cAMP-Adenosine Pathway in the Proximal Tubule

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ABSTRACT

The “extracellular cAMP-adenosine pathway” refers to the conversion of cAMP to AMP by ecto-phosphodiesterase, followed by metabolism of AMP to adenosine by ecto-5’-nucleotidase, with all the steps occurring in the extracellular compartment. This study investigated whether the extracellular cAMP-adenosine pathway exists in proximal tubules. Freshly isolated proximal tubules rapidly converted basolaterally administered cAMP to AMP and adenosine. Proximal tubular cells in culture (first passage) rapidly converted apically administered cAMP to AMP and adenosine. In both freshly isolated proximal tubules and cultured proximal tubular cells, conversion of cAMP to AMP and adenosine was affected by a broad-spectrum phosphodiesterase inhibitor (3-isobutyl-1-methylxanthine), and a blocker of ecto-5’-nucleotidase (α,β-methyleneadenosine-5’-diphosphate) in a manner consistent with exogenous cAMP being processed by the extracellular cAMP-adenosine pathway. In cultured proximal tubular cells, but not freshly isolated proximal tubules, stimulation of adenylyl cyclase increased extracellular concentrations of cAMP, AMP, and adenosine plus inosine, and these changes were also modulated by the inhibitors in a manner consistent with the extracellular cAMP-adenosine pathway. Conversion of renal interstitial (basolateral) cAMP and AMP to adenosine in vivo was shown by microdialysis coupled with ion trap mass spectrometry. Western blot analysis showed A1, A2A, and A3 receptors on both apical and basolateral proximal tubular membranes, with A1 and A2A receptors more highly expressed on basolateral compared with apical membranes. We conclude that cAMP that reaches either the apical or basolateral membranes of proximal tubular cells is converted in part to adenosine that has ready access to adenosine receptors.

Adenosine produced by renal cortical structures regulates several processes in the cortex, including blood flow, renin release, and electrolyte reabsorption. A1 receptors reduce and A2A receptors increase renal cortical blood flow. Infusion of A1 receptor agonists into the cortical interstitium decreases blood flow to superficial and deep nephrons (Agmon et al., 1993). A1 receptor-induced vasoconstriction is mostly caused by contraction of preglomerular microvessels (Holz and Steinhausen, 1987; Joyner et al., 1988); however, in juxtaglomerular nephrons, A1 receptors mediate vasoconstriction by contracting both preglomerular microvessels (Nishiyama et al., 2001) and efferent arterioles (Nishiyama et al., 1991). Recent experiments with A1 receptor knockout mice confirm a role for adenosine in mediating the reduction of renin release in response to increased sodium chloride delivery to the macula densa (Kim et al., 2005) or increased intrarenal perfusion pressure (Schweda et al., 2005).

A1 receptor activation inhibits renin release (Jackson, 1991). Recent experiments with A1 receptor knockout mice confirm a role for adenosine in mediating the reduction of renin release in response to increased sodium chloride delivery to the macula densa (Kim et al., 2005) or increased intrarenal perfusion pressure (Schweda et al., 2005).

In proximal tubular epithelial cells, adenosine via A1 receptors mediates enhancement of electrolyte transport. Stimulation of A1 receptors in cultured epithelial cells that express a proximal phenotype enhances Na+–glucose and Na+–phosphate symport (Coulson et al., 1991). Stimulation of A1 receptors in microperfused proximal tubules increases basolateral Na+–3HCO3− symport (Takeda et al., 1993), and glomerular filtration rate (Jackson, 1997, 2001), and potentiates postjunctional vasoconstrictor responses to renal sympathetic neurotransmission (Hedqvist and Fredholm, 1976; Hedqvist et al., 1978). In contrast, cortical A2A receptors, particularly those in efferent arterioles, mediate vasodilation (Hansen and Schnarrnann, 2003).

ABBREVIATIONS: IBMX, 3-isobutyl-1-methylxanthine; AMPCP, α,β-methyleneadenosine-5’-diphosphate; DPSPX, 1,3-dipropyl-8-p-sulfophenylxanthine; NBC-1, sodium bicarbonate cotransporter type 1; NHE-3, sodium hydrogen exchanger type 3; QC, quality control sample(s); CV, coefficient(s) of variation; LSD, least significant difference.
Because adenosine modulates processes in the renal cortex, it is important to understand the mechanisms of adenosine biosynthesis by cortical elements. Recently, we showed the ability of collecting duct cells to generate adenosine via the extracellular cAMP-adenosine pathway (Jackson et al., 2003). This pathway is defined as cAMP efflux from cells during activation of adenylyl cyclase, followed by the extracellular conversion of cAMP to adenosine by the serial actions of ecto-phosphodiesterase and ecto-5'-nucleotidase (Jackson and Dubey, 2001). This mechanism of extracellular adenosine production provides hormonal control of adenosine levels in the cell-surface biophase in which adenosine receptors reside. Tight coupling of the site of adenosine production to the site of adenosine receptors permits a low-capacity mechanism of adenosine biosynthesis to have a large impact on adenosine receptor activation (Jackson and Dubey, 2001). It is conceivable that the extracellular CAMP-adenosine pathway in proximal tubules is also a significant source of adenosine, both for proximal tubular epithelial cells and for other cellular elements in the renal cortex to which adenosine produced by the basolateral aspect of proximal tubules could diffuse. Egress of cAMP into the tubular lumen (across apical membrane) or interstitial space (across basolateral membrane) during activation of adenylyl cyclase could result in adenosine formation, provided that proximal tubular epithelial cells transport cAMP and express ecto-phosphodiesterase and ecto-5'-nucleotidase. Moreover, delivery of cAMP from distal sites (e.g., extrarenal via the bloodstream) could provide cAMP to the renal cortex for conversion to adenosine.

To test the hypothesis that the extracellular CAMP-adenosine pathway exists in proximal tubular epithelial cells, we examined the ability of freshly isolated proximal tubules and proximal tubular epithelial cells in culture to convert exogenous cAMP to AMP and adenosine. We also investigated whether stimulation of adenylyl cyclase increases extracellular levels of cAMP, AMP, and adenosine. Furthermore, we examined whether 3-isobutyl-1-methylxanthine (IBMX; a phosphodiesterase inhibitor that penetrates cell membranes) examined whether 3-isobutyl-1-methylxanthine (IBMX; a phosphodiesterase inhibitor that penetrates cell membranes) examined whether 3-isobutyl-1-methylxanthine (IBMX; a phosphodiesterase inhibitor that penetrates cell membranes) examined whether 3-isobutyl-1-methylxanthine (IBMX; a phosphodiesterase inhibitor that penetrates cell membranes) examined whether 3-isobutyl-1-methylxanthine (IBMX; a phosphodiesterase inhibitor that penetrates cell membranes) examined whether 3-isobutyl-1-methylxanthine (IBMX; a phosphodiesterase inhibitor that penetrates cell membranes) examined whether 3-isobutyl-1-methylxanthine (IBMX; a phosphodiesterase inhibitor that penetrates cell membranes) examined whether 3-isobutyl-1-methylxanthine (IBMX; a phosphodiesterase inhibitor that penetrates cell membranes) examined whether 3-isobutyl-1-methylxanthine (IBMX; a phosphodiesterase inhibitor that penetrates cell membranes) examined whether 3-isobutyl-1-methylxanthine (IBMX; a phosphodiesterase inhibitor that penetrates cell membranes) examined whether 3-isobutyl-1-methylxanthine (IBMX; a phosphodiesterase inhibitor that penetrates cell membranes) examined whether 3-isobutyl-1-methylxanthine (IBMX; a phosphodiesterase inhibitor that penetrates cell membranes) examined whether 3-isobutyl-1-methylxanthine (IBMX; a phosphodiesterase inhibitor that penetrates cell membranes) examined whether 3-isobutyl-1-methylxanthine (IBMX; a phosphodiesterase inhibitor that penetrates cell membranes) examined whether 3-isobutyl-1-methylxanthine (IBMX; a phosphodiesterase inhibitor that penetrates cell membranes) examined whether 3-isobutyl-1-methylxanthine (IBMX; a phosphodiesterase inhibitor that penetrates cell membranes) examined whether 3-isobutyl-1-methylxanthine (IBMX; a phosphodiesterase inhibitor that penetrates cell membranes) examined whether 3-isobutyl-1-methylxanthine (IBMX; a phosphodiesterase inhibitor that penetrates cell membranes) examined whether 3-isobutyl-1-methylxanthine (IBMX; a phosphodiesterase inhibitor that penetrates cell membranes) examined whether 3-isobutyl-1-methylxanthine (IBMX; a phosphodiesterase inhibitor that penetrates cell membranes) examined whether 3-isobutyl-1-methylxanthine (IBMX; a phosphodiesterase inhibitor that penetrates cell membranes) examined whether 3-isobutyl-1-methylxanthine (IBMX; a phosphodiesterase inhibitor that penetrates cell membranes) examined whether A1 receptors decreases Na+-dependent phosphate transport in proximal tubular cells (Cai et al., 1994, 1995).

Materials and Methods

Isolation of Proximal Tubules. Kidneys were harvested from adult male rats, and proximal tubules were rapidly isolated using a Percoll solution-centrifugation method described previously in detail by us (Jackson et al., 2003). Western blot studies established that freshly isolated proximal tubules did not express bumetanide-sensitive cotransporter type 1 (marker for thick ascending limb), thiazide-sensitive cotransporter (marker for distal tubules), or aquaporin-2 (marker for collecting duct) but did express aquaporin-1, sodium bicarbonate cotransporter type 1 (NBC-1), and sodium hydrogen exchanger type 3 (NHE-3) (markers for proximal tubules).

Isolation of Proximal Tubular Basolateral and Apical Membranes. Isolation of apical and basolateral membrane from kidney cortex was based on the methods described by Kinsella et al. (1979) and Biber et al. (1981). Kidneys were harvested from adult rats and placed in ice-cold phosphate-buffered saline. From this point forward, all the isolation procedures were performed at 4°C. The cortex was removed, minced into small pieces, and washed with ice-cold phosphate-buffered saline. The tissue was transferred to 8% sucrose in a Dounce glass homogenizer (Fisher, Pittsburgh, PA) with a tight-fitting pestle. The homogenate was centrifuged at 1000g for 10 min, and the supernatant (A) was collected. The pellet was reprocessed as above, and its supernatant (B) was collected. Supernatant B was combined and centrifuged at 9500g for 10 min, and the supernatant (C) and soft lighter portion of the pellet were centrifuged at 47,000g for 20 min in a Beckman (Fullerton, CA) Ti 90 rotor. The soft, lighter upper portion of the pellet was harvested and suspended in medium A (25 mM HEPES, 100 mM mannitol, 2 mM CaCl2, 1 mM MgCl2, and 1 mM MnCl2, adjusted to pH 7.0 with Tris) to the final protein concentration of 6 to 8 mg/ml. The suspension was left in an ice bath for 1 h and centrifuged at 1400g for 12 min. The supernatant (D) was saved and contained the apical membranes. The pellet was resuspended with medium A three times by suspending and centrifuging to reduce apical membrane contamination of the basolateral membranes in the pellet. Next, the pellet containing basolateral membranes was layered over a discontinuous sucrose gradient composed of 8% sucrose solution and 31% sucrose solution and spun at 90,000g for 60 min in a Beckman Ti 90 rotor. The 8 to 31% sucrose interface portion was harvested as basolateral membrane. Both apical and basolateral membrane portions were diluted in 25 mM HEPES buffer and spun down at 47,000g for 20 min. The pellets were dissolved in a lysis buffer (50 mM Tris with pH at 7.0, 2% SDS, and 10% glycerol) for Western blot analysis.

Western Blot Analysis. Electrophoresis, Western blot analysis, and immunostaining were performed as described previously (Jackson et al., 2003). Anti-bumetanide-sensitive cotransporter type 1, anti-thiazide-sensitive cotransporter, anti-aquaporin-1, and anti-aquaporin-2 antibodies were provided by Dr. Mark Knepper (National Institutes of Health, Bethesda, MD); anti-NBC-1, anti-NHE-3, anti-A2A-receptor, anti-A2B-receptor, and anti-A3-receptor antibodies were obtained from Chemicon (Temecula, CA); and anti-A1-receptor and anti-β-actin antibodies were obtained from Sigma.

Culture of Proximal Tubular Epithelial Cells. Proximal tubular epithelial cells in culture were obtained from freshly isolated proximal tubules using a method described previously by us for collecting duct cells (Jackson et al., 2003). All the experiments were performed in proximal tubular cells in first passage.

Studies in Freshly Isolated Proximal Tubules. Freshly isolated proximal tubules (approximately 0.2 mg) were incubated in capped tubes in 0.3 ml of L-15 medium in a shaking water bath at 37°C without or with exogenous cAMP at the indicated concentrations in the absence and presence of IBMX (1 mM), AMPCP (0.1 mM), or DPSPX (1 mM). The concentrations of various inhibitors

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were based on our previous published experience with inhibition of exogenous cAMP metabolism in cultured cardiac fibroblasts and vascular smooth muscle cells (Dubej et al., 1996, 2000). In addition, some proximal tubules were treated with stimulators of adenyl cyclase. Adenylyl cyclase was stimulated by treating the cells with a mixture of forskolin (10 μM; direct activator of adenyl cyclase), parathyroid hormone (1 mM; α-gene; β-adrenergic agonist), and fenoldopam (10 μM; agonist of dopamine type 1 receptors). Because we did not know which receptors were both expressed and coupled to stimulate adenylyl cyclase, we used the strategy of adding several agonists of adenylyl cyclase simultaneously. Just before adding the proximal tubules to the L-15 medium, the L-15 medium was bubbled with 100% oxygen. Just after adding the isolated proximal tubules, the heads of the cuffed tubes were flushed with 100% oxygen. After the indicated time of incubation, the isolated proximal tubules were centrifuged, and the supernatant was collected, transferred immediately into ice-cold tubes, and frozen at −70°C until purine levels were measured. The pellet was solubilized in 0.5 N NaOH, and protein content was measured by the copper bicinchoninic acid method.

Studies in Proximal Tubular Epithelial Cells in Culture. Monolayers of proximal tubular cells in culture were grown in 12-well culture plates. On the day of the experiment, the proximal tubular cells were washed with phosphate-buffered saline and then incubated (37°C under 5% carbon dioxide in cell incubator) with various treatments. Some proximal tubular cells were incubated for 0, 5, or 30 min with 0, 3, 10, or 30 μM cAMP in phosphate-buffered saline. In cells treated with 0, 3, 10, or 30 μM cAMP for “0” min, the phosphate-buffered saline containing cAMP was added to the cells and then quickly removed. Other proximal tubular cells were treated with 30 μM cAMP for 30 min in the presence and absence of IBMX, DPSPX, and AMPCP. In addition, some proximal tubular cells were treated for 30 min with stimulators of adenylyl cyclase (see above) and in the presence of absence of IBMX, DPSPX, and AMPCP. At the end of the specified incubation time, the conditioned phosphate-buffered saline was collected, transferred immediately into ice-cold tubes, and frozen at −70°C until purine levels were measured. At the end of the experiment, cells were solubilized in 0.5 N NaOH, and protein content was measured by the copper bicinchoninic acid method.

Analysis of Purines in Studies with Freshly Isolated Proximal Tubules and Proximal Tubular Cells in Culture. In vitro experiments using exogenous cAMP, purines were measured by high-pressure liquid chromatography using UV absorption as described previously (Jackson et al., 1996). In experiments with adenylyl cyclase activators, cAMP and adenosine concentrations were measured by high-pressure liquid chromatography using fluorescence detection (higher sensitivity relative to UV absorption method) as described previously (Jackson et al., 1996), and AMP was measured by high-pressure liquid chromatography using UV absorption.

In Vivo Microdialysis Experiments. Adult male rats were housed in the University of Pittsburgh Animal Care Facility for at least 1 week before the experiments. Animals were fed Pro Lab RMH 3000 rodent diet containing 0.26% sodium and 0.82% potassium and water ad libitum. On the day of the experiment, each animal was anesthetized with Inactin (100 mg/kg, i.p.) and placed on a Delphaphe isothermal pad. Body temperature was monitored with a rectal temperature probe and maintained at 37°C by adjusting a heat lamp positioned above the rat. A short section of polyethylene-240 tubing was placed in the trachea to facilitate respiration. A polyethylene-50 cannula was inserted into the left jugular vein, and 0.9% saline was flushed with 100% oxygen. After the indicated time of incubation, the isolated proximal tubules were centrifuged, and the supernatant was collected, transferred immediately into ice-cold tubes, and frozen at −70°C until purine levels were measured. The pellet was solubilized in 0.5 N NaOH, and protein content was measured by the copper bicinchoninic acid method.

Analysis of Purines in Dialysate Samples. Measuring purine levels in samples from in vivo studies is challenging because of the low levels of purines and the presence of potentially interfering substances. To ensure the accuracy of the results, we developed a novel assay for measuring purines using ion trap mass spectrometry. Because this is the first presentation of this assay, the assay is described in detail below.

The assay was developed using a ThermoFinnigan high-pressure liquid chromatographic system coupled to a ThermoFinnigan LC Q Duo ion trap mass spectrometer equipped with an electrospray ionization source (Thermo Electron Corporation, Waltham, MA). The analytical column was a C-18 Eclipse Zorbax XDB column (4.6 mm × 150 mm). The mobile phase flow rate was 0.5 ml/min. From 0 to 1.5 min, the mobile phase was 100% water. The mobile phase composition was then changed (linear gradient) to 90% water plus 10% methanol over 0.5 min and then kept constant at this composition for an additional 8 min. The mobile phase was acidified with 0.1% formic acid. The mass spectrometer was operated in the positive ion mode. Nitrogen was used both as sheath and auxiliary gas at a pressure optimized automatically for each of the analytes during tuning of each analyte. The spray voltage and the heated capillary temperatures were set at 5 kV and 270°C, respectively. The analytes were monitored using single ion monitoring: for AMP, mass-to-charge ratio (m/z) = 348; for hypoxanthine, m/z = 137; for adenosine and adenosine 9-β-D arabinofuranoside (internal standard), m/z = 268; and for inosine, m/z = 291. Samples were injected directly into the analytical system without previous sample preparation. For the first 5 min of the sample run, flow was diverted from the source to a waste bottle. This allowed for the removal of salts present in the sample and prevented blocking of the bore of the heated capillary by the nonvolatile salts. AMP, adenosine, inosine, hypoxanthine, and internal standard were prepared in phosphate-buffered saline. Dilutions were made in phosphate-buffered saline.

The assay was validated for AMP, adenosine, inosine, and hypoxanthine. A standard curve was generated in duplicate using the following concentrations: 0.5, 1, 2, 5, 10, 20, and 30 pg/μl. Twenty-seven quality control samples (QC) were processed along with the standard curve samples (nine QC each at the low, medium, and high range of the standard curve; i.e., 3, 12, and 25 pg/μl, respectively). This was repeated three times on different days and each time with freshly prepared solutions. From the QC, the intra-assay coefficient and interassay coefficient of variation (CV) were calculated.

The liquid chromatographic method afforded baseline separation of all four compounds and the internal standard. The retention times of AMP, adenosine, inosine, hypoxanthine, and internal standard (adenine 9-β-D arabinofuranoside) were 6.57, 7.02, 7.92, 8.76, and 9.47 min, respectively. No interfering peaks were detected in nonspiked (blank) samples. All analytes were detected at the lowest concentrations used (0.5 pg/μl), and all the compounds produced a linear relationship in the range used (0.5–30 pg/μl). The assay system could detect <25 pg injected on column of each of the analytes. The correlation coefficients (R²) of the standard curves used in the

Adenosine Biosynthesis in Proximal Tubules

1221

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TABLE 1
Regression equations and correlation coefficients for the calibration curves for the three different validations experiments (I, II, and III) of the different analytes

<table>
<thead>
<tr>
<th>Compound</th>
<th>Line</th>
<th>Correlation Coefficient</th>
</tr>
</thead>
<tbody>
<tr>
<td>AMP (I)</td>
<td>$y = 0.0029 + 0.0089x$</td>
<td>0.992</td>
</tr>
<tr>
<td>Hypoxanthine (I)</td>
<td>$y = -0.0038 + 0.0437x$</td>
<td>0.996</td>
</tr>
<tr>
<td>Adenosine (I)</td>
<td>$y = 0.0069 + 0.1559x$</td>
<td>0.995</td>
</tr>
<tr>
<td>Inosine (I)</td>
<td>$y = 0.00207 + 0.0252x$</td>
<td>0.965</td>
</tr>
<tr>
<td>AMP (II)</td>
<td>$y = -0.00305 + 0.0106x$</td>
<td>0.990</td>
</tr>
<tr>
<td>Hypoxanthine (II)</td>
<td>$y = 0.03938 + 0.0369x$</td>
<td>0.988</td>
</tr>
<tr>
<td>Adenosine (II)</td>
<td>$y = 0.0105 + 0.1241x$</td>
<td>0.987</td>
</tr>
<tr>
<td>Inosine (II)</td>
<td>$y = 0.0311 + 0.0327x$</td>
<td>0.982</td>
</tr>
<tr>
<td>AMP (III)</td>
<td>$y = -0.00932 + 0.00679x$</td>
<td>0.977</td>
</tr>
<tr>
<td>Hypoxanthine (III)</td>
<td>$y = -0.002935 + 0.02837x$</td>
<td>0.993</td>
</tr>
<tr>
<td>Adenosine (III)</td>
<td>$y = 0.02936 + 0.0916x$</td>
<td>0.977</td>
</tr>
<tr>
<td>Inosine (III)</td>
<td>$y = 0.01993 + 0.0264x$</td>
<td>0.990</td>
</tr>
</tbody>
</table>

validation are shown in Table 1. The standard curves were reproducible in the three independent experiments. The $R^2$ values for all the standard curves were 0.99. Table 1 also shows the regression equations for the standard curves for each analyte and for each experiment. The calculated slopes indicate that the assay detected adenosine and hypoxanthine with the highest sensitivity, followed by inosine and AMP.

Table 2 summarizes the intra-assay CV from QC for the three independent validation experiments for each of the compounds. The lowest concentration QC for AMP had CV ranging from 9.96 to 12.5%, the medium concentration QC from 4.3 to 12.2%, and the highest concentration QC from 6.77 to 8.65%. For hypoxanthine, the range for the low concentration QC was 7.52 to 16.6%, for medium concentration QC from 3.97 to 7.05%, and for high concentration QC from 4.12 to 7.99%. For adenosine, the range for low concentration QC was 6.29 to 11.3%, for medium concentration QC from 4.17 to 7.99%, and for high concentration QC from 3.97 to 7.05%. For hypoxanthine, the range for low concentration QC was 7.52 to 16.6%, for medium concentration QC from 6.77 to 8.65%. For hypoxanthine, the highest concentration QC from 6.77 to 8.65%.

The analytes were very stable as indicated by QC samples run 24 h after being at room temperature in the autosampler. For adenosine, the amounts calculated did not deviate more than 2.4, 4.9, and 10.0% from the average of the low, medium, and high QC concentrations, and had CV of 2.8, 2.2, and 4.6% for the low, medium, and high concentrations, respectively. For inosine, the deviations from the average of the QC were 10.3, 5.6, and 12.9%, with CV of 17.1, 12.5, and 6.3% for the low, medium, and high concentrations, respectively. For AMP, the deviations from the average of the QC were 14.7, 9.12, and 8.28%, with CV of 9.3, 6.29, and 5.37% for the low, medium, and high concentrations, respectively.

TABLE 2
Intra-assay coefficient of variation (CV expressed as a %) for the three different validation experiments (I, II, and III) of the different analytes

<table>
<thead>
<tr>
<th>Validation</th>
<th>AMP Low</th>
<th>AMP Medium</th>
<th>AMP High</th>
<th>Adenosine Low</th>
<th>Adenosine Medium</th>
<th>Adenosine High</th>
<th>Hypoxanthine Low</th>
<th>Hypoxanthine Medium</th>
<th>Hypoxanthine High</th>
<th>Inosine Low</th>
<th>Inosine Medium</th>
<th>Inosine High</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>11.5</td>
<td>4.30</td>
<td>6.77</td>
<td>12.5</td>
<td>12.2</td>
<td>8.43</td>
<td>9.37</td>
<td>8.29</td>
<td>7.31</td>
<td>15.6</td>
<td>4.58</td>
<td>10.0</td>
</tr>
<tr>
<td>II</td>
<td>11.3</td>
<td>4.97</td>
<td>5.22</td>
<td>7.69</td>
<td>5.51</td>
<td>4.98</td>
<td>7.52</td>
<td>3.97</td>
<td>5.37</td>
<td>15.6</td>
<td>6.05</td>
<td>11.9</td>
</tr>
<tr>
<td>III</td>
<td>16.8</td>
<td>6.05</td>
<td>4.12</td>
<td>11.9</td>
<td>7.05</td>
<td>7.99</td>
<td>7.52</td>
<td>3.97</td>
<td>5.37</td>
<td>15.6</td>
<td>4.58</td>
<td>10.0</td>
</tr>
</tbody>
</table>

L, low; M, medium; H, high.

Results

Studies in Freshly Isolated Proximal Tubules. Figure 1 illustrates the time-dependent (0–60 min) conversion of cAMP (30 μM) to AMP (Fig. 1A), adenosine (Fig. 1B), inosine (Fig. 1C), hypoxanthine (Fig. 1D), and xanthine (Fig. 1E) in proximal tubules. Addition of cAMP to proximal tubules caused a significant increase in the extracellular levels of AMP ($P < 0.001$), adenosine ($P < 0.001$), inosine ($P < 0.001$), hypoxanthine ($P = 0.002$), and xanthine ($P < 0.001$) (all comparisons with time control; i.e., proximal tubules incubated for the same period but without added cAMP). Extracellular levels of AMP and adenosine reached a plateau in approximately 5 min; however, extracellular levels of inosine, hypoxanthine, and xanthine increased over time of incubation.

Figure 2 illustrates the concentration-dependent (0–100 μM) conversion of AMP (20 min of incubation) to AMP (Fig. 2A), adenosine (Fig. 2B), inosine (Fig. 2C), hypoxanthine (Fig. 2D), and xanthine (Fig. 2E) in proximal tubules. Addition of increasing concentrations of cAMP to proximal tubules caused corresponding increases in the extracellular levels of AMP ($P < 0.001$), adenosine ($P < 0.001$), inosine ($P < 0.001$), hypoxanthine ($P = 0.001$) and xanthine ($P = 0.002$); however, the changes followed a different pattern compared with the changes in AMP, adenosine, and inosine. Low concentrations of cAMP increased hypoxanthine and xanthine levels. In contrast, higher concentrations of cAMP (i.e., those corresponding to significant accumulations of adenosine) suppressed levels of hypoxanthine and xanthine relative to the elevated levels.

Figure 3 summarizes the effects of inhibitors of phosphodiesterase (IBMX), ecto-phosphodiesterase (DPSPX), and ecto-5'-nucleotidase (AMPCP) on conversion of exogenous cAMP (30 μM for 20 min) to AMP (Fig. 3A), adenosine (Fig. 3B), and inosine (Fig. 3C) in freshly isolated proximal tu-
bules. IBMX and DPSPX decreased extracellular levels of AMP, adenosine, and inosine, and AMPCP decreased extracellular levels of adenosine and inosine but increased extracellular levels of AMP.

Studies in Proximal Tubular Epithelial Cells in Culture. Figure 4 illustrates conversion in proximal tubular cells in culture of exogenous cAMP to AMP (Fig. 4A) and adenosine (Fig. 4B) as a function of time (0, 5, and 30 min) and cAMP concentration (0, 3, 10, and 30 μM). Addition of increasing concentrations of cAMP to proximal tubular cells and incubating cells for increasing durations with cAMP caused corresponding increases in the extracellular levels of AMP and adenosine.

Figure 5 summarizes the effects of inhibitors of phosphodiesterase (IBMX), ecto-phosphodiesterase (DPSPX), and ecto-5'-nucleotidase (AMPCP) on conversion of exogenous cAMP (30 μM for 30 min) to AMP (Fig. 5A) and adenosine (Fig. 5B) in proximal tubular cells in culture. IBMX and DPSPX decreased extracellular levels of AMP and adenosine, and AMPCP decreased extracellular levels of adenosine but tended to increase levels of AMP.

When freshly isolated proximal tubules were incubated with activators of adenylyl cyclase, no increases in extracellular levels of cAMP, AMP, or adenosine were detected in the bulk incubation medium (data not shown). In contrast, proximal tubular cells in culture were markedly responsive to activators of adenylyl cyclase (Fig. 6, A–D). In this regard, stimulation of adenylyl cyclase greatly increased extracellular levels of cAMP, and this response was somewhat attenuated by IBMX, DPSPX, and AMPCP (Fig. 6, B–D, respectively). Stimulation of adenylyl cyclase did not increase extracellular levels of AMP in control cells (Fig. 6E), IBMX-treated cells (Fig. 6F), or DPSPX-treated cells (Fig. 6G). However, AMPCP increased baseline extracellular levels of AMP, and when ecto-5'-nucleotidase was blocked with AMPCP, stimulation of adenylyl cyclase markedly increased extracellular levels of AMP (Fig. 6H). As shown in Fig. 7A, stimulation of adenylyl cyclase in proximal tubular cells increased extracellular levels of adenosine plus inosine (main metabolite of adenosine), and this response was abolished by IBMX (Fig. 7B) and AMPCP (Fig. 7C).
Expression of Adenosine Receptor Subtypes in Apical Versus Basolateral Proximal Tubular Membranes. As shown in Fig. 8, apical membranes, relative to basolateral membranes, were enriched in NHE-3 (Fig. 8A) but expressed little NBC-1 relative to basolateral membranes (Fig. 8B), a finding consistent with the well accepted distribution of NHE-3 and NBC-1 in apical versus basolateral proximal tubular membranes. As shown in Fig. 9, although both apical and basolateral membranes expressed both A1 (Fig. 9A) and A2A (Fig. 9B) receptors, basolateral membranes clearly expressed higher levels of both of these adenosine receptor subtypes. We were unable to identify A2B receptors in either apical or basolateral proximal tubular membranes (data not shown). Unlike A1 or A2A receptors, A3 receptors were more abundant on apical versus basolateral membranes (Fig. 10).

In Vivo Microdialysis Experiments. As shown in Fig. 11, adding cAMP to the perfusate of microdialysis probes inserted into the renal cortex tripled the recovery of AMP (Fig. 11A) and adenosine (Fig. 11B) from the microdialysate. Addition of AMP to the perfusate of microdialysis probes inserted into the renal cortex increased the recovery of adenosine from the microdialysate by more than 15-fold (Fig. 11B).

Discussion

Ecto-5′-nucleotidase is tethered to the extracellular face of the plasma membrane and metabolizes AMP to adenosine (Zimmermann, 1992). Because stimulation of adenylyl cyclase causes egress of cAMP into the extracellular space (Barber and Butcher, 1981), if there is sufficient ecto-phosphodiesterase activity, stimulation of adenylyl cyclase would lead to the extracellular metabolism of cAMP to AMP and hence to adenosine. The production of cAMP, the substrate for the cAMP-adenosine pathway, could take place at remote sites and be delivered to cells via the bloodstream for metab-
olism to adenosine (hormonal model). Alternatively, cAMP production and egress could take place in a highly localized environment near the site of conversion of cAMP to adenosine (autocrine/paracrine model). Extracellular cAMP, whatever the source, would be converted to adenosine locally so that increases in cAMP levels at the cell membrane could give rise to significant concentrations of adenosine at the cell surface.

Evidence indicates that the kidney expresses an extracellular cAMP-adenosine pathway. Infusion of cAMP into isolated kidneys increases production of AMP, adenosine, and inosine, and the increases in AMP and adenosine are reduced by IBMX (phosphodiesterase inhibitor) and DPSPX (ecto-phosphodiesterase inhibitor) (Mi and Jackson, 1995). In addition, AMPCP (ecto-5'-nucleotidase inhibitor) blocks the increases in adenosine, but not AMP, caused by cAMP in isolated kidneys (Mi and Jackson, 1995). Moreover, cultured preglomerular vascular smooth muscle cells (Jackson et al., 1997) and collecting duct cells (Jackson et al., 2003) and freshly isolated preglomerular microvessels (Jackson and Mi, 2000) and collecting ducts (Jackson et al., 2003) express the cAMP-adenosine pathway.

\[\text{Fig. 3.}\] Freshly isolated proximal tubules were incubated with cAMP (30 \(\mu\)M) for 20 min with and without IBMX (1 mM), DPSPX (1 mM), or AMPCP (0.1 mM), and the amounts of AMP (A), adenosine (B), and inosine (C) were determined. Values are mean \(\pm\) S.E.M. of five preparations, and a and b indicate \(P < 0.05\) by Fisher’s LSD test compared with cAMP alone and cAMP + AMPCP, respectively.

\[\text{Fig. 4.}\] Line graph showing the time-dependent and concentration-dependent metabolism of cAMP to AMP (A) and adenosine (B) in proximal tubular cells in culture (first passage). Values are mean \(\pm\) S.E.M. for three preparations, and a and b indicate \(P < 0.05\) by Fisher’s LSD test compared with zero cAMP group at corresponding time and zero time within the given concentration group, respectively.

\[\text{Fig. 5.}\] Proximal tubular cells in culture (first passage) were incubated with cAMP (30 \(\mu\)M) for 30 min with and without IBMX (1 mM), DPSPX (1 mM), or AMPCP (0.1 mM), and the amounts of AMP (A) and adenosine (B) were determined. Values are mean \(\pm\) S.E.M. for three preparations, and a and b indicate \(P < 0.05\) by Fisher’s LSD test compared with cAMP alone and cAMP + AMPCP, respectively.
The present study supports the conclusion that renal epithelial cells in proximal tubules also express an extracellular cAMP-adenosine pathway. Addition of exogenous cAMP to isolated proximal tubules or proximal tubular cells in culture causes synthesis of AMP and adenosine. Importantly, the conversion of exogenous cAMP to AMP and adenosine is blocked by inhibition of phosphodiesterase (with IBMX) and ecto-phosphodiesterase (with DPSPX), and inhibition of ecto-5'-nucleotidase (with AMPCP) blocks the conversion of exogenous cAMP to adenosine and enhances the accumulation of AMP.

Because cAMP does not penetrate lipid bilayers, the conversion of exogenous cAMP to extracellular adenosine most likely is caused by extracellular metabolism of cAMP. This conclusion is supported by the results with AMPCP and DPSPX because AMPCP inhibits ecto-5'-nucleotidase but not cytosolic 5'-nucleotidase (Zimmermann, 1992), and DPSPX does not penetrate cell membranes (Tofovic et al., 1991). The ability of these inhibitors to attenuate the conversion of exogenous CAMP to extracellular adenosine and its metabolites is strong evidence for an extracellular site of cAMP metabolism. Moreover, the ability of AMPCP to increase extracellular AMP levels is consistent with an extracellular site of cAMP conversion. In this regard, if cAMP were metabolized to AMP intracellularly, inhibition of ecto-5'-nucleotidase would not prevent the further metabolism of AMP to adenosine and would not cause the accumulation of extracellular AMP.

The source of endogenous cAMP for processing to adenosine by proximal tubules could come from at least two non-exclusive sources. One source of cAMP would be the local egress of cAMP onto the proximal tubular cell surface after activation of proximal tubular adenyl cyclase (autocrine/paracrine model). Indeed, in proximal tubular cells in culture, but not in freshly isolated proximal tubules, activation of adenylyl cyclase causes accumulation of cAMP into the extracellular medium. Moreover, in proximal tubular cells in culture, when ecto-5'-nucleotidase is inhibited, AMP accumulates in the extracellular medium. The lack of extracellular AMP accumulation in the absence of ecto-5'-nucleotidase inhibition is consistent with the observation that proximal tubular cells express high levels of ecto-5'-nucleotidase (Takahashi et al., 1989), particularly on apical membranes (Vekaria et al., 2006), which would metabolize AMP to downstream purines and thus prevent the accumulation of AMP. It is noteworthy that the increase in extracellular cAMP induced by stimulation of adenylyl cyclase is reduced by IBMX, DPSPX, and AMPCP. This may be because of blockade of adenosine receptors by IBMX and DPSPX and inhibition of adenosine formation by AMPCP, thus reducing A2A receptor-mediated cAMP production. More important is the observation that stimulation of adenylyl cyclase in cultured proximal tubular cells significantly increases the extracellular accumulation of adenosine plus inosine (metabolite of adenosine) and that this increase is blocked by either inhibition of phosphodiesterase with IBMX or inhibition of ecto-5'-nucleotidase with AMPCP.

Unlike proximal tubular cells in culture, stimulation of adenylyl cyclase in isolated proximal tubules does not increase extracellular levels of cAMP in the bulk medium. This observation is consistent with the fact that cAMP is transported across the apical membrane into the tubular lumen via MRP4 (van Aubel et al., 2002), a direction of cAMP transport that would allow intracellular cAMP to reach the outer surface of the apical membrane. In contrast, cAMP is transported by OAT1 in basolateral membranes (Sekine et al., 1997), but only in the direction from extracellular to intracellular. Thus, stimulation of adenylyl cyclase would be expected to increase extracellular cAMP on apical, but not basolateral, membrane outer surfaces. In studies with
freshly isolated proximal tubules, the bulk medium would be
influenced nearly entirely by efflux across the basolateral
membrane because the ends of the tubular segments are
collapsed. In contrast, in proximal tubular cells in culture,
the bulk medium would be influenced nearly entirely by
efflux across the apical membrane because the basolateral
membrane is against the culture plate matrix.

The best explanation for the results with activators of
adenyllyl cyclase is that in proximal tubules, local stimulation
of adenylyl cyclase places cAMP on the extracellular aspect of
apical, but not basolateral, membranes. However, this does
not imply that the extracellular cAMP-adenosine pathway
only functions on apical proximal tubular surfaces. Another
source of cAMP for the extracellular cAMP-adenosine pathway
is cAMP released into the bloodstream from other organs
e.g., the liver) (Kuster et al., 1973), followed by filtration of
cAMP into proximal tubules and delivery of cAMP to the
cortical renal interstitial space via peritubular capillaries.
Thus, both the basolateral and apical proximal tubular mem-
branes would be bathed in systemically delivered cAMP,
which according to the present study should be converted in
part to adenosine. In addition, the basolateral proximal tu-
bular membranes may receive cAMP produced by adjacent
renal cell types in the renal cortex. Importantly, ecto-5'-
nucleotidase is present both on apical membranes in the
proximal tubules and in the interstitial compartment (Vek-
aria et al., 2006).

The present study shows that both A1 and A2A receptors
are enriched in basolateral membranes relative to apical
membranes. Importantly, both A1 and A2A receptors are cou-
ted to adenylyl cyclase via G proteins, and in proximal
tubules, adenylyl cyclase is located nearly exclusively on
basolateral, not apical, membranes (Hanson and Linas,
1995). These concepts would imply that adenosine formed
from cAMP within the renal interstitial compartment would
have more opportunity to affect tubular function than adeno-
sine formed from cAMP in the luminal compartment.
An important issue is whether cAMP is converted to AMP and adenosine in the renal cortical interstitial compartment of the intact kidney. To address this question, we placed cAMP and AMP into the renal interstitial compartment using a microdialysis probe and measured local production of AMP and adenosine via the same probe. Consistent with our results in isolated proximal tubules and consistent with the enrichment of A1 and A2A receptors in proximal tubular basolateral membranes, addition of cAMP to the renal cortical interstitial compartment caused a 3-fold increase in renal cortical interstitial levels of AMP and adenosine, and addition of AMP to the interstitial compartment caused a 15-fold increase in adenosine. These results strongly support the concept that in intact kidneys conversion of cAMP to adenosine via the extracellular cAMP-adenosine pathway provides renal cortical interstitial adenosine, which acts on basolateral adenosine receptors to modulate tubular function. The conversion of cAMP to adenosine in the renal cortical interstitial space probably involves multiple cell types, including proximal tubular cells, but also preglomerular vascular smooth muscle cells as previously shown by us (Jackson and Mi, 2000).

An important question is whether the extracellular cAMP-adenosine pathway influences renal biology. Because adenosine potently affects multiple renal physiological processes, it is likely that adenosine from any source, including the cAMP-adenosine pathway, alters renal biology. In support of this conclusion, our previous studies show that the extracellular cAMP-adenosine pathway inhibits glomerular mesangial cell proliferation and the production of collagen by mesangial
cells (Dubey et al., 1997). In the present study, the increase in extracellular cAMP induced by stimulation of adenylyl cyclase in cultured proximal tubular cells was reduced by blocking the extracellular-cAMP pathway with IBMX, DP-SPX, or AMPPCP. This finding is consistent with adenosine formed from the pathway activating A<sub>2A</sub> receptor-mediated cAMP production so that blocking the pathway reduces cAMP production.

In summary, the proximal tubule expresses an extracellular cAMP-adenosine pathway that provides extracellular adenosine. On the apical membrane, the source of cAMP is probably both cAMP locally released from proximal tubular adenosine. On the apical membrane, the source of cAMP is probably the cAMP-adenosine pathway that provides extracellular cAMP production. So that blocking the pathway reduces cAMP production.

References


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