Regulation of Renal Ectophosphodiesterase by Protein Kinase C and Sodium Diet

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Received November 17, 2007; accepted January 24, 2008

ABSTRACT

Kidneys metabolize arterial cAMP to adenosine by the sequential actions of ectophosphodiesterase (cAMP → AMP) and ecto-5′-nucleotidase (AMP → adenosine). In this study, we demonstrated that etheno-AMP (fluorescent AMP analog) is nearly completely converted to etheno-adenosine during a single pass through the isolated, perfused rat kidney indicating that ecto-5′-nucleotidase is not rate limiting. Therefore, we examined the regulation of ectophosphodiesterase. In 17 control kidneys pretreated with α,β-methylene-adenosine-5′-diphosphate (inhibitor of ecto-5′-nucleotidase to prevent AMP metabolism; 100 μM), addition of cAMP (10 μM) to the perfusate increased renal venous AMP from 0.6 ± 0.2 to 3.5 ± 0.5 nmol/min/g. Pretreatment of kidneys with phorbol 12-myristate 13-acetate (protein kinase C activator; 7.5 nM) increased renal vascular resistance and significantly augmented the cAMP-induced increase in renal venous AMP (from 0.8 ± 0.2 to 5.2 ± 0.7 nmol/min/g with cAMP). Pretreatment of kidneys with bisindolylmaleimide I (protein kinase C inhibitor; 3 μM) abrogated the effects of phorbol 12-myristate 13-acetate on both renovascular resistance and cAMP conversion to AMP. Compared with kidneys from rats fed a high-sodium diet (3.15%) for 1 week, in kidneys from rats fed a low-sodium diet (0.03%) the conversion of cAMP to AMP was attenuated (high sodium, from 1.0 ± 0.1 to 4.6 ± 0.4 nmol/min/g with cAMP; low sodium, from 0.5 ± 0.04 to 2.6 ± 0.04 nmol/min/g with cAMP). We conclude that the renal vasculature efficiently converts AMP to adenosine and that metabolism of cAMP to AMP is rate limiting and regulated acutely by protein kinase C and chronically by sodium intake.

The three classic biochemical mechanisms leading to extracellular adenosine are the intracellular ATP pathway (dephosphorylation of intracellular adenine nucleotides to adenosine followed by adenosine transport) (Schrader, 1991), the extracellular ATP pathway (dephosphorylation of extracellular adenine nucleotides to adenosine by ectoenzymes) (Gordon, 1986; Vallon, 2008), and the transmethylation pathway (the hydrolysis of S-adenosyl-l-homocysteine to l-homocysteine and adenosine by the enzyme S-adenosyl-l-homocysteine-hydrolase followed by adenosine transport) (Lloyd et al., 1988). We hypothesize yet another pathway to extracellular adenosine formation, i.e., the cAMP-adenosine pathway, that may provide fine-tuned hormonal modulation of adenosine levels by hormones (Jackson, 1991, 1997, 2001; Jackson and Dubey, 2001, 2004). Our hypothesis is that activation of adenylyl cyclase stimulates the cAMP-adenosine pathway, which has intracellular and extracellular sites of adenosine biosynthesis. The intracellular pathway is mediated by metabolism of cAMP to AMP and AMP to adenosine via cytosolic phosphodiesterase (PDE) and cytosolic 5′-nucleotidase (5′-NT), respectively, and the adenosine formed intracellularly would reach the extracellular space via facilitated transport. However, the quantitative impact of the intracellular cAMP-adenosine pathway would be reduced by the competition of cytosolic 5′-NT and adenylyl kinase for AMP and by the competition of nucleoside transport mechanisms with adenosine kinase for adenosine. Therefore, the extracellular arm of the cAMP-adenosine pathway may be quantitatively more significant.

We postulate that the extracellular cAMP-adenosine pathway is mediated by three spatially linked processes: efflux of cAMP, conversion of cAMP to AMP by ectophosphodiesterase (ecto-PDE), and hydrolysis of AMP to adenosine by ecto-5′-nucleotidase (ecto-5′-NT). Stimulation of adenylyl cyclase is consistently associated with efflux of cAMP into the extracellular compartment by an active transport mechanism (King and Mayer, 1974; Barber and Butcher, 1981), and ecto-5′-NT is a widely distributed enzyme tethered to the plasma membrane where cAMP breakdown is effectively arrested by adenosine kinase.
extracellular face of plasma membranes via a lipid-sugar linkage (Misumi et al., 1990; Zimmermann, 1992). Inasmuch as cAMP transport is robust and ecto-5'-NT efficiently metabolizes AMP to adenosine, stimulation of adenylyl cyclase would activate the extracellular metabolism of cAMP to AMP and hence to adenosine, provided that ecto-PDE is active. Because these reactions would take place in the quasisolid phase of the cell surface, this newly formed adenosine would then act in an autocrine or paracrine fashion to alter the local response to hormonal stimulation of adenylyl cyclase. In support of this concept, our studies demonstrate that hormone stimulation increases adenosine via the extracellular cAMP-adenosine pathway (Mi and Jackson, 1998; Dubey et al., 2000, 2001; Jackson et al., 2003, 2006).

Our investigations using the isolated, perfused rat kidney show that arterial cAMP causes a concentration-dependent increase in renal venous AMP and adenosine and that these increases in AMP and adenosine are inhibited by 1,3-isobutyl-1-methylxanthine (broad-spectrum PDE inhibitor) and 1,3-dipropyl-8-p-sulphophenylxanthine (ecto-PDE inhibitor at high concentrations), whereas the increases in adenosine, but not AMP, are blocked by α,β-methylene-adenosine-5'-diphosphate (AMPCP; ecto-5'-NT inhibitor) (Mi and Jackson, 1995). Thus, our previous experiments indicate that the isolated, perfused kidney contains both ecto-PDE and ecto-5'-NT activity available to arterially supplied cAMP. Because kidneys metabolize arterial cAMP to adenosine by the sequential actions of ectophosphodiesterase (cAMP → AMP) and ecto-5'-nucleotidase (AMP → adenosine), we decided to investigate the regulation of this pathway. In this regard, we first addressed the question as to whether ecto-5'-NT is rate limiting. Because ecto-5'-NT was not rate limiting, we next addressed the question of whether ecto-PDE activity could be acutely or chronically regulated. Reasoning by analogy, we chose to test the hypothesis that protein kinase C (PKC) or nitric oxide can acutely regulate renal ecto-PDE activity because previous studies demonstrated the acute regulation of ecto-5'-NT activity by protein kinase C (Kitakaze et al., 1995, 1997; Minamino et al., 1995; Siegfried et al., 1995) and nitric oxide (Siegfried et al., 1996; Ohata et al., 1998). We also decided to examine the effect of salt diet on renal ecto-PDE activity because sodium diet increases ecto-5'-NT activity in glomeruli (Satriano et al., 2006).

Materials and Methods

**Animals and Chemicals.** The experiments reported here used 111 adult male Sprague-Dawley rats obtained from Charles River (Wilmington, MA) that were housed at the University of Pittsburgh Animal Facility. All rats in protocols 1 to 6 were fed Prolab RMH 3000 (PMI Feeds, Inc., St. Louis, MO) containing 0.26% sodium and 0.82% potassium. Rats in protocol 7 were fed either a low-sodium (0.03%) or high-sodium (3.15%) diet (catalog numbers 5881 and 5883, respectively, from PMI Feeds). In addition, the rats on a low-sodium diet were treated with furosemide (100 mg/kg by gavage) for 3 days upon initiating the low-sodium diet to facilitate sodium depletion. All studies received prior approval by the University of Pittsburgh Animal Care and Use Committee. All chemicals were obtained from Sigma-Aldrich (St. Louis, MO) or obtained from Sigma-Aldrich (St. Louis, MO).

**Kidney Perfusion.** Rats were anesthetized (45 mg/kg sodium pentobarbital, i.p. injection), a midline incision was made, and the left kidney, left renal artery, abdominal aorta, and left ureter were dissected free from surrounding tissue. The left ureter was cannulated with polyethylene-10 tubing, the abdominal aorta below the left kidney was cannulated (polyethylene-50 tubing), the suprarenal aorta was ligated, and the left kidney was flushed with 2.5 ml/min oxygenated Tyrode’s solution containing 100 units/ml heparin. While maintaining perfusion, the left kidney was isolated and mounted in a water-jacketed organ chamber. The organ chamber was maintained at 37°C with a thermostatically controlled water circulation (Thermocirculator; Harvard Apparatus, South Natick, MA). Kidneys were perfused (5 ml/min) using a Harvard model 1310 peristaltic pump with Tyrode’s solution (composition: 137 mM NaCl, 2.7 mM KCl, 1.8 mM CaCl2, 1.1 mM MgCl2, 12 mM NaHCO3, 0.42 mM NaH2PO4, 5.6 mM d(+)-glucose) that was heated to 37°C with a warming coil, gassed with 95% O2 and 5% CO2, and passed through a bubble trap. A Statham pressure transducer (model P23ID; Statham Division, Gould Inc., Oxnard, CA) was connected to an access port located in the perfusion line immediately before the kidney so that perfusion pressure could be continuously monitored (Grass model 79D polygraph; Grass Instruments, Quincy, MA). The perfused kidneys were allowed to equilibrate for approximately 1.5 h before beginning an experiment.

**Protocol 1: Assessment of Ecto-5'-NT Activity.** After the equilibration period, five kidneys received increasing concentrations (0, 0.3, 0.8, 2.5, and 8.3 μM) of etheno-AMP in the perfusate. Each concentration was administered for 5 min, and venous perfusate was collected between 4 and 5 min into the administration of each concentration of etheno-AMP for subsequent analysis by high-pressure liquid chromatography with fluorescence detection of etheno-AMP and etheno-adenosine as described previously (Jackson et al., 1996).

**Protocols 2 to 6: Assessment of the Effects of Phorbol 12-Myristate 13-Acetate (Protocol 2), Phorbol 12-Myristate 13-Acetate + Bisindolylmaleimide I (Protocol 3), Angiotensin II (Protocol 4), Arginine Vasopressin (Protocol 5), and Acetylcholine (Protocol 6) on Ecto-PDE Activity.** After the equilibration period, kidneys received treatment with phorbol 12-myristate 13-acetate (PMA) (7.5 nM) (protocol 2), PMA (7.5 nM) + bisindolylmaleimide I (3 μM) (protocol 3), angiotensin II (Ang II) (10 nM) (protocol 4), arginine vasopressin (AVP) (10 nM) (protocol 5), or acetylcholine (1 μM) (protocol 6). Each protocol included a different group of control kidneys that were randomized into that protocol and that did not receive any of the aforementioned treatments. All kidneys were also treated with 100 μmol/l AMPCP, a specific inhibitor of ecto-5'-nucleotidase (Zimmermann, 1992), to inhibit the metabolism of AMP to adenosine and thereby increase the AMP signal. Our previously published results demonstrate that this concentration of AMPCP blocks AMP metabolism to adenosine in the isolated, perfused rat kidney (Mi and Jackson, 1995). Approximately 10 min after adding the pharmacological agents, a 1-min sample of venous perfusate was collected. Next, cAMP (10 μM) was added to the perfusate, and 4 min later, the venous perfusate was again collected for 1 min. Per fusate was immediately placed on ice and then frozen at −40°C for later analysis of AMP by high-pressure liquid chromatography with UV detection as described previously (Jackson et al., 1996).

Within each protocol, the appearance of AMP in the renal venous perfusate was compared in the absence (basal) and presence of cAMP by two-factor analysis of variance in which one factor was cAMP treatment (two levels, 0 versus 10 μM), and the second factor was pharmacological treatment (two levels, none versus treated). If a significant interaction between cAMP and pharmacological treatment was detected, multiple comparisons were performed by a Fisher’s LSD test. All statistical analyses were performed using the Number Cruncher Statistical System (Kaysville, UT), and all values in the text and figures refer to means ± S.E.M.

**Protocol 7: Assessment of the Effects of Salt Diet on Ecto-PDE Activity.** In this protocol, animals were randomized to either a low-sodium (0.03%) or high-sodium (3.15%) diet for 1 week, and then kidneys were perfused, and cAMP was administered as de-
scribed above but without any pharmacological treatments (i.e., these kidneys were treated exactly like the control kidneys in protocols 2–6).

Results

Protocol 1: Assessment of Ecto-5’-NT Activity. As shown in Fig. 1, there was a linear relationship (slope = 0.65) between the concentration of etheno-AMP added in the arterial perfusate and the concentration of etheno-adenosine ($r^2 = 0.9995; p = 0.0002$) appearing in the renal venous perfusate.

Protocols 2 to 6: Assessment of the Effects of PMA (Protocol 2), PMA + Bisindolylmaleimide I (Protocol 3), Ang II (Protocol 4), AVP (Protocol 5), and Acetylcholine (Protocol 6) on Ecto-PDE Activity. The left panels of Figs. 2 to 7 demonstrate that the addition of exogenous cAMP to the isolated, perfused rat kidney caused a reproducible and substantial increase (approximately 3–4-fold) in renal venous AMP. Acute treatment of kidneys with PMA, a protein kinase C activator, significantly enhanced renal perfusion pressure (Fig. 2, right) and significantly enhanced the increase in renal venous AMP induced by arterial cAMP (Fig. 2, left). However, in kidneys treated with bisindolylmaleimide I to block protein kinase C, PMA no longer had any effect on either cAMP-induced AMP in the renal venous perfusate (Fig. 3, left) or renal perfusion pressure (Fig. 3, right). Similar to PMA, acute treatment of perfused kidneys with either Ang II or AVP induced an increase in renal perfusion pressure (right panels of Figs. 4 and 5, respectively). However, unlike PMA, neither Ang II nor AVP had any detectable effect on cAMP-induced AMP in the renal venous perfusate (left panels of Figs. 4 and 5, respectively). Acetylcholine also did not affect cAMP-induced AMP in the renal venous perfusate and did not affect renal perfusion pressure (Fig. 6). The lack of effect of acetylcholine on perfusion pressure was possibly due to the lack of vascular tone in our model system. To verify that the renal vasculature was indeed sensitive to acetylcholine, in four separate perfused kidneys, Ang II was substituted to test for renal vasodilatation. In this experiment, Ang II (3 nM) increased perfusion pressure from 36 ± 4 to 62 ± 5 mm Hg. Addition of acetylcholine (1 μM) to the perfusate reversed the increase in perfusion pressure caused by Ang II (from 62 ± 5 to 40 ± 4 mm Hg).

![Image](https://example.com/image.png)

**Fig. 1.** Line graph illustrating the relationship between the concentrations of etheno-AMP added in the arterial perfusate and the concentration of etheno-adenosine and etheno-AMP appearing in the renal venous perfusate.

Protocol 7: Assessment of the Effects of Salt Diet on Ecto-PDE Activity. As shown in Fig. 7, there was a striking and statistically significant difference in the ability of arterial cAMP to increase renal venous AMP in kidneys obtained from animals chronically on a high- versus a low-sodium diet. Perfusion pressures were significantly lower during the cAMP administration; however, this effect was quantitatively very small.

Discussion

The present findings suggest that renal vascular ecto-5’-NT is unlikely to be rate limiting in the pathway of cAMP conversion to adenosine. Etheno-AMP is a fluorescent analog of AMP that can be converted to etheno-adenosine by ecto-5’-NT (Jamal et al., 1988). When added to the arterial perfusate, the slope of the relationship between etheno-AMP in the arterial perfusate and etheno-adenosine in the renal venous perfusate was 0.65, suggesting that the majority (at least 65%) of etheno-AMP entering the renal vasculature was converted to etheno-adenosine during a single pass through the perfused kidney. The remaining 35% either escaped the vascular compartment (for example, by filtration or diffusion) and therefore was not measured in the venous perfusate or was converted to metabolites other than etheno-adenosine or was not metabolized. However, fluorescent peaks other than etheno-AMP and etheno-adenosine were not detected on the chromatograms, and etheno-AMP in the renal venous perfusate was near the limit of detection. These findings suggested that nearly all of the etheno-AMP that did not escape the vascular compartment was indeed metabolized to etheno-adenosine in a single pass through the kidney. Therefore, in the pathway from cAMP to adenosine, ecto-5’-NT is very unlikely to be rate limiting because the conversion of AMP to adenosine is extremely efficient in the perfused kidney. Therefore, the rate-limiting step is possibly ecto-PDE activity.

If ecto-PDE is rate limiting in the pathway form cAMP to adenosine in the renal vasculature, then it is important to determine whether ecto-PDE activity can be regulated. In renal epithelial cells (Siegfried et al., 1995) and in myocardial tissue (Kitakaze et al., 1995; Minamino et al., 1995), stimulation of protein kinase C leads to activation of ecto-5’-NT. Therefore, it is conceivable that protein kinase C also activates ecto-PDE. Indeed, the results of the present study demonstrate that PMA, a classic protein kinase C activator, does increase the efficiency of conversion of cAMP to AMP in the isolated, perfused kidney. It is important to note that the effect of PMA on cAMP conversion to AMP is abrogated by pretreatment with bisindolylmaleimide I, a classic protein kinase C inhibitor. This finding lends support to the conclusion that protein kinase C does indeed activate ecto-PDE activity.

However, one caveat is that PMA causes renal vasoconstriction, which confounds the interpretation because it is conceivable that the effects of PMA are not direct but rather are mediated indirectly by changes in perfusion pressure. This issue cannot be resolved by blocking protein kinase C because blockade of protein kinase C also impairs PMA-induced vasoconstriction. It is important to note that concentrations of both Ang II and AVP that cause renal vasoconstriction similar in magnitude to that caused by PMA do not increase ecto-PDE activity. These results argue against any...
contribution of vasoconstriction per se in mediating the effects of PMA on ecto-PDE activity in intact kidneys. Because Ang II and AVP are known to stimulate protein kinase C, it might be expected that these agents also would activate ecto-PDE. There are at least two possible explanations for the lack of effects of Ang II and AVP on ecto-PDE. First, Ang II and AVP act on vascular smooth muscle cells, and it is conceivable that the effect of PMA is mediated by endothelial cells, vascular fibroblasts, renal interstitial cells, or epithelial cells. Second, there are multiple isozymes of protein kinase C in renal tissues, and it is possible that specific isozymes might be involved in the regulation of ecto-PDE activity.

Fig. 2. Effect of cAMP (10 μmol/l) on renal venous AMP (left) and renal perfusion pressure (right) by isolated, perfused rat kidneys in the absence (n = 17) and presence (n = 17) of PMA (protein kinase C activator; 7.5 nM). Kidneys were pretreated with AMPCP (ecto-5'-nucleotidase inhibitor; 100 μmol/l). a, p < 0.05 compared with basal/no PMA; b, p < 0.05 compared with basal/PMA; c, p < 0.05 compared with cAMP/no PMA (Fisher's LSD test).

Bisindolylmaleimide I HCL

Fig. 3. Effect of cAMP (10 μmol/l) on renal venous AMP (left) and renal perfusion pressure (right) by isolated, perfused rat kidneys in the presence of bisindolylmaleimide I (protein kinase C inhibitor; 3 μM) and absence (n = 4) and presence (n = 5) of PMA (protein kinase C activator; 7.5 nM). Kidneys were pretreated with AMPCP (ecto-5'-nucleotidase inhibitor; 100 μmol/l).

Fig. 4. Effect of cAMP (10 μmol/l) on renal venous AMP (left) and renal perfusion pressure (right) by isolated, perfused rat kidneys in the absence (n = 6) and presence (n = 6) of Ang II (10 nM). Kidneys were pretreated with AMPCP (ecto-5'-nucleotidase inhibitor; 100 μmol/l).
kinase C, and Ang II and AVP may activate a different set of isozymes relative to PMA.

The cellular mechanism via which protein kinase C stimulates ecto-PDE activity is unknown; however, it is possible that the mechanism by which protein kinase C activates ecto-PDE is similar to the mechanism by which protein kinase C activates ecto-5'-nucleotidase. Two observations support this hypothesis. 1) Both ecto-5'-nucleotidase and ecto-PDE are ectoenzymes; therefore, both enzymes must be stimulated by a mechanism that allows intracellular protein kinase C to stimulate an ectoenzyme with activity in the extracellular aspect of the enzyme. 2) Kitakaze et al. (1995) report that the time frame in which protein kinase C stimulates ecto-5'-nucleotidase is 10 to 15 min, which is identical to the time frame observed in the present study for stimulation of ecto-PDE activity by PMA. Protein kinase C isozymes
rapidly traffic to the membrane upon stimulation; therefore, it is possible that activation of protein kinase C may phosphorylate integral membrane proteins involved in allosteric regulation of both ecto-5’-nucleotidase and ecto-PDE.

The physiological significance of regulation of ecto-PDE activity by protein kinase C is also unknown. In the heart, modulation of ecto-5’-NT activity by protein kinase C mediates the effects of α1-adrenoceptors on ecto-5’-NT activity (Kitakaze et al., 1995). It is of interest as to whether the same is true regarding ecto-PDE regulation by protein kinase C, and future studies are required to address this hypothesis.

Ecto-5’-NT activity in kidneys and hearts is not only regulated by protein kinase C but is also modulated by nitric oxide (Siegfried et al., 1996; Obata et al., 1998). Therefore, it is conceivable that ecto-PDE is also regulated by the nitric oxide pathway. However, the results of the present study show that acetylcholine, an activator of the vascular nitric oxide pathway in the isolated, perfused rat kidney (Vargas et al., 1994), does not affect ecto-PDE activity, at least in the short term.

In the long term, sodium intake is generally a powerful modulator of renal function and biochemistry. Moreover, Satriano et al. (2006) report that a high-sodium diet increases ecto-5’-NT activity in glomeruli. Therefore, it is of interest to determine whether sodium intake affects the efficiency of conversion of CAMP to AMP in the kidney. Indeed, it seems that all of the maneuvers tested in the current study, chronic changes in sodium intake have the greatest influence on renal vascular ecto-PDE activity. This could be important physiologically because adenosine can profoundly affect renin release, renovascular tone, and sodium reabsorption (Jackson, 1997, 2001; Vallon et al., 2006). However, additional studies are required to elucidate the physiological function of changes in ecto-PDE activity in relation to sodium intake.

The fact that protein kinase C activates ecto-PDE activity may explain the increase in ecto-PDE activity in animals on a high-sodium diet. In this regard, studies by Zhuang et al. (2000) demonstrate that NaCl increases total PKC activity and induces PKCa, PKCβ, and PKCε translocation from the cytosol to the membrane in NIH 3T3 cells. These findings suggest that hyperosmotic conditions associated with high salt intake may activate both conventional and novel protein kinase C. Inasmuch as protein kinase C activates ecto-PDE, it is conceivable that the mechanism by which a high-salt diet increases ecto-PDE activity is via protein kinase C.

The molecular identity of ecto-PDE remains unknown and is not the subject of the current investigation. However, we recently investigated this issue (Jackson et al., 2007). In this regard, we tested, using an array of pharmacological inhibitors, whether ecto-PDE activity in the kidney is pharmacologically similar to one or more of the known 11 families of PDEs. Our findings excluded the possibility that PDE1, PDE2, PDE3, PDE4, PDE5, PDE6, PDE7, PDE9, PDE10, or PDE11 contribute to ecto-PDE activity in the kidney but were consistent with a possible role of PDE8. More recently, we have detected strong expression of PDE8 mRNA and protein in glomerular vascular smooth muscle cells (E.K. Jackson, unpublished data), so this hypothesis remains viable, but unproven.

In summary, we conclude that the renal vasculature efficiently converts AMP to adenosine and that metabolism of cAMP to AMP is rate limiting and regulated acutely by protein kinase C and chronically by sodium intake. Additional studies are required to elucidate the molecular identity of ecto-PDE and to determine the physiological significance of regulation of the enzyme by protein kinase C and sodium diet.

References
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