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Calcineurin mediates the angiotensin II-induced aldosterone synthesis in the adrenal glands by up-regulation of transcription of the *CYP11B2* gene

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Aldosterone synthesis in the zona glomerulosa of the adrenal gland is catalysed by aldosterone synthase (CYP11B2). The *CYP11B2* expression is induced by angiotensin II (Ang II), mediated by increase of intracellular Ca^{2+} level. Since calcineurin (CN) is an important mediator activated by Ca^{2+}, we investigated the issue of whether CN is involved in the Ang II-induced *CYP11B2* expression in human adrenocortical H295R cells. First, CN inhibitors, cyclosporine A (CysA) and tacrolimus (FK506) inhibited the Ang II-induced elevation of *CYP11B2* mRNA level. Second, enforced expression of a constitutively active CN increased the *CYP11B2* mRNA level. Third, depletion of CN by siRNA technique blocked the Ang II-induced elevation of *CYP11B2* mRNA level. Fourth, in reporter assays using a luciferase gene connected to a 5′-flanking region (from −134 to +43 bp) of the *hCYP11B2* gene, both CysA and FK506 inhibited the Ang II-mediated up-regulation of luciferase activity. Finally, activation of CN in living H295R cells following the Ang II treatment was confirmed using a fluorescence resonance energy transfer-based sensor. Taken together, we conclude that CN mediates the Ang II-induced aldosterone synthesis through up-regulation of the *CYP11B2* transcription.

Keywords: calciuum/cellular regulation/protein dephosphorylation/regulation/signal transduction/transcription.

Abbreviations: Ang II, anigiotensin II; ANOVA, analysis of variance; CaM, calmodulin; CaMK, CaM-dependent protein kinase; CN, calcineurin; CNΔA, constitutively active form of rat CN Aα; CRE, cAMP-response element; CREB, CRE-binding protein; CYP11B2, aldosterone synthase; CysA, cyclosporine A; FK506, tacrolimus; FRET, fluorescence resonance energy transfer; RAS, rennin-angiotensin system.

The renin–angiotensin system (RAS) is a major regulator of intravascular volume and blood pressure. Dysregulation of the RAS causes a type of hypertension (*1*, *2*). A key effecter in the RAS is the mineralocorticoid aldosterone, which acts on the distal convoluted tubule of kidney nephrons to increase sodium reabsorption, potassium excretion and water reabsorption through osmosis (*3*). Aldosterone synthesis in the zona glomerulosa of the adrenal gland is catalysed by a cytochrome P450 family enzyme, aldosterone synthase, which is encoded by the *CYP11B2* gene (*4*). The *CYP11B2* expression is induced by angiotensin II (Ang II) and potassium, mediated by increase of intracellular Ca^{2+} level (*5*).

Ang II acts via the type 1 Ang II (AT1) receptor, which is coupled to a G-protein family member, Gq. Triggering the receptor activates phospholipase C-β, resulting in increased levels of diacylglycerol and inositol triphosphate; the latter mobilizes intracellular Ca^{2+} stores (*6*). Protein kinase C (PKC) activated by diacylglycerol and Ca^{2+} does not play a major role in the regulation of *CYP11B2* expression (*7*). LeHoux and Lefebvre have reported that Ang II activates PKCα and PKCε in H295 cells, but PKCα and PKCε inhibit AngII-stimulated *CYP11B2* gene expression through mitogen-activeted protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) pathways (*8*). Ca^{2+} is a well-known secondary messenger that affects a number of calcium sensors such as calmodulin (CaM) and calpain. CaM in turn activates CaM-dependent protein kinases (CaMKs) and CaM-dependent serine/threonine protein phosphatase, calcineurin (CN) (*9*). The involvement of CaMKs in the Ang II-induced the *CYP11B2* expression has been extensively investigated by Rainey and his colleagues. Elevated Ca^{2+} level was found to activate CaMKI and CaMKIV (*10*). The Rainey's group has revealed *cis* elements in the 5′-flanking region of the *hCYP11B2* gene and related *trans*-acting factors that regulate the *CYP11B2* expression (*11*–*13*). Among them, the cAMP-response element (CRE) binds activating transcription factors, ATF-1 and ATF-2, and CRE binding protein (CREB) (*12*). Phosphorylation of ATF-1 and/or CREB by CaMKI and CaMKIV increases the ability of these factors to enhance gene transcription (*14*, *15*). Introduction of mutation in the CRE blocks the CaMKI-mediated elevation of *hCYP11B2* reporter activity (*10*). These findings suggest that activated CaMKI and CaMKIV phosphorylate ATF-1 and/or CREB leading to enhanced transcription of the *hCYP11B2* gene. On the contrary, the participation

of CN in the regulation of the *CYP11B2* expression remains to be elucidated.

A CN inhibitor, cyclosporine A (CysA) (*16*) is used as a potent immunosuppressive agent after organ transplantation and in the treatment of several autoimmune diseases. However, its clinical use is frequently complicated by hypertension (*17*). In rat models, CysA-induced hypertension is associated with large increases in plasma renin level with no parallel increments in plasma aldosterone level (*18*, *19*). In addition, CysA attenuates Ang II-stimulated aldosterone secretion in rat adrenal glomerulosa cells (*18*, *19*). These results suggest CN may participate in Ang II-stimulated aldosterone secretion in the adrenal glands.

Human adrenocortical cell line H295R is the only adrenal cell line that expresses all of the steroidogenic enzymes required for the synthesis of aldosterone and preserves the physiological regulation system of aldosterone secretion (*20*). Since intracellular Ca^{2+} level increases in response to Ang II treatment in H295R cells (*21* and this study) and CN inhibitors, CysA and tacrolimus (FK506) (*16*), blocked the Ang II-induced CYP11B2 expression in our preliminary study, we hypothesized that CN is involved in the signalling pathway from the Ang receptor to the gene expression of *CYP11B2*. The current study was undertaken to prove this hypothesis.

Materials and Methods

Materials

Ang II and CysA were purchased from Sigma. FK506 was from Calbiochem. Rat polyclonal antibody against CN-A was from Santa Cruz (sc-9070).

Culture of NIH H295R cells

Human adrenal gland H295R cells were cultured in RPMI-1640 containing 5% fetal bovine serum, 5 μg/ml of insulin, 10 μg/ml of transferrin, 30 nM sodium selenite, 10 nM hydrocortisone, 10 nM β-estradiol and 4 mM L-glutamine. Cells were used under 70–80% confluence. In the default condition, Ang II was treated at the concentration of 10^{-7} M for 8 h before cell harvest. CysA or FK506 was pretreated for 30 min before addition of Ang II in inhibition experiments.

Quantitative real-time PCR

Total RNA was extracted from H295R cells using a GenElute Mammalian total RNA Miniprep kit (Sigma) and reversely transcribed to cDNA using a High Capacity cDNA Reverse Transcription kit (Applied Biosystems). The cDNA was subjected to real time PCR using ABI 7700 for the quantification of *CYP11B2*, *CN* and glyceraldehydes-3-phosphate dehydrogenase (*GAPDH*) mRNA levels. Samples were amplified using FastStart Universal Probe Master (Roche) for *CYP11B2* mRNA. FastStart universal SYBR Green Master (Roche) was used for detection of *CN* mRNA and *GAPDH* mRNA. For *CYP11B2*, the following forward and reverse RT-PCR primers, which located upstream and downstream of the Taqman probe (Roche), respectively, were used to specifically amplify the cDNA, avoiding amplification of a similar sequence of *CYP11B1* cDNA (*22*). The PCR product was digested by *Xmn*I to confirm it to be of *CYP11B2*. *CYP11B1* does not have a digestive sequence for *Xmn*I between the primers. CYP11B2 forward: 5′-CATCCCAGCTGGGACATTGGTACAG-3′; CYP11B2 reverse: 5′-GTAGACCATCTTTATGTCCTCTTGA-3′. For CN, the following primers were used: CN forward: 5′-TATCTATACT CCGAGCCCACGAAGC-3′; CN reverse: 5′-TGAGCAGATGTTG AGGACATTTACCAG-3′. For GAPDH, the following primers were used: GAPDH forward: 5′-AATGACCCCTTCATTGACCT CAACT-3′; GAPDH reverse: 5′-TCCACGACGTACTCAGCGCA GCAT-3′. The conditions of PCR amplification consisted of 10 min at 95°C following 40 cycles of 15 s at 95°C, 60°C 1 min for *CYP11B2*; 10 min at 95°C following 40 cycles of 15 s at 95°C, 30 s at 55°C, 1 min at 72°C for *CN* and *GAPDH*. The quality of the *CN* and *GAPDH* PCR products was confirmed by the melting curve. Real-time PCR was carried out in parallel with the 10^7, 10^6 and 10^5 copies of *CYP11B2*, *CN* and *GAPDH* cDNA fragment as a template, and the quantity of cDNA in sample was calculated using a standard curve. The mRNA levels of *CYP11B2* and *CN* were normalized by the *GAPDH* mRNA level.

Adenovirus vector for constitutively active form of CN

An adenovirus vector encoding constitutively active form of rat CN Aα (CNΔA) (*23*) was constructed. Briefly, a pcDNA3-CNΔA fragment containing the cytomegarovirus promoter and SV40 polyadenylation signal was subcloned into the shuttle plasmid PHM5 (*24*). The constructed shuttle plasmid was then transferred into the E1 deletion region of the adenovirus vector plasmid pAdHM4 by an in vitro ligation method using the I-*Ceu*I and PI-*Sce*I sites generating pAdHM4-CNΔA. The resulting plasmid was then linealized by *Pac*I and transfected into H293 cells using Lipofectamine 2000 Regent (Invitrogen). Adenovirus vector for mock experiment (pAdHM4-Mock) was constructed in the same way except using non-inserted pcDNA3 instead of pcDNA3-CNΔA. The virus titres of AdHM4-CNΔA and AdHM4-Mock were adjusted to 10^7, 10^8,10^9 and 10^{10} pfu/ml.

Adenovirus vector for CN siRNA

Construction of adenovirus vector expressing CN siRNA was made according to the previous method (*24*). Briefly, the human U6 promoter sequence, containing a unique *Swa*I site around the transcription start site and an *Xba*I site downstream from the promoter, was amplified from H295R genomic DNA. The promoter sequence was introduced into the PHM5 plasmid, resulting in PHM5-hU6. To construct shuttle plasmids containing an shRNA-coding sequence against CN and luciferase as control, the following shRNA-coding oligonucleotides were synthesized, annealed, and cloned into the *Swa*I and *Xba*I site of PHM5-hU6. CN shRNA oligonucleotide, in which loop sequence is underlined: 5′-CCGCTTAGACCGAATTA ATGAGAGCTTCCTGTCACTCTCATTAATTCGGTCTAAGC CCTTTTTGGAAATCTAGAGGC-3′; luciferase shRNA oligonucleotide: 5′-CCGATTTCGAGTCGTCTTAATGTGCTTCCTG TCACACATTAAGACGACTCGAAATCCATTTTTGGAAATC TAGAGGC-3′. These constructed shuttle plasmids were then transferred into pAdHM4 as indicated above, resulting in pAdHM4-siRCN and pAdHM4-siRLcf. Adenovirus expressing siRNA constructs was prepared by the transfection of a *Pac*I-linealized shuttle plasmid into H293 cells. The virus titres of AdHM4-siRCN and AdHM4-siRLcf were adjusted to 10^9 pfu/ml.

Western blotting

H295R cells were lysed in 2× sample buffer [0.125 M Tris–HCl (pH 6.8), 4% SDS, 20% glycerol] and protein concentration was determined by a Bradford ULTRA reagent (Novexin) using bovine serum albumin as a standard. Samples with the same amount of protein were subjected to SDS–PAGE under a reduced condition using a 10% gel and then transferred to a PVDF membrane. The resulting membranes were incubated with a rat polyclonal antibody against CN-A (500× dilution), followed by horseradish peroxidase-conjugated anti-rabbit IgG. The protein band was visualized using an Immobilon Western Chemiluminescent HRP Substrate kit (Millipore).

Luciferase reporter gene assay

The human *CYP11B2* 5′-flanking region, containing −2015 bp relative to the transcription start site (*25*), was cloned into the promoterless pGL3-Basic luciferase reporter plasmid (Promega). Several 5′-flanking region deletion mutants were prepared using exonuclease III. One microgram of a mutant plasmid termed pGL3-134bp, containing −134 to +43 bp relative to the transcription start site, was transfected into subconfluent H295R cells using Transfast Transfection Reagent (Promega). The PRL-SV40 plasmid (Promega) was simultaneously transfected to monitor transfection efficiency. After transfection, the cells were cultured for 48 h, followed by pharmaceutical treatment. Luciferase activities were

measured with a luminometer using Dual-Luciferase Reporter Assay System (Promega). One microgram of pcDNA3-CNΔA or pcDNA3-Mock plasmid was cotransfected with the reporter plasmids and luciferase activities were measured as indicated above.

Measurement of intracellular calcium ion concentration

To monitor change in intracellular Ca^{2+} concentration following Ang II administration, 2 μM fluo-4/AM (Invitrogen) was loaded on H295R cells. After incubation at 37°C for 30 min, the cells were washed with medium and incubated for a further 30 min to allow complete de-esterification of intracellular AM ester. Fluorescence of individual cells was measured before and after Ang II stimulation on an Olympus Fluoview FV1000 confocal laser scanning microscope with excitation and emission wavelength, 488 and 516 nm, respectively. Fluorescence intensity at different time points was normalized by the intensity just before Ang II stimulation.

Analysis of intracellular CN activity by means of FRET

Intracellular CN activity was assayed using a fluorescence resonance energy transfer (FRET)-based CN activity sensor, in which the N-terminal regulatory domain of NFAT is sandwiched between cyan fluorescent protein (CFP) and yellow fluorescent protein (YFP) (*26*). In this system, CN activity can be monitored as increases in FRET due to dephosphorylation-dependent conformational changes in the NFAT region. The CN activity sensor gene was transfected into H295R cells cultured in 35-mm dishes. Cells were imaged on an Olympus Fluoview FV1000 with excitation wavelength at 440 nm and two emission wavelengths at 475 nm for CFP and 535 nm for YFP at room temperature. Pseudo colour images according to the ratio of yellow (*Y*)-to-cyan (*C*) were visualized at real time. The average emission ratio (*Y:C*) at various times was normalized by the ratio just before Ang II stimulation. CysA or FK506 was added 30 min prior to Ang II treatment in the inhibition studies.

Statistical analysis

Analysis of variance (ANOVA) was used to test differences among several means for significance. The Student's *t*-test was used to test differences between two means. When *P*-value was < 0.05, difference was considered significant.

Results

CN inhibitors blocked the Ang II-induced elevation of CYP11B2 mRNA level

First, we confirmed that Ang II enhances the gene expression of *CYP11B2* in human adrenal H295R cells. The amount of *CYP11B2* mRNA was quantified by real-time PCR as shown in 'Materials and Methods' section. Ang II increased the *CYP11B2* mRNA level in a dose-dependent manner (Fig. 1). The *CYP11B2* mRNA level was saturated at 10^{-7} M and reached 3-fold compared with the level in the absence of Ang II. The concentration of 10^{-7} M was employed as the default concentration of Ang II treatment afterward.

Next, we investigated the effects of CN inhibitors, CysA and FK506 on the Ang II-induced *CYP11B2* expression. CysA and FK506 had little effect in the absence of Ang II (Fig. 2, left side). In contrast, CysA and FK506 significantly reduced the Ang II-induced elevation of *CYP11B2* mRNA level in a dose-dependent manner (Fig. 2, right side). These consistent inhibitory effects of two different CN inhibitors suggested the involvement of CN in this pathway.

Constitutively active CN increased the CYP11B2 expression

Enforced expression of a constitutively active CN in H295R cells by means of infection of adenovirus

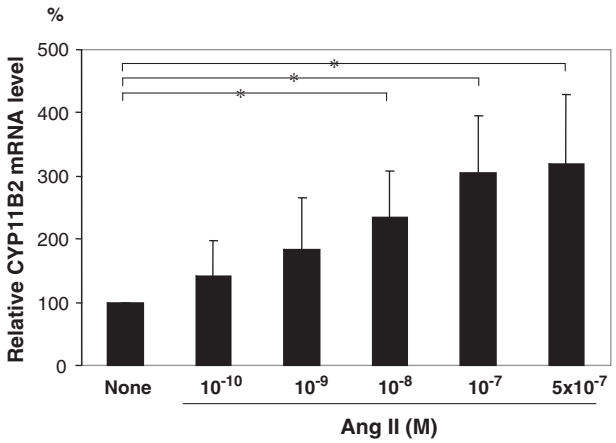

Fig. 1 Effects of Ang II on *CYP11B2* mRNA level in H295R cells. Indicated concentrations of Ang II were added onto cultured H295R cells and incubated for 8 h before cell harvest. Total RNA was extracted and reversely transcribed to cDNA as described in 'Materials and Methods' section. The cDNA was subjected to real time PCR for the quantification of *CYP11B2* and *GAPDH* mRNA level. The *CYP11B2* mRNA level was normalized by the *GAPDH* mRNA level. Data are expressed as percentage against *None* treatment. $*P < 0.05$, $n = 3$, One-way ANOVA test for differences among *None* and various concentrations of *Ang II*-treated groups yielded $P = 0.0054$.

vector-carrying CNΔA (*AdCNΔA*) dose-dependently increased the *CYP11B2* mRNA level, whereas a control virus, AdMock had no effect (Fig. 3A). When 10^8 pfu of AdCNΔA was infected, the *CYP11B2* mRNA level reached 6.3-fold compared with the mock infection of the same titre. Then, we investigated the effect of CN inhibitors on the CNΔA-induced elevation of *CYP11B2* mRNA level. As expected, CysA and FK 506 dramatically abolished the AdCNΔA-stimulated elevation of *CYP11B2* mRNA level (Fig. 3B). These results showed that over expression of CN activity in H295R cells increases *CYP11B2* mRNA level without stimulation of Ang II, indicating a positive regulatory role of CN in this signalling pathway.

Depletion of CN expression blocked the Ang II-induced CYP11B2 expression

In order to elucidate the participation of CN in the Ang II-stimulated signals, we explored the effect of loss of CN function by means of siRNA technique. First, we verified the effect of siRNA technique on CN expression in the mRNA and protein levels. Infection of 10^7 pfu of AdHM4-siRCN (*AdSiRCN*) into H295R cells knocked down *CN* mRNA level after 3 days, and reduced to <15% compared with the mock infection of the same titre (*AdSiRLcf*) (Fig. 4A). In addition, Ang II treatment had no effect on the *CN* mRNA level (Fig. 4A). To test the CN protein level, western blotting was performed. As shown in Fig. 4B, CN protein (*arrow*, 60 K) expression was markedly suppressed by the infection of AdHM4-siRCN compared to that by the infection of control AdHM4siRLcf. Ang II treatment also exhibited no effect on the CN protein level (Fig. 4B). Thus, our

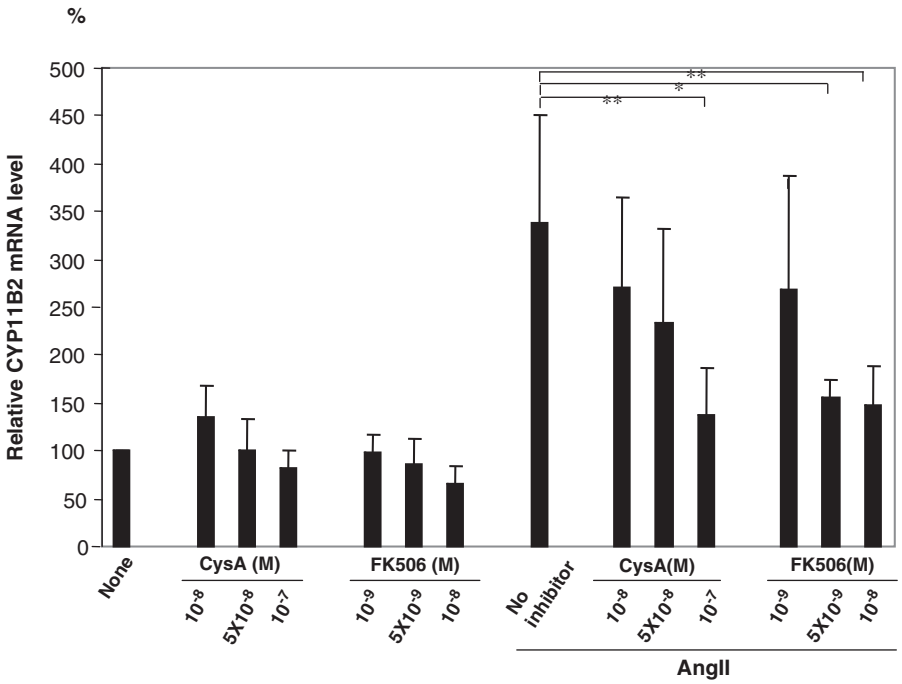

Fig. 2 Effects of CN inhibitors on the Ang II-induced elevation of CYP11B2 mRNA level. Indicated concentrations of CysA or FK506 were administrated in the absence or presence of 10^{-7} M Ang II. CysA and FK506 were added 30 min prior to Ang II. After addition of Ang II, cells were incubated for 8 h before cell harvest. *CYP11B2* and *GAPDH* mRNA levels were determined as Fig. 1. The *CYP11B2* mRNA level was normalized by the *GAPDH* mRNA level. Data are expressed as percentage against *None* treatment. $*P<0.05$, $**P<0.01$, $n=4$. One-way ANOVA test for differences among *No inhibitor* and various concentrations of *CysA* and *FK506*-treated groups in the presence of *Ang II* yielded $P=0.0034$ and 0.0006, respectively.

siRNA system efficiently suppresses the CN expression in H295R cells.

Then, we explored the effect of CN depletion on the Ang II-induced elevation of *CYP11B2* mRNA level. When AdHM4-siRCN (*AdSiRCN*) was infected, the Ang II-induced elevation of *CYP11B2* mRNA level was abolished, whereas control AdHM4-siRLcf virus had no effect (Fig. 4C). However, AdHM4-siRCN displayed no effect on the CYP11B2 mRNA level without Ang II treatment (Fig. 4C). These results indicated that depletion of CN expression blocks the Ang II-induced *CYP11B2* expression.

CN regulated the Ang II-induced reporter gene transcription

In order to investigate whether CN enhances the transcription of the *CYP11B2* gene, we performed reporter gene assay. The 5′-flanking promoter region (from −2015 to +43 bp relative to the transcription start site) of the human *CYP11B2* gene (*25*) was cloned into the pGL3 luciferase reporter plasmid and several deletion mutants were prepared using exonuclease III. A mutant (from −134 to +43 bp), containing the Ad5 and Ad1/CRE elements (*11*, *13*) to which transcription factors Nurr1 and ATF1/CREB bind, respectively, was responsible to Ang II treatment in a time dependent manner (Fig. 5A). After 3 h of Ang II treatment, the luciferase activity began to increase and reached the peak (2.5-fold as much as the basal level) at 8 h (Fig. 5A), and then decreased to the basal level at 24 h. CN inhibitors CysA and FK506 abolished the Ang II-induced luciferase activity in a dose dependent manner (Fig. 5B).

Next, we investigated the effect of constitutively-active CN on the expression of the *CYP11B2* reporter gene. Introduction of pcDNA3-CNΔA increased luciferase activity 6.25-fold compared to control experiment with pcDNA3-Mock (Fig. 5C). Furthermore, CysA and FK506 abolished the CNΔA-enhanced luciferase activity in a dose-dependent manner (Fig. 5C). These results indicate that CN is involved in the Ang II-induced *CYP11B2* reporter gene transcription and suggest that CN enhances the transcription of the *CYP11B2* gene mediated by the promoter region that was identified as responsible to Ang II stimulation (*11*, *13*).

CN was activated following the Ang II treatment in H295R cells

Oscillations of intracellular Ca^{2+} concentration were observed immediately after Ang II stimulation of H295R cells (data not shown), as demonstrated previously (*27*).

Next, we assessed the issue of whether CN is actually activated by Ang II treatment in living H295R cells. For this purpose, we employed a recently developed FRET-based CN activity sensor (*26*). Activated CN dephosphorylates NFAT, leading to its nuclear translocation and activation of target genes. This CN/NFAT pathway was first described in the activation of T cells upon antigen presentation to the T-cell receptor (*28*, *29*). Newman and Zhang designed a phosphatase activity-dependent

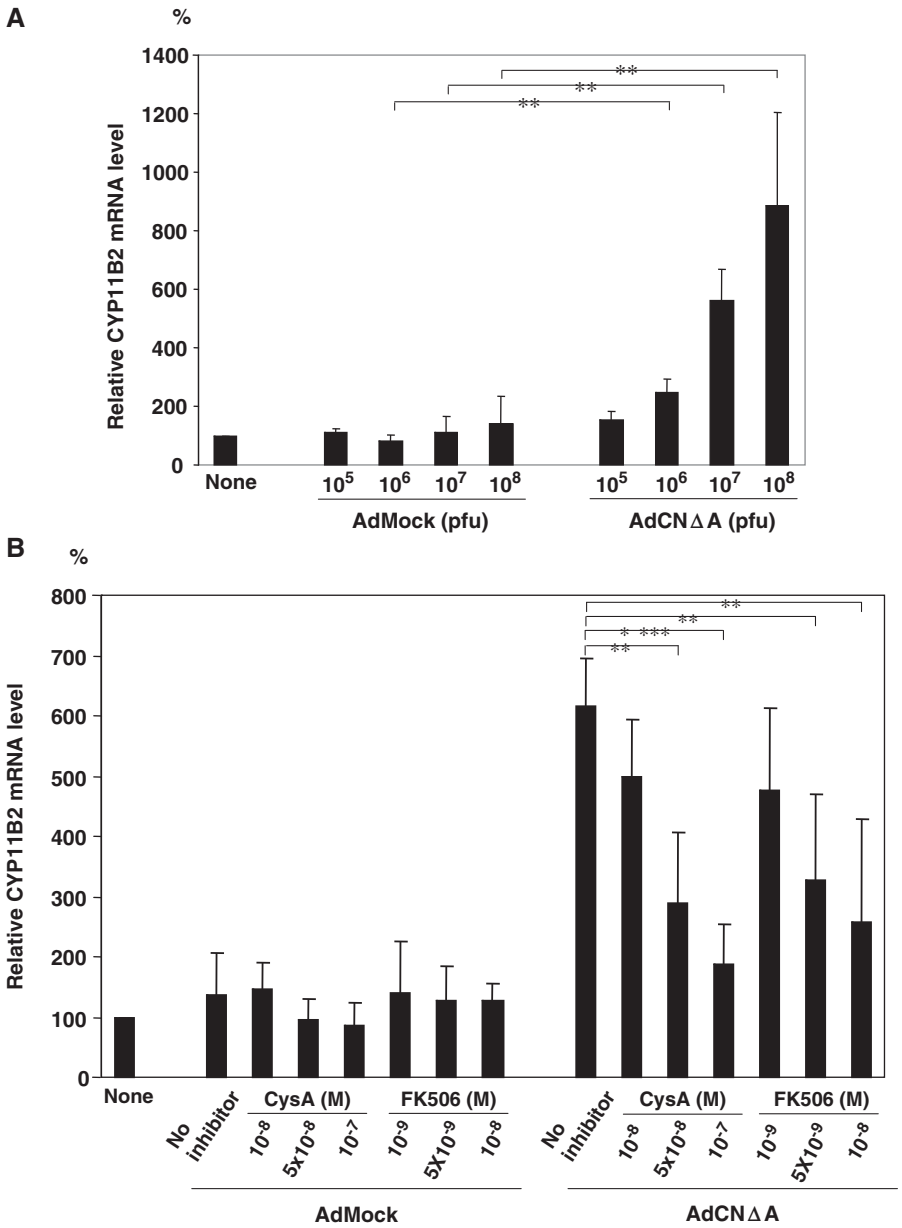

Fig. 3 Effects of enforced expression of constitutively active CN on *CYP11B* mRNA level. (A) H295R cells were infected with indicated titres of adenovirus vector carrying constitutively-active CN (*AdCNΔA*) and mock vector (*AdMock*). After infection, cells were incubated for 72 h before cell harvest. $^{**}P<0.01$, $n=4$. One-way ANOVA test for differences among *None* and various titres of *AdCNΔA*-treated groups yielded $P<0.0001$. (B) *AdCNΔA* or *AdMock* (10^7 pfu) was infected and incubated for 72 h, then indicated concentrations of CysA or FK506 were added to the cells and further incubated for 12 h before cell harvest. *CYP11B2* and *GAPDH* mRNA levels were determined by the same method as Fig. 1. The *CYP11B2* mRNA level was normalized by the *GAPDH* mRNA level. Data are expressed as percentage against *None* treatment. $^{**}P<0.01$, $^{****}P<0.0001$, $n=4$. One-way ANOVA test for differences among *No inhibitor* and various concentrations of *CysA* and *FK506*-treated groups in the presence of *AdCNΔA* yielded $P<0.0001$ and $=0.0061$, respectively.

molecular switch, which is composed of the N-terminal regulatory domain of NFAT sandwiched between CFP and YFP. In this reporter system, CN activity can be monitored as increases in FRET due to dephosphorylation-dependent conformational changes in the substrate region.

After transfection of H295R cells with the CN activity reporter gene, FRET was monitored following the treatment of Ang II. As shown in Fig. 6A, FRET was increased immediately and reached a maximum point within 5 min after addition of Ang II. After that, the highest level was kept over 15 min. When CN inhibitors, CysA and FK506 were added, the FRET signal was attenuated (Fig. 6A and B, respectively), indicating that the increase of FRET was mediated by CN. These results clearly demonstrated that CN is indeed activated in living H295R cells in response to Ang II stimulation.

Discussion

The major physiological regulators of aldosterone production in the zona glomerulosa of the adrenal gland are Ang II and potassium, mediated by increase of

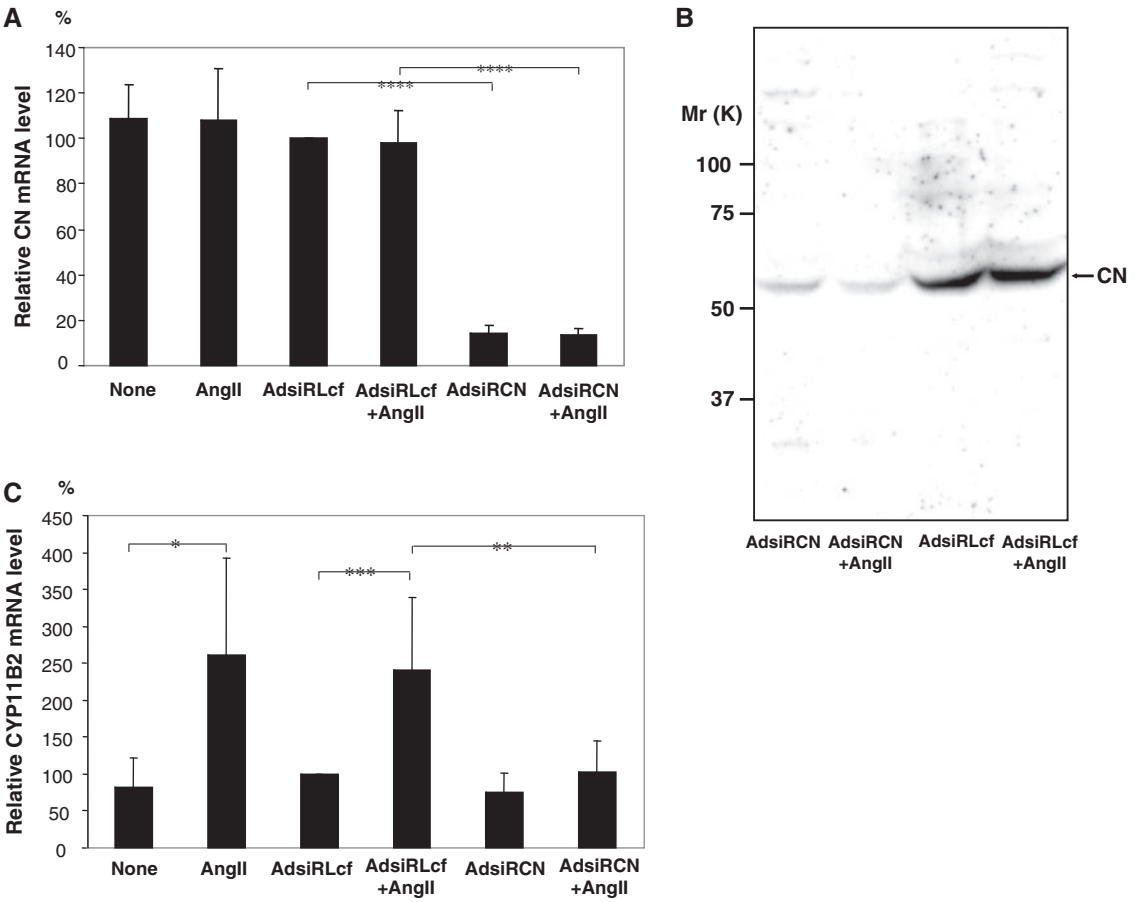

Fig. 4 Effects of depletion of CN on the Ang II-induced elevation of CYP11B2 mRNA level. H295R cells were infected with 10^7 pfu of adenovirus vector carrying CN shRNA (*AdSiRCN*) or irrelevant control vector (*AdSiRLcf*) and incubated for 5 days. Then, 10^{-7} M Ang II was added and further incubated for 8 h before cell harvest. (A) Down-regulation of CN mRNA level by the siRNA introduction. Following extraction of total RNA, *CN* and *GAPDH* mRNA levels were determined as Fig. 1. The *CN* mRNA level was normalized by the *GAPDH* mRNA level. Data are expressed as percentage against the *AdSiRLcf*-infected cells without Ang II treatment. $^{****}P<0.0001$, $n=9$. (B) Reduction of CN protein level by the siRNA treatment. Cell lysates corresponding to 99 μg protein were subjected to western blotting using a rat polyclonal antibody against CN-A. (C) Inhibition of the Ang II-induced elevation of *CYP11B2* mRNA level by the CN siRNA treatment. After extraction of total RNA, *CYP11B2* and *GAPDH* mRNA levels were determined as Fig. 1. The *CYP11B2* mRNA level was normalized by the *GAPDH* mRNA level. Data are expressed as percentage against the *AdSiRLcf*-infected cells without Ang II treatment. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$, $n=9$.

intracellular Ca^{2+} level (*5*). Ca^{2+} is a well-known secondary messenger that affects a number of calcium sensors including CaM. CaM activates CaM-dependent protein kinases (CaMKs) and CaM-dependent protein phosphatase, CN. Although CaMKs are known to participate in the signalling pathway of the Ang II-induced aldosterone (*10*), there is no report on the involvement of CN. Therefore, we investigated the issue whether CN is involved in the Ang II-induced *CYP11B2* expression.

In this study, we have elucidated the following four points to indicate the involvement of CN in the Ang II-induced *CYP11B2* expression in human adrenocortical H295R cells: (i) Ang II treatment enhances the CN activity level in the cells, (ii) CN inhibitors block the Ang II-induced elevation of *CYP11B2* mRNA level, (iii) constitutively active CN increases the *CYP11B2* mRNA level, (iv) knockdown of CN expression blocks the Ang II-induced elevation of *CYP11B2* mRNA level. The last result definitively demonstrates the actual role of CN in the signalling pathway of the Ang II-induced *CYP11B2* expression.

Next, we investigated the issue of whether *CYP11B2* transcription is enhanced by means of a reporter assay. We employed the luciferase gene as a reporter, which was connected to a 5′-flanking region (from −134 to +43 bp) of the *hCYP11B2* gene. Treatment with CN inhibitors suppresses the Ang II-induced elevation of luciferase activity. Furthermore, constitutively-active CN markedly enhances luciferase activity. These observations indicate that CN mediates the up-regulation of *CYP11B2* transcription, resulting in the increase of *CYP11B2* mRNA level.

The CN activation in the cells that was observed in the FRET experiment occurred immediately after Ang II stimulation in accordance with intracellular Ca^{2+} elevation. On the other hand, the *CYP11B2* transcription level that was analysed by a reporter gene activity gradually increased after 3 h and peaked at 8 h. This time lag suggests that some processes intervene between the CN activation and the up-regulation of *CYP11B2* transcription. It is reported that early response genes such as *NGFIB* and *Nurr1* are rapidly induced in H295R cells after AII stimulation, in

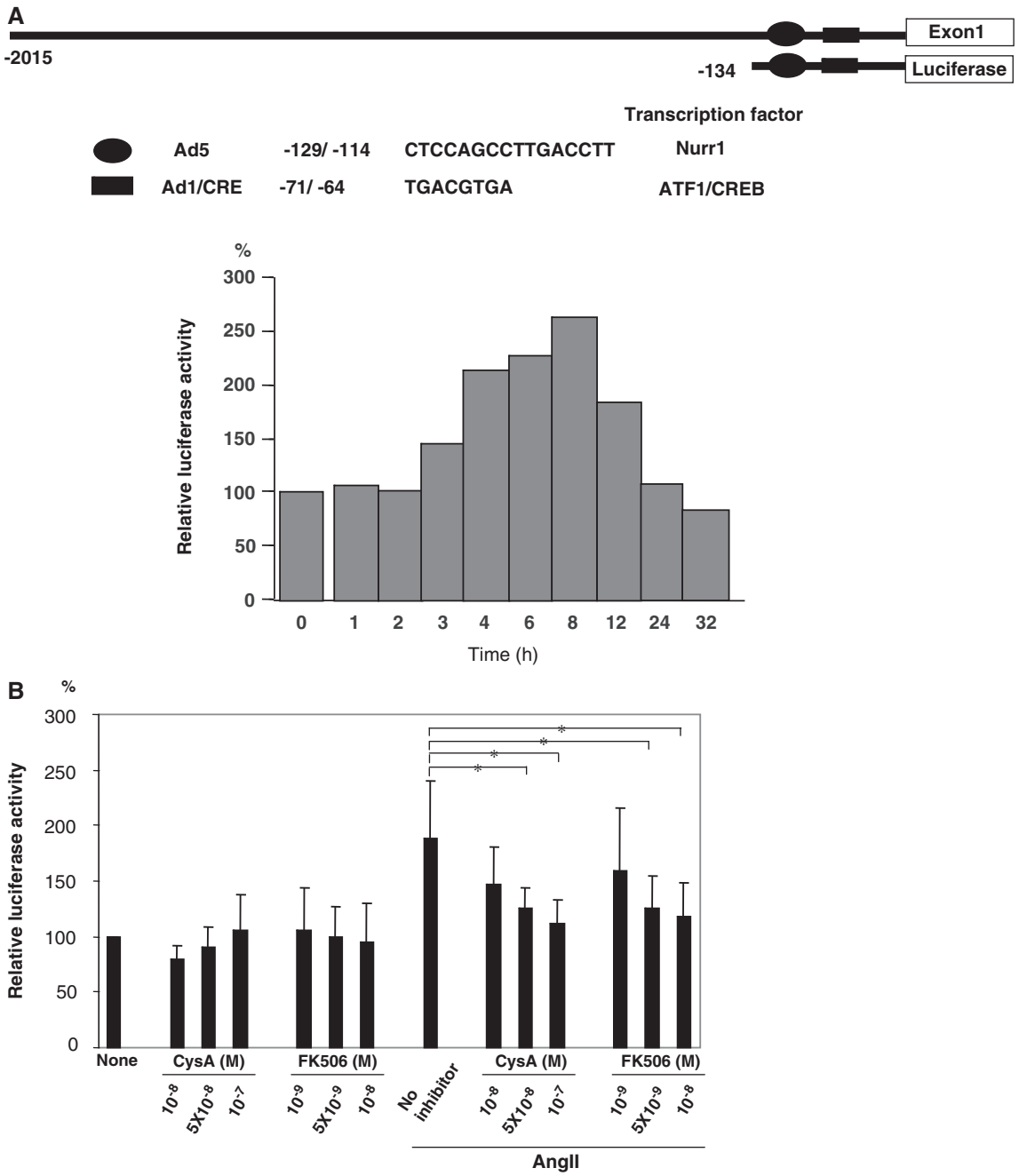

Fig. 5 Effects of CN on the Ang II-induced *hCYP11B2* reporter gene transcription. (A) Construct of the *hCYP11B2* reporter gene, in which luciferase gene is connected to a 5′-UTR region (from −134 to +43 bp) of the *hCYP11B2* gene, and the effects of Ang II treatment on the expression of the reporter gene. After transfection with 1 μg of pGL3-134bp reporter gene, H295R cells were incubated for 48 h. Then, 10^{-7} M Ang II was added and further incubated for indicated time before cell harvest. Luciferase activity was measured as described in 'Materials and Methods' section. Data are expressed as percentage against 0 h. (B) Effects of CysA and FK506 on the Ang II-induced reporter gene transcription. Indicated concentrations of CysA or FK506 were pretreated for 30 min prior to Ang II administration. After Ang II addition, cells were incubated for 8 h before cell harvest. Data are expressed as percentage against *None* treatment. $^{*}P<0.05$, $n=5$. One-way ANOVA test for differences among *No inhibitor* and various concentrations of *CysA* and *FK506*-treated groups in the presence of *Ang II* yielded $P=0.0183$ and 0.0523, respectively. (C) Effects of constitutively-active CN on the Ang II-induced reporter gene transcription. H295R cells were cotransfected with 1 μg of pcDNA3-CNΔA and pGL3-134bp reporter gene and incubated for 48 h. Then, cells were incubated with indicated concentrations of CysA or FK506 for 8 h before harvest. Data are expressed as percentage against *None* treatment. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$, $^{****}P<0.0001$, $n=5$. One-way ANOVA test for differences among *No inhibitor* and various concentrations of *CysA* and *FK506*-treated groups in the presence of *CNΔA* yielded $P=0.0007$ and 0.0002, respectively.

which the expression level of these genes reaches the peak at 3 h and returns to the basal level after 12 h (*30*). Considering that overexpression of NGFIB and Nurr1 induces the up-regulation of *CYP11B2* transcription (*13*), the rapid induction of the transcription factors could be involved in the late induction of the *CYP11B2* gene.

The induction of *CYP11B2* gene expression was much higher in the case of introduction of constitutively active form of CN than AII stimulation. Ang II

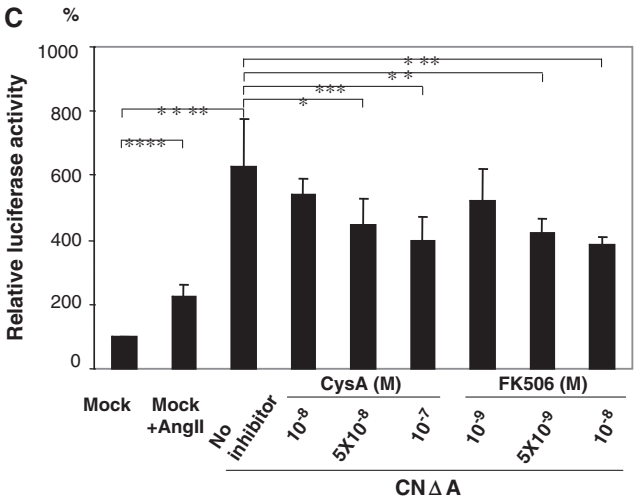

Fig. 5 Continued.

stimulates different intracellular signalling pathways in a different time course. Some of which enhance the gene expression of *CYP11B2*, but some cancel these induction pathways. Constitutively active form of CN may stimulate a particular pathway leading to the induction of the *CYP11B2* gene expression. Further investigation is needed to elucidate which pathway CN is involved in.

Ang II is known to induce cardiac hypertrophy (*31*). An important signalling pathway of this Ang II-induced myocyte hypertrophy is the CN/NFAT pathway (*32*). NFAT is also involved in the activation of T cells and the development of cardiac, skeletal muscle and nervous systems. Although NFAT was used as an artificial substrate of CN in this study, the real substrates of CN in the signalling pathway of Ang II-induced *CYP11B2* expression remain to be defined. There is no potential NFAT consensus binding site (GGAAAAT) (*33*) in the 5′-flanking region of the *hCYP11B2* gene. It is interesting to know what transcription factors are involved in the signalling pathway mediated by Ang II and CN in human adrenocortical cells, and this is currently under study in our laboratory.

In conclusion, we demonstrated that CN mediates the Ang II-induced aldosterone synthesis through upregulation of the *CYP11B2* transcription.

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Conflict of interest

None declared.

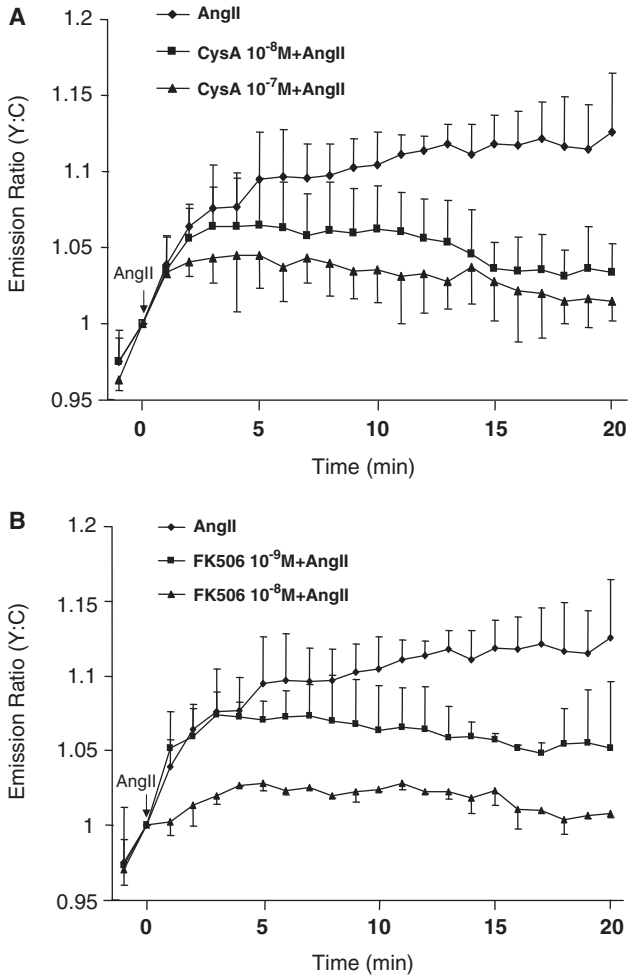

Fig. 6 Effects of Ang II on CN activity in living H295R cells. Activation of intracellular CN following Ang II treatment. The FRET-based CN activity sensor gene, in which the regulatory domain of NFAT is sandwiched between CFP and YFP, was transfected into H295R cells. After transfection, cells were incubated for 72 h. Then, cells were pretreated with indicated concentrations of CysA (A) and FK506 (B) before addition of Ang II. The emission ratio (Y:C) of FRET was measured before and after 10^{-7} M Ang II treatment.

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