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RGS2 inhibits β -adrenergic receptor-induced cardiomyocyte hypertrophy

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ABSTRACT

The chronic stimulation of certain G protein-coupled receptors promotes cardiomyocyte hypertrophy and thus plays a pivotal role in the development of human heart failure. The beta-adrenergic receptors (β -AR) are unique among these in that they signal via Gs, whereas others, such as the alpha1-adrenergic (α 1-AR) and endothelin-1 (ET-1) receptors, predominantly act through Gq. In this study, we investigated the potential role of regulator of G protein signalling 2 (RGS2) in modulating the hypertrophic effects of the β -AR agonist isoproterenol (ISO) in rat neonatal ventricular cardiomyocytes. We found that ISO-induced hypertrophy in rat neonatal ventricular myocytes was accompanied by the selective upregulation of RGS2 mRNA, with little or no change in RGS1, RGS3, RGS4 or RGS5. The adenylyl cyclase activator forskolin had a similar effect suggesting that it was mediated through cAMP production. To study the role of RGS2 upregulation in β -AR-dependent hypertrophy, cardiomyocytes were infected with adenovirus encoding RGS2 and assayed for cell growth, markers of hypertrophy, and β -AR signalling. ISO-induced increases in cell surface area were virtually eliminated by the overexpression of RGS2, as were increases in α -skeletal actin and atrial natriuretic peptide. RGS2 overexpression also significantly attenuated ISO-induced extracellular signal-regulated kinases 1 and 2 (ERK1/2) and Akt activation, which may account for, or contribute to, its observed antihypertrophic effects. In contrast, RGS2 overexpression significantly activated JNK MAP kinase, while decreasing the potency but not the maximal effect of ISO on cAMP accumulation. In conclusion, the present results suggest that RGS2 negatively regulates hypertrophy induced by β-AR activation and thus may play a protective role in cardiac hypertrophy.

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1. Introduction

RGS (regulator of G protein signalling) proteins are negative regulators of G protein signalling. They achieve this by accelerating the hydrolysis of GTP to GDP on the activated G protein, thereby converting it to its inactive state [1]. In addition to promoting G protein deactivation, RGS proteins may also negatively regulate signalling by blocking effector activation. All RGS proteins promote GTP hydrolysis by members of the G α i subfamily of G proteins, and a subset also produces such effects on G α q proteins [1].

RGS2 is a member of the B/R4 subfamily of RGS proteins which are relatively small and simple in structure. RGS2 is unique among the RGS proteins, since it binds to G α i proteins with relatively low affinity and thus selectively attenuates Gq-mediated signals [2]. RGS2 inhibits Gq-dependent phospholipase C β activation both by promoting GTP hydrolysis and also apparently by physically disrupting the G proteineffector interaction [1]. In addition to Gq, RGS2 can inhibit Gsstimulated adenylyl cyclase activity [3,4], albeit through a mechanism that does not appear to involve changes in the rate at which this G protein hydrolyzes GTP [4,5].

RGS2 is one of the predominant RGS proteins expressed in human heart [6]. Previously, we have shown that RGS2 expression in isolated neonatal rat cardiomyocytes is upregulated by Gq activation [7]. Furthermore, RGS2 overexpression attenuates cardiomyocyte hypertrophy induced by the activation of Gq-coupled receptors, whereas gene silencing of RGS2 by RNAi exacerbates Gq-mediated hypertrophy [9]. Mende et al. observed similar changes in adult ventricular myocytes [8], but also found that *in vivo* RGS2 ultimately may be downregulated subsequent to prolonged Gq activation [9]. Importantly, recent animal studies have substantiated the causal relationship between deficiency of RGS2 and cardiac hypertrophy. Takimoto et al. demonstrated that mice lacking RGS2 had a normal basal cardiac phenotype, yet responded rapidly to pressure overload, with increased myocardial Gq signalling, marked cardiac hypertrophy and failure, and early mortality [10]. Collectively, these observations

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suggest that RGS2 can protect against Gq-associated hypertrophic growth in cardiomyocytes, and that its loss contributes to the development of hypertrophy.

Signalling though the Gs-coupled β -AR is also able to stimulate cardiomyocyte hypertrophy, and RGS2 in other cells is upregulated by, and also can attenuate, signalling via Gs coupled receptors. Therefore, we hypothesized that signalling though the β -AR may regulate RGS2 expression in cardiomyocytes and that, in turn, RGS2 may regulate β-AR-induced cardiomyocyte hypertrophy. To test this, the effects of the β -AR agonist isoproterenol (ISO) on RGS2 expression, and the effects of overexpression of RGS2 on ISO-induced cardiomyocyte hypertrophy were examined in cultured rat cardiomyocytes. Notably, ISO treatment selectively induced RGS2 expression in primary rat cardiomyocytes. Furthermore, overexpression of RGS2 not only significantly attenuated ISO-induced cardiomyocyte hypertrophy, but also significantly blocked ISO-induced hypertrophic gene expression and attenuated ISO-induced ERK1/2 and Akt activation. These data indicate that RGS2 is important in regulating Gs-coupled hypertrophic responses in cardiomyocytes.

2. Materials and methods

2.1. Cell culture

Primary rat ventricular myocytes were isolated from 2-day-old Sprague–Dawley rats following previously described procedures [7]. The isolated myocytes were cultured in plating medium (supplementary material) for 2–3 days prior to all experiments. All studies using animals were approved by the University of Western Ontario Animal Care and Use Committee and complied with guidelines of the Canadian Council on Animal Care.

2.2. Recombinant adenovirus

RGS2 adenovirus was generated as described previously [7]. Myocytes were infected with adenovirus encoding either RGS2 (AdRGS2) or GFP (AdGFP) at a multiplicity of infection (MOI) of 10 under serum-free conditions. This MOI achieves exogenous gene delivery to \geq 95% of neonatal ventricular cells under these conditions. Expression levels were examined using immunoblotting (not shown). As is typically the case, adenovirally-expressed RGS2 was present in greater amounts than the endogenous protein by at least one order of magnitude. This is comparable to other published reports using RGS2 [8] and is likely to be due to the strong promoters (e.g. CMV) used.

2.3. RNA isolation and quantitative RT-PCR

Rat neonatal ventricular myocytes were serum-starved for 24 h prior to indicated treatments. Total RNA was isolated using Trizol reagent (Invitrogen) and first strand cDNA synthesis was performed using SuperScript[™] II reverse transcriptase (Invitrogen). Gene expression was determined in the DNA Engine Opticon 2 system (MJ Research) using SYBR green Jumpstart Taq ReadyMix DNA polymerase (Sigma). Primer sequences and PCR cycles used are indicated in supplementary Table S1. Melting curve analysis showed a single PCR product for each gene amplification. Relative cDNA levels were quantified using a standard curve of the respective genes and normalized to 18S rRNA measured in parallel. For β -myosin heavy chain expression, first strand cDNA synthesis was performed using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Gene expression was determined using Taqman® Universal PCR master mix, FAM[™]-labelled Taqman[®] probe for β-myosin heavy chain and VIC[™]-labeled Taqman[®] probe for GAPDH (Applied Biosystems).

2.4. Immunofluorescence detection

Rat neonatal ventricular myocytes grown on collagen-coated coverslips were serum-starved for 24 h before treatment with ISO (10 μ M) for 3 h, and fixation with periodate-lysine-paraformaldehyde for 30 min. Fixed cells were permeabilized with 0.3% Triton X-100 for 10 min and blocked with 5% BSA for 1 h. Cells were incubated overnight at 4 °C with a polyclonal goat antibody against the N-terminal 18 amino acid residues of RGS2 (1:250 dilution, Santa Cruz Biotechnology), followed by 1 h at room temperature with an antigoat antibody conjugated to Texas Red-X (1:1000 dilution, Molecular Probes). Following 3 washes with PBS, coverslips were mounted on slides with Immumount (Fisher Scientific, Nepean, ON, Canada) and examined using a Zeiss LSM 410 confocal microscope with a 566 nm laser and a rhodamine-Texas Red 630 nm filter for excitation and emission, respectively.

To analyze the effects of RGS2 on agonist-induced changes in cell morphology, myocytes that had been serum-starved for 24 h were infected with Ad-RGS2 or Ad-GFP (control). After 24 h, cells were treated with ISO (10 μ M) for 24 h and then fixed and incubated overnight at 4 °C with a murine antibody directed toward sarcomeric myosin heavy chain (1:5 dilution MF20 hybridoma). Using 10 μ g/ml rhodamine-conjugated sheep F(ab)'₂ anti-mouse IgG (Boehringer Mannheim GmbH, Mannheim, Germany) followed by Hoechst 33258 dye for nuclear morphology, cells were assessed for surface area as described previously [7] using Mocha software (Jandel, San Rafael, CA, USA).

2.5. Reporter gene assays

Cells were incubated overnight in DMEM/F-12, 1:1, 17 mM Hepes, 3 mM NaHCO₃, 2 mM L-glutamine, 50 µg/ml gentamycin, and 10% FBS before infection with Ad-RGS2 or Ad-GFP (control) in serum free medium. After 4 h, viral stocks were removed and myocytes were transfected using Effectene (Qiagen Inc., Mississauga, ON, Canada) with DMEM containing 2.5% calf serum, 5.0 µg of luciferase reporter plasmid comprising of either the α -skeletal actin (α -S. actin), α - or β myosin heavy chain (α MHC, or β MHC) plus 2.5 µg of CMV β -gal, as described previously [7]. After 24 h, cells were treated with ISO (10 µM) or ET-1 (10 nM) for a further 24 h and assessed by luciferase reporter gene assay. To control for potential differences in transfection efficiency among different myocyte cultures, luciferase activity was normalized to β -galactosidase activity. Data were obtained from at least 3 independent cell cultures using 3 replicate wells per condition tested.

2.6. Western blotting

Rat neonatal ventricular myocytes were infected with Ad-RGS2 or Ad-GFP (control) for 32 h and serum-starved for a further 16 h. After indicated treatments, myocytes were rinsed twice with ice-cold PBS and lysed in lysis buffer (20 mM Tris, 150 mM NaCl, 1% Triton X-100, 10% glycerol, 2 mM EDTA, 2 mM EGTA, 50 mM NaF, 200 µM Na₃VO₄, 10 mM Na₄P₂O₇ 40 mM β -glycerophosphate, 10 µg/ml leupeptin, 1 µM pepstatin A, 1 mM PMSF, and 1 µM calyculin A). Cell lysates were incubated on ice for 15 min and then centrifuged at top speed in a microcentrifuge at 4 °C for 15 min. Equal amounts of protein (30 µg per lane) were subjected to 10% SDS-PAGE, and then transferred to nitrocellulose membrane. Phospho-Akt (Ser 473), Phospho-ERK and phospho-JNK were determined using specific antibodies (1:1000 dilution, Cell Signalling Technology). The membrane was stripped at 45 °C for 15 min in 62.5 mM Tris-HCl (pH 6.8) containing 1% SDS, 100 mM β -mercaptoethanol and reprobed with the anti-total Akt, ERK and JNK antibodies (1:2000 dilution Cell Signalling Technology, Danvers, MA). In order to compare endogenous RGS2 protein levels following control, ISO and forskolin treatments, cells were lysed as

described above, and lysates (70 µg protein per lane), were separated on 12% SDS-PAGE, and then transferred to PVDF membrane. Membranes were blocked in PBS containing 5% skim dry milk and probed with antibody recognizing endogenous RGS2 (1:500 dilution, Sigma-Aldrich). After incubation with anti-chicken (GenWay Biotech, San Diego, CA, USA) secondary antibody, the immunoblot was visualized using chemiluminescence (Lumiglo Reserve[™] Chemiluminescent Substrate Kit, KPL).

2.7. Intracellular cAMP determination

Rat neonatal ventricular myocytes were infected with Ad-RGS2 or Ad-GFP (control). After 48 h, cells were preincubated with phosphodiesterase inhibitor IBMX (0.5 mM) and then treated with ISO for 90 s before being lysed. Lysates were frozen at -80 °C. cAMP levels were determined in thawed cell lysates using the cAMP Biotrak enzymeimmunoassay (EIA) system (GE Healthcare, Baie d'Urfé, Québec, Canada) according to the manufacturer's instructions. cAMP values were normalized to protein concentration.

2.8. [³H] Leucine incorporation

Protein synthesis levels in cardiomyocytes were determined by assessing the incorporation of labeled leucine. Following 24 h serum starvation, cardiomyocytes were treated with the indicated agents in the presence of [³H] leucine (1 μ Ci/ml, Amersham, GE Healthcare) for 24 h. Cells were then washed three times with ice-cold PBS and proteins were precipitated with 5% tricholoroacetic acid (TCA) for 30 min on ice. After two additional washes with 5% TCA, precipitates were solubilized in 0.2 N NaOH. The radioactivity of [³H] leucine incorporated into proteins was measured by liquid scintillation counting (Tri-Carb 2900TR).

2.9. Statistical analysis

Group data are presented as means \pm S.E.M and were statistically analyzed using one-way ANOVA. Differences were further evaluated by Fisher's protected least squares differences, which were considered significant at a value of *P*<0.05. Normalized data are presented as means \pm S.E.M and were analyzed using one sample *t* test. For MAP kinase activation, the enhancement of phosphorylated Akt, ERK and JNK in RGS2 overexpression vs. vector alone was compared using Student's *t* test. For cAMP accumulation data, differences in pEC₅₀ and *E*_{max} were compared using Student's *t* test.

3. Results

3.1. ISO induces RGS2 expression in neonatal ventricular myocytes

We investigated the ability of the β -AR agonist isoproterenol (ISO) to induce expression of endogenous RGS proteins in neonatal rat ventricular myocytes. Cells were treated with ISO (10 μ M) for up to 24 h and mRNA levels of RGS1, 2, 3, 4 and 5 were determined using quantitative RT-PCR. Of the five RGS proteins examined, only the RGS2 mRNA transcript was significantly enhanced by ISO treatment (Fig. 1A). The stimulation of RGS2 expression by ISO reached a maximum after 1 h and tapered off thereafter (Fig. 1A). These observations indicate that the β -AR agonist ISO selectively upregulates expression of RGS2 at the mRNA level in rat cardiomyocytes.

To determine whether the observed effects of ISO corresponded to changes in protein levels, we used both immunofluorescence and immunoblotting techniques to examine RGS2 protein expression in ISO-treated cardiomyocytes (10μ M). As expected, expression of RGS2 protein was clearly enhanced in the ISO-treated cells (Fig. 1B and C). These observations confirm that ISO selectively stimulates expression



Fig. 1. β-AR agonist ISO induces RGS2 expression in cultured cardiomyocytes. (A) Rat neonatal ventricular cardiomyocytes were serum-starved for 24 h followed by treatment with 10 μM ISO for the indicated time. Real-time RT-PCR was used to evaluate the expression of RGS1–5 mRNA, relative to 18s rRNA. Data from 10 to 12 independent experiments performed in duplicate are shown as means ± S.E.M. **P*<0.05; ***P*<0.01 (ISO-treated vs. untreated group). (B) Rat neonatal ventricular cardiomyocytes were serum starved for 24 h followed by treatment with 10 μM ISO for 3 h. The cells were then fixed followed by immunostaining using an anti-RGS2 antibody. All images were captured using Zeiss LSM 410 confocal microscope at 640× magnification and a resolution of 512×512. The images shown are representative of three independent experiments. (C) Endogenous RGS2 protein levels were visualized by immunobloting of neonatal ventricular cardiomyocyte lysates (70 μg protein/lane) following 3 h stimulation with ISO (10 μM) or forskolin (FSK, 10 μM). Shown is a representative of 3 independent experiments.

of RGS2 at the levels of both mRNA and protein in primary rat cardiomyocytes.

3.2. RGS2 is upregulated by both Gs and Gq signalling in cardiomyocytes

To better understand the signalling pathways that regulate RGS2 expression, we further examined the effects of agents that activate Gsand Gq-dependent signals in cardiomyocytes. Previously we showed that the α 1-AR agonist phenylephrine (PE) selectively upregulates RGS2, most likely through Gq [7], and here we show that another Gq activator, ET-1, similarly upregulates RGS2 mRNA expression (Fig. 2A). The effect of ET-1 differed from that of PE, however, in that the increase in RGS2 was accompanied by a comparable increase in RGS1 mRNA (Fig. 2A).

The observed effects of ISO (Fig. 1) and ET-1 (Fig. 2A) suggest that activation of Gs- and Gq-signalling pathways, respectively, may upregulate RGS2 expression. We have previously shown that phorbol myristate acetate (PMA), which activates protein kinase C

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Fig. 2. ET-1 and FSK induce RGS2 expression in cultured cardiomyocytes. Rat neonatal ventricular cardiomyocytes were serum-starved for 24 h followed by treatment with (A) ET-1 (10 nM) or (B) FSK (10 μ M) for the indicated time. Real-time RT-PCR was used to evaluate the expression of RGS1-5 mRNA, relative to 18s rRNA. Data from 10 to 12 independent experiments performed in duplicate are shown as means \pm S.E.M. **P*<0.05; ***P*<0.01 (ET-1-treated vs. untreated group).

(a downstream mediator of Gq signalling) is able to induce RGS2 expression in cultured neonatal myocytes [7]. Here we examined whether forskolin, which mimics Gs-signalling by activating the effector enzyme adenylyl cyclase, would be able to produce a similar effect. As shown in Fig. 2B, forskolin increased RGS2 mRNA, suggesting that signalling through Gs proteins can upregulate RGS2 in cardiomyocytes, as has been observed in other cell types [3,11].

Signalling through Gs- and Gq-coupled receptors presents a known hypertrophic stimulus to cultured cardiomyocytes [12]. Since RGS2 expression can be regulated by cell stresses which are independent of G protein signalling (e.g. heat shock, oxidative stress and DNA damage) in other cell types [13,14], we examined whether RGS2 expression in cardiomyocytes is sensitive specifically to Gprotein-mediated signals per se, or rather to any hypertrophic stimuli. This was achieved by treating cardiomyocytes with known stimulators of cardiomyocyte hypertrophy, whose actions are mediated by mechanisms which are independent of G protein signalling. Thus, cells were treated with IGF-1 (20 ng/ml), TNF-alpha (50 ng/ml) and aldosterone (100 nM) for up to 6 h. PE (10 μ M) was used as a positive control since we have previously shown that PE is able to induce hypertrophy in these cells [7]. As shown in Fig. 3A, RGS2 expression was not altered by these G protein independent hypertrophic stimuli (hypertrophic effects on the cells were confirmed by determining induction of beta myosin heavy chain expression, Fig. 3B), suggesting that RGS2 expression in cardiomyocytes is not a general marker of hypertrophy but rather is upregulated specifically through G protein pathways.



Fig. 3. Hypertrophic factors do not affect RGS2 expression in cultured cardiomyocytes. (A) Rat neonatal ventricular cardiomyocytes were serum-starved for 24 h followed by treatment with 20 ng/ml IGF-1, 50 ng/ml TNF- α , 100 nM aldosterone or 10 μ M PE (positive control) for the indicated time. (A) Real-time RT-PCR was used to evaluate the expression of RGS2 mRNA, relative to 18s rRNA. Data from 3 to 6 independent experiments performed in duplicate are shown as means \pm S.E.M. **P*<0.05; (PE-treated vs. vehicle-treated group). (B) After 24 h levels of β -myosin heavy chain were evaluated by real-time RT-PCR relative to GAPDH levels. Data are from 2 independent experiments performed in duplicate.

3.3. RGS2 attenuates the development of hypertrophy induced by ISO and ET-1

Despite activating different G protein signalling pathways, both ISO and ET-1 are able to promote cardiomyocyte hypertrophy [15– 18]. Overexpression of RGS2 is known to inhibit Gq-dependent cardiomyocyte hypertrophy [7,8], however, the effect of RGS2 on Gsstimulated cardiomyocyte hypertrophy has not been reported. To examine this we infected the cells with adenovirus encoding either RGS2 or GFP prior to treatment with either ISO (10 µM) or ET-1 (10 nM). In control cells, ISO and ET-1 treatment led to an increase in cell size (Fig. 4A and B), accompanied by expression of marker genes associated with cardiomyocyte hypertrophy (Fig. 5), whereas in cells infected with RGS2, these effects were reduced or eliminated. Although both agonists produced clear increases in cell size, the effect of ET-1 was more pronounced than that of ISO (Fig. 4). Similarly, the upregulation of genetic markers tended to be greater with ET-1, which produced increases in all four markers tested, while ISO produced statistically significant effects on α-skeletal actin and ANP but not on α - or β -myosin heavy chain (Fig. 5).

The different patterns produced by ET-1 and ISO supports the notion that they promote hypertrophy via different (albeit possibly overlapping) G protein-mediated signalling pathways. Notwithstanding these differences, neither agonist was found to increase cell size or upregulate markers of hypertrophy in cells infected with RGS2-adenovirus. It follows that RGS2 may block both Gq- and Gs-dependent signals that lead to cardiomyocyte hypertrophy. These observations suggest that RGS2 does not only inhibit Gq protein signalling-induced hypertrophy.



Fig. 4. RGS2 overexpression attenuates ISO-induced cardiomyocyte hypertrophy. (A)Rat neonatal ventricular myocytes infected for 24 h with either control (–) or RGS2-encoding adenovirus (+) were untreated (control), or treated with ET-1 (10 nM) or ISO (10 μ M) for 24 h were immunostained to reveal sarcomeric myosin heavy chain. (B) The surface areas of 50 or more cells per condition in each of three independent experiments were estimated as described previously [7], and presented as means \pm S.E.M. **P*<0.05; ***P*<0.01 (RGS2 vs. control adenovirus group).

3.4. Modulation of *β*-AR signalling pathways by RGS2

The primary action of Gs is to activate adenylyl cyclase, leading to accumulation of the second messenger cAMP. In order to examine the role of RGS2 on Gs mediated hypertrophic signalling in cardiomyocytes we measured ISO-stimulated intracellular cAMP following RGS2 or GFP infection. As expected, ISO treatment led to a robust, concentration-dependent accumulation of cAMP in cardiomyocytes (Fig. 6). RGS2 overexpression led to a significant reduction in the potency of ISO to stimulate cAMP, while there was no change in maximal response.

Both Gs and Gq coupled receptors are able to induce activation of MAP kinases in cardiomyocytes and this is, at least in part, responsible for mediating the hypertrophic effects of these stimuli [17,19–21]. We have previously shown that PE, acting through Gq, is able to stimulate ERK1/2 and JNK and that adenoviral infection of RGS2 modulates these effects [7]. In this study we wanted to determine the effect of RGS2 on MAP kinase activation through Gs coupled pathways. To test this, neonatal rat ventricular myocytes were treated with ISO subsequent to being infected with adenovirus encoding either GFP (control) or RGS2.

As expected, both Gq (ET-1, 10 nM) and Gs (ISO, 10 μ M) signalling significantly increased phosphorylation of ERK1/2 and JNK after 10 min (Fig. 7). Infection of cells with RGS2 adenovirus had no effect on basal ERK1/2 phosphorylation, however, it significantly attenuated ERK1/2 activation in response to both ET-1 and ISO (Fig. 7A). These results are similar to our previous observation that RGS2 attenuates ERK1/2 phosphorylation caused by the α 1-AR agonist phenylephrine [7]. In contrast to its effects on ERK1/2, we previously showed that RGS2 in cardiomyocytes is able to increase JNK phosphorylation [7]. When RGS2 was overexpressed in cells which were subsequently treated with ET-1 and ISO, JNK activation was further increased to a level consistent with an additive effect of the two treatments (Fig.7, RGS2 + ET-1/ISO), similar to the effect we previously observed with RGS2 and phenylephrine treatment [7]. These data indicate that RGS2 has different effects on ERK1/2 and JNK pathways in cardiomyocytes.

Previous studies have demonstrated that an alternative mechanism by which β -AR stimulation leads to cardiomyocyte hypertrophy is through activation (by phosphorylation) of the protein kinase Akt [22,23]. We examined the role of RGS2 in ISO-induced Akt phosphorylation subsequent to infection with adenovirus encoding either GFP (control) or RGS2. RGS2 overexpression had no effect on Akt phosphorylation while ISO (10 μ M, 10 min) led to a robust phosphorylation of Akt which was significantly attenuated by RGS2 infection (Fig. 8). These data demonstrate that RGS2 is able to limit signalling leading to Akt phosphorylation in cardiomyocytes and suggest that this pathway may also be involved in RGS2's inhibitory effect on ISO-induced cardiomyocyte hypertrophy.

3.5. ISO-induced cardiomyocyte hypertrophy is PKA-dependent

Chronic B-AR stimulation leads to increased intracellular cAMP levels and promotion of cardiomyocyte hypertrophy; however it is not fully understood whether these cAMP effects are mediated by protein kinase A (PKA) signalling or via Epac (Exchange protein activated by cAMP). To address this question, we measured ISO-induced protein synthesis in cardiomyocytes in the presence of a selective PKA inhibitor, H89. Isolated neonatal cardiomyocytes were pretreated with 10 μM H89 for 20 min, and then stimulated with 10 μM isoproterenol (ISO) for 24 h. Although pretreatment with H89 alone did not affect the basal level of protein synthesis, the ISO-induced increase in [³H]-leucine incorporation was significantly suppressed by this inhibitor (Fig. 9A). A similar effect was seen on cardiomyocyte cell size (data not shown). We also investigated whether a selective Epac activator (8-(4-Chlorophenylthio)-2'-O-methyladenosine 3',5'-cyclic monophosphate; 8-CPT-2Me-cAMP) or a selective PKA activator (8-(4-Chlorophenylthio)adenosine-3',5'-cyclic monophosphorothioate, Sp-isomer; sp-cAMP) could stimulate protein synthesis in these cells (Fig. 9B). Isolated cardiomyocytes were treated with either 10 μ M ISO as a positive control, 1 μ M sp-cAMP or 1 μ M 8-CPT-2Me-cAMP for 24 h in the presence of [³H]-leucine. A significant increase in protein synthesis was seen following treatment with the specific PKA activator, sp-cAMP, but not with the Epac activator, 8-CPT-2Me-cAMP. Taken together, these results suggest that the ISO-induced





Fig. 6. RGS2 overexpression modulated ISO-induced cAMP accumulation. Rat neonatal ventricular cardiomyocytes were infected with control (solid squares) or RGS2 (open triangles) adenovirus for 48 h, followed by treatment with ISO at the indicated concentrations for 90 s. Data represent one example of 4 separate experiments performed in triplicate. Potency (pEC₅₀) was significantly lower in RGS2-infected cells ($6.98 \pm 0.07, 6.49 \pm 0.11,$ GFP and RGS2 respectively, P = 0.005, n = 4). E_{max} was not different ($2364 \pm 290, 2162 \pm 232$ fmol/µg protein, GFP and RGS2 respectively, n = 4).

signal transduction pathway leading to cardiomyocyte hypertrophy is PKA-dependent.

4. Discussion

Previous studies examining RGS2 function in cardiac tissue and cultured cardiomyocytes have focused primarily on signalling through Gq coupled receptors. The aim of the present study was to determine the role of RGS2 in regulating Gs coupled hypertrophic stimuli in rat neonatal ventricular cardiomyocytes. Our data demonstrate that RGS2 mRNA and protein are upregulated in cardiomyocytes, not only by Gq coupled signalling, but also by ISO which acts through the Gs coupled β -adrenergic (β -AR) receptors. Furthermore, RGS2 is able to inhibit the hypertrophic effects of ISO in cultured cardiomyocytes, suggesting that RGS2 may have an important and heretofore unreported role in β -AR induced cardiac hypertrophy.

4.1. Gs coupled modulation of RGS2 expression

RGS2 expression is tightly regulated within cells and is highly inducible in response to both Gq (e.g. PE, ET-1, angiotensin II, ATP) and Gs (e.g. parathyroid hormone, glucose-dependent insulinotropic polypeptide, ISO) coupled responses in multiple cell types [3,11,24]. In cardiomyocytes, we and others [7,8], have demonstrated that Gq coupled signalling leads to a rapid induction of RGS2 mRNA and protein. The important finding in this study is that signalling through the Gs coupled β -AR also leads to a rapid (maximal at 1 h) induction of RGS2,

Fig. 5. RGS2 overexpression attenuates ISO-induced hypertrophic gene expression. (A) Rat neonatal ventricular cardiomyocytes were infected with control (white bars) or RGS2-encoding adenovirus (black bars). After 4 h incubation cells were transfected with reporter DNA constructs for the hypertrophic genes α -skeletal actin (α -S. actin), α -myosin heavy chain (α MHC) and β -myosin heavy chain (β MHC) for 24 h. Subsequently, cells were left untreated (control) or treated for 24 h ET-1 (10 nM) or ISO (10 μ M). Reporter gene activities were assessed by luminometry relative to an internal standard, and normalized to control values (no drug, control adenovirus). (B) Rat neonatal ventricular cardiomyocytes were infected with control (white bars) or RGS2-encoding adenovirus (black bars) for 24 h followed by treatment with ET-1 (10 nM) or ISO (10 μ M) for a further 24 h. Real-time RT-PCR was used to evaluate the expression of atrial natriuretic peptide (ANP) mRNA relative to 18S rRNA. Data represent means \pm S.E.M. of three to five independent experiments performed in triplicate. **P*<0.05; ***P*<0.01 (RGS2 vs. control adenovirus group).



Fig. 7. RGS2 overexpression modulates ISO-induced activation of MAP kinases. Rat neonatal ventricular cardiomyocytes were infected with control (white bars) or RGS2-adenovirus (black bars) for 32 h, and serum-starved for a further 16 h, followed by treatment with ET-1 (10 nM), or ISO (10 μ M) for 10 min. Cell lysates (30 μ g protein) were separated by SDS–PAGE, transferred to a nitrocellulose membrane, and the phosphorylation of (A) ERK and (B) JNK was detected by Western blotting with specific antibodies. The membrane was then stripped and the expression of total (A) ERK and (B) JNK was determined by Western reblotting with the specific antibodies. The immunoblots shown are representative of six independent experiments, the densitometric data of which are summarized in the bar graphs in the respective lower panels. Data is normalized to control values and expressed as fold increase over control. **P<0.01 (control vs. RGS2 adenovirus group).

and that forskolin, which mimics Gs signalling by activating adenylyl cyclase, has a similar effect. This finding is in agreement with a previous study in adult rat ventricular myocytes which found that both ISO $(2 \mu M)$ and forskolin $(10 \mu M)$ increased RGS2 mRNA after 3 h [8].

4.2. Inhibition of signalling through β -ARs

There is considerable evidence for the capacity of G protein coupled receptors which are coupled to Gq and Gs proteins to induce cardiomyocyte hypertrophy [12]. Previously, we have reported that



Fig. 8. RGS2 overexpression modulates ISO-induced activation of Akt. Rat neonatal ventricular cardiomyocytes were infected with control (white bars) or RGS2-adenovirus (black bars) for 32 h, and serum-starved for a further 16 h, followed by treatment with ISO (10 μ M) for 10 min. Cell lysates were separated by SDS-PAGE, transferred to a nitrocellulose membrane, and phosphorylated Akt was detected by Western blotting. The membrane was then stripped and the expression of total Akt was determined by Western reblotting. The immunoblots shown are representative of five independent experiments, the densitometric data of which are summarized in the bar graph in the lower panel. Data is normalized to control values and expressed as fold increase over control. **P<0.01 (control vs. ISO treatment), ##P<0.01 (control vs. RGS2 adenovirus group).

RGS2 overexpression is able to inhibit Gq-induced cardiomyocyte hypertrophy [7]. To date however, the role of RGS2 in regulating hypertrophic Gs-coupled responses has not been determined in cardiomyocytes. We evaluated this by examining the effect of RGS2 overexpression on β -AR-induced cardiomyocyte hypertrophy and gene expression. These experiments show that RGS2 is able to block the cellular hypertrophy, and ameliorate "fetal gene" expression induced by β -AR stimulation, suggesting that RGS2 is important in regulating β -AR-induced hypertrophy.

The mechanism by which RGS2 is able to inhibit signalling through Gs coupled receptors is not well understood and seems to be independent of any GAP effects [4,5]. Thus, despite a lack of effect on GTPase activity on isolated Gs, RGS2 is able to inhibit cAMP accumulation in endogenous and heterologously expressed systems [3,5]. In this study we found that RGS2 does regulate cAMP accumulation in cardiomyocytes, leading to a reduction in potency of the ISO response. There was no difference in maximal ISO-induced cAMP accumulation between RGS2 and GFP infected cells, thus the ability of RGS2 to inhibit cAMP production in these cells depends on the concentration of agonist present, with supersaturating doses producing equivalent effects in the absence and presence of RGS2.

4.3. Signalling pathways in β -AR-promoted hypertrophy

The pathways by which β -ARs lead to myocardial hypertrophy are not well understood and may vary depending on the model (e.g. adult vs. neonatal cardiomyocytes, *in vivo* vs. cell-based studies) and endpoint studied (e.g. gene expression vs. protein synthesis) [25,26].





Fig. 9. Effects of PKA inhibition and activation on ISO-stimulated protein synthesis in cardiomyocytes. (A) Serum-starved (24 h) neonatal ventricular cardiomyocytes were pretreated with 10 μ M H89 for 20 min. Cells were then incubated with 10 μ M ISO and [³H] leucine (1 μ Ci/ml) for 24 h. (B) Serum-starved (24 h) neonatal ventricular cardiomyocytes were treated with the PKA-specific activator 1 μ M sp-CAMP or the Epac-specific activator 1 μ M sp-CAMP or the Cardiom of [³H] leucine into the acid-precipitable cellular fraction was determined by liquid scintillation counting. Results are presented as means \pm S.E.M. from three to four independent experiments. **P*<0.05; ***P*<0.01 vs. vehicle.

The overall data suggest that multiple (possibly overlapping) β adrenergic pathways may be involved, including signals mediated via MAP kinases, increased intracellular Ca^{2+} due to Gs actions on L-type Ca^{2+} channels, and/or activation of the kinase Akt [22,23,27–29]. Despite these uncertainties, it would appear that the ubiquitous second messenger cAMP is the first molecule to initiate this signalling pathway. However, some controversy exists in whether hypertrophic signaling via cAMP is mediated by PKA, classically viewed as the main effector of cAMP in most eukaryotic cells, or by the Epac family proteins. Our results using the PKA-selective inhibitor H89 suggest that ISO-induced cAMP promotes hypertrophy in a PKA-dependent manner. This observation is supported by a previous study investigating ISO-induced cardiomyocyte hypertrophy, in which it was shown that treatment with H89 as well as a PKA-selective inhibitory cAMP analog, rpcAMP, also blocked ISO-induced protein synthesis [18]. Since there are no selective Epac inhibitors available, however, we cannot entirely rule out the possibility that Epac plays a role in mediating ISO-induced cAMP signaling in cardiomyocyte hypertrophy. Indeed, a recent study by Métrich et al. indicated that Epac1 expression increases in the rat myocardium following aortic constriction and that knockdown of Epac1 attenuates β-adrenergic receptor-induced hypertrophy [30]. Thus, it appears that more investigation is needed to fully understand the interplay of cAMP, PKA and Epac signalling in response to ISO-induced cardiomyocyte hypertrophy.

In order to examine the possible downstream pathways of the ISOinduced hypertrophic response which may be blocked by RGS2, we determined the effects of RGS2 overexpression on both Akt and MAP kinase activation by ISO in neonatal ventricular myocytes. These experiments found that ISO led to increased ERK and Akt phosphorylation, while infection of the cells with RGS2 adenovirus inhibited both of these signalling pathways. This suggests that the inhibition of hypertrophic signalling through β -ARs by RGS2 may be through combined effects on both MAP kinase and Akt pathways. The mechanism via which Akt is activated by β -AR is uncertain, although possible mechanisms include signals mediated via cAMP and Epac [31], as well as activation of Akt by G $\beta\gamma$ -stimulated PI3 kinase [32].

We found that RGS2 exerted complex effects on MAP kinase activation. In contrast to its inhibitory effect on receptor-promoted ERK phosphorylation, the overexpression of RGS2 led to increased JNK phosphorylation under basal-, ET-1- and ISO-stimulated conditions. In contrast RGS2 overexpression did not diminish but rather enhanced GPCR-dependent JNK phosphorylation, in agreement with our previous study [7]. The relative roles of the various MAP kinase pathways in cardiac hypertrophy have been studied in some detail. MAP kinases are traditionally associated with increased cell growth and proliferation following growth factor stimulation and therefore may be expected to be pro-hypertrophic in cardiac hypertrophy. In support of this hypothesis mice with cardiac-specific activation of the ERK pathway demonstrate a compensatory [33] or pathological [34] hypertrophy, while inhibition of the pathway leads to resistance to the development of hypertrophy following pressure overload [35].

In contrast to findings with ERK, mice in which the JNK or p38 pathway is activated do not develop cardiac hypertrophy but rather cardiomyopathy leading to diminished heart function [36]. Furthermore, dominant negative or knockout inactivation of these two pathways produces a hypertrophy which is similar or greater than wild type animals in response to pressure overload [37,38]. This suggests that increased p38 and JNK phosphorylation is protective against hypertrophy in vivo. Our findings in this study and a previous one [7] that RGS2 promotes p38 and INK phosphorylation in cardiomyocytes suggests that the activation of these proteins may be a contributing mechanism to the antihypertrophic function of RGS2 in vivo. Interestingly, a recent study by Zhang et al. [9] found that inhibiting RGS2 expression with RNAi led to a reduction in INK phosphorylation. This finding complements ours and provides further evidence that even basal levels of RGS2 may be important in maintaining basal phosphorylation of JNK, although the mechanism underlying this effect remains to be elucidated.

Any cell that undergoes hypertrophy must synthesize new protein in order to do so, and a study by Sadoshima et al. demonstrated that ISO-induced hypertrophy in neonatal rat ventricular cardiomyocytes was dependent upon the activation of the initiation factor eIF2Bɛ, which plays a rate-limiting role in the translation of mRNA into protein [39]. Recently, RGS2 has been shown to bind to this same initiation factor and to inhibit global protein synthesis *in vitro* and in cells, independent of its ability to regulate G protein-mediated signaling [40]. This suggests the intriguing possibility that direct effects on the translational machinery might contribute to the ability of RGS2 to block cardiomyocyte hypertrophy, although this remains to be tested.

4.4. Role of RGS2 in hypertrophy and heart failure

Both the present study and our previous one [7] have demonstrated an important role for RGS2 in cardiomyocyte hypertrophy, consistent with the work of Mende et al. [8], who similarly found that RGS2 is transiently upregulated following hypertrophic stimuli. However, the same group also showed that in the long term RGS2 expression appears to *decrease* in both predilated and end stage heart failure in two mouse models of cardiac hypertrophy with enhanced Gq/11 signalling (overexpression of constitutively active $G\alpha q$ and pressure overload due to aortic constriction) [9]. This suggests that long term reductions in RGS2 expression may contribute to the deregulation of multiple signalling pathways in heart failure. Still, even at the reduced levels observed in the latter study (~60-80% below controls) [9], RGS2 would likely still be one of the more highly expressed RGS proteins present in the myocardium [6,41] and thus might still afford a protective effect. That possibility is underscored by a recent study showing greatly increased susceptibility in RGS2 whole body knockout mice to pressure overload due to transverse aortic constriction, as mortality, pulmonary congestion, cardiomyocyte diameter, and cardiac hypertrophy were all increased in mice lacking RGS2 compared to wild type controls [10]. Such data argue strongly that RGS2 can mitigate cardiac hypertrophy in vivo, an effect that the present results suggest could reflect its ability to inhibit the detrimental effects of B-AR activation as well as Gq-mediated signalling.

In summary, this study has demonstrated that RGS2 is important in regulating cardiomyocyte hypertrophic signals through both Gq and Gs coupled receptors, and that the Gs response may be mediated through PKA, ERK and Akt. These findings suggest that RGS2 is a key regulatory protein in the development of cardiac hypertrophy through GPCRs.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.cellsig.2010.03.015.

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