ONLINE SUPPLEMENT:

ADENOSINE ACTIVATES A2b RECEPTORS AND ENHANCES CHLORIDE SECRETION IN KIDNEY INNER MEDULLARY COLLECTING DUCT CELLS

Madhumitha Rajagopal and Alan C. Pao

Division of Nephrology, Department of Medicine, Stanford University, Stanford, California 94305

Short Title: Adenosine Induces Chloride Secretion in IMCD Cells

Corresponding Author:

Alan C. Pao, M.D.

Division of Nephrology, Department of Medicine, Stanford University

780 Welch Road, Suite 106

Palo Alto, CA 94304

Tel: (650) 721-2245; Fax: (650) 721-3161

Email: paoman@stanford.edu

EXPANDED METHODS

Ussing Chamber Measurements

Cell sheets were mounted between the Lucite half chambers of the Ussing chamber apparatus and bathed in Krebs-Henseleit solution (in mmol/L: 140 NaCl, 25 $NaHCO_3$, 5 KCl, 5 glucose, 2 $CaCl_2$, and 1 $MgCl_2$) and gassed with a mixture of 95% O_2 and 5% CO_2. Transepithelial voltage (V_{te}) across cell sheets was clamped to 0 mV, and a set voltage pulse of 1 mV was applied across cell sheets for 200 ms every 20 s. The short-circuit current (I_{sc}) and transepithelial resistance (R_{te}) across cell sheets were continuously recorded using Acquire and Analyze Software (Physiological Instruments, San Diego, CA).

Western Blot Analysis

Seventy micrograms of protein from mIMCD-K2 lysates were resolved by 12% SDS-PAGE and transferred to PVDF membranes (BioRad, Hercules, CA). The membranes were immunostained with 1 μg/ml of anti-A2b receptor antibody (Alpha Diagnostics International, San Antonio, TX) for detection of A2b receptor immunoreactive protein. Membranes were incubated with horseradish peroxidase conjugated-secondary antibody (Amersham Biosciences, Piscataway, NH) and processed, as described previously [1].

cAMP Assay

Intracellular cAMP levels were measured using the Parameter cAMP ELISA assay kit (R & D Systems, Minneapolis, MN) after the apical side of cell sheets were treated with 10^{-5} mol/L adenosine or vehicle control. Cell sheets containing ~ 10^6 cells were lysed, and cAMP concentrations were determined per manufacturer instructions.

REFERENCES

1. Pao AC, McCormick JA, Li H, Siu J, Govaerts C, Bhalla V, Soundararajan R, Pearce D. NH2 terminus of serum and glucocorticoid-regulated kinase 1 binds to phosphoinositides and is essential for isoform-specific physiological functions. *Am J Physiol Renal Physiol.* 2007;292:F1741-1750.
2. Hall DA, Varney DM. Effect of vasopressin on electrical potential difference and chloride transport in mouse medullary thick ascending limb of Henle's loop. *J Clin Invest.* 1980;66:792-802.
3. Kriz W, Koepsell H. The structural organization of the mouse kidney. *Z Anat Entwicklungsgesch.* 1974;144:137-163.
4. Mejia R, Sands JM, Stephenson JL, Knepper MA. Renal actions of atrial natriuretic factor: a mathematical modeling study. *Am J Physiol.* 1989;257:F1146-1157.
5. Dickinson H, Moritz K, Wintour EM, Walker DW, Kett MM. A comparative study of renal function in the desert-adapted spiny mouse and the laboratory-adapted C57BL/6 mouse: response to dietary salt load. *Am J Physiol Renal Physiol.* 2007;293:F1093-1098.
6. Daly JW, Padgett WL. Agonist activity of 2- and 5'-substituted adenosine analogs and their N6-cycloalkyl derivatives at A1- and A2-adenosine receptors coupled to adenylate cyclase. *Biochem Pharmacol.* 1992;43:1089-1093.
7. Peakman MC, Hill SJ. Adenosine A2B-receptor-mediated cyclic AMP accumulation in primary rat astrocytes. *Br J Pharmacol.* 1994;111:191-198.
8. Yaar R, Jones MR, Chen JF, Ravid K. Animal models for the study of adenosine receptor function. *J Cell Physiol.* 2005;202:9-20.
9. Zhou QY, Li C, Olah ME, Johnson RA, Stiles GL, Civelli O. Molecular cloning and characterization of an adenosine receptor: the A3 adenosine receptor. *Proc Natl Acad Sci U S A.* 1992;89:7432-7436.
10. Klotz KN. Adenosine receptors and their ligands. *Naunyn Schmiedebergs Arch Pharmacol.* 2000;362:382-391.
11. Ongini E, Dionisotti S, Gessi S, Irenius E, Fredholm BB. Comparison of CGS 15943, ZM 241385 and SCH 58261 as antagonists at human adenosine receptors. *Naunyn Schmiedebergs Arch Pharmacol.* 1999;359:7-10.
12. Moresco RM, Todde S, Belloli S, Simonelli P, Panzacchi A, Rigamonti M, Galli-Kienle M, Fazio F. In vivo imaging of adenosine A2A receptors in rat and primate brain using [11C]SCH442416. *Eur J Nucl Med Mol Imaging.* 2005;32:405-413.
13. Borrmann T, Hinz S, Bertarelli DC, Li W, Florin NC, Scheiff AB, Muller CE. 1-alkyl-8-(piperazine-1-sulfonyl)phenylxanthines: development and characterization of adenosine A2B receptor antagonists and a new radioligand with subnanomolar affinity and subtype specificity. *J Med Chem.* 2009;52:3994-4006.

Table S1. Affinity of adenosine receptor agonists and antagonists for the four classes of adenosine receptors.

	K_i values (nmol/L)				
Agonist/Antagonist	A1	A2a	A2b	A3	References
Adenosine	73	150	5100	6500	[6-9]
CPA	2.3	790	18,600	43	[10]
CGS21680	290	27	89,000	67	[10]
NECA	14	20	2400	6.2	[10]
DPCPX	3.9	130	1000	4000	[10]
ZM241385	255	0.8	50	>10,000	[11]
SCH442416	1111	0.048	>10,000	>10,000	[12]
PSB 603	>10,000	>10,000	0.568	>10,000	[13]

Figure S1

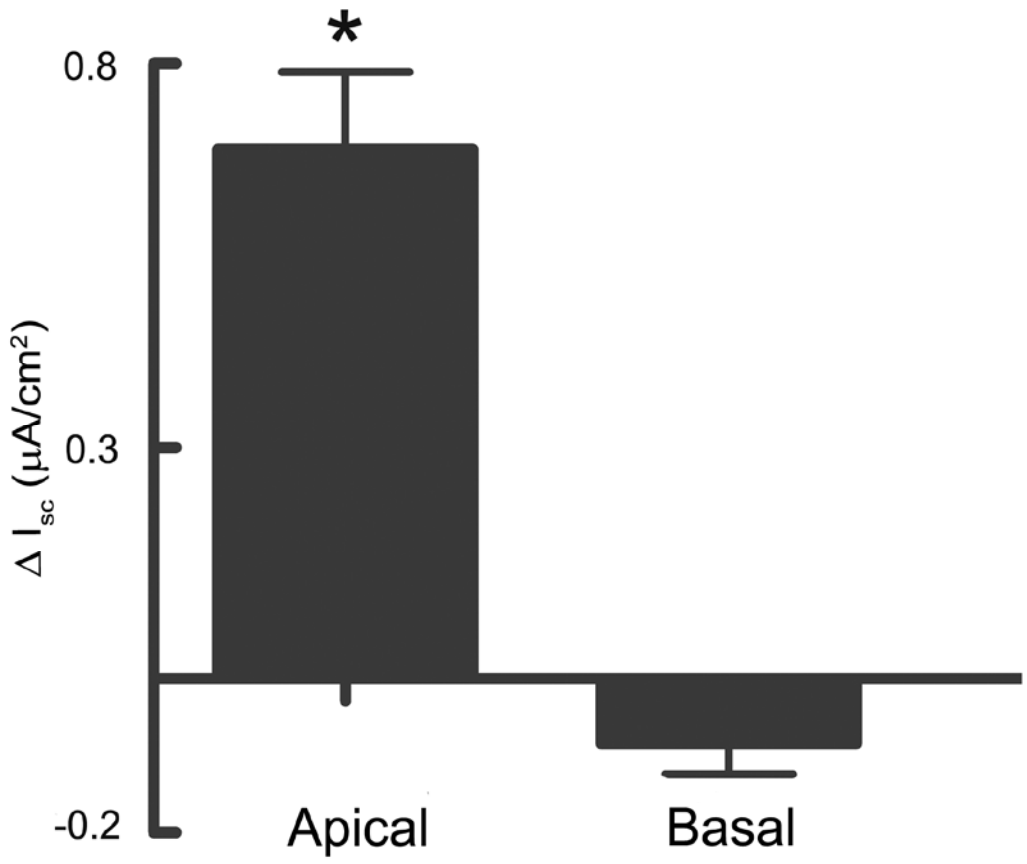

Figure S1. Sidedness of adenosine-induced I_{sc} across mIMCD-K2 cell sheets. Adenosine was added to either the apical or the basal side of paired cell sheets mounted in an Ussing chamber. ΔI_{sc}, change in I_{sc}. Values are mean ± SE (n = 13 filters). *P < 0.05.

Figure S2

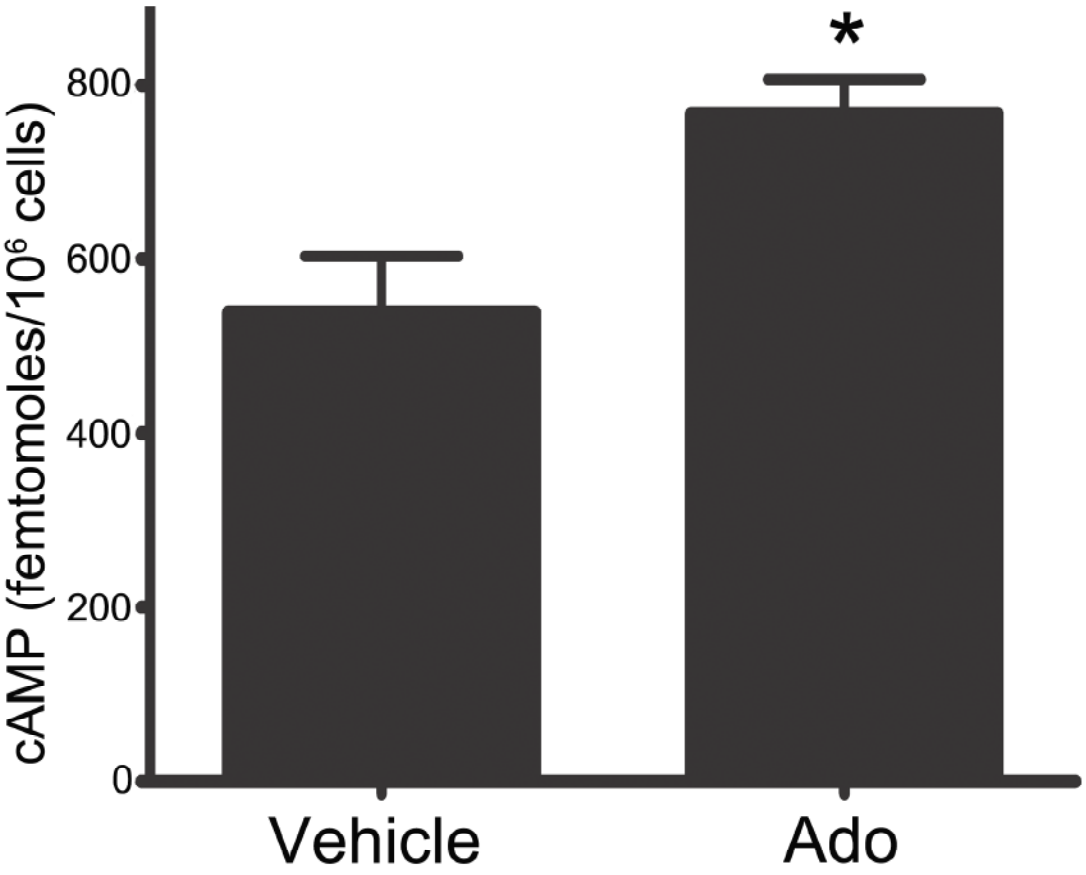

Figure S2. Effect of apical adenosine treatment on intracellular cAMP levels in mIMCD-K2 cells. Cell sheets treated with apical adenosine 10^{-5} mol/L (Ado) had significantly higher intracellular cAMP levels than cells treated with vehicle control. Values are mean ± SE (n = 6 cell sheets). *P <0.05.

Figure S3

Estimated surface area of mouse IMCD

Single collecting duct:
Internal diameter = 20 μm [2]
Length = 5.3 mm = 5300 μm [3]
$2\Pi rh = 2 \times \Pi \times 10\ \mu m \times 5300\ \mu m = 333{,}142.8\ \mu m^2 = 0.3331428\ mm^2$

The number of collecting ducts per kidney in a rodent model = 10,000 [4]

Surface area of mouse IMCD:
$0.3331428\ mm^2 \times 10{,}000$ collecting ducts x 2 kidneys = $6662.856\ mm^2$
$= 66.63\ cm^2$

Estimated Cl^- flux across mouse IMCD epithelium per day

Typical I_{sc} increase with 10^{-5} mol/L adenosine = 1.2 μA/cm^2
I_{sc} increase across mouse IMCD epithelium = 1.2 μA /cm^2 x 66.63 cm^2 = 80 μA
Using Faraday's constant, 26 μA = 1 μEq/hr, 80μA = 3.07 μEq/hr

3.07 μEq/hr x 24 hrs = 74 μEq of Cl^- secreted across mouse IMCD epithelium

Estimated Effect on Daily Fractional Excretion of Chloride (FE_{Cl})

Filtered Cl^- load in one day:
Serum [Cl^-] = 110 mEq/L = 110 μEq/mL
Creatinine clearance in a C57BL/6 mouse under high salt diet = 179 μl/min [5]
110 μEq/mL x 1 mL/1000 μL x 179 μL/min x 1440 min = 28,354 μEq Cl^- filtered

FE_{Cl} = 74 μEq of Cl^- secreted / 28,354 μEq Cl^- filtered = 0.261%

Figure S3. Mathematical modeling of the magnitude of adenosine-stimulated chloride flux extrapolated to the *in vivo* setting. Calculations listed are for the estimated adenosine-stimulated chloride flux across mouse IMCD epithelium and the estimated effect of adenosine-stimulated chloride secretion in the IMCD on daily fractional excretion of chloride (FE_{Cl}).