Inhibition of bicarbonate reabsorption in the rat proximal tubule by activation of luminal P2Y<sub>1</sub> receptors

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Bailey, Matthew A. Inhibition of bicarbonate reabsorption in the rat proximal tubule by activation of luminal P2Y<sub>1</sub> receptors. Am J Physiol Renal Physiol 287: F789–F796, 2004. First published June 1, 2004; 10.1152/ajprenal.00033.2004.—The present study used a stationary microperfusion technique to investigate in vivo the effect of P2Y<sub>1</sub> receptor activation on bicarbonate reabsorption in the rat proximal tubule. Proximal tubules were perfused with a bicarbonate Ringer solution before flow was stopped by means of an oil block. The recovery of lumen pH from the initial value (pH 8.0) to stationary values (pH ~6.7) was recorded by a H<sup>+</sup>-sensitive microelectrode inserted downstream of the perfusion pipette and oil block. The stationary pH value and the t<sub>1/2</sub> of pH recovery were used to calculate bicarbonate reabsorption (J<sub>NaHCO<sub>3</sub></sub>). Both EIPA and bafilomycin A1 caused significant reductions in proximal tubule J<sub>NaHCO<sub>3</sub></sub>, consistent with the established contributions of Na/H exchange and H<sup>+</sup>-ATPase to proximal tubule HCO<sub>3</sub> reabsorption. The nucleotides ADP and, to a lesser extent, ATP reduced J<sub>NaHCO<sub>3</sub></sub> but AMP and UTP were without effect. 2MeSADP, a highly selective agonist of the P2Y<sub>1</sub> receptor, reduced J<sub>NaHCO<sub>3</sub></sub> in a dose-dependent manner. MRS-2179, a P2Y<sub>1</sub> receptor-specific antagonist, abolished the effect of 2MeSADP. The mechanism is indirect, involving H<sup>+</sup>-sensitive short-circuit current in the native mammalian collecting duct principal cell (18). In contrast, the physiological ramifications of purinergic signaling in the proximal tubule are largely unknown. Purinergic receptors are certainly expressed in this segment (2, 3, 31), although functional confirmation has been limited to the measurement of calcium transients following application of nucleotides to the basolateral membrane.

Approximately 75% of filtered HCO<sub>3</sub> is reclaimed by the proximal tubule (15). The mechanism is indirect, involving H<sup>+</sup> secretion across the apical membrane coupled with basolateral reabsorption of HCO<sub>3</sub> (15). The major fraction of apical H<sup>+</sup> secretion is mediated by Na/H exchange, although H<sup>+</sup>-ATPase also contributes (15). Because such transporters can be regulated by purinergic receptor activation (1, 29), the present study investigated the effect of luminal applied extracellular nucleotides on proximal tubule HCO<sub>3</sub> reabsorption in vivo.

METHODS

Male Sprague-Dawley rats (n = 44; weight range 180–320 g) were anesthetized with an intraperitoneal injection of thiobitarbarbitual sodium (Inactin 100 mg/kg; Sigma) and prepared surgically for in vivo micropuncture experiments: the left jugular vein and carotid artery were cannulated for infusion of isotonic saline (3 ml/h) and blood sampling, respectively. A tracheotomy was performed and the bladder was catheterized for urine collection. The left kidney was exposed by flank incision and immobilized in a Perspex cup clamped to the operating table. During the experiments, body temperature was maintained at ~37°C. Stationary microperfusion of the proximal tubule. The technique employed in these experiments has been described in detail previously (23, 32). A proximal tubule was impaled by a perfusion micropipette and injection of FD&C green-colored saline was used to identify downstream loops of the proximal tubule. A H<sup>+</sup>-selective microelec-
trode was lowered onto the kidney surface for zero-voltage measurement before being inserted into the proximal tubule lumen downstream of the perfusion pipette. A second micropipette containing Sudan black-colored heavy mineral oil was inserted into a tubular segment between the perfusion pipette and microelectrode. For stationary measurements of proximal tubular H\(^+\) secretion, the inserted perfusion pipette was used to inject a control solution into the tubule lumen until the electrode recorded a large and stable voltage recorded a large and stable voltage. At this point, perfusion was stopped and a column of oil was injected to prevent downstream flow of either native tubule fluid or perfusate. Voltage measurements were recorded until stable reading was once again obtained. After control measurements, the first pipette was replaced by a second perfusion pipette containing the experimental perfusion solution (i.e., the control solution to which a chemical agent had been added). Recordings were again made. In some instances, the control pipette was reinserted to provide time control data. In a typical experiment, each nephron was perfused initially to pH (Fig. 1). For each perfusion, pH fell from the initial value of 8 to a stationary value (pHstat) of ~6.8. A one-phase exponential was fitted to this fall, allowing the half-time (t\(1/2\)) of proximal acidification to be generated. Bicarbonate reabsorption (J_{HCO_3}) was then calculated using the following equation (32)

\[
J_{HCO_3} = \frac{\ln(2)}{t_{1/2}} \times (\text{initial HCO}_3) - \text{stationary HCO}_3 \times r/2,
\]

where t\(1/2\) is the acidification half-time, initial HCO\(_3\) is 25 mmol/l, stationary HCO\(_3\) is calculated from stationary pH and arterial PCO\(_2\), and r is the lumen radius. Arterial PCO\(_2\) was 43.5 ± 0.9 mmHg. It should be noted that acidification measured under the present conditions consists of two components: acidification due to equilibration of the lumen perfusate with cortical CO\(_2\) and acidification due to proton secretion by the proximal tubule (13, 23). Both of these components will, in the first instance, occur concomitantly. CO\(_2\) equilibration is, however, both rapid and transient, being effectively complete within the first seconds of measurement. Thus the bicarbonate reabsorption reported here includes a small equilibration flux, assumed to be constant within the paired perfusions.

**Statistics.** Data are presented as means ± SE obtained from individual recordings under a given condition. Statistical comparisons were made using one-way ANOVA; the probability of 0.05 was taken as the limit of statistical significance. Specified point-to-point comparisons were made using Bonferroni’s test.

**RESULTS**

**Effect of EIPA and bafilomycin A1.** The methodology employed here yields values of bicarbonate reabsorption calculated from the rate and extent of lumen fluid acidification.

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*Fig. 1. Example of the data recording and transformation during perfusion of the same proximal tubule with control solution (solid line) and a solution containing 2MeSADP (100 μmol/l; dashed line). After perfusion (P) with the solution at pH 8 and block (B), H\(^+\) activity (mV) increases. Conversion to pH (B) allows the t\(1/2\) of acidification from initial pH to stationary pH (pHstat) to be measured: these values are used to calculate J_{HCO_3}.*
Because bicarbonate reabsorption in the rat proximal tubule is largely mediated by Na/H exchange and vacuolar H\(^+\)-ATPase (15), the present technique was validated using luminal application of inhibitors of these transporters, EIPA (200 \(\mu\)mol/l) and/or baflomycin A1 (1 \(\mu\)mol/l), respectively. As shown in Fig. 2, each agent significantly impaired \(J_{\text{HCO}_3}\). For EIPA, the inhibitory effect reflects significant reductions in the both the rate of acidification (\(t_{1/2}\)) and the maximum pH gradient achieved. For baflomycin A1, although pH\(_{\text{stat}}\) was significantly higher than in controls, the increase in \(t_{1/2}\) did not reach statistical significance. The inhibitory effect on \(J_{\text{HCO}_3}\) of perfusion with the two inhibitors together was significantly greater than perfusion with either EIPA or baflomycin A1 alone. The inhibitory effects on \(J_{\text{HCO}_3}\) recorded during blockade of Na/H exchange and/or H\(^+\)/H\(_{\text{ATPase}}\) were similar in magnitude to data obtained in the rat and mouse using direct quantitative measurement of bicarbonate flux (34, 35). The time control data were not different from the control data.

Effect of adenine and uridine nucleotides. A sample recording obtained during perfusion of the lumen with either ATP or ADP (both at 100 \(\mu\)mol/l) is shown in Fig. 3A. In Fig. 3B, the actions of AMP, ADP, and ATP on \(J_{\text{HCO}_3}\) are shown: AMP was without effect, whereas both ADP (\(P < 0.01\)) and ATP (\(P < 0.05\)) caused significant reductions in proximal HCO\(_3\) reabsorption. This agonist profile is consistent with that of P2Y\(_1\) receptors (27). In five proximal tubules, the P2Y\(_2\)/P2Y\(_4\) receptor agonist UTP was tested and found not to inhibit \(J_{\text{HCO}_3}\) (control 2.71 ± 0.43 \(\text{nmol/cm}^2\cdot\text{s}\); \(n = 15\); UTP 3.17 ± 0.56 \(\text{nmol/cm}^2\cdot\text{s}\); \(n = 20\); not significant).

Activation of P2Y\(_1\) receptors. A sample recording showing the effect of perfusion with 100 \(\mu\)M 2MeSADP, a potent and selective P2Y\(_1\) receptor agonist (27), is shown in Fig. 4A. This agent reduced \(J_{\text{HCO}_3}\) by \(\approx 50\%\), as shown in Fig. 4B. Most of this effect can be attributed to a reduction in \(t_{1/2}\) (Fig. 4C), i.e., in the rate of acidification, although pH\(_{\text{stat}}\) was significantly higher during perfusion with 2MeSADP than under control conditions (\(P < 0.01\)). The data shown in Fig. 4 represent the average of all recordings. Each tubule was, however, perfused several times, with \(\approx 1\)-min separating repeated applications. Figure 5 shows that the inhibition of \(J_{\text{HCO}_3}\) by 100 \(\mu\)M 2MeSADP was maintained over the separate time periods, suggesting minimal receptor desensitization within the time frame of these experiments.

These data suggest that P2Y\(_1\) receptors exert a controlling influence on proximal tubule bicarbonate reabsorption. Nevertheless, alternative hypotheses must be considered. First, commercial preparations of 2MeSADP are contaminated with small amounts of 2MeSAMP and thus the actions attributable...
to P2Y₁ receptor agonism could, in fact, reflect actions on the P1 receptor family. To assess this possibility, theophylline, a potent nonspecific antagonist of P1 receptors, was added to the perfusate. Theophylline (1 mmol/l) alone had no effect on $J_{\text{HCO}_3}$. Nor did it attenuate the inhibitory effect of 100 μM 2MeSADP (Fig. 6), even at this high concentration.

Second, 2MeSADP can activate (albeit with very low potency) P2Y₆ receptors (7), mRNA for which is found in rat proximal tubule (3). To assess this possibility, a selective, competitive inhibitor of the P2Y₁ receptor, MRS-2179 (5), was added to the perfusate (Fig. 7): at a concentration of 1 mmol/l, MRS-2179 increased $J_{\text{HCO}_3}$ significantly, suggesting basal ac-

Fig. 3. A: example recording showing acidification during paired perfusion with control (solid line) and either ATP (dashed line) or ADP (solid grey line). B: $J_{\text{HCO}_3}$ during perfusion with control ($n = 67$) and either AMP (100 μmol/l; $n = 30$), ADP (100 μmol/l; $n = 40$), or ATP (100 μmol/l; $n = 20$). Data are means ± SE of all individual recordings taken from 22 tubules in 4 rats; each tubule was perfused with control solution and then again with 1 experimental solution. *$P < 0.05$, **$P < 0.01$ vs. paired control. N.S., not significant.

Fig. 4. A: example recording showing acidification during paired perfusion with control (solid line) and 2MeSADP (100 μmol/l; dashed line). B: $J_{\text{HCO}_3}$ during perfusion with control ($n = 113$) and either time control (saline, $n = 52$) or 2MeSADP ($n = 88$). C: $t_{1/2}$ of pH recovery. D: stationary pH. Data are means ± SE of individual recordings taken from 31 tubules in 11 rats. **$P < 0.01$ vs. control.
Mechanism of inhibition. To investigate the mechanism by which $P_2Y_1$ receptor activation evoked reduced bicarbonate reabsorption, the proximal tubule was perfused in one series of experiments with three separate solutions: control, EIPA (200 μmol/l), and EIPA (200 μmol/l) plus 2MeSADP (100 μmol/l). The effect of a maximum inhibitory concentration of 2MeSADP was not additive to that of a maximum inhibitory concentration of EIPA (Fig. 10). This indicates that $P_2Y_1$ receptor activation impairs bicarbonate reabsorption in the proximal tubule via inhibition of Na/H exchange.

**DISCUSSION**

The rat proximal tubule expresses mRNA of four members of the $P_2Y$ receptor family (2, 3), three of which ($P_2Y_2$, $P_2Y_4$, and $P_2Y_6$) are pyrimidine selective. The fourth, $P_2Y_1$, is activated by purine nucleotides, particularly ADP (7). The confirmation of functional receptor expression through measurement of nucleotide-stimulated increases in $[Ca^{2+}]_i$ has thus far been limited to the basolateral membrane where $P_2Y_1$ is the predominant receptor subtype (2). Recent immunohistological data indicate that $P_2Y_1$ receptors are also expressed in the apical membrane of the rat proximal tubule (31): the present investigation demonstrates a functional role for apical $P_2Y_1$ receptors and suggests that luminal nucleotides are modulators of transepithelial bicarbonate transport.

The proximal tubule reclaims ~75% of filtered bicarbonate through Na/H exchanger- and H$^+$-ATPase-mediated proton secretion (15); both transporters are regulated by extracellular nucleotides in renal and nonrenal cell lines (1, 12, 16, 24, 29). The present study extended those observations made in isolated cells to a more complex biological system. The observed inhibition of bicarbonate reabsorption by the endogenous nucleotides ADP and, to a lesser extent, ATP, strongly implicates $P_2Y_1$ receptors in the regulation of proximal tubule transport processes. This supposition was confirmed by two observations: first, the greatest degree of inhibition was observed using 2MeSADP, the most potent agonist of $P_2Y_1$ receptors (27). Second, 2MeSADP was no longer able to inhibit $J_{\text{HCO}_3}$ in the presence of the $P_2Y_1$ receptor-specific antagonist, MRS-2179. Immunohistological data suggest that the only other $P_2$ receptor expressed in the apical membrane of the proximal tubule is $P2X_5$ (31), at which 2MeSADP is not an agonist (7). $P2X_5$ is activated by UTP (7), but this agent was without effect on $J_{\text{HCO}_3}$.
At maximal activation, P2Y<sub>1</sub> receptors reduce proximal tubule bicarbonate reabsorption by ~50%. This occurs at a lumen nucleotide concentration of >100 µmol/l, so it is doubtful whether such extensive inhibition is physiological. Moreover, because the proximal tubule is a leaky epithelium, it is possible that high concentrations of nucleotide gain access to sites other than the apical membrane.

Of greater physiological importance is the submaximal inhibition of proximal tubule bicarbonate reabsorption occurring between 1 and 10 µM 2MeSADP; at these concentrations, it is unlikely that significant leakage to the basolateral membrane could occur. Moreover, such concentrations of endogenous receptor agonist might be present in the tubule fluid. It is known, for example, that ATP is released across the apical membrane of isolated proximal tubule cells in quantities sufficient to achieve local concentrations in the low-micromolar range (28). Preliminary free-flow micropuncture studies indicate that the concentration of ATP in fluid samples harvested from the proximal tubule is ~250 nmol/l (28a), which, given the susceptibility of ATP to enzymatic degradation by proximal tubule ectonucleotidases (17), might underestimate significantly the actual concentration of nucleotide present at the apical membrane. Finally, it must be noted that the concentration of ADP in proximal tubule fluid has not yet been established: ATP, itself a P2Y<sub>1</sub> receptor agonist, provides a source for the more potent agonist ADP, thereby prolonging the regulation of proximal transport through P2Y<sub>1</sub> receptor activation.

In heterologous expression systems, the rat P2Y<sub>1</sub> receptor couples exclusively to activation of phospholipase C (26); inhibition of this pathway blocked the actions of 2MeSADP in the present study. Activation of protein kinase C inhibits NHE3 in transfected cells (12) and reduces bicarbonate (25) and volume (4) reabsorption in the microperfused proximal tubule. This latter finding is not universal, however, and may relate to the conditions of exposure (33) and the basal levels of protein kinase C.

The current study also advocates a role for protein kinase A as H-89 attenuated the effect of P2Y<sub>1</sub> receptor activation. Data
obtained from A6 cells reveal direct inhibition of NHE3 by a cAMP/protein kinase A-dependent pathway (1). However, direct coupling of P2Y1 receptors to Gq or Gi proteins is not evidenced (26), and thus the activation of protein kinase A by P2Y1 receptor activation may well be indirect, resulting from regulation of adenylate cyclase or phosphodiesterase by [Ca2+]i (9).

The nonadditive effects of 2MeSADP and EIPA suggest purinergic modulation of proximal tubule Na/H exchange and point, therefore, to inhibition of NHE3. Mechanistically, this could relate to a direct action on NHE3 by protein kinase A and/or protein kinase C, as stated above. This claim is buttressed by the fact that mutation of Ser552 and Ser605 in the rat NHE3 protein, which prevents phosphorylation of the transporter by protein kinase A (37), eliminates the inhibitory action of P2Y1 receptor activation (1). Indirect effects on Na/H exchange may involve modulation of basolateral transporters such as the Na-K-ATPase or Na/HCO3 cotransporter (30) by P2Y1 receptor activation and purinergic effects on carbonic anhydrase have also been reported (14).

In summary, luminal P2Y1 receptor activation inhibits bicarbonate reabsorption in the rat proximal tubule through a phospholipase C- and protein kinase A-dependent mechanism. The physiological ramifications of this inhibition are unresolved, but local control of NaHCO3 transport is implicated. One possibility is that coordinated activation of purinergic receptors in both apical and basolateral domains contributes to glomerulotubular balance processes. Increased filtration at the glomerulus leads to a concatenation of events that would stimulate NaHCO3 reabsorption to match filtered load: increased peritubular fluid viscosity promotes transepithelial bicarbonate flux, dependent on activation of basolateral P2 receptors (10), and increased tubular flow has been suggested to exert a diluting effect on luminal nucleotide (19), thereby alleviating inhibition of NaHCO3. Such events remain hypothetical. Nevertheless, the present study adds to the growing body of evidence advocating purinergic regulation of renal function.

Fig. 8. JHCO3 during paired perfusion with control and 2MeSADP at either 0.1 (n = 28), 1 (n = 49), 10 (n = 61), 100 (n = 88), or 1,000 (n = 35) µmol/l. Data are means ± SE of individual recordings taken from 53 tubules in 14 rats and are transformed to show the percentage inhibition of paired control values. **P < 0.01, *P < 0.05 vs. paired control (paired t-test).

Fig. 9. JHCO3 during perfusion with (A) control (n = 50), U-73122 (10 µmol/l; n = 45), 2MeSADP (100 µmol/l; n = 36), or both agents together (n = 52) and (B) control (n = 99), H89 (10 µmol/l; n = 25), 2MeSADP (100 µmol/l; n = 40), or both agents together (n = 65). Data are means ± SE of individual recordings taken from 37 tubules in 9 rats. **P < 0.01, *P < 0.05 vs. control (paired t-test).

Fig. 10. JHCO3 during paired perfusion of the same proximal tubule with control (n = 49), EIPA (200 µmol/l; n = 52), and EIPA + 100 µmol 2MeSADP (n = 63). Data are means ± SE of individual recordings taken from 28 tubules in 7 rats. **P < 0.01 vs. control.
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