C41

Angiotensin II inhibits SGLT-dependent glucose uptake in isolated jejunum by a process involving AT1-receptor stimulation at the brush border membrane

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Angiotensin II (AII) influences Na^+ and fluid transport across the small intestine, an effect mediated by AT1 and AT2 receptors. The observation that AII inhibits Na^+-dependent (SGLT-mediated) glucose transport in renal cells (Kawano et al. 2002; Han et al. 2003) raises the issue of whether AII affects enterocyte sugar transport. Of further interest is the possibility of local secretion and action of AII, since components of an intrinsic renin-angiotensin system (RAS) have been detected in jejunal mucosa (Cox et al. 1986; Duggan et al. 1989). The aims of our present study were to determine whether a local RAS is expressed in enterocytes per se, and to examine the influence of AII on glucose uptake across the luminal membrane.

Protein and mRNA expression of RAS components in villus enterocytes isolated from rat jejunal and ileal sections were measured using Western blotting and real-time PCR. Mucosal uptake of ^{14}C-glucose was determined using everted jejunal sacs with or without AII (0-100 nM) in the mucosal fluid.

Gene expression of angiotensin converting enzyme (ACE), angiotensinogen (AO), AT1 and AT2 receptors was detected in enterocytes, mRNA levels for AO, AT1 and AT2 receptors being 3.3-, 3.6- and 3.3-fold, respectively, greater in ileal compared to jejunal cells. Ileal:jejunal expression of AT1 and AO protein was 1.6 and 2.6, respectively. Immunocytochemistry revealed that AT1 receptors were expressed at both brush border and basolateral membranes. Phlorizin, a blocker of SGLT-mediated glucose transport, inhibited glucose uptake. The addition of AII to the mucosal fluid rapidly (within 2 min) also suppressed jejunal glucose uptake (12.0±0.4 and 2.8±0.1 nmol glucose/mg dry weight/min at AII = 0 and 100 nM, respectively, $p<0.001$ Student's unpaired t test), an effect that was dose dependent and which could be abolished by adding the AT1 receptor antagonist Losartan (1 μM) to mucosal fluid.

Our detection of an enterocyte RAS, together with the finding that AT1-receptor stimulation at the brush border membrane suppressed glucose uptake implies a hitherto unrecognized system for regulation of intestinal glucose transport. The higher RAS expression in ileum compared to jejunum may indicate differential inhibitory effects of the RAS system on glucose uptake in the two regions.

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C42

Effect of polarization and glucose concentration on glucose transport in H441 lung epithelial cells

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Glucose concentration in the fluid that lines the lumen of human airways (~0.5mM) is normally lower than that of blood (5mM). This implies that glucose is actively transported out of the airway lumen. We have also shown that people with hyperglycaemia have increased glucose levels in airway secretions (1). However, it is not known how glucose is transported into or out of the human airway lumen. Glucose transporters can be divided into two groups: the facilitative glucose transporters (GLUTs), and the sodium-glucose co-transporters (SGLTs). The aim of this study was to assess the relative contribution of glucose transporters to glucose transport in polarised and non-polarised human bronchiolar epithelial H441 cells, maintained at 1, 5, 10 and 15 mM glucose overnight, using phloretin (GLUT inhibitor), phlorizin (SGLT inhibitor) and ouabain (Na^+,K^+-ATPase inhibitor).

Functional transport studies were carried out over 10 min using ^3H-glucose. In non-polarized cells glucose transport was preferentially via GLUTs, with phloretin inhibiting ~90% of transport at all glucose concentrations (n = 6). Lowest levels of uptake were observed at 1 mM (18.4 ± 0.2 nmol glucose/mg protein), levels were raised at 5 and 10 mM (25.2 ± 0.2 and 26.5 ± 0.2 nmol glucose/mg protein, respectively) and maximal at 15 mM glucose (34.0 ± 1.6 nmol glucose/mg protein). We observed no ploridzin-inhibitable, SGLT-mediated glucose transport in non-polarised cells. Cells were polarised by growing as confluent monolayers at the air interface on permeable supports for 6-7 days. Under these conditions GLUT-mediated transport remained the principal mechanism of glucose uptake across both the basolateral and apical membrane. However, a significant SGLT-mediated transport component was observed across the basolateral membrane at all glucose concentrations studied (≥ 20%) and was also present across the apical membrane at 1 and 5 mM glucose. The effect of ouabain indicated that SGLT-mediated transport was coupled to the transmembrane Na^+ gradient at 1 and 5 mM glucose but not at 10 and 15 mM glucose. Furthermore, in contrast to non-polarised cells, total transport across both apical and basolateral membranes of polarised cells was lower at 15 mM glucose (apical 6.6 ± 0.2; basolateral 18.1 ± 0.6 nmol glucose/mg protein) than at 10 mM glucose (apical 24.6 ± 1.4; basolateral 44.0 ± 1.2 nmol glucose/mg protein, $p < 0.05$, n = 6).

These data indicate that both facilitative and active glucose transport systems are present in H441 lung epithelial cells and that both may have a role in transporting glucose out of the airway lumen. The process of cell polarisation induced SGLT-mediated glucose transport across apical and basolateral membranes. In addition, polarisation caused glucose uptake to be down-regulated when cells were subjected to 15mM glucose.

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C43

Interleukin 2 maintains surface expression of System L amino acid transporter in activated mouse T-lymphocytes

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Activation of T-cells during the immune response is accompanied by increases in cell growth and signalling through the nutrient-sensitive mTOR pathway (to downstream components of the mRNA translational machinery) only in the presence of external amino acids (Fumarola et al. 2005). T-cell activation is also associated with increased surface expression of CD98, the glycoprotein subunit of the System L amino acid transporter (Verrey, 2003). We are currently investigating whether this transporter upregulation lies upstream or downstream of mTOR pathway activation and also whether transporter expression correlates with the regulation of T-cell size by the cytokine interleukin 2 (IL-2).

Murine spleen-derived T-cells were activated via the T-cell receptor for 48h in suspension culture. Cells were subsequently incubated in the presence of either IL-2 (20 ng/ml) or medium alone for periods of up to 48h, with addition of a phosphotidylinositol 3-kinase (PI 3-K) inhibitor (LY294002; 10 μM) for the final 24h of certain experiments. System L transport function was assessed by cellular uptake of [^{3}H]-phenylalanine using [^{14}C]-inulin as an extracellular marker. CD98 surface expression was estimated by fluorescence activated cell sorting.

Activated T-cells incubated with IL-2 exhibit phenylalanine transport (K_m = 15 μM, V_{max} = 85 ± 9 pmol/10^6 cells/min; n = 3 cell preparations) with features characteristic of System L, notably a potent competitive inhibition by 2-amino-bicyclo [2,2,1] heptane-2-carboxylic acid (BCH; K_i = 9 μM) and a lack of Na^+ dependence. Phenylalanine transport was not affected by change in external pH between 6.2 and 8.6. CD98 surface expression and System L transport function were both substantially reduced (by > 80%) in activated T-cells deprived of IL-2 for 24h. CD98 surface expression was also downregulated by LY294002 treatment of T-cells maintained in IL-2: this process also correlated with reduced transport activity. Western blotting of T-cell membrane proteins revealed that changes in CD98 expression were reflected by similar, IL-2 regulated, changes in expression of the catalytic System L subunit protein LAT1.

The results show that activated T-cells express a functional System L transporter maintained at the cell surface by an IL-2 regulated, PI 3-kinase-dependent pathway. Regulated expression of this transporter may be important for control of T-cell growth and differentiation by the mTOR intracellular signalling pathway.

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C44

Dynamics of the fusion pore during exocytosis in mouse pancreatic acinar cells

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Enzyme secretion in pancreatic acinar cells is dependent on the exocytosis of secretory granules (1). After fusion with plasma membrane the granules remain close to the plasma membrane for many minutes and, at least in the early stages after fusion, the fusion pore is open (2). In other systems fusion pore dynamics have been observed and shown to be a factor in the release of granule contents (3). However, any dynamic to the behaviour of the fusion pore in acinar cells has not been studied. In the present study we have set out to determine if fusion pore dynamics are present in pancreatic acinar cells.

Fragments of mouse exocrine pancreas were prepared by a collagenase digestion method. In control experiments Lysine-fixable Texas Red (TR) and Fluorescein dextran (FD) were added to the extracellular solution before cell stimulation. These extracellular dyes then entered and labelled granules that had undergone exocytosis stimulated with ACh. The tissue fragments were then fixed in 4% PFA and the staining visualized with a confocal microscope. In experiments designed to study fusion pore dynamics, TR and FD application were separated in time. TR was added at the beginning of all experiments and, to probe for fusion pore closure, FD was added 2, 6 or 11 min after cell stimulation with ACh. To measure fusion pore dynamics in live cells we used 2-photon microscopy and visualised fusion pore dynamics by the simultaneous imaging of extracellular dyes sulforhodamine (SRB) and methoxypyrenetrisulfonic acid (MPTS).

In control experiments the simultaneous application of FD and TR before cell stimulation produced, as expected, identical patterns of staining. The FD/TR fluorescence ratio, measured in each individual granule, had a mean value of 0.84 ± 0.28 (mean ± SEM, n=175 granules) with no granules showing ratios lower than 0.2 indicating that granules are filled approximately equally with both dyes. When FD was added at later time points the FD/TR ratio subtly changed. For example, application of FD 2 min after stimulation gave a mean FD/TR ratio of 0.78 ± 0.43 (mean ± SEM, n=113). A Shapiro-Wilk normality test showed the control FD/TR ratio distribution was not significantly different from a Gaussian (P=0.2) but when FD was added after a 2 min delay the distribution was significantly skewed (P<0.05) with a predominance of low FD/TR ratios. For example, now 12.38% of granules had a ratio of less than 0.2 These small ratios indicate that these granules were filled predominantly with TR dye and we conclude that the fusion pore must have closed preventing entry of FD. Experiments using the 2-photon technique were performed in a similar manner, with SRB present throughout the experiment and the MPTS added at later time points to

probe for fusion pore closure. We then imaged the granules labelled with SRB only (indicative of fusion pore closure) and after a period of time were able to observe sudden filling of the granule with MPTS, indicating fusion pore reopening.
In conclusion, we show the fusion pore in acinar cells is dynamic. We hypothesize that this behaviour may be important in the regulation of enzyme release.

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SA23

Visualizing and manipulating phosphoinositide-mediated signalling in living cells

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Phosphoinositides are universal regulatory lipid molecules that control multiple signal transduction events and membrane dynamics within the cell. These lipids are present in tiny amounts in biological membranes and are formed and eliminated by multiple inositide kinase and phosphatase enzymes, found in specific cellular compartments. The highly localized roles of these molecules demand the development of methods that can resolve their changes with high temporal and spatial resolution, preferably in living cells. We have been working with protein domains that specifically recognize phosphoinositide isomers and created GFP fusion proteins to visualize lipid changes in intact cells. We also used these domains to interfere with the signalling function of these phospholipids. We will summarize our experience with these methods and review both their benefits and the pitfalls. We will also show new data on imaging of plasma membrane PtdIns4P with the OSH2 tandem PH domain – EGFP fusion protein. We demonstrate the specificity of this probe in vivo within the cells using a type-IV 5-phosphaatase enzyme to convert PtdIns(4,5)P2 to PtdIns4P and simultaneous imaging of PtdIns(4,5)P2 (with PLCd1PH-mRFP) and PtdIns4P (with OSH2-PH-tandem-EGFP). The value of this method to determine which PI 4-kinase enzyme(s) produce the PtdIns4P that is converted to PtdIns(4,5)P2 for agonist-induced Ca2+ signalling will be also shown. Finally, we will review our newest approaches to manipulate Ins(1,4,5)P3 and phosphoinositide levels in specific cellular compartments and will demonstrate how these techniques can help to understand the complexity of phosphoinositide-based signalling processes.

Where applicable, the authors confirm that the experiments described here conform with the Physiological Society ethical requirements.

SA24

Phosphatidylinositol metabolism and the regulation of polarized membrane traffic

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Significant roles for phosphatidylinositols (PtdIns) in membrane traffic have emerged in addition to their originally described function as intracellular second messengers. Distinct PtdIns species have been implicated in regulating interactions between the lipid bilayer and protein machinery involved in vesicle budding and traffic as well as in modulating cytoskeletal dynamics that affect transport kinetics. Efficient regulation of PtdIns production requires spatial and temporal control of PtdIns metabolism, and this is mediated in part by the subcellular distribution of phosphatidylinositol kinases (PIKs) and phosphatases. We are interested in the role of PtdIns metabolism in polarized biosynthetic delivery, and to this end, we have examined the effect of expressing individual PIKs and PtdIns phosphatases on lipid modulation and membrane traffic in a polarized renal epithelial cell system. Our studies have focused primarily on phosphatidylinositol 4- and 5-kinases, which sequentially metabolize PtdIns to phosphatidylinositol 4-phosphate and phosphatidylinositol 4,5-bisphosphate. Whereas the effects of PI4Ks on membrane traffic are most consistent with effects on the recruitment of trafficking machinery, overexpression of PI5K selectively stimulated surface delivery of an apical marker via a mechanism consistent with the involvement of actin comets. Our results suggest multiple mechanisms by which PtdIns synthesis and conversion differentially affect the surface delivery of apically- and basolaterally-destined proteins in polarized cells.

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SA25

Modulation of the epithelial Na^+ channel (ENaC) by phosphatidylinositides

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Activity of the epithelial Na^+ channel (ENaC) is limiting for Na^+ transport across many epithelia. Consequently, ENaC modulation is central to regulation of systemic blood volume and pressure. ENaC is an end-effector of diverse cellular signaling cascades, including those with phosphatidylinositide second messengers. In some instances, phospatidylinositides directly interact with ENaC to influence channel activity. $PI(4,5)P_2$ is permissive for ENaC opening having a direct effect on gating. Signaling from G protein coupled receptors and receptor tyrosine kinases decrease the membrane levels of $PI(4,5)P_2$ to decrease ENaC activity. $PI(3,4,5)P_3$, a second messenger in the aldosterone to ENaC signaling cascade, also affects channel gating through direct interactions. Rather than being permissive for gating, $PI(3,4,5)P_3$ increases ENaC open probability. The $PI(3,4,5)P_3$ binding site in ENaC involved in this regulation is localized to region just following the second transmembrane domain in the C-terminus of the γ-ENaC subunit. This domain contains several conserved positive charged residues. Substitution of these positive residues with neutral amino acids abolishes modulation by $PI(3,4,5)P_3$. In conclusions, our recent findings demonstrate that binding of phosphatidylinositides to ENaC directly regulate channel gating. This mode of channel regulation may be particularly important for ENaC responses to natriferic hormones, including aldosterone, involved in control of blood volume and pressure.

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SA26

Regulation of KCNQ potassium channels by phosphatidylinositols

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Voltage-gated Kv7 (KCNQ) potassium channels underlie important K^+ currents, including the neuronal M current and cardiac I_{Ks}. Recent work of several groups suggests high sensitivity of Kv7 channels to membrane phosphatidylinositol 4,5-bisphosphate (PIP_2) and puts forward the hypothesis that PIP_2 depletion underlie modulation of these channels by some $G_{g/11}$-coupled receptors, including muscarinic acetylcholine M1 and angiotensin II AT1 receptors. In this pathway, receptor stimulation results in activation of phospholipase C (PLC), which hydrolyzes PIP_2 into membrane-bound diacylglycerol (DAG) and soluble inositol trisphosphate (IP3) (reviewed by Delmas and Brown, 2005). Recent single-channel experiments suggest that PIP_2 acts as a stabilizer of channel opening by favouring the allosteric conformational change that opens the channel, such that channel open probability (P_o) is increased on the voltage-independent manner. It has also been demonstrated that different Kv7 channels display highly differential apparent affinity for PIP_2 (Li et al. 2005) and that different PIP_2 affinity may underlie highly diverse P_o of individual Kv7 channels in intact cells (for example both maximal P_o in cell-attached patches and apparent PIP_2 affinity of Kv7.4 are about 10 times lover then that of Kv7.3). I will discuss experiments that have led up to the establishment of the concept of PIP_2-sensitivity of Kv7 channels, its implications in health and disease as well as some unresolved problems in the field.

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