AuPS/ASB Meeting - Adelaide 2010

Symposium: Molecular physiology and membrane dynamics

Tuesday 30th November 2010 - The Gallery - 11:00

Chair: Jens Coorssen & Peter Thorn

Pre-, hemi- and postfusion stages of lamellar body (LB) exocytosis in the rat lung: mechanisms of regulation and implications for surfactant release

P. Dietl, Institute of General Physiology, Albert-Einstein-Allee 11, 89081 Ulm, Germany. (Introduced by Peter Thorn)

Exocytotic systems in biology are highly variable with regard to vesicle size, content solubility, dynamics of release and modes of stimulation. The alveolar type II cell is a paradigm for a “slow secreter”, with vesicles (lamellar bodies = LBs) of about 100-fold diameter (i.e. ≈ 1 million-fold volume) of a synaptic vesicle, and a poorly soluble, lipoprotein-like secretory product (surfactant). Due to their large size and sequential (rather than simultaneous) mode of release, LBs in type II cells are an ideal model system to elucidate single vesicle-related events in the course of exocytosis using live-cell imaging techniques. We have developed several fluorescence techniques that enable a “dissection” of the exocytotic process into various stages, based on quantum yield, solubility, diffusion and accumulation of dyes in different compartments according to biophysical properties and modes of application. In combination with other techniques, these methods allow to estimate with high spatial and temporal resolution the hemifusion lifetime, the instance of fusion pore formation, dynamics and physical forces of fusion pore expansion, and postfusion events in and around single fused LBs. We found that an elevation of the cytoplasmic Ca^{2+} concentration ($[Ca^{2+}]_c$) above ≈ 300 nmol/l is a stimulus for LB fusion events, where the amount of fusion correlates with the integrated $[Ca^{2+}]_c$ over time. Each fusion event is initiated by a hemifusion phase, i.e. a period of lipid merger between plasma and LB membrane, which can be detected by a decay of light intensity (SLID = scattered light intensity decrease) of the limiting LB membrane in darkfield microscopy. After fusion pore formation, LB contents remain within the fused LB, because the fusion pore opens slowly and surfactant is a hydrophopic material that does not immediately disintegrate. Cells expressing actin-GFP form a dense “actin coat” around the fused and swollen LB, and this actin coat formation is necessary for surfactant release throught the pore. When actin coat formation is prevented by removal of Ca^{2+} or by pharmacological treatment with Ca^{2+} channel blockers, surfactant release is inhibited. The dependence of actin coat formation and contraction on extracellular Ca^{2+} prompted to investigate localized $[Ca^{2+}]_c$ changes at the site of fusion. Fluo-4-fluorescence measurements revealed transient $[Ca^{2+}]_c$ elevations around single fused LBs subsequent to fusion pore formation (FACE = fusion-activated Ca^{2+} entry). Current experiments aim at elucidating the molecular components of Ca^{2+} entry in type II cells. We conclude that the postfusion phase plays an important active role and is rate-limiting for the release of surfactant. Ca^{2+} channels, which are selectively activated and/or accessible to the extracellular space during this phase account for a yet undetected postfusion Ca^{2+} signal, boosting release of vesicle contents. This type of Ca^{2+}-secretion-coupling may exist in all cell types, where vesicle content release by diffusion through a slowly expanding pore is not sufficient or fast enough.

Targeting membrane lipids to modulate amyloid precursor protein processing

B. Garner, Illawarra Health and Medical Research Institute, University of Wollongong, Wollongong, NSW 2522, Australia and School of Biological Sciences, University of Wollongong, Wollongong, NSW 2522, Australia.
(Introduced by Peter Thorn)

Inhibition of cerebral amyloid-β (Aβ) deposition represents a therapeutic target for Alzheimer's disease (AD). Aβ is derived from the amyloid precursor protein (APP) *via* two sequential cleavages that are mediated by β-secretase and the γ-secretase complex. Such amyloidogenic APP processing occurs in lipid raft microdomains of cell membranes (Vetrivel *et al.*, 2005) and it is known that modulating the distribution of cholesterol in lipid rafts can regulate APP processing and Aβ production (Simons *et al.*, 1998). Certain ATP-binding cassette (ABC) transporters regulate lipid transport across cell membranes and, as recent studies reveal, within membrane microdomains (Glaros *et al.*, 2005). We therefore examined the role that ABCA1, A2, A7 and G1 may play in regulating neuronal lipid homeostasis and APP processing. In addition, we directly modulated raft lipid composition using glycosphingolipid (GSL) synthesis inhibitors as another means to assess the impact membrane lipid composition has on APP processing. Our studies revealed that ABCA1, A2 and G1 were expressed in human neurons as was ABCA7, albeit at much lower levels. The same transporters were also expressed in human brain (Kim *et al.*, 2008). Cellular cholesterol efflux to apolipoprotein acceptors was accelerated by over-expressing ABCA1, A7 or G1 (but not A2) in HEK293 cells (Kim *et al.*, 2007, Chan *et al.*, 2008). Extracellular Aβ levels were reduced when CHO cells stably expressing human APP (CHO-APP) were transfected with ABCA1, A7 or G1 (but not A2); implying regulation of APP processing by ABC transporters was correlated with lipid efflux activity (Kim *et al.*, 2007). In very recent studies, we assessed the capacity of three ABCA1 mutants (that do not promote cholesterol efflux) to modulate APP processing and, unexpectedly, these also reduced Aβ production. Co-immunoprecipitation experiments indicated ABCA1 and APP physically interact which suggests a novel pathway by which ABCA1 may regulate APP processing. Using a different approach to modulate cellular lipid homeostasis, we reduced membrane GSL levels using synthetic ceramide analogues based on the D-1-phenyl-2-decanoylamino-3-morpholino-1-propanol (PDMP) structure that are established glucosylceramide synthase inhibitors. PDMP and related compounds PPMP and EtDO-P4 inhibited Aβ secretion from CHO-APP cells with approximate IC50 values of 15, 5 and 1 μM, respectively (Li *et al.*, 2010). In addition, EtDO-P4 inhibited endogenous Aβ production by human neurons. In conclusion, ABC transporter mediated modulation of APP processing may involve lipid–dependent and –independent processes. Our studies also provide novel information regarding the regulation of APP processing by synthetic ceramide analogues that could offer a novel therapeutic avenue to explore as a treatment for AD.

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The effect of membrane-active peptides on membrane dynamics and molecular order

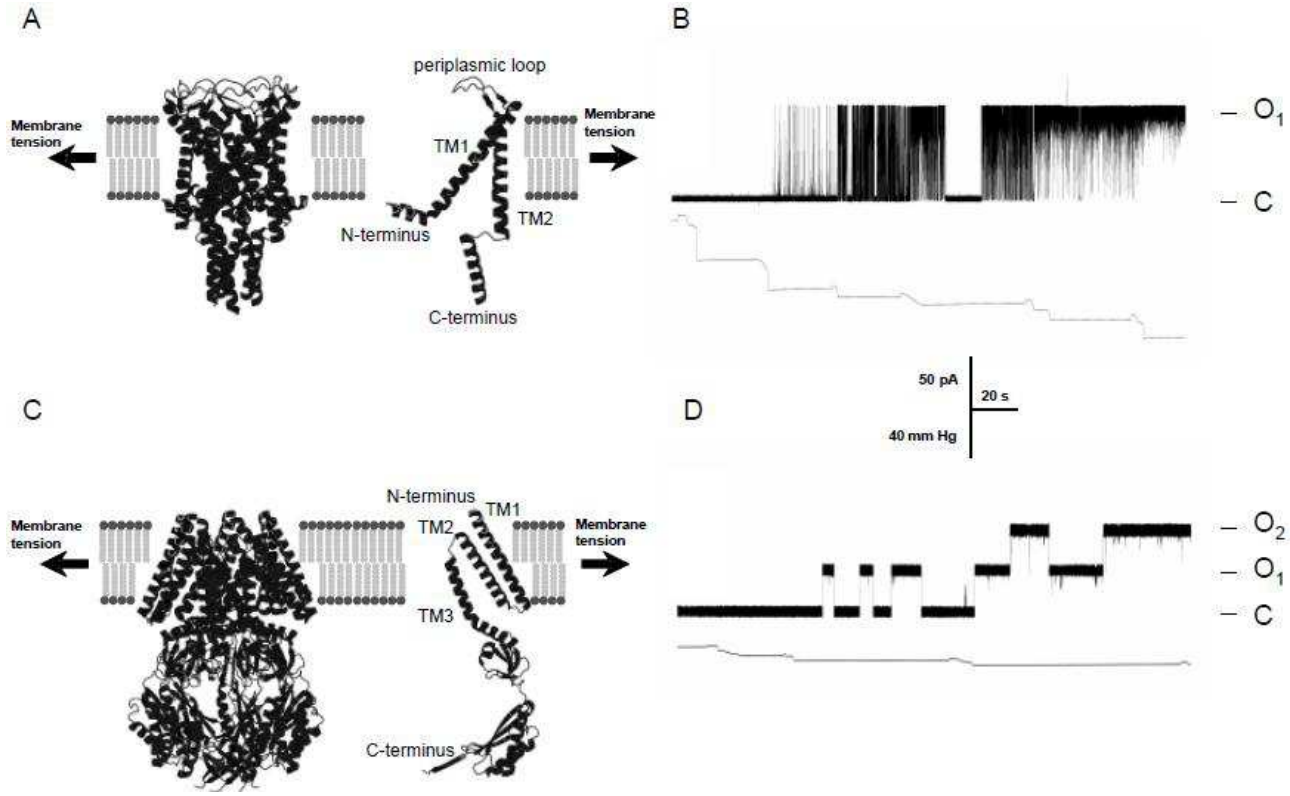
F. Separovic, School of Chemistry, Bio21 Institute, University of Melbourne, VIC 3010, Australia.

The results of solid-state NMR studies aimed at determining the orientation and location of antimicrobial peptides obtained from Australian tree frogs and amyloid peptides in phospholipid membranes will be discussed. The detailed structure of these peptides in membranes is difficult to determine as they disrupt the phospholipid bilayer. Solid-state NMR techniques are being used to determine the conformation and mobility of these pore-forming peptides in order to understand the mechanisms by which they exert their biological effect that leads to the disruption of biological membranes. Both static and magic angle spinning techniques have been applied to antimicrobial peptides in a range of model membranes, which reveal that the peptide activity is strongly dependent on the lipid composition of the bilayer and correlate with the selectivity for bacterial membranes. Similarly, the membrane interactions and structural changes of Aβ(1-42) and Aβ(1-40) from Alzheimer's disease are dependent on the presence of cholesterol and metal ions, which have been implicated in the disease. The data from both the amyloid and antimicrobial peptides reveal the importance of using appropriate membranes systems for studying membrane-active peptides.

Mechanisms of mechanosensation: Evolutionary origins of mechanosensitive ion channels

B. Martinac, Victor Chang Cardiac Research Institute, Lowy Packer Building, 405 Liverpool St, Darlinghurst, NSW 2010, Australia and St Vincent's Clinical School, University of New South Wales, NSW 2052, Australia.

Mechanosensitive (MS) ion channels are found in all types of living cells where they play an important role in mechanosensory transduction processes ranging from turgor control in bacteria and plant cells to hearing, touch, renal tubular function and blood pressure regulation in mammals. They convert mechanical stimuli acting upon membranes of biological cells into electrical or chemical signals (Hamill & Martinac, 2001). In the evolution of different life forms on Earth these ion channels may be among the oldest sensory transduction molecules that evolved as primary signalling elements in response to stimuli from the surrounding environment. The concept of ion channels gated by mechanical stimuli arose originally from studies of specialized mechanosensory neurons (Hamill & Martinac, 2001). Their discovery in embryonic chick skeletal muscle (Guharay & Sachs, 1984) and in frog muscle (Brehm *et al.*, 1984) over twenty five years ago demonstrated the existence of MS ion channels in many non-specialized types of cells. (Sachs, 1988) Instrumental for the discovery of MS channels was the invention of the patch clamp technique (Hamill *et al.*, 1981), which allowed the first direct measurements of single MS channel currents in a variety of non-specialized cells (Hamill & Martinac, 2001), including bacteria and archaea (Martinac, 2004). Studies of MS ion channels carried out over the last twenty five years have greatly contributed to our understanding of the molecular mechanisms underlying the physiology of mechanosensory transduction.



Bacterial MS channels. *(**A**) The structure of the pentameric MscL channel (left) and a channel monomer (right) from* M. tuberculosis *according to the 3D structural model. (**B**) A current trace of a single MscL channel reconstituted into azolectin liposomes (w/w protein/lipid of 1:2000) recorded at +30 mV pipette potential. The channel gated more frequently and remained longer open with increase in negative pressure applied to the patch-clamp pipette (trace shown below the channel current trace). (**C**) 3D structure of the MscS homoheptamer (left) and a channel monomer (right) from* E. coli. *(**D**) Current traces of two MscS channels reconstituted into azolectin liposomes (w/w protein/lipid of 1:1000) recorded at +30 mV pipette voltage. Increase in pipette suction (trace shown below the channel current trace) caused an increase in the activity of both channels. C and On denote the closed and open state of the n number of channels. (Modified from Martinac* et al., *2008)*

The cloning and structural determination of bacterial MscL and MscS channels (Figure), cloning and genetic analysis of the *mec* genes in *Cenorhabditis elegans*, genetic and functional studies of the TRP-type MS channels as well as functional and genetic studies of the TREK and TRAAK 2P-type K^+ MS ion channels continue to promote our understanding of the role that MS channels play in the physiology of mechanosensory transduction in living organisms (Venkatachalam & Montell, 2007). In recent years the scientific and medical community has become increasingly aware of the importance of aberrant mechanosensitive channels

contributing to pathophysiology of various diseases including heart failure and dysfunction, muscular dystrophy and polycystic kidney disease, to name a few (Venkatachalam & Montell, 2007; Martinac *et al.*, 2008). At present, MS channel proteins are at the focus of structural, spectroscopic, computational and functional studies aiming to understand the molecular basis of mechanosensory transduction in living cells.

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Molecules in motion: imaging peptides, their receptors and diffusion models

I.L. Gibbins, S. Goh, Y. DeGraaf, K. Hendy and J. Clarke, Anatomy & Histology and Centre for Neuroscience, Flinders University, GPO Box 2100, Adelaide, SA 5001, Australia. (Introduced by Peter Thorn)

Although neuropeptides and the G-protein coupled receptors (GPCRs) through which they operate have been well studied for more than 30 years now, many aspects of their function remain mysterious. When considering the roles of neuropeptides as transmitters in peripheral autonomic and sensory pathways, two questions remain largely unanswered: (1) can neuropeptides mediate non-synaptic neurotransmission? (2) how do neuropeptide signalling systems interact to modulate the excitability of neurons in a physiological milieu that includes a wide range of non-neural agents that also can affect neuronal excitability? For several years we have been examining these questions, focussing on interactions between substance P (SP) and angiotensin II (AngII) on prevertebral sympathetic neurons of guinea-pigs and on cell lines expressing NK1 receptors for SP or AT1A receptors for AngII. Intracellular electrophysiological recordings of guinea-pig coeliac ganglion neurons strongly suggest that receptors for SP and AngII converge on common intracellular signal transduction pathways to inhibit the same potassium channels to increase neuronal excitability. Based on combined electrophysiological and confocal microscopic analyses, most of SP released from collaterals of unmyelinated visceral nociceptive afferents probably acts non-synaptically. Mathematical modelling of SP diffusion using realistic morphological parameters derived from electron microscopy and direct measurements of SP diffusion coefficients with fluorescence correlation spectroscopy (FCS) or raster image correlation spectroscopy (RICS) show that physiological rates of afferent stimulation can generate concentrations of SP from non-synaptic release sites that are well within the range to affect the excitability of sympathetic neurons. Recently we have been using a confocal microscope with a high-speed resonant scanner and highly sensitive avalanche photodiodes to image the movement of EGFP-linked AngII receptors in CHO cells at rates of 20-25 frames/s. In addition to showing a considerable degree of constitutive internalisation of the receptors, these images have revealed the remarkably mobile nature of the cell membrane and the receptors it contains. Taken together, our data and models suggest that the environment within which peptides interact with their receptors is highly complex, such that they rarely occur under equilibrium conditions. In real life, it is most probable that sympathetic neurons are nearly always exposed to neuropeptides, peptide hormones and other agents at concentrations that increase their excitability significantly above a nominal resting levels. Somehow, the central nervous system must take this into account when regulating the degree of preganglionic drive to the peripheral neurons. Similarly, within the dorsal horn of the spinal cord, non-synaptic peptidergic transmission has the potential to greatly modify the processes underlying nociception.