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Research Article

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Indomethacin Secretion in the Isolated Perfused Proximal Straight Rabbit Tubule

Evidence for Two Parallel Transport Mechanisms

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Abstract

We studied indomethacin as a probe of anion transport across the isolated perfused proximal straight tubule of the rabbit and discovered that a substantial component of transport may occur by anion exchange at the basolateral membrane. Various perturbations involving direct or indirect dissipation of the cellular sodium gradient (ouabain, sodium- or potassium-free solutions, cooling to 18°C) resulted in only a 50% inhibition of indomethacin transport, which raised the question of a co-existent alternative pathway for secretion. Similarly, the anion exchange inhibitor, 4,4'-diisothiocyanostilbene (DIDS), diminished indomethacin secretion by only 50%. Cooling followed by DIDS or the reverse sequence resulted in additive inhibition such that the combination abolished active secretion of indomethacin.

We conclude that active secretion of indomethacin by the proximal straight tubule appears to be in part sodium gradient dependent; the remainder may be driven by an anion exchanger on the basolateral membrane.

Introduction

The secretion of a wide variety of both endogenous and exogenous organic anions is one of the excretory functions of the kidney (1, 2). The latter include numerous drugs. There is general agreement that the site of action of organic anion secretion is predominantly the proximal straight renal tubule, in particular the S2 segment (3). In contrast, the mechanism of secretion is much debated. Different carrier-mediated and passive transport processes have been suggested to be present across the basolateral as well as the luminal epithelial cell membrane. An active sodium-coupled anion transport process appears to be a primary candidate for the transfer of the anion from blood to cell (4, 5), whereas passive diffusion and/or an anion exchange mechanism have been suggested to play a role in transport across the luminal membrane (6). However, recent reviews on renal anion transport by Møller and Sheikh (1) and Grantham (2) illustrate that many other transport systems may also be involved in the secretion of organic anions.

The abovementioned characteristics of the transport system have derived, for the most part, from studies using *p*-aminohippurate (PAH)[1] as a probe. Although the choice of PAH as a prototype seems logical (low protein binding, high secretion rate, no intracellular metabolism, easy chemical determination), extrapolation of PAH studies to a general concept of anion secretory mechanisms may be incorrect for several reasons. First, to allow quantitation, PAH transport measurements are often performed at high concentrations of the probe. Thus, the transport mechanism(s) operative at such concentrations may not be applicable to drugs that are present in blood at much lower (pharmacological) levels. Second, there is evidence that the anion transport system may be divided into subsystems, each with specificity for certain anions (1). Moreover, the relative contribution of these multiple mechanisms to drug secretion may be dose dependent. Hence, data with PAH may not extrapolate well to other compounds. For these reasons we chose the isolated perfused rabbit proximal straight tubule (PST) to study the processes involved in the renal secretion of another organic anion, the NSAID indomethacin, which is highly bound to plasma proteins and is actively secreted. Moreover, we performed these studies using pharmacological concentrations of the drug. In doing so, we found evidence for two parallel transporters.

Methods

Isolated perfused tubule. The technique of perfusing the isolated PST was used as previously described by Burg (7, 8). Briefly, slices were cut from kidneys of 1.5–2.0-kg New Zealand White rabbits. A PST was dissected free hand in cooled (4°C) ultrafiltrate-like solution (in millimolar: 105, NaCl; 5, KCl; 25, $NaHCO_3$; 2.3, Na_2HPO_4; 1, $MgSO_4$; 10, sodium acetate; 5, alanine; 8.3, glucose; and 1.8, $CaCl_2$; and it was bubbled with 95% O_2/5% CO_2 to reach a pH of 7.40). Only cortical nephron segments of the PST were used. Tubules were bathed in the ultrafiltrate-like solution with 5 vol% of filtered fetal calf serum (Gibco, Grand Island, NY). The bath was exchanged continuously at a flow rate of ~ 0.5 ml/min. The tubule was sealed into a collection pipette using Sylgard No. 184 (Dow Corning Corp., Midland, MI). In one set of experiments a sodium-free solution was used. For this purpose the sodium ion was replaced by *N*-methyl-D-glucamine (Sigma Chemical Co., St. Louis, MO) (in millimolar: 140, *N*-methyl-D-glucamine Cl; 5, potassium acetate; 30, *N*-methyl-D-glucamine HCO_3 (made from *N*-methyl-D-glucamine Cl by bubbling with 20% CO_2); 2.3, KH_2PO_4; 1, magnesium acetate; 8.3, glucose; 5, alanine; 1.8, calcium acetate. Fetal calf serum was exhaustively dialyzed against double-distilled water to remove the sodium, and was then added to the bathing solution. Less

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1. *Abbreviations used in this paper:* DIDS, 4,4'-diisothiocyanostilbene; J_{indo}, indomethacin flux; J_v, volume flux; PAH, *p*-aminohippurate; PST, proximal straight tubule.

than 10^{-7} M of sodium, measured by atomic absorption, could be detected in this sodium-free bathing solution. The potassium-free medium used in another set of experiments was prepared as ultrafiltrate-like solution substituting 5 mM KCl by 5 mM NaCl. In this protocol, dialyzed fetal calf serum was used to prepare the bathing solution. In all experiments, indomethacin (Merck, Sharp & Dohme Research Laboratories, West Point, PA) as well as the competitive inhibitors sodium PAH (Eastman-Kodak Co., Rochester, NY), probenecid (Sigma Chemical Co.), ouabain (ICN, Irvine, CA), and 4,4'-diisothiocyanostilbene (DIDS) (lot 04065, Polysciences, Inc., Warrington, PA) were prepared in the bathing solution to the desired concentration. All pipettes used in the perfusion set-up were siliconized.

Transepithelial voltage (PD, in millivolts) was measured by means of an agarose Ringer's solution–calomel electrode series. Exhaustively dialyzed, tritiated inulin was added to the perfusate as a marker for volume flux (J_v).

Indomethacin transport measurement. A 45-min equilibration period preceded the actual perfusion experiments. Each indomethacin flux (J_{indo}) measurement (up to three per tubule) was bracketed by a measurement of J_v, and a 15-min equilibration separated each perturbation. J_v measurements were made with a 50-nl constant volume pipette, and a 110 nl constant volume pipette was used for indomethacin (see below). Only one collection for indomethacin measurement was made per perturbation to allow for multiple perturbations within the time span of one experiment in a tubule. The accuracy of such a single J_{indo} measurement was tested in four tubules by performing three consecutive periods of indomethacin collections (15-min equilibration, J_v, J_{indo}, and J_v). Thus, the interval between these J_{indo} measurements was comparable with that used in the actual experiments. The difference among these repetitive samples was 4.2±3.5% (mean±SD).

All indomethacin samples were immediately transferred to a glass tube (see below) and stored at 4°C in the dark.

Transfer and measurement of the indomethacin sample. The technique used to transfer the collected indomethacin sample has been described elsewhere (8). Briefly, during collection and transfer, evaporation of the sample was prevented by use of gamma terpinene, an oil that did not interfere with the indomethacin measurement. The 110-nl indomethacin sample was immediately transferred to a small glass tube containing 640 nl water (pH = 7.4) containing internal standard (phenylbutazone).

Samples were immediately assayed for indomethacin by HPLC as previously described (9). A semi-microbore column (10 μm) (Bondpak C18; Waters Assoc., Div. of Millipore Corp., Milford, MA) allowed small sample injections and increased the sensitivity of the measurements by increasing the concentration of drug in the detector. The mobile phase consisted of water and methanol (45:55). The flow rate was 130 μl/min, pumped with a modified Waters solvent delivery system (model 6000 A; Waters Assoc.). A micro flow cell (1.9 μl) was used in the ultraviolet (UV) detector (model 440; Waters Assoc.). Directly after the UV detector, 4 N NaOH was added to the mobile phase through a T-connector at a flow rate of 1.3 μl/min, thus maintaining an optimal pH of 12.75 in a reaction loop (130 μl, 64°C). Thus, indomethacin was hydrolyzed to a reaction product that could be measured at low concentrations by fluorescence detection using an excitation wavelength of 295 nm and emission wavelength of 376 nm (model 650-10 S; Perkin Elmer Corp., Instrument Div., Norwalk, CT). The intraassay variability of 10^{-7} and 10^{-6} M indomethacin standards was 5 and 3%, respectively. Day-to-day coefficients of variation were 6 and 2%, respectively. Recovery of indomethacin for collected and transferred samples was 99±12%.

Calculations. J_v was determined by the following formula: $J_v = (V_i - V_o)/L$, where J_v is rate of net volume flux in nl · mm^{-1} · min^{-1}, V_i is perfusion rate in nanoliters, V_o is collection rate in nanoliters, and L is tubular length in millimeters. The two J_vs per period that bracketed the J_{indo} collection were averaged. J_{indo} was determined by the following formula: $J_{indo} = [V_i$ (perfusate indo$_c$) $- V_o$ (collectate indo$_c$)$]/L$, where J_{indo} is rate of indomethacin secretion in fmol · mm^{-1} · min^{-1}, and indo$_c$ is measured concentration of indomethacin in micromolar. Since perfusate indo$_c$ is zero, this equation simplifies to: $J_{indo} = [-V_o$ (collectate indo$_c$)$]/L$.

Unless noted otherwise mean±SD are given. Statistical analysis was carried out using a (paired) rank test (Wilcoxon).

Results

Indomethacin secretion rate. The effect of various concentrations of indomethacin on J_v and PD is shown in Table I. The average tubular length was 2.5±0.6 mm. PD and J_v were measured during a control period and compared with a period with different bath concentrations of indomethacin ranging from 10^{-6} to 3×10^{-5} M. In 12 of these tubules, multiple (up to three per tubule) indomethacin concentrations were tested in random order. Table I shows that indomethacin did not significantly affect PD or J_v at the drug concentrations tested, although J_v tended to decrease at the highest bath concentrations.

J_{indo} rates were measured in 22 PST segments (length, 2.5±0.6 mm) at a bath concentration of 10^{-6} M. The indomethacin concentration in the collected perfusate was $2.9 \pm 1.3 \times 10^{-6}$ M, indicating bath-to-lumen transport against a chemical gradient; calculated J_{indo} averaged 16.8±8.8 fmol · mm^{-1} · min^{-1}. Mean PD and J_v were −1.4±0.4 mV and 0.39±0.15 nl · mm^{-1} · min^{-1}, respectively. Values for J_{indo} did not correlate with PD or J_v.

In 9 of these 22 tubules and in an additional 8 PST segments, J_{indo} was measured at higher bath concentrations of indomethacin (5×10^{-6}, 10^{-5}, 1.6×10^{-5}, and 3×10^{-5} M). J_{indo} increased with increasing bath concentrations of the drug (Fig. 1). If one assumes only one indomethacin transport site at the basolateral membrane, no intracellular metabolism of indomethacin, and an unrestricted one-way transfer of indomethacin across the luminal membrane, Michaelis-Menten kinetics can be applied to the curvilinear relationship between J_{indo} and bath drug concentration. The inset of Fig. 1 shows such a curve transformation. Although this derivation gives the impression that indomethacin secretion at least partially follows Michaelis-Menten kinetics, the deviation from a straight line is such that parallel or sequential transport mechanisms should not be excluded.

Effect of other organic anions on J_{indo}. To test whether the observed indomethacin secretion followed a similar transcellular pathway as described for other organic anions, the effect of two prototypic substrates for the organic acid transport system (PAH and probenecid) on J_{indo} was evaluated (Fig. 2).

Table I. Effect of Varying Indomethacin Bath Concentrations on PD and Water Reabsorption (J_v) in PST Segments

	PD		J_v	
[Indomethacin]$_{bath}$	Control	Indo	Control	Indo
	mV		*nl · mm^{-1} · min^{-1}*	
10^{-6} M (n = 7)	−1.4±0.3	−1.6±0.2	0.48±0.16	0.44±0.15
5×10^{-6} M (n = 7)	−1.2±0.4	−1.2±0.3	0.40±0.13	0.35±0.18
10^{-5} M (n = 11)	−1.3±0.4	−1.4±0.5	0.39±0.12	0.34±0.15
1.6×10^{-5} M (n = 6)	−1.5±0.4	−1.6±0.3	0.40±0.10	0.33±0.11
3×10^{-5} M (n = 6)	−1.5±0.4	−1.6±0.4	0.40±0.10	0.35±0.17

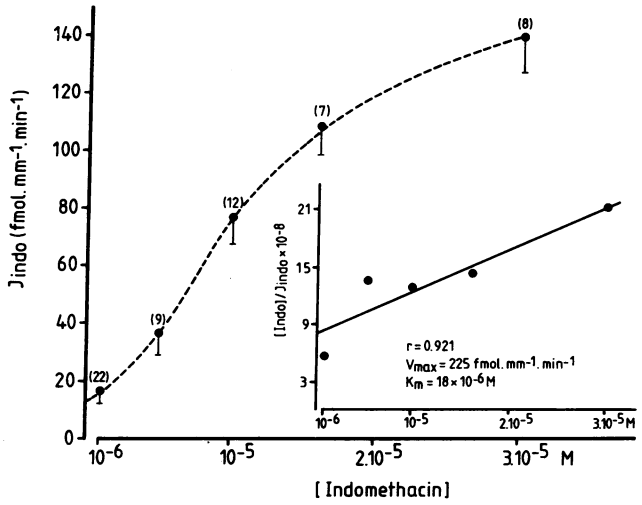

Figure 1. Effect (mean±SEM) of varying indomethacin bath concentrations on bath-to-lumen transport (J_{indo}) in the PST. The inset shows a curve transformation (Hanes plot). The values in parentheses represent the number of tubules studied at the given indomethacin concentration.

First, the effect of bath concentrations of PAH ranging from 6×10^{-5} to 4×10^{-3} M on indomethacin (10^{-6} M bath concentration) secretion was measured in seven PST. After a control measurement, J_{indo} was measured after adding PAH to the bath at the desired concentration, starting with either the highest or the lowest PAH concentration, followed by another two PAH concentrations in random order, and a recovery period. At these concentrations PAH had no significant effect on PD (−1.7±0.3 mV at the lowest to −1.8±0.1 mV at the highest bath concentrations). In contrast, J_v decreased by 33±16% of control at the highest PAH concentrations. At the end of the experiment J_v recovered to 82±10% of control values. With increasing bath concentrations of PAH, J_{indo} decreased reciprocally from a control value of 19.2±9.4 fmol · mm^{-1} · min^{-1} to < 17% of control J_{indo} at the highest PAH concentration used (4×10^{-3} M) (Fig. 2). At the end of the experiment, J_{indo} recovered to 103±30% of control values.

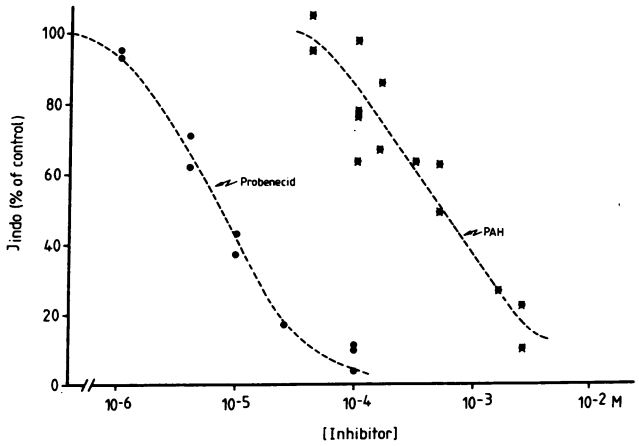

Figure 2. Effect of varying bath concentrations of probenecid and PAH on indomethacin (bath concentration, 10^{-6} M) transport rate (J_{indo}) in the PST, expressed as percent change compared with control.

In an additional five experiments a similar dose-dependent inhibition of indomethacin (10^{-6} M bath) secretion was observed by adding varying concentrations of probenecid to the bath (10^{-6} to 10^{-4} M). The highest probenecid bath concentrations had no effect on PD (−1.6±0.1 vs. −1.8±0.2 mV), nor on J_v (0.46±0.14 vs. 0.47±0.14 nl · mm^{-1} · min^{-1}). Fig. 2 shows that 10^{-6} M probenecid had no significant effect on control J_{indo} (18.4±10.5 fmol · mm^{-1} · min^{-1}), whereas 10^{-4} M nearly completely inhibited indomethacin secretion. In all experiments, complete recovery (92±5%) to control J_{indo} values was observed 30 min after withdrawing probenecid from the bathing solution. Apparently, both organic anions compete for indomethacin bath-to-lumen transport, and both PAH and probenecid are able to nearly completely abolish indomethacin secretion. Although the inhibition profile shows a similar pattern, probenecid is a much more potent inhibitor than PAH: ~ 100 times less probenecid than PAH is needed for half-maximal inhibition of J_{indo}.

Temperature dependency of J_{indo}. The effect of cooling to 18°C on J_{indo} is depicted in Fig. 3. In the first set of experiments (n = 12), bath temperature was lowered from 38° to 18°C. As also shown by others (10, 11), cooling to 18°C was sufficient to totally inhibit net volume reabsorption in these PST segments, which is reflected by the decrease of control J_v from 0.39±0.17 to −0.02±0.04 nl · mm^{-1} · min^{-1}. Similarly, cooling inhibited indomethacin secretion from 24.0±12.0 to 10.5±2.9 fmol · mm^{-1} · min^{-1}. However, as can be seen in Fig. 3, in none of the tested tubules was indomethacin transport completely abolished by cooling, the mean inhibition being 48±15%. To ascertain whether the inhibition of J_{indo} was stable with time, the cooling period was extended to 60 min in four of the above experiments. No further decrease in J_{indo} was observed comparing 15 min cooling (51±6%) with 40-min (51±7%) and with 1-h cooling (54±6%). In four of the cooling experiments, the bathing solution was reheated to 37°C, leading to a recovery of J_{indo} to 82±10% of control. In the remaining four experiments, probenecid (10^{-4} M) was added to the bath during continued cooling. This resulted in a further decrease of J_{indo} to < 15% of control, such that negligible amounts of indomethacin could be detected in the collected perfusate of three of the four PST segments.

To further explore the temperature dependency of indomethacin transport as related to active fluid reabsorption in the PST, an additional set of perfusion experiments was performed (n = 5). After control J_{indo} and J_v measurements at 38°C, the bath temperature was lowered in four consecutive steps (33°, 25°, 18°, and 12°C, respectively). In each of these

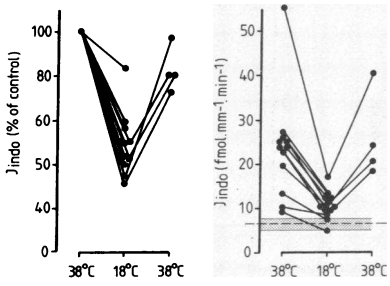

Figure 3. Effect of cooling (18°C) and recovery to control bath temperature (38°C) on bath-to-lumen indomethacin transport (J_{indo}) in the PST, expressed as percent change compared with control (*left*) and as absolute flux rates (*right*). The shaded area (*right*) indicates the mean±SD of the calculated flux rate, assuming the indomethacin concentration in the lumen equals that in the bathing solution (10^{-6} M).

periods J_{indo} and J_v were measured. J_v decreased from 0.45±15 to 0.30±0.08 (33°C), to 0.11±0.04 (25°C), and to 0.00±0.03 nl·mm^{-1}·min^{-1} at 18°C bath temperature. No further decrease of J_v was observed at 12°C (0.00±0.01). Whereas a maximum decrease of J_v was reached at the bath temperature of 18°C, J_{indo} inhibition at this temperature was 53±14% of control, consistent with prior experiments. By further decreasing bath temperature to 12°C, J_{indo} decreased to 31±6% of control values. Fig. 4 shows Arrhenius plots of both J_v and J_{indo} calculated from all tested bath temperatures. Assuming that J_v solely reflects active sodium transport across the basolateral membrane by Na^+,K^+-ATPase, one can calculate the activation energy of this enzyme to be between 15.5 and 22.6 kcal/mol (12). The profile of the Arrhenius plot for J_{indo} is difficult to interpret without knowledge of the carrier(s) that is (are) involved. However, the dissimilarity between the J_v and J_{indo} plots suggests that indomethacin secretion is not solely linked to an energy-dependent transport process.

Sodium dependency of J_{indo}. To more clearly distinguish between changes in indomethacin transport and changes in active sodium transport in the PST, a series of perturbations were used. First, the effect of adding 10^{-5} M ouabain to the bathing solution was tested in five tubules. After 10 min ouabain had completely inhibited water reabsorption in these tubules (control J_v, 0.40±0.20 to −0.04±0.03 nl·mm^{-1}·min^{-1}). J_{indo} (control, 36.7±12.4 fmol·mm^{-1}·min^{-1}), however, was inhibited by only 47±18% (Fig. 5, *left*). To inhibit active sodium transport by a different mechanism, we also substituted *N*-methyl-D-glucamine for sodium in the bath and perfusate. In three experiments with this sodium-free medium, J_v decreased from 0.33±0.10 (baseline) to −0.01±0.05 nl·mm^{-1}·min^{-1}. Again, J_{indo} (control, 47.1±13.4 fmol·mm^{-1}·min^{-1}) was inhibited submaximally by 36±3% (Fig. 5, *right*). In the last series of experiments (n = 3), bathing and perfusion solution were substituted by potassium-free media. J_v was totally inhibited (from 0.27±0.03 to 0.02±0.01 nl·mm^{-1}·min^{-1}) by this maneuver, whereas J_{indo} remained at a level 40±4% of control (34.7±13.1 fmol·ml^{-1}·min^{-1}). Like

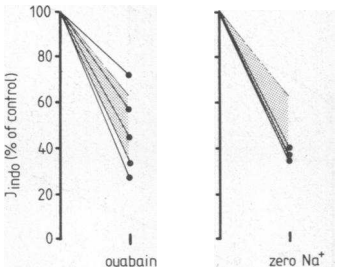

Figure 5. Effect of ouabain (bath concentration, 10^{-5} M) (*left*) and replacement of the bath and lumen Na^+ by *N*-methyl-D-glucamine (*right*) on indomethacin (bath concentration, 10^{-6} M) transport in the PST. The shaded areas represent the mean±SD change of indomethacin transport during cooling (18°C).

the results during 18°C cooling, the above perturbations completely inhibited sodium transport (estimated from J_v), whereas a distinct component of indomethacin transport remained uninhibited. These data raised the question of an indomethacin transport component independent of Na^+ transport.

Effect of DIDS on J_{indo}. Since it has been suggested that anion exchange may play a role in transport of anions in the PST (13–16), we evaluated the effects of an anion-exchange inhibitor (DIDS) on J_{indo}. To find an optimal dose of DIDS, dose-response experiments were first conducted. Fig. 6 shows the effect on J_{indo} of adding DIDS to the bathing solution of five PST segments. Consecutive increases in the dose of DIDS incrementally decreased J_{indo} to a maximum inhibition of 56±6% of control (23.4±2.5 fmol·mm^{-1}·min^{-1}). The plateau was reached with 10^{-5} M DIDS. At lower bath concentrations, DIDS had little effect on J_v; only at 10^{-4} M did DIDS tend to inhibit J_v, from 0.37±0.14 to 0.25±0.13 nl·mm^{-1}·min^{-1}.

In four PST segments the effect of 10^{-4} M DIDS followed by superimposed cooling of the bathing solution to 18°C was evaluated. DIDS marginally reduced J_v from 0.38±0.05 to 0.30±0.07 nl·mm^{-1}·min^{-1}; cooling further decreased J_v to −0.03±0.01 nl·mm^{-1}·min^{-1}. DIDS inhibited indomethacin transport to 54±6% of control values (30.4±4.5 fmol·mm^{-1}·min^{-1}, Fig. 7, *top*). Superimposed cooling further decreased J_{indo} to 14±3% of control. The effect of adding DIDS to a cooled tubule was evaluated in another four PST segments. Cooling decreased J_v from 0.40±0.20 to −0.03±0.04 nl·mm^{-1}·min^{-1}, whereas superimposed DIDS did not further alter J_v significantly (−0.01±0.05 nl·mm^{-1}·min^{-1}). Cooling inhibited J_{indo} to 45±4.5% of con-

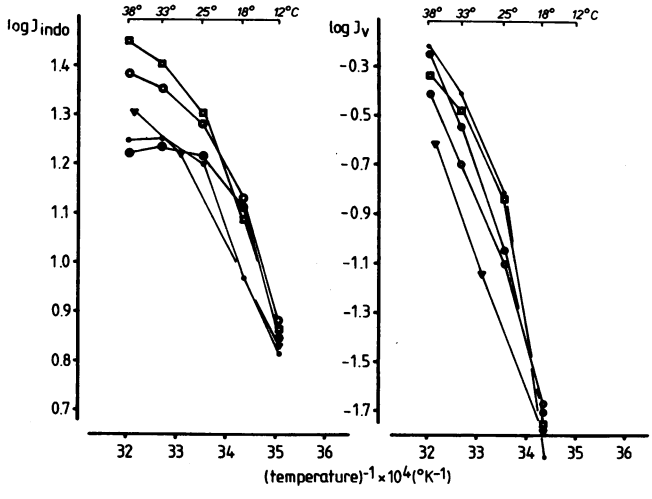

Figure 4. Arrhenius plots of bath-to-lumen indomethacin (bath concentration, 10^{-6} M) transport (*left*) and lumen-to-bath net water reabsorption (J_v, *right*) in the PST measured at different bath temperatures.

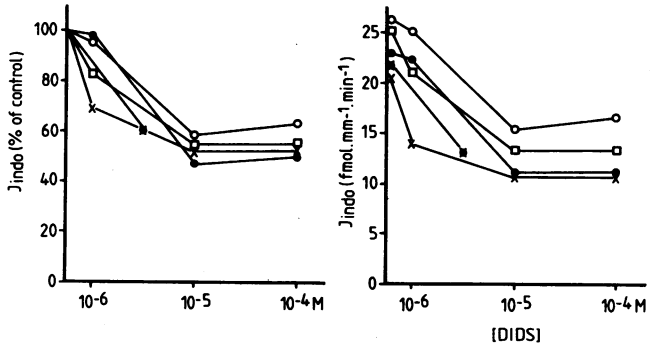

Figure 6. Inhibitory effect of increasing bath concentrations of DIDS on indomethacin (bath concentration, 10^{-6} M) transport in the PST, expressed as the percent change compared with control (*left*) and as absolute flux rates (*right*).

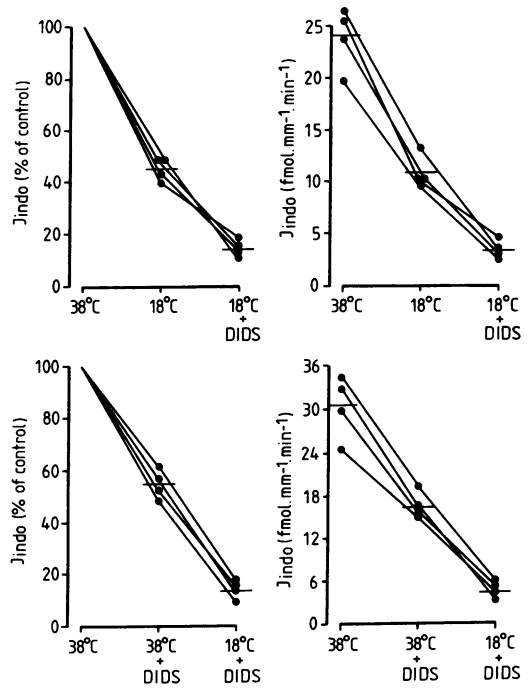

Figure 7. Effect of cooling (18°C) and superimposed DIDS (bath concentration, 10^{-4} M) on bath-to-lumen indomethacin (bath concentration, 10^{-6} M) transport in the PST (*top*), and the effect of DIDS and superimposed cooling on indomethacin transport (*bottom*). The plots on the left represent the percent change compared with control; the plots on the right represent the absolute J_{indo} rates.

trol (23.9±3.1 fmol · mm^{-1} · min^{-1}), whereas adding DIDS to the bath further inhibited J_{indo} to 14±3% of control, a value statistically not different from zero (Fig. 7, *bottom*).

Discussion

The present study documents the active secretion of the organic anion indomethacin across the rabbit renal PST, and addresses some of the underlying mechanism(s) for this transport. In general, indomethacin behaves like other organic anions tested for secretion by the PST. At the low (pharmacologic) bath concentrations used in this study, indomethacin was secreted into the lumen of the PST against a concentration gradient. The process appears to be saturable at higher bath concentrations of the drug; further, secretion is completely inhibited by probenecid and PAH, suggesting a transcellular route for indomethacin transport. The relatively high concentrations of competitors needed for inhibition indicate that indomethacin has one of the highest affinities for the carrier quantified to date (2). Various experimental approaches in the present study revealed dependence of indomethacin secretion on the sodium gradient. Interestingly, however, only about half of the indomethacin transport appeared susceptible to perturbations of this gradient, leaving a considerable amount of drug secretion via independent mechanism(s). The remaining portion of J_{indo} was eliminated by the anion-exchange inhibitor DIDS, raising the important question of whether a basolateral anion exchange process accounts for the other half of indomethacin secretion.

Before interpreting our observations in the context of the current concepts of anion transport by the PST, one should address the possible bias introduced by the study design and the choice of indomethacin as the studied compound. Three issues may have confounded our experiments: first, protein binding of indomethacin; second, cellular metabolism of indomethacin; and lastly, the absence of information on indomethacin transport processes across the luminal membrane.

As far as protein binding is concerned, indomethacin is highly bound to albumin, with various estimates ranging from 90 to > 99% (17, 18). For reasons of standardization and viability of the studied tubule, we added 5% fetal calf serum to the bath. In this setting, protein binding of indomethacin (10^{-6} M) was 65±3% (ultrafiltration technique using 14-mm MPS YMT filters [Amicon Corp., Danvers, MA]). The free amount of drug increased with higher bath concentrations of indomethacin (percent binding decreased to 50±4% at 10^{-4} M indomethacin). If one assumes that only the unbound fraction of the drug in the bathing solution is available for secretion, the actual V_{max} and K_m are lower than the values we calculated. However, correcting the dose-response curve for free concentrations of indomethacin did not affect the interpretation of Fig. 1. The assumption that free as opposed to total drug concentration is most relevant remains speculative, since no conclusive data are available as to whether the transporter is able to "strip" the anion from albumin, a process which is theoretically dependent upon the affinity of the anion for the transport system (19). Future studies should address this issue.

Neither PAH, probenecid, cooling (11°C), or ouabain affected the protein binding of indomethacin (61±3, 65±4, 62±3, and 61±5%, respectively). Hence, interpretation of these data would not be affected by protein binding. However, DIDS (10^{-4} M) increased the amount of free indomethacin (48±4% bound), possibly by competition for the albumin binding site. Not accounting for this effect would have served only to underestimate the inhibitory effects of DIDS on indomethacin transport, since (if only free drug is secreted) the higher free concentration of indomethacin in the bath during DIDS compared with control would have resulted in a higher J_{indo}. Interestingly, review of the literature concerning transport of PAH and other anions by the PST reveals no data concerning protein binding nor changes in binding caused by inhibitors in the bathing solution. Although protein binding of PAH is claimed to be very low (~ 25%) (20), large variations have been reported (21). As far as the current data are concerned, our analysis is in the most conservative fashion. Any unaccounted for effects of binding would only serve to amplify our findings.

Very little information is available concerning renal metabolism of indomethacin. In human pharmacokinetic studies, half of the clearance of indomethacin is accounted for by *O*-demethylation, and a smaller portion is *N*-deacylated (22). Most of the remainder of the active drug as well as the inactive metabolites are glucuronidated and excreted via kidney and bile. Whether glucuronidation occurs in the kidney is unknown. However, since only very low concentrations or no glucuronide conjugates are measured in serum, and large amounts have been detected in the urine, glucuronidation may well occur in the kidney (23). Indeed, we observed an extra peak (next to the solvent front) in chromatograms of the collected perfusate that was not present in the bathing solution. We have not yet been able to conclusively establish this peak to be indomethacin glucuronide (or another metabolite). This may be due to the extreme instability of the glucuronide,

which can readily dissociate to indomethacin and glucuronic acid (23). Relative to the indomethacin peak, the extra peak was small (~ 15%), and it did not appear to change significantly during the various perturbations used in our studies. Therefore, although we were not able to quantify tubular metabolism (if it exists), we felt it reasonable to assume that observed changes in indomethacin secretion were not the result of changes in PST intracellular metabolism.

All of the conclusions concerning indomethacin secretion in the present study are restricted to the basolateral membrane, based on the assumption that indomethacin movement across the luminal membrane is driven by passive diffusion (6) or alternatively by an anion exchange mechanism (16). Subsequent studies should characterize the transport step at this barrier more carefully.

How do our data on indomethacin transport contribute to current concepts of anion transport mechanisms in the PST? To date two models have been proposed. The first and oldest theory is that an anion, and in particular the prototype PAH, is secreted across the basolateral membrane via secondary active transport dependent upon Na^+-K^+ ATPase; anion then diffuses across the luminal membrane driven by the high intracellular concentration (6). Many studies in different species, using different experimental techniques and designs, have addressed the sodium dependency of this active basolateral anion transport. Some found PAH transport to decrease almost totally using either ouabain or sodium-free bathing solutions (3–5, 24–28). In the present study we consistently found a submaximal inhibition of J_{indo} by directly or indirectly removing the sodium gradient. The reason for persistence of J_{indo} in these settings compared to observations with other anions is unclear. In addition, it is uncertain as to whether our data are unique to indomethacin or represent a general aspect of anion transport. Because of our sensitive analytical techniques, we were able to use a relatively low concentration of anion (10^{-6} M) compared with other studies ($\geq 10^{-5}$ M). So doing may have revealed the existence of a nonsodium gradient–related carrier that is driven by anion or intracellular counter-anion concentrations rather than by the Na^+ gradient across the basolateral membrane. Because of the higher anion concentrations used by other investigators, such pathways may have been "swamped" and thereby undetected. In past reviews on the anion transport system, authors have suggested that the sodium gradient itself was not sufficient to account for the entire PAH gradient across the basolateral membrane (1, 2). This suggestion is supported by the results of the present study, in that we were not able to completely inhibit J_{indo} by dissipation of the sodium gradient.

In addition to sodium-dependent anion transport, Dantzler (16) has indirectly offered evidence that a 4-acetamido-4′-isothyocyanostilbene-2,2′-disulfonate (SITS)–inhibitable carrier mechanism may be present on the basolateral (as well as on the luminal) side of the tubule. Although experiments have suggested, to date none have clearly demonstrated the existence of such a carrier independent from sodium-dependent mechanisms. The present study offers data that are consistent with a second carrier (anion exchange?) at the basolateral membrane. The discontinuous shape of the dose-response curve, and the inability to completely inhibit J_{indo} by interference with the sodium gradient (whereas water reabsorption is abolished), together with the clear dissociation of the Arrhenius plots of J_v and J_{indo}, strongly suggest the presence of a second carrier (29). Moreover, probenecid and PAH can inhibit the transport process completely, thus making the possibility of paracellular indomethacin movement as an explanation for the uninhibited portion of J_{indo} highly unlikely. The inhibitory capacity of DIDS itself on J_{indo}, and the fact that the same inhibition can be achieved when a sodium gradient is absent (cooling), points again to the existence of two separate transport mechanisms on the basolateral membrane.

In this context it is important to discuss our reasons for using cooling to show sodium dependency during the DIDS experiments rather than ouabain or Na^+-free solutions. Though the latter two maneuvers are more commonly used to dissipate the sodium gradient, they affected the tubule preparation in our experiments so as to preclude performance of more than one perturbation. Perhaps indomethacin had a noxious effect such that superimposed ouabain or a Na^+-free environment adversely affected viability. Cooling, on the other hand, did not affect the stability of the preparation, as shown by the similarity of the J_{indo} measurements during cooling as well as the recovery data after reheating (Fig. 3). These practical justifications for use of cooling notwithstanding, this perturbation seems a valid probe of energy-dependent sodium transport. Shafer et al. have shown the similarity of cooling and ouabain on the PST (11). In addition, our data show equal abolishment of J_v by cooling as with ouabain and Na^+-free solutions. Moreover, inhibition of J_{indo} was similar with all three maneuvers. Thus, though additivity of effects of DIDS plus ouabain or DIDS plus Na^+-free solutions would represent ideal confirmation of the DIDS plus cooling experiments, we argue that the interpretation of the additive studies is not obviated by their absence.

In theory, DIDS could simply be a competitive inhibitor of the anion transport system as was also suggested by Koshier et al. (30). However, the fact that DIDS only minimally affected J_v, and the fact that DIDS could inhibit J_{indo} by at most 50%, makes this unlikely. It might be argued that albumin in the bathing solution may have blunted the effect of DIDS (15, 16, 30). This seems unlikely, since the final concentration of albumin in the bath (0.1%) was below that which reversed the inhibition of PAH transport caused by DIDS and SITS (15, 30). Furthermore, concentrations of DIDS were used an order of magnitude higher than those which caused maximal effects on indomethacin transport (Fig. 6). Thus, even if some of the DIDS were bound to albumin, it is likely that enough free concentration was available to cause a maximal effect.

Although the specific effect of DIDS on the PST is not known, its documented effect on anion exchange in the erythrocyte may in theory be extrapolated to the PST. If so, the second carrier for indomethacin transport on the basolateral membrane could be an anion exchanger. In this respect the second concept of anion transport across the proximal tubule proposed by Ullrich (31) is of interest. Several groups, using a variety of experimental preparations (kidney slices, membrane vesicles, in vivo microperfusion), found PAH transport across the basolateral membrane to be clearly independent of a sodium gradient (13, 31–34). Therefore, Ullrich et al. (14, 31, 36) and also Dantzler et al. (35) explain the effects of perturbations aimed at dissipating the sodium gradient to be the result of secondary changes in the tubular cells rather than their exerting a specific effect on the PAH carrier. Ullrich argues for a countertransport of anion (PAH) vs. OH^- at the basolateral membrane (14). Kasher et al. supported the model of an anion

exchange process for PAH transport across basolateral membrane vescicles (37). However, they concluded this transport was Na^+ dependent. As far as the DIDS-inhibitable component of J_{indo} in the present study, our data are consistent with the theory of basolateral anion exchange. The question still remains, however, as to why we were not able to completely inhibit indomethacin secretion with either DIDS or by totally inhibiting sodium transport (as measured by water reabsorption). As such, our data remain most consistent with two transport pathways for indomethacin. We cannot exclude the possibility that these phenomena are peculiar to use of the technique of the isolated perfused tubule. However, one can argue similarly that data obtained from isolated membranes is less physiological. Moreover, in support of the concept of two anion transport pathways, Lee et al. (38) observed in the isolated perfused rat kidney that furosemide secretion was best characterized by the presence of both a high affinity, low capacity system, as well as a low affinity, high capacity system. Further studies are clearly needed to resolve such issues.

In conclusion, the present study shows that the NSAID indomethacin is transcellularly secreted into the lumen of the PST by an anion transport mechanism like that described for PAH. The transport is characterized by a low transport rate and high affinity for the carrier. Half of the indomethacin secretion is blocked by maximal inhibition of sodium transport in the PST, whereas the remainder can be abolished by the anion exchange inhibitor DIDS. The data suggest that at pharmacological concentrations, indomethacin movement across the basolateral membrane of the PST is the result of sodium indomethacin co-transport as well as of indomethacin-anion exchange. Rather than explaining the mechanism of "active" basolateral anion transport as either totally sodium gradient dependent or as totally driven via anion countertransport, we feel that our data are most consistent with the simultaneous existence of both transport mechanisms on the basolateral membrane of the rabbit PST.

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References

1. Møller, J. V., and M. I. Sheikh. 1982. Renal organic anion transport system: pharmacological, physiological, and biochemical aspects. *Pharmacol. Rev.* 34:315–358.

2. Grantham, J. J. 1982. Studies of organic and cation transport in isolated segments of proximal tubules. *Kidney Int.* 22:519–525.

3. Woodhall, P. B., C. C. Tisher, C. A. Simonton, and R. R. Robinson. 1978. Relationship between para-aminohippurate secretion and cellular morphology in rabbit proximal tubules. *J. Clin. Invest.* 51:1320–1329.

4. Burg, M. B., and J. Orloff. 1962. Effect of strophanthidin on electrolyte content and PAH accumulation of rabbit kidney slices. *Am. J. Physiol.* 202:565–571.

5. Dantzler, W. H. 1969. Effects of K, Na, and ouabain on urate and PAH uptake by snake and chicken kidney slices. *Am. J. Physiol.* 217:1510–1519.

6. Tune, B. M., M. B. Burg, and C. S. Patlak. 1969. Characteristics of p-aminohippurate transport in proximal renal tubules. *Am. J. Physiol.* 217:1057–1063.

7. Burg, M. B., J. J. Grantham, M. Abramow, and J. Orloff. 1966. Preparation and study of fragments of single rabbit nephrons. *Am. J. Physiol.* 210:1293–1298.

8. de Zeeuw, D., D. C. Brater, and H. R. Jacobson. 1987. Micro-HPLC and transfer techniques for directly measuring drug (indomethacin) transport across the isolated perfused renal proximal straight tubule. *J. Pharmacol. Methods.* In press.

9. de Zeeuw, D., J. L. Leinfelder, and D. C. Brater. 1986. Highly sensitive measurement of indomethacin using a 'micro' HPLC technique combined with post-column in-line hydrolysis. *J. Chromatogr.* 380:157–162.

10. Biagi, B. A., and G. Giebisch. 1979. Temperature dependence of transepithelial potential in isolated perfused rabbit proximal tubules. *Am. J. Physiol.* 236:F302–F310.

11. Schafer, J. A., S. L. Troutman, and T. E. Andreoli. 1974. Volume reabsorption, transepithelial potential differences and ionic permeability properties in mammalian superficial proximal straight tubules. *J. Gen. Physiol.* 64:582–607.

12. Soltoff, S. P., and L. J. Mandel. 1984. Active ion transport in the renal proximal tubule. *J. Gen. Physiol.* 84:601–622.

13. Berner, W., and R. Kinne. 1976. Transport of p-aminohippuric acid by plasma membrane vesicles isolated from rat kidney cortex. *Pfluegers Arch. Eur. J. Physiol.* 361:269–277.

14. Ullrich, K. J. 1976. Renal tubular mechanisms of organic solute transport. *Kidney Int.* 9:134–148.

15. Hong, S. K., J. M. Goldinger, Y. K. Song, F. J. Koshier, and S. Lee. 1978. Effect of SITS on organic anion transport in the rabbit kidney cortical slice. *Am. J. Physiol.* 234:F302–F307.

16. Dantzler, W. H., and S. K. Bentley. 1980. Bath and lumen effects of SITS on PAH transport by isolated perfused renal tubules. *Am. J. Physiol.* 238:F16–F25.

17. Hvidberg, E., H. H. Lausen, and J. A. Jansen. 1972. Indomethacin: plasma concentrations and protein binding in man. *Eur. J. Clin. Pharmacol.* 4:119–124.

18. Rane, A., O. Oelz, J. C. Frolich, H. W. Seyberth, B. J. Sweetman, J. T. Watson, G. R. Wilkinson, and J. A. Oates. 1978. Relation between plasma concentration of indomethacin and its effects on prostaglandin synthesis and platelet aggregation in man. *Clin. Pharmacol. Ther.* 23:658–668.

19. Weiner, I. M. 1973. Transport of weak acids and bases. *In* Handbook of Physiology. J. Orloff and R. W. Berliner, editors. American Physiological Society, Washington, D.C. 521–554.

20. Donoso, V. S., and J. J. Grantham. 1986. Characteristics of renal p-aminohippurate and urate excretion in rabbits. *J. Lab. Clin. Med.* 107:315–321.

21. Richardson, D. R., S. E. Lee, C. W. Bryan, K. D. Nolph, and M. L. Zatzman. 1972. Variable protein binding of para-aminohippurate and ortho-iodohippurate. *Clin. Res.* 20:606. (Abstr.)

22. Duggan, D. E., A. F. Hogans, K. C. Kwan, and F. G. McMahon. 1972. The metabolism of indomethacin in man. *J. Pharmacol. Exp. Ther.* 181:563–575.

23. Faed, E. M. 1984. Properties of acyl glucuronides: implications for studies of the pharmacokinetics and metabolism of acidic drugs. *Drug Metab. Rev.* 15:1213–1249.

24. Chung, S. T., Y. S. Park, and S. K. Hong. 1970. Effects of cations on transport of weak organic acids in rabbit kidney slices. *Am. J. Physiol.* 219:30–33.

25. Grantham, J. J., P. B. Qualizza, and R. L. Irwin. 1974. Net fluid secretion in proximal straight renal tubules in vitro: role of PAH. *Am. J. Physiol.* 226:191–197.

26. Gerencser, G. A., and S. K. Hong. 1975. Roles of sodium and potassium on PAH transport in rabbit kidney slices. *Biochim. Biophys. Acta.* 406:108–119.

27. Spencer, A. M., J. Sack, and S. K. Hong. 1979. Relationship between PAH transport and Na-K-ATPase activity in the rabbit kidney. *Am. J. Physiol.* 236:F126–F130.

28. Maxild, J., J. Møller, and M. I. Skeikh. 1981. Involvement of Na-K-ATPase in PAH transport by rabbit kidney tissue. *J. Physiol. (Lond.).* 315:189–201.

29. Brot-Laroche, E., M. A. Serrano, B. Delhomme, and F. Alvarado. 1985. Different temperature sensitivity and cation specificity of two distinct d-glucose/Na-cotransport systems in the intestinal brush-border membrane. *Ann. NY Acad. Sci.* 456:47–50.

30. Koschier, F. J., M. F. Stokols, J. M. Goldinger, M. Acara, and S. K. Hong. 1980. Effect of DIDS on renal tubular transport. *Am. J. Physiol.* 238:F99–F106.

31. Ullrich, K. J., G. Rumrich, G. Fritzsch, and S. Klöss. 1987. Contraluminal para-aminohippurate (PAH) transport in the proximal tubule of the rat kidney. *Pfluegers Arch. Eur. J. Physiol.* 409:229–235.

32. Hayashi, H., and T. Hoshi. 1979. Sodium-dependence of p-aminohippurate transport by rat kidney cortex slices. *Arch. Int. Pharmacodyn. Ther.* 240:103–115.

33. Maxild, J., J. V. Møller, and M. I. Sheikh. 1981. An energy-dependent, sodium-independent component of active p-aminohippurate transport in rabbit renal cortex. *J. Physiol. (Lond.).* 310:273–283.

34. Sheikh, M. I., and J. V. Møller. 1983. Nature of Na^+-independent stimulation of renal transport of p-aminohippurate by exogenous metabolites. *Biochem. Pharmacol.* 32:2745–2749.

35. Dantzler, W. H., and S. K. Bentley. 1976. Low Na effects on PAH transport and permeabilities in isolated snake tubules. *Am. J. Physiol.* 230:256–262.

36. Ullrich, K. J., G. Capasso, G. Rumrich, F. Papavassiliou, and S. Klöss. 1977. Coupling between proximal tubular transport processes. *Pfluegers Arch. Eur. J. Physiol.* 368:245–252.

37. Kasher, Y. S., P. D. Holohan, and C. R. Ross. 1983. Na gradient-dependent p-aminohippurate transport in rat basolateral membrane vesicles. *J. Pharmacol. Exp. Ther.* 227:122–129.

38. Lee, L.-J., J. A. Cook, and D. E. Smith. 1986. Renal transport kinetics of furosemide in the isolated perfused rat kidney. *J. Pharmacokinet. Biopharm.* 14:157–174.