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Chair: Chen Chen and Peter Thorn

Complex interactions between ghrelin and obestatin in the regulation of GH secretion and food intake

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Ghrelin and obestatin derive from the same precursor, preproghrelin, but appear to exert antagonistic effects on food intake and growth hormone (GH) secretion. Ghrelin is a 28 amino-acid peptide with a unique acylation on a serine in position 3 which was isolated from the stomach as an endogenous ligand of the GHS-R (GH secretagogue receptor). It potently stimulates GH secretion and food intake. Obestatin is a 23 amino-acid peptide, also initially isolated from stomach on its property to inhibit food intake and as a ligand of the GPR 39. However, this latter results have not been reproduced convincingly and the nature of the obestatin receptor remains unknown. Ghrelin/obestatin interactions were assessed by measuring plasma peptide levels during voluntary food intake periods in ad libitum-fed mice or 24 h fasted mice. Whereas fasting resulted in elevated ghrelin levels, obestatin levels were significantly reduced, suggesting that both hormones are differentially regulated. Obestatin administration per se did not modify food intake. However, it inhibited ghrelin orexigenic effect as observed in fed but not in fasted mice. The relationship between acylated ghrelin, obestatin, and GH secretions was evaluated by iterative blood sampling every 20 min during 6 h in freely moving adult male rats. Plasma obestatin levels exhibited an ultradian pulsatility with a frequency slightly lower than acylated ghrelin and GH ones but ghrelin and obestatin levels were not strictly correlated. Obestatin administration inhibited ghrelin stimulation of GH levels in freely moving rats. However, it was ineffective when GH release was monitored in superfused pituitary explants. It was therefore of interest to assess peptide interactions at the hypothalamic levels. Patch-clamp recordings in slices from mediobasal hypothalamus of GHRH-GFP transgenic mice indicated that ghrelin clearly decreased GABAergic transmission in 62% of recorded GHRH neurons (n = 85). Obestatin had no effect on glutamatergic or GABAergic synaptic transmission but it blocked ghrelin-induced decrease of GABA responses.

Interactions between ghrelin and obestatin may be relevant in term of eating disorders such as *anorexia nervosa*, a strongly familial with genetic factors disease, which affects 0.3% of young girls with a mortality of 6% per decade. Family trios study of the three preproghrelin sequence single nucleotide polymorphisms were performed in 114 *anorexia nervosa* probands and their two parents, recruited in two specialized French centres. A transmission disequilibrium was observed for the Leu72Met SNP of the preproghrelin gene. When stratified by clinical subtype, this polymorphism was preferentially transmitted for the trios with a bingeing/purging proband. An excess of transmission of the Gln90Leu72 preproghrelin/obestatin haplotype in patients with *anorexia nervosa* was also observed. Thus, preproghrelin/obestatin polymorphisms may confer susceptibility to *anorexia nervosa*. Further analysis of ghrelin/obestatin interactions which represent an interesting component of the biological determinants of energy metabolism and feeding behavior, should contribute to the understanding of pathophysiological patterns in a highly redundant and homeostatic system such as the neuroendocrine control of growth and energy metabolism.

The behaviour and control of post exocytic vesicles

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Introduction. Classical work suggests that granule exocytosis in epithelial cells is followed by granule collapse into the plasma membrane and endocytic recovery of small clathrin-coated vesicles (Palade, 1975). Our recent work challenges this model and shows that granules don't collapse but persist at the plasma membrane for many minutes over which time the fusion pores remain open (Thorn *et al.*, 2004, Larina *et al.*, 2007). This suggests that post-fusion behaviour of the granule may influence release of granule content. Here we present evidence that F-actin and myosin 2 act in consort to maintain an open fusion pore.

Methods. Male CD-1 mice were killed according to the approved ethical procedures of The University of Queensland. The pancreas was excised and collagenase-digested to produce fragments of pancreatic tissue (see Thorn *et al.*,, 2004 for details). The tissue fragments were bathed in extracellular fluorescent dyes and either imaged live with 2-photon microscopy or after paraformaldehyde fixation with confocal microscopy. Cell exocytic responses were stimulated with acetylcholine (1 μM) and its action terminated 1 minute later by the application of atropine (10 μM) or cholesystokinin (15 pM). Upon exocytosis the extracellular fluorescent dye enters and therefore labels the granules. Using different dyes and different times of dye addition, we have developed methods to enable positive identification of whether the fusion pores are open or closed (see Larina *et al.*, 2007 for details).

Results. We initially used immunofluorescence to identify the localization of myosin 2 isoforms in cells within pancreatic tissue fragments. Our data indicate that myosin 2b is located in the basal region and myosin 2a is located in the apical region, coincident with the apical F-actin cytoskeleton. Using Western blot, we found that agonist stimulation increased myosin 2 phosphorylation to a peak level, after 4 minutes of ~3 times background levels. This elevated phosphorylation remained for many minutes, even after stimulation is terminated. Taken together these lines of evidence show that myosin 2a is a potential candidate to regulate agonist-evoked apical exocytosis.

We then studied the possible physiological functions of myosin 2 using our dye methods for identifying open and closed fusion pores and inhibiting myosin activity with 50 μM blebbistatin (a myosin ATPase inhibitor). Drug treatment increased the proportion of granules with a closed fusion pore. In control, we measured 22% of granules with closed fusion pores 5 minutes after stimulation compared to 58% with blebbistatin (measured from 6 independent experiments). ML-9 (a myosin light chain kinase blocker) similarly increased the proportion of closed fusion pores consistent with activation of myosin 2 *via* this kinase. The negative enantomer of blebbistatin did not change the numbers of closed fusion pores. In past work we have shown similar results with Latrunculin A treatment (inhibits F-actin formation, Larina *et al.*, 2007). Together this data indicate that F-actin and myosin 2 are necessary to maintain an open fusion pore.

Finally, we directly measured fusion pore lifetimes in living cells using a photobleaching protocol (Larina *et al.*, 2007). Here we determined the fusion pore lifetime. In the presence of blebbistatin the mean value of the pore lifetime was 4.86 minutes compared to 10.44 minutes in control (n = 16 and 33 granules respectively).

Conclusions. We here describe the actions of F-actin and myosin 2, probably myosin 2a, in maintaining an open fusion pore during exocytosis in secretory epithelial cells. Our work adds to a growing body of evidence that post-fusion events are important in regulating the exocytic process and may be important in the control of granule content release.

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Integrating studies of proteins and lipids: dissecting the mechanism of Ca^{2+}-triggered membrane fusion

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Ca^{2+}-triggered membrane fusion is the defining step of fast, regulated exocytosis, providing temporal and spatial control over the release of biologically active compounds. Despite recognising that the fusion machinery must include lipids and proteins working in concert, only more recently has the field begun to focus more equally on both these components. Thus, the mechanism by which Ca^{2+} triggers and modulates native membrane fusion is still poorly understood. We use a stage-specific preparation of Ca^{2+} sensitive, release-ready cortical vesicles (CV) isolated from sea urchin eggs that enables the tight coupling of quantitative functional (end-point and kinetic fusion assays) and molecular (protein and lipid) analyses necessary to dissect molecular mechanisms (Coorssen *et al*., 2003).

The stalk pore model proposes that bilayer merger proceeds rapidly *via* transient, high negative curvature intermediate membrane structures (Efrat *et al*., 2007). Consistent with this, cholesterol, a major CV membrane component, contributes to a critical local negative curvature that promotes formation of fusion intermediates (Churchward *et al*., 2005). Following depletion or sequestering of endogenous CV membrane cholesterol, structurally dissimilar lipids having intrinsic negative curvature ≥ cholesterol rescue the ability of CV to fuse but not fusion efficiency (Ca^{2+} sensitivity and kinetics; Churchward *et al*., 2008). Conversely, cholesterol- and sphingomyelin-enriched regions of the membrane regulate the efficiency of the fusion mechanism, presumably *via* spatial and functional organization of other critical lipids and proteins at the fusion site (Rogasevskaia & Coorssen, 2006). Critical proteins are thought to participate in Ca^{2+}-sensing, initiating membrane deformations and facilitating fusion pore expansion.

As an unbiased approach to identifying critical proteins, the effects of several thiol-reactive reagents on the homotypic fusion of isolated CV have been characterized - these reagents alkylate the free sulfhydryl groups on proteins and have been consistently shown to inhibit triggered fusion. We have however recently characterized an additional effect of the reagent, iodoacetamide (IA). IA treatment was found to enhance the Ca^{2+} sensitivity and kinetics of both CV-plasma membrane and CV–CV fusion (Furber *et al*., 2008). If Sr^{2+}, a weak Ca^{2+} mimetic, was used to trigger fusion the potentiation after IA treatment was even greater than that observed for Ca^{2+}; the maximal leftward shift in EC_{50} to ~600 μM $[Sr^{2+}]_{free}$ brings the triggering effect of this metal into a physiologically relevant range. This substantial effect on Sr^{2+} sensitivity is highly indicative that IA promotes fusion by acting on a thiol site that regulates a Ca^{2+}-sensing step of triggered fusion. Together with the known inhibitory roles of other thiol-reactive reagents, this implicates at least two distinct thiol sites in the fusion process: one involved in fusion competency (the ability of vesicles to fuse) and one that modulates fusion efficiency (Ca^{2+}-sensitivity and kinetics).

Capitalizing on the potentiating effect of IA, we have now identified other fluorescent thiol-reactive reagents with similar effects: treatment with Lucifer yellow iodoacetamide, monobromobimane or dibromobimane resulted in an average leftward shift in EC_{50} from 17.2±1.6μM to 8.9±1.9μM $[Ca^{2+}]_{free}$. These fluorescent reagents can be used to simultaneously enhance fusion and label proteins involved. Knowing that proteins involved in Ca^{2+}-sensing are likely to be situated in cholesterol-enriched areas of the CV membrane, we are narrowing the list of protein candidates by isolating these membrane fractions using density gradient centrifugation. 2D gel electrophoresis is then used to identify proteins potentially involved in the Ca^{2+}-triggering steps of membrane fusion.

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Receptor- and metabolite-mediated increase in $[Ca^{2+}]_i$ in rat pancreatic islet cells by free fatty acids

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Long-chain free fatty acids (FFAs) stimulate immediate insulin secretion from pancreatic islet β-cells. Constant exposure of β-cells to high level of FFAs evokes a clear dysfunction of β-cells *in vitro* and *in vivo*. Such dysfunction of β-cells occurs in type 2 diabetes. Recent work demonstrated that effects of FFAs on β-cells are achieved through two pass-ways mediated by intracellular metabolites of FFAs and membrane receptor GPR40. In order to clarify the signalling process of FFAs, role of intracellular metabolites and membrane receptor GPR40 in linoleic acid (LA)-induced increase in cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_i$) was investigated in primary cultured rat pancreatic β-cells loaded with Fura-2. Levels of $[Ca^{2+}]_i$ in islet cells were reflected by ratio of 510 nm emission fluorescent intensity with excitation wavelength of 340 and 380 nm at room temperature. LA (20 μM for 10 min) induced a transient peak (the first phase) and a subsequent strong, long-lasting (the second phase) increase in $[Ca^{2+}]_i$ in β-cells. Transient application (2 min) of LA induced a weak second phase increase in $[Ca^{2+}]_i$ without changing significantly the first phase increase. GW9508, a non-metabolic agonist of GPR40, mimicked the effects of transient 2 min LA application in producing a transient, strong first phase increase in $[Ca^{2+}]_i$. Inhibition of phospholipase C (PLC) by U73122 eliminated the first transient phase without changing the second phase increase in $[Ca^{2+}]_i$ in response to 10 min LA stimulation. In contrast, blockade of intracellular LA metabolism by acyl-CoA synthetase inhibitor, Triacsin C, suppressed the second phase but not the first phase increase in $[Ca^{2+}]_i$. The first phase increase in $[Ca^{2+}]_i$ was therefore due to activation of GPR40 and PLC system to induce a Ca^{2+} release from endoplasmic reticulum (ER) Ca^{2+} stores. This was then confirmed by its elimination by thapsigargin pre-treatment for 60 min. The second phase increase in $[Ca^{2+}]_i$ was composed of two parts: the minor one was suppressed by extracellular Ca^{2+} removal or by thapsigargin pre-treatment, suggesting a store-operated Ca^{2+} entry (SOCE); and the major component was not suppressed by either extracellular Ca^{2+} removal or thapsigargin pre-treatment but was eliminated after mobilization of mitochondrial Ca^{2+} by inducing mitochondrial membrane permeability transition pore (PTP) using triphenyltin. This indicates a large component of Ca^{2+} mobilization from mitochondrial compartment. In conclusion, LA (FFAs) mobilizes ER $InsP_3$-sensitive Ca^{2+} stores and induces the subsequent SOCE through activating GPR40 receptor, PLC system. Intracellular LA metabolites induce a release of mitochondrial Ca^{2+} mobilization in β-cells through an increase in mitochondrial membrane permeability. Increase in $[Ca^{2+}]_i$ contributes to the FFA-stimulated insulin secretion through Ca^{2+}-triggered exocytosis, whereas increase in mitochondrial membrane permeability and Ca^{2+} mobilization represents a dysfunction of mitochondrial, which may play a role in FFA-induced β-cell apoptosis and desensitization to glucose stimulation.

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