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Chair: Stefan Bröer and Phil Poronnik

Diabetes and ACE2; light on the dark side of the renin angiotensin system

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Activation of the renin-angiotensin system (RAS) plays a key role in the development and progression of diabetic complications. Historically, these actions have been linked to reduced signalling through angiotensin II (Ang II)-dependent pathways, as drugs that inhibit Angiotensin Converting Enzyme (ACE) or block the activation of the type 1 angiotensin (AT1) receptor are effective in attenuating the impact of diabetes. However, some the systemic effects of RAS blockade may be mediated by Ang 1–7, a potent vasodilator, with actions that antagonize or compensate those of Ang II. In the kidney heart and vasculature, Ang 1–7 is largely derived from the degradation of Ang II by the zinc-dependent carboxypeptidase, ACE2. Consequently, ACE2 KO mice have increased tissue levels of Ang II and reduced levels of Ang 1-7. ACE2 expression is also significantly modified in both experimental and clinical diabetes, potentially contributing to local activation of the RAS as well as some of the haemodynamic manifestations of diabetes, including hyperfiltration and albuminuria. In addition, ACE2 appears to be important in the development and progression of diabetes associated cardiac injury. These studies point to ACE2 as a complex, and site-specific modulator of diabetic complications, and shed light on the usually dark side of the RAS.

A GFP-based complementation screen for protein:protein interactions for the angiotensin type 1 receptor

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Angiotensin II (AngII) is a peptide hormone with important cardiovascular, endocrine and metabolic actions, including – vasoconstriction, water and salt homeostasis, neuromodulation, thirst, salt appetite, and stimulation of central sympathetic outflow. It promotes growth of vascular smooth muscle cells, cardiomyocytes and renal cells, and contributes to the hypertrophy of blood vessels, heart and kidney that is associated with cardiovascular disease. These actions are mediated by the type 1 angiotensin (AT1) receptor (AT1R), which couples to Gq/11 (protein kinase C and calcium) as well as modulating various ion channels and exchangers, activating soluble and receptor tyrosine kinases, and stimulating mitogen activated protein kinases. These responses show a distinct temporal arrangement, reflecting exquisite control mechanisms necessary to separate early events (vasoconstriction) from longer-term activities (cell growth). Following stimulation, AT1Rs are rapidly phosphorylated, desensitised and internalised in an arrestin-dependent manner via clathrin-coated pits and vesicles.

A major goal of my laboratory is to delineate the signalling and regulatory complexes formed at the AT1R to provide greater understanding of the temporal and spatial events underlying its signalling. To this end, we have developed an assay termed the Protein Complementation Assay (PCA) to screen for protein-protein associations in living cells. This fluorescence-based assay relies on the concept that two protein fragments (*e.g.,* the N- and C-terminal halves of yellow fluorescent protein (YFP), known as YFP1 and YFP2) will show no functional activity (*i.e.,* fluorescence) when expressed as separate entities. However, when the fragments are fused to two separate proteins that are able to interact, they are brought into close proximity such that appropriate re-folding can occur and a fluorescence signal is generated. This can be detected or quantified either by confocal microscopy, FACS or by plate reader-based methods. The pioneer of this assay, Professor Stephen Michnick (Université de Montreal, Canada), has provided the expression plasmids encoding YFP1 and YFP2 and has assisted us with establishing the assay.

After validating the assay on a known interaction between the AT1R and arrestin (following AngII stimulation, beta-arrestin2-YFP2 was recruited to the activated AT1R-YFP1, refolding of YFP occurred and fluorescence was observed), we have screened for novel AT1R interacting partners from a YFP2-cDNA library (subcloned by us from a human kidney library). After several rounds of screening (involving transfection of library pools and AT1R-YFP1, FACS to identified putative interactors and extraction of plasmid DNA, re-expression of candidates and finally DNA sequencing), we have identified some unique candidates, including proteins involved in: vesicular acidification, trafficking and endocytosis; post-translational modification and stability; and signalling. One of these, SUMO-1 (an ubiquitin-like protein involved in post-translational modification, location, and stability of proteins) has been confirmed by co-expression and co-immunoprecipitation as a bona fide component of the AngII-stimulated AT1R-arrestin complex. SUMO-1 is recruited to the receptor complex by modifying arrestin on a consensus motif ΦKXE (where Φ is a hydrophobic amino acid, K is the lysine to be modified, X is any amino acid, and E is glutamic acid) which is conserved in all arrestins identified to date.

These results show the power of unbiased screening to reveal the intricate networks that underlie intracellular communication and receptor/arrestin scaffolding and function. Such information is vital to the process of identifying appropriate targets to modify and control biology – particularly for AT1 receptors that contribute significantly to human health and disease.

Extracellular pH shifts and their consequences for secretory epithelial cells

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Introduction. Most, if not all, secretory granules maintain an acidic pH. which in many types of granules this is used to drive secondary-active uptake of contents into the granule (*e.g.* neurotransmitter uptake). In peptidergic granules the acid lumen is thought to act as a charge screen on between the proteins enabling a tighter packing of granule contents. Upon granule fusion, during exocytosis, protons are lost through the open fusion pore prior to the loss of other, heavier granule content. In this way, the loss of the acid gradient is one of the first events of exocytosis. Given the high mobility of the protons, release from a small granule into a large extracellular volume would be expected not to change the extracellular pH significantly, if at all. Where the extracellular environment is restricted, this might not be the case. There are however many extracellular environments where this might not be the case, particularly where the extracellular volume is restricted. Within hollow organs, it is conceivable that exocytotic release of protons from granules might contribute to the intra-organ pH environment. In organs with a restricted extracellular volume, regulated pH changes have been shown to occur as a result of the transport of acid into the extracellular environment (Chu & Montrose, 1995). Indeed in organs lined with mucous, pH changes within the restricted mucous volume have been shown and may well arise as part of the exocytic activity. We here test the hypothesis that whether exocytosis can lead to pH changes in the lumen of the exocrine pancreas.

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 & Parker, 2005 for details). The tissue fragments were bathed in extracellular fluorescent dyes and imaged live with 2-photon microscopy. Cell exocytic responses were stimulated with cholecystokinin (15, 20 or 100 pM). Upon exocytosis the extracellular fluorescent dye enters and therefore labels the granules. We used two different extracellular dyes; sulforhodamine B (SRB, an inert dye), 8-Hydroxypyrene-1,3,6-trisulfonic acid (HPTS, a pH sensitive dye, see Schwiening & Willoughby, 2002) and 8-Methoxypyrene-1,3,6-trisulfonic acid (MPTS used as an inert control for HPTS). We also used an intracellular calcium sensor, Fluo-4 AM. We calibrated the pH sensitivity of HPTS in the 2-photon microscope with 950 nm excitation light. Our estimated Kd, derived from the calibration was 6.79.

Results. Initial experiments were performed in the presence of extracellular 7 mM HEPES. Here we observed single exocytic events in response to 15 pM CCK. These events were seen as a sudden increase in SRB and HPTS fluorescence in the granule. In regions of interest in the lumens, immediately adjacent to the exocytic events, we observed little change in the SRB signal (in some cases a small increase due to dye binding to released proteinaceous content, Thorn & Parker, 2005). In contrast, we consistently observed small, transient decreases in HPTS fluorescence indicative of possible acidification. Applying our HPTS calibration to this data gave us an estimated mean decrease from 7.4 to 7.24 ± 0.03 (n = 68). To assess the unbuffered pH changes we removed HEPES from the extracellular solution. Again the luminal SRB changes were small or showed a slight increase. Now HPTS recorded greater pH changes (from 7.4 to a mean of 7.02 ± 0.03 n = 52). These luminal changes preceded the influx of SRB into the granule suggesting release of protons from the granule through an initial fusion pore too small to allow SRB entry. Control experiments with MPTS showed no changes. Experiments stimulating the cells with high CCK (100 pM) showed very large luminal acidifications. To determine if these extracellular pH changes affected cell responses we measured cytosolic calcium responses to CCK (with Fluo-4 AM) +/- extracellular HEPES. The responses were very different. For example the frequency of calcium oscillations in HEPES was 0.42 ± 0.03 Hz (n = 43) compared to 0.65 ± 0.06 Hz (n = 38) in the absence of HEPES (p < 0.001), supporting the idea that extracellular pH changes do have functional consequences for the cell.

Conclusions. What we show here is that proton release from secretory granules significantly acidifies the primary secretory output with pH drops of up to 0.4 pH units. This 10 fold increase in protons is an unprecedented change for an extracellular ion and we show this pH change is capable of modulating intracellular calcium levels. We conclude that the acid content of secretory granules has the potential for significant effects when released. In the pancreas we reveal a novel negative feedback mechanism in the integrative control of organ function.

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Purinergic regulation of epithelial Na^+ channels

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The epithelial Na^+ channels (ENaC) expressed in Na^+-absorbing tissues, such as the kidney collecting duct, distal colon and the respiratory epithelium, play an important role in Na^+ and fluid homeostasis, controlling blood pressure and regulating the level of alveolar fluid. Aberrations of ENaC function may lead to hypertension, hypotension, pulmonary edema and reduction of mucociliary clearance of the airways. The epithelia lining the lung, kidney and gut release nucleotides in response to physiological stimuli, activating P2Y purinergic receptors in either an autocrine or paracrine manner to regulate an array of physiological mechanisms, including ion transport by these tissues. In the aldosterone-sensitive segment of the kidney, activation of purinergic receptors at the apical or basolateral membranes significantly inhibits amiloride-sensitive Na^+ absorption mediated by the epithelial Na^+ channels (Vallon, 2008). Similarly, inhibition of ENaC during purinergic receptor activation has been reported in epithelia lining the lung and gut (Kunzelmann *et al.,* 2001; Matos *et al.,* 2007). The absence of any negative effect of nucleotides on the activity of ENaC in $P2Y_2$ knock-out mice (Matos *et al.,* 2007) suggests that $P2Y_2$ receptors may be responsible for purinergic regulation of ENaC. In the past decade, the $P2Y_2$ receptor-activated signaling mechanisms that regulate activity of ENaC have been extensively investigated. It has been reported that nucleotides may mediate their effect on ENaC via pertussis toxin-sensitive G-proteins (Kunzelmann *et al.,* 2002). Our recent studies, which used gene interference and gene expression techniques, suggest that free Gβγ-dimers, released from pertussis-sensitive G-proteins during $P2Y_2$ receptor activation, may mediate the purinergic regulation of ENaC activity. The signaling mechanism involved requires activity of PLCβ4, but appears to be independent of the traditional signaling effector molecules downstream of PLC, such as $[Ca^{2+}]_i$, PKC or MAP kinase. We conclude that depletion of membrane phophatydylinositol 4,5-biphosphate (PIP_2), induced by activity of PLC activated during $P2Y_2$ receptor stimulation, may play an important role in mediating downregulation of ENaC activity during $P2Y_2$ receptor activation.

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An insight into epithelial cells through rare disorders

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Disorders of amino acid transport have been very influential in the elucidation of amino acid absorption in the kidney and intestine. Molecular identification of the transport systems mutated in these disorders highlights the complexity of amino acid transport and demonstrates its links to more complex pathologies such as blood pressure regulation and epithelial cell differentiation. Hartnup disorder is an autosomal recessive disorder. Analysis in 17 families up to now confirms that SLC6A19 is the only gene involved in the disorder. A variety of alleles have been identified, of which only one (R240Q) did not abolish transport. This allele could be clarified after the discovery that SLC6A19 requires one of the two auxiliary proteins collectrin or ACE2 for surface expression. Collectrin is predominantly found in the kidney, whereas ACE2 is found predominantly in the intestine. The association of SLC6A19 with two different proteins in kidney and intestine offers an explanation for Hartnup disorder variants that affect only renal or only intestinal amino acid transport. Coexpression of SLC6A19(R240Q) with ACE2 or collectrin shows reduced transport activity, thereby explaining the onset of Hartnup disorder in individuals with this allele. Iminoglycinuria (IG) was first described fifty years ago as an autosomal recessive abnormality of renal transport of glycine and of the imino acids, proline and hydroxyproline. Hyperglycinuria (HG) has been attributed to heterozygosity of a putative defective glycine, proline and hydroxyproline transporter. Unconfirmed associations have been reported with hypertension, glycosuria, nephrolithiasis, and various neurological diseases. A candidate gene sequencing approach was applied in seven families first identified through newborn screening programs. Electrophysiological studies and a molecular splicing assay were used to demonstrate aberrant transporter function in affected families. Mislocalization of mutant aminoacid transporters and their normal physiological distribution was defined by immunofluorescence. We identified a common proline and glycine transporter as the major responsible gene and demonstrate consistent inheritance and functional studies. In some of the pedigrees the observed mutations of this transporter retained residual transport activity. In those cases the urinary phenotypes were modified by additional mutations in additional proline and glycine transporters. The model consistent with the observed pattern of inheritance is classical semi-dominant inheritance in which two inherited non-functional alleles of the major gene conferred the complete IG phenotype whereas one non-functional allele was sufficient to confer the HG phenotype. Despite the apparently simple urinary phenotypes of IG and HG, this study has revealed unexpected multigenic complexity as an explanation for the observed reduced penetrance. The contributions of mutations in multiple transporters in these discrete phenotypes provide a model for dissecting the molecular etiology of a major gene modified by genes with related functionalities.

Insights into renal albumin handling

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The appearance of albumin in the urine (microalbuminuria) is an important marker of renal impairment. The clinical importance of albuminuria as a major, independent risk factor for cardiovascular disease is now becoming widely appreciated and underscores the importance of understanding the mechanisms of albuminuria. The traditional view of albumin handling is that the glomerulus presents a charge and size selective barrier that prevents substantial amounts of albumin entering the tubules. The small amount of albumin that is filtered is rapidly reabsorbed by receptor-mediated endocytosis, broken down in the lysosomes and resultant amino acids returned to the blood. In disease, the glomerular filtration barrier is compromised, excess albumin leaks into the tubules which in turn disrupts the endocytic mechanism leading to microalbuminuria. This disruption of tubular endocytosis also implicates impaired lysosomal function in the development of microalbuminuria. This conventional paradigm is currently under challenge on two broad fronts. The first is the charge selectivity of the glomerular filtration barrier and the amount of albumin that crosses the barrier. The second is the nature of the mechanisms for the tubular uptake of albumin. Recent studies in transgenic animals have shown that removal of most of the negative charge from the glomerular capillary wall does not induce heavy albuminuria as predicted by the theory of glomerular charge selectivity. Real time *in vivo* imaging of albumin filtration in the normal kidney has challenged our view of glomerular permeability. These data suggest that albumin is highly permeable across the glomerular filtration barrier and that albumin is retrieved intact by an as yet uncharactersied high capacity retrieval pathway. Key to the understanding of these proposed pathways is the ability to distinguish between intact and degraded albumin as it is taken up by the proximal tubule. We have recently developed a method using a conjugate of albumin that only fluoresces when it is degraded to track the albumin degradation pathway in the nephron. Our data are consistent with significant levels of albumin being rapidly taken up and degraded by the proximal tubule and suggest that the conventional model of renal albumin handling may require re-examination.