interface with the antigen presenting cell. Recent evidence suggests they are actively signaling, however their role in signal initiation and propagation is still unclear. To mechanically control the agonist MCC-MHC per TCR cluster we use microcorrals of fluid supported lipid bilayer with a confined number of MCC-MHC and correspondingly bound TCR. T cell activation (measured by calcium flux) on these microcorrals is significantly reduced at low MCC-MHC concentrations, compared to cells off them. T cell activation depends not on the overall number of agonist-MHC present, but on their number per microcorral. At least two MCC-MHCs per corral are required to trigger T cell calcium flux. Cells that can cluster even more MCC-MHC per TCR cluster do not exhibit higher signaling. This result provides an extension to the heterodimer hypothesis, by which a direct complex between agonist-MHC-TCR and co-agonist-MHC-TCR is the functional signaling unit. It confirms that two MCC-MHC-TCR are required and suggests that they are sufficient for TCR nanocluster signaling.

3513-Pos Board B560

Spatial Mutation of The T Cell Immunological Synapse with Hybrid Protein and Membrane Patterned Surfaces

Cheng-han Yu[1], Niña C. Hartman[1], Jay T. Groves[1,2].
[1]Department of Chemistry, UC Berkeley, Berkeley, CA, USA, [2]Howard Hughes Medical Institute, Chevy Chase, MD, USA.

Reorganization of membrane components, such as membrane receptors and adhesion proteins, plays an important role in signal transduction *in vivo*. Patterned hybrid live cell-supported membrane junctions provide spatial controls over the lateral transport of signaling molecules inside the cell. Moreover, immobile protein patterns within hybrid cell junctions can serve as diffusion hindrances with selectivity to their specific ligand on cellular membranes. Here we combine protein and membrane patterning to study molecular assembly during immunological synapse formation. Specifically, we explore the effects of certain fixed obstacles on the overall intracellular actin flow and receptor transport processes. The micro-patterned membranes with the abilities to selectively retain signaling molecules in position enable us to explore dynamical sorting mechanisms in cellular membranes.

3514-Pos Board B561

NMR Footprinting Of Activating And Non-activating Monoclonal Antibodies On CD3 Indicate Dynamic Quaternary Structure Changes In The TCR Complex

Koh Takeuchi[1], Sun Taek Kim[2], Zhen-Yu J. Sun[1], Maki Touma[2], Amr Fahmy[1], Ellis L. Reinherz[2], Gerhard Wagner[1].
[1]Harvard Medical School, Boston, MA, USA, [2]Dana-Farber Cancer Institute and Harvard Medical School, Boston, MA, USA.

The T cell receptor (TCR) mediates antigen recognition and T cell activation via its dimeric αβ, CD3εγ, CD3εδ and CD3ζζ subunits, however, a structural mechanism relating both functions has remained elusive. Here, we determine the NMR footprints on CD3εγ of one non-agonist and two agonist anti-CD3 monoclonal antibodies (mAbs). The data indicate changes of the site-specific binding topology and the TCR quaternary structure upon activation. NMR cross-saturation and chemical shift mapping showed that agonist and non-agonistic mAbs have distinct binding sites on the CD3εγ heterodimer. Agonistic mAbs bind to the membrane distal CD3ε lobe, whereas a non-agonist mAb targets the cleft between CD3ε and CD3γ causing a non-native quaternary structure in TCRβ-CD3εγ module. Subsequent biological experiments confirmed that the difference in cell triggering is not linked to mAb affinity or CD3ε binding stoichiometry per TCR but to the difference in the binding epitope on CD3εγ. More importantly, an Fab that stabilizes an intact TCRβ-CD3εγ module inhibits antigen-dependent activation. These findings indicate that a dynamic but coordinated receptor quaternary structure change in T cell receptor is important for T cell activation, which offer new insights into functional integration within multi-subunit receptors and may guide design of immunosuppressive mAbs devoid of agonist activity.

3515-Pos Board B562

Pharmacological Properties of a Pore Induced by Rising in Intracellular Ca^{2+}

Robson X. Faria[1], Ricardo Augusto de Melo Reis[2], Cynthia Machado Cascabulho[1], Fernando Pires de Farias[1], Anael Viana Pinto Alberto[1], Andrea Henriques Pons[1], Luiz Anastacio Alves[1].
[1]FIOCRUZ, Rio de Janeiro, Brazil, [2]UFRJ, Rio de Janeiro, Brazil.

Abstract

Recent studies on the $P2X_7$ receptor in 2BH4 cells and peritoneal macrophages have demonstrated that a rise in intracellular Ca^{2+} concentration induces a pore opening similar to $P2X_7$ receptor pore. Herein, we have investigated whether the pore activated by rising in intracellular Ca^{2+} concentration is associated to $P2X_7$ receptor. Using patch clamp in cell attached, whole cell configuration and dye uptake, we measured the pore opening in cell types that express the $P2X_7$ receptor (2BH4 cells and peritoneal macrophages), and in cells that do not express this receptor (HEK-293 and IT45-RI cells). In 2BH4 cells, the stimulation with ionomycin (5-10μM) increased intracellular free Ca^{2+} concentration and induced pore formation with conductance of 421 ± 14 pS, $t_{1/2}$ for ethidium bromide (EB) uptake of 118 ± 17 s, and $t_{1/2}$ for Lucifer yellow (LY) of 122 ± 11 s. $P2X_7$ receptor antagonists did not block this effect. Stimulation of HEK-293 and IT45-RI cells resulted in pore formation with properties similar to those found for 2BH4 cells. Connexin hemichannels inhibitors (carbenoxolone and heptanol) also did not inhibit the pore induced effect following rise in intracellular Ca^{2+} concentration. However, 5-(N,N-hexamethylene)- amiloride (HMA), a $P2X_7$ receptor pore blocker, inhibited the induced pore. Moreover, intracellular signalling enzymes, such as calmodulin, phospholipase-C (PLC), mitogen-activated protein kinase (MAPK), and cytoskeleton components were important for the pore formation. Additionally, we confirmed the results obtained for electrophysiology by using the flow cytometry, and we discarded the possibility of cellular death induced by rise of intracellular Ca^{2+}, at the doses used by using lactate dehydrogenase (LDH) release assay. In conclusion, increased mobilization of intracellular Ca^{+2} induces a novel membrane pore pharmacologically different from the $P2X_7$ associated pore and hemichannel pore.

3516-Pos Board B563

Exercise training during diabetes minimizes loss of Rap2 and Rrad

Caronda Moore[1], Deniz U. Dincer[2], Chun-Hong Shao[1], Keshore R. Bidasee[1].
[1]University of Nebraska Medical Center, Department of Pharmacology and Experimental Neuroscience, Omaha, NE, USA, [2]Indiana University School of Medicine, Well's Center for Pediatric Research, Indianapolis, IN, USA.

Diabetes causes failure of multiple organs. However, molecular mechanisms underlying these defects remain incompletely characterized. Recent studies suggest that alterations in expression/function of GTPases maybe involved. This study was designed to determine whether expression of two of these GTPases, Rap2 and Rrad are altered during diabetes and whether exercise training could blunt these changes. Type 1 diabetes was induced in male Sprague-Dawley rats using streptozotocin (STZ). Three weeks after STZ injection, diabetic rats were divided into two groups. One group underwent exercise training (ExT) for four weeks while the other group remained sedentary. After seven weeks of diabetes, steady state levels of Rap2 protein decreased by 50% in heart, 40% reduction in brain, 30% reduction in liver, but there was no detectable change in kidneys. Steady state levels of Rrad protein was also reduced by 60% in heart, 20% in brain and 25% reduction in liver as well as by 50% in kidney during diabetes. Four weeks of ExT initiated three weeks after the onset of diabetes, attenuated the reduction in Rap2 and Rrad expression. Since Rap2 and Rrad are down stream effectors of the guanine nucleotide exchange protein, EPAC which is activated during diabetes, these data suggest that reduction in steady state levels of Rap2 and Rrad may serve to blunt the effect of persistent sympathetic activation. (Supported in part by minority supplement, NIH).

3517-Pos Board B564

Cardiac Hypertrophy In Diabetic Mice Is Prevented By Ablation Of The G-protein gα11

Sabine Groenke[1], Dieter Hoyer[1], Yüksel Korkmaz[1], Nina Wettschureck[2], Stefan Offermanns[2], Thomas M. Wilkie[3], Klaus Addicks[1], **Hannes Reuter**[1].
[1]University of Cologne, Cologne, Germany, [2]University of Heidelberg, Heidelberg, Germany, [3]UT Southwestern Medical Center, Dallas, TX, USA.

Large clinical studies provided evidence of a lower incidence of a reduced cardiac morbidity in diabetic patients treated with angiotensin type 1 (AT1) receptor blockers. The AT1 receptor is coupled to the Gq class of G-proteins, which stimulate proteinkinase C (PKC) via activation of phospholipase Cβ. To study the role of the Gq protein Gα11 and its signaling through PKC in diabetic heart disease, we induced diabetes in wildtype and Gα11 knockout mice using streptozotocin.

After eight weeks of stable hyperglycemia, cardiac morphology and function were assessed by echocardiography, myocardial expression and translocation of PKC isoforms by immunohistochemistry and immunoblot after tissue fractionation.

Wildtype mice (n=8) but not Gα11 knockout animals (n=8) developed ventricular hypertrophy upon induction of hyperglycemia. PKC isoforms α, βII, δ, ϵ, ζ and θ were all detected in myocardium of wildtype animals. Compared to normoglycemic control animals, PKC isoforms α and ζ showed increased expression levels in diabetic wildtype mice. In addition, PKC ζ was phosphorylated at Thr410/403 and showed strong translocation to nuclear membranes in cardiomyocytes of diabetic but not of control animals. Hearts from normoglycemic Gα11-knockout mice showed lower expression levels of PKC α and δ compared

to wildtype animals. Upon induction of hyperglycemia in knockouts, expression of PKC δ was only slightly increased to the level found in non-diabetic wild-types. Expression, phosphorylation and translocation of PKC isoforms, however, remained unaffected by the induction of diabetes in Gα11-deficient mice. We conclude that Gα11 is central to the induction of myocardial hypertrophy in type 1 diabetes. Activation of PKC α and ζ appear to be important pathways in hypertrophic signaling via Gα11. The inhibition of this pathway may in part explain the strong therapeutic benefit of AT1 receptor blockade in diabetic patients.

3518-Pos Board B565

Methylglyoxal Impairs Brain Microvascular Endothelial Cell Function In Vivo and In Vitro

Aydin Tay[1,*], William G. Mayhan[2,*], Denise Arrick[2], Chun-Hong Shao[1], Hong Sun[2], **Keshore R. Bidasee[1]**.
[1]University of Nebraska Medical Center, Department of Pharmacology and Experimental Neuroscience, Omaha, NE, USA, [2]University of Nebraska Medical Center, Department of Cellular and Integrative Physiology, Omaha, NE, USA.

The responsiveness of cerebral microvasculature to stimulation by intrinsic ligands is altered during diabetes. Studies attribute this to a defect in endothelial cell function. However, the etiology underlying endothelial cell dysfunction during diabetes remains poorly characterized. Here we show that methylglyoxal (MGO), a reactive carbonyl species whose production increases shortly after the onset of hyperglycemia, impairs rat brain endothelial cell function, *in vivo* and *in vitro*. When pial arterioles in anesthetized rats were exposed to adenosine diphosphate (10μM) and nitroglycerin (1μM), vessel diameters increased by 12.5 ± 0.7% and 22.0 ± 2.6%, respectively. Pre-treating arterioles with 25μM of freshly synthesized MGO for 30 min at 37^0C, significantly attenuated the response of vessels to stimulation by adenosine diphosphate (4.6 ± 1.1%), but the response to nitroglycerin was preserved (19.7 ± 1.7%). In live-cell confocal imaging employing relevant probes, acute exposure (15min) of rat brain microvascular endothelial cells to MGO (25μM-100μM) triggered dose-dependent increases in cytoplasmic and mitochondrial Ca^{2+} levels, and mitochondrial superoxide production. Acute exposure to MGO also reduced cytoplasmic nitric oxide level reminiscent of an increased cytoplasmic superoxide production as well. Incubating rat brain microvascular endothelial cells with MGO for 24hr at 37^0C MGO reduce cell viability in a dose-dependent manner with an EC_{50} of 75μM. MGO treatment also reduced the viability of brain microvascular smooth muscle cells, but the EC_{50} was shifted rightwards to 300μM. Taken collectively, these data suggest that increased production of MGO following early upregulation of semicarbazide-sensitive amine oxidase (also referred to as vascular adhesion protein1) may be an initiating cause for endothelial dysfunction during diabetes. (Work supported in part by grants from NIH to WGM and KRB)

3519-Pos Board B566

Adenosine A1 Receptor Stimulation in Mouse Heart Elicits Phosphorylation of Hsp27

Richard A. Fenton, Lynne M. Shea, Cecilia Doddi, James G. Dobson Jr.
Univ. Massachusetts Medical School, Worcester, MA, USA.

Adenosine in the heart manifests an anti-adrenergic action that limits adrenergic stimulation of the myocardium. One mechanism involves the stimulation of specific A_1 receptors (A1R) that reduces β-adrenergic-induced Gs protein cycling. This results in a reduction in adrenergic-enhanced adenylyl cyclase activity, cAMP levels, protein kinase A activation and contractile protein phosphorylation. Adenosine via A1R has been reported by this laboratory to induce the activation and translocation of PKCϵ to RACK2, and by others to induce phosphorylation and activation of p38-MAPKs. The latter exerts a negative inotropic effect possibly by phosphorylating heat shock protein 27 (Hsp27), which subsequently phosphorylates αB-crystallin resulting in a reduction in myofilament Ca^{2+} sensitivity. The hypothesis of this study is that another mechanism by which A1R limits contractile function involves the activation of a MAPK cascade and the phosphorylation of Hsp27. Mouse hearts were isolated and perfused with physiological saline. After stabilization, agents were administered for the designated times, whereupon hearts were freeze-clamped. Frozen heart proteins were resolved by SDS-PAGE, transferred and blotted with antibodies against Hsp27 and phospho-Hsp-27(Ser82). Stimulation of the heart with chlorocyclopentyladenosine (CCPA, 1μM), an A1R agonist, for 5 min increased the presence of phospho-Hsp27 by 2.6-fold, whereas treatment for 45 minutes increased it by 27-fold. Furthermore, 45 min of CCPA increased the ratio of phospho-Hsp27:total Hsp27 by 40-fold. Increases elicited by CCPA stimulation were inhibited by the A1R antagonist DPCPX (0.1 μM) and by the p38-MAPK inhibitor SB-203580 (2.64 μM). Together these data suggest that the A1R modulates heart function by eliciting the phosphorylation of Hsp27 by a signaling cascade involving p38-MAPK.

3520-Pos Board B567

Alteration of Gq-signaling in Human Heart Disease

Anke Marietta Scholz[1], Gregor Reither[2], Achim Heinz[3], Sandra Ruppenthal[1], Hans-Joachim Schaefers[4], Peter Lipp[1].
[1]Saarland University, Homburg (Saar), Germany, [2]European Molecular Biology Laboratory, Heidelberg, Germany, [3]Forschungszentrum Juelich, Juelich, Germany, [4]Saarland University Hospital, Homburg (Saar), Germany.

In this study we investigated putative correlations between key proteins of the Gq-coupled pathway and atrial arrhythmia (AA) in humans. For this purpose we used quantitative real time PCR to investigate transcription levels of various genes including: protein kinase C (PKC), phospholipase C and inositol-1,4,5-trisphosphate receptor. The mRNA was isolated from human auricles obtained from patients undergoing heart surgery that we divided into four groups:
1. patients with AA without cardio-specific medication
2. patients with AA receiving ACE- and/or beta-blocker
3. patients with sinus rhythm receiving ACE- and/or beta-blocker
4. patients with sinus rhythm without cardio-specific medication

Initial analysis of the mRNA appeared to suggest significant alterations of gene transcription with respect to the groups (1-4). Gene transcripts that were up-regulated during disease were found to be down-regulated in patients receiving cardio-specific medication. During atrial fibrillation structural remodeling occurs. Thus we investigated whether such remodeling was also reflected in the transcriptional activity of tissue specific marker genes. We found that e.g. the transcriptional activity of the PKCα gene strongly correlated with markers for endothelial cells and fibroblasts but did not show any correlation with myocyte specific markers. This finding suggested that in human atria PKCα is not expressed in the myocyte. Instead, transcriptional signals of this gene product most likely solely originate from non-muscle cells. Results such as the one described strongly suggest that qPCR analysis of entire cardiac tissue ought to be interpreted in light of possible changes of the tissue composition that usually accompanies cardiac diseases.

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Distinct Signaling Pathways Regulate Membrane Architecture And Drive Bone Stem Cell Differentiation

Beth Bragdon[1], Jeremy Bonor[1], Wesley Beamer[2], **Anja Nohe[1]**.
[1]University of Delaware, Newark, DE, USA, [2]The Jackson Laboratory, Bar Harbor, ME, USA.

Recent research demonstrates that membrane domains on the plasma membrane of cells and the underlying cytoskeleton regulate subsequent receptor mediated signaling. However less is known about how the three dimensional plasma membrane architecture created by proteins, lipids and cytoskeleton is regulated by the cell itself. Influencing the architecture of the plasma membrane by distinct signaling pathways may be one crucial mechanism for the cell to selectively respond to external signaling stimuli. Therefore, signaling pathways must be identified that affect its assembly.

Recently, we showed that membrane receptor and domain aggregation and their dynamics drive bone stem cell differentiation toward osteoblastic lineage commitment, however we failed to identify pathways necessary for lipid, protein and cytoskeletal assembly. In order to identify these novel roles of classical pathways, we employed the Family of Image Correlation Spectroscopy, Atomic Force Microscopy followed by Fractal Analysis, roughness calculations, and molecular biology techniques to primary bone marrow stromal cells isolated from mice; C57BL/6 (B6, control) and B6C3H-1-12 (1-12, high peak bone density).

Our results revealed two signaling pathways that affect membrane morphology. These signaling pathways are activated at distinct steps during osteoblast differentiation and communication with surrounding cells. While triggering the first signaling cascade altered the topography of the cell membrane by modifying the maximum valley depth, the second pathway caused a change in the perimeter and area of distinct membrane regions. Our data suggest that osteoblast precursor cells and osteoblasts undergo physical changes in the membrane dependent on their stage of differentiation. These variations in the topography were triggered by distinct signaling pathways causing a rearrangement of the plasma membrane. These changes may be crucial for the response of the cells to external stimuli received at various timeopoints during the differentiation event.

3522-Pos Board B569

Specific Inhibition of Protein Kinase B Protects Against Ischemic Myocardial Injury in Rat and Man

Josè Linares-Palomino, Mohammad Ali Husainy, John Dickenson, Vien Khach Lai, Nicholas Standen, **Manuel Galiñanes**.