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Analyses of the actin cytoskeleton using fluorescence resonance energy transfer (FRET)

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Actin is the principal component of microfilaments whose assembly/disassembly is essential for cell motility. It is present in the nucleus, where it may regulate gene expression. Cofilin is the principal regulator of actin assembly in cells. It can bind actin and translocate it into the nucleus during times of stress.

We used fluorescence resonance energy transfer (FRET) and confocal microscopy to analyse the interactions of cofilin and G-actin in the nucleus and cytoplasm. By measuring the rate of photobleaching of fluorescein-labeled actin ± Cy5-labeled cofilin, we show that most of the nuclear G-actin is bound to cofilin, but only half is bound in the cytoplasm. A significant proportion of cofilin in the nucleus and cytoplasm binds added TMR-labeled G-actin. These data suggest there is significantly more cofilin-G-actin complex and less free cofilin in the nucleus.

The actin cytoskeleton can also be probed in solution using FRET spectroscopy. This method can not only detect binding events but it can also detect structural changes in these proteins. We recently demonstrated that thymosin β_4 (tβ_4) binding induces spatial rearrangements within subdomains 1 and 2 of G-actin. Tβ_4 binding increases the distance between Gln-41 and Cys-374 of actin by 2 Å and decreases the distance between bound ATP (βATP at the NUC site) and Lys-61 by 1.9 Å. The distance between Cys-374 and Lys-61 is minimally affected. Our results favour a model where tβ_4 changes the orientation of actin subdomain 2. This conformational change presumably accounts for the reduced rate of nucleotide and amide hydrogen exchange from actin monomers.

Profilin binding to sub-micellar concentration of polyphosphoinositides PI(4,5)P_2 and PI(3,4,5)P_3

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Profilin is a small (12-14 kDa) actin binding protein which promotes filament turnover. Profilin is also involved in the signalling pathway linking the receptors in the cell membrane to the microfilament system within the cell. Profilin is thought to play critical roles in this signalling pathway through its interaction with phosphatidylinositol 4,5-bisphosphate [PI(4,5)P_2] and phosphatidylinositol 3,4,5-trisphosphate [PI(3,4,5)P_3] (Lu *et al.*, 1996). So far, profilin's interaction with polyphosphoinositides (PPI) has only been studied in micelles or small vesicles. Profilin binds with high affinity to small clusters of PI(4,5)P_2 molecules. The binding stoichiometry of PI(4,5)P_2 to profilin ranges from 5:1 to 10:1 (Goldschmidt-Clermont *et al.*, 1991). In the cell, PPI lipids are not structured as they are in micelles or small vesicles, therefore their interaction with profilin might be quite different. In this work, we investigated the interactions of profilin with sub-micellar concentrations of PI(4,5)P_2 and PI(3,4,5)P_3. We determined the relevant association/dissociation constant by fluorescence anisotropy when sub-micellar concentrations of fluorescently labelled PPI lipids bind to profilin. We show that the association/dissociation constant of profilin with sub-micellar concentrations of PPI lipids is significantly different to that of profilin with micelles or small vesicles. We also show that profilin binds more strongly to sub-micellar concentrations of PI(3,4,5)P_3 than to sub-micellar concentrations of PI(4,5)P_2.

Goldschmidt-Clermont, P.J., Kim, J.W., Machesky, L.M., Rhee, S.G. & Pollard, T.D. (1991) *Science 251*, 1231-3.

Lu, P.J., Shieh, W.R., Rhee, S.G., Yin, H.L. & Chen, C.S. (1996) *Biochemistry 35*, 14027-34.

Phospholipase Cγ is essential for activation of store-operated Ca^{2+} channels in liver cells

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Release of Ca^{2+} from intracellular stores in non-excitable cells results in activation of Ca^{2+} influx through so-called store-operated Ca^{2+} channels (SOCs) on the plasma membrane (Putney *et al.*, 2001). Activation of these channels occurs in response to a decrease in the concentration of Ca^{2+} in the lumen of the endoplasmic reticulum, and it does not depend on how this decrease in [Ca^{2+}] is initiated. The molecular mechanism that underlies this phenomenon is poorly defined. Phospholipase Cγ (PLCγ) has been previously shown to be either directly involved in activation of SOCs or to modulate their activity through the production of additional IP_3 in a number of cell lines (Patterson *et al.*, 2002). The identity of the SOCs regulated by PLCγ, however, has not been established.

In this work we used short interfering RNA (siRNA) to specifically reduce the expression of the genes encoding PLCγ1 and PLCγ2 and whole cell patch clamping technique to measure activation of store-operated Ca^{2+} current (I_{SOC}) in H4IIE liver cells. Immunofluorescence and Western blotting were employed to verify the effectiveness of siRNA and the time course of the knock down of PLCγ.

We have found that transfection of H4IIE liver cells with siRNA against PLCγ1 results in time dependent reduction of PLCγ1 protein with maximal effect apparent at 72-96 h. At the same time the amplitude of the I_{SOC} developed in response to intracellular perfusion with IP_3 in cells transfected with siRNA against either PLCγ 1 or 2 has decreased. The average maximal amplitude of I_{SOC} decreased from -3.3±0.2 pA/pF (n=23) in control cells to -2.3±0.3 pA/pF (n=15) in cells transfected with siRNA against PLCγ1 and to -1.5±0.25 pA/pF (n=13) in cells transfected with siRNA against PLCγ2. Co-transfection with two siRNAs against PLCγ1 and PLCγ2 together resulted in further reduction of the current to -0.65±0.17 pA/pF (n=14). Similar results were obtained when thapsigargin was used to activate I_{SOC} instead of IP_3. It is concluded that PLCγ is required for activation of I_{SOC} in liver cells, however, the catalytic activity of PLCγ in this process in not essential.

Putney, J.W., Jr., Broad, L.M., Braun, F.J., Lievremont, J.P. & Bird, G.S. (2001) **114**, 2223-2229.

Patterson, R.L., van Rossum, D.B., Ford, D.L., Hurt, K.J., Bae, S.S., Suh, P.G., Kurosaki, T., Snyder, S.H. & Gill, D.L. (2002) *Cell* **111**, 529-541.

Distinct characteristics of exocytosis in mouse pancreatic acinar cells

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Exocytosis, the fusion of a vesicle with the plasma membrane is the principal way a cell can release lipophobic substances to the outside environment. It is probable that the basic machinery of exocytosis is similar across different cell types. But recent studies have shown the process of exocytosis may be differently regulated in different cells. Here we describe novel characteristics of the prolonged (many minutes) exocytotic events in exocrine cells of the mouse pancreas.

Mice were humanely killed (in accord with local guidelines) and the pancreas gland removed. The gland was then incubated in collagenase (Worthington CLSPA) for 5-10 minutes at 37°C. The tissue was then resuspended in extracellular solution (containing [mM] NaCl 145, KCl 5, $MgCl_2$ 1, $CaCl_2$ 1, HEPES 10 – pH 7.4 NaOH) and gently triturated to produce a preparation of large clusters of acinar cells. The clusters were then placed on Poly-l-lysine coated coverslips. The cell clusters were imaged using a custom-built 2-photon microscope. Images were then processed using Metamorph software (Universal Imaging).

We imaged lobules and smaller fragments of mouse pancreatic tissue that retained the typical morphology of the intact exocrine glands. Inclusion of a fluorescent probe (Sulphorhodamine B or Oregon Green, Molecular Probes) in the extracellular bathing medium labelled acinar ducts and the extracellular space between cells, but dyes were excluded from the cell interior. Addition of ACh or the uncaging of caged CCh, with a flash of UV light, rapidly evoked fluorescence spots in the cell. These fluorescent spots were exclusively observed in the apical regions of cells, had the same diameter as secretory granules and had similar kinetics to the release of digestive enzymes. Our observations are therefore consistent with fluorescence labelling of zymogen granules. Using fluorescence recovery after photobleaching (FRAP) techniques we show that the fusion pore remains open for protracted periods of time (minutes) to allow free exchange between the aqueous granule lumen and the outside. Although, at later times, we show that granules do not take up extracellular dye indicating that the fusion pore can close. Finally, using lipophillic dyes, we show no evidence for interchange of lipid between the plasma membrane and the vesicle membrane during the lifetime of the vesicle.

We propose that these distinct characteristic of exocytosis in exocrine glands may represent adaptations to the characteristic physiological responses of these cells.

Synchronization of Ca^{2+} oscillations through interaction of intracellular Ca^{2+} stores and L-type Ca^{2+} channels

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Many lymphatic and blood vessels undergo spontaneous constriction-dilation cycle known as vasomotion. It has been shown that cyclical Ca^{2+} release from inositol 1,4,5-trisphosphate (IP_3) operated intracellular Ca^{2+} stores and influx of Ca^{2+} through L-Ca^{2+} channels underlie lymphatic vasomotion (Zhao & van Helden, 2003). Experimental observations show that blocking L-Ca^{2+} channels abolishes synchronous Ca^{2+} oscillations, leaving only asynchronous oscillations. Based on such experimental observations and theoretical studies, we have previously shown that L-Ca^{2+} channels form a long-range coupling link between oscillatory Ca^{2+} stores, and are essential for synchronization of store Ca^{2+} release (Imtiaz *et al.*, 2002; Zhao *et al.*, 2002). The present study examines this L-Ca^{2+} channel-mediated long-range coupling mechanism.

Synchronization of Ca^{2+} oscillations can occur through diffusion of Ca^{2+} or IP_3 through gap junctions. In the present study we investigate Ca^{2+} store entrainment through voltage dependent L-Ca^{2+} channel-mediated store Ca^{2+} release for a cell pair. Such a coupling mechanism is significantly more effective than the chemical coupling-based class of models, as membrane potential has a coupling effect over distances several orders of magnitude greater than either diffusion of Ca^{2+} or IP_3 through gap junctions (Imtiaz *et al.*, 2002).

We encapsulate experimental observations in a model where; 1) each local oscillator is composed of a cytosolic-store Ca^{2+} excitable system, 2) local Ca^{2+} oscillations are coupled to membrane potential, and, 3) membrane potential exerts a positive feedback on the local Ca^{2+} oscillator through Ca^{2+} influx through L-Ca^{2+} channels. We construct a coupled cell pair according to the schema outlined above.

We study the synchronization properties of the above cell pair system. It is shown that even weak electrical coupling is sufficient to synchronize heterogeneous cell pairs. A comparison is made between electrical and chemical coupling through diffusion of Ca^{2+} or IP_3. It is shown that chemical coupling is not effective when cells are weakly coupled and have different intrinsic frequencies. This is consistent with experimental observations where only asynchronous oscillations are observed during blockade of L-Ca^{2+} channels. The result of this study show that electrical coupling acting through L-Ca^{2+}-mediated modulation of store Ca^{2+} release is able to synchronize oscillations of cells even when cells are weakly coupled (or widely separated) and/or have different intrinsic frequencies of oscillation.

Imtiaz, M., Zhao, J., & van-Helden, D.F. (2002) *Proceedings of the Australian Physiological and Pharmacological Society* http://www.aups.org.au/Meetings/200211/abstracts/1148.html

Zhao, J., Imtiaz, M., and van Helden, D. (2002) *Proceedings of the Australian Physiological and Pharmacological Society* http://www.aups.org.au/Meetings/200211/abstracts/1149.html

Zhao, J. & van Helden, D.F. (2003) *British Journal of Pharmacology* **140**, 1399-1413.

Characterization of the of the electrical activity underlying spontaneous contractions in the mouse ureteropelvic junction

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The unique role of the upper urinary tract is to propel urine from the kidneys to the bladder for storage until micturition. The decreasing presence of 'atypical' smooth muscle cells (SMC) with distance from the renal fornix has long been correlated with a decreasing gradient in contraction frequency to suggest that these atypical SMC are the primary pacemaker cells underlying pyeloureteric motility. However, we have previously described the properties of a population of electrically active cells, with many of the morphological features of interstitial cells of Cajal (ICC) the pacemaker cells of the intestine, in the spontaneously active renal pelvis of the guinea pig which was absent in the electrically-quiescent ureter (Klemm *et al.*, 1999). These ICC-like cells were not immuno-reactive to c-Kit, but c-Kit positive cells have recently been described in the upper urinary tract of mouse, pig and human (Metzger *et al.*, 2005).

We have investigated the possible function of c-Kit positive cells in the urinary tract in portions of the mid renal pelvis from humanely killed mice using intracellular microelectrodes containing Lucifer Yellow and in single cells freshly dispersed from the ureteropelvic junction and proximal ureter, using conventional whole cell and single channel patch clamp techniques.

In approximately 60% of microelectrode recordings (at 33°C), muscle contractions (3.2±0.6 min^{-1}, n=9) were directly correlated in time with the discharge of an action potential which consisted of an initial spike followed by a prolonged plateau (1.2±0.3 s, n=9). These action potentials were blocked by nifedipine (1 μM) and the cells identified, *via* Lucifer Yellow filling, as spindle-shaped smooth muscle cells when viewed under a fluorescent microscope. The remaining recordings consisted of higher frequency (33±8 min^{-1}, n=9) electrical discharges which were not directly coupled to muscle contraction. These electrical discharges were recorded in both spindle-shaped smooth muscle cells as well as stellate-shaped cells as revealed by Lucifer Yellow. These high frequency electrical events were reduced only in amplitude and duration by nifedipine (1 μM).

Membrane depolarizing steps to potentials positive to -40 mV applied to single 'spindle-shaped' myocytes under voltage clamp (at 22°C) evoked a Ca^{2+} current upon which was superimposed a transient outward current (I_{Kto}), and a slowly developing outward current which inactivated little over 200 ms. I_{Kto} was 50% inactivated at a holding potential of -84.3 ±2.9 (n=3) mV and selectively blocked by 4-aminopyridine (1-3 mM). The little-inactivating outward current was blocked by tetraethylammonium (TEA 3 mM) or iberiotoxin (100 nM) suggesting that this current arose from the activation of large conductance Ca^{2+}-activated K^+ (BK_{Ca}) channels, which were readily recorded and characterized in excised membrane patches. In contrast, depolarization of stellate- or 'staghorn'-shaped cells to potentials positive to -40 mV evoked only a slowly-developing/decaying outward current that was partially blocked by TEA (2-20 mM). Some of these cells also displayed spontaneous transient inward currents which reversed near -10 mV.

We postulate that contractions of the mouse ureteropelvic junction arise from nifedipine-sensitive action potential discharge in smooth muscle cell bundles. In addition these action potentials could well be driven by pacemaker potentials generated in neighbouring c-Kit positive ICC-like cells observed under the fluorescent microscope.

Klemm, M.F., Exintaris, B. & Lang, R.J. (1999) *Journal of Physiology* **519**, 867-884

Metzger, R., Schuster, T., Till, H., Franke, F.-E. & Dietz, H.G. (2005) *Pediatric Surgery International* **21**, 169-174.