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Free communications: Membrane transport

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Chair: Phil Poronnik, Stefan Bröer

The effect of Ca^{2+} concentration on response to salinity stress in *Chara australis*

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It has been known for a long time that higher Ca^{2+} concentration in the medium ameliorates salinity stress (Hoffmann & Bisson, 1988; Shabala *et al.*, 2006). The mechanism of this effect, however, is not clearly understood. We used the charophyte cell system to resolve the effect of Ca^{2+} and high Na^+ on various membrane transporters. Charophytes are the sister plants to ancestors of higher plants. Their large cell size minimises the damage of electrode insertion and the electrical characteristics can be measured for many hours. Charophytes contain both salt-tolerant and salt sensitive genera.

We exposed salt-sensitive *Chara australis* to artificial pond water (APW) with 50 mM Na^+ and two Ca^{2+} concentrations: 0.1mM (high Na^+/low Ca^{2+}) and 1.0mM (high Na^+/high Ca^{2+}). To accustom the cells to lower turgor, they were initially exposed to sorbitol APW with osmolarity equivalent to that of 50mM NaCl. The membrane potential difference (PD) was voltage-clamped to bipolar staircase command to obtain current/voltage (I/V) profiles. These were then modelled to resolve the electrical characteristics of the proton pump, the inward and outward rectifiers and the background current (Beilby & Walker, 1996) as function of time in the two high salinity media.

Sorbitol APW. The I/V profiles were very similar to those in APW, despite of reduction of the turgor pressure. This lack of response contrasts with the turgor decrease-activation of the proton pump in the salt-tolerant charophyte *Lamprothamnium succinctum* (Al Khazaaly & Beilby, 2007). The results suggest that the *Chara* turgor sensor is not operating, or that the signal from the sensor to the pump is not communicated.

High Na^+/high Ca^{2+} APW. The resting PD changed from -227 ± 7 mV in sorbitol APW to -184 ± 20 mV. The resting PD exhibited small oscillations of amplitude 0.5 to 1.0mV. The background conductance increased about four times compared to that in APW and sorbitol APW. The channels passing the background current are thought to be like the NSCCs (Non selective cation permeable channels) found in higher plants (Shabala *et al.*, 2006), which provide one of the pathways for Na^+ to enter the cells. In this medium the proton pump exhibited activation, but the increased rate of proton pumping was not sufficient to keep the membrane PD from depolarising. Another response to increased salinity was the inability of the proton pump to withstand voltage clamp to levels far from the resting PD (Beilby, 2007). After voltage-clamps to levels more negative than about -280mV, the pump was inhibited for tens of minutes and recovered very slowly or not at all. This effect limited the span of the I/V characteristics.

High Na^+/low Ca^{2+} APW/ following high Na^+/high Ca^{2+} APW. The surviving cells became even more fragile under voltage-clamp protocols and hyperpolarised limit for the I/V protocol was made less negative at -230 mV. The resting PD depolarised to -150 ± 20 mV. The proton pumping rate declined, below that observed in APW, but the background conductance remained at the high level similar to that in high Na^+/high Ca^{2+} APW. The resting PD exhibited small oscillations 2 to 5mV.

High Na^+/low Ca^{2+} APW/ following sorbitol APW. These cells exhibited greater increase in the background conductance than the group pre-treated in High Na^+/high Ca^{2+} APW. The proton pump was totally inhibited in most of the cells with the resting PD of -100 ± 30 mV. Some cells exhibited repetitive spontaneous action potentials.

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Thermodynamics of ATP binding to the Na^+,K^+-ATPase

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The Na^+,K^+-ATPase is a P-type ATPase, found in all animal cells. The enzyme uses the free energy from the hydrolysis of ATP for transporting Na^+ and K^+ ions across the cell membrane. Previous work using stopped-flow fluorimetry suggests that the enzyme exists as a dimer with two gears of pumping depending on the number of ATP molecules bound (Clarke & Kane, in press). To test this hypothesis isothermal titration calorimetric (ITC) experiments have been performed to see if evidence for two ATP binding equilibria could be detected. Initial experiments showed heat signals due to a very slow ATP hydrolysis in addition to ATP binding. These signals disappeared, however, on adding the specific Na^+,K^+-ATPase inhibitor, ouabain, which blocks the enzyme in a phosphorylated state. The slow ATP hydrolysis must, therefore, be due to Na^+,K^+-ATPase activity stimulated by small amounts of divalent metal ions, which act as ATP cofactors for hydrolysis. The slow ATP hydrolysis could also be inhibited by the inclusion in the buffer of 10 mM CDTA, a strong Mg^{2+} chelator. Under these conditions any heat signals measured could be confidently attributed to ATP binding alone. ATP binding was also investigated by measuring the fluorescence anisotropy of the fluorescent ATP derivative, BODIPY FL ATP (Molecular Probes), over a range of concentrations at constant protein concentration. When the probe binds to Na^+,K^+-ATPase a significant increase in its fluorescence anisotropy occurs due to the reduced rotational mobility of the fluorophore. Analysis of both the ITC and fluorescence anisotropy data enabled ATP dissociation constants to be determined and a critical discussion of the dimer Na^+,K^+-ATPase hypothesis.

Clarke RJ & Kane DJ. (2007) *Biophysical Journal*, in press.

A single residue controls the substrate-induced conductance in the glutamine transporter SNAT3 (slc38a3)

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The glutamine transporter SNAT3 plays a pivotal role in several organs (Mackenzie & Erickson, 2004). In the liver it is involved in the uptake of glutamine, which serves as a central donor for ammonia dispatched in the urea cycle. In the brain SNAT3 releases glutamine from astrocytes and thereby forms part of the glutamate-glutamine cycle. In the kidney SNAT3 takes up glutamine into epithelial cells of the proximal tubule, where it is the principle donor of ammonia released in the urine to control plasma pH. The Mechanism of SNAT3 involves the cotransport of $1Na^+$ together with the substrate and the antiport of $1H^+$ (Broer *et al.*, 2002). As a result the SNAT3 transporter could be regarded as a glutamine-dependent Na^+/H^+ antiporter. Although the overall mechanism of the transporter is electroneutral, substrate-induced currents are observed when the transporter is expressed in *Xenopus laevis* oocytes.* These 'uncoupled' currents are carried by cations or protons, depending on the extracellular pH (Schneider *et al.*, 2007). To understand coupled and uncoupled movements of ions in the glutamine transporter SNAT3 we characterised site-directed mutants of the transporter. To identify residues that could be involved in Na^+-binding and translocation we compared the sequences of proton-coupled transporters (solute carrier family [slc] 36) with those of Na^+-coupled transporters (slc38). Residue Asn76 was selected for further analysis and changed into aspartate, glutamate, glutamine, histidine or serine. All of the mutants showed residual uptake of glutamine ranging from 10% to 40% of that of the wildtype. Subsequently, mutants were analysed in more detail by recording substrate-dependent currents, substrate-independent currents and by recording the intracellular pH using pH-sensitive microelectrodes. Mutation of Asn76 into any other residue completely abolished substrate-induced currents at pH 7.4, where they are largely carried by cations and at pH 8.4 where they are largely carried by protons. The glutamine-dependent Na^+/H^+ exchange activity by contrast was still readily detectable confirming the residual uptake of radioactive glutamine. The Asn76Gln replacement in particular showed significant coupled transport but no substrate-induced currents. Replacement of Asn76 by aspartate not only abolished substrate-induced currents but caused partial suppression of a substrate-independent conductance observed at pH 8.4. Removal of Na^+ renders wildtype and mutant transporters non-functional, due to the Na^+ cotransport mechanism. However, addition of glutamine in the absence of Na^+ caused a huge increase of the membrane conductance in case of the Asn76Asp mutation but not in the wildtype. A model explaining these observations in terms of an alternate access mechanism of the SNAT3 protein has been developed.

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*Procedures to remove oocytes were approved by the animal ethics committee of the ANU and the state of Rheinland-Pfalz.

Identification of early endocytic structures after stimulation of pancreatic acinar cells

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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 do not collapse but persist at the plasma membrane for many minutes over which time the fusion pores remain open (Thorn *et al.*, 2004). Most recently we have suggested that exocytosis is terminated by closure of the fusion pore not granule collapse (Larina *et al.* 2007). However, the next step, that of endocytic recovery of the granule membrane, still remains unclear. Past studies of endocytosis in epithelial cells have used extended stimulus times and followed endocytosis over hours (Oliver & Hand, 1978). In contrast, to identify the earliest endocytic events we use short stimulation times and now describe for the first time the recovery of large (~450 nm diameter) vesicles.

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 Horseradish Peroxidase (HRP, 50 mg/ml) for 30 minutes. Acetylcholine (1 mM) was applied at room temperature to the tissue fragments and its action terminated 1 minute later by the application of Atropine (10 mM). At various time points after stimulation, the tissue fragments were washed at 4^{o}C in extracellular buffer and then incubated in TRIS-buffered saline at pH 7.6 that contained diaminobenzidine (DAB, 1 mg/ml), ascorbic acid (50 mM) and H_2O_2 (0.012%) on ice. This protocol has been used to specifically stain endocytic structures; any compartment that is in continuity with the extracellular environment the ascorbic acid quenches the HRP-DAB reaction (Kirkham *et al*., 2005). After allowing 10 minutes for the HRP-DAB reaction the tissue fragments were fixed in glutaraldehyde (2.5%) and processed according to standard electron microscopy protocols. Thin (69 nm) tissue sections were then studied by transmission electron microscopy.

Results. In unstimulated tissue fragments the cells showed no evidence of granules fused with the plasma membrane. They did show some electron-dense DAB reaction product, localized to small, mulitvesicular bodies likely to be lysosomal compartments where endogenous oxidases may have reacted with the DAB. In stimulated tissue fragments, left to recover for 10 minutes prior to adding DAB, we observed unstained granules fused to the plasma membrane with open fusion pores; consistent with the protracted fusion events seen in live-cell experiments (Thorn *et al.*, 2004). Electron-dense DAB reaction product was now observed in large vesicles that were predominantly located towards the apical end of the cells. The measured diameter of these electron-dense vesicles was 450 ± 17 nm (mean diameter ± SEM, n = 44, 3 independent preparations). These labeled vesicles are statistically smaller in size compared to the total population of zymogen granules (748 ± 11 nm, n = 230, mean diameter ± SEM).

Conclusions. We here describe the primary endocytic structures formed immediately after stimulation of epithelial cells. Our technique unambiguously identifies recaptured vesicles that are no longer in continuity with the extracellular environment. The labeled structures we observe are surprisingly large and while this might suggest that whole granules are endocytically recaptured it is still unclear why they are smaller than the total population of zymogen granules.

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AKT and SGK-1 regulate albumin endocytosis *via* separate signalling pathways

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Albumin endocytosis in the renal proximal tubule occurs *via* a receptor medicated process that requires the coordinate regulation of a number of transmembrane and accessory proteins. Previously, we have demonstrated an essential role in albumin uptake for the Cl^- channel, ClC-5 as well as accessory proteins NHERF-2, Nedd4-2 and cofilin. The Serum Glucocorticoid Kinase 1 (SGK-1) is a member of the AGC kinase family of proteins that associates with both NHERF-2 and Nedd4-2. In addition, another AGC family member, AKT (v-Akt Murine Thymoma Viral Oncogene or Protein Kinase B), plays a role in the initial stages of albumin uptake *via* an association with the albumin receptor megalin. The roles of both these AGC kinases in regulating constitutive albumin uptake have yet to be determined.

In this study we investigated the roles of AKT and SGK-1 in albumin endocytosis using the well characterized opossum proximal tubule (OK) cell line. We then investigated whether pathophysiological concentrations of albumin regulated the levels of these kinases. To measure albumin uptake we used our standard protocol with Texas Red conjugated albumin (TR-albumin). Overexpression of dominant negative kinases or siRNA were used to manipulate the activity of the kinases in OK cells. We found that treatment of OK cells with AKT inhibitor I (Invitrogen) significantly reduced albumin uptake ($75 \pm 5\%$ compared to control; $n = 4$; $p < 0.05$), indicating a role for AKT in constitutive endocytosis in proximal tubule cells. Inhibition of the downstream ERK1/2 kinases with U1026 did not further reduce uptake (70 ± 2 % compared to cells with AKT silenced; $n = 3$), suggesting that AKT was upstream of ERK in this pathway. When the concentrations of albumin were increased to either 100 or 1000 μg/ml, Western blot analysis showed that total AKT was significantly reduced ($49 \pm 9\%$ and $62 \pm 13\%$, respectively, $n = 3$, $p < 0.05$). However, there was no change in the level of phosphorylated AKT indicating that the level of activity is maintained.

To investigate the role of SGK-1, we used both silencing and dominant negative strategies. When SGK-1 was silenced albumin uptake was reduced to $68 \pm 8\%$ ($n = 4$) of control levels. Similarly the kinase dead mutant of SGK-1 also reduced uptake to $81 \pm 3\%$ ($n = 4$). Both treatments significantly reduced the level of TR-albumin endocytosis ($n = 4$, $P < 0.05$) compared to control. Further, exposure of proximal tubule cells to increased levels of albumin (100 μg/ml or 1000 μg/ml) caused significant decreases in the levels of SGK protein in the cells ($87 \pm 3\%$ and $84 \pm 5\%$, respectively, $n = 3$, $p < 0.05$).

SGK and AKT can act *via* the same pathways. In order to determine if these two kinases were using the same pathway to inhibit albumin uptake, we treated cells silenced for SGK-1 with AKT inhibitor I (Invitrogen). This treatment resulted in a further significant reduction in the level of albumin uptake to ($50 \pm 7\%$, $n = 3$, $p < 0.05$) compared to cells silenced for SGK-1 ($68 \pm 8\%$, $n = 3$). This important result indicated that the effects of SGK-1 and AKT on albumin uptake are mediated by different signalling pathways. The activity of the sodium hydrogen exchanger NHE3 is required for albumin uptake. NHE3 is also inhibited by SGK-1. We therefore investigated whether SGK-1 was inhibiting albumin uptake by inhibiting NHE3. In control cells, the NHE exchange inhibitor EIPA (50 μM) reduced albumin uptake by $61 \pm 3\%$ ($n = 3$). When these experiments were repeated in cells in which SGK-1 had been silenced, there was no further inhibition of albumin uptake ($66 \pm 6\%$, $n = 3$, $p < 0.05$). This result suggests that SGK may inhibit albumin uptake by inhibiting NHE3.

This study shows that AGC kinases differentially regulate constitutive albumin uptake by using different signalling pathways. The alterations in protein levels in the presence of high albumin suggest that these proteins may be key players in mediating the changes in albumin handling in proteinuric renal disease.

Regulation of the epithelial Na^+ channel by caveolin

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Function of the epithelial Na^+ channels (ENaC) in the kidney and colon is essential for the regulation of Na^+ and fluid homeostasis, and the control of blood pressure. Membrane expression of ENaC is regulated by Nedd4-2, a ubiquitin protein ligase that induces ubiquitination of ENaC (Snyder *et al.*, 2004). It has been suggested that ubiquitinated-ENaC may undergo internalisation *via* clathrin-dependent endocytosis (Shimkets *et al.*, 1997). This notion is supported by the findings that ENaC subunits are localised in clathrin-coated vesicles (Wang *et al.*, 2006) and that they interact with several clathrin adaptor proteins (Staruschenko *et al.*, 2005; Wang *et al.*, 2006). ENaC subunits were also detected, however, in the detergent-insoluble glycosphingolipids- and chloresterol-enriched, membrane fraction which contains lipid rafts (Hill & Johnson, 2002). It is well established that caveolin is a structural protein that is essential for the formation of lipid rafts. A subsequent finding that ENaC and cavolin are colocalized (Jornot *et al.*, 2005) has prompted speculation that clathrin-independent endocytosis, mediated by caveolin, may also regulate membrane abundance of ENaC. In this study, we investigated the role of caveolin in the regulation of ENaC in Fisher rat thyroid (FRT) cells heterologously expressing ENaC grown on a permeable support. Activity of ENaC was measured as the amiloride-sensitive short-circuit current under open-circuit conditions (Lee *et al.*, 2007). Activity of caveolin in FRT cells was increaseded by co-transfecting the cells with caveolin constructs. Conversely, caveolin expression was reduced by transfecting the cell with an siRNA specific to caveolin isoform. Our findings suggest that caveolin is an important regulator of ENaC that controls its activity by regulating membrane expression of the channel.

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Determining albumin degradation rates in the proximal tubule

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Albuminuria is a marker of renal impairment and predicts poor cardiovascular and renal outcome in subjects with diabetes and hypertension. Albuminuria in itself is now recognised as a major mediator of tubular damage contributing to the progression of renal fibrosis and chronic kidney disease. Significant controversy persists around how albumin is handled by the kidney and how these pathways are affected during renal disease. Currently, there is no consensus as to how much albumin is processed by the proximal tubule and how many pathways are involved. However, it is widely accepted that a significant proportion of filtered albumin is endocytosed by the proximal tubule and undergoes proteolytic degradation to constituent amino acids in the proximal tubular epithelial cells (PTECs) to be reutilised by the body. Much research has focused on the molecular mechanisms underlying receptor-mediated albumin endocytosis. While this is of obvious importance, the other side of albumin processing in the proximal tubule, *i.e.,* degradation remains largely unexplored. Little experimental data exists regarding the dynamics of albumin degradation in the proximal tubule and how this pathway is affected by pathophysiological conditions or disease. This lack of data can be attributed to the difficulty in accurately and quantitatively assessing albumin degradation in a robust manner.

In this study, we have assessed the suitability of two auto-quenching fluorescent albumin conjugates, DQ-green-BSA and DQ-red-BSA as experimental tools to correlate the degradation of albumin with it's uptake in the proximal tubule both *in vitro* and *in vivo*. Initially, the characteristics of the DQ-BSA probes were assessed. In their native forms, DQ-BSA probes did not fluoresce above background levels. Fluorescence was detected after tryptic digestion of the probes in a time- and concentration- dependent manner. The probes were stable across a range of pHs, again only fluorescing after tryptic digest. Using a proximal tubular epithelial cell line (OK cells), albumin degradation in live cells was assessed. Cells were incubated with increasing amounts of DQ-BSA over a 3 hour timecourse. Fluorescence was detected in cell lysates in a time- and concentration-dependent manner. DQ-BSA fluorescence was reduced by the addition of increasing concentrations of unlabelled BSA, demonstrating the specificity of the pathway involved. The rate of albumin degradation was seen to be proportional to its uptake and at any given time point, approximately 40-50% of albumin had been degraded. Inhibition of the lysosomes with CHQ resulted in significant inhibition of albumin degradation in cells. Similarly, treatment of cells with an inhibitor of receptor mediated endocytosis, latrunculin A also significantly reduced albumin degradation. Confocal analysis revealed that DQ-BSA fluorescence was localised in large vesicles concentrated towards the baso-lateral surface of the PTECs. Together, these results indicated that DQ-BSA fluorescence was dependent on a receptor-mediated, lysosomal degradation pathway that had high affinity for albumin. This was also examined *in vivo*. Normal wistar rats were injected with 2mg DQ-BSA intra venously and sacrificed at 5, 10 and 20 minutes after injection. Kidneys were removed and fixed for sectioning. DQ-BSA fluorescence indicating albumin degradation in PTECs was evident at all timepoints examined. Further studies were performed to determine if albumin degradation was affected by pathophysiological conditions associated with CKD. In OK cells, albumin degradation was significantly decreased by pre-exposure to TGF-β1, high glucose and angiotensin II.

Together, these results have validated DQ-BSA probes as tools to analyse albumin degradation in the proximal tubular cells allowing correlation of albumin uptake and degradation showing that ~40-50% of reabsorbed albumin is being degraded at any given time. Further studies will allow us to delineate more fully the components of albumin uptake pathway and examine how this degradation pathway is modulated under disease conditions.

Carbonic anhydrase inhibition - a novel therapeutic strategy for renal disease

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Diabetic nephropathy is characterised by initial renal hypertrophy followed by progressive renal fibrosis eventually leading to end-stage renal disease. Research has focused on fibrotic processes and downstream effects of extracellular matrix accumulation, however, metabolic stress and hemodynamic factors (such as increased Angiotensin II (AngII) promote proximal tubular epithelial cell (PTEC) dysfunction prior to fibrosis. PTEC hypertrophy in diabetic nephropathy is associated with increases in the filtered load of Na^+. Primary transport functions of the proximal tubule are linked, in that availability of bicarbonate and activity of carbonic anhydrases (CAs) play a rate-limiting role by providing substrate for a number of these transporters. The cytosolic carbonic anhydrase (CAII) has been recently found to bind directly to membrane transporters to form so-called metabolons. Here the co-localisation of CAII with the transporters serves to maximise the HCO_3^- gradient for optimal transporter function.

This study seeks to delineate the hypertrophic effects from the fibrotic effects of AngII with a focus on the role of CA2 and whether modulation of CAs in the proximal tubule could have beneficial effects in preserving PTEC function under pathological conditions. The roles of CAs were investigated *in vitro* (HK-2 and OK cell lines) and *in vivo* (Ren2 rat model). Effects on CAII/IV expression were examined by Western blot and immunohistochemistry. Albumin uptake was measured using a fluorescent probe. The roles of CAs in AngII-induced PTEC hypertrophy and altered protein reabsorption were assessed using the CA inhibitors acetazolamide (ACZ) and ethoxyzolamide (ETZ). TGF-1 production was measured by ELISA.

Exposure of PTECs to AngII induced a significant hypertrophic response that was inhibited by the membrane permeable CA inhibitor ETZ but not membrane impermeant ACZ. This demonstrated that this effect was likely mediated by CAII. PTECs exposed to high glucose, AngII and TGF-β1 displayed impairment of normal protein reabsorption. This effect was significantly decreased in the presence of ETZ while the normal basal rate of protein reabsorption was unaffected. Under these conditions, protein expression of CAII was upregulated in PTECs. ETZ also significantly reduced high glucose-induced TGF-1 production in PTECs. In a Ren2, streptozotocin-induced diabetic rat model, elevated levels of CAII were detected both on Western blot and in the proximal tubules by immunohistochemistry.

These data clearly show that CAII plays a key role in mediating the hypertrophic response in PTEC and its levels are altered in diabetic nephropathy. Strategic inhibition of CAII may be of benefit in slowing the early onset diabetic nephropathy.