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Activation of adenylate cyclase by forskolin increases the protein stability of RCAN1 (DSCR1 or Adapt78)

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ABSTRACT

Overexpression of Regulator of Calcineurin 1 (RCAN1/DSCR1/Adapt78) is known to inhibit the calcineurin-NFAT dependent signaling pathway. In this report, we find that activation of adenylate cyclase by forskolin increases the expression of RCAN1 through the increase of the protein's half-life. The ability of forskolin to increase the accumulation of RCAN1 protein is significantly inhibited with protein kinase A inhibitors such as KT5720 and H-89. Furthermore, forskolin targets the central and C-terminal region of RCAN1 and enhances the inhibitory effect of RCAN1 on the calcineurin-mediated activation of NFAT. Our findings provide the first evidence that the accumulation of the RCAN1 protein by cAMP acts as an important regulatory mechanism in the control of the calcineurin-dependent cellular pathway.

Structured summary:
MINT-7262390: *PKA* (uniprotkb:P22694) *phosphorylates* (MI:0217) *RCAN1* (uniprotkb:P53805) by *protein kinase assay* (MI:0424)

1. Introduction

Regulator of Calcineurin 1 (RCAN1/DSCR1/Adapt78) was first identified as a Down Syndrome Critical Region 1 (DSCR1) gene located in a region of human chromosome 21, of which trisomy of the gene causes Down Syndrome (DS) [1–3]. The RCAN1 gene consists of seven exons, and alternatively spliced [4]. The major isoforms of RCAN1 protein are RCAN1-1, with the N-terminal amino acids encoded by exon 1, and RCAN1-4, with the N-terminal amino acids encoded by exon 4 [4]. The mRNA containing exon 1 encodes two potential translation initiation sites and might be translated into two proteins such as RCAN1-1S (short) and RCAN1-1L (long). All RCAN1 isoforms share 168 amino acids encoded by exons 5, 6, and 7 [4]. RCAN1 gene is expressed in many cell types and tissues such as brain, spinal cord, heart, liver and skeletal muscle and is elevated in the brains of Alzheimer disease patients (AD) [4,5]. Recent studies have indicated that RCAN1 plays an important role in calcineurin signaling by acting as a negative modulator through physical and functional association with calcineurin [5,6].

Calcineurin is a calcium/calmodulin-dependent serine/threonine phosphatase that has been involved in many cellular signaling processes such as synaptic plasticity and memory formation, T-cell activation and apoptosis, muscle growth and differentiation, and cardiac functions [7,8]. The most well characterized calcineurin substrate is the nuclear factor of activated T cell (NFAT) family of transcription factors [9]. Calcineurin activates the gene expression of several targets by directly dephosphorylating NFAT in cooperation with multiple partners including AP-1, MEF2, and GATA proteins [7,10–12]. Dysregulation of calcineurin activity has also been implicated with many forms of brain disease and injury, including Alzheimer disease (AD) and ischemia [13,14].

Recently, RCAN1 expression has been shown to be dynamically regulated by several intracellular signaling pathways [5,15–17]. Calcium and hydrogen peroxide regulates calcineurin-NFAT signaling pathway by inducing the mRNA expression of RCAN1-1S [5,15]. Glucocorticoids up-regulate the expression of RCAN1-1 in leukemic cells and Notch signaling down-regulates the expression of RCAN1-1 and RCAN1-4 in keratinocyte growth and differentiation [16,17]. After an ischemic insult, up-regulation of RCAN1-4 in the per-infarct cortex has been reported [18]. In

Abbreviations: RCAN1, Regulator of Calcineurin 1; PKA, protein kinase A; PMA, phorbol myristate acetate

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addition, posttranslational modification of RCAN1-1S and RCAN1-1L by phosphorylation can affect its protein stability via the ubiquitin–proteasome pathway [19,20]. Based on the above reports, it is likely that regulation of RCAN1 expression could potentially be associated with a wide range of cellular and tissue functions.

In the present study, we examined the possible effects of intracellular cAMP signaling pathway, which is important in neuronal activity, on the regulation of RCAN1 expression. We found that elevation of intracellular cAMP by forskolin increased the protein stability of RCAN1 through the increase of the protein's half-life. Furthermore, increase of RCAN1 accumulation by forskolin enhanced its inhibitory function on the calcineurin-mediated activation of NFAT transcription. Our data reveal a new regulatory mechanism of cAMP signaling on the negative regulation of calcineurin signaling pathway.

2. Materials and methods

2.1. Materials and expression vectors

Anti-HA, anti-GAPDH, and anti-GFP antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-phosphoserine antibody, forskolin, CPT-cAMP, H-89, 3-isobutyl-1-methylxanthine (IBMX), ionomycin, catalytic subunit of protein kinase A (PKA), and phorbol 12-myristate 13-acetate (PMA) were purchased from Sigma–Aldrich (St. Louis, MO). The expression vector for triple HA-human RCAN1-1S (NM_203417) was kindly provided by S. de la Luna. Expression vectors for wild-type and deletion construct of GFP-RCAN1-4 were a gift from B.A. Rothermel. The NFAT-driven reporter plasmid (pGL-IL2-luc) was kindly provided by G.R. Crabtree. A polymerase chain reaction (PCR)-based procedure was applied to generate point mutants in RCAN1 such as S108A, 112A, and S108/112A.

2.2. Cell culture

Human neuronal SH-SY5Y cells were maintained in DMEM supplemented with 10% FBS, penicillin, and streptomycin. Cells were transfected with the expression vectors by the LipofectAmine method (Invitrogen). After 24 h, cells were lysed in lysis buffer [20 mM Tris–Cl (pH 7.9), 1% Nonidet P-40, 150 mM NaCl, 1 mM EGTA, 10% glycerol, 1 mM Na_3VO_4, 1 μg/ml leupeptin, 1 μg/ml aprotinin, 10 mM NaF, and 0.2 mM phenylmethylsulfonyl fluoride].

2.3. Immunoprecipitation and immunoblotting

Cell lysates were subjected to immunoprecipitation with anti-HA antibody for 2 h, followed by adsorption to Sepharose-coupled protein G for 1 h. For immunoblot analysis, total cell lysates were separated by 10% SDS–PAGE, and the proteins were transferred to nitrocellulose membranes. The membranes were blocked in TBST buffer [20 mM Tris–Cl (pH 7.6), 137 mM NaCl, 0.05% Tween-20, and 3% non-fat dried milk] for 30 min and then incubated overnight at 4 °C in TBST buffer containing the appropriate antibodies.

2.4. Pulse-chase analysis

For experiments examining the metabolic stability of RCAN1, cells were preincubated for 1 h in Met/Cys-free DMEM and labeled for 10 min with 100 μCi/ml [^{35}S]-Met/Cys in Met/Cys-free DMEM. The cells were chased for the indicated time periods and the cell lysates were subjected to immunoprecipitation. The immunoprecipitates were resolved by SDS–PAGE, visualized with a phosphoimager, and quantified.

2.5. Reporter gene assays

SH-SY5Y cells were transfected with 0.5 μg of the each expression vectors by the LipofectAmine method (Invitrogen). Luciferase activity was measured using the luciferase assay system (Promega) and was normalized for transfection efficiency using a β-galactosidase expressing vector (pCMV5.LacZ) and the Galacto-Star system Perkin–Elmer).

2.6. RT-PCR

RNA preparation, reverse transcription, and PCR were performed as described previously [21]. The primers were: RCAN1 forward, 5′-GAGGAGGTGGACCTGCAGGACCTG-3′; RCAN1 reverse, 5′-TCAGCTGAGGTGGATCGGCGTGTAC-3′; GAPDH forward, 5′-ACCACAGTCCATGCCATCAC-3′; GAPDH reverse, 5′-TCCACCACCCTGTTGCTGTA-3′.

2.7. In vitro kinase assay

SH-SY5Y cells were transfected with the HA-RCAN1-1S and expressed for 24 h. Cell lysates were then subjected to immunoprecipitation with anti-IgG or anti-HA antibody for 2 h, followed by adsorption to Sepharose-coupled protein G for 1 h. After washing with lysis buffer, in vitro kinase assay was performed by incubating the immunoprecipitates with or without catalytic subunit of PKA in the reaction buffer [20 mM HEPES (pH 7.4), 10 mM $MgCl_2$, 2 mM DTT, 5 μCi [γ-^{32}P] ATP, and 100 μM ATP] at 30 °C for 30 min. The reaction was stopped by adding SDS sample buffer and subjected to SDS–PAGE. Phosphorylation was visualized by autoradiography.

3. Results and discussion

3.1. Forskolin increases the steady-state amount of RCAN1

To investigate whether the cyclic adenosine monophosphate (cAMP) signaling pathway could affect the steady-state level of RCAN1 protein, we ectopically expressed HA-tagged RCAN1-1S in neuronal SH-SY5Y cells and treated the cells with the adenylate cyclase activator, forskolin (Fig. 1A). We observed that forskolin increased the protein amount of RCAN1-1S in a time-dependent

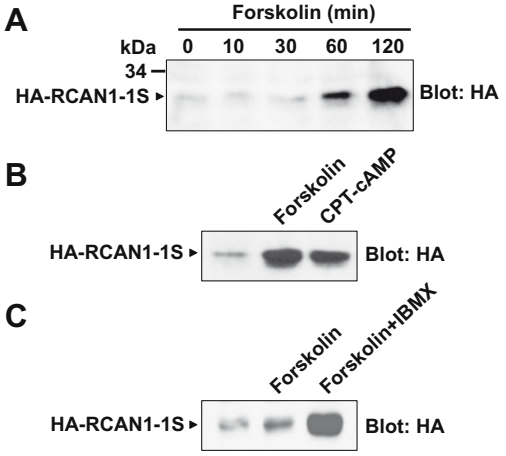

Fig. 1. Increased expression of RCAN1 by augmentation of intracellular cAMP levels. SH-SY5Y cells were transfected with HA-RCAN1-1S for 24 h. (A) Cells were treated with forskolin (10 μM) for the indicated times. (B) Cells were treated with forskolin (10 μM) and 8CPT-cAMP (100 μM) for 2 h. (C) Cells were treated with forskolin alone or together with IBMX (500 μM) for 2 h. The expression of HA-RCAN1-1S was assessed by immunoblotting with an anti-HA antibody.

manner (Fig. 1A). This increased amount of RCAN1-1S by forskolin was caused by augmentation of intracellular cAMP levels since the membrane permeable cAMP analogue 8CPT-cAMP, induced the accumulation of RCAN1-1S to the same extent as forskolin (Fig. 1B). Furthermore, treatment of 3-isobutyl-1-methylxanthine (IBMX), a phosphodiesterase inhibitor, together with forskolin, showed a significant synergistic effect on the increase in RCAN1-1S protein amount (Fig. 1C). Together, these results indicate that an increase of intracellular cAMP by forskolin increases the amount of RCAN1-1S.

3.2. Forskolin prolongs the half-life of RCAN1 protein

To test if significant accumulation in RCAN1-1S protein by forskolin was due to an increase in transcription, we performed RT-PCR. As shown in Fig. 2A, the level of RCAN1-1 mRNA was not significantly altered by forskolin treatment, suggesting that forskolin did not affect the transcription of RCAN1-1S. We next examined whether increased amount of RCAN1 by forskolin was caused by an increase in protein stability. To show that the accumulation of RCAN1-1S by forskolin was due to an increase in the half-life of the RCAN1 protein, we monitored the turnover rate of RCAN1-1S in the presence or absence of forskolin by pulse-chase analysis. As shown in Fig. 2B and C, treatment with forskolin significantly delayed the turnover rate of the newly synthesized RCAN1-1S protein. Therefore, we conclude that forskolin induces an accumulation of RCAN1-1S by increasing the protein's half-life.

3.3. Forskolin increases the stability of RCAN1 protein through a PKA-dependent mechanism

Next, we determined whether the cAMP-induced accumulation of RCAN1-1S protein might occur through a PKA-dependent intracellular signaling mechanism. As shown in Fig. 3A, preincubation of cells with a well-known PKA specific inhibitor, KT5720 inhibited the RCAN1-1S protein accumulation by either forskolin or 8CPT-cAMP, suggesting that cAMP-PKA signaling pathway was involved in the accumulation of RCAN1-1S protein.

We next explored whether forskolin increased the stability of RCAN1-1S protein through phosphorylation of RCAN1-1S. As shown in Fig. 3B, forskolin induced serine phosphorylation of RCAN1-1S. Furthermore, inhibition of PKA with H-89 blocked the serine phosphorylation of RCAN1-1S as well as the amount of RCAN1-1S suggesting that stability of RCAN1-1S protein was regulated by PKA (Fig. 3B). To confirm the phosphorylation of RCAN1-1S by PKA, we performed in vitro kinase assay. As shown in Fig. 3C, the immunoprecipitated RCAN1-1S was phosphorylated in the presence of catalytic subunit of PKA.

To further characterize whether the phosphorylation of RCAN1-1S was required for the increased accumulation by forskolin, we expressed the Ser mutants HA-RCAN1-1S (S108A) and S112A, as well as double mutant S108/112A, which were previously known to be phosphorylated by several kinases such as MEKK3, GSK3β, and ERK [22,23]. As shown in Fig. 3D, forskolin increased the protein amounts of these mutants of RCAN1-1S to the same extent as that of wild-type, indicating that these sites were not specifically targeted by forskolin.

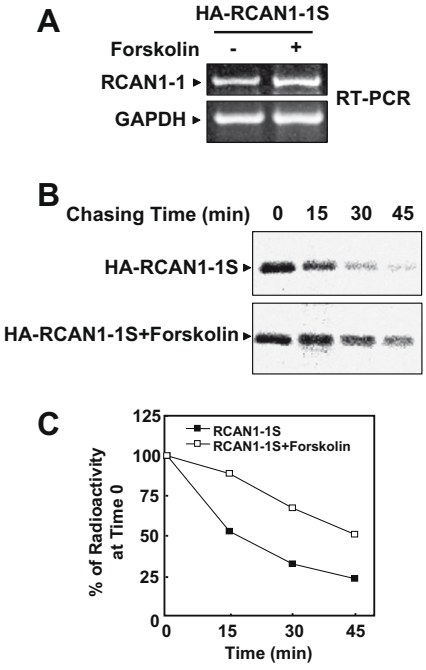

Fig. 2. Increased protein half-life of RCAN1-1S by forskolin. SH-SY5Y cells were transfected with HA-RCAN1-1S for 24 h. (A) Cells were incubated in the presence or absence of forskolin for 2 h followed by measuring mRNA levels of RCAN1-1S and GAPDH by RT-PCR. (B) Transfected cells were pulse labeled with [^{35}S]-Met/Cys and chased for the indicated times in the presence or absence of forskolin. Cell lysates were immunoprecipitated with an anti-HA antibody, and the [^{35}S]-labeled HA-RCAN1-1S proteins were visualized with a phosphoimager (B) and quantified (C).

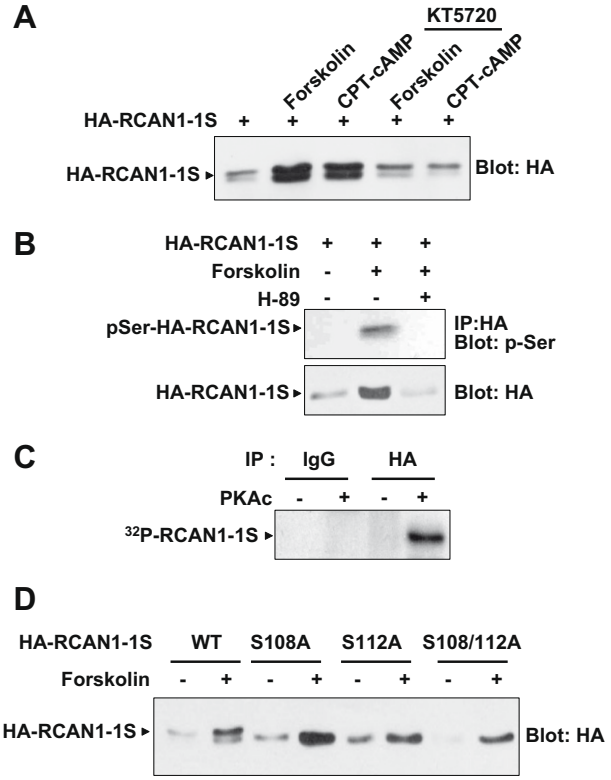

Fig. 3. Forskolin increases the steady-state level of RCAN1-1S protein through PKA. SH-SY5Y cells were transfected with the wild-type of HA-RCAN1-1S or with the point mutants constructs of HA-RCAN1-1S for 24 h. (A) Cells were preincubated with or without the indicated inhibitors KT5720 (3 μM) for 30 min followed by treatment with forskolin or 8CPT-cAMP for 2 h. (B) Cells were preincubated with H-89 (50 μM) for 30 min and the cell lysates were immunoprecipitated (IP) with an anti-HA antibody and immunoblotted with an anti-phophoserine (p-Ser) antibody. The expression of HA-RCAN1-1S was assessed by immunoblotting with anti-HA antibody. (C) Cell lysates were immunoprecipitated (IP) with an anti-IgG or anti-HA antibody. The immunoprecipitates were in vitro phosphorylated in the presence or absence of catalytic PKA and visualized by autoradiography. (D) Cells were treated with or without forskolin for 2 h and the expression of HA-RCAN1-1S were assessed by immunoblotting with the HA-antibody.

3.4. Forskolin targets the central and C-terminal region of RCAN1

We next explored whether increased amount of RCAN1-1S protein by forskolin is an isoform-specific effect. For this, we expressed GFP tagged RCAN1-4 and treated the cells with forskolin (Fig. 4A). As shown in Fig. 4A, we observed that forskolin increased the protein amount of RCAN1-4. Furthermore, inhibition of PKA with H-89 blocked the accumulation of RCAN1-4 suggesting that the accumulation of RCAN1 protein by forskolin is not an isoform-specific effect.

To determine which regions of RCAN1-4 protein are specifically targeted by forskolin for the increased protein accumulation, we expressed several deletion constructs encoding the RCAN1-4 fragments fused to GFP (Fig. 4B). We found that forskolin increased the protein amount of the RCAN1-4 fragments containing amino acids 30–197 and 90–197, as well as the full length RCAN1-4. However, forskolin had no effect on the expression of the RCAN1-4 fragments containing the 90 N-terminal amino acids, suggesting that the RCAN1-4 region critical for the increased protein stability by forskolin was within the amino acid residues 90–197. Since RCAN1-4 fragments containing amino acids 90–197 is common in RCAN1-1S and RCAN1-4 isoform (Fig. 4C), these results support that the effect of forskolin to induce the accumulation of RCAN1 protein is not an isoform-specific and might be applied to both isoforms.

3.5. Forskolin increases the inhibitory role of RCAN1 on NFAT activation through its central and C-terminal region

Activation of calcineurin by calcium stimulators using a combination of PMA and a Ca^{2+} ionophore dephosphorylates the transcription factor, NFAT resulting in NFAT translocation to the nucleus where it activates the transcription of target genes such as *IL2* [10,24]. Previous reports have suggested that RCAN1 can inhibit calcineurin-mediated NFAT-dependent gene transcription [5,6]. To elucidate the functional role of forskolin in increasing the stability of RCAN1 protein, we investigated whether forskolin could affect the inhibitory activity of RCAN1 on calcineurin-mediated NFAT luciferase activity of the *IL2* promoter. As shown in Fig. 5A, forskolin significantly enhanced the inhibitory effect of RCAN1-1S on calcineurin-mediated NFAT-transcriptional activity. The inhibitory effect of the double serine mutant (Ser108/112A) of RCAN1-1S on NFAT activation is the same extent as that of wild-type, suggesting that these sites were not specifically targeted for the enhanced inhibitory function of RCAN1-1S by forskolin (Fig. 5A). Furthermore, Western blot of Fig 5A indicates that forskolin enhanced inhibitory activity of RCAN1-1S on NFAT-dependent gene expression is due to an increase of the protein amount of RCAN1-1S.

Consistent with this result, forskolin could not affect the ability of the N-terminal fragment of RCAN1-4 (1–90) on NFAT-dependent *IL2* gene transcriptional activity (Fig. 5B), supporting that the enhanced inhibitory function of RCAN1 on NFAT-dependent transcription by forskolin was located in its central and C-terminal region. Taken together, these results indicate that forskolin enhances the inhibitory function of RCAN1 on calcineurin-induced NFAT-transcriptional activation by increasing protein accumu lation.

This study reports the first evidence that activation of adenylate cyclase by forskolin increases the expression of RCAN1 through increasing the half-life of the protein. We suggest that the ability

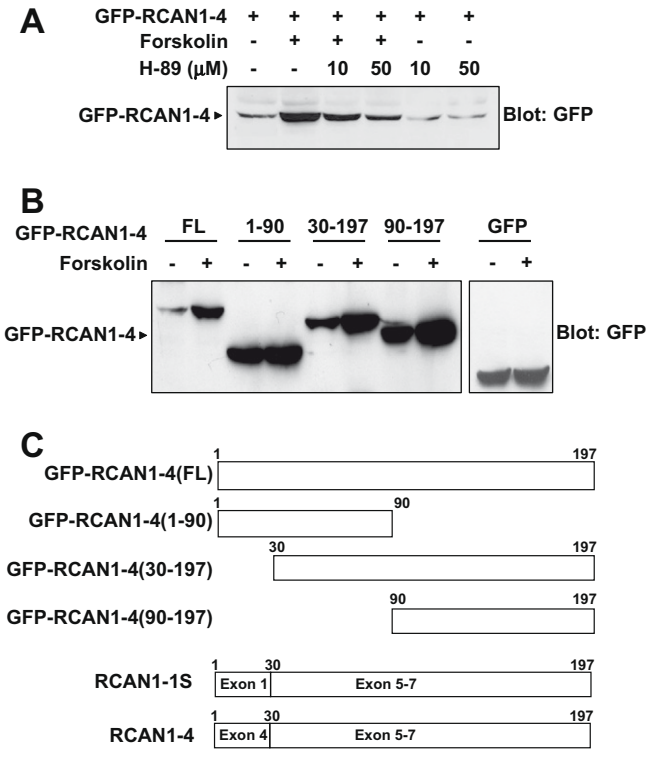

Fig. 4. Forskolin increases the protein amount of the central and C-terminal region of RCAN1. (A and B) SH-SY5Y cells were transfected with the indicated constructs of GFP-RCAN1-4 for 24 h. Cells were preincubated with or without H-89 for 30 min followed by treatment with forskolin for 2 h. The expression of GFP-RCAN1-4 was assessed by immunoblotting with the anti-GFP antibody. (C) Schematic diagram of RCAN1 constructs.

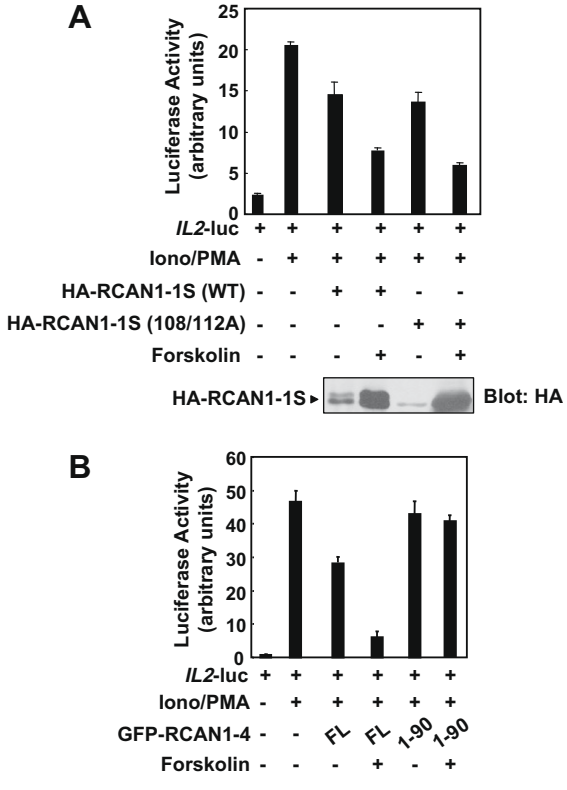

Fig. 5. Forskolin enhances RCAN1-dependent inhibition of NFAT-transcriptional activity. SH-SY5Y cells were transfected with 0.5 μg of *IL2*-luc reporter alone or in the presence of the indicated RCAN1 constructs for 24 h. Cells were treated with ionomycin (500 nM) plus PMA (10 ng/ml) for 6 h in the presence or absence of forskolin. The results are normalized with β-galactosidase activity and are represented as the means ± S.D. of three independent experiments.

of forskolin to increase the stability of RCAN1 protein results from a PKA-dependent intracellular signaling mechanism. Several recent report suggest the strong possibility that stability of short-lived RCAN1 might be continuously regulated by the proteasome through phosphorylation of RCAN1 [19,20,25]. For example, NF-kB inducing kinase (NIK), which has been identified as a binding partner of RCAN1 by yeast two hybrid screening, physically interacts with RCAN1 and can phosphorylate it [20]. The phosphorylation thus leads to an increase in protein stability by attenuating the proteasome-mediated degradation of RCAN1 [20]. In addition, phosphorylation also increases the rate of RCAN1 degradation [19]. In support of this, the SCF^{CDC4} E3 ubiquitin ligase complex has recently been identified as an enzyme that triggers the degradation of yeast Rcn1, a homologue of RCAN1 [26].

In the course of this study, we frequently detect exogenously expressed HA-tagged RCAN1-1S protein as two different electrophoretic bands presumably by phosphorylation and non-phosphorylation. In support of this, mutated forms of RCAN1-1S in which Ser 108 or Ser 112 are converted to Ala migrated faster by Western blot analysis as compared with wild-type RCAN1-1S (Fig. 3D). However, we have not been able to detect changes in mobility with the expression of GFP tagged RCAN1-4 (Fig. 4A and B). We speculate that the big size of GFP tag might function to inhibit the phosphorylation-dependent mobility shift of RCAN1-4 under our Western blot conditions.

Calcineurin is a Ca^{2+}/calmodulin-dependent serine/threonine phosphatase that coordinates a variety of cellular process [8,27]. Since RCAN1 was identified as a feedback inhibitor of the calcineurin signaling pathway [6], we set out to investigate the functional role of forskolin on the regulation of calcineurin activity by RCAN1. Many reports have indicated that calcineurin activated NFAT dephosphorylation is implicated in neuronal synaptic plasticity, cardiac hypertrophy, T-cell activation, and vascular development [28–31]. Our results show that the increased protein stability of RCAN1 by forskolin enhances the negative regulatory effect of RCAN1 on NFAT-dependent gene transcription. Thus it is likely that activation of cAMP-PKA may enable RCAN1 to more effectively oppose the calcineurin-mediated intracellular signaling pathway. Based on our observations, it is tempting to speculate that by inducing an accumulation of the RCAN1 protein, activation of cAMP-PKA pathway could be used as a therapeutic strategy to attenuate calcineurin activity, an event that is activated in diverse pathologies such as cerebral ischemia and neurodegenerative processes.

Acknowledgements

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