

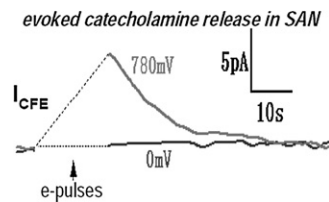
3544-Pos**A Role for Dysferlin and Agatoxin Sensitive Calcium Channels in the Calcium-Triggered Secretion of ATP Following Plasma Membrane Wounding**
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NIAAA/NIH, TS-06, MD, USA.

Mutations in dysferlin cause Limb-Girdle and Miyoshi Muscular Dystrophy. Dysferlin is structurally related to Otoferlin, a protein involved in the calcium trigger of neurotransmitters in cochlear hair cells. Dysferlin is a calcium-binding protein with a single membrane-spanning domain thought to be required for membrane wound repair. The specific function that dysferlin performs in wound healing is unknown. Here we report the activation of an intercellular signaling pathway in sea urchin embryos by membrane wounding that evokes calcium spikes in neighboring cells. This pathway was mimicked by ATP application, and inhibited by apyrase, cadmium, and omega-agatoxin IVA. Microinjection of dysferlin antisense morpholinos blocked this pathway; control morpholinos did not. Co-injection of mRNA encoding human dysferlin with the inhibitory morpholino rescued signaling activity. Our results indicate that membrane wounding triggers membrane depolarization, calcium influx through an agatoxin sensitive voltage-gated calcium channel, and ultimately the secretion of ATP. Moreover, dysferlin appears to mediate one of the essential steps in this signaling cascade.

3545-Pos**Evoked Catecholamine Release from Sympathetic Nerve Terminals in Cardiac Slices**J. Lu^{1,2}, Y. Xiu^{1,2}, Y. Mu^{1,2}, S. Guo^{1,2}, X.Y. Zhang¹, H.D. Xu¹, H.Q. Dou¹, Q. Li¹, X.J. Kang¹, L.N. Liu¹, Y.T. Yang¹, C.X. Zhang¹, L.H. Li², J.M. Cao², **Zhuan Zhou¹**.¹Peking University, Beijing, China, ²Peking Union Medical College, Beijing, China.

Neurotransmitter release from presynaptic cells can be recorded by postsynaptic potentials/currents in central nerve system. However, little is known about how to record nerve release in cardiovascular system in fresh tissue or in vivo. We have developed two novel methods for recording catecholamine release induced by nerve action potentials in cardiac slices and in vivo, respectively. Heart beating is regulated by peripheral (vegas and sympathetic) nerves, which innervate and release neurotransmitters in the heart. We are interested in how nerves regulate heart function. In the present study, we demonstrate first recordings from fresh cardiac slices. We have developed a novel method (termed cSEC) to record catecholamine release from rat and mouse sinus atrial nodes (SAN). Using micro carbon fiber electrode (CFE), catecholamine release from sympathetic nerve terminals were recorded as amperometric current (I_{CFE}) following depolarization made by field electric stimulation, high KCl, ACh, or hypoxia. The spatial mapping of evoked catecholamine signals is determined in SAN. Supported by NSFC and "973" program.

**3546-Pos****A Permissive Role for Protein Kinase A in Support of Epac Agonist-Stimulated Human Islet Insulin Secretion**George G. Holz¹, Oleg G. Chepurny¹, Colin A. Leech¹, Igor Dzshura¹, Grant G. Kelley¹, Michael W. Roe¹, Elvira Dzshura¹, Xiangquan Li¹, Frank Schwede², Hans-G. Genieser².¹State University of New York Upstate Medical University, Syracuse, NY, USA, ²BIOLOG Life Science Institute, Bremen, Germany.

Potential insulin secretagogue properties of an acetoxymethyl ester of a cAMP analog (8-pCPT-2'-O-Me-cAMP-AM) that activates the guanine nucleotide exchange factors Epac1 and Epac2 were assessed using isolated human islets of Langerhans. QPCR demonstrated that the predominant variant of Epac expressed in human islets was Epac2, although Epac1 was clearly detectable. Under conditions of islet perfusion, 8-pCPT-2'-O-Me-cAMP-AM (10 micromolar) potentiated 10 mM glucose-stimulated insulin secretion (GSIS), while failing to influence insulin secretion measured in the presence of 3 mM glucose. The secretagogue action of 8-pCPT-2'-O-Me-cAMP-AM was associated with K-ATP channel inhibition, depolarization and an increase of $[Ca^{2+}]_i$ measured in single beta cells or whole islets. As expected for an Epac-selective cAMP analog, 8-pCPT-2'-O-Me-cAMP-AM (10 micromolar) failed to stimulate phosphorylation of PKA substrates CREB and Kemptide in human islets. Further-

more, 8-pCPT-2'-O-Me-cAMP-AM (10 micromolar) had no significant ability to activate AKAR3, a PKA-regulated biosensor expressed in human islet cells by viral transduction. Surprisingly, treatment of human islets with an inhibitor of PKA activity (H-89, 10 micromolar), or treatment with a cAMP antagonist that blocks PKA activation (Rp-8-CPT-cAMPS; 200 micromolar), reduced GSIS measured in the absence of 8-pCPT-2'-O-Me-cAMP-AM. Furthermore, the action of 8-pCPT-2'-O-Me-cAMP-AM to potentiate GSIS was nearly abolished by H-89 and Rp-8-CPT-cAMPS. Thus, there exists a permissive role for PKA in support of glucose-stimulated and Epac-regulated human islet insulin secretion. We propose that this permissive action of PKA may be operative at the insulin secretory granule recruitment, priming, and/or post-priming steps of Ca^{2+} -dependent exocytosis.

3547-Pos**Tirf Microscopy Study of Exocytotic ATP Release from A549 Epithelial Cells**Irina Akopova¹, Sabina Tatur², Mariusz Grygorczyk², Ignacy Gryczynski¹, Zygmunt Gryczynski¹, Julian Borejdo¹, **Ryszard Grygorczyk²**.¹University of North Texas, Fort Worth, TX, USA, ²University of Montreal, Montreal, QC, Canada.

Extracellular nucleotides, via interaction with cell surface purinergic receptors, regulate multiple physiological processes in the lungs, including airway mucociliary clearance and surfactant secretion. Release of ATP from nonexcitable cells can be provoked by mechanical perturbations and cell-swelling, but the underlying mechanisms remain incompletely understood. We have shown previously that cell-swelling induced ATP secretion from A549 cells tightly correlates with intracellular Ca^{2+} elevations and sought to establish whether Ca^{2+} -dependent exocytosis is involved. In this study, 50% hypotonic shock-induced ATP release from A549 cells was examined by total internal reflection fluorescence (TIRF) microscopy in an attempt to directly visualize ATP-loaded vesicle movement, recruitment and fusion with the plasma membrane. Cells were loaded with quinacrine, a fluorescent marker of ATP intracellular storage sites, and time-lapse imaging was performed using the objective TIRF system. The time-course of fluorescence intensity changes of individual quinacrine-stained vesicles was evaluated during 1-2-min following hypoosmotic stimulation. Approximately 20%-30% of vesicles visible by TIRF at the cell base showed a quasi-instantaneous disappearance during the first minute post-stimulation, as expected for vesicle fusion and dispersal of their content. This was accompanied by recruitment of ~10% new vesicles into the evanescent field followed by their exocytosis. The hypotonic stimulus significantly (~5-fold) increased rate of exocytotic events compared to rate of spontaneous events in unstimulated cells. Exocytotic release mechanism is also consistent with ATP efflux measurements using luciferin-luciferase luminescence assay. Agents known to disrupt exocytotic process (brefeldin, monensin), or cytoskeleton (nocodazole, cytochalasin) reduced ATP release significantly (by up to 80%), while the release was completely blocked by N-ethylmaleimide (1 mM), and low (10°C) temperature. Thus, hypotonic shock-induced ATP secretion from A549 cells occurs mainly via Ca^{2+} -dependent exocytosis. Supported by CIHR and CCFR (RG), Emerging Technologies Fund Grant Texas (Z.G.), NIH-HL090786 (JB).

3548-Pos**Arachidonic Acid/ppara Enhancement of Ca^{2+} -Regulated Exocytosis in Antral Mucous Cells of Guinea Pig**

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Indomethacin (IDM, 10 μ M) enhanced the Ca^{2+} -regulated exocytosis stimulated by 1 μ M ACh in guinea-pig antral mucous cells, but not aspirin (ASA, 10 μ M). The differences in pharmacological actions between IDM and ASA suggest that IDM accumulates arachidonic acid (AA), which enhances Ca^{2+} -regulated exocytosis. AA (2 μ M) enhanced Ca^{2+} -regulated exocytosis in antral mucous cells similarly to IDM, moreover, an analogue of AA, AACOCF₃ (Arachidonyltrifluoromethyl ketone, a PLA₂ blocker) also enhanced it. These indicate that the Ca^{2+} -regulated exocytosis is directly enhanced by AA, not by the products of the AA cascade, such as PGs, LXs and LTs. We examined the effects of MK886 (an inhibitor of peroxisome proliferation activation receptor α , PPAR α) on the AA-induced enhancement of Ca^{2+} -regulated exocytosis, because AA is a natural ligand for PPAR α . MK-886 (40 μ M) abolished the enhancement of Ca^{2+} -regulated exocytosis induced by AA, IDM and AACOCF₃. Moreover, WY14643 (a PPAR α agonist) enhanced the Ca^{2+} -regulated exocytosis, similarly to AA. MK-886 decreased the frequency of the Ca^{2+} -regulated exocytosis activated by 1 μ M ACh or thapsigargin by 25-30%. Western blotting and immunohistochemical examinations demonstrated that PPAR α exists in antral mucous cells. Thus, ACh stimulates AA accumulation via

increases in $[Ca^{2+}]_i$, and then, AA activates PPAR α , which enhances Ca^{2+} -regulated exocytosis in antral mucous cells. A novel autocrine mechanism mediated via PPAR α maintains Ca^{2+} -regulated exocytosis of the antral mucous cells of guinea pig.

3549-Pos

Dopamine Production in the Pancreatic β -Cells: a Possible Autocrine Regulatory Mechanism for Insulin Secretion

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Glucose homeostasis is maintained by small clusters of hormone secreting cells in the pancreas: the pancreatic islets. Insulin secreting β -cells make for 90% of each islet and secrete insulin in a tightly regulated manner.

Scattered observations in the literature report that β -cells express the required machinery to synthesize and secrete dopamine. Other lines of evidence show that dopamine inhibits glucose stimulated insulin secretion (GSIS) in vitro, and the effect is mediated by the D2 isoform of the dopamine receptor. Yet, there is no evidence of dopaminergic neurons innervating pancreatic islets, and therefore, the biological relevance of such sensitivity is not clear.

We test the hypothesis that pancreatic islets produce dopamine from circulating precursor L-dopa and that the resulting dopamine is released as an autocrine inhibitory signal to regulate insulin secretion. We use microfluidic devices to maintain isolated intact islets viable during imaging experiments: we monitor islet metabolic activity by imaging of NAD(P)H autofluorescence with two photon excitation and we measure intracellular $[Ca^{2+}]_i$ oscillations by confocal microscopy. Our data from wild type and transgenic mice lacking D2 dopamine receptor support the hypothesis that dopamine is an autocrine regulator of GSIS. The results show that metabolic activity is not affected by dopamine. On the contrary, $[Ca^{2+}]_i$ oscillation frequency is reduced by both dopamine and L-dopa, suggesting that D2 receptor activation affects GSIS downstream of glucose metabolism.

This finding provides a new target for drug development in the treatment of diabetes and could help understanding the reported increased risk of developing type 2 diabetes by patients treated with antipsychotic drugs.

3550-Pos

Cholesterol Stabilizes the Fusion Pore of Rat Chromaffin Granules before Its Rapid Dilation

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Changes in cellular cholesterol level affect transmitter release but the role of cholesterol in the fusion machinery is not well understood. Using carbon fiber amperometry, we examined whether changes in cellular cholesterol level has any direct effect on the release of catecholamines from individual chromaffin granules. To avoid any possible effect of cholesterol perturbation on ion channels, exocytosis was stimulated directly via whole-cell dialysis of a Ca^{2+} -buffered solution. Cellular cholesterol level was either reduced by ~30% (via a cholesterol synthesis inhibitor and extracellular application of a cholesterol extractor) or increased by ~3 fold (via loading of cholesterol). Changes in cellular cholesterol level did not affect the rate of exocytosis, the quantal size or the kinetic parameters of the main amperometric spikes (which reflect the rapid release during and after the rapid dilation of the fusion pore). In contrast, cholesterol perturbation affected the amperometric foot signals (which reflect the catecholamine leakage via a semi-stable fusion pore). Reduction of cellular cholesterol destabilized the fusion pore while it was flickering (resulting in a decrease in the proportion of "stand-alone foot" signal) and before the onset of rapid dilation (resulting in a shortening of the foot signal). Elevation in cellular cholesterol level had opposite effects, suggesting that cholesterol elevation increased the stability of the semi-stable fusion pores. Acute extraction of cholesterol from the cytosolic side of the plasma membrane (via whole-cell dialysis of a cholesterol extractor) also shortened the foot signal and reduced the proportion of "stand-alone-foot" signals. However, acute extracellular application of cholesterol or its extractor did not affect the amperometric signals. We suggest that cholesterol on the cytosolic leaflet of the vesicular membranes constrained the fusion pores of chromaffin granules before the onset of rapid dilation.

3551-Pos

Integration of Electrical Stimulation together with Electrochemical Measurement of Quantal Exocytosis on Microchips

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We are developing microfabricated devices consisting of arrays of electrochemical electrodes in order to increase the throughput of single-cell measure-

ments of quantal exocytosis from neuroendocrine cells and to develop technology that allows simultaneous electrochemical detection and fluorescence imaging of single fusion events. One component of this effort is to develop on-chip methods for stimulating exocytosis from select cell population on the chip. Here we describe our efforts to use the same electrode to electrically stimulate the adjacent cell and subsequently measure exocytosis using amperometry. Voltage pulses were applied to planar electrodes while recording the membrane potential of an adjacent cell using a patch clamp pipette in current-clamp mode. We found that the threshold for eliciting action potentials is typically between 2.0 and 3.0 V for cells that are well adhered to Au electrodes (2.23 ± 0.49 V, 0.2 ms pulse, $n=20$ cells). Trains of stimuli, however, often lead to electroporation of the cell membrane, therefore we turned our attention to designing stimuli to promote efficient cell electroporation to trigger exocytosis upon Ca^{2+} influx from Ca^{2+} -buffered bath solutions. The amplifier was modified to allow it to transiently pass large currents to enable electroporation, yet record pA amperometric currents with low noise. We found that trains of voltage pulses of 5-8 V of 0.2-0.5 ms duration can reliably elicit Ca^{2+} -dependent exocytosis lasting for tens of seconds, presumably by eliciting electroporation of the cell membrane. Preliminary experiments with transparent electrodes and the fluorescent Ca^{2+} indicator fura-4F demonstrate a rise in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) upon electrical stimulation. Experiments are in progress to determine if this method allows one to clamp $[Ca^{2+}]_i$ to the Ca^{2+} level of the buffered bath solution. Supported by NIH grant NS048826.

3552-Pos

Quantitative Modeling of Synaptic Release at the Photoreceptor Synapse

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Exocytosis from the rod photoreceptor is stimulated by submicromolar calcium and exhibits an unusually shallow dependence on presynaptic calcium. This weak cooperativity may contribute to the linear relationship between calcium influx and release at photoreceptor synapses and contrasts with release at other ribbon and conventional synapses, which exhibit a fourth or fifth-order calcium dependence. To provide a quantitative description of the photoreceptor calcium sensor for exocytosis, we tested a family of conventional and allosteric computational models describing the final calcium-binding steps leading to exocytosis. Simulations were fit to two measures of release, evoked by flash-photolysis of caged calcium: exocytotic capacitance changes from individual rods and post-synaptic currents of second-order neurons. The best simulations supported the occupancy of only two calcium binding sites on the rod sensor rather than the typical four or five. For most models, the on-rates for calcium binding and maximal fusion rate were comparable to those of other neurons. However, the off-rates for calcium unbinding were unexpectedly slow. In addition to contributing to the high-affinity of the photoreceptor calcium sensor, slow calcium unbinding may support the fusion of vesicles located at a distance from calcium channels, perhaps located higher up the synaptic ribbon or away from the ribbon. In addition, partial sensor occupancy due to slow unbinding may further contribute to the linearization at this first synapse in vision.

3553-Pos

Inertia of Synaptic Vesicle Exocytosis

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Reliable synaptic vesicle exocytosis in primary hippocampal neurons depends on the number and availability of release-competent vesicles, their recharging with neurotransmitters and the kinetics of exo- endocytosis. We have analyzed the correlations between several exocytotic kinetic parameters by measuring FM-styryl dyes (FM 1-43, FM 4-64 and FM 5-95) discharge from electrically stimulated synapses: initial fluorescence, relative fluorescence loss, half-decay time and number of neighbors of each synapse. All terminals were maximally loaded and subsequently destained by three different stimulations, using 1200 action potentials (APs) at frequency of 40 Hz for loading and 600 APs at 30 Hz, 20 Hz and 10 Hz for destaining, respectively. Nerve terminals that contain more dye and thus more vesicles released styryl dyes slower compared to synapses that contain fewer vesicles. Furthermore, vesicle-rich synapses exhibited a lesser relative fluorescence loss than those with fewer vesicles. Interestingly, synapses with more neighbors matched these with high initial fluorescence. Computer model simulations revealed that the results of the exocytosis parameter measurements were not compromised by statistical and system-specific appraisal artifacts. The results of this study show that exocytosis is qualitatively