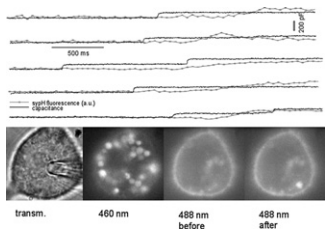
and quantitatively different in vesicle-rich and vesicle-poor synapses and depends on the spatial localisation of the synapse and their number of neighbors, respectively. This variation could be the basis for specific information-processing circuits in the hippocampus.

3554-Pos

Simultaneous Optical-Electrical Measurement of the Delay between formation of the Fusion Pore and Proton Equilibration in Exocytosis of Single Vesicles

Daniel Steinbrenner, Nadine Harmel, Nikolaj Klöcker, Jan C. Behrends.
University of Freiburg, Freiburg, Germany.

Optical detetction of neutralization of pH in granules or vesicles is often used to define exocytotic events. However, combined measurents of ensemble capacitance and pH-dependent vesicular fluorescence changes have suggested that the movement of protons only becomes possible after fusion pore expansion with a mean delay of $>$ 300 ms (1). To enhance the temporal resolution of such measurements, we have combined capacitance recordings of single vesicle fusion in RBL cells transfected with synapthpHluorin as a reporter of vesicular pH. To monitor cell capacitance steps due to exocytosis of single granules in whole cell patch-clamp mode, we used the piecewise linear technique. Internal solution contained 10 μM free Ca2+ and 300 μM GTPγS. Before establishing whole cell recordings, punctate fluorescence signals could be detected with excitation at 460 nm, while during perfusion with internal solution and excitation at 480 nm, punctate fluorescence signals gradually appeared at corresponding sites. Fluorescence increases clearly lagged capacitance steps by several 100 ms- seconds, supporting the idea that pH equilibration through the fusion pore is delayed.



(1) Barg et al.: Neuron, 33, 287-299, 2002.

Intracellular Channels

3555-Pos

Functional Properties of SR Cl^- and K^+ Channels during Postnatal Development of Cardiac Muscle

Maura Porta[1], Kristin Harris[1], Eric Winkel[1], Josefina Ramos-Franco[2], Rafael Mejia-Alvarez[1].
[1]Midwestern University, Downers Grove, IL, USA, [2]Rush University Medical Center, Chicago, IL, USA.

In adult (AD) heart, the sarcoplasmic reticulum (SR) contains Cl^- & K^+ channels presumably involved in controlling RyR-mediated SR Ca^{2+} release. These channels provide a countercurrent mechanism that attenuates the drop in Ca^{2+} driving force across the SR membrane, thereby preventing early termination of Ca^{2+} release. We showed that in newborn (NB), Ca^{2+} sparks occur with similar frequency than in AD but have shorter duration and smaller amplitude, implying an early termination of Ca^{2+} release. Although the functional properties of SR Cl^- & K^+ channels have been thoroughly described in AD, little is known about their presence and their role in NB. Consequently, we first tested the hypothesis that the early termination of Ca^{2+} release in NB coincides with absence/low density of SR Cl^- & K^+ channels at this stage. To this end, the heavy microsomal fraction was obtained from 5-days-old NB and AD rat hearts and SR Cl^- & K^+ channels were reconstituted into artificial planar lipid bilayers. Our results indicate that Cl^- & K^+ channels can be reconstituted from NB heavy SR microsomes with a similar success rate (number of SR channel incorporations / total number of bilayers) than in AD (~0.2 for Cl^- channels & ~0.1 for K^+ channels). Thus, an alternative mechanism would imply that in NB, smaller counterion fluxes result from different functional properties of SR Cl^- & K^+ channels. This assumption was tested by measuring their unitary conductance, open probability, and voltage dependence. The results in NB channels revealed no significant differences in any of these parameters in comparison to AD. Thus, we concluded that SR Cl^- & K^+ channels do not contribute to the developmental changes of Ca^{2+} release in NB cardiomyocytes. Supported by AHA-0655656Z to RMA.

3556-Pos

Role of TRIC-A Channel in Circulatory Function

Daiju Yamazaki[1], Satomi Kita[2], Shinji Komazaki[3], Aya Mishima[1], Miyuki Nishi[1], Takahiro Iwamoto[2], Hiroshi Takeshima[1].
[1]Grad. Sch. of Pharmaceutical Sci., Kyoto Univ., Kyoto, Japan, [2]Faculty of Med., Fukuoka Univ., Fukuoka, Japan, [3]Saitama Med. Univ., Saitama, Japan.

TRIC (trimeric intracellular cation) channels in the sarco-/endoplasmic reticulum likely act as counter-ion channels that conduct monovalent cations in a synchronized manner with release of stored Ca^{2+}. TRIC channel subtypes display differential expression patterns as TRIC-A is predominantly expressed in excitable tissues, including brain and muscle, and TRIC-B is present throughout many tissues. TRIC-A knockout mice are viable and fertile, while TRIC-B knockout mice exhibit neonatal lethality due to respiratory failure (Yamazaki et al., Development 2009), and double-knockout mice lacking both subtypes show embryonic cardiac failure (Yazawa et al., Nature, 2007). To resolve the physiological role of TRIC-A, we are currently focusing on abnormal circulatory function in TRIC-A-knockout mice during young adulthood. These mutant mice showed significant hypertension and bradycardia. Autonomic blocking agents (co-application of atropine and metoprolol) greatly improved the bradycardic condition without affecting hypertension in the mutant mice. This observation suggests that a hyperactive baroreceptor reflex leads to development of the bradycardic condition in the mutant mice. Blockers for vasoactive humoral factors, such as angiotensin, endothelin and vasopressin, did not significantly improve hypertension in the mutant mice, suggesting normal blood-vasopressor levels. Importantly, isometric tension measurements indicated that contractility is markedly impaired in aortic ring preparations from the mutant mice, and that acetylcholine-induced relaxation is hypersensitive in mutant mesenteric artery. Our results suggest a vital role for TRIC-A channels in the physiological regulation of vessel tonus by vascular smooth muscle and endothelial cells. To further examine the pathogenesis of hypertension at the molecular level, we plan to examine TRIC-A expression and agonist-evoked Ca^{2+} transients in smooth muscle and endothelial cells from TRIC-A-knockout and wild-type mice.

3557-Pos

Tic110 a Channel-Forming Protein at the Inner Envelope of Chloroplasts Electrophysiology and Regulation

Tom A. Goetze[1], Richard Wagner[1], Monica Balsera[2], Bettina Bölter[3], Jürgen Soll[3].
[1]University of Osnabrueck, Osnabrueck, Germany, [2]Paul Scherrer Institut, Villigen, Switzerland, [3]Ludwig-Maximilians-Universität, Munich, Germany.

Tic110 has been proposed to be a channel-forming protein at the inner envelope of chloroplasts whose function is essential for the import of proteins synthesized in the cytosol. Sequence features and topology determination experiments presently summarized suggest that Tic110 consists of six transmembrane helices. Its topology has been mapped by limited proteolysis experiments in combination with mass spectrometric determinations and cysteine modification analysis. Two hydrophobic transmembrane helices located in the N terminus serve as a signal for the localization of the protein to the membrane as shown previously. The other amphipathic transmembrane helices are located in the region composed of residues 92-959 in the pea sequence. This results in two regions in the intermembrane space localized to form supercomplexes with the TOC machinery and to receive the transit peptide of preproteins. A large region also resides in the stroma for interaction with proteins such as molecular chaperones. In addition to characterizing the topology of Tic110, we show that Ca2+ has a dramatic effect on channel activity in vitro and that the protein has a redox-active disulfide with the potential to interact with stromal thioredoxin.

3558-Pos

Luminal Ca^{2+} is a Major Sensitiser of Two-Pore Channels to NAADP

Samantha Jane Pitt[1], Tim Funnell[2], Michael X. Zhu[3], Mano Sitsapesan[1], Elisa Venturi[1], John Parrington[2], Magarida Ruas[2], Antony Galione[2], Rebecca Sitsapesan[1].
[1]University of Bristol, Bristol, United Kingdom, [2]University of Oxford, Oxford, United Kingdom, [3]The Ohio State University, Columbus, OH, USA.

It has been suggested that two-pore domain channels (TPCs) are the NAADP receptors responsible for NAADP-mediated Ca^{2+}-release from lysosome-related stores yet there is evidence that NAADP could also regulate RyR channels. We have therefore compared the effects of NAADP on native RyR1, RyR2 and purified human TPC2, reconstituted into artificial membranes under identical experimental conditions. Similar to RyR channels, we find that TPC2 behaves as an ion-channel permeable to both monovalent (300 ± 14pS; symmetrical 210 mM K^+; SD; n=3) and divalent cations (15 ± 2pS; 10μM *cis*/50 mM *trans* Ca^{2+};SD; n=5) with no evidence for anion permeability (in a 210mM *trans*: 510mM *cis* KCl gradient, the reversal potential coincides with the calculated value for a channel ideally selective for cations (E_{rev}=-23mV)). Addition of *trans* NAADP had no effect on TPC2, but *cis* ap-

plication induced marked channel activation. In symmetrical 210mM K^+ and 10μM Ca^{2+}, NAADP dose-dependently activated TPC2 channels with an EC_{50} of 500nM. Addition of 200μM *trans* Ca^{2+} significantly increased the sensitivity of TPC2, shifting the EC_{50} to 5nM. We have previously demonstrated that ligand-activation of RyR channels is also highly sensitive to luminal Ca^{2+} and therefore we have investigated how NAADP affects RyR1 and RyR2 in the presence of sensitising levels of luminal Ca^{2+}. Addition of NAADP ($\leq$1μM) did not affect RyR2 Po but slightly activated RyR1 (1μM NAADP increased Po from 0.022 ± 0.035 to 0.106 ± 0.147; SD, n=5). In contrast, larger increases in TPC2 Po (0.001 ± 0.002 to 0.4 ± 0.2; SD, n=3, $P<0.05$) could be elicited with much lower NAADP concentrations (10nM). Our study is the first to show that animal TPCs form functional, Ca^{2+}-permeable ion-channels. We also provide further evidence that TPC2 is capable of mediating NAADP-sensitive Ca^{2+}-release from acidic organelles but do not rule out a role for RyR1.
BHF supported

Membrane Transporters & Exchangers II

3559-Pos

Functional Reconstitution of Influenza a M2 (22-62)

Ted J. Ryser[1], Daniel Inouye[1], Spencer Funk[1], Emily Peterson[1], Mukesh Sharma[2], Timothy A. Cross[2], David D. Busath[1].
[1]Brigham Young University, Provo, UT, USA, [2]Florida State University, Tallahassee, FL, USA.

Amantadine-sensitive selective proton uptake by liposomes is currently the ideal method of demonstrating M2 functionality after reconstitution, validating structural determination of the reconstituted protein such as that carried out using solid state NMR (e.g. with M2 22-62, Sharma et al, this meeting). Under pH and Vm gradients, the truncated construct (which lacked 21 residues at the N-terminus and 35 at the C-terminus) was shown to transport protons at the same rate (21 H+/s at pH 6.5) as a similar construct, M2 (18-60), which elsewhere had been shown to transport similarly to other variants, including the full length protein. 100 μM amantadine was found to reduce transport by ~80%, and 10 μM amantadine or cyclooctylamine reduced transport by 50%. Transport was optimal at protein densities of 0.05-1.0% (weight peptide of weight protein and lipid). At 10%, transport was reduced, presumably due to density-dependent ion leakage. Reduction of pH to 5.0 increased transport. Rundown of total proton uptake after addition of valinomycin and CCCP, as detected by delaying application of valinomycin, indicate M2-induced K+ flux of <1 K+/s and that permeability (flux/concentration) of M2 22-62 to K+, relative to H+, is <10-7. Transport rate, amantadine and cyclooctylamine sensitivity, acid activation, and H+ selectivity are all consistent with full functionality of the reconstituted protein construct.

3560-Pos

***Yersinia* Translocon Complexes are Stabilized in Nanolipoprotein Particles (NLPs)**

Jenny A. Cappuccio[1], Craig D. Blanchette[1], Erin S. Arroyo[1], Angela K. Hinz[1], Feliza A. Bourguet[1], Todd A. Sulchek[2], Brent W. Segelke[1], Paul D. Hoeprich[1], Brett A. Chromy[1], Matthew A. Coleman[1].
[1]Lawrence Livrmore National Laboratory, Livermore, CA, USA, [2]Georgia Tech, Atlanta, GA, USA.

To enter host cells and evade host defenses, many gram-negative bacteria, including the plague pathogen, *Yersinia pestis*, utilize proteins that are able to interact with and enter host membranes. One particular mechanism for invasion is the type III secretion system, which is a highly ordered complex for injecting bacterial proteins into host cells, using a complex referred to as a translocon pore. Our results show cell-free expression of YopB and YopD was enhanced in the presence of liposomes or NLPs. However, liposomes containing YopB/D tended to aggregate and precipitate. In order to enable the study of the type III secretion proteins we have applied cell-free approaches for producing soluble *Y. pestis* membrane associated proteins YopB and YopD that are involved in the translocon pore as a complex supported by nanolipoprotein particles (NLPs). With addition of NLP, the YopB/D complex was rendered soluble. AFM showed that soluble YopB/D complex was associated with NLPs as measured by a height increase compared to NLPs not containing YopB/D. Preliminary AFM results also demonstrated binding between LcrV and YopB/D-NLPs which is indicative of proper folding in the NLP structure. Interaction studies of the YopB/D translocon embedded in a membrane with effectors such as LcrV may elucidate the poorly understood pore-forming event that helps this pathogen to evade the host defenses. Our method is applicable to other membrane proteins and represents a unique solution to solubility and purification problems.

3561-Pos

Location of Transmembrane Segments of Na^+/ca^{2+} Exchanger NCX1 Investigated with Chemical Crosslinkers

Xiaoyan Ren, Debora A. Nicoll, Kenneth D. Philipson.
UCLA, Los Angeles, CA, USA.

The sodium-calcium exchanger (NCX1) is a plasma membrane protein important in regulating calcium in cardiac myocytes. The topological model is comprised of nine transmembrane segments (TMs) and a large intracellular loop, which has two Ca^{2+} binding domains (CBD1 and CBD2), between TMs five and six. CBD1 and CBD2 have been crystallized recently and are important in regulating the function of NCX1. On the other hand, the three dimensional structure of the full length NCX1 is unknown. To gain insights into that 3-D structure, we performed cysteine crosslinking experiments. Pairs of amino acids in different TMs were mutated to cysteine on the backbone of cysteine-less NCX1. The mutated NCXs were expressed in an insect cell line and treated with cysteine-specific chemical crosslinkers followed by SDS-PAGE to determine the proximity of the introduced cysteines. The results allow us to place TMS I, IV and IX into the context of the other TMS. By combining our new results with our previous work (*J Biol Chem. 2006, 281: 22808-14; J Biol Chem. 2001, 276:194-9.*) , we propose that TMs II and VII, which contain a number of hydrophilic residues, are surrounded by the remaining TMs.

3562-Pos

Ca^{2+}-Induced Conformational Changes of Na^+-Ca^{2+} Exchanger Dimers: Role of Ca^{2+} Binding Domains

Michela Ottolia, Bernard Ribalet, John Scott, Kenneth D. Philipson.
Cardiovascular Research Laboratory, UCLA, Los Angeles, CA, USA.

The Na^+-Ca^{2+} exchanger is activated by the binding of cytoplasmic Ca^{2+} to two Ca^{2+} binding domains (CBD1 and CBD2). How binding of Ca^{2+} is translated into exchanger activation is unknown. We investigated Ca^{2+}-dependent movements as changes in FRET between exchanger dimers tagged with CFP or YFP at positions 266 within the large cytoplasmic loop of NCX1.1. The biophysical properties of the fluorescent exchangers are identical to those of the untagged NCX. Fluorescent exchangers were coexpressed in Xenopus oocytes from which plasma membrane sheets were isolated. Upon addition of Ca^{2+}, the coexpressed pair NCX-266CFP + NCX-266YFP showed an increase in FRET in a dose-dependent manner. Similar FRET changes were observed after mutating the Ca^{2+} coordination site in CBD2 (E516L). Exchanger E516L is not Ca^{2+} regulated. In contrast, mutating the Ca^{2+} coordination site in CBD1 (D421A, E451A and D500V) abolished FRET changes. These residues likely disrupt binding of Ca^{2+} to CBD1. Nevertheless, Ca^{2+} regulation of NCX is retained though with a substantial decrease in apparent affinity for Ca^{2+}. These results indicate that the Ca^{2+}-induced conformational changes of NCX dimers arise exclusively from the movement of CBD1. Peptides of Ca^{2+} binding domains, flanked by CFP and YFP, recapitulated the full length exchanger results: CBD1 showed movement upon Ca^{2+} addition while CBD2 did not. A peptide spanning CBD1-CBD2 displayed Ca^{2+}-dependent movement, which was abolished by mutating the Ca^{2+} coordination site in CBD1. Our results indicate the following: 1. Exchanger conformational changes are associated with the occupancy of a high affinity Ca^{2+} binding site exclusively within CBD1. 2. FRET studies confirm that the Na^+-Ca^{2+} exchanger exists as a dimer.

3563-Pos

The Role of Microscopic Interactions for Effective Antibiotic Delivery across the Bacterial Outer Membrane

Brigitte Ziervogel, Balasundaresan Dhakshnamoorthy, Lydia Blachowicz, Benoit Roux.
University of Chicago, Chicago, IL, USA.

Outer membrane protein F (OmpF) allows diffusion of beta-lactam antibiotics across the outer membrane of Gram-negative bacteria. The diffusion limit for translocating molecules is provided by the constriction zone, which defines both the channel diameter at the narrowest region, as well as electrostatic properties due to a unique arrangement of charged residues. Since reduced outer membrane permeability contributes to antimicrobial resistance, it is necessary to identify the role of drug-protein molecular interactions in antibiotic transfer in order to design antibiotics with improved diffusional characteristics. We have co-crystallized *E. coli* OmpF with various antibiotic molecules and observe the density corresponding to the antibiotic inside the OmpF pore. Results of this work give insights into how the charge distribution of the translocating molecule affects binding interactions within the OmpF constriction zone. Furthermore, functional assays and mutational analysis provide evidence that alteration of some key charged OmpF residues has an effect on bacterial cell survival. We are also using computational methods to model the pathways of diffusing antibiotics and measure their residence time in the OmpF pore.