

Full-length article

Inhibitory effect of ginsenoside Rb₁ on calcineurin signal pathway in cardiomyocyte hypertrophy induced by prostaglandin F_{2α}¹

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Key words

Rb₁; prostaglandin F_{2α}; cardiac hypertrophy; calcineurin

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Abstract

Aim: To examine the antihypertrophic effect of ginsenoside Rb₁ (Rb₁) induced by prostaglandin F_{2α} (PGF_{2α}) *in vitro* and to investigate the possible mechanisms involved in the calcineurin (CaN) signal transduction pathway. **Methods:** The cardiomyocyte hypertrophy induced by PGF_{2α} and the antihypertrophic effect of Rb₁ were evaluated in primary culture by measuring the cell diameter, protein content, and atrial natriuretic peptide (ANP) mRNA expression. ANP and CaN mRNA expressions, CaN and its downstream effectors NFAT₃ and GATA₄ protein expressions, and the intracellular free Ca²⁺ concentration ([Ca²⁺]_i) were assayed by RT-PCR, Western blot, and fluorescent determination using Fura 2/AM, respectively. **Results:** PGF_{2α} (100 nmol/L) significantly increased the cardiomyocyte diameter, protein content and [Ca²⁺]_i, and promoted ANP, CaN mRNA, and CaN/NFAT₃/GATA₄ protein expressions, which were inhibited by either Rb₁ in a concentration-dependent manner (50, 100, and 200 μg/mL) or *L*-arginine (1 mmol/L). *N*^G-nitro-*L*-arginine-methyl ester, a nitric oxide synthase inhibitor, could abolish the effects of *L*-arginine, but failed to change the effects of Rb₁ in the experiments above. **Conclusion:** The present data implicate that Rb₁ attenuates cardiac hypertrophy, the underlying mechanism may be involved in the inhibition of the Ca²⁺-CaN signal transduction pathway.

Introduction

Cardiac hypertrophy is recognized as an adaptive response characterized by the growth of individual cardiomyocytes in size rather than the increase in cell number. Initially beneficial, sustaining cardiac hypertrophy eventually leads to decompensation and results in dilated cardiomyopathy, arrhythmia, fibrotic disease, heart failure, and even sudden death^[1]. Furthermore, some studies have indicated that hypertrophy may not be required for a successful adaptation to increased workload^[2]. Clinical studies have found that several classes of drugs, including angiotensin-converting enzyme inhibitors, angiotensin II receptor blockers, calcium channel blockers^[3–5], and nitric oxide (NO)^[6] could have some beneficial effects in the prevention or treatment of car-

diac hypertrophy. However, more studies should be done to provide more therapeutic choices for cardiac hypertrophy.

It has been reported that ginsenosides extracted from the root of the herb *Panax ginseng* CA. They have many pharmacological effects, including increasing the activity of superoxide dismutase^[7] and protecting the brain and heart from ischemic and reperfusion injuries^[8,9]. Notably, ginsenoside Rb₁ (Rb₁), a major component in ginsenosides, has been shown to elevate the release of NO in rat ventricular myocytes^[10] and decrease intracellular free Ca²⁺ in cardiac myocytes and other tissues^[11–13], which indicates that Rb₁ may be a potential drug for anticardiac hypertrophy.

Prostaglandin F_{2α} (PGF_{2α}) has been shown to induce cardiac myocyte hypertrophy *in vitro* and cardiac growth *in vivo* and is a good candidate to mediate the growth of

cardiac cells^[14]. Meanwhile, Ca^{2+} signaling has been reported to play a critical role in the development of cardiac hypertrophy induced by various hypertrophic stimuli^[15]. The increased intracellular Ca^{2+} binds to calmodulin and regulates several downstream effectors, such as calcineurin (CaN), which is a key mediator of cardiac hypertrophy^[16,17]. CaN dephosphorylates the nuclear factor of activated T cells (NFAT), and then later translocates to the nucleus where it acts with other transcription factors (eg GATA₄) for the activation of downstream target genes to induce cardiac hypertrophy^[18,19]. A series of studies has shown that the neuroprotective activities and anti-aging function of Rb₁ were related to decreasing intracellular Ca^{2+} ^[11-13]. However, whether Rb₁ has antihypertrophic effects on cardiac hypertrophy and inhibits the Ca^{2+} -CaN signal pathway has not known as yet.

Materials and methods

Primary culture of myocytes Ventricular myocytes from 1–3-d-old rats (Animal Center of Institute of Surgery Research of the Third Military Medical University, Chongqing, China) were prepared and cultured for 48 h in Dulbecco's modified Eagle's medium (DMEM) containing 20% fetal bovine serum and 0.1 mmol/L bromodeoxyuridine (Sigma, St Louis, Missouri, USA) as described previously^[20]. The cells were adjusted to 1.5×10^6 – 3×10^6 cells/mL for measuring intracellular free calcium concentration ($[\text{Ca}^{2+}]_i$), or to 0.5×10^6 – 1×10^5 cells/mL for measuring cell diameter and protein content. The medium was replaced by serum-free DMEM for a further 48 h before the treatment of drugs. 100 nmol/L $\text{PGF}_{2\alpha}$ (Cayman Chemical, Ann Arbor, Michigan, USA) was used to stimulate the cardiomyocytes, Rb₁ with 99% purity and final concentrations of 50, 100, and 200 $\mu\text{g}/\text{mL}$ (Division of Chinese Material Medical and Natural Products, National Institute for the Control of Pharmaceutical and Biological Products, Ministry of Public Health, Beijing, China) and *L*-arginine with final concentration of 1 mmol/L (Alexis, Lausen, Switzerland) were used to investigate their antihypertrophic effects. *N*^G-nitro-*L*-arginine-methyl ester (*L*-NAME; Alexis, Lausen, Switzerland) 1 mmol/L was used to investigate the relationship between the antihypertrophic effects of Rb₁ and NO.

Measurement of cardiomyocytic diameters The cardiomyocytes were fixed in 4% polyformaldehyde solution and stained with HE. The diameter of single cells was measured by the BI2000 Imaging Analytic System (Chengdu Taimeng Sci-Tec, Chengdu, China). Five random fields (10–15 cells per field) from each slide were analyzed. The experiments were repeated 3 times.

Measurement of cardiomyocytic protein contents The cardiomyocytes were digested by trypsinase and counted. The cells were then washed 3 times with Hanks' balanced salt solution (HBSS) without Ca^{2+} and Mg^{2+} (D-HBSS; in mmol/L: NaCl 137.0, KCl 5.0, Na_2HPO_4 0.6, KH_2PO_4 0.4, NaHCO_3 3.0, glucose 5.6, pH 7.2) by centrifuging at $400 \times g$ for 2 min. The cardiomyocytes were homogenized with RIPA lysis buffer (Upstate, Lake Placid, New York, USA) and centrifuged at $12\,000 \times g$ for 20 min at 4 °C. The protein concentration in the supernatant was determined by the Bradford assay using bovine γ -globulin as the standard, then the protein concentration per cell was calculated.

Measurement of $[\text{Ca}^{2+}]_i$ The $[\text{Ca}^{2+}]_i$ was measured by the method described before^[21]. Briefly, the cells (1×10^6) were incubated in the medium with Fura 2/AM (5 $\mu\text{mol}/\text{L}$; Sigma, St Louis, USA) for 50 min at 37 °C, then washed 3 times with HBSS (D-HBSS plus 1.30 mmol/L CaCl_2 and 0.5 mmol/L MgCl_2) containing 0.2% bovine serum albumin by centrifuging at $500 \times g$ for 2 min. The fluorescence value from 1 mL cell suspension was measured by a Shimadzu RF-5000 spectrofluorometer (Kyoto, Japan) with dual excitation wavelengths at 340 and 380 nm and emission wavelengths at 510 nm. The $[\text{Ca}^{2+}]_i$ was calculated by the following equation: $[\text{Ca}^{2+}]_i = K_d \times (F - F_{\min}) / (F_{\max} - F)$. Here, K_d was the dissociation constant of Fura 2/AM for Ca^{2+} (about 224 nmol/L at 37 °C), F was the basal fluorescence value of the cells, F_{\max} was the fluorescence value under the presence of excess calcium in the cells due to the lysis of the cellular membrane caused by 0.98 g/L Triton-X 100 (Sigma, St Louis, Missouri, USA), F_{\min} was the fluorescence value under the presence of minimal calcium using 5 mmol/L ethyleneglycotetraacetic acid (EGTA) to chelate the Ca^{2+} in the cells after the lysis of the cellular membrane by Triton-X 100.

RT-PCR analysis of mRNA Total RNA was extracted from the cardiomyocytes by use of an RNeasy mini kit (Qiagen, Valencia, California, USA). RT-PCR was performed with an RT-PCR kit (Promega, San Jose, California, USA) according to the manufacturer's instructions. The nucleotide sequence of the primers were as follows^[22,23]: (i) atrial natriuretic peptide (ANP): sense 5'-GCC CTG AGC GAG CAG ACC GA-3', antisense 5'-CGG AAG CTG TTG CAG CCT A-3'; (ii) CaN: sense 5'-ACT GGC ATG CTC CCCAGC GGA-3', antisense 5'-GTG CCG TTA GTC TCT GAG GCG-3'; and (iii) β -actin: sense 5'-GAC TAC CTC ATG AAG ATC CTG ACC-3', antisense 5'-TGA TCT TCA TGG TGC TAG GAG CC-3'. The predicted products in size were 202, 244, and 423 bp, respectively. These primers were synthesized by Beijing Dingguo Biotech (Beijing, China). The following conditions of the RT-PCR reactions were met: (i) 1 cycle at 48 °C for 45

min, 94 °C for 2 min; (ii) 35 cycles at 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 1 min; and (iii) 72 °C for 7 min. The products were separated by electrophoresis on 1% agarose gel containing ethidium bromide, and photographed. The integral optical density values for each band of ANP, CaN, and β -actin on the gel were assayed by the BI2000 Imaging Analysis System (Chengdu Taimeng Sci-Tec, China). β -actin was used as an internal control for the semiquantitative assay.

Western blotting The protein (30 μ g) from cardiomyocytes separated by 10% SDS-PAGE was transferred onto polyvinylidene difluoride nylon membranes. The blots were probed with mouse anti-CaNA- α (1:200 dilution) or anti-NFAT₃ (1:100 dilution) or anti-GATA₄ antibodies (1:100 dilution; (Santa Cruz Biotechnology, Santa Cruz, California, USA), and then with horseradish peroxidase-conjugated goat anti-mouse immunoglobulin G (1:2500 dilution) antibodies. Immunodetection was carried out using the BI2000 Imaging Analysis System.

Statistical analysis All of the data were expressed as mean \pm SD and analyzed by either ANOVA or Student's *t*-test with SPSS 11.5 software (SPSS Inc, Chicago, Illinois, USA). Differences were considered statistically significant at *P*<0.05.

Results

Effects of Rb₁ on PGF_{2 α} -induced cardiomyocyte hypertrophy Light microscopic findings of the cardiomyocytes showed that the cardiomyocytes treated with PGF_{2 α} (100 nmol/L) became swollen and enlarged with undistinguishable borders among the cells (Figure 1B). Rb₁ (200 μ g/mL) markedly alleviated the morphological changes induced by PGF_{2 α} (Figure 1C). The addition of *L*-NAME (1 mmol/L) could not

antagonize the effect of Rb₁ on the hypertrophic myocyte (data not shown).

Table 1 showed that the diameters and protein contents of the cardiomyocytes treated with PGF_{2 α} significantly increased, compared with that of the control (*P*<0.01). The treatment of Rb₁ with a variety of concentrations (50, 100, and 200 μ g/mL) significantly relieved the changes induced by PGF_{2 α} in a concentration-dependent manner (*P*<0.05). *L*-arginine (1 mmol/L) also lowered these changes induced by PGF_{2 α} (*P*<0.01). *L*-NAME (1 mmol/L) abolished the effects of *L*-arginine, but failed to abolish the effects of Rb₁ (200 μ g/mL) on the cardiomyocyte diameter and protein content.

There was a low fundamental expression of ANP mRNA in the cardiac myocytes (0.005 \pm 0.002, *n*=3). PGF_{2 α} treatment obviously increased the ANP mRNA expression, which was significantly antagonized by Rb₁ (200 μ g/mL) treatment (Figure 2A).

Effects of Rb₁ on PGF_{2 α} -induced [Ca²⁺]_i in cardiomyocytes The resting [Ca²⁺]_i was 149.7 \pm 26.2 nmol/L (*n*=6), and it increased by 83% after the cardiomyocytes were treated with PGF_{2 α} (100 nmol/L) for 48 h (*P*<0.01). Treatments with either Rb₁ at the concentrations of 50, 100, and 200 μ g/mL or *L*-arginine (1 mmol/L) strongly blocked the [Ca²⁺]_i increase caused by PGF_{2 α} . Once again, *L*-NAME (1 mmol/L) treatment abolished the effect of *L*-arginine (*P*<0.01), but failed to antagonize the effect of Rb₁ (*P*>0.05; Figure 3).

Effects of Rb₁ on transcription of CaN and expressions of CaN, NFAT₃, and GATA₄ proteins from cardiomyocytes treated by PGF_{2 α} The relative CaN mRNA expression was 0.225 \pm 0.023 in the control and increased by 52% in the PGF_{2 α} -treated cardiomyocytes (Figure 2B; *n*=3, *P*<0.01). Similar treatments with PGF_{2 α} also significantly increased the expres-

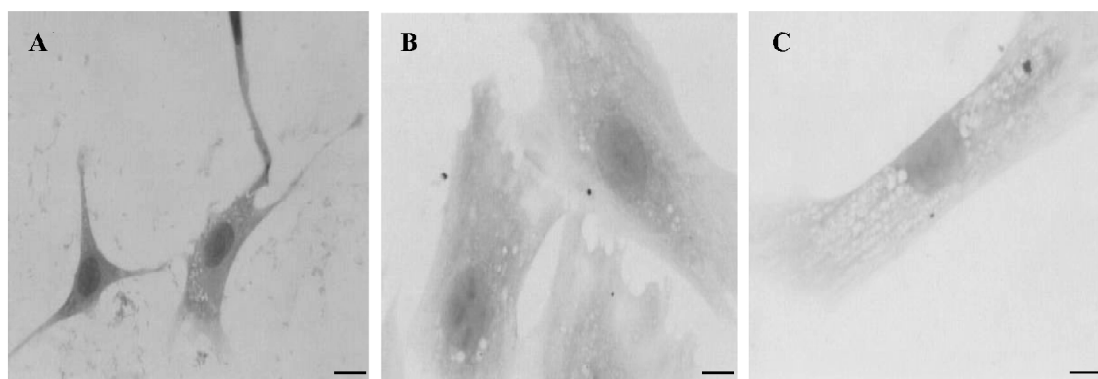


Figure 1. Effects of Rb₁ on cultured cardiomyocytes (HE \times 400). (A) control; (B) PGF_{2 α} (100 nmol/L)-pretreated group; (C) PGF_{2 α} (100 nmol/L) plus Rb₁ (200 μ g/mL)-pretreated group. Cardiomyocytes treated with PGF_{2 α} became swollen and enlarged with undistinguishable borders among the cells. Rb₁ markedly alleviated the morphological changes induced by PGF_{2 α} .

Table 1. Effects of Rb₁ on the changes of cardiomyocyte diameter (*n*=3, mean±SD) and protein level (*n*=6, mean±SD) induced by PGF_{2α}. ^c*P*<0.01 vs control; ^d*P*>0.05, ^e*P*<0.05, ^f*P*<0.01 vs PGF_{2α} (100 nmol/L); ^g*P*<0.01 vs *L*-NAME (1 mmol/L)+PGF_{2α} (100 nmol/L); ^h*P*<0.01 vs *L*-arginine (1 mmol/L)+PGF_{2α} (100 nmol/L).

Group	Cell diameter (μm)	Protein level (pg/cell)
Control	36±11	419.2±41.6
PGF _{2α} (100 nmol/L)	115±23 ^c	548.5±59.2 ^c
Rb ₁ 50 (μg/mL)+PGF _{2α} (100 nmol/L)	94±23 ^f	466.9±56.9 ^e
Rb ₁ 100 (μg/mL)+PGF _{2α} (100 nmol/L)	68±18 ^f	424.1±44.8 ^f
Rb ₁ 200 (μg/mL)+PGF _{2α} (100 nmol/L)	59±19 ^f	408.7±47.4 ^f
<i>L</i> -arginine (1 mmol/L)+PGF _{2α} (100 nmol/L)	58±17 ^f	397.9±45.8 ^f
<i>L</i> -NAME (1 mmol/L)+PGF _{2α} (100 nmol/L)	112±22 ^d	523.8±75.9 ^d
Rb ₁ 200 (μg/mL)+ <i>L</i> -NAME (1 mmol/L)+PGF _{2α} (100 nmol/L)	68±17 ⁱ	431.7±36.2 ⁱ
<i>L</i> -arginine (1 mmol/L)+ <i>L</i> -NAME (1 mmol/L)+PGF _{2α} (100 nmol/L)	108±14 ^l	539.7±36.3 ^l

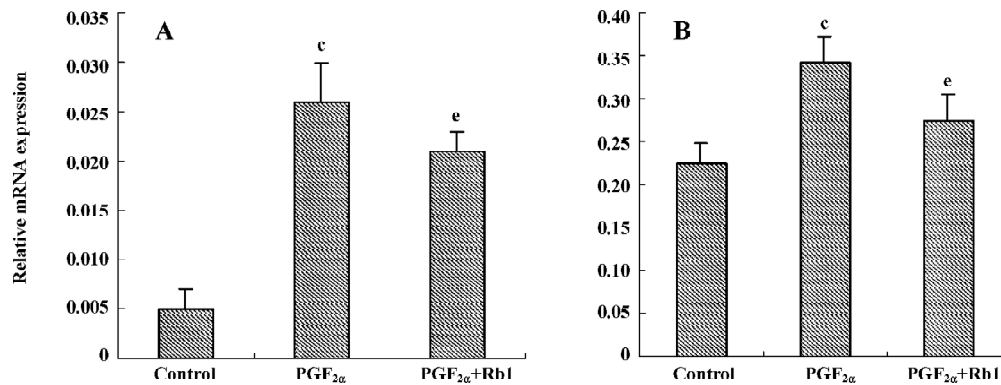


Figure 2. Effects of Rb₁ (200 μg/mL) on the upregulated transcription of ANP and CaN in cardiomyocytes treated with PGF_{2α} (100 nmol/L). (A) transcription of ANP relative to β-actin; (B) transcription of CaN relative to β-actin. *n*=3. Mean±SD. ^c*P*<0.01 vs control; ^e*P*<0.05 vs PGF_{2α}-treated group.

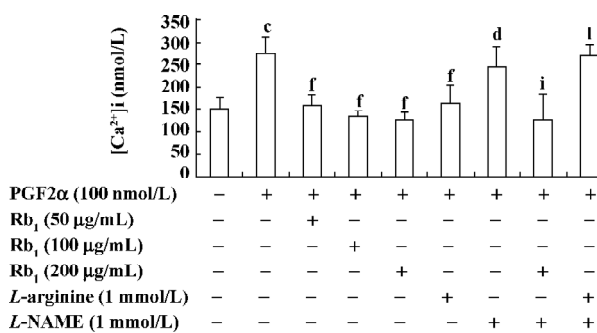


Figure 3. Effects of Rb₁ on the PGF_{2α}-induced [Ca²⁺]_i elevation from cardiomyocytes. Cultured cardiomyocytes were treated with PGF_{2α} (100 nmol/L) in the presence of a variety of Rb₁ concentrations (50, 100, and 200 μg/mL), *L*-arginine (1 mmol/L), and *L*-NAME (1 mmol/L). [Ca²⁺]_i changes of cardiomyocytes were calculated by the fluorescence method. *n*=6. Mean±SD. ^c*P*<0.01 vs control; ^d*P*>0.05, ^e*P*<0.01 vs PGF_{2α} (100 nmol/L); ^f*P*<0.01 vs *L*-NAME (1 mmol/L)+PGF_{2α} (100 nmol/L); ^g*P*<0.01 vs *L*-arginine (1 mmol/L)+PGF_{2α} (100 nmol/L).

sions of the CaN, NFAT₃, and GATA₄ proteins of cardiomyocytes. The treatment of Rb₁ (200 μg/mL) markedly decreased the mRNA expression of CaN and the protein expressions of CaN, NFAT₃, and GATA₄ from cardiomyocytes treated by PGF_{2α}. *L*-arginine (1 mmol/L) also inhibited the protein expressions of CaN and its downstream factors (*P*<0.05; Figure 4, *n*=3).

Discussion

It has been reported that the morphological changes of cardiomyocyte hypertrophy can be induced *in vitro* by stimulating cultured neonatal cardiomyocytes with various growth factors and cytokines, such as angiotensin II, endothelin-1, and PGF_{2α}, which was similar to those induced by pressure or volume load^[14,24-26]. The characteristic phenotype of hypertrophy following growth factor stimulation includes an increase of cell volume and protein synthesis

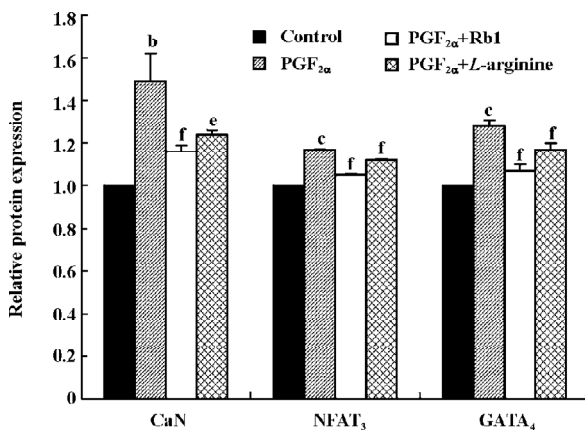


Figure 4. Effects of Rb₁ (200 μg/mL) on the increased expressions of CaN, NFAT₃, and GATA₄ in cardiomyocytes pretreated with PGF_{2α} (100 nmol/L). *n*=3. Mean±SD. ^b*P*<0.05, ^c*P*<0.01 vs control; ^e*P*<0.05, ^f*P*<0.01 vs PGF_{2α} 100 nmol/L.

with an accumulation of contractile proteins, organization of the contractile proteins into sarcomeric units, as well as the re-expression of fetal cardiac genes, including ANP^[27]. In this study, the findings from measuring the diameter, protein content, and ANP mRNA expression of the cardiac myocytes suggested that PGF_{2α} could induce cardiomyocyte hypertrophy resembling that described by Lai *et al*^[14], and that Rb₁ could significantly decrease the elevated cardiomyocyte volume, protein content, and ANP mRNA expression caused by PGF_{2α}.

Our results showed that PGF_{2α} induced cardiomyocyte hypertrophy with the elevating [Ca²⁺]_i level. Meanwhile, the antihypertrophic effects of Rb₁ were accompanied simultaneously with the alleviating [Ca²⁺]_i level. *L*-NAME, an NO synthase inhibitor, did not antagonize both effects of Rb₁ on hypertrophy and [Ca²⁺]_i of cardiomyocytes; on the contrary, it abolished the antihypertrophic and decreasing [Ca²⁺]_i effects of *L*-arginine. These results suggested that the directly decreasing [Ca²⁺]_i effect rather than the NO release may be responsible for the antihypertrophic effects of Rb₁.

In the past several years, a number of experiments have implicated that the CaN signal transduction pathway may play an important role in the cardiomyocyte hypertrophy process^[28]. We have previously reported that cardiac hypertrophy by PGF_{2α} may be mediated by the CaN signal pathway in rats^[29]. In the present paper, the fact that the transcription and expression of CaN, as well as the expression of the CaN downstream factors increased with elevating [Ca²⁺]_i under the stimulation of PGF_{2α} to the cardiomyocytes, which were blunted by Rb₁, suggested that the interference of the CaN signaling pathway might be involved in the

antihyper-trophic mechanisms of Rb₁.

L-arginine, an NO donor, was observed to inhibit cardiomyocyte hypertrophy^[30], which could be abolished by *L*-NAME. Surprisingly, *L*-NAME had no influence on either the antihypertrophic effect or on the inhibiting [Ca²⁺]_i rise from Rb₁. The results suggested that the antihypertrophic effect of Rb₁ might be different from *L*-arginine, but the relationship between the Rb₁ effects and NO still needs much investigation.

In conclusion, our study demonstrates that Rb₁ can alleviate cardiac hypertrophy *in vitro*, which may be mediated in part by an inhibitive effect on elevated [Ca²⁺]_i due to the inactivation of the CaN transduction pathway.

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