Role of Cyclic ADP-Ribose- Ca^{2+} Signaling in Mediating Renin Production and Release in As4.1 Cells

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Key Words

Juxtaglomerular Apparatus • Nucleotide • Calcium mobilization • Renin-angiotensin system • Renal hemodynamics

Abstract

The present study was designed to test the hypothesis that cyclic-ADP-ribose (cADPR) serves as a novel second messenger to mediate intracellular Ca^{2+} concentration in As4.1 cells, a prototype of renal juxtaglomerular cells, and thereby regulates the renin production and release. Western blot analysis showed that CD38, an enzyme responsible for the production of cADPR, was abundant in As4.1 cells. Using cADPR cycling assay, it was found that NaCl stimulated cADPR production in these cells, which was blocked by inhibition of ADP-ribosyl cyclase with nicotinamide. HPLC analysis showed that the conversion rate of β -NGD into cGDPR was dramatically increased by NaCl, which was attenuated by nicotinamide. Using fluorescent microscopic imaging analysis, NaCl (100 mM) was demonstrated to stimulate a rapid Ca^{2+} increase from the endoplasmic reticulum (ER), which was inhibited by a cADPR antagonist, 8-bromo-cADPR (30 μM), an inhibitor of ADP-ribosyl cyclase,

nicotinamide (6 mM), the ryanodine receptors blocker, ryanodine (30 μM), or a Ca^{2+} -induced Ca^{2+} release inhibitor, tetracaine (10 μM) by 70-90%. Finally, NaCl was found to significantly lower the renin production and release levels in As4.1 cells, which was accompanied by decreases in renin mRNA levels. Pretreatment of these cells with various inhibitors or blockers above significantly blocked the inhibitory effect of NaCl on renin production and release. These results indicate that cADPR-mediated Ca^{2+} signaling pathway is present in As4.1 cells and that this signaling pathway may play a contributing role in the regulation of renin production and release.

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Introduction

The renin-angiotensin system (RAS) is a major regulatory system controlling renal vascular tone, glomerular filtration, nephron fluid flow rates, electrolyte balance and systemic blood pressure [1]. It is well known that the rate-limiting enzyme in this hormonal cascade is renin, which is primarily synthesized and secreted into

circulation by renal juxtaglomerular (JG) cells, one of the components of the JG apparatus [1-2]. Renin production in JG cells can be stimulated by renal sympathetic nerve activity, low renal perfusion pressure and inhibited by endothelin and angiotensin (Ang) II [3-6]. In contrast to the regulated secretion of other substances, which are dependent on the increase in intracellular Ca^{2+} levels, decreases in intracellular Ca^{2+} levels or increases in cAMP or cGMP mediates the actions of these factors on renin production in response to different stimuli [7]. It has been suggested that a decrease in intracellular Ca^{2+} levels is a common signaling mechanism responsible for enhanced renin production and release. Using different indirect approaches to manipulate intracellular calcium concentrations, such as Ca^{2+} -mobilizing agents and increasing Ca^{2+} influx via store-operated Ca^{2+} channels, previous studies demonstrated the inhibitory role of Ca^{2+} on renin production or release [8-11]. However, so far little is known regarding which signaling pathway is responsible for the control of intracellular Ca^{2+} concentrations in these renal JG cells.

In previous studies in a variety of mammalian cells, Ca^{2+} signals were reported to be initiated by the influx of extracellular Ca^{2+} through Ca^{2+} channels in the plasma membrane (PM), followed by release of Ca^{2+} from the sarcoplasmic reticulum (SR) through 1, 4, 5-triphosphate (IP_3) receptors (IP_3R) and ryanodine receptors (RyR). Besides the well-studied IP_3 pathway, a novel Ca^{2+} mobilizing second messenger, cyclic ADP-ribose (cADPR) is also reported to play an important role in the control of intracellular Ca^{2+} signal through the activation of RyR. cADPR, an endogenous metabolite of nicotinamide adenine dinucleotide (NAD) via ADP-ribosyl cyclase, initiates Ca^{2+} -induced Ca^{2+} release (CICR) through activation of ryanodine (Rya) receptors (RyRs), which are completely independent of the IP_3 signaling pathway [12-16]. This cADPR-mediated signaling has been implicated in the regulation of a variety of cell functions or physiological processes, including insulin release, egg fertilization, cell proliferation, muscle contraction, neurotransmitter release and nitric oxide signaling [18]. Recent studies have demonstrated that cADPR-mediated Ca^{2+} signaling also plays an important role in the regulation of renal function by acting on different renal components, such as renal arteries [19], the early distal tubule [20] and renal mesangium [21]. However, so far little is known regarding whether the cADPR-mediated Ca^{2+} signaling pathway is involved in the regulation of renin production and release in renal JG cells.

By use of a renin-expressing clonal cell line (As4.1)

as a prototype of renal JG cells, the goal of this present study was to test the hypothesis that increased production cADPR and consequent Ca^{2+} mobilization contributes to the decrease in renin production and release in As4.1 cells. First, we identified and characterized the enzymatic pathway responsible for the production and metabolism of cADPR in As4.1 cells. Next, we quantified the intracellular cADPR levels using cADPR cycling assay and determined NaCl-induced changes. Moreover, we went to analyze the effects of NaCl on the activity of ADP-ribosyl cyclase by HPLC and determined the contribution of this cADPR-signaling pathway to Ca^{2+} release induced by NaCl using fluorescence microscopic imaging analysis. Finally, we confirmed that altered cADPR production was associated with production and release of renin in As4.1 cells. Our results suggest that cADPR-mediated Ca^{2+} signaling pathway may be importantly involved in the regulation of renin production and release.

Materials and Methods

Culture of As4.1 cells

Renin-expressing As4.1 cells (ATCC No. CRL2193) were obtained from the American Type Culture Collection (ATCC). As4.1 cells were developed via transgene-targeted oncogenesis in mice with a renin SV40 T antigen fusion construct and were purified from kidney neoplasm. This cell line expresses both the endogenous renin gene and the transgene and has been widely used as a model to study the cellular and molecular regulation of renin production and release [22-23]. In the present study, As4.1 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) with 4 mM L-glutamine adjusted to contain 18 mM sodium bicarbonate, 25 mM glucose, 90% (ATCC, Manassas, VA); 10% fetal Bovine serum (FBS) at 37°C in 5% CO_2 atmosphere. For basal cADPR level and cADP-ribosyl cyclase activity assays, after As4.1 cells reached 80% to 90% confluence, NaCl (100 mM) was added in culture medium for 60 min with or without 30 min pretreatment with an ADP-ribosylcyclase inhibitor, nicotinamide (6 mM, Nicot). For the measurement of renin release and production, As4.1 cells were pretreated with an ADP-ribosylcyclase inhibitor, nicotinamide (6 mM, Nicot), a CICR inhibitor, tetracaine (10 μM , TC) and a ryanodine receptor blocker, ryanodine (30 μM , Rya) for 30 min and then treated with NaCl (100 mM) for 1-6 hours. In these experiments, the concentration and time of NaCl treatment were chosen based on our preliminary experiments (data not shown). This condition can keep cell growth well and obtain significant effects of NaCl on renin production and renin release at 6 hours.

Western blot analysis

CD38 protein in As4.1 cells was detected by Western blot analysis [24]. Briefly, 20 μg proteins were subjected to SDS-

PAGE, transferred onto a nitrocellulose membrane, and blocked. The membrane was probed with primary monoclonal antibody anti-CD38 (1:1000 dilution, BD Biosciences Pharmingen, San Diego, CA) overnight at 4°C followed by incubation with horseradish peroxidase-labeled anti-mouse IgG. The immunoreactive bands were detected by chemiluminescence methods and visualized on Fuji medical X-ray film. For normalization, the blots were reprobed with alternative primary antibodies against the housekeeping protein β -actin (1:4000 dilution for 1 hours, Sigma). To quantitate the blots, the films with immunoactive blots were scanned and the intensity of corresponding protein bands was quantitated using UN-SCAN-IT gel (Silk Scientific Corporation, SPSS, Inc., Chicago, IL, USA).

Cycling assay for the intracellular cADPR level

The cADPR basal level in cultured As4.1 cells was determined as described previously [25]. After treatments, As4.1 cells were scraped by a plastic scraper and spun down by centrifugation. The centrifugal pellets were extracted with 0.5 ml of 0.6 M perchloric acid at 4°C. Perchloric acid was removed by mixing the aqueous sample with a solution containing 3:1 vol. of 1,1,2-trichlorotrifluoroethane to tri-*n*-octylamine. Following centrifugation for 10 minutes at 1500 X g, the aqueous layer containing cADPR was removed and incubated overnight at 37°C with an enzyme mixture containing: 0.44 unit/ml nucleotide pyrophosphatase, 12.5 units/ml alkaline phosphatase, 0.0625 unit/ml NADase, 2.5 mM $MgCl_2$ and 20 mM sodium phosphate (pH 8.0), all nucleotides except cADPR in the samples were hydrolysed.

To quantify cADPR concentration, a cycling reaction was conducted in 96-well plates. First, 0.1 ml of cADPR or other nucleotide samples were added into 50 μ l of reagent containing 0.3 μ g/ml ADP-ribosyl cyclase, 30 mM nicotinamide and 100 mM sodium phosphate (pH 8). This initiated the conversion of cADPR in the samples to NAD^+ . The conversion was allowed to proceed for 15 minutes at room temperature. The cycling reagent (0.1ml) was then added, which contained 2% ethanol, 100 μ g/ml alcohol dehydrogenase, 20 μ M resazurin, 10 μ g/ml diaphorase, 10 μ M FMN, 10 mM nicotinamide, 0.1 mg/ml BSA and 100 mM sodium phosphate (pH 8). The cycling reaction was allowed to proceed for 2–4 hours, and the increase in the resorufin fluorescence (with excitation at 544 nm and emission at 590 nm) was measured periodically using a fluorescence plate reader. With known concentrations of standards curve, quantitative measurements were performed.

HPLC assay of ADP-ribosyl cyclase activity in As4.1 cells

Homogenates, cytosol and microsome were prepared from As4.1 cells with a modified method as we described previously [26]. To determine ADP-ribosyl cyclase activity, the homogenate (100 μ g protein) was incubated with 100 μ M β -nicotinamide guanine dinucleotide (β -NGD⁺) at 37°C for 30 min. β -NGD⁺ was used as a substrate to determine ADP-ribosyl cyclase activity, because this enzyme converts NGD into cGDPR, but unlike cADPR, cGDPR cannot be hydrolyzed by cADPR hydrolase. The reaction mixtures were centrifuged at 4°C

through an Amicon microultrafilter at $13,800 \times g$ to remove proteins, and then analyzed by HPLC with a fluorescence detector (Hewlett-Packard 1090 HPLC system and 1046A spectrofluorometer). The excitation wavelength of 300 nm and the emission wavelength of 410 nm were used to detect the fluorescent products. All HPLC data were collected and analyzed by a Hewlett-Packard Chemstation.

Nucleotides were resolved on a 3- μ m Supelcosil LC-18 column (4.6×150 mm) with a 5- μ m Supelcosil LC-18 guard column (4.6×20 mm, Supelco, Bellefonte, BA). The injection volume was 20 μ l. The mobile phase consisted of 150 mM ammonium acetate (pH 5.5) containing 5% methanol (solvent A) and 50% methanol (solvent B). The solvent system was a linear gradient of 5% solvent B in A to 30% solvent B in A over 1 minute, held for 25 minutes, and then increased to 50% solvent B over 1 minute. The flow rate was 0.8 ml/min. Peak identities were confirmed by comigration with known standards. Quantitative measurements were performed by comparison of known concentrations of standards.

RNA extraction and real time RT-PCR

Total RNA was isolated from As4.1 cells using TRIzol reagent (GIBCO, Life Technologies, Carlsbad, CA) according to the protocol described by the manufacturer. The mRNA levels for renin were analyzed by real-time quantitative RT-PCR using a Bio-Rad iCycler system (Bio-Rad, Hercules, CA) according to the protocol described by the manufacturer. The mRNA level of renin was normalized to the 18S mRNA. The specific primers for renin based on the core sequence of rat renin cDNA (Accession number NM_031192) were 5'-GGG AGC CAA GGA GAA GAG A -3' (Forward) and 5'-TCC CAG GTC AAA GGA AAT GT-3' (Reverse).

Measurement of intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) in As4.1 cells

Determination of $[Ca^{2+}]_i$ in cultured As4.1 cells was performed using fura-2 as an indicator as described previously [26–27]. Briefly, the cells were cultured on a glass coverslip to reach 60% to 70% confluence and removed the culture medium. Next, the cells loaded with fura-2 acetoxymethyl ester (fura-2 AM, 5 μ M) (Molecular Probes, Eugene, OR, USA) at 37°C for 30 min. Then the cells were incubated for 20 min to allow the complete hydrolysis of intracellular fura-2-AM to fura-2 with a Ca^{2+} -free Hanks' buffered saline solution containing (in mM) NaCl, 137; KCl, 5.4; $NaHCO_3$, 4.2; Na_2HPO_4 , 3; KH_2PO_4 , 0.4; $MgCl_2$, 0.5; $MgSO_4$, 0.8; glucose, 10; HEPES, 10; pH 7.4. The coverslip was mounted horizontally on a Nikon Diaphoto TMD inverted microscope (Nikon, Japan). NaCl (100 mM) was added to the bath solution to induce Ca^{2+} release. To examine the specificity of NaCl and the role of the cADPR signaling pathway in NaCl-induced Ca^{2+} release, As4.1 cells were pretreated for 15 min with 8-bromo-cADPR (a cADPR antagonist, 30 μ M, 8-Br-cADPR), Nicot (6 mM), Rya (30 μ M), or TC (10 μ M). All these experiments were conducted in Ca^{2+} free Hank's buffer. A SPOT digital camera (SPOT RT Monochrome; Diagnostic Instruments) was used to record fura 2 fluorescence images of As4.1 cells. Metafluor imaging and analysis software (Universal Imaging) was used to acquire, digitize and store the images

Fig. 1. The presence of cADP-ribosylcyclase confirmed in As4.1 cells by Western blot analysis using anti-CD38 antibody. A. Immunoblot gel documents for CD38 and β -actin in homogenate, microsome and cytosol of As4.1 cells. B. Summarized data showing that the distribution of CD38 in microsome was higher than cytosol (n=6). * $P < 0.05$ vs. homogenate of As4.1 cells.

and data for off-line processing and statistical analysis. For a single measurement, fura 2 fluorescence in 10-20 cells was monitored to obtain average fluorescence changes. Usually, 6 measurements were performed for each group to reach statistical significance. F_{340}/F_{380} , a fluorescence ratio of excitation at 340 nm to that at 380 nm, was determined after background subtraction, and $[Ca^{2+}]_i$ was calculated by using the following equation: $[Ca^{2+}]_i = K_d \beta [(RR_{min})/(R_{min}R)]$, where K_d for the fura 2- Ca^{2+} complex is 224 nM; R is the fluorescence ratio (F_{340}/F_{380}); R_{max} and R_{min} are the maximal and minimal fluorescence ratios measured by addition of 10 μ M of Ca^{2+} ionophore ionomycin to Ca^{2+} -replete (2.5 mM $CaCl_2$) solution and Ca^{2+} -free (5 mM EGTA) solution, respectively; and β is the fluorescence ratio at 380-nm excitation determined at R_{min} and R_{max} , respectively. To detect whether IP_3 -mediated Ca^{2+} signaling pathway is also involved in the regulation of intracellular Ca^{2+} release, 2-APB (50 μ M), an antagonist of IP_3 and a phospholipase C (PLC) inhibitor U73122 (100 nM) were used to examine the NaCl-induced Ca^{2+} release. To detect whether NaCl-induced Ca^{2+} release is due to the increased osmolarity, experiments with several other osmolytes including urea (200 mM), sucrose (200 mM) and Na-gluconate (100 mM) were performed in this study.

Radioimmunoassay (RIA) for *renin* activity

The active renin content (renin release) and total (active and inactive) renin content were measured as described previously [28-29]. In brief, after culture of the As4.1 cells, the medium was removed and kept for renin release measurement, and the cell were washed twice with phosphate buffered saline (PBS) and then harvested and frozen in liquid nitrogen and store at -80°C . For measurement of active and total renin content, frozen cells were homogenized in 1 ml of buffer (pH 6.0) containing 2.6 mM ethylene diaminetetraacetate, 1.6 mM dithiothreitol, 3.4 mM 8-hydroxyquinoline sulfate, 0.2 mM phenylmethylsulfonyl fluoride, and 5 mM ammonium acetate.

Fig. 2. Effects of nicotinamide (Nicot) on NaCl-induced increase in cADPR level in As4.1 cells. Nicot (6 mM) was added in As4.1 cells 30 min before treatment with NaCl (100mM) for 60 min (n=8). * $P < 0.05$ vs. control, # $P < 0.05$ vs. NaCl treatment.

The homogenates were centrifuged at 12,000 g for 30 minutes, and the supernatant was removed. To measure total renin content in As4.1 cells, the samples (900 μ l) were incubated at 0°C for 60 min with 100 μ l of 4 mg/ml trypsin (Sigma) in 500 mM Tris buffer, pH=7.5, containing 5 mM $CaCl_2$, 0.1% NaCl azide, and 1% bovine serum albumin as described previously [28-29]. Soybean trypsin inhibitor (Sigma; 8 mg/ml final concentration) was added to stop the reaction. Thus, inactive renin in the samples was converted to active renin. Plasma of mice as the renin substrate was added into samples and the renin activity was determined by generation of angiotensin I from a plasma angiotensinogen substrate by radioimmunoassay

(RIA) kit (DiaSorin, MN, USA). In this assay, we treated As4.1 cells with Ang II (10^{-6} M) for 12 hours as a positive control to inhibit renin release and production.

Statistic analysis

Data are expressed as mean $\pm$ standard error of mean (SEM). The significance of the differences in mean values between and within multiple groups was examined using an analysis of variance for repeated measures followed by Duncan's multiple range tests. Student's *t* test was used to evaluate statistical significance of differences between two paired observations. *P* < 0.05 was considered statistically significant.

Results

Confirmation of CD38 presence in As4.1 cells

It has been reported that CD38 is expressed in variety of mammalian cells. However, it remains unknown whether CD38 exists in As4.1 cells. As shown in Figure 1A, the presence of a band at 45 kDa was demonstrated in the homogenate, cytosol and microsome fractions from these As4.1 cells. Additionally, the distribution of CD38 in microsome was much higher than cytosol fraction in agreement with previous studies that ADP-ribosyl cyclase is membrane-bound enzyme. These results are summarized in Figure 1B.

Effects of Nicot on NaCl-induced cADPR increase in As4.1 cells

As summarized in Figure 2, in the control As4.1 cells, the basal level of cADPR was 2.63 ± 0.55 pmol/mg protein. When As4.1 cells were treated with NaCl (100 mM) for 60 min, the intracellular cADPR levels substantially increased. Inhibition of ADP-ribosyl cyclase by Nicot (6 mM) significantly reduced the NaCl-induced cADPR generation, from 5.44 ± 0.32 pmol/mg protein to 3.12 ± 0.10 pmol/mg protein, a 43% maximal reduction.

ADP-ribosyl cyclase activity in As4.1 cells

By fluorescence HPLC analysis, cyclic GDP-ribose (cGDP) production converted from β -NGD⁺ by cultured As4.1 cell was measured, which indicated the ADP ribosyl cyclase activity in As4.1 cells. This product coeluted with the cGDP standard at a retention time of 2.2 minutes as shown in Figure 3A. Under control conditions, the enzymatic activity in As4.1 cell homogenates was 35.10 ± 1.67 pmol/min/mg protein. When As4.1 cells were treated with NaCl (100 mM) for 60 min, the conversion rate of β -NGD⁺ to cGDP was 44.29 ± 2.89 pmol/min/mg protein. In the presence of the ADP-ribosyl cyclase inhibitor, Nicot (6 mM), NaCl-induced increase in the conversion rate of β -NGD⁺ to cGDP was substantially inhibited by 67%. Additionally, to detect whether NaCl-induced this enzymatic activity is due to the increased

Fig. 4. NaCl-induced intracellular Ca²⁺ release in As4.1 cells. **A.** Representative fura 2 fluorescence ratio images (taken at excitation wavelengths of 340 and 380 nm (F340/F380)). Images were taken under resting conditions before (control) and after addition of NaCl in As4.1 cells (NaCl), or in the cells pretreated of cADPR antagonist, 8-Br-cADPR (30 μ M) (8-Br+NaCl). **B.** Representative recordings of [Ca²⁺]_i transients in the absence and presence of 8-Br-cADPR. **C:** Summarized data showing the NaCl-induced maximal increase in [Ca²⁺]_i in the absence or presence of 8-Br-cADPR (30 μ M); RyR blocker, Rya (30 μ M, $n = 6$); CICR inhibitor, TC (10 μ M, $n = 6$); ADP-ribosyl cyclase inhibitor, Nicot (6 mM, $n = 6$); an antagonist of IP₃, 2-APB (50 μ M) and a phospholipase C (PLC) inhibitor U73122 (100 nM). [#] $P < 0.05$ vs. NaCl treatment ($n=6$). **D:** Summarized data showing the effects of urea (200 mM), sucrose (200 mM) and Na-gluconate (100 mM) on the intracellular Ca²⁺ release ($n=6$), ^{*} $P < 0.05$ vs. control.

osmolarity or cell volume changes, other osmolytes including urea (200 mM), sucrose (200 mM) and Na-gluconate (100 mM) were used to treat As4.1 cells. It was found that they had no significant effects on the conversion rate of β -NGD⁺ to cGDPR in As4.1 cells (Figure 3C).

NaCl-induced Ca²⁺ release in the presence or absence of cADPR antagonist

As shown in Figure 4A, under resting condition, NaCl (100 mM) significantly increased fura 2 fluorescence ratio (images taken before and after 1 min of NaCl) in As4.1 cells bathed in Ca²⁺-free Hanks buffer with 1 mM EGTA, indicating a NaCl-induced intracellular Ca²⁺ release. Figure 4B presents typical Ca²⁺ transient recordings when the cells were incubated with NaCl (Control). It shows that NaCl produced a rapid Ca²⁺ rise in [Ca²⁺]_i from 108

± 23 nM to the maximal concentration at 1134 ± 148 nM in 1 minute. However, pretreatment of the cells with 8-Br-cADPR (30 μ M) markedly attenuated NaCl-induced Ca²⁺ response (Figure 4A and B). As shown in Figure 4B and C, the basal [Ca²⁺]_i was not significantly altered by 8-Br-cADPR, but the NaCl-induced increase in [Ca²⁺]_i (Δ [Ca²⁺]_i) was significantly attenuated to 250 ± 148 nM, a reduction of 78% ($n=6$). However, urea, sucrose or Na-gluconate did not induce intracellular Ca²⁺ release as shown in Figure 4D.

NaCl-induced Ca²⁺ release during RyR blockade, CICR suppression, or ADP-ribosyl cyclase inhibition

To further determine whether NaCl-induced Ca²⁺ release is associated with RyR-mediated Ca²⁺ mobilization in As4.1 cells, Rya as a RyR blocker and TC as a CICR

Fig. 5. Effects of inhibition of cADPR/RyR signaling pathway on renin release and total renin content using RIA assay. Summarized data showing the effects of NaCl on renin release (A) and total renin content (B) from As4.1 cells in the absence (V) or presence Nicot (6 mM), Rya (30 μ M) or TC (10 μ M) ($n = 6$). * $P < 0.05$ vs. control, # $P < 0.05$ vs. NaCl treatment.

inhibitor were used to pretreat As4.1 cells, and then the Ca^{2+} release response of those cells to NaCl was measured. In the presence of Rya (30 μ M) and TC (10 μ M), NaCl-stimulated Ca^{2+} release was significantly attenuated by 89% and 91%, respectively. Inhibition of cADPR production in As4.1 cells with Nicot (6 mM) also significantly reduced NaCl-induced Ca^{2+} release by 70% ($n = 6$). However, IP_3 antagonist 2-APB (50 μ M), and a phospholipase C (PLC) inhibitor U73122 (100 nM) only had small effects on NaCl-induced Ca^{2+} release. These results were summarized in Figure 4C.

Effect of inhibition of cADPR/RyR pathway on renin production and release

To determine whether that cADPR/RyR pathway is involved in the regulation of renin production and release in As4.1 cells, RIA assay was performed. As shown in Figure 5, treatment of these cells with NaCl (100mM) for 6 hours significantly decreased total renal content and renin release content which released to medium by 52% and 41%. However, Nicot, Rya and TC markedly restored both total renin content and renin release content. Based on the above results, we conclude that cADPR/RyR pathway is not only involved in the regulation of renin production but also in the regulation of renin activity in NaCl-treated As4.1 cells. Inhibition or blockade of the cADPR signaling system by Nicot, Rya and TC had no

Fig. 6. Effect of NaCl on the renin mRNA levels at different time treatments by real time RT PCR analysis ($n=6$). * $P < 0.05$ vs. control.

significant effects on the renin release and total renin content in As4.1 cells without NaCl treatment. It appears that cADPR primarily mediate the response of renin production or release to high NaCl stimulation in As4.1 cells. To further test whether NaCl-induced calcium release inhibits renin gene expression, real-time RT PCR analysis for renin was performed for different durations of NaCl treatment with a maximum of 6 hours. Figure 6 showed that basal renin mRNA levels slightly decrease in As4.1 cells with NaCl treatment at 4 hours and a 31.5% decrease at 6 hours.

Discussion

The present study was designed to test the hypothesis that increased production cADPR and Ca^{2+} mobilization contribute to the decreased renin production and release in As4.1 cells in response to stimuli such as increase in NaCl loading [30-33]. First, we identified and characterized the enzymatic pathway responsible for the production and metabolism for a novel Ca^{2+} mobilizing second messenger cADPR in As4.1 cells. Moreover, we demonstrated that NaCl-induced intracellular Ca^{2+} release in As4.1 cells is associated with enhanced cADPR in these cells. We found that the decreased intracellular Ca^{2+} concentrations by inhibition of cADPR/RyR-mediated Ca^{2+} -signaling pathway lead to increase in renin production and release. These results suggest that cADPR-mediated Ca^{2+} signaling pathway may importantly participate in the regulation of renin production and release.

It is well known that the ADP-ribosyl cyclase is a ubiquitous enzyme which converts NAD to cADPR and three homologues have been identified including two plasma membrane-associated mammalian proteins, CD38 [34] and CD157 (BST-1) [35-36]. CD38, a membrane-bound lymphocyte antigen, possesses ADP-ribosyl cyclase activity and is considered as the mammalian homolog of the ADP-ribosyl cyclase. In the present study, characterization of subcellular fractions from As4.1 cells revealed that the ADP-ribosyl cyclase and activity are primarily associated with the membrane fraction, while CD38 was also detected in cytosol fraction. Using fluorescence plate reader and HPLC analysis, basal cADPR level and the enzymatic activity were found to be stimulated by NaCl and inhibition of ADP-ribosyl cyclase blocked NaCl-induced increase ADP-ribosyl cyclase activity. These results conclude that ADP-ribosyl cyclase is present in these cells and that cADPR is produced in As4.1 cells.

To demonstrate the role of cADPR/RyR pathway-mediated Ca^{2+} release in As4.1 cells, we examined rapid Ca^{2+} transient response to NaCl in these cells. It was demonstrated that NaCl induced a rapid and transient increase in $[\text{Ca}^{2+}]_i$ in cultured As4.1 cells. Since the cells were bathed with Ca^{2+} -free solution, it is unlikely that Ca^{2+} entry participates in the effect of NaCl-induced Ca^{2+} increase. Therefore, Ca^{2+} release from intracellular store may be the primary resource of NaCl-induced Ca^{2+} increase. In the presence of 8-Br-cADPR, a cell membrane-permeable cADPR antagonist, NaCl-induced calcium release was significantly attenuated by 78.0%. Similar results were obtained when As4.1 cells were

pretreated with Nicot, an inhibitor of ADP-ribosyl cyclase. These results strongly indicate that NaCl-induced $[\text{Ca}^{2+}]_i$ increase is associated with the cADPR signaling mechanism. Furthermore, we determined the contribution of CICR and RyRs to Ca^{2+} response during NaCl-treated As4.1 cells. Either blockade of RyR by Rya or inhibition of CICR by TC markedly reduced NaCl-induced Ca^{2+} release to a similar extent to cADPR inhibition, suggesting that this cADPR pathway-associated component in the NaCl-induced Ca^{2+} response is CICR- and RyR-dependent [37-38].

However, NaCl-induced increase in $[\text{Ca}^{2+}]_i$ in As4.1 cells was not completely blocked by inhibition of this cADPR/RyR signaling pathway by different interventions. It is possible that IP_3 -mediated Ca^{2+} signaling pathway is also involved in the regulation of intracellular Ca^{2+} release. To address this issue, 2-APB, an antagonist of IP_3 and a phospholipase C (PLC) inhibitor U73122 were used to examine the NaCl-induced Ca^{2+} release. It was found that blockade of IP_3 pathway by 2-APB and U73122 only inhibited the NaCl-induced intracellular Ca^{2+} mobilization by 31% and 26% individually. On the basis of these results, it is suggested that the increase in Ca^{2+} release in As4.1 cells may be associated with both IP_3 and cADPR mechanisms, but cADPR-mediated Ca^{2+} signaling pathway represents one of major pathways producing Ca^{2+} increase in response to NaCl.

Previous studies have also addressed the signaling pathway responsible for the control of intracellular Ca^{2+} concentrations in As4.1 cells. Ryan and others [22] reported that the intracellular release pathway of Ca^{2+} contributing to CICR is mediated by the IP_3 receptor Ca^{2+} release channel due to mechanical distension of As4.1 cells. They concluded that the activation of $\text{Ca}^{2+}/\text{IP}_3$ pathway in response to stimulation may provide the basis for communicating a mechanical signal from the plasma membrane to the nucleus, ultimately leading to the control of renin production. The discrepancy from our findings could be due to the different experimental systems and different stimuli used.

To further explore whether the inhibitory action of renin production and release is directly associated with cADPR-mediated Ca^{2+} release from the ER in As4.1 cells, RIA was performed. It was found that NaCl markedly lowered renin production and release levels in these As4.1 cells. Pretreatment of renal with Nicot, Rya and TC significantly restored the inhibitory effect of NaCl on renin production and release in these cells. These results further confirm that cADPR/RyR mediated Ca^{2+} signaling pathway is present in As4.1 cells and plays a

contributing role on the regulation of renin production and release. Very recent studies by Klar J et al have reported that intracellular Ca^{2+} inhibits renin gene expression in As4.1 cells via a concerted action of inhibition of renin gene transcription and destabilization of renin mRNA [8]. It is imperative to know whether renin mRNA levels are changed by cADPR/RyR mediated Ca^{2+} signaling pathway during NaCl treatment. It was found that NaCl could decrease renin mRNA level when cell are exposed to NaCl for 6 hours. These results suggest that the inhibition of renin production by cADPR/RyR mediated Ca^{2+} release may be related to different regulatory events, not only to activation of renin synthetase, but also to changes in mRNA level due to destabilization of the renin mRNA or inhibition of renin gene transcription [8].

It should be noted that the primary goal of this study was to test the hypothesis that cADPR serves as a novel second messenger to mediate intracellular Ca^{2+} concentration in As4.1 cells. NaCl was used as one of stimuli to activate the production of cADPR. We did not attempt to extend these results to an *in vivo* situation where macula densa senses tubular NaCl concentrations inducing renin release from JG cells. We found that NaCl directly acts on As4.1 cells and induces Ca^{2+} release, which also contributes to the regulation of renin production and release in As4.1 cells. It seems that JG cells not only react to the signal from macula densa-extracted tubular NaCl changes, but also react to high concentrations of NaCl directly added to the cells if As4.1 and primary JG cells share the same mechanism. However, it needs to be further investigated in primary JG cells.

To determine whether NaCl-induced Ca^{2+} mobilization through cADPR pathway is associated with high osmolarity, additional group experiments were

performed by using urea, sucrose or Na-gluconate to increase extracellular osmolarity. It was found that all of them had no significant effect on intracellular Ca^{2+} release. It seems that NaCl-induced Ca^{2+} release in JG cells is not due to increased osmolarity under these experimental conditions. It is possible that this high extracellular NaCl concentration induced Ca^{2+} mobilization is associated with the activation of Cl^- movements under this condition, since blockade and attenuation of Cl^- channel activity have been reported to enhance renin production and release in JG cells [24, 39-41, 43]. There is a report indicating that increased production of cADPR by angiotensin II [42] could be attributed to this Cl^- channel-associated mechanism. In the present study, replacement of NaCl by urea or sucrose had no effect on cADPR production and Ca^{2+} release, indicating that the action of NaCl may not be associated with increased extracellular osmolarity. Furthermore, substitution of Na-gluconate for NaCl did not induce Ca^{2+} release. This suggests that Cl^- may participate in cADPR production, Ca^{2+} release and renin production regulation in these As4.1 cells.

Based on these results, we conclude that an enzymatic pathway for cADPR production and metabolism is present in As4.1 cells and that cADPR-mediated Ca^{2+} signaling pathway may importantly contribute to the regulation of renin production and release in these cells.

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