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Transepithelial urate transport by avian renal proximal tubule epithelium in primary culture

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Summary

Birds are uricotelic, and because they excrete urate by renal tubular secretion, they provide a convenient model for examination of this process. Primary monolayer cultures of the isolated renal proximal tubule epithelium from the domestic chicken, *Gallus gallus* L., were mounted in Ussing chambers where several substrates/inhibitors of renal organic anion transporters were tested for the sidedness and specificity of their effects on transepithelial urate transport. Transepithelial electrical resistance, electrical potential and sodium-dependent glucose current were monitored to detect nonspecific effects. Under control short-circuited conditions the ratio of unidirectional fluxes of [^{14}C]urate was found to be 3:1. Active net secretion was specifically inhibited by 1 mmol l^{-1} probenecid and 10 mmol l^{-1} *para*-aminohippuric acid (PAH). Bromocresol Green, cimetidine, nocodozole, cytochalasin D and ouabain also inhibited secretion but were toxic. Interstitial-side lithium (5 mmol l^{-1}) and glutarate (1 mmol l^{-1}) specifically blocked transport, but 10–100 μmol l^{-1} glutarate had no effect. Interstitial estrone sulfate (ES) stimulated urate secretion at 10 μmol l^{-1} but was inhibitory at 500 μmol l^{-1}. Active PAH secretion (5:1 flux ratio) was inhibited 34% by 330 μmol l^{-1} urate. ES (500 μmol l^{-1}) blocked the remainder. From the lumen side, glucose-free, Cl^--free and high K^+ (30 mmol l^{-1}) solutions, or an alkaline pH of 7.7 had no effect on urate transport and neither did several compounds known to be uricosuric. Lumen-side methotrexate (500 μmol l^{-1}) and MK571 (20 μmol l^{-1}) strongly inhibited urate secretion. MK571 had no effect from the interstitial side. RT-PCR revealed mRNA for OAT1-, OAT3-, MRP2- and MRP4-like organic anion transporters in chicken proximal epithelium.

Key words: renal secretion, organic anion, MK-571, *para*-aminohippuric acid, uric acid, chicken.

Introduction

Birds are uricotelic, i.e. urate is their major nitrogenous excretory product, and a well developed capacity for net urate secretion has been demonstrated in proximal tubules of both cortical (superficial reptilian-type) (Laverty and Dantzler, 1983) and transitional nephrons (Brokl et al., 1994). The capacity for net urate secretion is further evident from *in vivo* renal clearance measurements, with normal clearance ratios between 5-15 (Austic and Cole, 1972). Birds and certain mammals (humans, great apes) maintain substantial plasma urate concentrations (~300 μmol l^{-1}) predominantly through the actions of renal transport mechanisms. Most mammals, including humans, reabsorb virtually all filtered urate in the proximal convoluted tubule (98%). Evidence in mammalian systems indicates that uric acid is an efficient free radical scavenger (Becker et al., 1989; Green et al., 1986; Meadows et al., 1986; Becker et al., 1991; Peden et al., 1990), however, the beneficial effects from retention of urate must be carefully balanced by excretion to avoid detrimental effects such as gout (Scott, 1971). In fact, a urate secretory flux is also present and contributes to excretion (Gutman and Yu, 1972) such that, even in mammals, excreted urate may derive primarily from proximal tubule secretion (Gutman and Yu, 1972). About the same proportion of excreted urate is derived from tubular secretion in humans as in birds (about 73%) reflecting the importance of this transport process in urate homeostasis. In perfused straight proximal tubules of chicken transitional nephrons, robust net urate and *para*-aminohippuric acid (PAH) secretion are present together with slight mediated reabsorption of PAH, but there is no detectable mediated urate reabsorption (Brokl et al., 1994). Thus, the avian proximal tubule provides an excellent model for study of transepithelial urate secretion.

In birds and the urate-secreting mammals an organic anion transport system has been implicated in basolateral membrane (BLM) urate uptake, which is Na^+- and α-ketoglutarate (α-KG)-dependent (Brokl et al., 1994; Dantzler, 1969; Werner and Roch-Ramel, 1991). The mammalian PAH/α-KG

exchangers in the BLM have been identified as OAT-type (SLC22A; major facilitator family of organic anion transporters; Sweet et al., 1997) with urate a demonstrated substrate (Uwai et al., 1998). Human OAT3 has been strongly implicated in urate secretion by expression studies in *Xenopus* oocytes (Bakhiya et al., 2003). Both OAT1 and OAT3 transport urate, and OAT3 may have the higher affinity (Bakhiya et al., 2003). Some discrimination in the two transporters has been achieved with shared transported substrates for which affinities vary (see Wright and Dantzler, 2004).

Among the vertebrates, the modalities of urate transport across the apical membranes of the proximal tubule epithelium include electroneutral anion exchange (Enomoto et al., 2002; Guggino et al., 1983), electrogenic urate transport (Grassl, 2002a; Roch-Ramel et al., 1994) and primary active transport (Van Aubel et al., 2005). The last two processes have been associated with urate secretion, and NPT1 (sodium–phosphate transporter I; Uchino et al., 2000), Oat_v1 (voltage-dependent organic anion transporter 1; Jutabha et al., 2003) and MRP4 (multidrug resistance peptide 4; Van Aubel et al., 2005) have been proposed as candidate transporters. Chicken orthologs of NPT1 and Oat_v1 have yet to be reported, and no human ortholog of Oat_v1 has been found (Hediger et al., 2005). Overexpression of MRP4 in HEK293 cells revealed an ATP-dependent urate export capability (Van Aubel et al., 2005). The relationship of these processes in mammals to urate secretion is still unclear, and even less is known about the mechanisms of apical urate efflux in avian species (see Dantzler, 2002).

A general model of the mechanism of urate secretion by avian proximal tubule was recently reviewed by Dantzler (2005). This model represents the uric acid uptake at the BLM as two processes, one urate/α-KG exchange and a process that apparently exchanges urate for an unknown counteranion. The apical cell-to-lumen step is down an electrochemical gradient; however, neither apical nor BLM urate transporters have been fully defined. The initial step in urate secretion as determined from basolateral membrane vesicles (BLMV) of turkey kidney is consistent with the 'classical' organic anion (OA) uptake system of transporters (Grassl, 2002b).

In the present study, chicken proximal tubule epithelial cell primary monolayer cultures (PTCs) were used to investigate net active transepithelial transport of urate together with passive 'leak' fluxes under short-circuited conditions. The PTCs were shown to express mRNA for orthologs of OAT1, OAT3, MRP2 and MRP4. The data are consistent with OAT-like-mediated urate transport across the BLM. Active net urate secretion was not sensitive to apical membrane OH^-, Cl^-, or K^+ concentration gradients but was greatly reduced by inhibitors of MRP4.

Materials and methods

Animals

Kidneys were isolated from six to eight white leghorn chicks (domestic *Gallus gallus* L.) at 3–7 days of age for each cell culture preparation. The present study adheres to the newest Guiding Principles for Research as outlined by the American Physiological Society (2002). All investigations involving animals reported in this study were conducted in conformity with these principles, and the animal protocol was approved by the University of Connecticut IACUC (protocol #A04-107).

Solutions and chemicals

Hanks' balanced salt solution (HBSS) was purchased from Mediatech (Herndon, VA, USA). Krebs-Henseleit buffer was purchased from Sigma Chemicals (St Louis, MO, USA). This medium was supplemented with 4 mmol l^{-1} $NaHCO_3$ (pH 7.4). The final plating medium and maintenance medium consisted of Dulbecco's modified Eagle's medium–Hank's balanced salt solution (DMEM–Ham's) F12 supplemented with insulin/transferrin/selenium pre-mix (ITS; 5 μg ml^{-1} insulin, 5 μg ml^{-1} transferrin, 5 ng ml^{-1} selenite), 20 μmol l^{-1} ethanolamine, 300 μmol l^{-1} L-glutamine, and 10% fetal bovine serum (FBS). The saline solution used for Ussing chamber experiments contained (in mmol l^{-1}) 1.1 $CaCl_2$, 4.2 KCl, 0.3 $MgCl_2$, 0.4 $MgSO_4$, 120 NaCl, 0.4 NaH_2PO_4, 0.5 Na_2HPO_4, 1.0 glycine, 25 $NaHCO_3$ (pH 7.4 with 5% CO_2–95% O_2, 290 mosmol kg^{-1} H_2O). Additionally, 330 μmol l^{-1} urate and 5.5 mmol l^{-1} glucose were added to the saline solution in both lumen and interstitium at the start of each experiment (t=0).

PAH, probenecid, Bromcresol Green, ouabain, cimetidine, methotrexate (MTX), estrone sulfate (ES), ethanolamine, L-glutamine, oxonic acid, pyrazine, adenosine, nicotinic acid, nocodozole, cytochalasin D, and all components of the saline solution were purchased from Sigma (St Louis, MO, USA). DMEM–Ham's F-12 was from Mediatech (Herndon, VA, USA). FBS and lithium chloride were purchased from Fisher (Pittsburgh, PA, USA). ITS was purchased from Collaborative Biomedical Products (Bedford, MA, USA). Glutarate was purchased from Aldrich (Sheboygan, WI, USA). MK-571 was purchased from Biomol (Plymouth Meeting, PA, USA). Percoll was purchased from Amersham Pharmacia Biotech (Piscataway, NJ, USA).

Preparation of chicken PTCs

Chicken renal tubule segments were isolated and dispersed (Sutterlin and Laverty, 1998) and modified (Dudas and Renfro, 2001) as previously described. Briefly, kidneys were removed, rinsed in HBSS, cleaned of blood vessels, ducts and connective tissue, and minced. The tissue fragments were incubated in an enzyme solution containing collagenase A (0.13 U ml^{-1}) and dispase II (0.54 U ml^{-1}) at 37°C for 10 min. Nephron segments were further dissociated by trituration and filtration through a stainless steel sieve (380 μm). The dissociated tissue was rinsed three times with HBSS, with the last rinse containing DNase I (2161 U ml^{-1}), and resuspended in a 1:1 mixture of Percoll and 2× Krebs–Henseleit buffer. The suspension was centrifuged at 17,500 ***g*** and the high-density band consisting of small proximal tubule segments (probably a mixture from cortical, transitional and long-looped medullary nephrons) was removed, rinsed with HBSS, suspended in culture medium

with 10% serum, and plated on native rat-tail collagen as previously described (Dickman and Renfro, 1993). After 6 days in culture, the collagen gels were detached from the culture dishes, and after ~14 days, these floating collagen gels had been contracted by the epithelial monolayers to ~40% (17 mm to 10 mm diameter).

Ussing chamber studies

During days 15–29, transepithelial electrical characteristics and urate transport were measured. The tissues were supported by 150 μm nylon mesh and mounted in Ussing chambers as previously described (Gupta and Renfro, 1989). The saline bathing the luminal and interstitial sides of the tissue was maintained at 39°C, and continuously gassed (95% O_2, 5% CO_2) and stirred throughout the experiment.

Transepithelial electrical potentials (V_T) were determined with a pair of reference electrodes connected to the luminal and interstitial compartments via 3 M KCl–2% agar bridges. Electrode asymmetry was corrected at the beginning and end of each experiment with compensation for fluid resistance. Current was passed through Ag–AgCl electrodes connected to each compartment with 3 M KCl–2% agar bridges. Electrical properties were measured with a pair of computer-controlled, high-impedance automatic dual voltage clamps (DVC 1000; World Precision Instruments, Sarasota, Fl, USA). Transepithelial electrical resistance (TER) was determined from the change in V_T produced by a 10 μA current pulse. The sensitivity of the transepithelial current to 10^{-4} M phloridzin in the lumen side, a specific inhibitor of Na^+-dependent glucose transport, was determined at the end of every experiment in all tissues. This current (I_{glu}; sodium-dependent glucose current) served as a check on proximal tubule-like function and tissue vitality.

Determination of transepithelial urate fluxes

Tissues were continuously short-circuited during flux determinations (with the exceptions noted), i.e. there were no transepithelial electrical or chemical gradients. Short-circuit current was 4–5 μA cm^{-2} in these low-resistance tissues prior to glucose addition. Unidirectional tracer fluxes were initiated by the addition of 0.4 μCi [8-^{14}C]urate (ARC, St Louis, MO, USA) to the appropriate hemichamber. Duplicate 50 μl samples were taken from the unlabeled side every 30 min over a period of 1.5 h and replaced with equal volumes of unlabeled saline (see Fig. 1). The specific activity of the labeled solution was determined at the beginning and end of each experiment based on the urate concentration of 330 μM and radioactivity of the radioactive-side bathing medium.

Net flux was calculated as the difference between unidirectional secretory (interstitial-to-luminal) and reabsorptive (luminal-to-interstitial) fluxes. The unidirectional reabsorptive flux is also termed 'leak flux' reflecting a likely contribution of urate passage through the paracellular pathway (Brokl et al., 1994). Both control and treated tissues were paired monolayers from a single preparation. The monolayer cultures used in a given experiment were prepared from the same starting tissue at the same time and cultured under identical conditions. For statistical determinations this is referred to as one preparation.

Intracellular recordings

The cultured proximal tubule cell monolayers on collagen gels were affixed to 18 mm circular coverslips (Fisher, Pittsburgh, PA, USA) with chicken plasma/thrombin (1:1) clots and immersed in warmed (37°C) saline (same composition as used for Ussing chamber experiments; pH 7.4 with 5% CO_2, 95% O_2, 290 mosmol kg^{-1} H_2O). The coverslips with the attached monolayers were mounted on the stage of a Nikon Eclipse E-600 FN microscope (Nikon, Melville, NY, USA) and continuously perfused with saline. Cultures were visualized using infrared differential interference contrast (IR-DIC) microscopy. Electrodes were pulled from capillary tubing (Garner Glass N51A; Garner Glass Co., Claremont, CA, USA) using a Narishige multi-step electrode puller (Model PP-830) (Tritech Research, Los Angeles, CA, USA) and had resistances of ~30 MΩ. The electrode solution was 3 M KCl. Current-clamp recordings were made using an Axon Instruments 200B amplifier (Axon Instruments Inc., Foster City, CA, USA), and low pass filtered at 1 kHz. Currents were digitally sampled at 10 kHz and monitored with pCLAMP 8.0 software (Axon Instruments Inc.) running on a PC pentium computer.

To examine depolarization resulting from elevated luminal K^+ or the presence of luminal glucose we used a picospritzer (Picospritzer II, General Valve Corporation, Fairfield, NJ, USA) to locally apply either 30 mmol l^{-1} K^+, or glucose-free solution. Following a 2–4 s baseline recording, a brief pulse of 30 mmol l^{-1} K^+ (200 ms), or glucose-free solution (100 ms) was applied within ~100 μm of the punctured cell and the response of the cell was monitored for durations of ~25–35 s. Currents were identified using Clampfit 8 software (Axon Instruments).

Preparation of total mRNA from PTCs

Four PTCs, each containing ~10^7 cells were used for isolation of total RNA using the Qiagen RNeasy Midi Kit according to the manufacturer's instructions (Valencia, CA, USA). PTC mRNA was isolated from total RNA (~150 ng μl^{-1}) using the Qiagen Oligotex mRNA kit (Valencia, CA, USA). Final concentration of chicken PTC mRNA was 25 ng μl^{-1}.

RT-PCR for detecting known organic anion transporters in chick PTCs

Chick PTC mRNA was used for RT-PCR as described in the Qiagen one-step RT-PCR kit. The initial reverse transcription and subsequent PCR were carried out in a single step with primers (Table 1) against known sequences for OAT1-like (BBSRC Chick EST ID 603807902F1), OAT3-like (BBSRC Chick EST ID 603812145F1), MRP2 (GenBank accession XM_421698) and MRP4 (GenBank accession XM_416986) transporters from the domestic chicken. Since the OAT1 and OAT3 sequences in the database are only partial sequences and

Table 1. *Primer sequences used to amplify OAT1-like, OAT3-like, MRP2-like and MRP4-like cDNA from PTCs*

Transporter		Primer sequence
OAT1	Forward	5′-TGGTTCTCCACCAGCTTTGC-3′
	Reverse	5′-TTCAGGGAGGAAAAGAGCAGCG-3′
OAT3	Forward	5′-CCCTTCTTCCTCTTCTTCCTCG-3′
	Reverse	5′-TGGATCAGATAAATGCTGACCCC-3′
MRP2	Forward	5′-AAATCCTCCCTCACCAACTGCC-3′
	Reverse	5′-TTCGCCTTGCAGAGAAGACG-3′
MRP4	Forward	5′-CGAGGCAGTGGTTAGCATACGAC-3′
	Reverse	5′-CGCACTCAGTGGATCGTCCAA-3′

The primers were generated against known sequences for OAT1-like (BBSRC Chick EST ID 603807902F1), OAT3-like (BBSRC Chick EST ID 603812145F1), MRP2-like (GenBank accession no. XM_421698) and MRP4-like (GenBank accession no. XM_416986) transporters from the domestic chicken (*Gallus gallus*).

correspond to different regions of the mammalian OAT1 and OAT3 open reading frame, there is no certainty that these sequences correspond to unique OATs or whether they are from the same OAT-like transporter. They are termed OAT1- and OAT3-like since BLAST searches of the individual sequences found them to be most similar (~50% each) to human OAT1 and OAT3, respectively. In contrast, full sequences for chicken MRP2 and MRP4 transporters are available and amino acid alignments indicate that they are 60% and 86% similar to their human orthologs, respectively. The primers (0.6 μmol l^{-1}) and 25 ng of template mRNA were used in each RT-PCR reaction. The reverse transcription reaction was conducted at 50°C for 30 min followed by a 15-min incubation at 95°C to denature the reverse transcriptase. The PCR reaction was run for 38 cycles with a denaturing temperature of 94°C, an annealing temperature of 58.7°C (OAT1), 61.6°C (OAT3), 58.7°C (MRP2) or 56°C (MRP4), and an elongating temperature of 72°C. PCR products were separated on a 2% agarose gel and stained with Gel-Star (Fisher, Pittsburgh, PA, USA).

Statistics

Experimental results are expressed as means ± S.E.M. Sample means were compared with paired one-tailed Student's *t*-tests. Differences were judged significant if $P<0.05$.

Results

Demonstration of active transepithelial urate secretion

Fig. 1A is a representative plot illustrating net transepithelial urate secretion by chick PTCs under short-circuited conditions. Unidirectional fluxes were initiated by addition of [^{14}C]uric acid and 330 μmol l^{-1} non-radioactive urate at t=0. Transepithelial secretory flux approached steady state at 1.5 h reflecting the time necessary for equilibration of isotopic label with the transportable urate pool. V_T, TER and I_{glu} averaged

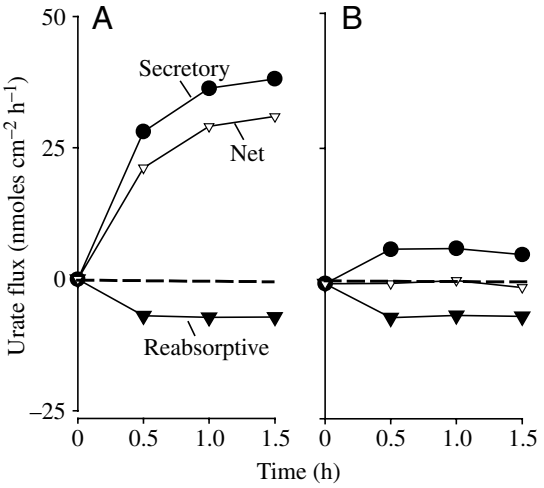

Fig. 1. Representative plot of unidirectional and net urate fluxes across chick PTCs as a function of time. The unidirectional fluxes include the interstitial-to-luminal secretory flux (secretory) and luminal-to-interstitial reabsorptive flux (reabsorptive; shown negative to indicate direction). Net flux is the difference between unidirectional fluxes. (A) Paired controls; (B) probenecid (1 mmol l^{-1}) added to interstitial and luminal sides at t=0. Fluxes approached steady state at t=1.0 h.

–1.25±0.12 mV (lumen negative), 29.19±1.82 Ω cm^2 and 11.59±1.10 μA cm^{-2}, respectively, in a series of replicative tests of probenecid (see Table 2), and these electrical data are representative of the values obtained for all of the experiments reported here. In the example shown, addition of 1 mmol l^{-1} probenecid to the interstitial and luminal sides of paired culture mates at t=0 (Fig. 1B; summary data in Table 2) decreased net transepithelial urate secretion to zero with no effect on electrical properties. Drug exposures were initiated at t=0 and electrical data recorded at t=1.5 h.

Pharmacological characterization

A general pharmacological characterization of the effects of a battery of inhibitors and (or) competitive substrates on active urate secretion is shown in Tables 2, 3 and 4. In the proximal tubule, alteration of the TER is indicative of a change in conductance through the paracellular shunts, and a change in I_{glu} can reflect a change in plasma membrane Na^+ electrochemical potential. Thus, a 'nonspecific' effect in this circumstance is defined as a change due to interaction of a compound with ATP production, Na/K-ATPase, membrane integrity, etc., rather than, or in addition to, specific interaction with a urate transporter. If a particular compound caused significant changes in TER, I_{glu}, or V_T, the direction of change is shown.

Interstitial-side effects

Others have provided evidence that urate shares an OA/PAH transport system in the avian kidney (Dantzler, 2002). High concentrations (2–10 mmol l^{-1}) of PAH in the bathing medium inhibit BLM urate uptake by both chicken isolated proximal tubules (Brokl et al., 1994) and turkey

Table 2. *Effect of probenecid, PAH, Bromocresol Green, and cimetidine on unidirectional secretory, unidirectional reabsorptive and net urate fluxes by chick PTCs*

Treatment	*N*	Side	Nonspecific	Secretory	Reabsorptive	Net
Control	4			100	29±13.3	70±13.3
Probenecid (1 mmol l^{-1})	4	Both	No	30±6.4*	36±14.2	–6±10.0*
Control	3			100	36±16.3	64±16.3
PAH (10 mmol l^{-1})	3	I	No	41±1.6*	54±17.8*	–13±18.1*
Control	5			100	36±8.6	63±8.6
Bromocresol Green (1 mmol l^{-1})	5	Both	↓TER	85±11.7	74±9.7*	10±10.2*
Control	7			100	44±6.7	55±6.7
Cimetidine (0.1 mmol l^{-1})	7	I	↓TER/↓I_{glu}	143±25.8*	98±19.2*	45±32.3

Data are presented as a percentage of the paired control secretory flux and are means ± S.E.M. (*N*=the number of preparations). The treatments were administered to either the interstitial side of the epithelium alone (I) or both the interstitial and luminal sides of the epithelium (both). Transepithelial resistance (TER) and phlorizin-sensitive glucose current (I_{glu}), measures of proximal tubule-like function and tissue viability, were monitored to determine if treatments were nonspecific. No effect of the treatment on TER or I_{glu} (No). ↓TER and ↓I_{glu} indicate a decrease in transepithelial resistance and phlorizin-sensitive glucose current, respectively (P<0.05, paired *t*-test). *Significantly different from paired control, P<0.05, paired *t*-test.

BLMV (Grassl, 2002b). As shown in Table 2, addition of PAH (10 mmol l^{-1}) to the interstitial side of PTCs in Ussing chambers completely abolished net transepithelial urate secretion (negative sign indicates net reabsorption) with no nonspecific effects. It should be noted that this high concentration of PAH in the interstitial side significantly stimulated unidirectional reabsorptive urate flux, an effect consistent with PAH entering the cell in exchange for intracellular urate.

Bromocresol Green, generally considered to be a high affinity substrate for proximal tubule OA secretion, inhibited net transepithelial urate secretion; however, its effect was also non-specific as it caused a significant increase in unidirectional reabsorptive (leak) flux together with a significant decrease in TER (Table 2).

The cationic OAT substrate, cimetidine, increased leak in both secretory and reabsorptive directions but, because of extremely high variance, had no effect on net urate transport (Table 2). Consistent with the increased leakiness, both TER and I_{glu} decreased, indicative of toxicity.

At the basolateral membrane, interaction of urate with at least OAT1- or OAT3-like transporters should be dependent upon exchange for intracellular α-KG. Thus, lithium (5 mmol l^{-1}), an inhibitor of Na^+–dicarboxylate cotransport should inhibit re-uptake of α-KG, depleting intracellular dicarboxylate concentration, and slowing urate secretion.

Table 3. *Effect of ouabain, lithium and glutarate on unidirectional secretory, unidirectional reabsorptive and net urate fluxes by chick PTCs*

Treatment	*N*	Side	Nonspecific	Secretory	Reabsorptive	Net
Control	4			100	36±6.5	63±6.5
Ouabain (0.1 mmol l^{-1})	4	I	↓I_{glu}	81±13.3	56±14.2	38±22.0*
Control	6			100	29±5.1	70±5.1
Lithium (5 mmol l^{-1})	6	I	No	73±8.0*	30±5.8	42±7.3*
Control	5			100	30±5.5	69±5.5
Glutarate (0.01 mmol l^{-1})	5	I	No	113±13.2	40±8.7	72±7.6
Control	4			100	44±7.7	55±7.7
Glutarate (0.1 mmol l^{-1})	4	I	No	103±19.8	49±9.7	54±14.1
Control	3			100	37±6.5	62±6.5
Glutarate (1 mmol l^{-1})	3	I	No	69±12.0	55±5.7	14±7.0*

Data are presented as a percentage of the paired control secretory flux and are means ± S.E.M. (*N*=the number of preparations). The treatments were administered to the interstitial side of the epithelium alone (I). Transepithelial resistance (TER) and phlorizin-sensitive glucose current (I_{glu}), measures of proximal tubule-like function and tissue viability, were monitored to determine if treatments were nonspecific. No effect of the treatment on TER or I_{glu} (No). ↓TER and ↓I_{glu} indicate a decrease in transepithelial resistance and phlorizin-sensitive glucose current, respectively (P<0.05, paired *t*-test). *Significantly different from paired control, P<0.05, paired *t*-test.

Table 3 shows that lithium, added to the interstitial side only of PTCs in Ussing chambers decreased net urate secretion by ~40%, mainly through decreasing the secretory flux. Lithium affected only urate secretion and had no effect on reabsorption or electrical properties. Consistent with the lithium effect, ouabain, an inhibitor of the Na^+/K^+ ATPase, also inhibited net transepithelial urate secretion by 40% and, as expected, significantly inhibited I_{glu}.

The inhibition by lithium of unidirectional and net transepithelial urate secretion in chick PTCs suggested that the well-characterized BLM tertiary active OA uptake system (Wright and Dantzler, 2004) was mediating urate uptake. However, acute addition of 10 or 100 μmol l^{-1} glutarate, the non-metabolized form of α-KG, to the interstitial bath had no effect on unidirectional secretory or reabsorptive urate fluxes in PTCs (Table 3). Addition of 1 mmol l^{-1} glutarate to only the luminal bath had no effect on urate fluxes (data not shown). To determine if 330 μmol l^{-1} urate (physiological concentration) was preventing the stimulatory effect of lower extracellular glutarate concentrations, the urate concentration in the bathing medium was reduced to 5.8 μmol l^{-1}. However, no stimulation of net transepithelial urate secretion resulted from the combination of lower urate and 10 μmol l^{-1} glutarate in the interstitial side (data not shown). This apparent lack of effect was probably due to production and efficient recycling of endogenous α-KG by Na^+/dicarboxylate cotransport since elevation of glutarate to 1 mmol l^{-1} in the interstitial solution inhibited 80% of net transepithelial urate secretion, as expected (Table·3).

The BLM OAT isoforms, OAT1 and OAT3, are OA/dicarboxylate exchangers (Bakhiya et al., 2003; Sweet et al., 2003) and can mediate the transport of several OAs, including PAH and urate (Bakhiya et al., 2003; Cha et al., 2001). ES in certain systems is a specific substrate of OAT3 but not OAT1 (see Van Aubel et al., 2000). Addition of ES to only the interstitial sides of PTCs significantly stimulated net transepithelial urate secretion at low concentration (10 μmol l^{-1}) and strongly inhibited urate secretion at higher concentrations (500 μmol l^{-1} and 2500 μmol l^{-1}) (Fig. 2). Interestingly, the urate reabsorptive flux was strongly stimulated by the highest ES concentration. This compound had no effect on electrical properties at any concentration.

Chick PTCs avidly secreted PAH (Fig. 3) exhibiting a 5:1 flux ratio in short-circuited conditions. Net PAH secretion was inhibited ~34% by addition of 330 μmol l^{-1} urate, and the remaining net flux was totally blocked by 500 μM ES. The latter, alone, blocked both the urate-sensitive and -insensitive net PAH secretion.

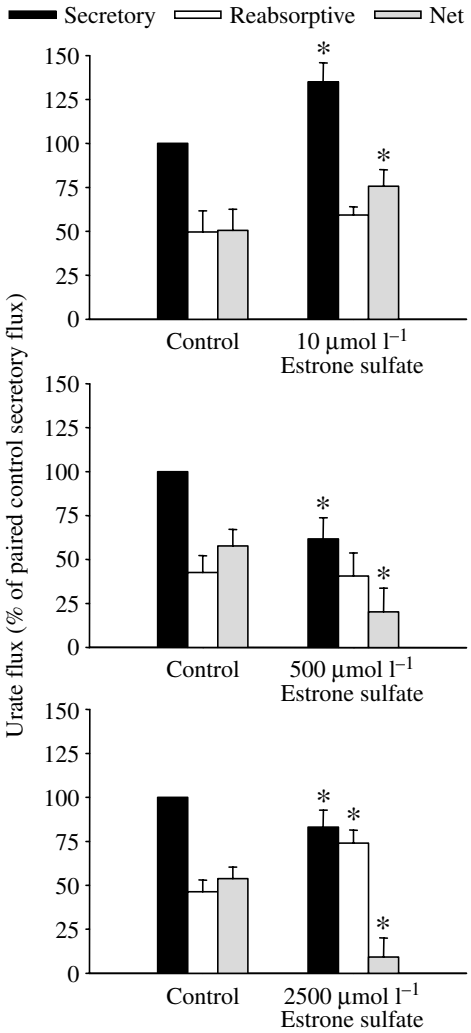

Fig. 2. Unidirectional secretory, unidirectional reabsorptive, and net urate fluxes by chick PTCs following treatment with estrone sulfate (10 μmol l^{-1}, 500 μmol l^{-1}, or 2500 μmol l^{-1}). Estrone sulfate was administered to the interstitial side of the epithelium only (at t=0). Data are presented as the percentage of the paired control secretory flux and are means ± S.E.M. of four (10 μmol l^{-1} and 500 μmol l^{-1}) and 10 (2500 μmol l^{-1}) preparations. *Significantly different from paired control flux (P<0.05, paired t-test).

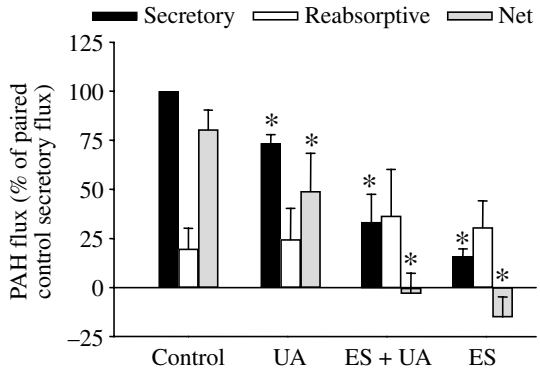

Fig. 3. Unidirectional secretory, unidirectional reabsorptive, and net PAH fluxes by chick PTCs following treatment with urate (330 μmol l^{-1}), urate and estrone sulfate in combination (ES + UA), or estrone sulfate (ES, 500 μmol l^{-1}) alone. All treatments were administered to the interstitial and luminal sides of the epithelium at t=–0.5 h. Data are presented as the percentage of the paired control secretory flux after 90 min exposure and are means ± S.E.M. of three preparations. *Significantly different from paired control flux (P<0.05, paired t-test).

Table 4. *Effect of various treatments on unidirectional secretory, unidirectional reabsorptive and net urate fluxes by chick PTCs*

Treatment	*N*	Side	Nonspecific	Secretory	Reabsorptive	Net
Control	3			100	34±5.9	65±5.9
K^+ (30 mmol l^{-1})	3	L	↓TER	133±19.2	59±13.3	73±17.3
Control	3			100	34±4.9	65±4.9
Glucose-free	3	L	$\downarrow I_{glu}$	116±15.9	36±4.8	80±13.6
Control	3			100	34±7.3	65±7.4
Cl^- free	3	L	No	89±15.3	44±11.3	45±16.0
Control	3			100	33±7.4	67±7.4
pH 7.7	3	L	$\downarrow I_{glu}$	125±17.2	49±14.9	75±5.6
Control	3			100	34±7.6	66±7.6
Oxonic acid (2.5 mmol l^{-1})	3	L	No	98±3.4	41±7.4	57±4.2
Control	3			100	33±6.2	66±6.2
Pyrazine (2 mmol l^{-1})	3	L	No	123±18.2	44±14.6	78±32.4
Control	4			100	31±4.2	68±4.2
Adenosine (2 mmol l^{-1})	4	L	No	112±15.7	39±11.0	72±18.6
Control	5			100	31±4.8	68±4.8
Nicotinic acid (0.3 mmol l^{-1})	5	L	No	82±8.4	34±7.6	48±15.3
Control	5			100	36±9.0	63±9.0
Nocodozole (0.03 mmol l^{-1})	5	Both	$\downarrow I_{glu}$	114±27.4	70±21.5*	44±8.5
Control	4			100	28±5.3	71±5.3
Cytochalsin D (10^{-3} mmol l^{-1})	4	Both	↓TER	124±15.3	120±38.9*	4±23.8*

Data are presented as a percentage of the paired control secretory flux and are means ± S.E.M. (*N*=the number of preparations). The treatments were administered to the luminal side of the epithelium alone (L) or both the interstitial and luminal sides of the epithelium (both). Transepithelial resistance (TER) and phlorizin-sensitive glucose current (I_{glu}), measures of proximal tubule-like function and tissue viability, were monitored to determine if treatments were nonspecific. No effect of the treatment on TER or I_{glu} (No). ↓TER and $\downarrow I_{glu}$ indicate a decrease in transepithelial resistance and phlorizin-sensitive glucose current, respectively ($P<0.05$, paired *t-test*). *Significantly different from paired control, $P<0.05$, paired *t*-test.

Lumen-side effects

Alterations in only the luminal fluid of several factors that are known to change urate transport in vertebrate brush-border membrane vesicles (BBMV; which facilitate both reabsorptive and secretory transport of urate) had no effect on net urate secretion by chick PTCs (Table 4). In all ion-substitution experiments the tissues were open-circuited. Substituting K^+ for Na^+ in the luminal bath (4.2–30 mmol l^{-1}) caused only a small change in V_{apical} (electrical potential across the apical (brush-border) membrane) from approx. –79 to –72 mV, whereas complete removal of luminal glucose shifted V_{apical} from –80.5 to –86.5 mV. In neither case were there effects on net transepithelial urate transport. The increased lumen [K^+] caused a significant decrease in TER, perhaps reflecting the increase in the transepithelial Na^+ gradient caused by the ion substitution. There was no effect on transepithelial urate transport of complete replacement of luminal Cl^- with gluconate (Table 4). Apparently, urate does not leave the cells in exchange for Cl^-. Luminal pH of the avian proximal tubule is slightly alkaline at ~7.62 (Laverty and Alberici, 1987), and in dog BBMV OH^-/urate anion exchange can occur (Kahn and Aronson, 1983). To assess whether a physiological luminal pH would alter urate transport, pH of the physiological saline solution bathing the luminal sides of PTCs in Ussing chambers was asymmetrically raised from 7.4 to 7.7 (a 50% decrease in [H^+]), again, with no effect on net transepithelial urate transport (Table 4). The significant drop in I_{glu} is probably due to increased apical Na^+/H^+ exchange and depletion of the apical membrane Na^+ gradient.

Also shown in Table 4, a series of substrates (oxonic acid, pyrazine, adenosine and nicotinic acid) known to interfere with urate reabsorption or cause uricosuria (Roch-Ramel et al., 1997), had no effect on PTC urate transport or transepithelial electrophysiological properties. These results coupled with the lack of effect of luminal Cl^- decrease the likelihood of a role for transporters such as URAT1 (urate transporter 1), the electroneutal urate/Cl^- exchanger thought to be responsible for urate reabsorption in humans (Enomoto et al., 2002) and the 74%-similar mouse renal-specific transporter (RST) which may mediate voltage-dependent urate transport (Imaoka et al., 2004).

Intracellular sequestration of OAs, subsequent bulk vesicular transport in the cytosol, and release into the tubule lumen by exocytosis have been suggested as part of the transepithelial OA transport process (Miller and Pritchard, 1994; Miller et al., 1993). In the chick PTCs concentrations of nocodozole (microtubule disrupter) or cytochalasin D (microfilament disruptor) known to interfere with vesicular trafficking were either ineffective or nonspecific (Table 4). Nocodozole significantly decreased I_{glu} and increased urate back leak but, because of very high variance, had no significant effect on net urate transport. Cytochalasin D significantly

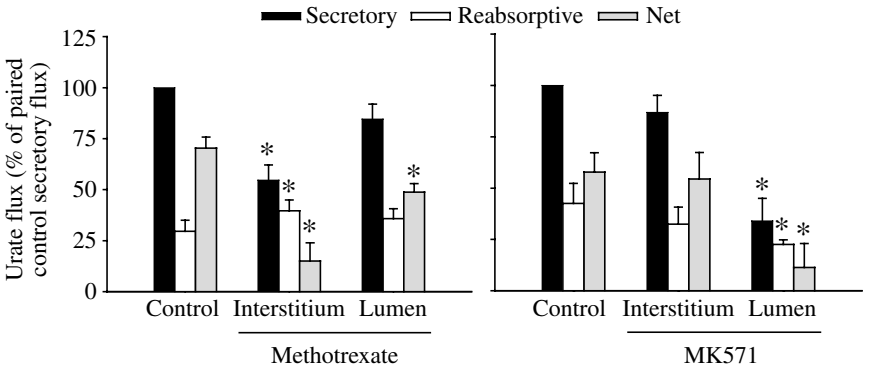

Fig. 4. Unidirectional secretory, unidirectional reabsorptive, and net urate fluxes by chick PTCs following treatment with methotrexate (500 μmol l^{-1}) or MK571 (20 μmol l^{-1}). Methotrexate (at t=–0.5 h) and MK571 (at t=0) were added to the interstitial or luminal sides of the epithelium. Data are presented as the percentage of the paired control secretory flux and are means ± S.E.M. of five preparations. *Significantly different from paired control flux (P<0.05, paired t-test).

decreased TER and increased urate back leak while totally blocking active urate secretion. The nonspecific effects of these disrupters make conclusions about bulk vesicular transport of urate in PTCs uncertain.

Because MRP4 resides in the brush border membrane (BBM) and is capable of primary active transport of urate (Van Aubel et al., 2005), known MRP4 substrates such as MTX (K_m ~100 μmol l^{-1} – 1 mmol l^{-1}; Russel et al., 2002) and MK571 (Sundkvist et al., 2000) should compete for urate transport sites. Addition of MTX (500 μmol l^{-1}) to the interstitium alone strongly inhibited net urate secretion by effectively reducing the secretory flux and stimulating the reabsorptive flux (Fig. 4). MTX also reduced net urate secretion from the lumen side, albeit not as effectively. OAT1 and OAT3 also have an affinity for MTX (Russel et al., 2002); therefore, to determine if luminal administration of 500 μmol l^{-1} MTX inhibited urate transport by interacting with the BLM OATs, the drug (500 μmol l^{-1}) was added to the lumen of PTCs for 1.5 h (no fluxes were run); the interstitial bath was then removed and used as the interstitial bath solution for measurement of urate transport by PTCs not previously treated with MTX. This particular treatment had no effect on net urate secretion (16±4.1 *versus* 16±4.3 nmoles cm^{-2} h^{-1}, N=4) indicating that MTX inhibition from the lumen side was due to interaction with an apical membrane site, and, thus, the drug may inhibit through either BLM or BBM transport pathways.

Addition of MK571 (20 μmol l^{-1}), a potent inhibitor of MRP4 (Jedlitschky et al., 2004), to the lumen of PTCs caused a dramatic reduction of net urate secretion (Fig. 4). Both unidirectional secretory and reabsorptive fluxes were significantly reduced. In contrast, MK571 had no effect from the interstitium.

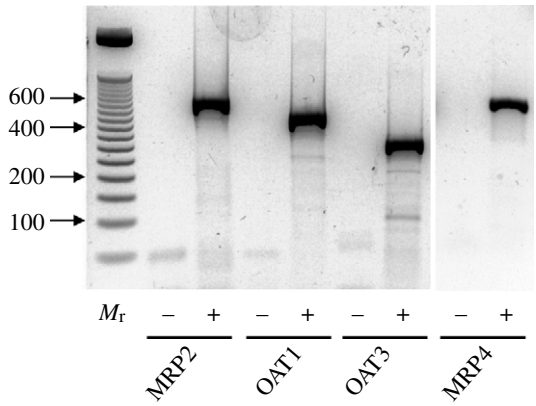

Fig. 5. RT-PCR to amplify chicken renal OAT1-like, OAT3-like, MRP2-like and MRP4-like cDNA. mRNA isolated from chick PTCs was reverse transcribed, and the resulting cDNA amplified using primers to chicken OAT1, OAT3, MRP2 and MRP4 (see Table 1). The RT-PCR products were run on a 2% agarose gel and stained with Gel-Star. The cDNA template was either present (+) or absent (–). No product was obtained when the cDNA template was omitted. Molecular size (base pairs) is indicated on the left.

Detection of mRNA for known organic anion transporters from PTCs

Specific primers (see Table 1) and RT-PCR were used to amplify cDNAs corresponding to OAT1-like, OAT3-like, MRP2 and MRP4 transporters from chick PTC mRNA (Fig. 5). In each case, a single cDNA product of the appropriate size (OAT1=483 bp; OAT3=344 bp; MRP2=586 bp; MRP4=565 bp) was identified on 2% agarose gels. No product was detected when the template mRNA was omitted.

Discussion

Transepithelial urate secretion by the chicken proximal tubule epithelium involves multiple membrane transporters in at least three cellular locations, i.e. the BLM, cytosolic trafficking and the BBM, and in addition the paracellular pathway. The specific transporters mediating the basolateral uptake of urate in the avian system most probably include one or more of the OAT-like avian orthologs demonstrated here by RT-PCR. Since both OAT1-like and OAT3-like genes are expressed in chicken proximal tubule epithelium (see Fig. 5) and these generally have overlapping substrate affinities (Wright and Dantzler, 2004), a specific urate transporter in the BLM, if one dominates, remains to be identified. In mouse and rabbit, PAH is transported by Oat1 and Oat3, but their OAT1 orthologs do not transport ES (see Wright and Dantzler, 2004). In the present study, urate blocked only about one-third of PAH transport, but ES blocked it all. Thus, in the chicken system PAH and urate appear to share only a part of the total OA secretory machinery whereas ES and PAH appear to totally overlap transporters at some point in

transepithelial transport. The potential for species variation in OAT-like transporter affinities is great; however, OAT3 may have the largest role in urate secretion in humans and rabbits (Bakhiya et al., 2003; Dantzler, 2005).

Lithium inhibits Na^+/dicarboxylate transport (Kekuda et al., 1999) and, in the present study, reduced net urate secretion by about one-half, through inhibition of the unidirectional secretory flux although the reabsorptive flux was unaffected. These data, in conjunction with the aforementioned PAH and probenecid sensitivity, are consistent with the presence of Na^+/dicarboxylate cotransport coupled with urate/α-KG exchange in the BLM as was demonstrated in mammals (Pritchard, 1990; Shimada et al., 1987) and confirmed in turkey BLMV (Grassl, 2002b). The physiological saline solution used for the present PTC transport studies was not supplemented with α-KG, so the lithium inhibition of urate secretion further supports urate exchange for intracellularly produced α-KG and recycling of the α-KG from the interstitial bath to the cell by Na^+/dicarboxylate cotransport. In the absence of extracellular α-KG, rabbit proximal tubule S2 segment uptake of OA is inhibited 25% by lithium, and the overall contribution of metabolically produced α-KG to OA secretion in rabbits is about 40% (Dantzler, 2002). In the chick PTCs, no stimulation of urate secretion by glutarate was observed, and it is possible that endogenous α-KG accumulates to a saturating concentration, precluding an effect of additional accumulation of glutarate. Lithium, however, would lead to depletion of intracellular α-KG and slowing of urate uptake. The inhibitory effect of high levels of interstitial glutarate (1 mmol l^{-1}, see Table 3) is consistent with glutarate/urate interaction.

Asymmetrical addition of 10 mmol l^{-1} PAH, 2.5 mmol l^{-1} ES, or 0.5 mmol l^{-1} MTX to the interstitial side of PTCs not only inhibited unidirectional secretory flux, it increased unidirectional reabsorptive flux with no nonspecific effects. This behavior (i) obviously cannot be due to increased non-mediated leak flux; (ii) is consistent with urate efflux, i.e. cell to interstitium, driven by reversal of an anion exchange mechanism and (iii) indicates that these substrates and urate interact with the same exchanger(s). These findings are consistent with the facts that PAH is accumulated intracellularly to twice the concentration of urate in isolated chicken proximal tubule fragments (Brokl et al., 1994) and PAH/urate exchange exists in turkey BLMV as demonstrated by Grassl (Grassl, 2002b). The mechanism by which lumen-to-cell flux was altered in this circumstance is not clear, however, urate must enter from the lumen against an electrochemical gradient, a phenomenon observed for PAH, but not urate, in perfused chicken proximal tubule (Brokl et al., 1994). This is apparently a very small component and perhaps not like the reabsorption process in mammals, in which luminal Cl^- exchanges for intracellular urate on URAT1 (Enomoto et al., 2002; Kahn and Aronson, 1983), because the present study revealed no effect of Cl^- removal from the luminal side on urate transport (see Table 4); and as already noted, urate/anion exchange could not be demonstrated in a recent study of avian BBMV (Grassl, 2002a).

A possible cell-to-lumen exit step in urate secretion has been characterized in turkey kidney (Grassl, 2002a). In isolated BBMV from these Galliforms a conductive uniporter facilitates diffusion of urate down its electrochemical gradient and is *trans*-stimulated and *cis*-inhibited by PAH. Likewise, PAH transport is driven by the electrical gradient and blocked by urate. Cl^- and pH gradients have no effect on BBMV uptake except secondarily through membrane diffusion potentials (Grassl, 2002a). There is no consensus as to the transporter(s) responsible for this step. In urate-secreting mammals the most likely transporters were recently reviewed (Hediger et al., 2005; Wright and Dantzler, 2004) and include MRP4 and Oat_v1 (Jutabha et al., 2003). The latter is 65% identical to human NPT1. Oat_v1 and NPT1 are located in proximal tubule BBM of pig and human, respectively, and can mediate electrogenic transport of PAH and urate (Busch et al., 1996; Uchino et al., 2000). However, no chicken orthologs of these two transporters have yet been identified; they are Cl^- sensitive and they do not have the BBMV urate transport properties, i.e. they are not electrogenic antiporters (Roch-Ramel et al., 1994).

MRP4 is a primary active export pump localized to the proximal tubule BBM and it mediates transport of MTX and urate (Van Aubel et al., 2005). In PTCs urate transport was inhibited by MTX from either the interstitial side or the luminal side. Luminal application of MK-571, a leukotriene C_4 receptor antagonist, dramatically reduced both urate secretory and reabsorptive flux. This compound is a known substrate for MRP2, MRP4 and the organic anion transporter oatp1 (Sundkvist et al., 2000), also known to be present in mammalian BBM; however, urate is not transported by MRP2 (Van Aubel et al., 2005). Interestingly, amino acid alignment of the chicken MRP4-like sequence against human MRP4 indicates 86% sequence similarity. However, the chicken MRP4-like sequence is 303 amino acids longer than human MRP4, containing additional amino acids at the extreme N terminus and from amino acid 1068 to amino acid 1148. If MRP4 is involved in the urate efflux step in the chicken proximal tubule it may be beneficial to investigate the structure–function relationship of MRP4 in this uricotelic species. The sensitivity of other possible urate transport pathways to MK-571 is not known. MK571 had no effect on urate transport from the interstitial side of the PTCs, providing, for the first time, a means for discrimination of BLM and BBM urate transport processes in intact epithelium.

In conclusion, the chick PTC system served to characterize urate transport in a predominantly secretory system amenable to the examination of the transepithelial aspect of urate transport. The chicken PTC culture system maintained excellent urate secretory capacity and should prove a useful tool for further study of this process, unclouded by the co-existence of a highly expressed mediated reabsorptive transport process. The pharmacological data and RT-PCR demonstration of expression in the PTCs of genes associated with OA and urate transport, presented here, should aid the ultimate identification of the participants in active tubular urate secretion. The data support the conclusion that avian renal

urate secretion is mediated by an OAT-like transporter in the BLM and provide a means of distinguishing the apical and basolateral membrane steps in proximal tubule epithelium.

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