channels and playing a role in cancer cell migration. Whereas, in breast cancer cells SK3/Orai1 complex in constitutively expressed into lipid-raft (Chantôme et al.,), in HCT-116 cells, only SK3 is localized in lipid-raft and the formation of SK3/Orai1/TRPC1 complex into lipid-raft is SOCE-dependent and essential for cell migration. Indeed, Orai1 and TRPC1 moved next to SK3 into lipid-raft only after depletion of intracellular calcium store. SK3 delocalization outside lipid-raft, causes by an alkyl-lipid Ohmline, is associated with a loss of function of the complex reducing cell migration associated to AKT / Rac pathway inhibition. Pharmacological or molecular biology approaches (siRNA) directed against SK3, Orai1 and TRPC1 abort AKT/Rac activation and thereby reduce cell motility. The concept of KRAS and PIK3CA as a marker of anti-EGFR antibodies resistance in colorectal cancer has been validated, we used HCT116 cell Line (KRAS, PIK3CA mute) to test cetuximab or EGF effect on cell migration, SOC influx and AKT pathway. Surprisingly, cetuximab, like EGF, potentiates SOCE entry and cell migration associated with activation of AKT/Rac pathway requiring the formation of SK3/Orai1/TRPC1 complex into lipid rafts. Then, SK3/Orai1/TRPC1 complex would be essential to the activation of AKT/Rac pathway promoting tumor development. Activation of this complex could be a mechanism of resistance to cetuximab, especially in cancer cells having a somatic KRAS mutation. Targeting of this complex by Ohmline could be a way to overcome this resistance. Tumor expression SK3/Orai1/TRPC1 could be a biomarker predictive of the efficacy of cetuximab in treating colorectal cancer in terms of tumor response and survival.

Chantôme A et al., Pivotal role of the lipid Raft SK3-Orai1 complex in human cancer cell migration and bone metastases. Cancer Res. 2013.

051

MODELING APPROACH OF CALCIUM SIGNALING AND MITOCHONDRIAL METABOLISM DURING CELLULAR INVASION BY SHIGELLA

Benjamin Wacquier

Unité de Chronobiologie Théorique, Faculté des Sciences, CP231, Université Libre de Bruxelles, Boulevard du Triomphe, B-1050 Brussels, BELGIUM

Shigellosis is the most widespread and lethal diarrheal disease: each year, it is responsible for more than one million deaths over the world. The bacteria causing this pathology, Shigella, invades intestinal epithelium cells and quickly modifies their actin network. Experiments showed that this network modification generates a reduction of diffusivity around the invasion area, which allows the emergence of a localized increase in cytosolic calcium in response to the stimulation of IP3 synthesis induced by the bacteria. A similar calcium response is observed in mitochondria of infected cells. In this work, we used mathematical modeling to test the assumption that the cytosolic calcium increase induces an increase in

mitochondrial calcium, activating ATP production. This ATP is in turn necessary to modify the actin network.

To this end, we combined and modified two existing mathematical models in order to describe the link between calcium dynamics and mitochondrial metabolism. Firstly, we investigated the relationships between the variations in calcium concentration in different organites (cytosol, endoplasmic reticulum and mitochondria). The model can correctly reproduce some experimental measurements. Moreover, it underscores the crucial role of mitochondria in maintaining cytosolic calcium oscillations. The model, consisting of 9 ordinary differential equations, reproduces the behavior of crucial mitochondrial variables such as NADH, ATP or membrane potential.

Then, we took diffusion into account in the model, in order to study calcium dynamics in cells invaded by Shigella. The model, based on 9 partial differential equations, correctly reproduces the localized calcium responses that are experimentally observed in the cytosol and in mitochondria. This validates the link that was assumed between both responses. The model predicts that the bacteria induce an increase in ATP production in the invasion area, confirming the initial assumption of the close relationship between calcium variations and mitochondrial metabolism. Nevertheless, the model suggests that the increase in ATP production is primarily caused by the increase in the basal level of calcium (via PLC and IP3-receptors recruitment) rather than by the calcium peak itself.

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A NEW TYPE OF KINETICS EXPLAINS CALCIUM RELEASE VIA RYANODINE RECEPTORS IN SMOOTH MUSCLE CELLS: MATHEMATICAL MODELING APPROACH

Norma C Perez-Rosas, Agustin Guerrero-Hernández, Moisés Santillán-Zerón

Cinvestav-IPN, México.

Calcium ions (Ca^{2+}) modify cell behavior by stabilizing conformational states of effector proteins. The increase of the cytosolic Ca^{2+} concentration is the triggering signal for many physiological responses. An excess of Ca^{2+} ions is, however, toxic, for this reason cells invest a substantial amount of energy to keep low the activity of this ion in the cytoplasm (near 100 nM). The Ca^{2+} gradient between the lumen of Sarcoplasmic Reticulum/Endoplasmic Reticulum and cytoplasm is so large that the opening of release channels should lead to an immediate reduction of the luminal [Ca^{2+}]. However, simultaneous recording of both luminal and intracellular [Ca^{2+}] showed that Ca^{2+} release process is more complex than previously envisioned. These experiments have revealed that intracellular Ca^{2+} increases without any significant reduction of luminal [Ca^{2+}]. This certainly is not easy to explain based on the principle of mass conservation. We have developed a simple deterministic mathematical model to explain this situation. The main contribution of this model is the recognition that experimental data cannot fitted using standard kinetics of Ca^{2+} binding to proteins. The solution was to assume a new type of kinetics that we called Kinetics on Demand (KonD), where the number of Ca^{2+} binding sites increases in response to the binding of Ca^{2+}. This situation is not totally unheard of since calsequestrin increases the number of Ca^{2+} binding sites depending on the polymerization state [1]. Model parameters were estimated from available experimental data. Stability analysis of the mathematical model shows that both kind of kinetics have a single stable state for those estimated parameter values. However, KonD is more effective in maintaining Ca^{2+} homeostasis than traditional saturable kinetics.

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053

AMPK- AND BECLIN 1-INDEPENDENT AUTOPHAGY INDUCED BY RESVERATROL NEEDS INTRACELLULAR Ca^{2+} SIGNALING

Ram Chandra Paudel, Tomas Luyten, Ludwig Missiaen, Humbert De Smedt, Jan B. Parys & Geert Bultynck

KU Leuven; Laboratory of Molecular and Cellular Signaling; Department of Cellular and Molecular Medicine; Campus Gasthuisberg O/N-I box 802, Herestraat 49, BE-3000 Leuven, Belgium

We previously showed that canonical mTOR- and Beclin 1-dependent autophagy elicited by either starvation or rapamycin lead to enhanced Ca^{2+} signaling from the endoplasmic reticulum. Sensitized IP3R-mediated Ca^{2+} signaling was essential for both the starvation and rapamycin-induced canonical autophagy [1, 2]. In this study, we wanted to examine the role of intracellular Ca^{2+} signaling in non-canonical autophagy induced by resveratrol, a polyphenol associated with various beneficial properties like anti-ageing and anti-oncogenic [3, 4]. Firstly, resveratrol induced autophagy in a time- and concentration-dependent manner. Treatment of HeLa cells with 100 μM resveratrol for 4h significantly induced autophagy flux by inhibiting mTOR as observed by activation of S6 ribosomal kinase, phosphorylated ribosomal protein- S6, and phosphorylation of ULK1. However, autophagy induction by resveratrol seemed non-canonical, since it was partly independent of Beclin 1. Secondly, resveratrol acutely induced Ca^{2+} release from the ER in intact, but not in permeabilized cells by a mechanism hitherto unknown. The resveratrol-induced Ca^{2+} release could be prevented by depletion of the ER Ca^{2+} stores using thapsigargin, by pre-incubation of U-73122, a pharmacological inhibitor of phospholipase C or by overexpression of IP3-5-phosphatase, an IP3-degrading enzyme. These results indicate that resveratrol induces IP3 production and Ca^{2+} release via IP3R. Thirdly, resveratrol activated AMPK in HeLa cells. However, inhibition of AMPK could not inhibit resveratrol-induced autophagy. Interestingly, pharmacological inhibitor of PLC, U-73122 and inositol monophosphatase inhibitor, LiCl could partly inhibit resveratrol-induced autophagy, indicating the partial role of resveratrol-induced Ca^{2+} release. The intracellular Ca^{2+} played an essential role in resveratrol-induced autophagy as chelation of intracellular Ca^{2+} by BAPTA-AM could completely inhibit resveratrol-induced autophagy. In conclusion, our results show that the resveratrol induces intracellular Ca^{2+} release via IP3R by promoting IP3 signaling and this partly plays a role in resveratrol-induced autophagy. Moreover, resveratrol-induced autophagy is completely dependent on intracellular Ca^{2+} signaling.

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054

A NOVEL MECHANISM FOR Ca^{2+} UPTAKE BY ENAMEL FORMING CELLS

Nurbaeva MK[1], Eckstein M[1], Feske S[2], Lacruz RS[1]

[1]Dept. Basic Science and Craniofacial Biology, New York University College of Dentistry; [2]Dept. of Pathology, New York University Langone Medical Center

Enamel development is a complex process that includes at least two main stages, secretory and maturation stages. Both growth stages are regulated by enamel epithelial cells termed ameloblasts whose main function is to build a hydroxyapatite-based mesh of crystals. In mineralized enamel Ca^{2+} is the most abundant element, yet the mechanisms used by ameloblasts to actively transport Ca^{2+} remain poorly understood. Patients with mutations to Ca^{2+}-release activated Ca^{2+} channel (CRAC) proteins STIM1 and ORAI1 present with severe combined immunodeficiency and display severely hypocalcified enamel. A link is thus established between autoimmune disease and enamel formation. The main aim of this present study is to investigate how ameloblasts utilize Ca^{2+} release channels and functional store operated Ca^{2+} entry (SOCE) mechanisms. Here we present evidence for Ca^{2+} signaling in rat secretory stage enamel organ (SSEO) and in maturation stage enamel organ (MSEO) cells. SSEO and MSEO cells were isolated from rat mandibular incisors using key anatomical landmarks as a reference to separate these stages. RT-PCR demonstrated that rat SSEO and MSEO cells express all isoforms of inositol 1,4,5-trisphosphate receptor (IP_3R) but that ryanodine receptor (RyR) transcript level, although present, is negligible. Moreover, SSEO and MSEO cells expressed Orai (1-3) and Stim (1-2), with Orai1 and Stim1 being the predominant isoforms. Immunofluorescence studies on paraffin sections showed that Stim1 was localized to areas overlapping the endoplasmic reticulum (ER) whereas Orai1 was localized to the plasma membrane. Fura-2 Ca^{2+} measurements by Flex Station identified that enamel organ cells from both developmental stages are equipped with functional SOCE. To identify CRAC characteristics, enamel cells were pre-treated with CRAC channel blockers (Synta-66, 3µM, BTP-2, 100nM, 2-APB, 50µM). In SSEO and MSEO cells, thapsigargin (1 µM) induced Ca^{2+} entry was significantly inhibited in pre-treated cells relative to controls (non-treated). Taken together, these data provide a novel mechanism for Ca^{2+} uptake by enamel cells whereby IP_3R is the main ER Ca^{2+} release channel with CRAC being an important pathway for Ca^{2+} entry into ameloblasts during enamel formation and crystal growth.

055

PHENOTYPE AND FUNCTION-SPECIFIC Ca^{2+} DYNAMICS IN PRIMARY HUMAN NEUTROPHILS

Katrin Hübner[1], Irina Surovtsova[1], Maria Hänsch[2] and Ursula Kummer[1]

[1]University of Heidelberg, Bioquant, Institute of Zoology, Im Neuenheimer Feld 267, 69120 Heidelberg, Germany; [2]University of Heidelberg, Institute of Immunology, Im Neuenheimer Feld 305, 69120 Heidelberg, Germany

Activating human neutrophilic leukocytes includes distinct processes such as adhesion, migration, degranulation and phagocytosis. These represent a variety of cellular states in which neutrophils translate chemotactic signals into state-dependent functional behaviour. Many of these processes are accompanied by substantial morphological changes whose observation is nicely facilitated by live cell imaging techniques. We recently studied single primary human neutrophils and applied multivariate live-cell imaging to investigate the cell-to-cell variability at the morphological as well as at the level of calcium dynamics. We observed that distinct functional states correlate well with specific calcium dynamics [1]. To investigate the mechanisms underlying these different calcium dynamics we have now developed a dynamic model of the integrin-induced activation of intracellular calcium signalling. We incorporated all major processes known to play a role in human neutrophils in detail. This also includes processes occuring in the mitochondria in addition to the well-studied uptake and release from the ER. Finally, transport processes at the plasma membrane are included as well. This model allows us to reproduce the experimentally observed phenomena and to analyse the dependence of the dynamics on the individual components of the signalling machinery.
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F-ACTIN INHIBITOR INDUCED INTRACELLULAR INFLUX OF CALCIUM ACROSS THE PLASMA MEMBRANE OF B CELLS REQUIRES EXPRESSION OF IGD

Martin Becker, Michael Reth and Palash Chandra Maity*

BIOSS Centre for Biological Signalling Studies, University of Freiburg, Department of Molecular Immunology, Institute of Biology III, University of Freiburg, Max-Planck-Institute of Immunobiology and Epigenetics, Freiburg

Elevation of cytoplasmic calcium levels is an early event of B cell antigen receptor (BCR) signaling. A BCR monomer consists of a membrane-bound antibody, non-covalently associated with the, immunoreceptor tyrosine-based activation motif (ITAM) containing, signaling subunits Igα and Igβ. Naïve B cells express two BCR isotypes, namely IgM and IgD, both having identical antigen specificity. Antigen binding to the BCR triggers a tyrosine-kinase cascade, leading to ITAM phosphorylation that is accompanied by two waves of cytoplasmic calcium influx and cytoskeleton remodeling. The first wave of calcium is

released from the endoplasmic reticulum (ER) as a consequence of inositol-triphosphate receptor (IP3R) activation. The second wave is generated by calcium influx across the plasma membrane (PM). The ER-resident Stim-1/-2 proteins sense the ER calcium store depletion and interact with PM-resident calcium channel Orai1 to allow influx from the extracellular space. Interestingly, disruption of the actin cytoskeleton, using the F-actin inhibitor latrunculin A (LatA) also induces calcium influx in a BCR dependent manner. However the molecular mechanism underlying LatA induced calcium signaling in B cells remains unclear. Here we show that LatA induced calcium influx originates primarily from the extracellular space and requires expression of the IgD-BCR, but not the IgM-BCR. Congruently, LatA induced calcium influx is impaired in IgDneg precursor B (pre-B) cells from the bone marrow and B1a cells from the peritoneal cavity. Surprisingly, LatA induced signaling, unlike BCR stimulation, is not accompanied by increased ITAM phosphorylation of Igα. In agreement with this we find that the Igβ subunit is essential for LatA induced calcium flux in B cells. Besides we find that LatA induced signaling is independent of both IP3R and Orai1, but sensitive to PM cholesterol content. Our data suggests the presence of an alternative plasma membrane calcium entry pathway in naïve B cells. Taken together, signaling through this pathway substantially differs from conventional BCR signaling, but requires the IgD-BCR. Thus our study also reveals a significant difference between the association of IgD and IgM-BCRs with the actin cytoskeleton.

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JUNCTATE DRIVES ER-PHAGOSOME MEMBRANE CONTACT SITE FORMATION THAT PROMOTES PERIPHAGOSOMAL Ca^{2+} MICRODOMAINS

Daniele Guido, Paula Nunes and Nicolas Demaurex

Department of Cell Physiology and Metabolism, University of Geneva, Geneva, Switzerland

Background: Ca^{2+} hotspots that boost phagocytosis are generated by Endoplasmic Reticulum-Phagosome membrane contact sites (ER-Ph MCS), and Stromal interaction molecule 1 (STIM1) is fundamental for Ca^{2+}-dependent recruitment of ER cisternae to phagosomes. Here, we sought to clarify which are the mechanisms that underlie ER-Ph MCS formation. Recent studies showed that STIM1 localization at ER-plasma membrane junctions is regulated by binding junctate via its luminal domain. Methods: To test the role of STIM1-mediated junctate binding in ER-Ph MCS formation, junctate-YFP was expressed in STIM1$^{-/-}$ knockout mouse embryonic fibroblast (MEFs) rendered phagocytic by ectopic expression of Fc-gamma-RIIA receptors. Confocal microscopy, Fura-2 and Fluo-8 imaging as well as electron microscopy were used to assess phagocytosis, global calcium, local calcium and MCS formation respectively. Results: Surprisingly, using confocal microscopy the overexpression of junctate-YFP in STIM1$^{-/-}$ MEFs revealed that junctate can increase the phagocytic capability independently of STIM1 and also the number of MCS around the phagosomes. In wild-type (WT) cells the exogenous expression of junctate also increased the phagocytic index but not the number of contact sites. The overexpression of junctate did not cause an increase in global calcium elevations in either WT or STIM1$^{-/-}$ MEFs. Ca^{2+} live imaging experiments, both in Ca^{2+} containing and Ca^{2+} free medium, show that junctate has a role in the regulation of periphagosomal Ca^{2+} microdomains. Finally electron microscopy showed that the overexpression of junctate, both in STIM1$^{-/-}$ and WT MEFs, can increase the length of the MCS around the phagosome but not their frequency.
Conclusions: These data indicate that junctate can replace the STIM1 pro-phagocytic function by creating Ca^{2+} microdomains around phagosomes.

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CHARACTERIZATION OF THE TRAFFICKING AND FUNCTIONAL PROPERTIES OF THE LONG STIM1 ISOFORM

Sophie Saüc[1,2], Monica Bulla[2], Ana Marchetti[2], Laurent Bernheim[1], Pierre Cosson[2], Maud Frieden[1,2] and Nicolas Demaurex[2]

From the Departments of [1]Basic Neurosciences and [2]Cell Physiology and Metabolism, Geneva Medical Center, 1, rue Michel Servet, 1211 Geneva 4, Switzerland

Store-Operated Ca^{2+} entry (SOCE) is a ubiquitous mechanism of Ca^{2+} entry involved in many cellular processes. SOCE is triggered by the Ca^{2+} depletion of the ER which initiates the oligomerization of the ER Ca^{2+} sensor STIM1 and its translocation to the plasma membrane (PM) where it aggregates into punctate structures corresponding to cortical ER subdomains (cER). Whereas in non-excitable cells SOCE takes 1-2 min to develop, in muscle cells influx occurs within 15-20 sec. The mechanism explaining the rapid Ca^{2+} influx of muscle remained unknown until our recent discovery of a longer STIM1L splice variant containing 106 additional amino acids. STIM1L interacts with actin filaments to form permanent clusters close to the PM that colocalize with Orai1 channels before ER depletion. Whether STIM1L remodels the ER to gate Orai channels is not established yet. As STIM1 and STIM1L are both expressed in muscle cells, we used a MEF cell line knocked-out for STIM1 and re-expressed either YFP-STIM1 or YFP-STIM1L to study the trafficking of each isoform independently. Using Ca^{2+} imaging and TIRF microscopy we observed that although STIM1L elicited a Ca^{2+} influx comparable to STIM1, it recruited much less additional PM clusters upon ER depletion and completely failed to enlarge PM clusters. Electron microscopy showed that unlike STIM1, STIM1L did not enlarge cER cisternae. Expression of a STIM1L mutant lacking the actin binding domain partially recapitulated the STIM1 phenotype, with no cluster enlargement but an increase of new PM clusters. Unexpectedly, Mn^{2+} quench experiments revealed that SOCE activation was slow (2 min) in MEF cells expressing either STIM1 or STIM1L together with Orai1. RFP-Orai1 channels were diffusely distributed at the PM in these cells and clustered with slow (1-3 min) kinetics upon store depletion. These results indicate that 1) STIM1L mediates SOCE without enlarging PM clusters or elongating cER cisternae, 2) cluster appearance and enlargement are 2 separable mechanisms, and 3) Orai1 clustering, rather than STIM1L recruitment at the PM, appears to be the rate-limiting step for SOCE.

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MOLECULAR BASIS OF SARCOPLASMIC RETICULUM ORGANIZATION IN SKELETAL MUSCLE FIBERS

Rossi D, Bencini C, Maritati M, Benini F, Lorenzini S, Pierantozzi E, Scarcella AM, Paolini C, Protasi F, Sorrentino V

Department of Molecular Medicine and Development, University of Siena, 53100 Siena, Italy.

The sarcoplasmic reticulum (SR) is a specialized form of endoplasmic reticulum that plays a key role in the regulation of muscle contraction by controlling release of Ca^{2+} following membrane depolarization in a process named excitation-contraction (e-c) coupling. In skeletal muscle, e-c coupling occurs at specialized intracellular junctions, named triads, where two terminal cisternae of the SR are positioned to flank, on opposite sites, a transverse (T)-tubule. The terminal cisternae membrane that faces the T-tubule represents the junctional SR. This is the SR region where the ryanodine receptors Ca^{2+} release channels and additional proteins, including triadin, junctin and calsequestrin, assemble into a large multi-protein complex. The domains where this machinery assembles are also referred to as Calcium Release Units (CRUs). The junctional SR is selectively aligned with the junction between the A and the I bands of the sarcomere and is in direct connection with the longitudinal SR, a large network
of tubules that are interconnected one with each other in forming a network that surrounds individually each single myofibril and that covers both the A and the I bands of the sarcomere. Regardless of the distinction in junctional and longitudinal domains, tubules and cisternae of the SR share a continuous lumen delimited by a single continuous membrane, thus corresponding to a single, though very large, organelle.

It would then appear that, to efficiently position the Ca^{2+} store close to the contractile apparatus, the entire SR is organized to surround each individual myofibril and that proteins of the CRUs are precisely targeted to the junctional SR. In the recent years we have started to identify and characterize some of the proteins that contribute to stabilize the SR around the myofibrils and to dissect the complex molecular mechanisms that target and organize some of the proteins of the CRU at the junctional SR. Current knowledge on these issues will be presented.

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A MUTATION IN THE CASQ1 GENE CAUSES A VACUOLAR MYOPATHY WITH ACCUMULATION OF SARCOPLASMIC RETICULUM PROTEIN AGGREGATES

Rossi D, Vezzani B, Galli L, Paolini C, Toniolo L, Pierantozzi E, Spinozzi S, Barone V, Pegoraro E, Bello L, Cenacchi G, Vattemi G, Tomelleri G, Ricci G, Siciliano G, Protasi F, Reggiani C, Sorrentino V

Molecular Medicine Section, Department of Molecular and Developmental Medicine, University of Siena, 53100 Siena, Italy

A missense mutation in the calsequestrin-1 gene (CASQ1) was found in a group of patients with a myopathy characterized by weakness, fatigue and the presence of large vacuoles containing characteristic inclusions resulting from the aggregation of sarcoplasmic reticulum (SR) proteins. The mutation affects a conserved amino acid residue located in one of the high-affinity Ca^{2+} binding sites of CASQ1 and alters the kinetics of Ca^{2+} release in muscle fibres. Expression of the mutated CASQ1 protein in COS-7 cells showed a markedly reduced ability in forming elongated polymers, while both in cultured myotubes and in in-vivo mouse fibres induced the formation of electron-dense SR vacuoles containing aggregates of the mutant CASQ1 protein that resemble those observed in muscle biopsies of patients. Altogether, these results support the view that a single missense mutation in the CASQ1 gene causes the formation of abnormal SR vacuoles containing aggregates of CASQ1 and other SR proteins, results in altered Ca^{2+} release in skeletal muscle fibres and, hence, is responsible for the clinical phenotype observed in these patients.

061

CONTROL OF MITOCHONDRIA MORPHOLOGY BY CCDC51

A. Paggio[1],A. Montagna[1] ,D. De Stefani[1], R. Rizzuto[1]

Department of Biomedical Sciences, CNR Neuroscience Institute, University of Padua, Padua, Italy

Mitochondria are dynamic organelles that fuse and divide to form constantly changing tubular networks in most eukaryotic cells. Furthermore, regulation of mitochondrial dynamics is crucial for the health of the cells, because they are essential for cellular development and homeostasis, as well as apoptosis. Defects of these functions are associated with several human genetic disorders and metabolic disease. The cytoplasmic dynamin-related GTPase DRP1 plays a key role in mitochondrial fission, while Mfn1, Mfn2 and Opa1 are involved in fusion reaction. Here, we analyze the role of the protein CCDC51, that seems to be implicated in mitochondrial dynamics. CCDC51 is a protein located on the inner membrane mitochondria, it contains two trans-membrane domains and it can forms oligomers. The overexpression of CCDC51 in Hela cells leads to a reduction of mitochondrial membrane potential and a consequent reduction of mitochondrial Ca^{2+} uptake, a decrease in ATP levels and an increase in ROS production. Upon overexpression of CCDC51, mitochondrial morphology was altered from filamentous to punctuate structure suggesting excessive mitochondrial fission. Also, a loss of functions of endogenous CCDC51 by short interfering RNA (siRNA) resulted in elongated mitochondria. In particular, when CCDC51 is silenced and DRP-1 is overexpressed, the GTPase is unable to induce mitochondrial fragmentation and mitochondria appear elongated. Our results demonstrate that CCDC51 is essential for the correct functions of DRP-1 and we propose a possible role of this protein in the fission of the mitochondrial inner membrane.

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THE ROLE OF THE MITOCHONDRIAL CALCIUM UNIPORTER IN THE CONTROL OF SKELETAL MUSCLE MASS

G. Gherardi[1], A. Raffaello[1], G. Pallafacchina[1,2], I. Zamparo[1], A. Braga[1], D. De Stefani[1], L. Zentilin[3], M. Giacca[3], C. Mammucari[1], R. Rizzuto[1,2]

[1]Department of Biomedical Science, University of Padova, Padova, Italy; [2]Institute of Neuroscience, Consiglio Nazionale delle Ricerche, Padova, Italy; [3]Molecular Medicine Laboratory, International Centre for Genetic Engineering and Biotechnology, Trieste, Italy

The second messenger Ca^{2+} plays a key role in mitochondrial function: cytosolic Ca^{2+} transients, generated by physiological stimuli, elicit mitochondrial Ca^{2+} ($mtCa^{2+}$) uptake which stimulates aerobic metabolism. Accordingly, high amplitude increases in mt [Ca^{2+}] are detected in skeletal muscle mitochondria during contraction. The highly selective channel responsible for Ca^{2+} entry into mitochondria is the Mitochondrial Calcium Uniporter (MCU), whose molecular identity has been described three years ago. More recently the role of MICU1 and MICU2 has been disclosed. MICU1 and MICU2 are direct modulators of the pore-forming subunit (MCU) with opposite effects on channel activity, and form a regulatory dimer. Importantly, mutations of MICU1 have been identified in individuals with a disease phenotype characterized by proximal myopathy, learning difficulties and a progressive extrapyramidal movement disorder. Here we show that MCU is both required and sufficient for muscle mass maintenance and that MCU exerts a protective effect against atrophy. Thus we suggest that MCU plays a crucial role in muscle trophism and therefore represents a possible target of clinical intervention.

063

FUNCTIONAL INTERPLAY BETWEEN NA^+ CHANNELS AND MITOCHONDRIAL Ca^{2+} TRANSPORTERS CONTROLS THE GLOBAL Ca^{2+} DYNAMICS AND OXIDATIVE METABOLISM IN PANCREATIC B CELLS

Iulia I. Nita[1], Michal Hershfinkel[1], Chase Kantor[2], Guy A. Rutter[2], Eli C. Lewis[3], Israel Sekler[1]

[1]Department of Physiology and Cell Biology, Faculty of Health Sciences, Ben Gurion University of the Negev, Israel; [2]Section of Cell Biology, Division of Diabetes, Endocrinology and Metabolism, Department of Medicine, Faculty of Medicine, Imperial College London, London, UK; [3]Department of Clinical Biochemistry, Faculty of Health Sciences, Ben Gurion University of the Negev, Israel

Na^+ channels are abundantly expressed in pancreatic B cells and activated by glucose. However, their role in regulating cytosolic and mitochondrial Ca^{2+} transients and thereby oxidative metabolism is poorly understood. We investigated the communication between Na^+ channels and mitochondrial Ca^{2+} transporters by combining fluorescent Na^+, Ca^{2+} and ATP imaging, electrophysiological analysis with tetrodotoxin (TTX) dependent block of the Na^+ channel and molecular manipulation of mitochondrial Ca^{2+} transporters. We show that TTX inhibits glucose dependent depolarization and blocks cytosolic Na^+ and Ca^{2+} responses and their propagation into mitochondria. Knockdown of the mitochondrial Ca^{2+} uniporter (MCU) expression largely blocked the TTX-sensitive mitochondrial Ca^{2+} influx. Furthermore, the TTX-dependent mitochondrial Ca^{2+} rise up regulated mitochondrial metabolism and enhanced ATP production. In addition, Na^+ dose responses analysis and knockdown of the mitochondrial Na^+/Ca^{2+} exchanger (NCLX) demonstrated that NCLX is tuned to sense and activated by TTX-sensitive cytosolic Na^+ rise. All together, our results indicate that Na^+ channels initiate cytosolic Na^+ and Ca^{2+} signals that are propagated by MCU and NCLX into mitochondria, thereby shaping both global Ca^{2+} transients and metabolism in pancreatic B cells.

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RASD1 REGULATES ACTIVATION OF TRPC4 VIA Gαi INDEPENDENT OF G PROTEIN COUPLED RECEPTOR

Jinhong Wie, Ju-Hong Jeon, Insuk So

Department of Physiology and Institute of Dermatological Science, Seoul National University College of Medicine, Seoul, Republic of Korea

Canonical transient receptor potential (TRPC) channels have six transmembrane (6-TM) domains and are Ca^{2+}-permeable and non-selective cation channels. It is generally speculated that TRPC channels are activated by stimulation of Gq-PLC-coupled receptors and oxidation. Activator of G-protein signaling1 (AGS1 or RASD1), the ras-related protein, interacts with Gi/Go and activates heterotrimeric G-protein signaling systems independent of G-protein-coupled receptor (GPCR). It is previously reported that AGS1 is related to GIRK channel and Ca^{2+} channel. However it is unknown whether AGS1 is associated with TRPC channels. We assumed that AGS1 might regulate TRPC4 channel, since AGS1 interacts with Gi/Go and TRPC4 is activated by Gi/o subunits. Here, we measured whole cell current of TRPC4/5 after the co-expression of TRPC4 or TRPC5 with constitutively active form of small GTPases in HEK293 cells. AGS1 (CA) mutant (Q to L) activated TRPC4 (38.8 ± 7.2 pA/pF) without GTPγS and independently of GPCR. Pertussis toxin (PTX), Gαi specific inhibitor, blocked RASD1-activated TRPC4 current (3.4 ± 1.6 pA/pF). When co-expressed with dominant negative Gαi protein subtype, TRPC4 activation by RASD1 was completely inhibited. With previous reports that TRPC4 is activated primarily by selective Gαi subunits rather than Gαq, these results suggest that AGS1 activates TRPC4 channel through modulating Gαi subunits and AGS1 is a new activator for TRPC4 channel.

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INVOLVEMENT OF T-TYPE CHANNELS IN ACCUMULATION OF CALCIUM STORES IN MOUSE EGGS

Carmen J. Williams, Miranda L. Bernhardt

Laboratory of Reproductive & Developmental Toxicology, National Institute of Environmental Health Sciences/NIH, PO Box 12233, MD E4-05, Research Triangle Park, NC, USA 27709

Changes in intracellular calcium following fertilization are crucial for egg activation and initiation of embryonic development. During mouse oocyte maturation, calcium levels increase within the endoplasmic reticulum (ER) during maturation from germinal vesicle (GV) stage oocytes to metaphase II-arrested (MII) eggs. This calcium increase contributes to the enhanced calcium-releasing ability of MII eggs compared to GV oocytes. Because mRNAs encoding the T-type calcium channel subunits Cacna1h and Cacna1i are expressed at relatively high levels in MII eggs, we hypothesized that one or both mediated calcium entry during oocyte maturation or fertilization. To test this idea, MII eggs were collected from Cacna1h$^{-/-}$ female mice, and calcium dynamics were examined. Following fertilization, the length of the first calcium transient was significantly shorter in Cacna1h$^{-/-}$ compared to Cacna1h$^{+/-}$ eggs, a finding that could be explained by a reduction in ER calcium stores. Indeed, there was a significant reduction in the total calcium content of Cacna1h$^{-/-}$ eggs as indicated by a lower amount of calcium released in response to ionomycin. Furthermore, when the sarco-ER calcium ATPase inhibitor, thapsigargin, was used to elicit calcium release, the difference between calcium stores in Cacna1h$^{-/-}$ eggs and Cacna1h$^{+/-}$ eggs was more substantial than with ionomycin. These findings indicated that ER and not other sources of intracellular calcium are primarily impacted by lack of CACNA1H. Calcium stores did not differ significantly between Cacna1h$^{-/-}$ and Cacna1h$^{+/-}$ GV oocytes, indicating that the difference in ER calcium stores arises during oocyte maturation. To determine whether reduced ER calcium is a direct consequence of loss of T-type channel activity during oocyte maturation, GV oocytes from wild type mice were matured in the presence or absence of the T-type channel inhibitor mibefradil. Both MI and MII oocytes showed dramatic reductions in thapsigargin-induced calcium release following this treatment. Culture in mibefradil did not impact calcium stores when oocytes were maintained in GV stage arrest using milrinone. Overall, these data show that calcium influx via T-type channels is one source of the maturation-associated increase in ER calcium stores required for a physiological calcium response at fertilization.

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IDENTIFICATION OF MEMBRANE TARGETING DOMAIN OF TRPC4 CHANNEL IRRELEVANT TO ITS FORMATION OF TETRAMERIC STRUCTURE

Insuk So, JongYun Myeon, Chansik Hong, Misun Kwak, Ju-Hong Jeon

Seoul national university graduate school, biomedical science building 101, 103 daehakro, Jongro-gu, Seoul 110-799, Korea

Canonical transient receptor potential (TPRC) channels are Ca^{2+}-permeable nonselective cation channels that are activated by a wide variety of stimuli, including a family of G protein coupled receptor (GPCRs). TRPC4 channel expresses in punctate distribution in the membrane. To find the regulating region of channel trafficking to the membrane, we generated deletion mutants of TRPC4 channel. We found that when either region of downstream of 20 amino acid of N-terminal or 700-730 amino acid was deleted, the mutants were retained in endoplasmic reticulum. By coexpression with wild type of TRPC4 with deletion mutants, we found that 23-29 amino acid of N-terminal regulates membrane trafficking. And by FRET method, we found that downstream of 99 amino acid of N-terminal and 700-730 amino acid region make assembly of TRPC4 tetramers. We inferred the candidate proteins that regulate or interact with 23-29 domain of TRPC4.

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RAGE MODULATES THE EXPRESSION OF S100 PROTEINS IN MELANOMA TUMORS

Varsha Meghnani, Anil Wagh, Benedict Law, Stefan Vetter and Estelle Leclerc

Department of Pharmaceutical Sciences, North Dakota State University, Fargo, ND 58102.

In recent years, the receptor for advanced glycation end products (RAGE) has emerged as a new potential therapeutic target in many diseases including cancer. We previously showed that RAGE levels were highly variable among human melanoma tumor samples, with up to 50-fold difference among samples. Based on these data and the work from others, we hypothesized that RAGE contributes to melanoma development in sub-populations of melanoma tumors where RAGE is over-expressed. To test our hypothesis, we transfected melanoma cells with RAGE and analyzed the effect of RAGE overexpression on the behavior of these cells. We showed that RAGE overexpressing cells exhibited increased anchorage independent proliferation and migration rates compared to control cells.

To further understand how RAGE controls tumor growth, we generated melanoma tumors in nude mice, and compared the growth of tumors established from WM115-RAGE cells (RAGE tumors) with those established from WM115-MOCK cells (MOCK tumors). We observed that the RAGE tumors cells grew faster than the control MOCK tumors. We also showed that an anti-RAGE antibody could reduce the growth of RAGE tumors, suggesting a role of RAGE ligands in increased melanoma tumor growth. Finally, we identified five S100 protein RAGE ligands (S100B, S100A2, S100A4, S100A6 and S100A10) that were upregulated in melanoma tumors, in response to RAGE overexpression.

In conclusion, our study suggests that RAGE contributes significantly to melanoma tumor growth through the up-regulation of its S100 protein ligands.

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CALCIUM ELECTROPORATION FOR TREATMENT OF CUTANEOUS METASTASES; A RANDOMIZED DOUBLE-BLINDED PHASE II STUDY

Hanne Falk, Louise Wichmann Matthiessen, Julie Gehl

Department of Oncology, Herlev Hospital, Denmark

Introduction: Electroporation is a method; using electric pulses to transient permeabilize the cell membrane, enabling normally non-permeable molecules to enter the cell. This is used in cancer treatment to facilitate the uptake of chemotherapy, such as bleomycin, into cancer cells (electrochemotherapy). In vitro and in vivo studies have shown that that the combination of calcium and electroporation increases the intracellular calcium concentration, which leads to death of cancer cells (Frandsen et al, cancer research 2012). This opens the possibility to use calcium instead of bleomycin in electroporation treatments.
Method: A randomized double-blinded phase II study has been initiated to compare calcium electroporation and electrochemotherapy with intratumoral bleomycin on patients with cutaneous metastases of any histology (clinicaltrials.gov ID-NCT01941901). The randomization is performed separately on each metastasis into two treatment arms: 1) Calcium is administered intratumorally followed by electroporation. 2) Bleomycin is administered intratumorally followed by electroporation. It is a once-only treatment and the randomization code will be revealed after six months of follow up. Efficacy and safety will be evaluated by tumor response, adverse event monitoring and by histological samples.
Provisional results: The study is still ongoing and five out of fifteen patients have been treated and twenty-six metastases are now evaluable for response. Provisional response data indicate that calcium electroporation has an effect on patients with cutaneous metastases. The data shows complete response in all metastases not previously irradiated, whereas previously irradiated metastases show mixed responses. Final response analysis awaits study completion. According to adverse events; calcium treated metastases have shown less hyperpigmentation than bleomycin treated metastases. There have been no serious side effects to the treatment so far.
Discussion: Preliminary data from this study indicate that calcium electroporation is a safe and efficient treatment. Comparison with bleomycin electroporation awaits final data. Calcium electroporation is a simple and inexpensive treatment. It may be used by various specialists as calcium is not cytotoxic in itself and can advantageously be used also in low-income countries. This study is expected to be important in the evolution of the feasibility, efficacy and safety of calcium electroporation.

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CALCIUM ELECTROPORATION OF SPHEROIDS INDUCE CELL DEATH IN CANCER CELL LINES, NOT A NORMAL CELL LINE

Stine Krog Frandsen[1,#], Laure Gibot[2,#], Moinecha Madi[2], Julie Gehl[1,¤], Marie-Pierre Rols[2,¤]

[1]*Department of Oncology, Copenhagen university Hospital Herlev, Denmark;* [2]*Institute of Pharmacology and Structural Biology, CNRS, Toulouse, France*

# These authors have contributed equally to this work
¤ Corresponding authors

Background: Electroporation (EP) utilises application of short, high voltage pulses to induce transient cell permeabilization allowing passage of otherwise non-permeant molecules into the cell cytosol. This method is used clinically in combination with chemotherapeutic drugs (e.g. bleomycin) and is in clinical trial in combination with calcium. Calcium electroporation has previously been proven very efficient in inducing tumor necrosis in vivo through acute ATP depletion [1].
Methods: Calcium electroporation has been applied to three different cancer-spheroids (bladder, breast, and colon) and spheroids of normal cells (fibroblasts). Spheroids were treated with calcium, bleomycin, EP, calcium electroporation, bleomycin electroporation, or untreated controls. Spheroid growth was measured before treatment and at day 2, 3, and 4, and intracellular ATP level at 1, 4, 24, and 72 hours after calcium electroporation.
Results/Discussion: Growth measurements showed significant size reduction in all three cancer-spheroids treated with either bleomycin electroporation or calcium electroporation. Interestingly, the normal fibroblast spheroids were not affected on growth by either calcium electroporation or bleomycin electroporation. Results were verified by live/death staining using calcein-AM and EthD-1.
ATP measurements showed dramatic decrease in intracellular ATP level already 1 hour after treatment with calcium electroporation in all four spheroids.
Conclusion: Calcium electroporation is an efficient anti-cancer treatment that affects cancer cells but not normal fibroblasts. This potentially new anti-cancer treatment where cell death is induced by something as simple as calcium has a high potential due to its simplicity, low cost and likely fewer side effects.
[1] S.K. Frandsen, H. Gissel, P. Hojman, T. Tramm, J. Eriksen, and J. Gehl, “Direct therapeutic applications of calcium electroporation to effectively induce tumor necrosis,” Cancer Res., vol. 72, pp. 1336-1341, 2012.

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THE ROLE OF TRPM2 CHANNELS IN PARACETAMOL-INDUCED LIVER DAMAGE

Ehsan Kheradpezhouh[1,2], Greg Barritt[2] and Grigori Rychkov[1,3]

[1]*Discipline of Physiology, School of Medical Sciences, University of Adelaide,* [2]*Discipline of Medical Biochemistry, School of Medicine, Flinders University;* [3]*Discipline of Medicine, School of Medicine, University of Adelaide and South Australian Health and Medical Research Institute, Australia*

Rise in the cytoplasmic free Ca^{2+} concentration ([Ca^{2+}cyt]) is considered one of the main pathways of cell damage caused by the overproduction of reactive oxygen species (ROS). The relationship between oxidative damage and Ca^{2+} is complex and not well understood, but removal of extracellular Ca^{2+} often prevents oxidative stress-induced cellular death, which suggests a significant role for Ca^{2+} permeable channels. Transient receptor potential melastatin 2 (TRPM2) channels have been shown to function as redox sensors and to contribute to ROS-induced [Ca^{2+}cyt] rise [1]. TRPM2 is expressed in most

tissues, including liver, and is found in both plasma membrane and lysosomes. Gating of TRPM2 by H_2O_2, other reactive oxygen species and ADPR may have significant implications in pathophysiological processes [2]. Our recent research suggests that TRPM2 channels are essential in the mechanism of paracetamol-induced liver damage [3]. Paracetamol is the most commonly used analgesic and antipyretic drug. At the same time, paracetamol overdose is the most common cause of acute liver failure and the leading cause of liver failure requiring liver transplantation in developed countries. In hepatocytes, paracetamol overdose causes formation of ROS, deregulation of Ca^{2+} homeostasis, covalent modification of proteins, lipid peroxidation, and DNA fragmentation. Using patch clamping, Ca^{2+} imaging and confocal microscopy we show that treatment of hepatocytes with paracetamol results in activation of Ca^{2+} entry and a cation current similar to that activated by H_2O_2 or the intracellular application of ADPR. Furthermore, paracetamol-induced liver damage in TRPM2 knock-out mice, assessed by liver histology and the concentration of blood liver enzymes is significantly attenuated compared to wild-type mice. Results suggest that [Ca^{2+}cyt] rise induced in liver by paracetamol overdose is mediated by TRPM2 channels and that their blockade may prove useful in treatment of paracetamol overdose and other oxidative stress-induced liver diseases.

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2. Perraud, A.L, et al. TRPM2 Ca^{2+} permeable cation channels: from gene to biological function. Cell Calcium, 2003 33:519-531.
3. Kheradpezhouh E, et al., TRPM2 channels mediate acetaminophen-induced liver damage. PNAS, 2014 111:3176-81

071

ANALYSIS OF ELEMENTARY Ca^{2+} SIGNALS IN T-LYMPHOCYTES EVOKED BY MICROINJECTION OF NAADP

E. Gattkowski, B.P. Diercks, A.H. Guse, I.M.A. Wolf

The Calcium Signaling Group, Department of Biochemistry and Molecular Cell Biology, University Medical Centre Hamburg-Eppendorf, Hamburg, Germany

Nicotinic acid adenine dinucleotide phosphate (NAADP) is the most potent Ca^{2+} releasing second messenger known today. It acts in the initial phase of Ca^{2+} signaling. Current candidates for the NAADP receptor/Ca^{2+} channel are ryanodine receptor 1 (RyR1), the two-pore channel 1/2 and TRP-ML1. Cytosolic NAADP-binding proteins might be signal transducers to activate one or more of these channels. In Jurkat T-Lymphocytes, we have obtained evidence for NAADP-binding proteins (Walseth et al., 2012, Messenger 1, 86) and activation of RyR1 by NAADP (Dammermann et al., 2009, Natl. Acad. Sci. U.S.A. 106, 10678). Goal of this study was high resolution Ca^{2+} imaging of elementary, spatially restricted Ca^{2+} signals observed upon microinjection of physiological NAADP concentrations. Our high resolution Ca^{2+} imaging system allows image acquisition of up to 50 fps at a spatial resolution of 153 nm. NAADP evoked local Ca^{2+} signals that propagated into global Ca^{2+} signals, often in the form of a Ca^{2+} wave spreading over the whole cell. Interestingly, propagation of Ca^{2+} signals proceeded in 2 different ways in different cells: slowly or in a more rapid manner. Signal propagation was characterized in these 2 groups of cells. In cells with slower signal propagation (n=17) we detected at 20(or 25) ms after NAADP injection 1.1 spots/cell with an area of 0.8±0.2 μm^2 (SEM) and $[Ca^{2+}]_i$ max of 142±16 nM (n=20). After 100 ms the number of spots increased to 1.9 with decreased area of 0.5±0.05 μm^2 and the $[Ca^{2+}]_i$ max raised to 279±21 nM (n=33). In cells with faster signal propagation (n=18) the number of spots/cell increased from 2.2 to 3.3 with a massive increased area from 0.6±0.1 to 8.6±2.7 μm^2 and a raised $[Ca^{2+}]_i$ max from 361±39 (n=35) to 719±21 nM (n=50). Mechanistically, both local and global Ca^{2+} signals were abolished in a RyR knock down clone (#10, Langhorst et al., 2004, Cell. Signal. 16,1283). These data, obtained at increased temporal resolution of approx. 50 fps, as compared to 7 fps (Dammermann & Guse, 2005, J. Biol. Chem. 280, 21394), suggest a major role for RyR1 in NAADP-evoked Ca^{2+} signals occurring as early as 20 ms post microinjection.

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TRPP2-DEPENDENT Ca^{2+} SIGNALING IN DORSO-LATERAL MESODERM IS REQUIRED FOR KIDNEY FIELD ESTABLISHMENT IN XENOPUS

Mélinée Futel[1,2], Catherine Leclerc[3,4,5†], Ronan Le Bouffant[1,2], Isabelle Buisson[1,2], Isabelle Néant[3,4,5], Muriel Umbhauer[1,2], Marc Moreau[3,4,5] and Jean François Riou[1,2†]

[1]Université Pierre et Marie Curie-Paris VI, Equipe "Signalisation et Morphogenèse", UMR7622-Biologie du Développement, 9, quai Saint-Bernard, 75005 Paris France; [2]CNRS, Equipe "Signalisation et Morphogenèse", UMR7622-Biologie du Développement, 9, quai Saint-Bernard, 75005 Paris, France; [3]Université Toulouse 3, Centre de Biologie du Développement, 118 route de Narbonne, F31062 Toulouse, Cedex 04, France; [4]CNRS UMR5547, Toulouse, F31062, France; [5]GDRE 731 Ca^{2+} toolkit coded proteins as drug targets in animal and plant cells

In Xenopus embryos kidney field (KF) specification is dependent on retinoic acid (RA) signaling and coincides with a dramatic increase of Ca^{2+} transients, but the role of Ca^{2+} signaling in the KF is unknown. We identified TRPP2, a TRP superfamily member of channel encoded by the *pkd2* gene, as a central component of Ca^{2+} signaling in the KF. In KF cells TRPP2 is not associated to primary cilia, showing that its role is distinct from its cilium function. TRPP2 is strongly expressed at the plasma membrane where it may regulate extracellular Ca^{2+} entry. Knockdown of *pkd2* in the KF results in the down-regulation of pax8, but not of the other KF genes lhx1, osr1 and 2. In addition, this inhibition of Ca^{2+} signaling by an inducible Ca^{2+} chelator also causes pax8 inhibition, and the pkd2 knockdown results in a severe inhibition of Ca^{2+} transients in KF explants. We propose that TRPP2-dependent Ca^{2+} signaling is a key component of pax8 regulation in the KF potentially downstream of a RA non-transcriptional control of TRPP2.

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ACTIVATION OF TRPM2 BY ADENOSINE 5'-DIPHOSPHORIBOSE: STRUCTURE-ACTIVITY RELATIONSHIP

R. Fliegert[1], C. Moreau[2], T. Kirchberger[1], J.M. Swarbrick[2], S.J. Bartlett[2], T. Yorgan[1], A. Bauche[1], A. Harneit[1], B.V.L. Potter[2], A.H. Guse[1]

[1]The Calcium Signalling Group, Department of Biochemistry and Cell Biology, Center of Experimental Medicine, University Medical Center Hamburg-Eppendorf, Martinistr. 52, 20246 Hamburg, Germany; [2]Wolfson Laboratory of Medicinal Chemistry, Department of Pharmacy and Pharmacology, University of Bath, Claverton Down, Bath BA2 7AY, UK

TRPM2, a non-selective Ca^{2+} permeable cation channel, has a C-terminal domain with homology to the mitochondrial ADPR pyrophosphatase NUDT9. Binding of adenosine 5'-diphosphoribose (ADPR) to this domain has been shown to activate the channel resulting in Ca^{2+} influx and membrane depolarization finally leading to cell death/apoptosis due to Ca^{2+} overload. Since ADPR is produced in cells in response to exposure to reactive oxygen species, the ADPR/TRPM2 signaling pathway has been implicated to be involved in the pathogenesis of neurodegenerative diseases like Alzheimer's, stroke, diabetes and ischemic heart disease making it an attractive novel target for intervention. However, research in this area is currently impeded by the lack of specific pharmacological tools. We developed an initial structure activity relationship for ADPR at TRPM2 by chemically synthesizing novel ADPR analogues with modifications in different parts of the molecule and evaluating their effect on the activation of TRPM2 by ADPR in whole-cell patch clamp experiments [1]. Introduction of hydrophobic substituents at C8 of the adenine turns ADPR analogues into antagonists, with the bulkier and flat aryl group in 8-Ph-ADPR being more effective than bromine in 8-Br-ADPR. N7 of the adenine base seems to be important for binding to the NUDT9H domain as evidenced by the reduced antagonist activity of 8-Br-7-deaza-ADPR when compared to 8-Br-ADPR. Removal of the 2'-hydroxyl group from the adenine ribose turned 8-Ph-ADPR into the most active antagonist we found: 8-Ph-2'-deoxy-ADPR. The lack of effect of ADP on ADPR mediated TRPM2 activation shows that the terminal ribose is important, but it can be partially replaced by the smaller cyclopentyl group. The charges of the pyrophosphate are necessary for antagonist activity since the pyrophosphate cannot be replaced by a non-charged bioisostere. However, substitution of the pyrophosphate and the terminal ribose by a sulfamate did result in some antagonist activity and this compound might be a lead for future development of more drug-like structures. We also demonstrate that the ADPR analogue 8-Ph-ADPR inhibits Ca^{2+} signaling and chemotaxis in primary human neutrophils, a process that as we have previously shown depends on the ADPR/TRPM2 signaling system.

[1] Moreau et al., J Med Chem 2013

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THE C-TERMINAL TRANSMEMBRANE DOMAIN OF BCL-2 IS ESSENTIAL FOR ITS INHIBITORY FUNCTION ON IP_3R CHANNELS

Hristina Ivanova, Giovanni Monaco, Ludwig Missiaen, Humbert De Smedt, Jan B. Parys, Geert Bultynck

KU Leuven - Herestraat 49, 3000 Leuven, Belgium

The anti-apoptotic Bcl-2 protein was shown to fulfill its pro-survival functions not only via inhibiting the pro-apoptotic members, but also via direct regulation of the Ca^{2+} signaling trough binding to the inositol 1,4,5-trisphosphate receptors (IP_3Rs). Bcl-2 inhibits IP_3R-mediated Ca^{2+} signaling by dual targeting of the central, modulatory domain (CMD) and the 6th trans-membrane domain of IP_3R channels (TMD-IP_3R). The N-terminal BH4 domain of Bcl-2 is essential for the binding to the CMD and thereby for inhibiting IP_3R-mediated Ca^{2+} release. The molecular determinants of the interaction between Bcl-2 and TMD-IP_3R are not yet established. We investigated the potential role of the hydrophobic cleft, involved in the binding to the pro-apoptotic Bcl-2 proteins. Yet, neither mutating the hydrophobic cleft (Bcl-2GR/AA) nor treatment with BH-3 mimetic drugs affected the binding of 3xFLAG-Bcl-2 to a GST-fusion protein covering the 6th TMD and the C-terminus of IP_3R1. Hence, Bcl-2 binding to TMD-IP_3R seems independent of its hydrophobic cleft. Further studies will include the study on the effect of 3xFLAG-Bcl-2 versus 3xFLAG-Bcl-2GR/AA overexpression and of BH3 mimetic drugs on IP3R-mediated Ca^{2+} release. In any case, another domain in Bcl-2 seems responsible for IP3R interaction. We anticipate a role for the C-terminal TMD of Bcl-2 for IP_3R binding, since 3xFLAG-Bcl-2ΔTMD, in contrast to the 3xFLAG-Bcl-2, failed to bind TMD-IP_3R, purified as a GST-fusion protein. Consistent with this, overexpression of 3xFLAG-Bcl-2ΔTMD, in contrast to wild-type 3xFLAG-Bcl-2, failed to inhibit agonist-induced IP3R-mediated Ca^{2+} release in COS cells. Further studies aim to elucidate the role of Bcl-2's TMD for protecting against Ca^{2+}-dependent apoptosis. We hypothesize that the C-terminal TMD of Bcl-2 is important for anchoring Bcl-2 to IP_3Rs, thereby increasing the local concentration of the BH4 domain of Bcl-2 in the neighborhood of the CMD of IP_3R channels. Hence, this mechanism may account for the efficient inhibition of IP_3Rs observed upon Bcl-2 overexpression, while the isolated BH4 domain inhibits IP_3Rs with relatively low efficiency (IC50 of ~30 μM).

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THE INVOLVEMENT OF CALCIUM IN AUXIN-REGULATED PIN ENDOCYTOSIS

Himschoot, Ellie[1], Costa, Alex[2], Bonza, Christina[2], De Vriese, Kjell[1], Krebs, Melanie[3], Schumacher, Karin[3], Kudla, Jorg[4], Sheen, Jen[5], Boudsocq, Marie[6], Robatzek, Silke[7], Yoshioka, Keiko[8], Beeckman, Tom[1], Friml, Jiri[9], Vanneste, Steffen[1]

[1]Department of Plant Systems Biology, VIB and Department of Plant Biotechnology and Bioinformatics, Ghent University, Technologiepark 927, 9052 Ghent, Belgium; [2]Department of Biosciences, University of Milan, 5 Via G. Celoria 26, 20133 Milan, Italy; [3]Ruprecht-Karls-Universität Heidelberg, Centre for Organismal Studies, Developmental Biology of Plants, Im Neuenheimer Feld

230, 69120 Heidelberg, Germany; [4]Institut für Biologie und Biotechnologie der Pflanzen, Universität Münster, Schlossplatz 4, 48149 Münster, Germany; [5]Department of Molecular Biology and Center for Computational and Integrative Biology, Massachusetts General Hospital, Boston MA 02114, USA; Department of Genetics, Harvard Medical School, Boston, MA 02114, USA [6]URGV, INRA-UEVE-CNRS, 2 rue Gaston Crémieux, 91057 Evry cedex, France; [7]The Sainsbury Laboratory, Norwich Research Park, Norwich NR4 7UH, United Kingdom; [8]Department of Cell and Systems Biology, University of Toronto, Toronto, Ontario, Canada M5S 3B2; [9]Institute of Science and Technology Austria, Am Campus 1, 3400 Klosterneuburg, Austria

The phytohormone auxin is an important regulator of multiple growth and developmental processes in plants. Each of these processes is characterized by a specific auxin distribution pattern throughout the plant tissue. PIN-FORMED (PIN) proteins function as auxin efflux carriers and are crucial players for the establishment of such distribution patterns. PINs are asymmetrically localized in the plasma membrane thereby mediating polar auxin transport. It was demonstrated that auxin can regulate the directionality of its own transport by inhibiting PIN endocytosis. Recently, the apoplast-localized auxin receptor AUXIN BINDING PROTEIN 1 (ABP1) has been connected to auxin-inhibited PIN endocytosis via Rho-related GTPases from plants (ROP) and ROP-interactive CRIB motif-containing protein (RIC) signalling. The second messenger calcium (Ca^{2+}) is known to be involved in several auxin-regulated processes like protoplast swelling, stomatal opening, and root elongation. Furthermore, it was demonstrated that auxin can rapidly induce Ca^{2+} signals through a yet unknown mechanism. Here, we evaluated the possible involvement of Ca^{2+} in auxin-induced inhibition of PIN endocytosis. We found that Ca^{2+} is required for the inhibitory effect of auxin on PIN endocytosis. On the other hand, increasing cytosolic Ca^{2+} concentrations by inhibition of Ca^{2+} ATPases is sufficient to inhibit PIN endocytosis. These observations are consistent with a model in which inhibition of PIN endocytosis by auxin is mediated by activation of Ca^{2+} signalling.

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A NOVEL TRIMERIC COMPLEX OF S100P, EZRIN AND IQGAP1 AND THEIR MUTUAL REGULATION

Rathangadhara Nammalwar, Annika Heil, Volker Gerke

Institute for medical biochemistry, Center for molecular biology of inflammation, Münster, Germany

Intracellular Calcium signaling is a key regulatory mechanism governing diverse physiological processes such as cellular motility, neuronal transmission, cytoskeleton organization and regulation of ion channels. A co-ordinated network of different effector proteins is indispensable for efficient functioning of Ca^{++} signaling. S100 proteins are the largest family amongst such Ca^{++} effectors. They act in response to elevated Ca^{++} levels by interacting with and modulating, in a Ca^{++} dependant manner, proteins which regulate a wide range of cellular processes. S100 target proteins include regulators of calcium homeostasis, cell migration, apoptosis, cytoskeletal proteins, enzymes, receptors and transcription factors. Although a number of S100 target proteins have been discovered, the list is constantly growing. The S100 family consists of 24 EF hand containing Ca^{++} binding proteins discovered to date. Our work focuses on S100P, a 95 amino acid, 10.4 kDa protein that can function by regulating cell cortex dynamics. We have identified two S100P targets in the cell, the membrane cytoskeleton linking molecule ezrin and the cytoplasmic scaffold protein IQGAP1.

Ezrin contains an N-terminal PI(4,5)P2 binding region by which it interacts with the cell membrane and a C-terminal F-actin binding region by which it interacts with the actin cytoskeleton. Therefore it can provide a crosslink between the membrane and the actin cytoskeleton. S100P interaction leads to an activation of ezrin that unmasks the F-actin binding site and thereby can trigger the membrane-F-actin crosslinking activity. IQGAP1, on the other hand is a multi-domain protein which recruits and regulates proteins such as the MEK1\2, Erk, Cdc42, Rac1, CXCR2, E-Cadherin and ß-catenin. S100P interacts with IQGAP1 resulting in the down regulation of IQGAP1 function as a signalling intermediate.

Here, we report a novel trimeric complex involving S100P, Ezrin and IQGAP1. Co-immunoprecipitation experiments have shown that these three proteins exist in a cellular complex. This provides for the first time, evidence for a direct intracellular link between ezrin and IQGAP1 and thus a mutual regulation of these important cell cortex regulators.

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NAADP MEDIATED Ca^{2+} SIGNALING AND REGULATES AUTOPHAGY GLUTAMATE-INDUCED IN ASTROCYTES

Gustavo J. S. Pereira[1], Manuela Antonioli[5], Hanako Hirata[1], Claudia Bincoletto[1], Grant Churchill[2], Sandip Patel[3], Mauro Piacentini[4], Gian M. Fimia[5] and Soraya S. Smaili[1]

[1]Department of Pharmacology, Federal University of São Paulo, (UNIFESP), São Paulo, Brazil; [2]Department of Pharmacology, University of Oxford, UK; [3]Department of Cell and Developmental Biology, University College London, London, UK; [4]Department of Biology, University of Rome Tor Vergata, Rome, Italy; [5]Department of Epidemiology and Preclinical Research, National Institute for Infectious Diseases IRCCS 'Lazzaro Spallanzani', Rome, Italy

Autophagy is an evolutionarily conserved lysosomal degradation pathway, yet the underlying mechanisms remain poorly understood. Nicotinic acid adenine dinucleotide phosphate (NAADP) is a potent Ca^{2+} mobilizing messenger in astrocytes, since NAADP releases Ca^{2+} from the endo-lysosomal system and this effect was mediated by the two-pore channels (TPCs), especially TPC type 2. Some independent studies have been shown that the specificity of NAADP-mediated Ca^{2+} release is inhibited by Ned-19. Furthermore, Glutamate has been considered as a potential NAADP-agonist in neuronal and glial cells. But, whether and how NAADP, Ned-19 or Glutamate act in astrocytes is unclear and the functions on autophagy pathway have yet to be defined. The effects of NAADP-AM, Ned-19 and Glutamate and their relationship between on autophagy regulation were studied. We showed that NAADP-AM and Glutamate induced autophagy, since increased LC3-II levels in a time dependent-manner and were potentiated by lysosomal inhibitors (E64d/Pepstatin A) treatment. However, Ned-19

didn't cause a further induced LC3-II accumulation after (E64d/Pepstatin A) treatment. Additionally, we found that NAADP and Glutamate activated autophagy mTOR independent via ACC/AMPK pathway whereas Ned-19 could block the increase in LC3-II formation Glutamate-mediated. This event was accompanied largely by a reversal in the Glutamate-mediated changes on AMPK activity, but was not able to modulate mTOR downstream proteins. Because TPCs might be NAADP-sensitive Ca^{2+}-permeable channel in lysosomes, we examined the effects of NAADP-AM or Glutamate on the fate of autophagosomes in TPC1 or 2-overexpressing cells. NAADP-AM indeed further induced the accumulation of both LC3-II and p62 in the both kind of cells. On the other hand, treatment with Glutamate decreased the levels of LC3-II and p62 in TPC2-overexpressing cells. Moreover, Glutamate and NAADP increased the red LC3 puncta (autolysosomes) in wild-type astrocytes cells, suggesting a probable autophagy induction. Taken together, our results further demonstrate that the activation of Glutamate/TPCs signaling for autophagy progression, indicating an agonist-specific recruitment of NAADP-sensitive Ca^{2+} stores, suggesting the potential physiological importance of TPCs in central nervous system.

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MORE THAN APOPTOSIS: BCL-2 TARGETING AND CONTROLLING RYANODINE RECEPTORS

Tim Vervliet[1], Elke Decrock[2], Jordi Molgó[3], Vincenzo Sorrentino[4], Ludwig Missiaen[1], Luc Leybaert[2], Humbert De Smedt[1], Nael Nadif Kasri[5], Jan B. Parys[1], Geert Bultynck[1]

[1]KU Leuven, Laboratory of Molecular and Cellular Signaling, Department of Cellular and Molecular Medicine, B-3000 Leuven, Belgium; [2]University of Ghent, Physiology Group, Department Basic Medical Sciences, B-9000 Ghent, Belgium; [3]Institut de Neurobiologie Alfred Fessard, CNRS, Laboratoire de Neurobiologie et Développement, 91198-Gif sur Yvette cedex, France; [4]University of Siena, Molecular Medicine Section, Department of Molecular and Developmental Medicine, and Interuniversitary Institute of Myology, 53100 Siena, Italy; [5]Donders Institute for Brain, Radboud University Medical Center Cognition and Behaviour, Department of Cognitive Neuroscience, 6500HB Nijmegen, The Netherlands

Anti-apoptotic B-cell lymphoma 2 (Bcl-2) proteins counteract apoptosis at the mitochondria by scaffolding pro-apoptotic Bcl-2-family members, but also act at the endoplasmic reticulum, controlling intracellular Ca^{2+} signalling. Bcl-2 suppresses Ca^{2+} release by targeting the inositol 1,4,5-trisphosphate receptor (IP_3R). The Bcl-2-binding site on the IP_3R shows striking similarities to a site present in all ryanodine receptor (RyR) isoforms. We now show that Bcl-2 interacts with RyRs in ectopic expression systems and in rat hippocampus. Detailed molecular studies (including SPR) revealed that Bcl-2, via its BH4, binds to purified RyR domains containing the putative binding site. Bcl-2 overexpression inhibited caffeine-induced Ca^{2+} release in RyR-expressing HEK293 cells. Consistent with the ability of the biotinylated BH4 domain to bind RyRs, a BH4-Bcl-2 peptide was sufficient to suppress RyR-mediated Ca^{2+} release in HEK293 cells and dissociated rat hippocampal neurons. Hence, these data indicate that besides IP_3Rs Bcl-2 targets RyR channels. Yet, while the BH4 domain of Bcl-XL fails to bind to and inhibit IP_3Rs, due to a critical conserved amino acid difference with BH4-Bcl-2 (i.e. Asp11 in Bcl-XL versus Lys17 in Bcl-2), BH4-Bcl-XL could target RyR channels, indicating that the binding determinants for complex formation with Bcl-2/Bcl-XL are similar for IP_3Rs and RyRs, but not identical. These data now set the stage for discovering novel biological functions for anti-apoptotic Bcl-2 proteins by targeting RyR channels in different cell types, including excitable cells.

079

INDUCTION OF Ca^{2+} FLUX AND APOPTOSIS BY THE MYCOTOXIN PHOMOXANTHONE A

Philip Böhler[#], Fabian Stuhldreier[#], David Schlütermann[#], Niklas Berleth[#], Stefan Drießen[#], Nora Hieke[#], Hendrik Niemann[§], Marian Frank[§], Peter Proksch[§], Sebastian Wesselborg[#], Björn Stork[#]

[#]Institute for Molecular Medicine I, University Hospital Düsseldorf, 40225 Düsseldorf, Germany; [§]Institute of Pharmaceutical Biology and Biotechnology, Heinrich Heine University, 40225 Düsseldorf, Germany

The mycotoxin Phomoxanthone A (PXA) is a small molecule with antibiotic activity against organisms as diverse as bacteria, algae, fungi, and animals. A recent study from our groups showed for the first time that PXA induces apoptosis in human cancer cell lines, including the highly apoptosis-resistant B lymphoma cell line DG75, with an IC50 of 0.1 – 0.5 µM and as soon as 4 h after treatment. Additionally, we showed in the same study that PXA has immune-stimulatory effects in human leukocytes. However, no study so far has shed light on the mechanism of action of PXA. Here, we report for the first time that PXA induces Ca^{2+} flux in human lymphoma cell lines including Jurkat J16 (T cell lymphoma) and Ramos (Burkitt lymphoma). Ca^{2+} is mobilised from both extracellular and intracellular stores. To investigate whether PXA-induced apoptosis is Ca^{2+} dependent, we pre-treated the cells with the cell-permeable Ca^{2+} chelator BAPTA-AM, which blocks PXA-induced Ca^{2+} mobilisation. Considering that mitochondrial Ca^{2+} overload is a known trigger of cell death, we used the mitochondrium-targeted fluorescent Ca^{2+} sensor Pericam in order to investigate the effect of PXA on mitochondrial Ca^{2+} levels. Additionally, we could show that PXA precipitates Ca^{2+} out of solution, indicating that it binds and possibly chelates Ca^{2+}. Taken together, these results led us to the conclusion that PXA could act as a Ca^{2+} ionophore.

080

IDENTIFICATION OF NOVEL Ca^{2+}-DEPENDENT ALG-2-INTERACTING TRANSMEMBRANE PROTEINS BY SEARCH BASED ON A NEWLY DEFINED BINDING MOTIF

Kyosuke Kojima, Kanae Sasaki, Wei Zhang, Takeshi Takahashi, Terunao Takahara, Hideki Shibata and Masatoshi Maki

Department of Applied Molecular Biosciences, Graduate School of Bioagricultural Sciences, Nagoya University, Nagoya 464-8601, Japan
Corresponding: mmaki@agr.nagoya-u.ac.jp

ALG-2, a 22-kDa penta-EF-hand protein, is involved in cell death, cancer, signal transduction, membrane trafficking, *etc*. by interacting Ca^{2+}-dependently with various proteins in mammalian cells. Most of known ALG-2-interacting proteins have proline-rich regions in which YP-repeated sequences (type 1 motif), P*X*PGF (type 2 motif), or both are commonly found. Searching for new interacting proteins by the yeast two-hybrid screen or mass-spec analysis of co-immunoprecipitates is not efficient for less abundant proteins. Assuming that low-quantity proteins also play important roles in cellular functions, non-biased abundance-independent search method is required. We previously identified CHERP (Ca^{2+} homeostasis ER protein; controversial concerning subcellular localization and function in ER or nucleus) as a novel ALG-2-interacting protein by *in silico* search of protein sequence databases using the binding motifs as a query [1] and experimentally demonstrated that CHERP and ALG-2 modulate alternative splicing of the IP_3R1 pre-mRNA [2]. However, the efficiency of this strategy was not satisfactory due to the presence of undesired very weak or no interacting proteins by *in vitro* binding assays. Refinement of binding motifs was necessary for improvement. Our X-ray crystal structural analyses have revealed that peptides of ALIX (type 1 motif) and Sec31A (type 2 motif) bind to different hydrophobic pockets [3, 4]. Based on the results of mutational analysis of the Sec31A peptide, an optimum binding sequence containing seven residues was newly defined as the type 2 motif and subjected to the database search. ER-transmembrane proteins, including SARAF (a negative regulator of SOCE), were selected for interaction analysis using the exogenously expressed epitope-tagged proteins and found to be co-immunoprecipitated with ALG-2 in the cell extracts containing Ca^{2+}. Furthermore, partial punctate co-localization was observed upon thapsigargin treatment between SARAF and ALG-2, each fused with a different type of fluorescent protein, by live cell imaging. ALG-2 and its interacting proteins might, or might not, modulate Ca^{2+} homeostasis in multiple ways.

(1) Osugi K et al (2012) *J Biochem* 151, 657; (2) Sasaki-Osugi K et al (2013) *J Biol Chem* 288, 33361; (3) Suzuki H et al (2008) *Structure* 16, 1562; (4) Takahashi T et al (2014) *in preparation*.

081

SHIGELLA INVASION OF EPITHELIAL CELLS INDUCES CYTOSOLIC LONG LASTING LOCAL CALCIUM RESPONSES

Guy Tran Van Nhieu[1], Bing Kai Liu[1,2], Jie Zhang[1,2], Fabienne Pierre[1,2], Sylvie Prigent[2], Philippe Sansonetti[3], Christophe Erneux[4], Jung Kuk Kim[5], Pann-Ghill Suh[5] Geneviève Dupont[6], Laurent Combettes[2]

[1]Inserm U1050, CIRB. Collège de France; [2]Université Paris-Sud, UMRS757, Orsay ; [3]Inserm U786, Institut Pasteur ; [4]IRIBHM, ULB, Campus Erasme, Brussels ; [5]Division of Molecular and Life Science, Pohang University, Korea ; [6]Unité de Chronobiologie Théorique. ULB. Brussels

Shigella, the agent of bacillary dysentery, invades epithelial cells by locally inducing actin reorganization. Bacterial invasion is determined by a type three secretion system (T3SS), which injects effectors from the bacterial cytoplasm directly into the host cell cytosol. T3SS effectors reorganize the cell cytoskeleton and promote invasion. Upon cell invasion, *Shigella* induces calcium (Ca^{2+})-signalling, but its role in invasion has remained unclear. Using high-speed fluorescence microscopy (HSFM), we found that invasive *Shigella, via* inositol 1, 4, 5-trisphosphate ($InsP_3$)- signalling and $InsP_3$ receptors, induces local Ca^{2+} transients at the site of bacterial invasion. These responses are restricted to microdomains of a few square microns and show atypically long durations (tens of seconds), which differentiate them from previously described puffs or sparks induced by low agonist concentrations described elsewhere. Fluorescence Recovery after Photo-bleaching (FRAP) experiments indicated that diffusion of small solutes was hindered at *Shigella*-invasion sites and that diffusion hindrance was dependent on bacterial-induced actin reorganization. Computational simulations and experimental challenge of the model supported the notion that the local accumulation of $InsP_3$ permitted by restricted diffusion and enrichment of $InsP_3$ receptors accounted for sustained local Ca^{2+} increases at entry sites. Thus, cytoskeletal reorganization through diffusion hindrance shapes the duration of local Ca^{2+} signals.
These atypical Ca^{2+} elevations induced sustained increases in mitochondrial Ca^{2+}. The implications of these confined and sustained Ca^{2+} signals induced by *Shigella* will be discussed.

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FROM STORE-OPERATED CALCIUM ENTRY EFFECTOR TO CELL KILLER…

Hardy E.[1], Doignon I.[2,1] and Dellis O.[1,2]

[1]University Paris 11, Bâtiment 400/443, 91405 Orsay France; [2]INSERM U757, Bâtiment 400/443, 91405 Orsay France

The Store-Operated Calcium Entry (SOCE) is an absolute requirement for leukocyte activation and the start of the immune response. During chronic inflammation process it has been shown that some leukocyte are sur-activated. Thus, SOCE inhibitors are subject of intensive studies: indeed, inhibition of the SOCE impairs the leukocyte activation and could serve as new therapies. In an attempt to find new SOCE effectors, we focussed our work on 2-APB by developing analogues. 2-APB is a well known inhibitor of SOCE, but lacks of specificity. Noteworthy, 2-APB potentiates the SOCE at 1-5 µM, and inhibits it at > 20 µM.

Using the T cell line Jurkat, and Indo-1 fluorescence, we characterize a new analogue we called C6 with the unique property to potentiate the SOCE. C6 increases the

SOCE of Jurkat cells by 200%. Furthermore, we showed with proliferation (MTS) and apoptosis (Tunel) tests that C6 is not toxic for non-activated Jurkat cells. On the contrary, while we expected a proliferation increase of phytohemagglutinin (PHA)-activated cells, we observed a decrease due to the induction of apoptosis in 25% of the cells after 24h. Thus, C6 could "kill" only activated T cells and we assumed it is due to an oversized Ca^{2+} influx the cells can not drive with.

We next tested C6 on Mantle Cell Lymphoma (MCL) cell lines. MCL is due to an over-expression of cyclin-D1 in a subfamily of B cells in lymph nodes allowing its proliferation. MCL Rec-1 cells, which express the higher Cyclin-D1 level, showed an increase of their resting cytosolic calcium concentration (rest$[Ca^{2+}]_{cyt}$) to 300 nM, values obtained by Jurkat cells when they are stimulated by PHA. Thus, Rec-1 cells seem to be in a pre-activated step. The amplitude of the SOCE was higher than in Jurkat cells. Without any stimulation, C6 was then able to induce the apoptosis of 20% of the Rec-1 cells in 24h.

This work shows the advantages of a SOCE potentiating agent over inhibitors: not toxic for non-activated cells, but since the rest$[Ca^{2+}]_{cyt}$ rises 200-300 nM, it is able to induce the cell death by apoptosis.

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Ca^{2+} DYNAMICS IN THE ENDOPLASMIC RETICULUM OF NERVOUS SYSTEM MONITORED WITH THE NEW FLUORESCENT Ca^{2+} SENSOR GAP1

Jonathan Rojo-Ruiz, Paloma Navas-Navarro, Macarena Rodríguez-Prados, María Teresa Alonso and Javier García-Sancho

Instituto de Biología y Genética Molecular (IBGM), Universidad de Valladolid & CSIC, Valladolid, Spain

Ca^{2+} dynamics in the endoplasmic reticulum (ER) is crucial for regulating key cellular functions and its alteration is associated with neurodegenerative diseases or aging. Therefore, accurate monitoring Ca^{2+} in the ER with appropriate tools is essential to study ER-Ca^{2+} dynamics. We have recently developed a new family of fluorescent Ca^{2+} indicators, dubbed GAP (GFP-Aequorin Protein). A low-affinity variant (GAP1) was generated to conform to the high Ca^{2+} content found in the ER. We demonstrated that ER-targeted GAP1 (erGAP1) is suited for optical imaging Ca^{2+} dynamics inside the ER (*see communication to session 5).* erGAP1 enabled monitoring $[Ca^{2+}]_{ER}$ changes in various types of primary neural cultures such as dorsal root ganglia (DRGs) or hippocampal neurons, both transduced with a herpes viral vector. Furthermore, we imaged ER-Ca^{2+} dynamics in astroglial cells, both in astrocyte cultures and in organotypic cortical slices using an astrocyte-specific adeno-associated viral vector.
In addition, we have generated several lines of transgenic mice that stably and robustly express erGAP1 in several neural tissues, including DRG, spinal cord, hippocampus, as well as cerebral and cerebellar cortex. In the transgenic neurons, erGAP1 displayed a signal-to-noise ratio and a dynamic range comparable to virally delivered erGAP1-neurons. Cultured hippocampal transgenic neurons displayed a robust erGAP1 expression in ER clearly visible in somata and dendrites. We showed reversible responses to $InsP_3$-agonists such as glutamate or to ryanodine receptor agonists such as caffeine. Finally, we are currently generating erGAP1 transgenic flies. Preliminary data on the expression and function of the Ca^{2+} sensor in the fly larvae nervous system will be presented.

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DECREASED PROTEIN UBIQUITINATION AND INCREASED BETA-CATENIN LEVEL IN HUNTINGTON'S DISEASE TRANSGENIC MICE MODEL YAC128

Czeredys M. and Kuznicki J.

International Institute of Molecular and Cell Biology in Warsaw, Laboratory of Neurodegeneration, Poland

Mutated huntingtin was shown to affect gene expression in the brain of Huntington's disease (HD) patients and in brains of HD models (Hodges et al. 2006; Czeredys et al. 2013). We found that the level of several genes and proteins involved directly (*Calb2*, *Cib1*, *Cib2*, and *Carl*) or indirectly (*CacyBP/SIP* and *Hap1*) in calcium homeostasis was at least 2-fold increased in the striatum of YAC128 mice. Some of these proteins, like calcyclin-binding protein (CacyBP/SIP), are involved in beta-catenin ubiquitination *via* Siah1 E3 ubquitinating enzyme (Matsuzawa and Reed, 2001). The aim of the present work was to establish if the changes of gene expression affect beta-catenin level in the striatum of HD mice. Using custom-made TaqMan low-density arrays we analyzed the mRNA level in the striatum and thalamus of wild type and HD YAC128 mice. Our arrays contained 96 genes, most of which are known to be involved in the protein ubiquitination and beta-catenin degradation. We detected impaired expression of genes involved in protein ubiquitination such as *Cul2*, *Cul4a*, *Mib1*, *Rbx1* and *Siah1b* in the striatum. The most statistically significant difference ($p<0.0006$) in mRNA level of YAC128 mice compared to the control mice was observed for beta-catenin gene. It was 1.3-fold increased in the striatum. Moreover, using western blotting we confirmed the increased level of beta-catenin protein and decreased total protein ubiquitination in the extracts from the striatum of YAC128 mice compared to wild type controls. The possible involvement of calcium and CacyBP/SIP in the observed changes in HD mice model is being analyzed.

085

TISSUE SPECIFIC DIFFERENCES OF MITOCHONDRIAL GAS PEDAL: CYTOSOLIC Ca^{2+} REGULATES THE MITOCHONDRIAL ENERGIZATION WITH PYRUVATE

F.N. Gellerich[1,2], Z. Gizatullina[1,2], T. Gainutdinow[2], J. Seppet[1], K. Mut[1], R. König[2], M. Riek-Burchard[2], G. Debska-Vielhaber[1], S. Vielhaber[1]

[1]Department of Neurology, Otto von Guericke University of Magdeburg and [2]Department for Behavioral Neurology, Leibniz Institute for Neurobiology, Magdeburg, Germany

See Abstract in Session 11

086

DROSOPHILA SEPTIN, PNUT IS A MODULATOR OF NEURONAL STORE OPERATED CALCIUM ENTRY

Bipan Kumar Deb, Trayambak Pathak and Gaiti Hasan

National Centre for Biological Sciences, Tata Institute of Fundamental Research, Bangalore, India

See Abstract in Session 7

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SORCIN LINKS CELL CYCLE, CALCIUM LEVELS IN ENDOPLASMIC RETICULUM AND MULTI DRUG RESISTANCE IN CANCER

Elena Poser[1], Vasiliki Lalioti[2], Andrea Ilari[3], Annarita Fiorillo[3], Ilaria Genovese[3], Gianni Colotti[3]

[1]Department of Biochemistry "A. Rossi Fanelli", Sapienza University of Rome, Piazzale Aldo Moro 5 00185 Roma, [2]Centro de Biologia Molecular Severo Ochoa (Consejo Superior de Investigaciones Cientificas), Madrid; [3]Institute of Biology, Molecular Medicine and NanoBiotechnology (IBMN-Italian National Council of Research), Rome

See Abstract in Session 4

088

CANCER STEM CELLS: Ca^{2+} INFLUX THROUGH STORE-OPERATED Ca^{2+} CHANNELS

Aulestia, F.J., Moreau, M. and Leclerc, C.

Centre de Biologie du Développement UMR 5547 118 route Narbone 31062 Toulouse-France.

See Abstract in Session 4

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Ca^{2+}-DEPENDENT MEMBRANE ORGANIZATION MEDIATED BY ANNEXIN A2

Milena Pejic[1], Patrick Drücker[2], David Grill[1], Hans-Joachim Galla[2], Volker Gerke[1]

[1]Institute of Medical Biochemistry, ZMBE, University of Muenster, von-Esmarch-Str. 56, D-48149 Muenster, Germany; [2]Institute of Biochemistry, University of Muenster, Wilhelm-Klemm-Str. 2, D- 48149 Muenster, Germany

See Abstract in Session 2

090

GLUCOSE AND NAADP TRIGGER ELEMENTARY INTRACELLULAR Ca^{2+} SIGNALS IN PANCREATIC ß-CELLS

Paula Maria Heister, Trevor Powell & Antony Galione

Department of Pharmacology, Mansfield Road, Oxford OX1 3QT, UK

See Abstract in Session 10

Conference Participants

Name	City and Country	E-mail address	ECS Membership
AGGARWAL Abhishek	Vienna, Austria	abhishek.aggarwal@meduniwien.ac.at	ECS Member
AIT GHEZALI Lamia	Bobigny, France		
AKL Haidar	Leuven, Belgium	haidar.akl@med.kuleuven.be	ECS Member
AL-MAWLA Ribal	Lyon, France	ribal.al-mawla@hotmail.com	
ALMEIDA Marianna	Aberdeen, U.K.	m.almeida@abdn.ac.uk	
ANTOS Christopher	Dresden, Germany	christopher.antos@crt-dresden.de	
ASGHAR Muhammad Yasir	Turku, Finland	myasghar@abo.fi	
ASTEGNO Alessandra	Verona, Italy	alessandra.astegno@univr.it	
AULESTIA Francisco	Toulouse, France	francisco.aulestia-araya@univ-tlse3.fr	
AZIMI Iman	Brisbane, Australia	i.azimi@uq.edu.au	ECS Member
BABIYCHUK Eduard	Bern, Suisse	edik@ana.unibe.ch	ECS Member
BECKER Martin	Freiburg, Germany	becker@ie-freiburg.mpg.de	
BERNARD David	Lyon, France	david.bernard@lyon.unicancer.fr	
BERRIDGE Michael	Cambridge, U.K.	michael.berridge@babraham.ac.uk	ECS Member
BLUM Robert	Wuerzburg, Germany	Blum_R@UKW.de	
BOCHATON-PIALLAT Marie-Luce	Geneva, Suisse	Marie-Luce.Piallat@unige.ch	
BOGESKI Ivan	Homburg, Germany	ivan.bogeski@uks.eu	ECS Member
BÖHLER Philip	Düsseldorf, Germany	philip.boehler@hhu.de	
BÖHM Johann	Strasbourg, France	johann@igbmc.fr	
BOOTMAN Martin D.	Milton Keynes, U.K.	martinbootman@yahoo.co.uk	ECS Member
BRAND Alex	Aberdeen, U.K.	a.brand@abdn.ac.uk	ECS Member
BRECHARD Sabrina	Luxembourg, Luxembourg	sabrina.brechard@uni.lu	ECS Member
BRESSON-BEPOLDIN Laurence	Bordeaux, France	laurence.bresson-bepoldin@inserm.fr	ECS Member
BRISSON Alain	Pessac, France	a.brisson@cbmn.u-bordeaux.fr	
BROHUS Malene	Aalborg, Denmark	mbrohus@bio.aau.dk	ECS Member
BULLA Monica	Geneva, Switzerland	monica.bulla@unige.ch	ECS Member
BULTYNCK, Geert	Leuven, Belgium	geert.bultynck@med.kuleuven.be	ECS Member
BURGOYNE Robert	Liverpool, U.K.	burgoyne@liv.ac.uk	
CANCELA Jose Manuel	Orsay, France	jose-manuel.cancela@u-psud.fr	ECS Member
CARONI Pico	Basel, Switzerland	caroni@fmi.ch	

CHARPENTIER Myriam	Norwich, U.K.	myriam.charpentier@jic.ac.uk	ECS Member
CHAZIN Walter	Nashville, USA	walter.j.chazin@vanderbilt.edu	ECS Member
CHEHAB Tala	Milton Keynes, U.K.	tala_chehab@yahoo.co.uk	ECS Member
CONSTANTIN Bruno	Poitiers, France	bruno.constantin@univ-poitiers.fr	ECS Member
CZEREDYS Magdalena	Warsaw, Poland	mczeredys@iimcb.gov.pl	
DE STEFANI Diego	Padova, Italy	diego.destefani@gmail.com	
DEB Bipan Kumar	Bangalore, India	bipankdeb@ncbs.res.in	ECS Member
DEBANNE Dominique	Marseille, France	dominique.debanne@univ-amu.fr	
DELETTRE Cecile	Montpellier, France	cecile.delettre@inserm.fr	
DELIOT Nadine	Poitiers, France		
DELLIS Olivier	Orsay, France	olivier.dellis@u-psud.fr	ECS Member
DELL'ORCO Daniele	Verona, Italy	daniele.dellorco@univr.it	ECS Member
DELPRAT	Montpellier, France	benjamin.delprat@inserm.fr	
DEMAUREX Nicolas	Geneva, Switzerland	nicolas.demaurex@unige.ch	ECS Member
DIERCKS Björn-Philip	Hamburg, Germany	p.diercks@uke.de	ECS Member
DISTELHORST Clark	Cleveland, USA	cwd@case.edu	ECS Member
DRAEGER Annette	Bern, Switzerland	draeger@ana.unibe.ch	ECS Member
DUCHEN Michael	London, U.K.	m.duchen@ucl.ac.uk	ECS Member
DUPONT Geneviève	Brussels, Belgium	gdupont@ulb.ac.be	ECS Member
EDEN Emily	London, U.K.	e.eden@ucl.ac.uk	
EMILIANI Valentina	Paris, France	valentina.emiliani@parisdescartes.fr	
ENYEDI Agnes	Budapest, Hungary	enyedi@biomembrane.hu	
FAGNI Laurent	Montpellier, France	laurent.fagni@igf.cnrs.fr	
FAJARDO Val Andrew	Waterloo, Canada	vafajard@gmail.com	
FALCKE Martin	Berlin, Germany	martin.falcke@mdc-berlin.de	
FALK Hanne	Herlev, Danmark	hanne.falk.hansen.01@regionh.dk	ECS Member
FLIEGERT Ralf	Hamburg, Germany	r.fliegert@uke.de	
FRANDSEN Stine Krog	Herlev, Denmark	Stine.Krog.Frandsen@regionh.dk	ECS Member
FRIEDEN Maud	Geneva, Switzerland	maud.frieden@unige.ch	ECS Member
GALIONE Antony	Oxford, UK	antony.galione@pharm.ox.ac.uk	ECS Member
GARCIA-SANCHO Javier	Valladolid, Spain	jgsancho@ibgm.uva.es	ECS Member
GARGUS J. Jay	Irvine, USA	jjgargus@uci.edu	ECS Member
GARRY Ambroise	Suresnes, France	ambroise.garry@fr.netgrs.com	ECS Member
GATTKOWSKI Ellen	Hamburg, Germany	ellen.gattkowski@studium.uni-hamburg.de	ECS Member
GEHL Julie	Herlev, Danmark	karen.julie.gehl@regionh.dk	ECS Member

GELLERICH Frank Norbert	Magdeburg, Germany	frank.gellerich@med.ovgu.de	
GERKE Volker	Muenster, Germany	gerke@uni-muenster.de	ECS Member
GHERARDI Gaia	Padua, Italy	gaia.gherardi88@gmail.com	ECS Member
GILL Donald	Hershey, USA	dlg49@psu.edu	ECS Member
GKIKA Dimitra	Lille, France	dimitra.gkika@univ-lille1.fr	
GOURIOU Yves	Lyon, France	yves.gouriou@inserm.fr	
GOROSTIZA Pau	Barcelona, Spain	pau.gorostiza@ub.edu	
GUEGUINOU Maxime	Tours, France	maxime.gueguinou-2@etu.univ-tours.fr	ECS Member
GUIDO Daniele	Geneva, Suisse	daniele.guido@unige.ch	ECS Member
GUSE Andreas H.	Hamburg, Germany	guse@uke.de	ECS Member
HAIECH Jacques	Illkirch, France	haiech@unistra.fr	ECS Member
HAJJAR Katherine	New York, USA	khajjar@med.cornell.edu	ECS Member
HAJNOCZKY Gyorgy	Philadelphia, USA	gyorgy.hajnoczky@jefferson.edu	
HAMMADI Mehdi	Bordeaux, France	mehdi.h28@gmail.com	ECS Member
HARDINGHAM Giles	Edinburgh, U.K.	giles.hardingham@ed.ac.uk	
HARTMANN Jana	Munich, Germany	jana.hartmann@lrz.tum.de	
HEGEDUS Luca	Vienna, Austria	hegedus_luca@yahoo.com	ECS Member
HEISTER Marie Paule	Oxford, UK	paula.heister12@ic.ac.uk	ECS Member
HEIZMANN Claus	Zurich, Switzerland	claus.heizmann@bluewin.ch	ECS Member
HIMSCHOOT Ellie	Gent, Belgium	elhim@psb.vib-ugent.be	
HUBNER Katrin	Heidelberg, Germany	katrin.huebner@bioquant.uni-heidelberg.de	
INOK Jang	Seoul, Korea		
INSUK So	Seoul, Korea	insuk@snu.ac.kr	
IVANOVA Hristina	Leuven, Belgium	hristina.ivanova@med.kuleuven.be	ECS Member
KALLAY Enikö	Vienna, Austria	enikoe.kallay@meduniwien.ac.at	ECS Member
KAWAMATA Hibiki	New York, USA	Hik2004@med.cornell.edu	
KILPATRICK Bethan	London, U.K.	bethan.kilpatrick.10@ucl.ac.uk	ECS Member
KOCH Karl-Wilhelm	Oldenburg, Germany	karl.w.koch@uni-oldenburg.de	ECS Member
KOERDT Sophia	Münster, Germany	s.koerdt@uni-muenster.de	
KREBS Joachim	Gottingen, Germany	jkrebs@nmr.mpibpc.mpg.de	ECS Member
KRIZANOVA Olga	Bratislava, Slovakia	olga.krizanova@savba.sk	
KUZNICKI Jacek	Warsaw, Poland	jacek@iimcb.gov.pl	ECS Member
La COUR Jonas	Copenhagen, Denmark	jonas@bio.ku.dk	
LECLERC Catherine	Toulouse, France	catherine.leclerc@univ-tlse3.fr	ECS Member
LECLERC Estelle	Fargo, USA	estelle.leclerc@ndsu.edu	ECS Member

LEE Hong Cheung	Hong Kong, China	leehoncheung@gmail.com	ECS Member
LESNIAK Wieslawa	Warsaw, Poland	w.lesniak@nencki.gov.pl	ECS Member
LI Fengzhi	Tianjin, China	lifengzhi1986@163.copm	
LINSE Sara	Lund, Sweden	sara.linse@biochemistry.lu.se	
LOEWEN Christopher	Vancouver, Canada	cloewen@mail.ubc.ca	
LORY Philippe	Montpellier, France	philippe.lory@igf.cnrs.fr	ECS Member
LUECK Katharina	London, U.K.	k.lueck@ucl.ac.uk	
MACHACA Khaled	Doha, Quatar	khm2002@qatar-med.cornell.edu	ECS Member
MAGNUSSON Melissa	Turku, Finland	memagnus@abo.fi	ECS Member
MAITY Palash Chandra	Freiburg, Germany	maity@immunbio.mpg.de	
MAJEWSKI Lukasz	Warsaw, Poland		
MAKI Masatoshi	Nagoya, Japon	mmaki@agr.nagoya-u.ac.jp	ECS Member
MANFREDI Giovanni	New York, USA	gim2004@med.cornell.edu	
MARINO Valerio	Verona, Italy	valerio.marino@univr.it	ECS Member
MARKOVA Jana	Bratislava, Slovakia	jana.markova@savba.sk	
McNEIL Paul	Augusta, USA	pmcneil@gru.edu	
MEDLER Kathryn	Buffalo, USA	kmedler@buffalo.edu	ECS Member
METHNER Axel	Mainu, Germany	axel.methner@gmail.com	ECS Member
MICHALAK Marek	Edmonton, Canada	marek.michalak@ualberta.ca	ECS Member
MIGNEN Olivier	Brest, France	olivier.mignen@univ-brest.fr	ECS Member
MILLER Andrew	Hong Kong, China	almiller@ust.hk	ECS Member
MOCCIA Francesco	Pavia, Italy	francesco.moccia@unipv.it	ECS Member
MONTEITH Greg	Brisbane, Australia	gregm@uq.edu.au	ECS Member
MOREAU Marc	Toulouse, France	marc.moreau@univ-tlse3.fr	ECS Member
MOSS Stephen	London, U.K.	s.moss@ucl.ac.uk	ECS Member
MYEONG JongYun	Seoul, Korea	cherubim0521@snu.ac.kr	ECS Member
NAMMALWAR Rathangadhara	Münster, Germany	chakrapa@uni-muenster.de	
NEANT Isabelle	Toulouse, France	neant@cict.fr	ECS Member
NITA Iulia	Beer Sheva, Israel	iulia.ioana.nita@gmail.com	ECS Member
NUNES Paula	Geneva, Switzerland	paula.nunes@unige.ch	
NURBAEVA Meerim	New York, USA	nurbaevameerim@googlemail.com	ECS Member
OVERGAARD Michael Toft	Aalborg, Denmark	mto@bio.aau.dk	ECS Member
PAGGIO Angela	Padua, Italy	angela.paggio@gmail.com	
PAPA Feroz	San Francisco, USA	frpapa@medicine.ucsf.edu	
PAPP Bela	Bobigny, France	bela.papp@stlouis.inserm.fr	ECS Member

PARYS Jan B.	Leuven, Belgium	jan.parys@med.kuleuven.be	ECS Member
PAUDEL Ram Chandra	Leuven, Belgium	ramchandra.paudel@med.kuleuven.be	ECS Member
PEJIC Milena	Münster, Germany	milena.pejic@uni-muenster.de	
PEREIRA Gustavo	Sao Paulo, Brazil	gujspharma@gmail.com	ECS Member
PEREZ ROSAS Norma	Apodaca, Mexique	ncperez@cinvestav.mx	
PETERS Amelia	Brisbane, Australia	a.peters1@uq.edu.au	ECS Member
POCHET Roland	Brussels, Belgium	rpochet@ulb.ac.be	ECS Member
POSER Elena	Rome, Italy	elena.poser@gmail.com	
POTIER-CARTEREAU	Tours, France	marie.potier-cartereau@univ-tours.fr	ECS Member
PREVARSKAYA Natacha	Villeneuve D'Ascq, France	natacha.prevarskaya@univ-lille1.fr	ECS Member
PULLI Ilari	Turku, Finland	ilari.pulli@abo.fi	
PUTNEY Jim	Research Triangle Park, USA	putney@niehs.nih.gov	ECS Member
RESENDE Rodrigo	Belo Horizonte, Brasil	rrresende@hotmail.com	ECS Member
RIETDORF Katja	Milton Keynes, U.K.	katja.rietdorf@gmail.com	ECS Member
RING Avi	Oslo, Norway	avi.ring@ffi.no	ECS Member
ROJO Jonathan	Valladolid, Spain	jonathan.rojo@ibgm.uva.es	
ROMANOV Roman	Stockholm, Sweden	roman.romanov@ki.se	ECS Member
RYAZANTSEVA Maria	St Petersburg, Russia	mariaandreevnar@gmail.com	ECS Member
RYCHKOV Grigori	Adelaide, Australia	grigori.rychkov@adelaide.edu.au	ECS Member
SANCHEZ Ana	Valladolid, Spain	asanchez@ibgm.uva.es	
SAÜC Sophie	Geneva, Switserland	sophie.sauc@unige.ch	ECS Member
SCHACHT Teresa	Mainz, Germany	teresa.84.boldt@googlemail.com	ECS Member
SCHENTEN Véronique	Luxembourg, Luxembourg	veronique.schenten@uni.lu	
SCHOENAUER Roman	Bern, Switzerland	roman.schoenauer@ana.unibe.ch	
SCHOLTEN Alexander	Oldenburg, Germany	alexander.scholten@uni-oldenburg.de	ECS Member
SCHWAB Albrecht	Münster, Germany	aschwab@uni-muenster.de	
SEAGAR Michael	Marseille, France	michael.seagar@univ-amu.fr	ECS Member
SEIMANDI Mathieu	Aix-en-Provence, France	rosa.graziani@neuroservice.com	
SHEN Yuequan	Tianjin, China	yuequan74@yahoo.com	
SHOSHAN-BARMATZ Varda	Beer Sheva, Israel	vardasb@bgu.ac.il	ECS Member
SIMMEN Thomas	Edmonton, Canada	tsimmen@ualberta.ca	
SIMPSON Alec	Liverpool, U.K.	awms@liv.ac.uk	ECS Member
SORRENTINO Vincenzo	Sienna, Italy	vincenzo.sorrentino@unisi.it	
SPEHR Marc	Aachen, Germany	m.spehr@sensorik.rwth-aachen.de	
STREHLER Emanuel	Rochester, USA	strehler.emanuel@mayo.edu	ECS Member

SULMANN Stefan	Oldenburg, Germany	stefan.sulmann@uni-oldenburg.de	
SZABADKAI Gyorgy	London, U.K.	g.szabadkai@ucl.ac.uk	ECS Member
TENNAKOON Samawansha	Vienna, Austria	samawansha.tennakoon@meduniwien.ac.at	ECS Member
THUL Rüdiger	Nottingham, U.K.	ruediger.thul@nottingham.ac.uk	ECS Member
TOLLE Fabrice	Luxembourg, Luxembourg		
TÖRNQUIST Kid	Turku, Finland	ktornqvi@abo.fi	ECS Member
TSIEN Richard W.	New York, USA	Richard.Tsien@nyumc.org	
VAN COPPENOLLE Fabien	Lyon, France	fabien.van-coppenolle@univ-lyon1.fr	ECS Member
VARDJAN Nina	Ljubljana, Slovenia	nina.vardjan@mf.uni-lj.si	ECS Member
VARGA Karolina	Budapest, Hungary	varga.kara@biomembrane.hu	ECS Member
VERVLOESSEM Tamara	Leuven, Belgium	tamara.vervloessem@med.kuleuven.be	ECS Member
WACQUIER Benjamin	Brussels, Belgium	benjamin.wacquier@gmail.com	ECS Member
WEBB Sara	Hong Kong, China	barnie@ust.hk	ECS Member
WEGIERSKI Tomasz	Warsaw, Poland	twegierski@iimcb.gov.pl	
WIE JingHong	Seoul, Korea	ontheball77@snu.ac.kr	
WILLIAMS Carmen	Research Triangle Park, USA	williamsc5@niehs.nih.gov	ECS Member
WOJDA Urszula	Warsaw, Poland	ulawojda@iimcb.gov.pl	ECS Member
WOLF Insa	Hamburg, Germany	iwolf@uke.de	
WOLFMEIER Heidi	Bern, Switzerland	heidi.wolfmeier@ana.unibe.ch	
YANG Xue	Tianjin, China	yangxue@nankai.edu.cn	
YAPA Kunsala	Brisbane, Australia	k.yapa@uq.edu.au	ECS Member
ZAHRADNIKOVA Alexandra	Bratislava, Slovakia	alexandra.zahradnikova@savba.sk	
ZHANG Mingdong	Stockholm, Sweden	mingdong.zhang@ki.se	
ZHANG Shenyuan	Temple, USA	shenyuan.zhang@tamhsc.edu	ECS Member
ZOREC Robert	Ljubljana, Slovenia	robert.zorec@mf.uni-lj.si	ECS Member