Renal Phosphodiesterase 4B Is Activated in the Dahl Salt-Sensitive Rat

Urmila Tawar, Kumar Kotlo, Shilpa Jain, Sagar Shukla, Suman Setty, Robert S. Danziger

Abstract—Reduced β-adrenergic signaling is associated with increased sympathoadrenal activity in hypertensive patients and animal models of hypertension. However, the mechanism that accounts for this characteristic decline in β-adrenergic signaling is unclear. In the present study, we investigated renal phosphodiesterase 4B, which metabolizes cAMP. Immunoblot analysis detected only the phosphodiesterase 4B isoform present in kidney tissue from spontaneously hypertensive rats, hypertensive Dahl salt-sensitive (SS) rats, and Dahl salt-resistant rats. The phosphorylated (activated) form of the protein was present at 2-fold greater levels in Dahl SS rats than in spontaneously hypertensive rats and Dahl salt-resistant rats, whereas the unphosphorylated form of the protein was reduced by approximately one half in SS animals. In accord with immunoblot data, rolipram-inhibitable cAMP hydrolyzing activity, a measure of PDE4 activity, was ≈3-fold greater in kidney cytosolic extracts from SS rats than in extracts from spontaneously hypertensive rats and salt-resistant rats. Phosphodiesterase 4B expression was detected by immunohistochemistry in the renal vasculature, proximal tubules, and distal tubules. These results raise the possibility that increased PDE4 activity, specifically phosphodiesterase 4B activity, reduces β-adrenergic signaling in the kidney and contributes to salt-sensitive hypertension in the Dahl SS rat. (Hypertension. 2008;51:762-766.)

Key Words: phosphodiesterase 4B ▪ cAMP ▪ Dahl rat ▪ β-adrenergic signaling

Activation of β-adrenoreceptors triggers the formation of cAMP through G protein–coupled adenylylate cyclase, which subsequently stimulates cAMP-dependent protein kinase A (PKA). cAMP phosphodiesterase (PDE) negatively regulates β-adrenoreceptor signaling by hydrolyzing cAMP and reducing PKA activity.1,2 Reduced vascular β-adrenergic responsiveness has been demonstrated in both hypertensive patients and rat models of genetic and acquired hypertension (see review3). This reduced responsiveness has been hypothesized to play an important role in the pathogenesis of hypertension. However, the mechanisms underlying reduced β-adrenergic signaling remain unclear. β-Adrenergic receptor number has been measured in different animal hypertension models, and the results have varied, with receptor number increasing, decreasing, or remaining unchanged in the heart and vasculature (see review4). Other evidence suggests that, in certain models of hypertension, expression of the stimulatory G protein Gs is reduced, and/or the expression of the inhibitory G protein Gi is increased.5–7 Another possibility, which we have investigated here, is that PDE activity may be increased in hypertension.

The PDE 4 family of PDEs, formerly known as cAMP-PDE and rolipram-sensitive PDE, is expressed in most cell types and is generally considered to account for most of the cAMP-hydrolyzing activity of cells (see review5). PDE4s are generated from 4 distinct genes (PDE4A through D), and each gene generates long and short splice variants using alternate splicing and promoters (see review6). Long PDE4 variants contain 2 conserved regions at the amino terminus known as upstream conserved regions 1 and 2, whereas short variants lack upstream conserved region 1 and may have a truncated upstream conserved region 2. PKA phosphorylates and activates many long PDE4 isoforms, whereas long and short forms are differentially regulated by extracellular signal regulated kinase–mediated phosphorylation of a conserved serine residue.8,10

The PDE4B family consists of 5 different isoforms (PDE4B1 through 5). PDE4B2 and PDE4B3 isoforms are strongly conserved among species, with the human and rat proteins being of identical length and sharing >90% of the amino acid sequence identity.11,12 PDE4B4, a 659-amino acid protein, is unique among the other long forms of PDE4B because of the presence of a 17-amino acid NH2-terminal region that regulates its catalytic activity. PDE4B4 is also unique, because, unlike the other forms, it is found exclusively in the cytosol of transfected COS7 cells.13 Recombinant PDE4B4 is activated through PKA-mediated phosphorylation at Ser-56.13 Recently, PDE4B5, a novel, super-short,
brain-specific cAMP PDE4 variant of which the isoform-
 specifying NH2-terminal region is identical to that of cAMP
 PDE4D6, has been reported.14 In the present study, we
 investigated the potential role of PDE4B in 2 rodent models
 of renal hypertension.

Materials and Methods

Animals and Treatments

Dahl salt-sensitive (SS) rats and spontaneously hypertensive rats
(SHRs) were used as models of renal hypertension. Male SHRs, Dahl
 hypertensive SS/Jr rats, and Dahl salt-resistant (SR/Jr) rats (200 to
 250 g) were purchased from Harlan (Indianapolis, Ind). Only males
 were studied to eliminate the confounding effects of the estrus cycle.
 Animals were housed at controlled temperatures (22°C to 25°C) and
 lighting (12-hour light/12-hour dark) with free access to food and
 water before experimentation. All of the animals used in this work
 received humane care in compliance with institutional animal care
 guidelines. All of the animals were maintained on high-salt diets
 (Purina 5501 chow with 8% NaCl) for 10 days before sacrifice.
 Rolipram and other reagents were purchased from Sigma Chemical
 Co unless otherwise noted.

Immunoblotting

On removal, brain and kidneys were fast-frozen in liquid nitrogen
 and dounce homogenized at 4°C in 50 mmol/L of Tris- HCl (pH 7.4),
 150 mmol/L of NaCl, 1 mmol/L of EDTA, 0.25% sodium deoxy-
 cholate, 1% Nonidet P-40, and protease inhibitor mixture. The
 homogenate was centrifuged at 13 000 rpm for 15 minutes at 4°C,
 and the supernatant was collected as the total protein fraction. The
 samples were boiled for 3 minutes in Laemmli buffer and subjected
 to electrophoresis in a 10% SDS-PAGE. The proteins were trans-
 ferred to a nitrocellulose membrane and then blocked in a Tris-
 buffered saline-Tween 20 (TBST) and incubated for 1 hour with
 Phospho-PDE4B4 Analysis

by Immunoprecipitations

Kidney lysates, prepared in the same manner as for immunoblotting,
 were incubated in lysis buffer containing 1 μg/mL of rabbit
 polyclonal PDE4B antibody with slow rotation on a rocker overnight
 at 4°C. Samples were then incubated with 50 μL of protein A/G
 agarose beads (Santa Cruz Biotechnology) for 2 hours under constant
 rotation. The samples were centrifuged, supernatants were removed,
 and agarose bead pellets were successively washed in PBS contain-
 ing 1.00%, 0.50%, and 0.05% Tween-20. The beads were resus-
 pended in Laemmli buffer, boiled for 3 minutes, and centrifuged.
 The supernatants were separated by SDS-PAGE and then subjected
 to immunoblotting with monoclonal anti-phospho serine-threonine
 antibody (BD Biosciences).

CAMP PDE Activity Assay

Rolipram-inhibitable cAMP-PDE activity was measured as de-
 scribed by Butcher and Sutherland13 and modified according to the
 method of Thompson and Appleman.15 Briefly, freshly isolated tissues
 were dounce homogenized in homogenization buffer (20 mmol/L of Tris-Cl
 [pH 7.2], 1 mmol/L of EDTA, 0.25 mol/L of sucrose, and protease inhibitors) with 15 strokes at full speed on ice.
 The homogenate was centrifuged at 59 000 rpm for 1 hour at 4°C,
 and the supernatant (cytosolic fraction) was collected. Cytosolic
 extracts were incubated for 30 minutes at 30°C with 1 mmol/L of
 cAMP in 20 mmol/L of Tris-Cl (pH 7.4) and 10 mmol/L of MgCl2.
 After this incubation was complete, 100 μl of snake venom (0.1
 mg/mL) was incubated with the reaction mixture for 30 minutes. The
 reaction was terminated by the addition of 50 μL of 50% trichloro-
 acetic acid. The samples were incubated on ice for 5 minutes
 and centrifuged at 5000 rpm for 5 minutes. The supernatant was then
 collected for measurement of phosphate content. Phosphate content
 was determined by incubating the sample with 1 mL of coloring
 reagent (FeSO4 and ammonium molybdate) for 1 minute and
 spectrophotometric measurement of the mixture at 740 nm. Protein
 was estimated using the Bradford method, and the phosphate content
 in the samples was extrapolated from a standard curve generated
 from known concentrations of KH2PO4.

Immunohistochemistry

A 2-step indirect immunoperoxidase staining technique was used.
 Briefly, isolated tissues were formalin fixed and embedded in
 paraffin. Sections (4 μm) were mounted on charged slides. The
 tissue sections were rehydrated in xylene followed by graded alcohol
 and then rinsed in a running water bath. Endogenous peroxidase
 activity was quenched by preincubating slides in 3% hydrogen
 peroxide. The tissue sections were then incubated with rabbit
 polyclonal antibody raised against the common carboxyl terminus of
 PDE4B for 1 hour. After an additional wash in TBST, the tissues
 were incubated with peroxidase-labeled polymer horseradish perox-
 idase-anti-rabbit (DAKO) for 30 minutes and washed again in TBST.
 Liquid diaminobenzidine+Substrate Buffer System (DAKO) was
 used to identify the bound antibody. The sections were finally
 washed in TBST, counterstained with a 50% dilution of Gill’s
 hematoxylin for 1 minute, dehydrated in graded alcohols and xylene,
 and mounted with a coverslip using Permount. All of the specimens
 were examined using a Nikon E600 microscope with Axiosplan
 objectives. Immunohistochemistry was performed with 2 negative
 control conditions as follows. In the first condition, the tissue was
 incubated with a blocking solution (5% normal goat serum) for
 30 minutes in the absence of the primary antibody. In the second
 condition, the tissue was incubated with the blocking solution for 30
 minutes, followed by a 1-hour incubation with a combination of
 PDE4 antibody (×4, Fabgenix International, Inc), which corre-
 sponds to the linear epitope against which the PDE4B antibody
 has been raised (1:100, Fabgenix International, Inc).

Images were acquired at ×400 and ×1000 using a Microlumina
 Ultra Resolution Scanning Digital Camera (3380×2700 pixels, Leaf
 Systems). In all of the cases, files were saved in tagged-image file
 format.

Statistics

Comparisons were by ANOVA and Student’s t test. Data are
 expressed as means±SDs. P<0.05 was significant.

Results

Phosphorylated PDE4B Is Increased in Kidneys
From Dahl SS Rats

Immunoblot analysis using an antibody to the “common”
carboxyl terminus of the PDE4B family revealed the presence
of PDE4B1, PDE4B2, PDE4B3, and PDE4B in brain
extracts from Dahl SS rats, Dahl SR rats, and SHRs (Figure
1). The levels of these 4 PDE4 isoforms did not differ
among strains. In contrast, only the PDE4B4 isoform could
be detected in kidney extracts from these animals. Unphospho-

ylated PDE4B4 was more abundant in the SHR and Dahl SS groups than in the Dahl SR group. Conversely, levels of phosphorylated PDE4B4 in the Dahl SS group were 2-fold greater than those in the Dahl SR and SHR groups. Similar results were obtained after immunoblot analysis of anti-PDE4B immunoprecipitates using an anti-phospho serine-threonine antibody (Figure 2). Interestingly, only the phosphorylated form of PDE4B4 was detected in the brain (Figure 1).

**PDE4 Activity Is Increased in Kidneys From Dahl SS Rats**

Next, rolipram-inhibitable cAMP-hydrolyzing activity was measured to determine whether increased PDE4B4 phosphorylation was associated with an increase in protein activity. Because PDE4B4 is the sole PDE4B isoform localized to the cytoplasm, rolipram-inhibitable activity was measured in cytosolic extracts. Hydrolyzing activity was 2- to 3-fold greater in Dahl SS kidneys than in the Dahl SR and SHR Kidneys (Figure 3).

**PDE4B4 Is Expressed in the Renal Tubules and Vasculature of SS Dahl Rats**

To investigate the distribution of PDE4B4 in the kidney, we performed immunohistochemistry using anti-PDE4B antibody (Figure 4). Although this antibody cross-reacts with all of the isoforms, we inferred that labeling primarily is of the PDE4B4 isoform, because this was the only isoform detected by immunoblot analysis using the same antibody. Strong fine diffuse granular cytoplasmic and nuclear PDE4B staining were seen in a majority of the proximal convoluted tubules and distal convoluted tubules in SS Dahl rats (Figure 4A). PDE4B immunostaining was also observed in a subset of cells within the glomerulus, as well as in the arterioles (Figure 4B). At higher magnification, focal granular cytoplasmic and nuclear PDE4B immunostaining were detected in endothelial cells, as well as in the smooth muscle of the arterioles and smaller arteries in the kidney (Figure 4C). In the glomerulus, a fraction of cells showed nuclei and cytoplasmic immunoreactivity. In addition, parietal epithelial cells of the glomerulus were positive in the nucleus and cytoplasm (Figure 4D).

**Discussion**

PDE4B transcripts have been detected in a variety of tissues, including heart and lung tissue (PDE4B3), liver tissue...
Our finding that PDE4B is also expressed in proximal tubules which we have shown expresses PDE4B in the Dahl SS rat.

Moreover, we show that phosphorylation of PDE4B4 is enhanced in the kidneys of hypertensive Dahl SS rats compared with those of SHR and Dahl SR rats. Phosphorylation at Ser-56 has been shown to activate the recombinant PDE4B4 in vitro. Until now, the in vivo phosphorylation of this isoform has not been reported. Although phosphorylation of PDE4B4 increased in Dahl SS animals, the combined levels of unphosphorylated and phosphorylated PDE4B4 were approximately the same as in the other 2 strains. This suggests that total levels of PDE4B4 (ie, unphosphorylated and phosphorylated) do not change and that the increase is because of phosphorylation of the unphosphorylated protein. The increase in the rolipram-inhibitable cAMP-PDE activity was similar in magnitude to the increase in phosphorylated PDE4B4. Together, these results are consistent with the report by Shepherd et al that phosphorylation of recombinant PDE4B4 increases activity in COS7 cells by \( \approx 60\% \).

This is the first report, to our knowledge, of the distribution of PDE4B4 in the kidney. PDE4B signal is detected in the vasculature, nephrons, and some glomerular cells. Rolipram has been shown to reduce renal vascular resistance and to increase glomerular filtration rate, urine flow rate, and urinary \( \text{Na}^+ \) in dogs. These effects were attributed to enhanced cAMP-mediated dilation of the renal vasculature, which we have shown expresses PDE4B in the Dahl SS rat. Our finding that PDE4B is also expressed in proximal tubules and distal tubules suggests that cAMP signaling may also be augmented by rolipram in renal tubules. The effect of cAMP in tubules remains controversial. Evidence suggests that \( \beta \)-adrenergic signaling promotes \( \text{Na}^+ \) excretion by facilitating a blood-lumen flux of \( \text{Na}^+ \), whereas other studies have indicated that \( \beta \)-adrenergic signaling inhibits \( \text{Na}^+ \) uptake in tubules by stimulating basolateral \( \text{Na/K} \) ATPase. Additional renal effects of rolipram that have been reported include regulation of the osmotic permeability response to antidiuretic hormone in neonatal tubules and renin release from juxtaglomerular cells.

Both the Dahl SS rats and SHRs are generally considered to be models of renal hypertension. Because the SHR is a model of salt-independent hypertension, these findings raise the possibility that PDE4 activity mediates renal adaptation to high salt and may play a pathogenic role specifically in SS hypertension. In accord with this possibility, rolipram is natriuretic in cirrhotic rats with ascites, a situation of enhanced sodium retention, but not in those without ascites.

Finally, our results also raise the possibility that increased cAMP PDE activity may account for \( \beta \)-adrenergic desensitization in arterial hypertension. Indeed, increased PDE4 activity has been shown to augment the primary vasoconstrictive action of norepinephrine by reducing intracellular cAMP levels and PKA activity in rodent models of shock and multiple organ dysfunction syndrome.

**Perspectives**

These results raise the possibility that \( \beta \)-adrenergic signaling in general and cAMP- PDE specifically contribute to the pathogenesis of SS hypertension in the Dahl rat by showing that activated or phosphorylated form of the PDE4B4 is predominant in the kidney of the Dahl SS rat. On the other hand, renal PDE4B activity was less abundant and the unphosphorylated form of PDE4B4 more abundant in the SHR rat, thereby eliciting the possibility that PDE4B contributes to hypertension in the Dahl SS but not SHR rat. This raises the specter that polymorphisms of PDE4B with increased activity may explain or contribute to SS hypertension in some patients and cAMP-PDE may be a therapeutic target for certain hypertensive patients (see review).

This study further highlights the complexity of \( \beta \)-adrenergic signaling, specifically highlighting that a unique feedback loop may exist in which PKA-mediated phosphorylations may reduce cAMP signaling in cells by activating PDE4B.

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**Disclosures**

None.
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