Extracellular Adenosine Stimulates Vacuolar ATPase–Dependent Proton Secretion in Medullary Intercalated Cells

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**ABSTRACT**

Acidosis is an important complication of AKI and CKD. Renal intercalated cells (ICs) express the proton pumping vacuolar H⁺-ATPase (V-ATPase) and are extensively involved in acid-base homeostasis. H⁺ secretion in type A intercalated cells (A-ICs) is regulated by apical vesicle recycling and stimulated by cAMP. In other cell types, cAMP is increased by extracellular agonists, including adenosine, through purinergic receptors. Adenosine is a Food and Drug Administration–approved drug, but very little is known about the effect of adenosine on IC function. Therefore, we investigated the role of adenosine in the regulation of V-ATPase in ICs. Intravenous treatment of mice with adenosine or agonists of ADORA2A and ADORA2B purinergic P1 receptors induced V-ATPase apical membrane accumulation in medullary A-ICs but not in cortical A-ICs or other IC subtypes. Both receptors are located in A-IC apical membranes, and adenosine injection increased urine adenosine concentration and decreased urine pH. Cell fractionation showed that adenosine or an ADORA2A or ADORA2B agonist induced V-ATPase translocation from vesicles to the plasma membrane and increased protein kinase A (PKA)–dependent protein phosphorylation in purified medullary ICs that were isolated from mice. Either ADORA2A or ADORA2B antagonists or the PKA inhibitor mPKI blocked these effects. Finally, a fluorescence pH assay showed that adenosine activates V-ATPase in isolated medullary ICs. Our study shows that medullary A-ICs respond to luminal adenosine through ADORA2A and ADORA2B receptors in a cAMP/PKA pathway–dependent mechanism to induce V-ATPase–dependent H⁺ secretion.

**Significance Statement**

Acidosis is an important complication of AKI and CKD. Renal intercalated cells (ICs) are major players in acid-base homeostasis via the proton pump, V-ATPase. V-ATPase activity is regulated by vesicle recycling, and cAMP induces its apical membrane accumulation. Adenosine increases cAMP in other cell types, but its effect on IC function is unknown. This study shows that adenosine stimulates V-ATPase–dependent proton secretion via the cAMP/PKA pathway through apical adenosine receptors in medullary ICs. Our results reveal a novel mechanism by which ICs respond to luminal agonists and provide a new biologic framework for a better understanding of the effect of adenosine-targeted therapeutics on kidney function.
proton secretion, and defective V-ATPase function in these cells leads to distal renal tubular acidosis.\textsuperscript{1–5} Although ICs play an integral part in maintaining blood pH within a very narrow viable range,\textsuperscript{1–5} the mechanisms by which these cells sense their extracellular environment to modulate their function still remain largely unknown.

The V-ATPase is composed of several subunits assembled into two domains: a transmembrane V\textsubscript{0} domain and a cytosolic V\textsubscript{1} domain.\textsuperscript{6,7} Certain V-ATPase subunits have multiple isoforms; subcellular localization of the V-ATPase is established through the assembly of a specific set of V-ATPase subunit isoforms. For example, subunits a4 and B1 are highly expressed in ICs. Proton secretion by A-ICs is regulated by V-ATPase recycling between subapical vesicles and the apical membrane.\textsuperscript{1,2,6,8–10} An increase in proton secretion correlates with an increase in apical V-ATPase accumulation, which leads to the formation and extension of apical microplicae.\textsuperscript{11–13}

Figure 1. Adenosine in vivo treatment induces V-ATPase apical membrane accumulation in A-ICs. Conventional IF labeling for the V-ATPase A subunit showed an increase in V-ATPase apical membrane labeling and a decrease in intracellular labeling in A-ICs from the inner stripe of the outer medulla in (B) adenosine-treated mice (1.5 \textmu mol/kg, 15 minutes; arrow) versus (A) saline-treated mice (arrow). Although the apical V-ATPase staining was brighter, the subapical region appeared darker after adenosine treatment, consistent with V-ATPase translocation from vesicles to the plasma membrane. Scale bars, 5 \textmu m. (C) Schematic representation of the criteria used to classify the cells after treatment. A-ICs that had V-ATPase intracellular (cytosolic and vesicular) staining were labeled “nonactivated” (\textminus), the ones that showed bright staining of V-ATPase in the apical membrane (and no or little intracellular staining) were named “activated cells” (++), and the ones that presented both patterns were labeled “partially activated cells” (+). (D) Percentage of the three populations of A-ICs after adenosine or saline treatments (n=10 animals per group). *P<0.05. (E and F) Confocal microscopy images showing the presence of A-ICs and the absence of other IC types in sections cut from the renal medulla. A-ICs were identified by their basolateral membrane AE1 (red) and apical V-ATPase labeling (green). Increased V-ATPase apical membrane labeling and decreased intracellular labeling were observed in medullary A-ICs after (F) adenosine treatment compared with (E) controls (ctr). Blue indicates the DNA marker 4\textsuperscript{,6}-diamidino-2-phenylindole (DAPI). Scale bars, 5 \textmu m.

Figure 2. Translocation of both a4 and B1 subunits to the apical membrane after adenosine in vivo treatment. Confocal Airyscan microscopy shows double labeling for (A and D) a4 (green) and (B and E) B1 (red). (C) In saline-treated mice, both subunits colocalize in intracellular vesicles located in the apical region of the A-ICs. Some B1 intracellular cytosolic staining is also seen throughout the cytoplasm. (F) In adenosine-treated mice, a4 and B1 colocalize in the apical membrane, which exhibits membrane protrusions known as microplicae (arrows). Note the presence of residual intracellular vesicles that are positive for a4 but not B1 in adenosine-treated mice. Blue indicates DAPI (4\textsuperscript{,6}-diamidino-2-phenylindole). Scale bars, 2.5 \textmu m.
This process is regulated by cAMP and protein kinase A (PKA).6,7,12,14–16

Here, we describe how the cAMP/PKA pathway participates in V-ATPase activation in A-ICs via purinergic signaling, a pathway that is gaining interest as a modulator of other renal functions.17–21 Deregulation of purinergic signaling is associated with renal pathologies.22–26 Purinergic receptors are present all along the renal tubule, and in the collecting duct, they contribute to the rapid, nonhormonal control of extracellular fluid volume via regulation of water and electrolyte transport in principal cells.17,20,25–29 However, there is limited knowledge on the purinergic regulation of the other major cell type of the collecting duct, the IC.

Renal epithelial cells release nucleotides, including ATP and ADP, and this process is activated by mechanical stimuli, ischemia, inflammation, and acute injury.23,25 These nucleotides are rapidly hydrolyzed by ectonucleotidases located along the renal tubule to produce adenosine in the luminal compartment. Adenosine activates purinergic P1 receptors: ADORA1 (A1), ADORA2A (A2A), ADORA2B (A2B), or ADORA3 (A3).20,29 Targeting adenosine receptors has strong prophylactic or therapeutic effects in murine models of AKI.23

Adenosine receptors are current targets for drug development, and adenosine itself has been used clinically since the 1940s.23,30–33 Purinergic modulators are being tested to treat kidney diseases.18,19,21,25,26 Given the role of adenosine receptors in cAMP signaling33 and the known role of cAMP/PKA in proton secreting cells, we set out to characterize the role of adenosine in the regulation of V-ATPase in ICs. Acidosis is a serious consequence of chronic and acute kidney malfunction. This study addresses critical mechanisms by which central players in the control of systemic acid-base balance, the ICs, respond to extracellular cues to modulate their function.

Figure 3. In situ PLA image shows that adenosine in vivo treatment induces the accumulation of assembled V-ATPases (B1 and a4 protein complexes) in the apical membrane. (A) In the control mouse, in situ PLA signal visualized by conventional wide-field microscopy (red) is detected in intracellular vesicles (arrows), but no signal is detected in the apical membrane. (B) In adenosine-treated mice, an intense reaction is shown in apical microvilli (arrows), with very little reaction in intracellular structures. Autofluorescence (green) was acquired to allow for the visualization of the entire tissue. Blue indicates DAPI (4′,6-diamidino-2-phenylindole). Scale bars, 10 μm; 5 μm in insets. (C) Changes in urine pH observed 15 minutes after saline or adenosine treatment (pHfinal–pHinitial=ΔpH per 15 minutes). Urine pH from saline-treated mice: initial pH =7.6±0.1 versus final pH =7.2±0.1; urine pH from adenosine-treated mice: initial pH =7.3±0.2 and final pH =6.3±0.3 (n=6 mice per group). *P<0.05.

Figure 4. Adenosine in vivo treatment induces V-ATPase redistribution from intracellular vesicles to the plasma membrane. Cell fractionation and Western blot analysis of the IC-specific V-ATPase a4 subunit in plasma membrane (Mem), cytoplasm (Cyt), and vesicle (Ves) fractions from kidney medulla of saline-treated (ctr), acidosis-treated (acid), and adenosine-treated (adeno) mice (1.5 μmol/kg, 15 minutes). To induce acidosis, mice were given NH4Cl in their drinking water. A significant increase in the amount of a4 located in the plasma membrane versus intracellular vesicles was observed after acidosis and adenosine treatments compared with control. Note that membrane-to-vesicle ratios were quantified for each sample, providing an internal normalization for each preparation. Ratios were then compared between samples (n=6 animals per group). *P<0.05 versus ctr.
RESULTS

In Vivo Adenosine Treatment Induces V-ATPase Apical Membrane Accumulation in Medullary A-ICs and Stimulates Urinary Acidification

Mice were injected with adenosine or saline alone. Here, we focused on the inner stripe of the outer medulla and the proximal inner medulla, which contain A-ICs and are devoid of bicarbonate-secreting pendrin-positive ICs. Immunofluorescence (IF) for the V-ATPase A subunit (V1 domain) showed labeling in the intracellular compartment of A-ICs in control mice (Figure 1A) as previously shown.1,34–37 In contrast, adenosine treatment induced V-ATPase apical accumulation in A-ICs (Figure 1B). A-ICs with V-ATPase intracellular staining were classified as “nonactivated” cells, the ones that showed bright apical membrane V-ATPase staining were named “activated cells,” and the ones that presented both patterns were labeled “partially activated cells” (Figure 1C). Adenosine induced a significant increase in the percentage of activated A-ICs and a decrease in the percentage of nonactivated A-ICs compared with control (Figure 1D). Double labeling for the anion exchanger AE1 and for V-ATPase was performed to confirm that the subtype of ICs in medullary kidney sections that respond to adenosine is A-ICs (Figure 1, E and F). In contrast, no detectable effect of adenosine was observed in V-ATPase localization in cortical A-ICs (AE1 positive) (Supplemental Figure 1) as well as pendrin-positive ICs (Supplemental Figure 2).

V-ATPase activity is regulated by assembly of the holoenzyme as well as by trafficking.7 Confocal Airyscan microscopy showed predominant colocalization of the B1 subunit (V1 domain), and the a4 subunit (V0 domain) in subapical vesicles in nonactivated A-ICs from control mice (Figure 2, A–C). Translocation of both a4 and B1 to the apical membrane was observed after adenosine injection (Figure 2, D–F). In contrast, many residual subapical vesicles were labeled for a4 but showed absent or weak B1 staining in activated A-ICs. Next, we applied an in situ proximity ligation assay (PLA) to kidney sections, which results in positive fluorescence signal only when a4 and B1 are very close together. This assay is, therefore, an indicator of V-ATPase assembly. Although an intracellular punctate staining was observed in nonactivated A-ICs, indicating assembled V-ATPase in vesicles (Figure 3A), a bright apical membrane staining occurred after adenosine treatment (Figure 3B). This indicates that assembled V-ATPases are located mainly at the plasma membrane in activated A-ICs. In addition, urine pH from adenosine-treated mice showed a significantly lower pH (Figure 3C).

Previous studies showed that A-ICs respond to systemic acidosis by accumulating the V-ATPase in their apical membrane.38–41 Here, as a positive control, we induced acidosis in mice, and we performed cell fractionation followed by Western blot of the renal medulla. Acidosis induced the redistribution of a4 from vesicle to plasma membrane fractions (Figure 4) and a decrease in urinary pH (Supplemental Figure 3). We observed a similar a4 membrane accumulation after in vivo adenosine treatment (Figure 4).

Immunogold electron microscopy (EM) labeling for the V-ATPase A subunit showed that, under control conditions, V-ATPase is mainly located in subapical vesicles of A-ICs, which exhibited very few and short microvilli (Figure 5A). In contrast, adenosine treatment induced V-ATPase relocalization into well developed apical microvilli (Figure 5B).
Moreover, the number of apical V-ATPase–associated gold particles per micrometer of membrane length (Figure 5C) and the length of microvilli (Figure 5D) significantly increased in adenosine-treated mice versus controls.

A2A and A2B Participate in Adenosine-Induced V-ATPase Membrane Accumulation

Adenosine receptor expression was analyzed by RT-PCR in EGFP+–ICs isolated by FACS from the kidney medulla (which mainly contains A-ICs) of B1-EGFP transgenic mice and in whole-kidney medulla extracts. A1, A2A, and A2B were detected in isolated medullary ICs (Figure 6A) (EGFP+). All adenosine receptors were detected in EGFP-negative cells, which correspond to all other medullary cell types, and in whole-medulla extracts. A1 decreases intracellular cAMP, whereas A2A and A2B are linked to Gs proteins and increase cAMP in other cell types. Because cAMP induces V-ATPase trafficking to the plasma membrane, we, therefore, investigated the role of A2A and A2B in regulating V-ATPase in A-ICs. IF studies using two antibodies recognizing different amino acid sequences for each receptor showed enrichment of both A2B (Figure 6B, Supplemental Figure 4) and A2A (Figure 6C, Supplemental Figure 5) in the IC apical membrane. No apical staining was observed after preincubation of the antibodies with their respective peptides (Supplemental Figures 4 and 5). Furthermore, there was an increase in urinary adenosine concentration after adenosine treatment (Figure 6D), indicating that injected adenosine reaches the luminal side of A-ICs.

Mice were then treated with agonists of A2B (BAY60–6583) or A2A (PSB0777), or saline. Both agonists induced V-ATPase apical accumulation in A-ICs, similar to the adenosine effect (Figure 7A). IF quantitative analysis revealed that both agonists increased the percentage of activated A-ICs and decreased the percentage of nonactivated A-ICs (Figure 7B). Cell fractionation studies of the renal medulla showed that both agonists induced the translocation of α4 from vesicles to the plasma membrane (Figure 7C). Immunogold EM studies revealed that these agonists induced an increase in V-ATPase–associated gold particles in the apical membrane of A-ICs (Figure 8, A and B). However, only the A2B agonist induced an increase in the apical membrane length (Figure 8).

Figure 6. A2A and A2B are located on the apical membrane of A-ICs, and adenosine treatment increases urinary adenosine concentration. (A) RT-PCR in EGFP+–ICs isolated by FACS from the medulla of B1-EGFP mice as well as in whole medulla (used as a positive control). A1, A2A, A2B, and A3 mRNAs were detected in EGFP-negative cells and whole-medulla extracts. A1, A2A, and A2B mRNAs were detected but A3 mRNA was not detected in isolated medullary ICs (EGFP+). No band or PCR product was detected in H2O and nRT (absence of reverse transcriptase) samples (used as negative controls). (B) Double labeling for A2B (red; using the Millipore antibody) and the V-ATPase A subunit (green). (C) Double labeling for A2A (red; using the Abcam antibody) and the V-ATPase A subunit (green). Both A2B and A2A are enriched in the apical membranes of A-ICs (arrows), which are identified by their positive labeling for the V-ATPase. Lower expression of both receptors was also observed in the apical membrane of principal cells (negative for the V-ATPase) and thick ascending limbs. Blue indicates DAPI (4,6-diamidino-2-phenylindole). Scale bar, 5 μm. (D) Adenosine in vivo treatment (1.5 μmol/kg, 15 minutes) induced a significant increase in urinary adenosine concentration normalized for osmolality compared with control (saline; n=7 mice per group). *P<0.05.
Figure 7. A2B and A2A agonist treatments in vivo induce V-ATPase apical membrane accumulation. (A) IF labeling for the V-ATPase A subunit in the kidney medulla of mice treated (1.5 μmol/kg, 15 minutes) with adenosine (adeno), A2B agonist (BAY60–6583; BAY), A2A agonist (PSB0777), or saline (ctr) imaged by conventional wide-field microscopy. Blue indicates DAPI (4',6-diamidino-2-phenylindole). Scale bars, 5 μm. (B) Quantification of the percentage of nonactivated (−), partially activated (+), and activated (++) A-ICs after the different treatments. A significant increase in the percentage of (++) A-ICs and a decrease in the percentage of (−) A-ICs were observed after adeno and agonist treatments (n=4 animals). *Comparison of (−) cells versus ctr (P<0.05); #comparison of (++) cells versus ctr (P<0.05). (C) Cell fractionation Western blot analysis of the IC-enriched V-ATPase a4 subunit in plasma membrane (Mem), cytoplasm (Cyt), and vesicle (Ves) fractions from the kidney medulla of mice treated in vivo with ctr, adeno, BAY, and PSB0777. A significant increase in the amount of a4 located in the plasma membrane versus intracellular vesicles was observed in all treatments compared with control. Note that membrane-to-vesicle ratios were quantified for each sample, providing an internal normalization for each preparation. Ratios were then compared between samples (n=5 animals per group). *P<0.05 versus ctr.
Adenosine Increases V-ATPase Proton Pumping Activity

To examine the effect of adenosine on V-ATPase proton pumping activity, we developed a functional intracellular pH (pHᵢ) assay in medullary EGFP⁺ cells using the pH-sensitive fluorescence dye (SNARF-5F-AM) (Figure 11A). pHᵢ increased when the solution was exchanged from 0 Na⁺ to NH₄Cl, and cells rapidly acidified after the solution was exchanged back to 0 Na⁺ (acid load). This was followed by an alkalinization toward control levels, showing Na⁺⁺- and bicarbonate-independent pHᵢ recovery (Figure 11B, inset). Adenosine increased the pHᵢ recovery rate compared with control (Figure 11, C and D), indicating V-ATPase activation. The V-ATPase-specific inhibitor, concanamycin A, prevented the adenosine-induced stimulation of pHᵢ recovery (Figure 11D). Altogether, these results indicate that, after an acid load induced by an NH₄Cl pulse, medullary ICs regulate their pHᵢ, and adenosine increases V-ATPase–dependent proton secretion in these cells.

DISCUSSION

Acidosis is a major comorbidity factor in AKI and CKD, and renal ICs play a major role in acid-base homeostasis. This study shows that extracellular adenosine stimulates V-ATPase–dependent proton secretion in medullary ICs via activation of the cAMP/PKA pathway through A2A and A2B located in their apical membrane. These results add adenosine to the list of extracellular “activators” of A-ICs, including aldosterone, angiotensin II, and bicarbonate.

Proton secretion by A-ICs is regulated by V-ATPase recycling between subapical vesicles and the apical membrane. In this study, we used for the first time a cell fractionation assay on whole-kidney medulla and EGFP⁺-ICs isolated from the medulla to characterize V-ATPase subcellular distribution. As a positive control, we showed V-ATPase membrane accumulation in acidicotic mice compared with controls, in agreement with the increased apical V-ATPase immunolabeling that was previously observed in ICs after acidosis. The direct effect of adenosine was shown in isolated EGFP⁺-ICs via activation of the cAMP/PKA pathway through A2A and A2B modulators. An enriched EGFP⁺ cell preparation was generated after FACS isolation (Figure 9A). Cell fractionation studies showed that adenosine (Figure 9, B–D) or the A2B (BAY60–6583) (Figure 9B) or A2A agonists (PSB0777) (Figure 9C) caused V-ATPase plasma membrane accumulation in ICs. The A2B (psb1115) (Figure 9B) and A2A (anr94) (Figure 9C) antagonists prevented the adenosine-induced V-ATPase membrane translocation. Moreover, a specific PKA inhibitor (mPKI) also blocked the adenosine-induced V-ATPase trafficking (Figure 9D).

Next, we analyzed PKA activation in medullary EGFP⁺-ICs by using an antibody that recognizes phosphorylated consensus motifs on PKA substrates. PKA activation was detected when ICs were exposed in vitro to the permeant analog of cAMP cpt-cAMP and adenosine (Figure 10A). Both the A2B (psb1115) and the A2A (anr94) antagonists prevented the adenosine-induced PKA activation. Furthermore, the A2A and A2B agonists induced PKA activation (Figure 10B).

Direct Effect of Adenosine in Isolated Medullary ICs

To determine whether the adenosine-induced V-ATPase membrane accumulation and microplicae growth that we observed in the intact animal were a direct effect or could have resulted from an indirect effect of adenosine, EGFP⁺-ICs isolated from the medulla were exposed in vitro to adenosine and A2B and A2A modulators. An enriched EGFP⁺ cell preparation was generated after FACS isolation (Figure 9A). Cell fractionation studies showed that adenosine (Figure 9B–D) or A2B agonists (BAY60–6583). (BAY) or the A2A agonist PSB0777 induced an increase in the density of V-ATPases at the plasma membrane. (C) However, only BAY induced the formation of apical microplicae and an associated increase in apical membrane length; >25 cells per group were analyzed. Data are expressed as means±SEM. *P<0.05 versus ctr.

**Figure 8.** In vivo treatment with both adenosine receptor agonists induces V-ATPase apical membrane accumulation, but only A2B activation induced the formation of microplicae in A-ICs. (A) Immunogold EM showing the V-ATPase A subunit labeled with 15-nm gold particles in A-IC from the inner stripe of the outer medulla. The V-ATPase is mainly located in the subapical cytoplasm and vesicles in control animals (ctr). Scale bar, 2.5 μm. (B) Treatments (1.5 μmol/kg intravenously, 15 minutes) with the A2B agonist BAY60–6583 (BAY) or the A2A agonist PSB0777 induced an increase in the density of V-ATPases at the plasma membrane. (C) However, only BAY induced the formation of apical microplicae and an associated increase in apical membrane length; >25 cells per group were analyzed. Data are expressed as means±SEM. *P<0.05 versus ctr.
treatment of rats with cAMP induces apical V-ATPase accumulation in A-ICs via PKA activation. In that previous report, V-ATPase labeling in B-ICs was not modulated by cAMP, in agreement with this study showing no effect of adenosine on pendrin-positive cells. Similarly, ANGII was reported to modulate V-ATPase trafficking in A-ICs but not B-ICs. In this study, cortical A-ICs also did not respond to adenosine. Whether the variable response to adenosine depends on the IC subtype or their location in the kidney remains an open question. Because A2A and A2B are located on the apical membrane, it is possible that a lower luminal adenosine concentration in the cortical versus medullary collecting duct is not sufficient to trigger a response in cortical ICs. Alternatively, each IC subtype was shown to express different sets of regulatory and scaffolding proteins, which could modulate their response to a single agonist.

PKA activity is linked to V-ATPase phosphorylation and/or its membrane recruitment or assembly in kidney cells and other tissues. We propose, therefore, that adenosine could induce the phosphorylation by PKA of one or more subunits of the V-ATPase or accessory proteins involved in its trafficking or assembly in medullary ICs. Our SNARF-based functional assay showed that the V-ATPase apical membrane accumulation induced by adenosine resulted in increased V-ATPase-dependent proton secretion. This was also indicated by urine acidification after adenosine injection. Future studies will be required to determine whether this urine acidification was solely due to increased proton secretion or if it was also secondary to modifications of the urine buffering capacity.

The increase in V-ATPase apical membrane density and the formation and elongation of apical microplicae induced by adenosine and an A2B agonist in medullary A-ICs are in agreement with our previous study showing a similar activating effect of luminal adenosine in epididymal clear cells. By contrast, A2A activation did not modify the morphology of the apical membrane, whereas an increased density of V-ATPase–associated gold particles at the membrane was still observed. These results suggest an incomplete activation of A-ICs via A2A compared with A2B. Further studies will be required to define the mechanisms underlying this difference in the response of medullary A-ICs to A2A or A2B activation.

Figure 9. Adenosine and A2A and A2B agonists induce the redistribution of V-ATPase from vesicles to plasma membrane in isolated EGFP⁺-ICs. (A) Representative pseudoblot of EGFP⁺-ICs isolated by FACS from the kidney medulla of B1-EGFP mice. (B) Cell fractionation Western blot analysis of the V-ATPase a4 subunit in plasma membrane (Mem), cytoplasm (Cyt), and vesicle (Ves) fractions from EGFP⁺-ICs incubated in vitro for 15 minutes with saline (ctr), adenosine (adeno; 600 μM), A2B agonist (BAY60–6583 [BAY]; 600 μM), and A2B antagonist (psb1151; 10 μM) together with adenosine. (C) EGFP⁺-ICs were incubated in vitro with ctr, adeno (600 μM), PSB0777 (600 μM), and A2A antagonist (anr94; 10 μM) together with adenosine. (D) EGFP⁺-ICs were incubated in vitro with ctr, adeno (600 μM), adenosine together with mPKI (10 μM), or mPKI alone. A significant increase in the amount of a4 located in the plasma membrane versus intracellular vesicles was observed in medullary ICs treated with adenosine and both agonists compared with control, and this effect was prevented by their respective antagonists and the PKA inhibitor mPKI (n=5). PE-A, phycoerythrin channel autofluorescence. *P<0.05 versus ctr.
Adenosine modulators are currently examined in the context of therapeutics. Adenosine is now recognized as a novel "immune checkpoint mediator" that interferes with antitumor immune responses. It is, therefore, clinically relevant to characterize the physiologic effects of adenosine on organ function. In particular, the protective effect of intrarenal adenosine through A2B and A2A receptors after kidney injury has gained significant attention over the past few years. Renal ischemia induces an increase in renal adenosine excretion, and renal adenosine levels are increased in diabetic nephropathy. This study provides evidence that adenosine has additional protective effects via activation of proton secretion by medullary A-ICs, which would contribute to reducing the acidosis that accompanies kidney malfunction. This will need to be considered in ongoing clinical studies that examine the effects of adenosine receptor modulators, such as A2B receptor antagonists, which are currently being tested for asthma and pulmonary disease treatments, as well as A2A receptor antagonists, which are in phase 1 clinical trial as cancer therapeutics.

In summary, this study shows that adenosine induces the trafficking of V-ATPase to the apical membrane of medullary A-ICs through activation of A2A and A2B receptors followed by stimulation of the cAMP/PKA pathway. Our results, thus, reveal a novel mechanism by which these cells respond to luminal purinergic agonists and provide a new biologic framework for a better understanding of the effects of future adenosine-targeted therapeutics on kidney function.

**CONCISE METHODS**

A complete description of the methods is in Supplemental Material.

**Animals**

Adult C57BL/6J wild-type male mice were purchased from Jackson Laboratories. In addition, B1-EGFP transgenic mice that express EGFP under the control of the ATP6V1B1 gene promoter were used. All procedures described were approved by the Massachusetts General Hospital Subcommittee on Research Animal Care and performed in accordance with the Massachusetts General Hospital Subcommittee on Research Animal Care and performed in accordance with The National Institutes of Health Guide for the Care and Use of Laboratory Animals. Animals were anesthetized (pentobarbital; 60 mg/kg intraperitoneally) and treated with saline solutions containing adenosine, agonists of ADORA2A or ADORA2B (1.5 μmol/kg body wt), or DMSO (0.1%). Acidosis was induced in mice by adding NH₄Cl in drinking water.

**IF and EM**

IF and EM immunogold were performed as previously described. IF images were acquired using a Bio-Rad Radiance 2000 confocal microscope, a Zeiss LSM800 confocal microscope equipped with Airyscan super-resolution capabilities, and Nikon Eclipse 90i epifluorescence microscope. Grids were examined in a JEOL1011 transmission electron microscope. The antibodies used are included in Supplemental Material.
RNA isolation, cDNA synthesis, and PCR were performed as described previously.\textsuperscript{42,66} The primers used are listed in Supplemental Material.

**Isolation of EGFP\(^+\)-ICs, Cell Fractionation, and Western Blot**

Isolation of EGFP\(^+\)-ICs from the inner stripe of the outer medulla and the proximal inner medulla of B1-EGFP mice was performed as previously described.\textsuperscript{42,45,67} Sorted EGFP\(^+\)-ICs were incubated in RPMI with different purinergic modulators (listed in Supplemental Material) for 15 minutes at 37°C. Cell fractionation of kidney medulla or EGFP\(^+\)-ICs was performed using a sequential centrifugation procedure.\textsuperscript{68–70}

Protein lysate preparation and immunoblotting procedures were performed as previously described.\textsuperscript{42,68–70}

**PLA**

*In situ* interactions were detected by using the PLA kit Duolink II.\textsuperscript{71} The probe anti-rabbit minus binds to the V-ATPase a4 subunit antibody, whereas the probe anti-mouse plus binds to the B1 subunit antibody.

**Adenosine Assay and Determination of Urine pH**

Adenosine levels were detected by using the BioVision kit. The pH was measured, immediately after urination, using pH microfiber paper strips.

**pH Measurements**

Proton secretion activity of ICs from B1-EGFP mice was measured using the pH sensitive dye, SNARF1, as previously described.\textsuperscript{53}

**Statistical Analyses**

Data were analyzed using one- or two-way ANOVA followed by a Tukey post hoc test. \( P < 0.05 \) was considered significant.

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