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Mediation of cimetidine secretion by P-glycoprotein and a novel H^+-coupled mechanism in cultured renal epithelial monolayers of LLC-PK_1 cells

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1 Previous studies have shown that the weak base, cimetidine, is actively secreted by the renal proximal tubule. In this study we have examined the transport of cimetidine by renal LLC-PK_1 epithelial cell monolayers.

2 In LLC-PK_1 cell monolayers the basal-to-apical flux of cimetidine was significantly greater than the apical-to basal flux, consistent with net secretion of cimetidine in a basal-to-apical direction.

3 Net secretion of cimetidine was significantly (70%) reduced by the addition of either 100 μM verapamil or 100 μM nifedipine to the apical membrane. The reduction in net secretion was the result of an inhibition of basal-to-apical flux; these agents had no effect upon flux in the apical-to-basal direction. These results suggest that cimetidine secretion is mediated primarily by P-glycoprotein located in the apical membrane. In addition we found no evidence of a role for organic cation antiport in the secretion of cimetidine.

4 In the presence of an inwardly directed proton gradient across the apical membrane (pH 6.0), cimetidine secretion was significantly reduced compared to that measured at an apical pH of 7.4. The reduction in net secretion at pH 6.0 was the result of a stimulation of cimetidine uptake across the apical membrane. This pH-dependent uptake mechanism was sensitive to inhibition by DIDS (100 μM).

5 Experiments with BCECF (2',7'-*bis*(2-carboxyethyl)-5(6)-carboxyfluorescein) loaded monolayers demonstrated that cimetidine influx across the apical membrane was associated with proton flow into the cell and was sensitive to inhibition by DIDS.

6 These results suggest that net secretion of cimetidine across the apical membrane is a function of the relative magnitudes of cimetidine secretion mediated by P-glycoprotein and cimetidine absorption mediated by a novel proton-coupled, DIDS-sensitive transport mechanism.

Keywords: Organic cation transport; LLC-PK_1 cells; P-glycoprotein; proton-coupled transport

Introduction

Cimetidine, an antagonist of the histamine H_2 receptor, has proved extremely effective in the treatment of peptic ulcer disease. The kidney is the primary route of cimetidine elimination from the body and since cimetidine usage is commonplace, a clear understanding of the renal handling of cimetidine and its interaction with other drugs for common excretory pathways is of considerable importance. Measurement of the renal clearance suggests that in addition to being filtered at the glomerulus, cimetidine is subject to tubular secretion along the length of the proximal tubule (Somogyi & Gugler, 1983; Pritchard & Miller, 1993).

Tubular secretion of cimetidine is thought to result from the interaction of cimetidine with two discrete transport systems; the first mediates cimetidine uptake into the epithelium across the basolateral membrane and the second mediates cimetidine exit across the apical membrane into the tubular lumen. The basolateral uptake step has been particularly well documented (Brändle & Greven, 1991; Ullrich, 1994), and may be mediated by the recently-cloned OCT1 transport protein (Gründemann *et al.*, 1994), but the nature of the apical exit step remains controversial. Cimetidine is an organic weak base which exists in both cationic and neutral form. Until recently cimetidine secretion across the apical membrane was thought to be mediated primarily by the secretion of the cationic form of cimetidine via organic cation-proton antiport (OC/H^+) (McKinney *et al.*, 1981; McKinney & Kunnemann, 1987; Gisclon *et al.*, 1987; Ott *et al.*, 1991) with little consideration being given to movement of the uncharged form of cimetidine. But now evidence is emerging to suggest that P-glycoprotein (Speeg *et al.*, 1992; Dutt *et al.*, 1994; Pan *et al.*, 1994), organic anion antiport (Cacini *et al.*, 1982; Gisclon *et al.*, 1989) and non-ionic diffusion (Boom *et al.*, 1994) of the uncharged form may all play a role in cimetidine secretion. To date, however, no systematic study of the contribution of these mechanisms to overall cimetidine secretion has been undertaken.

In this study we have investigated the involvement of these mechanisms in the secretion of cimetidine across epithelial monolayers of LLC-PK_1 cells. To do this, we have measured both transepithelial fluxes and initial rates of [^{3}H]-cimetidine uptake across the apical and basolateral membranes of cultured LLC-PK_1 cells grown on permeable filter supports. In addition we have investigated the coupling of cimetidine transport to proton flux using the pH-sensitive fluorescent probe 2',7' - *bis*(2 - carboxyethyl) - 5(6) - carboxyfluorescein (BCECF) (Dudley & Brown, 1995). The results suggest that cimetidine secretion across the apical membrane of LLC-PK_1 cells is mediated primarily by P-glycoprotein. In addition we found evidence for a novel DIDS-sensitive, pH-coupled reabsorptive mechanism at the apical membrane such that net secretion of cimetidine across this membrane appears to be a function of the relative magnitudes of the secretory and absorptive fluxes of cimetidine. Finally, evidence suggested that there was little contribution to cimetidine transport from either OC/H^+ antiport or from non-ionic diffusion.

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Methods

Cell culture

LLC-PK$_1$ cells were cultured in Medium 199 (with 1.25 g l^{-1} $NaHCO_3$ and L-glutamine) supplemented with 3% (v/v) foetal calf serum (FCS) and gentomycin (50 μg ml^{-1}). Cells were maintained in culture at 37°C in a 5% CO_2, 95% air atmosphere, with the culture media being replaced every 2 days. For intracellular pH studies, cell monolayers were grown on 12 mm diameter permeable filter supports; for transport studies, cells were grown on larger 24.5 mm diameter filter supports (Transwell polycarbonate cell culture inserts; Costar). Monolayer confluence was estimated by microscopy and measurement of epithelial resistance (R_T). Experiments were performed 5–7 days after seeding and 18–24 h after feeding.

Measurement of intracellular pH

Intracellular pH (pH_i) was measured as previously described (Thwaites *et al.*, 1993). Briefly, epithelial monolayers grown on 12 mm permeable filter inserts were loaded by incubation with BCECF-AM (5 μM) in both the apical and basolateral chambers for 30 min at 37°C in culture media. After loading, the inserts were placed in a 24 mm perfusion chamber located on the stage of an inverted fluorescence microscope (Nikon Diaphot). The apical and basolateral cell aspects were perfused independently using a compressed air driven system that allowed any combination of 6 apical and 6 basolateral solutions at a flow rate of 5 ml min^{-1}. At this flow rate, with the apical and basolateral bath volumes set at 0.5 ml and 1 ml, the composition of the apical and basolateral bath solutions could be changed in 6 and 12 s respectively. All experiments were performed at 37°C. Intracellular H^+ concentration was quantified by fluorescence (excitation at 440/490 nm, emission at 520 nm) from a group of 5–10 cells using a photon counting system (Newcastle Photometric Systems). Intracellular fluorescence was converted to pH_i by comparison with values obtained from an intracellular calibration curve using nigericin (10 μM) and high K^+ solutions (Thomas *et al.*, 1979).

Transport studies

Both initial rate of uptake across the apical and basolateral membranes and steady state transepithelial fluxes of [^{3}H]-cimetidine were measured essentially as described by Simmons (1990). For uptake studies, confluent monolayers of cells on filter inserts were gently washed with a modified Krebs buffer containing (mmol l^{-1}): NaCl 137, KCl 5.4, $MgSO_4$ 1.0, KH_2PO_4 0.3, NaH_2PO_4 0.3, HEPES 10, $CaCl_2$ 2.0, and glucose 10, buffered to either pH 7.4 or 8.4 with Tris. In experiments at pH 6.0, 10 mM MES replaced HEPES. The inserts were then suspended in fresh 6-well plates (Costar 3406) containing 2 ml of buffer (basolateral) and 1 ml of Krebs buffer added to the apical chamber. This set-up gave unrestricted access to both apical and basolateral cell aspects. The filters were then incubated for 15 min at 37°C. To initiate uptake cimetidine (0.5 Ci ml^{-1}; 25 μM) was added to either the apical or basolateral chamber as appropriate. Uptake was terminated by plunging each insert several times into a large volume (500 ml) of ice-cold Krebs buffer to remove extracellular isotope. The efficiency of this wash procedure was validated by use of [^{14}C]-mannitol as an extracellular space marker. The filters were removed from the inserts and cell-associated label determined by scintillation counting. Cellular accumulation is expressed as pmol l^{-1} cell water assuming an intracellular volume of 0.8 μl cm^{-2}. The volume of a monolayer ($\pi r^2 h$) was determined from the radius (r) of the filter and an estimate of cell height (h) measured by confocal microscopy. In order to measure steady state bidirectional fluxes (J_{a-b} and J_{b-a}), cell monolayers were washed and preincubated as described above. [^{3}H]-cimetidine (50 μM) was then added to either the apical or basolateral chamber with the same concentration of unlabelled cimetidine present in the contralateral chamber. The monolayers were incubated for 1 h at 37°C. At the end of the flux period the inserts were washed as described above and cell-associated radiolabel determined. To quantify tracer flux a 200 μl sample was taken from each chamber at $t=0$ and $t=60$ min. Cimetidine flux is expressed as nmol cm^{-2} h^{-1}.

In order to quantify the contribution of paracellular flux of cimetidine to transepithelial cimetidine flux, [^{14}C]-mannitol was present in the same chamber as [^{3}H]-cimetidine. ^{3}H and ^{14}C activities were determined by dual-label scintillation counting. Appropriate controls were made for channel spill over and quenching.

Statistical analysis

Data are expressed as mean ± s.e.mean. Statistical comparison of mean values was made with Student's t test (2-tailed solution) for paired or unpaired data as appropriate. For multiple comparisons, analysis of variance (ANOVA) was performed and significance assigned by use of either a Dunnet or a Bonferroni post test.

Materials

Cell culture media, supplements, and tissue culture plastic were from Life Technologies (Paisley, U.K.). Tissue culture inserts (12 mm and 24.5 mm diameter) were from Costar (High Wycombe, U.K.). BCECF-AM was purchased from Calbiochem (Nottingham, U.K.). [^{3}H]-cimetidine and [^{14}C]-mannitol were from Amersham (Little Chalfont, Bucks). Cimetidine was obtained from Aldrich (Gillingham, U.K.). All other biochemicals were supplied by Sigma (Poole, U.K.) or BDH (Lutterworth, U.K.).

Results

Transepithelial cimetidine fluxes in LLC-PK$_1$ cell monolayers

Figure 1 shows the transepithelial fluxes of cimetidine across confluent monolayers of LLC-PK$_1$ cells at 3 different apical pH values: At pH 7.4, J_{b-a} (1.18 ± 0.01 nmol cm^{-2} h^{-1}, $n=4$) was significantly larger than J_{a-b} (0.42 ± 0.02 nmol cm^{-2} h^{-1}, $n=4$, $P<0.001$); a result consistent with net cimetidine secretion in a basal to apical direction (0.76 ± 0.03 nmol cm^{-2} h^{-1}) in the absence of a pH gradient across the epithelium. Imposition of an inwardly directed H^+ gradient across the apical membrane

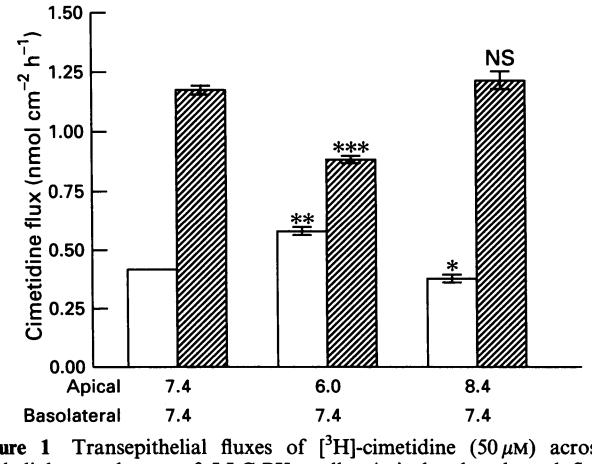

Figure 1 Transepithelial fluxes of [^{3}H]-cimetidine (50 μM) across epithelial monolayers of LLC-PK$_1$ cells. Apical-to-basolateral flux (open columns) and basolateral-to-apical flux (hatched columns) were measured over a 1 h period. The results are the mean ± s.e.mean of 4 separate experiments. (*$P<0.01$, **$P<0.005$ compared to J_{a-b} at pH 7.4 and ***$P<0.002$ compared to J_{b-a} at pH 7.4).

(apical bath pH 6.0), a condition which should stimulate OC/H^+ exchange, reduced net secretion of cimetidine by more than 50% compared to no proton gradient across the apical membrane (0.37 ± 0.04 vs 0.76 ± 0.03, $n=4$, $P<0.001$). The reduction in net secretion was the result of both a significant decrease in J_{b-a} (0.88 ± 0.01 vs 1.18 ± 0.02, $n=4$, $P<0.002$) and a significant increase in J_{a-b} (0.59 ± 0.02 vs 0.42 ± 0.02, $n=4$, $P<0.005$). In the presence of an outwardly directed H^+ gradient (apical bath pH 8.4), net cimetidine secretion (0.88 ± 0.02 nmol cm^{-2} h^{-1}, $n=4$) was significantly larger ($P<0.01$) than at either an apical pH of 6.0 or 7.4. The increase in net secretion at an apical pH of 8.4 was attributable solely to a decrease in J_{a-b} (0.33 ± 0.02 vs 0.42 ± 0.02, $n=4$, $P<0.01$); J_{b-a} was not significantly affected. The pH profile of cimetidine secretion across monolayers of LLC-PK$_1$ cells is thus not consistent with OC/H^+ antiport.

The effect of inhibitors of P-glycoprotein on transepithelial cimetidine flux

Recent evidence suggests that cimetidine may be a substrate for P-glycoprotein (Pan *et al.*, 1994). To investigate if cimetidine is transported across the apical membrane of LLC-PK$_1$ cell monolayers via P-glycoprotein, transepithelial fluxes of cimetidine (25 μM) were measured in the presence of two modulators of P-glycoprotein; nifedipine (100 μM) and verapamil (100 μM). In these experiments (Figure 2), net secretion of cimetidine at an apical pH of 7.4, in the absence of a significant pH gradient across the epithelium, was 0.36 ± 0.04 nmol cm^{-2} h^{-1} ($n=3$). Addition of either nifedipine or verapamil to the apical bathing solution resulted in an almost 70% reduction in net secretion of cimetidine to 0.13 ± 0.01 nmol cm^{-2} h^{-1} in the presence of nifedipine ($n=3$, $P<0.005$) and 0.10 ± 0.03 nmol cm^{-2} h^{-1} in the presence of verapamil ($n=3$, $P<0.005$). Both drugs reduced J_{b-a} (from 0.56 ± 0.02 to 0.39 ± 0.02 and 0.33 ± 0.01 respectively, $n=3$, $P<0.001$) but were without effect upon J_{a-b}. These results may be interpreted in support of a central role for P-glycoprotein in the secretion of cimetidine across the apical membrane of LLC-PK$_1$ cell monolayers.

The effect of DIDS upon cimetidine flux across the apical membrane

Evidence suggests that P-glycoprotein is not influenced by pH (Dutt *et al.*, 1994) yet the transepithelial fluxes of cimetidine show a clear pH dependence (Figure 1). To investigate this in further detail we measured initial rates of cimetidine uptake into LLC-PK$_1$ cell monolayers across both the apical and basolateral cell membranes at pH 7.4 and 6.0. Figure 3 shows that the initial rate of cimetidine uptake across the apical membrane was significantly greater in the presence of an inwardly directed proton gradient than at an apical pH of 7.4 (3.96 ± 0.05 vs 1.97 ± 0.01 μmol l^{-1} cell water 5 min^{-1}, $n=3$, $P<0.01$) and that the stimulation of uptake by an inwardly directed H^+ gradient was abolished by addition of 100 μM DIDS (4,4'-diisothiocyanostilbene-2-2'-disulphonic acid) to the apical compartment (2.07 ± 0.06 vs 3.96 ± 0.05 μmol l^{-1} cell water 5 min^{-1}, $n=3$, $P<0.01$). Initial rates of uptake of cimetidine across the basolateral membrane were not affected by a proton gradient (1.66 ± 0.05 vs 1.61 ± 0.04 μmol l^{-1} cell water 5 min^{-1}, $n=3$, $P>0.5$). Uptake of cimetidine across the basolateral membrane was not sensitive to DIDS addition to either the apical (Figure 3) or basolateral membrane (data not shown). These data suggest the presence of a uptake process for cimetidine at the apical membrane of LLC-PK$_1$ cells which is pH-sensitive and inhibited by DIDS.

Effect of DIDS upon transepithelial fluxes of cimetidine

To test whether a DIDS-sensitive uptake process was responsible for the reduction in cimetidine secretion at an apical pH of 6.0, the effect of DIDS upon the steady state transepithelial fluxes of cimetidine was measured (Figure 4). At an apical pH of 6.0, net secretion of cimetidine was reduced by 50% compared to net secretion at an apical pH of 7.4 (0.28 ± 0.03 vs 0.59 ± 0.03 nmol cm^{-2} h^{-1}, $n=3$, $P<0.002$). Addition of 100 μM DIDS to the apical bath completely abolished the reduction in net cimetidine secretion seen at an apical pH of 6.0 indeed in the presence of DIDS the net secretion of cimetidine was significantly greater than at pH 7.4 (0.92 ± 0.06 vs 0.59 ± 0.03 nmol cm^{-2} h^{-1}, $n=3$ $P<0.01$). The effect of DIDS (100 μM) upon net flux was the result of both a significant decrease in J_{a-b} and a concomitant increase in J_{b-a} (Figure 4).

Effects of cimetidine upon intracellular pH

The stimulation of cimetidine uptake at the apical membrane at pH 6.0 could result either from a cimetidine transport pathway sensitive to external pH or from the direct coupling of cimetidine to proton flow across the apical membrane. To distinguish between these two possibilities we investigated the effects of cimetidine

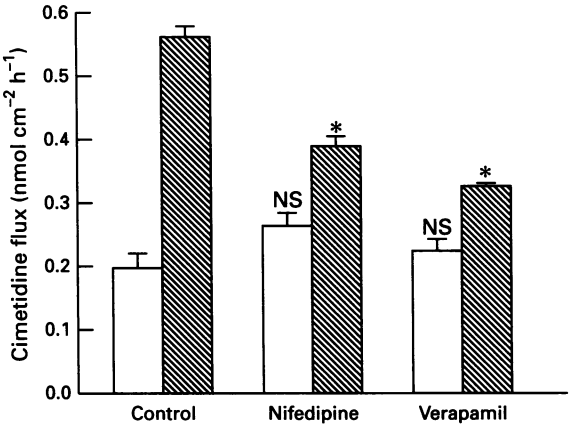

Figure 2 The effect of nifedipine (100 μM) and verapamil (100 μM) upon transepithelial fluxes of [^{3}H]-cimetidine (50 μM) across epithelial monolayers of LLC-PK$_1$ cells. Apical-to-basolateral flux (open columns) and basolateral-to-apical flux (hatched columns) were measured over a 1 h period. The results are the mean$\pm$s.e.mean of 3 separate experiments. *$P<0.001$ compared to control

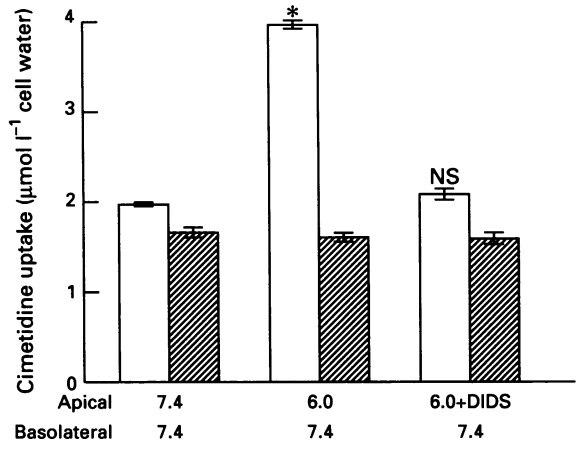

Figure 3 Initial rates of [^{3}H]-cimetidine uptake across the apical (open columns) and basolateral cell membranes (hatched columns) of LLC-PK$_1$ cells grown on permeable filter supports. Cimetidine uptake was determined in the absence of a pH gradient (pH 7.4); in the presence of an inwardly directed proton gradient (apical pH 6.0) and in the presence of an inwardly directed pH gradient plus 100 μM DIDS in the apical uptake solution. The results are the mean$\pm$s.e.mean of 3 separate determinations. *$P<0.01$ compared to pH 7.4.

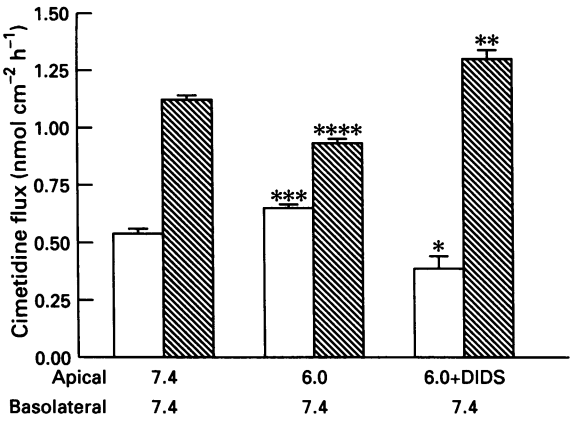

Figure 4 The effect of 100 μM DIDS upon the magnitude of the transepithelial fluxes of [^{3}H]-cimetidine at an apical pH of 6.0. Apical-to-basolateral flux (open columns) and basolateral-to-apical flux (hatched columns) were measured over a 1 h flux period in the presence of an inwardly directed proton gradient (apical pH 6.0) and in the presence of an inwardly directed pH gradient plus 100 μM DIDS in the apical uptake solution. The results are the mean ± s.e.mean of 3 separate determinations. *$P<0.05$, ***$P<0.005$ compared to J_{a-b} at pH 7.4; **$P<0.01$, ****$P<0.001$ compared to J_{b-a} at pH 7.4.

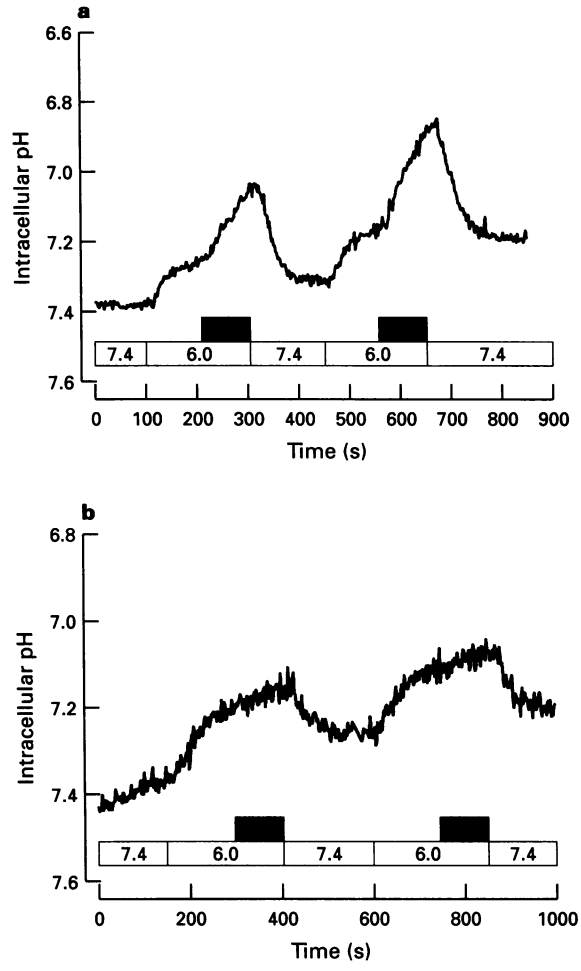

Figure 5 The effect of cimetidine (10 mM) upon intracellular pH. Intracellular pH was measured in monolayers of LLCPK$_1$ cells loaded with the pH-sensitive fluoroprobe BCECF. (a) The effect of apical addition of cimetidine at an apical perfusion pH of 6.0 (at solid bar); basolateral perfusion pH was constant at pH 7.4. A single trace representative of 7 separate experiments. (b) The effect of basolateral addition of cimetidine at pH 6.0 (at solid bar), apical perfusate pH was 7.4; a single trace representative of 7 separate experiments.

upon intracellular pH (pH_i). Figure 5a shows a representative trace of the effects of an apical perfusate pH of 6.0 and cimetidine upon pH_i. Initially the cell monolayer loaded with BCECF was perfused across both the apical and basolateral surfaces with Krebs at pH 7.4. Under these conditions pH_i was 7.39 (mean 7.41 ± 0.05, $n=7$). Imposition of an inwardly-directed H^+ gradient across the apical surface (apical perfusate pH 6.0, basolateral perfusate pH 7.4) resulted in a marked intracellular acidification (mean results: 7.41 ± 0.04 to 7.3 ± 0.04, $n=7$, $P<0.05$). Subsequent addition of 10 mM cimetidine at pH 6.0 to the apical perfusate resulted in a further significant acidification of pH_i from 7.3 ± 0.04 to 7.07 ± 0.06 ($n=7$, $P<0.001$). Removal of cimetidine from the apical perfusate and re-perfusion with pH 7.4 across the apical surface resulted in restoration of pH_i towards initial values. Repetition of the experimental protocol resulted in an almost identical response (Figure 5a). There was no significant effect of apical cimetidine upon pH_i at either an apical perfusate pH of 7.4 (mean results: 7.48 ± 0.04 vs 7.44 ± 0.06, $n=7$, $P>0.5$) or at an apical perfusate pH of 8.4 (7.52 ± 0.05 vs 7.49 ± 0.08, $n=7$, $P>0.5$) suggesting that a proton gradient and the cationic form of cimetidine are essential for maximal cimetidine uptake. Further evidence in favour of this conclusion is provided by the observation that when the external concentration of cimetidine in the cationic form is held constant at 8 mM the measured change in pH_i is strictly dependent upon the magnitude of the imposed proton gradient; at an external pH of 6.0 the change in pH_i was 0.25 ± 0.008 pH units ($n=3$); at pH 6.4 it was 0.14 ± 0.004 pH units and at pH 6.8 it was 0.09 ± 0.003 pH units. In the absence of a significant pH gradient across the apical membrane (apical pH 7.4) cimetidine uptake (Figure 3) was not associated with a change in pH_i or alternatively the change in pH_i was smaller than the buffer capacity of the cell and therefore not visible. Furthermore from these experiments we can conclude that the permeability of the apical membrane to the unionised form of cimetidine must be small since at pH 7.4 and at pH 8.4, where almost all the cimetidine is in the unionised form (80% at pH 7.4, 97.5% at pH 8.4) we see no effect upon intracellular pH. This contrasts with the marked effect upon intracellular pH found with other weak bases such as procainamide where permeation of the unionised form of weak base across the apical membrane followed by a reduction in intracellular proton concentration as the weak base becomes protonated results in an intracellular alkalinisation (Dudley & Brown, 1995).

In contrast to the marked effects of pH 6.0 and cimetidine upon pH_i at the apical surface, cimetidine was without effect at the basolateral membrane (Figure 5b). In this case, the pH_i of a representative cell monolayer at pH 7.4 was 7.43 (mean 7.44 ± 0.04, $n=7$). Imposition of an inwardly-directed H^+ gradient across the basolateral surface (apical perfusate pH 7.4, basolateral perfusate pH 6.0) resulted in a significant intracellular acidification (mean results: 7.44 ± 0.04 to 7.19 ± 0.03, $n=7$, $P<0.05$) however, in contrast to the apical membrane, no further acidification was seen on addition of cimetidine to the basolateral membrane (mean results: 7.19 ± 0.03 to 7.13 ± 0.07, $n=7$, $P>0.5$). Thus, despite altering the ratio of cationic to uncharged cimetidine from 0.25 to almost 4 we see no effect upon either the initial rate of uptake (Figure 3) or intracellular pH (Figure 5b) suggesting that the basolateral uptake mechanism for cimetidine does not see the degree of ionisation of cimetidine (Ullrich, 1994).

Effects of DIDS upon cimetidine-induced acidification

As DIDS affected both net flux and uptake of cimetidine across the apical membrane at pH 6.0, the effects of 100 μM DIDS upon pH_i were investigated. Figure 6a shows a re-

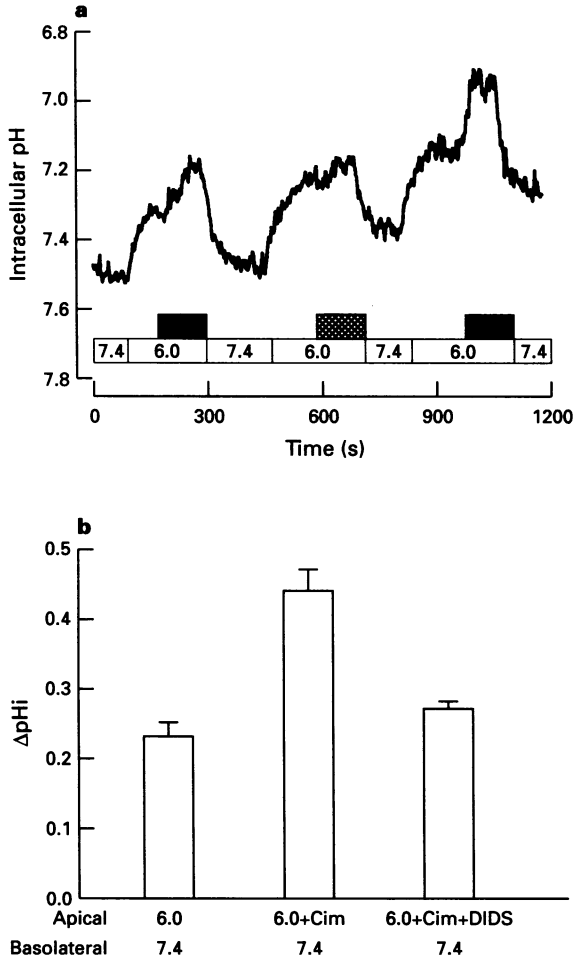

Figure 6 The effect of DIDS (100 μM) upon the cimetidine-induced acidification of pH_i. (a) The effect of apical addition of 10 mM cimetidine upon pH_i was measured at an apical perfusate pH of 6.0 in the absence (solid bar) and presence of 100 μM DIDS (cross-hatched bar). Basolateral perfusate pH was 7.4; a single trace representative of 4 separate experiments. (b) A summary of the effects of cimetidine (cim) and cimetidine + DIDS upon intracellular pH at the apical membrane. The results are expressed as the mean ± s.e.mean of 4 separate determinations.

presentative experiment and Figure 6b a summary of the mean changes in pH_i. At pH 7.4, mean intracellular pH was 7.48 ± 0.05 (n = 4). Perfusion of the apical membrane with pH 6.0 Krebs solution resulted in an acidification of pH_i by 0.23 ± 0.02 pH units compared to control. At an apical pH of 6.0, addition of cimetidine resulted in a further significant reduction in pH_i (0.44 ± 0.03 vs 0.23 ± 0.02 pH units, n = 4, P < 0.001) compared to pH 6.0. The presence of 100 μM DIDS in the apical perfusate at pH 6.0 completely abolished this further cimetidine-induced acidification (Figure 6a): Indeed, at an apical pH of 6.0, the change in pH_i in the presence of cimetidine plus DIDS (0.27 ± 0.03 pH units, n = 4) was not significantly different from the change induced by pH 6.0 alone (0.23 ± 0.02 pH units n = 4) (Figure 6b). The effect of DIDS upon the cimetidine-induced acidification was completely reversible; a second challenge with cimetidine at pH 6.0 in the absence of DIDS, resulted in a similar cimetidine-induced acidification as the first challenge (Figure 6a). The effects of DIDS were specific to the apical membrane; addition of DIDS to the basolateral membrane had no effect upon the response of the cell to apical addition of cimetidine at pH 6.0. Similarly perfusion of the apical membrane with DIDS was without effect upon resting pH_i or the response of monolayers to an apical perfusate of pH 6.0 in the absence of cimetidine.

Discussion

The principal findings of this study are: (1) cimetidine is secreted across epithelial monolayers of LLC-PK$_1$ cells grown on permeable filter supports; (2) cimetidine secretion appears to be mediated almost exclusively by P-glycoprotein located at the apical membrane; (3) the magnitude of net secretion is modified by the presence at the apical membrane of a novel DIDS-sensitive, pH-coupled uptake mechanism for cimetidine.

A central role for P-glycoprotein in the secretion of cimetidine across the apical membrane of LLC-PK$_1$ cells is shown by: the magnitude of the secretory flux in the absence of an external driving force, indicative of the involvement of an active transport step; the apparent pH-independence of the flux in the presence of DIDS and by the marked inhibition of basolateral to apical flux by apical addition of either verapamil or nifedipine, both potent modulators of P-glycoprotein activity (Hunter *et al.*, 1993). These findings are consistent with both the localization of P-glycoprotein to the apical membrane of the renal proximal tubule (Thiebaut *et al.*, 1987) and LLC-PK$_1$ cells (Horio *et al.*, 1990), its function as an active drug efflux pump (Gottesmann & Pastan, 1988) and the recent reports that cimetidine is a substrate for P-glycoprotein (Speeg *et al.*, 1992). Indeed, our findings in LLC-PK$_1$ cells provide clear experimental evidence in support of the proposal that P-glycoprotein plays an important role in the secretion of a wide range of organic cations in the proximal tubule (Nelson, 1988) and in the secretion of cimetidine in particular (Dutt *et al.*, 1994; Pan *et al.*, 1994).

A role for organic cation proton antiport in the transport of cimetidine has been well documented in renal brush border membrane vesicles (Gisclon *et al.*, 1987; McKinney & Kunneman, 1987), yet we found no evidence of a stimulation of cimetidine secretion across the apical membrane in the presence of an outwardly directed proton gradient. In fact we found the opposite, a significant inhibition of cimetidine secretion under these conditions, implying that organic cation proton antiport does not play a significant role in the secretion of cimetidine across epithelial monolayers of LLC-PK$_1$ cells. Similar conclusions were reached for cimetidine transport in isolated proximal tubule cells (Boom & Russel, 1993), procainamide transport in cultured renal LLC-PK$_1$ and OK cells (Dudley & Brown, 1995) and for TEA transport in LLC-PK$_1$ cells (McKinney *et al.*, 1988) – this despite the demonstration of TEA/H^+ antiport in isolated apical membrane vesicles of LLC-PK$_1$ cells (Innui *et al.*, 1985). If we accept that the organic cation proton antiporter and P-glycoprotein have similar broad substrate specificities (Horio *et al.*, 1988; Holohan *et al.*, 1992), then the apparent differences between transport in isolated membrane vesicles and intact epithelial cells may relate to: (1) The presence of a functional P-glycoprotein in intact cells (in membrane vesicles, in the absence of ATP, P-glycoprotein would be quiescent). (2) The level of expression of either system in the intact epithelium and (3) differences in the affinities of substrates for organic cation proton antiport and P-glycoprotein (Dellinger *et al.*, 1992; Dutt *et al.*, 1994; Wright *et al.*, 1995).

Measurement of the magnitude of cimetidine secretion at 3 different apical pH values revealed a clear pH-dependence of cimetidine exit across the apical membrane. Since P-glycoprotein has been reported to be pH-insensitive (Dutt *et al.*, 1994) and the pH profile of secretion was not consistent with organic cation antiport we set out to investigate the mechanism responsible for the apparent pH-dependence of cimetidine secretion. Initial measurements of transepithelial [^{3}H]-cimetidine fluxes suggested that the reduction in net secretion was predominately the result of a stimulation of the apical to basolateral flux of cimetidine at an apical pH of 6.0 compared to the flux at 7.4, and that the reduction in secretion could be completely reversed by the addition of DIDS to the apical membrane. These observations were confirmed by the measurement of initial rates of cimetidine uptake across the apical membrane. In agreement with the transepithelial flux data,

initial rates of uptake were both stimulated by an apical pH of 6.0 and were sensitive to inhibition by DIDS. Using a third experimental approach we measured directly the coupling between cimetidine uptake and proton flux. To do this we loaded cell monolayers with the pH sensitive dye, BCECF and measured the effects of cimetidine upon intracellular pH (Dudley & Brown, 1995). The results suggested that the pH-dependence of cimetidine uptake across the apical membrane was the result of a direct coupling of cimetidine flux to proton flux and that this transport process was sensitive to inhibition by DIDS. Thus, using three independent experimental approaches, we have provided clear evidence to identify the expression of a novel proton coupled, DIDS-sensitive cimetidine transporter at the apical membrane of LLC-PK$_1$ cells.

In summary, we have presented evidence to show that the organic cation cimetidine is secreted in a basal to apical direction across epithelial monolayers of LLC-PK$_1$ cells. The secretion of cimetidine across the apical membrane is consistent with secretion via an apical membrane located P-glycoprotein. There is no evidence of a role for organic cation proton antiport in mediating cimetidine secretion across LLC-PK$_1$ cell monolayers. Furthermore we have identified a novel proton-coupled, DIDS-sensitive reabsorptive mechanism at the apical membrane which is responsible for the apparent pH sensitivity of cimetidine secretion observed. If the expression of this transporter in *in vivo* proximal tubule mirrors that found in LLC-PK$_1$ cells then this mechanism may play an important role in determining the clearance of cimetidine. Clearly, further studies are necessary to identify the expression of this novel reabsorptive mechanism in intact proximal tubules, to determine its substrate specificity and to identify the transporter at a molecular level.

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