Beta-Adrenoreceptor mediated atria specific up-regulation of Regulator of G-protein Signaling (RGS) 5 in rodent atrium

by

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Your file Votre référence
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To my dear family,
for all their support, love, and encouragement

Abstract

Due to a 195 fold cardiac overexpression of ß$_2$-Adrenoreceptor (ß$_2$AR), the hearts of transgenic TG4 mice are chronically overstimulated and, indeed, show little stimulatory response to further ßAR agonists. Previous investigations had suggested that signaling from the overexpressed ß$_2$ARs was dampened in the atria of TG4 mice. Regulators of G-protein Signaling (RGSs) are a family of negative regulators of G-Protein Coupled Receptor (GPCR) signaling that are frequently induced by GPCR stimulation. Using an RT-PCR based strategy, we have identified RGS5 as being a candidate RGS that is up-regulated in the atria of TG4 mice. Northern blot analysis demonstrated that RGS5 levels are 2-3 fold higher in the atria of TG4 mice when compared to their non-transgenic littermates. To further characterize RGS5 expression, we generated an RGS5 specific anti-serum. As observed for RGS5 mRNA levels in TG4 mice, RGS5 protein levels were increased 2-3 fold in the atria of rats that were chronically treated with the ßAR agonist isoproterenol. Although high basal levels of RGS5 were detected, there was no observed increase in the levels of RGS5 mRNA or protein in the cardiac ventricles in response to chronic ßAR stimulation in either TG4 mice or in rats injected with isoproterenol. Western blot analysis further demonstrated that basal expression of RGS5 protein was confined to the heart and skeletal muscle. The expression of RGS5 in myogenic cells was confirmed by observing that RGS5 is expressed in the C2C12 skeletal muscle cell line. Finally, RGS5 remained undetectable in all other tissues examined even after 14 days of isoproterenol stimulation. These results indicate that RGS5 is a housekeeping RGS in the heart and in skeletal muscle but its ßAR mediated induction in the atria suggests that it also has a highly specialized function.

Résumé

A la suite d'une augmentation de 195 fois dans l'expression du récepteur β-Adrénergique (β_2 AR), les coeurs de souris TG4 transgéniques sont excessivement stimulés de façon chronique et, en conséquence, démontrent très peu de réponse à l'attachement de ligands additionnels à ce récepteur. Des études précédentes ont suggéré que la signalisation intracellulaire à partir des récepteurs β_2 AR, dont l'expression a été augmentée, avait diminuée dans l'oreillette des souris TG4. Les Régulateurs de Signalisation des Protéines G (**R**egulators of **G** Proteins **S**ignaling) constituent une famille de protéines servant à inhiber la signalisation provenant des récepteurs couplés à une protéine G, et sont fréquemment induites par une stimulation de ces récepteurs. Par la réaction de polymérisation en chaîne basée sur l'ARN (RT-PCR), nous avons identifié RGS5 comme étant un candidat adéquat dont l'expression pourrait être augmentée dans l'oreillette des souris TG4. Des analyses Northern ont démontré que les niveaux de RGS5 sont de 2 à 3 fois plus élevés dans l'oreillette des souris TG4, comparés à d'autres souris non-transgéniques provenant de la même portée. Afin de charactériser plus profondément l'expression de RGS5, nous avons fabriqué un anti-sérum spécifique à RGS5. Les niveaux protéiques de RGS5, tout comme ceux d'ARNm, ont augmenté de 2 à 3 fois dans l'oreillette de rats traités de façon chronique par l'Isoprotéranol, un agoniste du récepteur β-Adrénergique. Malgré qu'un niveau basal relativement élevé fut détecté, il n'y eut aucune augmentation dans le niveau d'ARNm ou de protéines de RGS5, en réponse à un stimulation chronique du récepteur β_2 AR, dans les ventricules des souris TG4 ou des rats soumis à des injections d'Isoprotéranol. Des analyses Western ont plus tard démontré que l'expression protéique basale de RGS5 était associée au coeur et au muscle squelettique.

L'expression de RGS5 dans des cellules myogéniques fut confirmée à la suite de l'observation que RGS5 est exprimée dans la lignée cellulaire de muscle squelettique C2C12. Enfin, RGS5 demeure indétectable dans tous les autres tissus analysés, et ceci même après 14 jours de stimulation par l'Isoprotéranol. Ces résultats indiquent que RGS5 est un Régulateur de Signalisation de Protéines G ayant une fonction basale dans le coeur et le muscle squelettique; cependant son induction, médiée par le récepteur β-Adrénergique suggère qu'il possède une fonction grandement spécifique dans l'oreillette. **(Traduit par Gaël Jean-Baptiste)**

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Preface: Contribution of Authors

In accordance with the guidelines for submitting a master's thesis, my thesis includes a literature review (Chapter 1) and a manuscript that has been submitted to Cardiovascular Research (Chapter 2). Regarding to the study "ß-Adrenoreceptor mediated up-regulation of Regulator of G-protein Signaling (RGS) 5 in rodent atrium", all of the experiments were performed by Xiao Yu Li with the following exceptions: RT-PCR, northern blot analysis and cAMP concentration measurement were performed by Dr. Rosemarie Panetta; rabbit RGS5 antisera were collected by Dr. Wei Song; with the purified RGS5-specific antibody, Gaël Jean-Baptiste repeated and confirmed the results of western blot analysis about RGS5 atrium specific up-regulation, Gaël also detected the expression of RGS5 in skeletal muscle, C2C12 cell line, human fetus heart with the purified antibody. Dr. Cynthia G. Goodyer provided us human fetus sample, and Dr. Jürgen F. Heubach, Dr. Ursula Ravens provided us the RNA extraction of TG4 mice.

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Chapter 1 Literature Review

1.1 G protein coupled receptor (GPCR) and G protein mediated signaling

1.1.1 G protein coupled receptor (GPCR)

G protein coupled receptor (GPCR) is a large family of heptahelical receptors located on the cell membrane that are activated by a variety of stimuli, such as light, odorants, ions, hormones and neurotransmitters, etc. By interacting with heterotrimeric G proteins, GPCRs convert extracellular information into intracellular signals [1-4]. GPCRs are involved in virtually all physiological responses and GPCR signaling defects can cause diverse pathophysiologies, including abnormal cell growth, blindness, oncogenesis, inflammation, asthma and heart disease [5-12]. Because of its wide-spread distribution and the key roles they play in signal transduction, the pharmaceutical importance of GPCRs can't be ignored, since >50% of all clinical drugs target GPCRs [13-15]. For example, β adrenergic receptor (βAR) blockers are used for re-sensitization of cardiac βAR in congestive heart failure treatment; β_2-adrenergic receptor agonists for asthma by promoting airway smooth muscle relaxation and cysteinyl leukotriene receptor antagonists are used to inhibit inflammation in the nasal mucosa and airways [12,16-19].

GPCRs are a structurally conserved family of receptors [6,20-23]. Although GPCRs consist of a single polypeptide chain of variable length, they nevertheless

contain a number of common structural characteristics, including seven transmembrane helical domains, alternating intracellular and extracellular loops, and extracellular N- terminus and intracellular C- terminal tail [24-26] (Figure1.5.1).

Based on the similarity of sequence and structure of GPCRs, they are divided into six classes: Class A Rhodopsin like, the biggest subfamily including the majority of GPCR identified to date; Class B Secretin like; Class C Metabotropic glutamate/pheromone; Class D Fungal pheromone; Class E cAMP receptors (*Dictyostelium*) and Frizzled/Smoothened family [20,24]. No matter which GPCR class is stimulated by extracellular agonist, they all share the same mechanism: the conformation of receptor changes to the activate state and thus they interact with heterotrimetric G proteins to turn on the downstream signaling cascades [25].

1.1.2 G protein

GPCRs are associated with heterotrimeric G proteins that are composed of three components, α (39-52kDa), β (35-37kDa), and γ subunit (6-10kDa). It is the α subunit that demonstrates the high affinity to guanine nucleotides, has intrinsic GTPase activity, and non-covalently binds with βγ dimer. The inactive form of G_α subunit is bound to GDP. When the G protein is activated, the α subnunit and βγ dimer separate from each other to activate the downstream signaling pathways [3,5,27-34]. In active form, the G_α subunit is bound to GTP.

To date, the genes encoding for α subunits are the most diverse among the three subunits of G proteins. At least 23 genes for α, 7 for β and 12 for γ subunits had been

identified [28]. Different allocation of 3 subunits leads to the diversity of heterotrimeric G protein effect [32-35].

The effectors of G_{α}-GTP subunit include adneylyl cyclase, phospholipase, and ion channels [36-38]. According to the amino acid sequence and effectors, α subunits can be classified into four subgroups [30,39], including $G_{\alpha s}$, $G_{\alpha i/o}$, $G_{\alpha q/11}$ and $G_{\alpha 12}$. Different classes regulate different effectors leading to different effects. For example, $G_{\alpha s}$ class can increase the activity of adenylyl cyclase and regulate Ca^{2+} channels. However, $G_{\alpha q}$ class can increase the activity of phospholipase Cβ leading to an increase of the second messangers, IP3 and diacylglycerol (DAG).

Like the α subunit, the βγ dimer affects signaling cascades by regulating different effectors, including K^{+} channels, phospholipase, adenylyl cyclase and kinases which serve for receptor desensitization [36-38].

Both α and γ subunit can be modified with adding a lipid moiety which allows for anchoring of the α subunit and the βγ dimer on the cytosolic surface of plasma membrane. This facilitates their interaction with intracellular parts of GPCR [27,29,36,38].

1.1.3 GPCR activation and G protein cycle

GPCRs have several dynamic states and the different conformations show different affinities for agonist [36-38]. In the agonist bound form, GPCR conformation is shifted to the activate state which causes the activation of heterotrimeric G proteins, G proteins play a key role in transducing the extracellular

information to a variety of cellular responses [40].

Because of the intrinsic GTPase activity of the G_α monomer, the GTP is hydrolyzed to GDP in order to return the G_α subunit to its inactive GDP bound form. The GDP bound G_α subunit has a high affinity for the $\beta\gamma$ dimer, so the heterotrimeric G protein is reassociated and signaling is stopped [27,41]. In fact, the hydrolysis of GTP-α subunit to GDP-α subunit is a very important mechanism that serves to regulate GPCR signaling [42].

1.1.4 GPCR desensitization

When GPCRs are continually exposed to agonist, the receptors show decreased sensitivity toward the same agonist to protect the cell from excessive stimulation [43-46]. To date homologous and heterologous desensitization of GPCR and negative feedback mechanisms have been found to explain the phenomenon [47-52].

1.1.4.1 Homologous desensitization

Usually homologous desensitization occurs very fast with a half-life ranging from milliseconds to a few seconds after specific agonist exposure. This desensitization mechanism involves phosphorylation of intracellular regions of the stimulated GPCR by second messenger dependent activated protein kinases and/or G protein-coupled receptor kinases (GRK) [47,48]. Extensive studies on β_2 adrenoreceptor (AR) have served to explain the mechanisms. Once β_2 AR is phosphorylated on serine residues by a specific GRK after challenge by agonist,

arrestin binds to the phosphorylated receptor to facilitate receptor endocytosis and prevent further activation of G proteins by the receptor. Both of protein kinases and GRKs serve to desensitize the receptor [47,48]. Arrestin is now known to be a family of 5 different proteins that serve to regulate GPCR signaling and intracellular trafficking [53-55].

1.1.4.2 Heterologous desensitization

Besides rapid homologous desensitization, heterologous desensitization is another important mechanism. Unlike the former one, which inhibits the given receptor's further stimulation by the same stimuli, the latter mechanism is non-agonist specific. Followed by activation of protein kinase A (PKA) and protein kinase C (PKC), which are mediated by $G_{\alpha s}$ and $G_{\alpha q}$ respectively, both stimulated and unstimulated GPCR may be phosphorylated. Arrestins will continue the downstream desensitization pathway to finish the two kinds of desentization [43-47,56].

1.1.4.3 Negative feedback

Continuous, long term exposure causes a decrease in the number of receptors on the cell surface. This phenomenon is caused because the phosphorylated receptors are transported into intracellular vesicle for degradation [57-62]; decrease in receptor mRNA level has also been reported for some GPCRs [46,63-65].

Besides the mechanisms of GPCR desensitization discussed above, regulator of G protein signaling (RGS), which accelerate the hydrolysis of GTP bound by G_{α}

monomer to promote termination the signaling process, is another important mechanism to decrease the GPCR signaling [66,67].

1.2 Regulators of G protein signaling (RGS) and RGS-like proteins

1.2.1 RGS

Regulators of G protein signaling (RGS) are key regulators of G protein activity through stimulating the intrinsic GTPase activity of GTP bound G_α subunits (G_i-and G_q-class) a minimum of 40 fold over basal level [66,68-70]. Only one RGS, RGS-PX1, has been bound to stimulate the GTPase activity of $G_{\alpha s}$ [71].

RGSs were first identified in yeast. Mutant yeast cells lacking the SST2 encoded RGS were found to have hyperresponsive pheromone receptor responses [72,73]. There are now over ~20 mammalian RGSs that have been identified [74-78]. The common point of these proteins is a conserved ~120 amino acid sequence [79], which is referred as the RGS box [40,80]. The sequence identity of the RGS box ranges from 20-80% [40,81]. But the remaining parts outside RGS box are mostly dissimilar in size and amino acid sequence [82-84].

Although the RGS boxes of most RGS proteins serve to fulfill the function of hydrolyzing the GTP of G_α-GTP [68,85,86], the non-RGS box part of most RGS is required for its complete function [87-89]. For example, the N-terminal non-RGS portion of RGS2 is required to inhibit adenylyl cyclase [88].

Besides negatively regulating G protein activity, many RGS proteins affect

signal transduction by other ways, they function as G-protein-regulated effectors, Gβγ scavengers or scaffold proteins that are involved in the assembly of large signaling complexes [84].

1.2.2 Subfamily of RGSs

RGS containing proteins have been grouped in 6 subfamilies based on sequence similarities within and outside the RGS sequence [81,82,84,90,91].

1.2.2.1 R4 RGS subfamily

The R4 RGSs is the largest RGS subfamily. This subfamily consists of RGS1, RGS2, RGS4, RGS5, RGS8, RGS13 and RGS18. These proteins have only ~200 amino acid residues and contain very little sequence outside the RGS box. In spite of the small size, RGSs in R4 subfamily have been shown to interact with other proteins [88,89,91-94]. For example, RGS4 is a very potent inhibitor of carbachol-mediated signaling. Compared to its ability to inhibit the effect of acetylcholine, bombesin and cholecystokinin, even though all three agonists activate the same G-protein [91], the N-terminal non-RGS region of RGS4 appears to mediate its interaction with specific GPCRs. The existence of a trimeric complex, which involves the RGS, the receptor and the G protein, accounts for the observed selectivity of the small RGSs [95]. In addition, the non-RGS sequences directly interact and inhibit some effector enzymes, such as adneyly cyclase [88].

Most of the remaining discussion will focus on this class of RGSs since my

studies were done on RGS5. I will nevertheless briefly introduce the other RGS subfamilies.

1.2.2.2 RGS3 subfamily

Because RGS domain of RGS3 is very similar as that of R4 RGSs, RGS3 is sometimes classified into the R4 family [81,82,90,96]. The controversy exists because there is a long isoform of RGS3 (~300aa) and N-terminal truncated isoforms of RGS3 (RGS3T and RGS3S) [97,98].

The members of RGS3 family are specific for G_i and G_q families. RGS3T is reported to be a potent inhibitor of G protein signaling [99], and it may also directly negatively inhibit the activity of adenylyl cyclase [88,97,99]. Besides its GAP activity for G_α monomers, RGS3 isoforms also showed inhibition of the $G\beta\gamma$ dimer-mediated activation of PLC, MAP kinase and phosphatidylinositol-3-kinase activities. By some unknown mechanism, RGS3T accumnulates in the nucleus where it apparently induces apoptosis [100,101]. PDZ-RGS3, another RGS3 isoform, binding to ephrin B receptors via its N-terminal PDZ domain, mediates reverse signaling through B ephrins by inhibiting chemokine GPCRs [102].

1.2.2.3 R7 RGS subfamily

R7 subfamily members are characterized by the presence of a unique G gamma-like domain (GGL) and Dihevelled/EGL-10/Pleckstrin domain [103,104]. RGS6, RGS7, RGS9 and RGS11 are the 4 known members of this subfamily.

Through their GGL domain, they form obligatory dimers with G protein subunit $G\beta_5$ *in vivo*. It is reported that $G\beta_5$ complexes of RGS6, RGS7 and RGS11 exhibit GAP activity to $G_{\alpha o}$ *in vitro* which serves to negatively regulate GPCR signaling [103-110].

1.2.2.4 R12 RGS subfamily

RGS12 and RGS14 belong to the R12 RGS subfamily. Due to splicing, RGS10 exists in multiple isoforms. The long isoform has been classified as a R12 member while several researchers classified small RGS10 isoforms to the R4 RGS subfamily [81,82,90]. The R12 RGSs are large proteins with C-terminal *GoLoco* motifs and multiple isoforms have been described for each member [111,112]. RGS12 and RGS14 interact with GDP-$G_{\alpha i1-3}$ through their *GoLoco* motifs and inhibit the dissociation of GDP from the α subunit [112-116]. They have also been reported to inhibit $G_{\alpha 12/13}$-mediated signaling by as of yet an unknown mechanism [114,117]. Another characteristic domain of RGS12 and RGS14 is the Rap-binding domain (RBD) that interact with Rap1 and Rap2, but the RBD function remains to be studied [115]. Besides the RBD and *GoLoco* domain, the largest RGS12 isoform contains a PDZ domain and a phosphotyrosine-binding (PTB) motif. It is its PTB motif that endows RGS12 with the ability to exert its inhibitory effect on the γ-aminobutyric $acid_B$ receptor regulated Ca^{2+} channels [118].

1.2.2.5 RZ RGS subfamily

To date, at least five RZ RGS isoforms encoded by *Rgs17*, *Rgs19* and *Rgs20*

belong to RZ family [119,120]: RGSZ2 is encoded by *Rgs17*; G_{α}-Interacting Protein (GAIP) and GAIP-v are transcripts of *Rgs19*; alternative splicing of *Rgs20* gives rise to RGSZ1 and Ret-RGS [77,119,120]. The N-terminal cysteine string domain is characteristic of all the members except RGSZ1 and Ret-RGS. They all can be anchored to the cell membrane through palmitoylation modification or a N-terminal transmembrane domain [121,122].

Compared with their rather weak GAP activity for $G_{\alpha q}$ family members, RZ RGSs preferentially interact with $G_{\alpha i}$ subunits. For example, GAIP specifically interacts with $G_{\alpha i3}$, which was verified by specific binding of *in vitro*-translated $G_{\alpha i3}$ with a GAIP-glutathione S-transferase fusion protein [77]. Furthermore, GAIP and RGSZ2 are unselective to the $G_{\alpha}i$ family. But to date, the favorite α subunit of RGSZ1 and Ret-RGS is the pertussis toxin insensitive $G_{\alpha z}$ [120,122-124].

1.2.2.6 RA RGS subfamily

Auxin and Conducin are genetically related homologues [96]. Although they contain RGS box, they do not appears to have G_{α} GAP activity [84]. But several reports indicate that those proteins display their non-RGS function through RGS box binding to target molecules [125,126].

1.2.3 other RGS-like proteins

A variety of other proteins also contain sequence of RGS box but they have not been classified as RGS yet. These proteins include the GPCR kinases (GRK1-7),

RGS-PX1 and a variety of RhoGEFs. It is of interest that the RGS box on p115RhoGEF and leukemia-associated RhoGEF (LARG) allow these proteins to interact with receptor activated $G_{\alpha 12}$ and $G_{\alpha 13}$ proteins [83,127].

1.2.4 Distribution of RGSs

RGS mRNAs are ubiquitously expressed in all tissues and cell types examined so far, but individual RGSs nevertheless show specific tissue distribution [83]. For example, RGS5 is abundantly expressed in heart, skeletal muscle, and at low levels in brain, placenta, liver, colon, and leukocytes etc.[128]. The lack of suitable specific RGS anti-sera has limited the study of the distribution of the RGSs.

1.2.5 Regulation of RGSs

1.2.5.1 Modulation of RGSs expression

The importance of regulating gene expression to RGS function is highlighted from studies in which the yeast RGS containing gene, SST2, has been investigated [129]. In these cells, both high and sub-threshold levels of GPCR agonist dramatically increase the expression of the SST2 gene. At low concentrations, the increase in SST2 protein (sst2p) modulates signaling to prevent receptor activation until a certain threshold of ligand concentration is present. At high concentration of ligand the increase in sst2p terminates signaling. Prolonged receptor stimulation leads to desensitisation by receptor phosphorylation and internalisation. The SST2 protein is then degraded to allow re-sensitisation. In fact, SST2 protein has a very short

half-life (~30min.) that is a common feature of most key regulatory proteins. Therefore certain mechanisms, such as transcriptional regulation, are used to maintain the appropriate levels of SST2 protein required to regulate GPCR signaling. Finally, yeast mutants devoid of SST2 show extreme hyper-responsive GPCR signaling and a greatly reduced ability to desensitize in the presence of continued agonist stimulation whereas cells overexpressing SST2 show a dramatic reduction or hypo-responsiveness in GPCR signaling.

Although the situation in mammalian cells is far more complex, a number of parallels can be drawn from yeast. For example, stimulation of GPCRs increases the expression of some and decreases the expression of other RGSs. Such alterations in the level of RGSs will lead to dramatic changes in GPCR signaling [130-134]. As noted with SST2, the decrease in the levels of some RGSs may also reflect the fact that the protein is no longer required to regulate signaling. Taken together, these observation suggest that RGSs or more precisely the mechanisms regulating the levels of RGS proteins, are part of complex regulatory network involved in modulating the timing, intensity and duration of GPCR signaling.

Different pathophysiological conditions also lead to an increase in RGS expression. For example, mRNA for RGS1, RGS4 and RGS16 have been reported to increase in the heart during sepsis [84,130]. Meanwhile, changed feeding behaviors affects the levels of RGS16 mRNA. Withholding food from rats or mice induced RGS16 expression in periportal hepatocytes, while post-prandial lead to rapid decrease in RGS16 mRNA expression [135].

Besides the up-regulation of RGSs, down-regulation of RGSs has also been reported. For example, microchip analysis and cDNA screening demonstrated that during *in vitro* capillary morphogenesis, RGS5 mRNA decreased [136].

1.2.5.2 Regulation in RGSs activity

Posttranslational modification may also play a key role in regulating RGS function. EGFR-mediated phosphorylation on residue Tyr (168) of RGS16 enhanced its GTPase accelerating (GAP) activity on $G_{\alpha i}$ [137]. But phosphorylation on residue Tyr (168,177) of RGS16 reduced its GAP activity [138].

Since they function as GTPase activating protein (GAP) through direct interaction with G_{α} subunit which is anchored at the inside of cellular membrane, RGSs have to show spatial relationship with their partner through certain mechanisms. Palmitoylation may be one of these mechanisms. Palmitoylated cystein-rich regions of GAIP had been reported to serve for its membrane anchoring and trafficking [122].

1.3 GPCRs distribution and function in the heart

At least 13 different GPCRs expressed in the heart and are particularly important for the homeostatic regulation of the cardiovascular system [139-141]. The sympathetic and parasympathetic nervous system control cardiac function by activating these receptors. The adrenoreceptor (AR) and muscarinic receptors are the most studied GPCRs in the heart. Other receptors including: endothelin, angiotensin II and opioid receptors whose physiological functions remain to be elucidated

[139,142].

1.3.1 Adrenoreceptor (AR)

The presence of α_{1A}-, α_{1B}-, α_{1D}-, β_1- and β_2-AR in the heart has been proved at mRNA level, protein level and by functional studies. In human myocardium, the ratio of α-AR and βAR is about 1:10, and β_1-AR comprises 75-80% of β-AR [143]. But β_1-: β_2-AR ratio is not totally same in atrium and ventricle: in atrium, it is about 60%:40% ~ 70%:30%, while it becomes 70%:30% ~ 80%: 20% in ventricle [141]. A number of studies has also reported the presence of α_2- and β_3-AR in the heart, but their importance remains to be determined [140,141].

The α_1-, β_1- and β_2-AR cause positive inotropic and chronotropic effect. α_1-AR interact with $G_{\alpha q/11}$ to activate PLC/IP3/DAG system, while β-ARs largely activated $G_{\alpha s}$ and function through cAMP/PKA system [141,143]. Phenylephrine stimulation of α_1-AR evokes 15-35% of the inotropic effect of βAR agonists [141].

Most notably, the distribution of β-ARs can be altered under the pathophysiological condition or by pharmacological treatment, in most cases, β_1-: β_2-AR ratio is shifted to β_2-AR because of the downregulation of β_1-AR. Usually the decrease of β-AR takes place in the ventricle [141,143].

1.3.2 Muscarinic receptor

M_2 muscarinic receptors are the principal receptors to cause negative iontropic effects in the heart. The receptors function largely by inhibiting adenylyl cyclase. The

importance of this receptor is seen in M_2 muscarinic receptor knockout mice, where cabachol can't reduce heart rate [144]. Nevertheless, unlike β-AR, there are no changes in the distribution and responsiveness of M_2 receptor in most cardiac disease [141].

1.3.3 GPCR abnormal signaling and heart disease

$G_{\alpha q}$ coupled GPCRs promote cardiac hypertrophy through the activation of hypertropic MAPK pathways: ERK JNKs and P38 MAPKs [145,146]. It has been demonstrated that blocking $G_{\alpha q}$ signaling can significantly attenuate the hypertropy and MAPK activation in response to pressure overload [147,148]. Interestingly, transgenic mice that lack endogenous catecholamine (nor-adrenaline and adrenaline) also show the same results [149], indicating that ARs in the heart contribute to the hypertropic response to the overload.

In the chronically failing heart, there are a number of abnormalities in cardiac β-AR signaling. To assist the failing myocardium, renin-angiotensin and sympathetic system are activated [150,151]. But the responsiveness to the continuous adrenergic stimulation gradually decreases. Successful utilization of β blockers therapy suggests β adrenoreceptor desensitization plays an important role in the progession of heart failure [16-19]. Although β_2-AR won't decrease in number like β_1-AR, its uncoupling from $G_{\alpha s}$ also results in decrease responsiveness. βARK1 is the G protein coupled receptor kinase that serves to desensitize the β_1AR in heart failure [143]. Indeed, inhibition of β-ARK1 serves to protect against heart failure.

1.4 RGSs in the heart

The heart expresses at least 9 G_α-subunits and at least 10 different small RGSs [140,152,153]. It remains to be determined which of the multiple GPCRs and/or G_α proteins are regulated by different RGSs. In addition, a number of RGSs are induced in the heart during cardiac diseases[84,130,154-156]. For example, RGS3 and RGS4 are induced in hypertrophy while RGS1, 4 and RGS16 are induced in response to sepsis. The increase in RGS may play an important role in the development of the hyporesponsive GPCRs that is observed in these syndromes.

1.4.1 Up regulation of RGS5 in the atria of transgenic mice with cardiac overexpression of β_2-AR (TG4 mice)

A number of transgenic animal models that overexpress adrenergic receptors (AR) in the heart have been developed [157-159]. In addition to serving as models of cardiopathologies, the study of these animals has also generated a great deal of insight on the basic physiology of cardiac AR signaling. TG4 mice, which have a 195 fold increase in cardiac β_2-AR levels, is a widely used model [160-162]. The overexpressed β_2-ARs are partly constitutively active and consequently the heart rate of these animals remains maximal even at rest. These animals demonstrate the concepts that at any given time a small portion of a pool of GPCR are spontaneously active. Inverse agonists were shown to block the spontaneously active β_2-AR suggesting that such compounds may have clinical value [161]. In spite of these observations, there is a good deal of experimental evidence to suggest that most of the

overexpressed β_2-AR do not properly couple to G_α proteins, especially in the left atria [162]. Since RGSs are capable of functionally uncoupling GPCRs and that a number of RGSs are induced in response to GPCR stimulation [163], we hypothesized that some RGSs may be up-regulated in the heart of TG4 animals.

Prior to my arrival, Dr. Greenwood's lab had shown that RGS5 mRNA is up-regulated 3 fold in the atria of TG4 mice. My project consisted of determining if β-AR stimulation was indeed responsible for the observed increase in RGS5 expression. To accomplish this, I purified RGS5 antiserum collected by Dr. Wei Song and examined the regulation of RGS5 gene expression in rat model treated with β-AR agonist using purified RGS5 specific antibody.

1.4.2 RGS5

The following is my introduction describing what is known about RGS5.

RGS5 belongs to the R4 RGS family, its sequence is quite similar to those of RGS1, RGS4 and RGS16 [84,130]. To date, the tissue reported expressing RGS5 include heart, aorta, stomach, lung, small intestine skeletal muscle, brain, pituitary, placenta, liver, colon developing pericytes and vascular smooth muscle, RGS5 expression is also detected in the cells of vascular system, brain capillary endothelium, choroid plexus, and leukocytes [67,128,164-168], but the results of different investigators were not consistent with each other except heart, skeletal muscle, moreover, all the reports about endogenous RGS5 are at the mRNA level other than protein level.

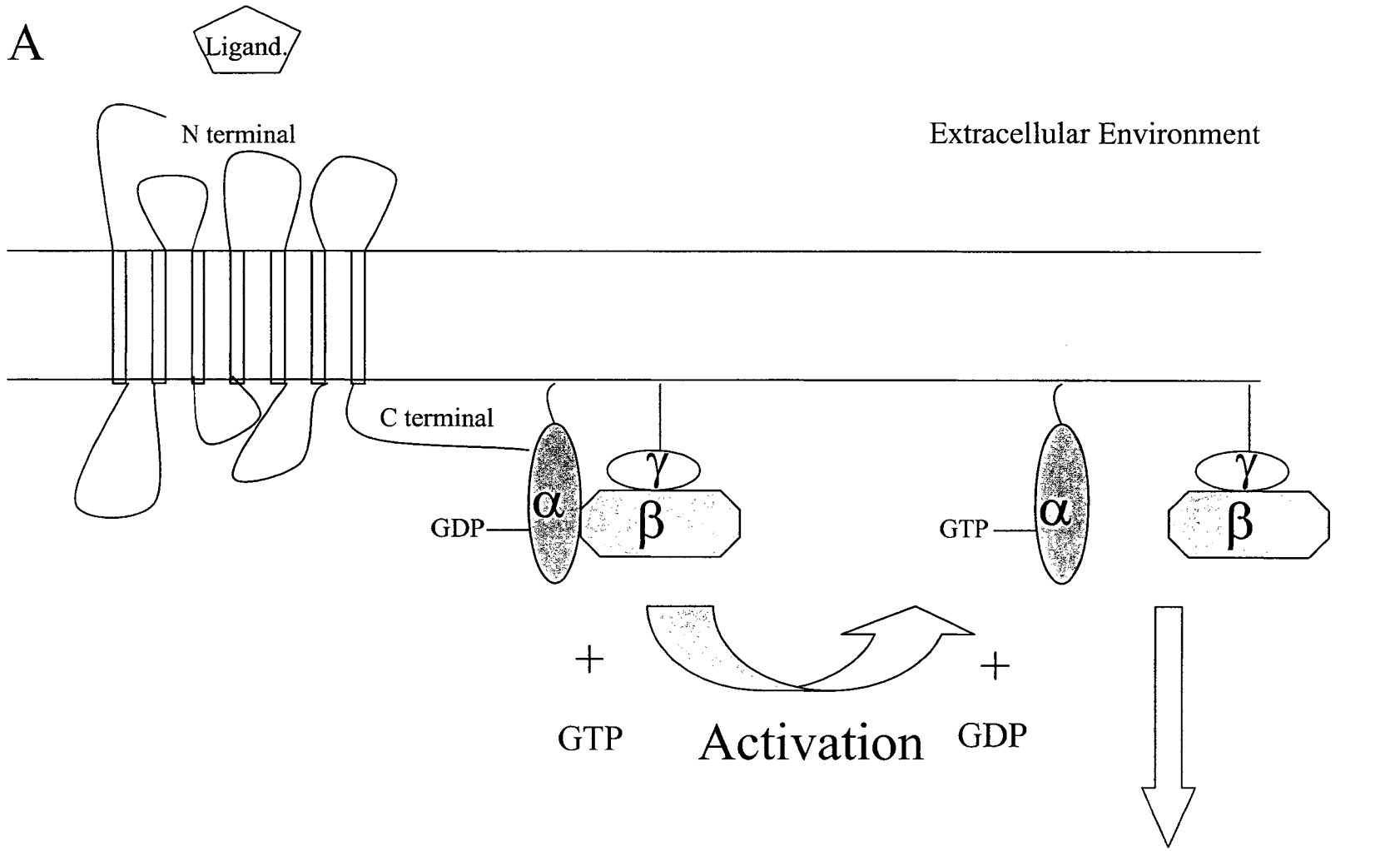
In vitro binding assays reveal that RGS5 bind to $G_{\alpha i1}$, $G_{\alpha i2}$, $G_{\alpha i3}$, $G_{\alpha o}$ and $G_{\alpha q}$ subunits of heterotrimeric G-proteins, but not to $G_{\alpha s}$ [167,169]. Angiotensin II-, endothelin-1-, sphingosine-1-phosphate-, and PDGF-induced ERK-2 phosphorylation were reduced by overexpressed RGS5 [165]. The Xenopus RGS5 orthologue negatively regulates the G-protein-mediated signaling pathway in developing embryos [170]. When expressed in 293T cells stably expressing angiotensin (Ang) AT1a receptors, the suppression of Ang II- and endothelin (ET)-1-induced intracellular Ca^{2+} transients by RGS5 depend on the RGS5 concentration [169]. Furthermore, RGS5 was downregulated in stroke-prone spontaneously hypertensive rats (SHRSP), indicate it may be a new stroke-related genes [166].

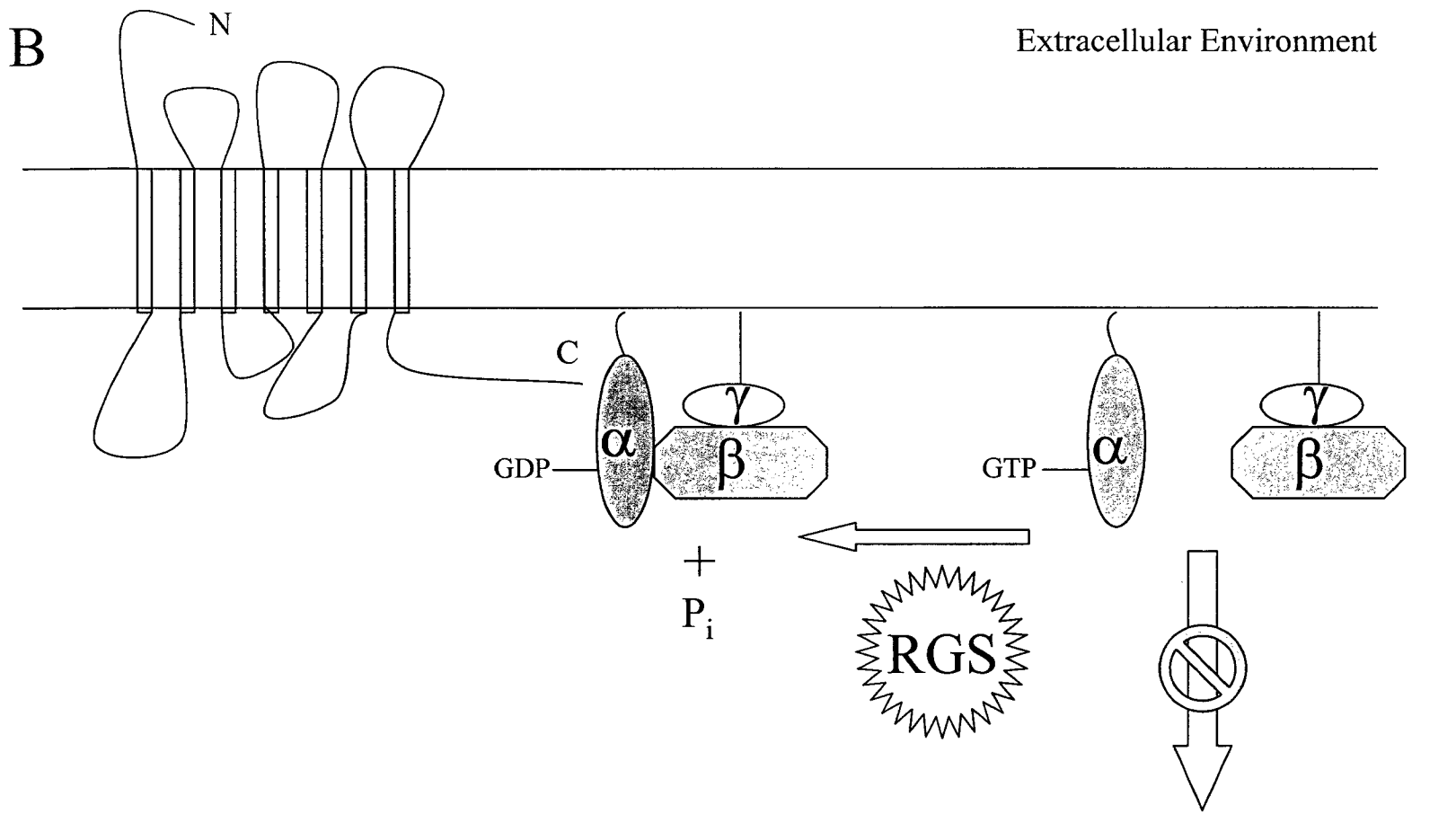
1.5 Figures

1.5.1 GPCR and G protein cycle

A. G protein coupled receptor (GPCR) is a large family of heptahelical receptors located on the cell membrane that are activated by a variety of stimuli. Upon activation, conformation of GPCR will shift to activate state. Heterotrimeric G protein disassociate to G_α subunit and $G_{\beta\gamma}$ dimer, at the same time, GDP is exchanged by GTP, both G_α-GTP and $G_{\beta\gamma}$ can stimulate the downstream effectors.

B. GTP-G_α hydrolysis can turn off GPCR signaling pathway through G_α reassociate with $G_{\beta\gamma}$. RGSs play a very important role in this procedure.


A
Ligand
N terminal
Extracellular Environment
C terminal
GDP
α
β
γ
GTP
α
β
γ
+
GTP
Activation
+
GDP
Downstream effectors


B
N
Extracellular Environment
C
α
β
γ
GDP
GTP
+
P_i
RGS
Downstream effectors

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Chapter 2: ß-Adrenoreceptor mediated up-regulation of Regulator of G-protein Signaling (RGS) 5 in rodent atrium.

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2.1 Introduction

Regulators of G-protein Signaling (RGS) are identified by the presence of a conserved 120-125 amino acid motif, which is referred to as the RGS box [1,2]. RGS containing proteins have been classified into six subfamilies. Most RGS containing proteins are relatively large proteins (> 300 residues) and they possess a number of previously identified sequence motifs that suggest specific functions. For example, GRK2 (or ßARK1) has a kinase domain that serves to phosphorylate GPCRs while its RGS box is capable of inhibiting $G_{\alpha q}$. In contrast, the RGSs of R4 the subfamily are small (ca. 200 residues). These RGSs negatively regulate G-Protein Coupled Receptor (GPCR)

signaling mainly by decreasing the levels of the receptor activated G_α-proteins [1,2]. Since this inhibition occurs without affecting the receptor per se, RGSs are strong candidates to be involved in mediating decreases in GPCR responsiveness that are not due to the internalisation of cell surface receptors. This role is strongly supported by the fact that a number of RGSs are induced in response to a wide variety of stimuli and that increased levels of RGSs lead to significant decreases in GPCR responsiveness.

The ß-Adrenoreceptors ($ß_1$- and $ß_2$AR) are the most powerful receptors for stimulating an increase in cardiac output in the failing heart [3]. Therefore the loss of ßAR responsiveness that occurs in chronic heart failure is a prominent feature in the progression of the disease. Although decreased ßAR responsiveness in some forms of cardiac failure is due to a loss of $ß_1$AR on the cell surface, there is no decrease in cell surface receptors associated with reduced ßAR responsiveness in sepsis and other forms of heart failure [4,5]. Up-regulation of RGSs may account for the hyporesponsiveness to vasoactive GPCR agonists that occur in cardiovascular tissues during sepsis and in the heart during heart failure. For example, RGS1, RGS4, RGS7 and RGS16 mRNA levels have been shown to increase in animal models of sepsis [4,6,7] while RGS3 and RGS4 levels are elevated in failing human hearts [8,9].

A number of transgenic animal models that overexpress ßAR in the heart have been developed [10]. In addition to serving as models of cardiopathologies, the study of these animals has also generated insight into the basic physiology of cardiac ßAR signaling. TG4 mice, which have a 195-fold increase in cardiac $ß_2$AR levels, is a widely used model [11-13]. The overexpressed $ß_2$AR are partly constitutively active and, consequently, the heart rate of these animals remains maximal even at rest. In spite of these observations,

there is experimental evidence to suggest that most of the overexpressed $ß_2$AR do not properly couple to G_α-proteins [13]. Since RGSs are capable of functionally uncoupling GPCRs and a number of RGSs are induced in response to GPCR stimulation, we hypothesized that some RGS(s) may be up regulated in the heart of TG4 animals. Using a degenerate RT-PCR based screen, we have identified RGS5 as being up regulated in the atria but not in the ventricles of TG4 mice. We further demonstrate that the chronic administration of the ßAR agonist isoproterenol to rats is sufficient to cause an increase in the levels of RGS5 in the atria.

2.2 Methods

2.2.1 Animals and tissues

TG4 and their non-transgenic littermates were generated and identified as previously described [13]. Wistar rats (male, 250-300g) were used to study the effects of bacterial LPS while Sprague Dawley (male, 280-320g) rats were used to study the effects of isoproterenol on RGS5 gene expression. The animals were given a single intra-peritoneal (i.p.) injection of 20 µg/kg of LPS and sacrificed 6 hours later. Isoproterenol was administered daily by i.p. injection at a dose of 2.4 mg/kg for 14 days and the animals were sacrificed 4 hours after the last treatment. Control animals were injected with the same volume of physiological saline. Human fetal tissue samples were obtained following therapeutic abortions (12 to 18 week fetal age). Tissues were quick frozen in dry ice and stored at -80^0C. All protocols conformed to the McGill University Animal Care and Research Ethics Committees and with the principles outlined in the Declaration of Helsinki.

2.2.2 Degenerate RT-PCR

Ventricles and left atria of TG4 mice and their non-transgenic littermates were rapidly dissected and snap-frozen in liquid nitrogen. RNA was isolated, reverse transcribed and amplified by PCR using degenerate RGS specific oligonucleotides as previously described [7]. The 240 base pair PCR fragments generated were cloned and divided into different groups based on the patterns obtained with a variety of restriction endonucleases. Amino acid sequences deduced from the DNA sequences were used to search the protein sequence database at GenBank [14].

2.2.3 Northern Blot Analysis

Twenty μg of total ventricular RNA and ten μg of total atrial RNA isolated from TG4 mice and their non-transgenic littermates were separated on a 1.5% agarose/formaldehyde gel and transferred onto a nylon membrane. The cloned 240 bp mouse RGS5 PCR product, corresponding to an internal portion of the RGS motif of RGS5 [7], was used as a specific probe to detect the RGS5 transcript, while the $ß_2$AR cDNA used as a probe was previously described [15]. Preparation of the [α-^{32}P]-dCTP radiolabelled DNA probes as well as the hybridization conditions used were previously described [7].

2.2.4 Production of RGS5 and RGS16 anti-serum

The entire coding sequences of RGS5 and RGS16 were amplified using the previously described RGS5 and RGS16 containing plasmids [7]. The following forward 5'-GGGATCCCCATGTGCAAAGGACTTGCAGCT -3' and reverse 5'-

CTCGAGCTACTTGATTAACTCCTGATA-3' primers were used for RGS5 and the following forward 5'-GGGATCCCCATGTGCCGCACCCTGGCCGCC-3' and reverse 5'-GCTCGAGTCAGGTGTGTGAGGGCTCGTC-3' primers were used for RGS16. The PCR products of the coding sequences of human (h)RGS5 and hRGS16 were individually subcloned as *BamH1-Xho1* fragments into pGEX-5X-3 (Amersham Bioscience). The plasmids were introduced into *E. coli* and recombinant GST-RGS5 and GST-RGS16 were produced and purified using glutathione-sepharose beads as recommended by the manufacturer (Sigma-Aldrich). The recombinant GST-RGS fusions were concentrated, emulsified in incomplete Freud's adjuvant and used to immunize rabbits. The anti-sera were collected and used 10 days following a third injection of GST-RGSs. The anti-RGS5 serum was further purified by antigen affinity chromatography using purified GST-RGS5 protein that was fixed to Affi-Gel 15 agarose beads as described by the manufacturer (BioRad).

2.2.5 Protein extraction and western blot analysis

Soluble protein was extracted from yeast by directly heating the NaOH treated cells to 95^0C in SDS-PAGE loading buffer as previously described [16]. Soluble protein was extracted from tissues using a Polytron in 2-3 volumes of ice-cold homogenizing buffer (50mM Tris-HCl, (pH 7.4), 0.2M sucrose, 1mM EDTA, 1mM PMSF, 1mM DTT, 1mM Benzamidine and 1% NP-40). Soluble protein was extracted from cultured C2C12 and HEK293A cells using 2-3 volumes of ice-cold lysis buffer (50mM Tris-HCl (pH 8.0), 0.1% SDS, 1mM PMSF, 1mM Benzamidine and 1% NP-40). Twenty to fifty μg of soluble protein were separated by SDS-PAGE, transferred to nitrocellulose membrane

and incubated with different primary anti-serum [16]. Commercially available mouse monoclonal anti-$ß_2$AR (Santa Cruz Technology) and rabbit anti-p44/p42 MAP kinase (Cell Signaling Biotechnology) were used as described by the manufacturer. HRP-conjugated secondary anti-sera were used and signals were subsequently detected with ECL plus (Amersham Bioscience) followed by exposure to X-ray film (Kodak X-Omat). The relative expression levels were determined using Multi-Analyst software (BioRad).

2.2.6 Mammalian cultured cells

The mouse skeletal muscle cell line C2C12 and HEK293A cells were grown in DMEM media containing 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin (pen-strep). HEK293A cells were transfected with pcDNA3.1 control vector or the pcDNA3.1 plasmid expressing RGS5 [7]. Stable cell lines were selected for and maintained by the addition of 400 μg/ml of Geneticin (G418).

2.2.7 Measurment of cAMP levels

SVEC4-10 (ATCC # CRL-2181) were cultured in Dulbecco's modified Eagle medium supplemented with 10 % FBS and were transfected at 70% confluency with the RGS5 expressing plasmid pcDNA3.1-hRGS5 or with the control vector pcDNA3.1 using lipofectamine. Twenty-four hours after transfection, the cells were subcultured into 6 well plates at a density of 10^5 cells/well. The cells were serum starved for 24 hours then treated with 50 μM 3-isobutyl-1-methylxanthine (IBMX) for 15 minutes prior to the addition of vehicle or 10^{-6}M isoproterenol [17]. Agonist stimulation of the cells was then terminated after 30 minutes by the addition 1 ml of ice -cold 75 % ethanol/16mM HCl

and the level of cAMP determined by radioimmune assay essentially as described by the manufacturer (DuPont NEN).

2.3 Results

2.3.1 RGS5 is differentially expressed in the heart and induced in the atria of TG4 mice

A degenerate RT-PCR based strategy was used to identify RGSs that are differentially expressed in the cardiac atria and the ventricles of transgenic TG4 mice that overexpress the $ß_2$AR. To search for potential RGSs, we isolated total RNA from the atria and ventricles of TG4 mice as well as their non-transgenic littermates. Samples of RNA from all the groups were reverse transcribed into DNA and subsequently amplified by PCR using degenerate RGS-specific oligos and twenty cloned PCR products were analyzed from each group. RGS5 was the only RGS identified in the screens. The number of RGS5 clones was higher in the ventricles of non-transgenic (8 out of 20) than in the corresponding atria (0 out of 20). In addition, the number of RGS5 clones was higher in the atria of the TG4 mice (5 out of 20) than in the atria of control animals (0 out of 20). To confirm the possibility that RGS5 may be unequally distributed in the heart as well as being up regulated in the atria of TG4 mice, northern blot analysis was performed using total RNA from each tissue. A single band corresponding to a 4.4 kb transcript was detected using RGS5 as a probe (Fig. 1A). The transcript was highly expressed in the ventricles of TG4 (Fig. 1A, lane 5) and their non-transgenic littermates (Fig. 1A, lane 4). In contrast, the level of RGS5 transcript was higher in the atria of TG4 animals (Fig. 1A, lane 2) than in the atria of control non-transgenic littermates (Fig. 1A, lane 1). Densitometric analysis, using 18S rRNA (Fig. 1B) to control for loading, revealed a 3-

fold increase in the basal levels of RGS5 levels in the ventricle over the atria in non-transgenic animals. In addition, there was a 2.6-fold increase in RGS5 levels in the atria of TG4 mice when compared to the levels observed in the atria of control animals. As a control, the blot was re-probed with a $ß_2$AR cDNA (Fig. 1C). The two characteristic transgene $ß_2$AR transcripts [11] were observed only in the RNA of TG4 animals (Fig. 1 C, lanes 2 and 5). These results indicate that RGS5 may be related $ß_2$AR overexpression with in the atria of TG4 mice; it is possible for RGS5 to regulate the increase in $ß_2$AR mediated signaling. The cardiac mouse model of $ß_2$AR overexpression is highly artificial. Therefore, general transferability of the results to more physiological conditions has to be shown. We continued our work on tissues, which were obtained from rats that were injected with the ßAR agonist isoproterenol. Furthermore, we generated an RGS5-specific anti-serum to characterize RGS5 expression on the protein level.

2.3.2 Generation and characterization of RGS5 specific anti-serum

Although the mRNAs for a number of RGSs have been shown to be present in the heart, very little is known regarding the expression of the RGS proteins [7,9,18-20]. This is in large part due to the lack of suitably specific anti-sera. To further characterize RGS5 expression, we generated an RGS5 specific anti-serum in rabbits using purified recombinant GST-RGS5 fusion protein. The resultant anti-serum was further enriched by affinity column chromatography. As a first step towards characterizing the RGS5 anti-serum we used extracts of yeast cells expressing RGS5 and RGS16, since we had previously demonstrated that these RGSs could be functionally expressed in these cells [15,16]. A prominent band having an estimated molecular weight (MW) of 23 kDa is

detected in extracts prepared from yeast cells expressing RGS5 (Fig. 2A, left panel). The size of the band corresponds to the calculated MW of 20.9 kDa for human RGS5. In addition, the anti-serum is specific for RGS5 since it did not recognize RGS16 in extracts of yeast cells expressing RGS16. As a control, we used an RGS16 anti-serum to demonstrate that a 25 kDa protein having the appropriate size for RGS16 (calculated MW of 22.6 kDa) is present in the extracts of yeast expressing RGS16 (Fig. 2A, right panel). Since RGS16 shares a high degree of sequence identity to both the RGS box and N-terminal non-RGS region of RGS5 [2,7], these results strongly suggest that the RGS5 anti-serum is specific for RGS5. As a further control, we demonstrate that the RGS5 protein is also detected by the anti-serum in extracts prepared from HEK293A cells that are transfected with a pcDNA vector expressing RGS5 but not in extracts from the same cells transfected with pcDNA alone (Fig 2B).

2.3.3 Distribution of RGS5 protein in the heart

Our results in Fig. 1 demonstrate that RGS5 mRNA is more abundant in the ventricles than in the left atrium of the mouse heart. To determine if the distribution of the RGS5 protein paralleled the distribution of its mRNA and to investigate the situation in rat heart, extracts were prepared from whole heart as well as isolated atria and ventricles and analyzed by western blot using our RGS5 anti-serum. Using yeast extracts expressing RGS5 as a control, RGS5 protein levels were found to be 3 fold higher in the ventricles than the atria (Fig 3A). As a control, we performed western blot analysis to determine the level of $ß_2$AR. A predominant band having an apparent MW of 70 kDa was detected in the heart using a $ß_2$AR specific anti-serum (Fig. 3A). The size of the protein is in agreement with previously published estimates for the receptor [21]. In contrast to

RGS5, $ß_2$AR was, as previously described, relatively evenly distributed in the atria and the ventricles [22].

2.3.4 Regulation of RGS5 protein in the heart

The results of our northern blot analysis suggested that the levels of RGS5 in the atria of TG4 mice with constitutive activity of the $ß_2$AR-signaling cascade were up regulated. To determine if chronic ßAR stimulation was sufficient for the observed increase, we examined the levels of RGS5 protein in the atria and ventricles of rats that had been chronically treated with the ßAR agonist, isoproterenol (2.4mg/kg-day), for 14 days. In these animals, the level of RGS5 protein was found to be increased 2-3 fold in atria (Fig. 3B). As observed in TG4 mice, very little increase of the level of RGS5 is seen in the ventricles of isoproterenol-treated rats. The levels of p42/p44 MAP kinase were determined to demonstrate that the observed increase in RGS5 was not due to a difference in the amount of protein analyzed.

2.3.5 Tissue distribution and regulation of RGS5 protein

In order to determine if RGS5 is expressed in other cell types by ßAR stimulation, western blot analysis was carried out using extracts that were prepared from a variety of different tissues that were obtained from both control and isoproterenol-treated animals. In control animals, RGS5 was detected in heart but not in thymus, brain, liver, lung, testes or kidney (Fig. 4A and B). We also examined if RGS5 could be up regulated in other tissues by ßAR agonist stimulation. RGS5 was not detected in tissues that were obtained from rats that received a daily injection of isoproterenol for 14 days (Fig. 4A).

This indicates that RGS5 is specifically up regulated only in the atria following ßAR stimulation.

Since some RGSs have been reported to be induced during sepsis [4,7], we examined the levels of RGS5 in a rat model of sepsis. RGS5 was not induced in any of the tissues examined indicating that it is unlikely to play a role in the altered GPCR responses associated with this syndrome (Fig. 4B).

2.3.6 Expression of RGS5 in skeletal muscle, cultured muscle cells and human fetal heart

Chen *et al.* [23] have previously reported that RGS5 mRNA is abundantly expressed in the heart and in skeletal muscle. Using our RGS5 anti-serum, we also detected RGS5 protein by western blot analysis in rat skeletal muscle (Fig. 5A). This 23-kDa protein had the same MW as the RGS5 that was detected in heart and in RGS5 expressing yeast cells. Because RGS5 is expressed in blood vessels [2,24,25] we used the mouse C2C12 skeletal muscle cell line to ascertain if RGS5 is expressed in skeletal muscle cells. A 23-kDa protein that has the same MW as muscle RGS5 was detected in growing C2C12 cells (Fig. 5B). This indicates that RGS5 is expressed in skeletal muscle cells and that C2C12 cells may be a good model in which to study the regulation and function of endogenously expressed RGS5.

We have also used extracts prepared from fetal tissues to determine if RGS5 is expressed in human heart. We examined heart and lung tissue from 6 different fetuses. We detected RGS5 in three of the hearts while RGS5 was not detected in the other three. Analysis of tissues obtained from fetus 1 and 2 is shown in Fig. 5C: RGS5 is detected in the heart from fetus 1 but not in the heart from fetus 2. As expected no RGS5 was

detected in the extracts obtained from the six lung tissues examined (two samples are shown in Fig. 5C). It is not known why RGS5 was detected in only three of the six fetal heart samples. There may variability in the quality of the different samples or it may be that RGS5 is weakly expressed and not easily detected in the early gestation tissues (12 to 18 week fetal age) that we used in this study. Nevertheless, our results demonstrate that RGS5 protein is expressed in human heart tissue.

2.3.7 Inhibition of ßAR signaling by RGS5

The endothelial derived cell line SVEC4-10 was used to assess the ability of RGS5 to inhibit ßAR signaling. The ßAR agonist isoproterenol induced a significant increase in intracellular cAMP in SVEC cells transiently transfected with the control vector pcDNA3.1 (Fig. 6). The increase in cAMP levels was reduced by 25% in cells transiently transfected with pcDNA3.1-hRGS5.

2.4 Discussion

RGS5 is a member of the R4 subfamily of RGS containing proteins. These RGSs are small (ca. 200 residues) and are characterized by the presence of an N-terminal region consisting of an amphipathic α-helix [26,27]. Overexpression of this class RGS in cultured mammalian cells has revealed that they are capable of inhibiting signaling from all GPCRs so far examined. In spite of these observations, it is largely assumed that the different RGSs will have specific functions [28]. Differential tissue-specific gene expression is an important factor that will serve to limit the role of the different RGSs. For example RGS5 expression is limited to the heart (Figs. 1,3,4), skeletal muscle (Fig.

5), smooth muscle, pericyte and a variety of sub-regions within the brain [2,23-25]. Therefore, RGS5 will be limited to regulate GPCR signaling within these cell types under normal condition. Further, there appears to be specific functions of any given RGS within any given cell. This is evident in yeast where gene knock-out studies have demonstrated that the RGS encoded by the *SST2* gene is strictly involved in regulating pheromone responses and does not regulate the signaling from the glucose sensing receptor [29]. In mammalian cells, the specificity of function may reside in the ability of individual RGSs to form of a trimeric complex with specific or subsets of specific receptors and G_{α}-proteins [30]. Clues as to the specificity of RGS5 function come from ribozyme knock down experiments in cultured smooth muscle cells [24]. A decrease in RGS5 levels caused a specific increase in Angiotensin II receptor signaling. On the other hand, a number of other studies suggest that heterologously expressed RGS5 can also regulate a variety of different GPCRs, including the endothelin 1 receptor, the somatostatin receptor 5, and the muscarinic acetylcholine receptor M2 [15,25,31,32]. It remains to be determined which of the 13 GPCRs and/or 10 G_{α}-proteins that are known to be expressed in the heart are regulated by RGS5 [3,19].

The levels of a number of RGSs are increased by a variety of stimuli, including GPCR agonists [1,4,7,29,33,34]. Many of the RGSs are induced in specific subsets of different cell types. For example, a number of RGSs are differentially regulated in a variety of regions of the rat brain following the induction of electroconvulsive seizures or the injection of amphetamine [35,36]. The simplest interpretation is that an increase in RGS levels in response to the stimulation of a given GPCR will serve as a negative feedback loop to limit signaling responses to the GPCR itself [34]. Conversely, the

increase in RGS levels may lead to decreased responsiveness for other GPCRs thereby serving to contribute to the phenomenon of heterologous desensitization. The tissue specific ßAR-mediated increase in RGS5 that we observe here in the atria (Figs. 4,5) suggests that RGS5 may be involved in regulating cell-specific GPCR signaling events, such as the GPCR-mediated release of ANF [37]. We found that the overexpression of RGS5 in cultured cells leads to a decrease in ßAR mediated signaling (Fig. 6). Although these results are suggestive, the exact role of increasing RGS5 in the atria will require the generation of specific transgenic animals or RGS5 inhibitors [28].

RGSs have also been shown to be increased in a number of cardiovascular diseases [2]. For example, RGS3 and RGS4 are elevated in congestive heart failure (CHF). These RGSs may therefore contribute to the decrease in ßAR-mediated responses that are observed in these hearts. RGS5 is not likely to play role in CHF since we do not observe an increase in its levels in the ventricles of rats that have been chronically stimulated with ßAR agonist isoproterenol (Fig. 4B). Nevertheless, the observed atrial specific regulation of RGS5 might be involved in diseases like atrial fibrillation, where GPCR signaling is attenuated [38].

2.5 Figures

Fig 2.5.1 Up-regulation of RGS5 in the atria of TG4 transgenic mice with cardiac overexpression of ß$_2$AR.

The hearts of TG4 mice and their non-transgenic littermates were separated into atria and ventricles. Total RNA was isolated and pooled from the left atria of 10 non-transgenic (lane 1) and from 12 TG4 (lane 2) mice. Similarly, RNA was isolated and pooled from the ventricles of 5 non-transgenic mice (lane 3), from 5 wild type parental C57BL6 mice (lane 4) and from 5 TG4 mice (lane 5). Northern blot analysis was then performed using 10 µg of atrial RNA and 20 µg of ventricle RNA. The blot was sequentially hybridized with (A) RGS5, (B) 18S rRNA and (C) ß$_2$AR specific [^{32}P]-labeled DNA probes.

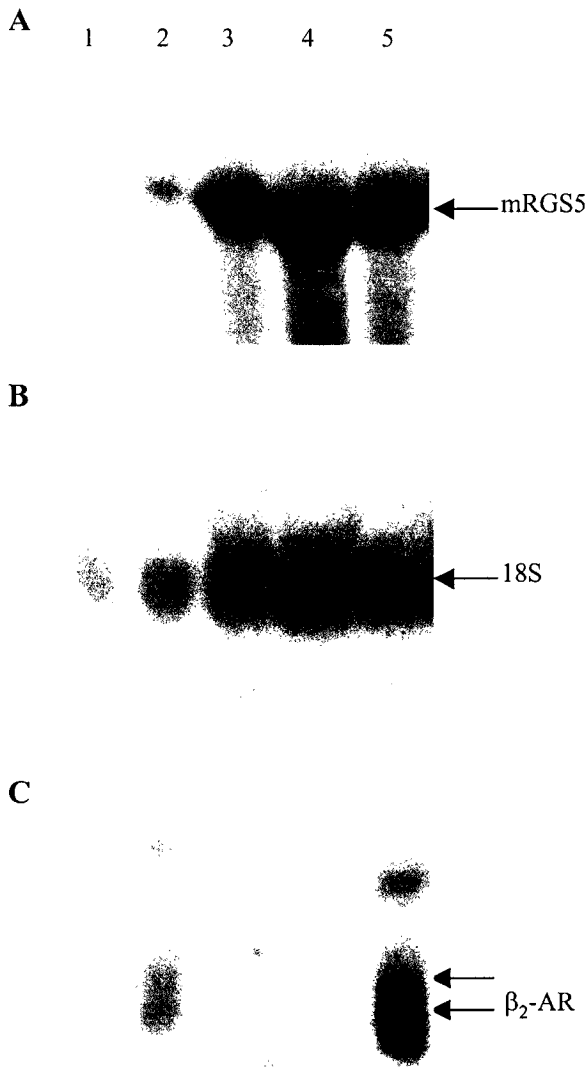

A
1
2
3
4
5
mRGS5
B
18S
C
β_2-AR

Fig. 2.5.2 Analysis of RGS5 anti-serum in yeast and HEK293A cells.

Western blot analysis was performed using protein extracts prepared from yeast and HEK293A overepressing RGS5. (A) depicts the results of western blots using extracts prepared from yeast cells expressing RGS5 or RGS16. The extracts were challenged with either the RGS5 anti-serum (left panel) or with RGS16 anti-serum (right panel). (B) shows the result of western blot analysis using protein extracted from HEK293A cells that were transfected with the mammalian expression pcDNA alone or pcDNA expressing a hRGS5 cDNA. As a control, 0.5 μg of protein extract from yeast cells expressing hRGS5 (YEAST) was included on the gel.

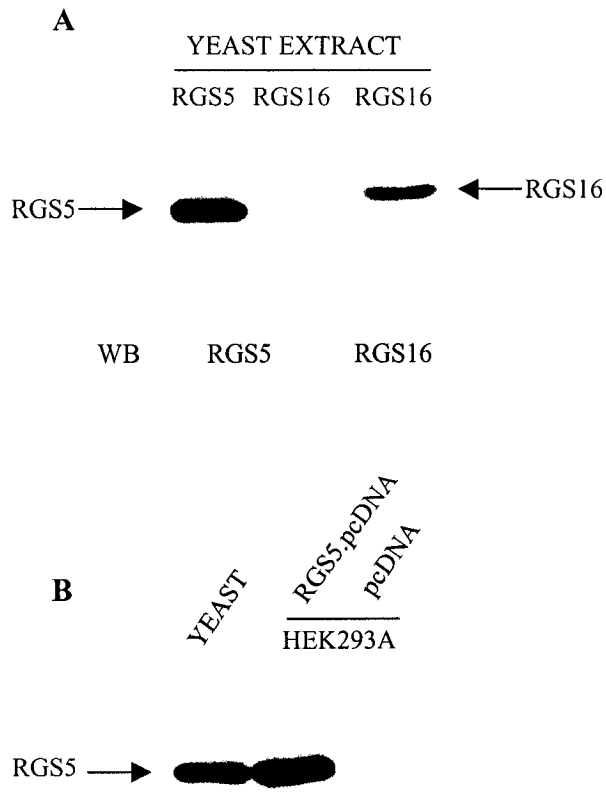
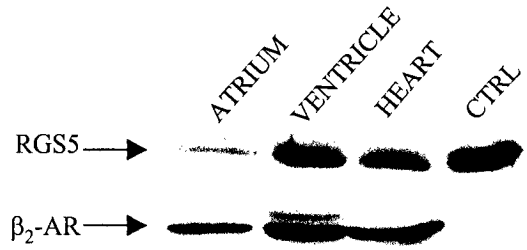

A
YEAST EXTRACT
RGS5 RGS16 RGS16
RGS5
RGS16
WB RGS5 RGS16
B
YEAST
RGS5.pcDNA
pcDNA
HEK293A
RGS5

Fig. 2.5.3 Differential distribution and ß$_2$-Adrenergic receptor mediated up-regulation of RGS5 in cardiac ventricles and atria.

The hearts of Sprague Dawley rats were surgically separated into atria and ventricles and soluble protein extracts were prepared for Western blot analysis. (A) depicts the levels of RGS5 detected in the atrium, ventricle and whole heart. The level of ß$_2$AR protein was determined in the same samples using a commercially available monoclonal antibody. (B) Sprague Dawley rats received a daily injection (i.p.) of isoproterenol (ISO) for 14 days (lanes labeled as 14) while the controls (lanes labeled 0) received saline alone. Fourteen days after treatment, the animals were sacrificed and RGS5 levels were determined in the ventricle and atrium by western blot using the RGS5 anti-serum. Two separate 14 day treated animals and one control animal are shown. Similar results were obtained in 3 separate experiments. The level of p44/p42 MAP kinase was determined in the same extracts using a commercially available anti-serum. CTRL, extracts from RGS5 expressing yeast cells.

A


ATRIUM
VENTRICLE
HEART
CTRL
RGS5
β_2-AR

B

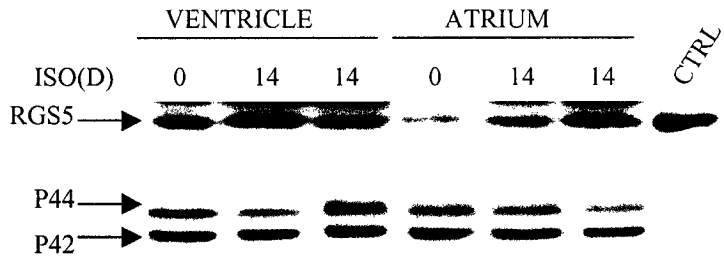

VENTRICLE
ATRIUM
CTRL
ISO(D)
0
14
14
0
14
14
RGS5
P44
P42

Fig. 2.5.4 Tissue distribution and the effect of ß adrenergic receptor stimulation and LPS on the levels of RGS5 protein

Western blot analysis using anti-RGS5 was performed on protein extracts prepared from a variety of different tissues. (A) Sprague Dawley rats received a daily injection (i.p.) of isoproterenol (ISO) and the animals were sacrificed fourteen (14) days later. Control animals (0) were injected with saline alone. (B) Wistar rats were injected (i.p.) with a single dose of bacterial lipopolysaccharide (LPS) and the animals were sacrificed six (+) hours later. Control animals (-) were injected with saline alone. CTRL, extracts from RGS5 expressing yeast cells. Similar results were obtained in 3 separate experiments.

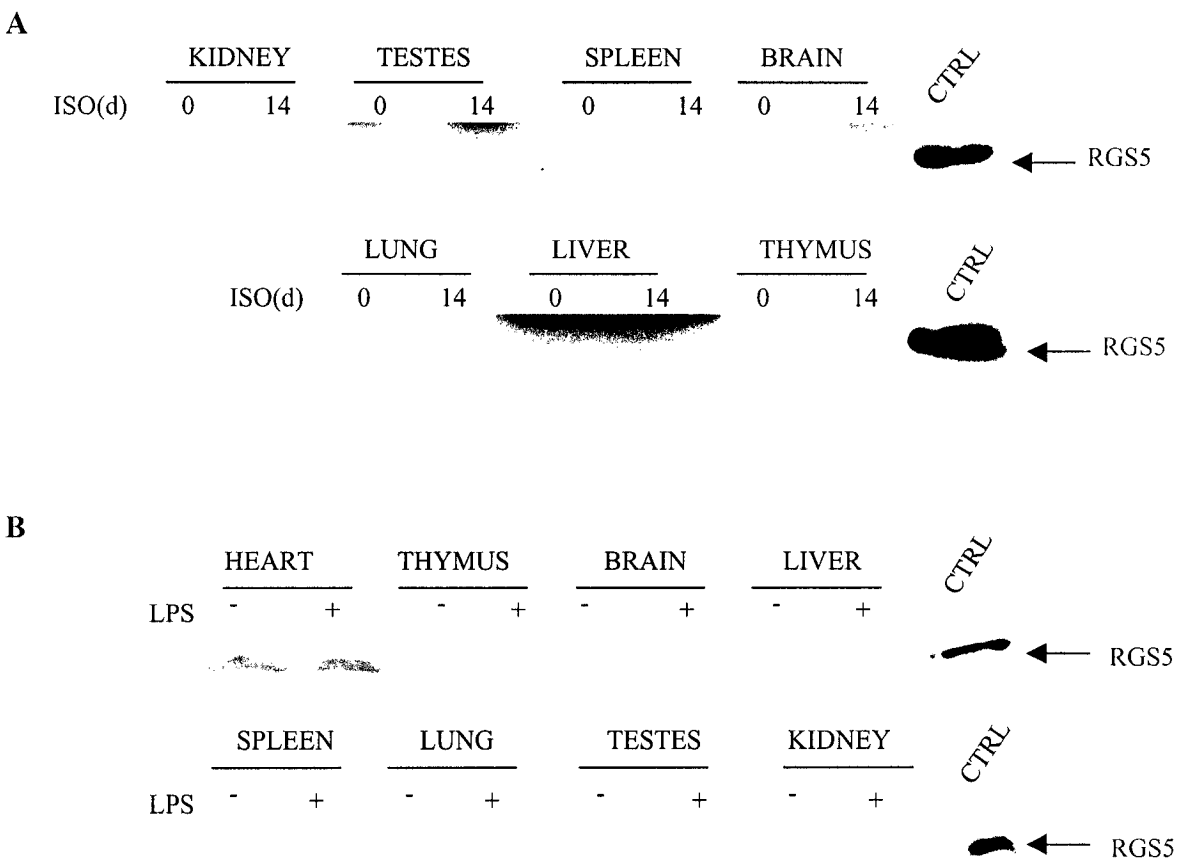

A
KIDNEY
TESTES
SPLEEN
BRAIN
ISO(d)
0
14
0
14
0
14
0
14
CTRL
RGS5
LUNG
LIVER
THYMUS
ISO(d)
0
14
0
14
0
14
CTRL
RGS5
B
HEART
THYMUS
BRAIN
LIVER
LPS
-
+
-
+
-
+
-
+
CTRL
RGS5
SPLEEN
LUNG
TESTES
KIDNEY
LPS
-
+
-
+
-
+
-
+
CTRL
RGS5

Fig. 2.5.5 Expression of RGS5 in muscle, cultured C2C12 cells and human fetal heart

(A), western blot analysis of extracts prepared from rat heart, skeletal muscle (MUSCLE) and testes using the RGS5 anti-serum. (B), western blot analysis for RGS5 expression in extracts prepared from four independent cultures of mouse C2C12 cells (labeled 1 to 4). Extract prepared from rat skeletal muscle (MUSCLE) is included as a control. (C), Protein extracts were prepared from human fetal tissues and analyzed for RGS5 expression by western blot. Both heart and lung tissues are shown from two different fetus (labeled 1 and 2). CTRL, extracts from RGS5 expressing yeast cells.

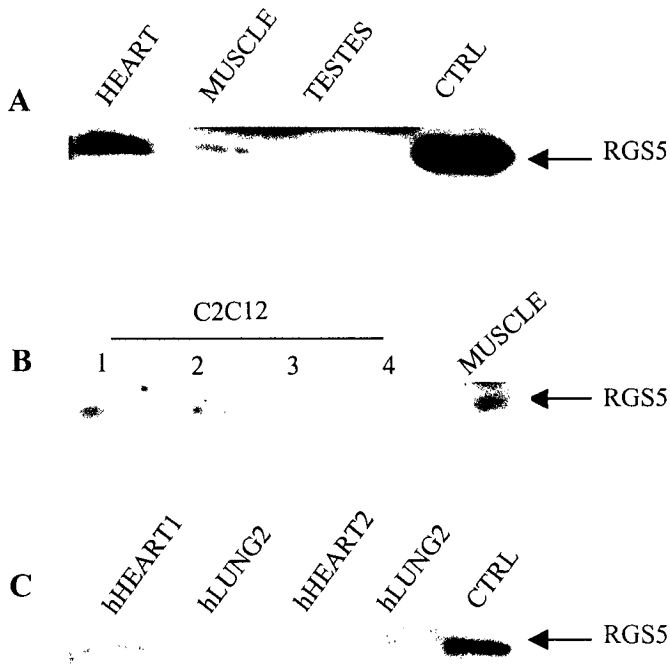
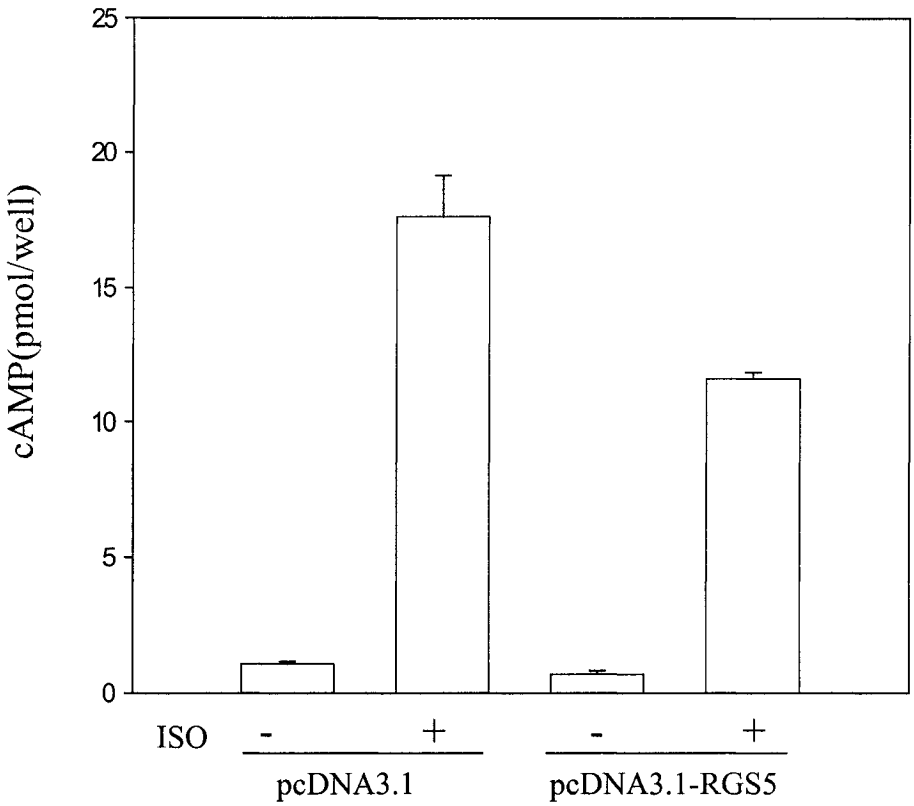

A
HEART
MUSCLE
TESTES
CTRL
RGS5
B
C2C12
1
2
3
4
MUSCLE
RGS5
C
hHEART1
hLUNG2
hHEART2
hLUNG2
CTRL
RGS5

Fig. 2.5.6 RGS5 inhibits ßAR-mediated increase in cAMP production in cultured SVEC4-10 cells.

Cells were transiently transfected with pcDNA3.1-RGS5 or with the vector alone (pcDNA3.1) and challenged with 10^{-6} M isoproterenol (+) or with vehicle alone (-) for 30 min. (0). The level of cAMP was determined in an equal number of plated cells and is expressed as pmol/well. Data are mean of triplicate assays.


25
20
15
10
5
0
cAMP(pmol/well)
ISO
-
+
-
+
pcDNA3.1
pcDNA3.1-RGS5

2.6 References

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Chapter 3: Future Direction

In this report, we found a candidate RGS that has possible inhibitory regulatory function on the β adrenoreceptor (AR) signaling pathway. Although we had shown that RGS5 is atrium specific up-regulated in chronically βAR stimulated rodents, we need further study to locate the possible signaling pathway that RGS5 is involved in. As C2C12 cell line endogenously expresses RGS5, therefore, we can use C2C12 and RGS5 overexpressing C2C12 as model, stimulate them with different agonist to detect downstream effectors of different GPCRs, for example, MAP kinase phosphorylation activity, we can expect RGS5 shows somewhat inhibitory effect on certain signaling cascade. Meanwhile, we can silence endogenous RGS5 through RNA interference (RNAi) to observe the physiological function of RGS5. We also can use primary cardiac cell culture as the second system to identify the interaction of RGS5 and specific GPCR. The identification of GPCR with which RGS5 interacts will help us understand the important role of RGS5 in heart disease.

Chapter 4 : Appendices

4.1 McGill University Animal use protocol