C46

Immunohistochemical localisation of a vacuolar-type H+-ATPase in porcine bronchi

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IntroductionIn order for the mechanisms by which the airways stay clear and free from infection to function normally, there must be strict control of the pH of the liquid bathing the luminal surface of the airway (pH_{ASL}). The mechanisms that regulate pH_{ASL} are not well understood, but our recent studies suggest that both glandular and surface epithelial regions are involved, and specifically that glandular secretions are relatively alkaline, whilst the surface epithelium secretes acid equivalents through the activity of bafilomycin A1-sensitive vacuolar type H^+-ATPase. (Inglis et al 2003). The aim of this study was to test the hypothesis that H^+-ATPase transporters are preferentially located in the plasma membrane of surface epithelial cells.Immunohistochemistry was carried out on sections of bronchus, which possess both glandular and surface epithelia, and these were fixed/embedded to see the presence and cellular distribution of subunits of the V1 sector of the vacuolar-type H^+-ATPase in the glands and epithelium. MethodCotswold pigs (15kg) were obtained from a local supplier, sedated with inhaled halothane, and killed with an intravenous overdose of pentobarbital sodium in accordance with UK and institutional regulations. Apical and middle lobes of the right and left lungs were excised and placed in KRB solution. Distal bronchi were carefully dissected free and fixed in a formalin solution. Porcine kidney was also dissected and placed in the fixative. As a secondary control rat kidneys were removed from animals that had been sacrificed for work in other areas of research following the recommended guidelines. The bronchi and kidney were embedded in paraffin wax prior to sectioning and immunohistochemistry using microwave antibody retrieval. Primary antibody was applied at a concentration of 1:1400.Positive and negative controls were carried out on rat kidney and porcine kidney, and also negative controls on porcine bronchi.ResultsResults in these tissues demonstrate the presence of a vacuolar-type H^+-ATPase in the apical surface of the epithelial cells in porcine bronchi. There also seems to be a presence in the submucosal glands, although this does appear to be much more diffuse throughout the cell. Positive and negative controls confirmed the efficacy of this technique.

SK Inglis, SM Wilson, RE Olver: Am J Physiol Lung Cell Mol Physiol 284: L855-L862, 2003

Wellcome Trust

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

C47

Stimulatory effect of *Scuterllariae Radix* extract on chloride secretion in human colonic T84 cells

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Scutellariae Radix (common name Huangqin), the dried root of *Scutellaria baicalensis* Georgi, has been frequently used in combination with other herbs in Traditional Chinese medicines for centuries. Our recent study has demonstrated that baicalein, the major flavonoid derived from the herb, stimulates Cl^- secretion across rat colonic mucosa (Ko *et al* 2002). The present study aims to investigate whether the herbal extract (Chen *et al* 2000) also stimulates anion secretion in human intestinal T84 cells and the underlying signaling pathways using the short-circuit current (I_{SC}) technique. Data are means ± S.E.M. of n observations.T84 cells were grown on permeable supports for 9 days using standard culture techniques. When clamped in the Ussing chambers and incubated in normal Krebs solution, T84 cells exhibited a basal I_{SC} of 1.5 ± 0.1 μAcm^{-2} and a transepithelial resistance of 627 ± 18 Ωcm^2 (n = 211). Apical addition of *Scutellariae Radix* extract (SRE) elicited a concentration-dependent increase in I_{SC} with an EC_{50} of 1.75 ± 0.16 mg/ml. Removal of extracellular Cl^- (n = 4) or basolateral addition of bumetanide (100 μM, n = 6) abolished the I_{SC} responses. The SRE-induced I_{SC} responses were sensitive to apical application of different Cl^- channel blockers such as DPC (100 μM), DIDS (100 μM) and glibenclamide (300 μM). The Cl^- secretory response to saturating concentration of forskolin (10 μM) was not further enhanced by SRE (n = 6). On the other hand, pre-treating the epithelia with the calcium mobilizing agent, thapsigargin (1 μM), did not prevent further increase in I_{SC} by SRE (n = 5). In contrary, the thapsigargin-evoked I_{SC} was potentiated by pre-treating the epithelia with SRE (n = 5). Moreover, SRE evoked an increase in cAMP production (n = 5). The SRE-evoked I_{SC} was sensitive to H89 (5 μM, n = 6), a protein kinase A (PKA) inhibitor and MDL 12330A (10 μM, n = 5), an adenylate cyclase inhibitor. In summary, the results indicate an increase in I_{SC} by SRE in T84 cells, which is due to Cl^- secretion. These data suggest that activation of adenylate cyclase, cAMP elevation and PKA pathway are involved in SRE's secretory action in T84 cells.

Chen ZY *et al.* (2000) *JAOCS* **77**, 73-77.

Ko WH *et al.* (2002). *Am J Physiol Gastrointest Liver Physiol* **282**, G508-518.

This work was supported by Earmarked Grant for Research, Research Grant Councils of Hong Kong (CUHK 4171/02M).

C48

Intracellular pH regulates the ATP-dependence of CFTR channel gating

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The cystic fibrosis transmembrane conductance regulator (CFTR) is a Cl^- channel with complex regulation. Channel gating is controlled by phosphorylation of the R domain and ATP binding and hydrolysis at two nucleotide-binding domains (NBDs). We previously demonstrated that intracellular pH (pH_i) regulates CFTR channel gating with acidic pH increasing open probability (P_o), but alkaline pH decreasing P_o and single-channel current amplitude (Chen *et al.* 2002). To understand better how pH_i regulates channel gating, we investigated the CFTR mutant CFTRΔR-S660A and the ATP-dependence of wild-type CFTR.We studied CFTR Cl^- channels in excised inside-out membrane patches from C127 cells expressing wild-type or mutant CFTR. The pipette (external) solution contained 10 mM Cl^- at pH 7.3. The bath (internal) solution contained 147 mM Cl^-, PKA (75 nM) and ATP (0.3 mM) at 37 °C. To adjust the bath solution to pH 8.3 and pH 6.3, we used Tris and H_2SO_4, respectively.To investigate the role of the R domain, we studied CFTRΔR-S660A, a mutant that deletes much of the R domain (residues 708-835) abolishing the PKA-dependence of CFTR. In contrast to wild-type CFTR, the P_o of CFTRΔR-S660A was low at both pH 7.3 and 8.3 (Student's paired t-test; $p = 0.36$). However, acidic pH_i stimulated greatly CFTRΔR-S660A (pH 7.3, $P_o = 0.06 \pm 0.01$; pH 6.3, $P_o = 0.26 \pm 0.06$; means ± SEM; $n = 6$; $p < 0.05$). These data suggest that the R domain might, in part, mediate the inhibitory, but not the stimulatory, effects of pH_i on CFTR channel gating.To examine the role of the NBDs, we studied the effects of $[MgATP]_i$ (0-10 mM, n = 6-7) on wild-type CFTR channel gating at different pH_i. The relationship between P_o and $[MgATP]_i$ was best fit by Michaelis-Menten functions at each pH_i. However, at pH 6.3, the K_m value was much reduced compared with those at pH 7.3 and 8.3 (pH 6.3, $K_m = 36$ μM; pH 7.3, $K_m = 90$ μM; pH 8.3, $K_m = 101$ μM). Moreover, the maximum P_o (P_{omax}) was increased at pH 6.3, but decreased at pH 8.3 (pH 6.3, $P_{omax} = 0.71$; pH 7.3, $P_{omax} = 0.61$; pH 8.3, $P_{omax} = 0.49$). These data suggest that pH_i might alter both the ATP affinity and ATPase activity of the NBDs. To understand better the effects of pH_i on the NBDs, we used Mg^{2+}-free bath solutions containing ATP (3 mM). In the absence of Mg^{2+}, P_o was greatly decreased at pH 7.3 and 8.3, but less reduced at pH 6.3 (n = 5-7). These data suggest several conclusions. First, acidic pH might enhance channel gating by increasing ATP binding to the NBDs. Second, alkaline pH might inhibit channel gating by slowing ATP hydrolysis at the NBDs. Third, amino acid residues in the NBDs might account for most of the pH_i dependence of CFTR channel gating.

Chen, J.H., Cai, Z. & Sheppard, D.N. (2002) *J. Physiol.* **544P**, 15P.

Supported by the CF Trust and the University of Bristol.

C49

Minimal effect on fluid absorption in vivo of E.Coli STA enterotoxin in the absence of Luminal Sodium ION: Further evidence against the epithelial cell chloride secretion model

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Heat stable enterotoxin (STa) from E.coli reduces net fluid absorption from rat proximal jejunum by interruption of sodium ion uptake. Stimulation by STa of epithelial cell chloride ion secretion could also contribute through associated fluid secretion. However, secretion is never detected in vivo with STa when a perfusate recovery method is used (Lucas et al., 2001). Experiments were therefore done under circumstances where sodium ion uptake and hence fluid uptake was minimised in the expectation that any underlying STa stimulated secretory process would become evident. Fluid uptake was investigated in vivo in anaesthetised (70 mg /kg i.p Sagatal) Wistar rats in recirculated perfused loops using recovered luminal volume to assess net fluid absorption. Loops of twenty five cm of proximal jejunum were perfused with buffers in which sodium ion was replaced by choline ion or by mannitol, with and without STa. In addition, some buffers contained 0.1 mM ethyl-iso-propyl-amiloride (EIPA) to inhibit any residual sodium ion uptake arising from nominally zero sodium ion perfusates. At the end of the experiment, the animals were humanely killed. Fluid absorption (mean +/-s.e (n)) from 150 mM saline was 56.1 +/- 10.0(6)μl/cm/hr v 28.6 +/- 4.5(7) μl/cm/hr in the presence of STa ($p<0.05$). Replacement of sodium ion by choline ion caused negative net fluid absorption of 13.3 +/- 4.0(7) μl/cm/hr, further reduced ($p<0.05$) to 30.4 +/- 5.5(6) μl/cm/hr when STa was added. When mannitol substituted for sodium chloride, negative fluid absorption was 11.9 +/- 4.9 (6) μl/cm/hr and increased to 32.4 +/- 8.2(5) μl/cm/hr by STa. To determine whether the smaller effect of STa in the absence of sodium ion was dependent still on residual sodium ion uptake, the experiments were repeated with the NHE-3 inhibitor EIPA additionally present. In the zero sodium ion perfusates with 01.uM EIPA negative fluid movement was 17.4 +/- 5.7(6) μl/cm/hr and 16.6 +/-3.4(6) μl/cm/hr when STa was present. These experiments indicate that when fluid uptake is inhibited by inhibiting sodium ion uptake, STa does not have the expected further effect on fluid movement through additional secretion. These observations make unlikely the hypothesised epithelial cell chloride ion secretion, proposed as the basis of enterotoxin action (Field et al.,1978).

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Field, M., Graf Jr., L.H., Laird, W.J. & Smith, P.L. (1978) Heat-stable enterotoxin of Escherichia coli: Proc natl Acad Sciences USA 75, 2800-2804.

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

C50

Regulation of a selective Na^+ conductance in H441 airway epithelial cells by cAMP

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Distal airway epithelia absorb Na^+ from the airway surface liquid and this process is acutely regulated by cAMP-dependent agonists (Olver *et al.* 2004). However, some groups attribute this response to an increase in sodium conductance (G_{Na}, see *e.g.* Collett *et al.* 2002) whilst others suggest that such agonists act by hyperpolarizing V_m and thus increasing the driving force for Na^+ entry (O'Grady *et al.* 2000). Here we report the effects of cAMP on the Na^+ conductance expressed by dexamethasone-stimulated H441 distal airway epithelial cells (Clunes *et al.* 2004), which has properties essentially identical to the conductance associated with co-expression of α, β and γ-ENaC (Canessa *et al.* 1994).Exposing the cells to a cocktail of cAMP-activating drugs caused a clear increase in G_{Na} (Fig 1A,C) whilst no such change in G_{Na} occurred in control cells (Fig 1B,C). The cAMP-activating drugs had no effect upon the reversal potential for the amiloride-sensitive component of the membrane current (control E_{rev} 74 ± 11 cf. cAMP-stimulated E_{rev} 82 ± 8 mV), and so this increase in conductance occurs with no change in Na^+ selectivity. In contrast, the cAMP cocktail caused a depolarising shift in the reversal potential for the total membrane current ($P<0.05$, ΔE_{rev} 18 ± 4 mV) indicating the response is associated with a depolarisation of *V*m.These data thus suggest that cAMP-stimulated Na^+ absorption reflects an increase in the activity and or surface abundance of highly selective sodium channels.

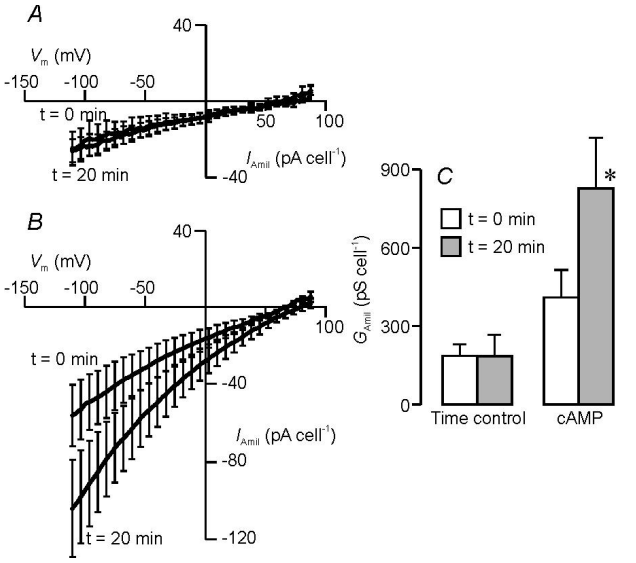

Figure 1. H441 cells were grown (~24 h) on glass coverslips in the presence of 200 nM dexamethasone before being studied using the perforated patch technique; all data are mean ± S.E.M. (n>4). The current – voltage relationship for the amiloride-sensitive current (I_{Amil}) measured at 0 and 20 min in control (A) or cAMP stimulated cells (B, 10 μM forskolin, 100 μM isobutylmethylxanthine, 1 mM N6,2′-O-dibutyryladenonsine 3′5′-cyclic monophoshate). C, Estimates of the amiloride-sensitive membrane conductance (G_{Amil}) from linear regression of data collected at negative holding potentials at 0 and 20 min. * denotes significance $P<0.05$ (Student's *t* test).

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Collett, A., Ramminger, S. J., Olver, R. E. & Wilson, S. M. (2002) *Am. J. Physiol. Lung Cell. Mol. Physiol.* 282, L621-L630.

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Olver, R. E., Walters, D. V. & Wilson, S. M. (2004). *Annu. Rev. Physiol.* in press.

This work was supported by grants from the Wellcome Trust, Tenovus Scotland and the Dale Fund.

C51

Simultaneous effects of nucleotides upon ion transport and intracellular calcium in H441 human bronchiolar epithelial cells

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Apical nucleotides have been shown to inhibit Na^+ transport in many absorptive epithelia, a response often attributed to $P2Y_2$ receptors which characteristically display equal sensitivity to ATP and UTP (Ramminger *et al.*, 1999) and allow these nucleotides to increase $[Ca^{2+}]_i$. We have now measured I_{SC} and $[Ca^{2+}]_i$ simultaneously and explored the effects of nucleotides upon the two parameters in H441 polarised cells. Apical ATP (Fig 1A) evoked a rapid transient in I_{SC}, followed by a larger sustained response that is accompanied by a comparable $[Ca^{2+}]_i$ response. In contrast, UTP (Fig 1B) evoked a transient increase in I_{SC} followed by a return to baseline, and was still accompanied by a large $[Ca^{2+}]_i$ signal. The different effects of ATP and UTP suggested the activation of separate receptor populations and not the proposed $P2Y_2$ subtype. Basolateral ATP and UTP evoked no discernible changes in I_{SC} or $[Ca^{2+}]_i$. Apical Adenosine consistently evoked a monophasic increase in I_{SC} without an accompanying $[Ca^{2+}]_i$ response (Fig 1C), a smaller response (⌣50% $P<0.05$ Students paired t test) was seen basolaterally. In the presence of the adenosine (A1) receptor antagonist, 8-cyclopentyl-1,3-dipropylxanthine (DPCPX), UTP evoked $[Ca^{2+}]_i$ and I_{SC} signals were unaltered, however, the ATP evoked change in I_{SC} was reduced ($P\leq0.05$) and not significantly different from that evoked by UTP; adenosine evoked no response. The sustained component of the I_{SC} response to ATP thus appears to be mediated by adenosine receptors, indicating that H441 cells do express only $P2Y_2$ receptors controlling both ion transport and $[Ca^{2+}]_i$ signalling in the apical membrane.

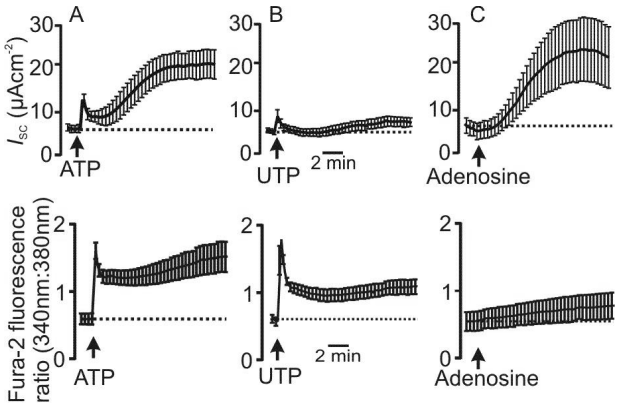

Figure 1. Effects of apical nucleotides upon I_{SC} and $[Ca^{2+}]_i$. H441 cells grown on permeable supports in dialysed media supplemented with dexamethasone (0.2μM) formed resistive monolayers within 3-5 days. They were loaded with the $[Ca^{2+}]_i$ dependent fluorescent dye, Fura-2 (3μM) and mounted in a miniature Ussing chamber to allow the effects of ATP, UTP and adenosine upon the spontaneous current and $[Ca^{2+}]_i$ to be explored simultaneously. The upper panels show (mean ± SEM) the responses in I_{SC} to 100μM apical ATP (n=10), UTP (n=9) and adenosine (n=6) respectively, whereas the lower panels show the simultaneous recorded responses in $[Ca^{2+}]_i$

Ramminger, S.J., Collett, A., Baines, D.L., Murphie, H., McAlroy, H.L., Olver, R.E., Inglis, S.K. & Wilson, S.M. (1999). P2Y2 receptor-mediated inhibition of ion transport in distal lung epithelial cells. *Br J Pharmacol.* **128**, 293-300.

Supported by a Wellcome Prize Studentship

C52

Thyroid hormone (T_3) potentiates the glucocorticoid-evoked increase in apical Na conductance (G_{Na}) *in H441 distal airway epithelial cells*

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Studies of fetal lambs have shown that thyroidectomy abolishes the adrenaline-evoked absorption of lung liquid that is normally seen during labour and birth. However, whilst this effect can be reversed by exogenous T_3, delivering T_3 to immature fetuses does not cause precocious absorption of lung liquid unless it is given with a glucocorticoid (Barker *et al.*, 1991). As this absorptive process is driven by active Na transport (Olver *et al.*, 2004), it thus appears that thyroid and glucocorticoid hormones are both important to the initiation and maintenance of the adult lungs' absorptive phenotype.Our earlier work shows that H441 distal airway epithelial cells do not transport Na if maintained in the absence of such hormones, but that 0.2 μM dexamethasone, a synthetic glucocorticoid, stimulates electrogenic Na transport by evoking expression of a selective apical Na conductance (Ramminger *et al.*, 2003). The present study therefore explored the possibility that T_3 may also play a role in this process and our data (Fig 1) show clearly that T_3 potentiates the dexamethasone-induced increase in G_{Na}. T_3 thus appear to be involved in the control of epithelial Na absorption (see also Pachá *et al.*, 1996), which may explain the abnormally high incidence of respiratory distress amongst congenitally hypothyroid infants (Cuestas *et al.*, 1976).

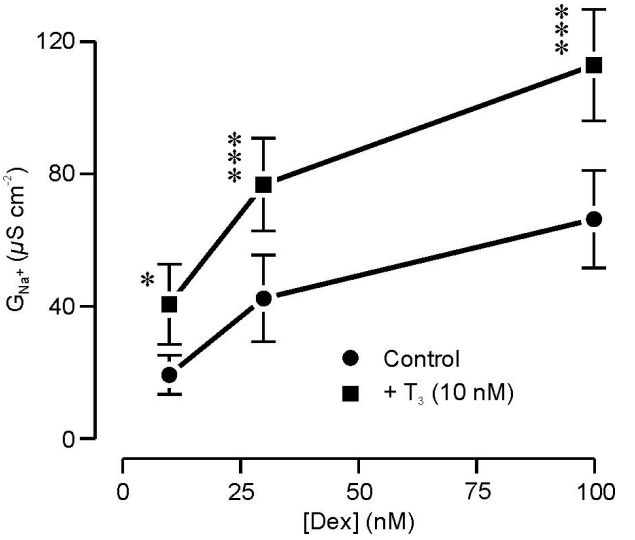

Fig. 1. H441 cells on Costar Snapwell membranes were grown for 7 days, by which time they had become incorporated into resistive epithelial sheets (R_t > 300 Ωcm^2). The cells were then mounted in Ussing chambers, bathed in cytoplasmic solution and basolaterally permeabilised using nystatin (75 mM). G_{Na} could then be quantified by measuring the amiloride-sensitive apical membrane currents evoked by imposing an inwardly directed Na gradient upon the permeabilised cells. The figure shows data (mean ± s.e.m., n = 5) for cells cultured in 10 nM, 30 nM or 100 nM dexamethasone, under control conditions and in the presence of 10 nM T_3. The medium used contained insulin, but no other hormones or growth factors were present. Asterisks denote significant effects of T_3 (* $P < 0.05$, *** $P < 0.001$, Student's paired t test).

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Cuestas, R. A., Lindall, A. & Engel, R. R. (1976). *N. Engl. J. Med.* **295**, 297-302.

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Pachá, J., Pohlová, I. & Zemanová, Z. (1996). *Gastroenterology* **111**, 1551-1557.

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Supported by grants from the Wellcome Trust and Tenovus Scotland

C53

Thyroid hormone binding interactions with rat liver membranes investigated using Surface Plasmon Resonance.

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The thyroid hormones T_3 and T_4 translocate the plasma membrane through multifunctional transporter proteins (Hennemann et al, 2001). In liver cells, the hormones may bind Triton X-100 soluble receptor proteins in the plasma membrane that channel T_3/T_4 to their transporters (Kemp & Taylor, 1997). A novel approach to studying this type of interaction is surface plasmon resonance (SPR) in which one member of a binding pair is immobilised to a sensor surface and the second member is passed over the immobilised partner. Binding is detected in real time as a change in refractive index at the sensor surface and is proportional to the change in mass due to binding.Sinusoidal membranes purified from livers of humanely-killed rats (Kemp & Taylor, 1997) were immobilised on an L1 sensor chip placed in a Biacore 3000 SPR system (Biacore AB, Uppsala, Sweden). Micromolar concentrations of T_3, T_4 and rT_3 in phosphate-buffered saline were injected over the immobilised membranes. SPR sensorgrams were double referenced with subtraction of non-specific binding to an unmodified sensor surface. Concentration dependent binding of T_3 and T_4 to the membranes was observed and analysed by a 1:1 Langmuir adsorption model using BioEvaluation software. For T_3; ka = 1.51 (±0.01) x 10^4 Ms^{-1}, kd = 0.029 (±0.004) s^{-1}, KD = 1.89 μM. For T_4; ka = 1.36 (± 0.16) x 10^4 Ms^{-1}, kd = 0.171 (±0.015) s^{-1}, KD = 12.6 μM. Here ka and kd represent the on/off rate constants of the interaction while KD is the equilibrium dissociation constant. rT_3 did not interact with liver membranes.T_3 - receptor interactions were also studied by injecting liver membranes over T_3 immobilised to a CM5 sensor surface (0.8 $pmol.mm^{-2}$); 15 μg membrane protein produced a maximum specific binding response (Figure 1). Membranes briefly treated with 0.5% Triton X-100 to solubilise T_3 receptors without loss of membrane vesicular integrity (Kemp & Taylor, 1997) showed a 98% reduction in binding. Crude Triton-extractable membrane protein displayed binding activity towards T_3 bound to the CM5 chip (see Figure 1). These results provide valuable kinetic information on real-time binding interactions of T_3 and T_4 with components of liver sinusoidal membrane. This work has demonstrated that SPR is of value in the identification and kinetic characterisation of physiologically important receptor - ligand binding interactions.

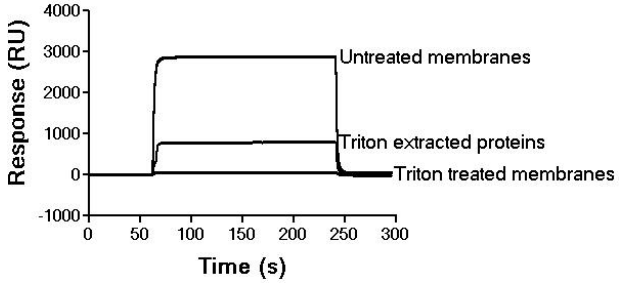

Figure 1. SPR sensorgram of interactions between T_3 immobilised to a CM5 surface and superfused liver sinusoidal membrane material. RU:- SPR Response Unit

Hennemann G. et al, (2001). Endocrine Reviews 22, 451-476

Kemp H.F. & Taylor P.M. (1997). Am. J. Physiol. 272, E809-E816

This work is supported by the Wellcome Trust and the University of Dundee.

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

C54

Thyroid hormone (T_3) does not modify glucocorticoid-evoked activation of the human α-ENaC promoter.

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Glucocorticoid hormones stimulate Na^+ transport in distal lung epithelial cells by increasing apical Na^+ conductance (G_{Na}) (Ramminger *et al.*, 2004) and such responses have been attributed to glucocorticoid receptor-mediated expression of the epithelial Na^+ channel α-subunit (α-ENaC) gene (Sayegh *et al.*, 1999). However, the glucocorticoid-evoked increases in G_{Na} seen in H441 distal airway cells are augmented by T_3 (Ramminger *et al.*, 2004), which accords with the view that glucocorticoid and thyroid hormones are both important to the development of the lungs' Na^+ absorbing phenotype (reviewed by Olver *et al.*, 2004). We have therefore explored the possibility that this effect of T_3 may reflect facilitation of glucocorticoid-evoked α-ENaC transcription (Otulakowski *et al.*, 1999).Our control data confirmed (Otulakowski *et al.*, 1999; Sayegh *et al.*, 1999) that dexamethasone, a synthetic glucocorticoid, can activate the α-ENaC promoter and showed that half maximal activation occurred at ~5 nM (Fig. 1). However, T_3, at a concentration that can clearly augment the effect of dexamethasone upon G_{Na} (Ramminger *et al.*, 2004), had no effect upon this response (Fig. 1). In contrast to earlier data from the rat α-ENaC promoter (Otulakowski *et al.*, 1999), the present results suggest that, in human cells, the potentiating effect of T_3 (Ramminger *et al.*, 2004) must reflect events downstream to transcription.

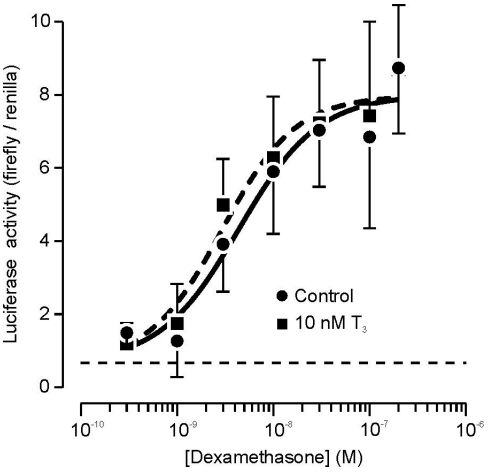

Fig 1. H441 cells were co-transfected (Ca_2PO_4 precipitation) with *(i)* a firefly luciferase reporter construct incorporating the 2.2 kb upstream region of the human α-ENaC gene which includes both transcription initiation sites (Sayegh et al., 1999) and *(ii)* a renilla luciferase construct included to control for transfection efficiency. Activation of the α-ENaC promoter would thus be signalled by formation of firefly luciferase. The plotted data (mean ± s.e.m., $n > 4$) show responses to dexamethasone (16 h) under control conditions and in the presence of 10 nM T_3. The sigmoid curves were fitted to the experimental data by least squares regression whilst the dashed lines shows the transcriptional activity measured in unstimulated cells.Supported by grants from the Wellcome Trust and Tenovus Scotland

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Sayegh, R., Auerbach, S. D., Li, X., Loftus, R. W., Husted, R. F., Stokes, J. B. & Thomas, C. P. (1999). *J. Biol. Chem.* **274**, 12431-12437.

Supported by grants from the Wellcome Trust and Tenovus Scotland

C55

Neuronal Plasticity in Peyer's patch in response to enteric parasitism

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Peyer's patches (PP) are sites of immune surveillance in the gastrointestinal tract. Nerve fibres underlie the follicular associated epithelium (FAE) in the dome of the PP (Krammer and Kulnel, 1993). The function(s) of such nerves has not been described although the enteric nervous system is known to regulate epithelial ion transport processes in normal and parasitised gut (Stead, 1992). The aim of this study was (1) to quantify the innervation of rat distal colonic lymphoid tissue from rats primed by *Fasciola hepatica* infection compared to uninfected controls and (2) to examine how electrical field stimulation (EFS) of nerves within PP modifies epithelial ion transport processes *in vitro*. Adult female Wistar rats were lightly anaesthetised (isofluorane) and orally infected with 20 metacercariae. This study was licensed by the Department of Health and approved by the Institutional Ethics Committee. For immunohistochemical analyses of nerves, five weeks post infection animals were euthanised by overdose of euthatal given by intraperitoneal injection. Post-mortem colonic PP from infected (n=8) and control (n=6) rats were collected, fixed, sectioned and mounted. Nerves were identified immunohistochemically using anti-GAP-43. Image analysis was performed using Image-Pro® software. In separate experiments, Wistar rats (200-300g) were humanely killed by cervical dislocation and colonic PP epithelium was mounted in Ussing chambers under voltage clamped conditions. The window area (0.28cm2) was completely occupied by Peyer's patch epithelium. Changes in short-circuit current (SCC) in response to electrical field simulation (EFS; 7v, 7Hz, 1ms, 1 min pulse train) and carbachol (CCh) (10^{-5}M) in PP epithelium were measured. All data are expressed as mean±s.e.m., comparison between infected and control means were made using unpaired two-tailed student t tests. Immunohistochemical analysis of PP nerves in normal rat colon showed 517±272 nerve fibres per mm^2 in the dome region (n=6) some adjacent to FAE. Numbers increased significantly with *F. hepatica* infection (935±87, n=8, P≤0.05). EFS of voltage clamped PP evoked a mean inward SCC of 35±6μA.cm^{-2} (n=16) which was accounted for, at least in part, by electrogenic chloride secretion. SCC responses were virtually abolished by tetrodoxin (TTX, 10^{-6}M). In contrast, ion transport responses to the directly acting secretagogue CCh (10^{-5}M) were not altered by TTX. These data indicate that the density of PP associated nerves are, as elsewhere in the gastrointestinal tract, raised as a consequence of parasitism. Furthermore, since these nerves regulate chloride secretion, their function may be to amplify protective responses of the parasitised host.

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This work was supported by the Irish Health Research Board

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

C56

The rabbit proton-coupled peptide transporter PepT1 functions as a multimer when expressed in *Xenopus* oocytes.

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The proton-coupled transporter PepT1 mediates the uptake of di-, tri-peptides and peptidomimetics in the intestine and kidney (reviewed by Meredith & Boyd 2000). Here we report the results of studies to test whether PepT1 acts as a functional monomer or multimer, using a combination of luminometry and transport assays in *Xenopus laevis* oocytes.Uptake studies were performed as previously reported (Meredith *et al.* 2000). The epitope FLAG (YDDDDDK) was inserted by PCR (at amino acid position 108, in the extracellular loop between TM3 and TM4, Covitz *et al.* 1998) to enable measurement of its expression level at the oocyte membrane by luminometry (Konstas *et al.* 2001). PepT1-FLAG showed normal expression and function compared to the non-tagged wild type transporter. In contrast a W294F-PepT1 mutant was expressed on the membrane but did not transport the dipeptide D-Phe-L-Gln.Whilst a constant amount of PepT1-FLAG cRNA (13.5 ng) was co-injected with an increasing amount W294F mutant cRNA (up to a 1:4 ratio), the amount of D-Phe-L-Gln transport was reduced, yet the expression levels of PepT1-FLAG remained constant. This implies that PepT1 functions as a multimer as co-expression of the non-functional W294F affects uptake by PepT1-FLAG. Data from a series of co-injection experiments were fitted with the Hill equation, and the stoichiometry determined to be 4.2±1.8, suggesting that PepT1 functional unit may be composed of 3 (min) to 7 (max) copies of the protein. Data were normalized and plotted as the fractional uptake versus the mole fraction of the mutant (Figure A) and two curves were found to fit the data equally well. These two curves represent the tetramer and the pentamer model, where two copies of PepT1-FLAG appear to be the minimal requirement for function (Figure B).

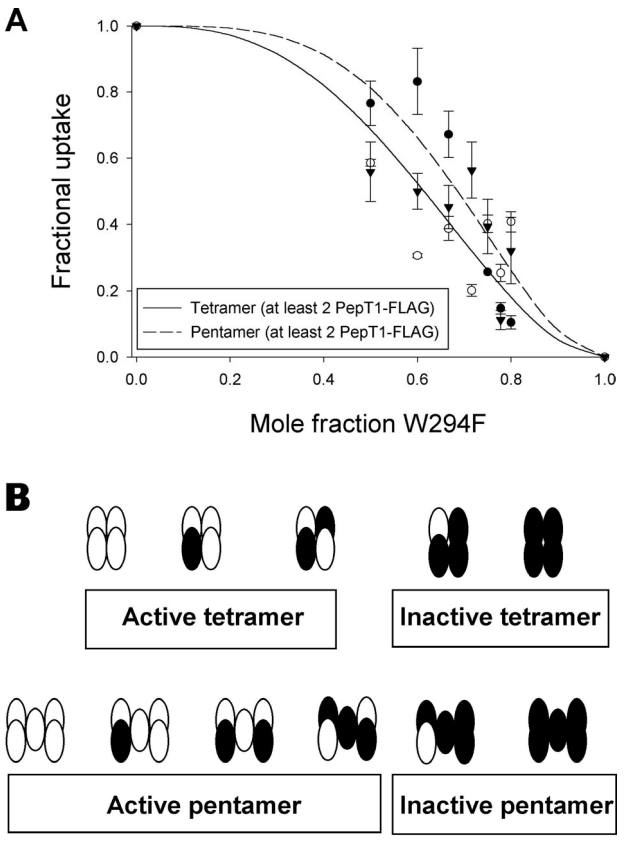

Figure A: Normalized data plotted as the fractional uptake of ^{3}H-D-Phe-L-Gln (0.42μM) versus the mole fraction (W294F/PepT1-FLAG+W294F) of the mutant. Data are mean ± SEM, n=5 oocytes. Lines represent theoretical fit for tetramer and pentamer.

Figure B: Possible ways that the PepT1-FLAG (open circles) and the W294F (filled circles) can be arranged in cases of tetramer and pentamer.Other 12 transmembrane domain transporters have recently shown to be multimers, including the human dopamine transporter (Hastrup *et al.* 2001). This study shows that PepT1 functions as a multimer, which will be highly relevant for structure-function modeling of the protein.

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Covitz KM *et al.* (1998) *Biochemistry* **37**, 15214-15221
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This work is generously funded by the Wellcome Trust.

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

C57

Estrogen-dependent upregulation of System A amino acid transport is associated with increased proliferation of breast cancer cells.

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Estrogens enhance proliferation of estrogen receptor positive (ER+) breast cancer cells, a process requiring a high supply of amino acids (Bhat & Vadgama, 2002). System A amino acid transport activity is expressed in abundance in tissues undergoing rapid growth and proliferation (e.g. tumor cells) and it has been suggested that increased proliferation and invasiveness of neoplastic cells is linked to changes in System A function (Singh et al 1996). Three isoforms of System A (SNAT1,2,4) have been identified, which have distinct substrate specificity and tissue expression patterns (Mackenzie & Erickson, 2004 for review). The aims of this study are to establish (i) which SNAT transporter isoforms are expressed in human breast cancer cells, (ii) whether System A is estrogen-sensitive in ER+ cells and (iii) whether suppressing activity of System A reduces growth and proliferation of tumor cells in culture. The MCF-7 (ER+) breast tumor cell line was cultured in Dulbecco's Modified Eagles Medium containing 10% fetal bovine serum. Na+-dependent uptake of [^{14}C]Me-AIB (methylaminoisobutyric acid, a selective System A substrate) was used as a functional assay of System A activity. Cell proliferation was assessed by monitoring total cell number using a Coulter counter.SNAT1 and 2 (but not SNAT4) mRNAs were detected in MCF-7 cells by RT-PCR. System A transport activity is upregulated by estrogen (10nM 17 β-estradiol) in MCF-7 cells (48% increase in V_{max} from 16.6 ± 0.3 nmol. mg protein^{-1}. 20 min^{-1} after 48h). K_m for MeAIB remained unchanged at 1.0 ± 0.1 mM, consistent with the up-regulation of SNAT2. Furthermore, blockade of System A transport using the non-metabolisable competitive substrate MeAIB prevents the accelerated cellular proliferation otherwise induced by estrogen treatment (see Figure 1). The results show that upregulation of System A transport activity by 17β-Estradiol in MCF-7 breast cancer cells is associated with enhanced cellular proliferation and also that blocking this transport activity arrests the proliferative effect of the hormone. These observations raise the possibility of targeting System A in therapies for breast tumors.

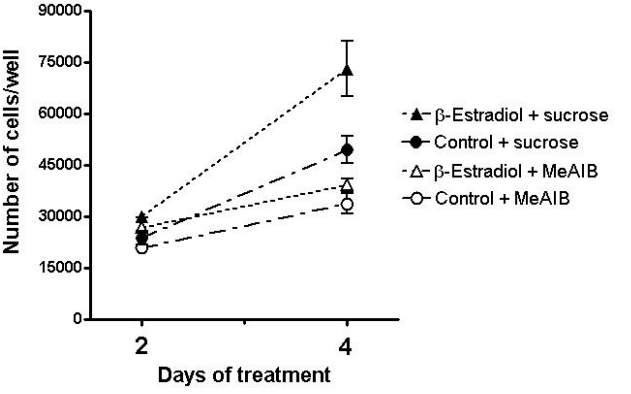

Figure 1. Effect of 10nM 17β-estradiol and MeAIB (20mM) on MCF-7 cell growth. Sucrose (20mM) is used as a control for

possible osmotic effects of MeAIB. Values are Mean ± SEM for 3 measurements; data are representative of 4 separate experiments.

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Supported by Tenovus Scotland.

C58

Amino acid signalling pathways in rat L6 skeletal myotubes: Pharmacological analysis of System A adaptive regulation

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An adaptive increase in the activity of the System A amino acid transporter is observed in various mammalian cell lines following prolonged amino acid (AA) deprivation. Recent publications from ourselves (Hyde *et al.* 2001) and other groups (Ling *et al.* 2001, Gazzola *et al.* 2001) have demonstrated that AA deprivation induces the increased expression of the sodium-coupled neutral amino acid transporter 2 (SNAT2), a functional System A transporter, at both the mRNA and protein level. The cellular events governing this response are poorly characterised, but a possibility is that amino acid-sensitive signal transduction pathways are modulated and lead to the altered expression of SNAT2. To address this possibility, we have studied System A adaptation in skeletal muscle cells in the presence of cell-permeable signal transduction inhibitors.Fully differentiated L6 myotubes were incubated in Earl's balanced salt solution in the presence or absence of an AA mix at physiological concentration for 4 h. AA deprivation over this time course leads to an ~ 4-fold increase in the transport of 10 μM [^{14}C]-methylaminoisobutyrate, a selective System A substrate. A variety of structurally distinct kinase inhibitors were selected for use in signalling studies, these included PD-098059 (50 μM), rapamycin (100 nM), SB-203580 (10 μM), wortmannin (100 nM), genistein (100 μM) and SB-216763 (5 μM). Of these compounds, only wortmannin, genistein and SB-216763 were shown to significantly inhibit the adaptive regulation of System A (by 38 ± 11.2 %, 59 ± 10.2 % and 50 ± 13.7 %, respectively; $p < 0.05$ in all cases, ANOVA). These results suggest that phosphoinositide-3-kinases, tyrosine kinases and glycogen synthase kinase-3 (GSK3), respective targets of the above three compounds, may play a role in amino acid signalling in L6 muscle cells.The potential involvement of GSK3 in System A adaptation is further indicated by similar effects of two additional GSK3 inhibitors, namely SB-415286 and Li^+ ions. Consistent with this, our recent experiments have demonstrated close parallels between the activities of glycogen synthase and System A in response to altered amino acid availability, indicative of a conserved amino acid sensing pathway with regulatory control over the two processes. The apparent involvement of GSK3 in this context is intriguing, since it has well documented roles in the control of glycogen and protein synthesis (Frame & Cohen, 2001).

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This work was supported by the BBSRC and GlaxoSmithKline plc