Adenosine Biosynthesis in the Collecting Duct

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ABSTRACT

Adenosine regulates tubular transport in collecting ducts (CDs); however, the sources of adenosine that modulate ion transport in CDs are unknown. 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 steps occurring in the extracellular compartment. The goal of this study was to assess whether the extracellular cAMP-adenosine pathway exists in CDs. Studies were conducted in both freshly isolated CDs and in CD cells in culture (first passage) that were derived from isolated CDs. Purity of CDs was confirmed by microscopy, by Western blotting for aquaporin-1, aquaporin-2, BSC-1 and TSC and by RT-PCR for adenosine receptors. Both freshly isolated CDs and CD cells in culture converted exogenous cAMP to AMP and adenosine. In both freshly isolated CDs and CD cells in culture, conversion of cAMP to AMP and adenosine was affected by a broad-spectrum phosphodiesterase inhibitor (3-isobutyl-1-methylxanthine), an ecto-phosphodiesterase inhibitor (1,3-dipropyl-8-p-sulfophenylxanthine) and a blocker of ecto-5’-nucleotidase (alpha, beta-methylene-adenosine-5’-diphosphate) in a manner consistent with exogenous cAMP being processed by the extracellular cAMP-adenosine pathway. In CD cells in culture, stimulation of adenylyl cyclase increased extracellular concentrations of cAMP, AMP and adenosine, and these changes were also modulated by the aforementioned inhibitors in a manner consistent with the extracellular cAMP-adenosine pathway. In conclusion, the extracellular cAMP-adenosine pathway is an important source of adenosine in CDs.
Several lines of evidence indicate that adenosine importantly regulates ion transport in collecting duct (CD) epithelial cells: 1) A1 receptors are more highly expressed in CDs compared with other nephron segments (Smith et al., 2001); 2) In A6 cells, a model system for transport in the CD, and in collecting ducts adenosine receptor agonists alter ion transport (Edwards and Spielman, 1994; Hoenderop et al., 1998; Lang et al., 1985; Ma and Ling, 1996; Macala and Hayslett, 2002; Schwiebert et al., 1992; Yagil et al., 1994; Yagil, 1990); and 3) Administration of selective A1 receptor antagonists increases sodium excretion without enhancing potassium excretion (Kuan et al., 1993), a pharmacological profile consistent with a diuretic action in CDs. Although endogenous adenosine significantly modulates transport in CDs, the sources of endogenous adenosine that mediate this effect are unknown.

The **extracellular cyclic AMP-adenosine 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 (ecto-PDE) and ecto-5’-nucleotidase (ecto-5’-NT) (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). There is now substantial evidence for a physiological role of the extracellular cyclic AMP-adenosine pathway in glomerular mesangial cells, renal and non-renal vascular smooth muscle cells, cardiac fibroblasts, cerebral microvessels, cerebral cortex, hippocampus, adipocytes and hepatocytes (Jackson and Dubey, 2001).

An important, yet untested, hypothesis is that the extracellular cAMP-adenosine pathway is a significant source of adenosine for CD epithelial cells. Activation of some types of hormone receptors
- for example dopamine type 1 receptors (Felder et al., 1993), beta_1-adrenoceptors (Hanson and Linas, 1995) and PTH receptors (Reshkin et al., 1991) - residing on apical and/or basolateral membranes of tubular epithelial cells stimulates adenylyl cyclase and increases intracellular levels of cAMP. Egress of cAMP into the tubular lumen (across apical membrane) and/or interstitial space (across basolateral membrane) during activation of adenylyl cyclase could result in adenosine formation, provided that CD epithelial cells transport cAMP and express ecto-phosphodiesterase and ecto-5’nucleotidase.

To test the hypothesis that the extracellular cAMP-adenosine pathway exists in CD epithelial cells, we examined the ability of freshly isolated CDs and CD 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. Finally, we examined whether 3-isobutyl-1-methylxanthine [IBMX; a PDE inhibitor that penetrates cell membranes (Beavo and Reifsnyder, 1990)], alpha, beta-methyleneadenosine-5'-diphosphate [AMPCP; an ecto-5'-NT inhibitor (Zimmermann, 1992)] and 1,3-dipropyl-8-p-sulfophenylxanthine [DPSPX; a xanthine that inhibits only ecto-PDE because it is restricted to the extracellular compartment and cannot gain access to intracellular PDE (Tofovic et al., 1991)] alter the conversion of exogenous and endogenous cAMP to AMP and adenosine.
METHODS

Isolation of Defined Nephron Segments. Kidneys were harvested from rats, placed in ice-cold L-15 medium, and halved longitudinally. L-15 was bubbled with 100% oxygen during all steps of the procedure. The cortex and outer and inner medulla were obtained, placed in separate dishes in ice-cold L-15 medium and cut into pieces. Samples were incubated at 37°C in 0.2% collagenase V in L-15 with shaking. Every 15 minutes, the supernatant was collected, centrifuged and poured back into the original tube and the pellet was collected and washed three times with 5 ml of ice-cold L-15 medium. This procedure was repeated until the original tissue was completely digested. The digest from the cortex and outer medulla were placed in 10 ml of ice-cold 45% Percoll solution, and the inner medulla was placed in 10 ml of ice-cold 35% Percoll solution. After mixing, suspensions of tubules were centrifuged at 10,000xg for 15 minutes at 4°C. After centrifugation, top bands from the cortex, outer medulla and inner medulla were collected, washed in 10 ml of ice-cold L-15 and suspended in 40%, 40% and 30% ice-cold Percoll solution, respectively. After mixing, the suspensions of tubules were centrifuged at 10,000g for 15 minutes at 4°C. Lower bands from the cortex and inner medulla were collected and washed four times with 10 ml of ice-cold L-15 medium. These fractions provide highly purified proximal convoluted tubules (PCTs) and inner medullary CDs, respectively. The top band from the outer medulla was resuspended in 35% ice-cold Percoll, mixed and centrifuged at 10,000g for 15 minutes at 4°C. The lower band was collected and washed four times with 10 ml of ice-cold L-15 medium. This fraction provide highly purified medullary thick ascending limbs (TALs).

Culture of CD Epithelial Cells. Freshly isolated CDs were washed in phosphate-buffered saline (PBS) without calcium and magnesium and incubated for 15 minutes with collagenase type IV (1 mg/ml in 5 ml of DMEM F12) in a shaking water bath at 4°C. Ten ml of DMEM F12 with 10% fetal calf serum (FCS) was added, and the sample was centrifuged. Pellets were re-suspended in
DMEM F12 with 10% FCS, and 1 ml of the suspension was added to 75 cm² flasks. Before adding cells, culture flasks were pre-conditioned by incubating with FCS for 30 minutes. The medium was changed after two days. After four days the cells were detached with tyrpsin/EDTA, washed and plated with DMEM F12 with 10% FCS. All experiments were performed in CD cells in first passage.

**Confirmation of Identity of CDs.** The identity of freshly isolated CDs was confirmed by Western blotting and by RT-PCR. With regard to Western blotting, we examined the putative CD segments for: 1) lack of expression of thiazide sensitive transporter (TSC), a protein expressed specifically in distal convoluted tubules (DCTs) (Knepper and Brooks, 2001); 2) lack of expression of bumetanide sensitive transporter type 1 (BSC-1), a protein expressed specifically in TALs (Knepper and Brooks, 2001); 3) lack of expression of aquaporin-1, a protein highly expressed in PCTs (Nielsen et al., 2002) but not expressed in CDs (Nielsen et al., 2002); and 4) the expression of aquaporin-2, a protein expressed specifically in CDs (Nielsen et al., 2002). In these experiments, we included in the Western blots positive controls for aquaporin-1 [purified (see above) freshly isolated PCTs], BSC-1 [purified (see above) freshly isolated TALs] and TSC (impure isolated tubules obtained as a by-product of the purification procedure described above that are enriched in DCTs).

With regard to RT-PCR-based identification of freshly isolated CDs, we compared the expression of mRNA for A₁, A₂A, A₂B and A₃ receptors in freshly isolated CDs, TALs and PCTs. In this regard, A₁ receptors are more highly expressed in CDs compared with other nephron segments (Smith et al., 2001) and it is likely that CDs express a unique profile of adenosine receptors relative to other nephron segments.

Since the CD cells in culture were derived from highly purified freshly isolated CDs, it was unnecessary to characterize those cells as stringently as the freshly isolated CDs. Nonetheless, we performed Western blots on CD cells in culture for aquaporin-2, BSC-1 and TSC.
**Western Blotting.** Electrophoresis, Western blotting and immunostaining were performed as previously described (Jackson et al., 2002). Antibodies (anti-BSC-1, anti-TSC, anti-aquaporin-1 and anti-aquaporin-2) were kindly provided by Dr. Mark Knepper.

**Extraction of RNA and RT-PCR.** Extraction of RNA and RT-PCR was performed as previously described (Jackson et al., 2002) and using the primer sequences listed in Table 1.

**Studies in Freshly Isolated CDs.** Freshly isolated CDs (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 in the absence of and presence of IBMX (1 mmole/liter), AMPCP (0.1 mmole/liter), and DPSPX (1 mmole/liter). The concentrations of various inhibitors were based on our previous published experience with inhibition of exogenous cAMP metabolism in cultured cardiac fibroblasts and vascular smooth muscle cells (Dubey et al., 1996; Dubey et al., 2000). Just before adding the CDs to the L-15 medium, the L-15 medium was bubbled with 100% oxygen. Just after adding the isolated CDs, the head space of the capped tubes was flushed with 100% oxygen. After 20 minutes of incubation, the isolated CDs 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 0.5N NaOH, and protein content was measured by the copper bicinchoninic acid method.

**Studies in CD Epithelial Cells in Culture.** Monolayers of CD cells in culture were grown in 12-well culture plates as described (see above). On the day of the experiment, the CD cells were washed with PBS and then incubated (37°C under 5% carbon dioxide in cell incubator) with various treatments. Some CD cells were incubated for 0, 5 or 30 minutes with either 0, 10, 30 or 100 μmoles/liter of cAMP in PBS. In cells treated with 0, 10, 30 or 100 μmoles/L of cAMP for “0” minutes, the PBS containing cAMP was added to the cells and then immediately removed. Other CD
cells were treated with 100 µmoles/L of cAMP for 30 minutes in the presence and absence of IBMX, DPSPX and AMPCP. In addition, some CD cells were treated for 30 minutes with stimulators of adenylyl cyclase and in the presence or absence of IBMX, DPSPX and AMPCP. Adenylyl cyclase was stimulated by treating the cells with a mixture of forskolin (10 µmoles/liter; direct activator of adenylyl cyclase), parathyroid hormone (1 µmoles/liter), isoproterenol (1 µmoles/liter; beta-adrenoceptor agonist) and fenoldapam (10 µmoles/liter; agonist of dopamine type 1 receptors). In our cell culture system, we did not know which receptors were both expressed and coupled to stimulate adenylyl cyclase. Therefore, we used the strategy of adding several agonists of adenylyl cyclase simultaneously. At the end of the specified incubation time, the conditioned PBS 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 0.5N NaOH, and protein content was measured by the copper bicinchoninic acid method.

**Analysis of Purines.** In experiments using exogenous cAMP, purines were measured by HPLC using UV absorption as previously described (Jackson et al., 1996). In experiments with adenyly cyclase activators, cAMP and adenosine concentrations were measured by HPLC using fluorescence detection (higher sensitivity relative to UV method) as previously described (Jackson et al., 1996) and AMP was measured by HPLC using UV absorption.

**Statistical Analysis.** Data were analyzed by 1-factor or 2-factor analysis of variance followed by a Fisher’s Least Significant Difference test. The criterion of significance was P<0.05. Results are presented as mean ± SEM.
RESULTS

**Confirmation of Identity of CDs.** Figure 1A illustrates a light micrograph of freshly isolated CDs. Figures 1B, 1C, 1D and 1E are Western blots using antibodies against aquaporin-1, aquaporin-2, BSC-1 and TSC, respectively. A clear signal for aquaporin-1, BSC-1 or TSC was not detected in freshly isolated CDs. The lack of signal in freshly isolated CDs was not due to an insensitive detection method because freshly isolated PCTs, TALs and DCTs tested strongly positive for aquaporin-1, BSC-1 and TSC, respectively. Importantly, freshly isolated CDs gave a strong signal for aquaporin-2, whereas freshly isolated PCTs and TALs did not. This expression profile was consistent with the conclusion that the freshly isolated tubules used in the present study were indeed CDs (Knepper and Brooks, 2001; Nielsen et al., 2002).

Figure 2A, 2B, 2C and 2D are ethidium bromide-stained agarose gels of RT-PCR products. Freshly isolated CDs yielded a strong signal for the 790 base-pair amplicon expected for the A1 receptor mRNA, whereas freshly isolated PCTs and TALs did not (Figure 2A). Freshly isolated CDs also provided a signal for the 615 base-pair amplicon expected for the A2A receptor mRNA; however, the signal was stronger for freshly isolated PCTs (Figure 2B). Freshly isolated CDs did not demonstrate expression of A2B receptor (1281 base-pair amplicon) or A3 receptor (640 base-pair amplicon) mRNA (Figures 2C and 2D, respectively), whereas freshly isolated PCTs clearly expressed A3 receptor mRNA. This expression profile was consistent with the conclusion that the freshly isolated tubules used in the present study were CDs since CDs are known to express more A1 receptors than other nephron segments (Smith et al., 2001) and since the profile of expression of adenosine receptors was unique compared with the other nephron segments.

Figure 3A illustrates a light micrograph of CD cells in culture. Figures 1B, 1C and 1D are Western blots using antibodies against aquaporin-2, BSC-1 and TSC, respectively. A clear signal for
aquaporin-2, but not BSC-1 nor TSC, was detected in CD cells in culture. This expression profile was consistent with the conclusion that the cells in culture used in the present study were indeed CD cells (Knepper and Brooks, 2001; Nielsen et al., 2002).

Studies in Freshly Isolated CDs. Figure 4 illustrates the concentration-dependent (0 to 100 µmoles/liter) conversion of exogenous cAMP to AMP (Figure 4A), adenosine (Figure 4B) and inosine (Figure 4C) in freshly isolated CDs. Addition of increasing concentrations of cAMP to CDs caused corresponding increases in the levels of AMP, adenosine and inosine (a metabolite of adenosine).

Figure 5 summarizes the effects of inhibitors of PDE (IBMX), ecto-PDE (DPSPX) and ecto-5'-NT (AMPCP) on conversion of exogenous cAMP (30 µmoles/liter) to AMP (Figure 5A), adenosine (Figure 5B) and inosine (Figure 5C) in freshly isolated CDs. IBMX and DPSPX decreased levels of AMP, adenosine and inosine, and AMPCP decreased levels of adenosine and inosine, but increased levels of AMP.

Studies in CD Epithelial Cells in Culture. Figure 6 illustrates conversion of exogenous cAMP to AMP (Figure 6A) and adenosine (Figure 6B) as a function of time (0, 5 and 30 minutes) and cAMP concentration (0, 10, 30 and 100 µmoles/liter). Addition of increasing concentrations of cAMP to CD cells caused corresponding increases in the levels of AMP. The increase in AMP after 30 minutes of incubation with cAMP was approximately the same as that obtained after 5 minutes of incubation, indicating rapid attainment of steady state with respect to AMP. In contrast, adenosine levels continued to increase with time as adenosine accumulated in the medium. Inosine levels were below the detection limit of our assay. As with freshly isolated CDs, the accumulation of AMP in the medium following addition of exogenous cAMP was concentration-dependent; however, the formation of adenosine appeared to saturate at concentrations of cAMP less than 30 µmoles/liter.

Figure 7 summarizes the effects of inhibitors of PDE (IBMX), ecto-PDE (DPSPX) and ecto-
5′-NT (AMPCP) on conversion of exogenous cAMP (100 µmoles/liter) to AMP (Figure 7A) and adenosine (Figure 7B) in CD cells in culture. Inosine levels were below the detection limit of our assay. IBMX and DPSPX decreased levels of AMP and adenosine and AMPCP decreased levels of adenosine, but tended to increase levels of AMP.

CD cells in culture were markedly responsive to activators of adenylyl cyclase (Figure 8). In this regard, stimulation of adenylyl cyclase increased extracellular levels of cAMP, particularly in the presence of IBMX, DPSPX and AMPCP (Figures 8B, 8C and 8D, respectively). Stimulation of adenylyl cyclase also increased extracellular levels of AMP (Figure 8E), a response that was blocked by IBMX (Figure 9F) and DPSPX (Figure 9G). AMPCP increased baseline levels of AMP and augmented the increase in AMP induced by stimulation of adenylyl cyclase (Figure 9H).

As shown in Figure 9A, stimulation of adenylyl cyclase in CD cells approximately doubled the extracellular levels of adenosine and this response was abolished by IBMX (Figure 9B) and DPSPX (Figure 9C). AMPCP interfered with the fluorescence-based HPLC assay for adenosine so we were unable to measure adenosine levels in CD cells treated with AMPCP.
DISCUSSION

Biosynthesis of adenosine occurs via at least four pathways. The transmethylation pathway is mediated by the enzyme SAH hydrolase and involves hydrolysis of S-adenosyl-L-homocysteine (SAH) to L-homocysteine and adenosine (Lloyd et al., 1988). The intracellular ATP pathway is activated when energy demand exceeds energy supply and is mediated by intracellular dephosphorylation of ATP to adenosine (Decking et al., 1997; Lloyd and Schrader, 1993; Schrader et al., 1977). The extracellular ATP pathway is activated by the release of adenine nucleotides from various cell types and is characterized by the metabolism of adenine nucleotides by ecto-enzymes (ecto-ATPases, ecto-ADPases, and ecto-5'-NT) that convert ATP to adenosine (Schwiebert and Kishore, 2001). This latter pathway is engaged when adenine nucleotides are released during sympathoadrenal activation, platelet aggregation and activation of cells by clotting factors, neutrophil interactions and catecholamines. The fourth mechanism of adenosine production is the cAMP-adenosine pathway, which involves conversion of cAMP to adenosine (Jackson and Dubey, 2001).

The cAMP-adenosine pathway is most amenable to physiological modulation by hormones (Jackson and Dubey, 2001). This mechanism has both intracellular and extracellular locations. Inside the cell, conversion of cAMP to AMP and AMP to adenosine is catalyzed via cytosolic PDE and cytosolic 5'-nucleotidase, respectively. In this case, adenosine thus formed reaches the extracellular space by way of facilitated transport. However, the biological significance of the intracellular cAMP-adenosine pathway is limited by the competition of cytosolic 5'-nucleotidase and adenylate kinase for AMP and by the competition of transport mechanisms with adenosine kinase for adenosine. Thus, the extracellular limb of the cyclic AMP-adenosine pathway is probably more important.

Many cell types express ecto-5'-NT, an enzyme that is tethered to the extracellular face of the plasma membrane via a lipid-sugar linkage and that efficiently metabolizes AMP to adenosine.
Since activation of adenylyl cyclase stimulates egress of cAMP into the extracellular space (Barber and Butcher, 1981; Rindler et al., 1978), provided sufficient levels of ecto-PDE exist, activation of adenylyl cyclase would lead to the extracellular metabolism of cAMP to AMP and hence to adenosine. Inasmuch as these biochemical processes would take place in a highly localized environment, adenosine formed by the extracellular cAMP-adenosine pathway would act in an autocrine/paracrine fashion to amplify, inhibit and/or expand the local response to hormonal stimulation of adenylyl cyclase. In this regard, modest increases in cAMP biosynthesis could give rise to significant concentrations of adenosine at the cell surface.

The vascular compartment of the kidney clearly expresses an extracellular cAMP-adenosine pathway. For example, infusion of cAMP into the isolated rat kidney causes a concentration-dependent enhancement in the renal secretion rates of AMP, adenosine and inosine, and the increases in AMP and adenosine secretion are attenuated by IBMX (PDE inhibitor) and DPSPX (ecto-PDE inhibitor) (Mi and Jackson, 1995). Moreover, AMPCP (ecto-5'-NT inhibitor) blocks the increases in adenosine, but not AMP, induced by cAMP in the isolated rat kidney (Mi and Jackson, 1995). Cultured preglomerular vascular smooth muscle cells (Jackson et al., 1997) and freshly isolated preglomerular microvessels (Jackson and Mi, 2000) also express the cAMP-adenosine pathway.

The results of the present study support the conclusion that renal epithelial cells in the CD express an extracellular cAMP-adenosine pathway. In this regard, addition of exogenous cAMP to freshly isolated CDs or CDs in culture results in the synthesis of AMP and adenosine. Moreover, the conversion of exogenous cAMP to AMP and adenosine is blocked by inhibition of PDE (with IBMX) and ecto-PDE (with DPSPX). Also, inhibition of ecto-5’-NT (with AMPCP) blocks the conversion of exogenous cAMP to adenosine while enhancing the accumulation of AMP.

In the present study, we examined the conversion of exogenous cAMP to extracellular purines.
Since cAMP is hydrophilic and does not penetrate lipid bilayers, the conversion of exogenous cAMP to extracellular adenosine most likely represents the results of transformations in the extracellular compartment. This conclusion is corroborated by the results with AMPCP and DPSPX. AMPCP inhibits ecto-5’-NT, but not cytosolic 5’-NT (Zimmermann, 1992), and DPSPX does not penetrate cell membranes (Tofovic et al., 1991). Thus, the ability of these inhibitors to attenuate the conversion of exogenous cAMP to extracellular adenosine and inosine is strong evidence for an extracellular site of cAMP metabolism. The ability of AMPCP to increase extracellular AMP levels is also consistent with an extracellular site of cAMP conversion to AMP. In this regard, if cAMP were converted to AMP intracellularly, inhibition of ecto-5’-NT would not prevent the further metabolism of AMP to adenosine and would not cause the accumulation of extracellular AMP.

Additional evidence for a functional extracellular cAMP-adenosine pathway is provided by the results with stimulation of adenylyl cyclase in CD cells in culture. Consistent with our hypothesis, activation of adenylyl cyclase causes accumulation of cAMP, AMP and adenosine in the extracellular medium. As expected, the increase in extracellular cAMP induced by stimulation of adenylyl cyclase is augmented by inhibition of PDE and ecto-PDE, whereas the increase in AMP and adenosine is blocked by inhibition of PDE and ecto-PDE. Moreover, the increase in AMP is enhanced by inhibition of ecto-5’-NT. This indicates that CD cells can: 1) synthesize large amounts of cAMP; 2) transport intracellular cAMP to the extracellular compartment; 3) convert extracellular cAMP to AMP; and 4) convert extracellular AMP to adenosine.

Importantly, inhibition of ecto-5’-NT augments the ability of stimulation of adenylyl cyclase to increase extracellular levels of cAMP. As demonstrated in the present study, CDs express high levels of adenosine A1 receptors, and it is well known that activation of A1 receptors inhibits adenylyl cyclase. Thus the ability of AMPCP to enhance the cAMP response suggests that A1 receptors are...
activated by endogenous adenosine to restrain adenylyl cyclase activity. Along these same lines, DPSPX is both an adenosine receptor antagonist (Tofovic et al., 1991) as well as an ecto-PDE inhibitor. Therefore, the enhanced cAMP response in the presence of DPSPX may be due to a combination of factors, i.e., inhibition of the degradation of extracellular cAMP, inhibition of adenosine formation and blockade of A₁ receptors.

The ability of CDs to support a cAMP-adenosine pathway has important implications for the role of adenosine in modulating transport in CDs. Activation of several types of hormone receptors in the kidney epithelium stimulates adenylyl cyclase and increases intracellular levels of cAMP (Felder et al., 1993; Hanson and Linas, 1995; Reshkin et al., 1991). This would increase the egress of cAMP across the apical membrane into the tubular lumen and across the basolateral membrane into the renal interstitial space (Barber and Butcher, 1981; Rindler et al., 1978). Conversion of cAMP to adenosine by the extracellular cAMP-adenosine pathway on the external face of apical and basolateral membranes of epithelial cells in CDs would provide adenosine in the local environment of adenosine receptors which would modulate the rate of tubular transport.

Exogenous cAMP added to monolayers of CD cells in culture would gain access mostly to the apical membrane. Thus the appearance of AMP and adenosine in the medium very quickly after adding exogenous cAMP to cultured CD cells indicates that apical membranes of CD cells express a functional extracellular cAMP-adenosine pathway. In contrast to monolayers of CD cells in culture, exogenous cAMP added to freshly isolated CDs would gain access first to the basolateral membrane and then diffuse more slowly via the partially open ends of the tubules to gain access to the apical membranes. In the present study, the appearance of AMP and adenosine after addition of exogenous cAMP to freshly isolated CDs was delayed by approximately 15 minutes (data not shown). This suggests that even in freshly isolated CDs, it is the apical membrane that converts cAMP to adenosine.
However, this inference must be evaluated by additional studies. A recent study in A6 distal nephron cells indicates that A$_1$ receptors on both the apical and basolateral membranes stimulate sodium transport (Macala and Hayslett, 2002); therefore, regardless of whether the conversion of cAMP to adenosine is restricted to the apical membrane or both occurs on both sides of CD cells, the adenosine so formed would have access to functional receptors.

It should be emphasized that this study applies only to the collecting duct. In this regard, it is highly likely that there are multiple sources of adenosine in the kidney and the sources of adenosine may differ quantitatively from site to site along the nephron. For example, recent evidence (Bell et al., 2003) indicates the release of ATP from basolateral membranes of macula densa cells. Released ATP could mediate signaling through P2 receptors; but it could also be another example of adenosine production from the hydrolysis of extracellular nucleotides playing a regulatory role, in this instance mediating tubuloglomerular feedback.

In summary, the present study indicates that inner medullary collecting ducts express an extracellular cAMP-adenosine pathway that provides adenosine to the cell surface whenever adenylyl cyclase is activated. It is conceivable that the extracellular cAMP-adenosine pathway importantly contributes to the regulation of epithelial transport in inner medullary collecting ducts.
REFERENCES


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FIGURE LEGENDS

Figure 1. Panel A: Light micrograph of freshly isolated collecting ducts. Panels B, C, D and E: Western blots of proteins extracted from freshly isolated proximal convoluted tubules (PCT), thick ascending limbs (TAL), collecting ducts (CD) and distal convoluted tubules (DCT) using antibodies against aquaporin-1 (Panel B), aquaporin-2 (Panel C), BSC-1 (Panel D) and TSC (Panel E).

Figure 2. RNA samples from freshly isolated proximal convoluted tubules (PCT; left three lanes), thick ascending limbs (TAL; middle three lanes) and collecting ducts (CD; right three lanes) were subjected to RT-PCR using primers to the A1 receptor (Panel A), A2A receptor (Panel B), A2B receptor (Panel C), A3 receptor (Panel D) and beta-actin (Panel E) mRNA. The primers and product sizes are given in Table 1. cDNA was run on an 1.2 % agarose gel and visualized with ethidium bromide staining.

Figure 3. Panel A: Light micrograph of collecting duct cells in culture (first passage). Panels B, C and D: Western blots of proteins extracted from collecting duct cells in culture using antibodies against aquaporin-2 (Panel B), BSC-1 (Panel C) and TSC (Panel D). Bracket indicates region on Western blot where signal from BSC-1 and TSC should be observed if expressed.

Figure 4. Line graph showing the concentration-dependent metabolism of cAMP to AMP (Panel A), adenosine (Panel B) and inosine (Panel C) in freshly isolated collecting ducts. “B” indicates basal levels. Values are means ± SEM of four to five different preparations. “a” indicates P<0.05 by Fisher’s LSD test compared with basal (B).
Figure 5 Freshly isolated collecting ducts were incubated with cAMP (100 µmoles/L) for 20 minutes with and without 3-isobutyl-1-methylxanthine (IBMX; 1 mmole/liter), 1,3-dipropyl-8-p-sulfophenylxanthine (DPSPX; 1 mmoles/liter) or alpha, beta-methyleneadenosine-5'-diphosphate (AMPCP; 0.1 mmoles/liter) and the amounts of AMP (Panel A), adenosine (Panel B) and inosine (Panel C), were determined. Values are means ± SEM of five preparations. “a” and “b” indicate P<0.05 by Fisher’s LSD test compared with cAMP and cAMP + AMPCP, respectively.

Figure 6. Line graph showing the time-dependent and concentration dependent metabolism of cAMP to AMP (Panel A) and adenosine (Panel B) in collecting duct cells in culture (first passage). Values are means ± SEM for three preparations. “a” indicates P<0.05 by Fisher’s LSD test compared with time control.

Figure 7. Collecting duct cells in culture (first passage) were incubated with cAMP (100 µmoles/liter) for 30 minutes with and without 3-isobutyl-1-methylxanthine (IBMX; 1 mmole/liter), 1,3-dipropyl-8-p-sulfophenylxanthine (DPSPX; 1 mmoles/liter) or alpha, beta-methyleneadenosine-5'-diphosphate (AMPCP; 0.1 mmoles/liter) and the amounts of AMP (Panel A) and adenosine (Panel B) were determined. Values are means ± SEM for three preparations. “a” and “b” indicate P<0.05 by Fisher’s LSD test compared with cAMP and cAMP + AMPCP, respectively.

Figure 8. Collecting duct cells in culture (first passage) were incubated with a mixture of forskolin (10 µmoles/liter; direct activator of adenylyl cyclase), parathyroid hormone (1 µmoles/liter), isoproterenol (1 µmoles/liter; beta-adrenoceptor agonist) and fenoldapam (10 µmoles/liter; agonist of dopamine type 1 receptors) for 30 minutes with and without 3-isobutyl-1-methylxanthine (IBMX; 1
mmole/liter), 1,3-dipropyl-8-p-sulfophenylxanthine (DPSPX; 1 mmoles/liter) or alpha, beta-methyleneadenosine-5'-diphosphate (AMPCP; 0.1 mmoles/liter) and the amounts of cAMP (Panels A, B, C and D) and AMP (Panels E, F, G and H) were determined. Values are means ± SEM for six preparations. “a” indicates comparison (P<0.05 by Fisher’s LSD test) between no adenylyl cyclase stimulation (-) versus with adenylyl cyclase stimulation (+) within a given treatment group. “b” indicates comparison (P<0.05 by Fisher’s LSD test) of the response with inhibitors with the response in the absence of inhibitors. AC, adenylyl cyclase. <DL, less than detection limit.

**Figure 9.** Collecting duct cells in culture (first passage) were incubated with a mixture of forskolin (10 µmoles/liter; direct activator of adenylyl cyclase), parathyroid hormone (1 µmoles/liter), isoproterenol (1 µmoles/liter; beta-adrenoceptor agonist) and fenoldapam (10 µmoles/liter; agonist of dopamine type 1 receptors) for 30 minutes without (Panel A) and with either 3-isobutyl-1-methylxanthine (IBMX; 1 mmole/liter; Panel B) or 1,3-dipropyl-8-p-sulfophenylxanthine (DPSPX; 1 mmoles/liter; Panel C) and the amounts of adenosine were determined. Values are means ± SEM for six preparations. “a” indicates comparison (P<0.05 by Fisher’s LSD test) between no adenylyl cyclase stimulation (-) versus with adenylyl cyclase stimulation (+) within a given treatment group. “b” and “c” indicate comparison (P<0.05 by Fisher’s LSD test) of the control response versus the response in the IBMX-treated and DPSPX-treated cells, respectively. AC, adenylyl cyclase.
Aquaporin-1 in Isolated Renal Tubules

Aquaporin-2 in Isolated Renal Tubules

BSC-1 in Isolated Renal Tubules

TSC in Isolated Renal Tubules
AQP-2 → 28 kDa

BSC-1

TSC
**A**

AMP

![Graph showing AMP levels over time](image)

**B**

ADENOSINE

![Graph showing ADENOSINE levels over time](image)
A. Control Cells

ADENOSINE (pmoles/mg protein)

0 100 200 300

AC Stimulation

- +

a,b,c

B. IBMX-Treated Cells

ADENOSINE (pmoles/mg protein)

0 100 200 300

AC Stimulation

- +

C. DPSPX-Treated Cells

ADENOSINE (pmoles/mg protein)

0 100 200 300

AC Stimulation

- +
Table 1  Adenosine receptor PCR primers and cDNA sizes.

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<th>Receptor</th>
<th>Accession Number</th>
<th>Primer</th>
<th>Nucleotides</th>
<th>Sequence 5’ to 3’</th>
<th>Product Size</th>
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